Elucidating the Role of Sorting Nexins in Skeletal Homeostasis

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This thesis is presented for the degree of Doctor of Philosophy of
The University of Western Australia

School of Surgery
The University of Western Australia

2017
Thesis Declaration

I, Audrey Chan, certify that:

This thesis has been substantially accomplished during enrolment in the degree.

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This research involving animals reported in this thesis followed The University of Western Australia and national standards for the care and use of laboratory animals.

The work described in this thesis was funded by grants from the National Health and Medical Research Council of Australia (NHMRC) APP1078280, a Department of Health Western Australia Merit Award and a UWA-UQ-A*STAR Trilateral Research Collaboration Award.
Technical assistance was kindly provided by R/O Euphemie Landao-Bassonga for histology and histomorphometry that is described in Chapter 5, and R/A Jamie Tan for the genotyping of SNX27 wild-type and knock-out mice.

This thesis contains published work and work prepared for publication, some of which has been co-authored.
Statement of candidate contribution

This thesis contains work that has been published.


**Location in thesis:** Chapters 5, 6 (shown in full as Appendix C)

**Student contribution to work:** Molecular and cellular experiments

**Co-author signatures and dates:**


**Location in thesis:** Chapter 7 (shown in full as Appendix D)

**Student contribution to work:** Figure 1, cellular experiments examining PTHR mutants

**Co-author signatures and dates:**

1. A/Prof Nathan Pavlos, certify that the student statements regarding their contribution to each of the works listed above are correct.

**Coordinating supervisor signature:**

**Date:** 23/01/2018
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Abstract

Growth and refurbishment of the vertebrate skeleton are strictly dependent on the coordinated cross-talk between bone-resident cells and their ability to respond to external stimuli via cell surface signalling receptors. Following agonist-induced stimulation, signalling receptors are internalised into early endosomes where they are sorted and then rerouted either back to the plasma membrane (i.e. recycling) or targeted to terminal lysosomes for degradation. The trafficking of signalling receptors must therefore be tightly regulated to ensure cellular homeostasis during skeletal development.

The Sorting Nexin (SNX) protein family has recently emerged as master regulators shown to govern endosomal sorting and recycling of transmembrane receptors and are unified by the possession of the Phox homology (PX) domain. This feature allows SNXs to bind to endosomal membranes where they then engage with luminal cargo proteins through specific interaction moieties (Teasdale and Collins, 2012). Although 34 SNX proteins have now been identified in humans, few have been functionally characterised. Moreover, the nature and number of SNX proteins expressed in bone resident cells as well as their potential physiological contribution to skeletal growth and homeostasis are presently unknown and thus are central to the investigation of this dissertation.

By combining quantitative expression analyses together with microscopy-based screens the entire SNX family was profiled in whole bone tissue and its individual cellular constituents (osteoclasts, osteoblasts and osteocytes). Whereas most SNXs were ubiquitously expressed in all bone cell types, some exhibited preferential cell and tissue expression patterns including SNX10 which was restricted to osteoclasts and SNX27, the most abundant SNX member expressed in total bone tissue. Unlike other members of the SNX family, SNX27 is unique, being the only member to house a post-synaptic 95, Dlg1, ZO-1 (PDZ) domain module and
interacts with cargo proteins possessing complementary PDZ-binding motifs within their carboxy-terminal tails.

To investigate the potential physiological role of SNX27 in skeletal growth and homeostasis, the bone phenotype SNX27-deficient mice was assessed. By combining micro-CT, histology and histomorphometry, SNX27-deficient mice were found to exhibit a severe form of osteochondrodysplasia, characterised by (i) a profound reduction in bone mass; (ii) reduced trabecular thickness and number and; (iii) an expansion of the epiphyseal growth plate. The bone phenotype was attributable, at least in part, to cell-autonomous defects in osteoblast differentiation and function in SNX27-deficient mice. This was reflected \textit{in vivo} by the reduced formation of new bone (osteoid) and confirmed \textit{in vitro} by the reduced capacity of SNX27-deficient osteoblasts to form mineralising bone nodules. Moreover, SNX27-deficient osteoblasts elicited sustained cAMP-dependent signalling and transcription following parathyroid hormone (PTH) stimulation. These observations hinted that PTH-signalling is intimately linked to SNX27, thus prompting the detailed investigation into the molecular interplay between SNX27 and the parathyroid hormone receptor type 1 (PTHR).

The PTHR is a prototypical class B family G-protein coupled receptor (GPCR) critical for the regulation of postnatal bone development, remodelling and mineral ion metabolism. Upon PTH stimulation, surface-resident PTHR elicits cyclic AMP (cAMP)-mediated activation of second messenger pathways (‘canonical’ signalling). Conversely, endocytosis of PTHR has long been considered to terminate receptor signalling at the plasma membrane, however PTHR (and other GPCRs) has been recently shown to generate cAMP within endosomes (‘non-canonical’ signalling) (Ferrandon et al., 2009). Therefore, PTHR-signalling is instead terminated by the post-endocytic retromer trafficking complex (Feinstein et al., 2011), although the molecular basis remained undefined.

PTHR possesses a carboxyl-terminal PDZ-binding motif (PDZbm; E-T-V-M) that is compatible with the binding signature of other SNX27 interacting cargo.
This led to the hypothesis that SNX27 acts as a molecular adaptor to link PTHR receptor signalling and recycling to retromer in a PDZ-dependent manner. This position was confirmed using a series of protein-protein interaction assays which demonstrated that the PDZbm was essential for SNX27-PTHR interaction and that the affinity of this association was enhanced in the presence of retromer. The spatiotemporal dynamics of this association was further monitored by confocal microscopy which captured the concomitant recruitment of SNX27 to PTHR-positive endosomes and their coordinated sequestration into de novo retromer positive recycling tubules. Moreover, the depletion of either SNX27 or retromer (VPS35) resulted in decreased PTHR surface levels and correlated with both sustained ‘non-canonical’ PTHR signalling and mis-trafficking of a subset of the receptor into degradative lysosomes.

To identify the minimal molecular determinants required for the PDZ-mediated interaction between SNX27 and PTHR, a series of alanine substitutions were introduced into the PTHR-PDZbm. By combining protein-protein interacting assays together with confocal microscopy, key glutamate residues upstream to the canonical PDZbm (i.e. E-W-E-T-V-M) were found to form critical electrostatic interactions with the conserved Arg<sup>58</sup> residue within the SNX27-PDZ domain. Cells expressing PTHR with the absence of this ‘electrostatic plug’ (i.e. PTHR-A6 mutants, A-W-A-T-V-M), or PDZbm (PTHR-ΔPDZbm) were found to have significantly impaired association(s) with SNX27. These mutations did not impair receptor internalisation, rather they increased the propensity of PTHR to mis-traffic toward degradative late-endolysosomes.

Collectively, the studies presented in this thesis unveil SNX27 (i) as a novel regulator of bone growth and homeostasis that; (ii) regulates PTHR signalling in osteoblasts by; (iii) directing the post-endocytic fate of the receptor and; (iv) acts as a molecular adaptor to simultaneously couple the receptor to the retromer recycling complex.
List of publications, abstracts and awards

Publications


Abstracts

2017

Chan A, Clairfeuille T, Collins BM, Pavlos NJ. The extreme C-terminus of PTHR encodes the key molecular determinants for PTHR-SNX27 interaction and trafficking; In: Lorne Protein Conference on Protein Structure and Function 2017; 5th – 9th February 2017; Lorne, Victoria, Poster Presentation.
2016

Chan A, Clairfeuille T, Collins BM, Pavlos NJ. The extreme C-terminus of PTHR encodes the key molecular determinants for PTHR-SNX27 interaction and trafficking; In: Combined Biological Sciences 26th Annual Meeting; 26th August 2016; Perth, Western Australia, Oral Presentation.

Chan A, Clairfeuille T, Collins BM, Pavlos NJ. The extreme C-terminus of PTHR encodes the key molecular determinants for PTHR-SNX27 interaction and trafficking; In: ANZBMS 2016 Annual Meeting: 21-24 August 2016; Gold Coast, Queensland, Australia, Plenary Poster #269

2015


**Trafficking to the Retromer for Postnatal Bone Growth**; In Australian Society for Medical Research, WA Symposium; 30th May 2015; Curtin University, Perth, Western Australia, Oral Presentation.

2014


Chan A, Landao-Bassonga E, Loo LS, Zheng MH, Hong WJ and Pavlos NJ. **Sorting nexin 27 (SNX27) is a crucial modulator of skeletal homeostasis**; In: 2nd EMBL Australia PhD Course; 30th June – 11th July 2014; Australian National University, Canberra New South Wales, Poster Presentation.

2013

Chan A, Landao-Bassonga E, Loo LS, Zheng MH, Hong WJ and Pavlos NJ. **Sorting nexin 27 (SNX27) is a crucial modulator of skeletal homeostasis**; In: Annual Combined Biological Sciences 23rd Annual Meeting; 30th August 2013; Perth, Western Australia, Poster Presentation (P46).

2012

Awards

2016

Combined Biological and Sciences Meeting (Perth, Aus) –

I. Harry Perkins Institute for Medical Research; (Award for Best Oral Presentation)

II. ThermoFisher Scientific; (Award for Best Manuscript)

Australian and New Zealand Bone and Mineral Society (Gold Coast, Aus) –

III. Christopher & Margie Nordin Young Investigator Poster Award

IV. Christine & T. Jack Martin Research Travel Grant

V. Travel Award

2015

American Society for Bone and Mineral Research (Seattle, USA) –

VI. Young Investigator Award, Seattle, Washington, USA

Australian and New Zealand Orthopaedic Research Society (Auckland, NZ) –

VII. PhD Student Award

VIII. Travel Award

Australian and New Zealand Bone and Mineral Society (Hobart, Aus) –

IX. Amgen-ANZBMS Outstanding Abstract Award

Australian Society for Medical Research (Perth, Aus) –

X. Murdoch University Award (Award for Best Oral Presentation)

2013
Combined Biological and Sciences Meeting (Perth, Aus) –

**XI. Coherent Scientific Runner Up Image Award**

**2012**

Australian and New Zealand Bone and Mineral Society (Perth, Aus)–

**XII. Roger Melick Young Investigator Award Runner Up**
Acknowledgements

This study was undertaken at the Centre for Orthopaedic Research, School of Surgery at The University of Western Australia, based at the Queen Elizabeth II Medical Centre, Nedlands.

I would first like to thank Associate Professor Nathan Pavlos for first accepting me as his Honours student and finding me worthy enough to continue these studies as his PhD student. Thank you for taking me under your wing and mentoring me side-by-side at the lab bench and from within the confocal microscopy room. Even though you were extremely busy, you always found the time to guide me in every aspect from the beginning to the end of my roller-coaster PhD. I have, and continue to learn an incredible amount from you and your incredibly intelligent brain and optimistic character.

To Winthrop Professor Ming Hao Zheng; thank you for your kind smile every time we meet and your over-enthusiastic perspective on all your projects. Thank you for your advice and support throughout my PhD and for inviting me to attend and participate in your international and local events at which I have befriended many ‘Winter-School’ and Chinese students that have visited Perth.

To Mrs. Euphemie Bassong-Landao; I cannot thank you enough for teaching me everything to do with research, from using a pipette to cell culture and embedding bones to histological staining. You have always been generous with your time and experience and for that I am forever grateful. Thank you for sharing your knowledge about both the lab and life, but most of all for sharing your warm smile every morning and afternoon, something that I still sorely miss to this day.

To Ms. Jamie Tan; a tremendous thank you for all your support and for maintaining the lab in an organised state (as I was probably one of those students who subconsciously maintained things in a chaotic balance). Thank you for always assisting me with my last-minute orders and for helping me (on more than a few
occasions) to find ‘mis-placed’ reagents. I will miss our non-research related conversations in the lab and tea room and our odd vents.

To my office buddies past and present; Guang Yi Li, Colin (Jun Jie) Gao, Pei Ying Ng, Ying Hua, Zhu Xiang, Louis (Tao) Wang, Kathrine (Lian Zhi) Chen, Aswin Beck and Suhaila Tahler. Thank you for your friendship and advice throughout the years, and for sharing your stories, humour and culture with me. Thank you for also making those failed experiments bearable and suggesting improvements to the methods or to simply shake your head with me. To the past Honours students, Anh Tran and Rebecca Weselman; thank you for joining us albeit for a (relatively) short time and being great students in the field of Orthopaedic Research.

To Professor Barry Iacopetta; thank you for your incredible support and encouragement throughout my PhD. To our colleagues at the Ear Sciences Centre and Cancer and Palliative Care Research and Evaluation Unit, thank you for your generous support when it came to sharing laboratory space and for your kind words of support and conversation from the office to the tea room.

To the Administration Team at the School of Surgery and in particular, Mrs. Belinda Seymour; thank you for your warm and genuine support throughout my Honours and PhD and for always encouraging students to consume as many Easter eggs and Christmas treats as they would like.

To the Centre for Microscopy, Characterisation and Analysis, in particular, Ms. Alysia Buckley and Mr. Paul Rigby; thank you for your tremendous training and support in the use and optimisation of the confocal microscope.

To our collaborators, A/Prof Brett Collins and A/Prof Rohan Teasdale and their laboratory at the Institute for Molecular Bioscience, University of Queensland; thank you tremendously for your generous support and advice over the past few years.
This research was supported by an Australian Government Research Training Program (RTP) Scholarship and the Ad Hoc Scholarship (School of Surgery, The University of Western Australia).
Special dedication

To my dad; you supported me to pursue knowledge and to never give up. You always stood behind me watching from afar to make sure that I could stand on my own two feet before you encouraged me to walk and explore.

To my brother; you always helped me when you could and always tried when you knew you couldn’t. As a true brother (and chemist), you even watched my video clip of dissolving a chemical which sizzled when it shouldn’t have, and when you couldn’t work out why, left me to my misery.

To my husband; you were there from the beginning to the end. You celebrated the little successes with me and when we should have partied to the big ones, we stayed in and watched a movie instead. You encouraged me to pursue my dreams and were supportive in every season and weather. When my own spirit fails me, you were and are my rock.

To mum; you were there from the moment I took my first breath. You taught me how to be strong when everything else convinced me that I’m not. You were the one who waited the longest for this; this thesis is dedicated to you.
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<tbody>
<tr>
<td>µCT</td>
<td>Micro-computed tomography</td>
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<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
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<td>AC</td>
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<td>ACVR1A/2B</td>
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<td>Ezrin-binding domain</td>
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<td>ERC</td>
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<td>HEK293</td>
<td>Human embryonic kidney 293</td>
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<td>HHM</td>
<td>Humoral hypercalcemia malignancy</td>
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<td>Hydroxymethylbilane synthase</td>
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<td>IL-1β, IL-6</td>
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<tr>
<td>PHEX</td>
<td>Phosphate-regulating neutral endopeptidase, X-linked</td>
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<td>Description</td>
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</tr>
<tr>
<td>PHTP</td>
<td>Primary hyperparathyroidism</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphoinositide</td>
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<td>Phosphoinositide 3,4-biphosphate</td>
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<td>Sortilin-related receptor with A-type repeat</td>
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<td>TGFβ</td>
<td>Transforming growth factor β</td>
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<td>TGN</td>
<td>Trans-Golgi network</td>
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<tr>
<td>TIP39</td>
<td>Tuberfundibular peptide-39</td>
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<tr>
<td>TMR</td>
<td>Tetramethylrhodamine</td>
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<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α (alpha)</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TRAP</td>
<td>Tartrate acid phosphatase</td>
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<tr>
<td>v-ATPase</td>
<td>Vacuolar-type H^+-ATPase</td>
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<td>V2R</td>
<td>Vasopressin receptor type 2</td>
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<tr>
<td>VEGFα</td>
<td>Vascular endothelial growth factor α (alpha)</td>
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<td>VPS</td>
<td>Vacuolar protein sorting</td>
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<tr>
<td>WASH</td>
<td>Wiskott-Aldrich syndrome protein and suppressor of cAMP repressor (SCAR) homology</td>
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<td>Wnt-Planar cell polarity</td>
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<td>Zonula occludens 1/2</td>
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<td>β1AR</td>
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<td>β2 adrenergic receptor</td>
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<td>β-Pix</td>
<td>p21-activated kinase interacting exchanging factor</td>
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CHAPTER

-1-

Introduction to the skeletal system
1. Introduction to the skeletal system

1.1. Introduction to bone

The principal function of the skeleton is to provide mechanical and physical support for the human body and its movements. Additionally, the bone marrow cavity housed within long bones also serves as a primary site for haematopoiesis (Harada and Rodan, 2003), while the bone matrix itself contains 99% of the body’s calcium reservoir in the form of hydroxyapatite \( \text{Ca}_{10}\left(\text{PO}_4\right)_6\left(\text{OH}\right)_2 \) with the remaining 1% circulating throughout the body and its tissues (Favus and Goltzman, 2013).

Despite its static appearance, bone is a highly dynamic organ that houses several specialised cells that drive skeletal growth and ensure both skeletal and mineral homeostasis. Chondrocytes are responsible for the production of cartilage and have crucial roles in the initial event of bone development and the formation of the endochondral skeleton. The bone is also populated with bone-excavating multinucleated osteoclasts that are responsible for the dissolution and removal of both the organic and inorganic components of cartilage and bone matrix; events which are required for (i) the removal of matrix during bone modelling, (ii) the removal of damaged bone during bone remodelling or (iii) the release of calcium into the bloodstream under hypocalcaemic conditions. The actions of osteoblasts oppose those of the osteoclast, where the former cells are primarily responsible for the deposition of a variety of extracellular proteins and mineral components that constitute the bone matrix. Following bone formation, osteoblasts may then become embedded within the bone matrix and terminally differentiate into osteocytes. Osteocytes are morphologically distinct from their predecessors and characteristically develop long dendrites that may extend across relatively long distances throughout the bone. These extensions serve to maintain direct cell-cell contacts with other skeletal-resident cells, including osteocytes, osteoblasts, osteoclasts and bone-lining cells, while also enabling osteocytes to detect micro-
damages that may occur to the bone matrix during skeletal loading, stress or injury. Additionally, the bone and its resident cells are supported through the function of peripheral cell populations, including bone-lining cells at the surface of the bone as well as those of the immune system, including T-cells and bone marrow macrophages (BMMs), the latter of which serve as precursors of the osteoclast.

Altogether, these bone resident cells collectively confer skeletal homeostasis by continually monitoring the physical integrity of the bone matrix and initiate the repair of damaged matrix through the coupled actions of the osteoclast and osteoblast. Bone remodelling is a highly dynamic process, where the balanced removal of the damaged matrix and the concomitant deposition of new bone has been estimated to result in the replacement of the entire skeleton every 10 years (Parfitt, 1980). Therefore, it is not surprising that skeletal disease often prevails in cases where skeletal homeostasis is severely compromised. Currently, 4.74 million Australians aged over 50 years are diagnosed with osteoporosis or osteopenia, the former being one of the most common skeletal diseases marked by low bone mass with a high risk of fracture due to brittle or weak bones while the latter is a milder form. By 2022, this is estimated to increase to a total of 6.2 million Australians, equating to a 31% increase from 2012. Together, these two skeletal diseases and their associated fractures have been predicted to accrue $33.6 billion over the next 10 years (2012-2022) to the Australian economy in direct and indirect costs for surgery and replacement implants, ambulance services, hospitalisations, rehabilitation and outpatient services. Furthermore, the current statistics claim that the frequency of one fracture occurring every 3.6 minutes will worsen to one in every 2.9 minutes by 2022 (Watts et al., 2013). Despite the urgency to reduce fracture incidence and improve skeletal health, most current therapies are aimed at preventing osteoclastic bone resorption without stimulating bone formation which leads to an ultimate accrual of damaged bone. In contrast to this, there are currently two therapies that promote the net formation of bone and have been approved for the treatment of osteoporosis (i.e. teriparatide and abaloparatide), however there are also limitations that accompany the use of these drugs including limited efficacy (~18-24
months). Therefore, researchers and clinicians alike are continuing to further our understanding into skeletal biology with the aim to develop more efficient therapies to combat not only osteoporosis, but a wide range of other skeletal diseases to improve global skeletal health.

1.2. Bone modelling (endochondral bone formation)

Skeletogenesis commences during the early stages of embryonic development, where the bones of the skeleton undergo events of modelling and growth until the early years of adulthood, after which the skeleton is continually remodelled throughout the individual’s lifetime. Osteogenesis, or the formation of new bone, may occur through one of two processes; intramembranous or endochondral ossification. The former most notably accounts for the formation of the craniofacial bones of the skull and involves the direct differentiation of mesenchymal stem cells (MSCs) into bone-forming osteoblasts (Yang, 2013). In contrast, most other bones of the skeleton are formed through endochondral ossification and are often collectively referred to as the bones of the endochondral skeleton (Mackie et al., 2011). This requires the initial formation of a cartilaginous template prior to it being remodelled and replaced by bone matrix by the highly-coordinated actions of chondrocytes, osteoblasts and osteoclasts. Another characteristic feature of endochondral bones is the presence of the epiphyseal growth plate between the two ossification centres, where its continual expansion growth is responsible for the longitudinal growth of the bone until its closure in early adulthood.

1.2.1. Role of the chondrocyte during skeletal development

Both intramembranous and endochondral ossification begin with the initial condensation of mesenchymal bi-potential stem cells (Figure 1.1A) and are capable of differentiating into cells of either the osteoblast or chondrocyte lineage. At this stage, these bi-potential progenitors express the chondrogenic transcription factor
Mesenchymal stem cell (MSC)
Perichondrial cell
Proliferating chondrocyte
Pre-hypertrophic chondrocyte
Hypertrophic chondrocyte

Figure 1.1.
Figure 1.1. Stages illustrating endochondral bone formation of the long bones in *Mus musculus*.

(A) Mesenchymal bi-potential progenitor cells undergo condensation at approximately embryonic day 11.5 (E11.5).

(B) Cells at the periphery (green) give rise to perichondrial cells that will later differentiate into articular chondrocytes, or osteoblasts, while the remaining cells (pink) commit toward the chondrogenic lineage and begin to deposit a cartilaginous matrix rich in collagen type II and proteoglycans.

(C) Cells within the centre of this mass undergo maturation and hypertrophy to become pre-hypertrophic (orange) and hypertrophic (brown) chondrocytes. Hypertrophic chondrocytes begin to express hypertrophic markers, including collagen type X and MMP13, and with the coordinated effort of osteoblast and osteoclast precursors that have migrated via the invading vasculature, the cartilaginous matrix is resorbed and mineralised, giving rise to the primary ossification centre at approximately E15.5.

(D) As the cartilage template elongates, an independent population of proliferating chondrocytes undergo hypertrophy and are joined by a second wave of vascularisation. This gives rise to the secondary ossification centre at either end of the developing bone at approximately postnatal day 7.

Adapted from Salazar, 2016 and Kizhemyakina, 2015.
SOX9, however this is lost following commitment to the osteoblast lineage (Figure 1.2). The stages of endochondral bone formation will be further described below.

Following condensation, the mass of cells at the centre and at the articular surface begin to commit to the chondrogenic lineage, while those surrounding the mass give rise to perichondrial cells that will later differentiate into osteoblasts (Figure 1.1B). The chondrocyte mass then begin to proliferate and secrete the main constituents of cartilage, namely collagens and proteoglycans, giving rise to a cartilaginous mould. Eventually, the cells within the centre begin to mature into hypertrophic chondrocytes and are marked with a considerable increase in cellular volume (Figure 1.1C). These cells also begin to express a unique set of genes that encode the enzymes and proteins required for the degradation of the surrounding matrix and invasion of blood vasculature, including matrix metalloproteinase 13 (MMP13) and vascular endothelial growth facture (VEGF) (Stickens, 2004, Inada et al., 2004, Maes et al., 2010). The primary ossification centre is then formed at the centre and marks the region where cartilage is degraded and is replaced with bone; an event that requires the coordinated actions of resorbing osteoclasts and bone-forming osteoblasts that have migrated to the site (as precursors) via the developing blood vasculature (Maes et al., 2010, Zelzer et al., 2002). As the bone continues to develop, a secondary ossification centre is formed at either end of the long bone where a discrete population of chondrocytes independently mature into hypertrophic chondrocytes and mediate the separate invasion of blood vessels (Figure 1.1D).

As the bone continues to grow, the continual removal of the cartilaginous matrix within the two ossification centres eventually gives rise to a refined region known as the epiphyseal growth plate (Figure 1.3A). Chondrocytes continue to remain as a highly organised structure where their continual proliferation and maturation is responsible for the longitudinal growth of the long bone until bone formation surpasses cartilage production (Wilsman et al., 1996, Noonan et al., 1998, Hunziker, 1994). The growth plate may be divided into five regions based on the state of chondrocyte differentiation and localised events (Figure 1.3B). The region
Figure 1.2. A schematic illustration of mesenchymal differentiation into cells of the osteoblast, chondrocyte and adipocyte lineages.

Mesenchymal stem cells (MSCs) give rise to cells of the osteoblast, chondrocyte and adipocyte lineages. Chondrogenic commitment of early SOX9+ progenitor cells and their subsequent maturation into hypertrophic chondrocytes are positively regulated by Wnt signalling. PTHrP-mediated signalling promotes chondrocyte proliferation while inhibiting chondrocyte hypertrophy, actions that oppose the effects of BMP-mediated signalling. Conversely, SOX9+ progenitor cells may also give rise to osteoblasts, where these cells sequentially express the major transcription factors, RUNX2 followed by OSX. Following bone formation, osteoblasts may either revert to become bone-lining cells (where PTH has been shown to induce re-activation into osteoblasts) or terminally differentiate into osteocytes. Alternatively, MSCs also give rise to adipocytes and are marked by the expression of major adipocyte transcription factor, PPARγ.

SOX9, Sry-related homeobox gene 9; RUNX2, runt-related transcription factor 2; OSX, osterix; PPARγ, peroxisome proliferator-activated receptor γ.

*Adapted from Kraenzlin, 2011.*
Figure 1.3.
Figure 1.3. Structural organisation of the growth plate in 4-week old mice.

(A) An image of the epiphyses of the mouse tibia sectioned longitudinally, stained with Goldner’s trichrome. Green/blue represents mineralised bone while red stains cartilage, unmineralised bone matrix and cells.

(B) Inset of (A), showing the structural organisation of the growth plate. Resting chondrocytes residing within the resting zone (RZ, zone 1) provide an indefinite supply of proliferating chondrocytes. Following the successive mitotic events of the proliferating chondrocytes residing within the proliferative zone (PZ, zone 2), these cells then intercalate and form distinct columnar stacks of chondrocytes and are a major determinant of the longitudinal growth of the long bone. These cells also secrete large quantities of collagen type II and proteoglycans that constitute a majority of the surrounding cartilaginous matrix. These cells then exit the cell cycle and mature, marked with cellular hypertrophy and are thus referred to as pre-hypertrophic and hypertrophic chondrocytes. These cells residing within the hypertrophic zone (HZ, zone 3) begin to express hypertrophic markers including collagen type X, MMP13, VEGFα and RANKL that attract and support osteoclast precursors to differentiate into osteoclasts, where they assist in the degradation of matrix in the zone of calcification (zone 4). Here, cartilage is resorbed and calcified by the actions of the osteoclast and hypertrophic chondrocytes, the latter of which eventually undergo apoptosis. Osteoprogenitors are recruited and subsequently differentiate into osteoblasts, where they then deposit and mineralise osteoid in the zone of ossification, marking the beginning of the primary spongiosa (zone 5). The accompanying graph also illustrates a brief list of the transcription factors and proteins expressed throughout the different zones.

MMP13, matrix metalloproteinase 13; VEGFα, vascular endothelial growth factor α; RANKL, receptor activator of nuclear factor kappa B ligand.

Adapted from Kozhemyakina, 2015 and Martin, 2016.
closest to the epiphysis is referred to as the ‘resting zone’ (Figure 1.3, RZ, labelled 1) providing an indefinite supply of progenitor chondrocytes. Below this region is the ‘proliferating zone’ (Figure 1.3, PZ, labelled 2) where, as the name suggests, these chondrocytes undergo successive mitotic division and intercalate to form characteristic columnar stacks that assist in the longitudinal growth of bone (Randall et al., 2012, Li and Dudley, 2009, Ahrens et al., 2009). Proliferating/columnar chondrocytes secrete abundant amounts of collagen type II and proteoglycans until eventually exiting the cell cycle to differentiate into pre-hypertrophic and hypertrophic chondrocytes found within the zone of maturation and hypertrophy (Figure 1.3, HZ, labelled 3). Maturing chondrocytes are marked by the substantial increase in cellular volume and secrete several characteristic proteins, including the exchange of collagen type II for type X, MMP13 and VEGF (Hattori et al., 2010). Both osteoblasts and hypertrophic chondrocytes initialise the deposition of hydroxyapatite crystals through the release of matrix vesicles (the latter being highly concentrated in calcium and phosphate) amongst the collagen fibres, generating the zone of calcification and degeneration (Figure 1.3, labelled 4). Hypertrophic chondrocytes eventually undergo apoptosis, while the matrix is remodelled into the mature trabecular network within the zone of ossification, a region that later develops into the primary spongiosa (Figure 1.3, labelled 5).

The initiation of chondrogenesis and the regulation of chondrocyte proliferation and differentiation associated with the formation of the growth plate are highly complex processes that require the tight coordination and crosstalk of several transcription factors and signalling pathways. These include the involvement of a variety of hormonal regulators and growth factors, such as thyroid hormones (Bassett and Williams, 2016), insulin-like growth factor-1 (IGF-1), fibroblast growth factors (FGFs) (Kronenberg, 2003, Kozhemyakina et al., 2015, Mackie et al., 2011), Wnt ligands (Liu et al., 2008), bone morphogenic proteins (BMPs) (Salazar et al., 2016), Indian hedgehog (Ihh), parathyroid hormone (PTH) and PTH-related protein (PTHrP) (Kronenberg, 2003). These regulators all function to modulate distinct stages of chondrocyte proliferation and differentiation, however, for the relevance to
this review, the role of transcription factors, SOX9 and RUNX2 and BMP signalling pathways will be briefly explained below, with a dedicated review of the PTH/PTHrP-dependent signalling pathway in Section 1.4.

1.2.1.1. **SOX9, the master regulator of chondrogenesis**

SOX9 belongs to the Sry-related homeobox protein family of transcription factors and marks all mesenchymal bi-potential progenitor cells that are able to commit to either the chondrogenic or osteoblastic lineages (Figure 1.2) (Akiyama et al., 2005, Yamashiro et al., 2004, Ducy et al., 1997, Otto et al., 1997, Bi et al., 1999). SOX9 is also considered as the master regulator of chondrogenesis, where it regulates the expression of several chondrogenic differentiation markers, including collagen type II, X, aggrecan and VEGFα (Dy et al., 2012, Hattori et al., 2010, Leung et al., 2011, Lefebvre et al., 1997, Lefebvre et al., 1998, Ng et al., 1997, Bell et al., 1997, Han and Lefebvre, 2008). Its importance is particularly highlighted in the phenotype of SOX9-null mice, where the skeleton of these mice is completely devoid of chondrocytes and cartilage (Bi et al., 1999, Akiyama et al., 2002).

SOX9 is expressed throughout chondrocyte differentiation and works in concert with other transcription factors to modulate proliferation and survival and delay hypertrophy (Ikegami et al., 2011, Dy et al., 2012), an event that depends on RUNX2 suppression (Yamashita et al., 2009, Zhou et al., 2006). Mice overexpressing SOX9 exhibit delayed chondrocyte hypertrophy partly by inducing the degradation of β-catenin and thus prevents Wnt-mediated chondrocyte differentiation (Figure 1.2) (Akiyama et al., 2004, Topol et al., 2009, Hartmann and Tabin, 2000, Enomoto-Iwamoto et al., 2002, Tamamura et al., 2005, Spater et al., 2006, Guo et al., 2004, Boyden et al., 2002, Gong et al., 2001). Additionally, SOX9 is able to upregulate its expression (Sekido and Lovell-Badge, 2008, Mead et al., 2013) as well as induce the expression of SOX5 and 6, where they work synergistically to maintain the expression of several chondrogenic genes (Smits et al., 2001, Akiyama et al., 2002, Lefebvre et al., 1998). In turn, SOX9 is also partly regulated by parathyroid hormone-related protein (PTHrP), where PTHrP promotes
chondrocyte proliferation (Bastepe et al., 2004, Chung et al., 1998) while also increasing the transcriptional activity of SOX9 via direct phosphorylation (Huang et al., 2001).

1.2.1.2. **RUNX2 and BMP2 signalling pathways in cartilage**

Runt-related transcription factor 2 (RUNX2), also known as core-binding factor subunit α1 (CBFα1) is expressed in both bi-potential progenitor cells at a later stage of mesenchymal condensation, as well as in perichondrial cells that later differentiate into osteoblasts (Figure 1.2). While the expression of RUNX2 declines during chondrogenesis, it later arises in maturing chondrocytes where its crucial roles in differentiation and hypertrophy are dependent on SOX9 suppression (Day et al., 2005, Hill et al., 2005, Kim et al., 1999, Otto et al., 1997, Ueta et al., 2001, Inada et al., 1999). RUNX2 is also able to modulate the expression of hypertrophic markers by binding directly to regulatory sequences present in several downstream targets, including Indian hedgehog (Ihh) (Yoshida et al., 2004), VEGFα (Zelzer et al., 2001), collagen type X (Drissi et al., 2003, Zheng et al., 2003) and MMP13 (Nishimura et al., 2012b, Selvamurugan et al., 2000). Moreover, RUNX2 is able to indirectly modulate chondrocyte hypertrophy via cross-talk with bone morphogenic proteins (BMP) and Wnt/β-catenin-dependent pathways (Hanai et al., 1999, Zhang et al., 2000, Javed et al., 2008, Dong et al., 2006).

BMP signalling is an evolutionary conserved pathway that coordinates several early developmental processes including body axis determination, tissue morphogenesis of several organ systems and determination of cell fate (Tuazon and Mullins, 2015, Salazar et al., 2016, Wang et al., 2014a). There are over 30 secreted ligands that have been recognised to elicit BMP-mediated signalling, including BMPs, growth/differentiation factors (GDFs), transforming growth factors (TGFs) and activins among others (Salazar et al., 2016).

With particular regards to BMPs, these ligands signal through both canonical and non-canonical pathways by binding to two dimers of type I and type
II BMP receptors. Specifically, there are three type I receptors (BMPR1A, BMPR1B and ACVR1A) and three type II receptors (BMPR2, ACVR2A and ACVR2B) that are capable of receiving BMP ligands. Upon binding to their heterotetrameric receptor complex at the cell surface, the constitutively-active type II BMP receptor phosphorylates the cytosolic tail of the type I receptor, leading to the activation of downstream regulatory-Smads (R-Smads, grouped as Smad1/4/8 or Smad2/3). These activated Smads then complex with co-mediator Smad (Smad4) and translocate to the nucleus where they regulate the transcription of target genes (Salazar et al., 2016, Wang et al., 2014a) (Figure 1.4A). Relatively less is known in regards to the non-canonical signalling pathways elicited by BMPs, however it most likely involves cross-talk with other pathways, including the MAPK, Akt and Wnt signalling pathways (Derynck and Zhang, 2003, Yamaguchi et al., 1995, Zhang, 2009, Akiyama et al., 2004, Jin et al., 2006, Topol et al., 2009).

Although BMPs were originally named after their ability to promote bone formation in cartilage (Wozney et al., 1988, Nogami and Urist, 1970, Urist and Strates, 1971), they have also been identified to direct chondrogenesis, including the initial condensation of MSCs, chondrocyte differentiation and maturation (Karamboulas et al., 2010, Yoon and Lyons, 2004, Liao et al., 2014, Shu et al., 2011). BMP signalling has been shown to have opposing effects on chondrogenesis depending on the state of cellular differentiation (Figure 1.2), where the inhibition of BMP signalling prevents the initial condensation of MSCs (Barna and Niswander, 2007) while also acting to accelerate chondrocyte hypertrophy in committed chondrocytes (Minina et al., 2002), a process that involves MAPK and Wnt signalling cross-talk (Akiyama et al., 2004, Jin et al., 2006, Topol et al., 2009). BMPs are also able to promote SOX9 expression and transcriptional activity to regulate chondrocyte proliferation and hypertrophy (Healy et al., 1999, Caron et al., 2013, Pan et al., 2009, Pan et al., 2008, Benazet et al., 2012), as well as inducing Ihh expression in pre-hypertrophic chondrocytes (Seki and Hata, 2004, Minina et al., 2002, Minina et al., 2001, Amano et al., 2008).
Figure 1.4. BMP and PTH signalling pathways

(A) BMP-mediated signalling: BMPs bind to a dimer of type I and II BMP receptors (BMPRI/II), where they then induce receptor activation. The canonical BMP signalling pathway involves the activation in R-Smads (either Smad1/5/8 or Smad2/3) where together with Co-Smads (commonly Smad4), translocate to the nucleus and regulate transcription of target genes. In addition to this, the activation of BMPRI/II may also induce the activation of the MAPK signalling pathway and the subsequent regulation of gene transcription.

(B) PTH-mediated signalling: PTH binds the amino-terminus region of PTHR type I (herein PTHR) where receptor activation results in the induction of G protein-dependent signalling. The predominant G protein activated is $G_s$, resulting in the activation of adenylyl cyclase (AC) and the generation of cyclic AMP (cAMP). This then results in the activation of PKA and subsequent downstream signalling cascades that ultimately regulate gene transcription. Additionally, PTHR activation has also been shown to induce $G_q$-dependent signalling cascades, resulting in the increase of intracellular calcium and the activation of PKC- and MAPK-dependent pathways that also regulate gene transcription. Following endocytosis, PTHR continues to generate cAMP, an event referred to as ‘non-canonical’ or endosomal signalling.

BMP, bone morphogenic protein; PTH, parathyroid hormone; PTHR, PTH receptor; $G_s$, G protein subunit $\alpha_s$; $G_q$, G protein subunit $\alpha_q$; AC, adenylyl cyclase; cAMP, cyclic AMP; PKA, protein kinase A; PKC, protein kinase C; DAG, diacylglycerol; PLC, phospholipase C; IP3, inositol triphosphate; MAPK, mitogen activating protein kinase.
1.3. Bone remodelling

Bone remodelling occurs throughout life and involves a tightly coordinated sequence of events that are necessary for the repair of bone. Old or damaged bone is continually replaced with newly-formed bone and as such, maintains the quality and integrity of the skeleton. The bone matrix may also be resorbed in the event where calcium and/or phosphate are required to be released into the blood stream as determined under hypocalcaemic or hypophosphatemic conditions. Bone remodelling events occur within localised regions of bone, also known as ‘bone modelling units’ (BMU) and involves the cooperate effort of a range of specialised cells, including osteoblasts, osteocytes, macrophages, osteoclasts and T cells (Sims and Martin, 2014, Frost, 1964). While the structure of BMUs differ between trabecular or cortical events, the fundamental concept remains the same; that bone resorption is tightly coordinated with the amount of new bone formed to ensure structural integrity and skeletal homeostasis (Sims and Martin, 2014, Delaisse, 2014).

Using human trabecular bone as a model for illustration, the sequence begins with the attraction of osteoclast precursors to the site to be resorbed (Figure 1.5). These precursors may migrate to the site from nearby capillaries or adjacent locations (e.g. bone marrow) and differentiate into bone-resorbing osteoclasts upon the stimulation of two crucial cytokines sourced from surrounding osteoblasts, osteocytes and T cells – macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B (RANK) ligand (RANKL) (Teitelbaum, 2007). Here, osteoclasts come in direct contact with the opposing bone matrix under a canopy of bone-lining cells or in mouse bone, tissue-specific macrophages (‘osteomacs’) (Chang et al., 2008) and proceed to resorb the bone matrix, an event that is thought to last 2-3 weeks (Sims and Martin, 2014). This is then followed by a lesser-characterised ‘reversal’ phase marked by the clearing of collagen and deposition of a cement line. The ‘formation’ phase is then initiated by the recruitment of osteoblast precursors to the localised site, where they differentiate into mature...
Figure 1.5. A schematic illustrating bone remodelling as it occurs within human trabecular bone.

In mice, bone remodelling takes place within a bone remodelling compartment at the trabecular surface. A canopy of either bone-lining cells or tissue-specific macrophages (osteomacs) is formed, allowing osteoclast precursors to migrate to the site through the vasculature and differentiate into osteoclasts upon the stimulation of M-CSF and RANKL, the latter of which is balanced by the secretion of the decoy receptor of RANK, OPG. Bone resorption typically last 2-3 weeks, where osteoclasts then undergo apoptosis. Under homeostatic conditions, this process is tightly coupled with the deposition of unmineralised bone matrix (osteoid) by osteoblasts, a process that may take 3-4 months. Following the completion of bone formation, osteoblasts may become embedded within the mineralising bone matrix and terminally differentiate into osteocytes.

M-CSF, macrophage colony stimulating factor; RANKL, receptor activator of nuclear factor kappa B ligand; OPG, osteoprotegrin.

Adapted from Sims and Martin, 2014.
osteoblasts and serve to deposit osteoid which is then mineralised over the course of 3-4 months (Sims and Martin, 2014).

1.3.1. A brief description of the osteoclast

Osteoclast precursors are derived from cells of the monocyte/macrophage lineage (Suda et al., 1999, Walker, 1975, Gothlin and Ericsson, 1976) and are recruited to the site of resorption through the action of a variety of cytokines, including M-CSF and RANKL, both of which together, are sufficient for osteoclastogenesis (Suda et al., 1999, Boyle et al., 2003, Pixley and Stanley, 2004, Teitelbaum, 2007). These cells subsequently fuse and differentiate to form multinucleated osteoclasts (Boyle et al., 2003) possessing anywhere between three to a hundred nuclei (Roodman, 1996) and are uniquely responsible for the resorption and dissolution of the organic and inorganic components of the bone matrix, leaving resorption pits or trails as visualised in in vitro bone surface cultures. Following the completion of the resorption phase, osteoclasts then undergo apoptosis.

Osteoclasts express a unique set of proteins that are involved in the formation and resorption processes, including cathepsin K (Ctsk), calcitonin receptor (CTR) and tartrate-resistant acid phosphatase (TRAP) (Boyle et al., 2003, Teitelbaum and Ross, 2003, Nicholson et al., 1986). While the exact function of TRAP during bone resorption remains unclear, it remains as a useful tool in identifying the presence of osteoclasts in vitro and in vivo (Filgueira, 2004, Suda et al., 1997). Osteoclasts formed on glass or plastic surfaces in vitro exhibit a low profile with an immense spreading of their cytoplasm across the cultured surface. These osteoclasts are said to reside in an ‘unpolarised’ state and interact with the cultured surface through actin-rich adhesion points referred to as ‘podosomes’ (Faccio et al., 2003). Osteoclasts cultured on bone surfaces however, adopt a ‘polarised’ state reaching between 10-80µm in height, where the substantial reorganisation of their actin cytoskeletal network gives rise to four distinct membrane domains – the functional secretory domain (FSD), basolateral domain(s) (BLD), sealing zone (SZ) and the ruffled border (RB) (Figure 1.6) (Teitelbaum,
Figure 1.6. A simplified illustration of the trafficking routes within an osteoclast.

When attached to the bone surface, the multi-nucleated osteoclast polarises where four distinct membrane domains are formed; the functional secretory domain (FSD), basolateral domain(s) (BLD), sealing zone (SZ) and the ruffled border (RB). The RB is the highly convoluted membrane that opposes the bone surface and forms the resorptive organelle of the osteoclast, where it is continually subjected to fusion events which release lysosomal enzymes (e.g. Ctsk) and proton pumps (e.g. v-ATPase) to facilitate the degradation of organic and inorganic components of the bone matrix (A, B). The osteoclast also uptakes resorption by-products through the centre region of the RB, where they are then digested and released at the FSD (C). In addition to these specialised events, there are several other membrane transport routes that occur within osteoclasts that are common to all cells, such as the biosynthetic route (transport of proteins from the ER/Golgi to the PM) through the use of endosomes (D, E).

(Inset B) A variety of molecular machinery are embedded into the membrane of the RB through fusion events, including v-ATPases and chloride channels (most notably in the form of CIC-7) that then secrete H+ and Cl- ions into the resorptive lacuna.
The ruffled border is a highly convoluted membrane and is the resorptive organelle of the osteoclast, found immediately opposing the bone matrix. The ruffled border is enclosed by the sealing zone, the latter of which is formed from the rearrangement and condensation of podosomes that form a tight attachments with both integrins (most commonly αvβ3 integrins) and RGD-motifs embedded within the bone matrix (McHugh et al., 2000, Faccio et al., 2003, Ross and Teitelbaum, 2005, Suda et al., 1992, Teitelbaum, 2007, Nakayama et al., 2011). The sealing zone acts as a ‘plug’, forming a microenvironment that encases the ruffled border and bone matrix for the localised degradation of bone matrix (Luxenburg et al., 2007).

Osteoclasts mediate the resorption of the inorganic (mineral) component of the bone matrix by continually secreting acid (H+) into the opposing resorptive lacuna, through the actions of H-ATPase and vacuolar (v)-ATPase pumps (Mattsson et al., 1994, Blair et al., 1989, Xu et al., 2007, Qin et al., 2012). Simultaneously, the continual fusion of lysosomal compartments with the ruffled membrane enable the release of degradative enzymes, including Ctsk, TRAP and MMPs, into the resorption lacuna, which mediate the degradation of the organic (collagen-rich) component of bone (Blair, 1998, Gelb et al., 1996, Teitelbaum et al., 1995, Goto et al., 2003). Being highly specialised bone-excavators, osteoclasts thus require a highly specialised set of machinery to generate and maintain the high demand of secretion and fusion events required for bone resorption (Zhao, 2012). Despite being the resorptive organelle of the cell, the ruffled border is also responsible for the removal of post-degradation by-products from the resorption pit, e.g. collagen fragments, minerals and ions that are released following bone degradation that may otherwise compromise osteoclast function (Vaaninen and Laitala-Leinonen, 2008). One way by which these by-products are removed is through the event of transcytosis, where collagen fragments are removed via membrane-delimited vesicles and subsequently released from the FSD (Salo et al., 1997, Stenbeck and Horton, 2004).
1.3.2. A brief description of the osteoblast

The osteoblast is the main cell responsible for bone formation and accounts for the second largest population of cells residing within bone (Capulli et al., 2014). As previously mentioned, both osteoblasts and chondrocytes are derived from mesenchymal bi-progenitor cells and though both express SOX9, cells that later commit to the osteoblast lineage lose SOX9 in favour of RUNX2 (Figure 1.2). For simplicity, the osteoblast lineage may be divided into four stages of differentiation – osteoblast progenitors, immature, committed/pre-osteoblasts and mature osteoblasts. While these boundaries between differential stages are not always clear, there are several observations that assist in differentiating these populations. Histologically, osteoprogenitors are RUNX2$^+$ and are often visualised as elongated and fibroblastic in vivo (Florencio-Silva et al., 2015). In contrast, mature and active osteoblasts are frequently found lining the bone surface, adopting a cuboidal-like morphology and often reside above a layer of osteoid that is rich in osteocalcin (OCN), osteonectin, osteopontin, bone sialoproteins (BSPs) and collagen type I (Long, 2012, Capulli et al., 2014). The cells transitioning between these two populations tend to be arbitrarily defined as the pre-osteoblast population for simplicity, where they maintain their ability to proliferate, express ALP and may express RUNX2$^+$ (early), or RUNX2$^+$OSX$^+$ (late/committed) (Capulli et al., 2014)(Pritchard, 1952; Owen, 1953; Owen and Macpherson, 1963).

Following osteoid deposition, osteoblasts then initialise the mineralisation of the surrounding matrix by secreting matrix vesicles (~100-200nm in diameter) (Anderson, 1969) that are rich in calcium and phosphate. Though the exact mechanism of mineralisation is still debatable, these nanospherical bodies are the source of the first hydroxyapatite crystals that form along the collagen fibers constituting the ECM (Anderson, 2003, Arsenault et al., 1991). Apart from undergoing apoptosis following the completion of bone formation (Jilka et al., 1998), osteoblasts may convert to quiescent, flat bone-lining cells, where they are thought to aid as a physical barrier to minimise the direct interaction between osteoclasts and the bone matrix (Del Fattore et al., 2011). However, osteoblasts are most notably
known for their ability to embed themselves within the mineralising bone matrix where they terminally differentiate into osteocytes (Manolagas, 2000).

Osteoblasts and osteocytes are the most abundant sources of M-CSF and RANKL required for osteoclastogenesis. Additionally, both osteoblasts and osteocytes balance the availability of RANKL with the simultaneous production of osteoprotegerin (OPG) which acts as a decoy receptor for RANKL (Simonet et al., 1997). In this way, the RANKL:OPG ratio is generally accepted as a key index of the overall balance between bone formation and resorption (Prideaux et al., 2016). Osteoblasts are also abundant sources of other cytokines that are capable of stimulating osteoclast differentiation and function, including PTHrP, interleukin 6 (IL-6) and tumour necrosis factor α (TNF-α) (Teitelbaum, 2007).

1.3.2.1. **RUNX2 and OSX as osteoblast transcription factors**

Similar to how SOX9 is the master regulator of chondrogenesis, RUNX2 is indispensable for osteoblastogenesis and is expressed throughout all stages of osteoblast differentiation (Ducy et al., 1997). RUNX2-null mice were found to be completely devoid of osteoblasts and bone formation, as well as lacking chondrocyte hypertrophy, the latter consistent with its dual role in modulating chondrocyte maturation (Komori et al., 1997, Otto et al., 1997). Moreover, mice with reduced (Adhami et al., 2015) or excessive (Liu et al., 2001) RUNX2 levels were found to have abnormal postnatal skeletal development, while humans with inactivating RUNX2 mutations presented with the abnormal development of craniofacial bones in the form of cleidocranial dysplasia (Mundlos, 1999), altogether confirming that RUNX2 is essential for both prenatal and postnatal skeletal development.

RUNX2 regulates the expression of several osteoblast genes, including ALP, collagen type I, bone sialoprotein, osteopontin, osteonectin, OCN, MMP13, RANKL and OPG (Fakhry et al., 2013, Harada et al., 1999, Kern et al., 2001, Ducy et al., 1997, Boumah et al., 2009), as well as directly inducing the expression of another crucial osteoblast transcription factor, OSX (Nishio et al., 2006). RUNX2
has also been shown to promote the expression of osteocyte markers, including
dentin matrix protein 1 (DMP1), Dickkopf-1 (DKK1) and sclerostin (SOST) (Fakhry
et al., 2013, Komori, 2011, Javed et al., 2010, James et al., 2006). In turn, the
transcriptional activity of RUNX2 is regulated by numerous co-factors and proteins,
including its positive regulation by MSX2 (Aioub et al., 2007, Liu and Lee, 2013),
PTH, fibroblast growth factors (FGFs), TGFβ and BMPs, and its negative regulation
by TNFα and transcription factor, Twist (Bialek et al., 2004, Liu and Lee, 2013).

OSX, previously known as Sp7, is a member of the zinc finger-containing
family of transcription factors and was initially identified as a BMP2-inducible target
(Nakashima et al., 2002). OSX has since been found to be crucial in both osteoblast
and osteocyte differentiation (Baek et al., 2009, Zhou et al., 2010) and lies
downstream of RUNX2. While RUNX2-null mice failed to express OSX, OSX-null
mice were found to express RUNX2, however their skeleton was marked with a lack
of mature osteoblasts and mineralised bone (Nakashima et al., 2002). Moreover, the
postnatal deletion of OSX resulted in increased fractures, decreased osteoblast
activity and impaired osteocyte maturation (Baek et al., 2009, Zhou et al., 2010),
indicating that OSX is a requirement for osteoblast commitment and differentiation.

Together with RUNX2, OSX is able to induce the expression of several
osteoblast markers, including collagen type I, OCN, ALP, BSP and OPG
(Nakashima et al., 2002, Fu et al., 2007, Kurata et al., 2007, Stein et al., 1996). In
turn, OSX is inducible by ascorbic acid and 1,25(OH)2D3 (Maehata et al., 2006, Xing
et al., 2007) and positively regulated by FGF, Wnt-β-catenin (Felber et al., 2015)
and BMP2-mediated pathways (Celil and Campbell, 2005, Lee et al., 1999, Lee et
al., 2003, Ulsamer et al., 2008). Interestingly, it has been reported that prolonged
PTH-mediated cAMP negatively regulated OSX expression (Hong et al., 2009,
1.3.2.2. BMP signalling in bone

Of the BMP family, BMP2, 4, 6, 7 and 9 are collectively considered to be osteogenic BMP ligands due to their ability to induce bone formation (Wang et al., 2014a, Luu et al., 2007, Nishimura et al., 2012a). In particular, BMP2 has been found to be indispensable for endochondral bone formation and its global depletion results in severe chondrodysplasia and early lethality (Shu et al., 2011). The conditional deletion of BMP2 in osteoprogenitors resulted in a skeletal phenotype of low bone mass with bones exhibiting a significant increase in spontaneous fractures that were unable to heal (Tsuji et al., 2006, Chappuis et al., 2012). Similarly, the conditional deletion of BMP2 in early or committed osteoblasts (2.3kb Col1a1 or OSX promoters) also resulted in mice with low bone mass, brittle bones and hypomineralised teeth (Yang et al., 2013, McBride et al., 2014, McBride-Gagyi et al., 2015, Feng et al., 2011). Both BMP2 and BMP4 are particularly important for osteogenesis (Bandyopadhyay et al., 2006) and for the anabolic action of PTH (Khan et al., 2016), while BMP7 is dispensable (Tsuji et al., 2010), indicating the differing roles of BMPs in the regulation of skeletal development and homeostasis.

In addition to its roles in chondrogenesis, BMP signalling also plays a crucial role in regulating osteoblast differentiation. Wnt/β-catenin signalling promotes the proliferation of osteoprogenitor cells while BMP signalling acts to oppose this and instead, promote osteoblast differentiation (Amedee et al., 1994, Hughes et al., 1995). In osteoblasts however, these two pathways then cooperate to suppress SOX9 and promote RUNX2- and OSX-mediated osteoblast differentiation and function (Lian et al., 2006, Bain et al., 2003, Rawadi et al., 2003, Day et al., 2005, Hill et al., 2005), a process that also involves cross-talk with the PI3K/AKT signalling pathway (Mukherjee and Rotwein, 2009). BMP2 may in turn, be regulated by β-catenin, having been found to possess a TCF/Lef response element within its promoter region (Zhang et al., 2013).
1.3.3. A brief role of the osteocyte

Nearing the completion of bone formation, osteoblasts may embed themselves within the bone matrix and terminally differentiate into osteocytes, the master regulators of skeletal homeostasis (Dallas and Bonewald, 2010). Osteocytes contribute to 95% of the resident cells within the skeleton and reside within the bone matrix in distinct lacunae. Despite this, osteocytes maintain direct cell-cell contacts through cellular extensions (dendrites) that travel throughout the lacunocanalicular network (Figure 1.5) (Dallas et al., 2013). Osteocytes are extremely important in sensing mechanical loading of the bone and oversee several processes that act to maintain skeletal homeostasis.

While osteocytes are somewhat isolated due to their location within the bone matrix, they are constantly surrounded by canaliculi fluid that flows throughout the canaliculi network and allows the passage of nutrients, oxygen and even signalling molecules throughout the osteocyte network. These characteristics of the osteocyte enable them to function as ‘mechanostats’ of the skeleton, where they are able to sense the mechanical loading that occurs on the bone matrix itself, or in the differences in fluid flow shear stress that follows intermittent loading to the bone (Weinbaum et al., 1994). Similarly, osteocytes are also able to detect micro-fractures within the bone matrix and therefore facilitates the translation of the released biochemical signals to initialise and modulate localised bone remodelling events (Dallas et al., 2013, Prideaux et al., 2016).

Osteocytes play key roles in modulating bone formation, particularly through Wnt/β-catenin and PTH-dependent pathways. Although highly complex, the Wnt/β-catenin pathway generally serves to promote osteoblast maturation and bone formation. Late-stage osteoblasts and osteocytes are known to express Wnt antagonists that serve to inhibit osteoblast differentiation and bone formation, including Dikkopft-1 (DKK1) and soluble frizzled proteins (sFRPs), while sclerostin (SOST) is exclusively expressed by mature osteocytes (Dallas et al., 2013, Bonewald and Johnson, 2008, Atkins et al., 2011). Furthermore, the increase in bone mass
following mechanical loading is partly dependent upon Wnt signalling and the concomitant decrease in Wnt antagonists (Lara-Castillo et al., 2015). In fact, van Buchem disease and sclerosteosis are both skeletal diseases marked with an absence of SOST, leading to a high bone mass phenotype and a substantial increase in osteoblast activity (Balemans et al., 2001, ten Dijke et al., 2008, Van Bezooijen et al., 2005). From this, SOST has now become a therapeutic target to promote bone formation and inhibit bone loss in the form of anti-SOST antibody therapies (McClung et al., 2014, Recker et al., 2015, MacNabb et al., 2016). In addition to this, osteocytes also serve as the primary source for RANKL in vivo, where deletion of RANKL in osteocytes resulted in severe osteopetrosis in mice (Nakashima et al., 2011).

Osteocytes have also been shown to express what was previously recognised as osteoclast-specific degradative enzymes, including Ctsk, carbonic anhydrase II (CAII), TRAP and ClC7 (Kogawa et al., 2013, Qing et al., 2012). This is thought to enable osteocytes to demineralise the bone matrix bordering their lacunae in order to release calcium during lactation, an event that is dependent on the actions of PTHrP (VanHouten et al., 2003, Qing et al., 2012, Kovacs, 2017).

1.4. PTHR signalling is a major signalling pathway in chondrocytes, osteoblasts and osteocytes

Skeletal homeostasis is dependent upon the tight coupling of osteoblasts, osteoclasts and osteocytes, as well as their ability to communicate with other cell populations. Several signalling pathways have been thoroughly investigated, within or beyond the context of bone, in an attempt to fully understand the mechanisms governing cellular differentiation, function and activity. Chondrocytes, osteoblasts and osteocytes are derived from a common mesenchymal progenitor cell and continue to share several signalling pathways following their commitment to their cell lineages. Most notable of these are the Wnt, BMP and PTH/PTHrP-mediated signalling pathways (amongst several others), where they regulate cellular
differentiation and function independently or through signalling cross-talk. The roles of Wnt and BMP have been comprehensively reviewed in (Baron and Kneissel, 2013, Salazar et al., 2016) therefore this section will focus on PTHR signalling which is central to the findings presented in this dissertation herein.

1.4.1. PTHR, PTH and PTHrP

The parathyroid hormone receptor (PTHR) belongs to the class B family of G protein-coupled receptors (GPCR) and normally resides at the PM of expressing cells. PTHR consists of an extracellular amino-terminal region that acts to receive its cognate ligands, followed by seven transmembrane domains and a cytoplasmic carboxy-terminal tail. Typical of most GPCRs, the cytoplasmic tail possesses seven serine residues that are phosphorylated by the action of G protein kinases (GRKs) following receptor activation which serve as a recruitment sites for β-arrestin to promptly engage and mediate receptor endocytosis (Gardella and Vilardaga, 2015).

There are two PTH-receptors currently recognised; PTHR type I (PTH1R, herein PTHR) and PTH2R, with the former being the best characterised. Briefly, PTH2R is expressed in a variety of tissues including the brain, testes, muscle and placenta and shares 51% homology to PTHR (Usdin, 1997, Usdin et al., 1995, Usdin et al., 1999, Usdin et al., 2000). Additionally, PTH2R possesses a slightly shorter carboxy-terminal tail, where it totals to 550 or 546 residues in the human or mouse, compared to PTHR (593 or 591 residues, respectively). PTH2R is also known to respond to an additional ligand, tuberfundibular peptide-39 (TIP39) and besides its role in spermatogenesis (Usdin et al., 2008), relatively little is known in regards to the downstream signalling pathways elicited, or the role of PTH2R in expressing tissues and will therefore not be further discussed for the purposes of this thesis.

PTHR is involved in the development and homeostasis of a variety of tissues, including the mammary glands, skin, pancreas, kidney and most importantly, the skeleton. Two endogenous ligands, PTH and PTHrP are able to bind to the extracellular region of PTHR and induce receptor activation. These ligands share
significant sequence conservation within the first 34 residues, where the amino-terminal fragments of these proteins (PTH(1-34), PTHrP(1-34) and PTHrP(1-36)) have shown comparable receptor binding and activation to their full-length counterparts (PTH(1-84) and PTHrP(1-141) (Potts et al., 1971, Nissenson et al., 1988, Juppner et al., 1991). While full-length PTH does not harbour any other notable domains besides its biologically-active amino-terminal region, full-length PTHrP consists of several downstream regions that participate in a variety of functions, including placental calcium transport (residues 67-86), nuclear localisation (residues 67-94) and an extended carboxy-terminal domain that has been implicated in mediating cell proliferation and survival (Toribio et al., 2010, De Miguel et al., 2001, Miao et al., 2008). Despite these differences, both PTH and PTHrP have been shown to play crucial yet distinct roles in skeletal development and homeostasis through their common receptor.

PTH is predominantly synthesised by chief cells within the parathyroid gland, where it is stored within secretory granules until its pulsatile release into the circulation under hypocalcaemic or hyperphosphatemic conditions (Evenepoel et al., 2016). As an endocrine hormone, PTH mainly exerts its effects on calcium and phosphate serum levels by inducing an increase in calcium reabsorption (and increase phosphate excretion) in the kidney and its release from the bone matrix through the resorptive actions of the osteoclast. Additionally, PTH also induces the production of calcitriol (biologically active vitamin D or 1,25(OH)_{2}D_{3}) in the kidney, which acts to increase calcium absorption through the intestine. Teriparatide (or PTH(1-34)) and PTH(1-84) have been recently joined by abaloparatide (a modified analogue of PTHrP(1-36)) as the only FDA-approved osteoanabolic therapies in the treatment of post-menopausal osteoporosis (Shirley, 2017, Chew and Clarke, 2017, Raisz, 2005, Neer et al., 2001, Greenspan et al., 2007). As discussed below, PTH exerts paradoxical actions on the bone, where intermittent or continuous exposure of PTH results in a net anabolic or catabolic effect on the skeleton, respectively.
PTHrP was first thought to act in a ‘PTH-like’ manner as a factor secreted from cancerous cells (Albright, 1941; Kukreja et al., 1980; Rude et al., 1981; Stewart et al., 1980; Burtis et al., 1990; Grill et al., 1991). Instead of functioning as an endocrine hormone, PTHrP is now widely recognised to act in a paracrine/autocrine manner and has since been shown to regulate the development and function of a variety of tissues. These include the event of tooth eruption (Philbrick et al., 1998, Wysolmerski et al., 2001), mammary gland development (Wysolmerski et al., 1998, Wysolmerski et al., 2001), placental calcium transport (Kovacs et al., 1996), regulation of vascular smooth muscle (Macgill et al., 1997, Qian et al., 1999) and skeletal development (Karaplis et al., 1994, Amizuka et al., 1994) (see Martin, 2016 for an extensive review). Following its synthesis and post-translational cleavage, the mature protein (PTHrP(1-141) in humans, or PTHrP(1-139) in mice) is secreted into the extracellular matrix where it is then able to bind to PTHR in adjacent cells.

1.4.2. PTHR signalling in skeletal development and remodelling

Upon binding to either PTH or PTHrP, PTHR activation predominantly leads to the subsequent activation of the adjacent $G_\alpha_s$ subunit (Figure 1.4). $G_\alpha_s$ activates adenylyl cyclase (AC) which is responsible for the generation of the secondary messenger molecule, cytosolic 3,5'-cyclic AMP (cAMP) within the cytosol. This then leads to the activation of downstream signalling effectors, including protein kinase A (PKA) and cAMP response element-binding protein (CREB) which translocates into the nucleus and modulates gene transcription. Additionally, PTHR activation has also been shown to activate $G_\alpha_q$, resulting in the activation of PM-resident signalling effector, phospholipase C (PLC) and the subsequent generation of inositol triphosphate (IP$_3$) and diacylglycerol (DAG). Protein kinase C (PKC) is also activated, leading to an increase in intracellular $[Ca^{2+}]_{i}$ and activation of PKC-dependent signalling cascades (Abou-Samra et al., 1992). Lastly, the activation of $G_{12/13}/$RhoA-mediated pathways have also been reported (Singh et al., 2005a), as well as $\beta$-arrestin-MAPK-dependent pathways (Gesty-Palmer et al., 2006, Sneddon and Friedman, 2007, Syme et al., 2005). Even though
there are multiple G protein-dependent pathways to be activated, which may contribute to skeletal homeostasis (Guo et al., 2010b), the Ga/cAMP signalling cascade remains as the major pathway modulating the anabolic actions on bone and phosphate reabsorption in the kidney (Guo et al., 2002, Bringhurst et al., 1993, Schipani et al., 1995).

The cell generally safeguards itself from prolonged receptor signalling by inducing the endocytosis of activated surface receptors. This remains true for PTHR, where the ‘canonical’ signalling of PTHR elicited at the cell surface is terminated following the recruitment of β-arrestin to the phosphorylated receptor tail and the subsequent internalisation of the activated receptor into early endosomes (Smith and Rajagopal, 2016). However, PTHR is amongst a growing list of GPCRs known to elicit ‘non-canonical’ signalling events from within the endosomal compartment, as shown through a combination of techniques including live cell confocal microscopy, Förster-resonance energy transfer (FRET), single molecular electron microscopy and the use of nanobodies (Calebiro et al., 2009, Feinstein et al., 2011, Feinstein et al., 2013, Ferrandon et al., 2009, Irannejad et al., 2013, Thomsen et al., 2016). These altogether provide evidence for the sustained production of cAMP following the endosomal entry of PTHR following activation by PTH but not PTHrP, as the latter rapidly dissociates from the receptor at the cell surface without inducing endocytosis (Dean et al., 2006, Dean et al., 2008a, Ferrandon et al., 2009). While the mechanism(s) involved in the termination of ‘non-canonical’ PTHR signalling are relatively less established, they are thought to involve ligand-receptor dissociation, acidification of the endosomal compartment and the retrieval of the receptor away from the endosome (Feinstein et al., 2011, Gidon et al., 2014). As the distinction between ‘canonical’ and ‘non-canonical’ PTHR signalling had only been appreciate in the past few years, the relevance of the two arms of PTHR signalling in skeletal homeostasis and regulation have yet to be determined.

