Protein homeostasis and sarcopenia: the influence of fasting and exercise on protein synthesis and degradation pathways

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ABSTRACT

Loss of skeletal muscle mass, strength and function that results from increasing age is known as sarcopenia. Sarcopenia results in frailty; the related falls and injuries necessitate long hospitalisation and recuperation times, which dramatically increases the health system cost burden as the relative distribution of our population ages. Thus there is a compelling need to understand the molecular mechanisms contributing to sarcopenia and develop effective therapeutic interventions to combat the effects. One of the many factors proposed to contribute to sarcopenia is disturbed protein turnover, which affects overall muscle homeostasis. The process of muscle homeostasis is dependent on a fine balance between protein synthesis and degradation (protein turnover), where atrophy results when the rate of protein breakdown exceeds that of synthesis. Disturbed protein homeostasis is implicated in sarcopenia.

The first part of this thesis investigated the main signalling pathway known to control protein turnover in skeletal muscle (the IGF-1/insulin signalling pathway). Specifically, this thesis quantified proteins and phosphoproteins involved in both protein synthesis, AKT(Ser473), S6K1(Thr389), rpS6(Ser235/236 and Ser240/244) and 4E-BP1(Thr37/46 and Ser65)) and protein degradation pathways, including proteasomal (MuRF1 and Atrogin-1) and autophagosomal degradation (ULK1(Ser757), LC3 and p62/SQSTM1) in young adult (aged 4 months(m)), middle-aged (15-18 m) and old (22 - 24 m) male C57BL/6J mice. Cohorts of young and old mice (aged 4 and 24 m) were also subjected to either 16 or 24 hours (h) of fasting to compare the impact of age on the response of these signalling pathways to food deprivation. One main finding of this study was the hyper-phosphorylation of S6K1 and rpS6 present in normal fed muscles, which coincided with the onset of sarcopenia (observed at 18 m of age). Another was that delayed dephosphorylation of AKT and mTORC1 signalling occurs following 16 h (but not 24 h) of fasting in sarcopenic muscle. Interestingly, this delay occurred concomitantly with a striking increase in proteins associated with an insoluble protein fraction (such as p62). These results highlight that perturbed protein turnover in ageing muscle can lead to inefficient protein homeostasis, which may further contribute to the progression of sarcopenia.

Secondly, this thesis investigated the role of IGF-1 as a therapeutic intervention for sarcopenia, via muscle-specific overexpression of the IGF-1 C2:Ea isoform in transgenic mice with and without voluntary wheel exercise. Life-long voluntary wheel running (unloaded wheel exercise) from 4 m of age prevented sarcopenia in select hind-limb muscles (including quadriceps) of male IGF-1 null littermates (FVB background) and proved to be of greater benefit compared with the muscle-specific
overexpression of IGF-1 C2:Ea.

The final chapters assessed the efficacy of two voluntary wheel running protocols (with applied resistance) to prevent or reduce sarcopenia in mouse models of ageing (using the C57BL/6J mouse strain) both in late life, and from mid-life. The short-term (10 week) resistance wheel running protocol initiated at approximately 25 m of age in male C57BL/6J mice increased soleus muscle mass, however failed to elicit muscle adaptations in larger hindlimb muscles, including the quadriceps or gastrocnemius. By contrast, resistance wheel exercise initiated prior to sarcopenia onset (at 15 m) in both male and female C57BL/6J mice prevented age-related loss of muscle mass (by 23 m of age) in a number of major hindlimb muscles (quadriceps, gastrocnemius, EDL and soleus). Resistance wheel exercise initiated from 15 m also improved a number of physiological parameters including myofibrillar mitochondrial content (and associated oxidative capacity) and select markers of autophagy. Importantly the maintenance of running activity, and thus muscle function, was maintained into old age.

These novel insights into the phenotypic and molecular basis for sarcopenia in addition to the benefits of exercise interventions on the ageing muscles are of considerable interest to the field of sarcopenia.
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EXPLANATORY NOTE

The regulations of the University of Western Australia allow for candidates for the Doctor of Philosophy degree (PhD) to present their work as a series of papers rather than as a conventional full text thesis. These may be papers that have already been published, papers that have been accepted for publication, papers that have been submitted for publication but not yet accepted, papers that are ready to be submitted, or any combination of these. However these publications must be tied together by a common theme and be integrated through a general introduction and discussion.

The Results for this thesis are comprised of 5 published papers and 2 manuscripts submitted for publication. These Results are brought together by a thesis Abstract, and an Introduction, followed by the Overall Aims and Hypotheses. Methodology is described in General Materials and Methods and also within the methods section specific to each publication/manuscript for each Results chapter. Finally, a General Discussion integrates the body of knowledge derived from this thesis and establishes the significance of this work.

Each Results chapter consists of a short overview and for the 5 published manuscripts the re-prints are incorporated into the Chapters with the original published page numbering, figures, tables, supplementary material and referencing system (and required formatting and spelling). The remaining submitted manuscripts are formatted specifically for the intended journal; each manuscript has an original referencing system with references listed at the end of each manuscript. All other parts of the thesis (Introduction, General Materials and Methods, Aims and Hypothesis, introductory pages for each manuscript and General Discussion) have a common referencing system and are listed at the end of the thesis.

NOTE: Prior to the start of 2016 my surname was “Soffe”. In early 2016 this was changed to “White” and thus authorship of papers involves this change of name.
LIST OF PUBLICATIONS

Publications are listed in chronological order, and are referred to in the Results Chapters by their publication number. My contribution to each is detailed in the specific Chapters.


DECLARATION

I hereby declare that the work contained within this Thesis is my own, except where the contributions of others have been acknowledged. Published research and work in preparation for publication, which has been co-authored is clearly labelled, and my individual contribution to the co-authored work is clearly stated.

__________________________
Zoe Rene White (previously Zoe Rene Soffe)

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CHAPTER ONE: GENERAL INTRODUCTION

Overview. This chapter consists of a comprehensive background and review of the literature pertinent to the present study.

1.1 Skeletal muscle

1.1.1 Structure and function

Skeletal muscle constitutes approximately 40% of human body mass and is a specialized, contractile tissue essential for movement, breathing, thermoregulation and the regulation of metabolism (Shavlakadze & Grounds 2006). Skeletal muscle tissue is comprised of striated, multinucleated cells called muscle fibres (or myofibres). There are three levels of connective tissue associated with myofibre organization, the endomysium, perimysium and epimysium (Gillies & Lieber 2011). At the macroscopic level, myofibres are arranged in parallel and individually encased in interstitial connective tissue referred to as the endomysium (Figure 1.1). The endomysium comprises a complex network of extracellular matrix proteins that house multiple structures, including blood and lymphatic vessels, nerve bundles and inflammatory cells (Grounds et al. 2005; Gillies & Lieber 2011). The many extracellular matrix molecules are essential for normal muscle function and also play major roles in muscle formation and tissue regeneration (Grounds 2008). Individual myofibres are further bundled together and covered in perimysium (also known as fascicles), before being assembled into whole muscle and encased in a thick layer of connective tissue known as the epimysium (Marieb & Hoehn 2016). Skeletal muscle is connected to the skeleton via connective tissue and tendons.

The cytoplasm and plasma membrane of myofibres are defined as the sarcoplasm and sarcolemma, respectively. A basement membrane, which encloses each myofibre and lies between the sarcolemma and endomysium, is comprised of interwoven collagen fibres and a network of specialised extracellular matrix proteins rich in laminins (Grounds et al. 2005). At the surface of the myofibre are many elongated myonuclei positioned immediately beneath the sarcolemma. Muscle satellite cells (undifferentiated mononuclear myogenic stem cells) are also localised at the periphery of the myofibre, residing outside the sarcolemma but beneath the basement membrane and act to facilitate growth and regeneration of the adult muscle fibre [reviewed in (Grounds et al. 2005)].
Chapter One: General Introduction

**Figure 1.1. Structure and architecture of skeletal muscle.** Skeletal muscle consists of myofibres arranged in bundles. The sarcoplasm of the myofibre is filled with striated myofibrils. A segment of the myofibril located between the two Z lines represents a sarcomere, and is the structural unit of the myofibre. Each sarcomere contains parallel arrays of contractile filaments, whose interdigitation produces a shortening of the muscle and generation of muscle force. Extracted from (Marieb & Hoehn 2016).

The major structural components of the myofibre are myofibrils, which are packed with highly organized contractile myofilaments (**Figure 1.1**). Each myofibre contains thousands of myofibrils arranged tightly in parallel. Under the light microscope, myofibrils are striated in appearance due to the alternating order of myosin and actin myofilaments and their varying refractive indices. The darker A bands are comprised of overlapping myosin and actin filaments, which together are of greater density than the actin filaments that form the lighter I bands (Barrett et al. 2010) (**Figure 1.1**). At the midsection of each A band is a lighter region known as the H zone, which marks the point at which myosin filaments no longer overlap with actin. The M line (M for middle, and comprised of the protein myomesin) further bisects the H zone and acts as anchorage for the thick myosin filaments (Barrett et al. 2010; Marieb & Hoehn 2016).

Each light I band is bisected by a Z disc and each segment of the myofibril located between two Z discs represents a sarcomere; these are the functional units of the myofibre responsible for contraction (Saladin 2008) (**Figure 1.1**). At the Z disc, many structural proteins help to further stabilise the contractile apparatus, and these include: actinin, to anchor actin filaments to the Z disc (not shown); the highly elastic titin, which provides scaffolding for the sarcomere by connecting each Z disc with the M line through its interaction with myosin; and desmin (not shown), which adds structure to the Z discs (Barrett et al. 2010; Hall & Guyton 2015; Marieb & Hoehn 2016).
Skeletal muscle is under voluntary nervous control (as part of the somatic nervous system), and the nerves that stimulate contraction are known as alpha motor neurons (Saladin 2008). The axons of these motor neurons extend from cell bodies located in the spinal cord, to the muscle periphery, where branching axon terminals attach to the surface of myofibres at the neuromuscular junction (NMJ) (Saladin 2008).

1.1.2 Muscle contraction

The arrangement and composition of myosin and actin myofilaments are essential for muscle contraction (Figure 1.2). Muscle myosin is comprised of both heavy and light chains, the latter of which has two globular heads that protrude from a backbone of interwoven polypeptide tails (Cooper 2000; Barrett et al. 2010; Marieb & Hoehn 2016). These heads are important for muscle contraction, as they contain both actin binding sites, as well as catalytic sites for ATP (adenosine triphosphate) hydrolysis, or the conversion of chemical energy in the form of ATP into mechanical work (Cooper 2000). The interacting actin filaments consist of several small G actin (globular) molecules polymerized to form filamentous actin (F actin) and are arranged into long two helical chains (Saladin 2008; McArdle et al. 2009) (Figure 1.2). In the groove of actin lies the protein tropomyosin, which in resting muscle covers the site where the myosin head interacts with actin. Each tropomyosin molecule further binds the protein troponin, which is a complex of three polypeptides; the troponin T subunit which binds troponin to tropomyosin, the C subunit that contains binding sites for calcium (Ca$^{2+}$), and the I subunit which inhibits the interaction between myosin and actin filaments through its action on both actin and tropomyosin (Cooper 2000; Guyton & Hall 2006; Barrett et al. 2010).

![Figure 1.2. Contractile myofilaments.](image)

The thick myosin filament (A) is composed of myosin molecules with two globular heads containing light chains and coiled portions composed of heavy chains at the tail end. The thin actin filaments (B) are composed of two helical chains of G actin molecules, with regulatory proteins troponin and tropomyosin. Figure extracted from (Saladin 2008; Marieb & Hoehn 2016).
The contraction of skeletal muscle is generated by the release of $\text{Ca}^{2+}$ from the sarcoplasmic reticulum upon alpha motor neuron stimulation of the myofibre. This process begins with an electrical signal from the nerve that is transmitted through acetylcholine release from the nerve into the NMJ to stimulate the myofibre (Guyton & Hall 2006; Marieb & Hoehn 2016). Activated acetylcholine receptors (AChR) on the motor end plate generate an action potential that is propagated along the muscle sarcolemma and down transverse (T) tubules, which are invaginations of the sarcolemma that enter the sarcoplasm and extend over the sarcoplasmic reticulum (Westerblad & Allen 2009; Marieb & Hoehn 2016). This rapid change in electrical potential alters the conformation and activation of voltage-gated calcium channels in the T tubule membrane (dihydropyridine receptors), which cause calcium release channels in the sarcoplasmic reticulum (ryanodine receptors) to open and release $\text{Ca}^{2+}$ into the sarcoplasm (Westerblad & Allen 2009; Marieb & Hoehn 2016). Subsequent binding of $\text{Ca}^{2+}$ to troponin C results in a weakening of the troponin I interaction with actin and simultaneously changes the conformation of tropomyosin, such that actin-binding sites are exposed and cross bridge formation (by binding of the myosin head to actin) can occur (Guyton & Hall 2006; Barrett et al. 2010).

Figure 1.3. Sliding filament model of muscle contraction. Extracted from (Marieb & Hoehn 2008).
Myosin and actin interaction and subsequent contraction is known as the sliding filament theory and is described in Figure 1.3. Briefly, cross-bridge formation causes a conformational change in the myosin head, which propels the actin filament towards the M line and leads to a shortening of sarcomere length (known as the power stroke) (Guyton & Hall 2006; Barrett et al. 2010). Energy for this process is derived from the hydrolysis of myosin-bound ATP to ADP (adenosine diphosphate) and a phosphate ion, releasing both products and freeing up the ATP binding site. Newly bound ATP causes the cross-bridge to detach from actin and allows hydrolysis of a new ATP molecule to begin the next power stroke. As long as intracellular Ca$^{2+}$ levels remain elevated and sufficient levels of ATP are available, muscle can repeatedly contract (Marieb & Hoehn 2016).

1.1.3 Skeletal muscle fibre types

Diverse populations of myofibre types accommodate the differing functional demands of skeletal muscles. Mammalian muscle contains four major myosin heavy chain (MHC) isoforms: slow (MHCI) and fast (MHCIIa, MHCIIx, MHCIIb). Myofibres expressing MHCI are termed type I fibres, whereas myofibres expressing MHCIIa, MHCIIx, and MHCIIb are termed type IIa, type IIx, and type IIb fibres, respectively (Westerblad et al. 2010). All four of these isoforms are present in the muscles of mice and rats, while only type I, IIa, and IIx fibres are present in those of humans (Smerdu et al. 1994; Westerblad et al. 2010).

