Functional significance of TRIM35 in haematopoietic and hepatic homeostasis

Submitted by

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ABSTRACT

Tri-partite motif (TRIM) proteins belong to a large family of highly conserved proteins. Their biological activity involves cellular proliferation, differentiation and immunity, and consequently they are associated with many different pathological conditions. The majority of TRIM proteins are functional E3 ubiquitin ligases with ubiquitination activity mediated through their RING domains. Ubiquitination is a post-translational modification that can regulate protein activity in several ways, making it a powerful cellular regulatory mechanism.

The focus of this thesis is TRIM35, a novel E3 ubiquitin ligase. TRIM35 was initially discovered during a spontaneous in vitro erythroid-to-myeloid lineage switch, suggesting a role for TRIM35 as a regulator of haematopoietic lineage commitment. Subsequently, TRIM35 has been identified as a regulator of the innate immune system and a key biomarker in hepatocellular carcinoma (HCC). However, the molecular mechanisms underlying TRIM35 activity and its role in these biological functions was unclear and, therefore, formed the basis of this research.

This study demonstrated that TRIM35 is capable of binding and ubiquitinating several SMAD proteins, thereby influencing TGF-β signalling. Unexpectedly, the TRIM35-mediated ubiquitination of the SMAD proteins did not affect protein levels. Rather, this study showed a non-proteolytic function for TRIM35 in regulating the activity of the Co-SMAD, SMAD4, responsible for transduction of all TGF-β cytokine signals to the nucleus. TRIM35-mediated ubiquitination affected the affinity of SMAD4 for other binding partners, and the nuclear translocation dynamics of SMAD4 in response to TGF-β stimulation. These data highlight a novel regulatory mechanism of TGF-β signalling through TRIM35-mediated ubiquitination.

Notably, TGF-β signalling is important in regulating quiescence and self-renewal of haematopoietic stem cells and as TRIM35 has previously been associated with several aspects of haematopoiesis, the haematopoietic compartment of TRIM35-/- mice was analysed.
This research demonstrated that loss of *TRIM35* resulted in a significant increase in the stem and progenitor cell compartments. In addition, erythropoiesis and lymphopoiesis were affected by loss of *TRIM35* in haematopoietic compartments of aged mice.

A recent study revealed TRIM35 was significantly down-regulated in patients with HCC. Combined with previous research revealing TRIM35 as a regulator of immunity, this prompted an investigation into a link between TRIM35, inflammation and HCC. This study compared the effect of induced liver injury using a choline-deficient ethionine-supplemented (CDE) diet on *TRIM35/-* mice. The high-fat diet resulted in a significantly higher induction of the acute phase response, a liver-specific response to injury, in *TRIM35/-* mice as demonstrated by an increase in serum levels of IL-6 and expression of *SERPINE1*, an acute phase protein and a TGF-β target gene. The CDE diet showed that loss of *TRIM35* is not sufficient to increase the susceptibility of mice to tumour development. However, *TRIM35/-* mice showed significant increases in symptoms related to the anti-inflammatory response, including accumulation of fat in the liver in the form of nodules, liver fibrosis, and higher serum levels of the anti-inflammatory cytokine, IL-4. Fibrosis and IL-4 expression are both induced by TGF-β signalling, further supporting a role for TRIM35 in modulating this pathway.

Data presented here significantly contributes to our understanding of the mechanism underlying TRIM35 activity. These results indicate that TRIM35 can significantly influence the inflammatory response, potentially by modulating TGF-β signalling. Due to the extensive roles of inflammation and TGF-β signalling in auto-immune diseases and cancer pathology, the identification of TRIM35 as a regulator of these processes provides novel opportunities for therapeutic approaches.
DECLARATION

I declare that, to the best of my knowledge, the work presented in this thesis:

- comprises my own work unless stated otherwise;
- has not been previously submitted or accepted for any other degree at this or any other institution;
- was accomplished during my enrolment with the University of Western Australia;
- was accomplished in adherence to relevant ethic and OGTR guidelines, and does not in any way violate any copyright, trademark or patent;
- acknowledges all sources and facilities used.

Research involving animal data reported in this thesis was assessed and approved by the Perkins Institute Animal Ethics Committee. Approved ethics: #AE005, #AE020.

Ms. Jennifer Beaumont (Leukemia Research group, Perkins Institute) had previously generated all constructs used here. In addition, she performed tail vein injections of 5-FU in mice. Ms. Kirsty Richardson (senior animal technician at BioResources facility) was responsible for administering anaesthesia on mice prior and during MRI scans and again at end-of-experiment, where she also collected blood through cardiac puncture on live animals. Dr. Caryn Elsegood performed IHC stains on liver sections collected from mice in CDE model.

Joanne van Vuuren

Dr. Louise Winteringham
ACKNOWLEDGEMENT

This thesis is the product of an incredibly memorable few years that I was fortunate enough to spend at the Perkins Institute surrounded and supported by a great number of people. Without their contributions, this would not have been possible and I would therefore like to express my gratitude.

To my supervisors:

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To the liver cancer lab, Caryn, Adam, Ken, and Robyn, as well as colleagues at Perkins, Evan, Kirsty, and Tom, thank you for your support, advice and contributions.

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# TABLE OF CONTENTS

Abstract .................................................................................................................. 1  
Declaration .............................................................................................................. 3  
Acknowledgement .................................................................................................... 4  
Table of contents ..................................................................................................... 6  
Abbreviations .......................................................................................................... 11  
List of Figures .......................................................................................................... 15  
List of Tables .......................................................................................................... 17  

CHAPTER 1 General Introduction ............................................................................ 18  
1.1.Tri-partite motif (TRIM) family of proteins ..................................................... 19  
1.1.1.RING-finger domain ..................................................................................... 19  
1.1.1.1.Ubiquitination by RING finger E3 ligases ................................................ 21  
1.1.1.2.UBiquitin-mediated proteolysis: K48-G76 poly-ubiquitination .................. 24  
1.1.1.3.UBiquitin-mediated proteolytic ubiquitination: Mono-ubiquitination ........ 25  
1.1.1.4.UBiquitin-mediated proteolytic ubiquitination: K63-G76 poly-ubiquitination . 26  
1.1.2.B-box domain ............................................................................................ 27  
1.1.3.Coiled-coil domain ..................................................................................... 28  
1.1.4.C-terminus variations found in TRIM proteins ............................................ 29  
1.2.TRIM proteins in immunity .............................................................................. 30  
1.2.1.Antiviral activities of TRIM proteins ........................................................... 30  
1.2.2.TRIM proteins in innate immune responses ................................................ 31  
1.2.3.TRIM proteins are implicated in numerous different pathologies ................. 34  
1.3.TRIM35, a novel E3 ubiquitin ligase ................................................................. 36  
1.3.1.Discovery and characterization ................................................................... 36  
1.3.2.Role of TRIM35 in haematopoiesis and immunology .................................. 37  
1.3.3.TRIM35 as a tumour suppressor .................................................................. 37  
1.4.TGF-β signalling ............................................................................................. 39  
1.4.1.Components of the TGF-β pathway ............................................................... 40  
1.4.2.Inhibition of TGF-β signalling mediated by proteolytic ubiquitination .......... 42  
1.4.3.Non-proteolytic ubiquitination of TGF-β signalling ..................................... 43  
1.4.4.Regulation of TGF-β signalling by transcription factors .............................. 44  
1.5.Haematopoiesis ............................................................................................... 46  
1.5.1.General overview of haematopoiesis .......................................................... 46  
1.5.2.TGF-β induces quiescence in hematopoietic stem cells .............................. 47  
1.5.3.HSC subtypes increase complexity of TGF-β signalling ............................. 48  
1.6.Liver disease ................................................................................................... 49  
1.6.1.Fatty liver disease ....................................................................................... 49  
1.6.2.Liver regeneration ....................................................................................... 50  
1.6.3.Inflammation and fibrosis ......................................................................... 50
Chapter 2 Materials and Methods ................................................................. 56

2.1. Materials ............................................................................................... 57
  2.1.1. Antibodies and chemicals ................................................................. 57
  2.1.2. Plasmids ......................................................................................... 57
  2.1.3. Cell lines ....................................................................................... 57
  2.1.4. Commonly used reagents and buffers ........................................... 57

2.2. Methods ................................................................................................ 61
  2.2.1. Molecular biology ........................................................................... 61
    2.2.1.1. RNA extraction ........................................................................ 61
    2.2.1.2. RT^2-Profiler ........................................................................... 62
    2.2.1.3. Real-time PCR ........................................................................ 63
  2.2.2. Cell biology .................................................................................... 64
    2.2.2.1. Cell culture ............................................................................... 64
    2.2.2.2. Transfections .......................................................................... 64
    2.2.2.3. Immunofluorescence ............................................................... 64
  2.2.3. Protein analysis ............................................................................... 66
    2.2.3.1. Cell lysis .................................................................................. 66
    2.2.3.2. Co-immunoprecipitation ......................................................... 66
    2.2.3.3. SDS-PAGE .............................................................................. 67
    2.2.3.4. Western blotting ...................................................................... 67
    2.2.3.5. Mass spectrometry .................................................................. 68
  2.2.4. Animals and animal procedures ................................................... 69
    2.2.4.1. Animal housing and care ......................................................... 69
    2.2.4.2.5-Fluorouracil (5-FU) injections ............................................ 69
    2.2.4.3. Anaesthesia ............................................................................. 70
    2.2.4.4. Magnetic Resonance imaging (MRI) scans .............................. 70
    2.2.4.5. Special dietary animal chow .................................................... 71
    2.2.4.6. Monitoring of mice on high-fat and CDE diet .......................... 71
    2.2.4.7. Euthanasia .............................................................................. 72
    2.2.4.8. Cardiac puncture ..................................................................... 72
    2.2.4.9. Harvesting of bone marrow and spleens for haematology ....... 72
    2.2.4.10. Isolation of livers, spleens and pancreases and sample preservation 73
  2.2.5. Biochemical analysis mouse samples ............................................ 73
    2.2.5.1. Hemavet ............................................................................... 73
    2.2.5.2. Flow cytometry ...................................................................... 73
CHAPTER 3 TRIM35 is a novel regulator of TGF-β signalling

3.1.Introduction ........................................................................................................ 77
3.1.1.TRIM35-mediated proteolytic regulation of signalling ....................................... 78
3.1.2.Protosome-independent function of TRIM35 ......................................................... 79
3.1.3.Project aims ........................................................................................................ 79
3.2.Methods .................................................................................................................. 81
3.3.Results .................................................................................................................... 82
3.3.1.TRIM35 binds SMAD proteins .............................................................................. 82
3.3.2.TRIM35 ubiquitinates SMAD proteins .................................................................. 85
3.3.3.TRIM35 alters binding partners of SMAD4 ........................................................ 89
3.3.4.TRIM35 delays nuclear uptake of SMAD4 after TGF-β treatment ....................... 91
3.4.Discussion .............................................................................................................. 94

CHAPTER 4 Role of TRIM35 in Haematopoiesis ......................................................... 99
4.1.Introduction ........................................................................................................... 100
4.1.1.Haematopoietic stem cells (HSC) ...................................................................... 100
4.1.1.1.Isolation and characterization of HSCs ......................................................... 101
4.1.1.2.Characterization of LT- and ST-HSCs ............................................................ 102
4.1.2.Lineage-associated progenitors ........................................................................... 105
4.1.2.1.Common lymphoid progenitor ...................................................................... 105
4.1.2.2.Common myeloid progenitor ........................................................................ 106
4.1.3.Project aims ....................................................................................................... 107
4.2.Methods ................................................................................................................ 108
4.3.Results .................................................................................................................. 110
4.3.1.Analysis of LSK population in haematopoietic compartments of adult mice ....... 110
4.3.1.1. *TRIM35*−/− mice have higher percentage LSK cells compared to WT mice ...... 110
4.3.1.2. Compared to WT, *TRIM35*−/− mice have higher percentage of HPCs ............. 112
4.3.1.2.1. Compared to WT, 8-12 week old *TRIM35*−/− mice have significantly higher percentages HPCs ............................................................. 112
4.3.1.2.2. Spleens of aged *TRIM35*−/− mice have higher percentages of HSCs, MPPs and HPCs than WT mice ......................................................... 112
4.3.1.3.Recovery of LSK populations after 5FU treatment ........................................ 115
4.3.1.3.1.LSK population in WT and *TRIM35*−/− mice show same response after 5FU treatment .................................................................................. 115
4.3.1.3.2. No significant difference in response of HSCs, MPPs or HPCs to 5FU treatment between WT and TRIM35/- mice .......................................................... 115

4.3.2. Analysis of erythro-myeloid progenitor populations in TRIM35/- mice .......... 118

4.3.2.1. Myeloid progenitor populations are increased in TRIM35/- mice ............ 118

4.3.2.2. Erythro-myeloid progenitor recovery after 5FU treatment ..................... 120

4.3.2.2.1. Sca1+ cKit+ population in WT and TRIM35/- mice show same response after 5FU treatment ................................................................. 120

4.3.2.2.2. WT and TRIM35/- erythro-myeloid progenitors show no significant differences after 5FU treatment .................................................... 120

4.3.3. Analysis of downstream differentiation pathways in TRIM35/- mice ............ 123

4.3.3.1. Erythroid maturation in WT and TRIM35/- mice .................................. 123

4.3.3.1.1. TRIM35/- foetal livers have significantly higher EryC population compared to WT ................................................................. 123

4.3.3.1.2. Erythroid maturation in TRIM35/- mice aged 8-12 weeks is unaffected ...... 125

4.3.3.1.3. Compared to WT, BM of aged TRIM35/- mice have lower percentages of EryA/EryB but higher percentages of EryC cells ........................................ 125

4.3.3.2. B cell maturation in WT and TRIM35/- mice ........................................ 128

4.3.3.2.1. Spleens of young adult TRIM35/- mice have higher percentage of mature B cells than WT ................................................................. 128

4.3.3.2.2. BM of aged TRIM35/- contain smaller percentages than WT of pre/pro-B cells and mature B cells .................................................................. 128

4.3.3.3. No difference in circulating blood cells between WT and TRIM35/- mice ...... 131

4.4. Discussion .................................................................................................................. 134

CHAPTER 5 Role of TRIM35 in liver disease ................................................................. 139

5.1. Introduction ............................................................................................................... 140

5.1.1. Liver disease ....................................................................................................... 140

5.1.2. Animal models for studying liver disease ......................................................... 140

5.1.3. Project aims ......................................................................................................... 142

5.2. Methods ..................................................................................................................... 143

5.3. Results ...................................................................................................................... 144

5.3.1. Effect of loss of TRIM35/- on control mice ..................................................... 144

5.3.1.1. TRIM35/- mice gain significantly more weight than WT mice on control diet... 144

5.3.1.2. Liver morphology of WT and TRIM35/- mice diet ........................................ 146

5.3.1.3. Analysis of liver fibrosis in WT and TRIM35/- mice ........................................ 146

5.3.2. Effect of control diet on inflammation in WT and TRIM35/- mice ................. 149

5.3.2.1. Analysis of inflammation in livers of TRIM35/- mice and WT mice .......... 149

5.3.2.2. Peripheral blood analysis of WT and TRIM35/- mice ................................. 149

5.3.2.3. TRIM35/- mice have significantly higher levels of circulating IL-4 and IL-6..... 152

5.3.2.4. Analysis of spleens and pancreases of WT and TRIM35/- mice .................. 154

9
5.3.3. Response of WT and TRIM35-/– mice to the CDE diet ........................................... 156
5.3.3.1. Development of liver tumours in mice on CDE diet ........................................... 156
5.3.3.2. WT and TRIM35-/– mice gain weight at equal rates on the CDE diet .............. 158
5.3.3.3. TRIM35-/– mice develop larger fat nodules than WT mice .............................. 160
5.3.3.4. Liver histology of WT and TRIM35-/– mice ..................................................... 163
5.3.3.5. TRIM35-/– mice develop more liver fibrosis compared to WT mice .......... 165
5.3.4. Effect of CDE diet on inflammation in WT and TRIM35-/– mice ................... 167
5.3.4.1. CDE diet induced liver inflammation ............................................................ 167
5.3.4.2. Analysis of haematopoietic cells in peripheral blood of mice fed CDE diet ...... 167
5.3.4.3. Loss of TRIM35-/– in mice results in higher levels of circulating IL-4 .......... 170
5.3.4.4. Analysis of spleens and pancreases between WT and TRIM35-/– mice on CDE diet ........................................................................................................................................... 172
5.3.5. Effect of loss of TRIM35 on TGF-β signalling in mice fed HF or CDE diet ...... 174
5.3.5.1. Analysis of TGF-β target genes in livers of TRIM35-/– mice on control diet ...... 176
5.3.5.2. Analysis of TGF-β target genes in livers of TRIM35-/– mice on CDE diet .......... 178

5.4. Discussion .................................................................................................................. 180

CHAPTER 6 Discussion ..................................................................................................... 186
6.1. General discussion ................................................................................................... 187
6.1.1. TRIM35 regulates TGF-β signalling through non-proteolytic ubiquitination ...... 187
6.1.2. TRIM35 plays a role in maintenance of HSCs and early progenitors ............ 188
6.1.3. Modulation of inflammatory responses by TRIM35 during chronic liver injury ...... 191
   6.1.3.1. TRIM35 is a regulator of adipose-related pro-inflammatory responses .... 193
   6.1.3.2. TRIM35 regulates immune responses responsible for hepatic fibrosis .... 194
6.2. Conclusion .................................................................................................................. 196

Appendix A ...................................................................................................................... 197
Appendix B ...................................................................................................................... 198
Appendix C ...................................................................................................................... 199
Appendix D ...................................................................................................................... 200
Appendix E ...................................................................................................................... 201
Appendix F ...................................................................................................................... 204
Appendix G ...................................................................................................................... 203
Appendix H ...................................................................................................................... 204

REFERENCES ................................................................................................................. 205
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-Phenylindole, Dihydrochloride</td>
</tr>
<tr>
<td>A</td>
<td>Acini</td>
</tr>
<tr>
<td>ACRVL1</td>
<td>Activin A Receptor Like Type 1</td>
</tr>
<tr>
<td>APL</td>
<td>Acute Promyelocytic Leukemia</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>AFLD</td>
<td>Alcoholic Fatty Liver Disease</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AMH</td>
<td>Anti-Müllerian hormone</td>
</tr>
<tr>
<td>AGM</td>
<td>Aorta-Gonad Mesonephros</td>
</tr>
<tr>
<td>Bmper</td>
<td>BMP Binding Endothelial Regulator</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenic Protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CDE</td>
<td>Choline-Deficient, Ethionine-supplemented</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co-Immunoprecipitation</td>
</tr>
<tr>
<td>CLP</td>
<td>Common Lymphoid Progenitor</td>
</tr>
<tr>
<td>Co-SMAD</td>
<td>Common mediator SMAD</td>
</tr>
<tr>
<td>CMP</td>
<td>Common Myeloid Progenitor</td>
</tr>
<tr>
<td>CDKI</td>
<td>Cyclin-Dependent Kinase</td>
</tr>
<tr>
<td>CDKI</td>
<td>Cyclin-Dependent Kinase Inhibitor</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>DAMP</td>
<td>Damage-Associated Molecular Pattern</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic Stem Cell</td>
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<tr>
<td>EMCV</td>
<td>Encephalomyocarditis Virus</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-Mesenchymal Transition</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>FMF</td>
<td>Familial Mediterranean Fever</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
</tr>
<tr>
<td>gr</td>
<td>Gram</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage-Colony Stimulating Factor</td>
</tr>
<tr>
<td>GMP</td>
<td>Granulocyte-Macrophage Progenitor</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>HPC</td>
<td>Haematopoietic Progenitor Cell</td>
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<td>HSC</td>
<td>Haematopoietic Stem Cell</td>
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<tr>
<td>HLS5</td>
<td>Hematopoietic lineage switch 5</td>
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<tr>
<td>HBV</td>
<td>Hepatitis B Virus</td>
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<tr>
<td>HCV</td>
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<tr>
<td>HCC</td>
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<tr>
<td>HF</td>
<td>High-fat</td>
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<td>HEK293T</td>
<td>Human Embryonic Kidney 293T</td>
</tr>
<tr>
<td>HFV</td>
<td>Human Foamy Virus</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>I-SMAD</td>
<td>Inhibitory SMAD</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon Regulatory Factor</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IL</td>
<td>Islets of Langerhans</td>
</tr>
<tr>
<td>Jab1</td>
<td>Jun Activation domain Binding protein 1</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>LPC</td>
<td>Liver Progenitor Cells</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal Repeat</td>
</tr>
<tr>
<td>LT-HSC</td>
<td>Long-term Haematopoietic Stem Cell</td>
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<tr>
<td>Ly-HSC</td>
<td>Lymphoid-biased Haematopoietic Stem Cell</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage Colony-Stimulating Factor</td>
</tr>
<tr>
<td>MAIR</td>
<td>Macrophage-derived Apoptosis-Inducing RBCC</td>
</tr>
<tr>
<td>MH1</td>
<td>MAD Homology 1</td>
</tr>
<tr>
<td>MH2</td>
<td>MAD Homology 2</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility complex</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MDa</td>
<td>Megadalton</td>
</tr>
<tr>
<td>MEP</td>
<td>Megakaryocyte Erythrocyte Progenitor</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram/s</td>
</tr>
<tr>
<td>μl</td>
<td>Microlitre/s</td>
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<td>mL</td>
<td>Millilitre/s</td>
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<td>mM</td>
<td>Millimolar</td>
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</table>
MAD  Mothers Against Decapentaplegic
mTGF-β  mouse Transforming Growth Factor-β
MPP  Multi-Potent Progenitor
My-HSC  Myeloid-biased Haematopoietic Stem Cell
NK  Natural Killer cells
NAFLD  Non-Alcoholic Fatty Liver Disease
NS1  Non-Structural Protein-1
N-MLV  N-tropic Murine Leukaemia Virus
NF-κB  Nuclear factor-κB
PHx  Partial Hepatectomy
PAMP  Pathogen-Associated Molecular Patterns
PRR  Pattern Recognition Receptors
PBS  Phosphate-buffered Saline
PEP  Phosphoenol Pyruvate
pg  picogram
PHD  Plant Homeodomain
PAI-1  Plasminogen activator inhibitor-1
PTEFb  Positive Transcription Elongation Factor b
PGRN  Progranulin
PCNA  Proliferating cell nuclear antigen
PML  Promyelocytic Leukemia
PKM1  Pyruvate Kinase isoform M1
PKM2  Pyruvate Kinase isoform M2
RT-PCR  Realtime-Polymerase Chain Reaction
R  Red Pulp
RP  Regulatory Particle
R-SMAD  Regulatory SMAD
RPA  Replication Protein A
RARα  Retinoic Acid Receptor α
RNA  Ribonucleic acid
RLR  RIG-1-like Receptor
RBCC  RING, B-box, Coiled-coil motif
ST-HSC  Short-term Haematopoietic Stem Cell
Stat1  Signal Transducer and Activator of Transcription 1
SCJ  Sister Chromatid Junctions
SCF  Skp1, Cullin and F-box protein
Smurf  SMAD Ubiquitination Regulatory Factor 1
SDS Sodium Dodecyl Sulfate
SDS-PAGE Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis
SLE Systemic Lupus Erythematosus
TAK Tat-Associated Kinase
TβR TGF-β Receptor
tPA Tissue Plasminogen Activator
TLR Toll-Like Receptor
TIF1 Transcriptional Intermediary Factor 1
TLS Translesion Synthesis
TRIM Tri-partite Motif
TBS Tris-buffered Saline
TBST Tris-buffered Saline with Tween
TNF-α Tumour Necrosis Factor-α
Th1 Type 1 helper T-cell
Th2 Type 2 helper T-cell
uPA Urokinase Plasminogen Activator
VP-16 Viral Particle-16
v/v volume/volume
w/v weight/volume
WB Western Blot
W White pulp
β-TrCP1 β-Transducin Repeat containing E3 ubiquitin protein ligase
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1.</td>
<td>Schematic representation of TRIM/RBCC family domain structures</td>
<td>20</td>
</tr>
<tr>
<td>Figure 1.2.</td>
<td>Schematic representation of ubiquitination pathway</td>
<td>22</td>
</tr>
<tr>
<td>Figure 1.3.</td>
<td>Schematic diagram of TGF-β signalling pathway</td>
<td>41</td>
</tr>
<tr>
<td>Figure 1.4.</td>
<td>Schematic diagram of different stages of inflammation involved in chronic liver injury</td>
<td>52</td>
</tr>
<tr>
<td>Figure 3.1.</td>
<td>TRIM35 interacts with SMAD proteins</td>
<td>83</td>
</tr>
<tr>
<td>Figure 3.2.</td>
<td>TRIM35 interaction with SMAD proteins is affected by active TGF-β signalling</td>
<td>84</td>
</tr>
<tr>
<td>Figure 3.3.</td>
<td>TRIM35 binds and ubiquitinates SMAD proteins</td>
<td>86</td>
</tr>
<tr>
<td>Figure 3.4.</td>
<td>TRIM35-mediated ubiquitination of SMAD proteins is regulated by TGF-β signalling</td>
<td>87</td>
</tr>
<tr>
<td>Figure 3.5.</td>
<td>TRIM35-mediated ubiquitination does not affect SMAD protein levels</td>
<td>88</td>
</tr>
<tr>
<td>Figure 3.6.</td>
<td>SMAD3 localization in NIH3T3 cells expressing pcDNA3 or TRIM35-Myc</td>
<td>92</td>
</tr>
<tr>
<td>Figure 3.7.</td>
<td>Quantification of nuclear SMAD4 in NIH3T3 cells expressing pcDNA3 or TRIM35-Myc</td>
<td>93</td>
</tr>
<tr>
<td>Figure 3.8.</td>
<td>Proposed model of TRIM35-mediated regulation of TGF-β signalling</td>
<td>98</td>
</tr>
<tr>
<td>Figure 4.1.</td>
<td>Schematic overview of surface markers used to identify different hematopoietic progenitor populations.</td>
<td>104</td>
</tr>
<tr>
<td>Figure 4.2.</td>
<td>Flow cytometry gating strategies for studying erythroblast maturation, myeloid progenitors and the LSK population</td>
<td>109</td>
</tr>
<tr>
<td>Figure 4.3.</td>
<td>Comparison of LSK population in haematopoietic compartments of WT and TRIM35-/− mice</td>
<td>111</td>
</tr>
<tr>
<td>Figure 4.4.</td>
<td>Distribution of HSC, MPP, HPC percentages in BM and spleens of WT and TRIM35-/− mice</td>
<td>114</td>
</tr>
<tr>
<td>Figure 4.5.</td>
<td>The response of LSK, HSC, MPP and HPC populations to 5-FU in BM of 8-12 week old mice</td>
<td>117</td>
</tr>
<tr>
<td>Figure 4.6.</td>
<td>Erythro-myeloid progenitors in BM of WT and TRIM35-/− mice aged 8-12 weeks</td>
<td>119</td>
</tr>
<tr>
<td>Figure 4.7.</td>
<td>Response to 5-FU treatment of erythro-myeloid progenitor population in BM of WT and TRIM35-/− mice aged 8-12 weeks</td>
<td>122</td>
</tr>
<tr>
<td>Figure 4.8.</td>
<td>Analysis of erythroid maturation in WT and TRIM35-/− E11 foetal livers</td>
<td>124</td>
</tr>
<tr>
<td>Figure 4.9.</td>
<td>Erythroid maturation measured in BM and spleens of WT and TRIM35-/− mice aged either 8-12 wks or older than 80 wks</td>
<td>127</td>
</tr>
<tr>
<td>Figure 4.10.</td>
<td>B cell maturation measured in BM and spleens of WT and TRIM35-/− mice aged 8-12 wks or older than 80 wks</td>
<td>130</td>
</tr>
</tbody>
</table>
Figure 4.1. Circulating blood composition of WT and TRIM35−/− mice aged 8-12 weeks as measured by Hemavet

Figure 4.2. Circulating blood composition of WT and TRIM35−/− mice aged 80 weeks or older as measured by Hemavet

Figure 5.1. Weight gain of WT and TRIM35−/− mice fed the control diet for 9 months

Figure 5.2. Liver morphology and histology of WT and TRIM35−/− mice fed the control diet for 9 months

Figure 5.3. Quantification of fibrosis in livers of WT and TRIM35−/− mice fed the control diet

Figure 5.4. Quantification of inflammatory cells in livers of WT and TRIM35−/− mice fed the control diet

Figure 5.5. Analysis of cellular composition of circulating blood of WT and TRIM35−/− mice fed the control diet

Figure 5.6. Analysis of cytokine levels in serum from WT and TRIM35−/− mice fed the control diet

Figure 5.7. Comparison of spleens and pancreases from WT and TRIM35−/− mice fed a control diet

Figure 5.8. MRI scanning revealed tumours in WT and TRIM35−/− mice

Figure 5.9. Weight gain of WT and TRIM35−/− mice fed the CDE diet for 9 months

Figure 5.10. Fat saturation in T1 MRI axial scans reveal presence of fat nodules in mice fed CDE diet

Figure 5.11. Onset and volumetrics of fat nodules in WT and TRIM35−/− mice fed the CDE diet

Figure 5.12. Liver morphology and histology of WT and TRIM35−/− mice fed the CDE diet for 9 months

Figure 5.13. Quantification of fibrosis in livers of WT and TRIM35−/− mice fed the CDE diet

Figure 5.14. Quantification of inflammatory cells in livers of WT and TRIM35−/− mice fed the CDE diet

Figure 5.15. Analysis of cellular composition of circulating blood of WT and TRIM35−/− mice fed the CDE diet

Figure 5.16. Analysis of cytokine levels in serum from WT and TRIM35−/− mice fed the CDE diet

Figure 5.17. Comparison of spleens and pancreases from WT and TRIM35−/− mice fed the CDE diet

Figure 5.18. RT2-Profiler analysis of TGF-β target genes in WT and TRIM35−/− mice fed a HF or CDE diet

Figure 5.19. TGF-β target gene expression in WT and TRIM35−/− mice fed the control diet

Figure 5.20. TGF-β target gene expression in WT and TRIM35−/− mice fed the CDE diet

Figure 6.1. Potential models for TRIM35 function in managing HSC population

Figure 6.2. Schematic diagram of inflammatory response during chronic liver injury and potential role of TRIM35
LIST OF TABLES

Table 1.1. TRIM proteins involved in viral restrictions and regulation of innate immune signaling 32
Table 1.2. TRIM proteins related diseases 35
Table 2.1 Summary of chemicals, reagents and consumables used for experiments presented in this thesis 58
Table 2.2 Antibodies used for experiments presented in this thesis 59
Table 2.3. Primers and Universal Probes used in RT-PCR experiments 63
Table 2.4 Composition of control and CDE diet 71
Table 3.1 Identification of SMAD4 binding partners that are altered in presence of TRIM35 90
Table 4.1. Comparison of HSC, MPP, and HPC populations in BM of WT and TRIM35/- mice measured as % of whole bone marrow (WBM) 113
Table 4.2 Response of WT and TRIM35/- LSK populations to 5FU treatment measured as % of whole bone marrow 116
Table 4.3. Composition of Erythro-Myeloid progenitors in BM of C56BL/6 mice aged 8-12 weeks measured as % of whole bone marrow 118
Table 4.4. Response of WT and TRIM35/- erythro-myeloid progenitors to 5FU measured as %WBM 121
Table 4.5. Erythroblast maturation in WT and TRIM35/- foetal livers measured as percentage of total cell population 123
Table 4.6. Distribution of erythroblast precursors in BM and spleens of youngs adult and aged, WT and TRIM35/- measured as total cells counted 126
Table 4.7. Distribution of B cell precursors in BM and spleens of young adult and aged WT and TRIM35/- mice measured as % of total cells counted 129
Table 5.1. Summary of 9 months of WT and TRIM35/- mice on HF and CDE diet 162
CHAPTER 1 GENERAL INTRODUCTION
1.1. TRI-PARTITE MOTIF (TRIM) FAMILY OF PROTEINS

To date, a total of 70 tri-partite motif (TRIM) proteins have been identified in humans that are encoded by 71 genes, several of which are clustered together in the chromosomal regions 6p21-23 and 11p15 [1, 2]. The TRIM proteins are involved in a range of cellular processes including cell proliferation, differentiation, development and apoptosis [3-5]. This large family of proteins is defined by a pattern of domains highly conserved in order and spacing [1]. Each member has a RING domain, one or two B-box motifs and a coiled-coil region (RBCC) prompting the classification of these proteins as the RBCC family (Figure 1.1) [1, 6]. The RING domain can be found in hundreds of other proteins, but the B-box domain is considered a signature domain of TRIM proteins. The RBCC domains are located at the amino-terminus (N-terminus) with the carboxy-terminus (C-terminus) of TRIM proteins being highly variable [2].

1.1.1. RING-finger domain

The RING domain is typically found within 10-20 amino acids of the first methionine in TRIM proteins and is a known mediator of ubiquitination, an enzymatic cascade resulting in the covalent attachment of ubiquitin to a substrate protein, most often targeting the protein for degradation [7]. Due to its critical role in transferring ubiquitin to heterologous substrates as well as to RING proteins themselves, the RING-finger is considered a defining characteristic of E3 ubiquitin ligases. Most TRIM proteins have been confirmed as functional E3 ubiquitin ligases [8]. Not all TRIMs have a RING domain (e.g. TRIM14, TRIM16 and TRIM29), however they are still considered part of the TRIM family due to the presence of the B-box and coiled-coil domains [1]. In addition, unlike other types of ubiquitin ligases that rely on the formation of multi-subunit complexes to catalyse ubiquitination [9], TRIM proteins can exert their ubiquitin ligase activity as a single protein [10, 11].
Figure 1. Schematic representation of TRIM/RBCC family domain structures. R, RING domain; B1, B-box type 1; B2, B-box type 2; CC, Coiled-coil domain. B-box 1 is in brackets as it is not present in all TRIM proteins. The most common C-terminal variations are represented on the right. LPD, WEVE, and LDYE are the amino acid sequences of the PRYSPRY domain. Inserted box shows consensus sequences for RING domain and both B-boxes. RING consensus: blue residues are involved in coordination of first zinc atom, red residues are involved in second zinc atom coordination. B-box 2: blue residues are involved in zinc atom coordination.
Apart from a small central β-sheet and α-helix, the RING domain is characterized by two zinc atoms that are held in place by an arrangement of cysteine and histidine residues producing a unique integrated structure called a ‘cross-brace’ motif [12, 13]. This motif is achieved by a linear series of 8 conserved cysteine (Cys) and histidine (His) residues: Cys-x2-Cys-x_{11-16}-Cys-x-His-x2-Cys-x2-Cys-x_{7-74}-Cys-x2-Cys, where x can be any amino acid. The first zinc atom is coordinated by cysteines 1, 2, 5 and 6 and the second by either a cysteine or histidine in positions 3, 4, 7 and 8 (Figure 1.1) [13, 14]. This linear structure can produce two RING subtypes, either C2 or H2, depending on whether the fifth coordination site has a cysteine (C2) or histidine (H2) residue [12], with the TRIM family belonging to the C2 subtype.