As previously mentioned, the paradoxical actions of PTH on the skeleton are dependent upon the duration of ligand exposure. Though the intermittent
administration of teriparatide (PTH(1-34)) has been shown to stimulate both bone resorption and formation, it ultimately results in a net anabolic effect on bone and a reduced risk of fracture in post-menopausal women and elderly men (Neer et al., 2001, Hodsman et al., 2006, Cosman, 2006, Thomas, 2006). The anabolic action of PTH is mainly the result of enhancing bone formation in existing BMUs by promoting osteoblast recruitment, differentiation and function and survival (Jilka, 2007, Jilka et al., 1999), while also inhibiting Wnt antagonist, SOST (Keller and Kneissel, 2005). These mechanisms of action are in stark contrast to the effects following long-term bisphosphonate therapy, which solely acts to inhibit bone resorption, leading to the accrual of poor quality bone.

Generally, PTH promotes the differentiation of committed osteoblasts without having much effect on the proliferation of uncommitted osteoprogenitor cells (Qin et al., 2005, Knopp et al., 2005, Datta et al., 2005, Datta et al., 2007, Wang et al., 2007b). This is marked with an upregulation of osteoblast transcription factors and extracellular matrix proteins, including ALP, RUNX2, OSX, OCN, OPN and collagen type I (Locklin et al., 2003, Ishizuya et al., 1997, Krishnan et al., 2003, Noda and Rodan, 1989). Additionally, the anabolic action of PTH is also greatly dependent upon its ability to suppress the expression of Wnt antagonists, SOST, DKK and sFRP1 (Kulkarni et al., 2005, Guo et al., 2010a). Intermittent PTH was also shown to promote osteoblast survival in a cAMP/PKA-dependent manner by increasing the expression of the survival gene, Bcl2 and decreasing the pro-apoptotic gene, Bad (Bellido et al., 2003, Amling et al., 1997, Yamamoto et al., 2007).

Interestingly, the cross-talk between PTH-mediated signalling and TGFβ/BMP signalling pathways has been shown to contribute to its anabolic effect on the skeleton. In addition to its influence on SOST expression, activated PTHR has also been shown to induce the internalisation of the resulting PTHR/LRP6 complex, resulting in enhanced BMP signalling (Yu et al., 2012). Complementing this, it was recently reported that PTH-null mice exhibited the reduced expression of BMPR2 during fracture healing that was dependent on cAMP/PKA/CREB
signalling, while also promoting RUNX2 expression via BMP-dependent signalling (Zhou et al., 2017). PTH has also been shown to promote the production of both BMP2/4 ligands (Khan et al., 2016).

In contrast to all this, the prolonged or continuous exposure to PTH (such as in the case of primary hyperparathyroidism) induces a net catabolic effect or loss of bone by disproportionately stimulating osteoclast resorption above that of bone formation while also subsequently resulting in hypercalcemia and hypophosphatemia. Though continuous PTH was shown to inhibit OSX expression in osteoblasts in a cAMP-dependent manner (Hong et al., 2009, Barbuto and Mitchell, 2013), it is more known for its indirect role in regulating osteoclastogenesis. Here, RANKL expression is increased in osteoblasts and osteocytes in a cAMP/PKA/CREB-dependent manner (O'Brien et al., 2008, Saini et al., 2013, Ma et al., 2001, Huang et al., 2004) while concomitantly decreasing OPG expression in a PKA/CREB/AP-1-dependent manner (Fu et al., 2002, Kondo et al., 2002). In addition to this, levels of monocyte chemoattractant protein-1 (MCP-1), a chemokine that promotes the migration of monocytes/macrophages to the site of interest, is continually elevated as a result of continuous PTH exposure as opposed to its transient increase during intermittent PTH stimulation (Li et al., 2007).

1.4.3. Genetically-modified animal models highlight PTHR signalling in skeletal development and postnatal homeostasis

Despite sharing a common receptor, the generation of a series of mice models comprehensively established that PTHrP, and not PTH played a critical role in skeletal development. Mice generated with the global depletion of PTHrP ($\text{Pthlh}^{-/-}$ null mice) died at birth from asphyxia due to the severe abnormalities in skeletal development. These mice presented with a severe phenotype of dwarfism where their long bones exhibited a severe reduction in chondrocyte proliferation, accelerated chondrocyte hypertrophy and matrix mineralisation and an increase in bone mass, cortical thickness and osteoblast numbers (Amizuka et al., 1994, Karaplis et al., 1994, Miao et al., 2002). Likewise, transgenic mice overexpressing PTHrP
specifically in chondrocytes (through the use of the collagen type II promoter) exhibited a predominantly cartilaginous skeleton due to substantial delays in chondrocyte differentiation and subsequent endochondral ossification (Weir et al., 1996). These early models revealed the foundational role of PTHrP in promoting chondrocyte proliferation while also delaying chondrocyte maturation and hypertrophy. This role of PTHrP in the developing bone was then further highlighted through an extraordinary chimeric mouse model generated by Chung and colleagues (Chung et al., 1998). Here, they were able to generate a series of chimeric mice expressing both $\text{PTH1R}^{+/+}$ and $\text{PTH1R}^{-/-}$ chondrocytes within the same growth plate. As a result, PTHrP was able to exert its action to promote proliferation and delay hypertrophy on $\text{PTH1R}^{+/+}$ chondrocytes, while adjacent $\text{PTH1R}^{-/-}$ chondrocytes continued to differentiate into hypertrophic chondrocytes due to their inability to respond to PTHrP.

In addition to this, it was later discovered that PTHrP worked in concert with Ihh to regulate chondrocyte differentiation in a negative feedback mechanism. The skeletal phenotype of $\text{Ihh}$-null mice was more severe than that of $\text{Pthlh}$-null mice, where pups died mid-gestation and exhibited severe dwarfism, ectopic cartilage mineralisation and abnormal endochondral ossification (St-Jacques et al., 1999). Similar to $\text{Pthlh}$-null mice, $\text{Ihh}$-null mice were also found to have a decrease in chondrocyte proliferation, accelerated hypertrophy and a complete absence of PTHrP, whereas the overexpression of Ihh consistently resulted in an increase in chondrocyte proliferation and delayed hypertrophy (St-Jacques et al., 1999). Together with the observations of double $\text{Ihh}^{-/-}/\text{Pthlh}^{-/-}$ mice, it was then confirmed that PTHrP functioned downstream of Ihh (Vortkamp et al., 1996, Karp et al., 2000). Interestingly, when $\text{Ihh}$-null mice were crossed with those expressing a constitutively active form of PTHR (as occurs in patients with Jansen’s metaphyseal chondrodysplasia; caPTHR), the premature maturation of chondrocytes, but not anomalies in proliferation, were able to be reversed, indicating that the mechanisms regulating these events are distinct (Karp et al., 2000). It is now established that the production of Ihh by maturing (pre-hypertrophic and hypertrophic chondrocytes)
induces PTHrP production by periarticular cells. PTHrP then acts on proliferating and early maturing chondrocytes that begin to express increasing levels of PTHR, to promote proliferation and delay hypertrophy. However, as these chondrocytes continue to proliferate and distance themselves from the source of PTHrP, PTHrP is no longer able to exert its actions on these cells and therefore undergo chondrocyte hypertrophy. The PTHrP/Ihh feedback mechanism therefore aims to spatially regulate chondrocyte proliferation and differentiation to enable the longitudinal growth of endochondral bones in a regulated manner (Kronenberg and Chung, 2001).

Studies involving the deletion or constitutive activation of PTHR provided additional insight into the combined efforts of PTH and PTHrP on the prenatal and postnatal skeleton. Firstly, the skeletal phenotype of PTH1R-null mice was more severe than that of Pthlh-null mice, where pups died mid-gestation and were marked with a predominantly mineralised skeleton (Lanske et al., 1996). Despite this, the similarities between the two models were indicative that PTHrP mediates its role in prenatal skeletal development through the actions of PTHR. In contrast, PTH-null mice were viable at birth and exhibited a milder skeletal phenotype, marked by a reduction in cartilage mineralisation and trabecular bone formation. Moreover, the skeletal phenotype of PTH+/Pthlh+/− mice was more severe than either of their single knock-out counterparts and therefore indicates that PTH does indeed play a role in skeletal development, albeit to a lesser degree than PTHrP (Miao et al., 2002). This also indicates that though PTH and PTHrP share a common receptor, the discrepancies between these mouse models suggests that PTH and PTHrP induce different downstream signalling cascades that may lie in (i) distinctions in ligand recognition, (ii) the presence of functional domains present in PTHrP and not in PTH, or (iii) an unidentified receptor that is capable of binding to either ligand (Martin, 2016, Evenepoel et al., 2016).

While it is clear that PTHrP (and to a lesser extent, PTH) play key roles in modulating the development of the prenatal skeleton, several mice models have also highlighted their roles in postnatally and in mineral ion homeostasis. Firstly, though
Pthlh<sup>−/−</sup> mice died at birth, their heterozygous littermates were viable without presenting any gross skeletal abnormalities. By 12-weeks of age however, Pthlh<sup>+/−</sup> mice were found to be haploinsufficient where they were found with decreased bone mass and trabecular thickness with decreased and disorganised bone formation (Amizuka et al., 1996). Furthermore, the deletion of Pthlh specifically in osteoblasts also generated viable pups with a skeletal phenotype that recapitulated the decrease in bone formation in Pthlh<sup>−/−</sup> mice (Miao et al., 2005). In both mice, osteoclast formation was also found to be reduced compared to their wild-type littermates, altogether confirming that PTHrP also plays a physiological role in bone remodelling (Martin, 2016). This is further emphasised through the observational differences in PTH<sup>−/−</sup> and PTH<sup>−/−</sup>/Pthlh<sup>−/−</sup> mice, where following bone marrow ablation (a method used to visualise the events of bone formation prior to bone resorption), bone formation, and osteoblast and osteoclast numbers were decreased in latter compared to PTH-null mice. This further supports the role of PTHrP in the recruitment of osteoblast and osteoclast progenitor cells to the site of remodelling (Zhu et al., 2013).

In addition to this, consistent with the role of PTH in regulating calcium and phosphate homeostasis, PTH<sup>−/−</sup> null mice presented with hypocalcemia and hyperphosphatemia while Pthlh-null mice did not, further distinguishing the roles in mineral ion homeostasis between the two endogenous ligands (Miao et al., 2002).

On the other hand, the generation of mice expressing caPTHr specifically in osteoblasts (Ono et al., 2012) and osteocytes (O'Brien et al., 2008) also provided some insight into the role of PTHR in skeletal homeostasis. In both cases, these mice presented with an increase in bone volume ratio, osteoblast and osteoclast number while also expressing an increase in Wnt ligands with a decrease in SOST (O'Brien et al., 2008, Ono et al., 2012). These results are consistent with the role of PTHR-mediated signalling in promoting the recruitment, proliferation and differentiation of osteoblast and osteoclast precursors to modulate coordinated bone formation and remodelling. In addition to this, the chondrocyte-specific deletion of PTHR in postnatal mice further showed the role of PTHR-mediated signalling in the maintenance of the growth plate, in particular, that PTHR activation is required to
continually sustain chondrocyte proliferation and prevent its premature closure (Hirai et al., 2011).

Lastly, mice homozygous for the deletion of Gaα in chondrocytes presented with some similarities with Pthlh-null mice, (Sakamoto et al., 2005). Mice generated with the deletion of Gaα deletion specifically in osteoblasts presented with a reduced number of mesenchymal progenitor cells and increased adipogenesis within the bone marrow (Sinha et al., 2014), whereas those with Gaα depletion in committed osteoblasts (through the use of OSX promoter) exhibited reduced osteoblast numbers due to the enhanced differentiation of osteoblasts as well as an increased expression of Wnt antagonists, DKK1 and SOST (Wu et al., 2011). These altogether support that the Gaα-cAMP-mediated role remains as the main mediator of both PTH and PTHrP actions in the growth plate and bone.

1.4.4. Human cases of PTHR-related disease

The first of the most severe cases of human skeletal dysplasia related to mutations in PTH1R is Blomstrand’s lethal chondrodysplasia and is the result from a mutation rendering the receptor non-functional (Jobert et al., 1998, Blomstrand et al., 1985, Karaplis et al., 1998, Zhang et al., 1998, Wysolmerski et al., 2001). Similar to the mice models previously described, these individuals died prenatally and were marked with dwarfism, advanced endochondral bone formation, lack of tooth eruption and mammary gland development. Conversely, mutations causing the constitutive (ligand-independent) activation of PTHR have been shown to result in Jansen’s metaphyseal chondrodysplasia (JMC), where the H223R mutation in particular has been used in the investigation of PTHR activation in the mouse models previously described. Individuals affected by JMC are characterised with short-limbed dwarfism and abnormal bone formation as well as hypercalcemia, hypophosphatemia and increased urinary excretion of cAMP (Parfitt et al., 1996, Kruse and Schutz, 1993, Schipani et al., 1997a, Schipani et al., 1995, Schipani et al., 1996). In addition to this, Eiken syndrome also results in the truncation of most of the cytoplasmic tail of PTHR encoding the region responsible for modulating
receptor signalling and endocytosis. The skeletal manifestations exhibited by the affected siblings were in fact distinct from both Blomstrand’s lethal chondrodysplasia and JMC, where they presented with severely delayed and disturbed bone ossification mainly affecting the hands and feet, and normal serum calcium and phosphate levels (Duchatelet et al., 2005). Although the occurrence in all of these diseases are extremely rare (Schipani and Provot, 2003) with no current treatment for the loss-of-function PTH1R mutations, there have been some encouraging results in the development of inverse agonists that are capable of binding to PTHR without eliciting downstream signalling cascades, which may become potential therapeutic agents in the treatment of the excessive activation of PTHR such as that of JMC (Cheloha et al., 2016). In addition to these severely debilitating diseases, there are other PTHR-related diseases that are of relatively lesser severity. These include enchondromatosis (including Ollier’s disease) which marked with the sporadic formation of ectopic cartilaginous tumours within the bone adjacent to the growth plate, and familial primary failure of tooth eruption, both of which have been attributed to mutations within PTH1R (Silve and Juppner, 2006, Couvineau et al., 2008, Decker et al., 2008).

In contrast, human diseases may also occur when ligands of PTHR are secreted in abnormal amounts. For instance, the manifestations of hypercalcemia, hypophosphatemia and the decrease in bone mass caused by the over-secretion of PTH as in the case of primary hyperparathyroidism are generally reversed following the parathyroidectomy (Fraser, 2009, Lewiecki and Miller, 2013, Bandeira et al., 2014). Moreover, the secretion of PTHrP in cases of humoral hypercalcemia of malignancy results in secondary hypercalcemia and increased bone resorption (Stewart, 2005). Due to the progressive stage of the disease, and the lack of an antibody directed toward PTHrP, the therapies currently used mainly target osteoclast resorption through the use of bisphosphonates or RANKL-directed antibody therapy.
In contrast to these cases, hypoparathyroidism results from the abnormally low secretion of PTH and commonly occurs following parathyroidectomy or in cases where mutations occur within the calcium sensing receptor (CaSR, of which acts to modulate PTH secretion from the parathyroid glands) (Shoback, 2008). As expected, these individuals present with hypocalcemia and the current therapy (besides calcium and phosphate supplements) is currently the administration of full-length PTH hormone (also known as Natpara®) (Mannstadt et al., 2013, Marcucci et al., 2016). In addition to this, there have also been several developments in the generation of long-acting PTH analogues (LA-PTH) that are able to activate PTHR without inducing the detrimental effects that are seen with continuous PTH exposure (Shimizu et al., 2016).

1.4.5. **Summary**

Overall, skeletal growth and homeostasis throughout postnatal life is dependent upon the coordinated activities between all skeletal-resident cell populations. Among the several signalling pathways that act to translate extracellular stimuli into cellular outcomes, PTHR-mediated signalling has been shown to be crucial during both skeletal development and currently remains as the only osteoanabolic therapy in the treatment for osteoporosis. Therefore, the efficacy of these therapies may be improved by further understanding the molecular mechanisms governing PTHR signalling.
CHAPTER

-2-

Membrane and protein trafficking
2. Membrane and protein trafficking

2.1. An introduction to membrane and protein trafficking

The cell is the fundamental unit of life, capable of both independent existence as a prokaryote or as part of a larger system forming tissues and organs within the eukaryotic organism. With the exception of the plasma membrane (PM), a distinguishing feature of eukaryotic cells is the formation of intracellular compartments, or organelles, through the use of biological membranes. This allows for the formation of microenvironments specialised for unique cellular functions and events, including protein synthesis (largely occurring within the endoplasmic reticulum, ER), modification or folding (often within the Golgi apparatus) and degradation (commonly through the lysosomal compartments). Although biological membranes are predominantly identified in the form of a phospholipid bilayer, they are also decorated with both integral (embedded) and peripheral proteins (Cooper, 2000) that participate in a variety of functions. Additionally, several of these membrane proteins also act as identity markers for the membranous compartment they reside on, including the well-characterised family of small Rab GTPases. Together with the skeletal network of the cell (composed of actin filaments and microtubules), these various components collectively serve to structurally organise the subcellular architecture of the cell. The phospholipid bilayer is inherently fluid in nature and allows for the lateral diffusion of proteins and lipids across the membrane, while not permitting the passive entry (or exit) of most molecules through the membrane (Nicolson, 2014, Edidin, 2003, Singer, 1974). Therefore, another evolutionary adaptation distinguishing eukaryotes from prokaryotes is the presence of a sophisticated intracellular transport apparatus, involving elaborate molecular machineries that facilitate the active and directed transport of cargo between organelles. It is therefore not surprising that the mechanisms governing cargo transport centralise around the use of biological membranes and their vesicular transport intermediaries (e.g. endosomes). The bi-directional flow and exchange of
membranes and cargo proteins within the living cell collectively constitute the fundamentals of membrane trafficking.

The concept of membrane trafficking was long assumed for several decades, however it was not until the pioneering studies led by Caro, Palade and Jamieson in the 1960’s-70’s that visually captured the export of cargo from pancreatic cells through the use of electron microscopy (Caro and Palade, 1964, Jamieson and Palade, 1967). Since then, the field of membrane trafficking now constitutes the characterisation of a vast library of transport machinery and mechanisms that participate across a variety of essential events in cellular function and homeostasis. For simplicity, this multifaceted system may be separated into different trafficking pathways, including the biosynthetic, endocytic, recycling and degradative pathways (Figure 2.1). These pathways however, are not mutually exclusive and cargo often converge at common organelles prior to reaching their final destination. The mechanisms governing the transport of cargo between organelles may be further simplified into a generalised sequence of events, where a specialised set of protein machinery facilitate each process (De Matteis and Luini, 2011) (Figure 2.2). Firstly, the cargo to be transported is recruited to the (donor) membrane through a direct interaction with the membrane or through the use of adaptor proteins, followed by the budding of the localised membrane (Figure 2.2A). The subsequent scission of this region gives rise to a relatively smaller carrier vesicle, where vesicular translocation is facilitated through the action of ATP-driven motor proteins (involving myosin, kinesin and/or dynein) along actin (short distances) or microtubule (long distances) -based networks (Figure 2.2B). Upon arrival, these carrier vesicles then tether and dock to the accepting membrane to which it then fuses with and thus, finalises cargo delivery (Figure 2.2C). While not emphasised in this illustration, there are numerous protein complexes that facilitate each process to ensure specificity and fidelity of cargo transport, as well as maintaining the membrane dynamics required. For example, the failure to transport cargo from the ER to the Golgi may induce an ER stress response within the cell and ultimately result in an overall deficiency in cargo delivery or export (Frakes and Dillin, 2017,
Figure 2.1.
Figure 2.1. Membrane trafficking at a glance.

Shown is a simplified illustration of the main trafficking pathways within a mammalian cell. Endocytic events (green arrow) occur at the plasma membrane (PM), where they generate primary endosomes which then undergo homotypic fusion to form the early endosome. EEA-1 and Rab5 are used as typical markers for the early endosome, where FYVE-containing proteins are additionally used as they bind to phosphatidylinositol 3-monophosphate (PI(3)P) lipids enriched across the endosomal membrane. Recycling of cargo (orange arrows) may occur from early endosomes in a Rab4-dependent manner, or following entry into the endosomal recycling compartment (ERC) in a Rab4- or Rab11-dependent manner. Additionally, cargoes may enter the Golgi apparatus in a retrograde manner (purple arrow), where TGN38 is commonly used as a trans-Golgi network marker, or GM130 as a cis-Golgi marker. Most cargoes destined for lysosomal degradation are retained within the early endosome as it eventually matures into the late endosome. The presence of PI3P is exchanged for PI(3,5)P$^2$ on the late endosomal membrane and acquires several markers including Rab7 and CD63, and upon fusion with lysosomes, Lamp1. Mucolipin 1 (ML1N) may be additionally used as a probe for PI(3,5)P$_2$ membranes to identify late endosomes. These organelles are often visualised to possess several intraluminal vesicles (ILVs) and are thus commonly referred to as multivesicular bodies (MVBs) or endosomes (MVEs). Biosynthesis of proteins usually occurs within the endoplasmic reticulum and passes through the Golgi for post-translational folding and modifications, gradually reaches its final destination (e.g. the cell surface) (blue arrows).

Adapted from De Matteis, 2011.
Figure 2.2. General steps involved in cargo transport via membrane carrier vesicles.
Cargoes integrated within the membrane (blue) or within the lumen are retrieved to a localised area where membrane budding is induced (A). Membrane scission then occurs (B), resulting in the formation of small carrier vesicles that translocate to their next destination with the assistance of motor proteins that track along actin- or microtubule-based networks. These vesicles are then tethered to their accepting membrane (C) where they then dock and fuse to the membrane.

*Adapted from De Matteis, 2011.*
Healy et al., 2012). Such is the case in individuals diagnosed with autosomal recessive forms of osteogenesis imperfecta, where mutations present within collagen type I chaperone proteins impair the export of collagen from the cell into the extracellular matrix (Forlino and Marini, 2016). In fact, the undesired failure to retrieve cargo from any organelle (such as in the failure of cargo recycling from endosomes) will inevitably affect its homeostatic expression and/or function and could lead to perturbations in overall cellular homeostasis.

As such, the transport of intracellular cargo must be strictly regulated in order to maintain cell function and homeostasis and unsurprisingly involves the coordinative effort of approximately 2,000 proteins (Gilchrist et al., 2006), or equivalent to 7% of the entire human proteome (Kim et al., 2014). This extensive library incorporates specialised proteins whose function involves, but are not limited to, cargo recognition and retrieval, membrane deformation and tubulation as well as those that participate in the architectural organisation of the endomembrane system. While only a small percentage of proteins involved in membrane trafficking have been thoroughly characterised, there have been several key protein families identified as crucial regulators, including the highly recognised family of Rab GTPases as one of the ‘master regulators’ of membrane trafficking. There are over 60 Rab proteins identified within the mammalian proteome, where they localise to distinct microdomains across several organelles throughout the endomembrane system (Sonnichsen et al., 2000). As such, Rab proteins are often utilised as markers of organelle identity as their localisation often correlates to their regulatory function (comprehensively reviewed in (Zerial and McBride, 2001, Stenmark, 2009)). For example, Rab5 is a specific resident on early endosomes and plays crucial roles in coordinating homotypic endosome-endosome fusion (Gorvel et al., 1991). By comparison, Rab7 is a common marker for late endosomes where it assists the maturation of Rab5-positive early endosomes into late endosomes (Bucci et al., 2000); the gradual acquisition of Rab7 and loss of Rab5 is a phenomenon referred to as ‘Rab conversion’ (Rink et al., 2005). In contrast, both Rab4 and Rab11 are commonly found on the membranes of smaller, recycling endosomes and thus often
co-localise with cargo proteins during recycling events (Sonnichsen et al., 2000). In more recent years, the protein family of Sorting Nexins (SNXs) has joined the Rab family as another family of crucial regulators in cargo transport, where SNX27 in particular, is the focus of this dissertation and is discussed in further detail in Section 2.2.

While the immense number of proteins regulating membrane trafficking permit for a certain level of plasticity and redundancy, an increasing number of Mendelian diseases have been attributed to mutations in trafficking proteins, or within the cargo protein itself, either of which results in disturbances in cargo transport (De Matteis and Luini, 2011). Unsurprisingly, mutations encoding ubiquitously-expressing or essential proteins (e.g. Rab proteins, and more recently, SNX proteins) often result in severe developmental impairments of which are prominent in cell types that rely heavily on protein secretion or export, such as neuronal and secretory cells (De Matteis and Luini, 2011, Schwarz et al., 2002, Cai et al., 2011, Im et al., 2013). Of relevance to bone, a considerable subset of skeletal dysplasias have been linked to the failure of cargo synthesis, transport and/or secretion, including the previously mentioned case of recessive forms of osteogenesis imperfecta, where collagen secretion is impaired due to mutations within the chaperone proteins Serpin H1 (\textit{SERPINH1}) and FKBP10 (\textit{FKBP10}), resulting in the appearance of ‘bowed’ lower limbs, a barrel chest and vertebral compressions (Schwarze et al., 2013, Forlino and Marini, 2016). In addition to this, spondyloepiphysyal dysplasia tarda (SEDT) shares similar characteristics where the transport of procollagen from the ER to Golgi is impaired within the chondrocyte (Gedeon et al., 1999, Tiller et al., 2001, Venditti et al., 2012). Lastly, there are also several skeletal diseases affecting the osteoclast, including osteopetrosis and pycnodysostosis where mutations affect the function of the vacuolar ATPase (v-ATPase) proton pump (Kornak, 2000, Lee, 2006, Frattini et al., 2000) and/or secretion of cathepsin K (Gelb et al., 1996, Saftig, 1998, Gowen, 1999), respectively. Therefore, understudying the molecular mechanisms governing intracellular
trafficking in skeletal heath and disease may open up new avenues to treat skeletal disorders.

2.1.1. Interacting through the plasma membrane

Aforementioned, a common feature in both prokaryotic and eukaryotic organisms is the presence of the PM which functions to physically separate the cytosolic components of the cell from its extracellular environment. Like all biological membranes, the PM is also equipped with a surface complement of proteins and macromolecules which enables it to continually sense and respond to the dynamic environment and external stimuli, the latter of which is often present in the form of ions, hormones, cytokines and other soluble ligands (Lodish et al., 2000a, Cooper, 2000). Thus, the PM can be viewed as the primary ‘interactive interface’ and communication platform between the cell and its environment. Of these, a range of transmembrane spanning surface receptors play fundamental roles in translating external cues into biochemical signalling cascades that lead to changes in cellular state and/or function.

The PM is a highly dynamic organelle and possesses the highest level of membrane turnover relative to other organelles and internalises between one to five rounds of its entirety, per hour (Steinman et al., 1983). Under these steady-state conditions, the PM undergoes a continual yet balanced removal and replenishment of its surface proteins and lipids (Alberts et al., 2002). In contrast, the receipt of signalling cues by its cognate surface receptor often induces the prompt internalisation of the activated receptor complex and leads to a further increase in the rate of membrane turnover (Sorkin and von Zastrow, 2009). Furthermore, this is a common yet crucial mechanism that aims to terminate receptor signalling by physically removing the surface receptor from the vicinity of PM-resident signalling effectors (Irannejad et al., 2015). While this remains true for most receptors, it has recently been challenged by a subset of G-protein coupled receptors (GPCRs), where in addition to surface signalling, entry into the early endosome is required for the induction of distinct signalling pathways and functional outcomes. This has therefore
given rise to the concept of the ‘signalling endosome’, where the receptor signalling remains active from within the endosomal compartment (von Zastrow and Sorkin, 2007, Sorkin and Von Zastrow, 2002, Pavlos and Friedman, 2017).

2.1.2. Endocytosis

Endocytosis is the process utilised by the cell to internalise localised regions of the PM and its surface complement of proteins and lipids. The term ‘endocytosis’ is often used as a generalisation for small-scaled events but it also encompasses several distinct mechanisms that have been traditionally categorised based on the use of the molecular scaffold, clathrin, as clathrin-mediated (CME) or clathrin-independent endocytosis (CIE), or through the use of flask-shaped pits (caveolae) in caveolae-mediated endocytosis (CavME). These mechanisms mediate the endocytic process of distinct targets where for example, many signalling receptors such as transferrin receptor (TfR), β2AR and PTHR undergo CME (Hanyaloglu and von Zastrow, 2008) while caveolae in CavME are lipid-rich, incorporating caveolin (specialised form of lipid raft), sphingolipids and cholesterol (Mayor and Pagano, 2007). There are also other distinctions in the pit formation and machinery involved, such as the typical involvement of large GTPase dynamin in the scission of clathrin-coated pits and larger endocytic processes (including macropinocytosis and phagocytosis) that are modulated independent of clathrin but require the polymerisation of actin (Mayor et al., 2014) and are further elaborated in Elkin, 2016.

Endocytic events at the PM involve the inward budding of the PM and following scission, result in the formation of small membrane-delimited vesicles referred to as primary endosomes. These then undergo repeated rounds of homotypic fusion to eventually form an early endosome (EE) (Salzman and Maxfield, 1988). Like any other membranous compartment, early endosomes are highly pleomorphic organelles consisting of a mosaic distribution of proteins and lipids across its surface, forming discrete sub-regional microdomains that mediate the recruitment and engagement of specific sorting and trafficking machinery (Gruenberg, 2001). In particular, early endosomes are enriched in phosphatidylinositol 3-monophosphate
(PI3P), to which several proteins (and by extension, their effector molecules) are recruited through action of PI3P-interacting domains, including Fab1/YOTB/Vac1/EEA1 (FYVE) and Phox homology (PX) domains, the latter an intrinsic feature of the Sorting Nexin (SNX) family of endocytic proteins (Section 2.2.) which are the focus of this investigation. (Lemmon, 2003, Balla, 2005, Gillooly et al., 2000, Klumperman and Raposo, 2014).

2.1.3. Receptor recycling

While endocytosis regulates several physiological processes of the cell by regulating the surface integration of membrane ion channels and glucose transporters, receptor-mediated endocytosis is perhaps one of the best understood forms of endocytosis. Upon the receipt of an appropriate stimulus, cell surface receptors are readily internalised and enter the early endosome, where they are then sorted for their next destination; either returning to the PM via recycling routes and thus resensitising the cell for further stimulation, or entering endolysosomal compartments for degradation and thus downregulating receptor levels (Figure 2.3). Under favourable conditions, many receptors are generally recycled to repopulate the PM and may occur rapidly from the early endosome (rapid or fast recycling) or following a detour to other organelles, including the Golgi apparatus (slow recycling) (Maxfield and McGraw, 2004, Bonifacino and Rojas, 2006, Johannes and Popoff, 2008, Chia et al., 2013). Recycling of receptors from the early endosome, or a similar compartment referred to as the ‘endosomal recycling compartment’ (ERC) commonly occur through the (rapid) Rab4- or (slightly slower) Rab11-dependent pathways (Huotari and Helenius, 2011, Maxfield and McGraw, 2004, Sonnichsen et al., 2000). Being both pleomorphic structures, the exact boundaries distinguishing the early endosome from the ERC remains blurred, where the ERC is thought to originate from both the transitioning body of the early endosome as well as existing as a separate entity (Goldenring, 2015). Additionally, receptors may be retrieved from the early endosome/ERC and transported toward the Golgi in a retrograde
Figure 2.3. Receptor endocytosis, recycling and degradation.

Shown is a simplified model of the sequential transport of surface-resident receptors. Following ligand-induced receptor activation, the cytosolic tail of the receptor is phosphorylated by GRKs where endocytosis is then promptly mediated by the action of β-arrestins. Once receptor-ligand complexes have entered the early endosome, the ligand dissociates and the receptor may be directly recycled to the plasma membrane (Rab4-dependent, green arrows) or detour to the Golgi prior to entering a slower recycling pathway (Rab11-dependent, red arrows). On the other hand, receptors that are not retrieved from the maturing endosomal compartment inevitably undergo lysosomal degradation.
manner, where they may be then directed toward the cell surface in a Rab11-dependent manner (Jones et al., 2006).

There are several characteristics of the endosomal compartment that assist in determining the favoured trafficking route and destination of the receptor in question. Firstly, the dissociation of many receptor-ligand complexes are pH sensitive, where dissociation commonly occurs under mildly acidic conditions upon reaching the early endosome (pH ~6.5) and thus favours the rapid recycling of receptors such as the transferrin receptor (Mellman, 1996, Maxfield and Yamashiro, 1987). Other receptor complexes have been reported to dissociate within the Golgi (pH ~6.0) or in the late endosome (LE) at slightly higher acidic conditions (pH ~5.5) and therefore favours slower recycling routes (Hullin-Matsuda et al., 2014, Rink et al., 2005, Huotari and Helenius, 2011. The pH sensitivity of the receptor-ligand complex also becomes important when considering a small subset of receptors that continually signal from within the endosome. For example, signals elicited from activated EGFR continues within the endosomal compartment until it is eventually degraded in lysosomes (Felder, 1990 #1719, Gorden et al., 1978).

The physical morphology and use of geometric volume-to-surface ratios also contributes to the favourable retrieval of receptors from the endosomal sorting station. As previously mentioned, the early endosome/ERC is characteristically pleomorphic and dynamic in structure where distinct endosomal recycling tubules can be seen to emanate from the surface of its largely spherical body. The spherical portion of the endosomal compartment may measure anywhere between 100-500nm in diameter (Huotari and Helenius, 2011) and its high volume-to-surface ratio maximises ligand dissociation (Maxfield and McGraw, 2004). In contrast, the narrow recycling tubules possess a high surface-to-volume ratio and thus are optimal in the recruitment and clustering of integrated receptors while minimising the loss of endosomal volume (Marsh et al., 1986, Maxfield and McGraw, 2004). The subsequent scission of these tubules later forms smaller transport vesicles which then
translocate to their next destination with the assistance of the actin- or microtubular

As the formation of these tubules is not energetically favourable, it is an
active process that requires highly specialised machinery to modulate membrane
deformation (Kozlov et al., 2014, Compeer and Boes, 2014). Moreover, the ability
to recruit receptors into these tubules for recycling requires the function of adaptor
proteins in cargo recognition and retrieval and is crucial in preventing their
undesirable endosomal persistence and entry into late endosomes/lysosomes. While
some receptors may exit the endosome through (unspecific) bulk membrane flow
(Mayer et al., 1993), most receptors harbour specific molecular motifs that assist in
cargo recognition. These recognition sequences may be encoded within the protein
sequence itself or act through post-translational modifications such as receptor
phosphorylation or ubiquitination (Babst, 2005, Babst, 2011, Hurley and Hanson,
2010, Henne et al., 2013, Raiborg and Stenmark, 2009, Bowman et al., 2016). An
important complex in modulating both endosomal tubule formation and cargo
retrieval is the Actin-Sorting Nexin 27-Retromer Tubule (ASRT) retromer complex,
discussed in detail in Section 2.2.2.

2.1.4. Receptor degradation

Receptor retrieval may occur at any stage during the transition of the early
endosome into the late endosome (Dunn et al., 1989, Pryor and Luzio, 2009) (where
certain receptors are specifically retrieved from these acidic compartments,
including CI-MPR). However, many receptors are not retrieved and are instead
retained within the maturing endosome and subsequently degraded upon heterotypic
fusion with lysosomes (Gruenberg and Maxfield, 1995). Furthermore, while this
may be the canonical pathway for certain receptors (e.g. epidermal growth factor
receptor, EGFR), the unregulated degradation of receptors may arise where there is
a deficiency in the transport machinery that would otherwise modulate receptor
retrieval. Many anomalies amongst the trafficking machinery have in fact been found
to perturb cellular and tissue homeostasis where they underlie a variety of Mendelian diseases (De Matteis and Luini, 2011).

Late endosomes are pre-lysosomal compartments that retain the large, spherical body of early endosomes and measure anywhere between 250-1000nm in diameter (Tooze and Hollinshead, 1992). Unlike early endosomes, these structures are generally concentrated within the perinuclear region of the cell, close to the microtubule organising centre (Matteoni and Kreis, 1987). Late endosomes also express an exclusive complement of surface proteins and lipids and are rich in triglycerides, cholesterol esters and PI(3,5)P\(_2\) (Odorizzi et al., 1998, Gary et al., 1998). Additionally, the maturation process of the early endosome to becoming a late endosome is also characteristically marked by the acquisition of Rab7 and concomitant loss of Rab5; an event described as ‘Rab conversion’ where each are commonly used as a marker for their respective compartments (Rink et al., 2005, Zerial and McBride, 2001, Shin et al., 2005, Christoforidis et al., 1999, Behnia and Munro, 2005, Jean and Kiger, 2012). Late endosomes are also marked with a relatively acidic luminal pH that, in addition to ligand dissociation and protein denaturation, creates an optimal environment for the activation of proteolytic enzymes for cargo degradation (Mellman et al., 1986, Fuchs et al., 1989). This is mediated through the combined effort of v-ATPase proton pumps at the membrane (responsible for secreting \(H^+\) into the lumen) as well as the continual delivery of acidic hydrolases and proteases sourced from the TGN (Bonifacino and Hurley, 2008, Pfeffer, 2009).

Late endosomes are also commonly described as multivesicular bodies (MVBs) or endosomes (MVEs) due to the incorporation of multiple intraluminal vesicles (ILVs) (Raiborg and Stenmark, 2009, Sachse et al., 2002, Maxfield and Yamashiro, 1987). ILVs are formed from the inward budding of the endosomal membrane and is a mechanism for incorporating integrated receptors (especially ubiquitinated proteins) into the lumen for degradation (Piper and Katzmann, 2007). As expected, the membranes and proteins of ILVs are susceptible to lysosomal
hydrolases, while those on the limiting membrane of late endosomes/MVBs remain stable through homotypic and heterotypic (lysosomal) fusion and are therefore used to identify these compartments. These include Rab7, lysosomal integral membrane glycoproteins (Lamp1/2) and CD63 (Pols and Klumperman, 2009, Staudt et al., 2016).

2.2. Sorting Nexins (SNXs) in endocytic protein trafficking and disease

Over the past two decades, the family of Sorting Nexins (SNXs) have emerged as crucial regulators in endocytic trafficking of transmembrane proteins across a variety of cells (Figure 2.4). As previously mentioned, SNXs are defined by the presence of a PX domain sharing >50% homology to the first SNX discovered (SNX1) (Kurten et al., 1996). As of current, the SNX family consists of 34 members (denoted SNX1 to SNX34), making it the largest family of PX domain-containing proteins within the mammalian proteome of which there are a total of 49 proteins thus far (Teasdale and Collins, 2012). The PX domain of most SNX proteins preferentially bind to PI3P present on the membranes of early endosomes, however a few SNXs have also shown the capacity to bind to other PI derivatives, including PI(3,4)P2 and PI(4,5)P2 (Teasdale and Collins, 2012). The differential distribution of certain PI derivatives at distinct organelles is achieved through the selective recruitment of kinases and phosphatases, as well as small Rab GTPases (Stenmark, 2009). Here, PIs collectively assist in the active remodelling of the lipid composition and identity of these membrane delimited organelles, while also clustering to form distinct microdomains that act as recruitment sites for PI-binding proteins that function to induce tubule formation and cargo retrieval.

Due to the ambiguous identification criteria initially proposed, there is a great diversity in protein function due to the fact that most members of the SNX protein family possess additional functional domains to their PX domain. As such, SNXs have been sub-categorised based on their secondary domains, including SNX-
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Figure 2.4.

SNX1
SNX2
SNX3
SNX4
SNX5
SNX6
SNX7
SNX8
SNX9
SNX10
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SNX31
SNX32
SNX33
SNX34

PX
FERM-like
PXA
BAR
Rho-GAP
Kinesin motor
RGS
PDZ
MIT
SH3 1
SH3 2
RUN
Unknown domain
The SNX proteins comprise of the largest subfamily of Phox homology (PX) proteins. The PX domain (orange) preferentially engages with phosphatidylinositol 3-monophosphate (PI(3)P, or PtdIns3P) enriched on the early endosomal membrane. While some SNX proteins solely possess a PX domain (SNX-PX proteins), most SNX members harbour additional secondary domains, including Bis/amphiphysin/Rvs (BAR) domains (SNX-BAR proteins), atypical 4.1/ezrin/radixin/moesin (FERM) domains (SNX-FERM proteins), PX-associated domains (SNX-PXA proteins), Src homology 3 domains (SNX-SH3 proteins), as well as others listed. Proteins and domains are shown to scale. PX, Phox homology; FERM, 4.1/ezrin/radixin/moesin; PXA, PX-associated domain; BAR, Bis/amphiphysin/Rvs; Rho-GAP, Rho-GTPase activating protein; RGS, regulatory of G protein signalling; PDZ, post-synaptic 95/discs large/zonula occludens-1; MIT, microtubule interacting domain; SH3, Src homology 3; RUN, RPIP8/UNC-14/ NESCA.

Adapted from Cullen, 2008 and Carlton et al., 2005.
BAR, SNX-SH3 (Src homology 3) and SNX-FERM (4/1/ezrin/radixin/moesin) to name a few, with SNX-PX referring to those that solely possess a PX domain (Teasdale and Collins, 2012). These additional domains provide these SNX proteins with the ability to simultaneously engage with lipids and/or other proteins and therefore, to function as adaptor molecules in membrane and protein trafficking. For example, SNX5 has been shown to modulate membrane ruffling in macrophages, SNX-BAR-, SNX3- and SNX27-retromer complexes are involved in cargo recognition and recycling (see Section 2.2.2), SNX6, SNX9 and SNX17 in Aβ production (Okada et al., 2010, Schobel et al., 2008, Lee et al., 2008), SNX19 as a chondrogenic factor in osteoarthritis (Kan et al., 2009) and SNX15, SNX16 and SNX24 in mammary, bladder and breast cancers (Osman et al., 2006, Watahiki et al., 2004, Krol et al., 2010, Wright et al., 2009).

2.2.1. Introducing Sorting Nexin 27

Among the Sorting Nexin family, SNX27 has received increasing attention in recent years. It was first identified to be a drug-inducible gene within the rat neocortex and was therefore originally named as methamphetamine responsive transcript 1 (mrt1) (Kajii et al., 2003, Fujiyama et al., 2003). SNX27 as it is now known, is ubiquitously expressed in a range of other tissues beyond that of the brain (Cai et al., 2011, Hayashi et al., 2012). Apart from its PX domain (which preferentially binds to PI3P), SNX27 also possesses two protein-protein interacting domains; an atypical FERM domain within its carboxy-terminal region (which it shares with SNX17 and SNX31) as well as being the only member of its family to possess a post-synaptic-95/Dlg/ZO-1 (PDZ) domain (Figure 2.5A). The accumulation of studies over the years have now established SNX27 as an adaptor protein involved in modulating the surface expression and endosomal recycling of a variety of cargoes.
Figure 2.5.

A. ASRT-Complex

B. PI3P-enriched endosomal membrane

Chapter 2 - Membrane and protein trafficking
Figure 2.5. Actin-Sorting Nexin 27-Retromer-Tubule Complex.

(A) The Actin-Sorting Nexin 27-Retromer-Tubule (ASRT) complex comprises of SNX27, retromer (VPS26, 29 and 35) bound to the Wiskott and Aldrich and SCAR homologue (WASH) complex. The WASH complex assists in the formation of actin-rich microdomains at the surface of the early endosome and recycling tubules, which facilitates to concentrate cargo to be recycled via these tubules.

(B) SNX27 engages with PI3P lipids at the endosomal membrane via its PX domain, while simultaneously engaging both retromer (VPS26) and PDZbm-containing cargo via its PDZ domain. The atypical FERM domain of SNX27 is also able to engage with cargo possessing N-P-x-Y/N-x-x-Y motifs, where x represents any residue.

Residues shown; N = Asn or asparagine; P = Pro or proline, Y = Tyr or tyrosine.

Adapted from Ghai et al., 2013; Lee et al., 2016
2.2.1.1. The atypical FERM domain

The atypical FERM domain of SNX27 is comprised of three modules (F1, F2 and F3), where F1 and F3 share structural similarities with ubiquitin- and phosphotyrosine-binding domains, respectively (Ghai et al., 2011). The slight truncation and therefore tertiary structure of the F2 module renders this domain ‘atypical’ from canonical form, however it does not impact the functional capacity for this domain to engage with its target proteins (Ghai et al., 2015). Here, the SNX27-FERM domain is able to recognise short sorting sequences conforming to Asn-Pro-Xaa-Try (N-P-\(x\)-Y) or N-\(x\)-x-x-Y motifs, where \(x\) represents any residue. Of the three SNX-FERM proteins (SNX17, SNX27 and SNX31), the FERM-mediated engagements of SNX17 remains as the best characterised and has since been shown to modulate the endosomal recycling of amyloid precursor protein (APP) (Lee et al., 2008), low-density lipoprotein receptors (LDLR) (Burden et al., 2004), LRP1 (Stockinger et al., 2002, van Kerkhof et al., 2005) and \(\beta\)-integrins (Bottcher et al., 2012, Steinberg et al., 2012). While the FERM domains of both SNX17 and SNX27 share comparable affinities for N-P-\(x\)-Y/N-\(x\)-x-Y motifs, SNX27 has been predominantly recognised to engage with proteins via its PDZ domain (see next section).

The F1 domain of SNX27 was initially thought to act as a ‘stand-alone’ Ras-association (RA) domain (Balana et al., 2013) however it was later found to be a component of the larger SNX27-FERM domain (Ghai et al., 2011). Belonging to the same Ras superfamily as small Rab-GTPases, Ras-GTPases are active when bound to GTP during which they are then able to engage with downstream effectors, including a variety of protein and lipid kinases (Wennerberg et al., 2005). As such, the role of SNX-FERM proteins may promote the formation of a ‘modular recruitment platform’ by coupling its capacity to sort and recruit cargo while simultaneously engaging with signalling molecules at the endosomal membrane. By extension, these SNX-FERM proteins may therefore play active roles in the dynamic modelling of the protein and lipid membrane composition in a spatiotemporal manner (Ghai and Collins, 2011, Ghai et al., 2011).
More recently, a recent report by Ghai and colleagues have described the exclusive lipid-binding property of the SNX27-FERM domain to bi- and tri-phosphorylated derivatives of PIs at the PM (Ghai et al., 2015). Under steady-state conditions, the PX domain of SNX27 is known to recruit to PI3P-enriched endosomal membranes. However, during the induction of cell polarisation (using the formation of an immunological synapse between (Jurkat) T cells and antigen presenting cells as a model), the FERM domain was found as a necessary component for the recruitment of SNX27 from endosomes to the cell surface and mediated its engagement with PI(4,5)P$_2$ (i.e. PIP2), PI(3,4,5P$_3$) (i.e. PIP3) and PI(3,4)P$_2$. As PIs are known to function as both biochemical markers on membranes as well as participate in key cell surface signalling events, this dynamic partitioning (i.e. surface-bound and endosomal-bound SNX27) and its active recruitment from endosomes further endorses the ability of SNX27 to merge endosomal trafficking with intracellular signalling (Ghai et al., 2015).

2.2.1.2. The PDZ domain

As mentioned, PDZ domains are one of the most common protein-protein interacting domains within the mammalian proteome and are central to governing intracellular signalling events (Nourry et al., 2003). These include playing crucial roles in cell polarity, intracellular organisation and structure, the clustering and trafficking of a variety of receptors and ion channels while also consolidating signalling complexes at specific membrane domains (Ye and Zhang, 2013). The majority of protein interactions of SNX27 have been shown to occur through its PDZ domain relative to its FERM domain, where SNX27 engages with target proteins harbouring a carboxy-terminal type I PDZbm as $x$-[S/T]-x-$\phi$, where $\phi$ represents any hydrophobic residue and $x$ as any residue (Steinberg et al., 2013, Balana et al., 2011). In addition to this, it is now known that the binding affinities in PDZ-mediated interactions may be additionally influenced by residues upstream to the canonical four terminal residues, up to position (-7), where position (0) represents the terminal residue (Birrane et al., 2003) as well as the phosphorylation of either the PDZ domain
or PDZbm (Cao et al., 1999, Weinman et al., 2007). This may assist to explain how PDZ domain-containing proteins may engage with several cargo proteins with selectivity without compromising specificity (Kalyoncu et al., 2010). Continuing studies may thus provide further insight as to how the cell is able to fine-tune the regulation of trafficking and/or signalling via post-translational modifications or through the involvement of upstream residues to the canonical PDZbm sequence.

To date, the numerous studies conducted have comprehensively validated the PDZ-mediated interaction between SNX27 and its various target proteins. These include potassium and ionotropic receptors within the brain (see Section 2.2.1.3.), glucose transporter 1 (GLUT1) and GLUT4 (Steinberg et al., 2013, Yang et al., 2015), insulin receptor (InsR) (Ghai et al., 2013), Na⁺/H⁺ exchanger 3 (NHE3) (Singh et al., 2015), tight junction protein, zonula occludens-2 (ZO-2) (Zimmerman et al., 2013), cytohesin-associated scaffolding protein (CASP) (MacNeil et al., 2007, Tompkins et al., 2014) and GPCRs, including β₁AR (Nooh and Bahouth, 2017, Nooh et al., 2016, Bowman et al., 2016) and β₂AR (Lauffer et al., 2010, Temkin et al., 2011, Bowman et al., 2016), somatostatin receptor 5 (Bauch et al., 2014), G protein-coupled receptor 17 (GPR17) (Meraviglia et al., 2016) and Fzd7 (Sun et al., 2016) amongst many others.

Interestingly however, is that the PDZ domain of SNX27 is capable of simultaneously engaging with retromer and its target protein to mediate endosomal recycling (Steinberg et al., 2013, Temkin et al., 2011, Cullen and Korswagen, 2012) (Figure 2.5A) (further elaborated in Section 2.2.2.). Although the PDZ domain of SNX27 closely follows that of canonical PDZ domains, it possesses an additional β-strand referred to as the ‘β3-β4 loop’ (Balana et al., 2011, Wang et al., 2010b) that is not present in NHERF proteins (a protein that bears a PDZ domain bearing the closest homology to that of SNX27) and is in fact responsible for engaging with the arrestin-like structure of VPS26A (Steinberg et al., 2013, Gallon et al., 2014). This interaction does not interfere with simultaneous cargo interaction, but rather enhances the affinity between SNX27 and its PDZbm-containing proteins and thus
supports the physiological relevance of the SNX27-retromer complex in cargo sorting (Gallon et al., 2014). Further adding to this, a mutation within VPS26A (p.K287X) associated with atypical Parkinsonism has been found to perturb VPS26A interaction with SNX27 (McMillan et al., 2016, Gustavsson et al., 2015).

2.2.1.3. SNX27 in the brain and neurological disease

Following its initial discovery, the most established role of SNX27 has since remained within the brain, where it plays crucial roles in neuronal plasticity, long-term potentiation and cognitive function by regulating the transport of ionotropic receptors and ion channels. One of the first SNX27-interacting cargoes identified belonged to the G protein-inwardly rectifying potassium (GIRK, or Kir3) channel family (Balana et al., 2013, Balana et al., 2011) which function to regulate neuronal excitability and responses to both learning processes and drug addiction (Huang et al., 2005, Kourrich et al., 2003, Signorini et al., 1997, Cruz et al., 2004, Beckstead et al., 2004). In addition to its engagement with GIRK2c/3 channels, Balana and colleagues identified residues upstream to the canonical PDZbm sequence that were essential for cargo selection where in particular, an acidic (or neutral) residue was required at position (-5) (Balana et al., 2013, Balana et al., 2011). As a result, the role of SNX27 in recycling and maintaining the surface expression of GIRK2c/3 channels has since been suggested to be a novel target in combating the neuronal hypersensitivity of cocaine addiction (Munoz and Slesinger, 2014, Balana et al., 2013).

In recent years, the role of SNX27 in modulating long-term potentiation is becoming increasingly understood, particularly in its regulatory role of ionotropic receptors N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. NMDA and AMPA receptors co-exist at the surface of the post-synaptic membrane where they are activated upon the binding of the neurotransmitter, glutamate by the pre-synaptic neuron. One consequence of repeated stimulation (e.g. during the processes of learning and memorising) is an increased integration of AMPA receptors at the post-synaptic
membrane, resulting in an ‘enhanced’ synapse that is capable of eliciting a stronger response during subsequent neuronal stimulations. Interestingly, heterozygous $SNX27$ mice were found to exhibit reduced neuronal plasticity and deficits in learning and memory, all of which were associated with the reduced expression in both NMDA and AMPA receptors (Wang et al., 2013, Cai et al., 2011). $SNX27$ was confirmed to modulate the surface expression of these ionotropic receptors in a PDZ-mediated manner, where the loss or overexpression of $SNX27$ favoured enhanced receptor degradation or recycling, respectively (Wang et al., 2013). Subsequently, two independent studies provided further insight into the role of $SNX27$ and long-term potentiation by directly modulating AMPA receptor trafficking that is dependent on the Ras-binding ability of $SNX27$ (Loo et al., 2014, Hussain et al., 2014).

The functional relevance of $SNX27$ in the brain also encompasses a list of neurodegenerative disease and will be briefly discussed here. Firstly, the brains of Down syndrome patients were found to have reduced levels of both $SNX27$ and AMPA receptors, as well as an increase in APP levels when compared to their age-matched controls (Wang et al., 2013). Here, these investigators implicated that these cognitive defects were linked to the increased expression of chromosome 21-encoded microRNA, miR-155, which was in turn, found to be a negative regulator of CCAAT/enhancer binding protein β (C/EBPβ), the latter being a transcriptional regulator of $SNX27$ (Wang et al., 2013). Additionally, $SNX27$ has been shown to regulate the amyloidogenic processing of APP in Alzheimer’s disease in both humans and mice. Wang and colleagues reported that the PDZ-mediated interaction between $SNX27$ and $\gamma$-secretase complex subunit, presenilin 1, acted to dissociate the complex and hence prevent the amyloidogenic processing of APP into neurotoxic $A\beta$ peptides (Wang et al., 2014b). An independent group also identified $SNX27$ to engage with APP trafficking component, sortilin-related receptor with A-type repeat (SorLA) to promote the endosome-to-PM trafficking of APP and consequently favouring the non-amyloidogenic processing of APP (Huang et al., 2016).
Lastly, there has only been one report describing the discovery of children from a consanguineous family possessing an SNX27 mutation rendering the protein non-functional (Damseh et al., 2015). Unsurprisingly, these siblings presented with infantile myoclonic epilepsy with evidence of neurodegeneration and died at an early age (ranging between 4 and 30 months of age) due to respiratory failure, similar to that of SNX27 global knockout mice (Cai et al., 2011). While the authors focused on assessing the extent to which SNX27 deficiency affected the brain, other systems including the skeletal system were not assessed.

2.2.2. **SNX27-retromer and the Actin-SNX27-Retromer Tubule Complex**

In addition to its role in establishing direct protein contacts through its PDZ domain, SNX27 is also known to complex with retromer to function as SNX27-retromer (Temkin et al., 2011, Gallon et al., 2014, Steinberg et al., 2013). Retromer itself, is a highly evolutionary conserved protein complex found to be a crucial modulator in membrane trafficking and cellular homeostasis. Initially named for its role in the retrograde transport (endosome-to-Golgi) of cation-independent mannose 6-phosphate receptor (CI-MPR) (Seaman et al., 1998, Seaman et al., 1997), its newly-discovered role in navigating the recycling routes (endosome-to-Golgi) of cargo proteins has now been regarded to be equally as important.

Yeast retromer is formed from two sub-complexes; a ‘cargo-recognition’ trimer of vacuolar protein sorting-26p (Vps26p), Vps29p and Vps35p complexed to a ‘membrane-bending’ dimer of Vps5p and Vps17p (Seaman et al., 1998, Horazdovsky et al., 1997). The trimeric complex is highly conserved in mammalian cells (as VPS26, VPS29 and VPS35) while the ‘membrane-bending’ sub-complex is a dimeric combination of Vps5p homologues, SNX1/2 with Vps17p homologues, SNX5/6 (Rojas et al., 2007, Wassmer et al., 2007) (Figure 2.6A). While both yeast and mammalian dimeric sub-complexes are capable of engaging with PI3P-enriched membranes through the PX domain, the SNX proteins possess an additional Bin/amphipathysin/Rvs (BAR) domain that assists in membrane sensing (Zimmerberg
Figure 2.6. SNX-BAR retromer.
A dimeric sub-complex of SNX1/2 with SNX5/6 (purple) engages with the trimeric sub-complex of vacuolar protein sorting 26 (VPS26), VPS29 and VPS35 (blue) at PI(3)P-enriched endosomal membranes, while VPS35 predominantly assists in cargo recognition (A, B). The BAR domain of the SNX-dimer both senses and assists in membrane deformation and tubule elongation (C).

Adapted from Bonifacio and Rojas, 2006; Cullen, 2008
and McLaughlin, 2004, Frost et al., 2008), where upon dimerization, a ‘rigid banana-shaped’ structure is formed (Peter et al., 2004). In addition to its ability to ‘sense’ curved membranes, mammalian retromer is also able to further drive membrane deformation and the elongation of tubules at early endosomes (Frost et al., 2008). As such, retromer is able to intrinsically modulate the recruitment and recycling of cargo proteins by influencing the formation and elongation of \textit{de novo} tubules at the endosomal membrane (Figure 2.6B, C).

Though retromer functions as an obligatory pentameric complex in yeast, the trimeric mammalian retromer sub-complex has been shown to form at least three distinct retromer complexes, as determined by the SNX protein it is bound to; SNX-BAR-retromer (van Weering et al., 2012, Seaman, 2012), SNX3-retromer (Lucas et al., 2016, Harterink et al., 2011, Harrison et al., 2014) and SNX27-retromer. The ability of retromer to associate with different SNX proteins (and associated complexes, e.g. WASH complex) is thought to contribute greatly to its diversity in cargo selection while also maintaining specificity across the multiple trafficking routes it has been shown to regulate.

The cooperative effort of SNX27 and retromer was first reported in the retrieval and recycling of internalised $\beta_2$AR; an interaction that was dependent on the presence of the PDZbm at the carboxy-terminus of the receptor (Lauffer et al., 2010, Temkin et al., 2011). High-resolution confocal images captured the PDZ-dependent sequestration of $\beta_2$AR into retromer-mediated endosomal tubules (Temkin et al., 2011), an event that has been recently found to be distinct from the bulk-flow recycling of $\beta_2$AR (Bowman et al., 2016).

Since then, important insight into the role of SNX27-retromer complex in cargo recycling has been gained through a seminal study by Steinberg and colleagues. Using a proteomics approach to survey the global SNX27-interactome, these investigators uncovered a multitude of surface proteins whose expressions were altered following the depletion of either SNX27 or VPS35 (retromer) (Steinberg et al., 2013). In particular, the surface expression of over 80 proteins
appeared to be co-dependent on the presence of both SNX27 and VPS35, alluding to its physiological role as SNX27-retromer in these scenarios. Among this list included the previously established $\beta_2$AR, as well as a variety of glucose, mineral and ion transporters and signalling receptors such as Fzds and LRPs (Steinberg et al., 2013).

Further adding to this, SNX27-retromer has more recently been shown to participate within a larger complex referred to as the Actin-SNX27-Retromer-Tubule (ASRT) complex. This complex consists of SNX27-retromer, as well as the Wiskott-Aldrich syndrome protein and SCAR homologue (WASH) complex and ankyrin repeat domain containing protein (ANKRD50) (Kvainickas et al., 2017, Temkin et al., 2011, Steinberg et al., 2013, Jia et al., 2012) (Figure 2.5) and provides crucial insight into the involvement of the actin dynamics used by SNX27-retromer (ASRT) complex in directing cargo recycling.

The WASH complex is a key endosomal activator of the actin nucleation Arp2/3 complex (Seaman et al., 2013) and consists of five subunits – WASH1, strumpellin, KIAA1033 (also known as SWIP), CCDC53 and FAM21 (Derivery et al., 2012). An important function of the WASH complex is to regulate the formation of branched actin networks at the surface of the endosome which results in the formation of discrete endosomal platforms (microdomains) (Derivery and Gautreau, 2015). In turn, these microdomains serve two functions; firstly as sites for the rapid polymerisation of actin, which provides the mechanical driving force for the elongation of recycling tubules (Derivery et al., 2009, Gomez et al., 2012) while also restricting the lateral diffusion of transmembrane cargoes across the endosomal membrane. Therefore, these two functions alone greatly encourage for the concentration of cargo and subsequent sorting into endosomal recycling tubules (Puthenveedu et al., 2010).

It is through the FAM21 subunit of the WASH complex that engages directly with both VPS35 and SNX27. Firstly, the unstructured carboxy-terminal tail of FAM21 (~1,100 residues) consists of 21 repeated LFa motifs that engage with
VPS35 (Goley et al., 2006, Harbour et al., 2012, Jia et al., 2012, Hao et al., 2013). This enables the WASH complex to engage with multiple retromer complexes and to therefore ‘sense’ areas enriched with cargo that await retrieval and therefore drive the formation of recycling tubules (Helfer et al., 2013, Li et al., 2016) (Figure 2.5B). Secondly, FAM21 has also been shown to interact with the carboxy-terminal SNX27-FERM domain and is in fact required for the localised recruitment of SNX27-retromer to endosomal subdomains and cargo retrieval (Lee et al., 2016b). Moreover, FAM21 was additionally found to prevent the misrouting of SNX27-retromer cargoes, GLUT1 and β2AR to the Golgi by regulating PI(4)P levels (Lee et al., 2016b). Therefore, the current model proposes that the Actin-SNX27-Retromer-Tubule (ASRT) complex comprising of SNX27-retromer, the WASH complex and actin, combines the various abilities possessed by its individual components to govern cargo sorting and recycling at endosomal recycling tubules.

Throughout the past few years, the attenuated role of the ASRT complex has been associated with a variety of diseases. For instance, a range of pathological viruses have been shown to utilise the host retromer to assist in virus replication and survival, including human papilloma virus (Popa et al., 2015, Lipovsky et al., 2013), human immunodeficiency virus (HIV-1) (Groppelli et al., 2014) and hepatitis C virus (Yin et al., 2016). Additionally, the relevance of retromer has also been demonstrated in two major neurodegenerative diseases. The lowered levels of VPS26 and VPS35 were detected in the late-onset of Alzheimer’s disease, where, similar to SNX27, retromer ultimately functions to prevent the improper cleavage of APP to Aβ peptides (Sullivan et al., 2011, Choy et al., 2012, Small and Petsko, 2015, Chu and Pratico, 2017). Additionally, retromer had also been shown to assist in the lysosomal delivery of cathepsin D (a cargo protein of CI-MPR), which therefore promotes the degradation of α-synuclein and subsequently prevents the formation of the characteristic Lewy Bodies observed in Parkinson’s disease (Follett et al., 2014).
2.2.3. Summary

In the cooperative effort to understand the complexity of the cell and how this knowledge can be used to prevent the progression of disease, there has been an increasing appreciation for the investigation of intracellular mechanisms that govern proper cellular function and homeostasis. In particular, recent developments in the field of membrane and protein trafficking have proved insightful, where the characteristics of several human diseases have been attributed to anomalies within trafficking machinery and/or pathways. In contrast to well-established protein families of membrane trafficking such as the family of small Rab GTPases, the SNX protein family is a relatively novel family where the exact function of many of its members remains to be fully elucidated. Based on the current literature available however, SNX27 is among a handful of SNX proteins that have been reported to function as key regulators in membrane and protein trafficking. Furthermore, despite having been predominantly assessed within the brain, the ubiquitous expression of SNX27 and its regulation of over 100 surface proteins almost certainly guarantees its relevance in additional cell types and tissues.
CHAPTER

– 3 –

Rationale, hypothesis and aims
3. **Rationale, hypothesis and aims**

3.1. **Rationale and hypothesis**

The skeleton is a crucial organ of the body that functions to provide mechanical support for physical movements as well as serving as the main calcium reservoir of higher vertebrates. Several highly-specialised cells reside within the bone matrix that drive skeletal growth and ensure the repair of bone through the process of remodelling. This process relies on the highly-coordinated cross-talk between bone-forming osteoblast, bone-resorbing osteoclasts as well as osteocytes that together, serve to maintain skeletal and mineral homeostasis. It is therefore not surprising that skeletal diseases arise when these resident cells become dysfunctional and thus lead to uncoupled cellular activity.

Intracellular trafficking is the highly sophisticated means by which molecules are transported within or secreted from the cell through the use of membrane-bound vesicular carriers. Membrane and protein trafficking have a particular prominence in skeletal biology, with a range of skeletal dysplasias arising due to genetic disruptions in trafficking proteins or their cargoes (Stenbeck and Coxon, 2014, Zhao, 2012). Often the most severe cases of skeletal disease occur when genetic mutations directly affect conserved trafficking complexes or components required for cell differentiation or function.

Over the past two decades, members of the SNX family of endocytic proteins have emerged as important regulators of membrane and protein trafficking. SNX proteins are defined by the presence of a PX domain that enables their engagement with PI(3)P lipids embedded within the membranes of early endosomes. Consisting of 34 members to date, SNX proteins are a diverse family of trafficking proteins which participate in a range of cargo transport processes in several cell types with specialised intracellular trafficking requirements (Cullen, 2008, Teasdale and Collins, 2012). Currently, the potential importance of SNX proteins in skeletal cell
function and homeostasis remains largely unclear, with SNX10 the only member implicated to date and is required for osteoclast function (Chen et al., 2012, Xu et al., 2013, Zhu et al., 2012). Indeed, a series of SNX10 mutations have been identified and are thought to account for 4% of cases of autosomal recessive osteopetrosis (Aker et al., 2012, Megarbane et al., 2013, Pangrazio et al., 2013); a percentage that is comparable to those caused by mutations within genes encoding RANKL/RANK (Tnfsf11/Tnfrsf11a) (Pangrazio et al., 2013). Whether there are additional members of the SNX family that confer cell specific functions and contribute to the regulation of bone homeostasis requires further investigation and is central to the overarching hypothesis of this dissertation:

**Hypothesis:** “Sorting Nexins are crucial regulators of skeletal cell function and bone homeostasis.”

Whereas SNX10 is a relatively unstructured member of the SNX family, possessing a single ‘extended PX’ domain, several SNXs harbour accessory domains that facilitate protein-protein interactions, thereby enabling simultaneous engagement with membrane proteins and intraluminal cargo(es) on individual endosomes. This is perhaps best exemplified by SNX27, one of a few SNXs shown to bind and direct the transport of a multitude of cell surface receptors (Steinberg et al., 2013). SNX27 is unique among its family, being the only member to harbour a protein-interacting PDZ domain through which it regulates the endosomal recycling and transport of several cargoes containing compatible carboxy-terminal PDZ-binding motifs. To date, most of what we understand about SNX27 has been gleaned from studies of SNX27 involvement in the trafficking and recycling of ionotropic AMPA and NMDA receptors in neurons. Until now however, the potential physiological involvement of SNX27 in skeletal cell function and bone homeostasis has not been previously explored.
3.2. Aims

The major goal of this dissertation was to evaluate the expression and functional role of SNXs in skeletal homeostasis.