These four different myofibre types can be phenotypically classified based on their contraction speed and resistance to fatigue: this is governed by the efficiency of myosin cross-bridges to catalyse the hydrolysis of ATP and the mechanisms that drive ATP production (either aerobic or anaerobic metabolism) [reviewed in (Westerblad et al. 2010)]. **Type I myofibres** are rich in mitochondria and oxidative enzymes, and rely on the aerobic synthesis of ATP via oxidative phosphorylation (or the breakdown of glucose to H$_2$O and CO$_2$, yeilding 38 molecules of ATP) (Atalay & Hänninen 2009). These oxidative myofibres hydrolyse ATP at a low rate (low ATPase activity) and are thus characterised as having slow, energy conserving contractile properties with high fatigue resistance (also known as slow-oxidative myofibres) [reviewed in (Bassel-Duby & Olson 2006)]. **Fast type IIb/IIx myofibres** in contrast, have high ATPase activity and can achieve much greater contraction velocities. Their low mitochondrial content however, mean that ATP production is derived solely from glycolytic metabolism (breakdown of glucose to pyruvate, yielding only 2 ATP per glucose molecule) (Atalay & Hänninen 2009). While glycolysis enables the fast production of ATP, which is suitable for short explosive movements, the accumulation of the pyruvate by-product lactic acid leads to high fatigability. In mice and rats, the **intermediate type IIa**
myofibre contains the metabolic properties of both oxidative and glycolytic myofibre types. These fibres have higher oxidative capacities and thus greater fatigue resistance than type IIB/IIX myofibres in the rat, and are also known as fast-oxidative myofibres (Baldwin et al. 1972).

Adult muscle contains varying mixtures of all four of these major myosin isoforms, and their relative distribution can vary according to species, anatomical site and functional use. For example in the adult mouse hindlimb, the EDL has minimal slow twitch myofibres and is comprised of 99% fast type myofibres (60% of which are IIB); and the soleus muscle is a mixture of fast-oxidative (60% type IIa) and slow type I myofibres (40%) (Chai et al. 2011) (Figure 1.4). While the majority of these larger muscles of the hindlimb are composed of fast type myofibres and enable dynamic movements, muscles such as the diaphragm and select postural muscles like the soleus and psoas contain higher percentages of slow type myofibres for more enduring and prolonged activity.

Figure 1.4. Myofibre distributions. Distribution of myofibre types in (A) the soleus and (B) the EDL of the young C57BL/6J mouse. Slow twitch, type I myosin (shown in red); Fast twitch, type IIa myosin (blue); Fast twitch type IIB myosin (green), detected by monoclonal antibodies to myosin and detected by immunofluorescence on transverse cryosections [extracted from (Chai et al. 2011)].

1.2 Regulation of muscle mass

Diverse populations of muscle fibres enable whole muscles to respond to a wide range of factors, such as physical activity, injury, nutrition and growth factor stimulation. By altering muscle morphology and protein content in response to such changes, skeletal muscle can adapt to many environmental demands. Anabolic skeletal muscle alterations can result in hypertrophy, such as in response to increased loading (section 1.2.1), while catabolic alterations, caused by aging, starvation or disease can result in muscle wasting (atrophy) (section 1.2.2).
1.2.1 Skeletal muscle hypertrophy

Skeletal muscle hypertrophy can be achieved by either an increase in the number and/or size of individual myofibres, and is associated with an increase in net myofibrillar protein content. The regulation of muscle mass and myofibre diameter in the adult myofibre is controlled by a dynamic interchange between anabolic and catabolic mechanisms, which regulate processes of protein synthesis and protein degradation, respectively [reviewed in (Glass 2003; Sandri 2008; Braun & Gautel 2011; Schiaffino et al. 2013)]. Thus, increased muscle mass can result from increased protein synthesis, decreased protein degradation, or a combination of both processes in the context of protein turnover.

1.2.2 Skeletal muscle atrophy

Conversely, skeletal muscle atrophy is characterized by reduced diameter of individual myofibres, myofibre death, reduced force production and increased fatigability (Jackman & Kandarian 2004). In most types of muscle atrophy, an upregulation of pathways that promote protein degradation exceed the ability of the cell to synthesize new protein, leading to a rapid loss of myofibrillar components and a decrease in net protein content (among other effects). Apart from starvation (Gomes et al. 2001), other prominent causes of myofibre atrophy are denervation (Chai et al. 2011; Cheng et al. 2013) and disuse (resulting from immobilization or muscle unloading) (Lecker et al. 1999), however atrophy can also occur in a number of disease states, such as cancer (Lecker et al. 2004), diabetes (Lecker et al. 2004; Sacheck et al. 2007), AIDS (Gonzalez-Cadavid et al. 1998), sepsis (Voisin et al. 1996), burns (Fang et al. 2002), or glucocorticoid administration (Chrysis & Underwood 1999), and progressively with age (Lexell et al. 1988; Barns et al. 2014).

Increased proteolysis in skeletal muscle has a distinct and well characterised transcriptional profile linked to the induction of the ubiquitin proteasome pathway (UPP) (Lecker et al. 2004). Although other pathways have also been implicated during atrophic conditions (such as autophagy), the two atrophy-related genes (atrogenes) commonly up regulated in these states are muscle atrophy F box (Fbxo32; also termed Atrogin-1 or MAFbx) and muscle RING finger-1 (MuRF1) (Bodine et al. 2001a; Gomes et al. 2001). Accordingly, the upregulation of atrogene expression in atrophic conditions is common to a wide range of catabolic situations, despite their induction by different factors (Lecker et al. 2004).

1.3 Age-related muscle atrophy (sarcopenia)

Muscle ageing in contrast, involves many processes involved in disuse and/or disease-induced atrophy, with progressive reduction in muscle mass and strength (known as
sarcopenia) that has important functional and metabolic consequences. The term ‘sarcopenia’ was coined by Irwin Rosenberg in 1989, from the Greek words *sarx* meaning flesh and *penia* meaning loss (Rosenberg 1997), and is widely used to define the age-related loss (atrophy) of skeletal muscle mass and function. For humans over the age of 75 years, muscle mass can be lost at a rate of ~0.6 – 0.7% per year in women and ~0.8 to 1% per year in men. This is associated with rapid decreases in muscle strength that can decline at a rate of 2.5-3% (women) or 3-4% (men) per year [reviewed in (Mitchell *et al.* 2015)], and can be greatly accelerated by physical inactivity and poor nutrition.

Muscle weakness contributes significantly to reduced mobility, diminished independence, and is associated with an increased risk of falls and bone fractures that necessitate longer hospitalisation and rehabilitation times, with immobilisation also further accelerating muscle loss. As a result, it is estimated that ~14% of the those aged between 65 and 75 years require help with routine household activities, and in individuals over the age of 85 this increases to 45% (Williams *et al.* 2002). The global population distribution is increasing in age and with Australia’s total population total already approximately 22 million, the estimated cost of falls in elderly individuals to the healthcare system will increase to $181 million by 2021 (Hendrie *et al.* 2004). Thus, there is a compelling need to understand the causes of sarcopenia and develop interventions to delay its onset and progression, and reduce its severity, in order to maintain optimal health and function of aging individuals.

**1.3.1 Pathogenesis and mechanisms of sarcopenia**

Age related changes to skeletal muscle are complex and the precise reasons for this pathogenesis vary. Morphologically an age-related decline in muscle mass can be attributed to both a loss and atrophy of individual myofibres, which in both men and women, is more profound in lower than upper limb muscle groups (Janssen *et al.* 2000). In humans, reduced myofibre number, which affects both type I and type II fibres can reach 30-40% by 80 years (Leeuwenburgh 2003). A concurrent decrease in the cross sectional area of individual myofibres is also a consequence of age, although appears to vary between myofibre types with type II myofibres undergoing preferential atrophy (Klitgaard *et al.* 1990). Indeed in men and women aged 85 - 97 years, type I and type II myofibres in the vastus lateralis were shown to be 25% and 57% smaller than for young aged 20-29 years, respectively (Andersen 2003). Within the subtypes of type II fibres, type Ila fibres atrophy preferentially compared with type IIb (Coggan *et al.* 1992).

Rodent models (both mice and rats) are useful for the study of sarcopenia. Irrespective
of rodent strain and species, sarcopenia can be identified as early as 18 months (m) (although typically by 24 m), and when compared to younger rodents (aged 6-12 m) have reduced mass of key hindlimb muscles such as the quadriceps, gastrocnemius, soleus, extensor digitorum longus (EDL), plantaris and tibialis anterior (TA) (Kimball et al. 2004; Paturi et al. 2010; Ibebunjo et al. 2013). The widely used C57BL/6J mouse strain has a healthy lifespan of approximately 24 m (they can live longer), with this age range being recommended as the upper limit for reliable sarcopenia studies (Sheard & Anderson 2012). As indicated below in Figure 1.5, relative to humans, a mouse aged 24 m is approximately equivalent to a 70-year old human (Flurkey et al. 2007). In C57BL/6J mice, reduced myofibre number and diameter contribute to total losses of muscle mass, although these can be highly variable between muscle types (Sheard & Anderson 2012). In both humans and rodents, an age-related decline in muscle strength or functional output, parallels this loss of mass (Larsson et al. 1979; Murray et al. 1980; Young et al. 1984; Murray et al. 1985; Ibebunjo et al. 2013).

![Figure 1.5. Lifespan equivalencies between mice and humans.](image)

The progression of sarcopenia can be influenced by multiple systemic and tissue specific parameters. For example, system wide changes include elevated inflammatory and cytokine levels (such as TNF and interleukins 1β and 6) (Visser et al. 2002), a lack of hormones (including testosterone or estrogen) and/or anabolic factors (e.g. insulin-
like growth factor -1 [IGF-1]) [reviewed in (Kamel et al. 2002; McMahon et al. 2011)], poor circulation and/or capillarization [reviewed in (Degens 1998)] and a lack of nutrition [reviewed in (Vetta et al. 1999; Visvanathan & Chapman 2009)]. Local factors include reduced physical activity combined with an increase in sedentary behaviour (Janssen et al. 2002; Budui et al. 2015), elevated free radical production and oxidative damage (Carmeli et al. 2002; Moylan & Reid 2007), mitochondrial dysfunction (Ibebunjo et al. 2013; Marzetti et al. 2013; Kruse et al. 2016) and the deterioration of neuromuscular junctions (NMJs), which contributes to the functional denervation of muscle (Courtney & Steinbach 1981; Arizono et al. 1984; Gambino et al. 1990; Deschenes et al. 2010; Valdez et al. 2010; Chai et al. 2011).

To date research has focussed on the regulation of muscle protein turnover (Baar et al. 2015; Markofski et al. 2015), in addition to the promotion of longevity via manipulation of the key mTORC1-signalling pathway by rapamycin (Zhang et al. 2014; Apelo et al. 2016) in normal aging rodents. However, the effects of food deprivation and exercise on these signalling pathways in aging muscles of mice have been less investigated and thus are the focus of my research. The research presented in this thesis investigated the main signalling pathway known to control protein turnover in muscle; the IGF-1/insulin/mTORC1 signalling pathway (summarised in Figure 1.6) that regulates protein synthesis and degradation pathways (ubiquitin proteasome and autophagy): these are discussed below, with reference to their role in ageing.

1.4 Insulin like growth factor 1 (IGF-1) signalling pathway

Both IGF-1 (an important growth factor) and insulin receptors (hereafter mainly referred to as IGF-1 signalling for simplicity) are key regulators of anabolism in skeletal muscle. Accordingly, IGF-1 signalling plays a role in myogenesis (Florini et al. 1996), developmental growth (Liu et al. 1993; Powell-Braxton et al. 1993), muscle metabolism (Singleton & Feldman 2001), regeneration in response to muscle damage (Pelosi et al. 2007), as well as hypertrophy in response to exercise (Devol et al. 1990). Evidence for the role of IGF-1 in growth and muscle development has been demonstrated via generation of transgenic mice carrying knockout mutations for IGF-1 signalling pathway [reviewed in (D’Ercole 1999)]. Mice lacking a functional IGF-1 gene (IGF-1<sup>-/-</sup>) die in the later stages of embryonic development (>95% perinatal death rate) and are born at a birth weight of approximately 60% of their wild-type siblings due to underdeveloped muscles, organs (lungs, brain and liver) and bones (Powell-Braxton et al. 1993). Given the strong anabolic effects exerted by IGF-1, it has been proposed as a promising therapeutic intervention for the prevention of muscle disease (both sarcopenia and the muscular dystrophies) [reviewed in (Lynch et al. 2005)].
The expression of IGF-1 is induced by growth hormone in multiple tissues, with approximately 75% of systemic IGF-1 production coming from the liver (Froesch et al. 1985). Local tissue expression of IGF-1 contributes to remaining circulating levels, of which skeletal muscle is a likely contributor [reviewed in (McMahon et al. 2011)]. In mammals the structure of IGF-1 is highly conserved, and its genetic sequence is described in a number of species, including human, rat and mouse (Rinderknecht & Humbel 1978; Bell et al. 1986; Rotwein et al. 1986; Shimatsu & Rotwein 1987). In rodents the IGF-1 gene is formed of 6 exons, from which a varied pool of IGF-1 mRNAs can be generated (Shimatsu & Rotwein 1987). Different promoters, transcription start sites on lead exons, post-transcriptional splicing of exons, polyadenylation sites, and final post-translational cleavage of IGF-1 precursor peptides, all contribute to the production of the mature and functional isoforms of IGF-1 [reviewed in (Shavlakadze et al. 2005)]. These isoforms are expressed locally in different tissues and their signalling capabilities are varied [reviewed in (Philippou et al. 2007)]. Briefly, IGF-1 mRNA transcripts can initiate from exons 1 or 2, which are also known as class 1 or class 2 variants, respectively, and can be further spliced to encode Ea or Eb terminal peptide sequences in rodents (Mutarò et al. 2001; Philippou et al. 2007). The main
IGF-1 mRNA splice variant expressed in skeletal muscle encodes the Class 1 Ea isoform (Musarò et al. 2001; Winn et al. 2002), and is commonly used in overexpression studies [reviewed in (Shavlakadze et al. 2005)]. Research on mice that overexpress the muscle-specific Class 1 Ea demonstrate that IGF-1 not only significantly enhances muscle growth in neonates, but also prevents sarcopenia and age-associated decreases to muscle strength (Musarò et al. 2001), improves the dystrophic phenotype when crossed with a mouse model of muscular dystrophy (mdx/IGF-1 (Class 1:Ea)) (Shavlakadze et al. 2004) and accelerates the growth and regeneration of new muscle following induced injury (Rabinovsky et al. 2003). Such protective effects have been attributed to an increase in levels of protein synthesis and a decrease in protein degradation regulated by the IGF-1 signalling pathway (Bodine et al. 2001b; Rommel et al. 2001; Stitt et al. 2004).