1.1.1.1. Ubiquitination by RING finger E3 ligases

Ubiquitination is involved in a wide range of biological processes including cell cycle progression, inflammatory response, and antigen presentation, usually through selective degradation of master regulators [11, 15, 16].

The multi-step process of ubiquitination is mediated by 3 types of enzymes: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases (Figure 1.2). E1 is encoded by a single highly conserved gene with two isoforms, E1a and E1b. Both isoforms lack specificity but are essential for cellular function as inactivation of E1 is lethal [16, 17]. On the other hand, there are at least 40 active E2 proteins in humans and roughly 600 E3 proteins, resulting in numerous E2/E3 combinations. Although E2 proteins share a conserved 150 residue domain, each is thought to execute distinct biological functions [18-20]. This biological specificity is thought to be determined by the preferences of E2s for certain E3s [21] as well as the type of ubiquitin chain formed [22, 23]. E3 ligases, in turn, provide increased specificity by recognising and binding only specific substrates [24].
Figure 1.2. Schematic representation of ubiquitination pathway. A) Ubiquitin is transferred to the E1 ubiquitin-activating enzyme through an ATP-driven reaction. In the second step, the ubiquitin is transferred to the active site cysteine of one of the 40 E2 ubiquitin-conjugating enzymes forming a second high-energy thiolester link. In the final step, an E3 ligase coordinates both the substrate and ubiquitin-bound E2 in its active site, thereby catalysing the transfer of ubiquitin to the substrate. The process can be repeated to generate several different types of ubiquitin chains. B) Different types of ubiquitin linkages can regulate different cellular processes.
The first step of ubiquitination is the ATP-dependent activation of ubiquitin, resulting in a high-energy thioester linkage between the C-terminal glycine residue of ubiquitin and the cysteine residue in the active site of the E1 protein (Figure 1.2). The ubiquitin molecule is then transferred to the active site cysteine of an E2 protein, producing a second high-energy thioester link. [25].

The final step of ubiquitination can be subdivided into two separate events: (1) the binding of an E3 ligase to the substrate via an ubiquitination signal and (2) the covalent ligation of one or more ubiquitin molecules to the substrate (Figure 1.2). Several mechanisms for substrate recognition have been observed. In some cases, a posttranslational modification such as phosphorylation of either the substrate or the E3 enzyme is sufficient for ubiquitination [26]. In other cases, a specific amino acid sequence can mediate E3 recognition, such as the N-terminal motif in mitotic cyclins [27]. In yeast, allosteric activation of E3 ubiquitin ligases, such as Ubr1, by small peptides has also been observed [28].

After recognition, the E3-substrate complex binds an ubiquitin-charged E2 and the E3 protein catalyses ubiquitin transfer to the substrate by coordinating the alignment of the ubiquitin-charged E2 and substrate within its active site. The ubiquitin molecule is usually transferred to the substrate by forming an isopeptide bond between the C-terminal glycine of ubiquitin with an internal lysine of the substrate or alternatively by forming a peptide bond with its N-terminus [29].

The addition of a single ubiquitin is called mono-ubiquitination, but E3 ligases can also mediate the addition of several ubiquitin molecules to different sites on the substrate resulting in multi-mono-ubiquitination. Most common, however, is the addition of ubiquitin to a growing chain of substrate-bound ubiquitin, usually by linking internal lysines of the most distal ubiquitin to the C-terminal of the newly added ubiquitin [30]. Ubiquitin contains 7
internal lysine residues that can be targeted for ubiquitination, generating poly-ubiquitin chains of different linkage types depending on which internal lysine is used [31].

1.1.1.2. Ubiquitin-mediated proteolysis: K48-G76 poly-ubiquitination

One of the best studied types of ubiquitin linkage is the poly-ubiquitin chain generated by linking lysine K48 of one ubiquitin to glycine G76 of the successive ubiquitin [30]. Extensive studies into proteolysis have shown that K48-G76 poly-ubiquitination specifically targets substrates for ATP-dependent degradation by the 26S proteasome [25, 30] and that a minimum of four ubiquitin molecules linked in a chain is required for recognition by the proteasome [32]. The 26S proteasome is a 2.5MDa complex composed of 32 subunits divided into a 20S proteolytic core and a 19S regulatory particle (RP) that regulates the activity of the 20S. The 20S and 19S sub-complexes exist either as the 26S complex or as independent complexes in both nuclear and cytoplasmic compartments [33].

The 19S RP caps the 20S core to form an active proteasome. The 19S RP is a 700kDa complex and is composed of rings of subunits that form a base and a lid [34]. Nine non-ATPase subunits are found in the lid whereas the base is made up of three non-ATPase subunits (Rpn1, Rpn2 and Rpn10) and six ATPase subunits (Rpt1-6) [34, 35]. The 19S ATPase subunits recognize and unfold the poly-ubiquitinated proteins before translocating them to the 20S proteolytic core [32, 36]. Also prior to their entry into the proteolytic centre, poly-ubiquitinated proteins are deubiquitinated by deubiquitinating enzymes (DUBs). Unfolding of the substrates as well as deubiquitination is essential for efficient degradation of the substrates [32].

The 20S core is a 670kDa complex consisting of 28 subunits. The core is a hollow cylindrical structure composed of two heptameric rings of α-subunits that sandwich two heptameric rings of β-subunits. (αββα) [37]. The α-subunits in the outer rings serve to
recognize substrates and direct them into the proteolytic centre [35, 37]. Once in the core, proteins are cleaved into peptides by the β-subunits and free ubiquitin is released.

Ubiquitin-dependent proteolysis is known to be essential in regulating many cellular processes through several mechanisms. The most straightforward mechanism of activation is the targeting of an inhibitor for degradation. As is the case for activation of nuclear factor-κB (NF-κB) transcription factor, where its inhibitor IkBα is targeted for degradation through K48-linked ubiquitination mediated by the E3 ligase, SCFβTrCP [38]. Another well-studied example of regulation through ubiquitination is cell cycle progression where several substrates, including cyclins, are targeted for degradation in a precisely timed mechanism to allow cells to progress through the different stages of mitosis [9, 26].

Since the initial discovery of ubiquitin-mediated proteolysis, several novel roles have been discovered that do not involve degradation of the targeted substrate.

1.1.1.3. Non-proteolytic ubiquitination: Mono-ubiquitination

Mono-ubiquitination was the first form of ubiquitination shown to mediate a non-proteolytic function [39]. It was determined that mono-ubiquitination, mediated by the Met-30 ubiquitin ligase, of the transactivation domain of viral protein-16 (VP-16) is essential for transactivity and leads to recruitment of positive transcription elongation factor (PTEFb) to target promoters [39, 40].

Several transcription factors have since been found to be positively regulated by mono-ubiquitination. Two important examples include p53 and FOXO4 [41, 42]. In response to cellular stress, p53 is mono-ubiquitinated by MDM2 which results in an increase in its transcriptional activity and stability [41, 43]. FOXO4 is also mono-ubiquitinated in response to cellular stress [42]. Once ubiquitinated, FOXO4 translocates to the nucleus where its transcriptional activity is subsequently increased [42, 44].
Mono-ubiquitination is also involved in DNA replication and DNA lesion repair, mediated by proliferating cell nuclear antigen (PCNA). After DNA damage has occurred, the ssDNA-binding replication protein A (RPA) binds at the stalled replication fork and recruits RAD18, a RING domain E3 ubiquitin ligase [45]. RAD18 and RAD6 subsequently mediate the mono-ubiquitination of PCNA at lysine K146 [46]. Once mono-ubiquitinated, PCNA recruits the less accurate translesion synthesis (TLS) polymerases (Polη and Polτ) to replace the replicative polymerases (Polδ and Polε) [47]. The TLS polymerases allow for DNA synthesis to continue for only a few nucleotides so as to bypass the lesion. Upon bypassing the lesion, the TLS polymerases are replaced by the replicative polymerases and normal DNA synthesis continues [46, 47].

1.1.1.4. Non-proteolytic ubiquitination: K63-G76 poly-ubiquitination

Further research has shown that not only mono-ubiquitination can mediate non-proteolytic functions, but certain poly-ubiquitin chains also regulate proteins in a proteasome-independent manner.

In contrast to K48-G76 poly-ubiquitin-mediated proteolysis, ubiquitin chains formed through the other 6 lysines present in ubiquitin are known to perform both proteolytic and non-proteolytic functions. In 1996, it was shown that the activity of the IκB kinase could be activated by poly-ubiquitination that did not involve proteasomal degradation [48]. Further studies revealed that this ubiquitination was mediated by TRAF6, a RING domain protein, and that it regulated IκB activity by generating K63-G76 poly-ubiquitin chains [49]. Since these initial discoveries, several examples of protein kinase activation by K63-G76 poly-ubiquitination have been identified that play a critical role in NFκB activation, such as the TNF pathway and the T-cell receptor pathway [50, 51].

K63-G76 poly-ubiquitination is also involved in DNA lesion repair through a mechanism similar to that of PCNA mono-ubiquitination [46, 52]. After PCNA is mono-
ubiquitinated, Ubc13/Mms and RAD5 can further extend the ubiquitin on PCNA to K63-linked poly-ubiquitin chains. Whereas mono-ubiquitinated PCNA promotes a low fidelity DNA repair, poly-ubiquitinated PCNA was found to mediate a more error-free pathway of DNA repair by activating an alternative lesion bypass system called template switch [52, 53]. Template switch involves recruitment of the DNA duplex as a template for DNA synthesis and the formation of structures called sister chromatid junctions (SCJs) [53].

In addition to its involvement in kinase activation and DNA repair, K63-G76 poly-ubiquitination is also involved in several areas of intracellular trafficking including endosomal degradation of MHC class 1 proteins [54], generation of vesicular bodies [55] and the process of autophagy [56].

1.1.2. B-box domain

Unlike the RING and coiled-coil domain, the B-box domains are considered critical determinants of the TRIM motif as they are not found in any other protein family. The B-box domains, also zinc-binding motifs, occur in two variations: B-box1 and B-box2 [1]. Although similar, they each have a distinct pattern of cysteine and histidine residues with the main difference being the second potential coordination residue which is a cysteine in B-box1 and a histidine in B-box2 [1]. Interestingly, if both B-box domains are present, B-box1 always precedes type 2, but if only one B-box is present, it is always a B-box2 [1].

Structural studies revealed that B-box2 coordinates only one zinc atom [57, 58]. It is assumed, due to structural similarities, that B-box1 is also capable of coordinating a singular zinc atom but there is no experimental evidence available. Despite no clear role, many of the TRIM proteins require intact B-boxes to be biologically functional [1]. An analysis of TRIM18 (MID1) showed that its B-boxes are mono-ubiquitinated and that B-box1 amplifies the E3 activity of the RING domain [59] but it is unknown whether this is a common feature amongst TRIM proteins.
1.1.3. Coiled-coil domain

The coiled-coil domain completes the tri-partite motif (Figure 1.1). Unlike its predecessors, the coiled-coil domain is not highly conserved. The only conserved characteristics are the hydrophobic amino acids, often leucine residues, which is required for the distinctive coiled coil packing system [60]. The predicted region of roughly 100 residues is frequently broken up into two or three separate coiled-coil motifs [1, 60]. A coiled-coil is a bundle of α-helices wound into a superhelix. A coiled-coil hallmark is the distinctive knobs-into-holes packing of amino acid side chains in the core of the bundle. In this arrangement, a residue from one helix (knob) packs into a space that is surrounded by four sidechains of the facing helix (hole) directly to the side of the equivalent residue of the facing helix [61, 62].

Functionally, the coiled-coil region is involved in promoting homo- and heterodimerisation, mediated by intertwining α-helices, and results in formation of large protein complexes [1]. Structural studies in TRIM69 have shown that a homodimer is formed in an anti-parallel orientation, where 3 α-helices in each protein orientate perpendicular of each other [63]. The dimerization between two monomers is mediated by the conserved hydrophobic residues indicating that homo- and heterodimerisation is a conserved mechanism within TRIM proteins. This is also supported by cross-linking studies that showed purified recombinant TRIM proteins formed dimers as well as higher oligomers [1, 63].

In TRIM proteins, the coiled-coil domain-mediated protein complexes were found to define specific subcellular compartments within the cell that do not correspond to other cellular structures such as the ER, mitochondria or endosomes [1, 64-66]. Cytoplasmic TRIM proteins were found to either associate with filaments (TRIM1, 2, 3 and 18) [1, 65, 67] or form ribbon-like structures (TRIM29) or ‘cytoplasmic bodies’ of variable sizes (e.g. TRIM4, 5, 12) sometimes around the nucleus (TRIM13) [1]. Nuclear TRIM proteins form structures described as ‘nuclear bodies’ (TRIM8, 19, 30 and 32) or ‘nuclear sticks’ (TRIM6) [1, 68, 69].
TRIM proteins that contain a BROMO domain in their C-terminus were found associated with specific chromatin regions [1]. This discovery is consistent with the chromatin remodelling function described for the BROMO domain protein family [70-72].

Disruption of the coiled-coil domain of TRIM proteins results in a diffuse localization pattern, making it the main mediator of the formation of the large protein complexes responsible for the subcellular compartments. However, disruption of the RING domain or B-boxes resulted in altered localization patterns that differed in shape and size, highlighting the importance of the RBCC pattern in maintaining proper cellular localization and protein function [1].

1.1.4. C-terminus variations found in TRIM proteins

Whilst the N-terminal tri-partite motif is characteristic of the TRIM protein family, the domains found in the C-terminus are more general and also found in otherwise unrelated proteins. In some cases, TRIM proteins do not have a defined C-terminus as the coding region is either limited to the tripartite motif or the C-terminal portion is not similar to any other known domain or protein [5, 73].

Almost two thirds of TRIM proteins have a B30.2 domain in the C-terminus (Figure 1.1) [5, 74]. The B30.2 domain consists of three sections named after the more conserved amino acid stretches: LDP (also known as PRY), WEVE and LDYE (also known as SPRY). This sequence specific nomenclature has resulted in the B30.2 domain also being referred to as the PRYSPRY domain. The PRYSPRY domains of TRIM1 and TRIM5α were found to be essential for targeting viral capsids after cell entry [75, 76] and variations in the TRIM5α PRYSPRY domain plays a role in dictating the retroviral specificity [76].

Two other domains frequently found in TRIM proteins are the NHL domain and the plant homeodomain (PHD)-associated BROMO domain. The NHL domain is named after the
Ncl-1, HT2A (TRIM32) and Lin41 proteins and consists of 2-6 repeats of a 40-residue sequence that assembles into a six-bladed β-propeller [77]. TRIM-NHL proteins have been linked to positive regulation of miRNA in mice [78] and C. Elegans [79].

The PHD-BROMO containing subfamily of TRIM proteins consists of 4 members, the transcriptional Intermediary Factor 1 (TIF1) proteins: TRIM24 (TIF1-α), TRIM28 (TIF1-β), TRIM33 (TIF1-γ) and TRIM66 (TIF1-δ) [80, 81]. The TIF1 proteins are highly conserved along the entire length of the sequence and are involved in transcription regulation through chromatin remodelling [4, 80]. Their involvement in transcriptional regulation is vital for embryonic development as well as tumour suppressor activity in hepatocellular carcinoma (HCC) [82].

1.2. TRIM PROTEINS IN IMMUNITY

Several TRIM proteins are induced by type I Interferon (IFN) stimulation [3, 83] and exhibit antiviral activities [75, 84]. Furthermore, an increasing number of TRIM proteins have been linked to innate immune responses through targeting of cellular proteins involved in pattern-recognition receptor (PRR) signalling [85-87]. Table 1.1 summarizes the role of TRIM proteins in innate immunity and antiviral restrictions.

1.2.1. Antiviral activities of TRIM proteins

TRIM proteins have been shown to restrict retrovirus replication at several different stages of infection. The two most common viruses linked to TRIM antiviral activity are Human immunodeficiency virus-1 (HIV-1) and N-tropic murine leukaemia virus (N-MLV) [88]. TRIM5α restricts both retroviruses, N-MLV and HIV-1, by inducing premature disassembly of the viral capsid after recognition by its C-terminal SPRY domain [75, 76]. TRIM22 inhibits the long terminal repeat (LTR)-driven transcription of HIV GAG p24, which is essential to form the viral capsid, thereby limiting HIV-1 replication [89]. TRIM56
was shown to inhibit the replication of the bovine diarrhoea virus by associating with its N-terminal protease [90].

TRIM21 introduced a completely novel mechanism of adenovirus neutralization. TRIM21 acts as a cytosolic IgG receptor that binds virus-coated IgG inside the cell and targets the whole IgG complex for degradation [91, 92]. Interestingly, TRIM19 has been shown to inhibit a wide range of viruses, including influenza A, human cytomegalovirus (CMV), herpes simplex virus-1 and HIV-1, through different mechanisms at different steps of infection and in a cell context specific manner [93-95].

Apart from inhibiting viral entry and replication, several TRIMs have also been implicated in inhibiting the release of viral particles. A comprehensive screen for antiviral activity showed that human TRIM8, 15, 18, 25, 26, 28 and 35 were capable of inhibiting MLV release [88] but the specific mechanisms were not investigated.

1.2.2. TRIM proteins in innate immune responses

The innate immune system is the first line of defence against invading microbial pathogens. These invading pathogens are recognized by the host cells through pattern-recognition receptors (PRRs) that can bind the pathogen-associated molecular patterns (PAMPs). PRRs can be grouped into at least three distinct families of proteins. The first is the membrane-bound Toll-like receptor (TLR) family that is expressed on antigen-presenting cells, such as dendritic cells and macrophages [96]. The second family is the cytoplasmic RIG-I-like receptor (RLR) family that can recognize RNA species produced in the cytoplasm of a variety of both immune and non-immune cell types [97]. The third group of PRRs is the NLR family that has more than 20 family members, several of which form the inflammasome to assist with the response to pathogen components, small particles, and diverse cellular stresses [98, 99].
<table>
<thead>
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<th>Activity</th>
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<td>No effect</td>
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<td>Mediates IFN-γ-induced autophagy</td>
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<td>TRIM5</td>
<td>Upregulation</td>
<td>Inhibits HIV and MLV entry by targeting viral capsid</td>
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<td></td>
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<td>Activates TAK1 through K63-linked poly-ubiquitin chains</td>
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<td>TRIM8</td>
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<td>Regulates NFκB activation by targeting TAK1 for K63-poly-ubiquitination</td>
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<td>NFκB activation when overexpressed</td>
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<td>Induces type I IFN secretion through regulation of RIG-I</td>
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<td>Increases IFN-γ-induced STAT1 activation</td>
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<td></td>
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<td>Mediates proteasomal degradation of IκBs and activates NFκB</td>
<td>[107, 108]</td>
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<td>Promotes degradation of antibody bound virus particles</td>
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<td>Promotes ubiquitination and proteasomal degradation of IRF3 and IRF7</td>
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<td>Inhibits IRF3 and PI3K interaction</td>
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<td>Inhibits HBV transcription by restricting core promoter activity</td>
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<td></td>
<td></td>
<td>Binds and inhibits all IKKs through ubiquitination</td>
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<td>Inhibits TLR4 signalling by promoting TAB2 and TAB3 degradation</td>
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<td>Targets PIA5y for degradation to activate NF-κB</td>
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<td>Promotes degradation of IRF7 to inhibit TLR7 and TLR9 signalling</td>
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<td>Inhibits TLR responses by targeting TRAF6 for degradation</td>
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<td>Activates TBK-1 signalling via K63-linked poly-ubiquitination of STING</td>
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<td>Regulates TRIF branch of TLR4 pathway</td>
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<td>TRIM65</td>
<td>No effect</td>
<td>Mediates IFN-γ-induced autophagy</td>
<td>[100]</td>
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</table>

MLV, N-tropic murine leukaemia virus; HIV, human immunodeficiency virus; HFV, Human Foamy virus; HBV, Hepatitis B virus; EMCV, encephalomyocarditis virus
Upon activation of these PRRs, several distinct signalling pathways are induced that lead to activation of two transcription factor families; the nuclear factor κB (NF-κB) and interferon regulatory factor (IRF). The activation of these families ultimately result in the increase of inflammatory cytokines, chemokines, and type I interferons (IFNs) required for antimicrobial activities. Several TRIM proteins have been linked to the activation of the signalling pathways that lead to induction of these cytokines and chemokines [85-87, 107]. A few are discussed here, but Table 1.1 provides a more extensive list.

TRIM20 is associated with the auto-inflammatory disease Familial Mediterranean fever (FMF) [107]. It has been suggested that FMF-associated mutations in TRIM20 results in an increase of NF-κB activation and caspase-1 dependent IL-1β production. Research show that TRIM20 is cleaved by caspase-1 and that the TRIM20 cleaved fragment interacts with IκBα and the RelA subunit of NF-κB. The interaction between TRIM20 and RelA increases the nuclear translocation of RelA, whereas its interaction with IκBα induces degradation of IκBα. The combined effect of these two actions is an increase in NF-κB activity [107].

RIG-I dependent sensing of viruses is dependent on TRIM25-mediated K63-linked poly-ubiquitination of RIG-I which increases its interaction with its viral targets [10]. In fact, TRIM25-deficient cells were unable to produce a cytokine response to viruses sensed by RIG-I [10]. In addition, the non-structural protein 1 (NS1) of the influenza A virus, known to suppress host antiviral responses, was shown to interact with TRIM25 and inhibit its E3 ligase activity [122]. This inhibits ubiquitination of RIG-I and its induction of the antiviral response.

IFN regulatory factor (IRF) 3 and 7 are considered master regulators of the type I IFN response and are often targeted in pathways aimed at regulating the immune response to avoid autoimmune diseases [123, 124]. IRFs induce transcription of the cytokines involved in the Type I IFN response following activation by the Toll-like receptors. TRIM21 is emerging
as an important regulator of the type I IFN response by targeting IRF3 and IRF7 for ubiquitin-mediated degradation [85, 109].

1.2.3. TRIM proteins are implicated in numerous different pathologies

As described previously, ubiquitination plays an important role in regulating cell signalling and cellular homeostasis, making ubiquitin ligases essential for survival. With such crucial responsibilities in cell maintenance, the risk of involvement in disease development is significantly high upon deregulation. TRIM proteins have been implicated in numerous pathological conditions including developmental disorders, neuro-degenerative diseases, viral infections, autoimmune diseases and cancer development [65, 82, 125-127]. A few examples are discussed below, but Table 1.2 provides a more comprehensive list.

TRIM32 expression is upregulated during muscle remodelling and myogenic differentiation and its expression is found primarily in cytoskeletal muscles. It is thought that, as TRIM32 ubiquitinates actin [128], it plays a role in myofibrillar protein turnover during muscle adaptation. It is not surprising therefore that a mutation in TRIM32 is linked to two forms of muscular dystrophy [128, 129]. TRIM32 has 6 C-terminal NHL domains. A homozygous point mutation (D487N) in one of these NHL domains is linked to limb-girdle muscular dystrophy type 2H as well as sarcotubular myopathy [130, 131]. Both diseases are characterized by vacuolar changes in the muscle cells and membrane degeneration.

Several TRIM proteins have been linked to cancer through fusions with other genes [69, 132, 133], including TRIM19 (also known as promyelocytic leukemia protein or PML) which was initially identified as part of a fused protein with retinoic acid receptor α (RARα) [127, 132]. Normally, TRIM19 localizes to the nucleus where it forms discrete nuclear structures, referred to as PML bodies. These PML bodies are known to interfere with the replication of various viruses including human foamy virus (HFV) [84], poliovirus [134], influenza [135] and rabies [95]. Due to a t15:17 chromosomal translocation, PML can be
<table>
<thead>
<tr>
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<th>Disease</th>
<th>Changes observed</th>
<th>References</th>
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<td>Glioblastoma</td>
<td>Gene deletion or loss of heterozygosity</td>
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<td></td>
<td>Laryngeal cancer</td>
<td>Correlation with nodal metastatic progression</td>
<td>[137]</td>
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<td>TRIM13</td>
<td>B-cell chronic lymphocytic leukaemia</td>
<td>Gene deletion</td>
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<td>Chronic lymphocytic leukaemia</td>
<td>Gene deletion</td>
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<tr>
<td>TRIM18</td>
<td>Optiz Syndrome type 1</td>
<td>Mutated in C-terminus</td>
<td>[65]</td>
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<td>TRIM19</td>
<td>Acute promyelocytic leukaemia (APL)</td>
<td>Chromosomal translocation: fusion with RARα</td>
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<td>TRIM20</td>
<td>Familial Mediterranean fever (FMF)</td>
<td>More than 60 types of mutation identified</td>
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<td>Sjogren's syndrome</td>
<td>Encodes autoantigen recognized by autoantibodies</td>
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<td>TRIM24</td>
<td>Myeloproliferative syndrome</td>
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<td>Liver cancer</td>
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<td>MDS-related AML</td>
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<td>Head and Neck squamous cell cancer</td>
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<td>[146, 147]</td>
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<td>High levels correlate with poor prognosis</td>
<td>[148]</td>
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<td></td>
<td>Endometrial cancer</td>
<td>Loss of expression</td>
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<td>Papillary thyroid carcinoma</td>
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<td>Lymphoma</td>
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<td>[153, 154]</td>
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<td>Bladder cancer</td>
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<td>Colon cancer</td>
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<td>Endometrial Cancer</td>
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<td>Sjogren's syndrome</td>
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fused to RARα [132]. The PML-RARα fused mRNA contains most of the PML sequence fused to a large part of RARα, including its DNA- and hormone-binding domains. This chimeric protein exhibits altered transactivation properties resulting in inhibition of differentiation of hematopoietic progenitors and subsequently results in acute promyelocytic leukaemia [127, 132].

In addition to direct associations in tumorigenic processes through translocations, several TRIM proteins have been implicated in various cancers through other mechanisms. For example, TRIM13, TRIM19, TRIM24, TRIM28 and TRIM29, can regulate the stability or transcriptional activity of the tumour suppressor p53 [176-179]. And although TRIM24−/− mice are viable, they specifically develop hepatocellular carcinoma (HCC) [143]. Further research into TRIM24 tumour suppressor activities showed that three of the TIF1 proteins work together in regulatory complexes to inhibit HCC [82].

Recently, TRIM35 has been linked to the development and progression of HCC through its interaction with the pyruvate kinase M2 (PKM2), as discussed below.

1.3. TRIM35, A NOVEL E3 UBIQUITIN LIGASE

1.3.1. Discovery and characterization

This project focuses on TRIM35, which was initially identified as a gene upregulated when erythroid cells spontaneously switched to myeloid cells in vitro. TRIM35 expression was detected in the monocyte phenotype and absent in erythroid cells [180]. In accordance with its role in lineage switching, TRIM35 was initially called hematopoietic lineage switch 5 (Hls5). A separate study independently identified TRIM35 as a macrophage-derived apoptosis-inducing RBCC protein (MAIR) upregulated in maturing macrophages after treatment with macrophage colony-stimulating factor (M-CSF). Characterisation of the TRIM35 protein structure revealed a 57 kDa protein that belongs to the subgroup of TRIM
proteins containing one B-box and a PRYSPRY domain in its C-terminus. The RING domain in TRIM35 has been confirmed as a functional E3 ubiquitin ligase [87, 181]. Since its initial discovery, several studies have investigated possible targets of TRIM35 and its potential role in immunity and disease pathology.

1.3.2. Role of TRIM35 in haematopoiesis and immunology

One of the first identified targets of TRIM35 was GATA-1, a transcription factor involved in erythropoiesis, the development of red blood cells [181, 182]. GATA-1 expression is essential for the maturation of red blood cells, with GATA-1 deficient cells unable to mature beyond the proerythroblast stage and the arrested precursors eventually undergoing apoptosis [182, 183]. TRIM35 was shown to reduce mRNA and protein levels of GATA-1 as well as affect the ability of GATA-1 to bind to DNA, thereby directly affecting transcription of GATA-1 target genes [181]. Interestingly, not all GATA-1 target genes were affected by TRIM35 expression. Although a significant reduction was visible in α and β globin transcripts, no change was observed in Epo-receptor or FOG-1 levels [181].

TRIM35 is also a regulator of the Toll-like receptor 7 and 9 (TLR7/9)-mediated type I IFN response through its association with the IFN regulatory factor 7 (IRF7) [87]. As mentioned previously, TLR7 is a critical inducer of the type I IFN response and requires tight regulation to avoid autoimmune diseases [184]. It has now been shown that activation of TLR7 and TLR9 results in an increase in TRIM35 expression [87]. TRIM35 subsequently induces K48-linked ubiquitination of IRF7, targeting it for proteasome-dependent degradation, thereby limiting the TLR7-mediated type I IFN response [87].

1.3.3. TRIM35 as a tumour suppressor

Overexpression of TRIM35 in Hela cells resulted in decreased proliferation, clonogenicity and tumorigenicity indicating that TRIM35 has tumour suppressor properties [185]. In addition, the TRIM35 gene in humans is located at 8p21 [185], a region known to
harbour a group of tumour suppressor genes commonly lost in several types of cancers [186, 187]. Moreover, a study investigating the deletion of several large chromosomal sections identified the genes in the 8p21-8p23 region to cooperatively inhibit tumorigenesis in mice, and when silenced, synergistically promote tumour growth [188].

A study in 2011 provided the first convincing link between the deletion of 8p22 and hepatocellular carcinoma (HCC) [170]. This study looked for copy number variations in 58 paired HCC tumours with adjacent non-tumorigenic tissue. TRIM35 was significantly downregulated in 60% of the tumour samples and the primary cause for loss of expression was due to the 8p22 chromosomal deletion. Furthermore, they found a correlation between decreased TRIM35 expression and severity of tumour grade, tumour size and serum α-fetoprotein (AFP) levels, an indicator of aggressive tumours [170, 189, 190].

More recently, TRIM35 was implicated in regulating the activity of pyruvate kinase isoform M2 (PKM2) in HCC [169]. This study showed that patients with low levels of TRIM35 and high levels of PKM2 harboured a more aggressive form of HCC, had a lower survival rate and shorter recurrence time [169]. A separate study showed that TRIM35 can interact directly with PKM2 but rather than targeting it for ubiquitin-mediated proteolysis, prevents phosphorylation of PKM2 at tyrosine residue 105 (Y105), thereby inhibiting the activation of PKM2 required to provide cancer cells with a metabolic advantage [191].

The two pyruvate kinase isoforms (M1 and M2) catalyse the transfer of a high-energy phosphate group from phosphoenol pyruvate (PEP) to generate pyruvate and ATP, which is the rate-limiting step in the glycolytic pathway. PKM1 is ubiquitously expressed, whereas PKM2 is restricted to cells with high rates of proliferation, such as found in embryos and undifferentiated tissues [192, 193]. Furthermore, PKM2 is highly expressed in many different types of cancer and high levels of PKM2 were found to correlate with elevated rates of glucose uptake and lactate production in the presence of oxygen [193, 194]. This aerobic
glycolysis is known as the Warburg effect and allows cancer cells to circumvent oxidative mitochondrial phosphorylation for energy production.

However, it is likely that this alternative but inefficient method of ATP production is not the only advantage gained by cancer cells with high PKM2 levels. It has been proposed that, in cancer cells, PKM2 forms the less active PKM2 dimers, resulting in accumulation of the upstream glucose metabolites [195]. These metabolites are then funnelled into biosynthesis of nucleotides and amino acids that are essential for rapid proliferation and cell growth, a characteristic of cancer cells [193, 195].

Apart from glycolysis, PKM2 also plays a transcriptional role in the regulation of oncogenes [196, 197]. PKM2 can direct the p300 transcriptional co-activator to HIF-responsive promoters, enhancing the hypoxic transcriptional response [196]. In addition, PKM2 can phosphorylate Histone H3, allowing for epidermal growth factor (EGF)-induced expression of genes involved in the cell cycle such as Cyclin D1 and c-Myc [197].

Collectively, these studies show that in addition to being a potential biomarker for HCC [169], TRIM35 could play a critical role in slowing down progression of cancer cells that rely on PKM2 for energy metabolism or oncogene regulation [191, 196, 197].

1.4. TGF-B SIGNALLING

To identify novel regulators of the transforming growth factor-β (TGF-β) pathway, Barrios-Rodiles et al. [198], developed a high throughput luminescence based assay to screen potential binding partners of the SMAD proteins. The SMAD proteins are the mediators of the TGF-β pathway [199]. TRIM35 was identified as a binding partner of several of the SMAD proteins, however the consequence of this interaction was not investigated and it is the focus of this study.
The TGF-β family regulates a wide range of cellular processes such as proliferation, differentiation, cell-cycle arrest and apoptosis [200-203]. Developmentally, TGF-β ligands regulate tissue growth and morphogenesis in the embryo [204-206], but activate cytostatic and cell death processes in mature tissue to maintain homeostasis [207-209]. The outcome of TGF-β signalling is dependent upon the context in which it occurs, including the cell type, cellular environment and presence or absence of other cytokines [210].

1.4.1. Components of the TGF-β pathway

The TGF-β superfamily consists of various signalling molecules, including TGF-β isoforms, nodals, activins and bone morphogenic proteins (BMPs). These cytokines bind a range of different receptors belonging to the TGF-β receptor family and as such, represent a remarkable regulatory versatility [203, 211].

Members of the TGF-β family induce signalling through heteromeric receptor complexes of Type I and Type II receptors [212, 213]. Type II receptors are responsible for recognizing and binding the ligands which allows these receptors to phosphorylate and activate the Type I receptors (Figure 1.3). The Type I receptors are responsible for activating the downstream signalling pathway through their kinase domain which phosphorylates the downstream signalling proteins, the SMADs. SMAD proteins are responsible for transferring the signal from the membrane-bound receptors to the nucleus (Figure 1.3) [199, 213].

