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Investigating the role of Bmi-1 in liver growth and function

Alex Kenneth Elder

PhD Thesis 2011

Supervisor: Dr Kristin Braun

Co-Supervisor: Professor Malcolm Alison

Centre for Cutaneous Research, Blizard Insitute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London
Declaration:

I, Alex Elder, confirm that the work presented in this thesis is my own, unless stated otherwise, and is in accordance with the University of London’s regulations for the degree of PhD. I also confirm that this thesis is the one upon which I expect to be examined.

Alex Elder
Abstract:

Bmi-1 is a member of the Polycomb group (PcG) family of transcriptional repressors, which are implicated in the maintenance of embryonic and adult stem cells. Previous reports indicate that Bmi-1 plays a key role in repressing the \textit{Ink4a/Arf} tumour suppressor locus. Overexpression of Bmi-1 in putative liver stem/progenitor cells leads to increased self-renewal \textit{in vitro} and tumourigenesis following transplantation into immunocompromised mice. However, the \textit{in vivo} functional requirement for Bmi-1 in liver development, homeostasis and regeneration has not been investigated. For this thesis, the consequences of \textit{Bmi-1} deletion for the post-natal murine liver were assessed using a knockout mouse model. Immunohistochemical techniques were first used to examine Bmi-1 expression in normal murine and human liver, and in human liver pathologies. Bmi-1 was highly expressed in biliary cells of both mouse and human liver, and at lower levels in some murine hepatocytes. Strong expression of Bmi-1 was observed in cytokeratin 19-positive oval cells in regenerating murine liver. \textit{Bmi-1} knockout mice exhibited structural abnormalities in the liver parenchyma, most strikingly the abnormal development of polyploidy in hepatocytes. \textit{Bmi-1} deletion did not result in impaired proliferation in mice, despite increased expression levels of cell cycle inhibitors p16\textsuperscript{Ink4a}, p19\textsuperscript{Arf} and p21. In contrast, an increase in hepatocyte proliferation was observed in 8 week old \textit{Bmi-1} deficient mice, which was correlated with upregulation of cyclin D1. Mel-18, a structural homologue of Bmi-1, was also expressed in murine cholangiocytes and was upregulated in the livers of \textit{Bmi-1} deficient mice during ageing, suggesting it may have been exerting a compensatory effect. Adult \textit{Bmi-1} knockout mice also exhibited excessive iron loading and abnormalities in the expression of key regulators of hepatic iron homeostasis.
# Table of Contents

Abstract: ........................................................................................................................................ 3

List of Figures: ................................................................................................................................. 9

List of Tables: ................................................................................................................................. 11

List of Abbreviations: ..................................................................................................................... 11

Acknowledgements: ....................................................................................................................... 15

Chapter 1. Introduction .................................................................................................................. 16

1.1. Liver anatomy and physiology: ............................................................................................... 18

1.2. Hepatocyte-mediated liver regeneration: ............................................................................... 20

1.3. Stem cell-mediated liver regeneration: .................................................................................. 20

1.3.1. Induction of the oval cell response: .................................................................................... 20

1.3.2. Proposed site of origin: ....................................................................................................... 21

1.3.3. Bilineage differentiation of oval cells: ............................................................................... 22

1.3.4. Oval cell markers ............................................................................................................... 24

1.3.5. Contribution of stem/progenitor cells to normal liver turnover: ....................................... 24

1.4. Liver cancer and disease: ....................................................................................................... 27

1.4.1. Liver cancers: ....................................................................................................................... 27

1.4.2. Contribution of oval cells to liver tumours: ....................................................................... 27

1.4.3. Liver cirrhosis: ..................................................................................................................... 28

1.5. Liver polyploidisation: .......................................................................................................... 28

1.5.1. Introduction to polyploidisation: ......................................................................................... 28

1.5.2. Mechanisms of polyploidisation: ....................................................................................... 30

1.5.3. Regulation of polyploidisation: .......................................................................................... 30

1.5.4. Ploidy reduction: ............................................................................................................... 31

1.5.5. Functional purpose of polyploidisation: ............................................................................. 32

1.5.6. Proliferative potential of polyploid hepatocytes: ............................................................... 33

1.5.7. Ploidy changes in liver disease and damage: ..................................................................... 34

1.5.8. Aneuploidy in hepatocytes: ............................................................................................... 35

1.6. Iron homeostasis: .................................................................................................................. 36

1.6.1. Introduction and iron absorption: ....................................................................................... 36

1.6.2. Iron uptake from plasma: .................................................................................................. 36

1.6.3. Iron export: ......................................................................................................................... 38

1.6.4. Control of iron homeostasis by hepcidin: ......................................................................... 39

1.6.5. Control of hepcidin expression: ......................................................................................... 41

1.6.6. Kupffer cells and iron metabolism: .................................................................................... 43

1.6.7. Iron overload: ..................................................................................................................... 43
1.7. Polycomb Group Genes: ................................................................. 46
  1.7.1. Introduction – the Polycomb family: ........................................ 46
  1.7.2. PRC complexes: ........................................................................ 48
  1.7.3. Repression by PcG proteins: ....................................................... 50
1.8. Bmi-1: ............................................................................................ 53
  1.8.1. Function of Bmi-1 within PRC1: ................................................. 53
  1.8.2. Bmi-1 knockout mice: ................................................................. 54
  1.8.3. Regulation of somatic stem cells by Bmi-1: .................................. 55
  1.8.4. Bmi-1 in tumourigenesis: ........................................................... 58
1.9. Regulation of the cell cycle by Bmi-1: .............................................. 59
  1.9.1. Introduction to the cell cycle: ....................................................... 59
  1.9.2. The Ink4a/Arf locus ................................................................... 61
  1.9.3. p21: ............................................................................................ 65
  1.9.4. Other Bmi-1 targets: ................................................................. 69
  1.9.5. Bmi-1 in mitochondrial function and the DNA damage response.... 70
1.10. Mel-18: .......................................................................................... 71
  1.10.1. Functions of Mel-18: ................................................................. 72
1.11. Other Bmi-1 homologues: ............................................................... 76
1.12. Polycomb genes and the liver: .......................................................... 77
  1.12.1 Polycomb genes and liver stem cells: ......................................... 77
  1.12.2. Polycomb genes in HCC and hepatocytes: ............................... 80
  1.12.3. Polycomb genes in CC and biliary diseases: ............................. 83
  1.12.4. Summary - Bmi-1 in liver tumours: .......................................... 84
1.13. Aims: ............................................................................................... 86

Chapter 2. Materials and Methods ............................................................ 89

  2.1. Mice and animal procedures: .......................................................... 90
    2.1.1. Generation of Bmi-1 knockout mice: ....................................... 90
    2.1.2. Animal husbandry and Home Office compliance: ................... 90
    2.1.3. BrdU injections: ...................................................................... 90
  2.2. Serum collection and analysis: ....................................................... 91
    2.2.1. Serum collection: ................................................................. 91
    2.2.2. Serum analysis: ................................................................. 91
  2.3. Genotyping of Bmi-1 knockout mice: ............................................ 91
    2.3.1. Isolation and preparation of DNA: ....................................... 91
    2.3.2. Genotyping PCR: ............................................................... 92
    2.3.3. DNA gel electrophoresis: .................................................. 93
2.4. Histology and Immunohistochemistry: .......................................................... 94
  2.4.1. Tissue collection and processing: ........................................................ 94
  2.4.2. Histological staining procedures: ....................................................... 95
  2.4.3. Primary antibodies used for immunohistochemistry: ......................... 95
  2.4.4. Immunoperoxidase methods: ............................................................. 96
  2.4.5. Quantification of BrdU, Ki67 and Cyclin D1 positive nuclei: ................ 99
  2.4.6. Quantification of Bmi-1 expression in human tumour and cirrhotic microarrays: ................................................................. 99
  2.4.7. Detection of mitochondrial mutations: ............................................. 99
2.5. Measurement of hepatocyte size parameters using touch preparations: .... 101
  2.5.1. Touch preparations: ........................................................................ 101
  2.5.2. Staining of touch preparations: ........................................................ 102
  2.5.3. Measurement of cell ploidy by analysis of touch preparation images: ... 102
2.6. Analysis of average hepatocyte centromere counts: .................................. 104
  2.6.1. Pan-centromeric in situ hybridisation: ............................................. 104
  2.6.2. Quantification of centromere numbers: ............................................ 105
2.7. Gene expression analysis in mouse liver tissue: ....................................... 105
  2.7.1. Sample collection: .......................................................................... 105
  2.7.2. RNA extraction: ............................................................................. 105
  2.7.3. cDNA synthesis: ............................................................................ 106
  2.7.4. Polymerase chain reaction (PCR): .................................................. 106
  2.7.5. Quantitative PCR (Q-PCR): ............................................................ 109
2.8. Western blotting: ................................................................................... 111
  2.8.1. Sample collection: .......................................................................... 111
  2.8.2. Quantification of protein concentration: ......................................... 111
  2.8.3. SDS Polyacrylamide gel electrophoresis (PAGE): ........................... 111
  2.8.4. Coomassie staining: ......................................................................... 114
  2.8.5. Western blotting: ............................................................................ 114
  2.8.6. Selection of housekeeping gene for western blotting: ...................... 117
  2.8.7. Densitometric analysis of western blots: ......................................... 117

Results and Discussion .................................................................................. 119

Chapter 3. Expression of Bmi-1 in normal liver and regenerating tissue .......... 120
  3.1. Reliability of anti-Bmi-1 antibodies: ....................................................... 121
  3.2. Bmi-1 protein is primarily expressed in biliary cells in normal murine liver: ... 122
     3.2.1. Bmi-1 is expressed in cholangiocytes: ........................................... 122
     3.2.2. Bmi-1 is expressed in hepatocytes: .............................................. 122
3.3. Bmi-1 protein is expressed in mouse oval cell nuclei: .......................... 125
3.4. Bmi-1 protein is expressed in some biliary cells in human liver: .............. 127
3.5. Bmi-1 protein is expressed in human cirrhotic liver tissue: ...................... 129
3.6. Expression of Bmi-1 in hepatic tumours: ........................................ 133
  3.6.1. Bmi-1 expression in human hepatocellular carcinomas: ..................... 133
  3.6.2. Bmi-1 expression in human cholangiocarcinomas: ........................... 134
3.7. Discussion: ....................................................................................... 138
3.8. Summary of Chapter: ......................................................................... 140

Chapter 4. Liver structure and function in  
Bmi-1 knockout mice .................. 141
4.1. Breeding and genotyping of Bmi-1 knockout mice: ................................ 142
4.2. Characterisation of Bmi-1 knockout mouse liver phenotype: ................. 146
  4.2.1. Liver function in Bmi-1 knockout mice: .......................................... 146
  4.2.2. Histology of Bmi-1 knockout mouse livers: .................................... 149
4.3. Bmi-1 knockout mice exhibit reduced hepatocyte size and ploidy: ......... 153
  4.3.1. Bmi-1 knockout mice have increased hepatocyte nuclear density: ....... 153
  4.3.2. Bmi-1 knockout mice have reduced hepatocyte cell and nuclear size: .... 155
  4.3.3. Bmi-1 knockout mouse hepatocytes exhibit fewer centromeres: ......... 160
  4.3.4. Hepatocyte binucleation is unimpaired in Bmi-1 knockout mice: ........ 162
  4.3.5. Bmi-1 knockout livers exhibit increased expression of the ploidy marker  
       Igfbp2: .......................................................................................... 163
  4.3.6. Bmi-1 knockout mouse livers have normal levels of phosphorylated Akt: 164
4.4. Expression changes of other Polycomb genes in Bmi-1 knockout mouse liver: 
 ..................................... ........................................................................ 166
  4.4.1. The Bmi-1 homologue Mel-18 is upregulated in Bmi-1 knockout mouse liver: 166
  4.4.2. Expression of Bmi-1 homologues Mblr and Nspc1 are not altered in Bmi-1  
       knockout liver: ............................................................................ 170
  4.4.3. Expression of other PRC members in Bmi-1 knockout mouse liver: ...... 170
4.5. Discussion: ....................................................................................... 172
  4.5.1. Liver function in Bmi-1 knockout mice: .......................................... 172
  4.5.2. Liver structure and polyploidisation in Bmi-1 knockout mice: .......... 172
  4.5.3. Similarities to mice with inactivated c-myc: .................................... 176
  4.5.4. Expression of other Polycomb genes in Bmi-1 knockout livers: .......... 177
4.6. Summary of Chapter: ......................................................................... 179

Chapter 5. The effect of Bmi-1 deletion on the cell cycle in murine liver ........ 180
5.1. Hepatocyte proliferation in Bmi-1 knockout mice: ................................ 181
5.2. Cholangiocyte proliferation in Bmi-1 knockout mice: ........................... 184
5.3. Expression changes of cyclin D1 and c-myc in Bmi-1 knockout mice: ........ 185
5.3.1. Cyclin D1 protein is overexpressed in Bmi-1 knockout mouse livers: .... 185
5.3.2. c-myc is overexpressed in Bmi-1 knockout mouse livers:.................. 188
5.4. Expression of cell cycle inhibitors in Bmi-1 knockout livers:................ 189
5.4.1. p16^{ink4a}, p19^{Arf} and p21 are upregulated in Bmi-1 knockout mouse liver: 189
5.4.2. Expression of p16^{ink4a} and p21 proteins are increased in Bmi-1 knockout livers:................................................................. 191
5.4.3. Localisation of p16^{ink4a} and p21 in Bmi-1 knockout livers:................ 193
5.5. Discussion:.................................................................................. 197
5.5.1. Changes in proliferative frequency in Bmi-1 knockout livers:................. 197
5.5.2. Upregulation of cyclin D1 and c-myc in Bmi-1 knockout livers:............. 200
5.5.3. Upregulation of p16^{ink4a} and p19^{Arf} in Bmi-1 knockout livers:......... 201
5.5.4. p21 expression in Bmi-1 knockout livers:.................................... 202
5.5.5. Similarities to mice with inactivated c-myc:........................................ 207
5.6. Summary of Chapter:......................................................................... 208

Chapter 6. Iron overload in Bmi-1 knockout mice ........................................ 211
6.1. Hepatic iron accumulation in Bmi-1 knockout mice:.............................. 212
6.2. Expression of iron regulatory genes in Bmi-1 knockout mice:.................. 215
6.2.1. Hepcidin antimicrobial peptide:.................................................. 217
6.2.2. Ferroportin:................................................................................... 218
6.2.3. Transferrin Receptor 1:................................................................. 218
6.2.4. Transferrin Receptor 2 and HFE:................................................. 219
6.2.5. Iron Regulatory Protein 2:........................................................... 220
6.2.6. Bone Morphogenetic Protein 6:................................................... 221
6.3. Serum iron analysis:............................................................................ 221
6.4. Discussion:........................................................................................ 224
6.4.1. Summary of iron overload phenotype:.......................................... 224
6.4.2. Potential causes of iron overload:................................................. 225
6.4.3. Histopathology of iron deposition in Bmi-1 knockout mice:............. 229
6.4.4. Iron overload and the cell cycle:................................................. 230
6.5. Summary of Chapter:.......................................................................... 231

Chapter 7. Final Discussion ........................................................................ 233
7.1. Conclusions:...................................................................................... 234
7.2. Discussion and Future Perspectives:.................................................... 235
7.2.1. Final discussion of Bmi-1 knockout liver phenotypes:....................... 238
7.2.2. Bmi-1 in hepatic stem cell mediated regeneration:............................ 240
7.2.3. Tumourigenesis in Bmi-1 knockout mice: .................................................. 242
7.3. Concluding paragraph: ............................................................................. 243

Bibliography: ..................................................................................................... 245

Appendix: ........................................................................................................... 267
A1. Lineage tracing using mitochondrial DNA mutations: .................................. 267
A2. Selection of reference gene for Q-RT-PCR experiments: .............................. 270
A3. Analysis of hepatocyte nuclear area using touch preparations: .................. 271

List of Figures:

Chapter 1. Introduction
Figure 1: Liver structure.................................................................................. 19
Figure 2: The murine oval cell response. .......................................................... 23
Figure 3: Polyploidisation in mammalian liver................................................. 29
Figure 4: Regulation of iron homeostasis by hepcidin. .................................... 40
Figure 5: Sequential model of PcG mediated gene silencing. ......................... 51
Figure 6: The mammalian cell cycle. ............................................................... 60
Figure 7: The murine Ink4a/Arf locus. .............................................................. 62

Chapter 3. Expression of Bmi-1 in normal liver and regenerating tissue
Figure 8: Bmi-1 is expressed in cholangiocytes and hepatocytes in murine liver. 124
Figure 9: Bmi-1 is expressed in CK19 positive murine oval cells.................... 126
Figure 10: Bmi-1 is expressed in cholangiocytes in human liver....................... 128
Figure 11: Bmi-1 expression in human cirrhotic livers. .................................... 132
Figure 12: Bmi-1 expression in human hepatocellular carcinomas.................. 136
Figure 13: Bmi-1 expression in human cholangiocarcinomas........................... 137

Chapter 4. Liver structure and function in Bmi-1 knockout mice
Figure 14: Breeding and genotyping of Bmi-1 knockout mice.......................... 144
Figure 15: Liver and body weights in Bmi-1 knockout mice............................. 145
Figure 16: Liver function biomarkers in Bmi-1 knockout mice......................... 148
Figure 17: Histology of newborn Bmi-1 knockout mouse livers....................... 150
Figure 18: Histology of adult Bmi-1 knockout mouse livers. .................. 151
Figure 19: Portal structure in Bmi-1 knockout mice. ......................... 152
Figure 20: Bmi-1 knockout mice have increased hepatocyte density. .. 154
Figure 21: Bmi-1 knockout mouse hepatocytes have reduced nuclear area. 157
Figure 22: Hepatocytes in 12 week old Bmi-1 knockout mice are smaller in size. ... 159
Figure 23: Bmi-1 knockout hepatocytes have reduced centromere numbers. .... 161
Figure 24: Binucleation is unimpaired in Bmi-1 knockout mouse hepatocytes. 162
Figure 25: Bmi-1 knockout mice express reduced levels of Igfbp2. ............ 163
Figure 26: Bmi-1 knockout mice exhibit normal levels of phosphorylated Akt. 165
Figure 27: Mel-18 is upregulated in Bmi-1 knockout mice during ageing. .... 168
Figure 28: Mel-18 is expressed in cholangiocytes in murine liver. .......... 169
Figure 29: Expression of other Polycomb genes in Bmi-1 knockout mice. ... 171

Chapter 5. The effect of Bmi-1 deletion on the cell cycle in murine liver

Figure 30: Bmi-1 knockout mice show altered hepatocyte proliferation. .... 183
Figure 31: Cholangiocytes in Bmi-1 knockout mice sporadically express Ki67. ... 184
Figure 32: Cyclin D1 is overexpressed in Bmi-1 knockout mice. ............... 187
Figure 33: c-myc mRNA is overexpressed in Bmi-1 knockout mice. ............. 188
Figure 34: p16\textsuperscript{ink4a}, p19\textsuperscript{Arf} and p21 are upregulated in Bmi-1 knockout mice. ....... 190
Figure 35: p16\textsuperscript{ink4a} and p21 protein are overexpressed in Bmi-1 knockout mice. ... 192
Figure 36: Localisation of p16\textsuperscript{ink4a} protein in Bmi-1 knockout mouse livers. ............. 194
Figure 37: Localisation of p21 protein in Bmi-1 knockout livers. ............... 195
Figure 38: The effects of Bmi-1 deletion on the cell cycle. ..................... 210

Chapter 6. Iron overload in Bmi-1 knockout mice

Figure 39: Kupffer cell pigmentation in Bmi-1 knockout mouse livers. ......... 213
Figure 40: Iron deposition in Bmi-1 knockout mouse livers. .................... 213
Figure 41: Altered expression of iron regulatory genes in Bmi-1 knockout mice. .. 216
Figure 42: Serum iron levels are unchanged in Bmi-1 knockout mice. ........... 223
Appendix

Figure 43: Application of mitochondrial lineage tracing techniques to Bmi-1 knockout mice.......................................................... 269

Figure 44: Analysis of nuclear area in hepatocyte touch preparations............... 273

List of Tables:

Chapter 1
Table 1: Polycomb Repressor Complexes..............................................................47

Chapter 3
Table 2: Bmi-1 expression in human cirrhotic livers...........................................130
Table 3: Bmi-1 expression in human liver tumours.............................................135

List of Abbreviations:

2-AAF – 2-acetylaminofluorine
ABCB1 – ATP binding cassette transporter B1
AFP – alpha fetoprotein
AL-uPA - albumin-urokinase type plasminogen activator
ALT – alanine aminotransferase
AP – alkaline phosphatase
APS – ammonium persulphate
AST – aspartate aminotransferase
ATP - adenosine-5’-triphosphate
BCOR – Bcl-6 co-repressor
Bmi-1 - B-cell specific Mo-MLV integration site 1
BMP – bone morphogenetic protein
BrdU - 5’-bromo-2-deoxyuridine
BRE – Bmi-1 responding element
CC – cholangiocellular carcinoma
CCl₄ – carbon tetrachloride
CDE diet – choline deficient, ethionine supplemented diet
Cdk – cyclin dependent kinase
ChIP – chromatin immunoprecipitation
CK – cytokeratin
COX – cytochrome c oxidase
DAB – diaminobenzidine
DDC - diethoxycarbonyl-1,4-dihydrocollidine
DDR – DNA damage response
DEN – diethylnitrosamine
Dlk – delta-like protein
DMT1 – divalent metal transporter 1
eEF2 – eukaryotic elongation factor 2
EPO – erythropoietin
Ercc1 - excision repair cross complementing 1
FACS – fluorescence activated cell sorting
Fah – fumarylacetoacetate hydrolase
Foxl1 – forkhead box I1
FPN – ferroportin
GAPDH – glyceraldehyde 3-phosphate dehydrogenase
GFAP – glial fibrillary acidic protein
GFP – green fluorescent protein
GGT – gamma-glutamyltransferase
GUS – β-glucuronidase
H3K27me3 – Trimethylation of histone H3 on lysine 27
H&E – haematoxylin and eosin
HAMP – hepcidin antimicrobial peptide
HCC – hepatocellular carcinoma
HC-CC – combined hepatocellular-cholangiocellular carcinoma
H-CFU-C – hepatic colony forming unit in culture
HFE – haemochromatosis protein
HJV – haemojuvelin
HMTase – histone methyltransferase
HPC – hepatic progenitor cell
HRP – horse radish peroxidase
HSC – haematopoietic stem cell
Igfbp2 – insulin growth factor binding protein 2
IRE – iron response element
IRP – iron regulatory protein
mBEC – mouse biliary epithelial cell
MBLR – Mel-18 and Bmi-1 like ring finger protein
Mdm2 – murine double minute 2
MEC – mammary epithelial cell
Mo-MLV – Moloney murine leukaemia virus
NBT – nitro blue tetrazolium
NOD/SCID – non-obese diabetic/severe combined immunodeficiency
NSPc1 – Nervous system polycomb 1
NTBI – non transferrin bound iron
PAGE – polyacrylamide gel electrophoresis
PBC – primary biliary cirrhosis
PBS – phosphate buffered saline
PcG – Polycomb group
PCNA – proliferating cell nuclear antigen
PCR – polymerase chain reaction
PHx – partial hepatectomy
PI3K - phosphatidylinositol 3-kinase
PMS – phenazine methosulphate
PRC – Polycomb repressor complex
PRE – Polycomb repressive element
Q-RT-PCR – quantitative reverse transcriptase polymerase chain reaction
Rb – retinoblastoma
ROS – reactive oxygen species
RPL27 – ribosomal protein L27
SDH – succinate dehydrogenase
SDS – sodium dodecyl sulphate
SHPC – small hepatic progenitor cell
shRNA – small hairpin RNA
siRNA – small interfering RNA
Sox9 – Sex determining region Y-box 9
Sox17 - sex determining region Y-box 17
SP – side population
TBST – tris buffered saline with Tween 20
TEMED – tetramethylethylenediamine
TERC – telomerase RNA component
TERT – telomerase reverse transcriptase
TfR – transferrin receptor
Thy-1 – thymus cell antigen 1
TTR – transthyretin
UTR – untranslated region
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Chapter 1

Introduction
1. Introduction:

During my PhD studies, I have addressed the role of the Polycomb group protein Bmi-1 in regulating hepatic growth and function. In particular, I have investigated alterations in hepatocellular ploidy, proliferation and iron accumulation following global deletion of Bmi-1 in mice. In addition, I have generated preliminary data suggesting that Bmi-1 is highly expressed during tissue regeneration in both murine oval cells and some human cirrhotic nodules, which will underpin future studies to investigate the role of Bmi-1 during hepatic repair.

The following introduction section will review the literature relevant to this work, with particular regard to the following topics:

1. The structure, regenerative capacity, and pathologies of the liver
2. Hepatocyte polyploidisation in the postnatal liver
3. The role of the liver in iron homeostasis
4. Bmi-1 and the Polycomb group family
5. Regulation of the cell cycle by Bmi-1
6. The Bmi-1 homologue, Mel-18
7. The function of Bmi-1 in hepatic cell populations
1.1. Liver anatomy and physiology:

The liver is the largest internal organ in the body and carries out a diverse array of functions, including detoxification of blood, production of bile and plasma proteins, carbohydrate storage and metabolism of lipids and amino acids (Wright & Alison, 1984). The bulk of the liver is formed from hepatocytes, which are arranged in plates separated by vascular sinusoids. The hepatocyte plates radiate out from the central vein to form the classical hexagonal liver lobule, which is the structural unit of the liver (Figure 1). At the vertices of the lobule are portal triads, consisting of terminal branches of the hepatic artery and portal vein, and the bile duct, formed from cholangiocytes. The endothelium lining liver sinusoids is fenestrated, permitting blood from the hepatic artery and portal vein to wash over the exposed apical surfaces of the hepatocytes before draining into the central vein. The space between the endothelium and hepatocytes is known as the space of Disse. This contains stellate cells, which are involved in liver fibrosis and vitamin A storage, and liver macrophages called Kupffer cells. The basal faces of the hepatocytes form bile canaliculi, which carry secreted bile to the main bile duct in the portal triad.
Figure 1: Liver structure.

a) The classical liver lobule. b) Haematoxylin & eosin stained paraffin-embedded mouse liver section, image at 100x magnification.
1.2. Hepatocyte-mediated liver regeneration:

The many essential functions carried out by the liver have led to the evolution of a great capacity for regeneration (Michalopoulos, 2007; Taub, 2004). Under resting conditions, the liver is a largely quiescent organ with a cell turnover of a year or more. A regenerative response mediated by hepatocyte proliferation can be initiated in rodents by using one of several available models to induce liver injury. The most commonly used is the two-thirds partial hepatectomy (PHx), which involves surgically removing two lobes (median and left lateral) of the liver while leaving the other lobes completely intact. This procedure can also be used in humans for tumour resection or repair following trauma. Alternatively, a hepatotoxic chemical such as carbon tetrachloride (CCl₄) can be used to cause necrosis of centrilobular cells. Following injury, the remaining liver cells enter the cell cycle and restore the complete liver mass within 5-7 days (in rodents). This process requires 90-95% of hepatocytes to undergo approximately two doublings, with a semi-synchronous peak in DNA synthesis observed at approximately 24h in rats (Grisham, 1962). Induction of DNA synthesis occurs later in non-parenchymal cells.

1.3. Stem cell-mediated liver regeneration:

1.3.1. Induction of the oval cell response:

There is strong evidence for the existence of a resident liver stem/progenitor cell compartment from experimental models for oval cell (or progenitor cell) activation in rodent livers (Duncan et al, 2009a; Santoni-Rugiu et al, 2005). Oval cells are small, transient, non-parenchymal cells with an ovoid shaped nucleus and high nuclear to cytoplasmic ratio. They are able to repopulate the liver in the absence of hepatocyte mediated regeneration. Models for oval cell activation tend to involve a combination of liver injury (usually two-thirds partial hepatectomy) and exposure to chemicals which prevent hepatocytes from proliferating in response to the damage, thus compromising
hepatocyte mediated regeneration processes. In the rat, the mitoinhibitory agent 2-acetylaminofluorene (2-AAF) is the most commonly used (Evarts et al, 1989; Tatematsu et al, 1984), although this does not induce a strong oval cell response in mice (Jelnes et al, 2007). Mouse models include partial hepatectomy in combination with the DNA alkylating agent dipin (Faktor et al, 1980). Alternatively, administration of carcinogenic choline-deficient, ethionine-supplemented (CDE) (Shinozuka et al, 1978) or diethoxycarbonyl-1,4-dihydrocollidine (DDC) (Presegger et al, 1999) diets can induce an oval cell response without the requirement for partial hepatectomy. The models used to activate oval cells in rodents are not suitable for humans, although ductular reactions of progenitor cells are found in several human liver diseases (Bird et al, 2008), including alcoholic and non-alcoholic fatty liver disease (Roskams et al, 2003). The cells appear in chronic and acute liver diseases, and are commonly referred to as hepatic progenitor cells (HPCs) instead of oval cells.