Specifically, the aims were to:

1. Establish a quantitative analysis of the relative expression and abundance of the SNX protein family in bone tissue and bone-resident cell types (osteoclasts, osteoblasts and osteocytes inclusive);

2. Identify candidate SNXs (i.e. SNX27) which may play potential physiological role(s) in skeletal homeostasis and to functionally characterise their contribution through the use of genetically-modified mice model(s);

3. Identify putative endocytic cargo (i.e. PTHR) regulated by the candidate SNX protein(s) (i.e. SNX27) and characterise the molecular mechanism and determinants that govern this interaction.
CHAPTER

- 4 -

Materials and Methods
4. **Materials and Methods**

4.1. **Reagents**

4.1.1. **Chemical reagents and general materials**

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<tr>
<td>Coverslips, 0.5mm Ø, thickness no. 0 (glass)</td>
<td>ProSciTech Pty Ltd., Australia</td>
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<td>Cetylpolyridinium chloride</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Chloroform (molecular grade)</td>
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<td>Coelentetrazine-H Substrate</td>
<td>Promega Corp., USA</td>
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<td>Dimethylsulphoxide (DMSO)</td>
<td>Merck Millipore Corp., Germany</td>
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<tr>
<td>Dibutyl phthalate</td>
<td>Sigma-Aldrich Co., USA</td>
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<tr>
<td>DNase I</td>
<td>Roche Diagnostics, Switzerland</td>
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<tr>
<td>Material</td>
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<tr>
<td>Ethanol (100%), molecular grade</td>
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<td>Ethylene diamine tetra-acetic acid (EDTA)</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Ethylene glycol-bis(2-aminoethylether)-tetra-acetic acid (EGTA)</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Ethylene glycol monomethyl ether (EGME)</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Fast green</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Fast red violet LB salt</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Ferric chloride</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Formaldehyde</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Glycerol, analytical grade</td>
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<td>Glycine</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Haematoxylin crystal</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>HEPES</td>
<td>Sigma-Aldrich Co., USA</td>
</tr>
<tr>
<td>Hybond™ C Nitrocellulose Membrane</td>
<td>GE Healthcare, USA</td>
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<tr>
<td>Hydrochloric acid (HCl, 37%)</td>
<td>Chem Supply, Australia</td>
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<tr>
<td>Isopropanol (100%), molecular grade</td>
<td>Sigma-Aldrich Co., USA</td>
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<tr>
<td>Light green</td>
<td>Sigma-Aldrich Co., USA</td>
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<tr>
<td>Magnesium chloride (MgCl₂)</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Methanol (100%), analytical grade</td>
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<td>Methyl methacrylate (MMA)</td>
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<td>Napthol AS-MX phosphate</td>
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<td>Neutral buffered formalin (10%)</td>
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<td>Nonidet™ P-40 (NP-40)</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Oil red O</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Orange G</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Paraformaldehyde (PFA)</td>
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<td>Perkadox 16</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Phenylmethylsulfonyl fluoride (PMSF)</td>
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<td>Phosphotungstic acid</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Ponceau 2R</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Ponceau S</td>
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<td>Potassium chloride (KCl)</td>
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<td>Potassium hydroxide (KOH)</td>
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<td>Potassium phosphate (KH₂PO₄)</td>
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<td>Sigma-Aldrich Co., USA</td>
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<td>Saponin</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Skim milk powder</td>
<td>Standard market brand</td>
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<td>Sodium acetate trihydrate</td>
<td>Sigma-Aldrich Co., USA</td>
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<td>Sodium chloride (NaCl)</td>
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<td>Sodium deoxycholate</td>
<td>Sigma-Aldrich Co., USA</td>
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<tr>
<td>Sodium dodecyl sulphate (SDS)</td>
<td>Sigma-Aldrich Co., USA</td>
</tr>
<tr>
<td>Sodium hydroxide (NaOH)</td>
<td>BDH Laboratory Supplies, England</td>
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<tr>
<td>Sodium orthovanadate</td>
<td>Sigma-Aldrich Co., USA</td>
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<tr>
<td>Sodium phosphate monobasic (NaH₂PO₄)</td>
<td>Sigma-Aldrich Co., USA</td>
</tr>
<tr>
<td>and dibasic (Na₂HPO₄)</td>
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<tr>
<td>Sodium tartrate dehydrate</td>
<td>Sigma-Aldrich Co., USA</td>
</tr>
<tr>
<td>TEMED (N, N, N’-tetramethyl-ethylenediamine)</td>
<td>Bio-Rad Laboratories, USA</td>
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<tr>
<td>Tinogard® TT</td>
<td>BASF, Germany</td>
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<tr>
<td>Trizma base</td>
<td>Sigma-Aldrich Co., USA</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Sigma-Aldrich Co., USA</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Sigma-Aldrich Co., USA</td>
</tr>
<tr>
<td>TRIzol® reagent</td>
<td>Thermo Fisher Scientific Inc., USA</td>
</tr>
<tr>
<td>Tween-20, molecular grade</td>
<td>MP Biomedicals, USA (?)</td>
</tr>
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Chapter 4 - Materials and Methods

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whatman® Chromatography paper 3MM</td>
<td>GE Healthcare, USA</td>
</tr>
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### 4.1.2. Tissue culture products and reagents

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-modification of Eagle’s medium (α-MEM)</td>
<td>Gibco by Thermo Fisher Scientific Inc., USA</td>
</tr>
<tr>
<td>Acrodisc® 25mm syringe filters with Supor® membrane, 0.2µm, 0.45µm and 0.8µm</td>
<td>Pall Corp., USA</td>
</tr>
<tr>
<td>β-glycerophosphate</td>
<td>Sigma-Aldrich Co., USA</td>
</tr>
<tr>
<td>3-isobutyl-1-methylxanthine (IBMX)</td>
<td>Sigma-Aldrich Co., USA</td>
</tr>
<tr>
<td>Ascorbic acid or L-ascorbate</td>
<td>Sigma-Aldrich Co., USA</td>
</tr>
<tr>
<td>Cell scraper (25cm)</td>
<td>Sarstedt, USA</td>
</tr>
<tr>
<td>Costar® cell culture plates</td>
<td>Corning Inc., USA</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Sigma-Aldrich Co., USA</td>
</tr>
<tr>
<td>Dimethylsulfoxide (DMSO)</td>
<td>Merck Millipore Corp., Germany</td>
</tr>
<tr>
<td>Dulbecco’s-modification of Eagle’s medium (DMEM), Glutamax or High Glucose</td>
<td>Gibco by Thermo Fisher Scientific Inc., USA</td>
</tr>
<tr>
<td>Falcon cell strainer, 100µm, nylon</td>
<td>BD Biosciences, USA</td>
</tr>
<tr>
<td>Foetal bovine serum (FBS)</td>
<td>Gibco by Thermo Fisher Scientific Inc., USA</td>
</tr>
<tr>
<td>Forskolin</td>
<td>Cell Signaling Technology Inc., USA</td>
</tr>
<tr>
<td>Geneticin® Selective Antibiotic (G418), 50mg/mL</td>
<td>Thermo Fisher Scientific Inc., USA</td>
</tr>
<tr>
<td>Glass bottom culture dish (35mm petri dish, 10mm microwell), no.15 coverglass</td>
<td>MatTek Corp., USA</td>
</tr>
</tbody>
</table>
### Chapter 4 - Materials and Methods

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hanks buffered salt solution (HBSS) buffer</td>
<td>Thermo Fisher Scientific Inc., USA</td>
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<tr>
<td>Isoproterenol hydrochloride</td>
<td>Sigma Aldrich Co., USA</td>
</tr>
<tr>
<td>L-Glutamine (200mM, 100X)</td>
<td>Gibco by Thermo Fisher Scientific Inc., USA</td>
</tr>
<tr>
<td>Parafilm “M”® laboratory film</td>
<td>Parafilm., USA</td>
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<tr>
<td>Penicillin-Streptomycin (10,000U/mL)</td>
<td>Gibco by Thermo Fisher Scientific Inc., USA</td>
</tr>
<tr>
<td>Puromycin dichloride</td>
<td>Sigma-Aldrich Co., USA</td>
</tr>
<tr>
<td>Rat tail collagen type I</td>
<td>In Vitro Technologies, Australia</td>
</tr>
<tr>
<td>TrypLE™ Express</td>
<td>Gibco by Thermo Fisher Scientific Inc., USA</td>
</tr>
<tr>
<td>Trypsin-EDTA (0.25%)</td>
<td>Gibco by Thermo Fisher Scientific Inc., USA</td>
</tr>
<tr>
<td>Cell culture flasks and plates (variety)</td>
<td>Corning Inc., USA</td>
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#### 4.1.3. Commercially purchased kits and molecular products

<table>
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<tr>
<th>Item</th>
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</thead>
<tbody>
<tr>
<td>100bp DNA ladder</td>
<td>Promega Corp., USA</td>
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<tr>
<td>6X DNA Blue/Orange loading dye</td>
<td>Promega Corp., USA</td>
</tr>
<tr>
<td>CellTiter 96® Aqueous One Solution Assay</td>
<td>Promega Corp., USA</td>
</tr>
<tr>
<td>Cyclic AMP XP™ Assay Kit</td>
<td>Cell Signaling Technology Inc., USA</td>
</tr>
<tr>
<td>DharmaFECT™ transfection reagent</td>
<td>Dharmaco Inc., USA</td>
</tr>
<tr>
<td>FuGENE® 6</td>
<td>Promega Corp., USA</td>
</tr>
<tr>
<td>GFP-Trap_A beads</td>
<td>ChromoTek Inc., USA</td>
</tr>
<tr>
<td>Leukocyte Alkaline Phosphatase Kit</td>
<td>Sigma-Aldrich Co., USA</td>
</tr>
<tr>
<td>Lipofectamine LTX and Plus Reagents</td>
<td>Thermo Fisher Scientific Inc., USA</td>
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</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse 1,25-dihydroxyvitamin D3 (DVD/DHVD3) ELISA Kit</td>
<td>Cusabio., USA</td>
</tr>
<tr>
<td>Mouse parathyroid hormone (PTH) ELISA Kit</td>
<td>Cusabio., USA</td>
</tr>
<tr>
<td>One Shot® TOP 10 Chemically Competent E. coli</td>
<td>Thermo Fisher Scientific Inc., USA</td>
</tr>
<tr>
<td>Poly-L-lysine solution</td>
<td>Sigma-Aldrich Co., USA</td>
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<tr>
<td>PureLink® HiPure Plasmid MidiPrep Kit</td>
<td>Thermo Fisher Scientific., USA</td>
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<tr>
<td>Precision Plus Protein™ Standards</td>
<td>Bio-Rad Laboratories, USA</td>
</tr>
<tr>
<td>ProLong® Diamond anti-fade mounting reagent</td>
<td>Molecular Probes by Thermo Fisher Scientific Inc., USA</td>
</tr>
<tr>
<td>RNase ERASE® solution</td>
<td>MP Biomedicals LLC, USA</td>
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<tr>
<td>SensiMix™ II Probe Kit</td>
<td>Bioline Reagents Ltd., UK</td>
</tr>
<tr>
<td>Superscript™ III First Strand Synthesis Kit</td>
<td>Thermo Fisher Scientific Inc., USA</td>
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<tr>
<td>SYBR® safe DNA gel stain</td>
<td>Thermo Fischer Scientific Inc., USA</td>
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<tr>
<td>Western Lightning™ Ultra Extra Sensitivity</td>
<td>Perkin-Elmer, USA</td>
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### 4.1.4. Commercially purchased antibodies

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<tbody>
<tr>
<td>Anti-α-tubulin, mouse mAb</td>
<td>Sigma-Aldrich Co., USA</td>
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<tr>
<td>Alexa Fluor® 488, 568, 647 goat anti-mouse IgG</td>
<td>Molecular Probes® Invitrogen, ThermoFisher Scientific Inc., USA</td>
</tr>
<tr>
<td>Alexa Fluor® 488, 568, 647 goat anti-rabbit IgG</td>
<td>Molecular Probes® Invitrogen, ThermoFisher Scientific Inc., USA</td>
</tr>
<tr>
<td>Alexa Fluor® rhodamine phalloidin, conjugated</td>
<td>Molecular Probes® Invitrogen, ThermoFisher Scientific Inc., USA</td>
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### Chapter 4 - Materials and Methods

<table>
<thead>
<tr>
<th>Antibody Description</th>
<th>Supplier</th>
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<tr>
<td>Anti-mouse IgG (whole molecule)-peroxidase, antibody produced in goat</td>
<td>Sigma-Aldrich Co., USA</td>
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<tr>
<td>Anti-rabbit IgG (whole molecule)-peroxidase, antibody produced in goat</td>
<td>Sigma-Aldrich Co., USA</td>
</tr>
<tr>
<td>Anti-collagen type II, rabbit pAb</td>
<td>Sigma-Aldrich Co., USA</td>
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<tr>
<td>Anti-collagen type X, mouse pAb</td>
<td>Quartett GmbH., Germany</td>
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<tr>
<td>Anti-connexin 43, rabbit pAb</td>
<td>Cell Signaling Technology Inc., USA</td>
</tr>
<tr>
<td>Anti-CD63 (H5C6), anti-human mouse mAb</td>
<td>Developmental Studies Hybridoma Bank, USA</td>
</tr>
<tr>
<td>Anti-EEA-1 (purified mouse IgG1, anti-human)</td>
<td>BD Biosciences Pharmigen, USA</td>
</tr>
<tr>
<td>Anti-FAM21C, rabbit pAb</td>
<td>Merck Millipore Corp., Germany</td>
</tr>
<tr>
<td>Anti-Flag (M2), mouse mAb</td>
<td>Sigma-Aldrich Co., USA</td>
</tr>
<tr>
<td>Anti-GFP (green fluorescent protein)</td>
<td>Enzo Life Sciences Inc., USA</td>
</tr>
<tr>
<td>Hoechst 33256 (bis-benzimide)</td>
<td>Molecular Probes® Invitrogen, ThermoFisher Scientific Inc., USA</td>
</tr>
<tr>
<td>Anti-Lamp-1, rabbit pAb</td>
<td>Abcam, UK</td>
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<tr>
<td>Anti-Myc</td>
<td>Merck Millipore Corp., Germany</td>
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<tr>
<td>Anti-TGN38 (trans-Golgi network-38), mouse mAb</td>
<td>BD Biosciences, USA</td>
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<tr>
<td>Anti-pan-AKT (C67E7), rabbit mAb</td>
<td>Cell Signaling Technology Inc., USA</td>
</tr>
<tr>
<td>Anti-pan-β-Catenin, rabbit mAb</td>
<td>Cell Signaling Technology Inc., USA</td>
</tr>
<tr>
<td>Anti-pan-ERK1/2, rabbit pAb</td>
<td>Promega Corp., USA</td>
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<tr>
<td>Anti-pan-Smad1, rabbit mAb</td>
<td>Cell Signaling Technology Inc., USA</td>
</tr>
<tr>
<td>Anti-phospho-AKT (Thr308) (D25E6) rabbit mAb</td>
<td>Cell Signaling Technology Inc., USA</td>
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<tr>
<td>Anti-phospho-β-Catenin (Ser675) rabbit pAb</td>
<td>Cell Signaling Technology Inc., USA</td>
</tr>
<tr>
<td>Anti-phospho-β-Catenin (Ser33/37/Thr41), rabbit mAb</td>
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<td>Item</td>
<td>Supplier</td>
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<td>----------------------------------------------------------------------</td>
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<tr>
<td>Anti-phospho-CREB (Ser133) (87G3) rabbit mAb</td>
<td>Cell Signaling Technology Inc., USA</td>
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<tr>
<td>Anti-phospho-ERK1/2 (E-4) mouse mAb</td>
<td>Santa Cruz Biotechnology Inc., USA</td>
</tr>
<tr>
<td>Anti-phospho-Smad1/5 (Ser463/465) (41D10) rabbit mAb</td>
<td>Cell Signaling Technology Inc., USA</td>
</tr>
<tr>
<td>Anti- (human) PTHrP (region 67-84) ‘R90’, IgG purified, rabbit pAb</td>
<td>Generated at St Vincent’s Institute, generously provided by E/Prof TJ (Jack) Martin (St. Vincent’s Institute of Medical Research, Melbourne VIC, Australia)</td>
</tr>
<tr>
<td>Anti- (human) PTHrP (region 1-33) ‘Kreman#158’, mouse mAb</td>
<td>Generated by Richard Kremen, generously provided by E/Prof TJ (Jack) Martin (St. Vincent’s Institute of Medical Research, Melbourne VIC, Australia)</td>
</tr>
<tr>
<td>Anti- (mouse) PTHrP (region 107-139) ‘R3959’, affinity purified, rabbit pAb</td>
<td>Custom made, generously provided by E/Prof TJ (Jack) Martin (St. Vincent’s Institute of Medical Research, Melbourne VIC, Australia)</td>
</tr>
<tr>
<td>Anti- (mouse) PTHrP (region 1-33) ‘Chugai’, IgG purified, mouse mAb</td>
<td>Generated by Chugai, generously provided by E/Prof TJ (Jack) Martin (St. Vincent’s Institute of Medical Research, Melbourne VIC, Australia)</td>
</tr>
<tr>
<td>Anti-SNX27, mouse mAb</td>
<td>Abcam, UK</td>
</tr>
<tr>
<td>Anti-VPS35 (B-5) mouse mAb</td>
<td>Santa Cruz Biotechnology Inc., USA</td>
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### 4.1.5. Cytokines and peptide fragments

<table>
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<th>Item</th>
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</thead>
<tbody>
<tr>
<td>Bone morphogenetic protein 2 (BMP2)</td>
<td>Abnova Corp., Taiwan</td>
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</tbody>
</table>
EGF-555 (epidermal growth factor, conjugated to Alexa Fluor®-555)
Macrophage colony stimulating factor (M-CSF)
Parathyroid hormone (PTH), fragment 1-34 (PTH(1-34))
Parathyroid hormone (PTH), fragment 1-34 (PTH(1-34))
PTH-related protein (PTHrP), variable lengths; including PTHrP(1-141), PTHrP(1-108), PTHrP(1-84) and PTHrP(1-36).

4.1.6. Plasmids and constructs

<table>
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<th>Plasmid</th>
<th>ID</th>
<th>Supplier</th>
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<tr>
<td>EGFP (enhanced green fluorescent protein) control vector</td>
<td>Originally purchased from Clontech Laboratories, Inc., USA,</td>
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<tr>
<td>Construct</td>
<td>Source Information</td>
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<td>-----------------------------------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------------</td>
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<tr>
<td>β-arrestin2-GFP</td>
<td>Addgene plasmid 35411 Gifted by Robert Lefkowitz (Addgene plasmid #35411)</td>
<td></td>
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<tr>
<td>SNX27(WT)GFP</td>
<td>Generously provided by Prof Wanjin Hong (Institute of Molecular and Cell Biology, A*STAR Singapore), as cited in Loo et al., 2014</td>
<td></td>
</tr>
<tr>
<td>SNX27(WT)mCherry</td>
<td>Generously provided by Prof Wanjin Hong (Institute of Molecular and Cell Biology, A*STAR Singapore), as cited in Loo et al., 2014</td>
<td></td>
</tr>
<tr>
<td>SNX27ΔPDZ-GFP</td>
<td>Generously provided by Prof Wanjin Hong (Institute of Molecular and Cell Biology, A*STAR Singapore), as cited in Loo et al., 2014</td>
<td></td>
</tr>
<tr>
<td>SNX27-H114A-GFP</td>
<td>Generated using QuikChange® Site-Directed Mutagenesis Kit (Agilent Technologies Inc., USA), generously gifted by Prof Wanjin Hong</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Institute of Molecular and Cell Biology, A*STAR Singapore).</td>
<td></td>
</tr>
<tr>
<td>Construct</td>
<td>Description</td>
<td>Source</td>
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<td>-------------</td>
<td>--------</td>
</tr>
<tr>
<td>pGIPZ non-targeting shRNA</td>
<td>RHS4346</td>
<td>Generated using lentivirus small hairpin RNA (shRNA) system (Thermo Fisher Scientific Inc., USA), generously gifted by A/Prof. Rohan Teasdale (Institute for Molecular Bioscience, University of Queensland).</td>
</tr>
<tr>
<td>pGIPZ shSNX27</td>
<td>V2LHS_237898</td>
<td>Generated using lentivirus small hairpin RNA (shRNA) system (Thermo Fisher Scientific Inc., USA), generously gifted by A/Prof. Rohan Teasdale (Institute for Molecular Bioscience, University of Queensland).</td>
</tr>
<tr>
<td>pGIPZ shVPS35</td>
<td>V2LHS_156301</td>
<td>Generated using lentivirus small hairpin RNA (shRNA) system (Thermo Fisher Scientific Inc., USA), generously gifted by A/Prof. Rohan Teasdale (Institute for Molecular Bioscience, University of Queensland).</td>
</tr>
<tr>
<td>GFP Rab5</td>
<td></td>
<td>Generously gifted from Marino Zerial (Max Planck Institute for Cell Biology and Genetics, Dresden, Germany), as</td>
</tr>
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</table>
### Chapter 4 - Materials and Methods

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
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<tbody>
<tr>
<td>GFP-Rab4b</td>
<td>Generously gifted by Dr. Henrik Martens (Synaptic Systems, Göttingen, Germany), as cited in <em>Pavlos et al., 2010.</em></td>
</tr>
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<td>mCherry-ML1N*2</td>
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the linker Arg-Leu-Ille-Ser-Gly-Ser according to the methods described in *Castro et al., 2005.*

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4.1.7. Oligonucleotide primers

All primers were generated using Roche Universal ProbeLibrary Assay Design Centre (online).

Upon arrival, oligos were resuspended to a final concentration of 100μM using DEPC-treated H₂O, diluted to a working stock of 20μM and stored at -20°C. All oligos were ordered through GeneWorks Pty Ltd., Australia, and all probes were purchased through Roche Diagnostics., Switzerland.

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Chapter 4 - Materials and Methods
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### Chapter 4 - Materials and Methods

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4.2. Buffers and solutions

All buffers and solutions were prepared using MilliQ double-distilled water (ddH₂O) and were sterilised via autoclave or filtering where applicable. All chemicals were weighed using AND EK200i weighing balance, and pH adjustments were carried using pH211 pH meter and concentrated HCl or NaOH, unless specified otherwise.

**β-glycerophosphate (0.4M)**

β-glycerophosphate disodium salt hydrate (powder) was reconstituted in 1X PBS (autoclaved) to a stock concentration of 0.4M. Solution was filtered through a 0.2µm filter, aliquoted and were stored at -20°C (long-term) or and kept at 4°C for re-use after thawing.

**Alizarin red solution (ARS, 40mM) (for in vitro mineralisation assay)**

Alizarin red (powder) was dissolved in MilliQ H₂O to a final concentration of 40mM, where the pH was adjusted to 4.1-4.3 with ammonium hydroxide (10%, v/v). Stock solution was filtered and stored at room temperature, protected from light.

**Alizarin red staining solution (ARS) (for whole mount staining)**
ARS was prepared as described above, and further diluted to 0.003% \((w/v)\) Alizarin red in 1\% \((w/v)\) KOH solution.

**Acetic acid (1\%, v/v)**

1\% \((v/v)\) acetic acid was made in MilliQ H\(_2\)O and stored at room temperature.

**Alcian blue staining solution (for whole mount staining)**

Alcian blue (powder) was made to 0.01\% \((w/v)\) using a 5:1 ratio of 96\% ethanol:glacial acetic acid.

**Ascorbic acid (5mg/mL)**

L-Ascorbic acid (powder) was reconstituted in autoclaved 1X PBS to a stock concentration of 5mg/mL. Stock solution was filtered through a 0.2\(\mu\)m filter, aliquoted and stored at -20\(^\circ\)C (long-term), and kept at 4\(^\circ\)C for re-use after thawing.

**BMP2 (10\(\mu\)g/mL)**

Pre-weighed BMP2 (powder) was reconstituted in 4mM HCl-0.1\% \((w/v)\) BSA (in MilliQ H\(_2\)O, filtered) to a stock concentration of 10\(\mu\)g/mL. Aliquots were stored at -20\(^\circ\)C (long-term) and kept at 4\(^\circ\)C for re-use following thawing.

**BSA-PBS Solution (3\%, 0.2\%, w/v)**

BSA (powder) was weighed and dissolved in autoclaved 1X PBS to a concentration of 3\% \((w/v)\) or 0.2\% \((w/v)\). Solution was filtered through 0.45\(\mu\)m filter prior to use and stored at 4\(^\circ\)C.

**Cetylpyridinium chloride (10\%, w/v) in 10mM sodium phosphate (pH 7.0)**

Cetylpyridinium chloride (powder) was weighed and added to 10mM sodium phosphate (pH 7.0), yielding a working solution of 10\% \((w/v)\). Solution was dissolved completely and stored at room temperature, protected from light.

**EDTA (10\%, w/v)**

EDTA (powder) was dissolved in MilliQ H\(_2\)O, and pH was adjusted to 7.4 using concentrated NaOH. Stock solution was stored at room temperature.
**Extraction buffer (for immunoprecipitation)**

A stock solution consisted of the following, to a final concentration of; HEPES-KOH [50mM], NaCl [150mM], MgCl\(_2\) [1mM] and EGTA [0.5mM]. Prior to use, a working solution was made following the fresh addition of PMSF [100µg/mL] and protease inhibitor [1X].

**Fast green (0.001%, w/v, for Safranin-O staining)**

Fast green (powder) was dissolved in MilliQ H\(_2\)O, to a final concentration of 0.001% (w/v). Solution was stored at room temperature.

**Forskolin (30mM)**

Pre-weighed forskolin (powder) was reconstituted in DMSO to a stock concentration of 30mM. Stock solution was stored at -20°C, and gently warmed and mixed well prior to use.

**HEPES-KOH (1M, pH 7.2-7.4)**

A stock solution of 1M HEPES was made in MilliQ H\(_2\)O and pH was adjusted to 7.2-7.4 using concentrated KOH. Solution was stored at 4 °C.

**IBMX (1M)**

Pre-weighed IBMX (powder) was reconstituted in DMSO to a stock concentration of 1M. Solution was stored at -20°C, and gently warmed and mixed well prior to use.

**Isoproterenol (0.1M)**

Isoproterenol (powder) was reconstituted in ethanol (100%) to a stock concentration of 0.1M. Stock solution was stored at -20°C.

**Light Green (0.2%, w/v, for Goldner’s trichrome staining)**

Light green (powder) was dissolved in 0.2% (v/v) acetic acid, and stored at room temperature.

**MMA-infiltration solution**
Made fresh, MMA-infiltration solution comprised of 89g MMA (methyl methacrylate), 10g dibuthyl phthalate, 1g perkadox 16 and 0.01g Tinogard® TT, and stored at 4°C.

Oil Red O staining solution

A stock solution of 8.6mM Oil red O was made in isopropanol and stored at room temperature protected from direct light.

A working solution of 5mM Oil red O was made by dissolving 6mL Oil red O (stock solution) in 4mL MilliQ H₂O. Working solution was filtered and used immediately.

Parformaldehyde (4% PFA, w/v)

PFA (powder) was measured and dissolved in 1X PBS under gentle heating, to a final concentration of 4% (w/v). Solution was filtered and aliquoted and stored at -20°C (long-term), or kept at 4°C for re-use following thawing.

Phosphate-buffered solution (PBS; 10X, 1X)

10X PBS was made as follows; NaCl [137mM], KCl [2.7mM], Na₂PO₄ [10mM] and KH₂PO₄ [2mM] in MilliQ H₂O. 10X PBS was diluted to 1X working solution in MilliQ H₂O and pH was adjusted to 7.4 using HCl and/or NaOH. Solution was then autoclaved for tissue culture use, and kept at room temperature.

Phosphotungstic acid-Orange G

Phosphotungstic acid (powder) and Orange G (powder) were dissolved in MilliQ H₂O to a final concentration of 2% (w/v) each. Solution was kept at room temperature.

PMSF (17.6mg/mL)

PMSF was dissolved in isopropanol (100%) to a stock concentration of 17.6mg/mL and stored at -20°C.

Ponceau S staining solution (0.1% (w/v) in 5% (v/v) acetic acid)

A 1X working solution consisted of the following: Ponceau S [0.1%, w/v] and acetic acid [5%, v/v], made in MilliQ H₂O. When not in use, solution was stored at 4°C.
**Ponceau Acid Fuschin**

Ponceau 2R (powder) was made to 1% (w/v) using MilliQ H$_2$O.

Acid Fuschin (powder) was made to 1% (w/v) using MilliQ H$_2$O.

A working solution was made fresh, consisting of 6mL 1% (w/v) Ponceau 2R, 2mL of 1% (w/v) Acid Fuschin solution and 9mL 2% (v/v) acetic acid, to 73mL MilliQ H$_2$O.

**Protease inhibitor (25X CPI, EDTA-free)**

Protease inhibitor stock solution was made by dissolving 1 tablet in 2mL MilliQ H$_2$O to make a stock solution of 25X (as per manufacturer’s instructions). Stock solution was stored at -20°C.

**Protein denaturing loading dye (4X)**

A 4X working solution consisted of the following; Glycerol [40%, v/v]; Tris-HCl, pH 6.8 [0.24M], SDS [8%, v/v], bromophenol blue [0.04%, w/v] and β-mercapethanol [5%, v/v], made in MilliQ H$_2$O. Loading dye was added to protein to a final concentration of 1X.

**Protein non-denaturing loading dye (2X)**

A 2X working solution consisted of the following; Glycerol [25%, v/v], Tris-HCl, pH 6.8 [62.5mM] and bromophenol blue [0.04%, w/v], in MilliQ H$_2$O. Loading dye was added to protein to a final concentration of 1X.

**PTH(1-34), PTH$^{TMR}$ and PTHrP (various lengths) (50µM)**

Commercially bought peptides (PTH(1-34) and PTH$^{TMR}$) were reconstituted in 0.01N acetic acid, to a stock concentration of 50µM. Aliquots were stored at -20°C and were not re-used following thawing.

PTHrP peptides were generously provided by Prof. TJ (Jack) Martin, and were reconstituted in 0.01N acetic acid to a stock concentration of 50µM. Aliquots were stored at -20°C and were not re-used following thawing.

**Rat tail collagen type I (0.15mg/mL in 5mM acetic acid)**
Rat tail collagen type I (0.15mg/mL) in 5mM acetic acid was prepared by mixing 0.6mL of 5mg/mL rat tail collagen type I (Sigma-Aldrich Co., USA) and 5mL of 0.02N acetic acid, to a total of 20mL in MilliQ H$_2$O. Solution was stored at 4°C and was used to coat culture surfaces up to 5 times prior to disposal.

**Ripa lysis buffer (protein extraction)**

A stock solution of 500mL consisted of the following; Tris-HCl, pH 7.4 [50mM], NaCl [150mM], NP-40 [1% v/v], sodium deoxycholate [0.5%, w/v] and SDS [0.1%, w/v] to a total of 500mL with MilliQ H$_2$O. Stock solution was stored at 4°C.

A working solution of protein lysis buffer (Ripa Ripa buffer) was made by the immediate addition of the following prior to lysis; PMSF [100µg/mL], protease inhibitor [1X], sodium orthovanadate [1mM] and DNase I [0.5mg/mL], to a total of 1mL in Ripa lysis buffer.

**Safranin O staining (0.1%, w/v)**

Safranin O (powder) was made to 0.1% (w/v) using MilliQ H$_2$O. Solution was stored at room temperature.

**Saponin (1%, 0.1%, w/v)**

Saponin (powder) was dissolved in MilliQ H$_2$O to a stock concentration of 1% (w/v).

A working solution of 0.1% (w/v) was made by diluting the stock solution in MilliQ H$_2$O, and filtered through a 0.45µm filter prior to use. Stock and working solutions were stored at 4°C.

**SDS (10% w/v)**

A stock solution was made by dissolving SDS in MilliQ H$_2$O to a concentration of 10% (w/v). Solution was stored at room temperature.

**Sodium phosphate monobasic, dibasic solution (pH 7.0)**

Working solutions of 10mM sodium phosphate monobasic, and 10mM sodium phosphate dibasic were made in MilliQ H$_2$O. The pH of 10mM sodium phosphate
dibasic was adjusted to 7.0 using 10mM sodium phosphate monobasic. The final solution was stored at room temperature protected from direct light.

**TRAP stain**

Buffer A: 6.8g Sodium acetate trihydrate [100mM], 5.8g sodium tartrate dehydrate [50mM], 1.1mL acetic acid (glacial) [0.22%], to a final volume of 500mL using MilliQ $H_2O$. pH was correctly adjusted to 5.0.

Buffer B: 50mg of Naphthol AS-MX phosphate was dissolved in 2.5mL of 2-ethoxyethanol (EGME), 300mg Fast Red-Violet LB salt, to a final volume of 500mL using MilliQ $H_2O$.

Buffer A and Buffer B were combined in equal ratios, filtered and aliquoted and stored at -20 ºC before use.

**Tris-buffered solution (TBS; 10X and 1X) and TBS-Tween (TBS-T; 1X)**

A 10X TBS stock solution consisted of the following; Trizma base [0.5M] and NaCl [1.5M], in MilliQ $H_2O$.

A 1X (TBS) working solution was made by diluting the 10X stock solution in MilliQ $H_2O$, and stored at room temperature. TBS-T consisted of an addition of Tween [0.1%, v/v].

**Tris-Cl solutions (1.5M, pH 8.8; 1.0M, pH 6.8)**

Stock solutions of 500mL were made by weighing Tris-HCl to the calculated amounts to make 1.5M and 1.0M solutions in MilliQ $H_2O$. Each solution was adjusted to the required pH using HCl and/or NaOH, filtered and stored at room temperature.

**Triton X-100**

Triton X-100 was added to MilliQ $H_2O$ to the final concentrations as indicated.

**Washing buffer (for immunoprecipitation)**
A working solution consisted of the following; HEPES-KOH [20mM], NaCl [150mM] and MgCl₂ [1mM]. Prior to use, the following were added freshly to solution: PMSF [100µg/mL] and Triton X-100 [0.2%, v/v].

**Weigert's Ferric Haematoxylin**

Solution A: 1% (w/v) Haematoxylin crystal was made in 96% ethanol.

Solution B: 4mL of 30% (v/v) ferric chloride was added to 1mL 37% (v/v) HCl, made to a total of 100mL MilliQ H₂O, and filtered.

Working solution was made fresh with equal ratios of Solution A and B.

**Western Blot SDS Running Buffer (10X, 1X)**

A 10X stock solution consisted of the following; Trizma base [0.25M], Glycine [1.92M], SDS [1% w/v], in MilliQ H₂O. Solution was kept at room temperature.

A 1X working solution was made by diluted 10X SDS stock in MilliQ H₂O. Solution was kept at room temperature.

**Western Blot Transfer Buffer (overnight transfer)**

A working solution consisted of the following; Trizma base [25mM], Glycine [192mM] and methanol [10%, v/v] in MilliQ H₂O. Solution was kept at room temperature, and was not re-used.

**Western Blot Transfer Buffer (semi-dry transfer)**

A working solution consisted of the following; Trizma base [48mM], Glycine [39mM] and methanol [20%, v/v] in MilliQ H₂O. Solution was kept at room temperature, and was not re-used.
4.3. Methods

4.3.1. Animals

The generation of SNX27−/− mice was previously described by Cai et al., 2011. All experimental procedures were approved by the Institutional Animal Ethics Committees (AEC) of UQ and UWA. Approval #RA/5/1/417, AEC #100/1399.

4.3.1.1. Micro-computed tomography of SNX27 mice

Animals were sacrificed by cervical dislocation according to AEC approval/guidelines, where the whole body (5-day-old pups) or the hind legs (4-week-old mice) were fixed in 70% ethanol, or 4% PFA, respectively, for 24-hours. The latter were then rinsed in 1X PBS and stored in 70% ethanol. 5-day-old pups were kept intact and placed into a 10mL syringe, while the left legs (of 4-week-old mice) were gently cleaned from the surrounding muscle tissue, tightly wrapped in parafilm and placed upright in a 1mL syringe. SkyScan1174 was used to perform micro-computed tomography (µCT) with the following settings; voltage (50kV), current (800µA), image pixel size (WT, 32.52µm; KO, 6.1µm), exposure (5,000ms), rotation step (WT, 0.5°; KO, 0.4°), flat field correction (ON), frame averaging (ON), random movement (ON), sharpening (40%), filter (aluminium, 0.5mm).

The acquired data was then reconstructed using NRecon Software (Bruker, Belgium), where the reference line was chosen at the growth plate. For measurements within the metaphyseal region of the proximal tibia, 50 frames distal to the growth plate baseline was used for reconstruction (with a total of 300 frames). For measurements of the proximal diaphysis (tibial cortical bone), frames 700-800 distal to the baseline were chosen for reconstruction (with a total of 100 frames). Reconstruction settings were set to the following; smoothing (2), beam hardening correction (32%), ring artefact correction (12), angular step (0.5°).

The CTAn Software (Bruker, Belgium) was then used to analyse the reconstructed images, where the region of interest (ROI) was defined by free-hand...
at a total of 3-7 levels. Auto-interpolation between these levels produced the total ROI. The reference line for trabecular bone was set at the point where the calcified cartilage ridges of the growth plate fuse together. Measurements were performed on 200 frames distal to the reference point, 5µm apart, at the trabecular bone specified by the ROI. For the cortical bone analysis, the ROI was specified by measuring the total volume minus the medullary cavity. Analysis parameters were set to the following:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Abbreviation (unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone volume ratio</td>
<td>Bone volume / total volume, BV/TV (%)</td>
</tr>
<tr>
<td>Trabecular thickness</td>
<td>Tb.Th (µm)</td>
</tr>
<tr>
<td>Trabecular number</td>
<td>Tb.N (1/mm)</td>
</tr>
<tr>
<td>Trabecular separation</td>
<td>Tb.Sp (µm)</td>
</tr>
</tbody>
</table>

4.3.2. Histology and histomorphometry

4.3.2.1. Whole mount staining

The outer skin and internal organs were removed from sacrificed mice prior to fixation in 90% ethanol for a minimum of 7 days prior to staining. Samples were incubated in Alcian blue staining solution (0.01%, w/v) for 3 days and post-fixed in 96% ethanol for 6 hours. Samples were then cleared in 2% (w/v) KOH overnight and subsequently incubated in Alizarin red staining solution (0.003%, w/v) for 24 hours. Samples were then cleared in 1% (w/v) KOH and placed in 20% (v/v) glycerine/1% (w/v) KOH solution for further clearing before imaging.

4.3.2.2. Sample preparation (MMA/paraffin embedding)

Samples were fixed in 4% PFA or 10% neutral buffered formalin for 24 hours at 4°C and washed in 1X PBS (pH 7.4) for 45 minutes.
For MMA (plastic) embedding, samples were increasingly dehydrated in ethanol (50%, 70%, 80%, 96% and 100%) for 2-3 hours each, and then subsequently in xylene (100%) for 13 hours at room-temperature under vacuum conditions, using a Leica TP1020 processor. Samples were then removed and infiltrated in MMA infiltration solution at 4ºC under vacuum conditions for 72 hours. Samples were then embedded in Teflon moulds filled with polymerisation mixture, sealed with metal lids and left to polymerise at 30ºC (using a water bath) for 9-15 hours. Embedded samples were then removed following polymerisation and sectioned at 5μm thickness using Leica RM2255 and placed onto glass slides. Immediately prior to staining, resin was removed from sections using MEA and xylene.

For paraffin (wax) embedding, fixed samples were decalcified using 10% EDTA (pH 7-7.4) for 4-7 days and subsequently washed in 1X PBS. Samples were then dehydrated in the Leica TP1020 processor in increasing concentrations of ethanol, and infiltrated in xylene and then molten wax (60ºC). Samples were then promptly removed and embedded in wax using Tissue-TEK III (Miles Scientific, USA).

### 4.3.2.3. Immunohistochemistry (IHC)

Paraffin-embedded sections were de-waxed and dehydrated, and incubated in protease XXIV (0.05% diluted in 1X PBS) for 10 minutes at 37ºC, followed by incubation in testicular hyaluronidase (0.1% in acetate buffer, pH 6) for 90 minutes at 37ºC. Sections were then incubated in ethanol (100%) for 10 minutes at room-temperature, rinsed in 1X PBS and blocked in 10% FBS for 1 hour at room-temperature. Samples were washed in 1X PBS and incubated with the indicated primary antibody (1/50 dilution in 0.5% (w/v), BSA-PBS) for 2 hours at room-temperature, washed in 1X PBS and incubated in secondary antibody (1/200 dilution in 0.5% (w/v), BSA-PBS) for 1 hour at room temperature. Sections were then incubated in DAB for 5 minutes, washed in 1X PBS and counterstained with Gills haematoxylin for 3 minutes, dehydrated using ethanol and mounted.
4.3.2.4. **Safranin O staining**

MMA-embedded sections were stained for fast green (0.001% w/v, fast green) for 3 minutes, rinsed in 1% (v/v) acetic acid and stained in Safranin O (0.1%, w/v) for 5 minutes. Sections were rinsed in ddH$_2$O, dehydrated and mounted.

4.3.2.5. **Goldner’s trichrome staining**

MMA-embedded sections were incubated in Weigert’s haematoxylin for 30 minutes, rinsed under running tap water for 10 minutes, and stained in Ponceau Acid Fuchsin for 30 minutes. Sections were rinsed in 1% (v/v) acetic acid and incubated in Phosphotungstic Acid-Orange G for 5 minutes, rinsed in 1% (v/v) acetic acid and stained with Light Green (0.2%, w/v) for 15 minutes. Sections were then rinsed in 1% (v/v) acetic acid for a total of 15 minutes, dehydrated in isopropanol, followed by methycyclohexane and mounted.

4.3.2.6. **Histomorphometric analyses**

Stained femur/tibial sections were scanned using ScanScope® XT (Nikon, Japan) and areas of the proximal metaphyses were visualised and analysed using ImageScope (Leica Biosystems Imaging Inc., GmbH) and BioQuant® Osteo Software (BioQuant Imaging Analysis Corp., GmbH), respectively.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Abbreviation (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue volume</td>
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<tr>
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<td>BV (mm$^2$)</td>
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</tr>
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<td>OS (mm)</td>
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<tr>
<td>Osteoid width</td>
<td>O.Wi (µm)</td>
</tr>
<tr>
<td>Osteoblast number</td>
<td>OB.N (OB/mm)</td>
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<tr>
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<td>OC.N (OC/mm)</td>
</tr>
<tr>
<td>Osteocyte number</td>
<td>OY.N (OY/mm)</td>
</tr>
</tbody>
</table>
4.3.3. Isolation of total bone for gene expression profiling

Genetically unmodified C57BL/6J mice were freshly sacrificed by cervical dislocation, in accordance to AEC guidelines. The long bones of the hind limbs (femorae and tibiae) were cleanly dissected from the surrounding tissue and rinsed in 1X PBS. Long bones were then quickly frozen in liquid nitrogen and were grinded through the use of a mortar and pestle (that had been previously autoclaved and treated with RNase ERASE® solution (MP Biomedicals, USA)).

4.3.4. RNA isolation, First-Strand Synthesis and quantitative PCR

The resulting (total bone) powder was then lysed with 1mL TRIzol® (or 200-500µL TRIzol® for cell lysate), incubated for 5 minutes at room-temperature and transferred into RNase/DNase-free Eppendorf tubes. Chloroform (molecular grade) was then added (200µL per 1mL TRIzol® used), where the tube was shaken vigorously by hand for 15 seconds and then incubated for 3 minutes at room-temperature. Samples were then centrifuged at 12,000g for 15 minutes at 4°C. The transparent aqueous (top) layer was then transferred to fresh RNase/DNase-free tubes where 100% isopropanol (molecular grade) was then added to precipitate the solublised RNA (500µL per 1mL TRIzol® used). The tube was inverted by hand and incubated for 10 minutes at room-temperature and then centrifuged at 12,000g for 10 minutes at 4°C to pellet the RNA. The aqueous layer was then removed and the pellet was washed in 70% ethanol (molecular grade) (1mL per 1mL TRIzol® used) and centrifuged at 7,500g for 5 minutes at 4°C. Ethanol was then removed and the pellet was left to air-dry before resuspending in DEPC-treated H₂O. RNA was then quantified using the Nanodrop 4000 (Thermo Fisher Scientific Inc., USA).

First-strand synthesis was then performed using SuperScript III First-Strand Synthesis Kit (ThermoFisher Scientific Inc., USA) according to manufacturer’s recommended protocol.
Briefly, 3μg of RNA was used per reaction, where one reaction consisted of 50μM oligo(dT)$_{20}$ (1μL), 10mM dNTP (1μL) in a total of 10μL DEPC-treated H$_2$O. Samples were then placed on the Veriti 96 Well Thermal Cycler for 5 minutes (65°C), and then immediately placed on ice for 1 minute. 10μL of RT2 per reaction was then added, consisting of 10X RT buffer (2μL), 25mM MgCl$_2$ (4μL), 0.1M DTT (2μL), 40U/μL RNaseOUT (1μL) and 200U/μL SuperScript™ III RT (1μL). Samples were then gently mixed, briefly centrifuged and incubated for 50 minutes (50°C), followed by 5 minutes (85°C), and then immediately placed on ice. 1μL 2U/μL Rnase H was then added per reaction, and incubated for 20 minutes (37°C). The resultant cDNA was then diluted appropriately in DEPC-treated H$_2$O and stored at -20°C until use.

Quantitative PCR was performed using SensiMixII™ Probe Kit, and Universal Probe Library (UPL) according to manufacturer’s recommended protocol, and analysed using BioRad CFX™ Connect Real-Time System and CFX™ Manager Software.

Briefly, a total volume of 25μL was achieved through the optimised to the final volumes of 1X SensiMix(dT), Universal ProbeLibrary Probe (200nM), forward primer (400nM), reverse primer (400nM), MgCl$_2$ (5mM) and cDNA (5μL) in DEPC-treated H$_2$O. Samples were placed into 96-well PCR plates, and run for 15 minutes (95°C), amplified for 15 seconds (95°C), 60 seconds (60°C) and 15 seconds (72°C) for 35 cycles.

4.3.5. Microarray hybridisation

As described in Section 4.3.4. and Section 4.3.6.1, RNA was extracted from cultured primary cells sourced from freshly sacrificed mice. Mouse BMMs were cultured in the presence of M-CSF (25ng/mL) but in the absence of RANKL, while mature osteoclasts were generated after 7-day culture in M-CSF (25ng/mL) and RANKL (100ng/mL). The quality and concentration of the RNA obtained were measured using the Nanodrop 4000 (Thermo Fisher Scientific Inc., USA).
cRNA preparation and array hybridisation was performed by Dr. Jennie Hui from the Department of Molecular Genetics at PathWest Laboratory of Medicine (Perth, Australia) using Illumina® BeadArray Technology.

Briefly, a total of 200ng of RNA was converted to biotinylated-cRNA using the Illumina® TotalPrep RNA Amplification Kit according to the manufacturer’s protocol. Reverse transcription was performed at 42°C for 2 hours to synthesise the first strand of cDNA. The second cDNA strand was synthesised by the addition of DNA polymerase I and RNase H, followed by an incubation at 16°C for 2 hours. The cDNA obtained was purified using a cDNA filter cartridge before undergoing \textit{in vitro} transcription with RNA polymerase at 37°C for 14 hours. \textit{In vitro} transcription resulted in the generation of multiple copies of biotinylated antisense RNA molecules from mRNA in the samples. The cRNA was purified and the eluted biotin-cRNA was evaluated for both quantity and quality prior to being loaded directly onto individual array spots on the Illumina® Mouse WG-6 v2.0 BeadChip and hybridised at 58°C for 19 hours. Hybridisation to each probe was assessed from an average of 30 separate beads, each of which contained identical oligonucleotide sequences. The BeadChip consisted of a total of 45,281 50-mer oligonucleotide probes, where 34,526 probes were targeted to Reference Sequence (RefSeq) transcripts, 4,922 probes targeted at RIKEN transcripts and the remaining 5,833 probes targeted to Meebo transcripts. Following hybridisation, the BeadChip was washed and fluorescently labelled and scanned in the Illumina® BeadArray Reader using Illumina® BeadScan image software.

The data generated was initially analysed using Illumina® BeadStudio 3 software. The raw expression levels were subjected to normalisation using the ‘quantile normalisation’ function in the software. To test for the occurrence of false positives, multiple testing corrections were performed using the ‘Benjamin-Hochberg False Discovery Rate’ function. The normalised data was then exported and further analysed using the GeneSpring GX 11.0 software (Agilent Technologies).
The expression of SNX genes were then listed relative to each other and displayed as a heatmap, where the colours red and blue arbitrarily represent an increase and decrease in relative expression, respectively.

4.3.6. **Primary cell culture**

All experimental procedures were approved by the Institutional Animal Ethics Committees of The University of Queensland and The University of Western Australia. Animals were sacrificed by cervical dislocation, and the femur and tibia were both isolated from 4-week-old mice by careful dissection to minimise the removal of the surrounding muscle tissue. The surrounding tissue and periosteal layer was removed by gentle scraping through the use of a scalpel, and rinsed in 1X PBS prior to the isolation of bone marrow. The spleen was also harvested from sacrificed mice for the isolation of monocytes/macrophages for osteoclastogenesis.

Primary human osteoclasts were also derived from giant cell tumour (GCT) tissue derived from bone, as described in Huang et al., 2000. All patients consented with the approval obtained from the Sir Charles Gairdner Hospital (SCGH) Human Ethics Committee.

4.3.6.1. **Primary osteoclast cell culture**

Murine osteoclast precursors (monocyte/macrophages) were isolated from spleen tissue through the use of a cell strainer (100µm) and cultured in complete medium (α-MEM + 10% FBS, 100U/mL penicillin, 100U/mL streptomycin) supplemented with M-CSF (25ng/mL dilution, or 1/20 dilution of conditioned medium). Following sub-confluency, cells were then trypsinised using TrypLE™ Express and re-seeded at 6x10³ cells/well (96-well) for osteoclastogenesis on plastic, glass coverslips or bone slices*. Osteoclastogenesis was induced following culture in complete medium supplemented with M-CSF (25ng/mL) dilution with RANKL (100µg/mL) stimulation every other day for up to 7 days.
Human-derived osteoclasts were harvested by mechanical disaggregation of the osteoclastoma tissue, and were cultured directly on bovine bone discs in the presence of recombinant human M-CSF and RANKL (100ng/mL).

*Bovine bone purchased from the butcher was thoroughly cleaned to remove any excess tissue or bone marrow. Once cleaning was completed, the bone was sliced into 0.75mm-thick sections using an IsoMet low speed saw (Beuhler) and diamond wafering blade (Biolabs Australia, Pty Ltd). 5mm-wide discs were punched from these slices using a single hole paper punch and stored in 70% ethanol at 4°C until required. Before use, the bovine bone discs were washed three times with serum-free α-MEM under sterile conditions to ensure the complete removal of ethanol. Prior to cell seeding, these bone slices were placed into the wells of a 96-well plate, submerged in 100µL of complete α-MEM and incubated at 37°C for at least 30 minutes. After the incubation, the media was removed and seeded with mouse BMMs or primary human osteoclasts.

4.3.6.2. Osteoclast TRAP staining

Mature osteoclasts cultured in 96-well plates were then fixed in 4% PFA, washed twice with 1X PBS and stained with pre-filtered TRAP stain for 30-60 minutes at 37°C. Stain was then removed and cells were washed with MilliQ H₂O prior to imaging with a Nikon Ti-E inverted motorised microscope (Nikon, Japan).

4.3.6.3. Primary osteoblast culture

Following the isolation of the long bone, the epiphyses were removed by a scalpel, and the bone marrow was then flushed using 25G needle and syringe, using complete-αMEM (10% FBS, 100U/mL streptomycin, 100U/mL penicillin). The isolated bone marrow was then resuspended and collected by centrifugation at 1,500 rpm for 5 minutes, seeded into T25 or T75 in complete-αMEM supplemented with ascorbic acid (50µg/mL) and maintained under standard cell culture conditions.
(humidified conditions at 37°C, 5% CO₂). Cells were sub-cultured or seeded for use when reaching confluency.

The remaining bone shafts were then chopped into 0.5-1mm fragments, where bone-derived osteoblasts were isolated as previously described by Bakker and Klein-Nulend, 2012 and Kular et al., 2015.. Following the successful explant of bone-derived osteoblasts onto the cultured surface (approximately 7-days following the undisturbed culture of bone chips), the cells were then removed from the culture surface through the use of trypsin-EDTA at 37°C for 5 minutes, followed by gentle scraping. Cells were then centrifuged at 1,500 rpm for 5 minutes, re-seeded and maintained in complete-αMEM supplemented with ascorbic acid (50µg/mL). Bone chips were discarded and not re-plated.

Calvarial osteoblasts were also isolated from sacrificed pups (~5-days-old) as described by Bakker and Klein-Nulend, 2012 and Kular et al., 2015.. Calvarial osteoblasts were cultured in complete-αMEM and sub-cultured following confluency, and cultured under standard cell culture conditions.

Where cells were required to be cryo-preserved, cells were centrifuged and resuspended in freezing media (92% FBS, 8% DMSO) and slowly frozen at the rate of -1°C/min, to a final temperature of -80°C, and stored in the gaseous phase of N₂, in accordance to the conventional protocol.

4.3.6.4. Osteoblast time-course stimulation assays

Once cultured primary osteoblasts reached confluency, cells were trypsinised as described above. Cells were then seeded at 1x10⁵ cells/well (12-well plate) for 24 hours, where they were then serum starved for 1 hour prior to stimulation with PTH(1-34) or PTHrP peptides (100nM), or BMP2 (100ng/mL), as indicated. In the case where osteoblasts were stimulated for 15 minutes or longer, (with the exception of BMP2), PTH or PTHrP (100nM) was added to the cells for 15 minutes, followed by ligand-washout (comprising of two washes with 1X PBS). Culture medium was then replaced with fresh complete-αMEM supplemented with
ascorbic acid (50µg/mL). Following the completion of the time-course, cells were washed with ice-cold 1X PBS and lysed with Ripa Ripa buffer, on ice for 20 minutes with gentle agitation. Cell lysates were then collected and centrifuged at 15,000 rpm for 30 minutes at 4°C. Post-nuclear supernatant was then removed and stored at -20°C, while the cell pellet (debris) was discarded.

4.3.6.5. Isolation of RNA from osteoblast cell cultures

Primary osteoblasts were cultured as described above, and seeded at 1x10^5 cells/well (12-well plate) for 24 hours, where they were then serum starved for 1 hour prior to stimulation with PTH(1-34) at 100nM at 37°C. Following 15 minutes post-stimulation, ligand-washout was performed as described above and cultured under normal complete-αMEM supplemented with ascorbic acid (50µg/mL), until the indicated times. Following the completion of the time course, cells were washed with ice-cold 1X PBS and lysed with TRIzol® for 5 minutes at room-temperature. RNA isolation, first-strand synthesis and qPCR were performed as described in Section 4.3.4.

4.3.6.6. Osteoblast alkaline phosphatase (ALP) assay

Primary osteoblasts were seeded at 4x10^4 cells/well (24-well plate) and maintained in osteogenic medium (complete α-MEM supplemented with 50µg/mL ascorbic acid, 2mM β-glycerophosphate, 1x10^{-8}M dexamethasone) with or without the presence of intermittent PTH (50nM) or BMP2 (30ng/mL) where indicated. Cell culture medium was replaced every other day, with a washout of PTH following 4 hours of stimulation. Cells were then fixed in Citrate-Acetone-Formaldehyde fixative and stained using the Leukocyte Alkaline Phosphatase Kit (Sigma-Aldrich Co., USA) according to the manufacturer’s protocol, to detect ALP activity.

4.3.6.7. Osteoblast mineralisation assay

Primary osteoblasts were cultured and maintained as described above, and fixed after 28 days using 4% PFA for 15 minutes at room temperature. Wells were
then rinsed with 1X PBS, followed by Milli-Q H₂O. Alizarin red staining solution (ARS, 40mM) was filtered prior to addition to each well, and incubated for 20 minutes at room temperature on a rocking platform. ARS was removed and wells were rinsed three times in Milli-Q H₂O for 5 minutes per wash, on a rocking platform. Wells were then left to air-dry prior to scanning and quantitation by de-staining (see below).

Bone nodules were then de-stained following the addition of 10% (w/v) cetylpyridinium chloride in 10mM sodium phosphate and incubated for 30 minutes at room temperature, on a rocking platform. The resulting stain was removed and read in duplicate at 595nm, in parallel with a standard of varying concentrations of ARS.

4.3.6.8. **Osteoblast proliferation assay**

Primary osteoblast were seeded into a 96-well plate at 8x10³ cells/well, in complete α-MEM supplemented with ascorbic acid (50µg/mL) for 48-hours prior to assessment. Osteoblast proliferation was then analysed using CellTiter 96® Aqueous One Solution Cell Proliferation (colourimetric) Assay according to the manufacturer’s protocol. After the initial 48-hours, the cell culture media was replaced with 100µL of fresh culture media with or without PTH(1-34) at the concentrations indicated. 20µL of CellTiter 96® Aqueous One Solution Cell Proliferation reagent was added per well, and a colourimetric measurement was taken immediately and following 4 hours of incubation, at 490nm.

4.3.6.9. **Oil red O assay**

Primary osteoblasts were cultured and maintained as described above, and fixed after 28 days using 4% PFA for 15 minutes at room temperature. Wells were then rinsed twice in Milli-Q H₂O, followed by the addition of isopropanol (60%) for 5 minutes at room temperature. Isopropanol was then removed and wells were allowed to air-dry completely prior to the addition of oil red O (working solution) and incubated for 10 minutes at room temperature. Oil red O solution was removed
and were immediately washed four times in Milli-Q H₂O. Images were then taken prior to de-staining (see below).

Cells were de-stained following the addition of isopropanol (100%) and incubated for 10 minutes at room temperature. The resulting stain was then removed and read in duplicate at 500nm.

4.3.7. Cell line culture

4.3.7.1. C2C12 cell line culture

The C2C12 (myoblast-like) cell line was purchased from ATCC and cultured in complete-DMEM (10% FBS) supplemented with 100U/mL penicillin, 100U/mL streptomycin. Culture medium was changed every other day, and sub-cultured following sub-confluency, using Trypsin-EDTA. RNA was extracted when cells reached sub-confluency, as described in Section 4.3.4.

4.3.7.2. MC3T3-E1 and UMR106-01 cell culture and transfection

MC3T3-E1 and UMR106-01 cell lines were purchased from ATCC and cultured in complete-MEM (10% FBS) supplemented with 100U/mL penicillin, 100U/mL streptomycin. Cells were maintained at 37°C in humidified conditions at 5% CO₂, with the culture medium changed every other day. Upon reaching sub-confluency, cells were then trypsinised using Trypsin-EDTA.

Cells were transfected using FuGENE® 6 (3:1 reagent:DNA ratio) according to manufacturer’s protocol.

4.3.7.3. MLO-Y4 cell line culture

The MLO-Y4 (osteocyte-like) cell line, generously provided by Dr Lynda Bonewald, University of Missouri-Kansas City, School of Dentistry, Department of Oral Biology, was cultured on rat tail collagen type I-coated T75 flasks until sub-confluency. Rat tail collagen type I was prepared in acetic acid to a concentration of 0.15mg/mL, and incubated on the surface of the culture flask for 1 hour and washed
with sterile 1X PBS prior to the addition of cells. Media was changed every 3-days using complete α-MEM (2.5% FBS, 2.5% FCS, 100U/mL penicillin, 100U/mL streptomycin). RNA extraction was conducted when cells reached sub-confluency (~60%), as described in Section 4.3.4.

4.3.7.4. OCY454 cell culture and transfection

The OCY454 cell line was generously gifted from Dr. Lynda Bonewald (Indiana Center for Musculoskeletal Health, Indianapolis USA) and cultured in complete-MEM (10% FBS) supplemented with 100U/mL penicillin, 100U/mL streptomycin. Cells were maintained at 33ºC in humidified conditions at 5% CO₂, and moved to 37ºC for 24 hours prior to transfection or differentiation. The culture medium changed every other day and upon reaching sub-confluency, cells were trypsinised using Trypsin-EDTA.

Cells were transfected using FuGENE® 6 (3:1 reagent:DNA ratio) according to manufacturer’s protocol.

4.3.7.5. HEK293 cell line transfection

Human embryonic kidney (herein HEK293) cells were maintained in complete DMEM-Glutamax High Glucose (10% FBS, 100U/mL penicillin, 100U/mL streptomycin) and maintained at 37ºC, in humidified conditions at 5% CO₂.

Stable cell lines were established using Lipofectamine LTX (ThermoFisher Scientific Inc., USA) as a transfection reagent, according to the manufacturer’s protocol. Following the successful expression of the protein of interest, stable cell lines were then derived through antibiotic selection using G418 (500μg/mL) and/or puromycin (2μg/mL) as appropriate.
4.3.7.6. **HEK293 cell line PTH/PTHRP time-course stimulation assay**

Transfected HEK293 cells were seeded at $7 \times 10^5$ cells/well (6-well plate) for 24 hours prior to stimulation. Cells were then serum starved for 1 hour prior to the addition of PTH(1-34) (100nM) for 15 minutes, followed by a washout after 15 minutes. Cells were then cultured under normal complete culture medium for the remaining time period. Following the completion of the time-course, cells were then washed with ice-cold 1X PBS and lysed in Ripa Ripa buffer and incubated on ice for 20 minutes with gentle agitation. Cell lysate was then collected and centrifuged at 15,000rpm for 20 minutes at 4°C, with the final post-nuclear supernatant (PNS) collected and stored at -20°C.

4.3.8. **Immunoblotting**

Protein concentrations were determined by Bradford Assay and subsequent dilutions were made in 4X or 2X loading dye and boiled at 99°C for 5 minutes. A total amount of 6μg – 10μg were used per lane, and resolved by SDS-PAGE (8-12% polyacrylamide gels) and transferred using semi-dry or overnight methods.

Western blot was then performed according to standard protocols. Briefly, protein membranes were blocked in 5% (w/v) skim milk for 1 hour at room temperature on a rocking platform, washed twice with TBS-T prior to incubation with primary antibodies at either 2 hours (room-temperature) or overnight (4°C) on a rocking platform. Membranes were then washed three times with TBS-T following incubation with secondary HRP-conjugated antibodies for 45 minutes (room-temperature). Membranes were then washed with TBS-T and TBS and detected using chemoluminescence.

Protein bands were semi-quantified using densitometry analyses using Adobe Photoshop CC (2014+).
4.3.9. Immunoprecipitation

HEK293 cells were seeded at 4x10⁶ cells/dish (10cm dish) for 24 hours prior to experiment. Cells were then transfected using Lipofectamine LTX as previously described.

After 48 hours post-transfection, cells were stimulated with PTH(1-34) (100nM) for 10 minutes. Cells were then rinsed in ice-cold 1X PBS and lysed in extraction buffer for 20 minutes on ice, with gentle agitation. Cell lysates were then homogenised using 25G syringe and centrifuged at 15,000rpm for 15 minutes at 4°C, with the cell pellet discarded. Triton X-100 was then added [final, 1%] and lysates were incubated for 1 hour at 4°C on a rotation wheel, followed by centrifugation at 100,000g for 1 hour at 4°C. The resulting lysate was incubated with GFP-Trap_A beads and incubated overnight at 4°C on a rotation wheel.

Protein/bead slurry was then centrifuged at 3,000rpm for 3 minutes at 4°C and the resulting supernatant was removed. Beads were then washed repeatedly and resuspended in 2X non-denaturing loading dye and boiled for 5 minutes at 95°C. The supernatant mixture was then removed by pipette and resolved by SDS-PAGE and blotted as previously described.

4.3.10. ELISA assay

4.3.10.1. Cyclic AMP assay

Primary osteoblasts were seeded at 6x10⁴ cells/well (96-well plate) for 24 hours prior to experiment. Cells were then serum starved for 1 hour prior to the addition of IBMX (200μM) for 30 minutes. Cells were then stimulated with PTH(1-34) (100nM), forskolin (10µM) or isoproterenol (1µM) for 15 minutes and subsequently analysed for cyclic AMP levels according to manufacturer's protocols, using the Cyclic AMP XPTM Assay Kit.
4.3.10.2. Serum-PTH assay

Sample blood was obtained from SNX27 wild-type and knock-out mice via heart puncture, centrifuged at 3,000 rpm for 5 minutes at 4°C and serum (top layer) was removed. Serum [PTH] was measured according to manufacturer’s protocol using the Mouse parathyroid hormone (PTH) ELISA Kit (Cusabio, USA). Briefly, serum was then diluted ¼ using sample diluent (provided), added to ELISA plate for 2 hours at 37°C. The solution was then removed and biotin was added for 1 hour at 37°C. The solution was again removed from the wells, and washed repeatedly prior to the addition of HRP-avidin to wells and incubated at 37°C. Following 1 hour, the solution was removed and the wells were washed repeatedly prior to the addition of TMB substrate for 30 minutes at 37°C. The reaction was then stopped and the plate was read at 450 nm.

4.3.10.3. Serum-Vitamin D3 assay

Sample blood was obtained as previously described. Serum [VitD₃] was measured according to manufacturer’s protocol using the Mouse 1,25-dihydroxyvitamin D3 (DVD/DHVD3) ELISA Kit (Cusabio, USA). Briefly, serum was diluted 1/40 using sample diluent (provided), added to ELISA plate at 1:1 ratio with HRP-conjugate solution (provided). The plate was incubated for 1 hour at 37°C, after which the solution was removed from wells and washed repeatedly prior to the addition of substrate A and B (1:1). The plate was then incubated for 15 minutes at 37°C. The reaction was then stopped and the plate was read at 450 nm.