The SMADs are named after their homologs SMA in C. Elegans and ‘mothers against decapentaplegic’ (MAD) in Drosophila [214]. SMAD proteins consist of two spherical domains called MH1 and MH2 that are connected by a linker region [215, 216]. Whereas the MH1 domain is responsible for DNA binding [215], MH2 can interact with a variety of other proteins that regulate the localisation of the SMADs, including the anchors for receptor activation [216], and nucleoporins for nucleocytoplasmic translocation [217].
Figure 1.3. Schematic diagram of TGF-β signalling pathway. TGF-β ligand binds Type II receptor and subsequently recruits Type I receptor. Upon close proximity, Type II receptor phosphorylates Type I receptor. In turn, Type I receptor phosphorylates one of the bound R-SMADs. The activated R-SMAD is released from cytoplasmic retention and can associate with SMAD4 (Co-SMAD) and other regulatory factors and translocate to the nucleus. Once in the nucleus, the transcriptional complex of SMADs and regulatory factors will induce cell-type and context specific gene expression.
The SMAD proteins can be classified into three functional families: the receptor-activated or regulatory SMADs (R-SMADs), inhibitory SMADs (I-SMADs) and common mediator SMAD (Co-SMAD or SMAD4) [211, 218]. The R-SMADs are the substrates for the Type I receptor kinase and are essential for TGF-β signalling [214, 219]. Following phosphorylation by the Type I receptors, the R-SMADs oligomerise with SMAD4 and other transcription factors, and upon entering the nucleus bind to the promoter regions of target genes or prevent transcription factors from binding (Figure 1.3) [215, 220, 221].

The I-SMADs, SMAD6 and SMAD7, are also induced by TGF-β family members and are responsible for a negative feedback loop of the TGF-β signal by competing with R-SMADs for interaction with the Type I receptor or SMAD4 as well as targeting receptors for degradation [211]. Apart from the autocrine negative feedback loop, I-SMADs expression can also be activated by epidermal growth factor (EGF) and IFNγ signalling allowing for trans-regulation between separate signalling pathways [222].

1.4.2. Inhibition of TGF-β signalling mediated by proteolytic ubiquitination

In addition to the I-SMADs, the activity of the R-SMADs is also controlled by ubiquitination. One of the first E3 ubiquitin ligases identified as a regulator of TGF-β signalling was the SMAD ubiquitination regulatory factor 1 (Smurf1). Smurf1 is a member of the NEDD4 family of HECT E3 ubiquitin ligases shown to target SMAD1 and SMAD5, mediators of BMP signalling, for proteasomal degradation [223]. Thus ubiquitination regulates the sensitivity of cells to the BMP signals by modulating the levels of SMADs available for signal transduction [223].

The identification of Smurf1 prompted subsequent studies to identify additional ubiquitin regulators of the TGF-β mediators. Yeast 2 hybrid-screening and BLAST searches led to the discovery of Smurf2, another NEDD4 E3 ubiquitin ligase [224, 225]. Smurf2 was shown to ubiquitinate and target SMAD1 and SMAD2 for proteasomal degradation [224,
As research into ubiquitination of the TGF-β mediators intensified, other E3 ubiquitin ligases were identified which can limit TGF-β signalling through ubiquitination of the R-SMADs [226, 227].

In addition to the R-SMADs, the Co-SMAD, SMAD4, is also known to be regulated through ubiquitination. SMAD4 is crucial for BMP and TGF-β pathways as both signals converge on SMAD4 before entering the nucleus. Therefore, targeting SMAD4 for degradation will significantly alter the cellular response to both cytokines. To date, two E3 ligases able to ubiquitinate SMAD4 have been identified [227, 228]. The first was the Jun activation domain binding protein 1 (Jab1), which is part of the COP9 signalling complex, that directly interacts with and ubiquitinates SMAD4. This results in decreased levels of SMAD4 and inhibition of TGF-β target gene expression [228]. The second complex capable of targeting SMAD4 for ubiquitin-mediated degradation is called SCFβ-TrCP1 and consists of three core subunits: Skp1, Cullin and F-box protein β-TrCP1, and several less crucial factors [227]. The F-box protein β-TrCP1 is an E3 ligase that binds and ubiquitinates SMAD4, catalysing its degradation thereby inhibiting TGF-β induced transcriptional activity [227].

The I-SMAD, SMAD7, also plays a key role in modulating TGF-β signalling through ubiquitination. Several studies demonstrated that SMAD7 functions as an adaptor protein for the E3 ligases, Smurf 1 and Smurf2, thereby facilitating ubiquitination and degradation of the TGF-β receptor type I (TβRI) [229, 230]. Smurf1 targets both TβRI and SMAD7, whereas Smurf2 has less effect on SMAD7 but effectively targets TβRI

1.4.3. Non-proteolytic ubiquitination of TGF-β signalling

Non-proteolytic ubiquitination has also been found to play a key role in regulating TGF-β signalling through SMAD2 [231], SMAD3 [232], and SMAD4 [233].
Ubiquitination of SMAD2 by the E3 ligase Itch did not affect SMAD2 protein levels, but instead was found to increase SMAD2 phosphorylation, induce complex formation between SMAD2 and TβRs, and increase TGF-β transcriptional activity [231]. Furthermore, loss of Itch was shown to reduce the sensitivity of cells to TGF-β signalling, implicating Itch as an important mediator of TGF-β signal transduction [231].

Non-proteolytic ubiquitination of SMAD3 involves multi mono-ubiquitination that does not affect its protein levels, but rather its ability to bind DNA and therefore induce TGF-β target gene transcription [232]. Although the ability of SMAD3 to directly bind DNA is affected, its association with other transcription factors that are capable of binding DNA and inducing gene expression is not altered [232].

Non-proteolytic ubiquitination of SMAD4 represents a diverse regulatory network. Previous research showed that SMAD4 can be mono-ubiquitinated at lysine K507, resulting in an increased affinity for SMAD3 [233]. The enhanced complex formation of SMAD3 and SMAD4 subsequently results in an increase of their transcriptional activity. Another study showed that lysine K519 of SMAD4 is the most important recipient of mono-ubiquitination [234]. However, mono-ubiquitination at this site was shown to disrupt the interaction with SMAD3, thereby limiting the signal transduction. Both studies underline the importance of ubiquitination as a mechanism for precise regulation of the TGF-β pathway.

1.4.4. Regulation of TGF-β signalling by transcription factors

Many signalling pathways are known to induce a cell-type specific response including the TGF-β pathway. The pleiotropic effects of TGF-β is well illustrated in haematopoiesis where TGF-β inhibits proliferation of hematopoietic progenitors [235, 236], but stimulates proliferation in lineage-committed cell lines [237-239].
The variety in cellular responses to TGF-β has been well documented, yet the key molecules responsible for determining the cell-type specific outcomes remain elusive in many cases. There are several theories surrounding the mechanism of cell-type specific responses to TGF-β signalling, all implicating a key role for transcription factors. Despite containing DNA-binding domains, SMAD proteins have a naturally low affinity for DNA and therefore rely on their cofactors for binding DNA as well as target specificity [240, 241]. This provides a potential mechanism for the cell-type specific responses to TGF-β.

Several studies have shown that a variety of transcription factors can interact with the SMADs, including members of the FOX, HOX and RUNX family [240]. This results in the formation of transcriptional complexes consisting of multiple proteins with DNA binding abilities. Promoter recognition is thereby complex-dependent requiring the correct consensus sequences at the appropriate distance and orientation to allow efficient induction of transcription [240, 241]. Through this mechanism, cell-type specificity is achieved by requiring the expression of specific transcription factors capable of interacting with the SMADs to form the transcriptional complex [240].

There is also evidence that a small set of cell-type specific master transcription factors is sufficient to direct SMADs to cell-type-specific binding sites. Master transcription factors were identified many years ago for their roles in establishing, maintaining and even reprogramming cellular identity [242, 243]. In 2011, several master transcription factors were linked to TGF-β signalling through their association with SMAD3 [237]. In this study, it was shown that SMAD3 associated and co-occupied the genome with Oct4 in embryonic stem cells (ESCs), Myod1 in myotubes and with PU.1 in pro-B cells. This was further supported by observed changes in expression of the genes bound by these master transcription factors and SMAD3 [237].
All of the evidence above suggests an important role for transcription factors in modulating the TGF-β signalling pathway, adding additional regulatory versatility. Moreover it suggests that TGF-β responses could be modified by interfering with the SMAD binding partners.

1.5. HAEMATOPOIESIS

1.5.1. General overview of haematopoiesis

Haematopoiesis describes the process by which all types of blood cells are formed. In mammals, initial primitive haematopoiesis takes place in the blood islands of the yolk sac of the embryo. Definitive haematopoiesis first arises in the aorta-gonad mesonephros (AGM) region [244], before migrating to the foetal liver, which is the primary haematopoietic organ during foetal development. Prior to birth, haematopoiesis will move to the bone marrow where it will continue to occur for the duration of the individual’s life [245, 246].

Continuous haematopoiesis is maintained by the presence of HSCs. HSCs are capable of self-renewal as well as differentiation into lineage restricted progenitors. Maintenance of HSCs, as well as differentiation into the downstream multipotent progenitors and subsequent lineages, is carefully regulated by complex networks of extracellular signals, cell-type specific transcription factors and signalling pathways [247-249].

HSCs can be stimulated by their environment to differentiate into either common myeloid progenitors (CMPs) or common lymphoid progenitors (CLP) [250, 251]. The CMPs enter lymphopoiesis, generating lymphocytes required for the adaptive immune system, such as T-cells and Natural Killer (NK) cells [252]. The CMPs mature into a large variety of cell types by entering one of several differentiation pathways, including thrombopoiesis (platelets), erythropoiesis (red blood cells), granulopoiesis (basophils, neutrophils and eosinophils) or monocytopoiesis (macrophages) [251].
1.5.2. TGF-β induces quiescence in hematopoietic stem cells

The TGF-β pathway plays a pivotal role in haematopoiesis by regulating proliferation, differentiation, and apoptosis, either positively or negatively depending on the maturation stage and environmental context of the target cell [253-255].

In haematopoietic stem cells (HSCs), TGF-β is considered a critical inhibitor of growth [235, 236]. However, this effect is concentration dependent as high levels were shown to inhibit HSCs, whereas low levels had a stimulatory effect [202, 239]. HSCs are generally quiescent and it is thought that autocrine TGF-β signalling is important in maintaining HSCs in the G₀ phase [256]. This was demonstrated by activation of HSCs in vitro, following inhibition of TGF-β signalling [256, 257].

Several mechanisms for TGF-β-mediated quiescence in HSCs have been proposed including changes in protein levels of cytokine receptors and cyclin-dependent kinase inhibitors. One study demonstrated that neutralization of TGF-β, with α-TGF-β antibodies, resulted in changes in the expression of key cytokine receptors [258, 259]. Conversely, cytokine receptors essential for controlling HSC differentiation and proliferation, such as IL-6, FLT3, and c-Kit, were up-regulated in cells treated with α-TGF-β [258, 259].

Several other studies have shown a link between TGF-β mediated quiescence and expression levels of cyclin-dependent kinase inhibitors (CDKIs). CDKIs regulate cell cycle progression by blocking the association between cyclins and cyclin-dependent kinases (CDKs). These cyclin/CDK complexes are essential for cells to progress through the different cell cycle checkpoints. TGF-β-mediated up-regulation of CDKIs results in stalled cell cycle progression, pushing cells into the G₀ phase instead. TGF-β has been linked to expression levels of several CDKIs, including p15^{INK4B}, p21^{WAF1} and p57^{KIP2} [260-262].
Previous research showed that treatment of primary human progenitor cells with TGF-β1, resulted in increased levels of p15\textsuperscript{INK4B} and p21\textsuperscript{WAF1} mRNA, resulting in an inhibition of proliferation [260, 261]. Furthermore, the presence of p57\textsuperscript{Kip2} was shown to be essential to induce cell cycle arrest in primary human progenitor cells after TGF-β1 treatment [263]. It was established that p57\textsuperscript{Kip2} is significantly up-regulated in these cells by TGF-β treatment before the TGF-β-induced cell cycle arrest [262].

1.5.3. HSC subtypes increase complexity of TGF-β signalling

Adding to the complexity is accumulating evidence that the HSC population is not homogeneous as previously thought, but heterogeneous consisting of cells with different self-renewal and differentiation abilities [264], that can respond differently to TGF-β [239]. Different research strategies have shown that the HSC compartment can be subdivided into myeloid-biased (My-HSCs) and lymphoid-biased (Ly-HSCs) stem cells [239, 264, 265].

In combination with other HSC markers, the My-HSCs and Ly-HSCs can be distinguished by their capacity to efflux Hoechst33342 [239]. Displaying Hoechst on whole bone marrow cells at two different emission wavelengths allows for identification of a side population (SP) that contains all the long-term HSCs [239, 266]. Although the canonical HSC markers are consistent within SP, there are clear differences in functional activity between different regions in SP, as measured by long-term reconstitution abilities [266, 267]. This discovery led to the identification of separate populations, an upper-SP and lower-SP, that had clear biases in maturation pathways [239]. Competitive transplant assays in mice showed that the lower-SP population had higher long-term reconstitution abilities, increased long-term (LT) HSCs in recipient mice, and, despite generating all haematopoietic cell types, generated more myeloid cells and is, therefore, thought to contain My-HSCs [239]. The upper-SP population also generates all haematopoietic cell types, but showed a clear
lymphoid cell bias and are, therefore, called Ly-HSCs [239]. Furthermore, My-HSCs were shown to be more quiescent whereas Ly-HSCs are more proliferative [239].

Subsequent treatment of My- and Ly-HSCs with TGF-β showed that although a high concentration inhibited both populations, lower concentrations induced proliferation and differentiation in the My-HSCs but still inhibited Ly-HSCs [239]. With so many different pathways and factors involved, it is clear that haematopoiesis relies on a complex molecular network with the TGF-β pathway as an integral regulatory component.

1.6. LIVER DISEASE

As mentioned previously, several studies have linked TRIM35 to onset of liver disease and more specifically to hepatocellular carcinoma (HCC) [169, 170, 191]. However, many questions still remain regarding the role of TRIM35 in liver disease and therefore part of this study was dedicated to further investigate the effect of TRIM35 on liver disease.

1.6.1. Fatty liver disease

The most common underlying causes for liver diseases are viral infections by hepatitis C (HCV) and hepatitis B virus (HBV), but alcohol abuse, obesity, and diabetes are increasingly major contributing factors [268].

Non-alcoholic fatty liver disease (NAFLD) is characterized by accumulation of lipids in the hepatocytes in the absence of alcohol abuse [269], whereas alcoholic fatty liver disease (AFLD) causes both nutritional disturbances and cellular toxicity due to oxidative stress [270]. Both these chronic liver diseases result in increases in protein, lipids and water in the hepatocytes, causing them to balloon [270]. Ballooning of hepatocytes is one of the characteristics of fatty liver disease and is called steatosis. Although steatosis is reversible, it can trigger cell death in hepatocytes. Hepatocyte cell death initiates a sequence of events through secretion of specific signalling molecules or by molecules released by the dying cells.
These damage-associated molecular patterns (DAMPs) work in a similar fashion as PAMPs and can activate the immune response through several types of receptors. The physiological response to hepatocyte death is to limit the extent of the damage, removing or repairing damaged cells, wound healing, protecting against further infection (in the case of viral infections), and to promote tissue repair and regeneration (Figure 1.4).

1.6.2. Liver regeneration

It is well-known that the liver possesses an enormous regenerative capacity and that it can restore a full liver after having two thirds removed by partial hepatectomy (PHx) [271, 272]. This is initially achieved by a few rounds of hepatocyte replication. However, hepatocyte replication is limited and might therefore not be sufficient or, depending on the injury, may even be impaired. In such cases, it has been proposed that a small population of bipotent liver progenitor cells (LPCs) that reside in the canals of Hering, can be triggered to proliferate and differentiate into either hepatocytes or cholangiocytes thereby regenerating the liver and restoring liver architecture [273]. If the liver is unable to regenerate through hepatocyte replication or the LPC activation after acute or chronic injury, it will result in rapid liver failure which can be fatal.

1.6.3. Inflammation and fibrosis

Hepatic cell death triggers, amongst other things, a complex inflammatory response through a vast array of signalling molecules and executed by a variety of cells (Figure 1.4). The resulting hepatitis is fundamental to disease progression and is considered a marker of the severity of liver disease [274].

The inflammatory response is initiated through activation of the stellate cells and hepatic macrophages, called Kupffer cells, through the release of DAMPs and PAMPs (Figure 1.4). Activated macrophages can be induced to differentiate into distinct phenotypes, depending on the cytokine milieu [275]. The DAMP and PAMP molecules as well as
secreted interferon-γ (IFN-γ) direct macrophage polarization towards the M1 pro-inflammatory phenotype. Pro-inflammatory M1 macrophages secrete IL-1, IL-12, IL-23, TNFα and reactive oxygen species (ROS) [276]. The released cytokines can mediate vascular permeability, allowing other pro-inflammatory cells, such as type 1 helper T-cells (Th1), natural killer (NK) cells and neutrophils, to be recruited and differentiate at the site of the injury [276]. During the pro-inflammatory response, the liver macrophages can phagocytose pathogens, apoptotic cells and cellular debris generated during tissue injury and remodelling, thereby facilitating tissue repair.

Although the pro-inflammatory response is essential for dealing with pathogens, it can be detrimental for patients if not tightly regulated and protracted. Excessive inflammation can result in tissue necrosis and inhibits epithelial repair in non-viral liver injuries [277, 278], therefore a persistent pro-inflammatory response can turn an acute injury into a chronic one.

Phagocytosis of apoptotic cells during the pro-inflammatory response can induce the polarization of macrophages to an M2 anti-inflammatory phenotype which subsequently promotes the resolution of inflammation [276, 279, 280]. Resolution of inflammation by macrophages prevents the continuous exposure of immune cells to immunostimulatory cytokines, thereby avoiding progression to chronic injury. The M2 anti-inflammatory macrophages secrete cytokines such as IL-4, IL-10, IL-13 and transforming growth factor-β1 (TGF-β1) that assist in shifting the immune response towards tissue repair [281, 282] through recruitment of eosinophils, basophils and type 2 helper T-cells (Th2) [283].

However, persistent production of cytokines and growth factors by the M2 macrophages is associated with insufficient tissue repair and results in the stimulation of hepatic stellate cells to transdifferentiate into fibrogenic myofibroblasts-like cells [284, 285]. Activated myofibroblasts are responsible for wound healing but can produce excessive amounts of extracellular matrix (ECM) which results in fibrosis.
Figure 1.4. Schematic diagram of different stages of inflammation involved with chronic liver injury. Hepatic damaging agents induce inflammatory reactions in several hepatic cell types. Damaged hepatocytes release inflammatory cytokines that activate the resident macrophages, Kupffer cells, into M1 macrophages and recruit activated type 1 helper T-cells (Th1). This inflammatory environment subsequently results in activation of hepatic stellate cells. Activated hepatic stellate cells produce additional cytokines that maintain their activated state. Eventually, phagocytosis of damaged hepatocytes results in polarization of the M1 macrophages into the M2 phenotype, causing a shift in secreted cytokines from pro-inflammatory to anti-inflammatory. Persistent liver injury and the anti-inflammatory response induce the transdifferentiation of the hepatic stellate cells into myofibroblast-like cells, which produce large quantities of extracellular matrix resulting in fibrosis.
The activation of the stellate cells as well as fibrogenesis is mediated by transforming growth factor-β (TGFβ) [286], whereas the continuous proliferation of the stellate cells is regulated by platelet-derived growth factor (PDGF) [287]. Other cytokines, such as TNFα, contribute to fibrogenesis by inhibiting apoptosis of the stellate cells which subsequently results in an increased population of stellate cells [288].

1.6.4. Cirrhosis and HCC

Although fibrosis can be damaging to the liver, it is still a reversible condition [289]. Apoptosis of damaged hepatocytes and the ECM-producing hepatic stellate cells as well as the production of matrix metalloproteinases (MMPs) that can digest ECM are essential in reversing fibrosis [290, 291].

Cirrhosis is a consequence of persistent fibrogenesis; however it is not simply characterized as extensive fibrosis. Rather, cirrhosis is characterized as a diffuse process that converts normal hepatic architecture into structurally abnormal nodules [292]. Diffuse refers to cirrhosis not being localized but involvement of the whole organ. Therefore focal lesion or nodules do not constitute cirrhosis and neither does diffuse nodularity without fibrosis [292]. The cirrhotic nodules, often referred to as regenerative nodules, lack the regular liver architecture, are surrounded by fibrosis and contain foci of hepatocyte dysplasia [293]. The dysplastic lesions can be further characterized by small cell or large cell dysplasia, in which small cell dysplasia are the predominant pre-neoplastic lesions [294].

Once fibrosis has progressed to cirrhosis it is no longer reversible but could possibly regress through degradation of the matrix and apoptosis of the activated stellate cells [295]. However, cirrhosis does not resolve completely and certain symptoms, such as fibrotic septa, will remain.
Currently, there is no unified proposed mechanism by which hepatocellular carcinoma is initiated. The prevailing view is that a large variety of genetic and environmental factors contribute and it develops as a consequence of changes to multiple pathways [296]. It is this heterogeneity of HCC that has made it difficult to develop effective and targeted therapies.

One of the more common mechanisms associated with the development of cancer, including HCC, is the accumulation of genetic and epigenetic changes that favour malignant transformations [296]. More recently, a ‘cancer platform’ has been proposed which describes a mechanism whereby cells require a minimum number of molecular alterations to develop the cancer phenotype [297]. The basis of this mechanism is the link between growth-promoting pathways and growth-inhibition pathways, such as senescence and apoptosis, where the latter is required to regulate the former to maintain homeostasis. Therefore, altering the growth-inhibition pathways results in uncontrolled growth [297].

1.7. PROJECT OUTLINE

Although it is clear that TRIM35 has significant impact on several biological processes as well as pathological conditions, the mechanism by which it exerts these influences remains unclear. Several pathways have been implicated as targets for TRIM35, but, at least in the case of the TGF-β pathway, it is not known how this interaction influences downstream signalling.

The overall aim of this project is to better understand the role TRIM35 plays in haematopoiesis, inflammation, and cancer progression and to determine if TRIM35 can influence TGF-β regulated outcomes.

To study the role of TRIM35 in regulating TGF-β signalling, this project focused on establishing the effect of TRIM35 expression on cellular responses to TGF-β in vitro using
several biochemical assays. In addition, a TRIM35 knockout (TRIM35−/−) mouse was
generated to analyse the effect of loss of TRIM35 in vivo.

To achieve this, the following aims were addressed:

1) Investigate the effect of TRIM35 on the TGF-β signalling pathway (Chapter 3),
2) Determine if loss of TRIM35 in mice affects haematopoiesis (Chapter 4),
3) Determine if loss of TRIM35 in mice i) increases their susceptibility to
hepatocellular carcinoma (HCC) in a mouse model specific for liver cancer
development and ii) influences the TGF-β signalling pathway (Chapter 5).
CHAPTER 2 MATERIALS AND METHODS
2.1. MATERIALS

2.1.1. Antibodies and chemicals

All chemicals and reagents used during experiments presented in this thesis were of Laboratory Reagent (LR) grade. Table 2.1 lists important chemicals, consumables, reagents and kits used for experiments presented here. Antibodies and the working dilutions used for Western blotting (WB), immunofluorescence (IF), flow cytometry and immunohistochemistry (IHC) are listed in Table 2.2.

2.1.2. Plasmids

The pcDNA3 plasmid expressing Myc-tagged TRIM35 was originally generated as described in Lalonde et al., 2003 [298]. The pcDNA3 plasmid expressing HA-tagged ubiquitin used in this study was originally described by Ingley et al., 2006 [299].

2.1.3. Cell lines

The human embryonic kidney (HEK) 293T cell line and NIH3T3 cell line were generously provided by Evan Ingley (Perkins Institute, Perth, Australia) and Leedman Group (Perkins Institute, Perth, Australia) respectively.

2.1.4. Commonly used reagents and buffers

Unless otherwise state, solutions listed here were stored at room temperature.

Tris-Buffered Saline with Tween (TBST) consisted of 150mM NaCl, 50mM Tris-Hcl pH 7.6, and 0.05% Tween-20.

Phosphate-Buffered Saline (PBS) consisted of 137mM NaCl, 2.7mM KCl, 10mM Na$_2$HPO$_4$, and 1.8mM KH$_2$PO$_4$. The pH was adjusted to 7.4 with 10M HCl prior to sterilisation through autoclaving.
### Table 2.1. Summary of chemicals, reagents and consumables used for experiments presented in this thesis

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<tr>
<td>Dulbecco's Modified Eagle's medium (DMEM)</td>
<td>Life Technologies #10313021</td>
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<td>Foetal calf serum (FCS)</td>
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<tr>
<td>Lipofectamine 2000</td>
<td>ThermoFisher #11668019</td>
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<tr>
<td>Mouse Transforming Growth Factor-β1 (mTGF-β1)</td>
<td>CST #5231</td>
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<tr>
<td>Penicillin-Streptomycin (5000 U/mL)</td>
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</tr>
<tr>
<td>Trypsin-EDTA (0.25%)</td>
<td>Life Technologies #25200056</td>
</tr>
<tr>
<td><strong>Complete Kits</strong></td>
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<tr>
<td>Cytokine 10-Plex Mouse panel for Luminex Platform</td>
<td>Invitrogen #LMC0001</td>
</tr>
<tr>
<td>Isolate II RNA Mini kit</td>
<td>Bioline #BIO-52072</td>
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<tr>
<td>mTGF-β RT2-Profiler PCR array</td>
<td>Qiagen #PAMM-235Z</td>
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Table 2.2. Antibodies used for experiments presented in this thesis.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier and catalogue number *</th>
<th>Source</th>
<th>Applications and working dilutions **</th>
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<tr>
<td><strong>Primary Antibodies</strong></td>
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<tr>
<td>SMAD1</td>
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<td>HA-tag</td>
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<td>Flow 1:400</td>
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<tr>
<td>PE-Cy7 Rat anti-mouse IgM</td>
<td>BD #552867</td>
<td>Rat, monoclonal</td>
<td>Flow 1:200</td>
</tr>
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</table>

* CST, Cell Signal Technology; Sigma, SigmaAldrich; BD, BD Bioscience; eBio, eBioscience, BioL, BioLegend
** WB, Western blot; IP, immunoprecipitation; IF, immunofluorescence; MS, mass spectrometry Flow, flow cytometry
Lyophilized mouse TGF-β1 was resuspended in 20mM citrate, pH3.0, containing 100mM NaCl, to a stock concentration of 50ug/μl. Solution was aliquoted and stored at -20°C. Immediately prior to use, aliquot was thawed and diluted to 5μg/mL in serum-free DMEM before being added to required amount of DMEM with 10% FCS to produce working concentration of 5ng/mL. Cells were lysed in RIPA lysis buffer which consisted of 150mM NaCl, 100mM Tris-HCl pH8.0, 1% (v/v) Triton-X100, 1% (w/v) Deoxycholate and 0.1% (w/v) SDS. Immediately prior to use, 1x complete EDTA-free protease inhibitor cocktail was added to lysis buffer.

For protein separation by gel electrophoresis, a running buffer was prepared consisting of 25mM Tris, 192mM glycine, and 3.5mM SDS in DI H2O.

Protein loading buffer was prepared as a 5x stock consisting of 80mM Tris-HCl pH6.8, 0.125% (w/v) SDS, 29.2 mM 2-mercaptoethanol, 7.5% (v/v) glycerol and 0.02% (w/v) bromophenol blue. Prior to use, buffer was diluted in protein samples to working concentration.

Coomassie Brilliant Blue R250 stain was prepared by dissolving 0.25% (w/v) Coomassie R250 in 10% (v/v) glacial acetic acid, 45% (v/v) Methanol and 45% (v/v) DI H2O.

Western blot (WB) transfer buffer consisted of 288gr glycine and 60.4gr Tris-HCl dissolved in 16L H2O, before adding 4L Methanol.

WB blocking and antibody dilution buffer consisted of 5% (w/v) skim milk in TBS-T.

Paraformaldehyde was prepared as a 37% stock solution by dissolving 1.85gr paraformaldehyde and 10M KOH in 3.5mL DI H2O. Mixture was heated in a 37°C waterbath to accelerate dissolving. The stock solution was stored at -20°C for up two weeks. Prior to use, solution was dissolved to working solution in DI H2O.
Immunofluorescence blocking and antibody dilution buffer consisted of 1% (w/v) bovine serum albumin (BSA) and 0.3% (v/v) Triton X-100 in PBS.

The MOWIOL mounting media was prepared by mixing 2.4 gr MOWIOL 4-88 with 6 gr of glycerol and adding 6 mL of DI H₂O. The suspension was incubated at room temperature with agitation for 3 hours until clear. 0.2 M Tris-HCl pH 8.5 was added before heating mixture to 50°C for 10 minutes. The remaining precipitate was removed by centrifugation at 5000 rcf for 15 minutes. The supernatant was removed and 1,4-diazabicyclo[2.2.2.]octane added at a concentration of 2.5% (w/v). The resulting media was aliquoted and stored at -20°C. Immediately prior to use, solution was heated to 65°C to keep media fluid.

For the 5-Fluorouracil (5-FU) (DBLaboratories) working stock, the 250 mg/mL stock was diluted to 12.5 mg/ml in sterilized DI H₂O immediately prior to injections.

2.2. METHODS

2.2.1. Molecular biology

2.2.1.1. RNA extraction

Snapfrozen liver tissue was disrupted in the TissueLyser II (Qiagen) with 5 mm stainless steel beads before adding the respective buffers of the RNA extraction methods discussed below.

For the RT²-Profiler™ PCR array for mouse TGF-β signalling targets (Qiagen), RNA was extracted from snapfrozen liver tissue with the Isolate II RNA Mini kit (Bioline, #BIO-52072) according to the manufacturer’s instruction.

For RT-PCR, RNA was extracted with TRIzol™ Reagent (Life Technologies). Liver tissue, disrupted with TissueLyser II (Qiagen), was resuspended in 1 mL TRIzol™. Samples were centrifuged at 10000 rpm for 5 minutes at 4°C and supernatant was transferred to new container where it was incubated for 5 minutes. 200 μl chloroform was added to each sample.
of 1mL TRIzol™ and incubated for 3 minutes at room temperature before centrifuging at 10000 rpm for 15 minutes at 4°C. The clear upper phase supernatant was transferred and 500μl isopropanol was added to each sample before incubating for 10 minutes at room temperature. RNA precipitate was pelleted by centrifuging at 10000rpm for 10 minutes at 4°C. Supernatant was discarded and RNA pellet was resuspended in 1mL 75% ethanol before centrifuging at 8000rpm for 5 minutes 4°C. Supernatant was discarded and RNA pellet left to dry for 10 minutes before being resuspended in 50μl RNase-free water.

Quantity and quality of RNA was measured with a NanoDrop® ND1000 spectrophotometer (Nanodrop, Wilmington USA). Instrument was operated with integrated software and blanked using the same buffer as the samples were resuspended in. The RNA concentration was measured at 260nm absorption and the quality determined with 260/280 and 260/230 ratios.

2.2.1.2. RT²-Profiler

The mouse TGF-β RT²-Profiler™ PCR array used was Format E, a 384 (4x96) well plate with dried assay in each well. Each plate contained 84 genes of interest, 5 housekeeping genes, 1 genomic DNA control, 3 reverse transcription controls and 3 positive PCR controls, with all genes repeated 4 times.

cDNA was first synthesised, using 0.5μg RNA from each sample, with the RT² First Strand kit supplied with the RT²-Profiler and the instructions provided by the manufacturer. RT² SYBR Green mastermix was added to each cDNA synthesis reaction before using supplied 384EZLoad cover to add samples to designated wells. Real-time PCR reaction was run on a Viia7™ (ThermoFisher) 384 well block according to manufacturer’s instructions and settings provided by RT²-Profiler protocol.
Data was analysed using the ΔΔC\(_T\) method where first ΔC\(_T\) (= C\(_T\)\text{(gene of interest)} - C\(_T\)\text{(housekeeping)}) was calculated for each gene by normalizing to the housekeeping gene. ΔΔC\(_T\) (=ΔC\(_T\)\text{(WT-CDE)} - ΔC\(_T\)\text{(WT-control)}) was calculated for experimental samples by normalizing to the sample from WT mice on control diet.

2.2.1.3. Real-time PCR

For all other gene expression analysis, cDNA was synthesised with SuperScript\textsuperscript{TM} III Reverse Transcriptase (Invitrogen) according to manufacturer’s instructions using 1μg of RNA from each sample. Required amount of RNA, 10mM dNTPs (Life Technologies), 200ng random primers (Promega) and DI \(H_2O\) were incubated for 5 minutes at 65°C. Tubes were spun down before adding First Strand buffer (5x), DTT, DMSO, Recombinant RNase Inhibitor RNaseOUT\textsuperscript{TM} (Invitrogen) and SuperScript\textsuperscript{TM} III RT (Invitrogen). Final mixture was incubated at 25°C for 5 minutes, 50°C for 60 minutes and 70°C for 15 minutes before cooling down to 4°C. cDNA was stored at -20°C until used in RT-PCR reaction.

RT-PCR was done with FastStart Universal Probe Master (ROX) and universal probes (Roche) according to manufacturer’s instructions. Universal probes and RT-PCR primers used for genes studied are described in Table 2.3.

### Table 2.3. Primers and Universal Probes used in RT-PCR experiments

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Forward primer</th>
<th>Reverse Primer</th>
<th>UPL probe</th>
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<td>ctcacaagtgactcaccag</td>
<td>#97</td>
</tr>
<tr>
<td>AMH</td>
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<td>#64</td>
</tr>
<tr>
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<td>ctgtgacacacaggttt</td>
<td>#56</td>
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<tr>
<td>c-Myc</td>
<td>ccagctgactgtaggagac</td>
<td>ccacacacacacactaattt</td>
<td>#77</td>
</tr>
<tr>
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<td>gcgtgtagagtacagaa</td>
<td>#69</td>
</tr>
<tr>
<td>Stat1</td>
<td>aaaaaaagcaggtgcttcag</td>
<td>tgctgctcaccaggaaggag</td>
<td>#69</td>
</tr>
</tbody>
</table>

*All primers and probes were chosen based on the Roche Universal Probe Library (UPL) Design centre recommendations*
2.2.2. Cell biology

2.2.2.1. Cell culture

NIH3T3 and human embryonic kidney (HEK)293T cells were cultured in high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM) (Life Technologies), supplemented with 10% (v/v) foetal calf serum (FCS) (Life Technologies), 2mM L-Glutamine (Life technologies) and 50U/mL Penicillin G and 50μg/mL Streptomycin (Pen Strep) antibiotics (Life Technologies). Cells were grown at 37°C in a humidified 5% CO₂ incubator.