1.3.2. Proposed site of origin:

The precise origin of oval/progenitor cells has not been conclusively established. Based on histological observations, they appear to arise from areas close to the canals of Hering (terminal biliary ducts of the liver, lined partly by cholangiocytes and hepatocytes) in rodents (Paku et al, 2001) and humans (Theise et al, 1999). However, it is unclear whether they originate from pre-existing liver stem/progenitor cells, or develop from other cell types following injury. A small cluster of cells has been identified within the portal region (two to four cells out of a total of 50,000) that express the embryonic stem cell markers Oct4, Nanog and Stat3, suggesting the presence a resident stem/progenitor cell pool (Tang et al, 2008). Label retaining assays in murine liver have identified putative stem cell compartments proximal to the portal areas: in the canals of Hering, intraductal cholangiocytes and peribiliary hepatocytes (Kuwahara et al, 2008), although it is possible that slower cycling stem cell populations are not initially labelled using this technique. Destruction of the biliary
epithelium by 4,4’-methylene dianiline (4,4’-diaminodiphenylmethane, DAPM) results in a complete absence of oval cell proliferation in 2-AAF/PHx treated rats (Petersen et al, 1997), suggesting a close relationship between oval cells and biliary epithelial cells. In support of this, a 3-dimensional reconstruction of human cirrhotic livers has also shown hepatocyte regenerative nodules linked directly to intralobular bile ducts by ductular reactions (Falkowski et al, 2003). However, there have not been any lineage tracing experiments which specifically demonstrate that mature cholangiocytes themselves can act as progenitor cells, although they do share several markers with oval cells (see section 1.3.4). Bone marrow cells have previously been reported as a potential source of oval cells (Petersen et al, 1999), however it has since been demonstrated that this is due to cell fusion and is not significant in liver regeneration (Wang et al, 2003b). There is also a body of evidence suggesting that glial fibrillary acidic protein (GFAP) positive quiescent hepatic stellate cells may be a type of epithelial progenitor. Following liver injury, green fluorescent protein (GFP) marked stellate cells from GFAP-Cre/GFP double transgenic mice become highly proliferative and express several oval cell markers, before repopulating areas of the parenchyma (Yang et al, 2008). Rats treated with the pyrrolizidine alkaloid retrorsine undergo liver regeneration mediated by small hepatocyte progenitor cells (SHPCs), which share characteristics of oval cells but are phenotypically distinct (Gordon et al, 2000). It therefore seems there is may be a multi-tiered progenitor cell system within the liver.

1.3.3. Bilineage differentiation of oval cells:

Following induction by the models described previously, oval cells proliferate in the periportal region of the liver and proceed to infiltrate the parenchyma (Alison et al, 1996) (Figure 2). This process is known as a ductular reaction. Oval cells are not observed in normal uninjured liver, or following the completion of regeneration in response to liver injury. It is therefore believed that they are transitional cells in a
lineage which can give rise to mature hepatocytes and biliary cells following injury. In vivo evidence for oval cell bipotentiality comes from studies using a Forkhead Box L1 (Foxl1)-Cre transgenic mouse crossed with a reporter line (Sackett et al, 2009). Foxl1 is a transcription factor which shows increased expression in DDC diet treated mice. Foxl1-Cre expressing cells are found in the periportal region following injury, and can give rise to progeny expressing either a hepatocyte marker (hepatocyte nuclear factor 4 alpha) or cholangiocyte marker (CK19). This suggests that Foxl1 expressing cells are bipotent progenitors, although it has not been demonstrated that an individual cell can give rise to both types of progeny. In humans, immunohistochemical analysis of cirrhotic livers has identified individual ductular reactions containing hepatocytes and cholangiocytes separated by transitional cells (Zhou et al, 2007).

Figure 2: The murine oval cell response.

Paraffin-embedded liver section from mouse treated with the DNA alkylating agent dipin. Section labelled with antibody against cytokeratin 19 (CK19). Labelled are CK19 positive oval cells (OC) and bile duct (BD). Scale bar 100 µm.
1.3.4. Oval cell markers

A number of markers have been used to identify oval cells in rodents (Bird et al, 2008)(Santoni-Rugiu et al, 2005), however these are often also expressed on some other cell populations within the liver. Briefly, these include markers for biliary cells (e.g. cytokeratins 7, 8, 18 & 19, OV-6, muscle pyruvate kinase, A6), hepatocytes (e.g. albumin), foetal hepatoblasts (e.g. alpha fetoprotein (AFP), delta-like protein (Dlk)). Oval cells have also been reported to express proteins common to non-liver cell populations, including haematopoietic cell markers c-kit, CD34 and thymus cell antigen 1 (Thy-1). Oval cells are difficult to study due to this phenotypic heterogeneity exhibited within the activated progenitor cell population. This heterogeneity is likely to be due in part the different degrees of differentiation along hepatocyte and biliary lineages (Zhou et al, 2007), and the emergence of other populations, potentially including Thy-1 positive myofibroblasts (Dezso et al, 2007), in regenerative responses. It seems that the oval cell response represents a heterogeneous population of liver cells that emerge in livers undergoing oval cell activation, including progenitor cells, biliary cells, stellate cells and fibroblasts. Hence the term ‘oval cell’ is not well defined as it does not represent a single cell type, and may not necessarily describe only cells with repopulating ability. It is also important to consider oval cell heterogeneity in different models and species. For example, oval cell reactions induced in the rat 2-AAF/PHx protocol express alpha-fetoprotein (AFP), whereas mouse oval cells from the DDC or CDE diet models do not (Jelnes et al, 2007).

1.3.5. Contribution of stem/progenitor cells to normal liver turnover:

Although the liver is primarily a quiescent organ, there is a gradual turnover of hepatocytes, with each cell having a lifespan of about 200-300 days (Duncan et al, 2009a). An early model for this was proposed by Zajicek et al, who observed migration of hepatocytes labelled with tritiated thymidine from the portal region to
central vein in rat livers (Zajicek et al, 1985). This led to the formulation of the 'streaming liver' hypothesis, whereby young hepatocytes originate near the portal region and then migrate through the liver, undergoing a differentiation process as they move. This is consistent with the idea of a stem/progenitor cell within the portal region, and would also explain the difference in gene expression patterns seen in periportal and pericentral hepatocytes. However, studies using tritiated thymidine are often criticised due to loss and subsequent re-utilisation of thymidine from nuclei, and low labelling indices (Bralet et al, 1994). The widespread belief has been that existing hepatocytes and cholangiocytes alone are responsible for liver turnover, and that there is little contribution from a stem/progenitor cell population (Duncan et al, 2009a). This is due in part to a study which retrovirally labelled proliferating hepatocytes following partial hepatectomy, and demonstrated that the labelled hepatocytes divide in isolated clusters with no evidence of migration (Bralet et al, 1994). This method only labelled approximately 1-5% of hepatocytes, and does not appear to rule out contribution of a stem/progenitor cell as this would not have been labelled in the first place. Another study has also reported similar results, by labelling a small proportion of hepatocytes which express human α1-antitrypsin (Kennedy et al, 1995). Over time, randomly scattered clusters of labelled hepatocytes were observed, again with no evidence of migration. The gene expression pattern in hepatocytes is also dependent on blood flow, as reversing the direction of the flow inverts the gene expression pattern in periportal and pericentral hepatocytes (Thurman & Kauffman, 1985). These studies all suggest that hepatocyte streaming does not occur in normal liver.

However, recent lineage tracing data has provided support for the involvement of stem cell populations in normal liver turnover. Fellous et al used histochemical techniques to identify patches of cells within normal tumour-adjacent human livers, which all have naturally occurring mutations in the mitochondrially encoded cytochrome c oxidase gene (Fellous et al, 2009a). Sequence analysis revealed that
cells within the same patch have identical mutations, suggesting that the patches are clonally derived. The patches also always originate from portal regions of the liver and extend out into the parenchyma, which suggests that they may be derived from a long-lived portal stem/progenitor cell which is able to accumulate the mutations. These data support a hypothesis that a portally-derived liver stem/progenitor cell is able to contribute to hepatocyte turnover in normal liver. Further support for this hypothesis has come from analysis of progenitors expressing sex determining region Y-box 9 (Sox9), a protein important in embryonic formation of several tissues, in murine models (Furuyama et al, 2011). The authors found Sox9 expression in all cholangiocytes in the liver, and then carried out tamoxifen induced lineage tracing to mark Sox9 expressing cells and their progeny with lacZ. Following tamoxifen injection, the majority of labelled cells are initially found in bile duct structures. Over time, the number of labelled hepatocytes gradually increases, and they appear to migrate slowly from the periportal region to the central vein. The biliary structures also maintain a population of Sox9 positive cells after 12 months, suggesting the cells are able to self-renew. The Sox9 positive cells also contribute to liver regeneration in some liver injury models. The number of lineage labelled hepatocytes increases following CCl₄ treatment, bile duct ligation and CDE diet administration. Interestingly, Sox9 expressing precursors appear to have limited ability to contribute to hepatocyte regeneration following a DDC supplemented diet, suggesting that other cell types are more important for the regeneration observed in this model. These data provide solid evidence that stem/progenitor cells located in the biliary tree contribute to hepatocyte turnover under resting conditions, in addition to replication of existing hepatocytes.
1.4. Liver cancer and disease:

1.4.1. Liver cancers:

There are two major cancers of the liver – hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) (Alison & Lovell, 2005). HCC is a malignant tumour which is composed of cells that resemble hepatocytes, but exhibit abnormalities in appearance. The majority arise in livers where hepatocyte damage and chronic inflammation leads to fibrosis. Infection with either hepatitis B or C virus is a major risk factor, along with alcohol abuse, metabolic liver disease and other conditions which lead to cirrhosis. CC is much less common, and is believed to arise from intrahepatic or extrahepatic biliary epithelium. Risk factors include chronic inflammation leading to biliary epithelial injuries, and impediment of bile flow.

1.4.2. Contribution of oval cells to liver tumours:

Progenitor cells and progenitor cell markers have also been identified in human liver cancers, leading to the suggestion that they may be involved in carcinogenesis. Many human liver tumours contain a mixture of mature cells and cells more phenotypically similar to HPCs, including cells that express progenitor cell markers such as OV-6 (Libbrecht & Roskams, 2002). A subset of tumours containing areas of both HCC and CC has also been observed, supporting the notion that a bipotent progenitor may be the tumour initiating cell (Theise et al, 2003). In rats fed with 2-AAF, hepatocytes labelled with β-galactosidase contribute to the formation of preneoplastic foci, demonstrating direct involvement of hepatocytes in carcinogenesis (Gournay et al, 2002). However, oval cells isolated from p53 null mice are able to produce HCCs in athymic nude mice (Dumble et al, 2002), suggesting there may be multiple potential tumour initiating cells.
1.4.3. Liver cirrhosis:

Liver cirrhosis occurs as a consequence of chronic liver injuries, including alcoholism, hepatitis B and C viruses and fatty liver diseases (Schuppan & Afdhal, 2008). It is characterised by advanced liver fibrosis, where there is replacement of injured liver tissue by fibrous connective tissue and scarring, leading to disruption of hepatic vasculature and end stage liver disease. The fibrosis is thought to be caused by activated myofibroblasts which arise from resident hepatic stellate cells (Brenner, 2009). There is some functional in vivo evidence that stellate cell and myofibroblast populations can be derived from the bone marrow in cirrhotic livers, based on transplantation of male bone marrow into lethally irradiated female mice (Russo et al, 2006). Cirrhotic tissue also contains regenerative nodules of hepatic parenchyma which are enclosed by areas of fibrosis, with proliferative biliary cells and ductular reactions within the fibrotic tissue. Recent lineage tracing using naturally occurring mitochondrial mutations suggests that regenerative nodules are clonally derived from hepatic progenitor cells within ductular reactions, as cells within both ductular reactions and the nodules all share identical mutations (Lin et al, 2010). Reconstruction of cirrhotic livers using serial sections has also demonstrated that regenerating hepatocyte buds are structurally associated with ductular reactions and the biliary tree (Falkowski et al, 2003).

1.5. Liver polyploidisation:

1.5.1. Introduction to polyploidisation:

The adult mammalian liver parenchyma consists of a heterogeneous mix of hepatocytes of varying ploidy, both mononuclear and binuclear. The process of polyploidisation begins at weaning (Margall-Ducos et al, 2007) and has been best characterised in the rat. The livers of newborn rats consist almost exclusively of diploid hepatocytes, which contain two copies of the genome (2n) (Guidotti et al,
Upon weaning, a population of diploid cells with two nuclei (binuclear) emerges, followed by the mononuclear tetraploid (4n) cells which form the bulk of the adult rat liver. This sequential process can continue further to generate binuclear 4n and mononuclear octaploid (8n) cells (Figure 3). The number of polyploid cells appears to increase with age in both rats (Enesco et al, 1991) and mice (Funk-Keenan et al, 2008). Polyploidy is correlated with cell size (Rajvanshi et al, 1998), and its onset occurs at a similar time to a shift from hyperplastic liver growth (increase in cell number) to hypertrophic growth (increase in cell size) (Funk-Keenan et al, 2008). The polyploidisation process is conserved in all mammalian species, however the degree to which it occurs varies dramatically. In mice, an estimated 80% of hepatocytes are binuclear, and cells with ploidy classes of 16n and beyond have been observed (Wright & Alison, 1984). In humans, the process is less extensive, with the proportion of mononuclear, diploid cells in the adult liver estimated at approximately 75% (Toyoda et al, 2005).

Figure 3: Polyploidisation in mammalian liver.
Schematic showing development of hepatocyte polyploidy from 2n mononuclear cells.
1.5.2. Mechanisms of polyploidisation:

The mechanism by which polyploid hepatocytes emerge in rat livers has been extensively studied (Guidotti et al, 2003). A proportion of 2n mononuclear hepatocytes undergo a process of incomplete cytokinesis, which leads to the formation of 2n binuclear cells. This occurs through the incomplete formation of a functional contractile actomyosin ring due to disorganised microtubule networks, leading to the abortion of cytokinesis in telophase (Margall-Ducos et al, 2007). The number of incomplete cytokinesis events increases dramatically following weaning, however this is not due to changes in carbohydrate intake due to an altered diet (Celton-Morizur et al, 2009). The binuclear cells formed have a critical role in the subsequent emergence of tetraploid cells (Guidotti et al, 2003). Both nuclei proceed through mitosis normally until metaphase, where a bipolar spindle is formed. This causes all the chromosomes to line up and segregate equally, leading to the formation of two 4n mononuclear cells following normal cytokinesis.

1.5.3. Regulation of polyploidisation:

The incomplete cytokinesis program is a feature of normal hepatocytes, but the signalling pathways which control it have not been well defined. Celton-Morizur et al demonstrated that the livers of rats treated with streptozotocin to destroy pancreatic β-cells, and hence reduce insulin levels, have reduced content of binuclear tetraploid hepatocytes and fewer incomplete cytokinesis events (Celton-Morizur et al, 2009). This also occurs in ob/ob mice, which have impaired insulin signalling. In contrast, injection of insulin into rats at the weaning stage leads to an increase in levels of tetraploid cells, suggesting insulin is involved in regulating liver tetraploidisation. This may be via the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, an important downstream mediator of insulin signalling. The authors discovered that direct inhibition of Akt phosphorylation in primary rat hepatocytes leads to a decrease in the
number of cytokinesis failure events, suggesting Akt may have a direct or indirect role in the generation of binuclear tetraploid hepatocytes. Akt upregulation also causes increased polyploidy in cultured vascular smooth muscle cells (Hixon et al, 2000). A potential role for the pro-proliferative transcription factor c-myc in the polyploidisation process has been established from observations that liver specific c-myc inactivation in mice leads to reduced cell ploidy (Baena et al, 2005). This supports earlier findings that the livers of mice overexpressing c-myc show a shift towards 4n and 8n nuclei (Kao et al, 1996).

1.5.4. Ploidy reduction:

It has recently been demonstrated that hepatocytes can undergo reductions in ploidy as well as increases. Fumarylacetoacetate hydrolase (Fah) is an enzyme involved in tyrosine catabolism, and its deficiency leads to the disease hereditary tyrosinaemia type I, which causes progressive liver failure (Overturf et al, 1996). Fah-/− mice provide an important model for hepatocyte transplantation and repopulation studies, as transplanted hepatocytes have a strong selective growth advantage. Transplantation of wild type bone marrow into fah-/− mice leads to the fusion of bone marrow cells with host hepatocytes (Wang et al, 2003b). These fusion derived hepatocytes are tetraploid, and are able to undergo ploidy reductions to form diploid cells (Duncan et al, 2009b). It has since been demonstrated that this also occurs with normal polyploid hepatocytes (Duncan et al, 2010). Fluorescence activated cell sorting (FACS) purified octaploid hepatocytes transplanted into an fah-/− mouse can reconstitute the liver with the same overall ploidy distribution as found in a normal mouse, suggesting the transplanted cells have undergone ploidy reversal. This also occurs in cultured hepatocytes, and is due to either multipolar or double mitoses (Duncan et al, 2010). It has not been established whether this process occurs in the liver under normal physiological conditions.
1.5.5. Functional purpose of polyploidisation:

Little is known about the functional purpose of hepatocyte polyploidisation. Polyploid rat hepatocytes have a greater degree of cytoplasmic complexity than diploid cells, with increased numbers of vacuoles, lysosomes and inclusions (Rajvanshi et al, 1998). They also exhibit increased cytochrome P450 activity compared to diploid cells, although diploid cells have higher levels of albumin synthesis. This suggests there may be some functional differences between diploid and polyploid hepatocytes. There have been suggestions that binucleate hepatocytes have increased protein synthesis capacity, based on the fact that they are more frequently immunopositive for retinol binding protein, a protein synthesised but not recycled in hepatocytes (Grizzi & Chiriva-Internati, 2007). The authors therefore interpret its expression as a marker for total protein synthesis, however this is not convincing as there is no evidence that protein synthesis is increased other than for retinol binding protein. Increased ploidy has been associated with increased mRNA synthesis of three key genes in megakaryocytes, which also undergo polyploidisation (Hancock et al, 1993). In another study, Anatskaya & Vinogradov tried to examine functional differences in polyploid cells by comparing gene expression microarray data from predominantly diploid tissue (human liver, mouse heart) with mainly polyploid tissue (mouse liver, human heart) (Anatskaya & Vinogradov, 2010). The polyploid (mouse) livers have increased expression of genes involved in key liver functions, including immunity, nitrogenous metabolism, redox state maintenance and metabolic adaptations to long term energy depletion. There is also an increase in gene expression related to cell survival pathways, reserve adenosine-5’-triphosphate (ATP) production and the switching of tissue specific functions to an energy saving mode. The authors suggest that polyploid cells have evolved to improve adaptation to stress and promote the performing of tissue specific functions in severe conditions. It appears uncertain whether the differences observed are merely due to variations between the species.
studied. The authors’ method of normalising a particular gene to its average expression across other tissues from that species should eliminate general species-specific upregulation of that gene. However, it is unclear if changes are specifically due to differences in ploidy between the tissues, or to other undefined differences in hepatic function between species. As such, these results do not provide conclusive evidence for improved function in polyploid cells.

1.5.6. Proliferative potential of polyploid hepatocytes:

Following partial hepatectomy, regenerating rat livers exhibit a large increase in the numbers of tetraploid and octaploid mononuclear cells, coupled with a dramatic decrease in the population of binuclear cells (Melchiorri et al, 1993). This is due to the replication of pre-existing binuclear cells to form mononuclear daughters (Gerlyng et al, 1993). In contrast, treatment with the mitogen lead nitrate results in increased polyploidisation via the induction of large numbers of binucleate cells, leading to liver growth (Melchiorri et al, 1993). The polyploid cells that emerge following partial hepatectomy remain in the liver for a prolonged period. These livers exhibit some senescence-like changes, including an increase in expression of senescence associated β-galactosidase and the cyclin dependent kinase (Cdk) inhibitor p21 (Sigal et al, 1999). Hepatocytes isolated from these livers following partial hepatectomy induced regeneration also have decreased proliferative capacity in vitro. Adult rat liver contains a minor population of small hepatocytes, which are mononuclear, diploid cells with a higher replicative potential than normal hepatocytes in culture (Asahina et al, 2006). Diploid rat hepatocytes also show increased DNA synthesis in response to hepatocyte growth factor, compared to polyploid cells (Rajvanshi et al, 1998). These results appear to support the general theory that advanced polyploidy represents a shift towards terminal differentiation, senescence and reduced proliferative capacity (Gupta, 2000). However, hepatocytes in all ploidy classes participate equally in repopulating mouse livers with hepatocellular damage following transplantation.
(Overturf et al, 1999; Weglarz et al, 2000). Hepatocytes formed by fusion with bone marrow cells, which are tetraploid by definition, can also fully repopulate the liver, and maintain proliferative ability following serial transplantation (Wang et al, 2003a). These data suggest that hepatocytes still retain proliferative capacity with increasing ploidy.

1.5.7. Ploidy changes in liver disease and damage:

Alterations in hepatocyte ploidy have been observed in a variety of liver diseases. Chronic viral hepatitis with fibrosis results in an increase in polyploid hepatocyte numbers, which correlates with the severity of the illness (Toyoda et al, 2005). The livers of carcinogen treated rats contain approximately 70% small diploid hepatocytes after 8 weeks, compared to only 10% in normal wild type animals (Schwarze et al, 1984). Carcinogenesis is characterised by the appearance of hyperplastic nodules, which are believed to represent neoplastic precursor lesions. These consist almost exclusively of diploid cells (Schwarze et al, 1984), suggesting that diploid hepatocytes are tumour precursor cells. This is in keeping with the theory that higher proliferative potential resides in diploid cells. However, the protocol used involves administering diethylnitrosamine (DEN) followed by partial hepatectomy and 2-AAF, which will induce oval cell proliferation, so it is possible that these are the tumour precursor cells rather than existing hepatocytes. Mice treated with DEN alone (Danielsen et al, 1988), or with \(N\)-methyl-\(N\)-nitrosourea and phenobarbital feeding (Sarafoff et al, 1986) both show a general increase in diploid cells and decrease in binuclearity in most tumour foci, although polyploid lesions were also observed, suggesting that diploid foci are not essential for tumour development. There is also a link between DNA damage and increased polyploidy. Excision repair cross complementing 1 (Ercc1) is part of a complex required for incision of damaged DNA during nucleotide excision repair. Mice with liver specific deletion of the \(Ercc1\) gene have dramatically increased polyploidy (Kirschner et al, 2007), suggesting that accumulated DNA damage may be a characteristic of increased polyploid cells observed in older mammals. However, the
fact that the polyploidisation process occurs following weaning in all normal animals suggests that it is not simply a consequence of disease or age related damage to the liver.

1.5.8. Aneuploidy in hepatocytes:

As described earlier, octaploid hepatocytes transplanted into damaged mouse liver will reconstitute the liver with a normal ploidy distribution (Duncan et al, 2010). The authors note that the majority of the donor cells are aneuploid following repopulation. This was also observed in the karyotypes of wild type mice. In yeast cells, aneuploidy can provide a selective advantage in response to environmental stress, and polyploidy and aneuploidy are common features of the best evolved yeast strains (Rancati et al, 2008). This has led to the theory that hepatocyte polyploidisation evolved as a mechanism to create aneuploidy via ploidy reversal (Duncan et al, 2010). This would create a genetically diverse pool of hepatocytes with variations in gene expression, from which the fittest strains would be selected following any liver injury. It could potentially allow selection of particular karyotypes with a degree of genetic resistance to specific liver diseases, or resistance to the harsh microenvironment which hepatocytes are exposed to.

In summary, the importance of hepatocyte polyploidisation is not well understood. The fact that it is a conserved process in mammals suggests its importance, and the need to learn more about the molecular regulators and functional consequences.
1.6. Iron homeostasis:

1.6.1. Introduction and iron absorption:

The liver plays a central role in the metabolism, storage and homeostasis of bodily iron (Graham et al, 2007; Papanikolaou & Pantopoulos, 2005). Iron is an essential trace element in all living cells, however is highly toxic when present in excess due to its ability to catalyse the formation of free radicals. It is therefore essential that iron homeostasis is tightly controlled. This is achieved at the level of iron absorption, as mammals do not have a mechanism for active iron secretion. Dietary iron is absorbed across the apical membrane of duodenal enterocytes through the broad specificity divalent metal transporter 1 (DMT1) (Gunshin et al, 1997). The absorbed iron is then exported into the plasma across the basolateral membrane, via the iron export protein ferroportin (Abboud & Haile, 2000). Excess intracellular iron is stored as ferritin, an iron storage protein which renders it non-toxic. The liver is the major site for the storage of iron, which occurs in hepatocytes (predominantly periportal) and Kupffer cells. Plasma iron is transported almost exclusively in complex with the glycoprotein transferrin, which maintains it in a soluble, non-toxic state.

1.6.2. Iron uptake from plasma:

Transferrin receptor 1:

Entry of plasma transferrin (diferric transferrin) into cells occurs via one of two transferrin receptors, with the predominant uptake in most cells being via transferrin receptor 1 (TfR1). Diferric transferrin binds to TfR1, which is then endocytosed (Dautry-Varsat et al, 1983). The iron now dissociates from transferrin in the more acidic environment of the endosome, and enters the cytoplasm via DMT1. Once internalised, iron is incorporated into iron-containing compounds such as haem, or stored as ferritin (Young et al, 1985).
**Regulation of TfR1 expression by iron response elements:**

The expression of TfR1 is post-transcriptionally regulated by iron levels, via iron response elements (IREs) in the 3’ untranslated region (UTR) (Casey et al, 1988). In iron depleted cells, iron regulatory proteins (IRPs) bind to the IRE and increase the stability of the mRNA, and hence expression of TfR1 (Pantopoulos, 2004). In cells where iron is abundant the reverse occurs; IRPs are inactivated to increase Tfr1 mRNA degradation. The 5’ UTR of ferritin mRNA also contains IREs which can be bound by IRPs, however in this case IRP binding blocks ferritin translation when iron levels are low. There are two IRPs, IRP1 and IRP2, both of which are only present in forms which will bind IREs in the absence of iron (Pantopoulos, 2004). Briefly, IRP1 is usually assembled as an iron-sulphate cluster, which degrades in iron starved cells to allow IRE binding. IRP2 is synthesised de novo following iron depletion, and then proteasomally degraded as iron levels increase. This regulatory system promotes increased transferrin bound iron uptake coupled with decreased iron storage when iron levels are low and the reverse when iron is plentiful. There are also other methods of regulating TfR1 expression, including increases in hypoxia, in response to cytokines, and in proliferating cells in response to increased cellular demand for iron during growth (Graham et al, 2007).

**Transferrin receptor 2:**

Transferrin receptor 2 (TfR2) is a homologue of TfR1, but has much more restricted tissue distribution with predominant expression in hepatocytes and haematopoietic cells (Kawabata et al, 1999). Its function has not been studied to the degree of TfR1, but it is believed to be involved in an alternative, low affinity method of iron uptake. The method of iron uptake by TfR2 is the same as TfR1, however the affinity of TfR2 for transferrin is 25-30 times lower than TfR1 (Kawabata et al, 2000). It is thought that the primary role of TfR2 is not actually iron uptake, as Tfr2 mutant mice still
accumulate hepatic iron (Fleming et al, 2002). Instead its key role may be in sensing iron levels and controlling iron metabolism through regulation of hepcidin expression (see section 1.6.4). Unlike Tfr1, Tfr2 mRNA expression is not believed to be regulated by intracellular iron levels, and has instead been shown to be altered during the cell cycle, with an increase found in proliferating cells (Kawabata et al, 2000).

*Non-transferrin-bound iron uptake:*

Small quantities of non-transferrin-bound iron (NTBI) can also exist in the plasma as a low molecular weight pool of chelates. Hepatocytes are the primary cells involved in uptake of these species (Craven et al, 1987). This is believed to be via DMT1, as NTBI uptake increases following overexpression of DMT1 protein, and membrane expression of DMT1 increases in iron depleted cultured cells (Shindo et al, 2006).

1.6.3. Iron export:

The ferrous iron transporter ferroportin (FPN) is the only known mediator of iron release from cells, and its inactivation in mice leads to iron accumulation in hepatocytes, macrophages and enterocytes (Donovan et al, 2005). Like ferritin, Fpn mRNA contains an IRE in the 5’UTR, so translation should be increased when iron is abundant. This is the case in iron overloaded hepatic, intestinal and monocytic cell lines, based on the use of a luciferase reporter gene flanked by the 5’ region (Lymboussaki et al, 2003). However, increased FPN mRNA levels are found in duodenal biopsies from patients with iron deficiency (Zoller et al, 2001), suggesting cell specific regulation of FPN expression. Mouse *in vivo* immunohistochemical data supports this, as iron deficiency leads to increased FPN expression in the duodenum, but decreased expression in liver Kupffer cells (Abboud & Haile, 2000). It is likely that other mechanisms of FPN regulation, including its degradation by hepcidin (see section 1.6.4), are more important in FPN regulation than the IRE.
1.6.4. Control of iron homeostasis by hepcidin:

Hepcidin, encoded by the *HAMP* (hepcidin antimicrobial peptide) gene, is a small circulating peptide produced in hepatocytes and is a key regulator of body iron homeostasis (Pigeon et al, 2001). Hepcidin is synthesised when iron levels are high, and hence reflects the level of iron sufficiency in the body (Papanikolaou & Pantopoulos, 2005). Increased hepcidin expression leads to a decrease in iron absorption in the duodenum, and retention of cellular iron stores. Correspondingly, mice with liver specific overexpression of hepcidin display severe symptoms of iron deficiency and anaemia (Nicolas et al, 2002a). When iron stores are depleted, or large amounts of iron are required for processes such as erythropoiesis, hepcidin expression drops. This causes an increase in iron absorption in the duodenum and release of iron from hepatocyte and macrophage stores. The mechanism for hepcidin action is thought to be via degradation of the iron export transporter ferroportin (FPN) (Figure 4a). Hepcidin has been shown to bind and internalise FPN *in vitro*, leading to ubiquitin mediated degradation (Nemeth et al, 2004b). This leads to a reduction in cellular iron export during iron sufficiency.
Figure 4: Regulation of iron homeostasis by hepcidin.

a) Hepcidin acts to degrade the iron exporter, ferroportin (FPN), preventing iron export from duodenal enterocytes and hepatocyte/macrophage stores. b) Proposed mechanism for sensing of bodily iron levels by Tfr1, Tfr2 and HFE. Under low iron conditions, HFE is able to bind to Tfr1. Increased levels of diferric transferrin (labelled Fe in diagram) stabilise Tfr2 and bind to Tfr1, displacing HFE. Tfr2 and HFE now facilitate the production of hepcidin.
1.6.5. Control of hepcidin expression:

Hepcidin expression is regulated at the transcriptional level both by hepatic and plasma iron concentrations. The precise pathway is not completely understood, however both transferrin receptors and the HFE protein are believed to be involved in sensing extracellular iron levels (Figure 4b). HFE is a major histocompatibility-type protein and is often mutated in iron overload disorders. HFE is able to complex with TfR1 and lower its affinity for transferrin (Feder et al, 1998), due to the fact that HFE and transferrin have overlapping binding sites on TfR1 (West et al, 2001). Mice with mutations in Tfr1 leading to a constitutive interaction of TfR1 with HFE suffer iron overload due to low hepcidin expression, while mice with a mutation preventing the HFE-TfR1 interaction express high hepcidin (Schmidt et al, 2008). This suggests that HFE is able to induce hepcidin expression when not in complex with TfR1. Given that the affinity of TfR1 for HFE is lower than for transferrin (West et al, 2001), it is likely that increased concentrations of diferric transferrin displace HFE from TfR1 and lead to induction of hepcidin expression.