4.3.11. Immunofluorescence and time-lapse confocal microscopy

Calvarial bones were isolated from freshly culled mice, in accordance to Animal Ethics Guidelines. The calvarial bone was then thoroughly cleaned and rinsed in 1X PBS, prior to fixation in 4% PFA for 24 hours, and stored at 4°C prior to immunostaining as described below.

HEK293 cells were seeded onto poly-L-lysine-coated glass coverslips at 2x10⁴ cells/well (96-well plate) for 48 hours. Cells were then serum starved for 1
hour prior to stimulation with PTH(1-34) (100nM) or PTH-TMR (100nM) for 0-120 minutes, with a washout following 15 minutes post-stimulation. Following the completion of the time-course, cells were then washed in ice-cold 1X PBS and fixed in 4% PFA (room-temperature). Cell surface PTH-TMR labelling was detected by subjecting cells to an ice block (4°C, 10 minutes) throughout the duration of agonist stimulation, washed in ice-cold 1X PBS and fixed in 4% PFA (room-temperature).

Cells were permeabilised with 0.1% Triton X-100 or 0.1% Saponin for 5 minutes and washed twice with 0.2% BSA-PBS to remove all traces of detergent. Cells were then subsequently blocked in BSA-PBS (3%) for 30 minutes (room-temperature) prior to incubation with primary antibodies (diluted in 0.2% BSA-PBS) for 1-1.5 hours (room-temperature). Cells were then thoroughly washed four times with 0.2% BSA-PBS, four times with 1X PBS, four times with 0.2% BSA-PBS and then incubated with secondary antibodies diluted in 1X PBS for 45 minutes (room-temperature). Cells were then washed again as previously described, and stained with Hoechst (1:5000 dilution) and mounted in ProLong® Diamond Antifade.

Detection of immunofluorescence was carried out using a Nikon A1Si confocal microscope running NIS-C Elements Software. For the detection of primary osteoclasts and HEK293 cells, a 40X or 60X oil immersion objective lens (Nikon, Japan) was used, where serial optical sections (z-stacks) of 0.5µm-1.0µm were used to reconstruct 2D or 3D projections in FIJI.

Time-lapse confocal microscopy was performed under controlled atmospheric conditions (5% CO₂, 37°C) in a Tokai Hit Stage Top incubator (INU2E-TIZ) as previously described in Ng et al., 2013.

4.3.12. Bioluminescence resonance energy transfer (BRET) assay

HEK293 cells stably expressing Rluc-PTHR were transiently transfected with SNX27-GFP constructs using Lipofectamine LTX. 24-hours post-transfection, cells were then trypsinised, washed three times in 1X PBS before resuspension in HBSS buffer and re-seeded onto white-bottom 96-well plates at 1.5x10⁵ cells/well.
Following an additional 24-hours, cells were then assessed for bioluminescence resonance energy transfer (BRET) using the PolarStar Optima Spectrofluorometer (BMG Labtechnologies) and accompanying software.

Briefly, 50μL of 10μM coelentetrazine-h substrate was added to the first well, and the background luminescence and fluorescence was detected over 10s. Immediately following this, PTH(1-34) (final [100nM]) was added to the well for 14 minutes, at which time the emission at both 530nm (EYFP or GFP) and 475nm (Renilla luciferase) were detected at 1s intervals, for a total of 60s. This was performed in triplicates, where the average readings for each triplicate were then used to determine the normalised BRET ratio. HEK293 cells expressing \text{Rluc-PTHr} and co-transfected with EYFP vector alone served as a non-interacting partner (negative control).

Normalised BRET ratio:

\[
\frac{\text{Emission (530nm)} - \text{Emission (475nm)} \times \text{cf}}{\text{Emission (475nm)}}
\]

Where,

\(\text{Emission (530nm)} = \text{sum of EYFP signal collected over 60s}\)

\(\text{Emission (475nm)} = \text{sum of Renilla luciferase signal collected over 60s}\)

\(\text{cf} = \text{the simple BRET ratio, i.e.}\)

\[
\frac{\text{Emission (530nm)}}{\text{Emission (475nm)}}
\]

The normalised BRET ratio eliminates data variability caused by fluctuations in light output, which can be a consequence of variations in assay volume, cell density and/or signal decay across the plate.

**4.3.13. Protein expression, purification and crystallisation**

Experiments describing protein purification, crystallisation, structure determination and isothermal titration calorimetry (ITC) were performed by A/Prof Brett Collins, Thomas Clairfeuille and colleagues from the Institute of Molecular
Bioscience, University of Queensland, Australia, as described in Chan et al., 2016, and below.

cDNAs of residues 9-327 of VPS26A (mouse) and 40-135 of SNX27 (rat) were cloned in a pMW172KanH6 plasmid downstream of a hexahistidine tag and in a pGEX4-T2, yielding an N-terminally GST-tagged protein. The QuikChange II Kit (Agilent Technologies) was used for site-directed mutagenesis. Proteins were expressed in *Escherichia coli* BL21 (D3) strain overnight at 20°C and purified using affinity chromatography followed by gel filtration. VPS26A was purified on a nickel-nitrilotriacetic acid gravity column and eluted with 300mM imidazole in buffer of 200mM NaCl and 20mM HEPES (pH 7). The SNX27 PDZ domain was purified on a glutathione-Sepharose gravity column and eluted after three hours of thrombin cleavage in 200mM NaCl/25mM Tris (pH 8) buffer. Proteins were then gel filtered using a Sepharose S200 16/60 column attached to an AKTA system (GE Healthcare, Waukesha, WI). For crystallisation, SNX27PDZ fractions were gel filtered into 20mM Bis/Tris plus 30mM NaCl (pH 6.5) buffer, pooled and concentrated and then directly mixed together to a 2:1 M ratio of SNX27 PDZ domain to PTHR peptide, where the final SNX27 PDZ concentration was 25mg/mL. Four 96-well crystallisation hanging-drop screens were set up using a Mosquito Liquid Handling robot (TTP LabTech, Melbourne, UK) at 20°C. Optimised diffraction-quality crystals were obtained using the sitting drop vapour diffusion method and a buffer containing 0.1M Bis/Tris (pH 5.5) and 2M ammonium sulphate.

4.3.14. Data collection and structure determination

Data were collected at 100K at beamline MX2 (Australian Synchrotron) and integrated and scaled with MOSFLM and SCALA. The structure was determined by molecular replacement using Phaser-MR (McCoy et al., 2007) with the SNX27 PDZ domain crystal structure as an input (Protein Data Bank [PDB code] 3QDO). A model was built using COOT (Emsley et al., 2010), followed by repeated refinement and model building with PHENIX, REFINE and COOT. All residues in the final model were built in accordance with Ramachandran statistics. The final structure
was solved at 0.95Å resolution and revealed electron density corresponding to all amino acids contained in the synthetic peptide but the Q residue. All structural figures were generated using PyMOL (www.pymol.org).

4.3.15. Isothermal calorimetry titration (ITC) assay

ITC experiments were performed on a MicroCal iTC200 instrument (Malvern, Malvern, UK) in 50mM Tris (pH 8)/100mM NaCl. The PTHR (Q^7-E^6-E^5-W^4-E^3-T^2-V^1-M^0) peptide at a concentration of 0.875mM was titrated into 50µM SNX27 PDZ domain WT or H114A (supplemented with 50µM VPS26A when required) protein solutions at 25°C. Data were processed using ORIGIN to extract the thermodynamic parameters ΔH and K_a (1/K_d) and the stoichiometry n. Here, ΔG and ΔS were derived from ΔG = -RT ln K_a and ΔG = ΔH – T ΔS.

4.3.16. Statistical analysis and data presentation

All data presented in this thesis is expressed as the mean ± standard error of the mean (SEM), where the results are representative of at least three independent experiments. In the case of the direct comparison between control and variable, an unpaired Student’s T-Test was performed, where * indicates p value < 0.05, ** p < 0.01, and *** p < 0.005.

4.4. Software and equipment

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Quantitative profiling of Sorting Nexins in bone identifies SNX27 as a crucial modulator in skeletal homeostasis
5. Quantitative profiling of Sorting Nexins in bone identifies SNX27 as a crucial modulator in skeletal homeostasis

5.1. Introduction

Skeletal development and homeostasis are dependent on the coordinated function of several highly-specialised resident cells, namely the chondrocyte, osteoclast, osteoblast and osteocyte (as described in Chapter 1). To ensure cellular cooperativity and homeostasis, each skeletal cell type has evolved sets of specialised intracellular machinery that facilitate the correct targeting and delivery of newly synthesised proteins as well as the secretion of proteolytic enzymes that collectively help build, shape and remodel the skeleton. This is particularly evident during longitudinal bone growth, which place high demands on intracellular trafficking in chondrocytes and osteoblasts in order to generate a cartilaginous template and ensure its ensuing mineralisation (Zhao, 2012).

The importance of intracellular trafficking during skeletal development is further exemplified by the increasing number of skeletal dysplasias that have been directly attributed to the disruption of vesicular transport machinery and/or the cargo (briefly mentioned in Chapter 1), such as osteogenesis imperfecta, whereby individuals present with ‘bowed’ bones, a barrel chest and vertebral compressions due to the inability of osteoblasts to synthesise or secrete collagen type 1 (Forlino and Marini, 2016).

While the importance of membrane and protein trafficking in skeletal homeostasis and disease has gained momentum over the past decade, we have only just recently begun to appreciate the complete spectrum and diversity of proteins that regulate intracellular trafficking processes unique to each skeletal cell type and function. One such family of trafficking proteins that have received increasing
attention are Sorting Nexins (SNXs). As reviewed in Chapter 2, SNXs bind endosomal membranes via their PX domain and thus facilitate endocytic transport. While 34 SNXs have been identified in human cells, few have been functionally characterised. Nonetheless, an increasing number of SNXs have been linked to human disease (extensively reviewed in (Teasdale and Collins, 2012)), including \textit{SNX10}, a short, PX-only domain-containing member in which mutations have been shown to correspond with malignant osteopetrosis (Aker et al., 2012, Megarbane et al., 2013, Pangrazio et al., 2013). While the involvement of SNX10 in osteopetrosis has helped to uncover a previously unappreciated link between SNX proteins and skeletal homeostasis, the exact nature and number of SNXs that potentially regulate skeletal cell function and bone homeostasis is presently unclear.

Therefore, this Chapter aimed to:

(i) Quantitatively determine the complement of SNXs expressed in bone tissue and skeletal cells and;

(ii) Functionally characterise candidate SNX members and evaluate their potential role in skeletal development using genetically-modified mice.

5.2. Results

To begin to address the potential contribution of SNX proteins in bone, the gene expression profile of SNX1-33 were systematically screened using mRNA isolated from both total bone and individual skeletal cell types, as described in Materials and Methods (Figure 5.1-5.2). Figure 5.1 illustrates the relative expression of SNXs in total bone as determined through quantitative PCR (qPCR), normalised to the reference gene hydroxymethylbilane synthase (HMBS) (Stephens et al., 2011), where an arbitrary line has been placed to identify relatively high- and low-expressing SNX proteins. Of the 33 SNXs expressed in bone tissue (SNX34 expression was unavailable to be assessed at the time), SNX27 was the most pronounced, followed by SNX21 and SNX25. In comparison, the evolutionary-
Figure 5.1. Gene profiling via qPCR reveals the differential expression of the SNX protein family in total bone.
C57BL/6J mice were sacrificed and the long bones (femorae and tibae) were cleanly dissected from the surrounding tissue. Total bone mRNA was isolated as described in Materials and Methods. First-strand synthesis was performed using SuperScript™ III Reverse Strand Synthesis Kit, according to manufacturer’s protocol. Quantitative PCR (qPCR, probe method) was then used to generate a relative gene expression profile of SNX1-33 and are presented as relative to the housekeeping gene, HMBS. Expression levels are shown on an arbitrary scale, with a line drawn at 1 to highlight those genes of relatively higher expression levels.
conserved SNX-BAR proteins (SNX1, 2, 5 and 6) were amongst those modestly-expressed. While this profile provided a simple overview of the relative abundance of SNXs in bone, it did not discriminate between skeletal cell populations. An initial micro-array analysis was performed using the Illumina® Mouse WG-6 v2.0 BeadChip, assessing most SNX members (with the exclusion of SNX23, 28 and 32) from mRNA isolated from primary cultures of bone marrow macrophages (BMM) cultured in the presence of M-CSF or in the additional presence of RANKL (100ng/mL) to generate mature osteoclasts (OC) (Figure 5.2A). Based on this assessment, SNX10 was most prominently expressed in osteoclasts and robustly upregulated during RANKL-induced osteoclastogenesis. Notably, the only other SNX protein that was upregulated during osteoclast differentiation was SNX27, albeit less pronounced that SNX10. In comparison, a few SNXs were found to be downregulated, including SNX13, followed by SNX2, 3 and 9. We next validated the SNX expression data by qPCR (Figure 5.2B). In addition to primary bone marrow macrophages and osteoclast cultures, qPCR was performed using mRNA sourced from two immortalised cell lines, namely C2C12 (early myoblast-like cell line, possessing osteoblast potential) and MLO-Y4 (osteocyte-like cell line) (Figure 5.2B). In keeping with our microarray data, expression of SNX10 was robustly upregulated in osteoclasts. In comparison, SNX5 was found to be abundantly expressed in BMMs although this was not reflected in our microarray data in Figure 5.2A, possibly owing to differences in the sensitivities of the two approaches used. In addition, the relative expressions of SNX1-6 (with the exception of SNX2 and 5) were found to be relatively stable between the different cell types, implying a general requirement of SNX-BAR proteins for all bone cells.

Since SNX10 and SNX27 were among the most abundant SNX family members expressed in osteoclasts and bone tissue, respectively, they were selected as potential candidates for more detailed characterisation. First, the tissue distribution of SNX10 and SNX27 were assessed through the use of the online gene annotation portal, bioGPS (Figure 5.3-5.4, as described in Wu et al., 2015). Consistent with the gene expressions generated from qPCR and micro-array
Figure 5.2. Gene profiling in individual skeletal-resident cell types identifies SNX10 and SNX27 amongst the highest expressing SNXs in osteoclasts.

(A) Bone marrow macrophages (BMM) were isolated from the bone marrow of long bones of freshly culled, genetically unmodified C57BL/6J mice and cultured in complete αMEM (supplemented with M-CSF) until sub-confluency. BMMs were then re-seeded and cultured under M-CSF alone (BMM) or in the presence of RANKL (100ng/mL) every other day, for 7 days to induce the formation of osteoclasts (OC). Illumina® BeadArray Microarray Technology was then used to assess the SNX gene expressions from BMM and OC mRNA extracted from in vitro cultures. Expression values are depicted arbitrarily as a heatmap, where SNX10 and SNX27 were found to be upregulated compared to other SNX proteins.

(B) mRNA was extracted from in vitro cultures of primary skeletal-resident cells (BMM, OC) as described above, and from myoblastic (C2C12) and osteocyte-like (MLO-Y4) cell lines, as described in Materials and Methods. qPCR was then performed to generate an expression profile of SNX1-33 and are shown relative to each other, normalised to the housekeeping gene, HMBS.
analyses, both SNX10 (Figure 5.3) and SNX27 (Figure 5.4) were found to be most prominently expressed in osteoclasts. Next, the endogenous expression and distribution of SNX10 and SNX27 was confirmed in human osteoclast-like cells at the protein level by immunofluorescence confocal microscopy using antibodies specific to SNX10 and SNX27 (Figure 5.5). Endogenous SNX10 (Figure 5.5A) and SNX27 (Figure 5.5B) were stained alongside filamentous actin (F-actin, red) and Hoechst (nuclear stain, blue) in the form of a Z-stack projection of 5 images, totalling 6µm thick. As expected both SNX10 and SNX27 were found to be abundantly expressed in osteoclastic cells, predominantly residing on puncta distributed throughout the cytosol whose size and distribution were most consistent with endosomes.

Collectively, these expression data indicate that SNX10 and SNX27 are highly expressed in osteoclasts and bone tissue, and thus suggest that these SNX members may play important roles in osteoclast function and/or skeletal homeostasis. Of these, SNX10 represented the most promising candidate for further characterisation, however during the course of this dissertation, the expression of SNX10 in osteoclasts was independently reported (Zhu et al., 2012) and subsequently shown that SNX10 deficiency corresponds with osteopetrosis (Ye et al., 2015). Nonetheless, because SNX27 had not been previously linked to bone it was chosen for further investigation.

To investigate the potential contribution of SNX27 in skeletal homeostasis, we examined the bone phenotype of SNX27-deficient mice previously generated by (Cai et al., 2011). Because SNX27-deficient mice die 4-6 weeks postnatally, studies were restricted to the first 4-weeks of postnatal bone growth. First, micro-computed tomography (µCT) and histology were employed to assess the skeletal phenotype of wild-type (WT), heterozygous (Het) and SNX27 globally-deficient (KO) mice (Figure 5.6). Consistent with previous reports (Cai et al., 2011) SNX27 knockout mice exhibited slightly smaller birth size and weight however there were no overt gross abnormalities observed at 5-days of age when compared to their wild-type or
Figure 5.3

SNX10 (Mus musculus)
Figure 5.3. Distribution of SNX10 across various tissues reveals its prevalent expression in mature osteoclasts.

The online gene annotation portal, BioGPS (as described by Wu et al., 2016) was used to assess the differential gene expression of SNX10 across a panel of 153 murine cell types and tissues, acquired via Affymetrix micro-array analysis (as described by Lattin et al., 2008). Values are shown normalised against an arbitrary scale (fluorescent intensity), where similar cell types are grouped according to bar colour.
Figure 5.4

SNX27 (Mus musculus)
Figure 5.4. Distribution of SNX27 across various tissues reveals its prevalent expression in mature osteoclasts.

The online gene annotation portal, BioGPS (as described by Wu et al., 2016) was used to assess the differential gene expression of SNX10 across a panel of 153 murine cell types and tissues, acquired via Affymetrix micro-array analysis (as described by Su et al., 2004). Values are shown normalised against an arbitrary scale (fluorescent intensity), where similar cell types are grouped according to bar colour.
Figure 5.5. SNX10 and SNX27 localise to endosomal puncta distributed throughout the cytosol of human osteoclasts.

Human osteoclasts were sourced from giant cell tumour biopsies, isolated and cultured *in vitro* on bone discs (A) or on glass (B) in the presence of M-CSF and RANKL (100ng/mL) for up to 10 days. Osteoclasts were then fixed in 4% PFA and immunostained for filamentous actin (F-actin, red), nuclear stain (Hoechst, blue) and SNX10 (A) or SNX27 (B), depicted in green. Images are shown as the product of a Z-stack projection (total 3-6µm thickness) with insets for F-actin (i) or SNX (ii) shown as insets. Scale bar represents 10 or 20µm as shown.
Figure 5.6. Globally-deficient SNX27 mice exhibit early growth retardation at 5 days post-partum.

(A) 5-day old SNX27 wild-type (WT), heterozygous (Het) and globally-deficient (knock-out, KO) mice were acquired in accordance with Animal Ethics Guidelines. Mice are shown to scale.

(B) Whole mount staining of 5-day old pups were performed as described in Materials and Methods. Alcian blue identifies cartilaginous matrix and Alizarin red identifies mineralised bone matrix.

(C) Micro-computed tomography (µCT) was used to reconstruct the mineralised skeleton of 5-day old pups as described in Materials and Methods. All images are shown to scale, where scale bar represents 2.5mm where indicated.
heterozygous littermates (Figure 5.6A). Whole mount staining (Figure 5.6B) and µCT reconstruction (Figure 5.6C) of the skeleton of 5-day old pups did not reveal any obvious abnormalities in the skeletal patterning of SNX27-deficient mice, where mineralised and cartilaginous matrix were determined using Alizarin red and Alcian blue staining, respectively (Figure 5.6B). By 4-weeks of age however, SNX27-deficient mice exhibited severe postnatal growth retardation, including a striking reduction in body size in keeping with earlier reports (Cai et al., 2011) (Figure 5.7A). Reflecting this, the long bones of SNX27-deficient mice were significantly shorter than their wild-type or heterozygous littermates (Figure 5.7B, C). Given the haplosufficiency of SNX27 in postnatal growth and life, studies conducted herein are restricted to comparisons between age and sex-matched wild-type and SNX27-deficient mice.

Next, the long bones (femur, Figure 5.8A and tibia, Figure 5.8B) of 4-week old wild-type and SNX27-deficient mice were examined by µCT. Analyses within the metaphyseal region of the long bone revealed the striking reduction in bone volume ratio (BV/TV, %), trabecular thickness (Tb.Th, mm) and number (Tb.N, 1/mm) accompanied by the significant increase in trabecular separation (Tb.Sp, mm) (Figure 5.8C-E). Additionally, the cortical thickness of SNX27-deficient mice was thinner when compared to wild-type controls, but no significant difference in the medullary volume (bone marrow cavity) was detected (Figure 5.9).

Next, histological analysis was performed on undecalcified tibial bone sections using Goldner’s trichrome staining (Figure 5.10). As illustrated in Figure 5.10A, the overall size of the SNX27 knockout bone was not only smaller than that of the wildtype but also less developed as evidenced by the conspicuous expansion of the epiphyseal growth plate. Histomorphometric analyses (using the BioQuant Software) further confirmed the dramatic decrease in bone parameters, including bone volume ratio (BV/TV, %), trabecular thickness (Tb.Th, mm) and number (Tb.N, 1/mm) and a concomitant increase in trabecular separation (Tb.Sp, mm) (Figure 5.10B-E). Together, these data demonstrate that SNX27-deficient mice
Figure 5.7. Globally-deficient SNX27 mice exhibit severe growth retardation by 4-weeks of age.

(A) 4-week old SNX27 wild-type (WT), heterozygous (Het) and globally-deficient (KO) mice were acquired in accordance with Animal Ethics Guidelines, as described in Materials and Methods. Mice are shown to scale. The gross morphology (B) and average length of isolated tibiae (C) revealed the significant shortening of SNX27-deficient bones when compared to those of wild-type littermates. n = 3 per group, ** p < 0.01.
Figure 5.8. Micro-computed tomography (µCT) analysis of long bones revealed the significant reduction of metaphyseal bone parameters in SNX27-deficient mice. 

(A, B) µCT reconstruction was performed on isolated femora (A) and tibiae (B) from wild-type (WT, i) or SNX27-deficient (KO, ii) littermates, as described in Materials and Methods. Images are shown as longitudinal cross-sections through the medial plane. Scale bar represents 0.5mm.

(C-F) µCT analyses was performed using CTAn, as described in Materials and Methods. SNX27-deficient mice were found to have significant decreases in bone volume ratio (BV/TV, %) (C), trabecular thickness (Tb.Th, mm) (D), trabecular number (Tb.N, 1/mm) (F) and an increase in trabecular separation (Tb.Sp, mm) (E). n = 4 per group, ** p < 0.01, *** p < 0.001.
Figure 5.9. Micro-computed tomography (µCT) analysis of long bones revealed further cortical thinning in SNX27-deficient mice. 

(A) µCT was performed on isolated tibiae as described in Materials and Methods. The diaphysis of reconstructed tibiae are shown as transverse cross-sections through the diaphysis of wild-type (WT) and SNX27-deficient (KO) littermates. Images are shown to scale, where scale bar represents 250µm. 

(B-D) µCT analysis of cortical bone are shown as total volume (TV, %, inclusive of bone marrow cavity) (D), bone volume (BV, %, exclusive of bone marrow cavity) (C) and medullary volume (TV-BV, medullary volume) (D). n = 4 per group, *** p < 0.001.
Figure 5.10. Histomorphometric analyses of proximal tibial sections confirmed the significant reduction in bone parameters of SNX27-deficient mice.

(A) Isolated tibial bones from 4-week old wild-type (WT) and SNX27-deficient (KO) mice were obtained and processed as described in Materials and Methods. Representative sections stained with Goldner’s trichrome are shown. Images are shown to scale, where scale bar represents 250µm.

(B-E) Histomorphometric analysis was performed as described in Materials and Methods, confirming the significant reduction in bone volume ratio (BV/TV, %) (B), trabecular thickness (Tb.Th, mm) (C), trabecular number (Tb.N, 1/mm) (E) and an increase in trabecular separation (Tb.Sp, mm) (D). \( n = 4 \) per group, * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \).
manifest a form of osteochondrodysplasia that is characterised by low-bone mass (osteopenia) and an abnormal growth plate expansion.

To begin to dissect out the potential cell-autonomous defects that might contribute to the observed bone phenotype the effect of SNX27 deletion on resident bone cells was further evaluated by histomorphometry. As SNX27 was previously found to be substantially upregulated following osteoclastogenesis (Figure 5.1-5.2), MMA-embedded tibial sections were first stained for TRAP-positive osteoclast using the Pararosaniline staining method (Figure 5.11). Surprisingly, no obvious differences in the morphology and/or activity of osteoclasts where observed within the metaphyseal regions of long bones of wild-type or SNX27-deficient mice (Figure 5.11A, B), with SNX27-deficient osteoclasts clearly retaining the ability to form sites of bone resorption along the trabecular surface (insets). Following quantification however, SNX27-deficient mice were found to have a significantly reduced number of osteoclasts within the metaphyseal region of the tibia when normalised to the total bone surface (Figure 5.11D), while presenting with a slightly higher number of osteoclasts within the epiphyseal region (secondary ossification centre) (Figure 5.12). These data suggest that the low-bone mass phenotype exhibited by SNX27-deficient mice was not likely attributable to anomalies within osteoclasts.

Next, the impact of SNX27-deficiency in osteoblasts was explored. Goldner’s trichrome-stained trabecular regions of the tibia were closely examined to identify any differences in osteoblast morphology and osteoid deposition (Figure 5.13A). Wild-type osteoblasts were found to adopt a polarised (cuboidal) morphology along the trabecular surface, accompanied by a uniform deposition of osteoid as shown in red/pink (Figure 5.13A, inset). In contrast, there was a drastic reduction in the number of osteoblasts lining the trabecular surface of bones isolated from SNX27-deficient mice (Figure 5.13B). This reduction corresponded with a net decrease in osteoid production, with both osteoid thickness and width significantly reduced (Figure 5.13A, D-E).
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Figure 5.11

A. 

WT 

KO 

B. 

C. OC.N

D. OC.N/BS

![Images of WT and KO tibias with bar charts showing OC.N and OC.N/BS counts]
Figure 5.11. Osteoclast numbers were significantly reduced within the metaphyseal region of the proximal tibia in SNX27-deficient mice.

(A) Isolated tibial bones from 4-week old wild-type (WT) and SNX27-deficient (KO) mice were obtained and processed for paraffin embedment as described in Materials and Methods. Sections were then stained to identify mature osteoclasts using the tartrate-resistant acid phosphatase (TRAP) staining method. Representative sections are shown, where the scale bar represents 250µm.

(B) TRAP-positive osteoclasts lining the trabecular surface (highlighted in insets) were comparable in morphology and size between WT and KO sections. Scale bar represents 100µm.

(C, D) Histomorphometric analysis was performed as described in Materials and Methods, and confirmed the significant reduction in osteoclast numbers (OC.N) (C) and density when normalised to bone surface (OC.N/BS) within the primary spongiosa. n = 4 per group, * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 5.12. Osteoclast density was increased within the secondary ossification centre in SNX27-deficient mice.

(A) Isolated tibial bones from 4-week old wild-type (WT) and SNX27-deficient (KO) mice were obtained and processed for paraffin embedment as described in Materials and Methods. Sections were then stained to identify mature osteoclasts using the tartrate-resistant acid phosphatase (TRAP) staining method. Representative sections are shown, where the scale bar represents 500 or 300µm as shown. 

(B-D) Histomorphometric analysis was performed as described in Materials and Methods, confirming the significant reduction in bone volume ratio (BV/TV, %) (B) within the secondary ossification centre (SOC). Despite no significant change between total osteoclast numbers (OC.N) (C), there was a significant increase in osteoclast density when normalised to bone surface (OC.N/BS) (D). 

n = 4 per group, * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 5.13. Osteoblast morphology and osteoid deposition was reduced within the proximal tibia of SNX27-deficient mice.

(A) Isolated tibiae from 4-week old wild-type (WT) and SNX27-deficient (KO) mice were obtained and processed as described in Materials and Methods. Representative regions of osteoblast-lined trabeculae are shown following Goldner’s trichrome staining, at 630X magnification. Here, osteoblast (red-stained cells) are shown overlaying osteoid (unmineralised bone matrix, red layer) and mineralised bone matrix (blue/green), with insets taken from boxed regions.

(B-E) Histomorphometric analysis was performed as described in Materials and Methods, confirming the significant reduction in osteoblast numbers (OB.N) (B) but not in osteoblast density when normalised to bone surface (OB.N/BS) (C). Both osteoid deposition, determined by osteoid thickness (O.Th) (D) and width (O.Wi) (E) were both significantly reduced in SNX27-deficient mice. $n = 4$ per group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. 
Following the completion of matrix synthesis and mineralisation, osteoblasts become embedded within the bone matrix and terminally differentiate into osteocytes (Dallas and Bonewald, 2010). Therefore, the osteocyte population was also assessed in both endochondral (cortical) and intramembranous (calvarial) bones taken from 4-week old mice (Figure 5.14). In general, SNX27-deficient mice were found to exhibit a significantly reduced population of osteocytes in both cortical and calvarial bone (Figure 5.14B, E), however this population was normalised when the osteocyte numbers where adjusted to total bone volume (Figure 5.14C, F).

In addition to the significant reduction in bone parameters, SNX27-deficient mice were also found to exhibit a striking expansion of the epiphyseal growth plate within the long bones (Figure 5.15A-C). Safranin O staining did not reveal any obvious anomalies in proteoglycan levels within the epiphyseal growth plate (Figure 5.15A), however the organisation of columnar chondrocytes appeared to be altered, albeit without affecting longitudinal directionality (Figure 5.15A, B). While the expansion was found to affect all chondrocytic zones, i.e. the resting zone (RZ), proliferating zone (PZ) and hypertrophic zone (HZ) (Figure 5.15D), the density of hypertrophic chondrocytes (measured as N/mm$^2$) appeared to be significantly reduced in SNX27-deficient mice (Figure 5.15 E). Additionally, the detection of collagen type X via immunohistochemistry (Figure 5.16B) further highlighted a noticeable difference in the cellular volume of SNX27-deficient hypertrophic chondrocytes and may contribute to the decreased chondrocyte population in the hypertrophic zone (Figure 5.15E). Despite this, there were no obvious deficiencies detected in the apparent staining intensity of either collagen type II or X deposited throughout the growth plate (Figure 5.16). Therefore, these data highlight that SNX27 functions within cells of the mesenchymal lineage to regulate osteoblast function as well as chondrocyte proliferation and/or maturation, where its depletion in these cells ultimately contributed to osteochondrodysplasia.
Figure 5.14

A. Cortical bone

B. **OY.N**

C. **OY.N/BV**

D. Calvarial bone

E. **OY.N**

F. **OY.N/BV**

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Figure 5.14. SNX27-deficient mice exhibit significant reductions in osteocyte densities in endochondral and intramembranous bones. Isolated tibiae from 4-week old wild-type (WT) and SNX27-deficient (KO) mice were obtained and processed as described in Materials and Methods. Representative regions of tibial (cortical bone, A) or calvarial (D) sections stained with Goldner’s trichrome are shown. Images are to scale, where scale bar represents 100µm where shown. Histomorphometric analysis was performed as described in Materials and Methods, revealing the significant reduction in total osteocyte numbers (OY.N) (B, E) but not population density when normalised to total bone volume (OY.N/BV) (C, F) in both cortical and calvarial bone of SNX27-deficient mice. n = 4 per group, * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 5.15

A. Safranin O

B. Goldner’s Trichrome

C. GP Height (tibia)

D. GP Height

E. Chondrocyte Density

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Figure 5.15. The epiphyseal growth plate is significantly expanded in the long bones of SNX27-deficient mice.

(A, B) Isolated tibial bones from 4-week old wild-type (WT) and SNX27-deficient (KO) mice were obtained and processed as described in Materials and Methods. Sections were then stained for Safranin O (A), staining proteoglycans (red) and mineralised bone (blue/green), or Goldner’s trichrome (B) showing the organisational zones of the chondrocyte populations.

(C) The total height of the growth plate was assessed by Aperio ImageScope Software, where the average height was calculated from 6 random regions (shown in A), per sample, using n = 4 per group.

(D, E) Histomorphometric analysis was performed as described in Materials and Methods, revealing the significant increase in height (µm) of the resting zone (RZ), proliferating zone (PZ) and hypertrophic zone (HZ) in SNX27-deficient mice (D). The densities of chondrocytes per region (calculated as the ratio of chondrocyte number to area (N/mm²)) was significantly greater in the RZ and PZ, but reduced in the HZ of SNX27-deficient mice (E). n = per group, * p < 0.05, p < 0.01, *** p < 0.001.
Figure 5.16. Collagen type II and X levels within the growth plate were comparable between wild-type and SNX27-deficient mice. Isolated tibiae from 4-week old wild-type (WT) and SNX27-deficient (KO) mice were obtained and processed for paraffin embedment as described in Materials and Methods. Immunohistochemistry was then used to identify collagen type II (A) or type X (B) expression levels. Representative sections are shown to scale, at 10X magnification.
In attempt to gain insight into the differential state of these bone cell populations, qPCR was then performed using total bone mRNA (inclusive of bone marrow and epiphyses) (Figure 5.17). With the exception of cathepsin K (Ctsk) (Figure 5.17A), there were minimal differences across osteoclast-marker genes, including calcitonin receptor (CTR), RANK and TRAP, confirming the previous observations of the osteoclast population *in vivo*. In contrast, there was no significant difference between RUNX2 or collagen type 1 expressions between wild-type and SNX27-deficient osteoblast/chondrocyte populations (Figure 5.17B). There was however, a significant reduction in osteocalcin (OCN) as well as sclerostin (SOST) expressions (Figure 5.17B, C). Lastly, while SOX9 was found to be significantly upregulated in SNX27-deficient bones, this is most likely to be a reflection of the increased growth plate present within these bones as opposed to wild-type samples, however this remains to be confirmed.

The substantial upregulation of SNX27 during osteoclast differentiation, yet lack of obvious anomalies affecting the osteoclast population as assessed *in vivo* prompted for the further investigation of osteoclastogenesis *in vitro*. Therefore, to confirm whether there was a direct role of SNX27 in regulating osteoclast proliferation and/or differentiation, osteoclast monocyte precursors were isolated from the spleen, cultured and differentiated to form osteoclasts *in vitro*, as described in Materials and Methods. When wild-type or SNX27-deficient precursors were cultured under the influence of M-CSF and RANKL, there were no obvious differences identified in either osteoclast differentiation (Figure 5.18A, B) or size (Figure 5.18C, D) under either end-point or dose-dependent conditions of RANKL administration. Furthermore, the resorptive capacity of formed osteoclasts were assessed following the generation of mature osteoclasts on bovine bone slices (Figure 5.18E). Confocal microscopy did not reveal any differences in osteoclast resorption capacity (as visualised through pit depth) or pattern (as emphasised through pit directionality).
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Figure 5.17

A. Osteoclast-related genes

B. Osteoblast-related genes

C. Osteocyte-related gene

D. Osteo/Chondro-related genes

* $p < 0.05$
** $p < 0.005$
*** $p < 0.001$
Figure 5.17. An overview of the gene expression profiles of skeletal-resident cell types using total bone derived from wild-type or SNX27-deficient mice.

Total bone mRNA was extracted from isolated long bones of freshly culled 4-week old wild-type (WT) and SNX27-deficient (KO) mice as described in Materials and Methods. qPCR (probe method) was then performed to profile the gene expression of osteoclast (A), osteoblast (B), osteocyte (C) or chondrocyte (D) marker genes, relative to housekeeping gene, HMBS. n = 3 per group, * p < 0.05, ** p < 0.01, *** p < 0.001.

(A) Osteoclast marker genes include cathepsin K (Ctsk), which was significantly increased, while calcitonin receptor (CTR), receptor activator of nuclear factor κ-B (RANK) or tartrate-resistant acid phosphatase (TRAP) were of comparable expressions.

(B) A significant downregulation of osteoblast marker gene, osteocalcin (OCN) was detected, but not for collagen type I (Col 1) or runt-related transcription factor 2 (RUNX2) expressions.

(C) Osteocyte-specific gene, sclerostin (SOST) was significantly downregulate, but not in late-stage/osteocyte marker genes, matrix extracellular phosphoglycoprotein (MEPE) or phosphate-regulating neutral endopeptidase, X-linked (PHEX) expressions.

(D) SOX9 was significantly increased in SNX27-deficient bones compared to wild-type.
Figure 5.18

A. WT KO

TRAP

B. WT KO

No. TRAP +ve cells per well (>3 nuclei)

C. RANKL (ng/ml)

0 12.5 25 50 100 200

WT KO

D. No. TRAP +ve cells per well (>3 nuclei)

#3-5 #6-10 #11+

No. of nuclei per well

E. WT KO

Resorption

Pit Depth Profile (XZ)
Figure 5.18. Culture of osteoclasts in vitro did not reveal any significant abnormalities in osteoclast differentiation or resorption between wild-type or SNX27-deficient cells.

(A, B) Bone marrow macrophages (BMM) were obtained from isolated spleen of freshly culled 4-week old wild-type (WT) and SNX27-deficient mice and cultured on plastic in the presence of M-CSF and RANKL (100ng/ml) as described in Materials and Methods. Osteoclasts were then fixed in 4% PFA and stained for TRAP (A) and quantified using FIJI (ImageJ, NIH) (B). n = 3 per group.

(C, D) Osteoclasts were then generated under dose-dependent RANKL concentrations, ranging from 0 to 200ng/mL of RANKL, administered every other day for a total of 7 days. Following TRAP staining and quantitation, there were no significant differences detected in osteoclast numbers or size. n = 3 per group.

(E) Osteoclasts were generated on bone slices as described in Materials and Methods. Following 10 days, osteoclasts were then stripped from the surface of the bone and the bone slice was imaged using the Nikon A1Si confocal microscope, revealing no significant abnormalities in osteoclast resorption or behaviour.
Next, to further characterise the intrinsic effects following SNX27-deficiency in osteoblasts, a gene profile of committed osteoblasts was established following the isolation and culture of osteoprogenitor cells in the presence of ascorbic acid in vitro, as described in Materials and Methods (Figure 5.19). Here, qPCR revealed that SNX27-deficient osteoblasts resided within a higher immature state of differentiation, as indicated through the higher levels of ALP, RUNX2, OSX and RANKL, while having a significantly reduced expression of late-stage markers, including MEPE and PHEX. To then confirm whether these cells were also deficient for osteoid deposition as previously indicated by histology (Figure 5.13), osteoblasts were cultured under osteogenic conditions (ascorbic acid 50µg/mL, β-glycerophosphate 3mM, dexamethasone 10nM) for up to 28-days in vitro (Figure 5.20). Despite higher levels of ALP expression (Figure 5.20A), there was an apparent deficiency in SNX27-deficient osteoblasts to form bone nodules in vitro, as determined through Alizarin red staining (Figure 5.20B). This was consistently observed across all sources of osteoblasts, i.e. bone-derived osteoblasts (shown), BMSCs and calvarial osteoblasts (not shown). In the attempt to rescue this mineralisation deficiency, this osteogenic assay was then repeated in the absence (Ctrl) or presence of anabolic agents; intermittent PTH (PTH) or bone morphogenic protein 2 (BMP2), as described in Materials and Methods (Figure 5.21). Despite both anabolic agents promoting mineralisation of WT osteoblasts in vitro, SNX27-deficient osteoblasts appeared unresponsive to PTH administration (Figure 5.21A, B). In stark contrast, SNX27-deficient osteoblasts appeared to exhibit an exaggerated response following BMP2 administration (Figure 5.21A, B).

These interesting results prompted the further investigation into the relationship between SNX27 and PTHR. PTHR is a crucial GPCR known to regulate mineral ion homeostasis, skeletal growth and development, and predominantly functions through the activation of Gαs-dependent downstream effectors. PTH is known to have opposing effects on bone, where intermittent PTH administration promotes a net anabolic effect on bone, while continuous PTH exposure results in a net catabolic effect. Therefore, to first investigate whether the absence of SNX27
Figure 5.19. Gene expression profiling identified decreases in osteoblast differentiation markers in SNX27-deficient osteoblasts cultured in vitro.

Osteoblasts were isolated from the bone marrow of freshly culled 4-week old wild-type and SNX27-deficient (KO) mice as described in Materials and Methods. Osteoblasts were cultured in complete αMEM supplemented with ascorbic acid (50µg/mL) for 14 days prior to mRNA extraction. qPCR determined that both MSC-derived and bone-derived (not shown) osteoblasts exhibited increases in pre-osteoblast markers, including alkaline phosphatase (ALP), osterix (OSX) and RANKL, with a decrease in late-stage markers, MEPE and PHEX. \( n = 3 \) per group, \( p < 0.05, ** p < 0.01, *** p < 0.001 \).
Figure 5.20. SNX27-deficient osteoblasts failed to form bone nodules under osteogenic conditions in vitro.

Osteoblasts were isolated from freshly culled 4-week old wild-type and SNX27-deficient (KO) mice as described in Materials and Methods. Osteoblasts were obtained from calvarial bone, bone fragments and MSCs (latter shown). Following culture under osteogenic conditions (complete αMEM supplemented with ascorbic acid (50µg/mL), β-glycerophosphate (3mM), dexamethasone (10nM)) for 28 days, cells were then fixed and stained for alkaline phosphatase (A) or alizarin red (B). Despite expressing sufficient ALP activity, SNX27-deficient osteoblasts failed to deposit bone nodules in vitro. n = 3 per group, * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 5.21. Intermittent PTH(1-34) induced bone nodule formation of wild-type but not SNX27-deficient osteoblasts cultured in vitro. Osteoblasts were isolated from freshly culled 4-week old wild-type and SNX27-deficient (KO) mice as described in Materials and Methods. Osteoblasts were obtained from calvarial bone, bone fragments and MSCs (latter shown). Cells were cultured under osteogenic conditions (complete αMEM supplemented with ascorbic acid (50µg/mL), β-glycerophosphate (3mM), dexamethasone (10nM)) (control), with additional PTH(1-34) (50nM) with ligand wash-out after 4 hours, or BMP2 (30ng/mL), for 28 days. Cells were then fixed and stained for alizarin red (A) and quantified following de-staining (B) as described in Materials and Methods. BMP2 but not intermittent PTH was able to induce bone nodule formation by SNX27-deficient osteoblasts. n = 3 per group, * p < 0.05, ** p < 0.01.
affected PTHR signalling and therefore its response to intermittent PTH, cAMP responses in osteoblasts were assessed in a dose-dependent manner through the use of an ELISA assay (Figure 5.22). Surprisingly, SNX27-deficient osteoblast exhibited an increased response to PTH following 15 minutes of agonist treatment, starting at 100nM (Figure 5.22A). This response was mirrored upon exposure to forskolin (a non-specific activator of adenylyl cyclase) but not isoproterenol (β2AR-specific agonist) (Figure 5.22B).

As these data indicate a net increase in cAMP levels in SNX27-deficient osteoblasts, its translation into the activation of downstream signalling effectors were then assessed through western blotting. Here, wild-type and SNX27-deficient osteoblasts were subjected to 15-minutes of PTH stimulation, followed by a ligand washout where appropriate, and assessed for phosphorylated (activated) CREB, AKT and ERK1/2 levels across 90 minutes from ligand addition (Figure 5.23). Consistently, SNX27-deficient osteoblasts exhibited the sustained activation of the transcription factor CREB (Figure 5.23B), and to a milder extent, activation of AKT (Figure 5.23C) and ERK1/2 (Figure 5.23C). Of particular interest, is that these events occurred within 5-30 minutes post-agonist addition, during which the activated surface receptor complex is internalised into early endosomes (see Chapter 6).

The sustained levels of cAMP and activation of secondary effector proteins, particularly the transcription factor CREB, then prompted the investigation of whether SNX27-deficient osteoblasts exhibited differential gene expressions in response to PTH. Osteoblasts were then cultured as described previously and subjected to 15 minutes of PTH stimulation followed by ligand washout, where cells were then harvested for mRNA following 1, 4 or 8 hours post-ligand addition (Figure 5.24). As previously shown (Figure 5.19), SNX27-deficient osteoblasts resided within a state of elevated ALP and collagen type 1 levels (Figure 5.24A), PTHR (Figure 5.24B), RUNX2 (Figure 5.24C), and RANKL (Figure 5.24D). While there was minor differences in the response to PTH following agonist addition (Figure 5.24B, C), the gene expressions of RANKL and OPG were substantially exaggerated.
Figure 5.22. SNX27-deficient osteoblasts exhibited sustained cAMP responses following the dose-dependent exposure to PTH(1-34). MSC-derived osteoblasts were isolated, cultured and stimulated with PTH(1-34) for 15 minutes prior to assessment of cAMP production, as described in Materials and Methods. SNX27-deficient (KO) osteoblast exhibited an increased response to PTH(1-34) under dose-dependent stimulation (A) and forskolin (10µM) (non-specific activator of adenylyl cyclase) (B) but not isoproterenol (1µM) (non-selective β-adrenergic receptor agonist). n = 3 per group, * p < 0.05, ** p < 0.01, *** p < 0.001.
Chapter 5 - SNX27 is a crucial modulator in skeletal homeostasis

Figure 5.23

A.

<table>
<thead>
<tr>
<th></th>
<th>WT (1-34)</th>
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<tr>
<td>PTH (1-34)</td>
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<td>p-CREB</td>
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<td>α-tubulin</td>
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B. Phospho-CREB:α-tubulin

C. Phospho-AKT:pan-AKT

D. Phospho-ERK1/2:pan-ERK1/2
Figure 5.23. SNX27-deficient osteoblasts exhibited sustained activation of \( \text{Ga}_\text{s} \)-dependent downstream signalling effectors during PTH(1-34) stimulation.

Wild-type (WT) and SNX27-deficient (KO) osteoblasts derived from MSCs and bone were cultured as described in Materials and Methods. Osteoblasts were then seeded 24-hours prior to stimulation with PTH(1-34) across 90 minutes, with ligand-washout after the initial 15 minutes of stimulation, where applicable (A). SNX27-deficient osteoblasts exhibited sustained activation of (phosphorylated) CREB (B), AKT (C) and to a lesser extent, ERK (D) when normalised to their respective controls, between 5-30 minutes post-stimulation. 

\( n = 3 \) per group, * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \).
Chapter 5 - SNX27 is a crucial modulator in skeletal homeostasis

Figure 5.24

A. Naive

B. PTHR

C. RUNX2

D. RANKL

E. OPG

F. Percentage change in RANKL and OPG

[Graphs showing relative fold expression for ALP, Col 1, WT, KO, Naive, 4hr PTH, 8hr PTH for each gene]
Figure 5.24. Expressions of PTH-response genes were appreciably up- or down-regulated in SNX27-deficient osteoblasts compared to wild-type, following PTH(1-34) stimulation.

Wild-type (WT) and SNX27-deficient (KO) osteoblasts derived from both MSCs and bone-derived (not shown) osteoblasts were isolated and cultured as described in Materials and Methods. Osteoblasts were seeded for 24-hours prior to stimulation with PTH(1-34) for 15 minutes, followed by ligand-washout for the indicated time period. Cells were then washed twice in 1X PBS, followed by mRNA extraction as described in Materials and Methods. qPCR was then performed and revealed the increased expression of ALP and collagen type I levels under basal conditions (A). SNX27-deficient osteoblasts also exhibited an increased downregulation of PTHR expression (B) following 4-hours post-PTH stimulation, and an increased upregulation of RANKL expression (D) and increased downregulation of OPG expression (E) following 8-hours post-PTH stimulation (F). n =2 per group.
in SNX27-deficient osteoblasts compared to wild-type controls (Figure 5.24D-F). This is suggestive only, as there were insufficient repeats conducted for this experiment. Nevertheless, these data collectively highlight that one intrinsic signalling pathway disrupted within SNX27-deficient osteoblasts is their ability to respond and regulate PTHR downstream $\text{G}_\alpha$-cAMP signalling cascades.

5.3. Discussion

5.3.1. Profiling SNXs in bone identifies potential regulators in skeletal homeostasis

The highly-conserved SNX protein family has become increasingly recognised as crucial modulators in membrane and protein trafficking in eukaryotic cells (Teasdale and Collins, 2012). At the commencement of this study however, the functional relevance of most SNX proteins in bone remained unknown. Therefore, the first aim of this study was to profile the differential gene expression across the SNX protein family within the context of bone and its resident cell types. While SNX10 and SNX27 were chosen for further investigation based on their substantial upregulation in osteoclastogenesis, with the latter being the highest expressed in total bone, there were several other notable members that were not pursued, but could potentially play regulatory role(s) in bone. These include SNX21 and SNX25, both of which joined SNX27 as amongst the few of the higher-expressing members in total bone, while SNX13 and SNX5 were found to be downregulated following osteoclastogenesis.

While the direct implication of most SNX proteins in bone remains to be determined, there have been several independent studies over the past few years that have shed light on their role in cellular homeostasis and endomembrane trafficking. For example, SNX25 has been shown to directly engage and modulate TGFβ-dependent receptor signalling by mediating clathrin-dependent endocytosis and lysosomal degradation of the TGFβ receptor (Hao et al., 2011), and could thus help explain its relative abundance in total bone. Additionally, SNX13 has been shown to
participate in regulating cell survival (Li et al., 2014, Zheng et al., 2006), and
together with SNX14, SNX19 and SNX25, form a novel group of ER-endolysosomal
tethering proteins (Henne et al., 2015). Lastly, the seemingly high expression of
SNX5 in macrophages as detected through qPCR has since been independently
validated to participate in macropinocytosis and membrane ruffling in macrophages,
both of which are vital processes required for antigen processing (Lim et al., 2015,
Lim et al., 2012).

Most notable of all however, was the independent characterisation of SNX10
in osteoclast function during the progression of this dissertation. Consistent with our
unpublished preliminary results, SNX10 was identified to be crucial for osteoclast
bone resorption, where it is now recognised to contribute to a small yet significant
proportion (~4%) of patients diagnosed with the severe form of osteopetrosis (Aker
et al., 2012, Megarbane et al., 2013, Pangrazio et al., 2013). While approximately
70% of osteopetrotic cases have been attributed to the loss of function of v-ATPase
α3 subunit (encoded by \textit{TCIRG1}) (Frattini et al., 2000) or H+/Cl− exchange
transporter 7 (encoded by \textit{CLCN7}) (Leisle et al., 2011), the proportion of cases
accountable to \textit{SNX10} mutations in on-par with those caused by mutations in
\textit{RANKL} (\textit{TNFSF11A}) or its receptor, \textit{RANK} (\textit{TNFRSF11A}) (Sobacchi et al., 2013).
All \textit{SNX10} mutations have so far been identified to reside within the only functional
domain within the protein; i.e. the PX domain (Aker et al., 2012, Pangrazio et al.,
2013). Though the exact molecular mechanism of SNX10 has not yet been defined,
the role of SNX10 is thought to affect the resorptive machinery of osteoclast (Chen
et al., 2012), as these patients are characterised to exhibit an ‘osteoclast-rich’ subtype
of osteopetrosis. Mice recently generated to exhibit the osteoclast-specific deletion
of SNX10 presented with a similar skeletal phenotype to that of human patients (Ye
et al., 2015). Interestingly however, is that the phenotype of globally-depleted
SNX10 mice was found to be a paradoxical combination of osteopetrosis and rickets,
where these mice also presented with a deficiency in calcium absorption through the
gastric epithelium. The rachitic phenotype of these global SNX10 KO mice (and life
span) was able to be ‘rescued’ through calcium diet supplementation, and may thus
be implemented in the future in the treatment of SNX10-dependent osteopetrotic cases co-presenting with a phenotype of rickets. Thus, this novel function of SNX10 in calcium absorption (Ye et al., 2015) further highlights the need to fully understand the functional role(s) of SNXs across various organs and tissues.

The relevant role of SNX27 in skeletal homeostasis was chosen for further characterisation, and begun with the analysis of the skeletal phenotype of globally-deficient SNX27 mice. SNX27 was originally identified as an inducible protein in the rat neocortex following the repeated exposure to psychostimulants (Kajii et al., 2003). The trafficking role of SNX27 still remains to be best characterised in the context of the brain, having crucial roles in modulating neuronal plasticity, cognitive function, memory and learning processes (see Chapter 2). Although several studies have demonstrated that SNX27 engages with a broad range of cargo, a pivotal study by Steinberg and colleagues identified SNX27 to regulate the surface expression of over 120 surface proteins in mammalian cells (Steinberg et al., 2013). Of these, the surface expression of 80 exerted co-dependence for retromer – a highly evolutionary-conserved heterotrimer involved in modulating protein transport within the endomembranous network. Despite having performed these experiments in HeLa cells (and thus do not share the same surface complement of receptors as skeletal cell types), Steinberg and colleagues reported the interaction and/or reduced expression of several crucial receptors required for skeletal development and homeostasis, including β2AR, Fzd and LRP co-receptors (Steinberg et al., 2013). Hence, these studies provide collective reasoning for the direct role of SNX27 in skeletal development and/or homeostasis.

5.3.2. The low-bone mass phenotype of SNX27-deficient mice was not attributable to enhanced osteoclast formation or resorption

Despite being up-regulated during RANKL-induced osteoclast formation, SNX27-deficient mice were found to exhibit reduced osteoclast numbers within the primary spongiosa. The precise reason for this discrepancy is presently unclear and warrants further investigation. The differentiation and function of osteoclasts are
normally tightly coupled to the osteoblast during bone modelling and remodelling processes, and so the global deficiency of SNX27 may confound any primary anomalies intrinsic to the osteoclast. For instance, the increased expression of osteoblast-derived (or even osteocyte-derived) RANKL under PTH-dependent and –independent conditions could potentially drive osteoclastogenesis under otherwise unfavourable conditions. The intrinsic role of SNX27 in osteoclast biology continues to remain as an ongoing focus for future studies, where the conditional deletion of SNX27 through the use of the Cathepsin K promoter is currently underway, and may provide accurate insight on the intrinsic role of SNX27 in osteoclast biology.

5.3.3. SNX27-deficient chondrocytes exhibited aberrations in chondrocyte organisation and hypertrophy

Of the primary skeletal cell types within bone, those of mesenchymal origin (i.e. chondrocytes, osteoblasts and osteocytes) appeared to be more drastically affected from the global deficiency of SNX27. In particular, one of the most striking observations from the initial characterisation of the bone phenotype exhibited by SNX27-deficient mice was the significant expansion of the epiphyseal growth plate. This was later identified to include (i) the peculiar organisation of the columnar chondrocytes and (ii) potentially larger hypertrophic chondrocytes than compared to wild-type littermates.

Firstly, in measuring the expansion of all major chondrocytic zones within the epiphyses of SNX27-deficient mice, there was an obvious peculiarity with the architectural organisation of the columns formed within the PZ. Based on the reports by Dodd in 1930, the canonical model of column formation can be described in four main steps (as summarised in (Romereim et al., 2014)), where proliferating chondrocytes first undergo mitosis across the lateral plane, followed by cellular rounding. These daughter chondrocytes then come within close proximity of each other and intercalate, ultimately resulting in the formation of a columnar stack parallel to the longitudinal direction of bone growth (Dodds, 1930, Romereim et al., 2014). Romereim and colleagues however, have proposed a recent alteration to this
model where following mitosis, these daughter cells form a transient adhesion surface that enables for immediate intercalation, and is in fact required for the final formation of the columnar stack (Romereim et al., 2014). This transient adhesion surface was found to be greatly enriched with the presence of β-catenin and cadherins, where disruption of the latter resulted in an increased distance between daughter cells and abnormal membrane protrusions that affected column formation. Although the assessment of adherens junctions or β-catenin-dependent signalling was not investigated within the population of chondrocytes, there were substantial differences observed in the latter following the time-dependent BMP2 stimulation of differentiated osteoblasts (see Section 5.3.5).

Despite having uncharacterised anomalies in the intercalation/formation of columnar stacks, chondrocytes depleted of SNX27 remained capable of exiting the cell cycle and undergoing hypertrophy. However, while the expansion of the RZ and PZ were accompanied by an expected increase in chondrocyte numbers, the HZ was instead found to have a reduced population of hypertrophic chondrocytes. Additionally, these chondrocytes were also seemingly larger in cellular volume than compared to their wild-type littermates, although no measurements in cellular volume were taken. While bone growth is ultimately achieved through the spatiotemporal regulation of chondrocyte proliferation, maturation and the deposition of extracellular matrix, the elongation of the long bones is driven mainly through chondrocyte hypertrophy (Hunziker, 1994). Therefore, these two disruptions observed within the growth plate may partly account for the disrupted organisation and growth of the developing bone in SNX27-deficient mice.

It is noteworthy to mention that the unregulated responses in PTHR-mediated signalling identified in SNX27-deficient osteoblasts are also likely to extend to chondrocyte populations. PTHR is expressed by both proliferating and pre-hypertrophic chondrocytes, where the regulation of the growth plate, and subsequent osteogenesis (bone formation) are driven through the key actions of the autocrine/paracrine factor, PTHrP. While the significant increased expression of
SOX9 in total mRNA isolated from SNX27-deficient mice could be a reflection of the mere expansion of the growth plate, it could also be the result from abnormalities in PTHrP-PTHR-mediated signalling, where PTHrP is known to promote chondrocyte proliferation while restraining maturation and hypertrophy, partly by inducing SOX9 phosphorylation in a Gαs-cAMP-dependent manner (Huang et al., 2001).

The generation of several mouse models have comprehensively established the role of PTHrP in regulating the growth plate and skeletal development. Pthlh-null or PTHR-deficient mice were found to exhibit accelerated chondrocyte maturation and premature growth plate closure (Amizuka et al., 1994; Karaplis et al., 1994; Lanske et al., 1996; Miao et al., 2002; Hirai et al., 2011) while chondrocytes expressing transgenic PTHrP or constitutively-active PTHR were found to have delayed chondrocyte hypertrophy and bone formation (Weir et al., 1996, Schipani et al., 1997b). Somewhat similar to these findings, SNX27-deficient mice were also found to possess an expansion of the growth plate, something that could be attributed to sustained PTHrP(1-141) signalling. SNX27-deficient osteoblasts were indeed found to possess sustained signalling upon intermittent exposure to PTHrP(1-141) (Appendix A, Figure 1), further supporting the inference that SNX27-deficient chondrocytes possess irregular PTHrP/PTHR-mediated signalling. It is important to note however, that the global disruption of SNX27 will inevitably affect a multitude of receptors and intracellular proteins and concomitant signalling pathways, thus making the phenotypic characterisation of these mice extremely difficult. For example, pathways known to cross-talk with PTHR, including Wnt/β-catenin and TGF β-dependent pathways are also likely to be affected.
5.3.4. **SNX27-deficient osteoblasts exhibited delayed osteoblast differentiation and impaired mineralisation capacity, partly attributed to the unregulated responses to intermittent PTH(1-34)**

An interesting finding in this thesis is that SNX27-deficient osteoblasts exhibit unregulated responses to intermittent PTH(1-34) stimulation. A gene profile established from the use of total bone samples did not reveal significant differences in RUNX2 or collagen type I expressions, suggesting that the commitment of MSCs toward the osteoblast lineage was largely unaffected in the absence of SNX27. Moreover, cellular proliferation assays under basal conditions, or under intermittent PTH exposure did not reveal any differences in proliferation of osteoblasts *in vitro* (Appendix B, Supplementary Figure 2), suggesting that the reduced number of osteoblasts quantified *in vivo* was not a reflection of defects in cellular proliferation.

SNX27-deficient mice did however, present with lower quantities of osteoid deposition *in vivo*, where the reduced capacity to mineralise under osteogenic conditions was mirrored in mineralisation assays conducted *in vitro*. While the apparent quiescent-like nature of SNX27-deficient osteoblasts *in vivo* could be due to either (i) inactivity of trabecular-lining osteoblasts, or (ii) decreased re-activation of bone-lining cells (which PTH has been shown to positively regulate (Kim et al., 2012)), further *in vitro* characterisation of these cells revealed SNX27-deficient committed osteoblasts to reside in a relatively higher immature state of differentiation (with higher expressions of ALP, RUNX2 and OSX) accompanied by the lower expressions of late-stage markers, including MEPE and PHEX, both of which are involved in matrix mineralisation, as well as the significant reduction in OCN levels detected in total bone samples. These together support the notion that SNX27 assists to regulate osteoblast differentiation and function.

The unanticipated difference observed in the response to intermittent PTH during the mineralisation assay *in vitro* then prompted for the further investigation into relationship between SNX27 and PTHR signalling. As PTHR activation predominantly results in the activation of the Gαs-cAMP-dependent pathway, cAMP generation was first examined. Indeed, SNX27-deficient osteoblasts were found to
sustain a significantly greater production of cAMP following 15 minutes of PTH exposure, of which resulted into the activation of signalling and gene regulators, AKT and CREB at the corresponding time period between 5-30 minutes post-stimulation. As expected, this also corresponded to an unregulated response in PTH-response genes, particularly emphasised through the substantial upregulation of RANKL, and concomitant downregulation of OPG.

Taken together, there are several skeletal attributes exhibited by SNX27-deficient mice that phenocopy animal models harbouring defects in PTHR- or \( \mathrm{G}_{\alpha_s} \)-mediated signalling, including decreased osteoblast differentiation, bone formation, and reduction in SOST (Wu et al., 2011, O'Brien et al., 2008), reduced cortical thickness (Calvi et al., 2001) as well as the expansion of the growth plate (described in Section 5.3.3). Despite this, there are also inconsistencies across these various mice models that highlight the complexity of the skeletal phenotype of SNX27-deficient mice. These include how SNX27-deficient mice failed to present with an obvious increase in osteoclast numbers and bone resorption, and a failure to present with the otherwise anticipated increase in osteoblast differentiation and trabecular bone formation (Calvi et al., 2001). Furthermore, while serum PTH levels from SNX27-deficient mice appeared consistent with individuals harbouring constitutively-active PTHR (Kruse and Schutz, 1993, Parfitt et al., 1996), serum \( 1,25(\mathrm{OH})_2\mathrm{D}_3 \) levels appeared slightly decreased (as opposed to an expected increase following sustained PTH-mediated signalling). Together with the lack of urine cAMP levels, these findings remain inconclusive, as there were a lack of samples attained due to the low survival rate of the SNX27-deficient mice.

Collectively, the lack of consistency between mice globally-depleted of SNX27 and models exhibiting PTH/PTHrP/PTHR mutations is not unexpected, as SNX27 regulates the trafficking and signalling of a plethora of other proteins. As previously mentioned, the skeletal phenotype of SNX27 is most likely to involve further aberrations in \( \beta_2\mathrm{AR} \) trafficking (Lauffer et al., 2010, Temkin et al., 2011) and its subsequent requirement for the PTH-mediated anabolic action in bone (Hanyu et
al., 2012), as well as the functional relevance of SNX27 in participating in receptor trafficking within the TGFβ superfamily (Qiu et al., 2010, Yin et al., 2013, Gleason et al., 2014). These are all likely to play a role in the final skeletal phenotype of SNX27-deficient mice, and highlight the critical role of SNX27 in skeletal development and homeostasis.

5.3.5. **SNX27-deficient osteoblasts exhibit altered BMP2-dependent signalling pathways**

BMP-mediated signalling plays crucial roles in regulating both chondrocyte and osteoblast differentiation and function. Originally named for its ability to induce bone formation in cartilage (Urist and Strates, 1971, Nogami and Urist, 1970, Wozney et al., 1988), human recombinant BMP has frequently been used as an osteoinductive agent used to assist fracture healing (Lo et al., 2012, Rosen, 2009). The mineralisation assay performed between wild-type and SNX27-deficient osteoblast cultures (Figure 5.21) revealed (i) an apparent lack of anabolic action by intermittent PTH as well as surprisingly, (ii) an exaggerated anabolic response to BMP2 administration.

BMPs may signal via canonical (Smad-dependent) or non-canonical signalling pathways, the latter often involving cross-talk with β-catenin, MAPK and AKT-dependent pathways. Therefore, to assess whether SNX27-deficient osteoblasts harboured any abnormalities in BMP2-mediated signalling, the activation of a range of effectors from these signalling cascades were then assessed through western blotting (Appendix B, Supplementary Figure 5). Over the course of 90 minutes, the canonical pathway (involving the phosphorylation of Smad1/5 at Ser463/465) appeared to be reduced in SNX27-deficient osteoblasts as compared to wild-type controls. Therefore, signalling effectors involved in BMP2-signalling cross-talk, including β-catenin, AKT, CREB and ERK1/2 were then assessed. Firstly, two phosphorylation sites of β-catenin were examined; Ser675, of which is phosphorylated by AKT and assists to stabilise cytosolic β-catenin (Hino et al., 2005, Taurin et al., 2006, Fang et al., 2007), and Ser33/37 and Thr41, these of which are
phosphorylated by GSK3β to promote the association of the E3 ligase complex to β-catenin and thus degradation via ubiquitination (Hart et al., 1999, Liu et al., 2002, Yost et al., 1996). This preliminary assessment of the balance between β-catenin activation and mark for degradation failed to present a clear mechanism by which SNX27-deficiency perturbs BMP2-mediated signalling in osteoblasts, as both Ser675 and Ser33/37/Thr41 were found to be relatively reduced when compared to wild-type controls. Additionally, there were also minimal differences in the activation of ERK1/2, also suggesting that the BMP2-MAPK signalling cross-talk may not be substantially affected following the depletion of SNX27. Lastly, and perhaps most striking of all, is the increased and sustained activation of AKT in SNX27-deficient osteoblasts following BMP2 administration, of which lasted throughout the 90 minute time course. To further add to this however, is that this did not appear to result in an increased activation of transcription factor, CREB.