Once cells reached required confluence, they were rinsed with PBS before being incubated with Trypsin-EDTA (0.25% Trypsin, 0.53mM EDTA) for 30 seconds at room temperature. Cells were resuspended in DMEM 10% FCS and counted manually with Neubauer hemocytometer counting chamber and amount of cells required was diluted in appropriate volume before transferring to culture flask or plate.

2.2.2.2. Transfections

HEK293T/NIH3T3 cells were transfected with Lipofectamine 2000 (Life Technologies) using protocol provided by manufacturer. Amount of DNA and Lipofectamine 2000 used varied depending on size of vessel used, as suggested by manufacturer’s protocol. Lipofectamine 2000 and DNA were incubated separately in serum-free DMEM for 5 minutes at room temperature before briefly being agitated with a vortex. Two mixtures were combined and incubated for another 20 minutes at room temperature before adding required amount to each dish/well of cells with serum-free DMEM. Cells were incubated with Lipofectamine/DNA mix for 14 hours before replacing media with DMEM 10% FCS and allowed another 10 hours of recovery before continuing with subsequent protocol.

2.2.2.3. Immunofluorescence

Prior to transfection, NIH3T3 cells were seeded on 10mm round coverslips (1.5H thickness) (Hurst Scientific) in 24-well plates at 0.1x10⁶ cells per well. Cells were allowed to
settle for 24 hours before transfection as described (2.2.2.2.). Another 24 hours later, cells were treated with 5ng/mL mTGF-β1 for 15, 30 or 60 minutes. After treatment, media was aspirated, cells were rinsed with PBS and fixed with 4% PFA for 15 minutes at room temperature. PFA was aspirated and cells rinsed with PBS three times for 5 minutes. Cells were blocked for 1 hour in IF blocking buffer (described in 2.1.) at room temperature.

Cells were incubated with primary antibodies (diluted in blocking buffer as described in Table 2.2) overnight at 4°C. Cells were rinsed three times for 5 minutes prior to incubation with fluorochrome-conjugated secondary antibody (see Table 2.2) for 1 hour at room temperature. DAPI was added to PBS and cells were rinsed for 5 minutes with DAPI/PBS and twice for 5 minutes with PBS only. Coverslips were mounted onto frosted microscope slides (Perth Scientific) using 15μl MOWIOL 4-88 mounting media (heated to 65°C). Mounting media was allowed to set overnight at 4°C before imaging with DeltaVision Elite™ (DV Elite) confocal microscope (GE Life Sciences).

For each coverslip, 30 individual cells were imaged with the DV Elite. For each cell, separate z-stacks were generated for each filter used (DAPI, TRITC and FITC). Deconvolution algorithm was applied using integrated software package before exporting for quantification.

ImageJ (Fiji) was used to quantify TRITC fluorescence in the nucleus of each cell. Secondary-only controls were used to determine pixel intensity threshold. Nuclear SMAD4 was quantified using the corrected total cell fluorescence (CTCF) method described in Mc Cloy et al., 2014 [300]. For each cell analysed, the DAPI z-stack was used to generate a nuclear region-of-interest (ROI-nuc), the TRITC stack was used to generate an ROI around whole cell (ROI-cell) and two background ROIs (ROI-Bk) were selected in the vicinity of the cell analysed. Each ROI was applied to the TRITC z-stack and integrated density, mean fluorescence and area of each ROI was measured.
The average mean fluorescence of the two ROI-Bk was determined for each slice of the z-stack and used to calculate the nuclear CTCF for each slice as:

\[
\text{CTCF}_{\text{Nuclear}} = \text{Integrated density}_{\text{ROI-Nuc}} - (\text{Area}_{\text{ROI-Nuc}} \times \text{Average Mean Fluorescence}_{\text{ROI-Bk}}).
\]

The sum of \( \text{CTCF}_{\text{nuclear}} \) of the individual slices was calculated to determine the nuclear CTCF of the whole z-stack (\( \sum \text{CTCF}_{\text{nuclear}} \)). The CTCF of the whole cell (\( \sum \text{CTCF}_{\text{cell}} \)) was calculated using the same method but with ROI-cell. The percentage of nuclear TRITC (nSMAD4) fluorescence was subsequently calculated as:

\[
n\text{SMAD4} = (\sum \text{CTCF}_{\text{nuclear}} / \sum \text{CTCF}_{\text{cell}}) \times 100.
\]

### 2.2.3. Protein analysis

#### 2.2.3.1. Cell lysis

293T cells were harvested as described in 2.2.2.1. and centrifuged for 5 minutes at 1000 rpm. Pelleted cells were resuspended in RIPA lysis buffer, with freshly added 1x complete Protein inhibitor cocktail (Roche), and lysed at 4°C for 1 hour. Cellular debris was removed by centrifuging samples for 5 minutes at 3000rpm. Supernatant, containing proteins, was transferred to a new tube. Protein concentrations were measured using microplate reader of Fluostar OPTIMA (BMG Labtech) spectrophotometer and Bradford protein assay (Bio-Rad). BSA (1mg/mL) was serially diluted to generate a standard and calculate protein concentrations of samples based on absorbance. Lysates were kept at -80°C for long-term storage unless used immediately in subsequent assays.

#### 2.2.3.2. Co-immunoprecipitation

Prior to transfection, 293T cells were seeded in 100mm dishes at 1x10^6 cells per dish. Plasmids were transfected 24 hours after seeding, as described (2.2.2.2.). Another 24 hours after transfection, cells were collected and lysed as described (2.2.3.1.).
From each sample, 20μl lysate was removed prior to Co-immunoprecipitation (Co-IP) to use as lysate control.

Remaining lysates were incubated with antibody against protein of interest (Table 2.2. for dilutions) and 150μg/mL Protein G Sepharose (Sigma) overnight at 4°C on rotating wheel to continuously agitate solution. Protein G-bound proteins were collected by centrifuging for 10 minutes at 10000rpm, removing supernatant and rinsing Protein G pellet three times with PBS. After final rinse, pellet was resuspended in equal volume 2x Protein loading buffer as was used for Protein G Sepharose.

2.2.3.3. SDS-PAGE

Cell lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels (resolving and stacking) for SDS-PAGE were prepared with a 30% acrylamide/bis acrylamide solution (37.5:1). Resolving gels consisted of 10% total acrylamide, 375mM Tris-HCl pH8.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate (APS), and 0.05% (v/v) tetramethylethlenediamine (TEMED). Stacking gels consisted of 4% total acrylamide, 125mM Tris-HCl pH6.0, 0.1% (w/v) SDS, 0.1% (w/v) APS and 0.1% (v/v) TEMED.

Prior to loading, protein loading buffer (see 2.1.) was added to 60μg of total protein from each sample and incubated for 5 minutes at 95°C. Proteins were separated at 150V in protein running buffer (see 2.1) using the Mini-PROTEAN Tetra Cell system (Bio-Rad) and the Precision Plus Protein™ Kaleidoscope™ Standard was used as reference for molecular weight.

2.2.3.4. Western blotting

Once separated, proteins were transferred to a Protran nitrocellulose membrane (Amersham) using wet transfer at 4°C, for 45 minutes at 110V in transfer buffer (see 2.1) using Criterion blotter system (Bio-Rad). Efficiency of transfer was determined by staining
with Ponceau S stain consisting of 0.1% (w/v) Ponceau S (Electran) and 5% (v/v) acetic acid in DI H₂O. Membranes were rinsed with TBST to remove Ponceau S before blocking for 1 hour at room temperature in WB blocking buffer.

Membranes were incubated with primary antibodies, diluted in fresh WB blocking buffer (see dilutions in Table 2.2.), overnight at 4°C. Unbound primary antibodies were removed by rinsing membranes four times for 5 minutes with TBST. Following rinse, membranes were incubated with HRP-conjugated secondary antibodies, also diluted in WB blocking buffer (dilutions in Table 2.2.), for 1 hour at room temperature and subsequently rinsed again as described previously. Bound antibodies were detected by applying Immobilon Western Chemiluminescent HRP substrate (Merck) for 1 minute on the membrane before exposing ECL Hyperfilm (Amersham). Signals were visualised by developing film in Agfa CP1000 automatic film processor (Agfa-Gevaert).

2.2.3.5. Mass spectrometry

293T cells were seeded in 150mm dishes at 5x10⁶ cells per dish before transfection with either control (pcDNA3) or pcDNA3-TRIM35-Myc plasmid (2.2.2.2.). 24 hours after transfection, cells were treated with 5ng/mL TGF-β1 for 1 hour or left untreated (control) before being lysed (2.2.3.1.). SMAD4 Co-IP was performed as described in 2.2.3.2. but using higher concentration α-SMAD4 antibody (1:100) and 200μg/mL Protein G Sepharose. Lysates were separated by SDS-PAGE (2.2.3.3.) and proteins were visualised on gel with Coomassie Brilliant Blue R250 (2.1). Using Precision Plus Protein Kaleidoscope Standard as guide, bands from each lane were excised from gel at four different size markers: ~75kDa, ~65kDa, ~50kDa, ~45kDa. Gel bands were submitted to Proteomics International Pty Ltd (Perth, Australia) for Matrix Assisted Laser Desorption/Ionization (MALDI)-time of flight (TOF) mass spectrometry with automatic database analysis using the Mascot search
algorithm and the MSPnr100 database (Swiss-Prot inclusive) (as described in Bringans et al., 2008 and Casey et al., 2017) [301, 302].

2.2.4. Animals and animal procedures

2.2.4.1. Animal housing and care

*TRIM35/-* mice were generated on a C57BL/6 background at the Walter and Eliza Hall Institute (WEHI) (Melbourne, Australia) by the laboratory of Prof. Warren Alexander before transportation to the Animal Resources Centre (ARC) (Murdoch, Perth, Australia) where the strains were maintained. Mice were housed and shipped in accordance with the ‘Australian Code of practice for the care and use of animals for scientific purposes’ as determined by the National Health and Medical Research Council (NHMRC) of Australia. All experimental procedures were approved by respective animal ethics committees of Royal Perth Hospital and Perkins Institute for Medical Research.

C57BL/6 mice were used as wildtype controls for the *TRIM35/-* mice. For the haematopoietic procedures, mice were shipped to the ARC facility at Royal Perth Hospital (Perth, Australia). The CDE model was conducted at the BioResources facility located in the Perkins Institute for Medical Research (Perth, Australia).

2.2.4.2. 5-Fluorouracil (5-FU) injections

Mice were weighed, to determine quantity of 5-FU required, and briefly placed in warming cabinet before being transferred to animal restraint used at BioResources facility (Perth, Australia). Once in restraint, tail was pulled through opening at the back and stabilized by Ms. Beaumont, the research assistant trained for specialized animal procedures. Tail was cleaned with gauze dampened in 70% alcohol. Mice were subsequently injected intravenously (i.v.) in the lateral tail vein with 5-FU at a dosage of 150mg/Kg (see 2.1.), similar to procedure described by Wang et al., 1997 [303], using 26G hypodermic needle
Mice were monitored for 15 minutes after injections to ensure there were no adverse reactions.

2.2.4.3. Anaesthesia

Inhalational anaesthesia, using isoflurane vaporiser, was administered according to the Australian Cancer Research Foundation (ACRF) Cancer Imaging Facility’s standard operating procedure (SOP) #1.07.01 (Administration of anaesthesia to mice for imaging purposes). BioResources technician, trained in anaesthesia administration, regulated isoflurane flow and supply of medical oxygen into induction chamber, pre-warmed to 30°C, where mice were kept until adequate depth of anaesthesia was obtained. Mice were briefly taken off anaesthesia to be transferred to imaging bed, where anaesthesia was continued through nosecone mask.

2.2.4.4. Magnetic Resonance imaging (MRI) scans

The abdominal region of mice were scanned with a 3.0 Tesla MRS 3000 preclinical magnetic resonance imaging (MRI) system (MR Solutions) at the ARCF Cancer Imaging Facility located in the Perkins Institute for Medical Research (Perth, Australia). MRI imaging bed was equipped to monitor breathing rate, heart rate and temperature thus allowing for anaesthesia adjustments during imaging to maintain optimal breathing rate around 40 per minute.

An initial scouting sequence (T1, coronal) was used to determine if mice were placed appropriately in front of coil to include whole liver. Mice were subsequently scanned using T1-weighted and T2-weighted sequences in axial, coronal and sagittal orientations. For T1-weighted axial, an additional sequence with fat saturation was added.
2.2.4.5. Special dietary animal chow

The choline-sufficient, high-fat (control) diet and choline-deficient ethionine-supplemented (CDE) diet were prepared by Specialtyfeeds (Perth, Australia). The compositions of the diets are listed in Table 2.4. Both diets contain 20% lard. Only the control diet is supplemented with 0.1875% choline chloride. The CDE diet contains 0.5% DL-ethionine. Both diets were stored at 4°C. Immediately prior to use, the CDE diet was reduced to 67% of its potency by mixing 75gr CDE with 35gr control diet (2:1). Mixture was blended for 1 minute in sterilized blender before adding 5mL water. Resulting mix was blended further until smooth consistency was achieved.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Communal</th>
<th>Control</th>
<th>CDE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (Acid)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Soy Protein Isolate</td>
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<td></td>
</tr>
<tr>
<td>Lard</td>
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<tr>
<td>Calcium Carbonate</td>
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<tr>
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<tr>
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2.2.4.6. Monitoring of mice on high-fat and CDE diet

Wildtype (WT) and TRIM35/-/- C57BL/6 mice, aged 5 weeks, were given regular chow for the first 7 days after arriving at the BioResources facility (Perth, Australia) to allow for acclimatisation. All mice were subsequently placed on the control diet for 7 days and weighed twice daily to ensure all mice reached a minimum weight of 18gr. Starting at day 8 (T=0), mice were given either the control (WT, n=9; TRIM35/-/-, n=10) or CDE diet (WT,
n=10; TRIM35-/-, n=11). As stipulated by the Perkins Institute for Medical Research Ethics committee, a mouse was euthanized if it exceeded the allowed 20% weight loss or attained a morbidity score of 3 or above. Mice were monitored and weighed twice daily during the first 21 days of the respective diets, during which mice are known to suffer significant weight loss on the CDE diet, and daily for the remainder of the experiment.

2.2.4.7. Euthanasia

Animals were euthanized by cervical dislocation at the end of the experiment, or if displaying significant weight loss or morbidity symptoms, according to the Perkins Institute for Medical Research Ethics committee SOP #1.08.01 (Euthanasia of the mouse – Cervical dislocation). Death was confirmed by checking pedal reflexes and respiration.

2.2.4.8. Cardiac puncture

Mice were administered anaesthesia (2.2.4.3.) in a zero dead space face mask. Blood was collected by Ms. Kirsty Richardson (senior animal technician at BioResources facility) via cardiac puncture on live animal using a 26G hypodermic needle and 1mL syringe (ThermoFisher). Blood was allowed to coagulate at room temperature for 30 minutes and serum was subsequently obtained by centrifuging samples at 13000g for 15 minutes at 4°C. Supernatant containing serum was aliquoted and stored at -80°C.

After euthanasia, the abdominal cavity was exposed and additional blood was immediately collected by cardiac puncture with a 26G hypodermic needle and 1mL syringe (ThermoFisher) and transferred to an EDTA-coated haematological tube. Samples were either analysed immediately or stored at 4°C until Hemavet was available.

2.2.4.9. Harvesting of bone marrow (BM) and spleens for haematology

Bone marrow (BM) was harvested from femurs of WT and TRIM35-/- C57BL/6 mice, aged 8-12 weeks or 80 weeks and older. Both femurs of each mouse were flushed with PBS,
containing 2% (v/v) FCS, using a 23G hypodermic needle and 3mL syringe (ThermoFisher). The collected BM cells from both femurs were pooled for each mouse.

Spleens were removed and transferred into PBS containing 2% (v/v) FCS. To generate single cell suspension and remove connective tissue, each spleen was pushed through a cell strainer (70μm) (Falcon) twice using plunger from 10mL syringe. Remaining cell clusters were disrupted by drawing suspension through 26G hypodermic needle using 3mL syringe (ThermoFisher).

2.2.4.10. Isolation of livers, spleens and pancreases and sample preservation

Pancreases from mice on control or CDE diet were located and removed first to prevent rapid enzymatic degradation. Pancreases, spleens and two liver lobes were fixed overnight in formalin and transferred into PBS before being sent for processing at CELLcentral in the University of Western Australia (Perth, Australia).

Two other separate liver lobes were frozen in OCT compound (VWR) in OCT moulds using liquid nitrogen, wrapped in aluminium foil and stored at -80°C. Small fragments of remaining liver lobes were snapfrozen in liquid nitrogen for RNA extraction and stored at -80°C.

2.2.5. Biochemical analysis of mouse samples

2.2.5.1. Hemavet

Cellular composition of blood was analysed using the murine profile on the Hemavet® (Drew Scientific), with access generously provided by Prof. Wally Langdon at the University of Western Australia.

2.2.5.2. Flow cytometry

BM and spleen cellular suspensions were centrifuged for 5 minutes at 1000 rpm, supernatant was removed and pellet was resuspended in PBS containing 2% (v/v) FCS.
Washes were repeated 3 times, before cells were counted manually with Neubauer hemocytometer counting chamber. Cell suspensions of 1x10^6 cells per sample were prepared in PBS with 2% (v/v) FCS containing fluorescent-conjugated flow cytometry antibodies (Table 2.2.) and incubated for 1 hour on ice, shielded from light.

For biotin-conjugated FcγRIII/II, the primary antibody was incubated as described above, followed by three rinses with PBS containing 2% (v/v) FCS after centrifugation for 5 minutes at 1000 rpm. Streptavidin-conjugated secondary was incubated for 30 minutes on ice whilst shielded from light.

Samples were again rinsed three times as described above, before being analysed on the FACSARia II (Becton Dickinson Bioscience, USA), operated by flow cytometry officer of Harry Perkins Institute for Medical Research.

**2.2.5.3. Cytokine analysis**

Cytokine composition of serum was analysed with the Mouse cytokine 10-plex panel (Invitrogen) on the Luminex 200 at the Centre for Microscopy, Characterisation and Analysis (CMCA) in the Perkins Institute for Medical Research (Perth, Australia).

The 10-plex cytokine panel utilizes a mixture of antibody-conjugated beads with defined spectral properties and fluorescence-conjugated antibodies to detect cytokines bound by the antibody-conjugated beads. This allows for simultaneous identification and quantification of ten cytokines by measuring the spectral properties of the beads and measuring the amount of fluorescence.

Standards and associated settings for detection were provided by manufacturer (Appendix E). Preparation of the 96 well filter plate was done in accordance with the manufacturer’s guidelines, utilizing a vacuum manifold (Millipore), before detection of cytokines with the Luminex 200 at CMCA (Perth, Australia).
2.2.5.4. Immunohistochemistry (IHC)

Formalin fixed livers, spleens and pancreases, as well as frozen livers in OCT compound (VWR), were sent to CELLcentral at the University of Western Australia (Perth, Australia) for paraffin embedding, sectioning and H&E staining. Formalin-fixed, paraffin-embedded samples were sectioned into 4μM sections and placed on Superfrost™ Plus microscopy slides. CELLcentral utilized automated processing to stain sections with H&E.

In addition, CELLcentral prepared unstained 4μM liver sections, which were subsequently used for CD45 staining. For CD45 staining, slides were dewaxed and rehydrated by incubating slides three times in Xylene for 5 minutes, three times in 100% Ethanol for 2 minutes, in 70% for 2 minutes and 10 minutes in DI H$_2$O. Slides were equilibrated in TBS before using wax pen to draw wax circles around sections. Antigen retrieval was done by incubating sections with 50μl Proteinase K (40μg/mL in TBS) for 10 minutes at room temperature. Endogenous peroxidase activity was blocked by incubating slides for 10 minutes in 3% (v/v) H$_2$O$_2$ in PBS, before rinsing again for 2 minutes in TBS bath. Slides were blocked in serum-free protein block (Dako) for 15 minutes before being rinsed again for 2 minutes in TBS. Sections were incubated with primary antibody against CD45 (details in Table 2.2.), diluted in Antibody Diluent (Dako) overnight in humidified chamber at 4 °C.

Slides were washed in TBS three times for 5 minutes before and after incubating sections with anti-rat HRP-conjugated secondary antibody (Table 2.2.) for 1 hour at room temperature. DAB solution (Dako) was prepared by adding 20μl of the DAB Chromogen to the provided substrate solution. Sections were incubated with DAB solution for 1 minute before being rinsed in TBS for 5 minutes, counterstained with hematoxylin for 3 minutes and rinsed in Scott’s tap water. Before mounting with DePeX (SigmaAldrich),
sections were dehydrated by washing for 1 minute each in 70% ethanol, three times in 100% ethanol and three times in Xylene.

Sirius Red and PanCk staining was performed by our collaborator Dr. Caryn Elsegood (Curtin University, Perth, Australia), utilizing protocols previously established in her laboratory as described in Elsegood et al., 2015 and Köhn et al., 2016 [304, 305].

2.2.6. Analysis of images

2.2.6.1. Volumetric analysis of MRI images

Fat nodules in mice fed the CDE diet were quantified using ITK-SNAP to generate 3D volumes (Appendix G). T1 axial scans were used to generate 3D renders of fat nodules and healthy liver (green), which was subsequently used to calculate total volume of liver consisting of fat nodules.

2.2.6.2. Imagescope and InForm analysis of IHC data

Stained sections were scanned with the Scanscope digital slide scanner at CMCA in the Perkins Institute for Medical Research. High-intensity positive pixels were quantified using Imagescope. In Imagescope, a region of interest was drawn around the sample of interest and visible artefacts (e.g. dust, dirt, etc) were excluded from the region to prevent false positives. Using the built-in positive pixel algorithm, the percentage of high-intensity positive pixels were calculated for each sample.

For the CD45 staining, the percentage of positive cells per section was detected and counted by our collaborator, Dr. Anne Kramer, using InForm software from PerkinElmer.
CHAPTER 3 TRIM35 IS A NOVEL REGULATOR OF TGF-β SIGNALLING
3.1. INTRODUCTION

TRIM35 is a novel E3 ubiquitin ligase likely to influence a number of different molecular pathways and functions [298]. The identification of TRIM35 binding partners, and therefore potential targets for TRIM35-mediated ubiquitination, contributes to elucidating its cellular and biological function. TRIM35 has previously been linked to the TGF-β signalling pathway via its interaction with the SMAD proteins [198]. TGF-β plays a significant role in several biological processes and is known to exert cell-type and environment-specific effects [239, 255]. Due to its essential role in homeostasis, it is not surprising that the TGF-β pathway is frequently deregulated in several pathological conditions [306-308].

This essential role in homeostasis and disease progression makes the identification of novel regulators of the TGF-β pathway of significant interest. Understanding this pathway and its regulators, will provide greater insight into the role of TGF-β in different biological processes, as well as identify potential novel therapeutic targets [307-309]. The aim of this study was to determine if TRIM35 regulates TGF-β signalling through ubiquitination of the SMAD proteins.

3.1.1. TRIM35-mediated proteolytic regulation of signalling

The importance of ubiquitin-mediated proteolytic regulation of the TGF-β signalling pathway was described previously (Chapter 1.4.2). The question raised in this chapter is whether TRIM35 affects TGF-β signalling through proteolytic ubiquitination of the signal transduction molecules.

To date, there are only a handful of examples of TRIM35-mediated ubiquitination influencing signalling pathways. One example is the TRIM35-mediated ubiquitination and regulation of GATA-1 activity [181]. Importantly, this work also demonstrated that TRIM35-mediated ubiquitination of GATA-1 is increased when GATA-1 is sumoylated, suggesting that TRIM35 can act as a Sumo targeted ubiquitin ligase (StUbl) (unpublished).
A second example of TRIM35-mediated proteolytic ubiquitination involves the Type I interferon (IFN) response [87], a pathway required for combating viral infection but in need of tight regulation to prevent auto-immune disease [310, 311]. A recent study showed that TRIM35 is part of a negative feedback loop responsibly for tightly regulating the Type I IFN response.

### 3.1.2. Proteosome-independent function of TRIM35

In addition to proteosomal-dependent degradation of target proteins, TRIM35 is able to regulate protein activity independent of proteosomal degradation [181, 191].

Recently, TRIM35 has been linked to HCC through its interaction with PKM2 [169, 191]. Rather than inducing proteosomal degradation, TRIM35-mediated ubiquitination inhibits PKM2 phosphorylation, an essential step for PKM2 to mediate metabolic advantages that allows energy production in cancer cells during hypoxic conditions [191].

Combined, these studies propose multiple mechanisms by which TRIM35 can regulate the activity of its target proteins.

### 3.1.3. Project aims

This chapter focuses on characterizing the role of TRIM35 in TGF-β signalling. A high throughput assay previously identified TRIM35 as a binding partner of some of the SMAD proteins. However, the function of this protein-protein interaction still remains unclear. It is possible that TRIM35 modulates TGF-β signalling by ubiquitinating SMAD proteins, targeting them for ubiquitin-mediated degradation.

Accordingly, to determine if TRIM35 is a novel regulator of the TGF-β pathway able to ubiquitinate and target SMAD proteins for degradation, the following aims were addressed:

1. Establish if TRIM35 binds and ubiquitinates SMAD proteins.
2. Determine if TRIM35-mediated ubiquitination targets SMAD proteins for degradation.

3. Determine the downstream signalling effects of the interaction between TRIM35 and SMAD proteins to establish a role for TRIM35 in TGF-β signalling.
3.2. METHODS

To study protein-protein interactions and TRIM35-mediated ubiquitination of the SMAD proteins, 293T cells were transiently transfected with either pcDNA3 or myc-tagged TRIM35, in combination with Ubiquitin-HA (Ub-HA). Cells were treated with 5ng/mL TGF-β 24 hours after transfection for 30 minutes, unless otherwise indicated. Following treatment, protein-protein interactions and presence of bound ubiquitin was determined by co-immunoprecipitation (Co-IP) and Western blot analysis (protocol outlined in Chapter 2).

Mass spectrometry analysis was performed after separating proteins by SDS-PAGE and staining bands with Coomassie R250. Bands were excised from the gel and analysed by Proteomics International (Perth).

For immunofluorescence (IF), NIH3T3 cells, stably transfected with either pcDNA3 or TRIM35-Myc, were seeded on 18mm diameter coverslips, treated with 5ng/mL TGF-β1 for 15, 30 or 60 minutes before continuing with IF protocol outlined in Chapter 2. Z-stacks were generated for 30 cells from each category using a Deltavision Elite confocal microscope. To quantify the percentage of nuclear SMAD4 (nSMAD), the DAPI signal was used to generate regions of interest (ROIs) for the nucleus across the z-stack of each cell imaged. These ROIs were used to determine the amount of SMAD4 signal in the nucleus in the TRITC channel. The cell boundaries were used to generate ROIs across the z-stack of each cell. These ROIs were subsequently applied to the TRITC channel to establish the total amount of SMAD4 signal in each cell to determine the percentage of nSMAD4.
3.3. RESULTS

3.3.1. TRIM35 binds SMAD proteins

A high throughput assay identified TRIM35 as a binding partner of several SMAD proteins [198]. To validate these protein-protein interactions, co-immunoprecipitation assays were performed on 293T cells overexpressing myc tagged TRIM35. As E3 ligases bind their targets through their RING-finger domain, the ability of the SMAD proteins to interact with a TRIM35 protein without the RING domain (TRIM35ΔN61) was also analysed.

The immunoprecipitation confirmed that TRIM35 interacts with SMAD1, SMAD3 and SMAD4 (Figure 3.1A). However, the ability of SMAD3 to pull-down TRIM35 was significantly lower than that of SMAD1 and SMAD4 (Figure 3.1A). Therefore, the focus of this study centred on the effect of TRIM35 on SMAD1 and SMAD4.

Interestingly, TRIM35ΔN61 was also capable of co-immunoprecipitating the SMAD4 (Figure 3.1B), suggesting that the interaction between TRIM35 and SMAD4 is not dependent upon the RING-finger domain. In addition, SMAD4 interacted with SMAD1, regardless of TRIM35 expression (Figure 3.1B).

To determine if TGF-β activation influenced the interaction between the SMAD proteins and TRIM35, the protein-protein interactions were assessed following treatment with 5ng/mL TGF-β1 for 30 minutes. In the presence of active TGF-β signalling, SMAD1 co-immunoprecipitates significantly more TRIM35 (Figure 3.2A). Conversely, active TGF-β signalling decreased the amount of TRIM35 co-immunoprecipitated by SMAD4 (Figure 3.2B).

These results confirmed that TRIM35 interacts with several SMAD proteins and these interactions are affected by activated TGF-β1 signalling, depending on the SMAD protein involved.
Figure 3.1. TRIM35 interacts with SMAD proteins. A) Co-immunoprecipitation of TRIM35 with SMAD1, SMAD3 and SMAD4 as indicated, followed by Western blotting with anti-myc antibody to recognise TRIM35. Cells transfected with pcDNA3 were used as control. (n=4 biological replicates) B) Co-immunoprecipitation of SMAD4 with TRIM35 and with the TRIM35 RING-finger mutant (TRIM35ΔN61). This was followed by Western blotting with anti-SMAD antibodies as indicated. (n=2 biological replicates)

IP, immune-precipitated.
Figure 3.2. TRIM35 interaction with SMAD proteins is affected by active TGF-β signalling. A) Co-immunoprecipitation of TRIM35 with SMAD1 from cells untreated or treated with 5ng/mL TGF-β1 for 30 minutes. This was followed by Western blotting with anti-SMAD1 and anti-myc to recognise TRIM35. (n=3 biological replicates) B) Co-immunoprecipitation of TRIM35 with SMAD4 from cells untreated or treated with 5ng/mL TGF-β1 for 30 minutes. This was followed by Western blotting with anti-SMAD4 and anti-myc to recognise TRIM35. (n=3 biological replicates)

IB, immune-blotted; IP, immune-precipitated
3.3.2. TRIM35 ubiquitinates SMAD proteins

To determine if TRIM35 ubiquitinates SMAD1 and SMAD4, myc-tagged TRIM35 and HA-tagged ubiquitin were overexpressed in 293T cells and the presence of ubiquitin bound to the SMAD proteins analysed. Increased TRIM35 expression resulted in an increase of ubiquitin bound to SMAD1 (Figure 3.3A) and SMAD4 (Figure 3.3B). This established TRIM35 does indeed ubiquitinate these SMAD proteins.

As we have established that TRIM35 ubiquitinates SMAD proteins, it was important to determine if TRIM35-mediated ubiquitination of SMAD proteins was influenced by TGF-β signalling. Figure 3.4A shows that, in the presence of TGF-β and ubiquitin, TRIM35-mediated ubiquitination of SMAD1 is lost and the interaction between TRIM35 and SMAD1 is significantly less.

In contrast, SMAD4 (Figure 3.4B) was ubiquitinated in the presence of TRIM35 regardless of the TGF-β treatment. However, the addition of ubiquitin resulted in a notable decrease in the interaction between SMAD4 and TRIM35.

Next, to determine if TRIM35-mediated ubiquitination of the SMAD proteins targets them for degradation, 10% of the whole cell lysate was collected before the co-immunoprecipitation and used for Western blot analysis (Figure 3.5). This showed that overexpression of TRIM35-Myc and Ub-HA does not affect SMAD1 (Figure 3.5A) or SMAD4 (Figure 3.5B) protein levels.

Although poly-ubiquitination can induce non-proteolytic affects, it has predominantly been associated with proteosome-dependent degradation. Therefore, these findings were unexpected and highlight a non-proteolytic role for TRIM35 in the TGF-β pathway. Two potential alternative functions were investigated: i) ubiquitin-regulated protein-protein interactions and ii) ubiquitin-mediated cellular localization.
Figure 3.3. TRIM35 binds and ubiquitinates SMAD proteins. Co-immunoprecipitation of ubiquitin, in the presence of TRIM35, with A) SMAD1 and B) SMAD4. After Co-IP, samples were used for Western blotting using antibodies indicated. (n=3 biological replicates)

IB, immune-blotted; IP, immune-precipitated
**Figure 3.4.** TRIM35-mediated ubiquitination of SMAD proteins is regulated by TGF-β signalling. Co-immunoprecipitation of ubiquitin with A) SMAD1 or B) SMAD4 in the absence or presence of TRIM35 in cells untreated or treated with 5ng/mL TGF-β1 for 30 minutes. (n=3 biological replicates)

*IB, immune-blotted; IP, immune-precipitated*
Figure 3.5. TRIM35-mediated ubiquitination does not affect SMAD proteins levels. Lysates of cells untreated or treated with 5ng/mL TGF-β for 30 min, in the absence or presence of TRIM35, was Western blotted to determine protein levels of A) SMAD1 or B) SMAD4. (n=3 biological replicates)

IB, immune-blotted; IP, immune-precipitated
3.3.3. TRIM35 alters binding partners of SMAD4

An alternative role for ubiquitination is to regulate protein interactions by either increasing [233] or decreasing [234] the affinity of the target protein for other proteins. SMAD4 is essential for TGF-β and BMP signalling and associates with several transcription factors to regulate the signal transduction. In addition, SMAD4 is known to be mono- and oligo-ubiquitinated for non-proteolytic purposes [233]. Thus, if TRIM35 can regulate SMAD4 activity, it is able to significantly alter the cellular response to all cytokines in the TGF-β family. Therefore, this study focused on identifying SMAD4 binding partners in the absence or presence of TRIM35-Myc.

To determine if ubiquitination affects the interaction between SMAD4 and other proteins, SMAD4 was immunoprecipitated from cells expressing either pcDNA3 or TRIM35, in the absence or presence of TGF-β. After separation by gel electrophoresis, proteins were stained, removed from the gel and analysed by MALDI-TOF.

Table 3.1 lists all the proteins identified by MALDI-TOF and the numbers per sample indicate the amount of times each protein was identified. Ubiquitination of SMAD4 by TRIM35 altered its ability to bind to several proteins. Moreover, when the TGF-β pathway was activated a different set of proteins were affected. As expected, several DNA-binding proteins were identified in each sample, including PHD domain and zinc finger containing proteins.