Mutations in Tfr2 in mice lead to reduced hepcidin expression, inferring that TfR2 is an upstream regulator of the hepcidin pathway (Kawabata et al, 2005). The exposure of cell lines to diferric transferrin leads an increase in both numbers and cell surface localisation of TfR2 (Deaglio et al, 2002). This means that increased transferrin levels act to stabilise TfR2, effectively allowing it to act as an iron sensor, and to facilitate hepcidin production in response to increased iron levels. HFE is able to form a complex with TfR2, suggesting that TfR2 and HFE could be involved in the same hepcidin regulatory pathway (Goswami & Andrews, 2006). This is supported by work in HepG2 cells stably expressing HFE, which usually increase hepcidin production in response to diferric transferrin (Gao et al, 2009). Depletion of TFR2 in these cells abolishes the increase in hepcidin production following treatment with diferric transferrin, supporting the notion that the TfR2-HFE complex is required for increasing
hepcidin synthesis. However, mice deficient for both *Hfe* and *Tfr2* exhibit more severe iron loading and greater reductions in hepcidin expression than mice deficient for only one of the proteins (Wallace et al, 2009). This suggests that the TfR2-HFE interaction may not be as critical *in vivo*, and that the two proteins can also regulate hepcidin production through independent pathways. The signals which regulate hepcidin expression downstream of the TfR1/TfR2/HFE pathway outlined above are not well understood. It is also not clear how intracellular iron levels are able to regulate hepcidin synthesis.

Another pathway which may play an important role in hepcidin regulation is the bone morphogenetic protein (BMP) signalling pathway. Increased BMP signalling upregulates hepcidin expression in hepatocytes, this is enhanced by haemojuvelin (HJV), a co-receptor for BMP ligands that is sometimes mutated in iron overload disorders (Babitt et al, 2006). In particular, expression of *Bmp6* is regulated by iron at the mRNA level, and *Bmp6*−/− mice exhibit a dramatic accumulation of iron in the liver and other tissues (Andriopoulos et al, 2009; Meynard et al, 2009). The mice also show decreased hepcidin expression, despite the iron overload, suggesting a specific role for BMP6 in regulating hepcidin expression. Both mRNA and protein levels of BMP6 also correlate with iron loading in mouse models, in a similar manner to hepcidin (Kautz et al, 2010).

Hepcidin production can also be regulated by signals other than iron levels. Erythropoiesis requires a large increase in the release of iron, both from stores and through iron absorption. Administration of the erythropoiesis stimulating agent erythropoietin (EPO) in mouse models leads to suppression of hepcidin expression, while the addition of erythropoiesis inhibitors results in increased hepcidin expression, despite anaemia induced by phlebotomy (Pak et al, 2006). It is therefore likely that regulators released during erythropoiesis signal to repress hepcidin expression and increase iron supply, although it is currently unclear what these are. Hepcidin is also
suppressed during hypoxia and anaemia (Nicolas et al, 2002b), precipitating the increase of iron absorption and release from stores. In contrast, hepcidin expression is upregulated during inflammation, this is likely to be mediated through cytokines including interleukin-6 (Nemeth et al, 2004a).

1.6.6. Kupffer cells and iron metabolism:

In addition to hepatocytes, the resident liver macrophages, Kupffer cells, have an important role in iron metabolism. Their major role is in the breakdown of haem, and subsequent iron release, from old erythrocytes which have been phagocytosed. The waste products of this are excreted in bile. Some excess iron can be stored in Kupffer cells as ferritin, although a large proportion appears to be exported back into the bloodstream (Kondo et al, 1988), supported by the fact that Kupffer cells express high levels of FPN (Delaby et al, 2005). FPN in Kupffer cells is mainly found in intracellular vesicles, which localise to the cell surface following erythrophagocytosis to allow for release of the recycled iron (Delaby et al, 2005). Kupffer cells can also obtain iron from transferrin when required (van Berkel et al, 1987).

1.6.7. Iron overload:

Primary iron overload

Haemochromatosis is a disorder characterised by high absorption of dietary iron, which leads to iron accumulation in tissues and long term organ damage. In the liver this can lead to fibrosis and cirrhosis, which in turn increases the risk of hepatocellular carcinoma. The iron overload tends to occur predominantly in the liver parenchyma, where it is more toxic, with Kupffer cells not exhibiting as severe a phenotype in the early stages of the disease (Papanikolaou & Pantopoulos, 2005). Iron deposits in overloaded tissues tend to occur as haemosiderin, a heterogeneous, insoluble iron storage compound thought to contain haem and degraded ferritin (Iancu, 2011). In most cases, haemochromatosis is hereditary and is commonly caused by mutations in
the *HFE* gene (Feder et al, 1996). *Hfe* knockout mice display the same iron overload phenotype as haemochromatosis patients (Zhou et al, 1998). Haemochromatosis patients and *Hfe* knockout mice both also exhibit low hepcidin mRNA expression levels, which are unexpected in cases of iron overload (Bridle et al, 2003). These results support a key role for HFE in the hepcidin regulation pathway. There are numerous other results which also imply that dysregulation of hepcidin expression is a key factor in haemochromatosis. The majority of mutations found in cases of juvenile haemochromatosis, in which severe iron overload is observed early in life, are in *HJV* (sometimes known as *HFE2*), which is also believed to regulate hepcidin levels. *Hjv* knockout mice exhibit increased liver iron loading and decreased hepcidin expression, possibly due to reductions in BMP signalling, as HJV is a co-receptor for BMP ligands (Babitt et al, 2006). Mutations in *TFR2*, which is thought to be an upstream regulator of hepcidin, ferroportin, which is targeted by hepcidin for degradation, and the *HAMP* gene itself are also observed in cases of haemochromatosis (Darshan et al, 2010).

Together, these observations all suggest that inappropriate reductions in hepcidin expression, and consequent increases in membrane ferroportin levels, are a common factor in haemochromatosis. This resulting rise in iron export through FPN causes increased plasma iron concentrations, and hence transferrin becomes saturated. This in turn increases the amount of NTBI in the plasma, which is quickly taken up by a variety of tissues, in particular hepatocytes in the liver through DMT1 (Chua et al, 2004). The excessive uptake of NTBI and transferrin is higher than the rate of iron loss due to increased FPN expression, so the liver will overload with iron.

**Secondary iron overload:**

Secondary iron overload is used to describe range of disorders where the primary iron regulatory pathways remain intact, but iron builds up for alternative reasons (Darshan et al, 2010). It can result from a number of factors, including excessive dietary iron, haemolysis or repeated blood transfusions applied to treat anaemia. Following
multiple transfusions, iron tends to localise primarily to Kupffer cells, although may gradually distribute to hepatocytes as well (Batts, 2007). This overload is more severe when combined with anaemias, such as β-thalassaemia, which also involve defective erythropoiesis and hence have increased production of erythroid regulators such as EPO (Ganz & Nemeth, 2011). As described earlier, erythropoiesis regulators have a suppressive effect on hepcidin expression, so patients with β-thalassaemia will have excessive iron absorption leading to iron overload.
1.7. Polycomb Group Genes:

1.7.1. Introduction – the Polycomb family:

Bmi-1 is a member of the Polycomb group (PcG) family of transcriptional repressors. PcG genes were first discovered in *Drosophila melanogaster* as epigenetic silencers of the *Hox* gene family, preserving cell fate and body patterning decisions in embryogenesis (Ringrose & Paro, 2004). Accumulation of PcG proteins has since been observed at many other chromosomal sites (Simon & Kingston, 2009), indicating that the PcG family represses many other genes in addition to the *Hox* family. Conserved homologues have since been found in vertebrates, with roles in maintaining stable transcriptional repression of a large number of target genes. The PcG proteins in mammals can be broadly classified into 2 primary complexes – Polycomb Repressor Complex (PRC) 1 and PRC2 (Table 1).
<table>
<thead>
<tr>
<th>Drosophila melanogaster</th>
<th>Mouse</th>
<th>Human</th>
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<tr>
<td><strong>PRC2</strong></td>
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<td>Esc (Extra sex combs)</td>
<td>Eed (4 isoforms)</td>
<td>EED (4 isoforms)</td>
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<td>E(z) (Enhancer of zeste)</td>
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<td>Su(z)12 (Suppressor of</td>
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<td>Rbbp4/RbAp48</td>
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<td>Rbbp7/RbAp46</td>
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<td>Pc (Polycomb)</td>
<td>Cbx2/M33</td>
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<td>Ring1B/Rnt2</td>
<td>RING1B/RNF2</td>
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<td>Psc (Posterior sex combs)</td>
<td>Bmi-1</td>
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<td>Mel-18/Rnf110/Pcgf1</td>
<td>MEL-18/RNF110/PCGF2</td>
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<td><strong>Other PcG Genes</strong></td>
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<tr>
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<td>PHF1</td>
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<td>Sfmbt1</td>
<td>SFMBT1</td>
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<tr>
<td>L(3)Mbt</td>
<td>L3Mbt1</td>
<td>L3MBTL1</td>
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**Table 1: Polycomb Repressor Complexes.**

Table of *Drosophila Melanogaster*, Mouse and Human Polycomb family members
1.7.2. PRC complexes:

**PRC2**

PRC2 in mammals consists of 3 core components: Eed, Suz12 and either Ezh1 or Ezh2. These are all essential for embryonic development, as mouse embryos which lack Eed, Ezh2 or Suz12 have gastrulation defects at 7-9 days postcoitus (Faust et al, 1995; O'Carroll et al, 2001; Pasini et al, 2004), and embryonic stem cells which lack Eed or Suz12 are unable to maintain pluripotency in vitro. Ezh2 is a histone methyltransferase (HMTase) which functions as the catalytic subunit of PRC2 (Muller et al, 2002). Ezh1, a homologue of Ezh2, also has HMTase activity (albeit less than Ezh2) and in some contexts can substitute for Ezh2 to form an alternative PRC2 complex, which can repress transcription in vitro and in vivo (Margueron et al, 2008). There may be some mechanistic differences between the complexes, as the Ezh1-PRC2 complex is able to compact polynucleosomes, while the Ezh2 complex cannot. It has been suggested that the Ezh2 containing PRC2 is associated strongly with proliferation, as it is strongly expressed in proliferative cell lines and is associated with markers of DNA replication, such as proliferating cell nuclear antigen (PCNA). Expression of the Ezh1-PRC2 complex is more predominant than Ezh2 in the adult mouse kidney, due to a decline in Ezh2 levels after birth, leading to the suggestion that Ezh1-PRC2 complex is associated more with non-proliferative adult tissues (Margueron et al, 2008). However, this is likely to be tissue specific, as expressed sequence tag based profiling shows large variations in the Ezh1/Ezh2 ratio between different adult tissues (Shen et al, 2008). The Ezh1-PRC2 complex also appears able to partially compensate for the loss of Ezh2 mediated methylation in Ezh2 null embryonic stem cells, as concurrent Ezh1 knockdown leads to the abolition of residual methylation (Shen et al, 2008).
Eed is a scaffold protein which physically links Ezh2 and the histone H3 substrate (Tie et al, 2007). There are four different isoforms of Eed; the largest of which, Eed1, is the predominant component of PRC2 (Kuzmichev et al, 2004). Incorporation of other isoforms leads to the formation of different PRC complexes, which the authors have designated PRC3 and PRC4. These appear to have different histone substrate specificities, which may help contribute to the diversity of polycomb targets (Kuzmichev et al, 2005). Suz12 is required for the HMTase activity of Ezh2 (Cao & Zhang, 2004), and is also involved in the recruitment of two other components, RbAp48/Rbbp4, and Aebp2, which can optimise enzymatic activity in vitro (Pasini et al, 2004).

**PRC1**

In *Drosophila*, PRC1 is formed from four components: Pc (Polycomb), Ph (Polyhomeotic), dRing and Psc (Posterior Sex Combs). In mammals, each of these proteins has two or more homologues (see Table 1), and these associate in various combinations to form a variety of PRC1 complexes. PRC1 is therefore not actually a single defined entity, but a broad term for a number of closely related complexes. The core component of PRC1 is an E3 ubiquitin ligase, Ring1b (also known as Rnf2), which is essential for survival as deletion causes embryonic lethality in mice (Voncken et al, 2003). PRC1 also contains homologues to Pc, Ph and Psc. The exact roles of each of these individual PRC complex members have not been fully elucidated. It has been demonstrated by tandem affinity purification that human PRC1 complexes contain only a single representative of the Psc and Pc homologues (Maertens et al, 2009).
1.7.3. Repression by PcG proteins:

The precise mechanism by which polycomb group proteins repress transcription in mammals is not particularly well understood. The canonical sequential model (Rajasekhar & Begemann, 2007) (Figure 5) contends that PRC2 is recruited to DNA, whereby it initiates transcriptional repression by trimethylating lysine 27 on histone H3 (H3K27me3). In Drosophila, PcG proteins are recruited to collections of binding sites known as Polycomb Repressive Elements (PRE) (Schuettengruber & Cavalli, 2009). These have not been defined in mammals despite numerous mapping studies, which appears to suggest that other molecules, such as transcription factors, may be more important for recruitment. None of the mammalian PRC2 members has a DNA binding sequence, so a PRC2 recruiter molecule must exist. In Drosophila, the Pleiohomeotic (Pho) protein can mediate interactions between PcG proteins and nucleosomes (Klymenko et al, 2006). The mammalian Pho homologue Yy1 is a candidate PRC2 recruiter, as Yy1 knockdown leads to the dislodging of Ezh2 in mouse myoblasts (Caretti et al, 2004). However, the overlap between Yy1 and PRC2 on chromatin in mouse embryonic stem cells is limited, suggesting this is not universal (Squazzo et al, 2006). Other molecules implicated as PcG protein recruiters include the transcription factors Oct4 and Nanog, long non-coding RNAs, and CpG binding proteins, as many PRC2 targets correspond to CpG islands or other CG rich regions (Simon & Kingston, 2009). It is possible that there is not a genome wide method of PcG recruitment, and that the mechanism varies at different targets.
Figure 5: Sequential model of PcG mediated gene silencing.

PRC2 is recruited to active chromatin, and the HMTase Ezh2 trimethylates histone H3 on lysine 27. This mark serves as a recruitment factor for PRC1, which ubiquitinates histone H2A on lysine 119 through the activity of the Ring1 E3 ubiquitin ligase. These modifications result in the long term maintenance of chromatin in a repressed state.

The H3K27me3 mark formed by PRC2 is believed to act in part as a recruitment mark for PRC1 (Simon & Kingston, 2009), which is needed for long term maintenance of repressive conditions. Knockdown of the PRC2 component Suz12 affects the chromatin binding and activity of PRC1 (Cao et al, 2005). There are however some examples of PRC1 accumulation without PRC2, for example recruitment by Xist non-coding RNA in X-inactivation (Schoeftner et al, 2006), demonstrating that independent methods of recruitment also exist. PRC1 is thought to act via monoubiquitination of histone H2A on K119, a modification associated with transcriptional repression (Wang et al, 2004). This is due to the ubiquitin ligase activity of Ring1b, removal of which leads to a large decrease in H2A ubiquitination in HeLa cells (Wang et al, 2004). It is not clear mechanistically how the action of PRC1 leads to long term transcriptional
silencing; theories include DNA polymerase pausing via ubiquitinated H2A, or chromatin compaction (Simon & Kingston, 2009).

The sequential model is likely to be an oversimplification of the actual mechanisms by which Polycomb proteins repress transcription, and more work is required to fully understand the complexities of PcG mediated silencing. This is in part due to the diversity of different PRC1 and PRC2 complexes that can form (Schuettengruber & Cavalli, 2009), which will have both distinct and overlapping functions in the repressive mechanism. One example involves recent *Drosophila* studies which have identified a distinct complex, known as dRAF, which contains dRing and Psc along with a histone lysine demethylase, Kdm2 (Lagarou et al, 2008). The addition of Kdm2 stimulates the H2A ubiquitination activity of dRing and Psc while the addition of Ph and Pc does not, suggesting that dRAF and not PRC1 may be the major ubiquitinating complex in *Drosophila*. dRAF has similarities to the Bcl-6 co-repressor (BCOR) complex found in mammals, which contains PcG proteins Ring1, Ring1b and the Psc homologue Nspc1, as well as another protein, the Kdm2 homologue Fbxl10, which contains a Jumonji C demethylase domain (Gearhart et al, 2006). This suggests there may also be two central members of the PRC1 family in mammals, although the specific functions and targeting of these have not been well defined. Recent work has also implicated the Jumonji C demethylase Jarid2 in the recruitment of PRC2 and modulation of its activity (Pasini et al, 2010; Peng et al, 2009). Another H3K4 demethylase, Rbp2/Jarid1a, can be recruited to PcG targets by PRC2 (Pasini et al, 2008), suggesting that tightly balanced, coordinated methylation and demethylation is important for PcG functions.
1.8. Bmi-1:

Bmi-1, a member of PRC1, was initially identified as an oncogene that cooperates with c-myc in the generation of mouse B cell lymphomas in Eµ-myc transgenic mice (van Lohuizen et al, 1991b). C-myc is a transcription factor that is commonly overexpressed in tumours and has a key role in the development of many human cancers. C-myc has a large collection of target genes and a range of different functions, a few of which include the ability to drive unrestricted cell proliferation, promote cell growth, reduce cell adhesion and, counter-intuitively given its proto-oncogene status, stimulating apoptosis (Adhikary & Eilers, 2005; Zeller et al, 2003). Eµ-myc transgenic mice highly express the c-myc gene specifically in B cells, leading to the development of pre-B cell lymphomas within 12 months (Verbeek et al, 1991). The infection of these mice with Moloney murine leukaemia virus (Mo-MLV) accelerates tumourigenesis, due to integration of the virus into specific sites and the activation of cooperating oncogenes. One of the sites most commonly occupied by the virus was a gene labelled B-cell specific Mo-MLV integration site 1, or Bmi-1 (van Lohuizen et al, 1991b). It was later discovered that Bmi-1 enhances tumourigenesis through inhibition of c-myc induced apoptosis, which represents a fail-safe following aberrant mitogenic signalling (Jacobs et al, 1999b). This is achieved through Bmi-1 mediated repression of the Ink4a/Arf locus (see section 1.9.2), as the presence of the Arf product p19Arf is required for c-myc induced apoptosis.

1.8.1. Function of Bmi-1 within PRC1:

Initial structural analysis of the Bmi-1 protein revealed the presence of motifs characteristic of transcription factors, including an N-terminal zinc finger and helix-turn-helix domain (van Lohuizen et al, 1991a). The protein was classified into the polycomb family of epigenetic repressors due to its sequence similarity with the Drosophila Psc protein. The precise function of Bmi-1 within mammalian polycomb
complexes has not been clearly defined. Bmi-1 is able to make multiple interactions with other PRC1 members, suggesting it may be important for PRC1 complex integrity (Cao et al, 2005). *In vitro* reconstitution of PRC1 complexes has demonstrated that the addition of Bmi-1 stimulates the E3 ubiquitin ligase activity of Ring1b, which is further enhanced by addition of other PRC1 members Ring1a and Cbx8 (Cao et al, 2005). Moreover, knockdown of *Bmi-1* reduces H2A ubiquitination in mouse fibroblasts, suggesting that Bmi-1 is an important contributory factor in PRC1 mediated ubiquitination.

1.8.2. *Bmi-1* knockout mice:

Many of the insights into Bmi-1 function have come from knockout mouse studies, first characterised by Van der Lugt et al (van der Lugt et al, 1994). *Bmi-1* knockout mice appear relatively normal at birth, although an estimated 50% are selectively cannibalised by the mothers. The mice that survive show stunted growth compared to wild type littermates, and survive for a maximum of 3-5 months. Their growth is characterised by the development of severe neurological, haematopoietic and skeletal defects. Neurological abnormalities include an ataxic gait which increases in severity with age, and abnormalities in the cerebellum including massive loss of neurons in the molecular layer. The mice suffer from haematopoietic abnormalities, including an involuted thymus caused by defective thymocyte maturation, smaller spleen and hypoplasia in regions of haematopoiesis, leading to a reduced haematopoietic cell count. These immunological defects lead to functional abnormalities, including a lack of response to interleukin-7, which usually induces pre-B-cell colonies. *Bmi-1* knockout mice exhibit defects in lymphopoiesis and myelopoiesis, but not erythropoiesis, suggesting Bmi-1 is only required within certain lineages of the haematopoietic system. Finally, the mice have skeletal abnormalities and posterior transformations along the anteroposterior axis, similar to those observed in *Hox* mutant mice. This is consistent with a function for Bmi-1 in regulating *Hox* genes, as
the *Drosophila* homologue Psc functions in a similar way. Following on from this initial characterisation, subsequent studies have revealed other defects in mice following *Bmi-1* knockdown. *Bmi-1* deficient mice have inhibited postnatal mammary gland development; in particular they usually lack terminal end buds, which contain stem cells and their progeny, leading to failure of the ductal epithelium to invade the fat pad (Pietersen et al, 2008a). However, these defects can be rescued by pregnancy when *Bmi-1* deficient tissue is transplanted into wild type mice, suggesting that hormones associated with pregnancy are able to overcome the effect of *Bmi-1* deletion. Retinas from *Bmi-1* knockout mice have smaller diameters than wild type littermates despite similar overall eye size, and this is associated with a reduction in progenitor cell number (Chatoo et al, 2010). *Bmi-1* knockout mice also suffer from cataracts and a premature ageing-like ocular phenotype (Chatoo et al, 2009). Examination of *Bmi-1* knockout pancreatic islets revealed a reduction in β-cell proliferation, which is associated with an increase in expression of the cell cycle inhibitor and *Bmi-1* target p16<sup>ink4a</sup> (see section 1.9.2) (Dhawan et al, 2009). This leads to a decrease in overall islet size as *Bmi-1* knockout mice age, with the β-cell mass similar to the wild type mice at birth, but dramatically reduced by 10 weeks of age. This is also associated with abnormal glucose homeostasis; *Bmi-1* knockout mice fail to reduce glucose levels to a baseline following glucose tolerance tests, and do not increase insulin levels in response to glucose injection. The loss of *Bmi-1* also affects the regenerative capacity of pancreatic β-cells, as *Bmi-1* knockout mice do not show any increase in β-cell proliferation or islet regeneration following exposure to streptozotocin, which induces β-cell destruction. Overall, the data from *Bmi-1* knockout mice demonstrates an important role for *Bmi-1* in the postnatal development of several adult tissues.

1.8.3. Regulation of somatic stem cells by Bmi-1:

The neurological and haematopoietic abnormalities of *Bmi-1* knockout mice are thought to be due to the essential function of *Bmi-1* in the self-renewal of stem cells.
Bmi-1 knockout mice are born with normal numbers of foetal liver haematopoietic stem cells (HSCs), however the numbers are dramatically reduced in adult mice due to the absence of self-renewal (Park et al, 2003). Forced overexpression of Bmi-1 in HSCs leads to the promotion of HSC self-renewal and expansion (Iwama et al, 2004) and HSCs from 5 week old Bmi-1 knockout mice are not able to significantly contribute to haematopoiesis following transplantation into an irradiated recipient (Park et al, 2003). Bmi-1 deficiency impairs the self-renewal of stem cells from foetal central nervous system telencephalon and peripheral neural crest stem cells in culture (Molofsky et al, 2003). Furthermore, Bmi-1 deficient mice have reduced frequencies of subventricular zone cells capable of forming neurospheres in culture, with their numbers becoming more depleted with age. Bmi-1 deletion does not significantly affect the colony forming potential of more restricted neural progenitors from the forebrain and gut, suggesting that Bmi-1 is specifically required for stem cell maintenance in these contexts (Molofsky et al, 2003). In contrast, Bmi-1 knockout mice have reduced numbers and expansion of immature granule cell precursors in vivo, leading to reduced cerebellar size (Leung et al, 2004). Immature granule cell precursors are more committed cerebellar precursor cells, so this work demonstrates that the function of Bmi-1 is not always limited to undifferentiated stem cells.

Roles for Bmi-1 in the regulation of stem cells from other tissues have also been elucidated. In the lung, bronchioalveolar stem cells isolated from Bmi-1 knockout mice show defects in proliferation and self-renewal in culture (Dovey et al, 2008), although, the mice themselves do not have obvious abnormalities in lung development. Lineage tracing using a Bmi-1-Cre-ER transgenic mouse, which expresses Cre from the Bmi-1 locus upon addition of tamoxifen, crossed to a strain with a conditional reporter, has identified Bmi-1 expressing stem cells near the bottom of crypts in the small intestine (Sangiorgi & Capecchi, 2008). These cells expand over time and can give rise to all the cell lineages in the intestinal epithelium. The authors then ablated the Bmi-1
positive cells by crossing the \textit{Bmi-1-Cre-ER} mice to mice with a conditional diphtheria
toxin allele expressed by the ubiquitous \textit{Rosa26} locus. The result of this was cell
death and the loss of crypts, demonstrating that the \textit{Bmi-1} expressing cells are
essential for crypt maintenance. A similar technique was used to label \textit{Bmi-1}
expressing cells in the pancreas, with the most abundant labelled population being a
subpopulation of acinar cells (Sangiorgi & Capecchi, 2009). Interestingly, the \textit{Bmi-1}
positive cells do not express markers for undifferentiated pancreatic progenitor cells,
and instead co-localise with markers for differentiated acinar, endothelial and
glucagon positive cells. This appears in contrast to the previously defined role of \textit{Bmi-1}
in the maintenance of undifferentiated adult stem cells. However, analysis of the
\textit{Bmi-1} expressing acinar cells revealed that the lineage persists for at least one year,
and is characterised by an expansion and then contraction of the population of
labelled cells. The \textit{Bmi-1} positive cells also show co-localisation for proliferation
markers, in support of previous work showing acinar cells can proliferate despite their
differentiated state. They also retain a 5’-bromo-2-deoxyuridine (BrdU) label for long
periods, demonstrating that the \textit{Bmi-1} expressing cells are not continuously
replicating, but are maintained until required. Ablation of the \textit{Bmi-1} lineage using
diphtheria toxin leads to an increase in mitosis among the acinar cell population,
demonstrating that the \textit{Bmi-1} expressing cells are not required for acinar cell
proliferation. These data all suggest that \textit{Bmi-1} may have a function in a
subpopulation of more differentiated cells, as well as in undifferentiated stem cells.

This is supported by data showing that \textit{Bmi-1} is expressed at varying levels in all cells
in wild type mouse mammary glands, as opposed to just a minority of stem cells
(Pietersen et al, 2008a). Injection of \textit{Bmi-1} knockout mammary epithelial cell (MEC)
populations, which include multiple cell types, into wild type mice does not result in
any growth. This led the authors to propose that the proliferation of more committed
progenitor cells within the MEC population is also impaired by \textit{Bmi-1} loss, in addition
to mammary stem cells. This would be in contrast to the neural forebrain, where restricted progenitors are unaffected by Bmi-1 depletion (Molofsky et al, 2003), although the authors did not specifically show that this is the case in an isolated population of mammary progenitor cells.

Overall, these data show that Bmi-1 is important for maintenance of self-renewal and proliferation in stem cells in a number of adult tissues, and that the requirement of Bmi-1 in more differentiated progenitor cells varies between different tissue types.

1.8.4. Bmi-1 in tumourigenesis:

Bmi-1 also has an important role in the regulation of cancer stem cells and tumourigenesis, as determined by a number of functional studies. Amongst these, Bmi-1 knockout leukaemic stem cells undergo proliferation arrest and differentiation *in vitro*, and are unable to repopulate secondary hosts, suggesting a key role for Bmi-1 in regulating proliferation of cancer stem cells as well as somatic stem cells (Lessard & Sauvageau, 2003). Bmi-1 deficient neural stem cells also give rise to lower grade gliomas when grafted into mice (Bruggeman et al, 2007). Bmi-1 is also overexpressed in medulloblastomas (Leung et al, 2004), and a crucial role for Bmi-1 in hedgehog-driven medulloblastoma expansion has been suggested due to the failure of medulloblastomas to develop in Bmi-1 knockout mice expressing the oncogenic hedgehog effector, SmoA1 (Michael et al, 2008). Bmi-1 knockout mice also exhibit decreases in lung tumour frequency, when crossed with mice carrying an oncogenic K-ras allele to provide an oncogenic stimulus. This is accompanied by decreased expansion of bronchioalveolar stem cells, which are thought to be the tumour initiating cells in K-ras induced lung tumours (Dovey et al, 2008). Consistent with these studies, Bmi-1 overexpression has been reported in tissue microarrays from several different types of human tumours (Sanchez-Beato et al, 2006). There are also a number of studies showing that high Bmi-1 expression is associated with more aggressive
tumour subtypes and poor long term survival, for example in oligodendroglial tumours (Hayry et al, 2008), non-small cell lung cancers (Vrzalikova et al, 2008) and metastatic melanomas (Mihic-Probst et al, 2007). There is also some work detailing improved survival and less aggressive phenotypes in Bmi-1 overexpressing tumours, including in breast cancers (Pietersen et al, 2008b), squamous cell carcinomas (Hayry et al, 2010) and endometrial carcinomas (Engelsen et al, 2008). These results appear to contradict the functional studies, and suggest that the role of Bmi-1 in tumour progression may be dependent on the context and tissue type of the tumours, although there are doubts over the reliability of anti-Bmi-1 antibodies that are used in many immunohistochemical studies (Sangiorgi & Capecchi, 2009).