Though this presents preliminary data into the alteration of BMP2-mediated signalling in SNX27-deficient osteoblasts, one key limitation is the lack in monitoring the gene response following BMP2 administration. While it is likely to expect an increase in osteogenic markers (e.g. OSX, Col1, OCN, BSP, MEPE and PHEX) due to the observed increase in bone nodule formation in vitro, it would be interesting to observe whether the enhanced response to BMP2 is also maintained amongst the chondrocyte population. For instance, BMP2 is known to synergise with SOX9 to enhance proliferation and hypertrophy (Caron et al., 2013), suggesting that SNX27-deficient chondrocytes may exhibit irregular responses in both PTHR- and BMP2-induced signalling pathways. The investigation into how SNX27 regulates BMP2 remains a subject for future investigation, and will be driven by the generation of the conditional depletion of SNX27 in chondrocytes (Col2 promoter) and osteoblasts (Col1, or OSX promoter).
5.3.6. Aberrations observed within the SNX27-deficient osteocyte population

While differences in proliferation, differentiation and function were assessed amongst the osteoblast population, analyses examining the deficiency of SNX27 in osteocytes was largely limited to histological observations in cortical and calvarial bones. As histomorphometric analyses revealed SNX27-deficient mice to exhibit reduced numbers of osteoblasts in vivo, this may be the key reason as to why these mice also presented with a significant reduction in osteocyte numbers in both cortical and calvarial bone. Furthermore, the reduced expression of osteocyte-associated genes MEPE, PHEX and SOST may also be reflective of this.

The abrogated PTHR signalling intrinsic to SNX27-deficient osteoblasts may have been inherited by the osteocyte population. As previously mentioned in Chapter 1, SOST is exclusively secreted by mature osteocytes and antagonises bone formation by targeting the Wnt/β-catenin signalling pathway. Additionally, PTH exerts its anabolic and catabolic effects on bone by regulating SOST levels, the former in a cAMP-dependent manner (Dallas et al., 2013, Ben-awadh et al., 2014, Kramer et al., 2010, Bellido et al., 2005, Keller and Kneissel, 2005). Intermittent exposure to PTH also acts to increase osteocyte density and prevent apoptosis in mice (Weinstein et al., 2010), however this was not assessed within the scope of this study.

In addition to this, an interesting observation into the expression of connexin 43 (Cx43) suggests that there are intrinsic defects within the osteocyte population following SNX27 depletion. Connexins are the unit proteins that form hexameric hemi-channels (also known as connexons) embedded at the cell surface and may further assemble into gap junctions in the event where hemi-channels meet from adjacent cells. Both hemi-channels and gap junctions enable the exchange of small molecules and signalling messengers <1.2kDa between cells and/or their extracellular environments, and include molecules such as cAMP, ATP, Ca$^{2+}$ and IP$_3$ (Jorgensen et al., 1997, Genetos et al., 2007, Vander Molen et al., 1996, Bivi et al.,...
2011, Segretain and Falk, 2004, Goodenough and Paul, 2003, Stains and Civitelli, 2005). The transfer of small molecules are then capable of eliciting downstream signalling responses in the accepting cell and therefore, enhances the level of communication between the osteocyte network (Bivi et al., 2012a, Bivi et al., 2011, Plotkin et al., 2008, Kanaporis et al., 2008). While there are over 20 connexins identified in mammals (Willecke et al., 2002), Cx43 is the most abundant in bone cells (i.e. osteoblasts, osteocytes and osteoclasts) where it plays crucial roles in skeletal homeostasis (Schirrmacher et al., 1992, Civitelli et al., 1993, Jones et al., 1993, Donahue et al., 1995, Mason et al., 1996).

As shown in Appendix B, Supplementary Figure 6, confocal microscopy was able to detect endogenous Cx43 within native osteocytes embedded within the calvarial bone. Compared to wild-type osteocytes, SNX27-deficient osteocytes presented with significantly reduced dendritic extensions and endogenous Cx43 expression. While the relationship between SNX27 and Cx43 was not further pursued within the scope of this study, the studies of Gja1-null mice possessed some similarities with SNX27-deficient mice. Osteoblast-specific Gja1-null mice presented with an osteopenic phenotype, abnormal osteoblast differentiation, reduced bone formation and a reduced anabolic response to PTH (Chung et al., 2006). Furthermore, the deletion of Gja1 in osteocytes and/or osteoblasts (Bivi et al., 2012b, Watkins et al., 2011, Plotkin et al., 2008) also established Cx43 as an important modulator in osteocyte apoptosis and osteoblast activity. Interestingly, mice heterozygous for a dominant negative Gja1 mutation led to an increase in osteoblast differentiation, function as well as bone marrow adipogenesis (Zappitelli et al., 2013, McLachlan et al., 2008, Flenniken et al., 2005), the latter of which was attributed to an increase in PPARγ2 expression and BMP2/4 signalling (Zappitelli et al., 2015). Therefore, the abnormal expression of Cx43 in SNX27-deficient mice may potentially contribute to the abnormal responses to PTH as well as the increased adipogenesis observed in osteoblast in vitro cultures (see next below).
5.3.7. SNX27 and its potential role in adipogenesis

An unexpected finding during the osteogenic (mineralisation) assay in vitro was the apparent increase in adipocytes generated in SNX27-deficient osteoblast cultures, as determined through ALP and Oil red O stains (Appendix B, Supplementary Figure 3A, B). Furthermore, this appeared to be reduced in the presence of intermittent PTH (Appendix B, Supplementary Figure 3C). Following this observation, mRNA was also extracted from these cultures, revealing substantially higher levels of two adipogenic transcription factors, PPARγ and c/EBPβ (Appendix B, Supplementary Figure 3D, E), however insufficient samples were obtained for statistical significance. c/EBPβ was in fact recently identified as a transcriptional regulator for SNX27 (Loo et al., 2014), and its upregulation in SNX27-deficient cells may be an attempt to compensate for the lack of SNX27 protein levels. While SNX27-deficient mice did not present with a grossly obvious increase in adipose tissue, these findings were not further pursued in vivo due to the failure of SNX27-deficient mice to survive past 4-weeks of age.

5.4. Summary

From establishing the gene expression profile of the entire SNX protein family in bone tissue and bone-resident cell populations, SNX27 was identified as a crucial modulator in skeletal homeostasis. SNX27-deficient mice exhibited a severe skeletal phenotype of osteochondrodysplasia, characterised with strikingly reduced bone mass and reduced osteoid deposition, partly due to intrinsic defects in osteoblast differentiation and function. Furthermore, osteoblasts derived from SNX27-deficient mice were found to exhibit aberrant PTHR signalling, where intermittent stimulation with PTH(1-34) led to the sustained generation of cAMP and activation of downstream signalling effectors, AKT and CREB. Therefore, these data have collectively identified SNX27 as a novel regulator in skeletal homeostasis through its intrinsic role in regulating PTHR signalling in osteoblasts.
SNX27 couples PTHR to the Retromer trafficking complex for signal termination
6. SNX27 couples PTHR to the Retromer trafficking complex for signal termination

6.1. Introduction

The growth and refurbishment of the vertebrate skeleton are strictly dependent on the coordinated cross-talk between bone-resident cells and their ability to respond to external stimuli via cell surface signalling receptors (Kronenberg, 2003, Sims and Martin, 2014). GPCRs form a large proportion of surface-resident receptors responsible for instructing bone cell differentiation and function, where the ligand-induced activation of the receptor and downstream G protein-dependent signalling pathways result in specific cellular outcomes. Among these is the class B parathyroid hormone receptor type 1 (PTHR) which is capable of G protein-dependent signalling following binding to either of its endogenous ligands; PTH or PTHrP. This predominantly results in the activation of the G\(_{\alpha_s}\)-cyclic AMP (cAMP) signalling pathway and to a lesser extent, G\(_{\alpha_q}\)-PKC-PLC-dependent signalling pathway as previously described in Chapter 1. Though PTHR is expressed in a range of tissues, it is best recognised for its role in skeletal development and regulation in mineral ion metabolism, where its abnormal expression and/or function has been found to correspond to several severe skeletal dysplasias and various metabolic syndromes in both human and mice. For instance, individuals diagnosed with JMC possess a mutation in \(PTHR\) resulting in its constitutive activation (Jansen, 1934, Juppner and Schipani, 1997), while Blomstrand’s lethal chondrodysplasia occurs as the result of a loss-of-function \(PTHR\) mutation and often leads to prenatal death (Blomstrand et al., 1985, Jobert et al., 1998, Oostra et al., 2000, Zhang et al., 1998). As reviewed by Schipani and Provot, individuals with either disease are marked with short-limbed dwarfism and severe abnormalities in skeletal growth and development, highlighting the importance of PTHR within the context of the skeleton (Schipani and Provot, 2003).
Within the physiological context of bone, both the magnitude and duration of PTHR signalling is central to its ability to induce opposing net effects on bone. This is best exemplified in patients with primary hyperparathyroidism (Fraser, 2009) and JMC (Schipani et al., 1996, Schipani et al., 1995) whereby persistent PTHR signalling indirectly increases osteoclast bone resorption through its actions on the osteoblast and osteocyte, resulting in hypercalcaemia and the net reduction in bone mass (Kruse and Schutz, 1993, Parfitt et al., 1996, Stein et al., 2013). In contrast, the intermittent administration of PTH (in the form of teriparatide, PTH(1-34) as FORTEO™) or more recently, a modified analogue of PTHrP (in the form of abaloparatide as TYMLOS™) induces a net anabolic response in bone by stimulating the activity and differentiation of osteoblasts and bone-lining cells (Kim et al., 2012, Jilka, 2007, Dobnig and Turner, 1995) and remain as the only FDA-approved anabolic therapies for the management of osteoporosis in post-menopausal women (Shirley, 2017, Chew and Clarke, 2017, Raisz, 2005, Neer et al., 2001, Greenspan et al., 2007). Therefore, understanding the molecular mechanisms involved in the temporal regulation of PTHR signalling may provide new avenues in tailoring PTH-based therapies in alleviating the consequences of prolonged PTH exposure.

Like other GPCRs, while the ligand-induced activation of surface resident PTHR induces immediate G protein-dependent signalling events at the cell surface, this is also promptly followed by the β-arrestin-dependent endocytic event of the signalling complex into early endosomes (Smith and Rajagopal, 2016). While this remains as the canonical mechanism which the cell utilises to terminate most GPCR signalling events, PTHR is among a small yet growing subset of GPCRs shown to exhibit persistent cAMP generation following endocytosis, a phenomenon referred to as ‘non-canonical’ signalling (Ferrandon et al., 2009, Feinstein et al., 2011, Pavlos and Friedman, 2017). Additional mechanisms are therefore required in the termination of endosomal PTHR signalling and include the intraluminal acidification of the endosome (Gidon et al., 2014) as well as the post-endocytic sorting of PTHR away from the endosomal compartment (Sorkin and von Zastrow, 2009, Elkin et al.,
2016). The latter results in the physical removal of the receptor away from adjacent signalling effectors, where the receptor may then be sorted for either its return to the cell surface or for lysosomal degradation (Alonso et al., 2011). Studies conducted in HEK293 cells by Feinstein and colleagues have recently demonstrated that the evolutionary-conserved trafficking complex retromer is recruited to PTHR-bearing endosomes and that PTHR signalling was prolonged in the absence of VPS35, an obligate subunit of retromer (Feinstein et al., 2011, Ferrandon et al., 2009). While these findings implicate the requirement of retromer to terminate the non-canonical signalling of PTHR from within the endosomal compartment, precisely how PTHR is recognised and subsequently retrieved during the post-endocytic sorting event is presently unclear, and hence forms the basis of this Chapter.

Retromer is an evolutionary-conserved trafficking complex that was first discovered to modulate the retrograde (endosome-to-TGN) transport of a variety of cargo receptors in yeast and mammalian cells (Teasdale and Collins, 2012), but has since also been found to facilitate the recycling of cargo (endosome-to-PM) following ligand-induced receptor endocytosis (Gallon and Cullen, 2015). Its ability to recognise a range of cargo while conferring specificity is partly attributed to the formation of three distinct retromer complexes with select members of the SNX family, i.e. SNX-BAR-retromer (Rojas et al., 2007, Wassmer et al., 2007), SNX3-retromer (Lucas et al., 2016, Harrison et al., 2014, Strochlic et al., 2007) and SNX27-retromer (Steinberg et al., 2013, Temkin et al., 2011) as described in Chapter 2. In particular, the role of SNX27-retromer has become increasingly important in the post-endocytic trafficking of a variety of cargo proteins and receptors, as emphasised by the recent SNX27-interactome generated by Steinberg and colleagues (Steinberg et al., 2013). These include β2-adrenergic receptor (β2AR), a crucial GPCR within the skeletal context which, similar to PTHR, undergoes ligand-induced receptor activation and subsequent β-arrestin-mediated endocytosis (Vilardaga et al., 2002). Following ligand-induced endocytosis, SNX27 was found to engage with β2AR via its carboxy-terminal PDZbm and modulate receptor entry into retromer-mediated tubules en route to the cell surface (Lauffer et al., 2010, Temkin et al., 2011).
Moreover, the retrieval of these activated receptors into retromer-dependent tubules was required for the termination of β2AR-endosomal signalling, and formed a distinct subset of receptors from those transported via bulk-recycling mechanisms (Tian et al., 2016, Bowman et al., 2016).

As described in the previous chapter, mice globally-lacking SNX27 exhibit a form of osteochondral dysplasia that is, in part, attributable to disruptions in PTHR signalling. While the overall skeletal phenotype of these mice is the likely result of the promiscuity of the SNX27-PDZ domain for a broad array of other PDZbm-containing cargo (Steinberg et al., 2013, Pfeffer, 2013), this observation that PTHR signalling is elevated in SNX27-deficient osteoblasts raised the hypothesis that the PTHR is a novel PDZ-binding cargo of SNX27. In keeping with this position, PTHR possesses a type I PDZbm at its extreme carboxy-terminus (Mahon et al., 2002) that has been shown to engage with the PDZ domain-containing protein, NHERF1/2 (Mahon et al., 2002, Sneddon et al., 2003, Wheeler et al., 2008, Mamonova et al., 2012). Therefore, by combining protein-protein interaction assays together with high-resolution confocal microscopy, this chapter sought to;

(i) Confirm the molecular interaction between PTHR and SNX27;
(ii) Characterise the molecular interplay between PTHR, SNX27 and the retromer trafficking complex;
(iii) Investigate the functional role of SNX27-retromer in PTH-induced PTHR trafficking and signalling.

### 6.2. Results

SNX27 engages with most of its cargo via its PDZ domain by recognising canonical type I PDZbm recognition sequences located within the extreme carboxy-terminus of interacting cargo, defined as [E]-[S/T]-x-ϕ, where x represents any residue and ϕ is any hydrophobic residue (Figure 6.1A). While PTHR has been
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Figure 6.1.
Figure 6.1. Sequence alignment of the canonical PDZbm of established and potential SNX27-interacting partners.

(A) The canonical type I PDZ-binding motif (PDZbm) displayed as four residues; E (Glu, glutamic acid), S/T (Ser/Thr, serine or threonine), x (any residue) and ϕ (any hydrophobic residue).

(B-E) The carboxy-terminal PDZbm of reported SNX27-interacting partners (B), PTH1R (C), Frizzled (Fzd) receptors (D) and other potential candidates (E). Colours are indicative of the level of conservation with the canonical PDZbm shown in (A).

*Alignment was conducted using Jalview Applet, as described in Waterhouse et al., 2009.*
reported to house a type I PDZbm conforming to these requirements (E-T-V-M) and is capable of engaging with NHERF1/2, its association with the PDZ domain of SNX27 had not been previously established. Interestingly, the PDZ domain of SNX27 shares strong sequence conservation with the PDZ domain(s) of NHERF proteins, i.e. both possess a conserved ‘GYGF’ motif that is essential for mediating PDZ-dependent interactions (Lee and Zheng, 2010). Sequence alignment of the PTHR-PDZbm with respect to the PDZbm of established SNX27-interacting partners using Jalview (Waterhouse et al., 2009) (Figure 6.1B, C) revealed that the PDZbm of PTHR conformed to the canonical binding signature (i.e. [E]-[S/T]-x-ϕ) of other SNX27-PDZ interacting cargoes. Importantly, the PDZbm of PTHR was highly conserved between mouse and humans, thus making the receptor an excellent fit as a bona fide SNX27-interacting cargo.

Along with PTHR, the carboxy-termini of other transmembrane proteins linked to bone homeostasis were similarly subjected to bioinformatic screening to identify other potential candidates for SNX27 engagement (Figure 6.1D, E). Members of the Frizzled (Fzd) receptor family are known to possess a carboxy-terminal PDZbm known to engage with PDZ domain-containing protein, Dishevelled, where the latter promotes downstream Wnt/β-catenin signalling central in skeletal development and homeostasis (Gao and Chen, 2010). Among these, Fzd1, 2, 4 and 7 all possess canonical type I PDZbms (Figure 6.1D) and represent likely candidates for SNX27 interaction. Moreover, other members of the Wnt signalling pathway were also identified as potential SNX27-interacting partners, including β-catenin and members of the LRP co-receptor family (Figure 6.1E). Together, these bioinformatical data illustrate the common existence of PDZbm sequences amongst several prominent transmembrane proteins involved in signal transduction, ion and nutrient metabolism within the skeleton and may all contribute to the global skeletal phenotype presented in SNX27-deficient mice. Among these, the role of PTHR in skeletal development and bone metabolism is well established was thus chosen as the primary focus of this chapter, while the role of SNX27 in relation to the other listed candidates remains as the focus of on-going investigations.
To test for a direct PDZ-mediated engagement between SNX27 and PTHR, a series of biochemical protein-protein interaction assays were performed. The first utilised the technique of bioluminescence resonance energy transfer (BRET), commonly used to monitor the proximity of two proteins in living cells. This highly sensitive assay yields a signal from the acceptor chromophore (in this case, GFP) after receiving the energy emitted from a bioluminescent donor molecule (commonly Renilla luciferase); an event that only occurs when the two proteins are within close proximity (<100Å). Therefore, the emission and magnitude of the detection signal are proportional to the intracellular association between the two proteins of interest.

For BRET assays, HEK293 cells stably expressing \( \text{R}_{\text{Lac}} \)-PTHR were transiently transfected with several SNX27-GFP fusion constructs (Figure 6.2A). These included SNX27\(^{\text{GFP}}\) wild-type (WT) or mutant proteins lacking the PDZ (\(\Delta\text{PDZ}\)) or PX (\(\Delta\text{PX}\)) protein- or lipid-interacting domains, respectively, or harbouring a His\(>\)Ala residue substitution at position 114 (H114A) known to perturb PDZ-mediated interactions (Lauffer et al., 2010). Importantly, the subcellular targeting of these fusion chimeras were all pre-validated by confocal microscopy, with both SNX27-WT and \(\text{R}_{\text{Lac}}\)-H114A proteins retaining their PX-mediated localisation to endosomal structures, while SNX27-\(\Delta\text{PX}\) mutant protein remained largely cytosolic (Figure 6.2B). Surprisingly and despite containing an intact PX domain, the localisation of SNX27-\(\Delta\text{PDZ}\) mutant protein was also predominantly cytosolic, suggesting that the PDZ domain may be a pre-requisite for correct SNX27 localisation.

Having validated the expression of subcellular targeting of SNX27\(^{\text{GFP}}\) WT and mutant variants, BRET assays were performed using HEK293,\(\text{R}_{\text{Lac}}\)-PTHR-expressing cells. As receptor endocytosis is rapidly induced within the initial 15-minutes post-agonist stimulation (Lodish et al., 2000b) and engagement of SNX27 with endosomal cargo is likely to occur during this time (Temkin et al., 2011), \(\text{R}_{\text{Lac}}\)-PTHR/SNX27\(^{\text{GFP}}\) co-expressing cells were stimulated with PTH(1-34) for 15 minutes to maximise the endosomal residence of PTHR prior to the detection of BRET signals (Figure 6.2C). SNX27-WT yielded the strongest BRET signal in the presence of \(\text{R}_{\text{Lac}}\)-PTHR relative to those expressing GFP alone, thus supporting the
Figure 6.2. Bioluminescence resonance energy transfer (BRET) confirms the direct protein interaction between SNX27-GFP and RLuc-PTHR constructs in HEK293 cells.

(A) Illustration of SNX27-GFP constructs used, where ΔPDZ and ΔPX indicate the removal of the PDZ and PX domains, respectively, and H114A represents His>Ala mutation at residue 114 within the SNX27-PDZ domain.

(B) HEK293 cells were transiently transfected to express GFP, or SNX27-GFP constructs, and re-seeded on poly-L-lysine-coated coverslips for 48-hours prior to fixation and imaging by confocal microscopy. Scale bar represents 10 μm.

(C) HEK293 cells stably expressing RLuc-PTHR were transiently transfected with SNX27-GFP constructs for 48-hours prior to BRET analysis, as described in Materials and Methods. Cells were stimulated with PTH(1-34) (100nM) for 15 minutes prior to signal detection. Graph represents normalised ratio of signal detection ± SD. Graph is representative of three individual experiments.
hypothesis of the physical association between the two proteins under physiological stimulation. In comparison, SNX27-ΔPDZ or –ΔPX mutant constructs failed to yield significant BRET signal above control (GFP-alone) cells indicating that an intact PDZ and/or PX domain are required to bring SNX27 into receptor proximity for ensuing direct interaction.

Next, to confirm this physical association, co-immunoprecipitation assays were performed using HEK293 cells stably expressing Myc-PTHR (Figure 6.3A, Appendix B, Supplementary Figure 8) or N-GFP-PTHR (Figure 6.3B, C). Following 15-minutes post-agonist stimulation, stimulated cells were lysed and SNX27-GFP constructs were captured using GFP-Trap_A beads. Consistent with the BRET assay, SNX27-WT was able to form a physical complex with PTHR, where this association was considerably reduced in the presence of the PDZ-H114A mutation (Figure 6.3A). As the association between SNX27 and PTHR was completely abolished in cells expressing SNX27-ΔPDZ, these data altogether confirm that this interaction is mediated through the PDZ domain. In parallel, the temporal association between exogenous N-GFP-PTHR with endogenous SNX27 was assessed by monitoring the formation of the associating complex across 60 minutes post-PTH stimulation (Figure 6.3B, C). While the direct interaction between SNX27 and PTHR was observed in the absence of ligand exposure (i.e. 0 minutes, likely reflecting the indiscriminate protein interaction upon cell lysis), SNX27-PTHR complex formation was markedly pronounced following ligand-induced stimulation, peaking at 30 minutes before tapering at 60 minutes. Altogether, these data confirm that PTHR is a novel SNX27-binding cargo and that this interaction is (i) PDZ-mediated and (ii) enhanced in response to ligand stimulation and/or receptor activation.

To investigate the functional involvement of SNX27 in PTHR trafficking and signalling, a HEK293 cell overexpression model was employed. The HEK293-PTHR overexpression system has been widely employed and validated as a reliable model for studying the trafficking and signalling kinetics of PTHR and other GPCRs (Feinstein et al., 2011, Ferrandon et al., 2009). For this, several stable PTHR-
Figure 6.3. Immunoprecipitation (IP) confirms the PDZ-mediated interaction between SNX27 and PTHR.

(A) HEK293 cells stably expressing Myc-PTHR were transiently transfected with GFP, or SNX27-GFP constructs for 48-hours prior to co-IP using GFP-Trap_A beads, as described in Materials and Methods. SNX27-WT (and to a lesser extent, SNX27-H114A) were confirmed to pull-down PTHR, but not SNX27-ΔPDZ. Right panel, total lysate (input 1%); left panel, bound lysate (10%); IB, immunoblot; IP, immunoprecipitation.

(B) HEK293 cells stably expressing N-GFP-PTHR were seeded for 48-hours prior to time-dependent PTH(1-34) (100nM) stimulation across 60 minutes, immediately followed by protein extraction and IP using an anti-SNX27 antibody. Endogenous SNX27 was confirmed to pull-down PTHR in a time-dependent manner.

(C) Semi-quantitative densitometry graph representing the relative association between PTHR and SNX27, as measured from (B).
expressing HEK293 clones were generated by transfecting either untagged or tagged human-PTHR fusion chimeras, including the previously mentioned RLuc-PTHR, Myc-PTHR and amino-terminal GFP-tagged PTHR (N-GFP-PTHR), all of which were maintained under antibiotic selection. Importantly, the expression and subcellular targeting of these constructs were all confirmed by confocal microscopy prior to their use in ensuing experiments described in this chapter.

First, the trafficking itinerary of PTHR was mapped by simultaneously monitoring the localisation of both the receptor (N-GFP-PTHR) and ligand (TMR-labelled PTH(1-34), herein PTH-TMR) in HEK293 cells with respect to a panel of well-characterised subcellular markers (Figure 6.4-6.5). At steady-state, N-GFP-PTHR localised exclusively to the cell surface (Figure 6.4B), where the formation of N-GFP-PTHR – PTH-TMR receptor-ligand complexes (regions of yellow overlap) remained restricted at the PM upon addition of PTH-TMR (100nM) under temperature restriction (i.e. 4°C ice block) (Figure 6.4B). The release of the temperature block, or addition of the ligand at 37°C resulted in the rapid internalisation of PTHR-PTH complexes into early endosomes (within 5 minutes) as identified by co-localisation with the early endosomal marker, EEA-1 (Figure 6.4A). Receptor sequestration within early endosomes was confirmed by the correlative line-scan analysis represented by the line-graph, where the overlap in fluorescent peak intensities corresponds to the degree of spatial co-localisation between each fluorescent channel. Following entry into early endosomes, many GPCRs are then directed back to the PM via post-endocytic sorting mechanisms. As retromer had been previously implicated in the post-endocytic recycling of PTHR (Feinstein et al., 2011) and β2AR (Lauffer et al., 2010, Temkin et al., 2011), PTHR-PTH complexes were then visualised in relation to endogenous retromer subunit, VPS35 (Figure 6.4C, D). Indeed, retromer was found to co-localise to PTHR-PTH complexes as early as 10 minutes and persist up to 30 minutes post-stimulation, suggesting that PTHR is recycled from endosomes via retromer recycling compartments. Following 60 minutes post-stimulation, a subpopulation of these endosomes appeared to cluster toward the perinuclear region identified as the Golgi apparatus (using the trans-Golgi marker, TGN38) (Figure
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Figure 6.4

N-GFP-PTHR  PTH-TMR  EEA-1  Merged

N-GFP-PTHR + PTH stimulation

Inset

Figure

100
200
Distance

EEA-1
PTHR
PTH

VPS35
PTHR
PTH

A.

B.

C.

D.

226
Figure 6.4. Ligand-bound PTHR internalised into EEA-1-positive endosomes and trafficked together with retromer following PTH(1-34) stimulation.

HEK293 cells stably expressing N-GFP-PTHR were cultured on poly-L-lysine-coated glass coverslips for 48-hours prior to time-dependent stimulation with PTH-TMR (100nM) for the indicated times, followed by a ligand washout after the initial 15 minutes, where applicable. Cells were incubated at 37°C during agonist stimulation, with the exception of time point 0 where PTH-TMR was added to cells at 4°C (ice) to identify the surface levels of PTHR. Following the completion of the time-course, cells were then washed in 1X PBS, fixed in 4% PFA and immunostained for endogenous EEA-1- (early endosomes), or VPS35 (retromer). Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens.

Proteins are represented as the following; N-GFP-PTHR (green) PTH-TMR (red), EEA-1 or VPS35 (blue), where nuclear staining (Hoechst, blue) is shown in the first column. PTHR/PTH co-localisation is depicted as yellow, and additional co-localisation with EEA-1 or VPS35 is depicted as white. Scale bar represents 10µm.

(Line scans); the relative fluorescent intensities taken from the indicated line (inset) are depicted as fluorescent peaks, where regions of peak-overlap indicate positive co-localisation between the markers shown.

(A) Surface-resident PTHR/PTH complexes internalised into EEA-1-positive endosomes at 5 minutes post-stimulation.
(B-D) Surface-resident PTHR/PTH complexes co-localised with endogenous VPS35 across 10-30 minutes post-stimulation.
6.5), and may be indicative of endosomal docking as opposed to its complete entry into the Golgi.

Next, the subcellular distribution of receptor-ligand complexes were assessed in relation to endogenous SNX27 (Figure 6.6). Under basal (agonist-naïve) conditions, endogenous SNX27 resided on intracellular vesicles that were highly reminiscent of endosomes (Figure 6.6, 0 (ice)). Following the brief exposure to PTH (5 minutes), PTHR was found to internalise into SNX27-positive vesicles, with a peak association at 15 minutes post-stimulation which then moderately declined thereafter, as determined by Pearson’s correlation coefficient (PCC) (Figure 6.6D). This was found to be mediated by the PDZ-PDZbm interaction, as there was a significant reduction in SNX27 association with receptor/ligand complexes in cells expressing PTHR-ΔPDZbm (Figure 6.7B, C). The nature of SNX27-bearing vesicles was confirmed to be of early endosomes through the use of a series of endosomal markers (Figure 6.8). As expected, SNX27-GFP (WT) co-localised with PTH-TMR to EEA-1+ and VPS35+ endosomes and endosomal recycling tubules (Figure 6.8A, B) as well as with the PI3P-binding 2x-FYVE construct (Figure 6.8C). In contrast, SNX27 did not co-localise to the Pi(3,4)P2-binding ML1N*2 construct, the latter of which is instead, enriched on endo-lysosomal compartments (Figure 6.8D). Following the identification of SNX27-retromer on endosomal recycling tubules (Figure 6.8B), the nature of these SNX27-retromer tubules was then further confirmed through the use of two Rab identity markers – Rab5 (early endosomes) and Rab4b (recycling endosomes) (Figure 6.9). SNX27 co-localised to Rab5-positive early endosomes as early as 5 minutes post-PTH stimulation, however its distribution shifted towards tubules that extended from Rab4b-positive recycling endosomes at time points that coincide with receptor recycling (i.e. 15-30 minutes, Figure 6.9A, C). The transition of an endosome bearing different Rab markers (in this case, early endosomes (Rab5) to recycling endosomes (Rab4b)) is also known as ‘Rab conversion’ (Figure 6.9B), and signifies known changes in endosomal membrane architecture that facilitates endosomal sorting and recycling (Stenmark, 2009).
Figure 6.5

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Figure 6.5. A subset of ligand-bound PTHR translocated toward the perinuclear TGN38-positive region following 60 minutes post-PTH(1-34) stimulation.

HEK293 cells stably expressing N-GFP-PTHR were cultured on poly-L-lysine-coated glass coverslips for 48-hours prior to time-dependent stimulation with PTH-TMR (100nM) for the indicated times, followed by a ligand washout after the initial 15 minutes, where applicable. Surface labelling with PTH-TMR (100nM) at 4°C is shown at time point, 0 (ice). Following the completion of the time-course, cells were then washed in 1X PBS, fixed in 4% PFA and immunostained for endogenous TGN38 (trans-Golgi network, TGN). Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens.

Proteins are represented as the following: N-GFP-PTHR (green) PTH-TMR (red), TGN38 (blue), where nuclear staining (Hoechst, blue) is shown in the first column. PTHR/PTH co-localisation is depicted as yellow, and additional co-localisation with TGN38 is depicted as white. Scale bar represents 10µm.

(A, B) A subpopulation of endocytic PTHR/PTH complexes were found to translocate toward the perinuclear (TGN38-positive) region of the cell at 60 minutes, but not at 5 minutes post-stimulation.
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Figure 6.6

Co-localisation between PTH-TR and SNX27

Pearson's correlation

Time (min)

0 30 60 90 120

0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8

A. N-GFP-PTHR + PTH stimulation
B. SNX27 + PTH-TMR + PTHR
C. SNX27 + SNX27
D. SNX27 + SNX27 + PTH-TMR + PTHR

0 (ice) 5 min 15 min 30 min
Figure 6.6. Endogenous SNX27 trafficked alongside ligand-bound PTHR across 60 minutes of PTH-TMR stimulation.

HEK293 cells stably expressing N-GFP-PTHR were cultured on poly-L-lysine-coated glass coverslips for 48-hours prior to time-dependent stimulation with PTH-TMR (100nM) for the indicated times, followed by a ligand washout after the initial 15 minutes, where applicable. Surface labelling with PTH-TMR (100nM) at 4°C is shown at time point, 0 (ice). Following the completion of the time-course, cells were then washed in 1X PBS, fixed in 4% PFA and immunostained for endogenous SNX27. Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens.

Proteins are represented as the following; N-GFP-PTHR (green) SNX27 (red), PTH-TMR (blue), where nuclear staining (Hoechst, blue) is shown in the first row (A). Co-localisation between PTHR and SNX27 is depicted as yellow, where additional co-localisation with PTH-TMR is depicted with white. Scale bar represents 10µM.

(A) Endogenous SNX27 was found on punctate structures throughout the 60 minute time-course.
(B, C) Following stimulation, PTH/PTHR internalised into SNX27-positive punctate and translocated toward the perinuclear region at 60 minutes post-stimulation.
(D) Pearson’s correlation coefficient (PCC) was measured between PTH-TMR and SNX27 across 120 minutes using FIJI (NIH, USA). Measurements shown are a representative average of 100 endosomes ± SEM.
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Figure 6.7. Truncation of the carboxy-terminal ΔPDZbm compromises SNX27 engagement at endosomes.

HEK293 cells stably expressing untagged-PTHR wild-type (PTHR) or truncated for the carboxy-terminal PDZbm (PTHR-ΔPDZbm) were cultured on poly-L-lysine-coated coverslips for 48-hours prior to time-dependent agonist stimulation with PTH-TMR (100nM) for 15 minutes. Stimulated cells were then washed in 1X PBS, fixed using 4% PFA and immunostained for endogenous SNX27. Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens. Proteins are represented as the following; SNX27 (green), PTH-TMR (red) where nuclear staining (Hoechst, blue) is shown in the third column. Receptor/ligand co-localisation are represented by PTH-TMR-positive punctate (red) and co-localisation between ligand-bound receptors and SNX27 are represented as yellow. Scale bar represents 10µm.

(A, B) PTHR (wild-type, A) or PTHR-ΔPDZbm (B) internalised into small punctate structures following the addition of PTH-TMR for 15 minutes.

(C) Pearson’s correlation coefficient (PCC) was used as a measurement of SNX27/PTH-TMR co-localisation, using FIJI (NIH, USA). Measurements shown are a representative average of 100 endosomes ± SEM.
Figure 6.8

A. SNX27-GFP

B. SNX27-GFP

C. SNX27-GFP

D. SNX27-GFP

15 minutes

Figure 6.8

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Figure 6.8. SNX27 co-localised with PI(3)P endosomal markers.

HEK293 cells stably expressing RLuc-PTHR were transiently transfected with SNX27-GFP and mCherry-2xFYVE or mCherry-ML1N*2 for 24-hours. Cells were then re-seeded onto poly-L-lysine-coated glass coverslips for 48-hours prior to 15-minutes agonist stimulation with PTH(1-34) or PTH-TMR (100nM). Cells were then washed in 1X PBS, fixed using 4% PFA and immunostained for endogenous EEA-1 (early endosomes) or VPS35 (retromer). Images were then taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens.

Proteins are represented as the following; SNX27-GFP (green), PTH-TMR, mCherry-2xFYVE or mCherry-ML1N*2 (red), EEA-1 or VPS35 (blue) where nuclear staining (Hoechst, blue) is shown in the first column. Co-localisation between (green/red) markers are depicted as yellow, while co-localisation between all three markers (green/red/blue) are depicted as white. Scale bar represents 10µm.

(Line scans); the relative fluorescent intensities taken from the indicated line (inset) are depicted as fluorescent peaks, where regions of peak-overlap indicate positive co-localisation between the markers shown.

(A, B) SNX27 co-localises with internalised PTHR/PTH complexes at EEA-1- and VPS35-positive early endosomes following 15-minutes post-stimulation.

(C, D) SNX27-retromer co-localises with PI(3)P-binding protein, 2xFYVE, but not with PI(3,5)P2-binding protein, MLN1*2 following 15 minutes post-stimulation.
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A. 

B. Rab conversion

C. Rab conversion

Figure 6.9

Rab-SNX27-VPS35

Early endosome

Recycling endosome

5 min

30 min

Rb-PTHR+ PTH(1-34) stimulation

Rb-PTHR+ PTH(1-34) stimulation

SNX27-mCherry

GFP-Rab5

GFP-Rab4b

VPS35
Figure 6.9. SNX27-retromer co-localised to Rab5-positive early endosomes and Rab4-positive recycling endosomes.

HEK293 cells stably expressing RLuc-PTHR were transiently transfected with mCherry-SNX27 and GFP-Rab5 or GFP-Rab4b for 24-hours. Cells were then re-seeded onto poly-L-lysine-coated glass coverslips for 48-hours prior to time-dependent stimulation with PTH(1-34) (100nM) for the indicated times, with ligand-washout following the initial 15-minutes of stimulation, where applicable. Cells were then washed in 1X PBS, fixed using 4% PFA and immunostained for endogenous VPS35 (retromer). Images were then taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens.

Proteins are represented as the following; GFP-Rab5 or GFP-Rab4b (green), mCherry-SNX27 (red), VPS35 (blue). Co-localisation between; Rab markers and SNX27 is depicted as yellow, Rab markers and retromer as cyan, SNX27 and retromer as magenta, or all three as white. Scale bar represents 10µm.

(A, i) SNX27-retromer was found to localise to discrete microdomains at the surface of GFP-Rab5-positive endosomes following 5-minutes post-stimulation.

(A, ii) SNX27-retromer localised to GFP-Rab4b-positive recycling endosomes following 30-minutes post-stimulation. Particularly, this occurred at discrete microdomains from which de novo endosomal tubules have begun to form.

(B) A schematic representation of ‘Rab conversion’ illustrating the events observed from (A) and described in Chapter 2.

(C) SNX27-retromer localised to regions that were positive for Rab5 as well as a hairline tubular extension that was positive for Rab4b but not Rab5 (yellow arrow).
Next, to further explore the spatiotemporal relationship that exists between SNX27 and PTHR, the behaviour of these two interacting proteins was monitored during agonist-induced receptor endocytosis by time-lapse confocal microscopy (Figure 6.10-6.11, Video 1). As expected, under agonist naïve conditions, N-GFP-PTHR localised almost exclusively to the PM whereas SNX27-mCherry resided primarily on endosomes (Figure 6.10A). By comparison, SNX27 and PTHR co-occupied endosomes following 15 minutes post-PTH stimulation. Upon closer inspection of an individual endosome bearing both SNX27 and PTHR, it was revealed that SNX27 concentrated at distinct microdomains of endosomal protrusions/tubulations (labelled 1, 2 and 3) (Figure 6.10B) that remained stable across the continuous tracking over 5 minutes (Figure 6.10C, D). Importantly, PTHR was also observed to co-localise with SNX27 in de novo tubules emanating from the endosomal surface following agonist-induced receptor internalisation (Figure 6.11B, C). This SNX27-PTHR endosomal tracing is in accordance with the reported function of SNX27 in the recognition and sorting of transmembrane receptors into endosomal recycling tubules (Varandas et al., 2016).

β-arrestin facilitates the endocytosis of GPCRs following receptor activation and has been implicated in promoting non-canonical signalling in a subset of GPCRs, including PTHR (Wehbi et al., 2013, Feinstein et al., 2011, Ferrandon et al., 2009). Interestingly, retromer subunit VPS26 contains an arrestin-like fold (Gallon et al., 2014, Shi et al., 2006, Aubry et al., 2009) that is predicted to engage with the cytosolic tail of PTHR (Feinstein et al., 2011). Therefore, to determine the order of recruitment between β-arrestin, SNX27 and retromer with PTHR, β-arrestin2-GFP, SNX27-mCherry and endogenous retromer (VPS35) were all visualised during the early phase of receptor endocytosis (Figure 6.12). Consistent with literature, (inactive) β-arrestin adopted an exclusive cytosolic distribution under basal conditions (Figure 6.12B), however following 15 minutes post-stimulation, β-arrestin was recruited to endosomes that were positive for both EEA-1 and SNX27-retromer (Figure 6.12A, C). β-arrestin was then found to dissociate from endosomes and predominantly return to its cytosolic state after 30 minutes post-stimulation (Figure 6.12D),
Figure 6.10

A. Naive Agonist PTH(1-34) 15 min

B. Merged

SNX27:mCherry

h-GFP-PTH

Chapter 6 - SNX27 couples PTH to the Retromer trafficking complex
Figure 6.10. Live cell confocal microscopy revealed the spatiotemporal interaction of PTHR with SNX27 at the endosome.

HEK293 cells stably expressing N-GFP-PTHR were transiently transfected with SNX27-mCherry for 24-hours. Cells were then re-seeded onto glass MaTek dishes for an additional 48-hours prior to real-time imaging at 37°C using the Nikon A1Si confocal microscope (Nikon, Japan) and Tokai Hit Stage Top incubator (INUG2E-TIZ) as described in Ng et al., 2013. N-GFP-PTHR is displayed as green, SNX27-mCherry as red and areas of co-localisation are depicted as yellow.

(A) Top, co-transfected cells were imaged under agonist-naïve conditions. Bottom, an independent set of co-transfected cells were imaged following 15 minutes post-PTH(1-34) stimulation (100nM).

(B) An individual endosome bearing both PTHR and SNX27 sourced from (A, inset), accompanied by a circumferential line-scan analysis of the fluorescent intensities at microdomains 1, 2 and 3.

(C, D) Still frames and relative fluorescence intensities at the endosome shown in (B) over 5 minutes of continuous imaging, with images taken at 15 second intervals. Regions of PTHR/SNX27 co-localisation are depicted as yellow.
Chapter 6 - SNX27 couples PTHR to the Retromer trafficking complex
Figure 6.11. Live cell confocal microscopy revealed the mobilisation of cytosolic SNX27 to PTHR-positive endosomes and their coordinate movement into de novo tubules.

HEK293 cells stably expressing N-GFP-PTHR were transiently transfected with SNX27-mCherry for 24-hours. Cells were then re-seeded onto glass MaTek dishes for an additional 48-hours prior to real-time imaging at 37°C using the Nikon A1Si confocal microscope (Nikon, Japan) and Tokai Hit Stage Top incubator (INUG2E-TIZ) as described in Ng et al., 2013. PTH(1-34) was added to cells to a final concentration of 100nM prior to imaging. N-GFP-PTHR is displayed as green, SNX27-mCherry as red and areas of co-localisation are depicted as yellow.

(A) Cells were imaged across 120 seconds following 15 minutes agonist-stimulation.

(B-D) Sourced from (A, inset), still images of an individual endosome bearing both PTHR and SNX27 shown at 10 second intervals across 75s (B, C) and extending to 6 minutes (D). The formation of a de novo tubule forms at ~40s seconds where SNX27/PTHR localised at discrete endosomal tubulations at 120s.
Chapter 6 - SNX27 couples PTHR to the Retromer trafficking complex
Endosomal residence of SNX27-retromer does not depend on the prior endosomal association of β-arrestin.

HEK293 cells stably expressing RLuc-PTHR were transiently transfected with β-arrestin2-GFP and SNX27-mCherry for 24-hours. Cells were then re-seeded onto poly-L-lysine-coated coverslips for 48-hours prior to time-dependent agonist-stimulation with PTH(1-34) (100nM) for the indicated times, with ligand-washout following the initial 15 minutes of stimulation, when applicable. Following the completion of the time-course, cells were then washed in 1X PBS, fixed in 4% PFA and then immunostained for endogenous EEA-1 or VPS35. Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens.

Proteins are represented as the following: β-arrestin2-GFP (green), SNX27-mCherry (red), EEA-1 or VPS35 (blue), where nuclear staining (Hoechst, blue) is shown in the first column. Co-localisation between β-arrestin and SNX27 is depicted as yellow, SNX27 and retromer as magenta, and all three as white. Scale bar represents 10µm.

(A) β-arrestin2 co-localised to EEA-1-positive puncta at 15 minutes following PTH(1-34) stimulation.

(B) Under agonist-naive conditions, β-arrestin2 predominantly adopted a cytosolic distribution while SNX27-retromer remained in complex and associated with endosomal puncta.

(C, D) Following 15 minutes post-PTH(1-34) stimulation, β-arrestin2 co-localised to endosomes of which were also occupied by SNX27-retromer (white). Following 30 minutes, β-arrestin2 dissociated from endosomes while SNX27-retromer continued to remain in complex at endosomes (D).

(E) Pearson's correlation coefficient (PCC) showing the agonist-dependent association between SNX27 and β-arrestin2 (green) but not between SNX27 and retromer (blue).
consistent with the transient role of β-arrestin during this early time period. While the formation of SNX27-retromer complexes was found to be independent of agonist exposure (Figure 6.12E), SNX27 only co-localised with β-arrestin during the time period at which PTHR internalisation was at its peak (Figure 6.12E, 15 minutes). Therefore, these data indicate that while β-arrestin and SNX27 occupied the same early endosomes, engagement between β-arrestin and PTHR precedes that of the SNX27-retromer recycling complex.

The observation that SNX27 engages with both PTHR and retromer on early endosomes raises the intriguing possibility that SNX27 serves as a molecular adaptor to couple PTHR to the retromer complex. To assess this, isothermal titration calorimetry (ITC) was performed to measure the binding of the putative carboxy-terminal PTHR-PDZbm to the SNX27-PDZ domain (Figure 6.13), where a synthetic peptide representing the extended carboxy-terminal PTHR-PDZbm was used (residues 587-593, Q7-E6-E5-W4-E3-T2-V1-M0, where 0 represents the terminal residue). The PTHR-PDZbm was found to associate with the SNX27-PDZ domain with a greater affinity (Kd ~ 6.3µM) than compared to two type I PDZbm of two cargoes trafficked by SNX27-retromer, GLUT1 (Kd ~ 17µM) and Kir3.3/GIRK3 (Kd ~ 154µM) (Gallon et al., 2014; Steinberg et al., 2013). Importantly, this binding was found to be allosterically enhanced upon association with VPS26 (Kd ~ 2.2µM), confirming the physiological relevance of SNX27-retromer and PTHR and is similar to what was previously observed with Kir3.3/GIRK3 (Gallon et al., 2014). By comparison, the PDZ binding-defective mutant of SNX27 (H114A) failed to bind PTHR-PDZbm (Figure 6.13A).

Next, to investigate the molecular determinants of PTHR-SNX27 engagement at the atomic level, the crystal structure of the SNX27-PDZ domain bound to the PTHR-PDZbm peptide was generated at an ultra-high resolution (0.95Å) (Figure 6.13B). The final model consists of residues 40-135 of the SNX27-PDZ domain, and residues 587-593 for the PTHR-PDZbm peptide as previously described. High-quality electron density maps demonstrated that the PDZbm of
Figure 6.13

A. Time (min)

B. SNX27PDZ-PTHR

C. SNX27PDZ-PTHR

D. VPS26
Figure 6.13. Crystal structure of the SNX27-PDZ domain bound to the PTHR-PDZbm peptide.

(A) The PTHR-PDZbm peptide binds directly to the SNX27-PDZ domain in vitro (blue), where the prior to engagement with VPS26A enhances this interaction (black). Top, raw ITC data; bottom, integrated normalised data and calculated $K_d$ values ±SD over three experiments. The SNX27-H114A mutation is shown as a negative control (orange).

(B) Electrostatic and ribbon representations of the SNX27-PDZ domain (gray) bound to PTHR peptide (yellow sticks) crystal structure. The PTHR-PDZbm peptide sequence is displayed in the middle, and its refined $2F_o-F_c$ electron density contoured at $2\sigma$ is shown in blue.

(C) Detailed view of the interaction surface between the SNX27-PDZ-binding cavity (gray) and PTHR (yellow). Critical contacts between residues governing the interaction are displayed as black dashed lines.

(D) Overlay of the SNX27-PDZ-VPS26A complex (PDB code 4P2A) with the SNX27-PDZ-PTHR complex structures highlights the different binding interfaces of both partners to SNX27. The SNX27-PDZ domain is shown in gray, VPS26A in orange, and the PTHR peptide in yellow.

*These studies were performed in collaboration with the Institute for Molecular Bioscience, at the University of Queensland.*
PTHR binds in a similar orientation to that previously published for SNX27-Kir3.3 (Balana et al., 2011). Furthermore, two glutamic acid side chains (Glu -5/E-5 and Glu -3/E-3) were found to associate with a basic patch on the SNX27 surface (Figure 6.13B), where in particular, Glu -3 is embraced between Asn 56 and Arg 58 of the SNX27-PDZ domain, and Glu -5 formed a salt bridge with Arg 58. Similarly present in association between SNX27-Kir3.3 (Gallon et al., 2014), these electrostatic interactions are thought to stabilise the engagement between the PTHR-PDZbm peptide within the PDZ cavity and thus provide a basis for the strong and favourable association shown through ITC (Figure 6.13A, C). In contrast, the Glu -6/E-6 and Gln -7/Q-7 residues were not well ordered within the crystal structure, suggesting that these residues are not directly implicated in the interaction between SNX27 and PTHR.

Furthermore, the carboxy-terminal PDZ triplet (T-V-M) was confirmed to be crucial for mediating interactions with the PDZ domain (Figure 6.13C), where the terminal Met 0/M 0 was found to form an array of hydrogen bonds with the backbone of the conserved GYGF residue stretch in SNX27, while Thr -2/T-2 forms a hydrogen bond with SNX27 His 114, explaining the requirement for a serine or threonine at this position. His 114, Asn 56 and Arg 58, along with the GYGF stretch in SNX27 are all highly conserved across a variety of species (Ye and Zhang, 2013, Chan et al., 2016).

Taken together, these data confirm that PTHR binds directly to the SNX27-PDZ domain, where this interaction site is located close to the VPS26-binding surface of SNX27 (Figure 6.13D) and is allosterically enhanced upon SNX27-VPS26 complex formation, similar to that with GLUT1 and Kir3.3/GIRK3 (Gallon et al., 2014).

Termination of non-canonical PTHR-mediated cAMP production requires retromer (Feinstein et al., 2011). Since the results described in this thesis thus far indicate that SNX27 functions as a molecular adaptor that directs PTHR to retromer, this implies that SNX27 may similarly function to restrain endosomal cAMP production elicited from the receptor. To assess this, plasmid-based GFP-tagged short hairpin RNAs (shRNA) targeting endogenous SNX27 (shSNX27) or VPS35 (shVPS35) were used to deplete endogenous levels in PTHR-expressing HEK293 cells (Figure 6.14A, B). Cell lines of HEK293 expressing these constructs were
Figure 6.14

A. 

<table>
<thead>
<tr>
<th>HEK293</th>
<th>Non-Targeting</th>
<th>SNX27</th>
<th>VPS35</th>
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<tbody>
<tr>
<td>SNX27</td>
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<tr>
<td>VPS35</td>
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<tr>
<td>α-tubulin</td>
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B. 

% Knockdown (Relative to endogenous)

- Non-Targeting: ![Image]
- shSNX27: ![Image]
- shVPS35: ![Image]

C. 

<table>
<thead>
<tr>
<th>PTH (1-34) 100nM</th>
<th>Non-targeting</th>
<th>shSNX27</th>
<th>shVPS35</th>
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- p-CREB: ![Image] (43kDa)
- α-tubulin: ![Image] (55kDa)
- p-AKT (308): ![Image] (60kDa)
- AKT: ![Image] (60kDa)
- p-ERK1/2: ![Image] (42kDa)
- ERK1/2: ![Image] (42kDa)
- SNX27: ![Image] (62kDa)
- VPS35: ![Image] (90kDa)
Figure 6.14. HEK293 cells depleted of SNX27 (shSNX27) or VPS35 (shVPS35) exhibited sustained downstream signalling pathways.

Stable PTHR-expressing HEK293 cells were maintained to express GFP-tagged short-hairpin RNA (shRNA) encoding a non-targeting sequence (non-targeting), SNX27 (shSNX27) or VPS35 (shVPS35).

(A, B) Protein lysate was extracted and blotted for knock-down efficiency. SNX27 was depleted by ~60%, while VPS35 was depleted by ~90% following shRNA stable selection, relative to cells expressing non-targeting shRNA.

(C) Stable HEK293 cells were then seeded for 24-hours prior to a time-dependent stimulation with PTH(1-34) (100nM) over 90 minutes, with ligand-washout following the initial 15-minutes of stimulation. Following the completion of the time course, cells were then washed in 1X PBS and protein lysate was then immediately collected as described in Materials and Methods. SDS-PAGE and western blotting was then conducted to assess downstream signalling pathways following PTHR activation. In comparison to cells expressing non-targeting shRNA, SNX27- and VPS35-depleted cells exhibited a substantial increase in CREB phosphorylation, and (to a lesser extent) AKT phosphorylation, during 5-30 minutes post-PTH stimulation.
maintained under antibiotic selection, where expression and knockdown (KD) efficacies were monitored by visualising the percentage of cells expressing the GFP-reporter (~80% in each cell line) and immunoblotting for residual SNX27 or VPS35 protein expression (Figure 6.14A, B). Although this did not result in complete depletion, SNX27 and VPS35 levels were found to be reduced by ~60% and 80%, respectively, compared to cells expressing shRNA non-targeting control cells. Following this, the effects of SNX27- or VPS35-depletion on PTHR-evoked cAMP-dependent signalling were then assessed by examining the activation (phosphorylation) of downstream signalling effectors by western blotting (Figure 6.14C). Consistent with that reported by Feinstein and colleagues, retromer depletion resulted in augmented signalling, an effect equally observed in SNX27-depleted cells. Specifically, the levels of phosphorylated CREB and AKT were markedly elevated, while ERK1/2 phosphorylation increased modestly, yet reproducibly. Furthermore, this signalling was most conspicuously prolonged within the 5-30 minute window immediately following PTH stimulation, a period of which coincided with the formation of SNX27-retromer transport tubules (Figure 6.8B) and was consistent with that shown in SNX27-deficient osteoblasts (Figure 5.23). Thus, taken together these data indicate that SNX27, like retromer, functions to restrict cAMP-evoked PTHR signalling at the endosomal level.

Two possible explanations could account for the elevated PTH-induced signalling in both SNX27- and retromer-depleted cells; either (i) increased surface availability of PTHR (i.e. increased receptor synthesis or delivery to the cell surface, or altered constitutive trafficking), or (ii) the delayed termination of non-canonical PTHR signalling generated from within endosomes. To therefore determine which of these represented these current events, the surface expression of PTHR was assessed through the use of PTH$_{TMR}$ surface labelling (Figure 6.15A). Intriguingly under resting conditions (i.e. temperature block at 4°C), it was revealed that SNX27- or VPS35-depletion significantly reduced the surface levels of PTHR (~50%) as measured by PTH$_{TMR}$ intensity (Figure 6.15B). Thus, the elevation of PTHR
Figure 6.1

A. 4°C

RLuc-PTHR + shRNA-GFP + surface labelling

B. % of control

Cell Surface Intensity

0 20 40 60 80 100 120 140

Non-Targeting

SNX27

SHPS35

+ shRNA-GFP + PTH-TMR

Merged

Chapter 6 - SNX27 couples PTHR to the Retromer trafficking complex
Figure 6.15. Knockdown of SNX27 or VPS35 significantly impaired surface expression levels of PTHR under basal conditions.

Stable PTHR-expressing HEK293 cells were maintained to express GFP-tagged short-hairpin RNA (shRNA) encoding a non-targeting sequence (non-targeting), SNX27 (shSNX27) or VPS35 (shVPS35). Cells were seeded onto poly-L-lysine-coated glass coverslips for 48-hours prior to surface labelling with PTH-TMR (100nM) at 4°C (time point, 0 ice). Cells were then washed in 1X PBS and fixed using 4% PFA. Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens.

Proteins are represented as the following; shRNA-GFP-positive cells (green), PTH-TMR (red) where nuclear staining (Hoechst, blue) is shown in the third column. Scale bar represents 10µm.

(A, B) Surface labelling of shRNA-GFP-positive cells revealed the significant reduction of PTHR surface expression (as represented by PTH-TMR labelling). Relative to cells expressing non-targeting shRNA, PTHR surface levels were reduced by ~50% and ~55% in SNX27- and VPS35-knockdown cells, respectively.
signalling observed in these cells were not due to increased levels of the receptor at the cell surface.

To further investigate whether the loss of either SNX27 or retromer affected the post-endocytic trafficking of PTHR, high-resolution confocal microscopy was employed to monitor the fate of internalised $\text{PTH}^{\text{TMR}}$ at various time points (up to 120 minutes post-stimulation) (Figure 6.16). Cells were stimulated with $\text{PTH}^{\text{TMR}}$ for 15 minutes, followed by a ligand-washout and observed in reference to the endogenous early endosome marker, EEA-1. In reference to adjacent un-transfected cells (lacking the shRNA-GFP reporter), receptor ligand complexes consistently internalised into EEA-1-positive early endosomes as early as 5 minutes post-agonist stimulation (Figure 6.16A). This was then followed by the decline of $\text{PTH}^{\text{TMR}}$ signal at 30 and 120 minutes post-agonist stimulation (Figure 6.16C, D), a period coinciding with the repopulation of PTHR at the cell surface (Alonso et al., 2011). In comparison, the reduced surface availability of PTHR precluded the ability to accurately trace the fate of internalised $\text{PTH}^{\text{TMR}}$ in SNX27-depleted cells (Figure 6.16). Despite this, distinct clustering of $\text{PTH}^{\text{TMR}}$ within the perinuclear region of these cells were observed at the later time point of 120 minutes (Figure 6.16D) that did not reside within EEA-1-positive structures. As this was not observed in adjacent control cells (Figure 6.16D, 6.17A), this was assumed to be a genuine phenomenon following the disruption of SNX27-retromer machinery. The nature of these compartments was then revealed to be of lysosomal origin, following positive staining for the integral membrane protein, Lamp-1 (Figure 6.17). Altogether, these data collectively suggest that the absence of functional SNX27-retromer machinery led to a general reduction of PTHR surface levels while increasing the residence-time of endosomal PTHR (as supported through the stalled entry of $\text{PTH}^{\text{TMR}}$ into lysosomes), thereby resulting in prolonged non-canonical signalling and activation of secondary signalling effectors.
Chapter 6 - SNX27 couples PTHR to the Retromer trafficking complex

Figure 6.16

Inset

Merged

EEA-1

PTH-TMR

SNX27 KD

5 min
15 min
30 min
120 min

RLuc-PTHR + shRNA-GFP + PTH stimulation
Figure 6.16. Comparative visualisation between shSNX27-GFP-negative and SNX27-depleted cells revealed impaired trafficking kinetics of PTHR across 120 minutes post-agonist stimulation.

Stable PTHR-expressing HEK293 cells were maintained to express GFP-tagged short-hairpin RNA (shRNA) encoding a non-targeting sequence (non-targeting), SNX27 (shSNX27) or VPS35 (shVPS35). Cells were seeded onto poly-L-lysine-coated glass coverslips for 48-hours prior to time-dependent stimulation with PTH-TMR (100nM) across 120 minutes, with ligand-washout following the initial 15 minutes of stimulation, where applicable. Cells were then washed in 1X PBS, fixed using 4% PFA and stained for endogenous EEA-1. Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens.

Proteins are represented as the following; shSNX27-GFP-positive cells (green), PTH-TMR (red), EEA-1 (cyan) where nuclear staining (Hoechst, blue) is shown in the fourth column. Scale bar represents 10µm.

(Line scans); the relative fluorescent intensities taken from the indicated line (inset) are depicted as fluorescent peaks, where regions of peak-overlap indicate positive co-localisation between the markers shown.

(A, B) Following 5-15 minutes post-stimulation, PTH-TMR internalised into EEA-1-positive endosomes in GFP-negative cells (inset, 1). In contrast, due to the reduced PTHR surface levels in SNX27-knockdown cells (GFP-positive cells, inset, 2), there was weak detection of PTH-TMR and localisation with EEA-1, as emphasised by the correlative line scan.

(C, D) Following 30-120 minutes post-stimulation, internalised PTH-TMR did not localise to EEA-1-positive endosomes in GFP-negative cells (inset, 1). Despite the considerably low levels of internalised PTH-TMR in SNX27-knockdown cells (inset, 2), a conspicuous accumulation of ligand was observed within the perinuclear region of the cell, which was negative for EEA-1 (D, inset, 2).
Chapter 6 - SNX27 couples PTHR to the Retromer trafficking complex

Figure 6.17

120 minutes
RLuc-PTHR + shRNA-GFP + PTH stimulation

Inset

Merged

LAMP-1

PTH-TMR

shRNA-GFP

Non-Targeting

SNX27 KD

VPS35 KD

A.

B.

C.
Figure 6.17. Delayed PTH-TMR degradation was observed in SNX27- and VPS35-knockdown cells.

Stable PTHR-expressing HEK293 cells were maintained to express GFP-tagged short-hairpin RNA (shRNA) encoding a non-targeting sequence (non-targeting), SNX27 (shSNX27) or VPS35 (shVPS35). Cells were seeded onto poly-L-lysine-coated glass coverslips for 48-hours prior to time-dependent stimulation with PTH-TMR (100nM) for 120 minutes, with ligand-washout following the initial 15 minutes of stimulation. Cells were then washed in 1X PBS, fixed using 4% PFA and stained for endogenous Lamp1 (lysosomal marker). Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens.

Proteins are represented as the following; shSNX27-GFP-positive cells (green), PTH-TMR (red), Lamp1 (cyan) where nuclear staining (Hoechst, blue) is shown in the fourth column. Scale bar represents 10µm.

(A) PTH-TMR was not detected in HEK293 cells expressing non-targeting shRNA following 120 minutes post-stimulation.

(B, C) PTH-TMR was found to accumulate within Lamp1-positive structures residing within the perinuclear region of SNX27- and VPS35-depleted cells. Sharing similarity with (A), PTH-TMR accumulations were not detected in adjacent GFP-negative cells.
6.3. Discussion

The traditional paradigm of GPCR activation involves a cyclical model and begins with the binding of the exogenous ligand to its cognate receptor at the cell surface. This results in the activation and conformational change of the receptor that induces the subsequent activation of Ga subunits at the plasma membrane, predominantly being either Ga\(_s\)/cAMP/PKA or Ga\(_q\)/PLC/PKC downstream signalling cascades. Under most circumstances, β-arrestin directs the endocytosis of the activated receptor, traditionally resulting in the termination of receptor signalling. The receptor may then be (directly or indirectly) recycled back to the PM or delivered to lysosomes for degradation. As some GPCRs have shown to exhibit ‘non-canonical’ signalling from following receptor endocytosis, the post-endocytic processes determining receptor fate become of increasing importance to safeguard against unregulated signalling events.

The evolutionary-conserved retromer complex has been shown to terminate endosomal signalling elicited by PTHR, potentially by directing the retrograde transport of PTHR from the endosome to the Golgi (Ferrandon et al., 2009, Feinstein et al., 2011). In accordance with this report, the data shown throughout this chapter have confirmed that retromer functions to restrain persistent signalling following PTH stimulation. In addition to this however, this chapter also provides comprehensive evidence for the role of SNX27, which acts in concert with retromer to facilitate the recognition and recycling of PTHR toward the PM. Together, SNX27-retromer serves as a ‘molecular brake’ to regulate the non-canonical signalling elicited by PTHR following PTH stimulation (Figure 6.18).

6.3.1. The carboxy-terminal type I PDZbm of PTHR engages with the PDZ domain of SNX27

The comprehensive series of protein-protein interaction assays presented throughout this chapter have confirmed the novel interaction between PTHR and SNX27 and that this is mediated through the carboxy-terminal type I PDZbm of
Figure 6.18. Illustrative model of the integral role of SNX27-retromer in the post-endocytic trafficking and signalling of PTHR following agonist-induced receptor internalisation.

(A) Upon PTH(1-34) stimulation, ligand-bound PTHR internalises into EEA-1-positive endosomes. Cytosolic SNX27 is then recruited to PTHR-positive endosomes and recruits PTHR into retromer-mediated tubules, from which it is then delivered to the cell surface in a Rab4b-dependent manner.

(B) While the agonist-induced internalisation of PTHR is not affected following the depletion of either SNX27 or retromer (VPS35), the absence of SNX27 (and retromer) abolishes the event of (PDZ-mediated) receptor recognition and entry into retromer-mediated tubules for receptor recycling. Endocytic PTHR is thus retained within the sorting endosome, where a subset is then inevitably degraded via the lysosomal pathway. These altogether indicate that the SNX27-retromer-mediated recycling route governing PTHR post-endocytic trafficking, functions to foremost retrieve and recycling endocytic PTHR toward the cell surface, thereby diverting PTHR away from unregulated lysosomal degradation.
PTHR and PDZ domain of SNX27. Moreover, the crystal structure generated of the SNX27-PDZ domain revealed that the β6 strand engages with the PTHR-PDZbm. The glutamic acid residue within the canonical four-residue PDZbm (Glu\(^3\)) as well as upstream residue (Glu\(^5\)) were found to form electrostatic interactions with the conserved Arg\(^{58}\) and Asn\(^{56}\) within the SNX27-PDZ domain. A previous report by Balana and colleagues have also shown that Glu\(^5\) within Kir3.3 is also crucial in determining the PDZ-mediated engagement with SNX27 (Balana et al., 2011), while the loss of either Glu\(^5\) and/or Glu\(^6\) in PTHR compromised its engagement with NHERF1 (Mahon and Segre, 2004). While the electrostatic interactions mediated by both Glu\(^3\) and Glu\(^5\) contributed to the high affinity of interaction between PTHR and SNX27 and were highly ordered within the crystal structure generated, the position of Glu\(^6\) was not well resolved, therefore indicating that it did not play an essential role in protein engagement. Altogether, these data strongly imply that amino acids residing upstream to the canonical four-residue PDZbm also facilitate SNX27-PDZ-mediated interactions. Precisely how these upstream residues influence the affinity of SNX27-PTHR engagement and post-endocytic receptor trafficking is the focus of Chapter 7.

In addition, the PDZ domain of SNX27 has been shown to engage with VPS26A via a β-hairpin that is not present within PDZ domain-containing protein, NHERF1 (Gallon et al., 2014). The formation of SNX27-retromer via this interaction does not compromise the additional engagement with PTHR, rather ITC assays revealed that SNX27-PTHR engagement was allosterically enhanced by a magnitude of 3-fold in the presence of VPS26A and therefore supports the physiological relevance of SNX27-retromer in modulating PTHR recognition and recycling.