1.4.1 Importance of PI3K/AKT signalling downstream of IGF-1

One of the intracellular signalling pathways activated upon IGF-1/insulin receptor stimulation is the phosphatidylinositol-3-kinase (PI3K) / protein kinase B (PKB or AKT) pathway (summarised in Figure 1.6). Many studies investigating muscle growth and homeostasis are focused on signalling through this pathway, and in skeletal muscle its activation is a crucial to the regulation of protein synthesis and hypertrophic growth, in addition to the prevention of muscle atrophy (Rommel et al. 2001; Glass 2003; Stitt et al. 2004; Sandri 2008). Tissue culture studies on myotubes of the classic myogenic C2C12 cell line have been used extensively to highlight the importance of PI3K/AKT signalling in skeletal muscle cells and its role in protein synthesis and hypertrophy (Bodine et al. 2001b; Rommel et al. 2001). Accordingly, the treatment of myotubes with IGF-1 induces hypertrophy concurrent with the phosphorylation and activation of AKT, whereas the pharmacological blockade of PI3K activity with the LY294002 inhibitor, which leads to the subsequent dephosphorylation of AKT, blocks IGF-1 induced hypertrophy in vitro (Bodine et al. 2001b; Rommel et al. 2001). Studies employing an in vivo approach also demonstrate that activation of PI3K following intramuscular transfection of the active Ras construct (RASV12C40), capable of PI3K-specific activation, induces hypertrophy in regenerating muscle (Murgia et al. 2000). Significant myofibre hypertrophy can also be achieved in adult mice conditionally induced to over-express constitutively active AKT (via tamoxifen induction) (Lai et al. 2004).

1.4.2 AKT activation by PI3K

There are three AKT isoforms in mammalian cells, AKT1, AKT2 and AKT3 and each is expressed in a tissue specific manner (Garofalo et al. 2003). For example, AKT1 and AKT2 are expressed in skeletal muscle (among other tissues), whereas AKT3
predominates in the brain [reviewed in (Nader 2005)]. Targeted deletions of AKT in genetically modified mouse models have helped to elucidate the specific functions of each isoform in vivo (Cho et al. 2001a; Cho et al. 2001b; Garofalo et al. 2003). Genetic ablation of AKT1 results in partial lethality and surviving mice are smaller than control neonates, which supports a essential role for AKT1 in developing muscle (Cho et al. 2001b). Accordingly, overexpression of AKT1 in skeletal muscle cells both in vivo and in vitro causes hypertrophy (Bodine et al. 2001b; Pallafacchina et al. 2002). In contrast, AKT2 null mice exhibit normal growth rates, are insulin resistant, and eventually develop a severe form of type II diabetes, which supports a role for AKT2 in the regulation of muscle metabolism (Cho et al. 2001a; Garofalo et al. 2003).

As shown in Figure 1.6, the intracellular signalling cascade leading to Akt activation (of either isoform) begins upon binding of IGF-1 (or insulin) to its receptor, which results in the autophosphorylation of IGF-1R β-subunits and recruitment of insulin receptor substrate-1 (IRS-1) to the cell membrane [reviewed in (Vivanco & Sawyers 2002; Glass 2003; Schiaffino & Mammucari 2011)]. Phosphorylation of IRS-1 recruits and activates PI3K, which converts phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3) to create a membrane binding site for AKT and the phosphoinositide-dependent kinase 1 (PDK1) [reviewed in (Coffer et al. 1998)]. Activation of AKT requires phosphorylation at both threonine and serine residues (Alessi et al. 1996). PDK1-dependent phosphorylation of AKT at Thr308 is essential for kinase activity (Alessi et al. 1997), however full AKT activation occurs following additional phosphorylation at Ser473 (Alessi et al. 1996). This is mediated by the mammalian target of rapamycin complex 2 (mTORC2) (Sarbassov et al. 2005).

In the regulation of protein turnover, increased phosphorylation and activation of AKT can induce muscle hypertrophy by up regulating pathways of protein synthesis through two mechanisms: 1) via phosphorylation and inhibition of glycogen synthase kinase 3β (GSK-3β) via the eukaryotic initiation factor 2B (eIF2B) (PI3K/AKT/GSK-3β pathway); and 2) by phosphorylation of mTORC1 (mammalian target of rapamycin complex 1) and its downstream targets (PI3K/AKT/mTORC1 pathway; see Figure 1.6) (Bodine et al. 2001b; Rommel et al. 2001). The focus of this thesis is on the latter pathway, which is discussed below in section 1.5.

Activated AKT also counteracts protein degradation pathways to reduce muscle atrophy (see Figure 1.6). AKT is a potent inhibitor of the ubiquitin proteasomal pathway (UPP), which acts via the direct phosphorylation of Forkhead Box O (FoxO) transcription factors (AKT-FoxO pathway) (Stitt et al. 2004; Mammucari et al. 2007; Zhao et al. 2007). The role of AKT as an inhibitor of autophagy has also been
implicated in skeletal muscle, although whether this action is exerted via inhibition of FoxO transcription factors by AKT (AKT/FoxO pathway) (Mammucari et al. 2007), or indirectly via the mTORC1/unc-51 like autophagy activating kinase 1 (ULK1) signalling pathway (Castets et al. 2013; Castets & Rüegg 2013) is disputed. The induction and regulation of both pathways of degradation are discussed further in sections 1.6 and 1.7.

1.4.3 AKT activity in sarcopenic muscle

Studies investigating AKT activity in ageing skeletal muscle have generated conflicting results. In humans Sandri et al. (2013) showed that both AKT(Ser473) phosphorylation and total protein amounts were similar in the vastus lateralis muscle of young (aged 18 – 30 years) and old (70 – 80 years) men and women (Sandri et al. 2013). Similar results for AKT phosphorylation have also been observed in some mouse studies: aged female C57BL/6J quadriceps muscles, between 3 and 29 m of age, albeit depressed total AKT protein amounts at 15 m of age (Barns et al. 2014). Conversely, Houtkooper et al. (2011) has demonstrated decreased AKT(Ser473) phosphorylation in the gastrocnemius muscles of 3 and 22 m old C57BL/6J mice (Houtkooper et al. 2011). Whereas Baar et al. (2016), observed that AKT activity decreases with age in skeletal muscles of fed male C57BL/6J mice between 6 and 30 m of age, but increased strikingly among female muscles of the same strain between 6 and 26 m of age (Baar et al. 2016). Further to this Baar et al. (2016) recently demonstrated additional abnormalities in ageing muscle, where AKT(Ser473) failed to dephosphorylate in response to 16 h of fasting in very old male (30 m) and female (26 m) muscle. This thesis further investigates the time-course of changes affecting AKT phosphorylation rates in aged male C57BL/6J mice during both fed and fasted states in order to measure fluctuating and basal levels of this pathway, in addition to its interaction with downstream mTORC1 signalling (discussed below) and interlinking pathways of protein degradation (Results Chapter 1).

1.5 Regulation of muscle protein synthesis

1.5.1 mTOR signalling

The mammalian target of rapamycin (mTOR) is a protein kinase that plays a key role in the regulation of protein synthesis, cell growth and metabolism (Sancak et al. 2010; Menon et al. 2014). mTOR binds two protein complexes, the rapamycin sensitive mTORC1, and the rapamycin-insensitive mTORC2 [reviewed in (Laplante & Sabatini 2009; Schiaffino & Mammucari 2011)]. The two complexes of mTOR have varied functions and contributions to the mTOR-signalling pathway and are summarised in Figure 1.7. In addition to mTOR, both complexes share the mammalian lethal with sec-
13 protein 8 (mLST8), DEP domain containing mTOR-interacting protein (DEPTOR) and the Tel2-interacting protein 1 (Tti1) and telomere maintenance 2 (Tel2) complex (Tti1/Tel2) [reviewed in (Laplante & Sabatini 2009; Johnson et al. 2013); (Kaizuka et al. 2010). The regulatory associated protein of mTOR (Raptor) and proline-rich Akt substrate 40 kDa (PRAS40) are unique to mTORC1, while the rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated map kinase-interacting protein 1 (mSin1) and protein observed with Rictor 1 and 2 (Protor1/2) are unique to mTORC2 [reviewed in (Johnson et al. 2013; Lamming et al. 2013; Li et al. 2014)].

Figure 1.7. The two mTOR complexes, their constituent proteins, upstream effectors and downstream processes. Extracted and adapted from (Ma & Blenis 2009; Bracho - Valdés et al. 2011; Laplante & Sabatini 2012; Johnson et al. 2013).

Much more is known about the upstream regulation and downstream output of mTORC1, than mTORC2 which is thought to be involved in organisation of the actin cytoskeleton (among others) (Jacinto et al. 2004) and more recently in autophagy (Mammucari et al. 2007; Kennedy & Lamming 2016). In comparison, mTORC1 drives a multitude of anabolic processes required for cell growth, including protein synthesis and energy metabolism, as well as inhibition of catabolic processes like autophagy (Laplante & Sabatini 2009; Sengupta et al. 2010; Johnson et al. 2013; Lamming et al. 2013; Li et al. 2014). Furthermore, while the muscle specific deletion of Rictor has no detrimental effects on muscle morphology, the assembly and function of mTORC1 is critical for skeletal muscle function. Mice deplete of Raptor do not survive past 190 days and develop dystrophic muscles (with distinct muscle atrophy) with diminished functional capacity and altered glucose metabolism (Bentzinger et al. 2008).

Dysregulation of mTORC1 is associated with many human diseases, including diabetes, cancer, obesity and neurodegeneration, and thus there are ongoing efforts to pharmacologically target this pathway. When bound to the 12kDa FK506-binding
protein (FKBP12), rapamycin potently inhibits mTORC1 activity and signalling to its downstream effectors [reviewed in (Wullschleger et al. 2006)]. The inhibitory effects of rapamycin have been confirmed to halt developmental growth (Drummond et al. 2009) and impair hypertrophy typically induced by synergist ablation (compensatory response) (Bodine et al. 2001b). Furthermore, the inhibition of mTORC1 activity with rapamycin has been widely proposed as an anti-aging intervention, with trials carried out in pre-clinical animal models (Harrison et al. 2009; Wilkinson et al. 2012; Flynn et al. 2013) and humans (Mannick et al. 2014). Accordingly, extended rapamycin treatment is shown to prolong life span in mice, even when treatment is initiated late in life (Harrison et al. 2009; Apelo et al. 2016) and has other beneficial effects including increased mobility (Wilkinson et al. 2012; Flynn et al. 2013).

![Figure 1.8. The signalling networks regulating mTORC1.](image)

The remainder of this thesis will focus on the regulation of only mTORC1. A variety of sources feed input into mTORC1, including IGF-1/insulin growth factor signalling, amino acid-sensing, and intracellular energy status and stressors, which have a profound effect on the downstream regulation of protein synthesis, and mediation of downstream effectors that act to regulate ribosomal biogenesis and the initiation and elongation of translation. These interacting signalling cascades are demonstrated in Figure 1.8 and discussed below in section 1.5.2.
1.5.2 Upstream regulators of mTORC1

1.5.2.1 Induction by growth factors (IGF-1/Insulin)

The mTORC1 pathway responds to growth factors from the IGF-1/insulin signalling pathway, which is mediated indirectly via PI3K/AKT activity. Levels of growth factors like IGF-1 and insulin are indicative of feeding status, and during normal fed conditions elevated levels of these growth factors promote positive anabolic processes like protein synthesis. As illustrated in Figure 1.8, mTORC1 is linked to the IGF-1 signalling pathway through AKT and its interaction with the tuberous sclerosis complex (TSC), which comprises TSC1 (also known as hamartin) and TSC2 (also known as tuberin) proteins [reviewed in (Laplante & Sabatini 2009)]. TSC1/2 functions as a guanosine triphosphatase activating protein (GAP) that regulates the guanosine triphosphate (GTP) loading state of Rheb (Ras homolog enriched in brain), and when active, TSC2 inhibits mTORC1 by promoting the conversion of Rheb-GTP (active) to Rheb-GDP (inactive) (Garami et al. 2003; Inoki et al. 2003; Tee et al. 2003). In contrast to TSC2, TSC1 is involved in complex stabilisation and lacks GAP activity (Liu et al. 2002). Activation of AKT leads to TSC2 phosphorylation on multiple residues including Ser939, Ser1086/1088, Thr1462 and Thr1422, which inhibit both complex and GAP activity and allow for the activated, GTP-bound form of Rheb to directly interact with and activate mTORC1 signalling (Inoki et al. 2002; Manning et al. 2002). The phosphorylation of TSC2 by AKT at all characterised phosphorylation sites are shown to be necessary for full activation of mTORC1 by insulin (Inoki et al. 2002). It should be noted that growth factors also regulate mTORC1 through mechanisms independent of the TSC complex, and active AKT can phosphorylate PRAS40 on residue Thr246 resulting in its dissociation from the complex and relief of its repressive hold on mTORC1 (not shown) (Sancak et al. 2007; Vander Haar et al. 2007). Regardless, TSC1/2 complex activity is shown to be imperative for the regulation of mTORC1 by growth factors and the loss of either protein leads to mTORC1 hyper activation, and in mice with a muscle specific knock out of TSC1 (TSCmKO), sustained rates of mTORC1 signalling and protein synthesis contribute to severe myopathy (attributed to an accumulation of autophagic substrates and dysfunctional organelles), as well as significant reductions to muscle function (Castets et al. 2013).