1.9. Regulation of the cell cycle by Bmi-1:

The downstream effects of Bmi-1 containing PRC1 complexes are due to transcriptional repression of a variety of targets, in particular cell cycle inhibitors such as p21 and the Ink4a/Arf locus, which encodes p16Ink4a and p19Arf. This section will introduce these proteins and their functions, and describe the evidence implicating Bmi-1 as a regulator of their expression.

1.9.1. Introduction to the cell cycle:

Proliferating cells proceed through the cell cycle in a highly regulated manner (Schafer, 1998; Yu et al, 2007) (Figure 6). Cells which are not actively cycling but have the potential to enter the cell cycle, such as the majority of hepatocytes, are termed as being in G0 phase. Cells that are cycling will proceed through G1 phase, S phase (DNA synthesis), G2 phase and M phase (mitosis). Cell cycle progression is tightly regulated, and is catalysed by complexes containing cyclin dependent kinases (Cdk) and their regulatory subunits, cyclins. The formation of Cdk-cyclin complexes is a requirement for Cdk activation and nuclear targeting. CdkS control cell cycle progression by phosphorylating and inactivating target substrates, such as
retinoblastoma (Rb), which controls entry into S-phase via the release of E2F transcription factors. Initial Rb phosphorylation occurs in mid G₁ by cyclin D family dependent kinases, this is enhanced later in G₁ by the Cdk2/cyclin E complex and maintained later in the cell cycle by cyclin A and B dependent Cdk5s. Numerous other molecules are also involved in cell cycle regulation, including the tumour suppressor gene p53 and the INK4 and WAF1/CIP/KIP families of Cdk inhibitors. The roles of p53 include the arrest of cells at the G₁/S and G₂/M checkpoints following DNA damage, achieved by activating a number of downstream targets, including the Cdk inhibitor p21\(^{Cip1/Waf1}\) (henceforth referred to as p21).

**Figure 6: The mammalian cell cycle.**

Simplified schematic showing the stages of the mammalian cell cycle and selected key cell cycle regulatory proteins.
1.9.2. The *Ink4a/Arf* locus

The *Ink4a/Arf* locus (also known as *Cdkn2a* locus) encodes the tumour suppressor proteins p16\(^{ink4a}\) and p19\(^{Arf}\) (p14\(^{Arf}\) in humans). The two proteins have distinct first exons spliced to a common second, however this is translated in an alternative reading frame so there is no sequence similarity between the two proteins (Gil & Peters, 2006; Kim & Sharpless, 2006) (Figure 7). An adjacent gene, *Cdkn2b*, encodes another tumour suppressor, p15\(^{ink4b}\). Both p15\(^{ink4b}\) and p16\(^{ink4a}\) are members of the INK4 family of Cdk inhibitors, which also includes p18\(^{ink4c}\) and p19\(^{ink4d}\) (a distinct protein from p19\(^{Arf}\)). All the INK4 proteins are highly conserved at the amino acid level and there appears to be very little biochemical difference between them (Sharpless, 2005). It is likely that functional differences in the proteins occur through differences in their expression patterns. For example, p18\(^{ink4c}\) and p19\(^{ink4d}\) appear to have a role in development, as they are widely expressed during mouse embryogenesis, while p16\(^{ink4a}\) and p15\(^{ink4b}\) are not (Zindy et al, 1997). p16\(^{ink4a}\) is associated with tumour suppression and senescence, as *p16^{ink4a}* knockout mice (which will also delete *p19^{Arf}* develop normally, but many spontaneously generate tumours by 29 weeks of age (Serrano et al, 1996). The INK4 members all act by blocking progression through the G1 phase of the cell cycle, specifically by inhibiting the activity of kinases that bind cyclin D (Cdk4 or Cdk6), but do not affect the functions of other Cdk/cyclin complexes (Serrano et al, 1993) (Hirai et al, 1995). INK4 proteins bind directly to Cdk4 or Cdk6 and induce a structural change, prevent the binding of the Cdns to cyclin D proteins (Russo et al, 1998). This is turn prevents Cdk4/6 mediated phosphorylation of Rb, leading to binding of E2Fs to hypophosphorylated Rb, and G\(_1\) arrest (Kim & Sharpless, 2006).

The *Arf* product does not share any homology with p16\(^{ink4a}\) as the proteins are encoded by alternative reading frames, and hence it exerts its tumour suppressor function via a different mechanism involving regulation of the murine double minute 2
Mdm2 - p53 pathway. Mdm2 is an oncogenic protein which binds to p53 and controls its function primarily through promoting p53 polyubiquitination and proteasomal degradation (Michael & Oren, 2003). Arf proteins are able to interact with Mdm2 and inhibit its activity, thus preventing degradation of p53 (Pomerantz et al, 1998).

Figure 7: The murine Ink4a/Arf locus.
Schematic showing the Ink4a/Arf (Cdkn2a) and adjacent Ink4b (Cdkn2b) loci, gene products and downstream targets.

Functions of p16\textsuperscript{Ink4a} and p19\textsuperscript{Arf}

p16\textsuperscript{Ink4a} and p19\textsuperscript{Arf} have both been implicated as biomarkers of ageing in rodents, as expression of Ink4a/Arf is dramatically increased in most tissues in older mice (26 months compared to 2.5 months), while other cell cycle inhibitors do not exhibit significant changes (Krishnamurthy et al, 2004). Levels of p16\textsuperscript{Ink4a} in haematopoietic stem cells increase as mice age, leading to decreased proliferation and more limited repopulating ability following bone marrow transplants (Janzen et al, 2006). These defects are rescued in p16\textsuperscript{Ink4a} null cells, suggesting a role for p16\textsuperscript{Ink4a} in stem cell
ageing. Age related increases in p16ink4a expression linked to decreased stem/progenitor cell function are also found in other cell types, including pancreatic islet cells, which have decreased regenerative potential (Krishnamurthy et al, 2006), and neural forebrain progenitors (Molofsky et al, 2006). Fibroblasts from Ink4a/Arf knockout mice do not senesce when explanted into culture, unlike normal cells, revealing a role for p16ink4a and p19Arf in senescence (Serrano et al, 1996). Mice with individual deletions of p16ink4a or p19Arf both have increased susceptibility to tumour formation, suggesting that both proteins are involved in mediating the tumour suppressive function of the Ink4a/Arf locus, although mice doubly deficient for p16ink4a and p19Arf show more dramatic tumour formation than the individual knockouts (Sharpless et al, 2004). In human tumours, mutations in INK4A/ARF are found frequently. Mutations of p16INK4A appear to be particularly important and have been observed in a large number of tumours (Forbes et al, 2006), including a number of cases that do not also mutate p14ARF or the adjacent INK4B locus (Kim & Sharpless, 2006). Although loss of p19Arf alone is oncogenic in mice, mutations in the p14ARF gene in human tumours that do not also affect p16ink4a are quite rare. This appears to suggest that p16ink4a is a more important tumour suppressor than p14Arf in humans, although some have pointed out that only a small portion of the N-terminus of p14Arf is actually required for the interaction with Mdm2, so mutations that actually disable p14Arf function would be less likely (Kim & Sharpless, 2006).

The Ink4a/Arf locus as a Bmi-1 target:

Some of the effects of Bmi-1 are thought to be mediated through repression of the Ink4a/Arf locus, as p16ink4a and p19Arf are upregulated in Bmi-1 null fibroblasts and lymphocytes, and removal of Ink4a/Arf partially rescues some of the haematopoietic and neural defects in Bmi-1 knockout mice. In particular, the Bmi-1−/−Ink4a−/− mice have normal cerebellar size and cell numbers, normal marker profiles on splenocytes and thymocytes (Jacobs et al, 1999a), and restored HSC self-renewal capacity (Oguro et
Ink4a/Arf deletion also rescues the mammary epithelial growth defects found in Bmi-1 knockout mice (Pietersen et al, 2008a). Chromatin immunoprecipitation (ChIP) analysis has revealed that Bmi-1 binds directly throughout the Ink4a/Arf locus in human and mouse cells (Bracken et al, 2007). The presence of PRC2 is required for the repressive effect, and Bmi-1 is displaced from the Ink4a/Arf locus in senescent cells following Ezh2 downregulation. Recent ChIP studies have demonstrated the existence of a particular region of the p16\textsuperscript{ink4a} promoter, labelled a Bmi-1 responding element (BRE), to which Bmi-1 directly binds (Meng et al, 2010). Interestingly, the authors found that Ring1b/Rnf2 is not recruited to this site in the p16\textsuperscript{ink4a} promoter, and that Ring1b overexpression does not affect the activity of a luciferase reporter linked to the p16\textsuperscript{ink4a} promoter sequence. This suggests that Bmi-1 mediated repression of p16\textsuperscript{ink4a} may be independent of the canonical Ring1b-containing PRC1.

There appears to be some species and cell-type specificity in Bmi-1 repression of the Ink4a/Arf promoter, with variability in binding of Bmi-1 to the p14\textsuperscript{Arf} promoter observed in human cells. Repression of Arf following Bmi-1 overexpression is found in cultured mouse embryonic fibroblasts, but not in human embryonic fibroblasts (Bracken et al, 2007). In contrast, increases in polycomb associated methylation of histone H3 are found throughout INK4A/ARF in human CD34+ bone marrow cells, with an associated loss of both p16\textsuperscript{ink4a} and p14\textsuperscript{Arf} expression (Bracken et al, 2007). The effects of cell culture on expression of p16\textsuperscript{ink4a} and p14/19\textsuperscript{Arf} has to be taken into account when analysing in vitro studies, backed up by the fact that the previous authors observed loss of Bmi-1 and the H3K27me3 mark from the Ink4a/Arf locus in mouse embryonic fibroblasts after three passages. However, in vivo data also demonstrates different effects following deletion of p16\textsuperscript{ink4a} versus p19\textsuperscript{Arf} in Bmi-1 knockout mice (Bruggeman et al, 2005). The p19\textsuperscript{Arf}/p53 pathway appears to be a general target of Bmi-1 repression in mice, as deletion of p19\textsuperscript{Arf} leads to partial rescue.
of a number of Bmi-1 knockout phenotypes in lymphoid cells and neural stem/progenitor cells. Loss of p16^{ink4a} alone does not rescue the phenotypes of some types of Bmi-1 deficient cells, including splenocytes, mouse embryonic fibroblasts and cerebellar granule neuron progenitors (Bruggeman et al, 2005). However, p16^{ink4a} deletion is required to completely alleviate defects in self-renewal and/or proliferation in Bmi-1 knockout neural stem cells, other neural progenitors and thymocytes, suggesting Bmi-1 mediated p16^{ink4a} repression is required in some cell types in mice. These data suggest that the contributions of p16^{ink4a} and p19^{Arf} repression in relaying Bmi-1 functions vary according to the cellular context.

1.9.3. p21:

p21, encoded by the Cdkn1a gene, is a member of the WAF1/Cip/Kip family of cell cycle inhibitors, which also includes p27^{Kip1} and p57^{Kip2}. It was initially discovered by multiple laboratories due to its interaction with Cdk2/cyclin E complexes, and was identified as a Cdk2 inhibitor (Weinberg & Denning, 2002). Further research revealed that p21 is induced in response to p53 expression, and can suppress the growth of tumour cells in culture (el-Deiry et al, 1993). p21 is an essential mediator of p53 dependent cell cycle arrest, as mice deficient for p21 show defects in p53 mediated G1 arrest in response to γ-radiation (Deng et al, 1995). It is not required for all aspects of p53 function and is also activated by several p53 independent mechanisms (Jung et al, 2010). The early research all marked p21 as purely a cell cycle inhibitor, however subsequent findings have shown that it is actually involved in multiple levels of cell cycle control, and can have both oncogenic and tumour suppressive effects.

Function of p21 in cell cycle progression:

An increase in p21 expression has generally been associated with growth arrest and terminal differentiation (Jung et al, 2010). Mice with a complete knockout of p21 develop normally, suggesting p21 is not required for normal differentiation (Deng et al,
1995), although it is possible that other family members may be able to compensate. However, p21 null embryonic fibroblasts isolated from these mice have deficiencies in G₁ arrest, supporting a role for p21 in regulating the G₁ checkpoint. p21 is able to inhibit the activity of different Cdk/cyclin complexes in \textit{in vitro} reconstitution assays, and its overexpression inhibits the proliferation of mammalian cells (Xiong et al, 1993). It appears that the inhibitory effects of p21 are mediated in particular through inhibition of complexes containing cdk2 and cyclin E or cyclin A. Cdk2/cyclin E complexes which contain p21 have been shown to be catalytically active when p21 is subsaturating, but become inactive as the levels of p21 are increased (Zhang et al, 1994). However, p21 also appears to have positive as well as negative effects on G₁ progression. Concurrent transfection of p21 or p27 with cyclin D1 and Cdk4 into cultured cells actually promotes formation of the Cdk4/cyclin D1 complexes, as determined by an immunoprecipitation-western assay (LaBaer et al, 1997). The authors observed very little complex assembly without p21 or p27 present. The complexes also have increased kinase activity at basal p21 levels, although this decreases when p21 is expressed at much higher levels. Moreover, another study has shown that mouse embryonic fibroblasts lacking p21 and p27 fail to assemble detectable Cdk/cyclin D complexes, and cannot direct cyclin D proteins to the nucleus (Cheng et al, 1999). Transient transfection of moderate levels of p27 into smooth muscle cells also causes an increase in proliferation (Kavurma & Khachigian, 2003). Taken together, these results demonstrate a dual role for p21 in both promoting assembly of active Cdk4/cyclin D complexes, but inhibiting their activity at higher stoichiometries. The sequestering of p21 and p27 into Cdk4/cyclin D complexes is also thought to help to relieve their inhibition of Cdk2/cyclin E complexes, thus facilitating cell cycle progression (Sherr & Roberts, 1999). In addition to its role as a cell cycle regulator, p21 has been reported to have both pro- and anti-apoptotic functions, which are likely to depend on the cellular context (Jung et al, 2010).
p21 in tumourigenesis:

p21 was originally considered a tumour suppressor, due to its anti-proliferative effects and regulation by p53. In the liver, loss of p21 accelerates hepatocarcinogenesis in the fah-/ mouse model (Willenbring et al, 2008), suggesting a tumour suppressor function for p21 in hepatic cells in this context. However, unlike p53 knockout mice, mice deficient in p21 have low levels of spontaneous tumour formation in the short term (Deng et al, 1995), although increased tumour incidence has been reported over a 2 year time frame (Martin-Caballero et al, 2001). Mutations in p21 are also uncommon in most human cancers (Weinberg & Denning, 2002), suggesting that p21 is not a general mediator of tumour suppression, in contrast to p53. Given the reported status of p21 as a cell cycle inhibitor, the preliminary expectation is that p21 levels would be decreased rather than overexpressed in tumours. However, this is not always the case. Overexpression of p21 has been noted in various squamous cell carcinomas, including in head and neck cancers (Erber et al, 1997; van Oijen et al, 1998) and also in breast cancers (Rey et al, 1998). The expression appears independent of functional p53, and in many cases correlates with expression of Ki67, leading to the conclusion that p21 overexpression is found in actively proliferating cells as well as differentiated cells (Erber et al, 1997). This suggests that the growth inhibitory function of p21 may be bypassed in some tumour cells. Van Oijen et al found that nuclear p21 expression in proliferating tumour cells usually occurs concurrently with overexpression of cyclin D1 in the same region (van Oijen et al, 1998), although this was not specifically demonstrated in a single cell. A correlation between expression of p21, cyclin D1 and the percentage of cells in S-phase is also found in breast cancers (Rey et al, 1998). These findings led van Oijen et al to suggest that cyclin D1 expression could overcome the anti-proliferative effects of excess p21 expression, which could possibly be due to the sequestering of excess p21 by cyclin D1 containing complexes. This theory has support from other studies.
showing a sequestering effect of cyclin D family members on p21. Russell et al demonstrated that elevated levels of cyclin D3 can sequester overexpressed p21 away from Cdk4/cyclin D1 and Cdk2 containing complexes in MCF-7 breast cancer cells, hence increasing the Cdk activity (Russell et al, 1999). They also found elevated p21 levels in cyclin D1 and cyclin D3 positive breast cancer samples. In addition to this, c-myc activation in cell lines leads to an increase in protein synthesis of cyclins D1 and D2, which in turn leads to increased binding of the cyclins to p27 and p21 (Perez-Roger et al, 1999). This proliferative effect of c-myc activation does not occur in cells from cyclin D1 or D2 null mouse embryos, showing that the sequestering function of cyclin D proteins is essential for c-myc induced cell cycle progression.

**Functions of p21 - summary:**

In summary, a major role of p21 appears to be as a negative regulator of the cell cycle through inhibition of Cdk/cyclin complexes. However, it can also have pro-growth functions, including its role as an assembly factor for Cdk4/cyclin D1 complexes, and as an inhibitor of apoptosis. It also seems to have both tumour-suppressive and oncogenic capabilities in different contexts. Which of these contradictory functions occurs seems to depend on a variety of factors, including the stoichiometry of the p21 protein in relation to other cell cycle regulatory molecules, the subcellular localisation of p21, and the cell system used in the study.

**p21 as a Bmi-1 target:**

The requirement of Bmi-1 for adult stem cell maintenance may be in part related to repression of the effects of activated Ink4a/Arf, however Bmi-1 must also regulate other genes, as deletion of Ink4a/Arf does not completely abrogate the phenotype of the Bmi-1 knockout mouse. For example, these double knockout mice still exhibit defects in the bone marrow microenvironment (Oguro et al, 2006), have only partial rescue of splenocyte and thymocyte numbers (Jacobs et al, 1999a), and do not show
improved growth or survival compared to Bmi-1 knockout mice (Molofsky et al, 2005). One other potential Bmi-1 target is p21. The knockdown of Bmi-1 in neural progenitor cells in vitro and in vivo causes rapid upregulation of p21, while p16\textsuperscript{ink4a} and p19\textsuperscript{Arf} remain relatively unaffected (Fasano et al, 2007). The anti-proliferative effect observed in the Bmi-1 depleted cells is rescued by co-transfection of a small hairpin RNA (shRNA) against p21, confirming that p21 is an important target in this system. It is possible in some cases that alterations in p21 expression following Bmi-1 knockdown are mediated through p19\textsuperscript{Arf} activation, as increased p19\textsuperscript{Arf} protein leads to the inhibition of Mdm2 activity and the stabilisation of p53, which in turn will cause increased transcription of p21. However the fact that transfection of shRNAs against Ink4a/Arf does not rescue the phenotype of Bmi-1 depleted neural progenitor cells (Fasano et al, 2007) suggests that p21 is a Bmi-1 target independent of repression of the Cdkn2a locus. This has since been demonstrated by ChIP studies showing that Bmi-1 directly binds the p21 promoter in cells isolated from wild type mouse cerebellum (Subkhankulova et al, 2010).

1.9.4. Other Bmi-1 targets:

In addition to the previously described role of Bmi-1 in repressing the expression of cell cycle inhibitors and Hox genes, there have been other reported targets for Bmi-1. Overexpression of BMI-1 in human mammary epithelial cells leads to immortalisation and an increase in telomerase activity; reporter assays confirmed that this is due to transcriptional activation of human telomerase reverse transcriptase (hTERT) (Dimri et al, 2002). hTERT is the catalytic subunit of telomerase, which also contains a telomerase RNA component (TERC). Telomerase is required particularly in germ cells and stem cells to prevent senescence by maintaining the length of telomeres, which are short repetitive sequences at the ends of chromosomes which shorten with each cell cycle. Telomerase activation is also therefore a common occurrence in tumour cells. The finding of a potential role for Bmi-1 in telomerase activation would be
consistent with the established Bmi-1 functions in promoting stem cell self-renewal and preventing senescence, and with its frequent upregulation in tumours. However, the authors also found that Bmi-1 does not induce telomerase in human fibroblasts, suggesting the function is cell type specific.

Bracken et al performed expression array analysis of human embryonic fibroblasts depleted for either of three PRC2 members or BMI-1, and identified over 300 potential polycomb targets based on expression changes (Bracken et al, 2006). Subsequently, ChIP-on-chip analysis using antibodies against PRC2 and PRC1 proteins, or the H3K27me3 mark validated many of these, and genome wide promoter array studies extended the number of potential Polycomb target genes to over 1000. These include genes involved in several key pathways in development and differentiation, including the Wnt, Notch, Hedgehog, transforming growth factor, and fibroblast growth factor signalling pathways, amongst others, although it has not specifically been demonstrated that Bmi-1-containing complexes regulate all these genes. Meng et al have since performed analysis of the total precipitated DNA content from a ChIP experiment using an anti-Bmi-1 antibody in HeLa cells (Meng et al, 2010). This revealed over 1500 genes potentially regulated by Bmi-1, including genes involved in the cell cycle, apoptosis and tissue specific development. These results are suggestive of a broader and more complex role for Bmi-1 in regulating a much more diverse array of cellular processes than simply progression of the cell cycle.

1.9.5. Bmi-1 in mitochondrial function and the DNA damage response

Recent work has also implicated Bmi-1 in the DNA damage response. Bone thymocytes isolated from Bmi-1 knockout mice have increased levels of reactive oxygen species (ROS), which are linked to impaired mitochondrial function (Liu et al, 2009). This is coupled with upregulation of components of the DNA damage response (DDR), including the protein kinase Chk2 which is involved in cell cycle arrest. Both
ROS-scavenging antioxidants and Chk2 deletion can partially rescue many of the defects in thymus and spleen found in Bmi-1 knockout mice, as well improve the colony forming ability of Bmi-1 null haematopoietic stem cells. The authors note that activation of the DDR has been implicated in stem/progenitor cell ageing and senescence, so the observation that Bmi-1 may have a role in regulating the DDR is consistent with the established role for Bmi-1 in stem cell maintenance. Bmi-1 is also recruited to DNA damage foci in HeLa cells, and Bmi-1 depleted cell lines have impaired homologous recombination-mediated repair of double strand breaks (Ginjala et al, 2011). Furthermore, both cell lines and primary primitive haematopoietic cells depleted for Bmi-1 show increased sensitivity to clastogenic agents, leading to spontaneous chromosome breakage and instability (Chagraoui et al, 2011). Bmi-1 knockout mice also die within 3 weeks of whole body irradiation, suggesting increased in vivo susceptibility to DNA damage. This is independent of increased ROS levels, as antioxidant treatment does not rescue the phenotype. Together, these studies suggest a new role for Bmi-1 in the maintenance of chromosome integrity.

1.10. Mel-18:

The Mel-18 gene product is structurally very similar to Bmi-1, sharing 93% homology in the N-terminal RING-finger and helix-turn-helix domains, and overall amino acid identity of 58% (Ishida et al, 1993), suggesting possible redundancy between the two proteins. Individual Mel-18 knockout mice show stunted growth, severe immunodeficiency and posterior transformations virtually identical to the Bmi-1 knockout mouse (Akasaka et al, 1996). They also exhibit reductions in both lymphoid progenitor numbers and their mitotic response to interleukin-7 (Akasaka et al, 1997) and reduced expansion of immature T progenitor cells (Miyazaki et al, 2005). These observations would appear to suggest that Bmi-1 and Mel-18 act in synergy in some cases. However, Mel-18 null mice do not show any of the cerebellar abnormalities seen in the Bmi-1 knockout, and have additional defects in the smooth muscle of the
colon. These unique phenotypes suggest some differences in the functions of Bmi-1 and Mel-18, despite their strong homology. Mice doubly deficient for Mel-18 and Bmi-1 die at embryonic day 9.5 with severe morphological abnormalities, including more severe skeletal transformations than in single knockout mice (Akasaka et al, 2001). Tandem affinity purification of Bmi-1 and Mel-18 shows they are found in nearly identical core complexes, interacting with other known PRC1 members, but never with each other (Wiederschain et al, 2007). In addition to this, simultaneous binding of both Mel-18-PRC1 and Bmi-1-PRC1 complexes has been observed at the INK4A/ARF locus in human fibroblasts (Maertens et al, 2009). Furthermore, shRNA mediated knockdown of either BMI-1 or MEL-18 leads to a reduction in the binding of the other protein at the INK4A/ARF locus. This suggests that binding of both complexes may be interdependent, and supports the idea that the complexes act synergistically in some cases. There do appear to be functional differences between the two complexes, as addition of Mel-18 is able to stabilise PRC1, but does not increase the E3 ubiquitin ligase activity of Ring1b, unlike Bmi-1 (Cao et al, 2005).

1.10.1. Functions of Mel-18:

Mel-18 as a tumour suppressor:

The exact role of Mel-18 in cell cycle regulation and tumour progression is a matter of debate. There is a body of evidence suggesting that Mel-18 functions as a tumour suppressor, in contrast to Bmi-1. Mel-18 was initially identified as a transcriptional negative regulator, with the recombinant protein able to directly bind to a specific DNA motif (Kanno et al, 1995). NIH 3T3 cells transfected with antisense MEL-18 RNA acquire tumourigenicity, suggesting Mel-18 has tumour suppressive activity. Bmi-1 and Mel-18 expression are inversely correlated in breast cancer cell lines and breast tumours, with the majority of samples having high Bmi-1 and low Mel-18, suggesting that the proteins have opposing roles (Guo et al, 2007b). This inverse correlation has
been noted in gastric cancers (Zhang et al, 2010), with increased Bmi-1 and low Mel-18 again associated with tumour progression (Lu et al, 2010). Low Mel-18 expression also correlates with poor survival in breast cancers (Guo et al, 2010) and prostate cancer (Wang et al, 2009). Primitive murine haematopoietic cells and human bone marrow cells express either Mel-18 or Bmi-1, but rarely both (Kajiume et al, 2009), again suggesting different functions within this context. This is supported by small interfering RNA (siRNA) knockdown experiments showing that the colony forming ability of putative murine haematopoietic stem cells is reduced by loss of Bmi-1, but increased by knockdown of Mel-18 (Kajiume et al, 2009). Isolated B cells from transgenic mice overexpressing Mel-18 exhibit suppressed proliferation combined with cell cycle arrest between G1 and S phase, suggesting that Mel-18 is a cell cycle inhibitor (Tetsu et al, 1998). This phenotype is accompanied by the downregulation of various proteins involved in the cell cycle, including c-myc, cyclins G2 and E, and the activities of Cdk 4&6. The B cell proliferation defects in the Mel-18 overexpressing mice are rescued by crossing the mice to c-myc overexpressing transgenic mice, identifying c-myc as an important mediator of Mel-18 cell cycle repression. Co-transfection of a Mel-18 plasmid with a reporter plasmid containing the upstream promoter of c-myc leads to a decrease in expression of the reporter, further supporting the view that c-myc is a downstream target of Mel-18. This repression does not occur when deletion mutants of the c-myc reporter are used, suggesting that Mel-18 directly binds the c-myc promoter in mice.

These results are supported by a thorough analysis of MEL-18 overexpression in human cells (Guo et al, 2007a). The authors found that overexpression of MEL-18 in human fibroblasts leads to inhibition of proliferation and cellular senescence, the opposite of the phenotype following Bmi-1 overexpression. The MEL-18 overexpressing cells exhibit downregulation of c-myc expression, but strikingly also a decrease in Bmi-1 protein and mRNA expression, and upregulation of p16INK4a.
Conversely, shRNA mediated *MEL-18* knockdown results in upregulation of Bmi-1. Further analysis revealed that the *BMI-1* promoter contains a binding site for c-myc, but not Mel-18, and use of ChIP showed binding of c-myc to this region. Reporter assays for *BMI-1* promoter activity demonstrate that c-myc can positively regulate *BMI-1* expression, and that Mel-18 negatively regulates *BMI-1* via repression of c-myc. However, addition of siRNAs against *Mel-18* to murine bone marrow cells does not affect expression of *Bmi-1* (Kajiume et al, 2009), suggesting that this effect is not universal.

Further work in human breast cancer cells has demonstrated that the repression of *BMI-1* by Mel-18 is accompanied by a reduction in Akt activity, as measured by levels of phosphorylated Akt, and also downregulation of cyclin D1, which can be activated by the Akt pathway (Guo et al, 2007b). Work by Lee et al, also in human breast cancer cells, has shown that Mel-18 mediated proliferation arrest occurs through downregulation of Cdk2 and Cdk4 activities (Lee et al, 2008). This is due to reductions in cyclin D1 expression and a phosphorylation mediated shift in the binding of p27 from Cdk4 to Cdk2, where it acts to inhibit cell cycle expression. These changes are mediated through Akt signalling without alterations in expression of *INK4A/ARF*, demonstrating that Mel-18 can also regulate the cell cycle independently of *INK4A/ARF*. The results obtained using the breast cancer model were supported by work in gastric cancer cell lines, which again show that Mel-18 can decrease Bmi-1 expression, leading to increased *p16\(^{\text{ink4a}}\) levels, a reduction in phosphorylated Akt, and a reduced transformation phenotype (Zhang et al, 2010). There is also evidence from a yeast two hybrid screen that Mel-18 can bind directly to cyclin D2, and may block its activity, as *Mel-18* knockdown causes increased proliferation in hamster ovarian cells (Chun et al, 2005). Overall, these studies suggest that Bmi-1 and Mel-18 have opposing functions, with senescent cells showing high Mel-18 expression and proliferating cells high Bmi-1 expression.
Mel-18 as an oncogene

Contrary to these results, a study by Wiederschain et al reports that overexpression of Bmi-1 or Mel-18 in Rat1 fibroblasts increases proliferation, with an enhanced phenotype observed when both are overexpressed together (Wiederschain et al, 2007). Different human cancer cell lines show varying sensitivities to shRNA mediated knockdown of BMI-1 or MEL-18. Some lines, such as U2OS human osteosarcoma, show decreased proliferation and colony formation only when both proteins are knocked down in combination. Others, including DAOY medulloblastoma and MCF7 breast cancer cells, are sensitive to individual knockdown of either BMI-1 or MEL-18, with a stronger phenotype observed when both are concurrently eliminated.