6.3.2. PTHR internalises into early endosomes and are sorted into SNX27-retromer-positive recycling tubules

Despite PTHR signalling being extensively studied over many decades, its visual itinerary following ligand-induced endocytosis is comparatively less well understood. As a prototypical GPCR, PTHR has been shown to internalise into EEA-
1-positive endosomes following stimulation with PTH, where a majority is then recycled back to the cell surface by 2 hours (Ferrandon et al., 2009, Feinstein et al., 2011, Alonso et al., 2011). Ubiquitin-mediated proteosomal degradation of the receptor has also been reported, being dependent on the balance between the activities of ubiquitinases and de-ubiquitinases (Alonso et al., 2011). As expected, the findings in this thesis are consistent with previous reports describing the prompt internalisation of PTHR into EEA-1- and retromer-positive endosomes upon PTH or PTH$^{TMR}$ stimulation (Feinstein et al., 2011). Additionally, both SNX27 and retromer were also found present on PTHR-bearing endosomes as early as 10 minutes, and retromer-mediated recycling tubules as early as 15 minutes post-agonist stimulation.

The recruitment of SNX27 to early endosomes is in accordance with the reported lipid preference of its PX domain which binds to PI3P-enriched endosomal membranes, but not the membranes of maturing endosomes bearing PI(3,5)P$_2$. This association was further confirmed through the use of early endosomal Rab marker, Rab5, where SNX27 and VPS35 localised to specific microdomains across the endosomal surface as early as 5 minutes post-agonist stimulation. Rab4 was also used to determine the nature of the SNX27-retromer tubules that contained internalised PTHR. While Rab5 decorated the surface of largely spherical early endosomes, Rab4 was found present on regions of de novo tubulation that were also cohabitated by SNX27 and VPS35, identifying these tubules as recycling tubules destined for the reintegration of cargo at the cell surface (van der Sluijs et al., 1992). Transferrin (Tfn) and its receptor (TfnR) are often used as a model for receptor recycling and have been shown to predominately use rapid (Rab4-dependent) recycling as well as slower (Rab11-dependent) recycling mechanisms (Yamashiro et al., 1984, Daro et al., 1996, Mellman, 1996, Sheff et al., 2002, Sheff et al., 1999). One limitation of this study is that the involvement of Rab11 was not characterised, and would provide potential insight into whether a subset of PTHR translocates to perinuclear recycling endosomes (or endosomal recycling compartments, ERC) located adjacent to the nucleus (Sheff et al., 2002), or enters the Golgi via retrograde transport as previously speculated.
The formation of these de novo tubules were captured using time-lapse confocal microscopy, where upon the ligand-induced internalisation of PTHR, SNX27 was observed to mobilise to these endosomes and coordinately direct PTHR to localised microdomains at which tubules were then formed. This therefore demonstrates that SNX27-retromer is actively recruited to early endosomes where it engages with PTHR and coordinately functions to direct the receptor into retromer-mediated recycling tubules. Furthermore, cells depleted of either SNX27 or VPS35 were found to exhibit a 50% reduction in PTHR surface expression levels under homeostatic conditions. While this could be the result of increased cellular stress and therefore reduced receptor synthesis, it could also be the result of insufficient trafficking machinery (i.e. SNX27-retromer) responsible for guiding constitutively internalised PTHR back to the PM.

Whereas SNX27-retromer directs PTHR sorting and recycling, it does not appear to participate in receptor internalisation as cells expressing either PTHR or PTHR-ΔPDZbm were equally capable of endocytosing the receptor upon PTH stimulation. Furthermore, the formation of the SNX27-retromer complex was found to be independent of PTH stimulation, existing as a stable complex throughout the time course of 60 minutes. This is most likely indicative of its wider physiological role in governing the intracellular trafficking of other PDZbm-containing cargo.

6.3.3. **SNX27-retromer is required for governing PTHR signalling and trafficking in a spatiotemporal manner**

The finding that SNX27-retromer governs the rapid recycling of PTHR following PTH-induced receptor endocytosis prompted the question: does SNX27, like retromer, regulate ‘non-canonical’ PTHR signalling at the endosome? This was addressed using an RNA interference approach (shRNA). As expected, the depletion of either SNX27 or VPS35 in PTHR-expressing HEK293 cells resulted in persistent endosomal signalling that was not attributed to increased PTHR surface expression levels (on the contrary, receptor levels were reduced by 50%). Consequently, the reduced surface availability of PTHR precluded accurate tracking of internalised
PTHR (represented by PTH\textsuperscript{TMR}) across the time period of 120 minutes post-stimulation by confocal microscopy. Moreover, the conspicuous accumulation of PTH\textsuperscript{TMR} in SNX27- or VPS35-depleted cells observed at 120 minutes (that were otherwise absent in control cells) was later confirmed to co-localise to lysosomal compartments. While the visualisation of PTH\textsuperscript{TMR} is a useful tool in tracing ligand-bound PTHR during and post-endocytosis, this technique has its limitations as PTH is known to dissociate from its receptor during endosomal acidification (Gidon et al., 2014). Nevertheless, these findings are clearly indicative that PTHR signalling continually ensues from within the endosomal compartment when SNX27-retromer is deficient. This resulted in an increased activation of signalling effectors downstream of G\textsubscript{\alpha}s, i.e. CREB and PKA during the time period at which receptor retrieval normally occurs (i.e. 5-30 minutes). Thus, deficiency of SNX27-retromer machinery results in (i) the failure to retrieve PTHR, (ii) persistent non-canonical signalling and (iii) an obvious delay in ligand degradation. The activation of PKA has been shown to subsequently contribute to the activation of v-ATPase proton pumps and therefore, the acidification of the endosomal lumen following PTHR activation. However, to what extent does SNX27-retromer deficiency and the resulting sustained PKA activation influence endosomal acidification is unknown, and requires further study.

Nevertheless, the physiological relevance of SNX27-retromer in PTHR signalling was evidenced demonstrated in Chapter 5, whereby SNX27-deficient osteoblasts exhibited coinciding persistent signalling within the period of 5-30 minutes post-PTH stimulation, even in the absence of ligand (i.e. ligand washout after 15 minutes). While the skeletal phenotype of SNX27-deficient mice is likely to be confounded with additional aberrations in cargo trafficking (and potentially signalling), SNX27-deficient osteoblasts possessed clear intrinsic anomalies in PTHR signalling downstream of the G\textsubscript{\alpha}s/cAMP/PKA/CREB signalling cascade. Furthermore, similar to SNX27- and VPS35-depleted HEK293 cells, SNX27-deficient osteoblasts were also shown to possess reduced surface receptor levels, as determined through PTH\textsuperscript{TMR} surface labelling (see Appendix B, Supplementary
Figure 14). While this potentially opposes the persistence of non-canonical PTHR signalling following PTH stimulation, the net effect in the population of osteoblasts at least, is a decrease in osteoblast differentiation, osteoid deposition and mineralisation in vitro and in vivo. The first of two notable limitations to these studies is that, though PTHR predominantly exercises $G_{\alpha_s}/cAMP$-dependent signalling following PTH stimulation, $G_{\alpha_q}$-mediated pathways were not assessed. Secondly, the use of PTHrP was not of particular focus within this chapter as it had previously been reported to dissociate rapidly from the cell surface without inducing receptor endocytosis or subsequent endosomal signalling (Ferrandon et al., 2009), however they had used PTHrP(1-36) which may not exert its full biological effects within the cell. As such, the trafficking and signalling outcomes following PTHrP-induced receptor activation have been briefly investigated and are described in Appendix A.

The finding that PTHR-expressing cells exhibit both canonical (at the cell surface) and non-canonical (within the endosome) signalling raises the question of whether these events are distinguishable by the cell and what functional relevance do they play? As described in (Tsvetanova et al., 2015), endocytosis of the activated receptor may confer spatial and temporal regulation of receptor signalling that is distinct from signal termination. Firstly, the removal of the receptor, its bound-ligand and its activated G protein subunit(s) from the cell surface may act to downregulate the sensitivity of the cell to external stimuli while also prolonging signalling events from within the endosomal compartment (Mullershausen et al., 2009, Calebiro et al., 2009, Ferrandon et al., 2009, Vilardaga et al., 2012). In addition to this, endocytosis may be required to elicit or enhance specific cellular responses by regulating the proximity of signalling effectors to the activated receptor and/or activated G proteins (Tsvetanova et al., 2015). This was shown through the use of adenylyl cyclases generated to reside specifically at the cell surface or endosomal membrane (Stierl et al., 2011, Tsvetanova and von Zastrow, 2014). Here, it was shown that cAMP generated at the endosome resulted in the transcription of response genes in a relatively more efficient manner than compared to cAMP generated at the PM. This may therefore ultimately assist the cell in distinguishing between ligands targeting a
common receptor and subsequent cellular outcomes based on their ability to promote receptor endocytosis. Furthermore, by inhibiting the endocytosis of activated β2AR, a decrease in CREB activation and subsequent transcription of specific β2AR-sensitive genes were observed without having any effect on net cAMP accumulation (Tsvetanova and von Zastrow, 2014).

Due to the recent recognition of the non-canonical signalling of PTHR, relatively little is known in regards to its functional relevance in bone. There have been several mouse models utilising caPTHR mutant initially identified in patients with Jansen’s metaphyseal chondrodysplasia (H223R) (Hanyu et al., 2012, Ono et al., 2012, O’Brien et al., 2008). Unlike the skeletal phenotype of SNX27-deficient mice described in Chapter 5, these three mice models consistently report the requirement of persistent PTHR signalling for (trabecular) bone formation. These mice were also found to exhibit an upregulation in bone turnover genes, including ALP and RANKL (Hanyu et al., 2012) that is partially consistent with that of SNX27-deficient mice. Furthermore and perhaps not surprising, is the fact that there is apparent cross-talk between β2AR and PTHR, where Hanyu and colleagues further showed that functional β2AR was required for intermittent PTH to execute its anabolic response on bone, and was only partially rescued following the overexpression of caPTHR (Hanyu et al., 2012). Whether the persistent signalling stemming from the H223R mutation is solely generated at the PM, endosome or is a combination of both, and how this affects PTHR trafficking remains to be investigated. In light of this, recent reports have described how the PTHR-mediated endosomal generation of cAMP is amplified when β2AR is simultaneously activated (Jean-Alphonse et al., 2017). In this instance, these investigators concluded that the activation of β2AR releases an additional pool of Gβγ subunits that are able to associate with PTHR, Gα, and β-arrestins at the endosomal level (potentially as a ‘megaplex’, see below). This results in Gβγ further sustaining adenylyl cyclase type 2 and therefore resulting in an increase in cAMP and activation of PKA and CREB (Jean-Alphonse et al., 2017). Therefore, given that β2AR is also an established
SNX27-interacting cargo, this β2AR-PTHR crosstalk may further account for the skeletal phenotype manifest in SNX27-deficient mice.

While the dual role of β-arrestin in both promoting and terminating GPCR signalling has become increasingly appreciated, recent reports have firmly established its role in modulating signalling complexes at the endosomal level. Upon mediating receptor endocytosis, β-arrestin has also been found to remain conformationally active following receptor dissociation where it is able to potentially amplify signalling by coupling its signalling effectors to a distant GPCR prior to β-arrestin returning to its quiescent (inactive) state (Lee et al., 2016a, Nuber et al., 2016). β-arrestin has also been shown to function as a scaffold molecule by recruiting various effector molecules to activated GPCRs, resulting in the formation of ‘signalosomes’ (Shenoy and Lefkowitz, 2011, DeFea, 2011, DeWire et al., 2008, Luttrell and Gesty-Palmer, 2010). In particular, Thomsen and colleagues have provided recent insight into the conformational variants of β-arrestin (Thomsen et al., 2016). In short, when β-arrestin is bound strongly to phosphorylated tail of the target GPCR, this generates an endosomal GPCR-Gαs-β-arrestin ‘megaplex’ that is able to sustain signalling from within the endosome without sterically interfering with G protein-receptor coupling (Thomsen et al., 2016). Furthermore, PTHR-expressing cells had been shown to produce exaggerated levels of cAMP in the presence of a dominant negative β-arrestin. This β-arrestin mutant is known to reside in an active state and exerts an enhanced affinity for both ligand-activated GPCRs and clathrin/AP-2 (Feinstein et al., 2011, Burtey et al., 2007). However, despite this mutant apparently inhibiting the association of retromer (VPS29) with endosomal PTHR, whether the increased cAMP generated is the result of canonical signalling or is the result of a ‘megaplex’ remains to be seen.

### 6.3.4. PTHR trafficking via the ASRT complex

During the course of this dissertation and following on from our recent publication (Chan et al., 2016) the role of SNX27 in PTHR trafficking and recycling was independently validated in a parallel study led by Freidman and co-workers.
(McGarvey et al., 2016). In this instance, the investigators used time lapse total internal reflection fluorescence (TIRF) to visually capture individual exocytic events of receptor re-integration at the cell surface, which were reduced upon SNX27 knockdown or upon the addition of Latrunculin A (an inhibitor of actin polymerisation). Therefore, not only did their this study confirm that SNX27-retromer modulated the post-endocytic recycling of PTHR but further suggested that this event was governed by the collective ‘Actin-Sorting Nexin 27-Retromer Tubule (ASRT) complex’ (McGarvey et al., 2016).

As described in Chapter 2, the ASRT complex has become increasingly recognised as a master regulator in membrane trafficking and has been implicated in a variety of diseases in both mouse and human. The ASRT complex is a working unit comprising of SNX27, retromer and the WASH complex, where its components co-ordinately function to deliver recognised cargo to localised, actin-rich sites at the endosomal membrane at which tubule formation is actively driven (Derivery and Gautreau, 2015). Furthermore, actin plays a general yet crucial role in the transport of carrier vesicles from one location to another (Figure 2.2) as well as maintaining the structural organisation of the organelles and in the shape of the cell itself. The data shown throughout this chapter have characterised the involvement of both SNX27 and retromer in governing PTHR signalling and trafficking, however it has not fully captured the participation of other crucial components, including the known involvement of actin polymerisation and the WASH complex. SNX27 was recently shown to engage with WASH complex subunit, FAM21 via its atypical FERM domain and as such, steer the transport SNX27-interacting cargo toward the cell surface (Lee et al., 2016b). In light of this, the localisation of FAM21 in relation to SNX27 and PTHR was assessed at 30 minutes post-PTH stimulation (see Appendix B, Supplementary Figure 11). FAM21 clearly co-localised well with SNX27-positive punctate that were also positive for PTHR, further supporting the role of FAM21 (and by extension, WASH complex) in modulating PTHR post-endocytic transport.
Mutations within genes encoding components of the ASRT complex have been shown to correlate with a variety of neurological diseases, including Alzheimer’s disease (Follett et al., 2014), Parkinson’s disease (Follett et al., 2016, McMillan et al., 2016), Down syndrome (Wang et al., 2013) and infantile epilepsy (Damseh et al., 2015). Furthermore, several SNX27-interacting cargoes have been identified to function within the neurological context, including governing the transport of β2AR (Lauffer et al., 2010, Temkin et al., 2011), GIRK/Kir3 channels (Munoz and Slesinger, 2014) and AMPA and NMDA receptors (Hussain et al., 2014, Cai et al., 2011, Loo et al., 2014). In addition, two papers have shown the potential roles of retromer subunit, VPS35 within the skeletal context. Firstly, hemizygous VPS35 mice were found to possess an osteoporotic skeletal phenotype due to an increase in osteoclast formation and resorption. Here, this was accounted for by the reduced retromer-mediated retrograde transport of RANK and therefore reduced termination of RANK signalling (Xia et al., 2013). Secondly, recent independent studies by Xiong et al., 2016 similarly demonstrated that mice possessing one VPS35 allele specifically in osteoblasts also exhibited an increase in PTHR signalling (CREB, AKT and ERK phosphorylation) in keeping with the findings described within this chapter. In this instance, sustained PTHR signalling was suggested to be due to decreased retrograde transport of PTHR and a concomitant increase in lysosomal degradation. Further, the investigators proposed that these signalling aberrations correlated with the ability of the protein phosphatase 1 (PP1) regulatory subunit 14C (PPP1R14C) to competitively engage with either VPS35 or PTHR. When PPP1R14C engages with retromer, PPP1R14C is unable to inhibit the dephosphorylating activity of PP1, therefore leaving PP1 free to dephosphorylate downstream signalling effectors (i.e. CREB, AKT and ERK) and to thus terminate PTHR signalling. As both retromer and PTHR were suggested to competitively bind PPP1R14C, the depletion of VPS35 therefore resulted in an increase in PTHR-PPP1R14C association which instead, promoted the inhibitory function of PPP1R14C on PP1 activity. The resulting prolongation of PTHR endosomal signalling resulted in the increase in RANKL/OPG expressions and accounted for
the catabolic activities of both primary osteoblasts and VPS35-depleted MC3T3 cells; an event that was reversed when PPP1R14C expression was depleted (Xiong et al., 2016). Therefore, these reports together with those described in this chapter (Chan et al., 2016) collectively unveil the ASRT complex as a key regulator of PTHR signalling and trafficking, and thus is essential for bone homeostasis.

Finally, there have been comprehensive studies describing the synthesis of modified PTH ligands that are capable of inducing differing magnitudes of PTHR downstream signalling, including the generation of long-acting PTH (LA-PTH), modified-PTH (M-PTH) and D-Trp\(^{12}\)-PTH(7-34), the latter of which acts as an inverse agonist to constitutively active forms of PTHR. These have been extensively reviewed in (Cheloha et al., 2016) and (Gardella and Vilardaga, 2015). However, in addition to these novel approaches to manipulate PTHR signalling, the findings presented in this chapter significantly contribute to the decades of collated knowledge of PTHR signalling and trafficking and provide further insight in how SNX27 may be used to fine-tune the magnitude of, or favour certain signalling events within the skeletal context.

6.3.5. **SNX27-independent trafficking of PTHR**

While the above studies clearly identify an important role for the ASRT complex in PTHR signalling and trafficking, McGarvey and colleagues reported an unexpected mechanism by which PTHR could be recycled to the PM in the absence of its carboxy-terminal PDZbm. As the recycling rates of both PTHR wild-type (PDZbm: ETVM) or mutant (PDZbm: ETVA) were shown to be comparable, the authors speculated that an alternative recycling mechanism must exist when engagement with ASRT machinery is deficient. The potential role of NHERF proteins was disregarded as the terminal alanine substitution had been shown to completely abolish its interaction with PDZbm-containing cargo (Hall et al., 1998, Mahon et al., 2002, Sneddon et al., 2003). Instead, they subsequently reported that PTHR may directly engage with VPS26A, and the latter then to SNX27 (albeit the interaction between PTHR-VPS26A was substantially weaker than that between
PTHR-SNX27). Lastly, an additional PDZ domain-containing protein, \( N- \)ethylmaleimide-sensitive factor (NSF) is known to govern the trafficking and surface expression of several receptors (Cong et al., 2001, Song et al., 1998, Nishimune et al., 1998, Wang et al., 2007a) and while protein engagement is mediated through the presence of a carboxy-terminal PDZbm, this conforms to [D/E]-[S/T]-[L/V]-x and may therefore interact with both PTHR wild-type and -ETVA mutant receptors.

Additionally, there are two other ways by which the localisation of PTHR is known to be regulated, both of which involve NHERF proteins. NHERF1 and NHERF2 are known to also engage with the carboxy-terminal PDZbm of PTHR, and have been found to modulate the cell surface retention of functional sub-populations of PTHR (i.e. apical and not basolateral) and its signalling preferences (\( \text{G} \alpha_q \) as compared to \( \text{G} \alpha_s \)) at the apical membrane of renal proximal tubule cells (Wang et al., 2010a, Mahon et al., 2002, Capuano et al., 2007, Wheeler et al., 2008, Sneddon et al., 2003, Traebert et al., 2000, Ba et al., 2003). Furthermore, \( \text{NHERF1} \)-null mice were found to exhibit intrinsic defects in osteoblast differentiation (Liu et al., 2012), suggesting that NHERFs may also play a role in modulating the signalling and/or trafficking of PTHR in osteoblasts. Unlike SNX proteins, NHERF proteins do not possess an intrinsic domain that enables them to localise to endosomal membranes, however they do possess a carboxy-terminal EBD which allows the interaction with the actin-binding protein, ezrin and therefore indirectly linking NHERF complexes with the actin cytoskeleton (Sneddon et al., 2003, Hernando et al., 2002, Mahon and Segre, 2004). Therefore, similar to its role in renal proximal tubule cells, the role(s) of NHERF1/2 are more likely to reflect its established role as a scaffolding protein rather than an endosomal adaptor protein such as that shown for SNX27. Furthermore, while both SNX27 and NHERF1 engage to PTHR through a common PDZbm, their role(s) are more likely to be complementary rather than competitive and potentially subject to different regulatory mechanisms. A recent report by Friedman and colleagues identified that PTH induced the phosphorylation of Thr\(^{591} \) (i.e. ETVM) within the canonical PDZbm of PTHR which subsequently inhibits NHERF-mediated interaction (Mamonova et al., 2017). However, two questions
arise; (i) in which context or regulatory mechanisms favour interaction with SNX27 or NHERF1 (e.g. tissue-specific expression of NHERFs, type of agonist, PTH or PTHrP and/or duration of exposure), and (ii) what is the exact role of NHERF1/2 in governing PTHR localisation and signalling in osteoblasts and potentially in osteocytes and chondrocytes? Answering these questions will provide further insight into how PTHR is regulated in different tissues and how these mechanisms may be manipulated to favour certain cellular outcomes.

Lastly, PTHR is known to possess a ‘KRK’ (Lys-Arg-Lys) motif within its juxtamembrane region of its carboxy-terminal tail that has been shown to bind to the FERM domain of ezrin without compromising NHERF-ezrin interaction (Mahon, 2009). The resulting ternary complex of NHERF, PTHR and ezrin were found to be essential for the apical (but not basolateral) expression and signalling of PTHR at the cell membrane of porcine-derived kidney cells. While the relationship between PTHR, NHERF and the actin cytoskeleton has been identified as crucial for the localisation of distinct sub-populations of PTHR in polarised kidney cells, whether this also applies within the context of bone cells is unknown. Furthermore, the atypical FERM domain of SNX27 is unlikely to engage with the ‘KRK’ motif of PTHR due to its location and failure to conform to the required N-P-x-Y/N-x-x-Y motif.

### 6.4. Summary

Through a series of protein-protein interaction assays and confocal microscopy, SNX27 was confirmed to directly engage with PTHR in a PDZ-mediated manner. Furthermore, the PDZ domain of SNX27 is responsible for coupling PTHR to the retromer complex to direct internalised receptors into retromer-mediated endosomal tubules for PTHR recycling. The depletion of the SNX27-retromer machinery in HEK293 cells led to persistent-activation of CREB and AKT phenocopying that observed in SNX27-deficient osteoblasts. This was later found to be due to disturbances in the post-endocytic trafficking kinetics of
internalised PTHR complexes; the failure to retrieve internalised receptors led to their retention within the signalling endosomes, where a portion were then mis-sorted for lysosomal degradation. Based on these findings, we conclude that SNX27 functions as a molecular adaptor that couples PTHR with retromer to safeguard endosomal PTHR recycling away from lysosomes.
CHAPTER 7

The ‘electrostatic plug’ of PTHR is essential for SNX27-mediated receptor sorting
The ‘electrostatic plug’ of PTHR is essential for SNX27-mediated receptor sorting

7.1. Introduction

GPCRs comprise of the largest family of transmembrane signalling receptors in the mammalian proteome and are crucial pharmacological drug targets for the treatment of a spectrum of diseases (Lagerstrom and Schioth, 2008). These receptors normally reside at the cell surface where binding to their cognate ligand induces downstream signalling cascades that translate into specific cellular outcomes. Cellular homeostasis is achieved through the concerted efforts of several sophisticated mechanisms that ensure the proper magnitude and duration of the elicited signal. Indeed, there are several pathological diseases known to occur in the event of constitutive GPCR activation or in the presence of loss-of-function mutations which render the GPCR non-functional.

The cell exerts spatiotemporal control of GPCR signalling through a series of sequential decisions that ultimately determine the post-endocytic fate of the receptor. Frequently following ligand-induced GPCR activation, the receptor promptly undergoes receptor endocytosis and enters the early endosome where it may then subsequently be sorted for recycling toward the cell surface, resulting in short-term signal attenuation and cellular resensitisation. To ensure specificity and efficiency of receptor recycling, this process is often aided through the presence of short linear recognition motifs encoded within the cargo protein, where in particular PDZbms have been identified as a common yet crucial sorting motif for the recycling of receptors from the endosomal compartment (Lee and Zheng, 2010, Ye and Zhang, 2013, Dunn and Ferguson, 2015). On the other hand, long-term signal attenuation is commonly achieved by routing the activated receptor for lysosomal degradation (Huotari and Helenius, 2011), where the best mechanisms governing this process involves the ubiquitin-mediated pathway and the endosomal sorting complexes
required for transport (ESCRT) complex (Henne et al., 2013). Here, the covalently-attached ubiquitin acts as a sorting motif for the ESCRT complex to recognise and cluster such labelled proteins at the limiting membrane of the maturing or late endosome. The membrane of the late endosome then buds inwardly to form intraluminal vesicles (ILVs) within the lumen of its parent compartment, the latter now referred to as a multivesicular body (MVB) (Piper and Katzmann, 2007, Gruenberg and Stenmark, 2004). MVBs then undergo fusion with lysosomes, where the contents of ILVs are then degraded through the activity of lysosomal proteases (Marchese et al., 2008, Dores and Trejo, 2014).

PDZ domain-containing proteins play crucial roles in modulating protein-protein interactions across a variety of biological processes (Ye and Zhang, 2013, Wang et al., 2010b). PDZ domain-containing proteins mediate endosomal sorting through the recognition of a canonical four-residue PDZbm typically encoded within the carboxy-termini of its interacting cargo and are present within several GPCRs (Hanyaloglu and von Zastrow, 2008, Romero et al., 2011). The data presented in Chapter 6 comprehensively demonstrated the functional role of SNX27 in the rapid recycling of PTHR from the early endosome and that the direct engagement between SNX27 and PTHR relies on the PDZ domain and PDZbm of each protein, respectively. Consistent with the role of PDZbms in cargo sorting, there is also evidence for these sorting motifs to divert cargo away from undesirable lysosomal degradation (Bonifacino and Traub, 2003, Steinberg et al., 2013). As shown in Chapter 6, PTHR lysosomal degradation was enhanced in the absence of either the PDZ domain or following the depletion of SNX27-retromer machinery (Chan et al., 2016). While the post-endocytic endosome-to-cell surface recycling of PTHR is now well-established, comparatively little is known regarding the terminal fate of the receptor upon reaching the end of its life-cycle. Nonetheless, the homeostatic expression of PTHR is thought to depend upon the balanced activity of ubiquitinases and de-ubiquitinases that are able to route PTHR toward the proteosome for degradation (Alonso et al., 2011).
The results of Chapter 6 suggest that PDZ-mediated engagement between SNX27 and PTHR is crucial for favouring PTHR recycling and preventing unregulated receptor degradation. However, whether structural residues upstream of the canonical SNX27-binding PDZbm “TVM” triplet or additional layers of regulation (e.g. phosphorylation) contribute to the strength of SNX27-PTHR engagement remains unclear. Therefore, the aims of this chapter were to;

(i) Map the key molecular determinants of the SNX27-PDZ-PTHR-PDZbm interaction and assess the potential participation of upstream amino acid residues and;

(ii) Determine the structure-function relationship of the identified residues on the post-endocytic transport and fate of PTHR.

7.2. Results

To map the minimal amino acid residues that contribute to SNX27-PTHR interaction, a series of PTHR mutants were generated with either a single or double alanine substitution throughout the extended carboxy-terminal PDZbm of PTHR, denoted PTHR-A1 to A7 (Figure 7.1). According to the conventional nomenclature, the terminal residue (Met, or M) is denoted as position 0 (Met\(^0\) or M\(^0\)), the second last position as -1 (Val\(^{-1}\) or V\(^{-1}\)) and so on. The canonical type I PDZbm remained intact in PTHR mutants A1 to A4 and A7, however PTHR-A4 possessed an additional alanine residue at position +1, which is known to cause a steric clash between SNX27 and its interacting cargo β₂AR (Temkin et al., 2011). Two additional mutants, PTHR-A5 and –A6 were generated in order to determine the extent to which the ‘electrostatic plug’ (i.e. electrostatic interactions between Glu\(^{-3}\)/Glu\(^{-5}\) (PTHR) and Arg\(^{58}\) (SNX27) (Chan et al., 2016, Clairfeuille et al., 2016, Balana et al., 2011)) participated in the interaction affinity between the two proteins. While the single alanine substitution at Glu\(^{-3}\) (PTHR-A5) was anticipated to impair the canonical PDZ-mediated engagement with SNX27, the complete removal of the
Figure 7.1. Sequential alanine substitutions throughout the extended carboxy-terminal PTHR-PDZbm.

Illustration of the series of PTHR mutant receptors generated following the sequential replacement of glutamic acidic residues (red) with alanine (blue) within the extended carboxy-terminal PTHR-PDZbm; Q-E-E-W-E-T-V-M (residues 586-593), with the exception of the PTHR-ΔPDZbm mutant where the terminal six residues of the PDZbm are removed and PTHR-A4 where an additional alanine is added to position (+1). Mutants are denoted PTHR-A1 to A7.
electrostatic interactions by double alanine substitution at both (-3) and (-5) positions were predicted to further compromise this association.

The binding affinities between the mutant PDZbm peptides (i.e. PTHR-A1 to –A7 mutants) and the SNX27-PDZ domain were determined through ITC (Figure 7.2). Relative to PTHR-WT ($K_a = 6.3\mu M \pm 0.1\mu M$) and with the exception of PTHR-A7 ($K_a = 2.0\mu M \pm 0.1\mu M$), the binding affinities for SNX27-PDZ of all other mutant peptides were found to be variably reduced. Substitution of Glu$^6$ for alanine (PTHR-A1) had minimal impact on its association with SNX27-PDZ, yielding a $K_a = 7.3\mu M \pm 1.0\mu M$. By comparison, the substitution of Glu$^5$ (PTHR-A2) or in combination with Glu$^6$ (PTHR-A3) resulted in slightly higher $K_a$ values ($K_a = 15\mu M \pm 0.8\mu M$ and $19\mu M \pm 3.0\mu M$, respectively), further supporting the findings reported in Chapter 6 that while Glu$^6$ may participate, it is not a pre-requisite for PTHR-SNX27 interaction. The additional alanine at position (+1) (PTHR-A4) completely abolished the interaction and as predicted, both PTHR-A5 and –A6 mutants were also unable to interact with the SNX27-PDZ domain. Altogether, these data confirm that the Glu$^3$ housed within the canonical four-residue PDZbm of PTHR is the major structural residue required for the PDZ-mediated interaction with SNX27, where Glu$^5$ may act to further stabilise this interaction (as both PTHR-A2 and –A3 were found to exhibit weaker affinities relative to PTHR-WT).

Next, confocal microscopy was employed to assess for any differences in SNX27 recognition of endosomal PTHR complexes (represented as PTH$^{TMR}$) following alanine substitutions (Figure 7.3). Here, HEK293 cells stably expressing (untagged) PTHR-A1 to –A7 and –ΔPDZbm mutants were stimulated with PTH$^{TMR}$ (100nM) for 15 minutes to maximise PTHR entry into endosomes. All ligand-bound PTHR mutants were found to readily internalise into endosomal-like puncta following agonist-stimulation, indicating that the PDZbm substitutions did not influence endocytosis (Figure 7.3A). Consistent with the ITC binding experiments, all PTHR mutants were found to exhibit variable reductions in endosomal SNX27-association. In particular, mutations affecting Glu$^3$ (i.e PTHR-A5 and –A6) or the
Figure 7.2. High-affinity binding of PTHR to SNX27 requires upstream glutamic acidic residues.

ITC experiments were performed to measure the binding affinity of the PTHR-PDZbm mutant peptides (PTHR-A1 to A7) to the SNX27-PDZ domain, as described in Materials and Methods. With the exception of PTHR-A7, the binding affinity of all mutants variably reduced. The substitution of Glu\(^{-3}\) residue abolished protein-protein interaction (i.e. PTHR-A5 and –A6), as did the addition of alanine (PTHR-A4). The substitution of Glu\(^{\pm}\) impaired protein-protein interactions albeit to varying degrees (i.e. PTHR-A2 and –A3).

These studies were performed in collaboration with the Institute for Molecular Bioscience, at the University of Queensland.
Figure 7.3

Chapter 7 - 'Electrostatic plug' is essential for SNX27-retromer-mediated sorting.
Figure 7.3. Substitution of upstream acidic residues impedes SNX27-PTHR engagement at endosomes.

(A) HEK293 cells stably expressing untagged-PTHR wild-type (WT), mutant (A1-A7) or truncated for the carboxy-terminal PDZbm (PTHR-ΔPDZbm) were cultured on poly-L-lysine-coated coverslips for 48-hours prior to time-dependent agonist stimulation with PTH\(^{-\text{TMR}}\) (100nM) for 15 minutes. Stimulated cells were then washed in 1X PBS, fixed using 4% PFA and immunostained for endogenous SNX27. Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens. Proteins are represented as the following; SNX27 (green), PTH\(^{-\text{TMR}}\) (red). Receptor/ligand co-localisation are represented by PTH\(^{-\text{TMR}}\)-positive punctate (red) and co-localisation between ligand-bound receptors and SNX27 are represented as yellow. Scale bar represents 1µm.

(B) Pearson’s correlation (PCC) was used as a measurement of SNX27/PTH\(^{-\text{TMR}}\) co-localisation, using FIJI (NIH, USA). Measurements shown are a representative average of 100 endosomes ± SEM.

** p < 0.01, *** p < 0.001.
complete removal of the extended PDZbm (PTHR-ΔPDZbm) significantly compromised the association with SNX27, as determined by quantitative co-localisation analyses by Pearson’s correlation (Figure 7.3B). These data altogether confirm that while the carboxy-terminal PDZbm of PTHR does not participate in the initial process of receptor endocytosis, Glu³ was found to be essential for mediating SNX27-engagement at the endosomal level *in cellulo*.

While the removal of Glu³ had the greatest effect on SNX27-mediated engagement with PTHR (PTHR-A5, -A6 and –ΔPDZbm), the additional substitution of Glu⁵ profoundly altered the morphology of ligand-bearing endosomes expressing either PTHR-A6 or –ΔPDZbm and was suggestive of the mis-trafficking of these internalised mutant receptors. To explore this possibility, the trafficking kinetics of N-GFP-PTHR-A6 was compared to that of N-GFP-PTHR-WT in HEK293 cells following ligand stimulation (Figure 7.4A). For this, each stable cell clone was stimulated with PTH-TMR (100nM) across the time course of 120 minutes, with a ligand washout following the initial 15 minutes of ligand exposure. As expected, PTHR-WT was predominantly distributed across the surface of the cell under steady-state conditions (Figure 7.4, surface 4°C) and rapidly internalised into endosomal-like structures following 15-30 minutes post-stimulation. While a subset of receptors could be seen to transit toward the perinuclear region of the cell by 60-minutes, the majority of receptors returned to the cell surface by 120-minutes (Figure 7.4A), by which time all internalised PTH-TMR had degraded and was therefore undetectable by the end of the time course (Figure 7.4B). In contrast, while PTHR-A6 mutants were readily found at the cell surface, this was at a considerably reduced level as determined through PTH-TMR surface labelling (Figure 7.4D). Similar to SNX27- and VPS35-depleted cells (Chapter 6), the reduced surface expression of PTHR-A6 mutants impaired the accurate tracing of internalised receptor-ligand complexes across the entire time course. Interestingly however, a substantial portion of (intracellular) PTHR-A6 was found to accumulate within enlarged endosomal-like structures in the absence of agonist-stimulation (Figure 7.4F, i) as well as at 120-minutes post-
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Figure 7.4. Comparative visualisation between N-GFP-PTHR-WT and N-GFP-PTHR-A6 revealed reduced mutant receptor surface levels and abnormal endosomes.

(A-D) HEK293 cells stably expressing N-GFP-PTHR (A) or N-GFP-PTHR-A6 (C) were cultured on poly-L-lysine-coated coverslips for 48-hours prior to time-dependent agonist stimulation with PTH-TMR (100nM) across 120 minutes, with ligand-washout following the initial 15 minutes of stimulation. Stimulated cells were then washed in 1X PBS, fixed using 4% PFA and imaged using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens. Scale bar represents 10µm.

(E, F) Images taken from A, C) under naïve conditions (i) or after 120-minutes post-stimulation (ii) in cells expressing PTHR-WT (E) or PTHR-A6 (F). Scale bar represents 5µm.
stimulation (Figure 7.4F, ii). In contrast, PTHR-WT receptors were found to reside at the cell surface during both of these time points (Figure 7.4E, i, ii).

The reduced surface levels of PTHR-A6 mutants may either reflect (i) reduced biosynthetic delivery or (ii) the increased endosomal sequestration and/or degradation as a consequence of the removal of the electrostatic plug. To distinguish between these possibilities, we assessed the subcellular localisation of PTHR-A6 mutants with respect to a panel of established subcellular compartment markers. First, the endosomal residence of receptor-ligand complexes was observed at 15 minutes post-PTH stimulation with endogenous EEA-1 (Figure 7.5). As expected, both PTHR-WT (Figure 7.5A) and –A6 mutants (Figure 7.5B, red arrow) internalised into early endosomes positive for EEA-1. However, the population of PTHR-A6-bearing early endosomes were amongst others that were negative for both PTH\textsubscript{TMR} and EEA-1 (Figure 7.5B, yellow arrow), implying that these receptors resided within endosomal compartments distinct from early endosomes and that they did not reside at the cell surface at the time of ligand exposure.

Next, the effect of the PTHR-A6 mutant on the recruitment of endogenous SNX27 to the endosome was further assessed by confocal microscopy at both 15 and 60 minutes post-PTH stimulation (Figure 7.6). As previously described in Chapter 6, peak association of SNX27 on PTHR-bearing endosomes was observed at 15 minutes post-stimulation (Figure 7.6A) and declined at 60 minutes (Figure 7.6C). In comparison, the association between endogenous SNX27 and internalised PTHR-A6 complexes was substantially reduced at both time points (Figure 7.6B, D) to the extent where there was limited recruitment of SNX27 to PTHR-A6-bearing endosomes and was otherwise predominantly distributed throughout the cytosol. Therefore, these data confirm that while both PTHR-WT and –A6 mutants were able to undergo ligand-binding and receptor endocytosis, the endosomal association of endogenous SNX27 was considerably weaker in the absence of the carboxy-terminal electrostatic plug.
Figure 7.5. The majority of N-GFP-PTHR-A6 mutants resided within EEA-1-negative endosomes.
HEK293 cells stably expressing N-GFP-PTHR (A) or N-GFP-PTHR-A6 (B) were cultured on poly-L-lysine-coated coverslips for 48-hours prior to time-dependent agonist stimulation with PTH-TMR (100nM) for 15 minutes. Stimulated cells were then washed in 1X PBS, fixed using 4% PFA and immunostained for endogenous EEA-1. Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens.
Proteins are represented as the following; PTHR (green), PTH-TMR (red) and endogenous EEA-1 (blue) where nuclear staining (Hoechst, blue) is shown in the first column. Receptor/ligand co-localisation are represented as yellow, and co-localisation between all three markers are shown as white. Scale bar represents 5µm.
(Line scans); the relative fluorescent intensities taken from the indicated line (inset) are depicted as fluorescent peaks, where regions of peak-overlap indicate positive co-localisation between the markers shown.
Figure 7.6: 

- 

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Figure 7.6. Ligand-bound N-GFP-PTHR-A6 endosomal complexes were substantially devoid of cytosolic SNX27 endosomal recruitment.

HEK293 cells stably expressing N-GFP-PTHR (A, C) or N-GFP-PTHR-A6 (B, D) were cultured on poly-L-lysine-coated coverslips for 48-hours prior to time-dependent agonist stimulation with PTH-TMR (100nM) for 15 minutes (A, B) or 60 minutes (C, D) following a ligand-washout following the initial 15 minutes of stimulation. Stimulated cells were then washed in 1X PBS, fixed using 4% PFA and immunostained for endogenous SNX27. Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens.

Proteins are represented as the following; PTHR (green), PTH-TMR (red) and endogenous SNX27 (blue) where nuclear staining (Hoechst, blue) is shown in the first column. Receptor/ligand co-localisation are represented as yellow, and co-localisation between all three markers are shown as white. Scale bar represents 5µm. 

(Line scans); the relative fluorescent intensities taken from the indicated line (inset) are depicted as fluorescent peaks, where regions of peak-overlap indicate positive co-localisation between the markers shown.
Next, to explore the dynamic relationship between SNX27 and PTHR-WT or –A6 mutants, the fluorescently-tagged fusion chimeras were monitored by time-lapse confocal microscopy (Figure 7.7). For this, HEK293 cells were transiently co-transfected with either \textsuperscript{N}-GFP-PTHR-WT or –A6 together with SNX27\textsuperscript{mCherry} and then stimulated with PTH\textsuperscript{TMR} for 15 minutes prior to imaging (where images were taken at 15 second intervals). As expected, SNX27 was recruited to endosomes bearing wild-type PTHR, typically associating with PTHR at specific endosomal microdomains that later developed into de novo recycling tubules (Figure 7.7A-C, Videos 2, 4). By comparison, in cells expressing PTHR-A6, the morphology of PTHR-bearing endosomes appeared abnormally spherical, lacking clear endosomal microdomains or evidence of membrane tubule formation (Figure 7.7D, Videos 3, 5). Interestingly, while SNX27 was observable on the surface of the endosomes harbouring the PTHR-A6 variant, its association and behaviour was intermittent, possibly reflecting its inability to recognise or engage with the compromised PTHR-A6 PDZbm (Figure 7.7E, F, Video 3). Together, these data further confirm that the electrostatic plug within the extended PTHR-PDZbm is a crucial molecular determinant for SNX27-cargo recognition and stabilisation at the endosomal membrane.

If SNX27 is unable to recognise or engage with PTHR-A6 at the endosome as is required for its delivery to the cell surface, then what is the post-endocytic fate of the mutant receptor? To address this, the intracellular trafficking itinerary of PTHR-A6 was further mapped with respect to a panel of well-established subcellular markers. Firstly, cells expressing PTHR-WT or –A6 mutant receptors were assessed in relation to trans-Golgi marker, TGN38, following 90 minutes post-agonist stimulation (Figure 7.8). As expected, a subset of ligand-bound WT receptors was observed to translocate toward the perinuclear region of the cell, reminiscent of either the ERC or trans-Golgi region (Figure 7.8A). On the other hand, yet similar to what was previously observed, a majority of PTHR-A6 mutants (not bound to PTH\textsuperscript{TMR}) were found to reside within enlarged endosomal structures distributed throughout the cytosol (Figure 7.8B). The differences in PTHR distribution between...
Figure 7.7

A. N-GFP-PTHR-WT (top) + SNX27-mCherry (bottom)

B. N-GFP-PTHR-A6 (top) + SNX27-mCherry (bottom)

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Figure 7.7. Real-time confocal microscopy captured differences in SNX27-PTHR-A6 association at endosomes.
HEK293 cells stably expressing N-GFP-PTHR-WT or N-GFP-PTHR-A6 were transiently transfected with SNX27-mCherry for 24-hours. Cells were then re-seeded onto glass MaTek dishes for an additional 48-hours prior to real-time imaging at 37°C using the Nikon A1Si confocal microscope (Nikon, Japan) and Tokai Hit Stage Top incubator (INUG2E-TIZ) as described in Ng et al., 2013. PTH(1-34) was added to cells to a final concentration of 100nM prior to imaging. N-GFP-PTHR is displayed as green, SNX27-mCherry as red and areas of co-localisation are depicted as yellow.
Still images of PTHR-bearing endosomes across 90 seconds (captured at 15 second intervals).
(A, B, C) Yellow arrows indicate discrete regions of endosomal budding and are were tightly coordinated with SNX27 residence.
(D, E, F) Yellow arrows indicate SNX27 presence on PTHR-A6-bearing endosomes.
Figure 7.8. PTHR-A6 mutants were not found to translocate toward the perinuclear region after 90-minutes post-PTH stimulation.

HEK293 cells stably expressing \textsuperscript{N-GFP-}PTHR (A) or \textsuperscript{N-GFP-}PTHR-A6 (B) were cultured on poly-L-lysine-coated coverslips for 48-hours prior to time-dependent agonist stimulation with PTH\textsuperscript{TMR} (100nM) for 90 minutes, with a ligand-washout following the initial 15 minutes of stimulation. Cells were then washed in 1X PBS, fixed using 4% PFA and immunostained for endogenous TGN38. Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens. Proteins are represented as the following; PTHR (green), PTH\textsuperscript{TMR} (red) and endogenous TGN38 (blue) where nuclear staining (Hoechst, blue) is shown in the first column. Receptor/ligand co-localisation are represented as yellow, and co-localisation between all three markers are shown as white. Scale bar represents 10µm.
PTHR-WT and A6 mutant receptors therefore suggested that a substantial subpopulation of PTHR-A6 receptors are not routed for recycling (either via the ERC or Golgi) and may have been instead, targeted for terminal degradation along the late-endosomal/lysosomal pathway. Therefore, PTHR localisation was then compared relative to the endogenous multivesicular body (MVB) marker, CD63 (Figure 7.9), (where the time period of 30 minutes was chosen to maximise the detection of PTH\textsuperscript{TMR} with PTHR-A6 mutant receptors). Unsurprisingly, a majority of internalised PTHR-WT receptors remained bound to PTH\textsuperscript{TMR} within the endosomal compartment and expressed minimal association with CD63-positive structures (Figure 7.9A, C). In contrast, endosomes bearing PTHR-A6 mutants formed two distinct sub-populations; (i) those that were expressed at the cell surface and internalised together with PTH\textsuperscript{TMR} (green arrow) and (ii) a subset that resided within CD63\textsuperscript{+} endolysosomal compartments (yellow arrow) (Figure 7.9B, C).

Despite the removal of the Glu\textsuperscript{3}/Glu\textsuperscript{5} residues, PTHR-A6 mutant receptors were still found to be expressed at the cell surface (albeit to a lesser extent than PTHR-WT receptors), therefore concluding that the electrostatic plug is not essential for the biosynthetic delivery of the receptor to the PM. One possible explanation for the reduced receptor expression of PTHR-A6 stems from the interesting observation of excessive membrane ruffling as seen in Video 5. It was therefore hypothesised that PTHR-A6 mutants were subjected to constitutive internalisation and degradation independently of agonist exposure. To confirm this, PTHR-expressing cells were then immunostained for two endolysosomal markers, Lamp-1 and CD63 under agonist-naïve conditions (Figure 7.10). Similar to the findings shown in Figure 7.9, the enlarged structures bearing the majority of PTHR-A6 receptors resided within Lamp-1\textsuperscript{+}CD63\textsuperscript{+} endolysosomal compartments (Figure 7.10B, C). This confirmed that PTHR-A6 mutants were subjected to constitutive degradation in the absence of ligand stimulation and could therefore contribute to the reduced surface expression of PTHR-A6 at the cell surface.
Figure 7.9

30 minutes

A.

WT

N-GFP-PTHR

PTH^TMR

CD63

Merged

B.

A6

C.

Pearson's Correlation Coefficient

WT

A6

CD63/PTHR

CD63/TMR

PTHR/TMR

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Figure 7.9. A substantial proportion of unbound PTHR-A6 mutants associated with MVB marker, CD63 during agonist-stimulation.

HEK293 cells stably expressing N-GFP-PTHR or N-GFP-PTHR-A6 were cultured on poly-L-lysine-coated coverslips for 48-hours prior to time-dependent agonist stimulation with PTH-TMR (100nM) for 30 minutes, with a ligand-washout following the initial 15 minutes of stimulation. Cells were then washed in 1X PBS, fixed using 4% PFA and immunostained for endogenous CD63. Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens. Proteins are represented as the following; PTHR (green), PTH-TMR (red) and endogenous CD63 (blue) where nuclear staining (Hoechst, blue) is shown in the first column. Receptor/ligand co-localisation are represented as yellow, and co-localisation between PTHR/CD63 are represented as cyan. Scale bar represents 10µm.

(A) Ligand-bound PTHR-WT complexes did not localise to CD63-positive structures (green arrow).

(B) Some endosomal PTHR-A6 complexes weakly associated with CD63-positive structures (green arrow) while most unbound PTHR-A6 receptors strongly associated with enlarged CD63-positive structures (yellow arrow).

(C) Pearson’s correlation (PCC) was used as a measurement of co-localisation between the markers indicated, using FIJI (NIH, USA). Measurements shown are a representative average of 100 endosomes ± SEM. *** p < 0.001.
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Figure 7.10

C.

N-GFP-PTHR

Merged

CD63

Lamp1

Lamp1/CD63

WT

A6

0.8

0.6

0.4

0.2

0

Pearson's Correlation

WT

A6

***

***

***

Naive
Figure 7.10. The majority of PTHR-A6-bearing endosomes localised with endolysosomal markers, Lamp-1 and CD63 under agonist-naïve conditions.

HEK293 cells stably expressing N-GFP-PTHR or N-GFP-PTHR-A6 were cultured on poly-L-lysine-coated coverslips for 48-hours prior to fixation with 4% PFA. Cells were immunostained for endogenous Lamp-1 or CD63. Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens.

Proteins are represented as the following; PTHR (green), endogenous Lamp-1 (blue) and endogenous CD63 (red) where nuclear staining (Hoechst, blue) is shown in the first column. Co-localisation between PTHR/Lamp-1 is shown as cyan, PTHR/CD63 as yellow, Lamp-1/CD63 as magenta and all three markers as white. Scale bar represents 10µm.

(A) The majority of PTHR-WT were found to reside at the cell surface in the absence of ligand exposure.

(B) Some PTHR-A6 mutants resided at the cell surface, while most accumulated within Lamp-1/CD63-positive endolysosomal structures.

(C) Pearson’s correlation (PCC) was used as a measurement of co-localisation between the markers indicated, using FIJI (NIH, USA). Measurements shown are a representative average of 100 endosomes ± SEM. *** or ### p < 0.001.
Collectively, the data presented throughout this chapter has confirmed the critical involvement of the extended PDZbm of PTHR, where specifically, Glu\(^{-3}\) and Glu\(^{-5}\) residues, in the form the electrostatic plug, engages with conserved residues within the SNX27-PDZ domain and are crucial for SNX27-mediated recognition of PTHR and subsequent receptor retrieval at the endosomal level. Removal of these electrostatic interactions (as in the case of PTHR-A6) severely compromised the ability of SNX27 to engage with endosomal PTHR and instead, resulted in the unregulated degradation of PTHR-A6 in either the presence or absence of PTH stimulation (Figure 7.11).

7.3. Discussion

The results presented in Chapter 6 had identified the novel PDZ-mediated interaction between SNX27 and PTHR. While the PTHR-PDZbm acts as a crucial recognition motif for engagement with SNX27, it was hypothesised that residues upstream of the canonical four-residue sequence also participated in determining the affinity of cargo interaction. This posture stemmed, in part, from previous reports describing the role of upstream Glu\(^{-5}/Glu^{-6}\) residues of the PDZbm influencing the affinity between SNX27-Kir3.3 and NHERF1-PTHR (Balana et al., 2011, Mahon and Segre, 2004). Furthermore, the structural data presented in Chapter 6 implied that glutamic acidic residues Glu\(^{-3}\) and Glu\(^{-5}\) together formed electrostatic interactions with Arg\(^{58}\) (Balana et al., 2011), an arrangement referred to here as an ‘electrostatic plug’ (Clairfeuille et al., 2016). To further elucidate the mechanisms underlying the interaction between SNX27 and PTHR, this chapter therefore sought to (i) characterise the role of canonical (Glu\(^{-3}\)) and upstream (Glu\(^{-5}/Glu^{-6}\)) glutamic acidic residues and (ii) assess its effects on determining the post-endocytic fate of PTHR.
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Figure 7.11

A. SNX27-retromer recycling (fast)

Early endosome
Endo-lysosomal degradation?

B. Deficiency of 'electrostatic clamp' (PTHR-A6)

Early endosome
PTHR-A6 Receptor degradation

PTHR A6
β-arrestin
SNX27
Rab11
Rab7
Rab4
Rab5

ILVs
Cytosol
Plasma membrane
Figure 7.11 Illustrative model of the integral role of the electrostatic interactions mediated by Glu$^3$/Glu$^5$ (‘electrostatic plug’) in the SNX27-mediated sorting and recycling of PTHR.

(A) PTH(1-34) stimulation induces PTHR internalisation into EEA-1-positive endosomes. SNX27-retromer then recruits to these endosomes and direct the entry of PTHR into retromer-mediated tubules to facilitate receptor recycling.

(B) The absence of the electrostatic plug (i.e. PTHR-A6) does not affect endocytosis but instead, compromises SNX27-mediated engagement with endosomal PTHR. This results in an increased propensity for PTHR-A6 mutants to enter MVB/lysosomes for receptor degradation.
7.3.1. Electrostatic interactions mediated by the canonical Glu\(^{-3}\) residue and upstream Glu\(^{-5}\) residue within the PTHR-PDZbm forms an electrostatic plug that is crucial for SNX27-mediated interaction

In order to determine which glutamic acidic residues were essential for mediating cargo selectivity and/or affinity for SNX27, a series of PTHR mutants (A1 to A7) were generated. Specifically, the removal of either Glu\(^{-3}\) (PTHR-A5) or in conjunction with Glu\(^{-5}\) (PTHR-A6) completely abolished interactions with SNX27 at both the molecular and endosomal level, therefore confirming that Glu\(^{-3}\) is a major determinant in this interaction. In contrast, the Glu\(^{-5}\) residue played an auxiliary role in stabilising the SNX27-mediated interaction upstream of the PDZ cavity, while this interaction was minimally affected in the alanine substitution of Glu\(^{-6}\) alone. In support of this, it was later characterised that in addition to forming contacts with Asn\(^{56}\)/Arg\(^{58}\), Glu\(^{-3}\) also formed a hydrogen bond with Ser\(^{82}\) (Clairfeuille et al., 2016).

7.3.2. Phosphorylation of Ser/Thr residues within the extended PDZbm may serve as an additional regulatory switch for SNX27 preferences

The recent report by Clairfeuille and colleagues was the first to identify a positive role of serine or threonine phosphorylation in PDZ-mediated interactions, therefore further elucidating the molecular interactions governing SNX27-mediated engagements. Firstly, they confirmed that within the extended PDZbm of PTHR, the substitution of Glu\(^{-5}\) for phosphorylated Serine (pSer) formed sufficient electrostatic and hydrogen bonds with the conserved Asn\(^{56}\), Arg\(^{58}\) and Ser\(^{82}\) residues of SNX27. Furthermore, several SNX27-interacting cargoes (including β\(_2\)AR) possessed Ser/Thr residues in the place of Glu\(^{-5}\)/Glu\(^{-6}\) that, when phosphorylated, increased the affinity of SNX27 engagement. For example, the substitution of pSer at position (-6) or (-5) within the β\(_2\)AR-PDZbm was found to increase affinity for SNX27 (and furthermore in the presence of VPS26A), while the phosphorylation of Thr\(^{-2}\) sterically impaired this interaction. This positive regulation of SNX27-PDZ-mediated interaction (and its allosteric enhancement in the presence of VPS26A) was consistently observed amongst several other SNX27-interacting proteins such as 5-
HT₄R and NMDA receptors, GluN1 and GluN2B. Therefore, the ability to enhance SNX27-mediated interactions via selective phosphorylation is therefore highly likely to serve as a mechanism to fine-tune the dynamics of these interactions under certain contexts (Clairfeuille et al., 2016). As a result of this discovery, these investigators identified a list of 432 putative binding partners that could potentially interact with SNX27-retromer through this mechanism. Moreover, they have proposed a classification system based on the presence of glutamic acid and serine/threonine residues that ultimately determine the magnitude of SNX27-interaction (see Appendix D for complete manuscript and map of binding candidates). In brief, Class 1 lists proteins that may undergo constitutive or strong associations with SNX27 under physiological conditions, including known SNX27-interacting proteins, PTHR and DGKζ; Class 2 lists those possessing Ser/Thr in positions (-3), (-5) and/or (-6) that may increase PDZ-interaction affinities upon phosphorylation and include MRP4, Fzd7 and β₂AR; and Class 3 lists proteins lacking Glu⁵ and possess limited binding affinities in the absence of Ser/Thr phosphorylation, including 5-HT₄R (Clairfeuille et al., 2016).

While the data presented in this study and that discussed above have provided significant insight into the mechanisms governing SNX27- (and PDZ-) mediated interactions, several questions still remain to be answered. These include (i) validating the role of SNX27-retromer in governing the endosomal recycling of this extensive list of candidate cargoes, (ii) identifying which kinases and phosphatases are involved in this process and (iii) whether their activity can be manipulated to favour certain cellular outcomes, e.g. to promote cargo recycling or signal termination (Clairfeuille et al., 2016). Interestingly, a follow-up study by Mamonova and colleagues have recently shown that the functional effect of PDZbm-phosphorylation may also apply to other PDZ domain-containing proteins. Using NHERF1 as an example, they showed that PTH(1-34) induced the phosphorylation of Thr² within the PTHR-PDZbm which resulted in a significant reduction in NHERF-PTH interaction. Furthermore, the series of alanine substitutions at Glu³, Glu⁵ and Glu⁶ utilised throughout this chapter also resulted in variable decreases in
NHERF-mediated affinity for PTHR, with the NHERF-PTHR interaction completely abolished following the removal of the electrostatic plug (Glu\(^3\)/Glu\(^5\)) (Mamonova et al., 2017). Therefore, while the regulatory role of PDZbm-phosphorylation remains to be fully elucidated, these studies provide substantial insight into the mechanisms governing SNX27- and NHERF-mediated interactions with PTHR as well as highlighting potential avenues by which these interactions may be manipulated in an attempt to favour certain physiological outcomes.

### 7.3.3. SNX27 failed to retrieve PTHR-A6 mutant receptors from the endosomal compartment

Using confocal microscopy, the association of SNX27 with the PTHR-A6 mutant was observed to be dramatically reduced and was particularly emphasised through the real-time capture of the lack of SNX27 engagement with PTHR-A6 at the endosomal level. Despite the generally larger size of PTHR-A6-positive endosomes compared to that of PTHR-WT-bearing endosomes, there was an obvious lack of endosomal tubulation, further indicating that PTHR-A6 mutants were not retrieved from endosomal compartments in a manner dependent on SNX27-retromer tubule formation. Lastly, the reduced translocation of PTHR-A6 mutants towards the perinuclear ERC or Golgi compartment was observed, where instead, PTHR-A6 mutants were found to be mis-directed toward CD63\(^*\)/Lamp1\(^*\) endosomal compartments.

While the data presented in this chapter clearly support the increased degradation of PTHR-A6 mutants under both basal and agonist-stimulated conditions, there are several limitations to this study. Firstly, with the exception of SNX27, the relative distribution or involvement of other components of the ASRT complex (i.e. retromer or WASH complex) were not assessed. Furthermore, while the electrostatic plug is a requirement for SNX27-mediated engagement, its relative importance in the endosomal signalling of PTHR was also not investigated. Based on the previous Chapter, it might be assumed that PTHR-A6 mutants would exhibit sustained signalling events compared to that elicited by PTHR-WT due to the
increased endosomal residence of the mutant receptor. In this regard, validating the consequential effect of endosomal signalling in the compromised engagement between SNX27 and PTHR-A6, and in particular, whether this remains consistent within the context of bone cells remains an important step in developing molecular enhancers or inhibitors that may fine-tune this engagement to favour certain signalling events and cellular outcomes.

While these data collectively imply that SNX27 plays a major role in directing PTHR recycling, and that this is perturbed in the absence of the electrostatic plug, the generation of de novo endosomal tubules were still observed in PTHR-A6-expressing cells following PTH-stimulation (Videos 4, 5). This suggests that in the absence of SNX27-mediated receptor retrieval, alternative routes may be able to partially compensate in PTHR recycling. As suggested previously in Chapter 6, these alternative mechanisms may involve the direct engagement between retromer and PTHR or an additional PDZ domain-containing protein such as NSF, but would not include the role of NHERFs (as NHERFs have been also shown to require the electrostatic plug within the PTHR-PDZbm (Mamonova et al., 2017)). In addition to this, studies from Nooh and colleagues have recently provided interesting insight in identifying a ‘transplantable recycling motif’ that enables the PDZ-independent recycling of β1AR (Nooh et al., 2016). Here, this 20-residue linker was not found to resemble either PDZbm or N-P-x-Y recognition sequences and when added to the carboxy-terminal of β1AR, with (β1AR[+20]) or without (β1ARΔPDZ[+20]) an intact PDZbm, β1AR was still able to recycle efficiently to the cell surface following agonist stimulation. This was attributed to the specific 20-residue sequence, as randomly extending the cytoplasmic tail of β1AR impaired receptor recycling (Nooh et al., 2016). Furthermore, there are some proteins listed within the SNX27-interactome that do not possess either PDZbm or N-P-x-Y motifs, thus raising the question of how SNX27-retromer modulates their intracellular transport (Steinberg et al., 2013). While it remains plausible that these proteins may still recycle through indirect interactions, findings from Nooh and colleagues have further highlighted
that there may certainly be additional motifs governing SNX27-retromer interactions that have yet to be discovered.

Interestingly, while both β1AR and β2AR recycle via PDZ-dependent mechanisms (Nooh et al., 2014, Lauffer et al., 2010, Temkin et al., 2011), β1AR was found to possess an additional sorting ‘barcode’, determined by the PKA-mediated phosphorylation of Ser312 that in turn, required the prior recruitment of PKA by PDZ domain-containing protein, SAP97 (Nooh et al., 2016, Gardner et al., 2007). Therefore, they concluded that a ‘two-barcode endosomal verification system’ may assist to distinguish between β1AR and β2AR prior to entry into retromer-mediated recycling tubules. This stems from two main observations, that (i) inhibition of Ser312 phosphorylation prevented the recycling of β1AR, even in the presence of its PDZbm and (ii) though the recycling of both receptors relies upon ASRT machinery, depletion of FKBP15 (a FAM21C endosomal interacting protein) inhibited β1AR but not β2AR recycling (Nooh and Bahouth, 2017). Furthermore, in a brief screen, these authors proposed that this ‘two step verification’ system could potentially apply to calcitonin receptor (CTR), as CTR possesses both a type I PDZbm and a PKA substrate Ser/Thr motif for phosphorylation (Figure 6.1) (Nooh and Bahouth, 2017). Therefore, due to its known role in regulating osteoclast differentiation, it would be interesting to further investigate whether SNX27 also plays a role in CTR trafficking and signalling, as well as whether this mechanism may potentially apply to the sorting of wild-type receptors from PTHR-A6 mutants.

7.3.4. PTHR-A6 mutant receptors were found to exhibit increased constitutive internalisation and lysosomal degradation

Despite the absence of the electrostatic plug, a subpopulation of PTHR-A6 mutants were found at the cell surface and readily internalised into early endosomes upon PTH\textsuperscript{TMR} stimulation, therefore confirming that the carboxy-terminal PDZbm is not an essential requirement for PM delivery. Furthermore, PTHR-A6 mutants were not found to accumulate within the ER or Golgi (suggesting that receptor folding and protein export were not impaired). There was however, a distinct
subpopulation of PTHR-A6 mutants residing within endolysosomal compartments during steady-state and following agonist exposure, indicative that PTHR-A6 mutants underwent substantial unregulated receptor degradation compared to that of wild-type receptors. As explained above, the most obvious explanation for this is the reduced affinity of SNX27 for PTHR-A6, therefore resulting in the failure to retrieve PTHR-A6 from the maturing endosome. As such, these mutant receptors would then be directed for incorporation into CD63+ MVBs and undergo lysosomal degradation.

While the degradation of PTHR has not been the subject of comprehensive investigation, one main contributor in the incorporation of cargoes into ILVs involves the role of the ESCRT complex. This generally requires the ubiquitination of cargo prior to its entry into ILVs, however the state of PTHR-WT or PTHR-A6 ubiquitination was also not assessed in this chapter. An additional protein, arrestin domain-containing protein 3 (ARRDC3) has been shown to associate with a sub-complex of ESCRT complex, ESCRT-0 to promote endosomal retention of β2AR. In doing so, ARRDC3 prevented the entry of β2AR into SNX27-mediated retromer tubules and therefore prolonged cAMP production, interestingly without affecting β2AR degradation (Tian et al., 2016). The role of ARRDC3 however, appears to be receptor-specific as it has been shown to promote ubiquitination and degradation of another GPCR, PAR1 (Dores et al., 2015). Nevertheless, the functional role of ESCRT complex and potentially ARRDC3 in modulating PTHR trafficking and signalling remains an interesting line of future investigation.

Several GPCRs undergo constitutive (agonist-independent) internalisation at the PM mediated through the addition of ubiquitin to their carboxy-terminal tail. This then directs them for degradation through lysosomes and/or proteasomes and occurs as a part of normal receptor turnover (Skieterska et al., 2017). In addition to this, receptors may also be targeted for ubiquitin-mediated degradation following agonist exposure. Using β2AR as an example, acute agonist stimulation has been shown to undergo rapid recycling (Pippig et al., 1995), while prolonged agonist exposure has been shown to promote long-term signal attenuation by directing β2AR
for lysosomal degradation (Moore et al., 2007, Shenoy et al., 2008). In the latter scenario, β2AR was found to undergo ubiquitination at the cell surface prior to its entry into late endosomal compartments (6 hours of continuous agonist exposure) (Hanyaloglu and von Zastrow, 2007, Shenoy et al., 2008). Despite this, β2AR may be retrieved from late endosomes and recycled to the cell surface following the removal of ubiquitin by two deubiquitinases, USP20 and USP33 (Berthouze et al., 2009). Therefore, it was proposed that in the case of β2AR at least, that rapid recycling is thought to occur through the rapid dephosphorylation of the cytoplasmic tail while slower recycling mechanisms may be linked with the activities of deubiquitinases (Berthouze et al., 2009, Pippig et al., 1995). Despite not having been investigated here, the fate of PTHR is determined through the reversible ubiquitination of PTHR by deubiquitinase, USP2 (Alonso et al., 2011). However, this regulatory mechanism is most likely to prevail during slower recycling as compared to the rapid, SNX27-retromer-mediated recycling of PTHR (Chan et al., 2016, McGarvey et al., 2016, Pavlos and Friedman, 2017). Similar to β2AR, PTHR also undergoes ubiquitination at the cell surface (Alonso et al., 2011), therefore it is tempting to speculate that PTHR-A6 could potentially reside in a prolonged state of ubiquitination following internalisation, which would assist in explaining why receptor degradation is favoured over receptor recycling, although this remains to be confirmed.