1.5.2.2 Induction by amino acid supply

In addition to its activation by growth factors, mTORC1 is controlled by the supply of amino acids. Reduced intake of amino acids starves the cell of substrates required for anabolic processes like protein synthesis. The amino acid leucine has been identified as a key amino acid implicated in mTORC1 activation (Drummond & Rasmussen 2008). In humans, the ingestion of essential amino acids (highly enriched in leucine)
stimulates an acute increase in mTOR phosphorylation and the activation of downstream markers of protein synthesis in skeletal muscle (Fujita et al. 2007; Dickinson et al. 2011). Similarly in animals, rapamycin administration prior to an increase in amino acid availability has been shown to block downstream markers of mTORC1 and inhibit this process (Anthony et al. 2000; Kimball et al. 2000).

In contrast to mTORC1 induction by IGF-1/insulin, the TSC1/2 complex is not essential in the regulation of mTORC1 by amino acids. Accordingly, in cells depleted of TSC2, the mTORC1 pathway remain sensitive to the removal of amino acids but not growth factors (Smith et al. 2005; Roccio et al. 2006). The regulation of mTORC1 in response to amino acid supply is poorly understood, although the recruitment of mTORC1 to a lysosomal membrane where it can interact with Rheb appears to be crucial to this process (Sancak et al. 2008; Sancak et al. 2010). Tethered to the lysosomal membrane is the multi-protein complex known as the Ragulator (Sancak et al. 2010), which acts as a docking site for Rag GTPase heterodimers (Sancak et al. 2008) (not shown). In mammalian cells there are four Rag proteins, RagA, RagB, RagC and RagD, that form heterodimers consisting of the functionally redundant RagA or RagB paired with either RagC or RagD (Sekiguchi et al. 2001; Sancak et al. 2008) (see Figure 1.8). Each heterodimer pair has opposing nucleotide loading states, which are dependent on the presence of amino acids (Laplante & Sabatini 2009). Thus, when cells are deprived of amino acids, RagA/B is bound to GDP and RagC/D to GTP (Sancak et al. 2008). Amino acid stimulation conversely promotes the GTP loading state of the RagA/B heterodimer, which in turn enables the recruitment of Raptor (and thus the mTORC1 complex) to the lysosomal surface, where the GTP-loaded Rheb plays a role in the final steps towards mTORC1 activation (Sancak et al. 2008; Sancak et al. 2010) (see Figure 1.8).

1.5.2.3 Intracellular energy status and stress

The balance of energy generation and consumption is fundamental to all cells. In skeletal muscle, the generation of intracellular energy, which can be derived from glucose, influences mTORC1 signalling through indirect mechanisms. From glucose, ATP is produced from a number of processes including, glycolysis (cytoplasm), the Krebs cycle and oxidative phosphorylation in resident mitochondria (Atalay & Hänninen 2009). The adenosine monophosphate (AMP)-activated protein kinase (or AMPK) is a crucial sensor of energy status and becomes activated when the ratio of cellular ADP or AMP/ATP increases, for example during sustained exercise. Indeed, the activation of AMPK following the administration of 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR), a chemical mimic of exercise, results in decreased rates of protein synthesis and repressed mTORC1-induced signalling in skeletal muscle, both
in vivo and in vitro (Bolster et al. 2002; Williamson et al. 2006). Similarly, a decrease in the ATP/AMP ratio as occurs during aerobic exercise also leads to AMPK phosphorylation and limits anabolic processes, via mTORC1 inhibition in an attempt to restore intracellular energy [reviewed in (Hardie et al. 2012)]. AMPK is shown to relay such changes in intracellular energy status to mTORC1 via two targets (see Figure 1.8). Firstly, AMPK can directly phosphorylate TSC2 on residues Thr1227 and Ser1345 leading to its activation, which as stated above stimulates the GAP activity of Rheb (or the conversion of Rheb into its GTP (active) or GDP (inactive) state) and thus the inhibition of mTORC1 (Inoki et al. 2003) (see Figure 1.8). Secondly, AMPK can also inhibit mTORC1 directly via Raptor phosphorylation at Ser722 and Ser792, thus leading to its dissociation from the complex (Gwinn et al. 2008).

While long-term hypoxia (or oxygen depletion) can also result in energy depletion, and thus contributes to AMPK activation, short-term hypoxic stress can also rapidly induce the inhibition of mTORC1 via REDD1 (regulated in development and DNA damage responses 1) signalling by its subsequent activation of TSC2 [reviewed in (Ma & Blenis 2009)] (see Figure 1.8).

1.5.2.4 Mechanical stimulation (mechanotransduction)

It is proposed that mechanical stimulation (exercise) also promotes increased mTORC1 signalling through additional mechanisms (not shown). Studies by Baar and Esser (1999) demonstrated that electrical stimulation over 6 weeks is sufficient to increase S6K1 phosphorylation. Increased phosphorylation rates observed 6 h post stimulation was also highly correlated with the hypertrophic response exhibited by both TA and EDL muscles (Baar & Esser 1999). These findings were further extended upon by Bodine et al. (2001) using a model of functional overload in the plantaris muscle induced by surgical removal of the soleus and gastrocnemius muscles, where resultant adaptive hypertrophy was accompanied by an increase in S6K1 phosphorylation (Bodine et al. 2001b). Moreover mTORC1 specific inhibition with rapamycin prevented the hypertrophic increase in plantaris muscle weight (Bodine et al. 2001b). When taken together, these observations demonstrate that activity of the mTORC1 complex is an essential step towards promoting growth in response to mechanical stimuli.

The role of mechanotransduction on mTORC1 signalling is suggested to act via an AKT-independent pathway involving both phosphatidic acid (PA) and phospholipase D (PLD) [reviewed in (Hornberger 2011)], although this remains controversial. PA has been shown to bind directly to the FRB domain on mTORC1 and stimulate downstream activation of S6K1 (Fang et al. 2003), and is reported to interact with mTOR in a manner that is competitive with FKBP12-Rapamycin domain-binding (Toschi et al. 2008).
The incubation of EDL muscles \textit{ex vivo} in a PA phosphatase inhibitor is able to promote an intramuscular increase in the concentration of PA and S6K1(Thr389) phosphorylation, an effect that can be abolished via rapamycin treatment. Furthermore, utilisation of an \textit{ex vivo} model of mechanical stimulation can induce an early transient increase in PLD and a sustained increase in PA activity, which was again correlated with increased S6K1 phosphorylation (Hornberger \textit{et al.} 2006). PA can be synthesised from phosphotidylcholine by PLD, and studies demonstrate that GTP-activated Rheb can bind to and activate PLD in order to facilitate this process (Sun \textit{et al.} 2008).

\subsection*{1.5.2.5 Protein synthesis downstream of mTORC1}

Once activated, mTORC1 stimulates protein synthesis. The translation of new proteins requires the recruitment of ribosomes to the 5’ end of mRNA, and this process is facilitated by a number of translation initiation factors [reviewed in (Hay \& Sonenberg 2004)]. This process is promoted by mTORC1 via the activation of both S6 kinase 1 (S6K1) at Thr389, Thr229, Ser404 and Thr70 and the eukaryotic translation initiation factor (eIF) 4E-binding protein (4E-BP1 also known as phosphorylated heat-and-acid stable protein; PHAS-1) at Thr36/37, Ser65 and Thr70 via recruitment of the Raptor adaptor protein (Nojima \textit{et al.} 2003). The phosphorylation and activation of S6K1 by mTORC1 promotes ribosomal biogenesis through the phosphorylation and activation of ribosomal protein S6 (rpS6) at Ser235, Ser236, Ser240 and Ser244, which further promotes the translation of mRNAs for ribosomal proteins (Krieg \textit{et al.} 1988; Thomas 2000; Jastrzebski \textit{et al.} 2007). The 5’ ends of transcribed mRNAs have a cap structure (mRNA 7-methylguanosine) that can be recognised by the eukaryotic translation initiation factor (eIF) 4E [reviewed in (Hay \& Sonenberg 2004)]. The activation and phosphorylation of 4E-BP1 by mTORC1 releases its inhibitory hold on eIF4E and allows the subsequent recruitment of eIF4G and eIF4A (or assembly of the eIF4F complex) to the 5’ end of mRNA [reviewed in (Ma \& Blenis 2009)]. Following cap binding, helicase activity of the eIF4F complex acts to unwind mRNA, which can be further enhanced by eIF4B [reviewed in (Wang \& Proud 2006)]. Indeed, S6K1 phosphorylation of eIF4B, recruits both eIF4B and eIF4A to the eIF4F complex (Holz \textit{et al.} 2005). S6K1 phosphorylation is also shown to further direct eIF4B into a complex with eIF3, where it can interact with the 40S ribosomal subunit (as part of the 43S translation pre-initiation complex), ternary complex association, and thus the recruitment of ribosomes to mRNA (Holz \textit{et al.} 2005). Collectively, these processes lead to the synthesis of new proteins.

Hence, S6K1 and its downstream effectors rpS6 and 4E-BP1 phosphorylation are often used as an in vivo and in vitro readout of mTORC1 activity and to assess rates of protein synthesis. While several studies have demonstrated that increased
phosphorylation of mTORC1 effectors (AKT, mTOR, S6K1 and 4E-BP1) are involved in the modulation of muscle protein synthesis rates (MPS) (Fry et al. 2011; Drummond et al. 2013), it must be noted however, that a disconnect between mTORC1 signalling protein activation and active protein synthesis rates have also been reported (Greenhaff et al. 2008).

1.5.2.6 Protein synthesis, mTORC1 signalling and sarcopenia

Muscle protein synthesis is regulated by the responsiveness of skeletal muscle to anabolic stimuli, including food intake and exercise (see Figure 1.8). The reduced capacity of elderly humans to induce protein synthesis in response to nutrition or physical activity has been termed anabolic resistance. Indeed, there are reports that older men and women (average age 70 years) exhibit blunted or delayed phosphorylation of AKT(Ser473), mTOR(Ser2448), S6K1(Thr389) and 4E-BP1(Thr37/46) following an acute bout of resistance exercise (10 repetitions at 45% of 1-repetition max (1RM) and 8 sets of 10 repetitions at 70% of 1RM) compared with younger individuals (aged 27 years) (Fry et al. 2011). Age related deficits to mTOR, S6K1 (Parkington et al. 2004) and 4E-BP1 phosphorylation (Funai et al. 2006) have also been reported in old (aged 30 m), but not young adult (aged 6 m) male rats following a single bout of high frequency electrical stimulation. Reduced ribosomal biogenesis (reduced rDNA transcription), despite increased ribosomal protein gene expression, has also been linked to blunted muscle hypertrophy in plantaris muscles of old (aged 25 m), but not young (aged 5 m) male mice, in response to 14 days of bilateral synergist ablation, although no protein data for mTORC1 signalling were reported (Kirby et al. 2015). Similar attenuations of anabolic signalling are also observed following amino acid ingestion. Indeed, in young adult men (with an average age of 28 years), activity of S6K1(Thr389) and 4E-BP1(Thr37/46) phosphorylation rates were readily stimulated following the consumption of a 10 g dose of essential amino acids (including leucine), whereas in older men (~70 years) the amino acid response is dampened (Cuthbertson et al. 2005). The responsiveness of mTORC1 and/or muscle protein synthesis rates to amino acid supplementation in elderly individuals can however be overcome by increasing leucine content (Caspersen et al. 2012), which has also been shown to improve the protein synthetic response as well as S6K1(Thr389) phosphorylation in young (24 years) and old (70 years) men when supplemented post resistance exercise (6 x 8 knee-extension repetitions at 75% of 1RM) (Atherton et al. 2016). Together these data suggest that the ability to increase protein synthesis and/or translational capacity may be compromised in aged skeletal muscle.

Interestingly, age-related muscle atrophy is also characterised by the hyper
phosphorylation of mTORC1 and its downstream targets (S6K1 and/or rpS6) in rodents (Barns et al. 2014; Baar et al. 2016) and ageing humans (Markofski et al. 2015), although whether this contributes to an increase in rates of protein synthesis is currently unknown. This thesis further investigates the time-course of changes affecting mTORC1 signalling in aged male C57BL/6J mice during both fed and fasted states to measure fluctuating and basal levels of this pathway (Results Chapter 1).

1.6 Regulation of protein degradation

In skeletal muscle, two major integrative catabolic systems oppose protein synthesis and result in protein degradation and both are activated in response to atrophic conditions, 1) the ubiquitin proteasome pathway (sections 1.6.1) and 2) the autophagy lysosome pathway (sections 1.6.2). The regulation of both pathways and their implications in ageing are described below.

1.6.1 Ubiquitin proteasomal pathway (UPP)

The UPP is responsible for the breakdown of contractile myofibrillar proteins and long-lived soluble proteins, and plays a major role in muscle remodeling during wasting conditions (as described in section 1.2.2). UPP-mediated catabolism is also essential to maintain amino acid reserves during prolonged periods of starvation and turns over damaged or misfolded proteins [reviewed in (Bonaldo & Sandri 2013b)]. The UPP consists of a number of integrated actions that link chains of ubiquitin (Ub) to proteins via an ATP dependent process (ubiquitination), thus flagging them for degradation. There are three key processes required to link Ub chains to protein substrates (Figure 1.9). At the apex of ubiquitination are the E1 or Ub-activating enzymes that use ATP to activate and link reactive Ub molecules to E1 enzymes. Activated Ub is then transferred to an E2 Ub conjugating enzyme, which acts as an escort for the transport of activated Ub to an array of E3 Ub ligases [reviewed in (Varshavsky 1997; Tisdale 2005; Lecker et al. 2006; Löw 2011)]. As Figure 1.9 also demonstrates, these ligases catalyse the transfer of activated Ub to the protein substrate, and progressive rounds of E3-mediated Ub-ligation results in the attachment of a polyubiquitininated chain. Proteins flagged for degradation are recognised by the 26S proteasome, which comprises two 19S components, and one 20S component. The 19S components contain multiple ATPases to facilitate Ub removal and linearization of proteins for loading into the central core of the proteasome (the 20S) where it is digested into peptides. Peptidases in the cytoplasm degrade the resultant peptides into amino acids for further processing by the cell [reviewed in (Varshavsky 1997; Tisdale 2005; Lecker et al. 2006; Löw 2011)].