Furthermore, downregulation of either BMI-1 or MEL-18 suppresses growth of human medulloblastoma xenografts in vivo, suggesting both have oncogenic functions. Transcriptional profiling also shows a significant overlap in genes regulated by Bmi-1 and Mel-18 in medulloblastoma, supporting the idea of complementary functions for the two proteins in this context. It appears that Mel-18 can also repress transcription of p16\textsuperscript{ink4a}, as mouse embryonic fibroblasts overexpress p16\textsuperscript{ink4a} mRNA following Mel-18 knockdown (Jacobs et al, 1999a). p16\textsuperscript{ink4a} derepression without changes in BMI-1 expression is seen following MEL-18 knockdown in human fibroblasts (Maertens et al, 2009), with the authors also showing that Mel-18 binds to the INK4A/ARF locus with a similar pattern to Bmi-1. There is also evidence from tissue microarray immunolabelling that Mel-18 is upregulated in some types of human tumour samples compared to normal tissue, including in hepatocellular carcinoma (Sanchez-Beato et al, 2006).

Summary of Mel-18/Bmi-1 relationship:

In summary, the work described above suggests that the oncogenic capacity of Mel-18 and functional redundancy with Bmi-1 can differ in different cellular contexts. The
high levels of homology between Bmi-1 and Mel-18 would appear to suggest similar functions, and this appears to be the case in some contexts, supported by the fact that the knockout mice display many overlapping phenotypes. However, other work has clearly demonstrated that Mel-18 can act as an inhibitor of proliferation in a variety of tumour cell types. It is possible that the Mel-18 and Bmi-1 function similarly in normal development, but contribute to cancer growth and survival differently. It seems there may be multiple methods by which Mel-18 can regulate the cell cycle and tumour progression; including by acting to repress transcription of genes such as *Ink4a/Arf* as part of a polycomb complex analogous to Bmi-1, and by downregulating Bmi-1 expression via repression of c-myc. It is not clear which signalling pathways or cellular contexts causes these seemingly contradictory functions of Mel-18 to occur, although they may be dependent on the level of c-myc expression. Given that the tumour suppressor function of Mel-18 seems in part to be due repression of Bmi-1 via c-myc, it is possible that Mel-18 will be unable to perform this role in tumour cells with overexpressed c-myc.

1.1. Other Bmi-1 homologues:

In addition to Mel-18, two other Bmi-1 homologues have been characterised in mammals: Mel-18-and-Bmi-1-like Ring finger protein (MBLR) and Nervous System Polycomb 1 (Nspc1). MBLR acts a transcriptional repressor when transfected into cultured cells and can directly interact with Ring1b/Rnf2 (Akasaka et al, 2002). Nspc1 expression was initially discovered in neural crest structures in mouse embryos (Nunes et al, 2001) and also has transcriptional repressive activity (Gong et al, 2005). It is able to repress *p21* transcription *in vivo* (Gong et al, 2006) and can cooperate with Ezh2 and DNA methyltransferase 1 in repressing *Hox* genes via H2A ubiquitination (Wu et al, 2008). It is also part of the mammalian BCOR ubiquitinating complex (discussed in section 1.7.3), which may be an important source of PcG mediated ubiquitination. Any functional relationship or redundancy between these
proteins and Bmi-1 has not yet been established. At least two other proteins homologous to Bmi-1 exist; Pcgf3 and Pcgf5 (Sauvageau & Sauvageau, 2010), however these have not been characterised.

1.12. Polycomb genes and the liver:

1.12.1 Polycomb genes and liver stem cells:

There has not been a large amount of investigation into the role of Bmi-1 and other polycomb genes in the liver, with functional studies limited primarily to in vitro work. A key study was carried out by Chiba et al, who examined the role of Bmi-1 in a putative stem/progenitor cell fraction derived from foetal murine liver (Chiba et al, 2007). These cells were sorted based on expression of five markers (c-kit-, CD29+, CD49f+, CD45-, Ter-119-), and previously showed increased enrichment of hepatic colony forming units in culture (H-CFU-C) (Suzuki et al, 2000). Overexpression of Bmi-1 in these cells results in larger colonies, and an increased number of albumin and CK7 double-positive cells, which suggests bilineage potential. Conversely, shRNA mediated Bmi-1 knockdown causes a four-fold decrease in the number of colonies. These effects do not occur in fractions which lack H-CFU-C capacity, suggesting a specific role for Bmi-1 in liver stem cells. Putative stem cells transduced with Bmi-1 demonstrate increased colony growth in soft agar and form tumour nodules with combined CC-HCC characteristics in immunodeficient mice, suggesting that enhanced self-renewal due to Bmi-1 overexpression drives transformation.

Later studies examined the role of Bmi-1 in hepatic stem cells using foetal liver cells positive for delta like protein (Dlk), a marker of early hepatic stem/progenitor cells. Chiba et al found a higher expression of Bmi-1 in Dlk+ cells than in Dlk- fractions, however were still able to isolate comparable numbers of Dlk+ cells from the foetal livers of Bmi-1 knockout mice, indicating that Bmi-1 is not required for development of the hepatic components of foetal liver (Chiba et al, 2010). In culture, loss of Bmi-1 has
a small effect on the size but not number of colonies formed from Dlk+ cells, with the
effect significant after 14 days in culture. This effect appears to be smaller than in the
authors' previous work on shRNA mediated knockdown for Bmi-1 in a hepatic
stem/progenitor cell fraction (Chiba et al, 2007). More dramatic is the reduction in the
number of albumin/cytokeratin 7 (CK7) double positive cells in colonies obtained from
Bmi-1 null Dlk+ cells. These double positive cells are thought to represent hepatic
progenitor cells due to their ability to give rise to hepatocyte and cholangiocyte
lineages. The proportion of Dlk+ cells in Bmi-1 deficient colonies also declines at a
greater rate than in wild type colonies, suggesting a progressive loss of stem cells.
The Bmi-1 null Dlk+ cells do however maintain some ability to give rise to albumin
positive (hepatocyte) and CK7 positive (cholangiocyte) lineages, which suggests that
Bmi-1 does play a role in the maintenance of stem cells in foetal liver, but is not
critical. It is important to underline that these studies were carried out in cells from
foetal liver. Bmi-1 knockout mice appear relatively normal at birth, and only exhibit
stronger phenotypes in other organs postnatally (van der Lugt et al, 1994). It is
possible that more dramatic effects may be observed in adult liver cells, in line with
what is observed in the haematopoietic system (Park et al, 2003). However, Chiba et
al also found that Bmi-1 knockout mice treated with a DDC diet show a reduction in
oval cell numbers, but not a complete loss, suggesting Bmi-1 may not be critical in
adult liver stem cells either.

Bmi-1 knockout Dlk+ cells have increased expression of p16Ink4a and p19Arf, and ChIP
assays in wild type cells have demonstrated that Bmi-1 directly binds the Ink4a/Arf
locus in liver progenitor cells (Chiba et al, 2010). Ink4a/Arf null Dlk+ cells have both
increased colony forming ability and increased numbers of bipotent albumin and CK7
double positive cells compared to wild types. Dlk+ cells with concurrent knockdown of
both Bmi-1 and Ink4a/Arf show small but significant decreases in colony sizes and
numbers of bipotent cells compared to cells deficient in Ink4a/Arf alone. The colony
sizes and bipotent cell numbers appear much larger than in Bmi-1/- cells, suggesting that deletion of Ink4a/Arf largely, but not completely, rescues the defects found in Bmi-1 knockout Dlk+ cells. This suggests that the Ink4a/Arf locus is a major Bmi-1 target in liver progenitor cells. However, overexpression of Bmi-1 in Ink4a/Arf null Dlk+ cells increases colony size, but not number nor the frequency of bipotent cells. These Bmi-1 overexpressing cells are also able to form tumours when transplanted into non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, unlike Ink4a/Arf null cells without transduction of Bmi-1. These results indicate that while Ink4a/Arf is a Bmi-1 target in liver progenitor cells, it is not the only one, and its derepression is not responsible for the tumourigenic potential of Bmi-1. The authors then performed gene expression analysis on Bmi-1 transduced Ink4a/Arf null cells to identify a number of other targets. Gene ontology analysis revealed that many downregulated genes are involved in metabolism or transport, and include genes involved in hepatocyte maturation, suggesting Bmi-1 suppresses hepatocyte maturation and differentiation. Many upregulated genes are associated with the maintenance of stemness features, migration and development. In particular, the authors found that Bmi-1 binds directly to the promoter of Sox17 (sex determining region Y-box 17), suggesting a novel target for Bmi-1 in hepatic stem cells. In support of this, overexpression of Sox17 impairs the colony forming ability of wild type Dlk+ cells, and transduction of Sox17 suppresses the tumourigenic capacity of Bmi-1 overexpressing Ink4a/Arf null Dlk+ cells. Sox17 is a Wnt antagonist expressed in the early endoderm, suggesting activation of the Wnt pathway may mediate some effects of Bmi-1.

Another paper from the same group looked at the effect of the PRC2 member Ezh2 on Dlk+ hepatic stem/progenitor cells (Aoki et al, 2010). Ezh2 knockdown in Dlk+ cells causes a slight decrease in total colony numbers and a more pronounced drop in the number of large colonies. This phenotype is similar to that found in Bmi-1 knockdown
cells, but more dramatic. Ezh2 depletion also appears to induce a shift towards hepatocyte differentiation, as indicated by an increase in the number of albumin positive cells. As with Bmi-1, ChIP studies demonstrated that Ezh2 binds directly to the Ink4a/Arf locus. Knockdown of Ezh2 in Ink4a/Arf null Dlk+ cells partially abrogates the colony forming defects found in Ezh2 depleted cells, however this is smaller than for Bmi-1 knockdown. This suggests that other targets exist for Ezh2 in hepatic stem/progenitor cells.

Overall, these data indicate that Bmi-1, along with Ezh2, has a role in regulating liver stem cells and tumourigenesis, similar to its function in several other tissues. This is achieved in part through repression of the Ink4a/Arf locus, however other targets also exist. The effect of Bmi-1 knockdown on hepatic stem/progenitor cells appears generally to be less dramatic than that found in other tissues, such as the haematopoietic system.

1.12.2. Polycomb genes in HCC and hepatocytes:

Bmi-1 overexpression was initially reported in HCC using cDNA microarray analysis to identify suitable markers for HCC diagnosis (Neo et al, 2004). Wang et al have since found high expression of Bmi-1 in 29.9% of HCC specimens examined using immunohistochemistry, with more positive staining in well differentiated tumours (Wang et al, 2008). They also observed that high Bmi-1 expression is a prognostic factor for poor overall survival. This seems unexpected, given that well differentiated tumours are generally thought to be less aggressive. However, they report cytoplasmic Bmi-1 staining, whereas the expectation based on other studies and the role of Bmi-1 as an epigenetic regulator is for nuclear localisation. Some of the data are backed up by western blotting, but it is not specified how many samples this was performed on. Nuclear Bmi-1 expression has been observed in several HCC cell lines (Effendi et al, 2010), and the authors also found overexpression in HCC clinical
samples, in particular in well differentiated and early HCC. This is correlated with overexpression of ATP binding cassette transporter B1 (ABCB1), a protein reported to be expressed on stem cells and cells displaying resistance to anti-cancer drugs, although this would appear to suggest a worse prognosis. Another study reports nuclear expression of both Bmi-1 and the PRC2 member Ezh2 in HCC and combined hepatocellular-cholangiocellular carcinoma (HC-CC) (Sasaki et al, 2008a). The authors found Bmi-1 staining in most cells within the CC component of HC-CC, and heterogeneous staining within HCC samples. The expression pattern of Ezh2 is similar, but appears in a smaller number of cells to Bmi-1. Both proteins show higher expression in poorly differentiated tumours, in contrast to the results of Effendi et al and Wang et al described previously. Expression of Bmi-1, and in particular Ezh2, is associated with both increased proliferation, as indicated by cyclin A expression, and vascular invasion. siRNA mediated knockdown of BMI-1 and EZH2 also leads to a decrease in proliferation of the HCC cell lines HepG2 and Huh7. A correlation between high Bmi-1 and high Ezh2 expression has also been reported independently (Yonemitsu et al, 2009). In this case, no correlation between expression of Bmi-1 or Ezh2 and survival or Ki67 expression was found. Another study has also found increased Ezh2 mRNA expression in HCC samples, correlating with increased portal vein invasion but not survival rate (Sudo et al, 2005).

It has also been observed that Bmi-1 is preferentially expressed in side population (SP) cells from the HCC cell lines Huh7 and PLC/PRF/5 (Chiba et al, 2008). SP cells are characterised by the ability to efflux Hoechst 33342 dye, which is thought to represent stem cell behaviour, so are believed to represent minor populations with cancer stem cell ability. BMI-1 knockdown in both HCC cell lines decreases the number of SP cells, while stable overexpression of BMI-1 in Huh7 leads to an increased SP. BMI-1 transduced SP cells show increased tumourigenicity when xenografted into NOD/SCID mice, while BMI-1 knockdown has the opposite effect.
This appears to be via INK4A/ARF, as mRNA levels of the gene products increase in BMI-1 depleted PLC/PRF/5 cells. Another study has also implicated Bmi-1 as an oncogene in HCC independent of INK4A/ARF (Xu et al, 2009). The authors again found upregulation of BMI-1 mRNA in HCC samples, but did not see expression changes in INK4A/ARF. They also found that BMI-1 knockdown leads to growth inhibition of three human HCC cell lines (SK-Hep1, Huh7, Hep3B), with INK4A/ARF expression again unaffected in any of these lines. This result is not relevant in Huh7 and SK-Hep1, which have either a deletion (SK-Hep1) or strong promoter methylation (Huh7) of INK4A/ARF, but appears to suggest Bmi-1 acts independently of INK4A/ARF in Hep3B. The authors then established a mouse model to examine the role of Bmi-1 in hepatocarcinogenesis in vivo. Transgenic mice stably overexpressing either Bmi-1 or RasV12 (an activated form of N-Ras) alone in hepatocytes did not show enhanced tumour development, however co-expression of both induced tumours in the majority of animals. This demonstrates that Bmi-1 and RasV12 can cooperate to form liver tumours in vivo. Surprisingly, mRNA expression of p16\textsuperscript{Ink4a} and p19\textsuperscript{Arf} was also increased in the tumours. This seems inconsistent with the established role of Bmi-1 in repressing the Ink4a/Arf locus and with the proliferative phenotype of the tumours. It is unclear why this would be the case, as there is no previous literature suggesting that Bmi-1 overexpression can lead to increased expression of Ink4a/Arf. The increase in Ink4a/Arf is not due to RasV12, as adenovirus mediated expression of RasV12 in hepatocytes has little effect on Ink4a/Arf levels. Examination of the effects of V5 tagged Bmi-1 overexpression in primary hepatocytes also revealed little effect on cell senescence, levels of p16\textsuperscript{Ink4a} protein or p19\textsuperscript{Arf} mRNA, although p16\textsuperscript{Ink4a} mRNA was upregulated. The reasons for this were not established, although the expression level of V5 tagged Bmi-1 appears very low compared to endogenous Bmi-1, so it is unclear how much effect it would really have. In summary, this study suggests that the function of Bmi-1 in hepatocytes and liver tumourigenesis is independent of Ink4a/Arf repression. This does not appear
universal to all hepatic tumour cells, as Chiba et al report increased mRNA levels of $p16^{\text{Ink4a}}$ and $p14^{\text{ARF}}$ in PLC/PRF/5 cells depleted of Bmi-1 (Chiba et al, 2008).

1.12.3. Polycomb genes in CC and biliary diseases:

Bmi-1 expression has been reported within normal cholangiocytes in both mouse and human livers. Examination of liver tissue from a Bmi-1-GFP-knock-in mouse showed strong GFP tagged Bmi-1 expression in the portal region, and possible weaker expression within the parenchyma (Hosen et al, 2007). Sasaki et al observed Bmi-1 expression in biliary cells in human livers (Sasaki et al, 2006). They also noted that decreased Bmi-1 expression is associated with damaged bile ducts in human livers with primary biliary cirrhosis (PBC), an autoimmune disease leading to bile duct loss, cholestasis and liver failure. This appears to be related to oxidative stress, a candidate cause of senescence in PBC cells, as oxidative stress causes a decrease in Bmi-1 expression in mouse biliary epithelial cells (mBECs). Loss of Bmi-1 was also associated with an increase in expression of $p16^{\text{Ink4a}}$ in PBC cells in vivo, suggesting that Bmi-1 regulates $p16^{\text{Ink4a}}$ expression in cholangiocytes, in contrast to what has been reported in hepatocytes. In agreement with this, Bmi-1 knockdown in mBECs results in decreased proliferation, increased senescence and increased $p16^{\text{Ink4a}}$ expression. In contrast, knockdown of Ezh2 in mBECs slightly reduces nuclear Bmi-1 expression, but has no effect on proliferation or senescence.

Constant Bmi-1 expression has been noted in biliary epithelia of both non-neoplastic and neoplastic tissue, and invasive CC (Sasaki et al, 2008b). However, similar to HCCs, increasing Ezh2 expression characterises more invasive tumours, with expression progressively increasing from low grade biliary dysplasias up to invasive carcinomas. This correlates with a decrease in $p16^{\text{Ink4a}}$ protein levels and increase in promoter methylation, suggesting that the overexpression of Ezh2 contributes to a drop in $p16^{\text{Ink4a}}$ in multi-step carcinogenesis. This was supported by in vitro data
demonstrating that knockdown of \textit{EZH2} in CC cell lines decreases both \textit{p16^{INK4A}} promoter methylation and Ezh2 binding. The same group has later reported that \textit{BMI-1} mRNA is upregulated in intrahepatic cholangiocarcinoma compared to non-tumour liver samples (Sasaki et al, 2009). This comparison seems unlikely to be particularly meaningful, as the non-tumour samples will consist mainly of hepatocytes, while CC is thought to be derived from biliary cells. There does not appear to be any conclusive data on whether Bmi-1 is overexpressed in CC cells when compared to normal cholangiocytes. The authors also observed that \textit{BMI-1} knockdown in CC cell lines leads to decreased colony formation, decreased proliferation and increased senescence, suggesting that Bmi-1 may have a function in CC progression. However, there are no data on whether the loss of Bmi-1 would also affect these parameters in normal cholangiocytes. Given that Bmi-1 is expressed in normal cholangiocytes, it seems logical that it would also be found in CC samples, and hence may not be specifically relevant to the progression of the disease.

\textbf{1.12.4. Summary - Bmi-1 in liver tumours:}

In conclusion, the data seem to suggest roles for Bmi-1 and Ezh2 in malignant progression of both HCC and CC, as their overexpression has been found in several liver cancers, and both tumour cell lines and putative stem cell fractions transfected with Bmi-1 have increased tumourigenicity when transplanted into mice. Bmi-1 overexpression has been associated with poorly differentiated tumour cells, increased proliferation and invasion; although other work has marked Bmi-1 overexpression as a factor in well differentiated cells and the early stages of hepatocarcinogenesis, which appears contradictory. A general problem with many studies is that they are based almost entirely on immunohistochemical analysis. Inconsistencies in the staining pattern observed using commercially available anti-Bmi-1 antibodies have been reported in pancreatic tissue (Sangiorgi & Capecchi, 2009), making it difficult to assess how robust many of these studies are. The validity of Bmi-1 and Ezh2
overexpression as prognostic factors for long term survival is therefore not clear. Functional data in mice suggests overexpressed Bmi-1 alone cannot induce tumours \textit{in vivo}, but that the presence of an activated form of Ras is also required.
1.13. Aims:

There is growing acknowledgement of the importance of understanding the role of epigenetic regulators, as they can impact on multiple signalling pathways. From the evidence summarised in the introduction, it can be concluded that Bmi-1 has an important role in adult stem cell maintenance in vitro. Following on from this, defects of varying severity have been reported in virtually all murine Bmi-1 knockout tissues examined. Hepatic cell populations appear to exhibit impairments in proliferation and self-renewal in vitro following Bmi-1 depletion, along the same lines as those observed in cells derived from other organs. Despite this fact, there has been no detailed assessment of the function of Bmi-1 in hepatic tissue in vivo. Only in vivo models permit assessment of tissue development, disease progression and wound healing. The work detailed in this thesis aims to address this issue with regard to the following objectives:

Objectives: To investigate the role of Bmi-1 in hepatic growth and function in vivo.

1. To examine the evidence for Bmi-1 expression in rodent and human hepatic tissue, under homeostatic conditions and during perturbed tissue growth.

2. To assess the effect of Bmi-1 deletion on postnatal murine liver structure, function and development in vivo.

The results section of the thesis is presented in four chapters which address the above objectives as follows:
Objective 1:

Chapter 3: Expression of Bmi-1 in normal liver and regenerating tissue

As a precursor to functional *in vivo* studies, it was first considered important to ascertain the precise expression pattern of Bmi-1 in murine liver, as immunohistochemical studies thus far have predominantly examined Bmi-1 expression in human hepatic tissue. Bmi-1 expression was also assessed in murine oval cells and human cirrhotic liver tissue, to investigate whether Bmi-1 is likely to have a function in hepatic repair, and in human tumour samples.

Objective 2:

Chapter 4: Liver structure and function in *Bmi-1* knockout mice

*Bmi-1* deletion in mice leads to structural and functional defects in a variety of adult organs, including in neural tissue, the haematopoietic system, mammary glands and pancreatic islets. However, there has been no investigation into abnormalities in the livers of *Bmi-1* knockout mice. This chapter examines liver structure and function in both newborn and adult *Bmi-1* knockout mice. In particular, abnormalities in hepatocyte size in *Bmi-1* knockout mice were indicative of impairment in the postnatal developmental process of polyploidisation. The expression of other Polycomb group genes in *Bmi-1* knockout livers was also examined to determine whether functional redundancy could be a contributory or compensatory factor impacting the phenotype.

Chapter 5: The effect of *Bmi-1* deletion on the cell cycle in murine liver

*Bmi-1* depletion leads to proliferative defects in putative hepatic stem/progenitor cells populations and liver tumour cells *in vitro*. Impaired proliferation is also observed in other tissues from *Bmi-1* knockout mice. This is due in part to the fact that the most well characterised targets of Bmi-1 are cell cycle inhibitors, in particular those encoded by the *Ink4a/Arf* locus. This chapter attempts to investigate the proliferative
ability of hepatocytes and cholangiocytes following $Bmi-1$ deletion $in$ $vivo$, and to correlate this phenotype with expression changes of key cell cycle regulators.

Chapter 6: Iron overload in $Bmi-1$ knockout mice

Observations in hepatic tissue from $Bmi-1$ knockout mice suggested the possibility that the mice were suffering from iron overload, a phenotype which has not previously been described in any other $Bmi-1$ deficient cell populations $in$ $vitro$ or $in$ $vivo$. This chapter provides preliminary analysis of this phenotype, and assesses expression changes in key members of iron regulatory pathways.
Chapter 2

Materials and Methods
2. Materials and Methods:

2.1. Mice and animal procedures:

2.1.1. Generation of Bmi-1 knockout mice:

*Bmi-1 +/-* mice generated on a FVB/N background (van der Lught et al, 1994) (mice provided by Professor Silvia Marino) were intercrossed to generate knockout offspring (*Bmi-1 -/-*) together with wild type control littermates (*Bmi-1 +/-*).

2.1.2. Animal husbandry and Home Office compliance:

All mice were bred, maintained and experimentally treated in accordance with the Animal (Scientific Procedures Act) 1986. Mice were housed in pathogen free facilities located at Queen Mary, University of London that have been licensed by the Home Office.

I have completed modules 1-4 of the Home Office training course and hold a personal license (PIL 70/24337). All animal procedures were performed under project licenses PPL 70/7166 and PPL 70/6452.

2.1.3. BrdU injections:

To be able to detect cells that were going through S-phase of the cell cycle, 5-bromo-2-deoxyuridine (BrdU) (Sigma) was injected into the intraperitoneal cavity at 100 mg BrdU per kg body weight. Mice were killed by a Schedule 1 method and tissues were collected approximately 1 hour after BrdU injection.

The BrdU stock (10 mg/ml) was made by dissolving BrdU powder in phosphate buffered saline (PBS) (Sigma, made by adding 1 PBS tablet per 200 ml distilled water), which was then sterile filtered, aliquotted and stored at -20°C.


2.2. Serum collection and analysis:

2.2.1. Serum collection:

Blood samples were collected by cardiac puncture using a 25G needle from Bmi-1 wild type and knockout mice anaesthetised with carbon dioxide. All injections were performed by staff at the animal facility. The samples were placed immediately on ice in 1.5 ml microcentrifuge tubes and left for approximately 20 min to allow clotting to occur. The tubes were then centrifuged at 3000 rpm for 15 min, and the serum (supernatant) was removed and placed in a separate tube for storage. The samples were stored at -80°C until required for analysis.

2.2.2. Serum analysis:

Serum samples were sent to the Royal Veterinary College Diagnostic Laboratories for analysis of serum iron, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP) and total bilirubin.

2.3. Genotyping of Bmi-1 knockout mice:

2.3.1. Isolation and preparation of DNA:

Small ear snips were taken from newly weaned mice litters at approximately 3 weeks of age. The samples were digested in 50 µl ear buffer (1 M Tris-HCl pH 8.0, 2 M NaCl, 10% (wt/vol) sodium dodecyl sulphate (SDS), 10 mg/ml Proteinase K (Sigma)) at 55°C for a total of 3 h, with vortexing after 1 h. The samples were diluted with 150 µl of distilled water, and heated at 95°C for 10 min to inactivate Proteinase K. Digested samples were stored at -20°C until required for polymerase chain reaction (PCR) analysis.
2.3.2. Genotyping PCR:

Digested ear snip DNA samples were subjected to PCR analyses using the following reagents and conditions:

2.3.2.1. Primers

Genotyping primers were designed by Dr Danielle Lavery and purchased from Sigma-Aldrich. Primer stocks were diluted to a final concentration of 5 µM in Nuclease Free Water (Ambion).

Wild type forward: CTGCCAATGGCTCCAATGAAGAC

Wild type reverse: CAAACTCCTAACACAACTTGGC

Wild type expected product size: 323 bp

Knockout forward: CTCTCGATGAGCTGATGCTTTG

Knockout reverse: GAGTACTTCTACACAGCCATCG

Knockout expected product size: 446 bp

2.3.2.2. PCR reaction mix

2 µl DNA sample

2 µl Wild type forward and reverse primer mix (stock concentration 5 µM)

2 µl Knockout forward and reverse primer mix (stock concentration 5 µM)

2.5 µl 10x reaction buffer (supplied with enzyme)

0.5 µl 10 mM dNTP mix (Invitrogen)

1.25 µl magnesium chloride (supplied with enzyme)

0.1 µl Platinum Pfx DNA Polymerase (Invitrogen)
14.65 µl ddH$_2$O

2.3.2.3. Cycling conditions

1) 94°C, 4 min

2) 94°C, 30 sec

3) 60°C, 30 sec

4) 72°C, 1 min

5) To step 2, 39 times

6) 4°C forever

2.3.3. DNA gel electrophoresis:

A 2% (wt/vol) agarose gel was prepared by dissolving agarose (Fisher) in 1x TAE buffer (diluted from 50x stock; 2 M Tris, 1 M Acetic acid, 0.05 M EDTA, pH 8.3) in a microwave. Ethidium bromide (Sigma, final concentration 0.4 µg/ml) was added to the gel mixture following cooling, and the gel was poured and left to set. A 10 µl sample of the completed PCR reaction was added to 1µl of TrackIt Cyan/Orange Loading Buffer (Invitrogen) diluted 50% with glycerol, and the samples were loaded onto the gel. Sizes of the amplified fragments were elucidated by including a lane loaded with 5 µl of 1 Kb Plus DNA ladder (Invitrogen). TAE (1x) was used as the running buffer for electrophoresis at 100 V. DNA fragments were visualised with an ultraviolet transilluminator (Uvitec), and the gel was photographed.
2.4. Histology and Immunohistochemistry:

2.4.1. Tissue collection and processing:

2.4.1.1. Human liver samples

Paraffin-embedded human tumour-adjacent normal liver and cirrhotic liver tissue blocks were provided from a tissue bank maintained by my second supervisor Professor Malcolm Alison under the Human Tissues Act (Ethics: 06/Q0604/40). Paraffin-embedded human liver tissue arrays comprising a panel of hepatocellular carcinoma (Grades I,II,III), cholangiocarcinoma (Grades I,II,III), normal and tumour-adjacent samples, and cirrhotic livers were purchased from US Biomax, Inc (Product codes LV242, T032, LV805, LV8011, BC03119).