While the exact mechanisms governing PTHR trafficking have yet to be elucidated, there is a great potential to develop tailored therapies to favour receptor signalling or long-term signal attenuation. For example, by manipulating the activities of SNX27-retromer, or the balance of receptor ubiquitination or deubiquitination, receptor degradation or recycling could be fine-tuned in patients exhibiting constitutively active forms of PTHR, excessive production of PTH (in the event of hyperparathyroidism) or to prolong PTHR signalling in the event of hypoparathyroidism. Furthermore, by further elucidating the role of SNX27-retromer within the skeletal context, innovative small molecule therapies may be developed to manipulate the affinity of SNX27-retromer for PTHR to favour bone
anabolism over catabolism and therefore provide an alternative avenue for the treatment of a variety of skeletal diseases.

7.4. Summary

By investigating the molecular determinants governing SNX27-PTHR interaction, two glutamic acidic residues (Glu\textsuperscript{-3}/Glu\textsuperscript{-5}) within the extended carboxy-terminal PTHR-PDZbm were identified to form crucial electrostatic interactions with conserved residues within the PDZ domain of SNX27. These interactions, referred to as the electrostatic plug, were found to play determining roles in the endosomal retrieval of PTHR following PTH stimulation. Substituting these two residues for alanine (PTHR-A6 mutants) resulted in the failure of SNX27 to interact with PTHR at the surface of endosomes, which subsequently led to the inevitable lysosomal degradation of PTHR. Therefore, the interactions facilitated by the electrostatic plug determine the PDZ-mediated engagement between SNX27 and PTHR and are required for subsequent receptor recycling.
CHAPTER

- 8 -

General summary, limitations and future directions
8. General summary, limitations and future directions

8.1. General summary

The skeleton is a crucial organ of the body that serves multiple functions, including the provision of mechanical support for physical movements as well as functioning as the main calcium reservoir and primary site for haematopoiesis. Despite its static appearance, the skeleton is a dynamic organ that is remodelled throughout adult life through the coordinated activities of its specialised resident cells. Skeletal homeostasis is thus greatly dependent on the coordinated cross-talk between chondrocytes, osteoblasts, osteoclasts and osteocytes, with the loss of intercellular coupling commonly contributing to the pathogenesis of a variety of skeletal disease. Membrane and protein trafficking encompasses the highly sophisticated and evolutionary conserved mechanisms by which the cell organises the transport of intracellular cargoes. Here, molecules are transported within the cell, or exported into the extracellular environment through the use of membrane-bound vesicular carriers such as endosomes (Stenbeck and Coxon, 2014). Cellular homeostasis in all tissues is highly dependent on the fidelity of these processes; this is particularly emphasised in the range of skeletal dysplasias arising from the disruption in the function of conserved trafficking proteins (Zhao, 2012, De Matteis and Luini, 2011). Often, the most severe cases of skeletal disease follow from genetic mutations disturbing intracellular trafficking processes that act to regulate cellular differentiation and/or function, including spondyloepiphyseal dysplasia tarda (SEDT), osteogenesis imperfecta, osteopetrosis and osteoporosis, all of which affect osteoblast, osteoclast and chondrocyte function (Forlino and Marini, 2016, Boyadjiev et al., 2011, Venditti et al., 2012).

Over the past two decades, members of the SNX protein family have emerged as indispensable regulators of intracellular trafficking along the endocytic
pathway. Currently 34 members have been identified in mammalian cells, all defined by the presence of the PX domain which enables SNXs to localise to endosomal membranes. Along with the PX domain, several members also possess accessory functional domains that facilitate their association with a variety of proteins and lipids, thereby conferring structural and functional diversity across the SNX family. Despite SNXs having been implicated in multiple endocytic trafficking processes in a variety of cell types, SNX10 remains the only SNX family member thus far to be linked to skeletal homeostasis and disease. Mutations within \textit{SNX10} account for approximately 4\% of individuals with infantile osteopetrosis (Aker et al., 2012, Megarbane et al., 2013, Pangrazio et al., 2013) which has been attributed to osteoclast dysfunction and impairment in endocytosis (Chen et al., 2012, Ye et al., 2013, Zhu et al., 2012). Up until now, whether other SNXs are similarly involved in bone homeostasis and disease was unknown and thus was central to the investigation of this thesis.

To address this information gap, the first aim of the study was to assess the relative expression of SNX proteins in bone tissue and its resident cells through micro-array and qPCR analyses. Using this two pronged approach, SNX10 and SNX27 were identified among the few SNX members found to be upregulated during RANKL-induced osteoclast differentiation. Of these, SNX27 was the most abundant SNX protein expressed within total bone. As SNX27 is unique among the SNX family, i.e. it is the only member to house a PDZ domain and had not been previously implicated in skeletal cell function or bone homeostasis, it was then chosen for more detailed investigation.

To assess the potential physiological contribution of SNX27 to skeletal development and homeostasis, the bone phenotype of SNX27 global knock-out mice was explored. By combining micro-CT with histological assessment, SNX27-deficient mice were shown to exhibit a previously unappreciated form of osteochondral dysplasia characterised by drastically reduced trabecular bone mass (osteopenic), reduced cortical thickness and an abnormal expansion of the

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epiphyseal growth plate. Histomorphometric and ex vivo cell cultures studies further revealed that the bone phenotype was partly attributable to the intrinsic defects in osteoblast differentiation and function, which was reflected by the reduced deposition of osteoid in vivo and decreased bone nodule formation in vitro. Subsequent in vitro experiments further demonstrated that SNX27-deficient osteoblasts elicited sustained cAMP generation and activation of downstream effector molecules (including CREB, AKT and ERK) following intermittent PTH stimulation, implying that an intimate relationship exists between SNX27 and PTHR signalling in osteoblasts.

Indeed, using a series of complementary biochemical and cell biological assays, PTHR was identified as a novel SNX27-interacting cargo; an engagement that coincided with the recruitment of the retromer trafficking complex. Specifically, the PDZ domain of SNX27 formed strong interactions with the carboxy-terminal PDZbm of PTHR while simultaneously engaging with retromer through its subunit, VPS26A. As such, these data collectively illustrated the role of the SNX27-retromer complex in directing endosomal PTHR into retromer-mediated tubules for receptor recycling and preservation. Detailed structural studies further unveiled key electrostatic interactions (‘electrostatic plug’) between glutamic acidic residues in the ‘extended’ PDZbm of PTHR (Glu⁻³/Glu⁻⁵) and conserved residues within the PDZ domain of SNX27. These amino acid residues were found to be an important prerequisite for both the affinity and specificity of this protein-protein engagement. Reflecting this, removal of the PDZ-mediated engagement between SNX27-retromer and PTHR (following either the SNX27- or VPS35-depletion, or mutagenesis of the electrostatic plug) impaired PTHR retrieval from the early endosome, resulting in (i) prolonged receptor retention in endosomes; (ii) sustained non-canonical endosomal signalling and (iii) an increased propensity to mis-traffic the receptor toward degradative lysosomes. Based on these collective observations we conclude that SNX27 couples PTHR to the retromer trafficking complex in to regulate endosomal PTHR signalling in osteoblasts during postnatal bone growth.
The publication of these findings, as well as those of an independent group (McGarvey et al., 2016) have contributed significantly to the relationship between the ASRT complex and PTHR signalling and trafficking. The current model, as described in Pavlos and Friedman, 2016 (Figure 8.1), describe that the post-endocytic trafficking of activated PTHR may occur through three different routes. The data presented in this thesis have characterised the predominant pathway governing PTHR recycling, i.e. the rapid ASRT-dependent recycling route via Rab4-positive recycling endosomes. In addition to this, endosomal PTHR may also transit via the Golgi and subsequently recycle to the cell surface in a slower, retrograde-dependent pathway. Lastly, Friedman and colleagues had reported the possibility of PTHR recycling to occur via an ASRT-independent mechanism, which may involve another PDZ domain-containing protein known as NSF (McGarvey et al., 2016). While collectively, these findings have begun to map the intricate relationship and regulation between PTHR trafficking and signalling, there are several limitations and avenues that have yet to be explored, and will be discussed below.

8.2. Limitations and future directions

While the results of this dissertation firmly establish a role for SNX27 in PTHR signalling and trafficking, several questions remain to be addressed. For instance, does SNX27-PTHR interaction exhibit a ligand bias for PTH over PTHrP? Furthermore, during the course of this study we and others demonstrated that SNX27 functions as a major endosomal sorting hub capable of interacting with a multitude of structurally and functionally diverse PDZbm-containing cargo proteins (Steinberg et al., 2013, Clairfeuille et al., 2016), many of which are known regulators of skeletal homeostasis including (but not limited to) receptors of the canonical Wnt signalling pathway, BMPRs and gap junctions (e.g. Cx43). Therefore, how many of these receptors are governed by the SNX27-retromer complex? Do these receptors synergise with PTHR signalling and thus contribute to the net bone phenotype
Figure 8.1. Alternative modes of GPCR trafficking along the endocytic network.

* Taken from Figure 2. Pavlos & Friedman, 2016.

**Key:**
- PTHR
- PTH
- β-Arrestin
- SNX27
- Retromer
- WASH
- PDZ adaptor
- Rab4
- Rab5
- Rab7
- Rab11

**Diagram Description:**
- **Plasma membrane**
- **Cytosol**
- **Early endosome**
- **Endo-lysosomal degradation?**
- **Intraluminal vesicles (ILVs)**
- **Trans-Golgi network (TGN)**

**Legend:**
- **ASRT-dependent recycling** (rapid)
- **ASRT-independent recycling** (rapid)
- **Retrograde transport** (slow)

**Textual Description:**
Upon ligand-induced activation, GPCRs are internalized into early endosomes for sorting. At the early endosome, GPCRs containing C-terminal PDZ binding motifs are recognized by compatible PDZ-adaptor proteins such as SNX27, which works in conjunction with retromer and the WASH complex (Inset). Following sorting, receptors are packaged into recycling tubules which emanate and bud from the membranes of early endosomes. These recycling intermediaries transport GPCRs back to the plasma membrane leading to resensitization of the cell surface. Alternatively, GPCRs marked for degradation are sequestered into intraluminal vesicles (ILVs) of multivesicular endosomes that fuse with lysosomes, resulting in receptor proteolysis and downregulation at the cell surface. Membrane retrieval of endocytosed PTHR occurs via three divergent trafficking itineraries: (i) ASRT-mediated endosome to plasma-membrane (PM) recycling (green), (ii) ASRT-independent endosome to PM recycling (blue), or (iii) retrograde endosome to trans-Golgi network (TGN) to PM recycling (red). This GPCR signaling-trafficking cycle is superimposed by the recruitment and functional transition of small Rab GTPases: early endosome (Rab5), late-endosome/lysosome (Rab7), and recycling endosomes (Rab4 and Rab11).
observed in SNX27-deficient mice? These intriguing possibilities are discussed below.

8.2.1. Does SNX27 differentiate between PTH vs PTHrP-mediated PTHR signalling?

While our studies demonstrate that loss of SNX27 alters PTHR signalling in osteoblasts, it is noteworthy that PTHR is also expressed by osteocytes and chondrocytes and thus the SNX27-retromer likely plays an equally important role in modulating the functions of these skeletal resident cells. This is perhaps best evidenced by the pronounced expansion of the epiphyseal growth plate observed in SNX27-deficient mice owing to aberrant chondrocyte proliferation. Unlike PTH-induced PTHR signalling which primarily drives anabolic responses in osteoblasts during bone remodelling, the cellular actions of chondrocytes are largely dictated by the paracrine/autocrine effects of PTHrP during skeletal growth. It has been previously reported that whereas PTH-mediated PTHR signalling is terminated by retromer (Feinstein et al., 2011), PTHrP-mediated signalling utilises an alternative pathway, implying ligand bias for PTH towards non-canonical endosomal signalling and alternative mechanisms of signal termination (Ferrandon et al., 2009, Dean et al., 2008b). As such, it remains unclear whether SNX27 similarly influences PTHrP-elicited PTHR signalling. To answer this, we have recently embarked on a collaboration with E/Prof TJ (Jack) Martin and A/Prof Natalie Sims (St Vincent’s Institute of Medical Research, Melbourne VIC) to explore the relationship between SNX27 and PTHrP-mediated signalling (preliminary data presented as Appendix A). Similar to that observed following exposure to PTH(1-34), osteoblasts derived from SNX27-deficient mice yielded an increase in CREB and AKT activation following PTHrP(1-141) stimulation (Appendix A, Figure 1). This finding suggests that SNX27 indiscriminately modulates both PTH/PTHrP ligand-induced PTHR signalling. Future studies will further address the significance of SNX27 on PTHR signalling in chondrocytes using chondrocytes isolated from globally-deficient SNX27 mice. To unequivocally assess the cell-autonomous roles of SNX27 in chondrocytes, we will also assess the skeletal phenotype of conditional knockout
mice (e.g. collagen type II-Cre driven mouse model) to specifically delete SNX27 in chondrocytes.

Of interest, the preliminary findings presented in Appendix A, are in discordance with previous reports investigating the trafficking kinetics of PTHR following PTHrP stimulation. It is noteworthy to mention that in this study the use of PTHrP(1-36) (as opposed to its physiological full-length PTHrP(1-141)) was deemed equivalent on the basis that PTHrP(1-36) had been shown to possess comparable receptor binding affinity and signalling to its full-length counterpart (Kemp et al., 1987, Nissenson et al., 1988, Li et al., 2012). However, unlike PTH(1-34), PTHrP(1-36) was reported to bind, yet rapidly dissociate from surface-resident PTHR without inducing receptor endocytosis, resulting in a transient cAMP response localised to the cell surface (Dean et al., 2008b, Ferrandon et al., 2009). Despite sharing a common receptor, the observed differences in signalling were attributed to the affinity each ligand exhibited for the two high-affinity ligand-binding conformations of PTHR, denoted as \( R^0 \) and \( R^G \) conformations of PTHR; both PTH(1-34) and PTHrP(1-36) expressed comparable affinities for the G protein-bound \( R^G \) conformation, however PTH(1-34) exhibited an affinity that was several-fold higher for the G protein-independent \( R^0 \) conformation than expressed by PTHrP(1-36) (Dean et al., 2008b). In this way, it is thought that internalised PTH-PTHr complexes are able to induce endosomal signalling by catalytically activating multiple \( G_\alpha \) subunits by repeatedly shifting between its \( R^0 \) and \( R^G \) states without ligand dissociation (Rodbell, 1997, Dean et al., 2008b, Gardella and Vilardaga, 2015). However, despite consistent reports describing the inability of PTHrP(1-36) to induce receptor endocytosis, one limitation of these experiments is that PTHrP(1-36) may not be fully representative of the biological activity of PTHrP(1-141) under physiological conditions or different target tissues, particularly within the skeletal context (Martin, 2016, Gardella and Vilardaga, 2015).

Therefore, to further assess this, we have recently examined PTHrP(1-141)-mediated receptor internalisation in PTHR-expressing HEK293 cells following the
exposure to a series of PTHrP variants for 15 minutes (Appendix A, Figure 1.2). Preliminary observations suggest that while the rates of PTHR internalisation are variable across the different forms of PTHrP, both PTHrP(1-36) and PTHrP(1-141) are capable of inducing PTHR endocytosis, most notable at 15 minutes post-stimulation (Appendix A, Figure 2). In particular, a subset of endosomal PTHR co-localises to EEA-1-positive endosomes following PTHrP(1-141) stimulation, an event that is not likely to be a result of coincidental co-localisation as this was not seen at 60 minutes post-agonist stimulation. Furthermore, to directly visualise the internalisation of PTHrP itself, a series of PTHrP antibodies were tested on HEK293 cells that had been exposed to PTHrP(1-141) at 4°C (Appendix A, Figure 3). Of these, the ‘R90’ antibody exhibited best specificity as determined by its co-localisation to the cell surface of PTHR-expressing cells (arrow). Furthermore, the R90 antibody successfully identified retromer-positive endosomes specifically in PTHrP-expressing cells following 15 minutes post-stimulation. By 60 minutes however, the R90 antibody no longer stained these endosomes but instead labelled enlarged endosomal compartments of an unknown identity. Notwithstanding the specificity limitations of the R90 antibody, this suggests, contrary to previous reports, that PTHrP(1-141) is capable of eliciting PTHR endocytosis. If this finding holds true then it raises several interesting questions; first, if PTHrP(1-141) internalises together with PTHR, does this also result in endosomal signalling? In addition, the downstream functional domains of PTHrP have been shown to elicit specific cellular responses in calcium regulation and nuclear localisation; therefore, at which point do the trafficking routes or sorting of PTHrP and PTHR diverge? Finally, if PTHrP does not induce PTHR endocytosis, is there an unidentified receptor that facilitates ligand entry independently of PTHR? Future experiments will focus on determining (i) the differences in PTHR signalling and trafficking between PTH and full-length PTHrP and (ii) the role of SNX27 in these events in order to fully understand the physiological actions of PTHrP in bone.
8.2.2. Can SNX27 be therapeutically targeted to modulate PTHR signalling?

Characterisation of the underlying mechanisms regulating PTHR signalling with respect to (i) ligand bias, and (ii) canonical vs. endosomal signalling will enable us to further understand the paradoxical actions of PTH (and PTHrP) in bone. Historical reports investigating either the constitutive activation of PTHR or its loss of function, have largely focused on whether the net increase or decrease in PTHR-elicited signalling contributes to the phenotype of the disease. However, in light of the novel findings described throughout this dissertation – that SNX27-retromer plays a protective role against prolonged endosomal PTHR signalling, future investigations should, therefore, focus on distinguishing which of the two arms of PTHR signalling (i.e. canonical or endosomal) and its individual contribution to the regulation of distinct cellular outcomes. In doing so, this will assist in designing peptides and tailored therapies aimed at specifically targeting SNX27-PTHR interactions in the hope of tilting PTHR signalling in favour of bone anabolism.

One such approach involves the design of membrane-permeable small molecule inhibitors of protein-protein interactions. For example, the PDZ binding cavity is a well-defined pocket that has long been an attractive target for the development of small molecule inhibitors. Furthermore, the electrostatic interactions mediated by Glü³/Glu⁵ are determining factors in the affinity and specificity of the SNX27-PTHR interaction, and therefore, may be considered key to the design of potential inhibitory peptides. In fact, small molecule inhibitors have already been shown to effectively inhibit PDZ-mediated interactions and downstream signalling events by targeting the PDZ binding cavities of Dishevelled, Pick1 and AF6 (Grandy et al., 2009, Lee et al., 2009, Thorsen et al., 2010, Vargas et al., 2014). Similarly, a series of small molecules targeting the GYGF loop within the PDZ domain of NHERF1 were shown to compete for the interaction with the carboxy-terminal PDZbm peptide of cystic fibrosis transmembrane regulator (CFTR) (Mayasundari et al., 2008) and PTHR (Fitzpatrick et al., 2014). While these studies may provide a basis on which to model small inhibitory peptides against the PDZ cavity of SNX27,
it is important to acknowledge that these peptides will likely influence other PDZ-mediated SNX27 interactions. An alternative approach, therefore, is to design complementary molecules or peptides that specifically target the extended PTHR-PDZbm, thereby preventing or prolonging SNX27-PTHR engagement, signalling and recycling. However this will need to be designed in such a way that it does not comprise the PDZ-mediated regulation of PTHR by other PDZ domain-containing proteins, including NHERF and/or (potentially) NSF proteins. Nevertheless, if realised, these two approaches could prove feasible in preventing prolonged PTHR signalling or to delay receptor resensitisation.

To a certain extent, both ligand-independent and –dependent PTHR activation share similar physiological outcomes. In cases where PTHR signalling occurs independently of ligand exposure (i.e. constitutive forms of PTHR in individuals with JMC and Eiken syndrome), these small molecule inhibitors could prove as an effective means to block SNX27-mediated interactions to favour receptor degradation and long-term signal attenuation. However, in cases where the continuous exposure to PTHR agonists occurs (e.g. primary hyperparathyroidism (PHPT) and humoral hypercalcemia of malignancy (HHM)), designing stabilising proteins to improve the affinity between SNX27-PTHR interactions to prolong engagement and favour ‘slower’ recycling rates may prove effective to terminate endosomal signalling and delay cellular resensitisation. Aforementioned, one must first unequivocally define which arms of signalling determine the paradoxical effects mediated by PTH-induced PTHR signalling and which downstream signalling cascades are involved in eliciting these distinct cellular outcomes. This is particularly emphasised as patients of JMC, Eiken syndrome, PHPT and HHM all share persistent signalling, hypercalcemia and skeletal abnormalities, while also exhibiting distinct phenotypic characteristics (Fraser, 2009, Stewart, 2005, Schipani et al., 1995, Schipani et al., 1996, Kruse and Schutz, 1993).
8.2.3. The potential role of SNX27 in other signalling pathways in bone

To date, combined bioinformatics and proteomic analyses estimate the SNX27-interactome to exceed over 400 proteins (Steinberg et al., 2013, Clairfeuille et al., 2016). Among these putative SNX27 interacting cargoes, a subset of proteins have previously established roles in skeletal homeostasis (Figure 6.1). This raises questions as to precisely how many of these candidate PDZbm-containing proteins are \textit{bona fide} SNX27-interactors? And like PTHR, does the loss of these associations contribute to the net bone phenotype observed in SNX27-deficient mice?

Firstly, β-catenin possesses a carboxy-terminal PDZbm (i.e. DTDL) that has been previously shown to interact with NHERF1. NHERF1 binds both β-catenin and platelet-derived growth factor-receptor β (PDGF-Rβ) and, together in complex with N-cadherin at the cell surface, facilitates cell migration via PDGF-mediated signalling (Theisen et al., 2007). While the direct relationship between SNX27 and β-catenin was not investigated within the scope of this study, preliminary data shown in Appendix B has indicated that SNX27 is likely to participate in the regulation of BMPR/β-catenin signalling cross-talk. Following BMP2 stimulation across a time-course of 90 minutes, SNX27-deficient osteoblasts were found to have aberrant β-catenin phosphorylation (at both activating and de-activating sites), as well as AKT and CREB activation (Appendix B, Supplementary Figure 5). The SNX27-interactome established by Steinberg and colleagues found the surface expression of BMP receptors to be altered following SNX27- and/or VPS35-knockdown (Steinberg et al., 2013). Specifically, type I BMP receptors, i.e. ACVR1B and TGFβRI were both downregulated following SNX27-retromer depletion, while BMPR1A was only downregulated upon SNX27-knockdown. In contrast, the surface expression of type II BMP receptors were not found to be altered; an observation that could be partially explained due to the different mechanisms regulating the internalisation and/or recycling between type I and II receptors. In a study of BMP receptors in \textit{C. elegans}, Sma-6 (equivalent to type I receptors) internalised in a clathrin-dependent manner while DAF-4 (equivalent to type II receptors) internalised in a clathrin-independent manner (Gleason et al., 2014, Di...
Guglielmo et al., 2003, Hartung et al., 2006). Furthermore, retromer was found to directly engage with Sma-6 and prevent receptor mis-sorting to lysosomes (Gleason et al., 2014). Upon establishing whether SNXs were also involved in this process, they identified the surface expression of Sma-6 to be altered following SNX3, but not SNX27-knockdown, suggesting that SNX3-retromer may be implicated in this process. Together with the data presented in Supplementary Figure 5, these data imply that SNX27-retromer may regulate the signalling and trafficking of BMP receptors or signalling cross-talk via interactions with β-catenin.

Dishevelled (Dvl) is another PDZ domain-containing protein that engages with components of the Wnt signalling pathway, including β-catenin (Song and Gelmann, 2005) and Fzd receptors (Chen et al., 2003, Yu et al., 2007). As SNX27 and Dvl both possess PDZ domains, the role of Dvl in regulating Wnt signalling may provide some insight into how SNX27 could also modulate this pathway. Following receptor activation, Dvl translocates to the Wnt-Fzd-LRP5/6 ternary complex (Wong et al., 2003) where it acts to recruit Axin/GSK3β (Cong et al., 2004, Zeng et al., 2008). This aids in both the phosphorylation of LRP5/6 and the destabilisation of the destruction complex. Interestingly, PTHR has also been shown to promote LRP6 phosphorylation and the recruitment of Axin/GSK3β (Wan et al., 2008), however whether SNX27 indirectly assists in this process through its interaction with PTHR is not known. Another interesting observation is that LRP4, but not LRP5/6 possesses a compatible PDZbm that could mediate interactions with SNX27 (Figure 6.1). LRP4 is known to antagonise Wnt signalling in bone, where LRP4-deficient mice exhibited an increase in bone mass, bone formation and impaired bone resorption, partly due to its role to elicit SOST-mediated action on bone (Xiong et al., 2015). Again, whether SNX27 participates in the retention of LRP4 at the cell surface, or mediates LRP receptor endocytosis has yet to be investigated.

Dvl is also known to facilitate the formation of the Wnt signalling complex at the endosome (referred to as the Wnt signalosome) through its actions as a scaffolding protein (Feng and Gao, 2015). The Wnt signalosome promotes ligand
degradation while also prolonging β-catenin activity by continuing to engage with Axin/GSK3β, thereby preventing the formation of the destruction complex (Seto and Bellen, 2006, Blitzer and Nusse, 2006, Taelman et al., 2010, Dobrowolski et al., 2012). While it is unknown whether SNX27 is also able to act as a scaffolding protein in a similar manner, it is most likely to participate in Wnt signalling through its interactions with Fzd receptors. Fzd1, 2, 4 and 7 receptors all possess PDZbsms that could be recognised by SNX27 (Figure 6.1). While Dvl had been shown to direct receptor endocytosis of Fzd4 (Chen et al., 2003), SNX27 had been recently confirmed to direct the endocytosis and degradation of Fzd7 in a PDZ-mediated manner, ultimately leading to the downregulation of both Wnt signalling and cell migration (Sun et al., 2016). Therefore, despite the limited knowledge of SNX27 in the regulation of Wnt signalling, the protein- and lipid-binding properties of its PDZ and FERM domains could enable SNX27 to participate in multiple aspects of receptor retention, endocytosis and/or sorting, through direct interactions with Fzd or LRP4 receptors, or indirectly by modulating PTHR-LRP5/6 cross-talk.

Lastly, SNX27 could play an unidentified role in regulating Cx43 expression in bone-resident cells. As briefly mentioned in Chapter 5, Cx43 plays crucial roles in skeletal development by regulating osteoblast and osteocyte differentiation and function. In particular, deletion of Cx43 in osteoblasts impaired osteoblast differentiation and its anabolic response to PTH (Chung et al., 2006), potentially by sequestering β-arrestin away from PTHR and thereby prolonging canonical signalling (Bivi et al., 2011). In contrast, mice heterozygous for a dominant negative Cx43 (G60S) mutant, were found to exhibit a contradictory increase in osteoblast function, an increase in BMP2/4 signalling and bone marrow adipogenesis (Zappitelli et al., 2013, Zappitelli et al., 2015). As these findings share some similarities those identified in SNX27-deficient mice (Appendix B, Supplementary Figure 3, 5), confocal microscopy was used to probe for the endogenous expression of Cx43 in native calvarial osteocytes (Appendix B, Supplementary Figure 6). Preliminary analyses revealed that SNX27-deficient mice exhibited a significant reduction in both Cx43 hemi-channels and length of dendritic extensions, suggesting
that Cx43 signalling and/or trafficking is impaired and thus may also contribute to
the skeletal phenotype of these mice. As Cx43 possesses an incompatible (type II)
PDZbm, it is unlikely to mediate a direct relationship with SNX27. Instead, Cx43
has been shown to associate with PDZ domain-containing proteins, ZO-1 and ZO-2
(Singh et al., 2005b), the latter having been established as an SNX27-interacting
protein (Zimmerman et al., 2013) that in addition to its PDZ domain, also possesses
a carboxy-terminal type I PDZbm. Therefore it is tempting to speculate that SNX27
indirectly modulates Cx43 through its engagement with ZO-2. Future studies will
address this intriguing possibility.

8.3. Summary

Collectively, the findings presented throughout this dissertation have
unmasked a previously unappreciated role for SNX27 in osteoblast function and
skeletal homeostasis. In particular, they have helped unveil the missing molecular
link between PTHr signalling and the retromer trafficking complex. As such, the
data and concepts described here may assist in the future development of potential
therapies to combat anomalies related to PTHr signalling as observed in a range of
bone and mineral diseases. Finally, given that the complement of SNX27 binding
cargo has recently been expanded beyond 400 putative transmembrane proteins, a
challenge remains to decipher the exact number of cargo which operate through the
SNX27 ‘sorting nexus’ and thus, like PTHr, contribute to the growth, maintenance
and homeostasis of the skeleton.
CHAPTER

- 9 -

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9. References


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APPENDIX

- A -

Preliminary data of PTHrP-mediated PTHR trafficking
Appendix A, Figure 1

A.

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<th>KO</th>
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<tr>
<td>VPS35</td>
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<tr>
<td>p-CREB</td>
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<tr>
<td>α-tubulin</td>
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<td>p-ERK1/2</td>
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B.

**PRE-PRO**

**PTH**

Placental Ca\(^2+\)

NLS

C-terminal

PTh

SVSEHQLMHLNLCNLHLSNMRVEWLRLKLOQDVNF

PTHrP

AVSEHQLDEKSKSIQDLRFRHLLHIALIETHA

C.

Continuous incubation time (min)

D.

Incubation time after wash-out (min)

E.

Incubation time after wash-out (min)
Appendix A. Figure 1. PTHrP-induced PTHR signalling in SNX27-deficient osteoblasts and PTHR-expressing HEK293 cells.  

(A) Wild-type (WT) and SNX27-deficient (KO) bone-derived osteoblasts cells were isolated and cultured as described in Materials and Methods. Osteoblast were then seeded 24-hours prior to stimulation with PTHrP(1-141) (100nM) across 90 minutes, with al ligand washout following 15-minutes stimulation, where applicable. Protein was then extracted from ells as described in Materials and Methods, and assessed for the activation of downstream signalling effectors, namely phosphorylated CREB, phosphorylated AKT and phosphorylated ERK1/2.

(B) Illustrative map of full-length PTHrP. Variable lengths of PTHrP were generously provided by E/Prof TJ (Jack) Martin, St Vincent’s Institute of Medical Research, Melbourne VIC. These include full-length PTHrP(1-141), ΔC-terminal region (PTHrP(1-108)), ΔNLS and C-terminal regions (PTHrP(1-84) and PTH-like signalling region (PTHrP(1-36)). Highlighted residues indicate conservation between PTH and PTHrP within the initial 34 residues of the PTH-like signalling region (1-34).

(C-E) RLuc-PTHR-expressing HEK293 cells were cultured and stimulated with PTH(1-34) or variable PTHrP proteins as described in Materials and Methods. Cells were either exposed to PTH or PTHrP throughout the time course (B) or were subject to ligand washout following 15 minutes post-agonist stimulation (C, D). Protein was extracted and assessed for phosphorylated ERK1/2.
Appendix A, Figure 2

A.  

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<th>60 minutes</th>
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<td>Merged</td>
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B.  

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</tr>
<tr>
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Appendix A, Figure 2. Ligand-induced internalisation of PTHR differs between PTH(1-34) and variable lengths of PTHrP.

HEK293 cells stably expressing N-GFP-PTHR were cultured on poly-L-lysine-coated glass coverslips for 48-hours prior to PTH(1-34) stimulation (100nM), or PTHrP for 15 minutes, followed by ligand washout. After 60 minutes post-agonist stimulation, cells were then washed in 1X PBS, fixed in 4% PFA and immunostained for endogenous VPS35 or EEA-1 (red) and Hoechst (nuclear stain). Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using the 60X oil lens.

(A) PTH(1-34) induced PTHR endocytosis into retromer-positive endosomes after 15 minutes post-agonist stimulation, where a subpopulation of PTHR-bearing endosomes translocated toward the perinuclear region (top panel). In contrast, PTHrP-induced receptor internalisation varied, where the PTH-like PTHrP(1-36) fragment appeared to induce receptor internalisation the most.

(B) Despite PTHR remaining predominantly at the cell surface (A), a smaller subset of PTHR-bearing endosomes could be seen to reside within EEA-1-positive endosomes at 15 minutes, but not 60 minutes post-PTHrP(1-141) stimulation.
Appendix A, Figure 3

**Appendix A - Preliminary data of PTHrP-mediated PTHR trafficking**
Appendix A, Figure 3. Validating the specificity of PTHrP antibodies in HEK293 cells.

HEK293 cells stably expressing N-GFP-PTHr were surface labelled with PTHrP(1-141) (100 nM) at 4°C for 10 minutes. Cells were then rinsed in 1X PBS, fixed in 4% PFA and immunostained for PTHrP using PTHrP antibodies generated from the laboratory of E/Prof TJ (Jack) Martin (shown in red). Filamentous actin (F-Actin) and Hoechst (nuclear) stain were also used, according to manufacturer’s recommendations. Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 40X oil lens. Scale bar represents 10µm.

All anti-PTHrP antibodies were used at a starting dilution of 1:100. Both rabbit pAb anti- (mouse) PTHrP (region 107-139) ‘R3959’ (A) and mouse mAb anti- (mouse) PTHrP (region 1-33) ‘Kreman#158’ (B) antibodies were not found to express any obvious specificity for PTHrP(1-141). Mouse mAb anti- (human) PTHrP (region 1-33) ‘Chugai’ (C) and rabbit pAb anti- (human) PTHrP (region 67-84) ‘R90’ (D) antibodies were found to express semi-specificity as visualised by N-GFP-PTHR co-localisation (orange arrows).
Appendix A, Figure 4. Full-length PTHrP internalises with PTHR into retromer-positive endosomes.

HEK293 cells stably expressing N-GFP-PTHR were cultured on poly-l-lysine-coated glass coverslips for 48-hours prior to PTH(1-34) stimulation (100nM), or PTHrP for 15 minutes, followed by ligand washout. Following 60 minutes post-agonist stimulation, cells were then washed in 1X PBS, fixed in 4% PFA and immunostained for endogenous VPS35 (blue), PTHrP (R90 antibody, 1:100 shown in red) and Hoechst (nuclear stain). Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using the 60X oil lens.

Both PTH(1-34) and PTHrP(1-141) induced receptor internalisation into retromer-positive endosomes by 15 minutes post-stimulation. PTH-PTHR complexes translocated toward the perinuclear region after 60 minutes post-stimulation, while PTHrP(1-14) was found to dissociate from PTHR and accumulate into enlarged endosomal structures of unknown identity.
APPENDIX

– B –

Supplementary Figures
Supplementary Figure 1.

A. 

B. 

C. 

D. 

E. 

Appendix B - Supplementary Figures
Supplementary Figure 1. Three-dimensional micro-computed tomographic (µCT) skeletal reconstructions of 4-week old SNX27 littermates
Micro-computed tomographic (µCT) was used to reconstruct the mineralised skeleton of 5-day old SNX27 pups (shown in Figure 5.6) (A), skull (B, C), forelimbs (D) and spine (E).
Supplementary Figure 2. Cell culture and proliferation assay of primary osteoblasts.

(A) Bone marrow was flushed from isolated long bones of freshly culled mice, and cultured for osteoblastogenesis as described in Materials and Methods. The cortical bone was diced into small pieces (~1mm in length) and digested as described in Bone Research Protocols (Helfrich and Ralston, 2013). Treated bone chips were then placed into T25 flasks and cultured in complete αMEM (supplemented with ascorbic acid, 50µg/mL) and left undisturbed for 5 days at 37°C, 5% CO₂. When osteoblasts began to explant from the bone chips, cells were passaged and bone chips were discarded. Images were taken with an inverted microscope using the 4X objective lens.

(B) Wild-type (WT) and SNX27-deficient (KO) osteoblasts were cultured in complete αMEM (supplemented with ascorbic acid, 50µg/mL). Cells were then seeded into a 96-well plate at 8x10³ cells/well for 48-hours prior to assessment. Cell culture medium was then changed, with or without the addition of PTH(1-34) (100nM). Osteoblast proliferation was then assessed using CellTiter 96® Aqueous One Solution Cell Proliferation (colourimetric) Assay, following the manufacturer’s protocol. Colourimetric measurements were then taken after 4 hours of incubation, at 490nm. Measurements are shown as an average of n = 3 replicates ± SEM, and is representative of 3 individual experiments.
Supplementary Figure 3.

A. ALP

B. Oil red O

C. Adipogenic potential

D. c/EBPβ

E. PPARγ
Supplementary Figure 3. SNX27-deficient osteoblasts exhibited increased adipogenesis following 28-days of culture under osteogenic conditions. 

(A-C) Primary osteoblasts were cultured in complete αMEM (supplemented with ascorbic acid, 50µg/mL, 3mM β-glycerophosphate, 10nM dexamethasone) for 28 days, as described in Materials and Methods. Cells were then fixed using Citrate-Acetone-Formaldehyde fixative and stained for alkaline phosphatase activity using the Leukocyte Alkaline Phosphatase Kit (Sigma-Aldrich Co., USA), according to manufacturer’s protocol (A). Alternatively, adjacent wells were stimulated with intermittent PTH(1-34) (50nM) throughout the 28-day culture before being fixed in 4% PFA and stained for lipid droplets using Oil red O staining procedure (B), as described in Materials and Methods. Oil red O-positive droplets were counted (C), where measurements are represented as an average of n = 3 replicates ± SEM, and is representative of 2 individual experiments.

(D, E) Total mRNA was extracted from wild-type (WT) and SNX27-deficient (KO) osteoblasts cultured under osteogenic conditions as described above. Gene expressions of two adipogenic markers, c/EBPβ (D) and PPARγ (E) were assessed using qPCR, and normalised to HMBS. Measurements are shown as an average of n = 3 replicates, ± SEM, and is representative of 2 individual experiments.
Supplementary Figure 4. Serum levels of PTH and Vitamin D (1,25(OH)₂D₃) of 4-week old wild-type (WT) and SNX27-deficient (KO) mice.

Blood from wild-type (WT) and SNX27-deficient (KO) mice were obtained via heart puncture, and serum levels of PTH (A) and Vitamin D (B) were assessed using commercially available ELISA kits (Cusabio, USA) as described in Materials and Methods. Measurements are an average of n = 3, ± SEM per group.
Supplementary Figure 5. SNX27-deficient osteoblasts exhibit altered downstream signalling following BMP2 stimulation.

Primary osteoblast from wild-type (WT) and SNX27-deficient (KO) mice were cultured as described in Materials and Methods. Osteoblasts were seeded at 1x10^5 cells/well (12-well plate) for 24-hours prior to stimulation with BMP2 (100ng/mL) for the time points indicated. Following the completion of the time course, cells were then washed in 1X PBS and protein was then promptly extracted using Ripa Ripa Lysis Buffer.

The activation of various signalling effectors were assessed via western blot, including the activation (Ser675) and deactivation (Ser33/37/Thr41) of β-catenin, and activation (by phosphorylation) of Smad1/5, AKT, ERK and CREB. Blots are shown as a representative of n = 2 individual experiments.
Supplementary Figure 6. SNX27-deficient osteocytes have reduced connexin43 (Cx43) number, distribution and reduced dendrites.

(A) Calvarial bone was harvested from sacrificed wild-type (WT) and SNX27-deficient (KO) 4-week old mice, and promptly cleaned and fixed in 4% PFA. Calvarial bone was then stained for endogenous filamentous actin (F-actin, red), endogenous Cx43 (green) and nuclear stain (Hoechst, blue). Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using the 60X oil lens. Scale bar represents 10µm.

(B) The number of dendrites (solid bar) and hemichannels (dotted bar) are averages ± SEM, taken from approximately 2-30 cells per sample, from a total of 3 samples per group.
Supplementary Figure 7. SNX27-deficient osteoblasts did not exhibit aberrant EGF trafficking.

Osteoblasts derived from wild-type (SNX27\(^{+/+}\)) or SNX27-deficient (SNX27\(^{-/-}\)) mice were stimulated with epidermal growth factor (EGF) conjugated to Alexa Fluor™ 555 (EGF-555) for 15 minutes, followed by ligand washout. Cells were then incubated for the time periods indicated, fixed in 4% PFA and immunostained for endogenous VPS35. EGF binds to its receptor (EGFR) at the cell surface (2 min, ice) and internalises into early endosomes by 15 minutes. EGF/EGFR is known to traffic as a complex and is commonly used as a prototypical marker for trafficking along the degradation route.
Supplementary Figure 8. **Myc-PTHR expresses and localises correctly in HEK293 cells.**

Stable clones of **Myc-PTHR**-expressing HEK293 cells were maintained in the presence of antibiotic selection. To validate the expression and localisation of **Myc-PTHR**, cells were stimulated with **PTH-TMR** (red) for 15 minutes, washed in 1X PBS and fixed in 4% PFA. Cells were then immunostained for endogenous **VPS35** (cyan) and PTHR using an anti-Myc antibody (green). Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens.
Supplementary Figure 9. Cyclic AMP production following PTH-TMR or PTH(1-34) stimulation in UMR106-01 cells.

UMR106-01 cells were seeded in triplicate in 12-wells and cultured in complete αMEM for 24-hours prior to the experiment. Cells were then cultured in the presence of isobutylmethylxanthine (IBMX, 1mM) for 60 minutes, followed by the addition of PTH-TMR or PTH(1-34) at the indicated concentrations, for a further 12 minutes. Culture medium was then removed and acidified alcohol was added to the wells. Cells were then reconstituted in assay buffer and cAMP production was determined relative to a standard curve, as described in Hammonds et al., 1989.
Supplementary Figure 10. SNX27 requires the PX and PDZ domains for correct localisation to endosomes.

PTH-expressing HEK293 cells were transiently transfected to express GFP-tagged SNX27 wild-type (WT) or mutant proteins lacking the PX (ΔPX) or PDZ (ΔPDZ) domains. Cells were cultured on poly-L-lysine-coated coverslips for 48-hours prior to stimulation with PTH–TMR (100nM) (red) for 30 minutes (in the presence of ligand washout following the initial 15 minutes of stimulation). Cells were then washed in 1X PBS, fixed in 4% PFA and immunostained for endogenous VPS35 (blue) or nuclear stain (Hoechst, blue). Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens. Cells expressing SNX27-WT localised to PTH-bearing endosomes that were also present for retromer. SNX27ΔPX and SNX27ΔPDZ were both distributed throughout the cytosol and failed to localise to endosomal membranes.
Supplementary Figure 11. SNX27 and FAM21 co-localise to PTHR-bearing endosomes.

HEK293 stably expressing N-GFP-PTHR were cultured in poly-L-lysine-coated coverslips for 48-hours prior to 30 minutes of PTH(1-34) (100nM) stimulation (in the presence of ligand washout following the initial 15 minutes of stimulation). Cells were then washed in 1X PBS, fixed in 4% PFA and immunostained for endogenous SNX27 (red), FAM21 (blue) and nuclear stain (Hoechst, blue). Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 60X oil lens. Scale bar represents 10µm.

SNX27 and FAM21 localised to similar endosomal microdomains containing PTHR, at which endosomal tubulations were also present.
Supplementary Figure 12. PTHR trafficking in MC3T3-E1 and UMR106-01 cells.

MC3T3-E1 and UMR106-01 cells were transiently transfected to express N-GFP-PTHR for 24 hours prior to re-seeding onto poly-L-lysine-coated glass coverslips. Following 48-hours, cells were then stimulated with PTH-TMR (100nM) (red) for 15 minutes, followed by ligand washout. Cells were then incubated for the time periods indicated, where they were then washed in 1X PBS, fixed in 4% PFA and immunostained for endogenous VPS35 (blue) and nuclear stain (Hoechst, blue). Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using a 40X oil lens. Scale bar represents 10µm. PTHR bound and internalised with PTH-TMR into retromer-positive endosomes in both MC3T3 and UMR106 cells. These receptor-ligand complexes were then found to translocate to the juxtanuclear region at 90 minutes post-stimulation, where retromer moderately co-resided.
Supplementary Figure 13. PTHR trafficking in OCY454 cells.  
Osteocytic OCY454 cells were maintained in 33°C prior to transfer to 37°C where they were transiently transfected to express N-GFP-PTHR. Cells were then re-seeded onto poly-L-lysine-coated glass coverslips for 48-hours, where they were then stimulated with PTH-TMR (100nM) (red) for 15 minutes, followed by ligand washout. Cells were then incubated for the time periods indicated, fixed in 4% PFA and immunostained for endogenous VPS35 (blue) and nuclear stain (Hoechst, blue). Images were taken using the Nikon A1Si confocal microscope (Nikon, Japan) using the 40X oil lens. Scale bar represents 10µm.  
Surface-resident PTHR internalised with PTH-TMR into retromer-positive endosomes as early as 5 minutes. Receptor-ligand complexes proceeded to accumulate within concentrated compartments within the perinuclear region of the cell by 60 minutes.
Supplementary Figure 14. SNX27-deficient osteoblasts exhibit reduced surface PTHR expression.

(A) MSC-differentiated osteoblasts from wild-type (WT) or SNX27-deficient (SNX27-/-) mice were cultured on coverslips and the surface expression of PTHR was assessed using PTH$_{\text{TMR}}$ surface labelling at 4°C for 10 minutes. Scale bar represents 10µm.

(B) Quantification of total fluorescence in each cell was normalised to the mean fluorescence detected in WT osteoblasts and expressed as a percentage of WT ±SEM (n = 10), ** p < 0.01.
APPENDIX

-C-

Publication

Chan et al., 2016
Sorting nexin 27 couples PTHR trafficking to retromer for signal regulation in osteoblasts during bone growth

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ABSTRACT The parathyroid hormone 1 receptor (PTHr) is central to the process of bone formation and remodelling. PTHr signaling requires receptor internalization into endosomes, which is then terminated by recycling or degradation. Here we show that sorting nexin 27 (SNX27) functions as an adaptor that couples PTHR to the retromer trafficking complex. SNX27 binds directly to the C-terminal PDZ-binding motif of PTHR, wiring it to retromer for endosomal sorting. The structure of SNX27 bound to the PTHR motif reveals a high-affinity interface involving conserved electrostatic interactions. Mechanistically, depletion of SNX27 or retromer augments intracellular PTHR signaling in endosomes. Osteoblasts genetically lacking SNX27 show similar disruptions in PTHR signaling and greatly reduced capacity for bone mineralization, contributing to profound skeletal deficits in SNX27-knockout mice. Taken together, our data support a critical role for SNX27-retromer mediated transport of PTHR in normal bone development.

INTRODUCTION

Growth and refurbishment of the vertebrate skeleton are strictly dependent on the coordinated cross-talk between bone-resident cells and their ability to respond to external stimuli via cell surface signaling receptors (Kromberg, 2003; Sims and Martin, 2014). The type 1 parathyroid hormone receptor (PTHR), a member of the class B G protein-coupled receptor (GPCR) family, is one of the best characterized and most important signaling receptors, modulating bone remodeling and mineral ion homeostasis (Garred and Vilardebo, 2015). In mammals, PTHR is primarily present in bone cells and kidney proximal tubules, where it exerts its physiological function(s) in blood calcium and phosphate metabolism and tissue development through the endocrine and paracrine/autocrine actions of two distinct peptide ligands, PTH and PTH-related protein (PTHrP; McCauley and Martin, 2012; Garred and Vilardebo, 2015). Not surprisingly, abnormal expression and/or function of PTHR correspond with severe bone dysmorphisms (formed skeletal dysplasias and various metabolic syndromes in both humans and mice (Karaplis and Goltzman, 2000). Specifically, disruption in PTHR signaling affects differentiation of cartilage-synthesizing chondrocytes and bone-forming osteoblasts (OBs), leading to alterations in postnatal growth and development of skeletal long bones. Intermittent injection of a PTH analogue is used in the clinical management of osteoporosis. Thus factors that influence PTHR signaling are highly sought after for the development of new therapeutic targets for the treatment of osteoporosis and other metabolic bone and mineral diseases (Martin and Saaman, 2007; Krawczyn and Maier, 2011).

PTH is released by the parathyroid glands in response to low serum calcium levels, which stimulate PTHR in OBs and activate parallel signaling pathways through trimeric G protein subunits Gα1 and...
Gsk3. The Gsk3 cascade activates phosphatase C, leading to insulin-like growth factor (IGF) activation, and subsequent mitogen-activated protein kinase (MAPK)–mediated cell proliferation. Gsk3 activates adenylyl cyclase to produce cAMP, stimulating the protein kinase A (PKA) pathway, transcription factor activation, and production of receptor activator of NF-κB (RANKL), eventually stimulating RANKL-expressing osteoclasts (bone-degrading cells). These signaling cascades in cells result in the phosphorylation of downstream targets, most notably cAMP–response element-binding protein (CREB), the kinase Akt, and MAPKs, including extracellular-signal-regulated kinase ERK1/2. As with other GPCRs, PTHR-evoked cAMP production has long been believed to originate almost exclusively at the cell membrane, with signal termination achieved upon receptor phosphorylation via G protein receptor kinases, β-arrestin-mediated internalization into early endosomes, and lysosomal degradation, that is, "canonical" GPCR signaling. However, it is now known that cAMP accumulation persists long after PTHR is internalized into endosomes (Ferrando et al., 2009). This "noncanonical" endosomal GPCR signaling involves an elaborate ensemble of proteins, which function to promote PTHR-mediated cAMP accumulation, including G proteins, β-arrestins, and small Rab GTPases (Rabs), which facilitate endocytosis and trafficking of the receptor to early endosomes (Vandala et al., 2012). Here cAMP generation persists until final signal arrest from the receptor upon PKA-driven intrastu- rnal acidification and the recruitment of the estrogen receptor protein complex (Ferrandon et al., 2009; Feinstein et al., 2011; Goldin et al., 2014).

Retron is an evolutionarily conserved endosomal protein complex consisting of a core consisting of vacuolar protease sorting 26 (VPS26), VPS29, and VPS35 (Collins, 2008). Retron plays a central role in endosomal membrane trafficking and is best recognized for regulating retrogade endosome-to-trans-Golgi network (TGN) trafficking of transmembrane cargos (Seaman, 2012; Gallon and Cullen, 2015). Recently, however, it has been shown that retron plays an equally important role in recycling of endocytosed protein to the plasma membrane (PM) via a complex with sorting nexin 27 (SNX27; Tomkin et al., 2011; Steinberg et al., 2013). SNX27 is a member of a large family of proteins containing a membrane-binding phospho-homology domain (Cullin and Konigswagen, 2012). It also possesses a C-terminal 41/42 amino acid (EYKD)–like domain that mediates binding to transmembrane cargos containing the N–N–N–X–N–X–X sequence (Ghali et al., 2011) and a unique N–terminal posttranslational sorting density 95/kilodalton large/nuclear locus–1 (P62D) domain. The P62D domain binds to type 1 P62D-binding motifs (P62Dm/s) with the consensus [TS]x-x-x (where x is a bulky hydrophobic amino acid) to mediate endosomal trafficking (Jaubert et al., 2004; Lunn et al., 2007; Lauffer et al., 2010; Balana et al., 2011; Cai et al., 2011; Tomkin et al., 2011; Valdes et al., 2011; Hayashi et al., 2012; Wang et al., 2013; Luo et al., 2014; SNX27 and retron form a complex to mediate the trafficking of many P62Dm-containing cargos, and cargo interactions with SNX27 are allosterically enhanced by the retron subunit VPS26 binding directly to the P62D domain (Steinberg et al., 2013; Gallon et al., 2014). Although SNX27–ret- ron dissection has been associated with neuronal defects in mouse models and human disease (Muhammad et al., 2008; Cai et al., 2011; Villarino-Guez et al., 2011; Zimprich et al., 2011; Wang et al., 2013, 2014; Luo et al., 2014; Danseh et al., 2015), the range of cargos bound by the SNX27–retromer complex (Steinberg et al., 2013) points to a much broader physiological role.

Here we use a broad range of structural and cellular approaches combined with mouse knockouts models to demonstrate that the SNX27–retromer complex plays a central role in PTHR signaling and retron-mediated endosomal recycling during bone growth and remodeling in vivo. PTHR is coupled to the retron complex via the structurally unique SNX27 P62D domain. We show that the assembly of this complex occurs on endosomes in response to PTH stimulation, where it directs PTHR to recycling to the cell surface, and provide evidence that the PTHR–SNX27–retromer association is physiologically required to restrict PTH signaling in OCLs, where disruption of SNX27 impairs OB activity and contributes to a severe growth and maturation defect in the skeleton of SNX27-deficient mice. Thus we propose that SNX27 serves as an endosomal P62D–cargo acaptor that links PTHR to the retron traf- ficking complex to regulate PTHR signaling during postnatal bone development.

RESULTS

SNX27 P62D domain interacts with PTHR in endosomes after PTH stimulation

The P62D motif (VPS26) encoded at the C-terminus of PTHR is highly conserved across species and similar to other known SNX27– P62D-binding proteins including the potassium channel KIR3.3, making PTHR a potential SNX27–P62D–interacting cargo (Figure 1, A and B). To test this, we coexpressed C-terminal green fluorescent protein (GFP)–tagged full-length PTHR and mutant variants of SNX27 lacking either the entire P62D domain (ΔVPSD) or a single–aminoacid point substitution (H114A) known to destabilize P62D–associated SNX27 cargos (Lauffer et al., 2010; Gallon et al., 2014) in a human embryonic kidney cell line consisting of vacuolar protease sorting 26 (VPS26), VPS29, and VPS35 (Collins, 2008). Retron plays a central role in endosomal membrane trafficking and is best recognized for regulating retrogade endosome-to-trans-Golgi network (TGN) trafficking of transmembrane cargos (Seaman, 2012; Gallon and Cullen, 2015). Recently, however, it has been shown that retron plays an equally important role in recycling of endocytosed protein to the plasma membrane (PM) via a complex with sorting nexin 27 (SNX27; Tomkin et al., 2011; Steinberg et al., 2013). SNX27 is a member of a large family of proteins containing a membrane-binding phospho-homology domain (Cullin and Konigswagen, 2012). It also possesses a C-terminal 41/42 amino acid (EYKD)–like domain that mediates binding to transmembrane cargos containing the N–N–N–X–N–X–X sequence (Ghali et al., 2011) and a unique N–terminal posttranslational sorting density 95/kilodalton large/nuclear locus–1 (P62D) domain. The P62D domain binds to type 1 P62D-binding motifs (P62Dm/s) with the consensus [TS]x-x-x (where x is a bulky hydrophobic amino acid) to mediate endosomal trafficking (Jaubert et al., 2004; Lunn et al., 2007; Lauffer et al., 2010; Balana et al., 2011; Cai et al., 2011; Tomkin et al., 2011; Valdes et al., 2011; Hayashi et al., 2012; Wang et al., 2013; Luo et al., 2014; SNX27 and retron form a complex to mediate the trafficking of many P62Dm-containing cargos, and cargo interactions with SNX27 are allosterically enhanced by the retron subunit VPS26 binding directly to the P62D domain (Steinberg et al., 2013; Gallon et al., 2014). Although SNX27–retromer dissection has been associated with neuronal defects in mouse models and human disease (Muhammad et al., 2008; Cai et al., 2011; Villarino-Guez et al., 2011; Zimprich et al., 2011; Wang et al., 2013, 2014; Luo et al., 2014; Danseh et al., 2015), the range of cargos bound by the SNX27–retromer complex (Steinberg et al., 2013) points to a much broader physiological role.
SNX27 interacts with internalized PTHR after activation. (A) Schematic drawing of PTHR, highlighting the PDZ domain at the intracellular C-terminus. (B) Sequence conservation of the PDZ domain of PTHR between species, together with published PDZ domains of known SNX27-interacting partners (sNIR and Kv3.3, using julienne 2. (C, D) Schematic illustration and expression of C-terminally GFP-tagged SNX27 (full-length) (FL), PDE truncation (PDZ2) and H114A mutant proteins in HEK293 cells. Bar, 10 µm. (E) Immunoprecipitation of HEK293 stably expressing mYFP-PTHR, with transient transfection of GFP-SNX27 (FL), GFP-SNX27PDZ2, or GFP-SNX27H114A. Blots are representative of one independent experiment. (F) After agonist- and time-dependent colocalization of mYFP-PTHR, PDE14A, and endogenous SNX27 in HEK293 cells. Bar, 10 µm. (G) Comparative colocalization analysis of internalized PDE14A and SNX27 on endosomes in stably HEK293 cells expressing PTHR or PTHR + PDZ2m at 15 min post-stimulation. Bar, 15 µm. (H) Magnified boxed region. Bar, 1 µm. **p < 0.001.

SNX27 links PTHR to the retromer complex

To define the precise steps in PTHR recycling and/or delivery cascade in which SNX27 operates, we further mapped its intracellular trafficking itinerary in relation to established components of the PTHR-endocytic machinery because SNX27 (Tamkin et al., 2011) and PTHR (Froemegen et al., 2011) have been independently shown to traffic along retromer-associated recycling pathways. We first assessed for colocalization between SNX27–PTHR and retromer after PTHR stimulation. We observed extensive colocalization between SNX27 (SNX27-GFP), PTHR (as monitored by PTHR-YFP), and retromer (using antibodies against endogenous VPS35; Arighi et al., 2004) on endosomes 15 min after PTHR stimulation (Figure 3A). This close overlap was verified by measuring the fluorescence intensity peaks between respective image channels by correlative line-scan analyses (Figure 3B). SNX27-retromer association appeared independent of the PTHR endocytosis adaptor β-arrestin (β-arrestin), whose fluorescence overlap with SNX27 (here SNX27-GFP) was found to decrease over time.
whereas, SNX27-extracellular association remained stable throughout the stimulation period (Figure 3, C and D), consistent with the view that they form part of the same endocytic recycling complex (Steinberg et al., 2013; Gallon et al., 2014).

The PDZ-dependent SNX27-PTHR interaction, together with the stable colocalization observed between these binding partners and retromer, suggested that SNX27 serves as a physical platform to scaffold PTHR to the retromer complex. To assess this, we used isothermal titration calorimetry (ITC) to measure the binding of the putative C-terminal PTHR PDZ3 to the SNX27 PDZ2 domain. We used a synthetic peptide representing the C-terminal eight residues of the PTHR (C-terminal FAMKTVVMK) and in comparison to the type 1 PDZ3s of GLUT1 and K9.3.3, two cargoes trafficked by the SNX27-retromer pathway (Steinberg et al., 2013), the PTHR PDZ3 bound more tightly to the SNX27 PDZ domain (Kd = 6.3 μM, compared with 17 and 154 μM for K9.3.3 and GLUT1, respectively; Figure 4A). Of importance, this binding is allosterically enhanced upon association with VPS26A (Kd = 2.2 μM; enthalpic increase from 8 μl to −19.7 kcal/mol), confirming that the SNX27-retromer complex physiologically forms a trimeric complex with PTHR. This allosteric enhancement is similar to what we previously observed for K9.3.3 (Gallon et al., 2014). By comparison, the PDZ binding-defective mutant of SNX27 (H1144) showed a dramatically reduced binding signal, as expected.

To investigate PTHR recognition at the atomic level, we next determined the crystal structure of the SNX27 PD2 domain bound to a PTHR peptide at ultrahigh resolution (0.95 Å; Figure 4B and Table 1). The final model contains residues 40–135 for the SNX27 PD2 domain and 501–593 for the PTHR peptide (EEWETVMK). Using the common nomenclature for PDZ domain sequences, this corresponds to residues 6–10 for High-quality electron density maps demonstrate that the PDZ2m of PTHR binds in a similar orientation as in the previously published SNX27 PD2-Kir3.3 complex (Balana et al., 2011). The PTHR peptide forms an antiparallel β-sheet interaction with PTHR, encompass the conserved Arg10 side chain of SNX27. This interaction could explain both the relatively high affinity of this peptide (and the similar K9.3.3; Gallon et al., 2014) for SNX27 and the highly favorable enthalpy of association measured by ITC (Figure 4B).

The Glu7 and Gln2 residues were not well ordered in our crystal, suggesting that these amino acids are not directly implicated in SNX27 recognition. In detail (Figure 4C), our structure confirms that the C-terminal PDZ3, tetratricopeptide (TPM) is crucial for the interaction. The terminal carboxyl group of Met12 establishes an array of hydrogen bonds with the backbone of the conserved GYGYG residue stretch in SNX27, whereas Thr1 forms a hydrogen bond with SNX27 His12, explaining the requirement for serine or threonine at this position of the motif. In addition to intermolecular β-sheet main-chain contacts, PDZ recognition extends upstream of the canonical type PDZ interaction and is mediated mainly by the two Glu7 and Gln2 residues. Glu10 is hyperbranched between SNX27 Arg5 and Arg10, and Glu10 similarly forms a salt bridge with Arg5. This network of interactions cooperatively stabilizes the PTHR peptide in the PDZ cavity, burying a surface area of 470 Å² (PDBePISA server, www.ebi.ac.uk), and provides a basis for strong and specific association (Figure 4C). The Aa10 and

**FIGURE 2:** Spatial-temporal interaction of PTHR with SNX27 after endocytosis. (A) Time-lapse confocal microscopy of live HBKG93 cells stably expressing GFP-PTHR and SNX27 mCherry. Interaction between GFP-PTHR and SNX27 mCherry is shown at both the basal state (agonist naive) and 15 min poststimulation with 100 nM PTH(1-34). Bar, 10 μm. (B) From A showing the localization and cell quantification line scan analysis of SNX27 mCherry to PTHR-positive endosomal microdomains, starting at 0. Bar, 1 μm. (C, D) Time-lapse dynamics of HBKG93 cells stably expressing GFP-PTHR and SNX27 mCherry after stimulation with 100 nM PTH(1-34) across 5 min (C) and intensity plot over 300 s × 300 s (D). (E) Mobilization of GFP-PTHR and SNX27 mCherry to endosomes after stimulation with 100 nM PTH(1-34) across 120 s. Bar, 1 μm. (F) Beneath region showing the recruitment of SNX27 mCherry to tubular endosomal protrusions occupied by GFP-PTHR (arrows). Bar, 1 μm. (G) Fluorescence intensity tracking of ruffling between GFP-PTHR and SNX27 mCherry across 360 s after agonist stimulation.

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**FIGURE 4:** Molecular dynamics of PTHR binding to SNX27. (A) Superposition of the PTHR PDZ3 (PDB 1mfn) and SNX27 PD2 (PDB 5m84) structures. Residues 40–135 of SNX27 PD2 and 501–593 of PTHR are colored in green and black, respectively. The C-terminal PDZ3 of SNX27 (red) interacts with the N-terminal PDZ3 of PTHR (gray) and in comparison to the type 1 PDZ3s of GLUT1 and K9.3.3, two cargoes trafficked by the SNX27-retromer pathway (Steinberg et al., 2013), the PTHR PDZ3 bound more tightly to the SNX27 PDZ domain (Kd = 6.3 μM, compared with 17 and 154 μM for K9.3.3 and GLUT1, respectively; Figure 4A). Of importance, this binding is allosterically enhanced upon association with VPS26A (Kd = 2.2 μM; enthalpic increase from 8 μl to −19.7 kcal/mol), confirming that the SNX27-retromer complex physiologically forms a trimeric complex with PTHR. This allosteric enhancement is similar to what we previously observed for K9.3.3 (Gallon et al., 2014). By comparison, the PDZ binding-defective mutant of SNX27 (H1144) showed a dramatically reduced binding signal, as expected.

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Snx27

A

B

C

D

FIGURE 3: PTHR is transported in SNX27-retrorner-decorated endosomal tubules. (A) Immunofluorescence staining of HEK293 cells stably expressing PTHR stimulated with PTH₁R or FFFM or for 15 min. Bar, 10 μm. Inserts: co-localization of SNX27 (green), PTHR (red), and VPS35 (blue), Bar, 1 μm. (B) Correlative line-scan analysis of SNX27 (red), PTHR (red), and VPS35 (green) as shown in the merged inset in A. (C) HEK293 cells stably expressing PTHR were transiently cotransfected with β-arrestin-2LacZ and SNX27-GFP, images show the recruitment of β-arrestin-2LacZ with SNX27-GFP and VPS35 at 15 and 30 min poststimulation with 100 nM PTH(1-34), Bar, 10 μm. (D) Colocalization analysis of SNX27-GFP and VPS35 across 60 min. Values are mean ± SEM and representative of three independent experiments.

Arp2/3 of SNX27 are conserved across species, as is the CYFIP stretch that mediates binding to all PI3Kε triplets (Ye and Zhang, 2013) (Figure 4D). To confirm the structural basis of SNX27 binding to the PTHR, we mutated the PTHR sequence by either adding an Ala to the Cterminus or altering the Glu residue to Ala. Both alterations abolished binding to the PTHR peptides, according to IFC (Figure 4A). Taken together, these data confirm that PTHR binds directly to the SNX27 PDZ domain and that this interaction site is located close to the VPS35-binding surface of SNX27 (Figure 4E) and is allosterically coupled to SNX27-VPS35 association, as for GSK1 and Kif3.3 (Gallien et al., 2014).