Interestingly, several proteins bound to SMAD4 in control samples, were not detected in samples expressing TRIM35 (Table 3.1). Two of these proteins, Leucine-rich repeat-containing protein p53 (LRRC53) and centrosome-associated protein E (CENPE), were detected in pcDNA3-expressing cells before and after treatment with TGF-β, but were not present in TRIM35-expressing cells, regardless of TGF-β treatment. Additionally, four other proteins were identified only in untreated pcDNA3-expressing cells and three proteins were
only detected in TGF-β-treated pcDNA3-expressing cells. None of these proteins were identified in TRIM35-expressing cells.

One protein that was found at a high frequency only in untreated TRIM35-expressing cells, is the histone-lysine N-methyltransferase EHMT2. This protein was identified 7 times in pull down assay of SMAD4 in untreated TRIM35-expressing cells, but was not detected in any of the other samples. Additionally, one protein, Zinc finger protein 439 (ZNF439), was identified solely in TRIM35 expressing cells treated with TGF-β.

These results confirm that TRIM35-mediated ubiquitination can regulate the binding partners of SMAD4. However, it is likely that there are additional binding partners that are affected by TRIM35, that could not be identified under the current assay conditions.

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3.3.4. TRIM35 delays nuclear uptake of SMAD4 after TGF-β treatment

The second possible function of non-proteolytic regulation of the TGF-β pathway by TRIM35 that was investigated, was ubiquitin-mediated changes in SMAD4 subcellular localization. Ubiquitin-mediated induction of nuclear translocation has previously been reported [312]. To determine if TRIM35-mediated ubiquitination of SMAD4 alters its translocation into the nucleus, NIH3T3 cells, stably transfected with pcDNA3 (control) or TRIM35-Myc, were treated with TGF-β for 15, 30 or 60 minutes. Localization of SMAD4 and TRIM35 was visualized by immunofluorescence and the nuclear SMAD4 (nSMAD4) was quantified in 30 cells of each cell type and treatment using ImageJ.

In control (pcDNA3) NIH3T3 cells, SMAD4 is visible in the nucleus and cytoplasm, regardless of TGF-β treatment (Figure 3.6). Quantification of nuclear SMAD4 (nSMAD4) showed no significant changes in nSMAD4 between any of the timepoints in control cells after TGF-β treatment with averages ranging between 22-24% (Figure 3.7).

TRIM35 formed ribbon-like structures in the nucleus and cytoplasm of untreated cells and cells treated for 15 minutes with TGF-β, whereas aggregates were detected at 30 minutes post-treatment (Figure 3.6). In cells with increased TRIM35 expression, SMAD4 showed similar ribbon like structures and aggregates. Quantification showed that in the presence of TRIM35, nSMAD4 percentages were significantly reduced at 15 and 60 minutes post-treatment, with averages of 20.9% and 18.5% respectively, compared to control cells which had averages of 23.6% and 23.4% at the respective timepoints (Figure 3.7). However, at 30 minutes, cells expressing TRIM35 had a significantly higher percentage of nSMAD4, with an average of 26.0%, compared to control cells which had an average of 22.0%.

Overall, these results demonstrate that TRIM35 affects the localization dynamic of SMAD4 after TGF-β treatment.
Figure 3.6. SMAD4 localization in NIH3T3 cells expressing pcDNA3 (control) or TRIM35-Myc.

NIH3T3 were transiently transfected with pcDNA3 or TRIM35-Myc and treated with TGF-β, 24 hours after transfection, for indicated times. Localization of SMAD4 (red) and TRIM35-Myc (green) was studied using fluorescent antibodies and DAPI to stain the nucleus (n=30 cells counted for each condition).

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<td>TRIM35-Myc</td>
<td><img src="image10" alt="Image" /></td>
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Figure 3.7. Quantification of nuclear SMAD4 in NIH3T3 cells expressing pcDNA3 or TRIM35-Myc. SMAD4 fluorescence was quantified in the nucleus and whole cell in NIH3T3 cells and percentage of nuclear SMAD4 was calculated. In TRIM35-expressing cells, there was significantly lower percentages of nuclear SMAD4 in the nucleus at 15 min and 60 min post-TGF-β treatment, but significantly higher percentage at 30 min. Data presented as mean±SEM. Statistical significance was determined using multiple t-test with *p<0.05 **p<0.001.
3.4. DISCUSSION

This study set out to determine if TRIM35 altered TGF-β signalling via its interaction with SMAD proteins. SMAD proteins had been identified as binding partners of TRIM35 in a high throughput luminescence-based screen [198], but the consequences of these interactions have not been explored. This study demonstrates that TRIM35 mediates poly-ubiquitination of SMAD1, SMAD3 and SMAD4, but unexpectedly, did not affect their protein levels. Rather, this study identified a non-proteolytic function for TRIM35.

However, it was not determined if TRIM35 is directly responsible for the ubiquitination or instead acts as a mediator for a secondary E3 ubiquitin ligase. Considering that TRIM35 can still bind SMAD4, even without its RING domain, it is plausible that TRIM35 simply facilitates ubiquitination through other ligases. Unfortunately, due to time constraints, this study did not investigate whether the RING domain mutant of TRIM35 is capable of still increasing ubiquitination of SMAD4 to establish the causal link.

The interaction between SMAD4 and TRIM35 was of particular interest, due to the crucial role of SMAD4 in transferring signals from all TGF-β cytokines to the nucleus. Therefore, this study focused specifically on characterizing the interaction between TRIM35 and SMAD4. Two different aspects of SMAD4 biology were studied in detail: i) its affinity for specific binding partners, which could alter subsequent gene expression and ii) the translocation of SMAD4 into the nucleus, which would affect its ability to regulate gene expression.

Due to the ease of culturing and efficiency in transfections, this part of the project utilized 293T HEK and NIH3T3 cell lines. Although these cell lines are not physiologically relevant to the cell types studied in the subsequent chapters (i.e. haematopoietic and hepatic cell types), the results gained from the studies performed in these cell lines provide a proof-of-principle mechanism for TRIM35 function.
Ubiquitin-mediated regulation of SMAD4 binding partners has been previously documented [233], so the possibility that TRIM35-mediated ubiquitination of SMAD4 would alter its interaction with other proteins was investigated. In the presence of TRIM35, several changes in binding partners were identified, including an increase in the affinity of SMAD4 for histone-lysine N-methyltransferase 2 (EHMT2). EHMT2, also known as G9a methyltransferase, plays an essential role in gene suppression through dimethylation of histone H3 at lysine-9 [313]. One possibility is that TRIM35-mediated ubiquitination of SMAD4 could affect the expression of TGF-β target genes by recruiting EHMT2 to the SMAD4/transcription factors protein complex required for inducing gene expression.

TRIM35 also affected the affinity of SMAD4 for key proteins involved in the regulation of gene expression. Depending on whether TRIM35 was present or absent, SMAD4 associated with different DNA-binding proteins, i.e. Zinc finger proteins [314], or chromatin-remodelling proteins, i.e. PHD finger proteins [315]. These DNA binding proteins differ in their affinity for specific genes and therefore the genes targeted by SMAD4 can be regulated by determining which of these are recruited to the transcription protein complex.

Although several important genes were identified using this approach, there were some unexpected omissions, notably, SMAD3. SMAD3 is known to bind SMAD4 before entering the nucleus, after treatment with TGF-β1. Yet, none of the SMAD proteins were detected in SMAD4 pull-down assays from cells treated with TGF-β1. It is possible that the conditions used for this assay were not optimal for this interaction, but it also implies that there will be additional interactions beyond those identified in this study.
A second potential mechanism of ubiquitin-mediated regulation of SMAD4 activity explored in this study focused on cellular localization of SMAD4 in the presence of TRIM35. Data presented here showed that TRIM35 affected the dynamics of SMAD4 nuclear localization after TGF-β1 treatment (Figure 3.8). Strikingly, SMAD4 in control NIH3T3 was not affected by TGF-β1 treatment, whereas the TRIM35-expressing cells displayed a much more dynamic pattern with levels of nuclear SMAD4 oscillating significantly.

There are several possible explanations for the change in SMAD4 localization in TRIM35-expressing cells. Ubiquitin-induced localization of proteins has previously been observed [312] and it is, therefore, possible that TRIM35-mediated ubiquitination directly alters the ability of SMAD4 to localize to the nucleus. A second possibility involves the potential changes in binding partners discussed above. Although SMAD4 is capable of localizing to the nucleus, interaction of specific proteins can either enhance or inhibit this process [237, 316]. Therefore, by altering the proteins capable of binding SMAD4, it is possible that TRIM35 could regulate the localization of SMAD4.

This study focused specifically on the influence of TRIM35-mediated ubiquitination of the Co-SMAD, SMAD4 as it is crucial for all TGF-β cytokine signals. However, SMAD1 and SMAD2/3, R-SMADs for the BMP and TGF-β1 signalling pathways respectively, were also shown to be ubiquitinated by TRIM35 without affecting their protein levels and may, therefore, also be important in TRIM35-mediated regulation of the TGF-β pathway. Indeed, studies into the ubiquitination of R-SMADs have shown that ubiquitination of SMAD1 sensitized cells to TGF-β1, thereby establishing another regulatory mechanism [223]. This is particularly important as TRIM35-mediated ubiquitination of SMAD1 was inhibited in the presence of TGF-β. It is possible that TRIM35 may influence the balance between BMP and TGF-β signalling with significant functional consequences.
Additionally, previous studies into proteolytic and non-proteolytic ubiquitination showed the importance of the type of poly-ubiquitin chain, with K48-G76 linked chains predominantly involved with proteosomal degradation but K63-G76 linked chains associated with non-proteolytic functions [25, 49, 55]. Although beyond the scope of this study, it would be of interest to investigate the type of poly-ubiquitination chains produced by TRIM35 and whether this differs depending on the target protein or cellular environment.

Non-proteolytic functions of TRIM35-mediated poly-ubiquitination include inhibition of phosphorylation [191]. Although not investigated in this study, it would be of significant interest to determine if TRIM35-mediated ubiquitination of the SMAD proteins affected their ability to be phosphorylated and therefore activated. If this is found to be the case, this would result in an inhibition of the signal transduction as phosphorylation of the SMAD proteins is essential for their downstream signalling.

Based on the data presented in this chapter, the following model is proposed for TRIM35 regulation of TGF-β signalling (Figure 3.8). In the absence of TGF-β signalling, TRIM35 ubiquitimates the R-SMADs and the Co-SMAD, SMAD4. Instead of targeting the SMADs for proteosomal degradation, TRIM35-mediated ubiquitination affects their affinity to bind specific transcription factors, thereby altering the genes targeted for transcriptional activation. The pleiotropic effects of TGF-β are influenced by the specific transcription factors that bind SMAD4. Therefore, regulating the affinity of SMAD4 for these transcription factors presents an opportunity to regulate the functional outcomes of TGF-β signalling. It should be noted that as TGF-β has cell-type and environment-specific effects, the ability of TRIM35 to change the signalling pathway might also be dependent on cell type and additional external signals.
Figure 3.8. Proposed model of TRIM35-mediated regulation of TGF-β signalling. As previously discussed, the TGF-β cytokine induces association of the Type I and Type II TGF-β receptors. This results in cross-phosphorylation and activation. In absence of TRIM35 (left), TGF-β mediated signalling results in phosphorylation of R-SMADs which subsequently associate with the Co-SMAD, SMAD4. Once bound to SMAD4, the protein complex translocates to the nucleus where it associates with other factors to induce expression of specific TGF-β target genes. In the presence of TRIM35 (right), it is proposed that the SMAD proteins are ubiquitinated prior to TGF-β signalling. This ubiquitination results in a change in the proteins capable of binding the SMAD protein complex, thereby inducing a different set of genes and eliciting a different cellular response.
CHAPTER 4 ROLE OF TRIM35 IN HAEMATOPOIESIS
4.1. INTRODUCTION

TRIM35 was identified as one of several proteins involved in haematopoietic lineage switching between erythroblasts and monoblasts, with TRIM35 expression restricted to the monocyte lineage [298]. Furthermore, TRIM35 overexpression suppressed erythroid maturation by inhibiting proliferation and interfering with GATA-1 mediated transcription [181]. Additionally, TRIM35 has been linked to the TGF-β pathway [198], a key regulator of haematopoietic stem cell (HSC) quiescence [257]. Several TRIM proteins have been linked to immunological disorders and TRIM35 itself has also been linked to the innate immune system [87]. Building on the current knowledge of haematopoiesis and defining the possible role of regulators such as TRIM35 and TGF-β is essential for understanding the fundamental mechanisms involved in haematological pathologies, such as leukaemia, as well as immunological disorders. Furthering our understanding of its role could provide valuable insights into such pathologies.

4.1.1. Haematopoietic stem cells (HSC)

Haematopoietic stem cells (HSCs) are found in extremely low numbers in foetal livers, cord blood and adult bone marrow, and yet they perform a vital function in maintaining life-long production of mature blood cells [317]. Their self-renewal and differentiation abilities allow them to fully repopulate the hematopoietic compartment of an individual, as demonstrated by cytotoxic depletion [318] and retroviral marking studies [319]. This repopulation ability and their biological significance make HSCs extremely appealing for the study of stem cells and gene regulation. Furthermore, a comprehensive understanding of HSC regulatory pathways and fate decisions would have significant clinical consequences for bone marrow transplantations, immune diseases and other hematopoietic related pathologies. Therefore, isolation and characterization of HSCs has been the focus of extensive research.
4.1.1.1. Isolation and characterization of HSCs

The initial search for HSCs utilized cell separation assays, based on size and density, and colony assays [320, 321]. However, these methods were limited by lack of assays that could measure all types of blood cell developmental outcomes or the self-renewal abilities of HSC. The introduction of flow cytometry and identification of lineage cell surface markers significantly enhanced our ability to isolate primitive progenitors [250, 251, 322-326]. One defining experiment showed that B-cell lineage-engrafting cells actually lacked the B-lineage marker [325, 326]. If the B-lineage progenitor lacked the B-cell marker, then surface markers for the other committed lineages could also be absent. This resulted in a cocktail of 7-10 antibodies against lineage (Lin) markers that are used in flow cytometry to exclude committed progenitors from the stem cell pool [250, 251, 322-326].

Two of the first positive markers that identified murine HSCs in adult bone marrow, were stem cell antigen 1 (Sca1) and c-Kit [248, 327]. Sca1, or lymphocyte activation protein-6A (Ly-6A), is an 18-kDa antigen first identified on activated lymphocytes [328]. Isolation of Sca1+ cells from the Lin- population, resulted in enrichment of a progenitor population with significant repopulation abilities, with only 30 cells required to rescue 50% of lethally irradiated mice [327]. By adding Sca1 as a marker to the Lin- population, it was determined that the Lin-Sca1+ population contained long-term self-renewing HSCs (LT-HSC), short-term renewing HSCs (ST-HSC) that are only capable of ~10 weeks of self-renewal, and the downstream multipotent progenitors (MPP) [329, 330].

Sca1 was also shown to upregulate expression of c-kit, a transmembrane tyrosine kinase receptor for Stem Cell Factor (SCF) [331], which was already linked to HSCs in separate studies [332]. Subsequent research showed the HSCs and progenitors can be enriched by combining c-Kit as a marker with the Lin-Sca1+ population [248]. Colony-forming and reconstitution assays showed that the most primitive population of the bone
marrow compartment resides in the Lin\(^-\)Sca1\(^+\)c-kit\(^+\) fraction, which is also referred to as the LSK population [248, 327, 333].

**4.1.1.2. Characterization of LT- and ST-HSCs**

Initially, it was thought that the LSK population was homogenous, predominantly consisting of LT-HSCs. Recently, the LSK population has been shown to be heterogeneous, containing HSCs and progenitors with varying self-renewal and repopulation capabilities [251, 334, 335]. Several markers were identified that can assist in isolating the HSC population, including Thy-1.1 and CD34 [249, 336]. Although these studies showed that the LT-HSC population resided in the LSKThy1.1\(^{lo}\) or LSKCD34\(^-\) fraction, only a small proportion of these cells were capable of long-term multi-lineage reconstitution [249, 337].

In 2005, two studies were published that used different combinations of cell surface markers to further characterize the heterogeneity of the LSK population, enabling researchers to enrich for LT-HSCs or ST-HSCs for clinical use [334, 338]. One of these studies examined LSK cells lacking the cytokine polypeptide deformylase (fms)-like tyrosine kinase receptor 3 (flt3) that overlapped with the LSKThy1.1\(^{lo}\) phenotype and, therefore, contained all LT-HSCs [334]. Previous research suggested that short-term stem and progenitor activity was found in LSKCD34\(^+\) and LSKflt3\(^+\) compartments, respectively [249, 339]. However, the LSKCD34\(^+\) population was very heterogeneous [249] and LSKflt3\(^+\) showed short-term reconstitution abilities, but was biased toward the lymphoid lineages [339]. Therefore, to separate the LT-HSCs from ST-HSCs, this study combined CD34 and flt3 markers to compare the self-renewal and repopulation abilities of LSK cells with different levels of CD34 and flt3 [249, 339]. This led to the identification of three distinct groups in the LSK population: LSKCD34\(^-\)flt3\(^-\), LSKCD34\(^+\)flt3\(^-\), and LSKCD34\(^+\)flt3\(^+\) [334].

The LT-HSCs, with lifelong reconstitution and self-renewal abilities, were enriched with LSKCD34\(^-\)flt3\(^-\) [334]. The LSKCD34\(^+\)flt3\(^-\) and LSKCD34\(^+\)flt3\(^+\) populations both
contained cells with efficient and rapid haematopoietic repopulation abilities, 3 weeks after transplantation [334]. However, neither exhibited lifelong repopulation capacity. Furthermore, the LSKCD34+flt3− population’s contribution to the peripheral blood levels was enhanced, compared to total bone marrow transplants, at 10 weeks after transplant, whereas the LSKCD34+flt3+ population’s contribution decreased almost 10-fold [334], indicating the latter was the more committed MPP population and the LSKCD34+flt3− population harboured the ST-HSCs.

The second study from 2005 to investigate the murine HSC compartments within the LSK population was the first to identify a family of cell surface receptors, the SLAM family, that can be used to distinguish stem cells from progenitors [338]. The SLAM family consists of 10-11 cell surface receptors capable of regulating activation and proliferation of lymphocytes [340, 341]. Gene expression analyses comparing LSKThy1.1lo HSCs, Thy1.1loMac1loCD4loMPPs, and CD45+ total bone marrow, showed that HSCs, but not MPPs or the lineage restricted hematopoietic progenitor cells (HPCs), expressed CD150 (Figure 4.1). CD48 was expressed by the HPCs, but not the MPPs or HSCs, while CD244 was found on the MPPs but not the HSCs or HPCs (Figure 4.1) [338]. This established a correlation between SLAM receptors expression levels and maturation of progenitors, where increased CD150 expression correlated with self-renewal ability and CD48 correlated negatively.

This discovery led to further enrichment of the LT-HSC population as almost half the LSKCD150+CD48− cells had long-term multi-lineage reconstitution abilities [335, 338]. Previous studies using conventional markers achieved only 20% engraftment [249, 337]. Interestingly, loss of CD150 does not affect HSCs in mice as CD150−/− showed normal haematopoiesis according to the composition of bone marrow, blood, spleen and thymus [338, 342]. Furthermore, CD150−/− HSC transplantations in irradiated mice had similar HSC frequencies and reconstituting potential compared to wildtype (WT) mice [338]. Therefore,
Figure 4.1. Schematic overview of surface markers used to identify different hematopoietic progenitor populations. By using the SLAM family of markers (CD150, CD48, and CD244), it is possible to distinguish between the hematopoietic stem cell (HSC), multi-potent progenitor (MPP) and the lineage committed hematopoietic progenitor cells (HPC), the common lymphoid progenitor (CLP) and common myeloid progenitor (CMP). Additional markers can be used to further analyze the CMP compartment. The use of CD34 and FcγRII/III allows for identification of the CMP population, and the more lineage restricted megakaryocyte-erythrocyte progenitor (MEP) and granulocyte-macrophage progenitor (GMP). [251, 335, 338]
CD150 is not essential for HSC maintenance or function, but is thought to regulate more subtle aspects of HSC biology.

### 4.1.2. Lineage-associated progenitors

The classical haematopoiesis model suggests that the first lineage-determining decision occurs after HSCs have been stimulated to generate haematopoietic progenitor cells (HPCs), which are common myeloid (CMPs) or common lymphoid progenitors (CLPs). According to this model, CMPs give rise to megakaryocytes and erythrocytes (MegE) as well as granulocytes and macrophages (GM) with virtually no B-cell potential [251]. CLPs, in turn, give rise to all types of lymphocytes, including natural killer (NK) cells, B-cells and T-cells, with no detectable potential for myeloid differentiation [250, 343]. The only cells that can arise from both CLPs and CMPs are dendritic cells (DCs) and DCs are therefore not classed as either lymphoid or myeloid [344].

#### 4.1.2.1. Common lymphoid progenitor

The ability to identify and isolate HSCs using cell surface markers prompted the search for downstream lineage-committed progenitors. In adult human bone marrow, a population was identified with markers Lin⁻c-Kit⁻Thy-1⁻CD10⁺CD34⁺ that could give rise to clonal progenitors for B-, NK cells as well as lymphoid DCs, but it was not shown whether they could give rise to T-cells [345]. On the other hand, in murine thymus a thymic precursor population identified as c-Kit⁺Thy-1⁻lo was shown to give rise to all lymphoid cells [346, 347].

To further characterize lymphoid progenitors in the immature c-Kit⁺Thy-1⁻lo population, research focused on Interleukin-7 (IL-7), a cytokine essential for T-cell and B-cell development by maintaining cell survival and supporting proliferation [348, 349]. IL-7 requires the presence of the IL-7 receptor α chain (IL-7Rα) and the common cytokine receptor γ chain [350]. Therefore, by using IL-7Rα expression as a marker, it became
possible to identify a population of cells with extensive and rapid short-term lymphoid-restricted reconstitution activity [250]. Clonogenic analysis of these Lin°Sca-1°c-Kit°Thy-1°IL7R° cells showed that they are able to differentiate into both B- and T-cells, thereby representing the earliest known lymphoid-restricted progenitor population [250].

4.1.2.2. Common myeloid progenitor

Recent evidence suggests that, unlike the classical haematopoiesis model, most CMPs are already biased to either erythroid or myeloid lineages and the fate of these CMPs lie with earlier progenitors [351]. This study showed that transplantation of most single CMPs resulted in either only erythrocytes or only myeloid cells, with only a small fraction of the CMP population having bipotential capacity [351]. Further research, using fluorescently labelled progenitors, showed that the MPPs were in fact capable of giving rise to the erythro-myeloid lineages [351]. This suggests that, in addition to the distinction between erythro-myeloid and lymphoid lineages being determined at MPP level, the commitment to either megakaryocyte/erythrocyte or granulocyte/macrophage lineages also occurs within the MPP population [251, 351].

The heterogeneous CMP population has been shown to contain the two myeloid lineage-specific progenitors: the granulocyte-macrophage lineage-restricted progenitor (GMP) and the megakaryocyte-erythrocyte lineage-restricted progenitor (MEP) [251]. Characterization of the CMP population showed that the myeloerythroid colony-forming units (CFUs) were found exclusively in the Lin°cKit° fraction [251]. HSC’s can be removed from this fraction by excluding the ScaI° population. By combining the myelomonocytic marker, Fcγ receptor-II/III (FcγR) and CD34, the Lin°ScaI°cKit° population can be divided into CMPs, GMPs and MEPs as determined by the abilities of the isolated cells to produce distinct colony types in methylcellulose (Figure 4.1) [251]. This study shows that 90% of cells isolated with Lin°ScaI°cKit°FcγR°CD34° produced a colony distribution similar to that
of cells from the LSK population. On the other hand, 90% of Lin\(^-\)Sca\(_1\)cKit\(^+\)FcγR\(_{lo}\)CD34\(^-\) cells produced exclusively CFU-megakaryocyte (CFU-Meg), burst-forming units-erythroid (BFU-E) or CFU-MegE and therefore contains the MEPs. The GMP population was found in the Lin\(^-\)Sca\(_1\)cKit\(^+\)FcγR\(_{lo}\)CD34\(^+\) fraction, as 90% of this population gave rise to CFU-macrophages (CFU-M), CFU-granulocytes (CFU-G) or CFU-granulocytes/macrophages (CFU-GM) (Figure 4.1) [251].

4.1.3. Project aims

TRIM35 was initially identified for its role in lineage switching between erythroid and monoblastoid phenotypes, where TRIM35 expression was restricted to monocyte cells. Therefore, we hypothesized that in the absence of TRIM35 there would be a shift in the differentiation pathways, favouring the erythro-myeloid pathway in early stages and the erythrocyte maturation pathway later on.

The main aim was to determine whether TRIM35 can influence erythro-myeloid lineage commitment in differentiating cells using a TRIM35\(^-/-\) mouse model. As TRIM35 has been linked to TGF-β signalling, a pathway known to play a crucial role in the differentiation and proliferation of HSCs, this study also studied the HSC’s, downstream progenitors, erythro-myeloid progenitors, as well as erythroblast, and B-cell populations ex vivo.

To determine the role of TRIM35 in haematopoiesis; the project was divided into the following aims, which were to determine if loss of TRIM35:

1. Affected the hematopoietic stem cells (HSCs) in the murine haematopoietic compartments
2. Affected the erythro-myeloid progenitor populations
3. Altered erythroblast or B-cell maturation
4. Affected circulating blood composition of adult mice
4.2. METHODS

To study the different stem cells, progenitors and differentiation pathways *ex vivo*, cells collected from the haematopoietic compartments (bone marrow and spleens) of C57BL/6 wildtype and *TRIM35*-/- mice were compared in male mice, aged 8-12 weeks or mice older than 80 weeks.

The HSC population and erythroid-myeloid progenitors were analysed by staining collected cells with fluorescent antibodies for LSK/CD48/CD150 or LSK/CD34/FcγRI/III. The reconstitution abilities of wildtype and *TRIM35*-/- HSCs were analysed by injecting mice aged 8-12 weeks intravenously with the cytotoxic drug, 5-Fluorouracil (5FU). 5FU depletes the more committed progenitor populations in mice, stimulating the HSCs to divide and replenish these progenitors. Bone marrow (BM) samples were collected at day 0, 4, 8 and 15 post-treatment and circulating blood was collected for analysis with a Hemavet®. Progenitor populations in the BM were analysed by staining harvested bone marrow cells, with either LSK/CD48/CD150 or LSK/CD34/FcγR.

Circulating blood was collected by cardiac puncture and the cellular composition analysed with a Hemavet®. Erythroid progenitors were analysed by flow cytometry in foetal livers (E11), as well as bone marrow and spleens of adult mice using TER119 and CD71 fluorescent antibodies. Lymphopoiesis in adult bone marrow and spleens was analysed by flow cytometry with B220 and IgM fluorescent antibodies.

All flow cytometry gating strategies used in this chapter are described in Figure 4.2.
Flow cytometry gating strategies for studying erythroid maturation, B-cell maturation, myeloid progenitors, and the LSK population.

**Figure 4.2.** Flow cytometry gating strategies for studying erythroid maturation, B-cell maturation, myeloid progenitors, and the LSK population.

**Myeloid Progenitors**
- Lin^−^ Sca1^−^ cKit^+^ CD34^+^ FcyRll^−^ - MEP
- Lin^−^ Sca1^−^ cKit^+^ CD34^+^ FcyRll^−^ - GMP
- Lin^−^ Sca1^−^ cKit^+^ CD34^+^ FcyRll^−^ - CMP
- Lin^−^ Sca1^−^ cKit^+^ CD34^+^ FcyRll^−^ - HPC

**Lympopoiesis**
- B220^−^ IgM^−^ - pre/pro-B cells
- B220^−^ IgM^−^ - immature B cells
- B220^−^ IgM^−^ - mature B cells

**Erythroid Maturation**
- Ter119^−^ CD71^−^ EryA
- Ter119^−^ CD71^−^ EryB
- Ter119^−^ CD71^−^ EryC (most mature)

**LSK**
- Lin^−^ Sca1^−^ cKit^+^ CD150^+^ CD48^−^ HSC
- Lin^−^ Sca1^−^ cKit^+^ CD150^+^ CD48^+^ MPP
- Lin^−^ Sca1^−^ cKit^+^ CD150^+^ CD48^+^ HPC

**Lineage Markers**
- Sca1
- cKit
- LSK
- CD150
- CD48
4.3. RESULTS

4.3.1. Analysis of LSK population in haematopoietic compartments of adult mice

The murine LSK population in mice is known to be heterogeneous, containing HSCs and MPPs. To determine if this population was affected by the loss of TRIM35, cells collected from bone marrow and spleens of wildtype (WT) and TRIM35-/- mice, aged either 8-12 weeks or 80 weeks, were stained for the LSK markers and the LSK population, as determined by flow cytometry, was measured as percentage of the total cells counted in bone marrow or spleens.

4.3.1.1. TRIM35-/- mice have higher percentage LSK cells compared to WT mice

In WT mice, aged 8-12 weeks, the LSK population represents 0.39%±0.33% (SEM) of the total bone marrow population and 0.77%±0.044% (SEM) of the spleen (Figure 4.3A). On the other hand, 8-12 week old TRIM35-/- mice had a 1.5-fold increase in the bone marrow compared to WT mice, with an average percentage of 0.62%±0.047% (SEM). The spleens of TRIM35-/- mice had a 2.5-fold increase compared to WT mice, with an average of 1.9%±0.085% (SEM). The higher percentages observed in the TRIM35-/- mice were statistically significant compared to WT mice with p <0.05, as determined by Mann-Whitney t-test (Figure 4.3A).

In aged WT mice, aged 80 weeks or older, the LSK population is 0.66%±0.129% (SEM) of the total population in the bone marrow and 0.77%±0.133% (SEM) of the total cellular population of the spleen (Figure 4.3B). TRIM35-/- mice of the same age range had an average of 0.75%±0.116% (SEM) LSK cells in the bone marrow, similar to what was observed for WT mice. However, the spleens of TRIM35-/- 80 week old mice, with an average of 1.3%±0.148% (SEM), had a 1.7-fold increase compared to WT mice, which is significantly higher than WT mice with p <0.05 (as measured by t-test).
Figure 4.3. Comparison of LSK population in haematopoietic compartments of WT and TRIM35-/- mice. The LSK population in the bone marrow and spleens of WT and TRIM35-/- mice was measured, using flow cytometry, in mice aged 8-12 weeks (A) or 80 weeks or older (B). Data is represented as mean±SEM. For mice aged 8-12 weeks: WT: n=6; TRIM35-/-: n=12. For mice aged 80 weeks or older: WT: n=9; TRIM35-/-: n=15. Significance was determined using Mann-Whitney t-test with * p<0.05, ** p<0.01 and *** p <0.001.
4.3.1.2. Compared to WT, *TRIM35/-* mice have higher percentage of HPCs

To further analyse the effect of TRIM35 on progenitors residing in the LSK population, BM and spleens from WT and *TRIM35/-* mice were stained with the LSK markers as well as the SLAM markers, CD48 and CD150. The addition of the SLAM markers allow for enrichment of the HSCs as well as isolating the MPPs and HPCs for additional comparisons.

4.3.1.2.1. Compared to WT, 8-12 week old *TRIM35/-* mice have a significantly higher percentage of HPCs

BM from WT mice, aged 8-12 weeks, displayed a similar distribution of percentages of HSCs, MPPs, and HPCs, as defined by the LSK and SLAM markers, compared to those found in C567BL/6 mice, 8-12 week old [335] or 7-10 week old [353], in previous studies (Figure 4.4A and Table 4.1) [335, 352, 353]. In the spleens of WT mice, aged 8-12 weeks, a similar distribution of the HSC, MPP and HPC percentages were observed as in the BM (Figure 4.4A and Appendix A).

The BM of 8-12 week old *TRIM35/-* mice showed a significant increase of the HSC and HPC population (See Figure 4.4A and Table 4.1), with the HSC population increased by 2-fold compared to WT. Interestingly, the intermediate MPP population shows no statistically significant change. The spleens of the *TRIM35/-* mice contained a significantly higher HPC population compared to WT, with a ~2.5-fold increase (Figure 4.4A). However, the HSC and MPP populations in the spleens were not significantly different compared to WT mice.

4.3.1.2.2. Spleens of aged *TRIM35/-* mice have higher percentages of HSCs, MPPs and HPCs than WT mice

In the BM and spleens of 80 week or older WT mice, the HSC, MPP, and HPC percentages displayed a similar distribution as their younger counterparts (Figure 4.4B and Table 4.1). The percentages of HSCs, MPPs, and HPCs in the BM of *TRIM35/-* mice, aged 80 weeks or older, showed no statistically significant changes compared to WT mice of the
same age group (Figure 4.4B and Table 4.1). Spleens from \( \text{TRIM35}^-/- \) mice, on the other hand, showed significant increases in all three of the subpopulations residing within the LSK population (Figure 4.4B and Appendix A). HSCs and HPCs showed an almost 2-fold increase in \( \text{TRIM35}^-/- \) mice compared to WT. In spleens of aged \( \text{TRIM35}^-/- \) mice, the HSCs represented 0.03\%, compared to 0.016\% in WT mice. The HPC population in \( \text{TRIM35}^-/- \) spleens was 0.65\%, compared to 0.331\% in WT. The MPP population was increased 3-fold in \( \text{TRIM35}^-/- \) mice, which had an average of 0.044\% compared to 0.014\% in WT mice (Figure 4.4B and Appendix A).

| Table 4.1. Comparison of HSC, MPP, and HPC populations in BM of WT and \( \text{TRIM35}^-/- \) C576BL/6 mice measured as \% of whole bone marrow (WBM) (mean±SEM) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cell type       | Markers         | Literature      | 8-12 week old mice | 80 week old mice |
|                 |                 | WT | \( \text{TRIM35}^-/- \) | WT | \( \text{TRIM35}^-/- \) |
| HSC             | LSKCD150'CD48' + | 0.008 to 0.022 | 0.026±0.001 | 0.058±0.009 | 0.016±0.002 | 0.030±0.005 |
| MPP             | LSKCD150'CD48' - | 0.040 to 0.048 | 0.014±0.001 | 0.029±0.006 | 0.014±0.005 | 0.044±0.012 |
| HPC             | LSKCD150'CD48'  | 0.113 to 0.230 | 0.270±0.024 | 0.436±0.031 | 0.331±0.056 | 0.650±0.058 |

*Literature values obtained from Oguro et al. 2013, Ng et al. 2010 and Ng et al. 2012 [335, 352, 353]. HSC, haematopoietic stem cells; MPP, multi-potent progenitors; HPC, haematopoietic progenitor cells. Data presented as mean±SEM.*
Figure 4.4. Distribution of HSC, MPP, and HPC percentages in BM and spleens of WT and TRIM35-/- mice.
The HSC, MPP and HPC populations were analysed in the bone marrow and spleens of WT and TRIM35-/- mice, aged 8-12 weeks (A) or 80 weeks or older (B), using flow cytometry strategy outlined in Figure 4.2. Data is represented as mean±SEM. For mice aged 8-12 weeks: WT: n=6; TRIM35-/-: n=12. For mice aged 80 weeks or older; WT: n=9; TRIM35-/-: n=15. Significance was determined with multiple t-test using the Holm-Sidak method with * p<0.05 and ** p<0.01.