Varied E2-E3 pairings degrade a wide variety of proteins, and the proportion of these
ligases vary between tissues and in response to different physiological conditions (Bonaldo & Sandri 2013a). The two most characterised E3 ubiquitin ligases present in skeletal muscle are MuRF1 and Atrogin-1 (Bodine et al. 2001a), which are up regulated in a variety of muscle wasting conditions (section 1.2.2). Mice depleted of either MuRF1 or Atrogin-1 are resistant to denervation induced muscle atrophy (Bodine et al. 2001a). Similarly, the knockdown of Atrogin-1 under fasting conditions can prevent muscle wasting (Cong et al. 2010). Both E3 ubiquitin ligases have distinct roles in skeletal muscle maintenance: MuRF1 facilitates the ubiquitination of both myosin heavy and light chains (Clarke et al. 2007; Cohen et al. 2009) and muscle actin (Polge et al. 2011); whereas Atrogin-1 (although less defined) is proposed to play a role in the control of protein synthesis via the degradation of eIF factor 3 subunit F (Lagirand-Cantaloube et al. 2008; Csibi et al. 2010), myogenic signalling through MyoD degradation (Csibi et al. 2010), as well as the degradation of other structural proteins (Lokireddy et al. 2012). Additional E3 ubiquitin ligases are also activated under atrophic conditions in skeletal muscle and aid in the removal of damaged intracellular proteins or alternatively inhibit anabolic processes. Although not the focus of this thesis, they are extensively reviewed by (Sandri 2013; Schiaffino et al. 2013).

Figure 1.9. The ubiquitin proteasomal pathway (UPP). Three biochemical steps leading to the conjugation of ubiquitin to a protein substrate and degradation via the proteasome. Figure adapted from (Lecker et al. 2006; Bonaldo & Sandri 2013b).

1.6.1.1 Regulation of the UPP by AKT

Upregulation of the UPP in skeletal muscle is mediated by AKT, and the Forkhead Box O (FoxO) family of transcription factors (see Figure 1.8). There are three FoxO isoforms present in skeletal muscle, FoxO1, FoxO3 and FoxO4 (Milan et al. 2015). Atrophic muscle is characterised by decreased PI3K/AKT activity and an upregulation of atrogenes, which occurs via the dephosphorylation (activation) of FoxO transcription factors and translocation from their inactive cytosolic state to the nucleus where they are transcriptionally active (Sandri et al. 2004; Stitt et al. 2004). The induction of FoxO
transcription factors is essential for myofibre atrophy, and for the induction of MuRF1 and Atrogin-1 (Sandri et al. 2004; Sandri et al. 2006). Accordingly, in both adult skeletal muscle and in cultured myotubes, the overexpression of constitutively active FoxO3 causes an up regulation of atrogenes and leads to extensive muscle atrophy (Sandri et al. 2004). Myogenin, a key transcription factor regulating muscle development and myogenesis, is also required for the full activation of MuRF1 and Atrogin-1 following denervation (Moresi et al. 2010).

Conversely, activated AKT phosphorylates FoxO on multiple conserved sites leading to an inhibition of their transcriptional function and their exclusion from the nucleus, thus blocking this pathway of protein degradation (Brunet et al. 1999). Treatment of atrophying myotubes with IGF-1 supresses atrogene upregulation by preventing activation of FoxO transcription factors (Stitt et al. 2004). Moreover, the transfection of myotubes with either constitutively active PI3K or AKT blocks the upregulation of atrophy related genes to further demonstrate that the anabolic (or anti-atrophic effects) of IGF-1 rely on PI3K/AKT activity (Stitt et al. 2004). These effects have been further demonstrated in vivo, for example in fasted mice, Atrogin-1 promoter activation is blocked following transfection of constitutively active AKT (Sandri et al. 2004) and expression of both MuRF1 and Atrogin-1 are suppressed following IGF-1 injection into short-term denervated muscle, concurrent with reduced rates of myofibre atrophy (Stitt et al. 2004).

### 1.6.1.2 Age-related changes to the UPP

The maintenance of proteasome activity and subsequent protein turnover is necessary for proper cellular function, and decreased proteasomal activity are associated with accumulation of protein aggregates and disturbed muscle homeostasis (Wong & Cuervo 2010). While reduced proteasomal activity has been reported in multiple tissue types, including the heart, liver and brain (Patterson et al. 2007; Koga et al. 2011; Löw 2011)], controversy exists regarding whether the UPP is elevated, depressed or unaltered with age. Down regulated transcription is reported for E2 conjugating enzymes and E3 ubiquitin ligases (including MuRF1 and Atrogin-1) (DeRuisseau et al. 2005; Edstrom et al. 2006), in addition to decreased expression of critical 26S proteasomal subunits and/or their damage by posttranslational modification (Hwee et al. 2014) to contribute to the functional decline of the proteasome in sarcopenic rodent muscles. In other rodent studies, constitutive levels of these crucial components are reported to either be higher (Cai et al. 2004; Clavel et al. 2006; Hepple et al. 2008; Altun et al. 2010; Barns et al. 2014) or, in some circumstances, fail to up regulate in response to induced stress (Altun et al. 2010). In humans, proteasomal activity in ageing is further diversified, and the ubiquitin proteasomal system has been shown to
function similarly between adult and old muscles (Bossola et al. 2008), although increased levels of intramuscular ubiquitin have also been reported (Cai et al. 2004). Regarding regulation of the E3 ubiquitin ligases, Atrogin-1 expression is shown to remain stable with age (Whitman et al. 2005; Raue et al. 2007; Fry et al. 2013), while the regulation of MuRF1 has been shown to either increase (Raue et al. 2007) or remain stable (Whitman et al. 2005; Fry et al. 2013). Thus, changes to the UPP in skeletal muscle and how they alter protein turnover during ageing are still unclear: furthermore this pathway has not been characterized in the context of protein turnover in a number of ageing studies (which largely focus on protein synthesis). For additional discussion see Chapter Three: Results Chapter 1.

1.6.2 Autophagy

Complimentary to the UPP, autophagy is particularly important in tissues containing long-lived post-mitotic cells such as skeletal muscle. The word autophagy is derived from the Greek roots auto meaning self, and phagy meaning eating, and refers to the catabolic process by which cellular constituents are transported to lysosomes for selective degradation (Ravikumar et al. 2010). There are three types of autophagy present in mammalian cells; macroautophagy, microautophagy and chaperone-mediated autophagy, however, this thesis will focus mainly on macroautophagy (herein referred to as autophagy). Autophagy operates at basal levels in skeletal muscle and is required for the homeostatic turnover of cytoplasmic components including unfolded and toxic protein aggregates and dysfunctional organelles (including mitochondria) to reduce cytotoxicity and maintain optimal cellular function (Masiero et al. 2009). Genetic proof that a sufficient level of autophagy is required for healthy muscle function comes from a mouse model with a muscle specific deletion of the autophagy gene 7 (Atg7) (Masiero et al. 2009). These mice display muscle wasting and decreased force, accumulation of abnormal mitochondria, disorganization of sarcomeric reticulum and enhanced muscle loss after fasting and denervation. Autophagy dysregulation also leads to severe myopathies like Pompe and Danon diseases, which are characterised by vacuolated fibres, dysfunctional mitochondria and impaired muscle function [reviewed in (Levine & Kroemer 2008; Temiz et al. 2009)].

1.6.2.1 Autophagy machinery

The initial steps of autophagy include vesicle formation (or nucleation) and expansion (elongation) of the isolation membrane, or phagophore (Figure 1.10). Fusion of the phagophore results in the complete sequestration of cytoplasmic components within a double-membranous isolation structure, or autophagosome. This is followed by fusion of the autophagosome with a lysosome to form an autophagolysosome, where the
encapsulated material, together with the inner membrane is degraded by lysosomal hydrolases (Figure 1.10) (Xie & Klionsky 2007; Hosokawa et al. 2009; Glick et al. 2010).

Figure 1.10. Key regulatory complexes involved in mammalian autophagosomal formation, fusion and degradation. Adapted from (Mizushima et al. 2010).

There are over 30 autophagy related genes (Atg) genes shown to regulate autophagy induction and autophagosome formation in mammalian cells (Hall & Sanes 1993) and key genes are indicated in Figure 1.10 (Mizushima et al. 2010). These include formation of the ULK1/Atg1 complex that signals to downstream autophagic machinery in response to upstream signals from mTORC1, and the PI3K Class III signalling complex that mediates vesicle induction and nucleation (Vps34, P150, Beclin1, Atg14, and Ambra1) (Mizushima et al. 2010). Further elongation of the phagophore and closure of the autophagosome requires the coordination of both Atg12-Atg5 and LC3-Atg8 (microtubule-associated protein light chain 3) conjugation systems; the mechanisms of which necessitate two ubiquitin-like reactions [reviewed in (Eskelinen, 2005; Rubinsztein et al. 2011; He & Klionsky 2009)]. In the first, the combined actions of Atg7 and Atg10 (which act like E1 and E2 ubiquitin-like activating enzymes, respectively), conjugate Atg12 to Atg5. The Atg12-Atg5 conjugate then binds to Atg16L1 and the newly produced E3-like enzyme complex translocates to the developing autophagosomal membrane (Tanida et al. 2004; Eskelinen 2005; He & Klionsky 2009; Rubinsztein et al. 2011). The Atg12-Atg5-Atg16L1 complex eventually dissociates from the completed autophagosome, although is required for localising the second Atg8/LC3 conjugation system to the autophagosomal membrane. Atg8/LC3 is cleaved by the cysteine protease Atg4 to generate the cytosolic form of LC3 (LC3I), which is further conjugated to the target lipid phosphatidylethanolamine in a process that requires Atg7 and the additional E2-like activating enzyme, Atg3 (Yang & Klionsky 2010). The lipidated, autophagosome-specific form of LC3 (LC3II) is localised to both inner and outer membranes of the maturing phagophore, and unlike the Atg12-Atg5-
Atg16L1 complex that dissociates from the completed autophagosome, remains bound to the inner membrane (He & Klionsky 2009; Mizushima et al. 2010). Upon fusion with the lysosome, Atg4b cleaves the LC3II present on the outer membrane, and remaining LC3II on the inner membrane is degraded by lysosomal hydrolases (Mizushima et al. 2010). In mammalian cells, lysosomal proteins, lysosome-associated membrane protein 2 (LAMP-2) and the small GTPase Rab7 are required for lysosomal fusion (He & Klionsky 2009).

Autophagy was originally thought to be a bulk degradation system, although it is now known that adaptor proteins can select specific targets for degradation, either by shuttling them to the growing autophagosome and/or binding targeted substrates to autophagosomal membrane proteins for degradation. The autophagosome-bound LC3 functions as a receptor to one such protein, sequestosome 1 (p62/SQSTM1), which regulates a variety of signalling pathways [reviewed in (Bitto et al. 2014)]. P62 can bind to LC3 and be preferentially degraded by the autophagy process; as a result, marked accumulation of p62 has been observed in a number of cell types, including skeletal muscle during autophagy insufficiency (Komatsu et al. 2007; Pankiv et al. 2007; Ogata et al. 2010; Castets et al. 2013). Since p62 also contains a ubiquitin-binding domain, it is shown that ubiquitinated proteins can also be recruited and incorporated into the autophagosome, again via LC3 interaction (Pankiv et al. 2007).

Damaged mitochondria can also be selectively targeted for lysosomal degradation, via the process of mitophagy (Schiaffino et al. 2013). In mammals, mitophagy initiation relies on the recruitment and activation of the E3 ubiquitin ligase parkin, by PINK1 (PTEN-induced putative kinase protein kinase 1), which permits the attachment of a polyubiquitin chain to the outer membrane of damaged or dysfunctional mitochondria. Autophagy adaptor proteins including p62 incorporate mitochondria flagged for degradation into autophagic vesicles [reviewed in (Narendra & Youle 2011; Youle & Narendra 2011; Jin & Youle 2012)]. Additional regulatory proteins, Bcl-2/adenovirus E1B 19-kDa interacting protein 3 (Bnip3) and its homologue Bnip3L, are also reported to bind directly to LC3 and further recruit damaged mitochondria to the autophagosome for selected degradation (Novak et al. 2010; Hanna et al. 2012).

1.6.2.2 Autophagy induction

Autophagy can be induced in response to severe catabolic conditions like denervation or starvation (fasting), as well as during oxidative stress, DNA damage, hypoxia, mitochondrial damage and following exercise (Levine & Kroemer 2008). In mammalian cells, the classical pathway regulating autophagy acts via mTORC1 (Jung et al. 2009). The inhibition of mTORC1 via fasting or administration of rapamycin is sufficient to
induce autophagic activity in most cell types (including skeletal muscle) (Zhao et al. 2007; Ogata et al. 2010; Castets et al. 2013), and relies on the release of mTORC1-mediated phosphorylation of ULK1 at its inhibitory residue (Ser757) with subsequent phosphorylation of ULK1 on Ser555, Ser317 and Ser777 by AMPK (Jung et al. 2009; Egan et al. 2011; Kim et al. 2011; Bujak et al. 2015). This promotes the assembly and activation of the ULK1/Atg13/FIP200 complex and thus promotes the induction of autophagy (see Figure 1.6). The roles of mTORC1 as both an inhibitor and activator of autophagy in skeletal muscle have been demonstrated in TSCmKO mice, which lack TSC1 and thus the endogenous inhibition of mTORC1 (Castets et al. 2013). Resultant hyperactivity of mTORC1 signalling and ULK1 phosphorylation (Ser757) strongly inhibits autophagic flux, and while prolonged starvation (a usual inducer of autophagy) fails to stimulate autophagy in TSCmKO mice, treatment with rapamycin can directly suppress mTORC1 signalling, dephosphorylate ULK1 at Ser757, and restore autophagic clearance (Castets et al. 2013; Castets & Rüegg 2013).