2.4.1.2. Mouse liver samples

Freshly collected liver samples from Bmi-1 wild type and knockout mice killed using schedule 1 methods were immediately placed in neutral buffered formalin (NBF; 3.7% formaldehyde, 0.03 M sodium phosphate monobasic anhydrous, 0.05 M sodium phosphate dibasic anhydrous). Samples were fixed for a minimum of 24 h in NBF, and transferred to 70% ethanol for storage prior to tissue processing and paraffin embedding, which was carried out by the Blizard Institute Pathology Core Facility. To assess Bmi-1 expression in murine oval cells, archival paraffin blocks containing livers collected from albumin-urokinase type plasminogen activator (AL-uPA) transgenic mice (Braun et al, 2003) were obtained from Eric Sandgren (University of Wisconsin-Madison). Blocks from mice treated with dipin (Braun & Sandgren, 2000) were also obtained from Eric Sandgren. Tissue sections were subsequently cut from paraffin blocks at a depth of 5 µM onto Superfrost Plus microscope slides (VWR) for use in immunostaining procedures. Some section cutting was carried out by the Blizard Institute Pathology Core Facility.
2.4.2. Histological staining procedures:

The following stains were carried out on paraffin-embedded liver sections from Bmi-1 wild type and knockout mice by the Blizard Institute Pathology Core Facility: Haematoxylin and Eosin (H&E), Picro Sirius Red, Perls Prussian Blue.

2.4.2.1. Cell counting in Bmi-1 wild type and knockout mouse sections

The number of hepatocyte nuclei in a 40x microscope field was recorded for 5 randomly photographed fields in each H&E stained mouse liver section. Results were averaged and assessed for significant differences using a 2-tailed Student’s t-test, with significance recognised at p<0.05.

2.4.3. Primary antibodies used for immunohistochemistry:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Species</th>
<th>Company</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmi-1</td>
<td>Mouse/human Bmi-1</td>
<td>Mouse monoclonal</td>
<td>Millipore 05-637</td>
<td>1:100</td>
</tr>
<tr>
<td>Mel-18 (H-115)</td>
<td>Human/mouse Mel-18</td>
<td>Rabbit polyclonal</td>
<td>Santa Cruz SC-10744</td>
<td>1:100</td>
</tr>
<tr>
<td>Troma3</td>
<td>Mouse cytokeratin 19</td>
<td>Rat monoclonal</td>
<td>Cancer Research UK</td>
<td>1:25</td>
</tr>
<tr>
<td>BrdU</td>
<td>BrdU</td>
<td>Rat monoclonal</td>
<td>Oxford Biotech 0030</td>
<td>1.100</td>
</tr>
<tr>
<td>Ki67</td>
<td>Human/mouse Ki67</td>
<td>Rabbit polyclonal</td>
<td>Novacastra NCL-Ki67p</td>
<td>1.1000</td>
</tr>
<tr>
<td>p16 (M-156)</td>
<td>Mouse p16H44</td>
<td>Rabbit polyclonal</td>
<td>Santa Cruz SC-1207</td>
<td>1.1000</td>
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<tr>
<td>p21</td>
<td>Human/mouse p21</td>
<td>Rabbit polyclonal</td>
<td>Abcam ab7960</td>
<td>1.750</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>Human/mouse cyclin D1</td>
<td>Rabbit monoclonal</td>
<td>Cell Signaling 2978</td>
<td>1.50</td>
</tr>
<tr>
<td>Mouse IgG1 isotype control</td>
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<td>Mouse monoclonal</td>
<td>Millipore CBL600</td>
<td>Same as primary being tested</td>
</tr>
<tr>
<td>Rabbit IgG isotype control</td>
<td>n/a</td>
<td>Rabbit monoclonal</td>
<td>Cell Signaling 3900</td>
<td>Same as primary being tested</td>
</tr>
<tr>
<td>A6</td>
<td>Mouse oval cells</td>
<td>Mouse monoclonal</td>
<td>Valentina Factor, NIH</td>
<td>1.25</td>
</tr>
</tbody>
</table>
2.4.4. Immunoperoxidase methods:

2.4.4.1. Deparaffination and rehydration

Slides containing paraffin sections were dewaxed and rehydrated by incubation through a xylene and graded ethanol (EtOH) series:

Dewaxing:

Xylene 1 (5 min)
Xylene 2 (5 min)
100% EtOH 1 (3 min)
100% EtOH 2 (3 min)
90% EtOH (3 min)
75% EtOH (3 min)
Distilled water (3 min)

2.4.4.2. Antigen retrieval

Antigen unmasking was carried out by boiling slides in 10 mM tri-sodium citrate pH 6.0 with 0.1% Tween20. The citrate solution was pre-heated in a microwave, after which the slide rack was added and the slides heated for 15-20 min. The slide container was left to cool for 20 min at room temperature, and the slides were then rinsed in phosphate buffered saline containing 0.1% Tween20 (PBST). Endogenous peroxidases were then quenched by incubation in 3% hydrogen peroxide in methanol for 10 min.
2.4.4.3. Single antigen labelling

Slides were blocked for 1 hour using blocking solutions from the mouse-on-mouse (M.O.M.) kit (Vector Laboratories) for anti-Bmi-1 antibody, or normal serum from the appropriate species-specific Vectastain Elite ABC kit (Vector Laboratories) for other antibodies, as per the manufacturer’s instructions. Primary antibodies were diluted in blocking solution and incubated overnight at 4°C, and slides were subsequently washed in PBST for 3x 5 min. Slides were then incubated with either Vector Universal ImmPress reagent (Vector Laboratories, for anti-Bmi-1 antibody only), or secondary antibody and subsequent ABC reagents supplied with the appropriate mouse, rabbit or rat Vectastain ABC kit (Vector Laboratories), diluted in PBS and normal serum as per the manufacturer’s instructions. Following further washes in PBST (3x 5 min), Diaminobenzidine (DAB) (Vector Laboratories) was used to visualise the specific binding. The DAB staining was timed and monitored under a microscope, and the slides were placed in distilled water when the staining intensity reached a suitable level. The same DAB incubation time was used for each slide examined. Slides were counterstained in haematoxylin for approximately 5 sec and placed in distilled water prior to mounting.

2.4.4.4. Mounting and examination

Dehydration was carried out by immersing slides through graded ethanol and xylene solutions, in the reverse of the procedure described in section 2.4.4.1, and slides were mounted in DPX mounting medium (Sigma). Slides were examined and photographed using a Leica DM 5000 B microscope with a Leica DFC 490 camera. Sections with species matched isotype control antibodies substituted for the primary antibody were also included in all staining procedures. No staining was observed on slides incubated without primary antibody or with isotype control antibody in all experiments included in this thesis.
2.4.4.5. Double labelling of Bmi-1 and CK19

Double antigen labelling for Bmi-1 and CK19 was achieved by sequentially staining slides for the two antigens. The single staining procedure was performed for the anti-Bmi-1 antibody as described in section 2.4.4.3. Prior to haematoxylin counterstaining, slides were subjected to a second antigen retrieval step (boiling in tri-sodium citrate pH 6.0 for 10 min) and blocked for 20 min using normal serum from the Vectastain Elite ABC-AP Rat IgG kit (Vector Laboratories). Sections were incubated with anti-CK19 antibody diluted in blocking serum for 1 h at room temperature. Specific binding was visualised using the Vectastain ABC-AP kit, as per the manufacturer’s instructions, followed by incubation with BCIP/NBT substrate (Roche) diluted 1:50 in NTMT buffer (100 mM Tris-HCl pH 8.5, 100 mM NaCl, 50 mM MgCl2, 0.1% Tween 20) until staining was observed. Slides were counterstained using nuclear fast red (Sigma), dehydrated and mounted in DPX.

2.4.4.6. BrdU staining

Paraffin sections from blocks obtained from BrdU injected mice were deparaffinised and hydrated through xylenes and graded alcohols, and placed in PBST for 5 min. The slides were then microwaved in tri-sodium citrate solution for 10 min as described in 3.4.4.2, cooled at room temperature for 20 min and rinsed in PBST. Slides were then incubated in pre-warmed 4N HCl for 30 min at 37°C, and subsequently washed in borate buffer pH 7.6 (85% 0.2 M boric acid, 15% 0.05 M sodium diborate) for 3 min. Sections were digested in 0.01% trypsin (PAA) in 0.05 M Tris-HCl pH 7.5 for 3 min at 37°C and washed in dH2O. Endogenous peroxidases were quenched using 3% hydrogen peroxide in methanol for 10 min, and slides were blocked in normal serum for 20 min. Sections were incubated in anti-BrdU primary antibody diluted in blocking serum overnight at 4°C, and specific binding was visualised using the Vectastain Elite ABC Rat IgG kit (Vector Laboratories) and DAB as per the manufacturer’s
instructions. Slides were counterstained in haematoxylin for 5 sec, dehydrated through graded alcohols and mounted in DPX.

2.4.5. Quantification of BrdU, Ki67 and Cyclin D1 positive nuclei:

Slides containing mouse liver tissue from completed BrdU, Ki67 or Cyclin D1 staining procedures were labelled blindly, and 5 randomly selected images were captured per slide at 40x magnification. The numbers of nuclei positive for BrdU or Ki67 were recorded for each image, and the mean of all images was calculated to obtain an average per mouse. These were divided by the total number of hepatocyte nuclei per field for that mouse (calculated as described in section 2.4.2.1) to obtain a value for the percentage of cells expressing BrdU or Ki67. Significance was assessed at p<0.05 using a 2-tailed student’s t-test.

2.4.6. Quantification of Bmi-1 expression in human tumour and cirrhotic microarrays:

Human tissue microarrays containing cores from patients with liver pathologies (see section 2.4.1.1.) were stained for Bmi-1 as described in section 2.4.4.3. All individual cores were photographed, and the images were blindly labelled. All images were examined and scored as either positive or negative for Bmi-1, in both hepatocytes and cholangiocytes if applicable. Significant differences between wild type and tumour/cirrhotic samples were assessed using a chi-squared test (http://people.ku.edu/~preacher/chisq/chisq.htm), with significance recognised at p<0.05.

2.4.7. Detection of mitochondrial mutations:

2.4.7.1. Frozen sections

Freshly excised mouse livers were embedded in O.C.T. (Fisher) on dry ice. Frozen sections (thickness 10 µm) were cut using an OTF5000 cryostat (Bright Instrument Co Ltd) and stored at -80°C until required.
2.4.7.2. Solutions for COX and SDH staining

All solutions for cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) histochemistry were prepared in 0.2M phosphate buffer pH 7.0:

19.5 ml 0.2 M Sodium phosphate dibasic (Sigma)
30.5 ml 0.2 M Sodium phosphate monobasic (Sigma)

Stored at -20°C

500 µM cytochrome c:
0.031 g cytochrome c (Sigma) in 5 ml phosphate buffer

Stored at -20°C in 250 µl aliquots

5 µM diaminobenzidine (DAB):
0.0288 g DAB (Sigma) in 16 ml phosphate buffer

Stored at -20°C in 800 µl aliquots

1.875 mM Nitro blue tetrazolium (NBT):
0.0245 g NBT (Sigma) in 16 ml phosphate buffer

Stored at -20°C in 800 µl aliquots

1.3 M Sodium succinate:
2.11 g sodium succinate (Sigma) in 6 ml phosphate buffer

Stored at -20°C in 300 µl aliquots
2 mM Phenazine methosulphate (PMS):

1.84 mg PMS (Sigma) in 3 ml phosphate buffer

Stored at -20°C in 150 µl aliquots, kept away from light

2.4.7.3. COX/SDH Staining Protocol

Frozen liver sections were removed from a -80°C freezer and thawed at room temperature for approximately 30 min. COX staining solution was prepared by adding 200 µl of cytochrome c solution to 800 µl of DAB solution. Slides were incubated in COX staining solution for 15 min at 37°C. Sections were washed twice in PBST, and incubated in SDH staining solution (800 µl NBT solution, 100 µl sodium succinate solution, 100 µl PMS solution) for approximately 30 min at 37°C. The staining intensity was periodically monitored under a microscope to ensure the SDH counterstain did not mask the COX staining. Slides were then washed twice in PBS, dehydrated through graded alcohols (described in section 2.4.4.4.) and mounted in DPX.

2.5. Measurement of hepatocyte size parameters using touch preparations:

2.5.1. Touch preparations:

*Bmi-1* wild type and knockout mice were killed using schedule 1 methods, and the livers excised. Small pieces of liver (approx 5-10 mm wide) were placed in a solution of 5 mg collagenase type I (Lorne Laboratories) dissolved in 10 ml distilled water, and incubated at 37°C for 20 min. The liver pieces were then removed from the collagenase and placed into a petri dish. A scalpel was used to cut through a liver segment, and the freshly cut face was immediately touched firmly against a Superfrost Plus microscope slide (VWR). The touching procedure was repeated multiple times with the same piece to fill the slide with cell preparations. The slide was then air dried for approximately 5 sec, and placed straight into a fixative solution of 95% ethanol for 15 min. The whole procedure was repeated for other pieces on fresh slides to obtain
multiple slides from each mouse. The slides were transferred to PBST prior to staining.

2.5.2. Staining of touch preparations:

The slides were incubated in methylene blue solution (0.4 g methylene blue (Sigma) in 100 ml of 50% methanol) for 5 min and then rinsed 3 times in distilled water. The slides were placed into haematoxylin solution for 1 min, and then dehydrated through graded ethanol and xylenes prior to mounting in DPX.

2.5.3. Measurement of cell ploidy by analysis of touch preparation images:

2.5.3.1. Photography and image preparation

The touch preparation slides were photographed using a Leica DM 5000 B microscope with a Leica DFC 490 camera at 400x magnification and 1088x816 resolution. Images were taken at the same time with the same exposure settings. Images were taken at random from areas of the slide where the cells had adhered adequately, resulting in a minimum of 20 images per mouse across multiple slides. The images were all randomly labelled with a numbered code to allow for blind analysis.

2.5.3.2. Counting of binucleate cells

The number of binucleate and mononucleate cells in the blindly labelled images were counted manually using the cell counter feature of ImageJ software, a public domain, Java based imaging program developed at the National Institutes of Health (http://rsb.info.nih.gov/ij/index.html). The percentage of binucleate cells was calculated by dividing the binucleate count by the total cell number count, and the results were averaged for Bmi-1 wild type and knockout mice, with significance assessed using a 2-tailed student’s t-test.
2.5.3.3. Analysis of hepatocyte nuclear size

The average nuclear size of the blindly labelled touch preparation images was analysed using ImageJ software. All images were analysed using consistent parameters and threshold values. The image was converted to 8-bit greyscale, and an appropriate threshold value used to highlight the nuclei to be studied. The nucleus counter feature was then used to obtain output data on the number of selected nuclei and their average area. This measurement was repeated twice for each image, with two different values (75 and 100 pixels) for the minimum particle size to be included in the analysis. Threshold values were defined in pixels, and the results subsequently converted to $\mu\text{m}^2$. This procedure was also repeated with a minimum particle size threshold of 44 $\mu\text{m}^2$ (425 pixels) to count the number of large hepatocyte nuclei present in the touch preparation. The nuclear area measurements from the blindly labelled images were matched with the actual image information, and an average area calculated for $Bmi-1$ wild type and knockout mice. Significance was assessed using a 2-tailed student's t-test.

2.5.3.4. Measurement of hepatocyte cell size

Hepatocyte cell sizes were quantified from randomly selected, blindly labelled touch prep images using ImageJ software. A box measuring 500 by 500 pixels was placed in the top left corner of the image. All hepatocytes which completely fell within this area were manually outlined using the Freehand Selection tool, and the Multi Measure tool was used to obtain area measurements for each selected cell. A total of 200 individual hepatocytes each were measured from $Bmi-1$ wild type and knockout mice, across a minimum of 10 different images. The area measurements were averaged, and significance assessed using a 2-tailed student's t-test at $p<0.05$. 

103
2.6. Analysis of average hepatocyte centromere counts

2.6.1. Pan-centromeric in situ hybridisation

Hepatocyte centromere numbers were estimated using pan-centromeric in situ hybridisation on paraffin embedded sections. This procedure was carried out with assistance from Rosemary Jeffery, Cancer Research UK. Sections were deparaffinised in xylene (2 x 5 min), and endogenous peroxidases were then blocked in 0.18% hydrogen peroxide (2.4 ml in 400 ml absolute alcohol), prior to dehydration in graded alcohols to water. The sections were permeabilised in 1 M sodium thiocyanate (Sigma) for 10 min at 80°C, and washed 2x in PBS for 10 min. The sections were now digested in 0.4% pepsin (Sigma) in 0.1 M hydrochloric acid for 15 min at 37°C, the reaction was stopped in double concentrated PBS with 0.2% glycine for 5 min and the slides washed in PBS for 5 min. The sections were fixed in freshly prepared 4% paraformaldehyde for 2 min, washed 3x in PBS, and dehydrated through graded alcohols. 15 µl of mouse pan-centromeric probe mixture (Cambio), which had been pre-warmed at 37°C, was added to the air dried sections. The sections were then covered with a glass coverslip, and sealed with rubber cement. The slides were denatured on a metal plate in a 60°C oven for 10 min, and placed overnight in a moist chamber at 37°C. The coverslips were now removed, and slides washed in 0.5x standard sodium citrate (SSC; diluted from 20x solution containing 87.6 g sodium chloride, 44.1 g sodium citrate in 500 ml distilled water, pH 7.4) for 5 min at 37°C, followed by PBS washes. Anti-fluorescein-POD antibody fragments (Roche) were applied for 45 min (diluted 1.150 in PBS), and the slides washed 3x in PBS. Staining was visualised using DAB (Vector Laboratories). The slides were then washed in PBS, counterstained in haematoxylin, dehydrated through graded alcohols and xylenes, and mounted in DPX.
2.6.2. Quantification of centromere numbers

Centromere numbers were quantified using images of randomly selected 400x microscope fields of slides exhibiting successful pan-centromeric in situ labelling. The number of visible centromeres in hepatocyte nuclei falling within the selected field were counted. Any nuclei which were not clearly identifiable as individual hepatocyte nuclei were not included. A total of 645 hepatocytes across four mice of each genotype were counted.

2.7. Gene expression analysis in mouse liver tissue:

2.7.1. Sample collection:

Small pieces of freshly collected mouse liver samples (approximately 5 mm diameter) were immediately submerged in 6 ml of RNAlater (Qiagen). Samples were stored for a maximum of 3 days at 4°C until RNA extraction, and any unused were moved to -20°C for long term storage.

2.7.2. RNA extraction:

RNA was extracted using the RNeasy Mini kit (Qiagen). All centrifugation steps in this procedure were carried out at 13000 rpm at room temperature. Samples collected in 3.6.1 were homogenised in 600 µl RLT buffer with added β-mercaptoethanol using a glass homogeniser, and centrifuged for 2 min in a Qiashredder column (Qiagen). The resulting lysate was mixed with an equal volume of 70% ethanol, and 700 µl of the mixture was transferred to an RNeasy spin column and centrifuged at 10000 rpm for 15 seconds at room temperature. On column DNase digestion was now performed using an RNase-Free DNase Set (Qiagen). The column was washed by adding 350 µl of buffer RW1 and centrifuging for 15 seconds. Flowthrough material was discarded after each centrifugation step. The spin column membrane was now incubated with DNase I solution (10 µl DNase I in 70 µl RDD buffer) for 15 min at room temperature,
and then centrifuged for 15 seconds following addition of 350 µl of buffer RW1. The spin column was now washed twice by adding 500 µl of buffer RPE and centrifuging for 15 seconds (1\textsuperscript{st} wash) and 2 min (2\textsuperscript{nd} wash), and the empty tube centrifuged again for 1 min. RNA was eluted from the column into a 1.5 ml microcentrifuge tube by adding 50 µl of RNase free water and centrifuging for 1 min. The RNA concentration was determined using a NanoDrop® Spectrophotometer (Thermo Scientific), using distilled water as the blank control.

2.7.3. cDNA synthesis:

First-strand cDNA was synthesised using SuperScript RT III First Strand Synthesis System (Invitrogen). 1 µg of RNA extracted in 3.6.2 was added to a nuclease free microcentrifuge tube along with 125 ng of random primer mix, 1 µl of 10 mM dNTP mix and Nuclease Free Water (Ambion) up to a total volume of 13 µl. The mixture was heated for 5 min at 65\textdegree C and incubated on ice for 5 min. To the mixture were added 4 µl 5x First Strand Buffer, 1 µl dithiothreitol (DTT), 1 µl RNase Out Recombinant RNase Inhibitor (Invitrogen) and 1 µl Superscript III RT. Appropriate control reactions without Superscript III RT were also included. The solution was gently mixed by pipetting, and incubated at 50\textdegree C for 45 min. The reaction was inactivated by heating at 70\textdegree C for 15 min. The cDNA obtained was diluted 1:10 in nuclease free water prior to PCR/Q-PCR analysis.

2.7.4. Polymerase chain reaction (PCR):

Qualitative expression of selected genes was identified using the following primers and reaction:

2.7.4.1. Table of primers

Primers were designed by myself and Dr Danielle Lavery.
<table>
<thead>
<tr>
<th>Gene Target</th>
<th>Forward (F) and Reverse (R) Primer Sequence</th>
<th>Annealing Temperature</th>
<th>Size of amplified fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse β-actin</td>
<td>F: GAGAAGCTGCTGTATGGTGTGCTC&lt;br&gt;R: CCATACCCAAAGGAAGGCTG</td>
<td>62°C</td>
<td>169</td>
</tr>
<tr>
<td>Mouse Glyceraldehyde 3-phosphate Dehydrogenase (Gapdh)</td>
<td>F: GGTCGGGTTGGAAGGATTGG&lt;br&gt;R: GCCGTGGGTAGAGTACTGAATACTGGAAC</td>
<td>62°C</td>
<td>148</td>
</tr>
<tr>
<td>Mouse Ribosomal 18S</td>
<td>F: CGGCCGGCTTTGGTGAACCTGTAGAT&lt;br&gt;R: TATGCCACGGGCTAGACTACCTGA</td>
<td>62°C</td>
<td>112</td>
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<tr>
<td>Mouse Eukaryotic Elongation Factor 2 (eEF2)</td>
<td>F: GCCCTTTGATAGGTCTGTCTGT&lt;br&gt;R: CCATACCTCAAGGTGTCTCTG</td>
<td>62°C</td>
<td>130</td>
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<tr>
<td>Mouse Ribosomal Protein L27 (Rpl27)</td>
<td>F: GAACATTGAGATGGCAGCCTG&lt;br&gt;R: GGAATTGGTTACCGAACCTGTTG</td>
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<td>130</td>
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<tr>
<td>Mouse β-glucuronidase (Gus)</td>
<td>F: CAAGATACCGACATGAGAGTGG&lt;br&gt;R: CACTCTGGACCAGCTTGTAATG</td>
<td>62°C</td>
<td>142</td>
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<td>Mouse Bmi-1</td>
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<td>143</td>
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<td>Mouse Mel-18</td>
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<td>155</td>
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<td>Mouse Mblr</td>
<td>F: GAATGAGCACCCTTTCTTGC&lt;br&gt;R: CTTGGGTATCGATAGGTGAAC</td>
<td>58°C</td>
<td>140</td>
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<td>Mouse Nspc1</td>
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<td>Mouse Scmh1</td>
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<td>163</td>
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<td>Mouse Ring1</td>
<td>F: GAGAAAGTTCTGGAAGGTTGTCG&lt;br&gt;R: CTCCTTTTGTCTCTGCCTCCTTGTGCTG</td>
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<td>Mouse Rnf2</td>
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<td>62°C</td>
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<tr>
<td>Gene Name</td>
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<td>Reverse Primers</td>
<td>Annealing Temp</td>
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<td>-------------------------</td>
<td>----------------------------------</td>
<td>----------------------------------</td>
<td>----------------</td>
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<tr>
<td>Mouse <em>Eed</em></td>
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<td>R: CGCAGACAGCTATGGAGGATGC</td>
<td>65°C</td>
</tr>
<tr>
<td>Mouse <em>p16</em>&lt;sup&gt;ink4a&lt;/sup&gt;</td>
<td>F: CAGACCGACGGGCATAGCCTTCAG</td>
<td>R: CACATGCTAGACAGCCTAGCATC</td>
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</tr>
<tr>
<td>Mouse <em>p19</em>&lt;sup&gt;nm&lt;/sup&gt;</td>
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<td>R: GAACGCAAATATCGACAGATGTC</td>
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</tr>
<tr>
<td>Mouse <em>p21</em></td>
<td>F: CAGATCCACAGCGATATCCAGAC</td>
<td>R: CGCAAACGCTCAGCTGACAG</td>
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</tr>
<tr>
<td>Mouse <em>c-myc</em></td>
<td>F: CAGCAGCGACTCGGCTGAGAGAG</td>
<td>R: GCACCTCTGAGGACAGGTGG</td>
<td>65°C</td>
</tr>
<tr>
<td>Mouse <em>H&lt;sub&gt;amp&lt;/sub&gt;</em></td>
<td>F: GCAGAAACAGAACAGCTGATG</td>
<td>R: GTCGCAAAGGCTGAGCTCTGAGTGL</td>
<td>65°C</td>
</tr>
<tr>
<td>Mouse <em>Hfe</em></td>
<td>F: CCACAGTAAGGTCAGAAGTTGA</td>
<td>R: GCAGAATTCCAGGTATCTTCGC</td>
<td>65°C</td>
</tr>
<tr>
<td>Mouse Ferroportin (<em>Fpn</em>)</td>
<td>F: GACCAAGGCAAGAGATCACC</td>
<td>R: GCTGTTTCCATAGAGTTCGCCACC</td>
<td>62°C</td>
</tr>
<tr>
<td>Mouse Transferrin Receptor 1 (<em>Tfr1</em>)</td>
<td>F: CTAGGACCTGGTGATCCATACAG</td>
<td>R: GTTCCATGCTAGGACAGCTTCCACC</td>
<td>60°C</td>
</tr>
<tr>
<td>Mouse Transferrin Receptor 2 (<em>Tfr2</em>)</td>
<td>F: GTCAFCAATGGGTTAGCACCTC</td>
<td>R: GTCCAGGGCTCAGTACACACAG</td>
<td>65°C</td>
</tr>
<tr>
<td>Mouse Bone Morphogenetic Protein 6 (<em>Bmp6</em>)</td>
<td>F: GTGGAGTACGACAGAGGTTC</td>
<td>R: CACCCACAGTGCCTTGAGTAC</td>
<td>58°C</td>
</tr>
<tr>
<td>Mouse Insulin growth factor binding protein 2 (<em>Igfbp2</em>)</td>
<td>F: CCACAGCGAGTGGCACAGACTG</td>
<td>R: GCTCCCTCATGCTGACTTGAG</td>
<td>65°C</td>
</tr>
</tbody>
</table>

### 2.7.4.2. PCR reaction mix

2 µl DNA sample

2 µl forward and reverse primer mix (5 µM stock concentration)

2.5 µl 10x reaction buffer (supplied with enzyme)

0.5 µl 10 mM dNTPs (Invitrogen)

1.25 µl magnesium chloride (supplied with enzyme)

0.1 µl Platinum Taq DNA polymerase (Invitrogen)

16.65 µl ddH<sub>2</sub>O
2.7.4.3. **Cycling conditions**

1) 94°C, 4 min

2) 94°C, 30 sec

3) Annealing temperature, 30 sec

4) 72°C, 1 min

5) To step 2, 39 times

6) 4°C forever

2.7.4.4. **Gel electrophoresis**

See section 2.3.3

2.7.5. **Quantitative PCR (Q-PCR):**

2.7.5.1. **Generation of standards**

Primers were tested by qualitative PCR as described in 2.7.4, and products were purified using a QIAquick PCR purification kit (Qiagen). 10 µl of PCR product was mixed with 50 µl of buffer PB, and placed in a QIAquick spin column. The column was centrifuged at 13000 rpm for 60 seconds at room temperature, and the flowthrough discarded. The spin column was washed by adding 750 µl of buffer PE and centrifuging at 13000 rpm for 2 min, followed by an additional 1 min centrifugation of the empty tube. DNA was eluted from the column into a microcentrifuge tube by adding 50 µl of buffer EB, and centrifuging for 1 min. The concentration of purified products was quantified using a NanoDrop® Spectrophotometer, and the samples were subsequently diluted 1:2 with Nuclease Free Water (Ambion). The absolute concentration of each standard was calculated by dividing the molecular weight of the amplicon (OligoCalc: http://www.basic.northwestern.edu/biotools/oligocalc.html) by
Avogadro’s number, and dividing this number by the concentration of the original standard to obtain a value expressed as molecules (transcripts) of gene of interest per µl. Prior to each Q-PCR experiment, original standards were serially diluted 1:5 to obtain twelve standards of decreasing concentration. The last nine of these dilutions were included in the Q-PCR experiment to allow creation of a standard curve.

2.7.5.2. Q-PCR reaction

Q-PCR reactions were set up in 96 well Q-PCR plates (Applied Biosystems) using the following reaction mix with 2 µl of cDNA sample, and data was collected and analysed using the AB7500 Fast Real Time PCR System (Applied Biosystems):

8.125 µl ddH₂O

2 µl reverse and forward primer mix (5 µM stock concentrations)

0.375 µl ROX dye, diluted 1.500 (Stratagene, supplied with SYBR green)

12.5 µl Brilliant II SYBR green master mix (Stratagene)

2.7.5.3. Analysis of results and normalisation

A standard curve was plotted by AB7500 System Software using the standards generated in section 2.7.5.1. Absolute expression values of unknown samples were quantified using this graph. All reactions were run in triplicate, and the mean of each triplicate was used as the expression value for each gene measured. Two programs; geNorm (http://medgen.ugent.be/~jvdesomp/genorm/) (Vandesompele et al, 2002) and Normfinder (http://www.mdl.dk/publicationsnormfinder.htm) (Andersen et al, 2004), were used to validate the stability of four candidate reference genes (β-actin, Gapdh, eEf2 and Gus). Subsequently, absolute expression values for genes of interest were normalised by dividing by the absolute eEf2 expression (see appendix section A2 for details of reference gene selection). A two-tailed student’s t-test was
used to assess significant differences between wild type and knockout mice at each
time point, with significance recognised when p<0.05.