HPC, haematopoietic progenitor cells; HSC, haematopoietic stem cells; MPP, multipotent progenitors
4.3.1.3. Recovery of LSK populations after 5FU treatment

An increase of HSCs and progenitors in TRIM35/- could indicate a role for TRIM35 in modulating self-renewal and proliferation. To determine if loss of TRIM35 affects the repopulation ability of HSC after cytotoxic depletion, WT mice and TRIM35/- mice aged 8-12 weeks, were treated with 5FU. Changes in the LSK population and subpopulations in the BM were analysed at 4, 8 and 15 days post injection. Statistical significance between timepoints was determined with one-way ANOVA and between genotypes with Mann-Whitney t-test, with significance determined as p<0.05.

4.3.1.3.1. LSK population in WT and TRIM35/- mice show same response after 5FU treatment

The percentage of LSK cells in BM of WT mice showed a statistically significant increase at day 4 and day 8, compared to the percentages at day 0. By day 15, the LSK percentage had recovered to its initial value (Figure 4.5 and Table 4.2). The only significant difference between the WT and TRIM35/- percentage of LSK cells was found at day 0. There were no statistical significantly differences in the days post-treatment.

4.3.1.3.2. No significant difference in response of HSCs, MPPs or HPCs to 5FU treatment between WT and TRIM35/- mice

The HSC population in BM of WT mice showed a significant 6-fold increase in percentage of whole bone marrow (WBM) at day 4 post-treatment, compared to day 0. At day 8, the HSC percentage was still significantly higher than day 0, but by day 15 had returned to the initial values (Figure 4.5 and Table 4.2). The WT MPP population showed no statistically significant differences between the timepoints (Figure 4.5 and Table 4.2). The HPC population in WT mice showed a significant 2.5-fold increase at day 8 post-5FU before decreasing significantly by day 15 (Figure 4.5 and Table 4.2). These results are consistent
with 5FU depletion of progenitors inducing a stimulus of HSC proliferation at day 4 post-5FU followed later by an increase in percentage of the lineage-restricted HPCs.

Compared to WT population percentages, the TRIM35/- HSC population was significantly higher at day 0, but not at day 4, 8 or 15 post 5FU treatment (Figure 4.5 and Table 4.2). Despite the difference in pattern of TRIM35/- MPP percentages across the timepoints, no statistical significances were found between WT and TRIM35/- MPPs at any of the days measured. Compared to WT mice HPCs, the only statistical significance was found at day 0, where the TRIM35/- percentage of the HPC population was found to be significantly higher than WT.

<table>
<thead>
<tr>
<th>Table 4.2. Response of WT and TRIM35/- LSK populations to 5FU treatment measured as % of whole bone marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell type</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
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</tr>
<tr>
<td>HPC</td>
</tr>
<tr>
<td>Sample size (n)</td>
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<td></td>
</tr>
</tbody>
</table>

HSC, haematopoietic stem cells; MPP, multi-potent progenitors; HPC, haematopoietic progenitor cells.

Data presented as mean±SEM.
Figure 4.5. The response of LSK, HSC, MPP, and HPC populations to 5-FU in BM of 8-12 week old mice. To determine the effect of 5FU on the LSK population and its subpopulations, WT and TRIM35−/− mice aged 8-12 weeks were injected with 5FU and the bone marrow was collected at day 4, 8, and 15 post-treatment. The different populations in the bone marrow of these mice were analysed using the flow cytometry strategy as outlined in Figure 4.2. Data is presented as mean±SEM.

HPC, haematopoietic progenitor cells; HSC, haematopoietic stem cells; MPP, multipotent progenitors
4.3.2. Analysis of erythro-myeloid progenitor populations in TRIM35-/- mice

Due to its role in lineage switching within the erythro-myeloid lineages, it is essential to determine if loss of TRIM35 affects the erythro-myeloid differentiation pathway. The CMP population and its two downstream progenitors, GMP and MEP, were analysed in BM of mice aged 8-12 weeks. Statistical significance was determined with Mann-Whitney t-test.

4.3.2.1. Myeloid progenitor populations are increased in TRIM35-/- mice

In WT mice, the Lin^Sca1^-cKit^+ population accounted for 4.2% of the whole BM (Figure 4.6B). This population is further divided into CMPs, GMPs and MEPs which accounted for 0.012%, 0.196%, and 0.438% respectively of WT BM (Figure 4.6B and Table 4.3). Remainder of Lin^Sca1^-cKit^+ population (CD34^FcγRII/III^) is uncharacterized. Interestingly, in this study, the CMP and GMP populations were lower than described in the literature for C57BL/6 mice, whereas the MEP percentage was higher (Table 4.3).

In TRIM35-/- mice, the percentage of cKit^Sca1^- cells was 7.6%, which was significantly higher compared to WT, constituting a 1.8-fold increase (Figure 4.6B and Table 4.3). This increase in the overall myeloid progenitor population was further reflected in statistically significant increases in all three of the subpopulations (Figure 4.6B and Table 4.3). The highest increase in percentage was found in the TRIM35-/- GMP population, which showed an 11-fold higher percentage. The CMP and MEP populations showed increases of 8-fold and 2-fold respectively, compared to WT populations.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Markers</th>
<th>Literature %</th>
<th>WT</th>
<th>TRIM35-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythro-Myeloid precursor</td>
<td>Lin^Sca1^-cKit^+</td>
<td>~7.00</td>
<td>4.252±0.549</td>
<td>7.628±0.981</td>
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<tr>
<td>CMP</td>
<td>Lin^Sca1^-cKit^+CD34^-FcγRII/III^-</td>
<td>0.20 to 0.34</td>
<td>0.012±0.002</td>
<td>0.104±0.026</td>
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<tr>
<td>GMP</td>
<td>Lin^Sca1^-cKit^+CD34^-FcγRII/III^-</td>
<td>0.40 to 0.48</td>
<td>0.196±0.025</td>
<td>2.194±0.503</td>
</tr>
<tr>
<td>MEP</td>
<td>Lin^Sca1^-cKit^+CD34^-FcγRII/III^-</td>
<td>0.10 to 0.14</td>
<td>0.438±0.080</td>
<td>0.897±0.113</td>
</tr>
</tbody>
</table>

*Table 4.3. Comparison of Erythro-Myeloid progenitors in BM of C576BL/6 mice aged 8-12 weeks measured as % of whole bone marrow.

Literature values obtained from Akashi et al. 2000 [251]. CMP, common myeloid progenitor; GMP, granulocyte-macrophage restricted progenitor; MEP, megakaryocyte-erythrocyte restricted progenitor. Data presented as mean±SEM.
Figure 4.6. Erythro-Myeloid progenitors in BM of WT and TRIM35-/- mice aged 8-12 weeks.
A) The different erythro-myeloid progenitor populations were analysed in the bone marrow of WT and TRIM35-/- mice aged 8-12 weeks. B) Quantification of the different erythro-myeloid populations in the bone marrow of WT and TRIM35-/- mice, aged 8-12 weeks. Data is presented as mean±SEM. Statistical significance was calculated with multiple t-test using Holm-Sidak method, with * p<0.05 and ** p<0.01.
CMP, clonal myeloid progenitors; GMP, granulocyte and myeloid progenitors; MEP, megakaryocyte and erythrocyte progenitors.
4.3.2.2. Erythro-myeloid progenitor recovery after 5FU treatment

With the significant changes observed in the TRIM35/- myeloid progenitors, the next step was to determine if this increase in numbers changed their response to 5FU. As described above, 8-12 week old WT and TRIM35/- mice were treated with 5FU and the BM myeloid progenitors analysed at day 4, 8 and 15 post injection, using LSK, CD34 and FcγRII/III antibodies. Statistical significance was determined with one-way ANOVA, with significance determined as p<0.05.

4.3.2.2.1. Sca1+cKit+ population in WT and TRIM35/- mice show same response after 5FU treatment

The WT Sca1+cKit+ population showed little change at day 4 post-treatment, but a 2-fold increase by day 8. By day 15, the percentage of Sca1+cKit+ cells had decreased to similar values as day 0 (Figure 4.7 and Table 4.4). However, none of the changes post-treatment were found to be statistically significant, compared to untreated WT mice, as determined by one-way ANOVA.

The TRIM35/- Sca1+cKit+ population at day 4 post-treatment is half the size compared to day 0. At day 8 and day 15, the TRIM35/- population follows the same pattern as the WT cells, with no significant differences visible (Figure 4.7 and Table 4.4). Between WT and TRIM35/-, no significant differences were found at any of the time points as measured by t-test.

4.3.2.2.2. WT and TRIM35/- erythro-myeloid progenitors show no significant differences after 5FU treatment

In WT mice, the CMP population showed a significant 5-fold increase at day 4 compared to day 0 (Figure 4.7 and Table 4.4). At day 8, the population showed a slight decrease, but was still significantly higher than day 0. At day 15 post-treatment, the WT CMP population had returned to percentages comparable with day 0. The WT GMP population also
displayed a significant increase of 8.5-fold at day 4 (Figure 4.7 and Table 4.4) then decreased by day 8 and day 15 post-treatment. The WT MEP population changed little at day 4, but doubled by day 8 post-treatment, although this was not statistically higher than the population at day 0 (Figure 4.7 and Table 4.4). At day 15, the MEP population had returned to values similar to day 0.

In contrast, the elevated TRIM35/-/ CMP population was halved on day 4 compared to the population at day 0 (Figure 4.7 and Table 4.4). No significant change was observed thereafter. Between WT and TRIM35/-, there was a significant difference at day 0 only. The elevated TRIM35/-/ GMP population decreased slightly at day 4 and was significantly lower on day 8 and day 15 post-treatment (Figure 4.7 and Table 4.4). However, between WT and TRIM35/-/ GMP populations, the only significant difference was at day 0. Similarly, the higher TRIM35/-/ MEP population on day 0 decreased to a third of the initial percentage by day 4. A transient increase was observed on day 8. However, none of the MEP changes were found to be statistically significant between timepoints. The only significant difference between WT and TRIM35/-/ MEP populations was found at day 0 with a significantly higher TRIM35/-/ population.

| Table 4.4. Response of WT and TRIM35/-/ erythro-myeloid progenitors to 5FU treatment measured as % WBM |
|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Cell type     | Days post 5FU treatment |
|               | 0             | 4             | 8             | 15            |
| Sca1+ cKit+   |               |               |               |               |
| WT            | 4.252±0.549   | 3.441±0.641   | 9.598±1.717   | 5.742±1.357   |
| TRIM35/-      | 7.628±0.981   | 3.365±0.878   | 10.948±3.622  | 7.641±1.529   |
| CMP           |               |               |               |               |
| WT            | 0.012±0.002   | 0.064±0.009   | 0.046±0.005   | 0.015±0.006   |
| TRIM35/-      | 0.104±0.026   | 0.051±0.003   | 0.060±0.015   | 0.057±0.021   |
| GMP           |               |               |               |               |
| WT            | 0.196±0.025   | 1.702±0.318   | 0.587±0.132   | 0.603±0.174   |
| TRIM35/-      | 2.194±0.503   | 1.772±0.546   | 0.642±0.108   | 0.932±0.366   |
| MEP           |               |               |               |               |
| WT            | 0.438±0.080   | 0.334±0.072   | 0.835±0.112   | 0.270±0.145   |
| TRIM35/-      | 0.897±0.113   | 0.277±0.047   | 1.088±0.480   | 0.581±0.182   |
| Sample size (n) |           |               |               |               |
| WT            | 4             | 4             | 4             | 7             |
| TRIM35/-      | 5             | 3             | 4             | 6             |

CMP, common myeloid progenitor; GMP, granulocyte-macrophage restricted progenitor; MEP, megakaryocyte-erythrocyte restricted progenitor. Data presented as mean±SEM.
Figure 4.7. Response to 5FU treatment of erythro-myeloid progenitor populations in BM of WT and TRIM35−/− mice aged 8-12 weeks. To determine the effect of 5FU on the erythro-myeloid progenitor populations, WT and TRIM35−/− mice aged 8-12 weeks were injected with 5FU and the bone marrow was collected at day 4, 8, and 15 post-treatment. The different populations in the bone marrow of these mice were analysed using the flow cytometry strategy as outlined in Figure 4.2. Data is presented as mean±SEM.

CMP, clonal myeloid progenitors; GMP, granulocyte and myeloid progenitors; MEP, megakaryocyte and erythroid progenitors.
4.3.3. Analysis of downstream differentiation pathways in TRIM35-/- mice

4.3.3.1. Erythroid maturation in WT and TRIM35-/- mice

To determine if loss of TRIM35-/- affected erythroid maturation in murine haematopoietic compartments, BM and spleens from 8-12 week or 80 week old mice as well as foetal livers were analysed using the erythroid maturation cell surface markers, CD71 and TER119 (Figure 4.2). The pro-erythroblasts (pro-E) are defined by TER119<sup>CD71<sub>hi</sub></sup>, whereas the less mature EryA and EryB erythroblasts are found in the TER119<sup>+</sup>CD71<sup>hi</sup> population. The most mature EryC erythroblast is characterized by TER119<sup>+</sup> CD71<sup>-</sup>. The different populations were analysed as percentage of total cells measured and statistical significance determined by multiple t-test using the Holm-Sidak method.

4.3.3.1.1. TRIM35-/- foetal livers have significantly higher EryC population compared to WT

Foetal livers were collected at E11 from pregnant WT or TRIM35-/- females and the foetal livers from each female pooled for analysis.

WT foetal livers had an average of 15.1% ProE cells, 42.8% EryA and EryB population, and 8.7% of the EryC population (Figure 4.8 and Table 4.5), which was higher than previously reported [354]. In comparison, TRIM35-/- foetal livers had an average of 11.8% ProE, and 32.1% of the EryA/EryB population, none of which were significantly different to the WT foetal livers (Figure 4.8 and Table 4.5). The TRIM35-/- foetal livers, however, had a significant increase of the EryC population with an average of 26.2%.

| Table 4.5. Erythroblast maturation in WT and TRIM35-/- foetal livers measured as percentage of total cell population |
|------------------|-----------------|-----------------|-----------------|
| Cell type        | Markers         | WT (mean±SEM)   | TRIM35-/- (mean±SEM) |
| ProE             | TER119<sup>+</sup>CD71<sup>hi</sup> | 15.125±1.130 | 11.832±2.468 |
| EryA/EryB        | TER119<sup>+</sup>CD71<sup>hi</sup> | 42.750±3.121 | 32.100±5.115 |
| EryC             | TER119<sup>+</sup>CD71<sup>-</sup> | 8.697±1.700  | 26.167±5.648 |

Pro-E, pro-erythroblasts; EryA/EryB and EryC, different subsets of maturing erythroblasts. Data presented as mean±SEM.
Figure 4.8. Analysis of erythroid maturation in WT and TRIM35-/- E11 foetal livers.
A) Different erythroblast populations were identified using CD71/TER119 markers on cells from foetal livers collected from WT (n=4 pregnant females) or TRIM35-/- (n=3 pregnant females) mice. B) Comparison of the different erythroblast populations showed no significant difference between WT and TRIM35-/- foetal livers, with the exception of the EryC population. Data is presented as mean±SEM. * p<0.005
4.3.3.1.2. Erythroid maturation in TRIM35-/ mice aged 8-12 weeks is unaffected

The BM of WT mice, aged 8-12 weeks, contained an average of 1.0% ProE cells, 19.4% EryA/EryB cells, and 28.1% EryC cells (Figure 4.9A and Table 4.6). The spleens of WT mice had a lower ProE and EryA/EryB population, with an average of only 0.36% and 4.2% respectively, but a higher percentage of EryC cells with an average of 43.8% (Figure 4.9A and Table 4.6).

The BM of TRIM35-/ mice, aged 8-12 weeks, had similar values to the BM of WT mice, with averages of 1.2% ProE cells, 16.9% EryA/EryB cells and 31.3% EryC cells (Figure 4.9A and Table 4.6). No statistical differences were observed between the WT and TRIM35-/ populations. Similarly, the spleens of TRIM35-/ mice had comparable populations as spleens from WT mice, with averages of 0.32% ProE cells, 3.7% EryA/EryB cells and 38.4% EryC cells with no statistically significant differences between WT and TRIM35-/ populations (Figure 4.9A and Table 4.6).

4.3.3.1.3. Compared to WT, BM of aged TRIM35-/ mice have lower percentages of EryA/EryB but higher percentages of EryC cells

The BM of WT mice aged 80 weeks or older had an average percentage of ProE cells of only 0.16%, whereas the EryA/EryB and EryC populations represented 23.2% and 24.5% respectively (Figure 4.9B and Table 4.6). The spleens from aged WT mice had an average of 0.02% of ProE cells whereas the EryA/EryB and EryC populations represented 5.1% and 45.6% of cells counted respectively.

Compared to WT aged mice, the BM of TRIM35-/ mice had a significantly lower EryA/EryB population but a significantly higher EryC population (Figure 4.9B and Table 4.6). The EryA/EryB population in the BM of aged TRIM35-/ mice accounted for 17.8% and the EryC population represented 41.4%. The BM of TRIM35-/ mice aged 80 weeks or older
had an average ProE population of 0.27% (Figure 4.9B and Table 4.6), which was not significantly different from WT.

Compared to WT mice of 80 weeks or older, the spleens of aged TRIM35-/- mice showed no statistically significant changes. The spleens of TRIM35-/- aged mice contained an average ProE population of 0.04%, EryA/EryB population of 4.7% and the EryC population accounted for 47.5%. (Figure 4.9B and Table 4.6).

<table>
<thead>
<tr>
<th>Cell type</th>
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<th>Bone marrow</th>
<th>Spleen</th>
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<tr>
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</tr>
<tr>
<td>EryA/EryB</td>
<td>TER119'CD71hi</td>
<td>WT</td>
<td>19.432±1.443</td>
<td>16.898±1.464</td>
</tr>
<tr>
<td>EryC</td>
<td>TER119'CD71hi</td>
<td>WT</td>
<td>28.056±3.139</td>
<td>31.298±2.895</td>
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</tbody>
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<table>
<thead>
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<th>Mice aged 80 weeks or older</th>
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<tr>
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<td>TER119'CD71hi</td>
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</tr>
<tr>
<td>EryC</td>
<td>TER119'CD71hi</td>
<td>WT</td>
</tr>
</tbody>
</table>

ProE, pro-erythroblasts; EryA/EryB and EryC, different subsets of maturing erythroblasts. Data presented as mean±SEM.
Figure 4.9. Erythroid maturation measured in BM and spleens of WT and TRIM35−/− mice aged either 8-12 weeks or older than 80 weeks. Using TER119 and CD71 as markers, the erythroid populations in the bone marrow and spleens was compared between WT and TRIM35−/− mice aged 8-12 weeks (A) or 80 weeks or older (B). Data is represented as mean±SEM with WT: n=18 and TRIM35−/−: n=22. Significance was determined by multiple t-test using Holm-Sidak method with * p<0.05 and ** p<0.01.
4.3.3.2. B cell maturation in WT and TRIM35-/- mice

To further investigate the potential role of TRIM35 in downstream haematopoiesis, the effect of loss of TRIM35 on the downstream populations of the lymphoid lineage was investigated. B cell maturation was measured in the BM and spleens of WT and TRIM35-/- mice aged 8-12 weeks or older than 80 weeks. The different B cell populations were characterized using expression levels of the cell surface markers B220 and IgM with the pre/pro-B cell population characterized as B220medIgM-, the immature B cell population as B220medIgM+ and the mature B cell population as B220hiIgM+. All populations are presented as percentage of total cells counted by flow cytometry and statistical significance determined by multiple t-test using the Holm-Sidak method.

4.3.3.2.1. Spleens of young adult TRIM35-/- mice have a higher percentage of mature B cells than WT

In the BM of WT mice aged 8-12 weeks, the pre/pro-B-cells constituted an average 5.8% of the whole BM, the immature B cell population was an average of only 1.2% whereas the mature B cell population counted an average of 2.3% of the whole BM (Figure 4.10A). In contrast, the spleens had an average pre/pro-B cell population of 2.2%, an average immature B cell population of 2.1% and a mature B cell population of 10.9% (Figure 4.10A).

The only significant difference in lymphoid precursors detected between WT and TRIM35-/- mice, was a higher percentage of the mature B cell population, with an average of 17.4%, in the spleens of the TRIM35-/- mice.

4.3.3.2.2. BM of aged TRIM35-/- contain smaller percentages than WT of pre/pro-B cells and mature B cells

The BM of WT mice, aged 80 weeks or older, had an average pre/pro-B cell population of 5.1%, immature B cell population of 1.1% and mature B cell population of 1.6%, similar to what was observed in 8-12 week old WT mice (Figure 4.10A and B). The spleens of aged
WT mice had an average pre/pro-B cell population of 2.6%, immature B cell population of 3.5% and mature B cell population of 5.3%, which is very close to that of the younger WT mice (Figure 4.10A and B).

In the BM of TRIM35/- mice, aged at least 80 weeks, significant decreases were observed in the pre/pro-B cell and mature B cell populations, but not the immature B cells (Figure 4.10B). TRIM35/- mice had an average pre/pro-B cell population of 3.2% and mature B cell population of 1.1%, whereas the average immature B cell population was 0.90% (Figure 4.10B). In contrast, no significant differences were detected in the spleens of aged TRIM35/- mice compared to WT mice of the same age group (Figure 4.10B).

<table>
<thead>
<tr>
<th>Table 4.7. Distribution of B cell precursors in BM and spleens of young adult and aged WT and TRIM35/- mice measured as % of total cells counted</th>
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</thead>
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<tr>
<td>Immature B cell</td>
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<tr>
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</table>

Data presented as mean±SEM.
Figure 4.10. B cell maturation measured in BM and spleens of WT and TRIM35−/− mice aged 8-12 weeks or older than 80 weeks. Using B220 and IgM as markers, the pre/pro-B cell, immature B-cell and mature B-cell populations in the bone marrow and spleens of WT and TRIM35−/− mice aged 8-12 weeks (A) or 80 weeks or older (B) was analysed with flow cytometry. Data is represented as mean±SEM with WT: n=18 and TRIM35−/−: n=22. Significance was determined by multiple t-test using the Solm-Hidak method with * p<0.05 and ** p<0.01.
4.3.3.3. No difference in circulating blood cells between WT and TRIM35-/- mice

As TRIM35 was shown to affect HSCs (Figure 4.4), as well as certain progenitor (Figure 4.6) and precursor populations (Figure 4.9 and Figure 4.10), the effect of TRIM35 deletion on the composition of circulating blood of adult mice was subsequently analysed. Blood samples from WT and TRIM35-/- mice, aged 8-12 week olds or aged 80 weeks and older, were analysed and compared.

In WT mice, aged 8-12 weeks old or 80 weeks and older, the circulating blood cellular populations as well as hematocrit and haemoglobin, were all within the normal range (Figure 4.11, Figure 4.12 and Appendix B). Circulating blood from TRIM35-/- mice, aged 8-12 weeks or 80 weeks and older, also fell within the normal range and showed no statistical difference with the WT populations, as measured by t-test (Figure 4.11, Figure 4.12 and Appendix B), indicating that the effect of TRIM35 does not extend to mature circulating blood cells.
Figure 4.11. Circulating blood composition of WT and TRIM35-/- mice aged 8-12 weeks as measured by Hemavet®. No significant differences between WT and TRIM35-/- were visible in any of the cell types (top two rows) or quantities of haematocrit or haemoglobin (bottom row). Statistical significance was measured with unpaired t-test. (WT: n=17; TRIM35-/-: n=22)
Figure 4.12. Circulating blood composition of WT and TRIM35-/- mice aged 80 weeks or older as measured by Hemavet®. No significant differences between WT and TRIM35-/- were visible in any of the cell types (top two rows) or quantities of haematocrit or haemoglobin (bottom row). Statistical significance was measured with unpaired t-test. (WT: n=10; TRIM35-/-: n=15)
4.4. DISCUSSION

TRIM35 was shown to be involved in lineage switching within the erythro-myeloid lineage in haematopoiesis and is a known regulator of GATA-1, an essential transcription factor for erythroid maturation [181, 298]. The aim of this study was to investigate the role of TRIM35 in haematopoiesis and determine if it is a key player in haematopoietic lineage commitment. To understand potential influences over the whole lifespan of the mice, the effect of loss of TRIM35 was studied in embryonic tissue, sexually matured adults (8-12 weeks) and aged mice (80 weeks or older). In this study, the loss of \textit{TRIM35/-} was shown to affect HSCs and early progenitors in mice, but surprisingly had little effect on mature blood cells.

Haematopoiesis begins with HSC maturing into early progenitor cells such as MPPs and HPCs. Data presented in this chapter illustrate that the loss of \textit{TRIM35} results in an increase of the LSK population in the BM and spleens of 8-12 week old mice. Furthermore, this increase of the overall LSK population was reflected in increases of the LSK subpopulations, HSCs and HPCs, in the BM of \textit{TRIM35/-} mice. In the spleens, there was also a significant increase of the HPC population in \textit{TRIM35/-} mice, whereas the MPP population was unaffected by loss of \textit{TRIM35}. Interestingly, in 80 week old mice there were no significant differences in the BM, but all of the LSK subpopulations were significantly increased, including the MPP population, in the spleens.

The increases observed in mice lacking \textit{TRIM35}, suggests a potential role for TRIM35 in regulating HSC self-renewal and progenitor proliferation. Previous research has shown that HSC quiescence is regulated by TGF-\(\beta\) [235]. The possibility that TRIM35 acts as a novel
regulator of TGF-β pathway (Chapter 3) is particularly intriguing in the context of maintaining HSC quiescence.

This study measured the percentages of cells within specific populations. Increases in these ratios may indicate increased proliferation, but is not definitive. To confirm whether TRIM35-/- HSCs and HPCs are more proliferative, cell cycle analyses should be performed on the different cell types. This will confirm whether larger percentages of cells in TRIM35-/- have entered the cell cycle. Additionally, this study did not investigate the differentiation ability of TRIM35-/- HSCs by colonies or BM reconstitution of lethally-irradiated mice. In light of TRIM35 being linked to a more monocyte phenotype, it would be of interest to determine if the HSCs in TRIM35-/- mice are already biased towards the myeloid lineage, or whether these cells are able to fully reconstitute the haematopoietic compartment of irradiated mice.

In young adult WT mice, the depletion of progenitors with 5FU resulted in a significant increase of the HSC and MPP populations in the BM at day 4 post treatment but by day 8 both these populations had returned to normal levels. The more committed HPC population however, showed a significant increase at day 8 post treatment before returning to normal levels at day 15. This result illustrates a wave of maturation activity after 5FU-mediated progenitor depletion. Although the HSC, MPP and HPC proportions were raised in TRIM35-/-mice at day 0, there were no significant differences at any other time point post-5FU treatment.

These results indicate that despite playing a potential role in negatively regulating proliferation of stem cells and progenitors, loss of TRIM35 did not alter the response of the haematopoietic system to the stress induced by 5FU. This observation implies a context-specific role for TRIM35 in regulating HSC and progenitor activity.
TRIM35 has predominantly been linked to cell types within the erythro-myeloid lineage [181, 298]. To determine if TRIM35 regulates differentiation within these pathways, the effect of loss of TRIM35 on CMPs and its progeny, GMPs and MEPs was studied. Data presented here show the BM of young adult TRIM35-/− mice contained significantly higher percentages of CMP, MEP and GMP populations than WT BM. Importantly, the largest difference was observed in the GMP population, which was 11-fold higher than that of WT mice, while the TRIM35-/− MEP population was 2-fold larger than WT. This suggests that TRIM35 plays a key role in the maturation of progenitors which produce mature erythroid and myeloid cells.

As with the LSK populations, our study into the erythro-myeloid progenitors focused on percentages of cells within specific populations. Cell cycle analysis would be required to determine whether these progenitors have increased proliferative capacity compared to WT progenitors.

Depletion of proliferating progenitors in WT mice with 5FU resulted in significant increases in all the erythro-myeloid progenitors, CMPs, MEPs, and GMPs. In marked contrast, TRIM35-/− progenitors showed a completely different pattern with significant decreases observed in CMPs, MEP, and GMPs. The unexpected decrease of TRIM35-/− erythro-myeloid progenitors after 5FU treatment again indicates a possible context-specific function for TRIM35. In this case, the stress-induced proliferation stimulus resulted in reduced proliferation of TRIM35-/− progenitors, with none recovering to their initial values by day 15.
TRIM35 expression was initially linked to a monocyte phenotype and in fact, TRIM35 overexpression was shown to be able to inhibit erythropoiesis [181]. To determine to what extent TRIM35 can influence erythroid maturation, erythroblast populations were examined in different haematopoietic compartments of young adult and aged WT and TRIM35-/- mice as well as foetal livers, the primary source of erythroid progenitors and precursors in the foetus. Significant changes in different erythroblast subpopulations were detected, primarily in TRIM35-/- foetal livers, as well as the BM and spleens of aged TRIM35-/- mice compared to WT. However, no difference due to loss of TRIM35 was observed in the BM or spleens of young adult mice. This indicates that TRIM35 does play a role in modulating erythroid maturation, under specific conditions.

To establish whether the lymphoid lineage is affected by loss of TRIM35, B-cell maturation was also studied in the haematopoietic compartments of young adult and aged WT and TRIM35-/- mice. Significant changes were detected in some subpopulations of B cell precursors. Again, this observation suggests that the effect of TRIM may be context-dependent.

To definitively establish the role of TRIM35 in erythroid and B cell maturation, a more in-depth analysis of the differentiation pathways is required, such as colony assays where the stimulatory environment of progenitors can be tightly regulated using different cytokines. The resulting composition of differentiated cell types resulting from WT or TRIM35-/- progenitors will further assist in understanding the role TRIM35 plays in regulating these pathways.

Comparison of circulating blood cells between young adult and aged WT and TRIM35-/- mice, revealed no significant differences in cell numbers or hematocrit and haemoglobin values. Given the differences observed in HSCs and progenitors/precursors, this result was surprising. One interpretation of these data is that despite changes in the stem and progenitor
compartments in TRIM35-/ mice, some complimentary mechanism exist which ensures normal blood cell levels are maintained in absence of TRIM35. One potential compensating mechanism could be overlapping functions between TRIM proteins, as several TRIM proteins have been linked to haematopoiesis. However, to date, there is no evidence that suggests this is the case and it is generally considered that, despite their conserved domains, the TRIM proteins all have distinct functions. Potential compensation between TRIM proteins would require further research into these specific roles of the various proteins.

In conclusion the data presented in this chapter indicate a role for TRIM35 in regulating HSCs and several progenitors. However, TRIM35 may not be essential for regulation of the mature blood cell types. This implies a very specific role for TRIM35 in the maturation of haematopoietic cells. Further research is clearly required to elucidate the role of TRIM35 in haematopoiesis. The precise role of TRIM35 in managing the HSC and progenitor populations needs to be addressed in more detail, and whether loss of TRIM35 does indeed result in increase of cellular proliferation. If loss of TRIM35 does increase self-renewal of HSCs, as well as proliferation of progenitors, this would provide additional evidence that TRIM35’s role in TGF-β signalling may influence stem cell quiescence and subsequent proliferation of progenitors.

In this study, TRIM35 has been linked to TGF-β signalling through its interaction with several SMAD proteins. Therefore, one possible explanation for the context-specific effects observed in TRIM35-/ mice could be explained by its role in regulating the TGF-β pathway. Unfortunately, attempts to sort the different HSC and progenitor populations and extract RNA after treatment with TGF-β, was unsuccessful. Future attempts to analyse the gene expression differences in these populations after TGF-β treatment will significantly enhance our understanding of the role of TRIM35 in haematopoiesis.
CHAPTER 5 ROLE OF TRIM35 IN LIVER DISEASE
5.1. INTRODUCTION

Several studies have recently linked TRIM35 to hepatocellular carcinoma (HCC) based on genetic profiles of HCC patient samples [169, 170, 188]. Furthermore, it was shown that TRIM35 is capable of interacting with PKM2, a pyruvate kinase isoenzyme essential for cancer cell growth [191]. Despite the strong evidence that TRIM35 plays a role in HCC development, it remains unclear whether loss of TRIM35 is causal and initiates liver disease, or consequential and exacerbates or promotes progression of the disease.

5.1.1. Liver disease

Despite major advances in the management of liver disease, its incidence rate and prevalence is still steadily increasing [355]. Chronic liver disease can lead to cirrhosis and in some cases to hepatocellular carcinoma (HCC), the leading causes of worldwide morbidity and mortality [268, 355]. Liver cirrhosis places patients at risk of deadly hepatic failure. In fact, half of the patients with alcoholic fatty liver disease that have progressed to cirrhosis will not survive [356]. In addition, 80-90% of patients with cirrhotic livers will develop HCC, which is the fifth most common cancer and accounts for at least 600 000 deaths worldwide per annum [268].

Currently, the most reliable treatment for liver failure is liver transplant, which is limited by donor availability. This makes the development of better methods for early diagnosis and preventive measures even more important in the effort to reduce liver disease mortality.

5.1.2. Animal models for studying liver disease

The progression of HCC can be broken down into distinct phases, including steatosis, inflammation, fibrosis, and cirrhosis (see 1.6). Understanding the different mechanisms that regulate each step is essential for developing effective and targeted therapies. To investigate the separate stages of HCC as well as the role played by the LPCs, several animal models
have been developed. In this project, we utilized the choline-deficient ethionine-supplemented (CDE) diet to study the effect of the carcinogen on the liver during a long-term study [357].

The CDE diet was originally developed to mimic human conditions of fatty liver disease in rats [358]. Very early on it was shown that a choline-deficient, high-fat diet is capable of inducing fat accumulation in the hepatocytes [359]. By combining the choline-deficiency with a carcinogen such as ethionine, a significant increase in liver damage can be observed within two weeks on the diet compared to mice fed ethionine with a choline-sufficient diet [358, 359]. The CDE diet is relevant for studying human disease as it induces inflammation and eventually LPC proliferation that is observed in human chronic liver disease [360].