Literature regarding autophagy regulation in skeletal muscle has been extensively debated. Initially, the overexpression of constitutively active FoxO3 (but not rapamycin) in C2C12 myotubes and adult myofibres was shown to increase autophagosome formation and induce the expression of many key autophagy genes (including LC3, Gaparapl1, Ulk2, Atg4b, Bnip3 and Beclin1) typically up regulated following fasting or denervation (Mammucari et al. 2007; Zhao et al. 2007). FoxO3, which can also be phosphorylated by AMPK on residues Ser413 and Ser588 under fasting conditions (Sanchez et al. 2012), was thus believed to be the key regulator of autophagy in skeletal muscle. Later studies in mice with the muscle specific depletion of Raptor (RAmKO), which have inhibition of both mTORC1 and FoxO signalling as well as hyper-phosphorylation of AKT, have helped to further elucidate the role of these proteins in skeletal muscle (Bentzinger et al. 2008). The acute activation of AKT in fasted adult muscle and muscle cell cultures leads to an inhibition of autophagosome formation, halts the conversion of LC3 from the cytosolic form (LC3I) to the lipidated membrane bound form of LC3 (LC3II), and prevents the rise in transcript levels of select autophagy genes (including, LC3, Gaparapl1 and Bnip3) (Mammucari et al. 2007). Conversely, even with AKT hyperactivity, mouse muscles depleted of Raptor have sustained autophagic activity (even during normal fed conditions), which implies that mTORC1 inactivation may also be key driver of autophagic activity that does not rely on FoxO activation (Bentzinger et al. 2008).

To further complicate autophagy signalling in skeletal muscle, upstream mTORC2-mediated inhibition of AKT has also been implicated in autophagy (not shown), whereby rictor knockdown via RNAi, significantly induces autophagosome formation in
skeletal muscle (Mammucari et al. 2007). The role of mTORC2 as mediator of autophagy however requires further research and is thus not discussed further.

### 1.6.2.3 Autophagy, ageing and sarcopenia

Age-related changes to autophagy and a role in sarcopenia are yet to be fully characterised, despite proposals that a decline in key regulatory proteins of autophagy lead to a general imbalance between the rates of protein damage and protein turnover in skeletal muscle. These changes have been shown, in part, to reflect deficiencies in the formation of autophagosomes, with a majority of studies demonstrating a down-regulation of LC3I and II, or reduced LC3II/I ratios in skeletal muscles of ageing rodents and humans (McMullen et al. 2009; Wohlgemuth et al. 2010; Fry et al. 2013; Carnio et al. 2014). Decreased expression and/or protein amounts of LAMP2 (Wohlgemuth et al. 2010; Kim et al. 2013) and an increase in p62 and/or ubiquitin protein amounts in aged muscles, suggest both the delayed fusion of autophagosomes with lysosomes and a subsequent increase of accumulated protein substrates in the muscle’s cytosol, respectively (Komatsu et al. 2007). Indeed, autophagy insufficiency is commonly shown to contribute significantly to the posttranslational modification of proteins and organelles in ageing cells, leading to an age-related accumulation of damaged cellular components, non-degradable lysosome-bound lipofuscin, damaged mitochondria, and indigestible protein aggregates (Terman et al. 2007) which can have a significant impact on muscle homeostasis. Changes to key regulatory markers of autophagy in aged skeletal muscle are further discussed in Chapter Three: Results Chapter 1.

### 1.7 Chronic exercise as a therapeutic intervention for sarcopenia

It is well established that reduction to physical activity contribute to age-related muscle loss and is associated with increased disability. Indeed, elderly humans who are physically inactive experience a sharper decline in skeletal muscle mass and function than those who remain physically active throughout life. Identification of effective interventions that preserve muscle mass and/or function in elderly individuals is an important public health challenge. Several types of exercise are recommended to reduce sarcopenia; 1) resistance (strength) training, 2) aerobic or endurance exercise and 3) flexibility and/or balance training (typically used in combination with endurance and resistance forms). The impact of both endurance (or aerobic) and resistance forms of exercise and their benefits towards the prevention of sarcopenia is discussed below (with further discussion in Results Chapters 2 - 6).

The physiological benefits of resistance exercise (also known as strength training) for the elderly are numerous and this has been highlighted as the best form of exercise to prevent sarcopenia and promote muscle hypertrophy. In humans (aged > 60), marked
gains in strength (Fiatarone et al. 1990; Pyka et al. 1994; Bamman et al. 2003; Melov et al. 2007; Leenders et al. 2013), muscle mass (whole muscle and myofibre CSA) (Pyka et al. 1994; Bamman et al. 2003; Leenders et al. 2013) and functional mobility (Fiatarone et al. 1990), have been observed after progressive resistance training programs ranging in duration from 8 weeks to 1 year. High intensity strength training consisting of concentric and eccentric lifting (up to 80% of 1RM), can also lead to significant gains in muscle size (mid thigh) and gait speed even among frail men and women aged up to the age of 96 years (Fiatarone et al. 1990).

Aerobic exercise in contrast has not typically received a lot of support as a countermeasure for sarcopenia, which likely results from the capacity of aerobic protocols to induce metabolic changes in muscle rather than increasing muscle mass and/or strength (Sipila & Suominen 1995; Jubrias et al. 2001a; Izquierdo et al. 2004). However several studies have demonstrated the potential role for endurance forms of exercise to prevent sarcopenia. In particular, 12 weeks of cycling (3-4 days/week) promotes similar increases in muscle size and strength among young and old men (aged 20 and 74 years) (Harber et al. 2012). Other aerobic training programs on a cycle ergometer ranging in duration from 12 weeks to 16 weeks (3/4 days/week) also observed increases to quadriceps CSA or upper leg muscle mass that ranges between 4 and 12% in old men (aged 75 years) and women (aged 70-71 years) (Harber et al. 2009; Konopka et al. 2010; Lovell et al. 2010). One study even observed a greater hypertrophic response in quadriceps muscles of old (aged 74 years) compared with young men engaged in 6 months of walking/jogging (5 days/week) (Schwartz et al. 1991).

While increases to muscle strength and hypertrophy, brought on by increased protein accretion are the main phenotypic outcomes of resistance (and some endurance) exercise programs in young and old humans, both types of training bestow multiple physiological benefits on skeletal muscle. Perhaps the most pronounced changes in the elderly, includes increases to intramuscular mitochondrial content and function.

### 1.7.1 Mitochondrial adaptations

Endurance exercise of sufficient intensity, duration and frequency (at least 60% of VO$_{2\ max}$ for 12 – 24 weeks at 3/4 sessions/week) can significantly increase aerobic capacity in elderly men and women (ranging in age from 65 to 80 years) (Conley et al. 2013; Konopka et al. 2013). Increases to intramuscular protein amounts of mitochondrial biogenesis markers (including peroxisome proliferative activated receptor gamma coactivator 1 alpha (PGC-1α), citrate synthase, succinate dehydrogenase (SDH) and cytochrome c oxidase, subunit IV (COX4)) (Konopka et al. 2013), mitochondrial quality
control via fusion and fission (Mitofusin 1 and 2, and Fission 1) (Konopka et al. 2013) and capacity for increased oxidative phosphorylation (or increase in maximal mitochondrial ATP production) (Conley et al. 2013) contribute to these improvements in aged muscle. Similarly, reports in aged male rats (aged 26 m) demonstrate that 6 weeks of treadmill running (at 60% of VO$_2$ max) for 60 minutes a day is sufficient to increase mitochondrial biogenesis, as demonstrated by increased PGC-1α, SDH and COX4 gastrocnemius protein content, increased mtDNA abundance and citrate synthase activity, as well as the restoration of fusion and fission protein amounts to a similar level observed in young (aged 3 m) rats (Koltai et al. 2012).

Resistance training by contrast is not as commonly shown to promote improvements to mitochondrial function. However, among elderly humans (average age of 68.5 years) resistance exercise for 14 weeks (at 80% of 1RM, 3 times weekly) has shown to increase complex 4 oxidative enzymes in the vastus lateralis muscle (which reflects an increase efficiency of the electron transport chain) and reduced levels oxidative damage to DNA (Parise et al. 2005). Moreover, Melov et al. (2007) demonstrated that the mitochondrial transcriptome signatures of elderly individuals (aged 65 – 80 years) following 24 weeks of whole body resistance training (2 times weekly, up to 80% of 1RM), was altered to more closely resemble that of younger muscles (aged 18 – 27 years) (Melov et al. 2007).

1.7.2 Protein turnover

While increased and/or maintained muscle mass is the goal of long-term exercise regimes in ageing muscle, very few studies have investigated the intracellular signalling pathways associated with protein synthesis and degradation. A review of this literature has been included below.

1.7.2.1 AKT/mTORC1 and protein synthesis

There are very few studies in elderly humans and rodents that have explored the response of the AKT/mTORC1 protein synthesis pathway following chronic training regimes. However, following 12 weeks of resistance exercise (up to 75-80% of 1RM; 3 times weekly) similar levels of AKT(Ser473) (Farnfield et al. 2011), S6K1(Thr389) (Farnfield et al. 2011; Li et al. 2012) and rpS6(Ser235/236) (Farnfield et al. 2011), 4E-BP1(Thr37/46) (Li et al. 2012) phosphorylation rates have been reported in the vastus lateralis muscles of aged men (60 – 86 years), as well as younger individuals (18 – 41 years). Protocols in rodents measuring anabolic signalling in aged muscle following long-term resistance protocols in contrast are limited and inconsistent. Luo et al. (2013) demonstrated that 9 weeks of tail weighted resistance training (1 m ladder at 85 degree incline, with increasing tail weights) decreased AKT(Ser473) and mTOR(Ser2448)
phosphorylation (Luo et al. 2013). While in the gastrocnemius muscles of male B6C3F1 mice (aged 16 – 19 m) endurance wheel running for 3 m increased activity of AKT(Ser473) and total amounts of mTOR, with no change in S6K1 total protein. No changes to phosphorylation rates of downstream mTORC1 effectors were reported (Reynolds et al. 2004). Accordingly, treadmill training (up to 60 min at 28 metres per minute) significantly increased mTOR(Ser2448) phosphorylation after 8 weeks, when exercise was performed 5 days per week, however no total mTOR was measured to reflect overall activation rates, and no measurements of downstream mTORC1 markers were reported (Pasini et al. 2012). Thus further research is required to adequately determine the activity of AKT and mTORC1 signalling in aged muscles in response to long-term exercise interventions.

1.7.2.2 Protein degradation: autophagy and the UPP

Data concerning the effect of exercise on the UPP in aged skeletal muscle are also limited, although at least in humans appear to be in agreement. In human muscle, high intensity resistance training in elderly women (aged 76 - 86), 3 days/week over 12 weeks did not induce any changes in MuRF1 and Atrogin-1 mRNA relative to age-matched sedentary controls (Williamson et al. 2010; Greig et al. 2011). Similar findings have been reported following 12 weeks of aerobic exercise (20 - 45 minutes per session, 3-5 days/week) in elderly women (average age 70 years) (Konopka et al. 2010). Life-long exercise in elderly men (aged 65 – 79 years), with self-reported activity levels over the past 30 years, also showed that expression levels of MuRF1 and Atrogin-1 were similar, compared to healthy seniors who only undertook routine household activities throughout the same time period (aged 65 – 74 years) (Zampieri et al. 2014).

In contrast to the UPP, autophagy is markedly affected by exercise. It should be noted that a majority of available data exploring the effects of chronic exercise on the variety of skeletal muscle types has been mainly obtained from rodent studies. Indeed, EDL and gastrocnemius hypertrophy following 8 weeks of treadmill running (40 min per day, 5 days/week at a 5° incline) in 12 m ICR mice has been shown to increase protein amounts of LC3II in both muscles, as well as Beclin-1 and Atg7 in EDL (Kim et al. 2013). Life-long wheel running in male Fischer 344 rats (from 11 weeks to 24 m of age) lead to an upregulation of lysosomal associated membrane 2 (LAMP-2) expression, and Atg7 and Atg9 protein amounts, concurrent with increased plantaris muscle weight (Wohlgemuth et al. 2010). Similarly, 9 weeks of tail weighted resistance training (1 m ladder at 85 degree incline, with increasing tail weights) increased Beclin-1, Atg5, Atg7 and decreased p62 in hypertrophied gastrocnemius muscles of aged male Sprague Dawley rats (18 – 20 m old) (Luo et al. 2013). It has been proposed that the training
induced increase of autophagy in ageing muscle acts to recycle damaged mitochondria and organelles, as well as misfolded proteins brought about by increased contractile activity.

1.8 **Significance of this thesis**

Thus, in light of the literature reviewed above this thesis aims to characterise protein synthesis and degradation pathways in ageing skeletal muscle and further investigate the ability of exercise (both endurance and resistance forms) to attenuate sarcopenia. Specific aims and hypothesis tested in this thesis are listed below.
THESIS AIMS

Overall aims:

1. Understand age-related changes in the pathways that regulate protein synthesis and degradation in murine skeletal muscles, as these pathways may become targets for potential therapeutic interventions for sarcopenia.

2. Assess the efficacy of voluntary wheel running protocols (both unloaded and with resistance) in mouse models of ageing, and their ability to prevent sarcopenia in old mice.

Specific aims:

1. Analyse the activity of anabolic (IGF-1/insulin/mTORC1) and catabolic (UPP and autophagy) signalling pathway in sarcopenic mouse muscles under both fed and fasted conditions (Results Chapter 1).

2. Examine the effects of muscle-specific IGF-1 (Class 2: Ea) overexpression, combined with life-long unloaded voluntary wheel exercise, as therapeutic interventions for sarcopenia (Results Chapter 2).

3. Develop a protocol of voluntary resistance wheel exercise to test the running ability of very old mice and assess the efficacy of this protocol to promote muscle hypertrophy and improve muscle function when used as a short-term intervention later in life (Results Chapter 3).

4. Evaluate the efficacy of voluntary resistance wheel exercise when initiated from mid-life (prior to sarcopenia onset), to attenuate sarcopenia and to prevent potential changes in the signalling pathways in muscles associated with normal ageing in sedentary mice (Results Chapter 4).
THESIS HYPOTHESES

Overall Hypotheses:

1. The dysregulation of protein turnover plays a fundamental role in the development and progression of sarcopenia and is associated in changes in related signalling pathways in old muscles.

2. Overexpression of IGF-1 as well as exercise can ameliorate sarcopenia.

Specific hypotheses:

1. Signalling pathways that drive protein turnover are impaired in ageing skeletal muscle and contributes to an accumulation of damaged and malfunctioning proteins (Results Chapter 1).

2. Local overexpression of the IGF-1 Class 2: Ea isoform in skeletal muscle and/or lifelong unloaded voluntary wheel exercise will prevent sarcopenia (Results Chapter 2).

3. A short-term, voluntary resistance wheel exercise protocol will be sufficient to induce muscle hypertrophy even when initiated in very old mice (Results Chapter 3).