2.8. Western blotting:

2.8.1. Sample collection:

Whole livers were excised from Bmi-1 wild type and knockout mice, and a small piece
(approximate diameter 0.5 ml) was placed into a glass homogeniser containing 400 µl
of RIPA buffer (0.75 ml 2M NaCl, 0.5 ml 10% sodium deoxycholate solution, 0.1 ml
10% SDS solution, 0.5 ml 1M Tris pH 8.0, 8.05 ml ddH₂O, 0.1 ml Triton X-100, 1
Protease Inhibitor Cocktail Tablet (Roche). The sample was homogenised until
liquefied, and placed into a cooled 1.5 ml microcentrifuge tube on ice. The tubes were
then centrifuged at 13000 rpm for 25 min at 4°C, and the supernatant was transferred
to a fresh tube. Samples were stored at -80°C prior to usage.

2.8.2. Quantification of protein concentration:

Aliquots of the protein samples were diluted 1:10 in RIPA buffer prior to quantification
to ensure the concentrations were within the working range of the assay kit. Protein
concentrations were measured using the colourimetric Bio-Rad DC Protein assay as
per the manufacturer’s instructions. Protein standards of 1.5, 1, 0.75, 0.5, 0.25 and 0
mg/ml were prepared by diluting bovine serum albumin (BSA, Sigma) in RIPA buffer.
5 µl of each standard and sample was added to a 96 well microtitre plate, followed by
25 µl of assay reagent A’ and 200 µl of assay reagent B. The plate was left at room
temperature for 15 min, and absorbance was read at 650 nm using a Wallac Victor²
1420 Multilabel Counter plate reader (Perkin Elmer).

2.8.3. SDS Polyacrylamide gel electrophoresis (PAGE):

SDS-PAGE and subsequent western blotting was carried out using the Bio-Rad Mini
Trans-Blot Cell system and accessories.
2.8.3.1. Preparation of gels

Resolving gel mixtures were prepared as follows:

10% gel:

4.0 ml ddH$_2$O
3.3 ml 30% Protogel (Fisher)
2.5 ml 1.5 M Tris pH 8.8
0.1 ml 10% SDS
0.1 ml 10% ammonium persulphate (APS) solution (Sigma)
4 µl Tetramethylethylenediamine (TEMED) (Sigma)

15% gel (used only for p16$^{ink4a}$ and p21 antibodies):

2.3 ml ddH$_2$O
5 ml 30% Protogel
2.5 ml 1.5 M Tris pH 8.8
0.1 ml 10% SDS
0.1 ml 10% APS
4 µl TEMED

The resolving gel solution was poured into the gel casting apparatus, and 1 ml of isopropanol was added on top. Once the gel solidified, the isopropanol was poured off and the gel rinsed in ddH$_2$O.
Stacking gel mix:

2.1 ml ddH₂O

0.5 ml 30% Protogel

0.38 ml 0.5 M Tris pH 6.8

0.03 ml 10% SDS

0.03 ml 10% APS

3 µl TEMED

The stacking gel mixture was poured on top of the resolving gel, and a plastic comb inserted into the solution. Once solidified, the gel used immediately or stored wrapped in wet tissue paper and cling film at 4°C until required for SDS-PAGE.

2.8.3.2. Preparation of gel apparatus

The comb was removed from the polymerised gel, and the gel assembled with the electrophoresis unit. The gel tank was filled with 1x running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS), made by diluting 100 ml of 10x stock solution (250 mM Tris, 1920 mM glycine, 1 l dH₂O) in 900 ml of dH₂O and adding 10 ml of 10% SDS.

2.8.3.3. Preparation of protein samples

50 µg of protein sample as added to a 1.5 ml microcentrifuge tube, using the protein concentrations measured in section 2.8.2 to calculate the volume of sample required. The samples were made up to 15 µl by adding RIPA buffer, and then 3 µl of 6x Laemmli buffer (6% (w/v) SDS, 180 mM Tris-HCl pH 6.8, 15% β-mercaptoethanol, 60% glycerol, 0.02 % bromophenol blue)(Sigma) was added to each sample. The samples were boiled at 95°C for 5 min prior to loading. A sample containing no protein was also included as a negative control.
2.8.3.4. Gel electrophoresis

The protein samples were loaded into the gel along with 5 µl of Precision Plus Protein Standards (Bio-Rad). The gel was run at room temperature at 80 V until the proteins had reached the resolving gel, and then increased to 100 V. The electrophoresis was stopped when the marker band reached the bottom of the gel.

2.8.4. Coomassie staining:

Following electrophoresis, the gel apparatus was disassembled and the stacking gel removed using a scalpel. The gel was immediately rinsed in Tris buffered saline-Tween buffer (TBST; 50 mM Tris, 150 mM NaCl, 0.1% Tween-20), transferred to Coomassie solution (0.25 g Coomassie powder (Fisher), 10 ml glacial acetic acid, 45 ml methanol, 45 ml dH₂O) and incubated overnight with agitation. The gel was destained in multiple changes of destain solution (10 ml glacial acetic acid, 45 ml methanol, 45 ml dH₂O) until the protein bands were clearly visible (approximately 5 hours). The gel was dried onto filter paper using a Model 583 Gel Dryer (Bio-Rad). The stained gel was used to adjust the volume of protein sample loaded for future experiments to ensure equal loading of each sample.

2.8.5. Western blotting:

2.8.5.1. Transfer

Transfer buffer was prepared by mixing 100 ml of 10x running buffer solution (section 2.8.3.2) with 200 ml methanol and 700 ml dH₂O, and stored at 4°C until required. Following gel electrophoresis, the apparatus was disassembled, excess gel cut off, and the gel placed in 1x transfer buffer along with sponges and blotting paper. Polyvinylidene fluoride (PVDF) membrane (Bio-Rad) was cut to size and immersed briefly in 100% methanol, before being soaked in 1x transfer buffer for 10 min. The gel transfer unit was assembled as per the manufacturer’s instructions, and the tank filled.
with 1x transfer buffer. The sponges, blotting paper, gel and PVDF membrane were placed into the apparatus in the correct orientation. Transfer was carried out at 110 V for 1 h at 4°C. The membrane was washed in TBST prior to the blotting procedure. For initial test blots, the quality of transfer was assessed by staining the membrane with Ponceau solution (0.2% (w/v) Ponceau S (Sigma) in 3% trichloroacetic acid) and then rinsing in dH₂O.

2.8.5.2. Blotting

The membrane was incubated in blocking solution (5% w/v BSA, 5% (w/v) powdered milk in TBST) for a minimum of 1 h with agitation (N.B. BSA was not included in block for anti-Erk1/2 antibody) and subsequently incubated overnight at 4°C in primary antibody diluted in blocking solution. The membrane was washed 4x in TBST for a minimum total time of 1 h, and incubated in blocking solution containing the appropriate mouse, rabbit or goat horse radish peroxidase (HRP) conjugated secondary antibody (Dako) for 1 h at room temperature. The membrane was washed in TBST for a minimum of 3x 10 min prior to detection.

2.8.5.3. Detection

ECL chemiluminescent reagents:

Solution A:

2ml 250 mM luminal (Sigma)

440 µl 90 mM p-coumaric acid (Sigma)

100 ml 100 mM Tris pH 8.6

Solution B:

120 µl 30% Hydrogen peroxide
100 ml 100 mM Tris pH 8.6

ECL solutions A and B were mixed 1:1 before usage, and incubated with the membrane for 1 min. The membrane was wrapped in cling film and transferred to a cassette. The membrane was exposed to a sheet of ECL Hyperfilm (GE Healthcare) in a dark room initially for 30 sec, and then subsequently for an appropriate period depending on the strength of the signal. Films were developed using an automated Hyperprocessor (Amersham Pharmacia Biotech). Membranes were transferred to TBST for storage at 4°C.

2.8.5.4. Blot stripping

Used blots were stripped by incubating with ReBlot Plus Mild Antibody Stripping Solution (Millipore) at room temperature for approximately 15 min (incubation time dependent on strength of original signal). The blot was washed in TBST, and submitted to the blotting and subsequent detection procedures described in section 2.8.5.2.
### Antibodies used for western blotting

<table>
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</thead>
<tbody>
<tr>
<td>Bmi-1 (Mouse)</td>
<td>Bmi-1 (Mouse)</td>
<td>Millipore</td>
<td>05-637</td>
</tr>
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<td>Mel-18 (Goat)</td>
<td>Mel-18 (Goat)</td>
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<tr>
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<tr>
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<td>Abcam ab8226</td>
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#### 2.8.6. Selection of housekeeping gene for western blotting:

Equal volumes of protein samples from Bmi-1 wild type and knockout mice of varying ages (based on Coomassie staining, see section 2.8.4) were loaded onto gels and transferred as described previously. The membranes were blotted with antibodies against three commonly used reference genes: α-tubulin, Erk1/2 and β-actin. Erk1/2 was selected as the optimal reference gene for future experiments based on the detection of strong, consistent signals for each lane, the intensities of which correlated with the protein loading observed on the Coomassie stained gel.

#### 2.8.7. Densitometric analysis of western blots:

The intensity of bands on western blots was quantified on scanned images of the blot using ImageJ software. Blot scans were converted to 8-bit greyscale images, and the threshold feature of ImageJ was used to highlight the bands. Only bands from the
same blot image were directly compared to prevent differences in image processing from affecting the results. The band to be measured was outlined using a manually drawn rectangle, and ImageJ used to measure the integrated density within this region. The rectangle was then moved to enclose the next band, and the measurement repeated. These were normalised by dividing by the intensity of housekeeping gene (Erk1/2) bands from a stripped and re-probed blot. The mean intensity was calculated to obtain averages for Bmi-1 wild type and knockout mice, and significance assessed at p<0.05 using a 2-tailed student's t-test.
Results and Discussion
Chapter 3

Expression of Bmi-1 in normal liver and regenerating tissue
3. Expression of Bmi-1 in normal liver and regenerating tissue:

3.1. Reliability of anti-Bmi-1 antibodies:

Problems with commercially available anti-Bmi-1 antibodies (and also in situ hybridisation) have previously been described, with the absence of a reliable Bmi-1 staining pattern reported in pancreatic tissue (Sangiorgi & Capecchi, 2009). This may be reflected by the contradictory staining patterns observed in some previous studies of Bmi-1 expression in tumour patients, where cytoplasmic staining has been reported (Wang et al, 2008), despite the fact that Bmi-1 is believed to be a nuclear protein. In agreement with this, we observed that some older archival paraffin blocks had high levels of background staining. These problems were exacerbated in mouse tissue, as the most prominently used antibody is a mouse monoclonal. To generate optimal immunohistochemistry data, multiple lots of anti-Bmi-1 antibody (Millipore) were tested to find one which gave the most robust and consistent staining pattern in paraffin sections made from freshly collected liver tissue. The selected lot was then used for all future experiments. The validity of these staining results in mouse was assured by including control tissue from Bmi-1 knockout mice, which do not express any residual protein, in all experiments. These tissues were taken from littermates of the wild type animals used, and were identically fixed and processed to eliminate variability. Only experiments which yielded no Bmi-1 labelling in Bmi-1 knockout tissue were considered for analysis.
3.2. **Bmi-1 protein is primarily expressed in biliary cells in normal murine liver:**

3.2.1. **Bmi-1 is expressed in cholangiocytes:**

To begin elucidating the functional consequences of Bmi-1 expression in liver, the first step was to confirm the protein expression pattern of Bmi-1 in normal mouse liver. Paraffin-embedded liver sections from multiple 8 week old *Bmi-1* wild type and knockout mice were stained for Bmi-1 using indirect immunohistochemistry (Figure 8). Bmi-1 is reported to be a predominantly nuclear protein (Chiba et al, 2007), so any background cytoplasmic staining was discounted. Bmi-1 expression was consistently high in the cholangiocytes that form bile ducts in the portal regions in all tissue sections examined (Figure 8a). Bmi-1 staining was absent in all nuclei in the *Bmi-1* knockout tissue, demonstrating that antibody reactivity is specific to the Bmi-1 epitope (Figure 8b). The expression pattern for Bmi-1 is consistent with that previously observed in the *Bmi-1-GFP* knock-in mouse, which showed strong expression in the biliary region (Hosen et al, 2007). The expression pattern of Bmi-1 is also broadly in agreement with a proposed role for Bmi-1 in adult stem cell maintenance, as liver stem cells are believed to derive from the intrahepatic biliary tree (canals of Hering).

3.2.2. **Bmi-1 is expressed in hepatocytes:**

The question of whether Bmi-1 is also expressed in hepatocytes has previously remained unclear. The *Bmi-1-GFP* knock-in mouse appears to show some expression within the hepatic parenchyma (Hosen et al, 2007), and faint Bmi-1 expression in human hepatocytes has also previously been reported (Sasaki et al, 2006). A direct comparison of Bmi-1 stained hepatocyte nuclei from wild type and *Bmi-1* knockout mice appeared to show light staining in some wild type nuclei, suggesting low level Bmi-1 expression in some hepatocytes (Figure 8a,b). However, levels of hepatocellular Bmi-1 staining were difficult to evaluate conclusively, due to the aforementioned issues with commercially available anti-Bmi-1 antibodies (Section
3.1). Because of this, it was decided to have sections independently stained and assessed by Dr. Bradley Spencer-Dene of the Experimental Pathology Core Facility at Cancer Research UK. Wild type and Bmi-1 knockout murine liver sections (n=3) were submitted for staining and analysis. Some hepatocytes in wild type mice clearly exhibited Bmi-1 expression within particular subnuclear compartments (Figure 8c,e). This expression was not definitively identifiable in all hepatocytes, and labelling was absent in all sections from Bmi-1 knockout mice (Figure 8d,f). A similar nuclear punctate pattern of Bmi-1 expression is also seen in HCC cell lines (Effendi et al, 2010) and cultured putative murine hepatic/stem progenitor cells (Chiba et al, 2007). Electron immunomicroscopy has also demonstrated that polycomb proteins are concentrated at the perichromatin compartment within the nucleus (Cmarko et al, 2003). These data demonstrate that Bmi-1 is strongly expressed in the nuclei of murine cholangiocytes, and is expressed at lower levels within some hepatocytes.
Figure 8: Bmi-1 is expressed in cholangiocytes and hepatocytes in murine liver. Labelling of paraffin-embedded 8 week old mouse liver sections using indirect immunohistochemistry to detect Bmi-1. Antibodies were visualised with DAB and counterstained with haematoxylin. a) Tissue from wild type mouse showing expression of Bmi-1 in biliary cells (black arrow) and hepatocytes (red arrow). b) Immunolabelling is absent in cholangiocytes and hepatocytes in Bmi-1 knockout mouse. c,d) Tissue from Bmi-1 wild type (c) and knockout (d) mice, again showing expression in wild type cholangiocytes (black arrow) and punctate nuclear labelling pattern in some hepatocytes (red arrow). e,f) Enlarged images of selected hepatocyte nuclei from e) wild type and f) Bmi-1 knockout mouse. Scale bars 50 µm.
3.3. Bmi-1 protein is expressed in mouse oval cell nuclei:

Bmi-1 has been previously associated with maintenance of stem cell populations, both in cells from foetal liver (Chiba et al, 2010) and in many other tissues (see introduction). To begin investigating a potential role for Bmi-1 in the adult liver stem cell response, Bmi-1 protein expression was assessed by immunohistochemical methods in tissue sections from mice undergoing an oval cell response. Oval cells are believed to be derived from resident liver stem cells. The tissues stained were obtained from archival paraffin-embedded blocks from two different mouse models of oval cell induction. The first were from transgenic mice with an albumin-urokinase type plasminogen activator transgene (AL-uPA), which develop liver disease following uPA expression in hepatocytes (Braun et al, 2003). The second set of blocks was from mice treated with the DNA alkylating agent dipin, which induces an oval cell response (Braun & Sandgren, 2000). Double-label immunohistochemistry revealed high Bmi-1 expression in the nuclei of oval cells in tissue sections from several mice, using both models of oval cell activation (Figure 9) (n=5 from each line). Oval cells were identified by parenchymal localisation, nuclear shape and staining for cytokeratin 19 (CK19), a marker for bile ducts and oval cells (Jelnes et al, 2007). CK19 gave the same ductal cell labelling pattern as the more commonly used A6 antibody (Figure 9d) (A6 antibody kindly supplied by Dr Valentina Factor, NIH, Bethesda, MD, USA). Bmi-1 was expressed in all oval cell nuclei within a CK19 positive ductular reaction. These results are the first to demonstrate that Bmi-1 is strongly expressed in oval cells from two different mouse models, suggesting a possible functional relevance for Bmi-1 in the hepatic stem cell response.
Figure 9: Bmi-1 is expressed in CK19 positive murine oval cells.

Labelling of paraffin-embedded liver sections from mice treated with dipin (a,b,c,d) and from AL-uPA transgenic mice (e,f) using indirect immunohistochemistry. Sections co-labelled with Bmi-1 (brown) and CK19 (blue) antibodies and counterstained with nuclear fast red, except d) which was labelled with A6 antibody (brown) and counterstained with haematoxylin. a,e) Negative control slides with isotype control antibody instead of anti-Bmi-1, with absence (a) and presence (d) of anti-CK19 antibody. b,c,e) Bmi-1 positive oval cells (black arrow). d) A6 positive oval cells (black arrow). Scale bars in a,b): 200 µm; in c,e,f): 50 µm; in d): 100 µm.
3.4. Bmi-1 protein is expressed in some biliary cells in human liver:

To determine whether the expression pattern observed in mouse liver was similar in humans, paraffin-embedded sections of tumour-adjacent normal human liver (provided by Professor Malcolm Alison with appropriate ethical approval) were stained for Bmi-1 (n=3) (Figure 10). As in mouse, nuclear labelling was observed for Bmi-1 in cholangiocytes (Figure 10b). However, unlike in the mouse samples examined, there appeared to be a degree of heterogeneity in the intensity of staining in individual cholangiocytes, with some nuclei displaying very strong staining and some very weak/absent staining. Bmi-1 expression was not obvious in hepatocytes in human tissue, unlike in mouse, although this was difficult to evaluate conclusively due to the lack of Bmi-1 depleted human tissue for comparison. These data were supported by staining commercially purchased hepatic tissue microarrays (US Biomax) which contained normal and tumour-adjacent hepatic tissue. Interestingly, a number of bile ducts (7/21) in normal hepatic tumour microarray cores appeared not to express Bmi-1 (Figure 10c). This suggests that some human bile ducts may not express Bmi-1, in contrast to mice. Other cores on the same slide did show immunoreactivity for Bmi-1 (Figure 10d), suggesting that the antibody was working correctly under the staining conditions. However, the possibility that the cores were not all identically fixed cannot be ruled out, which could cause variability in the staining intensity.

In summary, these results demonstrate that strong Bmi-1 expression is found in some cholangiocytes in the livers of different species, and that experiments in mice are likely to have relevance to human tissue.
**Figure 10: Bmi-1 is expressed in cholangiocytes in human liver.**

a,b) Labelling of paraffin-embedded human sections using indirect immunohistochemistry to detect Bmi-1. Antibodies were visualised with DAB and sections counterstained with haematoxylin. a) Isotype control demonstrating absence of staining in cholangiocytes (black arrow) b) Tissue labelled with anti-Bmi-1 antibody, showing cholangiocytes both negative (black arrow) and positive (red arrow) for Bmi-1. c,d) Normal liver cores from tissue microarrays labelled for Bmi-1, showing bile ducts without (c) and with (d) Bmi-1 immunolabelling. Scale bars 50 µm.
3.5. Bmi-1 protein is expressed in human cirrhotic liver tissue:

Liver cirrhosis is a consequence of hepatic injury, including alcoholism, hepatitis and fatty liver diseases. Cirrhotic livers contain regions of advanced liver fibrosis, which involves the replacement of injured hepatic tissue by fibrous connective tissue. Found within the fibrotic regions are ductular reactions, which are believed to contain hepatic progenitor cells. These are thought to be derived from cells in the terminal biliary ducts (canals of Hering) (Theise et al, 1999). Cirrhotic tissue also contains regenerative nodules; these are regions of hepatocytes undergoing clonal proliferation following liver damage. As such, cirrhotic tissue provides a model to examine human cells undergoing a localised hepatic regenerative response. Expression changes in Bmi-1 have not previously been assessed in human cirrhotic livers. To address this question, paraffin-embedded tissue arrays containing samples from liver cirrhosis patients (US Biomax) were stained for Bmi-1 (Figure 11) and scored for Bmi-1 expression (Table 2).

Bmi-1 expression was assessed in ductal cells within fibrotic tissue, which were histologically identified on some cores on the microarray (Figure 11a,b). 59% of these cores exhibited ductal cells with positive Bmi-1 labelling. However, this does not represent a significant increase in frequency compared with bile ducts in normal liver samples (Table 2). Bmi-1 staining was also scored in hepatocyte regenerative nodules, with 22% exhibiting Bmi-1 expression. This represents a significant difference in comparison to normal hepatocytic samples, none of which expressed detectable Bmi-1 (Table 2). These results were supplemented with sections from cirrhotic liver patients containing much larger areas of tissue, provided by Professor Malcolm Alison (n=5). These again exhibited heterogeneity in Bmi-1 staining, with the presence and absence of Bmi-1 observed in both regenerative nodules and ductal cells (Figure 11 c-f). Together, these results provide some evidence that Bmi-1 may be involved in both hepatocyte mediated regeneration and progenitor cell responses.
in human tissue. However, it is unlikely to be critical, as Bmi-1 expression was not observed in the majority of regenerative nodules examined. As with the tissue array cores with normal hepatic tissue, it cannot be ruled out that fixation differences between cores could account for some of the heterogeneity observed in the Bmi-1 staining.

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<td>Cirrhotic ductal cells</td>
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<td>12</td>
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<table>
<thead>
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<td>38</td>
</tr>
<tr>
<td>p=0.0208 *</td>
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**Table 2: Bmi-1 expression in human cirrhotic livers.**

Table showing number of tumour samples of each type positive and negative for Bmi-1 immunolabelling. p values calculated for each table using a chi-squared test. *=significant at p<0.05.*
Figure 11 (overleaf): Bmi-1 expression in human cirrhotic livers.

Cirrhotic liver samples were labelled using indirect immunohistochemistry to detect Bmi-1. Antibodies were visualised with DAB and sections counterstained with haematoxylin. a,b) Human cirrhotic cores from tissue microarray, with absence (a) and presence (b) of Bmi-1 expression in ductular epithelial cells (black arrow) and hepatocyte regenerative nodules (red arrow), scale bars 50 µm. c-f) Sections from human cirrhotic patients. c) Negative control tissue section incubated with isotype control antibody. d) Cirrhotic liver with absence of labelling for Bmi-1. e) Bmi-1 expression in cholangiocytes (black arrow), but not in regenerative nodules (red arrow). f) Bmi-1 expression in both cholangiocytes and adjacent regenerative nodule. Scale bars 100 µm.
Figure 11: Bmi-1 expression in human cirrhotic livers.

See previous page for figure legend.
3.6. Expression of Bmi-1 in hepatic tumours:

3.6.1. Bmi-1 expression in human hepatocellular carcinomas:

As described in the introduction (section 1.12.2), Bmi-1 overexpression has been reported in human liver cancers. However, it is unclear whether Bmi-1 expression correlates with tumour progression, as different groups have reported contrasting results. Some have shown a correlation between Bmi-1 expression and well differentiated tumours (Effendi et al, 2010; Wang et al, 2008), while others report high Bmi-1 expression in poorly differentiated tumours (Sasaki et al, 2008a). This may be in part due to previously described issues with the consistency of anti-Bmi-1 antibodies. To try and address this issue, liver cancer tissue array slides (US Biomax) containing different grades of hepatocellular carcinomas (HCCs) and normal or tumour-adjacent hepatic tissue were stained to detect expression of Bmi-1. These arrays eliminate the possibility of any variability in staining procedures between different slides, although conversely it is not possible to ensure that all cores are identically fixed. The tumour cores were graded by the manufacturer as Grade I (well differentiated), Grade II (moderately differentiated) or Grade III (poorly differentiated). Images of the tumour cores were blindly labelled and scored as positive or negative for Bmi-1 expression (Table 3). Significance was assessed using a chi-squared test, and representative images are shown in Figure 12. Bmi-1 was significantly upregulated in HCC samples compared to normal or tumour-adjacent liver. However, only 40% of the tumour samples examined expressed Bmi-1, indicating high Bmi-1 expression is not essential for tumour development. In contrast to some previously published results, there was no significant difference in Bmi-1 expression between different tumour grades. This suggests that Bmi-1 is unlikely to be a prognostic factor for more aggressive tumours, although increased sample numbers (especially Grade I tumours) will be required to confirm this conclusion. It also cannot be guaranteed that all tumour cores on the array were identically fixed, which could cause variability in the
staining intensity. This makes it difficult to draw robust conclusions from both our data and published studies on whether Bmi-1 is a suitable prognostic factor for HCC.

3.6.2. Bmi-1 expression in human cholangiocarcinomas:

The tissue arrays obtained from US Biomax also contained various human CC samples, which were also stained for Bmi-1 to see whether any alterations in Bmi-1 could be detected in CC samples compared to normal biliary cells (Figure 13) (Table 3). Bmi-1 was expressed in 56% of CC samples examined. Given that a third of the normal bile ducts scored did not exhibit Bmi-1 expression, this does not represent a significant difference based on a chi-squared test. There is also no correlation between tumour grade and Bmi-1 expression. As Bmi-1 appeared to be expressed in some biliary cells in normal livers, it seems that Bmi-1 expression in CC cells may not represent an abnormality. As such, it is unclear whether it would have any relevance to the progression of the disease, as CC is believed to be derived from cholangiocytes.
Table 3: Bmi-1 expression in human liver tumours.

Table showing the number of tumour samples of each type positive and negative for Bmi-1 immunolabelling. p values calculated for each table using chi-squared test. *=significant at p<0.05

Figure 12 (overleaf): Bmi-1 expression in human hepatocellular carcinomas.

Selected cores from paraffin-embedded human HCC tissue microarrays labelled with indirect immunohistochemistry to detect Bmi-1. Antibodies were visualised with DAB and sections counterstained with haematoxylin. a) Negative staining in normal hepatic tissue. b) Grade I HCC sample showing light immunoreactivity for Bmi-1. c,d) Grade II HCC samples, both negative (c) and positive (d) for Bmi-1. e,f) Grade III HCC samples, both negative (e) and positive (f) for Bmi-1. Scale bars 50 µm.
Figure 12: Bmi-1 expression in human hepatocellular carcinomas.

See previous page for figure legend.
Figure 13: Bmi-1 expression in human cholangiocarcinomas.

Selected cores from paraffin-embedded human CC tissue microarrays labelled using indirect immunohistochemistry to detect Bmi-1. Antibodies were visualised with DAB and sections counterstained with haematoxylin. a,b) Examples of Grade II CC samples without (a) and with (b) Bmi-1 expression. Scale bars 50 µm.
3.7. Discussion:

Immunohistochemical analysis of murine liver tissue demonstrated strong Bmi-1 expression in all cholangiocytes. Bmi-1 staining in hepatocytes was more difficult to conclusively evaluate, as commercially available anti-Bmi-1 antibodies did not always give a consistent staining pattern (see section 3.1). To address this issue, murine liver tissue sections were independently analysed by Dr. Bradley Spencer-Dene of the Experimental Pathology Core Facility, Cancer Research UK. From these data sets, it can be concluded that Bmi-1 is expressed in some hepatocytes at a lower level than cholangiocytes. The robustness of these results was assured by the inclusion of isotype controls, and more importantly, the absence of labelling in all Bmi-1 knockout mouse tissue used as a negative control. The data could be improved in the future by assessing Bmi-1 expression in pure cholangiocyte and hepatocyte cell populations freshly isolated from murine livers (Cho, 2002; Klingmüller et al, 2006), or by obtaining cells from tissue sections using laser capture microdissection. Western blotting and/or Q-RT-PCR could then be used to define more precisely the level of Bmi-1 expression within these hepatic sub-populations.

It is interesting that Bmi-1 expression was observed in all murine cholangiocytes and some hepatocytes in vivo, given that both in vitro work in liver cell populations and in vivo work in other tissues have predominantly associated high levels of Bmi-1 expression with stem and progenitor cell populations (see introduction). Although stem cells within the liver are thought to reside within the portal region, it has not generally been believed that mature cholangiocytes themselves have stem/progenitor cell function, either in terms of contributing to normal hepatocyte turnover or the oval cell response. However cholangiocytes and hepatocytes do maintain the ability to proliferate extensively and give rise to identical daughter cells during regenerative responses, so can be considered as unipotent stem/progenitor cells. There is also recent lineage tracing based evidence that Sox9 positive cells within biliary structures
can both self-renew and give rise to hepatocytes during normal liver turnover (Furuyama et al, 2011), providing support for the theory that some cells within the biliary population do have stem/progenitor cell activity, although not necessarily mature cholangiocytes. Interestingly, in both normal and cirrhotic human liver tissue, some biliary cholangiocytes and cells involved in ductular reactions appeared to express Bmi-1 at higher levels than others. This raises the question of whether the Bmi-1 expressing human cholangiocytes represent cells with stem/progenitor cell properties, which may contribute to normal liver turnover or to regenerative nodules in cirrhosis. There is lineage tracing evidence that regenerative nodules can be clonally derived from progenitor cells within ductal reactions (Lin et al, 2010), and 3-dimensional reconstructions have also shown hepatocyte buds linked to mature bile ducts in cirrhotic livers (Falkowski et al, 2003). From our studies, Bmi-1 was expressed in all murine oval cells, suggesting a link between Bmi-1 expression and cells with stem/progenitor cell properties in murine liver. These questions could be addressed in future by performing lineage tracing of Bmi-1 expressing cells in liver using Bmi-1-Cre-ER transgenic mice, as already carried out in intestine and pancreas (Sangiorgi & Capecchi, 2008; Sangiorgi & Capecchi, 2009). This would provide clearer insight into the fate and function of Bmi-1 expressing cells within the liver.