In short term studies using the CDE diet, mice will progress through steatosis, inflammation and subsequent LPC proliferation [360]. Applying the same diet for long-term will result in fibrosis, cirrhosis and eventually HCC [361]. Unfortunately, in its original form, this diet has severe effects on mice, including in excess of 20% body weight loss, high morbidity rate, including decreases in movement and grooming. To address these concerns and to meet welfare guidelines, a trial was undertaken investigating the effectiveness of lower dosages of the CDE diet by mixing it with high-fat chow [362]. The addition of the high-fat chow reduced the amount of weight loss, thereby reducing the morbidity of mice fed the CDE diet. By using the LPC response as a measure for effectiveness, this study showed that a CDE diet reduced to 75% or 65% in potency, is equally effective in inducing a LPC response as a full-strength CDE diet during a short-term study (3 weeks), but with a large reduction in morbidity and mortality of the animals [362]. The control mice are given a choline-sufficient, high-fat diet.
5.1.3. Project aims

Inflammation of the liver is a key step in the development of liver disease and progression to HCC. Importantly, TRIM35 has previously been linked to both inflammation [61] and occurrence of HCC [87, 169, 191]. Additionally, research presented in Chapter 3 shows TRIM35 is able to regulate TGF-β signalling, a pathway known to be essential in promoting liver disease progression. Therefore, it is hypothesized that loss of TRIM35 could influence HCC development and progression. No direct link between loss of TRIM35 and HCC has yet been established. Therefore, the focus of this project was to investigate whether loss of TRIM35 affects the development of HCC in vivo.

To definitively determine the role of TRIM35 in HCC in vivo, this project utilized WT and TRIM35-/- mice fed either a high-fat (control), or 67% choline-deficient, ethionine supplemented diet supplemented with high-fat chow (CDE).

This project sought to achieve the following aims:

1. Establish if loss of TRIM35 can induce symptoms of liver disease in mice fed a control diet.
2. Determine if loss of TRIM35 increases the incidence rate and severity of HCC in mice fed the CDE diet.
3. Investigate the role of TRIM35 in TGF-β signalling in the liver by analysing the TGF-β response in livers of WT and TRIM35-/- mice fed the control or CDE diet.
5.2. METHODS

To monitor the effect of loss of TRIM35/- on liver disease progression and HCC development, wildtype and TRIM35/- mice, aged 5-6 weeks, were fed either control or CDE diet for the duration of 9 months. Upon arrival in the animal facility, all mice were first given the control diet for 7 days. On day 8 after arrival (T=0), mice were given their respective diets. During the first three weeks, mice were weighed daily. After 21 days, they had regained their initial weight and were weighed twice weekly for the remainder of the experiment. Progression and disease onset were monitored with monthly MRI scans, starting at 5 months on the diet.

Upon reaching the endpoint, mice were sacrificed and blood samples were collected. Circulating blood cell populations were analysed using a Hemavet® and the remainder of the blood was allowed to coagulate to obtain serum. Serum was used for quantifying cytokines using the mouse 10-plex Luminex kit from Invitrogen and the Luminex 200 at the Centre for Microscopy, Characterisation and Analysis (CMCA) located at the Perkins Institute.

The pancreas, spleen, and a kidney were fixed overnight in 4% paraformaldehyde (PFA) before transferring to PBS. Each liver lobe was appropriated differently: one lobe was fixed overnight in 4% PFA and two lobes were snap frozen in OCT in liquid nitrogen. Samples fixed in 4% PFA were processed, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) by CELLCentral at the University of Western Australia. All sections were imaged at 40x magnification with a ScanscopeXT at the Centre for Microscopy, Characterisation and Analysis (CMCA).

The remaining lobes were used to extract RNA, using Bioline RNA extraction kit, which was subsequently used to measure mRNA levels of TGF-β target genes using a RT2-Profiler kit from Qiagen. Based on this initial screen, the expression levels of several other genes were validated by RT-PCR.
5.3. RESULTS

5.3.1. Effect of loss of TRIM35-/- on control mice

The control diet does not result in HCC but, as it is high in fat, it can induce symptoms related to early stages of chronic liver disease, such as hepatocytes ballooning and inflammation [363]. Therefore this study analysed the effect of control diet on WT (n=9) and TRIM35-/- (n=10) mice to determine if loss of TRIM35 is sufficient to increase symptoms of liver injury, such as inflammation.

5.3.1.1. TRIM35-/- mice gain significantly more weight than WT mice on control diet

WT and TRIM35-/- mice fed the control diet were weighed twice a week for the duration of the 9 month study to measure the extent of weight gain.

Not surprisingly, WT and TRIM35-/- mice gained a significant amount of weight during the initial months on the diet, but eventually stabilized. Interestingly, the TRIM35-/- mice had an overall higher body weight on the control diet than the WT mice (as measured by two-way ANOVA) (Figure 5.1A).

To determine if the rate of weight gain was different between WT and TRIM35-/- mice, the daily average weight gain was calculated from T=0 to end of experiment (Figure 5.1B). Compared to the daily average gain of 0.115gr/day of WT mice, the daily average gain of 0.131gr/day observed in TRIM35-/- mice fed the control diet was found to be statistically higher (determined with Mann-Whitney t-test) (Figure 5.1B). These results indicate an unexpected novel role for TRIM35 influencing the rate of fat accumulation.
Figure 5.1. Weight gain of WT and TRIM35-/- mice fed the control diet for 9 months. Mice were given 7 days to acclimatise, thus day 8 is T=0. A) The average body weight of TRIM35-/- mice (n=10) on the control diet was significantly higher than WT mice (n=9) (as measured by two-way ANOVA) B) The daily average body weight gain (gr/day) of WT and TRIM35-/- mice on the control diet was calculated. TRIM35-/- mice had a significantly higher average daily weight gain than WT mice (determined by Mann-Whitney t-test). Data is presented as mean±SEM. *p<0.05
5.3.1.2. Liver morphology of WT and TRIM35-/- mice diet

To study the morphology of the livers of WT and TRIM35-/- mice on the high-fat diet, sections of the livers were stained with H&E.

Visual comparison of livers of WT and TRIM35-/- mice suggested TRIM35-/- mice had slightly larger livers (Figure 5.2A). H&E staining of WT and TRIM35-/- livers showed no differences in liver architecture (Figure 5.2B). WT and TRIM35-/- livers both show ballooning of hepatocytes (asterisks), an indication of fat accumulation which was expected.

5.3.1.3. Analysis of liver fibrosis in WT and TRIM35-/- mice

A step in the progression of chronic liver injury is fibrosis. Therefore, to measure the extent of damage caused by 9 months on the control diet, WT and TRIM35-/- liver sections were stained with Sirius Red and the extent of staining was quantified using Imagescope.

WT and TRIM35-/- mice fed the control diet showed signs of mild fibrosis throughout the visible liver steatosis, as expected of mice enduring a long-term high-fat diet (Figure 5.3A). Quantification of the high intensity pixels showed that there were no statistically significant differences between WT and TRIM35-/- (Figure 5.3B).
Figure 5.2. Liver morphology and histology of WT and TRIM35-/- mice fed the control diet for 9 months. A) Livers of WT and TRIM35-/- mice show no obvious difference in morphology. B) Liver histology shows no obvious differences between WT and TRIM35-/- mice fed the control diet. Ballooning of hepatocytes (asterisks) is clearly visible in H&E stains of liver sections from WT and TRIM35-/- mice. WT, n=9; TRIM35-/-, n=10.
Figure 5.3. Quantification of fibrosis in livers of WT and TRIM35-/− mice fed the control diet. A) Liver sections were stained with Sirius Red to visualize collagen deposits, an indicator of fibrosis. WT and TRIM35-/− mice showed intense staining amongst the visible steatosis. Bar represents 50μm. B) Quantification of the Sirius Red staining showed no significant differences between WT and TRIM35-/− mice (measured by t-test). Data is presented as mean±SEM. WT, n=9; TRIM35-/−, n=10.
5.3.2. Effect of control diet on inflammation in WT and TRIM35-/- mice

A high-fat diet is known to induce inflammation in adipose and hepatic tissue in mice [363] and previous research has implicated TRIM35 as a regulator of the immune system [87, 181]. As obesity is associated with inflammation [364] and TRIM35-/- mice on the control diet gained significantly more weight than WT mice, investigating changes in the inflammatory response in TRIM35-/- mice was of interest in the context.

5.3.2.1. Analysis of inflammation in livers of TRIM35-/- mice and WT mice

To compare hepatic inflammation in WT and TRIM35-/- mice fed the control diet, liver sections were stained CD45 to identify infiltrating immune cells and the amount of strongly positive pixels were quantified with Imagescope. Additionally, percentage of CD45 positive cells was analysed with InForm (Appendix D).

Livers of WT and TRIM35-/- mice showed CD45 positive cells scattered throughout the liver in a diffuse rather than localized pattern (Figure 5.4A). Quantification showed that WT mice had an average of 0.55% CD45 positive pixels and TRIM35-/- mice had an average 0.97% (Figure 5.4B). Although there was a definite trend towards increased inflammation in TRIM35-/-, t-test analysis showed that the observed increase of CD45 positive cells in livers of TRIM35-/- was not significant (Figure 5.4B).

5.3.2.2. Peripheral blood analysis of WT and TRIM35-/- mice

Peripheral blood from WT and TRIM35-/- mice, fed the control diet for 9 months, was analysed to determine if there were changes in haematopoietic cells.

Hemavet® analysis showed no statistically significant differences in any immune cells between WT and TRIM35-/- mice fed the control diet (Figure 5.5). In addition, the average measurements of all mice were within the empirically determined ranges for mice (Appendix C).
Figure 5.4. Quantification of inflammatory cells in livers of WT and TRIM35-/- mice fed the control diet. A) Liver sections were stained with CD45 to visualize inflammatory cells. WT and TRIM35-/- mice contained CD45 positive cells dispersed throughout the liver. Bar represents 50μm. B) Quantification of the CD45 staining showed no significant differences between WT and TRIM35-/- mice (measured by t-test). Data is presented as mean±SEM. WT, n=9; TRIM35-/-, n=10.
Figure 5.5. Analysis of cellular composition of circulating blood of WT and TRIM35-/− mice fed the control diet. The cellular composition of circulating blood, collected from WT and TRIM35-/− mice fed the control diet for 9 months, was analysed with Hemavet®. The different populations and measurements were compared between WT and TRIM35-/− and no statistically significant differences were found (measured by t-test). Data is presented as mean±SEM. WT, n=9; TRIM35-/-, n=10.
5.3.2.3. *TRIM35*/- mice have significantly higher levels of circulating IL-4 and IL-6

Cytokine production and subsequent release by inflammatory cells plays an indispensable role in inflammation. Thus, to further investigate the effect of loss of *TRIM35* on the inflammatory response induced by the control diet, cytokine levels in serum from WT and *TRIM35*/- mice were analysed using the Luminex 10-plex assay. Appendix E shows standards used to calculate concentrations.

Two cytokines, GM-CSF and IL-2, could not be detected. Of the eight cytokines measured, six showed no significant difference between WT and *TRIM35*/- mice fed the control diet for 9 months (Figure 5.6).

However, IL-4 and IL-6 were shown to be significantly higher in *TRIM35*/- mice compared to WT. IL-4 could not be detected in WT mice, whereas *TRIM35*/- mice showed an average concentration of 0.60pg/mL. WT mice had an average IL-6 concentration of 0.55pg/mL and *TRIM35*/- mice had an average of 2.2pg/mL, constituting a 4-fold increase in concentration.

Interestingly, these two cytokines belong to opposite types of inflammatory responses with IL-6 associated with the initial acute phase pro-inflammatory response and IL-4 associated with the anti-inflammatory response induced after chronic injury.
Figure 5.6. Analysis of cytokine levels in serum from WT and TRIM35−/− mice fed the control diet. Cytokine concentrations in serum from WT and TRIM35−/− mice were measured with a Luminex 10-plex assay. GM-CSF and IL-2 were undetectable. TRIM35−/− mice had significantly higher levels of IL-4 and IL-6 compared to WT (measured by t-test). Data is presented as mean±SEM. *p<0.05. WT, n=9; TRIM35−/−, n=10.
5.3.2.4. Analysis of spleens and pancreases of WT and TRIM35-/- mice

Obesity and inflammation are also known to affect the spleen and pancreas and can eventually lead to diabetes [365, 366]. This study showed that loss of TRIM35 results in increased body weight (Figure 5.1) as well an increase in circulating inflammatory markers (Figure 5.6) in mice fed the control diet. Therefore, to further analyse the effect of loss of TRIM35, this study compared the spleens and pancreases of WT and TRIM35-/- mice fed the control diet for 9 months. Sections of spleens and pancreases were stained with H&E to visualise changes in organ architecture.

Surprisingly, despite the previous observed differences in obesity and inflammatory cytokines, visual inspection revealed no obvious morphological differences between WT and TRIM35-/- spleen and pancreases (Figure 5.7).

The spleens of WT and TRIM35-/- mice on the control diet show no abnormalities in the white (W) or red (R) pulp (Figure 5.7A). The pancreases also showed no abnormalities and no obvious differences were observed between WT or TRIM35-/- mice involving either the exocrine acini (A) or endocrine islets of Langerhans (IL) (Figure 5.7B).
Figure 5.7. Comparison of spleens and pancreases from WT and TRIM35-/- mice fed a control diet. Spleens and pancreas sections were stained with H&E for comparison between WT and TRIM35-/- mice fed the control diet for 9 months. A) Spleens of WT and TRIM35-/- mice show no significant differences in morphology and white (W) and red (R) pulp appear normal in both mice. B) Pancreases of WT and TRIM35-/- mice show no significant differences. The endocrine islets of Langerhans (IL) and exocrine acini (A) appear normal in WT and TRIM35-/- mice. Squares indicate area used for higher magnification. WT, n=9; TRIM35-/-, n=10.
5.3.3. Response of WT and TRIM35/- mice to the CDE diet

Several studies have linked loss of TRIM35 to the development, progression and severity of HCC [169, 170, 191]. However, it remains unclear whether loss of TRIM35 is a causal factor in HCC or a consequential event that increases tumorigenicity. To investigate if loss of TRIM35 is sufficient to increase susceptibility of mice for HCC, this study investigated the effect of the 67% CDE diet on WT (n=10) and TRIM35/- mice (n=11). Unfortunately, two of the TRIM35/- mice lost significant amount of weight, without visible causes, and were subsequently excluded, resulting in 9 TRIM35/- mice left for comparison.

5.3.3.1. Development of liver tumours in mice on CDE diet

MRI scanning showed 3 WT mice (out of 11) and 1 TRIM35/- mouse (out of 11) developed tumours on the CDE diet (Figure 5.8). The tumour-bearing TRIM35/- mouse was culled after 7 months on the CDE diet due to a sudden decrease in body weight which reached the allowed percentage body weight loss of 20%.

The tumours in both WT (Figure 5.8A) and TRIM35/- (Figure 5.8B) mice were visible at 5 months (yellow arrows in Figure 5.8 indicate large tumours). To determine if there was a difference between the volume of the tumours in WT and TRIM35/- mice fed the CDE, the T1 images were used to render 3D volumes of the tumours and livers using ITK-SNAP and the percentage of liver tissue consisting of tumours calculated (Appendix G shows an example of volumetric analysis).

WT mice had an average tumour volume of 25% of the total liver volume after 9 months on the CDE diet, whereas the TRIM35/- mouse had a tumour volume of 34.8% of the total liver after 7 months on the CDE diet. However, due to low numbers of mice that developed tumours, it is impossible to determine if there is a statistically significant difference between WT and TRIM35/- fed the CDE diet.
Figure 5.8. MRI scanning revealed tumours in WT and TRIM35-/− mice. Tumours appear hyperintense in a T2 MRI scan compared to surrounding dark liver tissue. Yellow arrows indicate large tumours visible in a WT (A) and TRIM35-/− (B) mouse in both T2 axial and T2 coronal scans at their respective endpoint scans. In WT mice, no tumours were visible at the first scan at 5 months on the diet, whereas the TRIM35-/− mouse already had multiple large tumours visible. Number of mice with tumours: WT, n=3 (out of 10); TRIM35-/−, n=1 (out of 11).
5.3.3.2. WT and TRIM35-/- mice gain weight at equal rates on the CDE diet

Although there was no significant difference in tumour development between WT and TRIM35-/- mice, the possibility remains that loss of TRIM35 can exacerbate symptoms of liver disease. Therefore, other indicators of chronic liver injury were investigated. For the duration of the 9 month CDE diet, WT and TRIM35-/- mice were weighed twice daily as a measure of animal well-being.

Both WT and TRIM35-/- mice showed initial weight gain before stabilizing, with no significant difference in overall body weight (Figure 5.9A). To determine if the rate of weight gain was affected, the daily average weight gain (gr/day) was calculated (Figure 5.9B). There were no significant differences in daily weight gain between WT mice, 0.078gr/day, and TRIM35-/- mice, 0.083gr/day (Figure 5.9B).
Figure 5.9. Weight gain of WT and TRIM35-/- mice fed the CDE diet for 9 months. A) The average body weight of WT and TRIM35-/- did not differ significantly (determined by two-way ANOVA). B) Daily average weight gain (g/day) was calculated for WT and TRIM35-/- mice on the CDE diet for 9 months. There was no significant difference in the rate of body weight gain between WT and TRIM35-/- mice (determined by t-test). Data is presented as mean±SEM. WT, n=8; TRIM35-/-, n=8.
5.3.3.3. *TRIM35/-* mice develop larger fat nodules than WT mice

In addition to tumours, MRI scanning revealed that WT and *TRIM35/-* mice fed the CDE diet also developed small high-intensity lesions (Figure 5.10). Using T1 axial scans with fat saturation, which suppresses the signal produced by fat and not tumours, it was confirmed that these lesions are fat nodules and present in both WT (Figure 5.10, red arrows) and *TRIM35/-* (Figure 5.10, yellow arrows) mice.

![Figure 5.10. Fat saturation in T1 MRI axial scans reveal presence of fat nodules in mice fed the CDE diet. MRI scans of mice fed CDE diet for 9 months show presence of hyperintense nodules in T1 scans in WT and *TRIM35/-* mice. By applying fat saturation to the T1 sequence, the hyperintense signal is lost, thereby establishing that these nodules consist predominantly of fat. Red arrows indicate hyperintense nodule in WT mice which subsequently appears dark after fat saturation. Yellow arrows show the same effect in *TRIM35/-* mice.](image)
The percentage of WT and *TRIM35*-/- mice with nodules visible at each timepoint was counted and graphed (Table 5.1 and Figure 5.11A). WT and *TRIM35*-/- mice with tumours were excluded from these counts as the size of the tumours made it impossible to distinguish the presence or volume of fat nodules.

None of the WT mice had visible nodules until 6 months on the CDE diet (Figure 5.11A). At 7 months, more than 50% of the WT mice had fat nodules and by the end of the experiment, 70% had nodules visible. In contrast, *TRIM35*-/- mice had nodules visible at 5 months. Furthermore, at 6 months, more than 50% of *TRIM35*-/- mice had nodules and by the end of the experiment 100% of the mice had visible nodules (Figure 5.11A). T-test analysis confirmed that this difference was not significant (p<0.05).

Next to determine if there is a difference in the volume of the fat nodules between WT and *TRIM35*-/- mice fed the CDE diet, ITK-SNAP was used to generate 3D volumes of the fat nodules and livers of each mouse at each time point (Appendix H shows example of volume measurements over time). The fat nodule volumes are presented as percentage of total liver volume.

WT mice showed a slow increase in the percentage of fat nodules and at 9 months the nodules only averaged 2% of total liver volume (Table 5.1 and Figure 5.11B). In contrast, *TRIM35*-/- mice showed a more rapid increase in fat nodules and reached an average of ~10% of total liver volume by 9 months. Two-way ANOVA analysis of the averages of WT and *TRIM35*-/- at the different timepoints, confirmed that the percentage of fat nodule volume was significantly higher in *TRIM35*-/- mice (p<0.01).
Table 5.1. Summary of 9 months of WT and TRIM35-/− mice on CDE diet

<table>
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<td>0.164</td>
<td>0.469</td>
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</tr>
<tr>
<td>TRIM35-/− (n=8)</td>
<td># of mice with nodules</td>
<td>2</td>
<td>6</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>average % fat nodules</td>
<td>0.54</td>
<td>1.669</td>
<td>3.085</td>
<td>5.392</td>
</tr>
</tbody>
</table>

Figure 5.11. Onset and volumetrics of fat nodules in WT and TRIM35-/− mice fed the CDE diet. A) Percentage of WT and TRIM35-/− mice that showed visible nodules at each timepoint scan on the CDE diet. There were significantly more TRIM35-/− mice with visible nodules than WT mice. B) Percentage of liver volume consisting of fat nodules was measured at each timepoint scan. TRIM35-/− mice had a significantly higher average of fat nodule volume compared to WT. Statistical differences measured with (A) t-test or (B) two-way ANOVA. *p<0.05 **p<0.01. WT, n=8; TRIM35-/−, n=8.
5.3.3.4. Liver histology of WT and TRIM35-/- mice

To study the morphology of the CDE diet livers of WT and TRIM35-/- mice, sections of the livers were stained with H&E for analysis.

The fat nodules, previously observed by MRI, were clearly visible on the surface of livers from WT and TRIM35-/- mice after 9 months on the CDE diet (Figure 5.12A).

H&E staining of WT and TRIM35-/- livers showed ballooning of hepatocytes (asterisks) as well as the boundaries between the fat nodules and residual tissue (black arrowheads) (Figure 5.12B). The inset show the distribution of several nodules in WT (left) and TRIM35-/- mice (right) with the nodules appearing pale compared to surrounding tissue due to dense accumulation of fat in these regions (Figure 5.12B, insert). Visual comparison of H&E sections indicate livers from TRIM35-/- mice contain more fat nodules than WT mice (Figure 5.12B, insert). However, due to the densely packed nature of the fat nodules in livers, with boundaries between adjacent nodules not always clear, quantification of the number of nodules would not be accurate.
Figure 5.12. Liver morphology and histology of WT and TRIM35-/- mice fed the CDE diet for 9 months. A) Livers of WT and TRIM35-/- mice show no obvious difference in morphology with the fat nodules appearing as yellow lesions on livers of WT and TRIM35-/- mice. B) H&E staining of livers clearly show presence of fat nodules (insert) in WT and TRIM35-/- mice with clear boundaries (black arrowheads) between fat nodules and residual tissues. No significant differences were visible between WT and TRIM35-/- mice.

WT, n=8; TRIM35-/-, n=8.
5.3.3.5. *TRIM35-/-* mice develop more liver fibrosis compared to WT mice

To determine if loss of *TRIM35* predisposes mice to liver fibrosis, livers from WT and *TRIM35-/-* mice, fed the CDE diet for 9 months, were stained with Sirius Red to visualize collagen deposition. High intensity staining was quantified using Imagescope.

Both WT and *TRIM35-/-* mice had clear signs of fibrosis throughout their livers (Figure 5.13A), with significant collagen deposition visible between the ballooning hepatocytes. Interestingly, fibrosis was observed around and occasionally inside the visible fat nodules.

Quantification of high-intensity pixels showed that livers from WT mice had an average positive pixel percentage of 0.0009%, whereas *TRIM35-/-* mice had an average of 0.0017%, which is almost a 2-fold increase (Figure 5.13B). T-test analysis confirmed that the increase observed in *TRIM35-/-* mice was significant (p<0.05).

It is possible that the increase of liver fibrosis seen in *TRIM35-/-* is a consequence of the high volume of fat nodules observed. However, a direct link between the fat nodules and fibrosis is not immediately obvious as fibrosis was also observed in areas outside the fat nodules.
Figure 5.13. Quantification of fibrosis in livers of WT and TRIM35-/- mice fed the CDE diet. A) Liver sections were stained with Sirius Red to visualize collagen deposits (arrows), an indicator of fibrosis. WT and TRIM35-/- mice on the CDE diet showed intense staining throughout the liver, around the fat nodules as well as inside the nodules. Bar represents 50μm. B) Quantification of the Sirius Red staining showed significant differences between WT and TRIM35-/- mice (measured by t-test). Data is presented as mean±SEM. *p<0.05. WT, n=8; TRIM35-/-, n=8.
5.3.4. Effect of CDE diet on inflammation in WT and TRIM35-/- mice

Inflammation plays a key role in liver disease progression and HCC development. The CDE model is able to induce hepatic inflammation, closely mimicking clinical conditions of chronic injury and subsequent HCC development. As there is an established link between TRIM35 and immunity, it was of interest to analyse the development of CDE-induced inflammation in livers of TRIM35-/- mice.

5.3.4.1. CDE diet induced liver inflammation

To compare the extent of liver inflammation between WT and TRIM35-/- mice fed the CDE diet, liver sections were stained with CD45 to visualize all inflammatory cells and the positively stained cells were quantified using Imagescope. Additionally, percentage of CD45 positive cells was analysed with InForm (Appendix D).

Livers from WT and TRIM35-/- mice show small clusters of CD45-positive cells throughout the livers (Figure 5.14A). Visual inspection revealed no obvious differences in quantity or localization of the CD45-positive cells.

Imagescope quantification confirmed that there was no statistically significant difference in CD45-positive cells between livers from WT and TRIM35-/- mice, with both having an average positive pixels percentage of 0.5% (Figure 5.14B).

5.3.4.2. Analysis of haematopoietic cells in peripheral blood of mice fed CDE diet

Blood samples from WT and TRIM35-/- mice, fed the CDE diet for 9 months, were compared to determine if loss of TRIM35 affected the composition of haematopoietic cells in the peripheral blood during chronic liver injury.

Hemavet® analysis showed no significant differences between WT and TRIM35-/- mice (Figure 5.15), and the values measured all fell within the empirically determined normal range of mice (Appendix C).
Figure 5.14. Quantification of inflammatory cells in livers of WT and TRIM35-/- mice fed the CDE diet. A) Liver sections from WT and TRIM35-/- mice were stained with CD45 to visualize inflammatory cells. WT and TRIM35-/- mice contained small clusters of CD45 positive cells throughout the livers. There were no obvious differences between WT and TRIM35-/- mice. Bar represents 50μm. B) Quantification of the CD45 staining showed no significant differences between WT and TRIM35-/- mice (measured by t-test). Data is presented as mean±SEM. WT, n=8; TRIM35-/-, n=8.
Figure 5.15. Analysis of cellular composition of circulating blood of WT and TRIM35−/− mice fed the CDE diet. The cellular composition of circulating blood, collected from WT and TRIM35−/− mice fed the CDE diet for 9 months, was analysed with a Hemavet®. The different populations and measurements were compared between WT and TRIM35−/− and no statistical significant differences were found (measured by t-test). Data is presented as mean±SEM. WT, n=8; TRIM35−/−, n=8.
5.3.4.3. Loss of TRIM35-/ in mice results in higher levels of circulating IL-4

To further investigate the effect of loss of TRIM35 on CDE-induced inflammation, circulating cytokines in the serum of WT and TRIM35-/ mice were compared.

Of the ten cytokines detected, 9 showed no significant differences between WT and TRIM35-/ mice fed the CDE diet (Figure 5.16).

However, the anti-inflammatory cytokine, IL-4, was found to be significantly higher in TRIM35-/ mice compared to WT mice fed the CDE diet (Figure 5.16). WT mice had an average IL-4 level of 0.59 gr/mL, whereas TRIM35-/ mice had an average of 2.73 gr/mL, constituting a 4.5 fold increase.

Interestingly, TNFα, IL-6 and IL-12 levels in TRIM35-/ mice fed the CDE diet were higher than that of WT, however the increases were not found to be significant. Surprisingly, despite significant differences in fibrosis observed in the livers of TRIM35-/ mice compared to WT, none of the other anti-inflammatory cytokines were affected.
Figure 5.16. Analysis of cytokine levels in serum from WT and TRIM35-/- mice fed the CDE diet. The different levels of cytokines in the serum from WT and TRIM35-/- mice fed the CDE were measured, after 9 months on their respective diets, with a Luminex 10-plex assay. GM-CSF and IL-2 were undetectable. TRIM35-/- mice had significantly higher levels of IL-4 compared to WT (measured by t-test). Data is presented as mean±SEM. *p<0.05. WT, n=8; TRIM35-/-, n=8.
5.3.4.4. Analysis of spleens and pancreases between WT and TRIM35-/− mice on the CDE diet

Next, the spleens and pancreases of WT and TRIM35-/− mice on the CDE diet were investigated for inflammatory changes. Sections of both organs were stained with H&E and visually assessed.

Visual assessment of the spleens of WT and TRIM35-/− mice fed the CDE diet showed no obvious differences (Figure 5.17A). In addition, the white (W) and red (R) pulp from the WT and TRIM35-/− spleens appear unaffected by the CDE diet (Figure 5.17A).

In the pancreases of mice fed the CDE diet, the endocrine islets of Langerhans (IL) and exocrine acini (A) show no significant differences between WT and TRIM35-/− mice (Figure 5.17B).

Interestingly, the pancreases of WT and TRIM35-/− mice contained areas resembling the fat accumulation (F) seen in the liver (Figure 5.17B). Visual assessment did not show any obvious differences between WT and TRIM35-/− mice in the amount of fat accumulation. However, to confirm this, these fatty areas would need to be quantified using either a specific stain, such as Oil Red O, or software capable of distinguishing between different types of tissue (e.g. fatty cells, endocrine islets of Langerhans and inflammatory lesions) within a region of interest.

The pancreases of WT and TRIM35-/− mice fed the CDE diet also contained areas that resembled lesions of inflammatory cells (red arrowheads, Figure 5.17B). However, visual analysis showed no significant difference in the presence, size or quantity of these lesions between WT and TRIM35-/− mice as all samples from both groups contained several lesions of various sizes throughout the pancreas. Whether these lesions contain inflammatory cells still needs to be confirmed with additional histological stains.
Figure 5.17. Comparison of spleens and pancreases from WT and TRIM35-/- mice fed CDE diet. Spleens and pancreases were collected from WT and TRIM35-/- mice after 9 months on CDE diet and stained with H&E for analysis. A) Spleens of WT and TRIM35-/- mice show no significant differences in morphology. White (W) and red (R) pulp appear normal in WT and TRIM35-/- mice after 9 months on CDE diet. B) Pancreases of WT and TRIM35-/- mice show fat accumulation (F) amongst exocrine acini (A) and infiltrating inflammatory cells (red arrowheads). The endocrine islets of Langerhans (IL) appear normal, although surrounded by the inflammatory lesions. Squares indicate area used for higher magnification. WT, n=8; TRIM35-/-, n=8.
5.3.5. Effect of loss of TRIM35 on TGF-β signalling in mice fed HF or CDE diet

The role of TGF-β signalling in development and progression of liver disease through regulation of inflammation is well established [281, 282]. Significantly, data presented in Chapter 3 of this thesis identified a novel regulatory role for TRIM35 in the TGF-β pathway, it was therefore of significant interest to investigate potential changes in TGF-β signalling in WT and TRIM35-/- mice either on the control diet or the CDE diet.

To study the effect on TGF-β signalling, RNA was extracted from the livers of WT and TRIM35-/- mice fed the control or CDE diet. One sample from each group was used for an initial screen of TGF-β target genes with the RT²-Profiler assay, to identify significant changes in target gene expression between WT and TRIM35-/- mice. The fold change was calculated using the ΔΔCₜ method and normalizing to the WT mice fed the control diet.

The initial screen identified several genes of interest (Figure 5.18). Six of the most interesting genes were selected for further analysis by RT-PCR Acvrl1, Amh, Bmpr, c-Myc, Serpine1 and Stat1. Unfortunately, it was not possible to complete the assays for all the genes displaying noteworthy fold changes within the given timeframe.
Figure 5.18. RT²-Profiler analysis of TGF-β target genes in WT and TRIM35-/- mice fed a HF or CDE diet. Analysis of TGF-β target genes using one sample from each dietary and genotype group, identified several potential genes that are significantly altered in mice fed a CDE diet as well as in TRIM35-/- regardless of diet. Data shown is fold change normalized to the WT mice fed the HF diet. Several genes were selected for further validation with RT-PCR.
5.3.5.1. Analysis of TGF-β target genes in livers of TRIM35/- mice on control diet

The expression levels of the selected target genes in livers of WT and TRIM35/- mice were analysed with RT-PCR using β-Actin as housekeeping gene as a reference control. Fold change of the gene expression in TRIM35/- mice was determined with the ΔΔCt method by normalizing to WT expression levels.

Of the six genes studied, only two genes showed a significant difference in TRIM35/- mice compared to the WT mice fed the control diet (Figure 5.19).

Compared to WT mice, livers from TRIM35/- mice on the control diet showed a significant 3-fold increase in expression of Bmpr, an inhibitor of the BMP signalling pathway. Additionally, TRIM35/- mice had a significant 4-fold increase, compared to WT mice, of SERPINE1, a gene that encodes an inhibitor of fibrinolysis (Figure 5.19).

Higher expression levels of Anti-Müllerian hormone (AMH) and the protoc-oncogene, c-Myc, were also measured in livers of TRIM35/- mice compared to WT mice fed the control diet. However, possibly due to low number of samples, these increases were not statistically significant.
Figure 5.19. TGF-β target gene expression in WT and TRIM35/- mice fed the control diet. Gene expression of TGF-β target genes was analysed using RNA from livers of WT and TRIM35/- mice fed the HF diet for 9 months. RT-PCR revealed significantly higher levels of Bmper and SERPINE1 in TRIM35/- mice. WT, n=9; TRIM35/-, n=10.
5.3.5.2. Analysis of TGF-β target genes in livers of TRIM35-/- mice on CDE diet

Expression levels of the selected TGF-β target genes in livers of TRIM35-/- and WT mice fed the CDE diet, were analysed as described above. Fold change of the gene expression in TRIM35-/- mice fed the CDE diet was determined by normalizing to WT levels.

Comparison of TGF-β target gene expression between livers from WT and TRIM35-/- mice fed the CDE diet showed four genes to be significantly downregulated in TRIM35-/- mice (Figure 5.20).

In TRIM35-/- mice fed the CDE diet, Activin A Receptor Like Type 1 (ACVRL1) expression was half that of WT mice on the same diet (-1-fold change) (Figure 5.20). Expression levels of c-Myc and Stat1 were also significantly down regulated in TRIM35-/- mice, with decreases of 1.5-fold and 1.3-fold respectively. The largest difference found was in AMH expression, which showed a 38-fold decrease in TRIM35-/- mice compared to WT on the CDE diet (Figure 5.20).