4. Initiation of voluntary resistance wheel exercise prior to the onset of sarcopenia will prevent sarcopenia with associated benefits at the molecular level for protein homeostasis (Results Chapter 4).
CHAPTER TWO: GENERAL MATERIALS AND METHODS

This chapter records all materials and methods used in the main study chapters.

2.1 Animals

2.1.1 Maintenance

All strains of mice were obtained from breeding colonies established at the Animal Resources Centre (ARC), Perth, Western Australia, and housed under pathogen free conditions at (1) the Pre-Clinical Facility (PCF) at the University of Western Australia or (2) AgResearch Ltd. Hamilton, New Zealand. Mice were maintained on a 12-h light-dark cycle, at 22°C, both individually and in groups (see specific chapters), in standard cages, with free access to meat-free rat and mouse diet (protein, 20%; total fat, 4.8%; total fibre, 28.8%; total carbohydrate, 59.4%) fortified with vitamins and minerals (Specialty Feeds, Perth, Western Australia), and free access to drinking water. All animal procedures were approved and conducted in accordance with the guidelines of the University of Western Australia Animal Ethics Committee, National Health and Medical Research Council, Australia and the Ruakura Animal Ethics Committee, Hamilton, New Zealand.

2.1.2 Strains

C57BL/6J. Age-related changes to skeletal muscle were studied in the C57BL/6J mouse strain. According to lifespan data available from the Jackson Laboratory and prior publications (Chai et al. 2011; Sheard & Anderson 2012; Sandri et al. 2013; Soffe et al. 2016). C57BL/6J mice have an average lifespan of just over 2 years (www.jax.org/research-and-faculty/research-labs/the-harrison-lab/gerontology/life-span-as-a-biomarker), with 50% survival by 28 months and with 24 m being recommended as the upper limit for reliable ageing studies. For comparison, a 24-month-old mouse is roughly the equivalent of a 70-year-old human (Flurkey et al. 2007), see Figure 1.5.

IGF-1(C2:Ea) transgenic mice. Transgenic IGF-1(C2:Ea) mice were generated at the European Molecular Biology Laboratory, Rome, Italy by N. Winn and N. Rosenthal. The MLC/IGF-1(C2:Ea) expression construct was generated by cloning the mouse IGF-1(C2:Ea) cDNA sequences into the previously described skeletal muscle-specific expression cassette containing the myosin light chain (MLC) 1/3 promoter, a SV40 polyadenylation signal, followed by the MLC1/3 enhancer sequence (Rosenthal et al. 1989; Musarò et al. 2001; Shavlakadze et al. 2010). FVB mice were used as embryo donors. Positive founders were bred to FVB wild-type mice and positive transgenic mice were selected by PCR from tail digests. Littermate null (FVB) mice were used as controls. The IGF-1(C2:Ea) (FVB background) heterozygous colony was established at the Animal Resource Centre in Perth, Western Australia under pathogen-free
conditions. Mice were shipped to AgResearch, Hamilton, New Zealand at 3 m of age, after one month of quarantine.

2.2 Resistance wheel exercise setup, Perth

Mice subjected to resistance wheel exercise were housed individually in Lafayette Mouse Activity Wheel Chambers (23.5 cm × 35.3 cm; Model 80820; Lafayette Instrument, IN, USA) equipped with a 12.7 cm diameter exercise wheel with a 5.72 cm wide running surface (Model 80820RW, Lafayette) and an adjustable servo-brake (Model 86070-B1) to control resistance application and wheel function (Figure 2.1). Each chamber was equipped with an activity wheel counter (Model 86070A) to monitor wheel revolution, distance travelled (set at 0.40 m/revolution) and speed (m/min). The Activity Wheel Monitoring (AWM) Software (Model 86065) was used to record all data sets. Wheel loading was determined by hanging known weights on each individual wheel and adjusting the brake to hold each selected weight (per manufacturer’s instructions). See Chapters 5 and 6 for specific loading protocols.

2.3 Voluntary wheel running (no resistance) setup, New Zealand

Mice exercising on running wheels with no resistance applied were housed individually in standard cages equipped with a 12 cm mouse wheel secured to the cage top with grip ties (Figure 2.2). A bidirectional bicycle computer (Union-10, Marwi Taiwan Industrial Co., Ltd) was mounted to a 10 cm length of PVC pipe (3.5 cm in diameter) with the surplus wire tucked inside the pipe to prevent mice from chewing the wire. A magnet was glued to the exterior of the wheel and in line with the transducer that was secured with grip ties to the top of the cage (Figure 2.2). Mice had free access to these wheels at all times. To record data for analysis, the monitors were reset to zero once each month and data for distance (km), duration (min), and the maximum and average speed (km/h) were recorded over a 24 h period (as described in McMahon et al.)
Chapter Two: General Materials and Methods

2.4 Animal anaesthesia

Mice sampled at UWA were killed by cervical dislocation while under terminal anaesthesia (2% v/v Attane isoflurane, Bomac, NSW, Australia, 400mL NO₂ and 1.5L O₂) and mice sampled at AgResearch, New Zealand were killed by CO₂ inhalation followed by cervical dislocation, due to differing animal ethics requirements.

2.5 Tissue sampling and processing for histology

Muscles were dissected out, cut transversely in the mid-region and mounted onto tragacanth gum (Sigma-Aldrich Pty Ltd, Sydney, Australia) and frozen in liquid nitrogen-cooled isopentane (Sigma-Aldrich Pty Ltd, Sydney, Australia). Frozen samples were stored at -80°C prior to processing. Transverse frozen sections (8 µm) were cut on a cryostat (CM3050, Leica Biosystems, Nussloch, Baden-Württemberg, Germany) at -20°C, collected onto charged slides (SF41296SP, SuperFrost Plus, Lomb Scientific Pty Ltd. NSW, Australia), air dried for 15 minutes at room temperature and stored at -20°C until processing.

2.5.1 Haematoxylin and eosin staining

Frozen sections were placed directly into Harris’ Haemotoxylin (HH500, Amber Scientific, Midvale, Western Australia) (15 sec) and washed in tap water. Haematoxylin stained slides were placed in 70% ethanol (3 min), stained with 0.1% eosin (10 sec), washed and dehydrated in 3 exchanges of 100% ethanol (3 min each) and passed through 3 exchanges of xylene (3 min each). Slides were mounted with DPX mountant for microscopy (06522, Sigma-Aldrich Pty Ltd, Sydney, Australia), air-dried and stored at room temperature.
2.5.2 Nicotinamide adenine dinucleotide nitro-blue tetrazolium (NADH-TR) staining

Frozen sections were warmed to room temperature, and circled with a DAKO wax pen. A 1:1 mixture of NBT (Nitro-Blue Tetrazolium) solution (2 mg/mL; NBT/0.05M TRIS pH 7.6) (N6876, Sigma-Aldrich Pty Ltd, Sydney, Australia) and NADH (Nicotinamide adenine dinucleotide, reduced) solution (1.6 mg/mL; NADH/0.05M TRIS pH 7.6) was added to tissue sections and incubated for 30 min at 37°C. Incubated sections were washed with three exchanges of tap or deionized water, and unbound NBT removed with three exchanges of 30, 60 and 90% acetone-deionized water in increasing, then decreasing concentration. Sections were left in 90% acetone until a faint purple cloud could be seen over the section, then sections were rinsed with three exchanges of tap or deionized water and mounted with DPX mountant for microscopy (06522, Sigma-Aldrich Pty Ltd, Sydney, Australia), air-dried and stored at room temperature.

2.5.3 Masson's trichrome stain

Frozen muscle sections were rehydrated for 1 min in dH2O. Sections were stained in Weigert’s Haemotoxylin (1:1 dilution of Solution A: 1% alcoholic (95% EtOH) haematoxylin and Solution B: 30 ml 40% (w/v) FeCl3, 10 mL conc. HCL made up to 1L with dH2O) for 10 min, followed a 3 x 2 min rinse in dH2O. Slides were then transferred into Ponceau-Fuchsin (1% Ponceau 2R in 1% acetic acid and 1% Acid Fuchsin in 1% acetic acid) for 5 min, and the excess rinsed in tap water (3 x 2 min washes) before collagen staining was differentiated in a 1% (w/v) mixture of phosphomolybdic acid in distilled water (until collagen no longer appears red). Sections were then rinsed in 3 x 2 min in dH2O, placed in a 1% (w/v) of Light Green in 1% acetic acid for 1 min and then rinsed in 1% acetic acid for 1 min, before blotting the excess carefully with filter paper. Sections were dehydrated in 3 exchanges of 100% ethanol (3 min each) and passed through 3 exchanges of xylene (3 min each). Slides were mounted with DPX mountant for microscopy (06522, Sigma-Aldrich Pty Ltd, Sydney, Australia), air-dried and stored at room temperature.

2.5.4 BODIPY 493/503 stain

Frozen muscle sections were fixed in 4% paraformaldehyde for 20 minutes at room temperature and then washed 3 x 1 min in PBS. Slides were then incubated in 2 µg/mL of BODIPY (493/503) (Invitrogen, Australia) in PBS for 30 minutes (taken from the stock solution of 2 mg/mL BODIPY (493/503) diluted in Ethanol) and washed 3 x 1 min in PBS. 5 µl/mL Hoechst 33258 (861405, Sigma-Aldrich Pty Ltd, Sydney, Australia) in PBS was added to each section for 2 min and then rinsed in 3 x 1 min in PBS. Slides were mounted using DAKO (S3023, Carpinteria, CA, USA). Fluorescent stained
sections were captured using a Nikon Eclipse Ti inverter microscope equipped with Nikon DS-Fi2 camera (Nikon Corporation, Tokyo, Japan). Images were captured using NIS-Elements BR 4.1 software. Morphometric analyses were carried out with ImagePro Plus 4.5 (Media Cybernetics, MD, USA) software.

2.5.5 Immunofluorescence staining

Frozen sections were warmed to room temperature and rehydrated in PBS. Sections were blot dried and circled with a DAKO wax pen. Nonspecific antibody binding was blocked with 5% (v/v) foetal calf serum diluted in PBS for 1 h at room temperature. Polyclonal rabbit anti-PAN Laminin antibody (L9393, Sigma-Aldrich Pty Ltd, Sydney, Australia) was diluted in 0.5% BSA-PBS 0.1 M glycine (1:300) was applied to the sections and incubated overnight at 4°C, then washed 3 x 5 min in PBS. Primary antibody was omitted on the control sections of each slide. The primary antibody was detected by goat anti-rabbit ALEXA594 (A-11012, Invitrogen, Molecular Probes, Oregon, USA) diluted in 0.1% BSA-PBS (1:500) for 1 h at room temperature and followed by 3 x 5 min washes in PBS. Slides were mounted using DAKO (S3023, Carpinteria, CA, USA). Fluorescent stained sections were captured using a Nikon Eclipse Ti inverter microscope equipped with Nikon DS-Fi2 camera (Nikon Corporation, Tokyo, Japan). Images were captured using NIS-Elements BR 4.1 software. Morphometric analyses were carried out with ImagePro Plus 4.5 (Media Cybernetics, MD, USA) software.

2.6 Image acquisition

Tiled images of transverse muscle sections stained with H&E, NADH-TR and Laminin were captured at 10x magnification using a Nikon Eclipse Ti inverter microscope equipped with Nikon DS-Fi2 camera (Nikon Corporation, Tokyo, Japan) for bright field imaging (H&E and NADH-TR) and CoolSNAP EZ camera (Roper Scientific Photometrics, Ottobrunn, Germany) for fluorescence imaging (laminin). Images were captured using NIS-Elements BR 4.1 software. Non-tiled images of transverse muscle sections were captured at 40 × magnification using a Nikon 90i microscope equipped with Nikon DS-Fi2 camera. Images were captured using NIS-Elements AR 3.0 software (Laboratory Imaging Ltd., Czechoslovakia). Colour enhancements on H&E and laminin images were performed using Adobe Photoshop (Adobe Systems Incorporated) Version 7. All morphometric analyses were carried out with ImagePro Plus 4.5 (Media Cybernetics, MD, USA) software.
2.7 Immunoblotting

2.7.1 Protein extraction

Protein was extracted from quadriceps muscles as detailed in (Shavlakadze et al. 2013b). Muscles were ground in liquid nitrogen, and powder homogenised (9001271, Tissue Ruptor, Qiagen VIC, Australia) in ice-cold PBS, 1% NP40, 1mM EDTA buffer, supplemented with complete EDTA free protease inhibitor and PhosSTOP phosphatase inhibitor tablets (Roche, Manheim, Germany), and centrifuged at 13,000g for 20 min at 4°C. The supernatant represents the 1% NP40 soluble protein fraction. Resultant pellets were resuspended in a buffer containing 20 mM HEPES (pH 7.5) and 4% SDS, supplemented with protease and phosphatase inhibitor tablets (Roche, Manheim, Germany) and solubilized by sonication 4 x 5 s bursts at 40% amplitude (VCX130, Vibra Cell, Sonics & Materials Inc.) followed by centrifugation at 19,600xg for 10 min at 16°C.

2.7.2 Protein quantitation

The soluble protein fraction was quantified using a Bradford Protein Assay. Protein samples were quantified with reference to BSA protein standards of known concentrations using acetylated BSA as stock (10 mg/ml). Dilutions (100, 200, 300, 400 and 500 µg/mL) were prepared by serial dilutions of BSA (1 mg/mL in 0.01 M PBS). Each standard (10 µl) was aliquoted in duplicate into a 96-well-plate. Protein samples were diluted 1:20 in PBS and each sample was then aliquoted in triplicate onto the same plate. Bio-Rad solution (200 µl) (500-0006, Bio-Rad, NSW, Australia) was added to each well, incubated for 5 min on a shaker at room temperature and measured at 595 nm absorbance using absorbance reader (EL808, BioTek Instruments, Inc., Winooski, VT, USA). Concentrations of protein samples were extrapolated from the BSA standard curve.