Depletion of SNX27-retrorner causes sustained PTHR signaling

Because termination of cAMP generation from endosomal PTHR requires retrorner (Feinstein et al., 2011) and the results described so far indicate that SNX27 is the molecular adaptor that directs PTHR to retrorner, we next asked whether SNX27 also functions to restrict cAMP signaling initiated by the receptor. For this, we used plasmid-based short hairpin RNAs (shRNAs) against SNX27 and VPS35 to deplete SNX27 and retrorner expression in PTHR-expressing HEK293 cells and assessed the effects on PTHR-activated cAMP signaling by examining the phosphorylation of several downstream second messenger pathways, including CREB, AKT, and MAPK (p-ERK1/2). Transfection and knockdown (KD) efficiencies were monitored by visualizing the percentage of cells expressing the GFP-retrorner KD cells using PTH₁R as a probe. Intriguingly, under resting conditions (i.e., temperature block 37°C, 10 min), we found that depletion of either SNX27 or VPS35 protein led to a reduction in PTH₁R-PTHR surface levels (up to ~50% as quantified by PTH₁R-intensity) compared with cells expressing nontargeting control shRNA (Figure 5, D and E). Thus, the elevated PTHR signaling observed in SNX27- or retrorner-depleted cells did not originate from enhanced levels of receptor at the cell surface. We next probed whether loss of either SNX27 or retrorner routes PTH₁R-PTHR trafficking to a distinct anoxic/gut compartment by monitoring the fate of internalized PTH₁R over various time points (up to 120 min) after PTH stimulation and washout in SNX27 and retrorner-depleted cells in reference to the early endosome marker EEA-1. In PTH₁R-stimulated PTHR-HA293 cells expressing the nontargeting shRNA (unpublished data) or neighboring cells lading expression of the SNX27 shRNA GFP reporter, PTHR consistently internalized into EEA-1-positive early endosomes at 5 min poststimulation (Figure 5, F and G) before entering VPS35-positive endosomes (typically within 10–30 min), with PTH₁R signal dissipating by 60–120 min poststimulation/ washout, a period that coincided with reoccupation of the receptor back to the PM (unpublished data). In comparison, whereas the low cell surface levels of PTH₁R (unpublished results) in SNX27 and retrorner KD cells (Figure 5, F and G) at time points immediately after administration of the PTH analogue (5–30 min), we did; however, observe PTH₁R signal in vesicular clusters at the perinuclear
region of SNX27 and retromer KD cells at later time points (60 min). These accumulated and were most conspicuous 120 min after PTH
stimulation (Figure 5, F and H). These perinuclear clusters were distinct from early endosomes, as they lacked EEA-1 and were rarely
observed in neighboring non-transfected or non-transfected iSNX27 control cells but were frequent in cells depleted of either SNX27 or
VS535 (Figure 5, F and H), implying that this was a general phenomenon upon disruption of the SNX27-retromer machinery. To reveal the
nature of these PTH-KD-containing compartments, we per-
formed immunostaining against various subcellular markers, including the lysosomal integral membrane protein (LAMP1; Figure 5H), which identified the population of perinuclear vesicles as lysosomes. These observations imply that down-regulation of SNX27 and retromer expression reduces PTHR surface levels, leading to an increase in sustained endosomal signaling, and stalls the entry of PTH into degradative lysosomes.

OBs lacking SNX27 display overactive PTH signaling and impaired mineralization activity in vitro

To confirm whether the observed disturbances in PTHR signaling in SNX27-retromer-depleted HDX293 cells translated physiologically, we further assessed the integrity of PTHR signaling in OBs (which natively express PTHR) (isolated from SNX27- deficient SNX27−/− mice (Cai et al., 2011)).

First, we checked the functional consequence of SNX27 ablation on PTH-induced cAMP accumulation across a range of PTH concentra-
tions. We observed a significant elevation in PTH-induced cAMP accumulation in OBs derived from bone marrow mesenchymal stem cells (BMSCs) from SNX27−/− mice compared with those from wild-type (WT) littermates (Figure 6A). We then probe PTH-associated secondary messenger signaling path-
ways including pCR6, pAKT, and MAPK (pERK1/2), and found again that they were significantly increased in the early phase (5–30 min) of PTH stimulation (Figure 6B–D) despite reduced cell surface PTH-PTHrP levels (Supplemental Figure S5). Because PTHR also operates through the paracrine/autocrine cAMP-dependent PTHP in OBs (Maio et al., 2005), we also checked whether SNX27 deficiency altered PTH- or PTHrP-activated PTHrP signaling. In this instance, PTHrP(1–34) stimulation in-
duced elevated CREB and AKT phosphorylation profiles (most evident between the 5- and 30-min time points) in OBs derived from
SNX27−/− mice compared with WT controls (Figure 6B).

Importantly, we noted that the intensity of CREB phosphorylation was consistently more pronounced in OBs stimulated with PTHrP than in those exposed to PTH over the 90-min stimulation period (Figure 6C), possibly reflecting structural differences between PTHrP(1–34) and PTHrP(1–36) peptides or distinctions in the activation/deactivation kinetics, as previously reported for these two ligand systems (Ferrando et al., 2009).

PTH signaling is critical to OB differentiation and bone anabolic function (Datta and Aboobaker, 2009). Therefore, to study the cum-
ulative effect of PTHrP desensitization on the function of SNX27-OBs, we assessed their differentiation and bone formation activity in vitro. Whereas the number of alkaline phosphatase (ALP)-expressing OBs obtained from BMSC-derived bone cell populations was largely indistinguishable between WT and SNX27 knockout lattenuates

FIGURE 4: Structure of the SNX27 PDZ domain bound to the PTHR PDZ binding motif. (A) The PTHR PDZ2 domain peptide binds directly to the SNX27 PDZ domain in vitro (blue), and prior engagement by VS526A facilitates this binding (black). Top, raw ITC data; bottom, integrated normalized data and calculated Kd values with SEs over three experiments. The SNX27 N144A mutation is shown as a negative control (orange). PTHF mutant peptides PTHR E99GA (purple) and PTHR A201 (pink) fail to bind the SNX27 PDZ domain. (B) Electroporated and ribbon representations of the SNX27 PDZ domain (gray) bound to PTHR peptide (yellow). Stick crystal structure. The PTHR peptide PDZ domain sequence is displayed in the middle, and its refined 2F_o – F_c electron density contoured at 2σ is shown in blue. (C) Detailed view of the interaction interface between the SNX27 PDZ domain binding cavity (gray) and PTHR (yellow). Critical contacts between residues within the interaction are displayed as black dashed lines. (D) Alignment of amino acid sequences of SNX27 PDZ domains from various species and residues involved in binding to PTHR. Matching residues with the human sequence are depicted as dots. Alignment was generated using CLC BIO software. (E) Overlay of the SNX27 PDZ-VP526A complex (PDZ core 4P24) with the SNX27 PDZ-PTHrP complex (this study) structures highlights the different binding interfaces of both partners to SNX27. The SNX27 PDZ domain is shown in gray, VP526 (VS526A) in orange, and the PTHR peptide in yellow.
A. Data collection

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B. Refinement

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TABLE 1: Summary of crystallographic structure determination statistics.

SNX27−/− Ols compared with controls (Figure 6A), whereas the expression levels of retinon (VPS55) remained constant between WT and SNX27−/− Ols (Supplementary Figure S6). Taken together, these data imply that the absence of SNX27 leads to non-overlapping endosomal PTHR1 signaling, failed terminal differentiation of Ols, and thus impaired mineralization function.

Postnatal SNX27−/− knockout mice exhibit skeletal growth deficits and reduced bone mass

PTHR1 is a crucial regulator of postnatal bone development and remodeling. Because our studies identify PTHR1 as a novel SNX27−/− interacting cargo and a role for SNX27 in bone morphogenesis has not been described, we further assessed the pathophysiologic consequences of SNX27 ablation on the mouse skeleton. SNX27−/− mice die at 4 wk postpartum (Can et al., 2011). Skeletal examination of PS SNX27−/− mice by whole-mount preparations and three-dimensional (3D) micro-computed tomography (μCT) reconstruction revealed overall impairment in skeletal growth in SNX27−/− mice (Figure 7, A and B). Reflecting this, SNX27−/− deficient mice were macroscopically smaller and exhibited shortened of limbs and tails and smaller skull sizes that were of reduced bone density compared with their WT and heterozygous littermates. These developmental disturbances extended to long bones (femur and tibia), forelimbs (humerus, ulna and radius), and spines of 4-wk-old SNX2−/− mice (Figure 7, C-F). Detailed linear longitudinal reconstruction and assessment of microarchitectural bone parameters of femora and tibia isolated from 4-week-old, sex-matched littermates revealed that bone trabeculation within the metaphyseal regions was drastically reduced in SNX27−/− mice (Figure 8, A and C). In addition, severe cortical thinning was observed in cross-sectional views in long bones of SNX27−/− mice compared with their littermate controls (Figure 5B). These profound reductions in trabecular bone volume (TBV/TV) and trabecular number (Tb.N) and thickness (Tb.Th) were also verified by histomorphometric analysis (Figure 8, D and E).

To distinguish whether the stastically reduced bone mass might result from enhanced bone resorption by osteoclasts or reduced bone formation by Ols, we analyzed the number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts at the trabecular plane of SNX27−/− bones. We found that the osteoclast number per bone surface (CS/BS) (mm) was only marginally decreased in the bones of SNX27−/− mice compared with WT littermates (Figure 8, F and G). In comparison, histomorphometric assessment of the number of Ols occupying trabecular bone surfaces (O.N./BS) revealed that they were substantially reduced in the long bones of SNX27−/− mice (Figure 8, F and H). Reflecting this, the total deposition (BS/BS) and thickness (O.W/BS) of reasonably deposited unmineralized bone (osteoid) was equally reduced in SNX27−/− mice (Figure 8, F). Therefore, the decrease in bone mass in SNX27−/− deficient mice results from inadequate osteoblastic differentiation and bone formation and not enhanced bone resorption.

Finally, in addition to the observed microarchitectural changes in bone density, there was a conspicuous enlargement of the gap between the trabeculae and femoral diaphyses and epiphysis (Figure 5A). Histological assessment of long bones revealed that this cartilaginous expansion arose from postnatal delays in the formation of the secondary ossification center and epiphyseal growth plates in SNX27−/− mice (Figure 8, D and I). Tibial and femoral growth plates of SNX27−/− mice were ~50% longer than in WT (Figure 8K), as confirmed by immunohistochemistry for the chondrocytic marker type II collagen (Figure 8J). Despite these anomalies, the characteristic columnar cellular organization and proteoglycan levels (periodic acid-Schiff [PAS])
and Safarian O staining of the growth plate layers were generally well preserved. Viewed collectively, these data indicate that the maturation of the postnatal skeleton in Snk27-deficient mice is severely delayed due, at least in part, to growth plate abnormalities and reduced osteoblastic differentiation and bone formation.

**DISCUSSION**

Here we report multiple lines of evidence to indicate that Snk27 functions as an endosomal cargo adaptor for PTHR, a clinically important GPCR central to normal bone homeostasis and systemic calcium regulation. We show that Snk27 associates with endosomes bearing PTHR after agonist-induced internalization, where it binds directly to PTHR with high affinity, and simultaneously scaffolds the receptor to the retromer complex. Recruitment of PTHR to Snk27-retromer–recycling tubules restricts PTHR-evoked cAMP signaling, a prerequisite for PTHR signal regulation and function in OEs during postnatal bone growth and remodeling.

The spatiotemporal segregation of GPCR signaling in endosomes is now recognized as a key process in the cell's response to different stimuli (von Zastrow and Sorlin, 2007; Sorlin and von Zastrow, 2009). In the case of PTHR, it has been suggested that activation by PTH can promote sustained endosomal signaling but PTH-IR cannot (Vilardaga et al., 2014). Several reports have shown that the endosome also acts as the site for PTHR signaling desensitization after PTHA-mediated intracellular acidification and recruitment of select endocytic read-throughs (Feinstein et al., 2011; Gibson et al., 2014; Vilardaga et al., 2014). Our work confirms the central role of retromer in terminating cAMP production by PTHR. However, until now, the molecular basis for this was incompletely understood. It was suggested that retromer likely controlled retrograde trafficking of PTHR to the TGN (Feinstein et al., 2011) and might associate with PTHR via either the structural similarity of the VPS26 retromer subunit to β-arrestin (Shi et al., 2006; Colins, 2008; Aubry et al., 2009) or binding of an aromatic-containing PNN sequence to the retromer VPS35 subunit, although no direct interactions were shown (Feinstein et al., 2011; Vilardaga et al., 2014). Our results clearly show that Snk27 serves as an endosomal platform to integrate PTHR signal activation and retromer-mediated termination. We propose that Snk27 not only confers spatiotemporal control over endosomal PTHR signaling, but that it also directs PTHR to the retromer for recycling back to the plasma membrane (possibly via the TGN), thus providing a mechanistic basis for Snk27-retromer-mediated signal attenuation (Figure 9).
FIGURE 6: SNX27 is required for PTHR signal transmission in OBLs and OIB differentiation. (A) Cyclic AMP assay performed on SNX27 WT and SNX27 KO OBLs stimulated for 10 min with increasing concentrations of PTH(1-34). (B, C) SNX27 WT and SNX27 KO OBLs were stimulated with 100 nM PTH(1-34) (B) or 100 nM PTH(1-141) (C), for 15 min, followed by a washout. Signaling pathways (CREB, AKT, ERK) were assessed through Western blotting. (D) Densitometry quantification of protein bands from SNX27 WT and SNX27 KO Western blots (B) were conducted using Photoshop 2014 and expressed as a percentage with respect to their respective control. (E) ALP and alizarin red staining (ARS) of SNX27 WT and SNX27 KO BMSC-derived OBLs cultured in vitro under mineralizing conditions (50 ng/ml TGF-β1, 2 mM β-glycerophosphate, 10 μM dexamethasone) for 21 d. (F) Quantification of ARS-positive nodules per square millimeter using ImageJ. (G) Calvarial OBLs isolated from SNX27 WT and SNX27 KO were cultured under mineralizing conditions (+) for 21 d and stained with ALP or ARS. (H) mRNA expression from BMSC-derived SNX27 WT and SNX27 KO OBLs was analyzed through quantitative PCR, using HMB2 as a reference gene. *p < 0.05, **p < 0.01, and ***p < 0.001.
As with other SNX27-interacting cargo, for example, Kiss13 (Balane et al., 2011), our findings demonstrate that PTHR cargo recognition is achieved via its C-terminal PDZ domain, which binds with high affinity to the N-terminal PDZ domain of SNX27. Of importance, the PDZ domain not only discriminates SNX27 from other SNX family members, but it also serves as an interaction module to simultaneously wire PTHR to the retromer core subunit VPS26, VPS29 binding additionally stabilizes and enhances the affinity of the SNX27 PDZ domain for the PTHR PDZ domain, a feature common to other SNX27-retromer-interacting cargo housing PDZs (Gallon et al., 2014). Similar to Kiss13 (PESE; Balane et al., 2011), the PTHR houses a four-amino acid stretch (CED) encoded immediately upstream of the canonical PDZ tripeptide (VVM) that encompasses two acidic glutamate residues, at positions -3 and -5, that form a strong electrostatic interaction with the conserved Arg6 side chain of the SNX27 PDZ domain. Precisely how this terminal acidic sequence accounts for the preferential binding of affinity of the PTHR observed over other known SNX27 PDZ-domain bearing cargo (Cherfils et al., unpublished data) forms the subject of our future studies.

Together with previous work describing a role for the retromer complex in endosomal PTHR trafficking (Feinstein et al., 2011), our morphological and signaling analyses best support a function for SNX27 as a molecular adaptor for recruiting PTHR into retromer-decorated tubules for endosome-to-cell surface recycling. SNX27-mediated transport thus attenuates sustained endosome-associated PTHR signaling. This conclusion is supported by our evidence that genetic ablation of SNX27 leads to enhanced cAMP accumulation and subsequent overactivation of downstream signaling cascades upon PTHR stimulation, most evident at times that parallel receptor internalization into SNX27 and retromer-bearing endosomes in PTHR-expressing HEK293 cells. We further show that depletion of the SNX27 or retromer leads to a reduction in PTHR-PTHR cell surface levels, presumably reflecting reduced recycling rates or mistraficking of the receptor (e.g., into lysosomes) as observed for other SNX27 PDZ-domain-bearing cargoes (Steinberg et al., 2013; Gallon et al., 2014). In fact, during the course of this investigation, depletion of either SNX27 or retromer (VPS28) was independently shown to correlate with a 50% decrease in the rate of PTHR recycling at the cell surface following agonist stimulation (J. McGenity and P.A. Friedman, personal communication).

Interestingly, despite these reduced cell surface levels, PTHR-induced PTHR signaling remains elevated upon SNX27 and retromer disruption. This implies that the levels of PTHR remaining on the cell surface are sufficient to elicit signal sensitization when ligands are added at saturating concentrations. It also implies that newly synthesized PTHR or compensatory retention mechanisms might help to maintain PTHR levels when SNX27-retromer expression becomes limited. For example, cytosolic NHERF proteins (NHERF1,2) contain PDZ modules that also bind to the PTHR PDZ domain (Malcon et al., 2002; Sneddon et al., 2003) and have been reported to regulate the membrane retention, trafficking, and desensitization of PTHR (Wang et al., 2007, 2008; Andrus et al., 2011; Wheller et al., 2011). Of interest, mice lacking NHERF1 (Shenolikar et al., 2002) and patients harboring NHERF1 mutations (Kaim et al., 2008) manifest bone abnormalities (osteopenia and osteosclerosis; Weinman et al., 2006; Liu et al., 2012) that are attributed, in part, to overactivated PTH
signaling and impaired mineralization capacity in Ols (Liu et al., 2012), in keeping with the skeletal deficits observed in Snx27−/− mice in our study. The precise interrelationship that exists between PTHR, NHEF1, and Snx27−/− is unclear, but it appears that at least two PIZZ-dependent modes of PTHR trafficking can operate in vertebrates to modulate cell surface levels—one regulated by NHEF1 (Audria et al., 2013) and the other presided over by Snx27, as shown in this study.

Snx27 disruption in humans (Gompels et al., 2013) and mice (Coli et al., 2011) manifests in severe growth and developmental disturbances (as shown here) and also correlates with net alterations in the expression of many critical neuronal proteins (Coli et al., 2011; Wang et al., 2013, 2014; Lou et al., 2014). Although Snx27 is expressed in many tissues (including bone; Chan and Pavlos, unpublished data), studies characterizing the effect of its disruption have been largely restricted to its role in the impairment of neuronal function. Our finding that Snx27 deficiency leads to severe skeletal dysplasia expands the physiological importance of Snx27 to bone morphogenesis. Of interest, the skeletal dysmorphisms exhibited in Snx27−/− mice bear some resemblance to those in humans (Schipani et al., 1991, 1992) and mice (Konishi et al., 1994; Lameke et al., 1999; Calvi et al., 2001; Miao et al., 2002) with disruptions in PTH and associated G protein-dependent signaling pathways (Mausmann et al., 2006; Wu et al., 2011). On one hand, the reduced cortical bone density, increased oC4P accumulation, overactivation of associated secondary messenger signaling cascades (pCREB, pAKT, and pBAD20), along with the unregulated transcriptional expression of PTH-responsive genes observed in both unstimulated Ols and total bone, phenocopies those observed in osteoblastic cells from mice bearing constitutively active mutations in the PTHR gene (Calvi et al., 2001) and/or deletion of Gt (Wu et al., 2011). On the other hand, the expansion of the cartilaginous growth plate (as opposed to its premature closure) clearly distinguishes Snx27−/− mice from those with disrupted PTHR signaling pathways alone (Calvi et al., 2001).
Kobayashi et al., 2002; Miao et al., 2002; Hiroi et al., 2011). Thus additional SNX27-signaling cargo must contribute to the bone phenotype. Indeed, the JASPER (Laffler et al., 2010; Temkin et al., 2011), an established SNX27-binding GPCR, is functionally required for the anabolic action of PTHrP on bone (Bienaymé et al., 2012). Receptors of other notable bone morphogens, including transforming growth factor β (TGFβ), BMPs, and Wnts, which interact with PTHrP and traffic via the retromer recycling pathway, may similarly add to the net bone phenotype (Cui et al., 2010; Hartenstein et al., 2011; Yin et al., 2013; Glisson et al., 2014). Irrespective of the exact number of cargoes involved, our studies clearly demonstrate that PTHrP is a physiologically important SNX27-PPO2 cargo with central roles in bone growth and remodeling.

Unraveling the fundamental mechanisms governing PTHrP signal- ing has been a focus of intensive experimental and pharmacological research (McCueley and Martin, 2012; Gordelia and Vrabcega, 2013). The identification of new molecules, such as SNX27, that directly modulate PTHrP signaling, trafficking, and function may open up new avenues for the development of more effective therapeutic agents that are applicable not only to disorders of bone metabolism like osteoporosis but also extend to wider metabolic syndromes.

**MATERIALS AND METHODS**

**Antibodies, peptides, and constructs**

We used the following materials from the respective suppliers: phospho-CREB, phospho-ERK1/2, phospho-PTHrP(1-34), phospho-JNK, phospho-ATG4, and phospho-ERK1/2 (Cell Signaling Technology, Danvers, MA); PTHrP, phospho-ERK1/2, and V5-tagged PTHrP (Santa Cruz Biotechnology, Dallas, TX); total ERK1/2 (Promega, Madison, WI); EEA1 and TGN38 (BD Biosciences, Franklin Lakes, NJ); SNX27, Lamp1 (Abcam, Cambridge, United Kingdom); anti-Myc (Merck, Milwaukee, Germantown, MD); collagen II, α-tubulin, peroxidase-conjugated goat anti-mouse immunoglobulin G (1:500), goat anti-rabbit IgG (1:500), goat anti-rabbit IgG (1:500), Sigma-Aldrich, St. Louis, MO); Hoechst 33258 (Thermo Fisher Scientific, Waltham, MA); 1,3,5-triaminophenylisocyanate (PITM1-34) was purchased from Sigma-Aldrich. A tetramethylrhodamine-labeled parathyroid hormone (PTH(1-34)) by which TMR was added to the examining group of Ly51 of PTHrP(1-34) was synthesized from Genescript (Piscataway, NJ). Constructs were as follows: β-arrestin2-GFP (Addgene plasmid 35411); SNX27(2-150), SNX27(2-150)M1R, and SNX27(2-150)R246 (previously described in Luo et al., 2014); and SNX27/H114A (generated using QuikChange Site-Directed Mutagenesis Kit, Agilent Technologies, Santa Clara, CA). Stable knockdown HEK293 cell lines were generated using a lentiviral shRNA system (Thermo Fisher Scientific) with pGIPZ shRNA-non-targeting (R8-53436), pGIPZ shRNA1 SNX27 (R8-237989), and pGIPZ shRNA2 SNX27 (R8-156301).β-Raf and c-Myc proteins were previously described (Pavlov et al., 2010). The mCherry-M1R2 was generated by Genescript and subcloned into pCAGGShuttle. The mCherry-SNX27 was generated by subcloning 2xFYVEmCherry (pEFPp) 2xFYVEmCherry as described in Karr et al. (2010). For construction of 2xFYVEmCherry, the N-terminus of the PTHrP (residues 61–101) was replaced with the enhanced GFP sequence preceded by the linker Arg-Lys-Ser-Gly-Ser according to the methods described in Castro et al. (2005). Unliganded PTHrP and PTHrP/PO2LmCherry were generated by Genescript. JPhospho-M1R2 was generated by ChemGenes Technologies (Rockville, MD).

Animals

The generation of SNX27−/− mice was described previously (Cai et al., 2011). This study was performed in strict accordance with the Animal Welfare Act 2002 (Western Australia) and requirements of the eighth (2013) edition of the Australian code for the care and use of animals for scientific purposes. All of the animals were handled according to institutional animal care protocols approved by the Animal Ethics Committee of the University of Western Australia (Approval No. RA/3/100/1999).

Cell culture and transfection

All cell culture products were purchased from Thermo Fisher Scientific. Primary mouse OIBs were isolated from B6SJCF1 and calvarial bone as described previously (Baekker and Klein-Nulend, 2012). Kular et al., 2015) and cultured in complete αMEM (10% fetal bovine serum [FBS], 100 U/ml penicillin, 100 U/ml streptomycin). HEK293 cells were cultured in complete DMEM-Glutamax (10% FBS, 2 μM l-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin) and maintained in humidified conditions of 5% CO2 at 37°C. Cells were transfected using Lipofectamine LTX (Thermo Fisher Scientific) according to the manufacturer’s protocol. Stable cell lines were derived through antibiotic selection of neomycin/1418 at 500 μg/ml or puromycin at 2 μg/ml.

Immunoprecipitation

The respective plasmids were transfected into HEK293 cells line stably expressing the PTHrP using Lipofectamine LTX according to manufacturer’s protocol. Cells were incubated for 48 h, followed by the addition of PTHrP(1-34) (100 nm) for 10 min. Cells were washed twice with ice-cold 1× phosphate-buffered saline (PBS) and lysed in nonde-naturing extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM MgCl2, 0.5 mM ethylene glycol tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1× complete protease inhibitor [CI], 0.1% Triton X-100) on ice. Cell lysis was homogenized and centrifuged at
15,000 rpm for 15 min at 4°C. Cleared lysates were incubated with GO-P-Trap-A beads (ChromatoTek, Finspong, Malmö, Sweden) overnight at 4°C. Beads were washed in washing buffer (20 mM HEPES-KOH pH 7.2, 150 mM NaCl, 1 mM MgCl₂, 1 mM IPMSF, 0.5% Triton X-100, boiled, in 2× non-reducing loading buffer, and resolved by SDS-PAGE (12%).

**Immunofluorescence and time-lapse confocal microscopy**

Stably transfected HEK293 cells were cultured on poly-L-lysine-coated glass coverslips for 48 h and serum starved for 1 h before stimulation with PTH(1-34) (100 nM) or PTH(1-34) (100 nM) over a time course of 0–120 min. After 15 min of stimulation, cells were washed twice with PBS and replaced with complete medium. Cell surface PTH(1-34) fluorescence was detected by subjecting cells to an ice block (4°C for 10 min) and washing and then fixing (4% paraformaldehyde/PFA). Samples were then permeabilized with 0.1% Triton X-100 for 1 h and stained with Alexa dyes.

**Histology and histomorphometry**

Lox primoral bone and distal femur samples from 1-mo-old wild-type and knockdown littermates were processed using a Leica TP1020 processor (Leica Biosystems, Nussloch, Germany). Lox for preparation for either methyl methacrylate (MMA) or paraffin embedding according to standard protocols. Sections were also stained using a Leica Biosystems RM2155. Automated Microtome for MMA-embedded samples or a Bicor 3005 at a thickness of 5 μm. Sections were then stained with Toluidine blue and stained with Fast Green for collagen 2 according to standard protocols. Sections were further stained using a Leica Biosystems Aquarius ScanScope.

**Immunohistochemistry**

A histological analysis was performed using BioQuant D200, version 13.26 (Biorquant Image Analysis, Nashville, TN).

**Mineralization and ALP assay**

Mineralization assays were conducted using 4 × 10⁵ cells/well (24-well plate) in complete MEM supplemented with osteogenic medium, that is, 1% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine, 100 nM sodium selenite, 2 mM L-proline, 100 nM dexamethasone, and 50 μM ascorbic acid (PAA Lab). Cells were then stained with Alizarin Red S solution (Sigma-Aldrich) and analyzed using an Image-Pro Plus program (National Institute of Health, Bethesda, MD). ALP activity was visualized using a LeukoBlue Alkaline Phosphatase kit (Sigma-Aldrich).

**Cyclic AMP assay**

Primary OSEs were seeded into 96-well plates at a density of 1.2 × 10⁴ cells/well and allowed to grow for 21 days. Cells were then stimulated with forskolin (1 μM) for 45 min. Forskolin treatment resulted in a statistically significant increase in cyclic AMP (CAMP) levels, as determined by a CAMP assay kit (Cell Signaling Technology).

**Data collection and structure determination**

Data were collected at 100 K at beamline MX2 at the Australian Synchrotron, Australia. The structure was determined by molecular replacement using Phaser 3.10 (McCoy et al., 2007) with the PDB code 2D0X as the search model. The model was refined with X-PLOR and Coot. All residues in the model were built in accordance with R.m.s.d. statistics. The final model was solved at 0.15 Å resolution and revealed electron density corresponding to all amino acids contained in the synthetic peptide but the C² residue. All structural figures were generated using PyMOL (www.pymol.org).

**Isothermal titration calorimetry**

ITC experiments were performed on a MicroCal ITC200 instrument (Malvern, Malvern, United Kingdom) in 50 mM Tris (pH 8.1) buffer. The PTHrP (Glu1-34)Val1 peptide at a concentration of 0.275 mM was titrated into 50 μM SNA27 POZ domain III in 50 mM Tris buffer supplemented with 50 μM PMSF when required. The protein solutions were diluted to 5°C. Data were processed using ORIGIN to extract the thermodynamic parameters and the stoichiometry. Here, N and S are derived from N-S and N-S with equimolar mixing.

**References**

(All references are cited in the original document.)
bands were quantified by densitometry analysis using Photoshop 2014 (Adobe, San Jose, CA; data shown are representative of at least three independent experiments and expressed as mean ± SEM).

**RNA and quantitative PCR**

For quantifying gene expression, RNA samples were extracted using TRIzol (Thermo Fisher Scientific). First-strand synthesis was conducted using 1-2 μg of total RNA using SuperScript II RT Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Quantitative PCR was performed using a SensiMix I Probe Kit (BioLine Reagents, London, United Kingdom) and the Universal ProbeLibrary (Roche Diagnostics) and analyzed using a CFX Connect Real-Time System and CFX Manager Software (BioRad, Hercules, CA). Relative fold expression was normalized to β-actin (ACTB), TbpMen and hydroxymethylbilane synthase (HMBS) sample controls using the primer sequences shown in Table 2.

**Skeletal staining and micro-computed tomography**

Killed F5 mice were skinned, eviscerated, and fixed in 10% ethanol for 7 d and prepared for whole-mount staining with Alcian Blue and Alizarin Red S according to the protocol detailed in Lin et al. (2015). Whole-mount pυCT of F5 mouse was performed using SkyScan 1176 (Bruker, Kontich, Belgium) at 45 kV and 556 mA with a pixel size of 1.77 μm. For 0.1-μmol mass, μCT was performed on the left proximal tibia and distal femur metaphysis and diaphysis for trabecular and cortical bone analysis, respectively, using SkyScan 1174 (Bruker) at 50 kV and 300 mA with a pixel size of 6.1 μm. All images were reconstructed using the SkyScan NRecon program version 1.1 and analyzed using SkyScan CTAn software (Bruker).

**Statistical analysis and data presentation**

Results were statistically analyzed using a two-tailed t-test using Prism 5 (GraphPad Software, La Jolla, CA). All data shown are representative of at least three independent experiments and expressed as mean ± SEM.

**PDB accession codes**

Structural data are deposited in the PDB under accession number 4ZLJ. Raw diffraction data are available at the Diffraction Images Repository (http://sncdiffraction.ims.u-psud.fr).

**ACKNOWLEDGMENTS**

We thank T. J. Martin for the critical reading of the manuscript. All microscopy was carried out using facilities at the Centre for Microscopy, Characterisation, and Analysis, University of Western Australia. We also acknowledge the assistance with X-ray diffraction data collection of the resources and staff of the University of Queensland Remote Operation Crystalisation (UQ ROCK) and the Australian Synchrotron.

**REFERENCES**


APPENDIX

-D-

Publication

Clairfeuille et al., 2016
A molecular code for endosomal recycling of phosphorylated cargos by the SNX27–retromer complex

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Recycling of internalized receptors from endosomal compartments is essential for the receptors’ cell-surface homostasis. Sorting nexin 27 (SNX27) cooperates with the retromer complex in the recycling of proteins containing type I PDZ-DxG-Z01 (PDZ)-binding motifs. Here we define specific acidic amino acid sequences upstream of the PDZ-binding motif required for high-affinity engagement of the human SNX27 PDZ domain. However, a subset of SNX27 ligands, such as the β2 adrenergic receptor and N-methyl-D-aspartate (NMDA) receptor, lack these sequence determinants. Instead, we identified conserved sites of phosphorylation that substitute for acidic residues and dramatically enhance SNX27 interactions. This newly identified mechanism suggests a likely regulatory switch for PDZ interaction and protein transport by the SNX27–retromer complex. Defining this SNX27 binding code allowed us to classify more than 400 potential SNX27 ligands with broad functional implications in signal transduction, neuronal plasticity and metabolic transport.

Endocytic recycling of internalized transmembrane proteins is essential for their homostasis, but the mechanisms that regulate sequence-dependent recycling are still poorly understood. The retromer trafficking complex recycles cargos containing PDZ-domain binding motifs via the adaptor protein SNX27. PDZ-domain proteins are one of the most common protein-protein interaction scaffolds found in proteins controlling cell trafficking and signaling networks1–3, and they play a major role in the clustering of neuronal receptors within the post-synaptic density (PSD)4,5. The canonical mechanism of PDZ-domain interaction involves a conserved cavity that recognizes different classes of PDZ-binding motifs (PDZboxes) found at the C termini of ligand proteins. Type 1 motifs have the sequence [ST][A/V][X][Φ] (in which Φ represents any hydrophilic residue) and are often found within the cytoplasmic tails of transmembrane proteins6. Whereas the C-terminal tripeptidic is essential for binding, specificity is often enhanced by upstream sequences7,8.

Noncanonical PDZ-domain binding is less common and involves extensions to the core PDZ fold that promote interactions with specific protein partners9. SNX27 uses both canonical and noncanonical PDZ interfaces to simultaneously engage type 1 PDZ ligands and the endosomal trafficking hub–complex retromer (a heterotrimer of VPS35, VPS26 and VPS29) via the VPS26 subunit10 (Fig. 1a). SNX27 thus acts as a cargo adaptor for retromer-mediated transport from endosomes to the cell surface8,11,12. Cargos include G-protein-coupled receptors (GPCRs) such as the β2 adrenergic receptor (β2AR)13,14,15 and parathyroid hormone receptor (PTHR)15,16, and proteins involved in neuronal plasticity including the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)16,17, NMDA receptors (NMDARs)17,18, and 5-hydroxytryptamine 4 receptors (5-HT4R)19, among others20–22. SNX27 and retromer are expressed in multiple tissues but play particularly prominent roles in neurons. Altered expression and mutations are linked to Alzheimer’s disease, frontotemporal dementia, Down syndrome and infantile hyperglycemia23–25, and lowered expression of SNX27 or retromer reduces the surface levels of β2ARs, NMDARs and AMPARs and results in the correlated synaptic defects26,27,28. SNX27 and retromer also play a role in bone morphogenesis through trafficking of the PTHR1 in osteoblasts29,30. Generally, however, it remains poorly understood as to how SNX27–retromer efficiently recruits diverse endosomal cargos (and accessory proteins), which are often transported in response to activation by different cell stimuli.

Here we set out to define sequence requirements for PDZbox binding to the SNX27–retromer complex. We found that cargos fall into two distinct categories of high- and low-affinity binders, on the basis of the positions of acidic side chains upstream of the C-terminal PDZ box. High affinity–cargo receptors (acids reside located closer to the amino acid residues upstream of the C terminus that are able to clamp a conserved arginine on the SNX27 surface. Intriguingly, many SNX27 ligands, including the β2AR and NMDARs, have these acidic side chains but instead possess conserved sites of serine and threonine phosphorylation. Data both in vitro and in cells, together with the crystal structures of SNX27

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Received 25 March; accepted 11 August; published online 5 September 2016; doi:10.1038/nmb3290
bound to different phosphopeptides, show that phosphorylation of these residues mimics acidic side chains required for high-affinity binding and further suggest a novel layer of post-translational regulation in endosomal recycling. In silico analyses of the human genome provide a comprehensive inventory of probable SNX27–receptor interactions and suggest that there are hundreds of potential proteins recycled by SNX27–receptors, either constitutively or after phosphorylation.

RESULTS
Specific sequences in PDZ domains control association with SNX27
A common feature seen in previous structures of the PDZ domains of PTHR and Kir3.3 bound to SNX27 is the presence of what we refer to as an "electrostatic plug," in which two glutamate side chains at the -3 and -5 positions of the cargo peptide embrace the conserved SNX27 residue Arg38 (refs. 13, 14) (Fig. 1b). In addition, SNX27 possesses an extended surface of positive electrostatic potential adjacent to the -3 and -6 peptide side chains. We predicted that PDZ domain sequences containing the acidic pair of side chains at positions -3 and -5 would bind more strongly than those without, and that acidic side chains at position -6 might enhance the binding affinity through delocalized electrostatic association.

To define the relative contribution of each acidic residue in binding to SNX27, we used the PTHR PDZ2 as a model peptide21 and measured the affinities of a series of alanine mutations (Fig. 1c and Supplementary Table 1). The results showed that: (i) altering the Glu(-6) residue (PDZ2 A1) did not greatly reduce SNX27 binding, (ii) Glu(-5) substitution (PDZ2 A2), which was predicted to destabilize the electrostatic plug, led to a modest decrease in affinity by a factor of 4, (iii) the elimination of both Glu(-3) and Glu(-6) (PDZ2 A3) reduced the binding affinity even further. Hence it appears that whereas Glu(-3) is most important, a combined negative potential at both -5 and -6 can further enhance interaction. Because the PDZ binding pocket is surrounded by positively charged side chains near the N terminus of the bound peptide (Fig. 1b), and the acidic residue at -6 is not conserved in the PTHR complex (or in the D7GK complex, as shown below), we propose that a localized electrostatic complementarity between N-terminal PDZ2 acidic side chains and the basic surface of SNX27 is not strictly required but can enhance peptide interactions. We previously showed that a glutamate-to-alanine mutation at position -3 (PTHr A1 or A4) on a C-terminal alanine addition (PTHr A4) prevents association with SNX27 (ref. 11) (shown in Fig. 1c for comparison), thus indicating that Glu(-3) and its contacts with Arg38 and Asp56 are essential for complex formation, as in the register of the C-terminal PDZ2 (Fig. 1c).

We tested the importance of specific PDZ2 sequences in SNX27–receptor association in cells. We stimulated HJK35 cells stably expressing untagged PTHR with fluorescent emethyhydrocinnamic (TAMO)-labeled parathyroid hormone (PTH)1-34 analog (TAMOPTH) to induce activation and receptor internalization. Unbound TAMO-PTh-bound PTHR showed prominent association with SNX27 at endosomes after several minutes, as observed previously22 (Fig. 1d). However, when C-terminal sequences of PTHR were mutated at the -3 and -5 positions or the PDZ2 was removed altogether13, we found that PTHR was readily internalized after stimulation and localized to endocytic compartments; however, both mutants showed similar defects in colocalization with SNX27, as determined by Pearson's correlation coefficient (R2) (Fig. 1e).

Electrostatic interactions promote high-affinity binding to SNX27
We next tested the binding of three small ligands known to be associated with the SNX27–receptor pathways: (i) β1A3, (ii) diacylglycerol kinase ζ (DGKζ), a soluble SNX27-binding enzyme20, and (iii) the scaffolding protein kinase D-1 interacting substrate of 220 kDa (KIDINS220)3. The corresponding PDZ2 peptide sequences showed large differences in their ability to bind SNX27. PDZ β1AR association was severely detectable (Kd ~ 200 μM), whereas both KIDINS220 and DGKζ interacted strongly (Kd ~ 6.5 μM and 2 μM, respectively) (Fig. 2a,b and Supplementary Table 1). Mutation of the SNX27 peptide-binding pocket (H111A) blocked all interactions, thus confirming the specificity. The magnitude of the enthalpic energy release (ΔH) after DGKζ or KIDINS220 binding was many times greater that after β1AR binding, thus suggesting that electrostatic bonds make a larger contribution to the interactions. We observed similar results for the PDZ domain and leucine-rich repeat protein phosphatase 1 (PHLPP) and PH1P2 sequences that our bioinformatics studies predicted should bind (data shown) and that have also been observed in procomics3 (Fig. 2c and Supplementary Table 1). VHS2 allosterically improved binding to these PDZ2s, as shown previously for GLUT1, Kir3.3 (ref. 8) and PTHR1,2, and this effect was markedly pronounced for β1AR.

To confirm that upstream acidic residues promote high-affinity PDZ2 domain interaction with SNX27, we mutated the -3 and -6 residues in β1AR and DGKζ from ST to LL and from ED to SS, respectively (Fig. 2a). In DGKζ, this mutation caused a decrease in affinity (Kd from 2.0 to 5.4 μM). The reverse substitution of Glu(-5) and Glu(-6) in β1AR dramatically improved binding (Kd from 280 μM to 19 μM) (Fig. 2a). Overall, these results showed that acidic residues in the -3 and -6 positions are not strictly required but can enhance the affinity of PDZ2 domain interaction with SNX27.

Structure of the SNX27 PDZ domain bound to DGKζ
To investigate PDZ2 domain recognition by SNX27 in greater depth, we determined the crystal structure of the SNX27 PDZ domain bound to the PDZ domain from DGKζ at 1.1 Å resolution (Fig. 3 and Table 1). The first Arg(-7) residue in the DGKζ peptide was not visible in the electron density, and the Glu(-6) side chain had relatively poor density and does not interact directly with the PDZ domain, thus strengthening the idea that residues upstream of the -5 position are not strictly associated with SNX27 (Fig. 3a). The three DGKζ C-terminal residues engage SNX27 in the canonical orientation where the terminal carbonyl group of Val98 forms critical hydrogen bonds with the backbone of SNX27 Tyr53 and Gly54 while its side chain is embraced by a hydrophobic cavity underneath Thr(-2) and SNX27 His114 forms a hydrogen bond (Fig. 3c) that is essential for complex formation.

Determining SNX27–PDZ2 structures at atomic resolution allowed us to compare the detailed molecular aspects of PTHR(13), Kir3.3 (ref. 14) and DGKζ binding (Fig. 3b). All peptides have a Glu(-3) residue that is unshifted between SNX27 Arg58 and Asp56 and also forms a hydrogen bond with Ser82. These strong contacts explain why Glu(-3) is the major determinant of selectivity. For other prominent feature of this interaction is how Arg58 establishes contacts with both Glu(-3) and Glu(-5) or Asp(-5) of these peptides, thus forming an electrostatic plug. The side chains shared among these three residues lock the peptides in place upstream of the PDZ cavity. Altogether, these combined structures show that the Glu(-3) residue is essential for binding, whereas the electrostatic plug involving an acidic side chain at the PDZ2–receptor position provides a mechanism for enhanced selectivity.

To confirm the importance of the electrostatic plug in DGKζ, we tested the ability of DGKζ mutants to competitively precipitate SNX27 in [arbaclo Tetr. Whereas GFP-DGKζ precipitated SNX27, constructs...
Figure 1: High-affinity binding of PTHr to SNX27 rebramer requires upstream acidic residues. (a) Cartoon illustration of the endosomal recycling pathway mediated by the SNX27-rebramer complex. Endocytosed cargo organelles (often stimulated by ligand binding and cationization) associate with SNX27 within the endosome, are sorted into tubulovesicular membrane domains through allosteric coupling with the rebramer complex and return to the cell surface, avoiding degradation within the late endosome or lysosome compartment. (b) Structure of the SNX27 PZ domain bound to PTHr peptides from the Kx6.3 potassium channel (green), PTHr1-340 (magenta) and PTHr1-340 (magenta), showing two glutamate residues in the +3 and +5 positions interacting with a basic patch, thus potentially stabilizing the complex. (c) ITC experiments measuring binding of the PTHr PZ2b mutants to the SNX27 PZ domain. Data from ref. 13 are shown for comparison and are marked with an asterisk. (d) Top: Fluorescence microscopy showing colocalization of SNX27 (green) with internalized PTHrP-PTHrR ligand-receptor complexes after 5 min against stimulation with 100 nM PTHrP (magenta). Scale bar, 10 μm. Bottom: confocal line-scan analysis of SNX27 and PTHrP intensity profiles, as shown in the merged inset. Scale bar, 1 μm; a.u., arbitrary units. (e) Fluorescent microscopy of PTHr constructs containing C-terminal PZ2base (as indicated in c), showing differential endosomal recruitment with SNX27 after 15 min against stimulation and endocytosis. Scale bars, 1 μm. Right: colocalization analysis (Pearson's coefficient, R) of SNX27 with internalized PTHrP. Values are mean R ± s.e.m. (n = 10 cells, 200 endosomes for each receptor. **P < 0.01, ***P < 0.001 by one-tailed Student's t test).
modified in their PDZ1 triple did not (ARTAV, T927A and T927I) (Fig. 3d). Mutation of the −3 side chain (E926A or D926A) abolished the interaction with SNX27, whereas alteration of the −5 side chain (D824A) decreased but did not abolish binding, thus indicating that the −3 acidic side chain, compared with the essential −3 residue, plays an important but auxiliary role. This finding also correlated with a mild reduction in binding affinity determined by isothermal titration calorimetry (ITC) when the −5 and −3 residues were altered (E926S/D924S; ED SS) (Supplementary Table 1).

PDZ2bm phosphorylation promotes interaction with SNX27

Trafficking is frequently regulated by post-translational modifications including ubiquitination and phosphorylation. Whereas SNX27 regulates PDZ-motif-dependent endosomal recycling of GPCRs such as β2AR8 and 5-HT1AR7, as well as AMPARs and NMDARs10–14, notably, none of these important cargo proteins have acidic residues required for forming the stable electrostatic plug with Arg166. However, these proteins do possess sites of potential phosphorylation (Fig. 4a). We hypothesized that introduction of a phospho group at the critical −3, −5 or −6 positions might substitute for essential acidic residues and consequently promote binding to the SNX27 PDZ domain.

Phosphorylation of β2AR resulted in two antagonist effects depending on the location: phosphorylation of Ser−6 or Thr−5 increased binding affinity (from Kd > 200 μM to 94 and 104 μM respectively), whereas phosphorylation of Ser−2 abolished interaction, presumably by causing a steric clash with the SNX27 His114 side chain (Figs. 4c and 4d). This result is in line with our results showing that phosphotyrosine mutation of the −2 position of DGR5 (T927D) abrogates SNX27 PDZ binding (Fig. 3d). Additional re-arrangement induced improvement binding to β2AR-pS−6 (in which pS denotes phosphoserine) from 94 μM to 17 μM, thus again demonstrating that allostery can make β2AR a higher-affinity ligand (Fig. 4b and Supplementary Table 1).

To analyze the effect of phosphorylation on SNX27 association, we performed NMR titrations of [15N]−labeled SNX27 PDZ domain with β2AR, β2AR-pS−2 and β2AR-pS−6 peptides (Fig. 4c and Supplementary Fig. 1). On the basis of backbone resonance assignments performed previously15, we mapped residues that showed the largest changes in chemical shifts onto the SNX27 PDZ surface. Both native β2AR and β2AR-pS−2 peptides associated only weakly with the SNX27 PDZ binding pocket, and only at the site where the C-terminal carboxyl group is constrained. In contrast, β2AR-pS−6 titration strongly affected residues located around the entire PDZbm interacting surface (Fig. 4c). This result supports the idea that negatively charged residues and phosphorylation of upstream sequences promote the coordinated binding of peptides in the extended binding groove. Interestingly, residues in the β-hairpin of SNX27 that bind to VPS26 (ref. 6) also appeared to be affected by β2AR-pS−6 association, thus suggesting a link between the VPS26 and peptide-binding sites that may relate to the allosteric coupling. Altogether, these data suggest phosphorylation of Ser−6, Ser−5 or Ser−2 provide regulatory switches that trigger or prevent SNX27−receptor trafficking of the β2AR.

Phosphorylation regulates SNX27 binding to glutamate receptors NMDARs and AMPARs in neurons control glutamatergic synaptic transmission16, and neuronal trafficking is strongly influenced by phosphorylation of their intracellular domains10,11,13. SNX27−receptor colocalizes with glutamate receptors in neuronal postsynaptic densities10,11,12,13,17,18 and forms complexes with these receptors, as determined by communoprecipitation. We also observed proximal localization with GluN2B in dendrites (by using proximity ligation assays (PLA) (Supplementary Fig. 2). Because, similarly to β2AR, glutamate receptors are actively phosphorylated at their C termini in vivo (Fig. 4a), we sought to test whether phosphorylation might provide a regulatory mechanism controlling SNX27 interactions.
The PDZ2bm from the NMDAR subunit GluN1 interacted only weakly with SNX27 in its native form, but double phosphorylation on Ser(−5) and Ser(−5) greatly improved binding (Fig. 5b and Supplementary Table 1), thus suggesting phosphorylation of GluN1 at these sites mimics the electrostatic plug for SNX27 Arg588. Similarly, phosphorylation of GluN2B at Ser(−5) and Ser(−6) also conferred a much higher affinity, thus confirming that phosphorylation mimics the contribution of aspartate or glutamate residues found in constitutively high-affinity ligands (Fig. 5b and Supplementary Table 1).

Retromer association enhances binding to both peptides. To confirm the binding of NMDARs and SNX27 in cells, we performed pull-downs of Myc-SNX27 with glutathione S-transferase (GST)-tagged GluN1 or GluN2B C-terminus tail proteins with phosphorylation-defective (ΔA) or phosphomimetic (ΔD) mutations. Compared with the wild-type GluN2B, the phosphomimetic mutation at (−5) or (−6) improved binding. The effect was even more pronounced for GluN1, whose binding was difficult to detect for both the wild-type peptide and the phosphodefective mutant but was dramatically enhanced by the phosphomimetic mutations at (−5) and (−6) sites (Fig. 5c). This result supports the idea that certain HSV1 sequences, such as GluN1, that lack an acidic residue at −3 are unable to interact with SNX27 unless they are phosphorylated, whereas cargos, such as GluN2B, that possess this acidic residue can be greatly enhanced by phosphorylation. The identities of the −3, −5, and −6 residues in these cargos are strictly conserved, thus implying that they are functionally important (Fig. 5d).

Previous studies have suggested a role for SNX27-retromer in transport of the AMPAR11,13. However, we did not detect an affinity for AMPAR peptides in native or phosphorylated states (Supplementary Fig. 3). GluA1 possesses a type 1 PDZ motif (ISSL) but lacks the acidic side chain at the −3 site and does not bind strongly in the SNX27 PDZ2 domain, even in the presence of VPS26. GluA2 has a type II motif (IQXL) and also does not associate prominently with SNX27, even after phosphorylation of Ser(−5) or Tyr(−7). On the basis of structural considerations, it is clear that although GluA1 may be physically able to associate, albeit weakly because of the lack of the acidic −2 site chain, the binding of GluA2 is precluded because it lacks the key Ser(−2) or Thr(−2) side chain required for interacting with SNX27 His14. In the cortex of all postsynaptic receptor trafficking, we propose that NMDARs are vastly more efficient cargos than AMPARs for SNX27-mediated recycling.

Mechanism of SNX27 binding to phosphorylated PDZ2bms

On the basis of our results with NMDARs and NMDARs, we scanned known SNX27 binders for other sequences potentially enhanced by phosphorylation at the −2 or −5 or −6 positions and tested two of these: the sorting nexin SNX14 (ref. 9) and the GPCR 5-HT2AR (Fig. 6a and Supplementary Table 1). A phosphomimetic SNX14 sequence showed strong binding to SNX27, as expected (Kd = 8 μM), whereas a phosphorylated version of the 5-HT2AR bound to SNX27 with an affinity nearly four-fold greater than the native sequence and was enhanced to an Kd of 1.2 μM by the allosteric effect of VS26 binding. This result further supports the role of PDZ2bm phosphorylation in conferring high affinity interaction with SNX27 by mimicking key acidic residues. To confirm the mechanism of phosphorylation-enhanced binding, we determined the high-resolution crystal structure of the complex between the SNX27 PDZ domain and phosphorylated 5-HT2AR PDZ2bm (Fig. 6b and Table 1). The crystal structure shows that the phosphomimetic Ser(−5) cooperates with the acidic Glu(−3) side chain in enabling the clamping of SNX27 Arg588, as is done by glutamates and asparaginates of other PDZ2bms (Fig. 6b).

We next confirmed the structural basis for enhanced SNX27 affinity via phosphorylation of the critical PDZ2bm −3 side chain. We solved the crystal structures of two different peptide complexes:
Table 1 Data collection and refinement statistics

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<td>0.965 (0.858)</td>
<td>0.98 (0.769)</td>
<td>0.796 (0.690)</td>
<td>0.989 (0.826)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>96.8 (96.6)</td>
<td>100 (100)</td>
<td>99.7 (100)</td>
<td>96.7 (96.4)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>13.2 (12.9)</td>
<td>14.2 (14.2)</td>
<td>13.9 (15.9)</td>
<td>7.1 (6.2)</td>
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<tr>
<td>Refined</td>
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<tr>
<td>Resolution (Å)</td>
<td>14.94-1.10 (1.17-1.10)</td>
<td>37.26-1.60 (1.72-1.62)</td>
<td>10.2-0.85 (0.87-0.85)</td>
<td>36.7-1.32 (1.35-1.32)</td>
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<td>Rmerge</td>
<td>0.062 (0.78)</td>
<td>0.170 (0.242)</td>
<td>0.230 (0.363)</td>
<td>0.163 (0.179)</td>
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<tr>
<td>Rmerge (all i, j)</td>
<td>0.178 (0.242)</td>
<td>0.197 (0.242)</td>
<td>0.312 (0.418)</td>
<td>0.163 (0.178)</td>
</tr>
<tr>
<td>No. atoms</td>
<td>Protein</td>
<td>80,457 (2,650,2,590,154)</td>
<td>14,225 / 715 (2,940,158)</td>
<td>86,890 / 2,000,440,149</td>
</tr>
<tr>
<td></td>
<td></td>
<td>86,890 / 2,000,440,149</td>
<td>23,811 / 2,000,142,250</td>
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</tr>
<tr>
<td>Average δ factor (°)</td>
<td>17.7</td>
<td>27.1</td>
<td>10.9</td>
<td>19.2</td>
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<tr>
<td>R.m.s. deviations</td>
<td>Bond lengths (Å)</td>
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<td>0.011</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>Bond angles (°)</td>
<td>1.217</td>
<td>1.336</td>
<td>2.06</td>
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</table>

*Values in parentheses are for high-resolution shell.

Bioinformatic discovery of SNX27-interacting proteins

Combining our data with analysis of previously identified SNX27-interacting proteins (Supplementary Table 2), we defined three major classes of PDZ2 domains containing ligands, in which the identities of the last seven amino acids of the protein influence binding affinity. Acidic residues or phosphorylated side chains at the −5 position (and to a lesser extent −6) positively enhance SNX27 affinity (Supplementary Fig. 5 and Supplementary Table 1). Modulating these three positions yields predictable classes of SNX27 binders (Fig. 7a). Class 1a peptides are strong interactions that clamp Arg88 to SNX27 and are likely to constitutively associate under physiological conditions. Class 2a binders possess motifs for which binding is enhanced by phosphorylation at the −5 position, whereas class 2b and class 2c peptides contain potential sites of phosphorylation at the −3 position that is critical for SNX27 binding. Our data also suggest that additional acidic or phosphorylated residues upstream at the −6 position positively influence association through local electrostatic interactions with SNX27 classes 1a, 2b, and 2c. Class 3e binders are ligands that bind relatively weakly to SNX27. Finally, class 3b binders are those that have threonine or serine at the −3 position and are unlikely to bind to SNX27 without phosphorylation.

These newly defined consensus motifs allowed us to search the human genome and identify 415 putative ligand and cargo molecules...
that possess the molecular signatures required to bind the SNX27-retromer complex (Supplementary Fig. 5 and Supplementary Table 3). A number of these have previously been identified, but our bioinformatics results suggest that most of the SNX27 PDZ interactions remains to be explored. In agreement with our predictions, the greatest proportion of previously identified ligands fell within the classes of proteins that we predicted to have the highest affinity for the SNX27-retromer complex. To begin to test these predictions, we analyzed the levels of several receptors in HeLa cells with SNX27 deleted by CRISPR-Cas9. In agreement with defective recycling and lysosomal turnover, the levels of AMPA and PDGFRβ were decreased in SNX27-knockout cells, as was the sorting motif SNX14 (Fig. 7B). SNX27 is a member of a distinct sorting nexus subfamily localized at endosomes at steady state[28,47], but whether it undergoes plasma-membrane trafficking similarly to other transmembrane cargos or is simply stabilized at endosomal compartments by interaction with SNX27 is unclear. Interestingly, the solute carrier EAAT1 (or SLC1A3) does not appear to be degraded in SNX27-knockout cells, although a decrease has previously been observed in proteomics studies involving SNX27 knockdown.
Figure 5. Phosphorylation of the GluM and GluR2B subunits of NMDA receptors triggers interaction with SNX27. (a) Fluorescence microscopy showing hippocampal neurons transfected with GFP-GluR2B (green) and Myc-SNX27 (magenta). Arrowheads indicate co-localization of these two proteins in dendritic spines (top). Scale bars, 50 μm (top) and 5 μm (bottom). (b) ITC experiments comparing the binding profiles of both normal and phosphorylated NMDA receptor peptides to free or VPS26-engaged SNX27 PDZ domain. Peptide sequences used in each experiment are indicated on the right. (c) GST-pulldown experiments from HEK-293 cells transfected with Myc-SNX27 and GST-GluM or GST-GluR2B C-terminal tails, including wild-type, PDZ-mutant, phospho-mutant (AA) or phospho-kinetic (DD) mutants. Unlabeled images are shown in Supplementary Figure 7. (d) amino acid conservation of the PTHIR, GluM, and GluR2B PDZ domains. PDZ domains were aligned with the closest 100 homologs identified by BLAST searching. The alignment was made with CLC sequence viewer.

DISCUSSION
We investigated the molecular aspects of cargo and accessory-protein binding to the endosomal trafficking complex SNX27–retromer. The combined action of Arg36, Asp46 and Ser56 in SNX27 provides a key binding site for a negatively charged acidic side chain at the −3 site of ligand PDZ domains that is essential for interaction. This result correlates with proteomics data suggesting that the majority of identified SNX27 interactors that possess a PDZ motif have an acidic residue at the −3 position. In addition, Arg36 can accommodate binding to adjacent acidic side chains in the peptide −5 position, in an arrangement that we refer to as the electrostatic plug. To a lesser extent, acidic side chains in the −6 position also promote SNX27 interaction through general electrostatic interactions. Previous work by Ishida and colleagues on G-proteins inwardly rectifying potassium channels supports these findings. Our data show that retromer allosterically enhances binding to SNX27 for all cargo and ligand PDZ domains, and association in the context of the membrane bilayer. In addition to lipid interactions of the SNX27 PX and FYM domains, coordinates the physiological...
association between these binding partners in the endosome. Notably, many proteins identified in SNX27 protrinsics do not possess PDZ motifs; some possess NPYX sequences15, whereas others may be recruited through as-yet-undetermined mechanisms.

The most striking observation is that the binding of many PDZ2bm to SNX27 is enhanced by phosphorylation. Our data show that phosphorylated groups add negative charges that, when placed in −3, −5 and −6 positions, promote SNX27–retromer association. In contrast, phosphorylation of the −2 residue inhibits binding because the phosphate group is sterically precluded. Phosphorylation of the −2 residue in type 1 PHD of several proteins, including NM23A and β2AR44–45, has previously been found to negatively regulate binding to other PDZ domains. Only one previous structure of a PDZ domain bound to a phosphorylated peptide has been determined, describing the complex between the PDZ domain of L1CAM and a type 2 PDZ2bm from syndecan-1 tyrosine-phosphorylated at the −1 position30. Binding studies have also been performed with the ZO-1 PDZ domain binding type 2 PDZ2bm from claudin-3 tyrosine-phosphorylated at the −6 residue35. In the former case, phosphorylation alters the tyrosine-binding conformation but has no effect on binding affinity, whereas in the latter case, phosphorylation inhibits the interaction. Although inhibitory effects of phosphorylation are well established, a positive role in PDZ interactions has not previously been observed, to our knowledge. The dramatic enhancement of PDZ2bm binding to SNX27 suggests an important and previously unidentified role of post-translational modification in promoting PDZ–domain interactions.

Structural studies demonstrated how phosphorylation mimics the electrostatic plug centered on Arg98, thereby potentiating SNX27 binding. The consistent enhancement of binding by phosphorylation of highly conserved sequences indicates that it almost certainly represents a functional signal for regulating cargo transport along the SNX27–retromer pathway. The PhosphositePlus Database (http://phosphosite.org) confirms that many of the predicted ligands are phosphorylated at the required sites, although the signals leading to these modifications remain unknown (for example, Fig. 6a). Many transmembrane cargos are modified at their C termini by kinases and phosphatases46, and an important question will be to determine which enzymes are involved in regulating SNX27–retromer interaction and what effects these modifications have on endosomal transport. For example, GRR2 phosphorylates β1AR at both Ser(−2) and Ser(−6) after agonist stimulation47, although it is unknown whether this particular kinase modulates endocytic recycling.

Our results define three broad classes of SNX27 PDZ2bm ligands: strongly or weakly associated cargos with or without acidic side chains at the −3, −5 and −6 positions, respectively, and other cargos whose association is controlled by phosphorylation. We speculate that this electrostatic code defines a specificity for SNX27–retromer association that determines the efficiency and timing of endosomal recycling, in which some cargos are recycled constitutively, and others are recycled efficiently under only certain conditions of cellular stimulation that cause their phosphorylation. A pathway analysis of the molecules that we identified in the SNX27 interac...
Figure 7. Classification of the SNX27 putative PDZ interactions, on the basis of C-terminal sequences. (a) Schematic representation of the SNX27 PDZ binding pocket (brown) and the molecular contacts (orange shaded lines) established with strong PDZ ligands (black). The presence of acidic residues or phosphate head groups (green) in positions –3, –5 or –6 introduces a negative charge and binding to the SNX27 PDZ domain. For all PDZ ligands, phosphorylation of the Ser(–2) or Thr(–2) residue disrupts binding to the PDZ domain. The three PDZ Dom classes were subclassified and defined on the basis of continuous sequences emerging from a Rfindings (bottom). a represents sequence, b sequence, c sequence, d sequence or e sequence. (b) Immunoblot analysis showing steady-state levels of putative SNX27 cargo after SNX27 knockdown (KD) by CRISPR-Cas9 editing. Whole cell lysates from control HeLa cells (stably expressing Cas9) and SNX27-knockout cells were probed for different PDZ domain-containing cargo by western blotting. Nait-KATPase is a control plasma-membrane protein; beta tubulin is a loading control. (c) Schematic representation of the different functions of PDZ domains trafficked by SNX27. The 432 putative interacting proteins curated from our human proteome screen were analyzed by Gene Ontology (GO) process. The top 250 enriched pathways (on the basis of false discovery rate) were manually classified into functional groups and are depicted as spheres. The proteins composing the network are also annotated according to their molecular functions (bottom right histogram) (additional data in Supplementary Table 4).

Supports a prominent role of SNX27–retromer in trafficking cargos involved in synaptic function and cell signaling, as suggested by previous studies (Fig. 7c and Supplementary Tables 3 and 4). Surprisingly, however, the largest subset of putative ligands includes members of the metabolite transporters or solute carrier family, which comprises transmembrane proteins whose trafficking is very poorly understood despite their major importance in human physiology. The biological functions of many putative SNX27-binding proteins are unknown, thus suggesting that new discoveries regarding SNX27’s role in endosomal transport and signaling are yet to come. It is also notable that some pathogens interact with SNX27 through similar mechanisms and are likely to compete with and perturb normal transport processes.

In summary, our study describes an adaptable molecular code controlling SNX27–retromer recruitment of many different cargos including GPCRs, ion channels, solute carriers, and soluble signaling and regulatory proteins. In a broader context, our data provide new mechanistic insights into how PDZ domains can fine-tune the dynamics of protein–protein interactions network.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Accession codes. Coordinates and structure factors of the SNX27 PDZ domain in complex with different PDZ domain peptides have been deposited in the Protein Data Bank under accession codes PDB 5Z9L (with DGKp peptide), PDB 5Z9F (with phosphorylated S-1H4a3J peptide), PDB 5Z9A (with LRR3CB (peptide) and PDB 5Z9B (peptide).
ARTICLES


ONLINE METHODS

Peptides. All synthetic peptides used for ITC and X-ray crystallography were from GenScript. Peptides were dissolved in water and diluted to 50 mM Tris pH 7.4, 100 mM NaCl, 20 mM Mg-Br, pH 5.4, buffer for use in acetylation experiments. A streptavidin-conjugated horseradish peroxidase (D710TMB), in which 25% ITC was used in 50 mM Tris, 20 mM Mg-Br, 500 mM NaCl, 1 M LEDA (123799), and Supplementary Table 3. The functional classification of proteins shown in Figure 7c was defined on the basis of analysis with the Goteborg database from MitoCarta. The 452 genes obtained by searching the human proteome were provisioned for the ITC experiments, in 30 mM NaCl, 20 mM Mg-Br, 500 mM NaCl, 500 mM NaCl, 1 M LEDA (123799), and Supplementary Table 3. The functional classification of proteins shown in Figure 7c was defined on the basis of analysis with the Goteborg database from MitoCarta. 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cells were transfected with three gRNA constructs targeting SNX27 from Santa Cruz Biotechnology (sc-421504). Transfected cells were isolated on the basis of an encoded GFP reporter with single-cell FACS, and the resultant transfectant cell lines were confirmed to express SNX27 by western blot. 

Analysis of protein levels for putative SNX27 cargos in SNX27-knockout HeLa cells. 

HeLa Carr and SNX27−/− cell lines were cultured in high-glucose Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% foetal bovine serum (FBS), 5 μg/ml penicillin and streptomycin and 2 mM l-glutamine and maintained in 3% CO₂ at 37°C. For protein extraction, cells were washed with cold PBS and homogenized in buffer containing 20 mM HEPES, pH 7.4, 250 mM sucrose, 1 mM EDTA, 10 mM MgCl₂, 30 mM NaCl, 2 mM Na₃VO₄, 0.5 mM ATR and protease-inhibitor cocktail with 22 U/ml ganciclovir. lysates were centrifuged at 390×g for 5 min to remove cell debris and nuclei. The resulting supernatants were subjected to hydroxyapatite (HAP) assays (Thermo Scientific) to determine protein concentration. Equivalent amounts of protein per sample were re-suspended in SDS-PAGE according to procedures performed in previous studies. Proteins were transferred onto Immobilon-HDFV membranes (Millipore), and the membranes were blocked with 5% BSA or 5% skim milk in PBS containing 0.1% Tween-20 for 1 h at room temperature. Membranes were then incubated with primary antibodies against VHR (1:1000; A57671, Alomone), SNX27 (1:1000; ABC7789, Alomone), ATP-α (1:1000; 3A2.3G1, Alomone), SNX14 (1:1000; 1B24759S, Sigma-Aldrich) and B-actin (1:1000; T6074, Sigma-Aldrich) overnight at 4°C. Membranes were washed with PBS containing 0.1% Tween-20 before being incubated with HRP conjugated anti-mouse secondary antibody (1:10000; 620-65-2, LiCor, Lincoln, USA). All antibodies are validated on membranes (data not shown). Signals in blotting experiments or fluorescence signals were detected with a Super Signal FCL detection kit (Thermo Scientific) or Odyssey Infrared Imaging System (LI-COR).

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