Interestingly, Bmper expression was almost 2-fold higher in TRIM35-/- mice compared to WT on the CDE diet, but this difference was not statistically significant. SERPINE1 expression levels in livers of TRIM35-/- mice was almost half that of WT mice on the CDE diet, but this was also not statistically significant.
Figure 5.20. TGF-β target gene expression in WT and TRIM35-/- mice fed the CDE diet. Gene expression of TGF-β target genes was analysed using RNA from livers of WT and TRIM35-/- mice fed the CDE diet for 9 months. RT-PCR revealed significantly lower expression levels of ACVRL1, AMH, c-Myc and Stat1. WT, n=8; TRIM35-/-, n=8.
5.4. DISCUSSION

This study aimed to clarify the role of TRIM35 in the development and progression of liver disease and subsequent HCC. To achieve this, mice were fed either a high-fat or CDE diet, known to induce liver injury in a manner that mimics liver disease and clinical progression of HCC. The CDE diet was reduced in potency, by addition of choline-sufficient high-fat chow, to ensure mice stayed within the required weight loss parameters mandated by animal ethics protocols. Consequently, the control mice were fed a choline-sufficient high-fat chow. Therefore, in addition to studying the effect of the CDE diet, this model provided the opportunity to study the effects of TRIM35 loss when a diet high in fat is consumed. This in itself is relevant, as high-fat diets are known to result in fatty liver disease which can progress to HCC [363].

Data presented in this chapter clearly show a role for TRIM35 in limiting the onset of symptoms associated with chronic liver disease. Specifically, fat accumulation and the inflammatory responses involved in regulating disease onset were found to be affected by the loss of TRIM35.

Mice fed both the control and CDE diet accumulated more fat-related metabolic changes in TRIM35/- mice compared to WT mice, including a higher rate of weight gain and increased amount of hepatic fat nodules. However, the extent of the influence that TRIM35 has on fat metabolism, as well as the mechanism by which this occurs, still remains unclear.

Importantly, livers of TRIM35/- mice fed the CDE diet also had significantly higher levels of fibrosis. Fibrosis is a consequence of excessive inflammation and although reversible, chronic injury will promote the progression of fibrosis to cirrhosis and eventually
HCC. Interestingly, higher expression levels of SERPINE1 was observed in livers of TRIM35-/− mice fed the control diet. SERPINE1 encodes an acute phase protein, plasminogen activator inhibitor-1 (PAI-1), which is a serine protease inhibitor that inhibits tissue (tPA) and urokinase plasminogen activator (uPA), thereby making PAI-1 an inhibitor of fibrinolysis (the breakdown of fibrin) [367, 368]. It is possible that increased levels of SERPINE1 could facilitate the increased fibrosis associated with loss of TRIM35.

The most compelling result from this study is the link between TRIM35 and the different types of inflammatory responses. Previous research only implicated TRIM35 in the innate immune response, whereas data presented here indicates that TRIM35 plays a more complex role in regulating excessive inflammation. In the control mice, loss of TRIM35 resulted in significantly higher levels of IL-6 and IL-4.

In the liver, IL-6 is a key mediator of the acute phase response in areas of acute and chronic inflammation [369, 370]. The acute phase response and associated proteins are involved in limiting tissue damage as well as facilitating tissue repair and regeneration in response to injury [370].

The significant weight gain and increase of serum IL-6 in TRIM35-/− mice fed the control diet is consistent with previous findings that showed correlation between obesity and serum IL-6 [364, 371]. In obese patients, the adipokine progranulin (PGRN) induces adipose tissue to produce IL-6, resulting in the recruitment of pro-inflammatory cells, which can lead to insulin resistance [372]. The results presented here suggest a role for TRIM35 in reducing the acute phase response and pro-inflammatory response induced by the control diet through restriction of IL-6 expression levels. However, it is unclear whether TRIM35 regulation of
IL-6 production occurs in adipose and/or hepatic tissue as this study only studied serum levels and both types of tissue are capable of producing IL-6 but do so via different pathways.

*TRIM35/-* mice also had significantly higher levels of serum IL-4 on the CDE diet. IL-4 is a cytokine involved in inducing differentiation of naive T helper cells into type 2 (Th2) helper cells, which are involved in wound repair. Furthermore, IL-4 is involved in stimulating macrophage differentiation into M2 macrophages [276]. Activated macrophages subsequently secrete factors such as IL-10 and TGF-β that assist in reducing pathological inflammation. This resolution of inflammation is required to prevent over stimulation of the pro-inflammatory response. However, persistent production of anti-inflammatory cytokines such as IL-4 by M2 macrophages is known to contribute to fibrosis [285].

The role of IL-4 in resolving inflammation might explain why *TRIM35/-* mice with high levels of IL-4, on the control and CDE diet, do not have an increase of inflammatory cells in the liver as was initially expected. It is possible that the high levels of IL-4 in *TRIM35/-* mice mediated a resolution of the strong immune response that was expected by recruiting basophils, eosinophils and Th2 cells.

This increase in IL-4 further supports the hypothesis that TRIM35 plays a role in the early stages of liver disease and provides additional evidence of a role for TRIM35 in regulating the immune response.

The molecular mechanism by which TRIM35 regulates IL-6 and IL-4 expression remains unclear. Several molecular pathways for induction of IL-6 expression have been described, including signalling by TGF-β1 and IFN-γ [373, 374]. IL-4 is expressed in various cell types, including macrophages, basophils and Th2 cells, and several different mechanisms have been described that result in induction of IL-4 expression [375, 376]. In addition, IL-4 levels can also be upregulated through IL-6 expression, which means that the observed
increase of IL-4 in the TRIM35-/− mice could be an indirect consequence of the upregulated IL-6 levels in the absence of TRIM35.

To fully understand how TRIM35 regulates the expression of these cytokines, *in vitro* characterisation of TRIM35-/− inflammatory cells is required. Comparison of protein levels and gene expression in WT and TRIM35-/− inflammatory cells will provide insight into the regulatory pathways affected and therefore provide a potential mechanism for TRIM35 activity. *In vitro* analysis of inflammatory cells, including routinely cultured macrophages that can be used to study overexpression or knockdown of TRIM35, will allow to further investigate the role of TRIM35 in the immune response in the liver after injury.

It is also important to note that the hepatic cells themselves secrete factors involved in initiating the immune response and attracting the necessary inflammatory cells. Therefore, the effect of hepatocyte-specific knockdown of TRIM35 in mice should also be explored.

Taken together, the data from TRIM35-/− mice clearly show that loss of TRIM35 affects the inflammatory responses involved with chronic liver injury. But the localized effect of loss of TRIM35 on adipose and hepatic inflammatory responses requires further characterization. By comparing the type of inflammatory cells and the cytokine levels of adipose and hepatic tissue of WT and TRIM35-/−, the overall effect of loss of TRIM35 on the local inflammatory responses will be more obvious. Pro-inflammatory cells would include NK cells, Th1 cells and neutrophils, whereas the anti-inflammatory response would result in recruitment of basophils, eosinophils and Th2 cells. Of particular interest would be to determine the presence of M1 or M2 macrophages, to establish if loss of TRIM35-/− results in an increase of the anti-inflammatory response as suggested by the high levels of circulating IL-4. This will also establish whether the role of TRIM35 in inflammation varies among the types of tissue.
There is also the possibility that loss of *TRIM35* affected the pro-inflammatory response in mice fed the CDE diet. However, as the pro-inflammatory response occurs during the initial stages of CDE diet-induced liver injury, it was not detectable after 9 months. To determine if loss of *TRIM35* affects the pro-inflammatory response induced by the CDE diet, the effect of the diet during the first three weeks needs to be investigated. Specifically, it would require extensive investigation at several timepoints during the initial stages of liver injury as it is not clear when the pro-inflammatory response is initiated.

Unexpectedly, loss of TRIM35 did not result in a significant increase of number of mice fed the CDE diet with HCC, which suggests that loss of TRIM35 is not sufficient to predispose mice to eventual onset of HCC in the model used. However, the onset of tumours in the TRIM35-/- mice was earlier compared to the tumours in WT mice. Unfortunately, due to the low number of mice that developed tumours, it is not possible to statistically determine the significance. Taken together with, the increase of fat nodules and fibrosis in TRIM35-/- mice fed the CDE diet, this does indicate a potentially significant role for TRIM35 in the early stages of liver disease.

Considering the increase of symptoms relating to liver disease observed in *TRIM35-/-* mice on the control and CDE diet, as well as the strong link established between TRIM35 and HCC in previous studies, it remains of interest to determine whether loss of *TRIM35* affects the severity of HCC, despite not affecting onset of tumours.

To ensure that animals remained healthy we could only use 67% CDE in their diet. Although this has been shown to induce LPC activation, an indicator of initiation of liver disease, it appears sub-optimal for HCC development. The main welfare issue is excessive weight loss at the initiation of the diet, most likely due to the smell or flavour imparted by the
ethionine supplement. Several other variations of the CDE diet, including addition of the ethionine to the water rather than chow, have been trialled and appear to be effective in inducing HCC with less weight loss. This model may provide better insight into the role of TRIM35 in liver disease.

In addition, the LPC activation was studied in livers from WT and TRIM35/- mice, fed either the control or CDE diet, using PanCK staining (Appendix F). Unfortunately, due to technical issues, it was not possible to quantify the amount of LPC’s in the different livers and therefore not possible to establish the effect of loss of TRIM35 on the activation of LPC’s in response to the control or CDE diet.

In conclusion, the results presented here show that TRIM35 is an important regulator of the inflammatory responses associated with metabolic changes. By regulating expression levels of key cytokines that promote recruitment of inflammatory cells, TRIM35 may act as a restraint on inflammation preventing liver disease progression.
CHAPTER 6 DISCUSSION
6.1. GENERAL DISCUSSION

The main objective of this project was to investigate the molecular activity of TRIM35 and its biological functions. TRIM35 is a relatively unknown E3 ubiquitin ligase, initially discovered for its role in haematopoiesis. Since its discovery, TRIM35 has been identified as a regulator of the innate immune response [87] as well as a potential tumour suppressor in hepatocellular carcinoma (HCC) [169, 170]. At a molecular level, TRIM35 has been identified as a binding partner of the mediators of the TGF-β pathway, the SMAD proteins [198].

The aims of this study focused on characterizing the role of TRIM35 in TGF-β signalling and determining whether this interaction contributes to its role in haematopoiesis, inflammation and HCC. The data presented in this study significantly improves our understanding of TRIM35 activity in several biological contexts and presents interesting opportunities for TRIM35 as a therapeutic target. Data presented in this thesis demonstrate that TRIM35 regulates TGF-β signalling through the SMADs in an ubiquitin-mediated non-proteolytic manner. Analysis of TRIM35 in haematopoiesis identified a role for TRIM35 in maintenance of HSCs and early progenitors. Finally, TRIM35 was found to have a significant role in regulating the immune response induced during chronic liver injury.

6.1.1. TRIM35 regulates TGF-β signalling through non-proteolytic ubiquitination

The data presented here identifies TRIM35 as a regulator of TGF-β signalling through interaction and non-proteolytic ubiquitination of several SMAD proteins. Further characterization of the interaction between TRIM35 and the Co-SMAD, SMAD4, showed that TRIM35 overexpression alters the affinity for specific binding partners of SMAD4.

In addition, after treatment with TGF-β1 and in the presence of TRIM35, SMAD4 displayed significant changes in nuclear localisation.
The ability of TRIM35 to alter rather than inhibit the TGF-β pathway, makes TRIM35 an important regulator. TGF-β signalling is a known inhibitor of cell growth and in certain circumstances can trigger cell death [207, 263, 377]. However, in tumorigenic circumstances, TGF-β signalling more commonly results in epithelial-mesenchymal transition (EMT) [378-380]. Therefore, the discovery of a novel regulator potentially capable of changing the signalling outcome of TGF-β, raises the possibility of targeted therapy which could involve shifting the TGF-β signal away from EMT induction as well as inducing a tumour suppressive response. As the TGF-β pathway is altered in tumorigenic cells, it is possible that the ability of TRIM35 to interact with the SMADs and mediate appropriate signalling is inhibited or altered under these circumstances.

The ability of TRIM35 to regulate the outcome of TGF-β signalling introduces novel opportunities for therapeutic approaches targeted at modulating TGF-β signalling, as mediators as well as regulators of the pathway are often targeted in an effort to inhibit the oncogenic effect of TGF-β.

6.1.2. TRIM35 plays a role in maintenance of HSCs and early progenitors

One of the initial biological processes linked to TRIM35 was haematopoiesis, where it was proposed that TRIM35 plays a role in distinguishing between the erythroid and myeloid lineages [181, 298]. Analysis of haematopoietic compartments of TRIM35/-/ mice (Chapter 4), indicated no significant effect on the erythro-myeloid differentiation pathway in 8-12 week old mice, but showed significant changes in mice aged 80 weeks or older, implicating a regulatory effect of TRIM35 in matured erythro-myeloid differentiation. Furthermore, the bone marrow (BM) of TRIM35/-/ mice had a higher percentage HSCs and early progenitors compared to WT, thereby implying a key role for TRIM35 in maintaining HSC quiescence. This potential role of TRIM35 in HSC regulation aligns with the known role of TGF-β in haematopoiesis, as TGF-β has been shown to be predominantly involved in inducing
quiescence in HSCs thereby sustaining the HSC pool throughout the life of an individual [256, 257].

Interestingly, previous research showed that the outcome of TGF-β signalling in HSCs is determined by the ability of SMAD2/3 to interact with either Tif1γ or SMAD4 [381]. The association of SMAD2/3 with Tif1γ results in erythro-myeloid differentiation after stimulation by TGF-β, whereas the association with SMAD4 results in growth inhibition (Figure 6.1A). Therefore, regulation of Tif1γ and SMAD4 expression levels or their affinity for SMAD2/3 can regulate the outcome of TGF-β signalling in HSCs. Taken together, the TRIM35-SMAD interaction data and the finding that TRIM35 can regulate the HSC compartment, raises an interesting potential model for the role of TRIM35 in HSC development (Figure 6.1A). Loss of TRIM35 resulted in a larger percentage of HSCs in the BM, which suggests that TRIM35 restricts their population size, potentially through altering the affinity between SMAD2/3 and SMAD4 resulting in growth inhibition rather than differentiation (Figure 6.1A).

Additionally, a previous study showed long-term HSCs harbour a myeloid or lymphoid bias [239]. Based on the ability of LSK cells to efflux Hoechst stain, a clear distinction can be made between myeloid-biased (My-HSC) and lymphoid-biased HSCs (Ly-HSC). Characterization of these two subpopulations revealed My-HSCs to be quiescent, but proliferative in the presence of TGF-β1 (Figure 6.1B) [239]. In contrast, Ly-HSCs proved more proliferative but were subsequently inhibited by TGF-β1. Thus TGF-β regulates the balance between the two HSC subpopulations. Loss of TRIM35 resulted in a significantly higher percentage of HSCs, as well as common myeloid progenitors (CMP) (Figure 4.3 and Figure 4.5). Therefore, it is possible that TRIM35 influences the TGF-β regulated balance between My- and Ly-HSC resulting in the observed increase in CMP (Figure 6.1B).
Figure 6.1. Potential models for TRIM35 function in managing HSC population. A) Previous molecular studies have shown that the outcome of TGF-β signaling in HSCs is regulated by the interaction of SMAD2/3 with Tif1γ and SMAD4. Data from this study suggests that TRIM35 regulates the affinity of SMAD proteins for other factors and plays a role in HSC quiescence. Therefore, it is possible that TRIM35 increases affinity of SMAD2/3 for SMAD4, thereby inducing growth inhibition. B) The higher percentage of HSCs and CMPs in BM of TRIM35−/− mice can be due to a shift in the My-HSC population. My-HSCs are stimulated by TGF-β to proliferate, whereas Ly-HSCs are inhibited. C) Loss of TRIM35 can affect several cell types within the endosteal or vascular niche of the BM that also regulate quiescence and activation of HSCs. Osteoblasts are required to maintain HSCs, whereas stromal cells and endothelium cells of sinusoids activate HSCs and facilitate their release. The mouse model used in this study was a full knockdown of TRIM35 and therefore all of the components of the niche could potentially be affected.
Finally, the differences observed in the haematopoietic compartments of TRIM35-/- mice could also be explained by changes in the HSC niche in the BM (Figure 6.1C). Quiescent HSCs are found in the endosteal niche, where they are kept quiescent through autocrine and paracrine TGF-β signalling as well as TPO production from nearby osteoblasts [382, 383]. Active HSCs however, are found towards the centre of the BM where stromal and endothelial cells produce a range of activating factors that stimulate differentiation and release of HSCs into circulating blood [382, 384]. As the niche plays a crucial role in regulating the activity of HSCs, it is possible that the changes in HSCs and progenitors observed in the TRIM35-/- mice are a consequence of changes in the cell types associated with the different niches and their subsequent cytokine production.

6.1.3. Modulation of inflammatory responses by TRIM35 during chronic liver injury

The role of TRIM35 in inflammation and liver disease was investigated using TRIM35-/- mice fed a choline-deficient ethionine-supplemented (CDE) diet or a control diet which is normal chow with a high-fat (HF) content (discussed in 5.1.4.2.). The HF diet is known to induce a pro-inflammatory response in mice [363] whereas the CDE diet mimics the different steps of hepatocellular carcinoma (HCC), including hepatic inflammation, fibrosis, and cirrhosis [360, 361]. The results presented in Chapter 5 clearly indicate a role for TRIM35 in modulating the immune response including altering circulating inflammatory cytokines and inducing physiological symptoms associated with excessive inflammation such as obesity and fibrosis.

During liver injury, the cytokines affected by loss of TRIM35, IL-4 and IL-6, are known to play several key roles in the pro- and anti-inflammatory responses, including activation of hepatic stellate cells (SCs), transdifferentiation of SCs into fibrogenic myofibroblasts and polarization of M1 macrophages to an M2 phenotype (Figure 6.2) [385-387].
Causes of liver injury

- Ethanol abuse
  - Gut permeability
  - Ethanol metabolism
- HCV
  - Protein cleavage
- Metabolic syndrome
  - Impaired glucose metabolism
  - Fat accumulation
  - Impaired lipid metabolism

<table>
<thead>
<tr>
<th>Causes of liver injury</th>
<th>Ethanol abuse</th>
<th>HCV</th>
<th>Metabolic syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol abuse</td>
<td>Gut permeability</td>
<td>Ethanol metabolism</td>
<td>Protein cleavage</td>
</tr>
</tbody>
</table>

**Figure 6.2. Schematic diagram of inflammatory response during chronic liver injury and potential role of TRIM35.**

Data from this study showed that loss of *TRIM35* resulted in significant changes in the inflammatory environment induced by fat accumulation and carcinogen administration due to the HF and CDE diet. Specifically, levels of serum IL-6 and IL-4 were significantly increased in *TRIM35−/−* mice, indicating a role for TRIM35 in restricting the production of these cytokines. During liver injury, IL-6 and IL-4 are involved in several processes, including activation and trans-differentiation of stellate cells, recruitment of T-cells and macrophage polarization. Additionally, our research showed TRIM35 can regulate TGF-β, providing another potential mechanism for the action of TRIM35 in regulating liver inflammation. TGF-β is especially crucial for ECM synthesis by myofibroblasts. Based on the observed increase of fibrosis in *TRIM35−/−* mice, it is possible that TRIM35 restricts ECM synthesis in myofibroblasts and does so by modulating the cellular response to TGF-β. Th1, type 1 helper T-cell; Th2, type 2 helper T-cell; ECM, extracellular matrix.
It is noteworthy that TGF-β1 plays an important role in several stages of the hepatic inflammatory response to chronic liver injury, importantly it is essential for fibrosis (Figure 6.2) [388]. These data suggest several possible opportunities where TRIM35 can modulate the immune response.

6.1.3.1. TRIM35 is a regulator of adipose-related pro-inflammatory responses

The significantly higher body weight and serum IL-6 levels observed in TRIM35-/− mice indicate an important role for TRIM35 in regulating the immune response and subsequent symptoms induced by the HF diet [363]. As a pro-inflammatory cytokine, IL-6 is involved in recruitment of inflammatory cells, such as type 1 helper T-cells (Th1) and neutrophils, required for clearing pathogens or cellular debris. The results presented in Chapter 5 indicate that TRIM35 is normally capable of maintaining appropriate levels of IL-6, thereby limiting the extent of the pro-inflammatory response induced by excessive fat accumulation. However, the mechanism by which TRIM35 is responsible for maintaining IL-6 expression remains unclear.

Previous research showed that an increase of fat mass in adipocytes, prior to body mass increase, results in elevated serum levels of pro-inflammatory cytokines [389] and elevated serum pro-inflammatory cytokines predict future weight gain [390]. Additionally, a positive correlation has been found between body mass and accumulation of macrophages in adipose tissue, with these macrophages being the primary source of cytokines [391]. It is possible, therefore that the observed increase of IL-6 levels in TRIM35-/− mice fed the HF diet is due to a change in the cytokine production of the adipose macrophages. Furthermore, in adipose tissue, IL-6 has been shown to regulate the expression levels of IL-4 receptors and influence the cellular response of cells to IL-4, resulting in polarization of these macrophages to an M2 phenotype [387], thus implicating TRIM35 as a novel regulator of macrophage polarization.
In addition, hepatic macrophages are also capable of inducing IL-6 expression after liver injury induced by fat accumulation (Figure 6.2) [392]. In the liver context, IL-6 expression is associated with the acute phase response (discussed in Chapter 5), which is considered a predictor of obesity and type 2 diabetes [368, 393]. Previous research determined that adipose inflammation precedes that of hepatic tissue in mice fed a HF diet for extended periods, with hepatic inflammation not observed till 40 weeks on the diet [363]. This implies that elevated IL-6 levels in TRIM35-/- mice are a direct result of adipose inflammation. However, analysis of gene expression in hepatic tissue showed significant increase of SERPINE1, a gene encoding an acute phase protein (see 5.3.5.1), indicating that there is also activity in the hepatic tissue.

6.1.3.2. TRIM35 regulates immune responses responsible for hepatic fibrosis

By inducing more extensive hepatic damage through the CDE diet, this study revealed a significant difference in the anti-inflammatory response and subsequent fibrosis between WT and TRIM35-/- mice.

During the early stages of liver injury, IL-6 mediates the pro-inflammatory response by activating hepatic SCs and recruiting neutrophils, Th1 cells and NK cells (Figure 6.2) [394, 395]. Although IL-6 was not found to be significantly different in TRIM35-/- mice compared to WT after 9 months on the CDE diet, it remains possible that there was a significant pro-inflammatory response in TRIM35-/- during the early part of the study that went undetected. Especially considering the high levels of IL-6 observed in TRIM35-/- mice fed the HF diet. An early peak in pro-inflammatory IL-6 could explain the increase in the anti-inflammatory response observed in TRIM35-/- mice fed the CDE diet (Figure 6.2). A higher level of pro-inflammatory cytokines would elicit a stronger anti-inflammatory response in mice fed the CDE diet by inducing an increase in M1 to M2 polarization (Figure 6.2). This suggests a role for TRIM35 in regulating cytokine production in macrophages and in restricting polarization,
thereby acting as an important inhibitor of the early stages of liver disease. It is noteworthy that TRIM35 has previously been associated with increased macrophage activation [180].

The development of fibrosis requires a complex network of cytokines, produced by various cell types, which regulate the activity of the hepatic stellate cells (SCs) in the liver (Figure 6.2). IL-4 is a crucial inducer of transdifferentiation of SCs into myofibroblast-like cells that are induced to produce extracellular matrix (ECM) during chronic liver injury resulting in fibrotic tissue [282, 285]. The increased levels of fibrosis observed in TRIM35-/− mice fed the CDE diet is potentially due to the elevated levels of serum IL-4 observed in these mice.

In the liver, IL-4 is predominantly produced by the M2 macrophages after chronic injury induces a shift from pro-inflammatory to anti-inflammatory. As discussed above, it is possible that this increase in the anti-inflammatory IL-4 occurred as a response to an increase in pro-inflammatory cytokines. Alternatively, it is possible that TRIM35 acts to limit the production of IL-4 by M2 macrophages, thereby restricting the anti-inflammatory response thus preventing chronic injury resulting in fibrosis. Data from mice fed the HF and CDE diet provide evidence supporting both scenarios and further investigation of the adipose and hepatic tissue in both these mouse models would be required to categorically define the role of TRIM35 in this process.

The fact that TRIM35 is able to influence TGF-β signalling provides another possible explanation for the changes observed. TGF-β is involved in both pro- and anti-inflammatory responses during chronic liver injury (Figure 6.2) [286, 288, 396]. In the context of liver inflammation, TGF-β is best known for its role in inducing the transdifferentiation of the SCs into myofibroblasts and stimulating these cells to produce collagen [397-399]. Therefore, the ability of TRIM35 to modulate the cellular response to TGF-β could potentially alter these
processes, resulting in delayed onset of fibrosis during chronic liver injury. Further analysis of the liver pathology is required to determine whether this is indeed the case.

6.2. CONCLUSION

In summary, the research presented here identified TRIM35 as a regulator of TGF-\(\beta\) signalling, but rather than inhibiting the pathway, TRIM35 was able to alter the outcome of TGF-\(\beta\) signalling. Comparison of the haematopoietic compartments of WT and \textit{TRIM35-/-} mice confirmed that TRIM35 can influence HSC and CMP cells. It is noteworthy that TGF-\(\beta\) is a well-recognised regulator of haematopoiesis, in particular HSC differentiation. Finally, the effect of depleting \textit{TRIM35} in a model of induced liver injury revealed significant changes in inflammatory cytokines, which are critical mediators of liver disease.

Due to the link between inflammation and an increasing number of metabolic syndromes [400, 401], the ability of TRIM35 to modulate the inflammatory response is of significance. Furthermore, the link between TRIM35 and TGF-\(\beta\) provides possibilities for novel therapeutic approaches aimed at utilizing the pleiotropic effects of TGF-\(\beta\) to reduce morbidity. Considering the strong connection between TGF-\(\beta\) signalling and fibrosis in several tissues [402–404], this approach could have potential benefits in several pathological conditions.
## APPENDIX A

### Distribution of LSK populations in WT and TRIM35-/- mice as % total cells counted (mean±SEM)

#### Mice aged 8-12 weeks

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Markers</th>
<th>WT</th>
<th>TRIM35-/-</th>
<th>WT</th>
<th>TRIM35-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSK</td>
<td>Lin-Sca1+c-Kit+</td>
<td>0.3848±0.033</td>
<td>0.6161±0.047</td>
<td>0.7657±0.044</td>
<td>1.865±0.085</td>
</tr>
<tr>
<td>HSC</td>
<td>LSKCD150+CD48-</td>
<td>0.0259±0.001</td>
<td>0.0576±0.009</td>
<td>0.0348±0.003</td>
<td>0.0558±0.008</td>
</tr>
<tr>
<td>MPP</td>
<td>LSKCD150-CD48-</td>
<td>0.0135±0.001</td>
<td>0.0292±0.006</td>
<td>0.0172±0.001</td>
<td>0.039±0.008</td>
</tr>
<tr>
<td>HPC</td>
<td>LSKCD150-CD48+</td>
<td>0.2704±0.024</td>
<td>0.4358±0.031</td>
<td>0.3656±0.022</td>
<td>0.9012±0.088</td>
</tr>
</tbody>
</table>

#### Mice aged 80 weeks or older

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Markers</th>
<th>WT</th>
<th>TRIM35-/-</th>
<th>WT</th>
<th>TRIM35-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSK</td>
<td>Lin-Sca1+c-Kit+</td>
<td>0.6558±0.129</td>
<td>0.7474±0.116</td>
<td>0.7711±0.133</td>
<td>1.302±0.148</td>
</tr>
<tr>
<td>HSC</td>
<td>LSKCD150+CD48-</td>
<td>0.0138±0.004</td>
<td>0.0231±0.004</td>
<td>0.0156±0.002</td>
<td>0.0297±0.005</td>
</tr>
<tr>
<td>MPP</td>
<td>LSKCD150-CD48-</td>
<td>0.0131±0.003</td>
<td>0.0235±0.005</td>
<td>0.0144±0.005</td>
<td>0.0444±0.012</td>
</tr>
<tr>
<td>HPC</td>
<td>LSKCD150-CD48+</td>
<td>0.2992±0.030</td>
<td>0.3458±0.048</td>
<td>0.3307±0.056</td>
<td>0.6497±0.058</td>
</tr>
</tbody>
</table>
## APPENDIX B

**Hemavet analysis of WT and TRIM35-/- mice aged 8-12 weeks or 80 weeks and older**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Normal Range</th>
<th>mice aged 8-12 weeks</th>
<th>mice aged 80 weeks or older</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cells (x10^12 cells/L)</td>
<td>6.36 - 9.42</td>
<td>8.600±0.480</td>
<td>8.423±0.386</td>
</tr>
<tr>
<td>White blood cells (x10^9 cells/L)</td>
<td>1.8 - 10.7</td>
<td>5.200±0.961</td>
<td>5.204±0.570</td>
</tr>
<tr>
<td>Lymphocytes (% of total pop)</td>
<td>55.8 - 91.6</td>
<td>75.60±0.7.403</td>
<td>71.59±3.506</td>
</tr>
<tr>
<td>Neutrophils (% of total pop)</td>
<td>6.6 - 38.9</td>
<td>13.29±4.612</td>
<td>19.55±7.237</td>
</tr>
<tr>
<td>Eosinophils (% of total pop)</td>
<td>0.0 - 3.9</td>
<td>3.812±0.621</td>
<td>1.414±1.501</td>
</tr>
<tr>
<td>Monocytes (% of total pop)</td>
<td>0.0 - 7.5</td>
<td>3.918±0.331</td>
<td>5.718±2.047</td>
</tr>
<tr>
<td>Basophils (% of total pop)</td>
<td>0.0 - 2.0</td>
<td>0.371±0.186</td>
<td>0.4100±0.276</td>
</tr>
<tr>
<td>Platelets (x10^9 cells/L)</td>
<td>592 - 2972</td>
<td>1219±290.4</td>
<td>1106±438.5</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>110 - 151</td>
<td>132.8±1.758</td>
<td>111.5±13.46</td>
</tr>
</tbody>
</table>
**APPENDIX C**

Hemavet analysis of WT and TRIM35/- mice fed either HF or CDE diet for 9 months

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Normal Range</th>
<th>WT</th>
<th>TRIM35/-</th>
<th>WT</th>
<th>TRIM35/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cells (x10^12 cells/L)</td>
<td>6.36 - 9.42</td>
<td>9.19±0.420</td>
<td>7.69±0.589</td>
<td>8.55±0.310</td>
<td>7.95±0.562</td>
</tr>
<tr>
<td>White blood cells (x10^9 cells/L)</td>
<td>1.8 - 10.7</td>
<td>7.66±1.404</td>
<td>7.26±1.696</td>
<td>5.90±1.505</td>
<td>8.02±1.542</td>
</tr>
<tr>
<td>Lymphocytes (% of total pop)</td>
<td>55.8 - 91.6</td>
<td>69.01±1.578</td>
<td>67.13±2.596</td>
<td>64.54±2.164</td>
<td>68.40±2.252</td>
</tr>
<tr>
<td>Neutrophils (% of total pop)</td>
<td>6.6 - 38.9</td>
<td>24.59±1.569</td>
<td>26.00±1.707</td>
<td>28.87±2.780</td>
<td>25.84±2.029</td>
</tr>
<tr>
<td>Eosinophils (% of total pop)</td>
<td>0.0 - 3.9</td>
<td>1.93±0.478</td>
<td>2.55±0.934</td>
<td>2.50±0.671</td>
<td>2.17±0.702</td>
</tr>
<tr>
<td>Monocytes (% of total pop)</td>
<td>0.0 - 7.5</td>
<td>3.54±0.447</td>
<td>3.32±0.613</td>
<td>3.25±0.222</td>
<td>2.95±0.585</td>
</tr>
<tr>
<td>Basophils (% of total pop)</td>
<td>0.0 - 2.0</td>
<td>0.91±0.153</td>
<td>0.99±0.343</td>
<td>1.08±0.343</td>
<td>0.63±0.253</td>
</tr>
<tr>
<td>Platelets (x10^9 cells/L)</td>
<td>592 - 2972</td>
<td>409.2±60.17</td>
<td>361.5±57.22</td>
<td>579.3±55.04</td>
<td>457.0±93.73</td>
</tr>
<tr>
<td>Hematocrit (L/L)</td>
<td>0.35 - 0.454</td>
<td>0.42±0.020</td>
<td>0.37±0.029</td>
<td>0.42±0.014</td>
<td>0.39±0.029</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>110 - 151</td>
<td>126.4±6.189</td>
<td>107.8±8.772</td>
<td>121.7±4.767</td>
<td>115.3±7.236</td>
</tr>
</tbody>
</table>
APPENDIX D

Percentage CD45 positive cells, as measured by InForm analysis software, in WT and TRIM35-/− mice fed either the control diet (A) or the CDE diet (B) for 9 months.
Standards of cytokines used to calculate sample concentrations, as measured with Luminex assay.
**APPENDIX F**

PanCK staining of WT and *TRIM35/-/-* mice fed either control or CDE diet for 9 months

LPC activation in the livers of WT and *TRIM35/-/-* mice, fed either the control or CDE diet for 9 months, was analysed using PanCK staining on liver sections. Red arrows indicate small, PanCK positive cells that are potential LPCs. Black arrowheads indicate boundaries of fat nodules.
APPENDIX G

Volumetric analysis of fat nodules in mice fed the CDE diet for 9 months. T1 MRI scans are used to distinguish fat nodules (red) from remaining liver (green) in ITK-SNAP. A 3D volume is rendered which can be used to calculate percentage of liver that consists of fat nodules.
Example of volumetric measurements done for a TRIM35−/− mice fed the CDE diet for the duration of the 9-month experiment. Fat nodule (red) volume was calculated as total of the whole liver (green).
REFERENCES


Ro52/Trim21 induces tissue inflammation.

Pyrin, a Caire fever protein, is cleaved by caspase.


operate to induce cell cycle arrest and control of cell growth by ectodermin, a cancer.


Uchida, N. and I.L. Weissman, Searching for hematopoietic stem cells: evidence that Thy-1.1 to Lin-Sca-1+ cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. Journal of Experimental Medicine, 1992. 175(1): p. 175-184.