Insoluble protein fractions were quantified with DC™ protein assay (5000112, Bio-Rad, NSW, Australia). Protein samples were quantitated with reference to BSA protein standards using acetylated BSA as stock (10 mg/mL). Dilutions (100, 200, 300, 400 and 500 µg/mL) were prepared by serial dilutions of BSA (1 mg/mL in Insoluble Extraction Buffer (as per above)). Each standard (40 µl) was aliquoted in duplicate into a 96-well-plate. Protein samples were diluted 1:20 in Insoluble Extraction Buffer and each sample was then aliquoted in triplicate onto the same plate. Reagent A (25 µL), followed by 150 µl of Reagent B was added to each well, incubated for 5 min on a shaker at room temperature and measured at 750 nm absorbance using an absorbance reader (EL808, BioTek Instruments, Inc., Winooski, VT, USA). Concentrations of protein samples were extrapolated from the BSA standard curve.
2.7.3 Sample preparation

Protein samples (soluble and insoluble) were prepared by adding 3x protein loading buffer (0.19 M TRIS pH 6.8; 6% (w/v) SDS, 30% (v/v) glycerol, 0.03% (w/v) Bromophenol Blue, 0.3 M DTT) and then denatured at 95°C for 5 min. Extracted protein (10-20 µL) was then resolved on 4-15% SDS-PAGE TGX gels in 1 x Tris/Glycine/SDS Electrophoresis Buffer (1610771, Bio-Rad, NSW, Australia) at 250 volts for 20 mins. Precision Plus Protein Kaleidoscope prestained protein standards (#1610375, Bio-Rad, NSW, Australia) (5 µL per well) were included on each gel. Protein was transferred onto nitrocellulose membranes (170-4158, Bio-Rad, NSW, Australia) using the Trans Turbo Blot system (Bio-Rad, NSW, Australia) (BioRad protocol list; mixed molecular weight (2.5A – 25V – 7 min)). Ponceau S (P7170-1L; Sigma) was used to check the quality of protein transfer and to act as a loading control for insoluble protein fractions as normal loading controls (GAPDH and tubulin) were not applicable.

2.7.4 Protein localisation

Following transfer, nitrocellulose membranes were washed 3 x 5 min in TBST (20 mM TRIS, 150 mM NaCl, 0.1% Tween-20, pH 7.5). Nonspecific antibody binding was blocked with 5% (w/v) skim milk in TBST for 1 h at room temperature. Excess milk was rinsed in three exchanges of TBST (5 min each), and primary antibodies diluted in 5% BSA (w/v) in TBST were added to each membrane and incubated overnight at 4°C. Primary antibodies used and their recommended dilutions for immunoblotting are shown in Table 2.1. Following incubation, membranes were washed 3 x 5 min in TBST. Donkey anti-rabbit, donkey anti-mouse, and rabbit anti-goat HRP-conjugated antibodies (Thermo Fisher Scientific, MA, USA) diluted 1:10000 in 5% (w/v) skim milk in TBST were used to detect primary antibodies (Table 2.2). Membranes were incubated in secondary antibody for 1 h at room temperature, followed by 3 x 5 min washes in TBST. Chemiluminescence signal was detected using the Luminata Classico HRP substrate, Luminata Crescendo HRP substrate (WBUC0100; WBLUR0100, Merck Millpore, Billerica, MA, USA) or the Western Lightning Ultra Extreme Sensitivity Chemiluminescence Substrate (NEL111001EA, Perkin Elmer, Waltham, BA, USA) for proteins in high, medium or low abundance, respectively. Chemiluminescence signal was captured using the ChemiDoc MP Imaging System (Bio-Rad, NSW, Australia) and digital images generated.

2.7.5 Membrane stripping and re-probing

Membranes were washed 3 x 5 min in TBST to remove the chemiluminescent substrate, then incubated in stripping buffer (21059, Restore Western Blot Stripping Buffer; Thermo Scientific, MA, USA) for 15 min at room temperature, washed 3 x 5 min
in TBST, re-blocked in 5% milk (w/v) in TBST, and probed with primary antibody. Stripping protocols were only used for the detection of loading controls (GAPDH and Tubulin) or total (but not phosphorylated) proteins listed in Table 2.1.

### Table 2.1. List of primary antibodies used for immunoblotting

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Host Species</th>
<th>Dilution</th>
<th>Catalogue Number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-AKT (Ser473)</td>
<td>Rabbit (Polyclonal)</td>
<td>1:1000</td>
<td>9271</td>
<td>Cell Signalling Technology (CST) (Danvers, Massachusetts)</td>
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<tr>
<td>Total AKT</td>
<td>Rabbit (Polyclonal)</td>
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<td>9272</td>
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<tr>
<td>Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E)</td>
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<td>Phospho-S6 Ribosomal Protein (Ser240/244) (D6878)</td>
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<tr>
<td>S6 Ribosomal Protein (5G10)</td>
<td>Rabbit (Polyclonal)</td>
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<tr>
<td>Phospho-p44/42 MAP Kinase (Thr202/Tyr204)</td>
<td>Rabbit (Polyclonal)</td>
<td>1:1000</td>
<td>9101</td>
<td></td>
</tr>
<tr>
<td>P44/42 MAP Kinase</td>
<td>Rabbit (Polyclonal)</td>
<td>1:1000</td>
<td>9102</td>
<td></td>
</tr>
<tr>
<td>Phospho-4E-BP1 (Thr37/46) (236B4)</td>
<td>Rabbit (Polyclonal)</td>
<td>1:1000</td>
<td>2855</td>
<td></td>
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<tr>
<td>Phospho-4E-BP1 (Ser65)</td>
<td>Rabbit (Polyclonal)</td>
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<td>9451</td>
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<tr>
<td>4E-BP1</td>
<td>Rabbit (Polyclonal)</td>
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<td>Phospho-p70 S6 Kinase (Thr389)</td>
<td>Rabbit (Polyclonal)</td>
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<td>9205</td>
<td></td>
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<tr>
<td>p70 S6 Kinase</td>
<td>Rabbit (Polyclonal)</td>
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<td>9202</td>
<td></td>
</tr>
<tr>
<td>Phospho-ULK1</td>
<td>Rabbit (Polyclonal)</td>
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<td>6888</td>
<td></td>
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<tr>
<td>ULK1 (D8H5)</td>
<td>Rabbit (Polyclonal)</td>
<td>1:1000</td>
<td>8054</td>
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<tr>
<td>LC3B</td>
<td>Rabbit (Polyclonal)</td>
<td>1:1000</td>
<td>2775</td>
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<tr>
<td>SQSTM1/p62</td>
<td>Rabbit (Polyclonal)</td>
<td>1:1000</td>
<td>5114</td>
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<tr>
<td>MuRF1/Trim63</td>
<td>Goat (Polyclonal)</td>
<td>1:2000</td>
<td>AF5366</td>
<td>R&amp;D Systems (Minneapolis, USA)</td>
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<tr>
<td>Anti-α-Tubulin (Clone B-5-1-2)</td>
<td>Mouse (Monoclonal)</td>
<td>1:2000</td>
<td>T5168</td>
<td>Sigma-Aldrich Pty Ltd (Sydney, Australia)</td>
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<tr>
<td>GAPDH (14C10)</td>
<td>Rabbit (Monoclonal)</td>
<td>1:1000</td>
<td>2118</td>
<td>CST</td>
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### Table 2.2. List of secondary antibodies used for immunoblotting

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Catalogue Number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Goat F(ab)2 Fragment Specific</td>
<td>31403</td>
<td>Thermo Fisher Scientific, MA, USA</td>
</tr>
<tr>
<td>Anti-Mouse IgG (H+L)</td>
<td>31430</td>
<td></td>
</tr>
<tr>
<td>Anti-Rabbit IgG (H+L)</td>
<td>31460</td>
<td></td>
</tr>
</tbody>
</table>

#### 2.7.6 Image analysis

Resultant images were converted into a TIFF format and densitometry performed using ImageJ software. A common sample was always loaded onto each gel to normalize for detection efficiencies across membranes. Proteins that were immunoblotted on the
2.8 RNA extraction and quantitative real time PCR (qRT-PCR)

RNA was extracted using the RNeasy® Fibrous Tissue Mini Kit (74704, Qiagen VIC, Australia). In brief, 20-30 mg of ground quadriceps tissue powder was homogenised in 300 µL of Buffer RLT (1:100 β-mercaptoethanol) (9001271, Tissue Ruptor, Qiagen VIC, Australia). RNase-free water (590 µL) and of proteinase K (10 µL) was then added to each homogenate, mixed and then incubated at 55°C for 10 min, followed by centrifugation at 10 000 x g for 3 min. Supernatant was transferred to a new tube, and supplemented with 0.5 volumes of 96-100% ethanol followed by gentle mixing. Lysate (700 µL) was transferred to an RNeasy Mini column and centrifuged for 15 s at 8 000 xg, where the flow though was discarded, and the entire process repeated until all lysate was used. DNase 1 (10 µL) in RDD Buffer (70 µL) was then added to the RNeasy membrane and incubated at room temperature for 15 min. Columns were washed with Buffer RW1 (350 µL) and centrifuged for 15 s at 8 000 xg and the flow through discarded. Buffer RPE (500 µL) was then added to the RNeasy column, centrifuged at 8 000 g for 15 s and the flow-through discarded. This step was then repeated, with centrifugation increased to 2 min at 8 000 xg. The RNeasy column was placed in a new 1.5 ml tube and 40 µL of RNase-free water added and centrifuged for 1 min at 8 000 xg. RNA was aliquoted and stored at -80°C until required.

Quality and concentration of RNA was quantitated on the NanoDrop Spectrophotometer (ND-1000, NanoDrop, Wilmington, Delaware, USA). RNA (1 µg) was reverse transcribed using a QuantiTect Reverse Transcription Kit (205311, Qiagen, VIC, Australia). RNA was diluted in RNase free water to a total 12 µL. DNA Wipeout Buffer (2 µL) was added to each sample and placed in a thermal cycler (PTC-100 Programmable Thermal Controller, MJ Research Inc., St. Bruno (Quebec), Canada) for 2 min at 42°C. To each reaction, 1 µL of Quantscript Reverse Transcriptase, 4 µL of 5 x Quantscript RT Buffer, 1 µL RT Primer Mix and 1 µL of RNase free water was added (6 µL total), incubated at 42°C for 15 min, followed immediately by a 3 min incubation at 95°C to inactivate the Quantscript Reverse Transcriptase. The resultant cDNA was diluted with 100 µL of RNase free water and stored at 4°C until required.

QuantiTect primers purchased from Qiagen are listed in Table 2.3. qRT-PCR was performed using SYBR green chemistry (GoTaq qPCR Master Mix; Promega, Madison, WI, USA) on a Rotorgene-Q qPCR thermal cycler (Qiagen, VIC, Australia). Gene expression in quadriceps muscles was normalized to the geometric mean of either
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Gapdh and Ppia or Tbp1 and Hprt expression values (Vandesompele et al. 2002; Barns et al. 2014).

Table 2.3. List of Qiagen qRT-PCR Primers

<table>
<thead>
<tr>
<th>Genes of Interest</th>
<th>Qiagen Catalogue Number</th>
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</thead>
<tbody>
<tr>
<td>Nicotinic acetylcholine receptor δ subunit (Chnrd)</td>
<td>QT00199472</td>
</tr>
<tr>
<td>Nicotinic acetylcholine receptor γ subunit (Chnrg)</td>
<td>QT00100268</td>
</tr>
<tr>
<td>Growth arrest and DNA damage-inducible 45 α (Gadd45α)</td>
<td>QT00249655</td>
</tr>
<tr>
<td>Muscle, skeletal, receptor tyrosine kinase (Musk)</td>
<td>QT00197792</td>
</tr>
<tr>
<td>Myogenin (Myog)</td>
<td>QT00112378</td>
</tr>
<tr>
<td>Runt-related Transcription Factor-1 (Runx1)</td>
<td>QT00100380</td>
</tr>
<tr>
<td>Muscle RING finger protein-1 (MuRF1)</td>
<td>QT00291991</td>
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<tr>
<td>Muscle atrophy F-box protein 32 (Fbxo32/Atrogin-1)</td>
<td>QT00158543</td>
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<tr>
<td>Sequestosome 1 (p62/SQSTM1)</td>
<td>QT00127855</td>
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<tr>
<td>Hypoxanthine phosphoribosyl-transferase 1 (Hprt1)</td>
<td>QT00166768</td>
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<tr>
<td>Peptidylprolyl isomerase A (Ppia)</td>
<td>QT00247709</td>
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<tr>
<td>TATA box binding protein (Tbp)</td>
<td>QT00198443</td>
</tr>
<tr>
<td>Glyceraldehyde-3 phosphate dehydrogenase (GAPDH)</td>
<td>QT01658692</td>
</tr>
</tbody>
</table>

Standards for each primer set were made using the Wizard® SV Gel and PCR Clean-up System (A9281/2/5, Promega, Madison, WI, USA). Membrane Binding Solution (50 µL) was added to 50 µL of PCR product, mixed well, added to the Wizard® SV minicolumn, centrifuged at maximum speed (19 000 xg) for 1 min and the flow through discarded. Membrane Wash Solution (700 µL) was added to the spin column and centrifuged for 1 min at maximum speed, followed by an additional 400 µL of Membrane Wash Solution and centrifugation for 1 min. To a new tube, 50 µL of dH₂O was added to the column membrane and centrifuged for 1 min at maximum speed. Total DNA was quantitated on the NanoDrop Spectrophotometer (ND-1000, NanoDrop, Wilmington, Delaware, USA) and a 10-point standard curve generated from serial dilutions of total product.

2.9 Citrate synthase activity

Citrate synthase (CS) activity was measured in both quadriceps and gastrocnemius muscles as described elsewhere (Terrill et al. 2013). In brief, snap frozen muscles were ground in liquid nitrogen and homogenized in a buffer containing 5 mM HEPES (pH 8), 1 mM EGTA, 5 mM MgCl₂, 1 mM dithiothreitol, and 0.1% TritonX-100. After centrifugation, protein concentrations were measured using the DC protein assay (Bio-Rad, NSW, Australia) as above, and all samples were frozen at −80°C until analysis. Samples were defrosted and aliquoted into a 96 well plate. Tris buffer (0.1 M, pH 8) was added to each well, and both a 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) solution
and acetyl CoA solution added and the plate vortexed. A solution containing oxaloacetate was added to initiate the reaction and assayed immediately; absorbance was recorded at 412 nm every 30 s for 5 min. The protein concentration of each sample was again measured using the DC protein assay, to give a measurement of nmol.min\(^{-1}\).mg protein\(^{-1}\) as described above in section 2.9.2.