There are also other published examples of Bmi-1 expression in more differentiated cell types. In the pancreas, the Bmi-1+ lineage in Bmi-1-Cre-ER transgenic mice has been reported to co-localise with differentiation markers for acinar cells, glucagon positive cells and endothelial cells, as opposed to pancreatic progenitor markers Nestin and Pdx1 (Sangiorgi & Capecchi, 2009). These pancreatic acinar cells also maintain the ability to proliferate despite their differentiated state, similar to hepatocytes and cholangiocytes in the liver. Bmi-1 expression has also been identified in all cells in the mouse mammary gland, as opposed to just stem cell subpopulations (Pietersen et al, 2008a). Together with the Bmi-1 expression pattern in liver described
above, these data suggest that Bmi-1 could have unspecified roles in more differentiated cell populations in addition to stem cells.

3.8. Summary of Chapter:

In summary, this chapter has demonstrated that Bmi-1 is expressed in cholangiocytes in normal murine and human liver, and appears to be expressed at lower levels in some murine hepatocytes. In addition to this, Bmi-1 is expressed in murine oval cells, along with some ductal progenitors and hepatocyte regenerative nodules in human cirrhotic livers. These results suggest that Bmi-1 may have a function in various forms of liver regeneration, a hypothesis that will be addressed in future experiments (see final discussion section 7.2.2). The expression of Bmi-1 in hepatic tumours was also examined. Bmi-1 was overexpressed in HCC samples compared to normal tissue, but no correlation was observed with tumour grade. As such, it is uncertain whether Bmi-1 is a suitable prognostic factor for HCC.
Chapter 4

Liver structure and function in *Bmi-1* knockout mice
4. Liver structure and function in Bmi-1 knockout mice:

4.1. Breeding and genotyping of Bmi-1 knockout mice:

To begin characterising the effects of global in vivo deletion of Bmi-1 on liver physiology, Bmi-1 knockout mice were generated by inter-crossing Bmi-1 +/- mice (generously provided by Professor Silvia Marino) (van der Lugt et al, 1994). Ear snips from the resulting progeny were genotyped using primers designed to distinguish the wild type and knockout DNA sequences. The genotyping results indicated repeated successful generation of Bmi-1 knockout mice (Figure 14a). To confirm that Bmi-1 knockout mice did not express any residual protein, western blotting was used to assess Bmi-1 protein levels in whole mouse liver extracts at 8 weeks of age. These results demonstrated expression of Bmi-1 protein in the livers of wild type mice, and a complete absence in the knockout mice (Figure 14b).

Newborn Bmi-1 knockout mice were obtained at a frequency of 16.9% (Figure 14c), which does not differ from expected Mendelian ratios based on a chi-squared test (p=0.283). Following genotyping at 3-4 weeks of age, the frequency of Bmi-1 knockout mice was 11.0%, which deviates significantly from expected Mendelian ratios (p<0.001). This is consistent with previously published results reporting that knockout mice are selectively cannibalised by the mothers (van der Lugt et al, 1994). The Bmi-1 knockout mice were outwardly normal in appearance at birth and at the postnatal day 7 time point. By 3 weeks of age, the knockout mice appeared noticeably smaller than wild type littermates. This is illustrated by growth curves comparing the body weight of wild type and knockout mice (Figure 15a). The knockouts also began to exhibit previously characterised abnormalities, most notably an ataxic gait and hunched phenotype due to skeletal transformations.

Studies of other organs have demonstrated that Bmi-1 knockout mice are relatively normal at birth, and then display phenotypes which become progressively worse with
age (van der Lugt et al, 1994). Because of this, it was decided to focus analysis on Bmi-1 knockout livers from adult mice, although livers from newborn mice and postnatal day 7 were also initially examined. The Bmi-1 knockout mice have previously been reported to live to 3-5 months of age (van der Lugt et al, 1994), although in our animal colony, the mice appeared generally unwell by 12 weeks of age, so it was decided not to keep them alive beyond this stage. To reduce the possibility of secondary effects due to the illness of the mice, an earlier adult time point at which the mice appeared healthier (8 weeks) was also chosen for analysis. Despite this, the fact that the mice are complete knockouts as opposed to liver specific Bmi-1 knockouts means that secondary effects cannot be ruled out in the subsequent work.
Figure 14: Breeding and genotyping of Bmi-1 knockout mice.

a) Representative photograph of agarose gel showing results of genotyping PCR from mice ear snips. Lower band represents Bmi-1 wild type allele (product size 323 bp); upper band is knockout allele (product size 446 bp). Labelled are examples of Bmi-1 wild type (Wt), heterozygote (Het) and knockout (KO) mice. b) Western blotting for Bmi-1 on whole liver protein extracts from 8 week old Wt and KO mice. Erk1/2 was used as a housekeeping gene. c) Table of obtained frequencies of Bmi-1 knockout mice.
Figure 15: Liver and body weights in *Bmi-1* knockout mice.

a) Growth rates of *Bmi-1* wild type (Wt) and knockout (KO) mice. Points represent body weight of an individual mouse at one particular time point. b) Liver to body weight ratio in 12 week old male *Bmi-1* Wt and KO mice (n=5), p value calculated using 2-tailed Student's t-test.
4.2. Characterisation of \textit{Bmi-1} knockout mouse liver phenotype:

4.2.1. Liver function in \textit{Bmi-1} knockout mice:

The livers in \textit{Bmi-1} knockout mice were smaller than in wild type littermates due to the reduced size of the mice. However, the liver to body weight ratio tends to remain constant in mammals, and any alterations can be an indicator of abnormal liver physiology. The liver to body weight ratio was calculated for \textit{Bmi-1} knockout mice at 8 and 12 weeks of age, and no significant differences were observed (Figure 15b).

To further assess abnormalities in the hepatic function of \textit{Bmi-1} knockout mice, serum from 8 week old mice was analysed for commonly used biomarkers indicative for liver disease or dysfunction (Fox et al, 2006) at the Royal Veterinary College, Hawkshead. The results are shown in Figure 16. Serum levels of albumin, the primary protein constituent of blood plasma, were normal in \textit{Bmi-1} knockout mice (Figure 16a), demonstrating that the synthetic capacity of the knockout livers is not diminished. Total bilirubin levels were also no different in \textit{Bmi-1} knockout mice compared to wild types (Figure 16b). Bilirubin is a breakdown product of haem which is excreted by the liver, and can be increased in liver diseases such as cholestasis. \textit{Bmi-1} knockout mice showed elevated levels of alkaline phosphatase (AP) compared to wild types (Figure 16c). AP is often elevated in cases of bile duct obstruction or cholestasis, suggesting that there may be some impairment of bile flow in \textit{Bmi-1} knockout mice. However, serum AP can also become elevated in bone diseases (Johnston, 1999), so a liver-specific phenotype will require further confirmation. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are both enzymes present in liver parenchymal cells, and are commonly used as markers of liver injury, as hepatocyte damage and necrosis causes them to leak into the blood. They tend to be greatly increased following acute liver injury, and more moderately increased in chronic diseases (Johnston, 1999). Levels of ALT appeared to be slightly increased in \textit{Bmi-1} knockout
mice (Figure 16d), however this is not statistically significant (p=0.097). AST levels were significantly increased in the knockout mice compared to wild types (Figure 16e). These results are indicative of some liver damage in Bmi-1 knockout mice, however it should be noted that unlike ALT, AST also shows significant activity in cardiac muscle, so can be indicative of cardiac muscle damage as well as hepatocellular damage (Fox et al, 2006).
Figure 16: Liver function biomarkers in Bmi-1 knockout mice.

Serum was collected from Bmi-1 wild type (Wt) and knockout (KO) mice and analysed for levels of a) albumin, b) total bilirubin, c) alkaline phosphatase (AP), d) alanine aminotransferase (ALT), e) aspartate aminotransferase (AST). a,b,c) n=3 Wt, 4 KO mice. d,e) n=4 Wt, 5 KO mice. * p<0.05 using 2-tailed Student’s t-test.
4.2.2. Histology of Bmi-1 knockout mouse livers:

To assess the effects of Bmi-1 deletion on liver histology, liver tissue sections from newborn, postnatal day 7, 8 week old and 12 week old Bmi-1 knockout mice were stained with haematoxylin and eosin (H&E) for histological assessment. The sections were also blindly labelled and analysed by my second supervisor Professor Malcolm Alison, a recognised expert in mouse hepatic pathology. Histologically, livers from newborn Bmi-1 knockout mice did not present any detectable phenotype (Figure 17a,b). Given that Bmi-1 was found to be highly expressed in cholangiocytes, the morphology of portal regions in Bmi-1 knockout mice was closely examined to assess whether loss of Bmi-1 had any effect on biliary organisation. Tissue sections from wild type and knockout mice were labelled with an antibody against the bile duct marker cytokeratin 19 (CK19) (Figure 17c,d). Newborn Bmi-1 knockout mice exhibited normal CK19 labelling with no obvious abnormalities in cholangiocyte appearance or biliary structure. The same results were observed in mice at postnatal day 7, with no difference between wild types and knockouts (data not shown).

In adult mice, at both 8 and 12 weeks of age, the Bmi-1 null mice exhibited cellular disorganisation within the hepatic parenchyma (Figure 18). Blind analysis consistently identified Bmi-1 knockout livers as having an abundance of smaller hepatocytes, which appeared more uniform in size than in wild type livers. CK19 labelling did not uncover any noticeable differences in either cellular structure or distribution of bile ducts within the liver parenchyma in 8 week old Bmi-1 knockout mice (Figure 19a-d). Picro Sirius red staining also demonstrated normal deposition of collagen surrounding the biliary regions in Bmi-1 knockout mice, with no evidence of fibrosis (Figure 19e,f). Taken together, these data suggest bile duct development is not noticeably affected by deletion of Bmi-1.
**Figure 17: Histology of newborn Bmi-1 knockout mouse livers.**

a,b) H&E stained paraffin-embedded sections from newborn Bmi-1 wild type (a) and knockout (b) mice. c,d) Paraffin-embedded sections labelled using indirect immunohistochemistry to detect cytokeratin 19 in Bmi-1 wild type (c) and knockout (d) mouse livers, showing bile duct labelling (black arrows). Antibodies visualised with DAB. Scale bars 100 µm.
Figure 18: Histology of adult *Bmi-1* knockout mouse livers.

Haematoxylin and eosin (H&E) stained paraffin-embedded liver sections from 8 week old *Bmi-1* wild type (left panels) and knockout mice (right panels). Images at 100x, 200x and 400x magnification, all scale bars 50 µm. BD = bile duct, CV = central vein.
Figure 19: Portal structure in *Bmi-1* knockout mice.

a-d) Paraffin-embedded liver sections from 8 week old *Bmi-1* wild type and knockout mice labelled using indirect immunohistochemistry to detect cytokeratin 19. Antibody visualised using DAB, and sections counterstained with haematoxylin. Images shown at low (a,b) and high (c,d) magnification. e,f) Picro Sirius red stained paraffin-embedded liver sections from 8 week old mice, to detect collagen fibres. All scale bars 100 µm. BD = bile duct, CV = central vein.
4.3. *Bmi-1* knockout mice exhibit reduced hepatocyte size and ploidy:

4.3.1. *Bmi-1* knockout mice have increased hepatocyte nuclear density:

Initial qualitative assessment of H&E stained sections from *Bmi-1* knockout mice appeared to show abnormalities in the organisation of the liver parenchyma, with many hepatocytes appearing smaller and more uniform in size than in the wild type livers. This was unexpected, given that Bmi-1 was expressed most highly in cholangiocytes in normal murine liver. It was therefore decided to further characterise the hepatocellular abnormalities. To quantify the phenotype, the ‘Cell counter’ feature of ImageJ software (http://rsbweb.nih.gov/ij/) was used to count the total number of nuclei per 400x microscope field in H&E stained sections from *Bmi-1* wild type and knockout mice, at both 8 and 12 weeks of age. This analysis revealed a significant increase in the number of nuclei per field in *Bmi-1* knockout mice at both time points (Figure 20).
Figure 20: *Bmi-1* knockout mice have increased hepatocyte density.

Average number of hepatocyte nuclei in a 400x microscope field for *Bmi-1* wild type (Wt) and knockout (KO) mouse liver tissue. Hepatocyte numbers were counted in 5 randomly selected fields of an H&E stained section for each mouse and averaged. a) 8 week old mice (n=10 animals per group). b) 12 week old mice (n=4). Data are means ± standard deviation, *p<0.05 using 2-tailed Student’s t-test.
4.3.2. *Bmi-1* knockout mice have reduced hepatocyte cell and nuclear size:

The histological observation that *Bmi-1* knockout livers had larger numbers of small hepatocytes led to the hypothesis that these mice showed impaired development of polyploidy. Cell ploidy is correlated with cellular and nuclear size (Rajvanshi et al, 1998), so any reductions in ploidy would potentially provide an explanation for the increased hepatocyte numbers, and hence reduced cell size, observed in the knockout liver sections. The DNA content of a cell is correlated with the size of the nucleus, and cell/nuclear size has been previously used as a measure of polyploidy in a number of publications (Nunez et al, 2000) (Chipchase et al, 2003). It has also been noted that measurement of nuclear size correlates well with FACS analysis of DNA content (Danielsen et al, 1986; Sigal et al, 1999). To investigate further, it was first decided to quantify hepatocyte cell and nuclear size to confirm that these parameters were reduced in *Bmi-1* knockout mice. The polyploidisation process begins around weaning and develops as the mice age (Celton-Morizur et al, 2009), so 12 week old mice were used to assess this phenotype. Nuclear area measurements were carried out on images of hepatocyte touch preparations from freshly isolated livers, using ImageJ software (see appendix section A3 for more detail of procedure). Analysis of a minimum of 20 randomly selected microscope field images per mouse revealed a significant reduction in average nuclear area of *Bmi-1* knockout mouse hepatocytes (Figure 21a). A ranking of the average nuclear areas in individual images illustrates a clear divide between *Bmi-1* wild type and knockout mice (Figure 21b). These results suggested that the average nuclear ploidy in the *Bmi-1* knockout hepatocytes may have been reduced. A similar reduction in average nuclear area was also observed in preliminary analysis of *Bmi-1* knockout mice at 8 weeks of age (data not shown), indicating that the phenotype is not restricted to the most aged animals.

To further characterise the phenotype, the image analysis was repeated for five randomly chosen images per mouse to count the number of nuclei per field classed
above a threshold value of 44 µm². This value was chosen to select only the largest nuclei, based on previous analysis of nuclear area distribution and ploidy in wild type hepatocytes (Nunez et al, 2000). The counts were expressed as a percentage of total nuclei, and are shown in Figure 21c. An average of 12% of wild type hepatocyte nuclei had an area above the threshold, whereas these larger nuclei were rarely observed in hepatocytes from Bmi-1 knockout mice. This result demonstrates a virtual absence of large hepatocyte nuclei, which are potentially of higher ploidy classes, in 12 week old Bmi-1 knockout mice.
Figure 21: *Bmi-1* knockout mouse hepatocytes have reduced nuclear area.

a) Average nuclear area (µm²) of hepatocytes isolated by touch preparation from *Bmi-1* wild type (Wt) and knockout (KO) mice at 12 weeks of age. n=3 female mice, 20 images per mouse. Individual images were averaged to obtain mean nuclear area per mouse. Data presented are means of individual mice ± standard deviation. b) Ranking of all analysed images from highest to lowest average nuclear area. Red = wild type mice, blue = *Bmi-1* knockout. c) Percentage of touch preparation hepatocyte nuclei above 44 µm² in size. n=3 female mice, 5 images per mouse. Data are means of individual mice ± standard deviation, * p<0.05 using 2-tailed Student's t-test.
To confirm whether the reduced hepatocyte nuclear size in Bmi-1 knockout mice correlated with cell size, randomly selected, blindly labelled touch preparation images were used to measure hepatocyte areas. Hepatocytes falling within a defined region of the image were manually outlined (Figure 22a), and the area of each cell calculated using ImageJ software. Hepatocytes from Bmi-1 knockout mice had significantly decreased cell areas compared to wild type littermates (Figure 22b). The distribution of individual hepatocyte areas is shown in Figures 22 c & d, showing an absence of larger hepatocytes in Bmi-1 knockout mice. This also demonstrates that the hepatocytes in the wild type mice show a greater diversity in size, while the Bmi-1 knockout hepatocytes are more uniform. This agrees with the initial observations in H&E stained sections. These results support the hypothesis that polyploidisation is impaired in Bmi-1 knockout mice, given the correlation between cell size and ploidy (Epstein, 1967). They also suggest that the reason for the increased number of nuclei per microscope field in Bmi-1 knockout mice is because the hepatocytes are smaller on average.
Figure 22: Hepatocytes in 12 week old *Bmi-1* knockout mice are smaller in size.

a) Example of hepatocyte area calculation procedure. All hepatocytes falling completely within a 500x500 pixel area of the touch preparation image were manually outlined, and their area calculated using ImageJ software. Scale bar 50 µm. 

b) Average area of hepatocytes in *Bmi-1* wild type (Wt) and knockout (KO) mice. Data represent mean ± standard deviation. n=200 hepatocytes. *p<0.05 using 2 tailed Student’s t-test.

c,d) Number of individual hepatocytes falling within defined area classes for c) wild type and d) *Bmi-1* knockout mice.
4.3.3. *Bmi-1* knockout mouse hepatocytes exhibit fewer centromeres:

To provide evidence that the reduced hepatocyte nuclear size phenotype was related to a decrease in ploidy, *in situ* hybridisation using a pan-centromeric probe was carried out to allow estimation of the chromosome content of wild type and *Bmi-1* knockout hepatocytes. This method has previously been used to analyse ploidy in hepatocytes (Mayhew et al., 2005). As expected, the larger hepatocytes found in wild type mice appeared to have greater numbers of visible centromeres (Figure 23a,b). This was quantified by counting the number of centromeres per hepatocyte nucleus, which showed a significant reduction in average centromere numbers in *Bmi-1* knockout mice (Figure 23c). Plots of the distribution of centromere numbers in individual hepatocytes demonstrate a near absence of nuclei with high centromere numbers in *Bmi-1* knockout mice (Figure 23d,e). Together, these results provide support for the hypothesis that polyploidisation is impaired in *Bmi-1* knockout mouse hepatocytes.
Figure 23: Bmi-1 knockout hepatocytes have reduced centromere numbers.

In situ hybridisation on paraffin embedded 12 week old wild type (a) and Bmi-1 knockout (b) murine liver sections using a pan-centromeric probe, indirectly visualised using DAB. Scale bars 10 μm. c) Quantification of average numbers of visible centromeres per hepatocyte nucleus. Data presented are means of individual mice ± standard deviation. n=4 mice, with a total of 645 hepatocytes of each genotype assessed. Significance assessed using 2-tailed Student’s t-test. d,e) Distribution of individual centromere counts for wild type and Bmi-1 knockout mice.
4.3.4. Hepatocyte binucleation is unimpaired in \( Bmi-1 \) knockout mice:

One well characterised pathway by which polyploid nuclei develop is via the formation of binucleated cells (Guidotti et al, 2003). To assess whether the binucleation process was impaired in \( Bmi-1 \) knockout mice, binucleate cell numbers were manually counted using the previously described touch preparation images. This analysis demonstrated no significant reduction in the frequency of binucleate cells at 12 weeks of age (Figure 24). This result suggests that \( Bmi-1 \) knockout hepatocytes are still able to become binucleate at the same frequency as wild type cells, and that any reduction in the ploidy of \( Bmi-1 \) knockout hepatocyte nuclei did not occur through impaired binucleation.

**Figure 24: Binucleation is unimpaired in \( Bmi-1 \) knockout mouse hepatocytes.**

The numbers of binucleate hepatocytes in touch preparation images from 12 week old female mice (minimum 20 images per mouse) were manually counted, and expressed as a percentage of the total number of cells. \( n=3 \) wild type (Wt) and 3 \( Bmi-1 \) knockout (KO) mice. Data presented are means of individual mice ± standard deviation. Significance assessed using 2-tailed Student’s t-test.
4.3.5. *Bmi-1* knockout livers exhibit increased expression of the ploidy marker *Igfbp2*.

To provide further evidence for a reduction in polyploidisation in *Bmi-1* knockout mice, the expression levels of insulin growth factor binding protein 2 (*Igfbp2*) were examined. *Igfbp2* mRNA expression has previously been proposed as a marker of polyploid cells, as it is upregulated in polyploid nuclei (Kirschner et al, 2007). Quantitative reverse transcriptase PCR (Q-RT-PCR) analysis revealed reduced levels of *Igfbp2* mRNA in *Bmi-1* knockout mouse livers at 12 weeks of age (Figure 25), supporting the previous conclusion that there are fewer polyploid hepatocytes.

![Figure 25: *Bmi-1* knockout mice express reduced levels of *Igfbp2.*](image)

Q-RT-PCR data on cDNA made from mRNA extracted from *Bmi-1* wild type (Wt) and knockout (KO) whole mouse livers for the polyploidy marker *Igfbp2*. Values expressed as transcripts of *Igfbp2* per transcript of reference gene (*eEft2*). Data are means ± SD from 3 animals per group. * p<0.05 using 2-tailed Student’s t-test.
4.3.6. *Bmi-1* knockout mouse livers have normal levels of phosphorylated Akt:

The polyploidisation process is driven by a program of incomplete cytokinesis, but the signals which control this have not been fully elucidated. It seems that insulin may regulate the process via the Akt signalling pathway, as inhibition of Akt signalling in primary rat hepatocytes leads to a reduction in the number of cytokinesis failure events (Celton-Morizur et al, 2009). It has also been observed that depletion of *Bmi-1* can lead to a decrease in the levels of phosphorylated (active) Akt in breast (Guo et al, 2007b) and pancreatic (Song et al, 2010) cancer cell lines. To assess the hypothesis that reductions in Akt signalling could be the cause of the decreased polyploidisation observed in *Bmi-1* knockout mice, levels of phosphorylated Akt (Ser473) protein in whole liver extracts from 8 week old mice were examined using western blotting. No apparent reduction in phospho-Akt levels was observed in mice at 8 weeks of age (Figure 26a), this was quantified by densitometric analysis of the blot (Figure 26b). This finding indicates that any reduction in polyploidisation in *Bmi-1* knockout mouse livers is not due to a reduction in Akt activity.
Figure 26: *Bmi-1* knockout mice exhibit normal levels of phosphorylated Akt.

a) Western blotting of whole liver protein extracts from 8 week old *Bmi-1* wild type and knockout mice for phosphorylated Akt (Ser473) (pAkt). Total Erk1/2 was used as a housekeeping gene. b) Densitometric analysis of blot photograph using ImageJ. Data expressed as mean ± SD of the integrated density of pAkt band divided by Erk1/2 band. Significance assessed using 2-tailed Student’s t-test.
4.4. Expression changes of other Polycomb genes in *Bmi-1* knockout mouse liver:

4.4.1. The *Bmi-1* homologue *Mel-18* is upregulated in *Bmi-1* knockout mouse liver:

The *Bmi-1* knockout mice appeared to present a relatively subtle abnormal hepatic phenotype in comparison to other organs, especially in cholangiocytes where *Bmi-1* was strongly expressed in wild type mice. These observations raised the question of whether other polycomb family members are able to compensate for the loss of *Bmi-1*. The most obvious candidate is the partial *Bmi-1* homologue, *Mel-18*, which shows a large degree of sequence homology to *Bmi-1* and may be functionally redundant in some, but not all, cases (discussed in introduction section 1.10). Potential *Mel-18* compensation was first investigated by using Q-RT-PCR to examine gene expression levels of both *Bmi-1* and *Mel-18* mRNA in *Bmi-1* wild type and knockout mice at three time points. As expected, *Bmi-1* expression was absent in livers of *Bmi-1* knockout mice, while in wild type mice mRNA levels did not change significantly between time points (Figure 27a). *Mel-18* expression was highest in newborn wild type mice, and then decreased dramatically in adult mice (Figure 27b). Newborn *Bmi-1* knockout mice expressed *Mel-18* at similar levels to wild type mice, however the adult knockout mice exhibited upregulation of *Mel-18* relative to the wild types (Figure 27b). This *Mel-18* upregulation was small but significant at 8 weeks of age, and greater in 12 week old mice. To confirm that the increased *Mel-18* mRNA translated to increased protein expression, protein levels of Mel-18 were assessed by western blotting on whole liver extracts. The blots were quantified using densitometry, using Erk1/2 levels as a reference gene. In contrast to the Q-RT-PCR results, there was no upregulation of *Mel-18* protein in 8 week old *Bmi-1* knockout mice (Figure 27c,e). However, mice at 12 weeks of age showed an approximate doubling of *Mel-18* protein expression levels relative to the wild type mice (Figure 27d,e), confirming the Q-RT-PCR result. One
potential explanation is that Mel-18 is being upregulated in the older knockout mice to compensate for the loss of Bmi-1.

To assess whether Mel-18 and Bmi-1 are expressed within the same cellular compartments, the localisation of Mel-18 protein in adult murine liver was assessed using immunohistochemistry (Figure 28). No labelling was observed in slides incubated with an isotype control antibody (Figure 28a). The expression pattern of Mel-18 was virtually identical to that of Bmi-1, with high Mel-18 immunoreactivity observed in all cholangiocytes (Figure 28b). Due to the sequence similarity between Mel-18 and Bmi-1, it was considered possible that the anti-Mel-18 antibody was cross-reacting with Bmi-1. To rule this out, liver sections from Bmi-1 knockout mice were also immunolabelled for Mel-18. The same expression pattern as in the wild type mice was observed (Figure 28c), demonstrating that the anti-Mel-18 antibody was not cross-reacting with Bmi-1. There did not appear to be any clear evidence of Mel-18 protein upregulation in biliary cells of Bmi-1 knockout mice using immunohistochemistry, however it is likely that the antibody is not sensitive enough to detect changes in protein level using this method. Together, these results suggest that Mel-18 may be able to compensate for the absence of Bmi-1, given that potential redundancy between the two proteins has previously been reported (see introduction section 1.10). This may account for the apparent absence of structural biliary abnormalities observed in Bmi-1 knockout mice.
Figure 27: Mel-18 is upregulated in Bmi-1 knockout mice during ageing.

a,b) Q-RT-PCR data on cDNA made from mRNA extracted from Bmi-1 wild type (Wt) and knockout (KO) whole mouse livers from newborn (n=5 animals per group), 8 week (n=7) and 12 week (n=3) old mice, for expression of Bmi-1 (a) and Mel-18 (b). Values expressed as transcripts of gene of interest per transcript of reference gene (eEF2). Data are means ± SD from n animals per group. c,d) Western blotting on whole liver protein extracts from 8 week (c) and 12 week old (d) Wt and KO mice for Mel-18. e) Densitometric analysis of Mel-18 blot photographs using ImageJ software (n=3). Data expressed as mean ± SD of the integrated density of Mel-18 band divided by Erk1/2 band. Significance assessed using 2-tailed Student's t-test, * p<0.05.
**Figure 28: Mel-18 is expressed in cholangiocytes in murine liver.**

Paraffin-embedded sections of 12 week old murine livers labelled using indirect immunohistochemistry to detect Mel-18; antibody visualised with DAB and sections counterstained with haematoxylin. a) Section incubated with isotype control antibody. b) Wild type mouse. c) *Bmi-1* knockout mouse. Mel-18 expression was observed in bile ducts (black arrow). All scale bars 50 µm.
4.4.2. Expression of Bmi-1 homologues *Mbl* and *Nsps1* are not altered in *Bmi-1* knockout liver:

In addition to Mel-18, there are two other published Bmi-1 homologues; MBLR and Nspc1. To investigate whether expression changes in these genes could have a compensatory effect following loss of Bmi-1, mRNA levels of *Mbl* and *Nsps1* were assessed by Q-RT-PCR in 8 week old *Bmi-1* wild type and knockout mouse livers. Both *Mbl* and *Nsps1* were expressed in murine liver, however no alterations in the expression of either mRNA were observed in the knockout liver relative to the wild type (Figure 29a,b). It is possible the presence of MBLR and Nspc1 in *Bmi-1* knockout livers allows them to perform some compensatory functions, however there is no published data suggesting these proteins exhibit functional redundancy with Bmi-1, so it is not possible to draw firm conclusions on this issue.

4.4.3. Expression of other PRC members in *Bmi-1* knockout mouse liver:

There have been no previous studies of the expression changes in other PRC members following *Bmi-1* knockdown in mice, so preliminary Q-RT-PCR studies were performed to ascertain whether other PRC complex members were differentially expressed in *Bmi-1* knockout mouse livers. Initially, the PRC2 members *Eed, Ezh1, Ezh2* and *Suz12*, were chosen for analysis as these are essential core components of the complex. No changes in expression of any of these genes were detected in 8 week old *Bmi-1* knockout livers relative to wild types (Figure 29c). Preliminary analysis of the expression of other PRC1 members was carried out on a single pair of wild type and knockout mice at 8 weeks of age. For the majority of genes, no significant changes in expression were observed (Figure 29d). It was therefore decided not to continue with further analysis of these PRC1 members. There was however an upregulation of the PRC1 member *Cbx7* in the *Bmi-1* knockout livers at 8 weeks of age, which was confirmed in samples from multiple mice (Figure 29e). This suggests
that increased *Cbx7* expression could be part of a compensatory response to the deletion of *Bmi-1*.

**Figure 29: Expression of other Polycomb genes in *Bmi-1* knockout mice.**

Q-RT-PCR data using cDNA made from mRNA extracted from *Bmi-1* wild type (Wt) and knockout (KO) whole mouse livers. Data expressed as transcripts of gene of interest per transcript of reference gene (*eEif2*). a) *Mblr* (*n*=3). b) *Nspc1* (*n*=3). c) Core PRC2 genes in 8 week old mice (*n*=3, all p values >0.05). d) Preliminary analysis of PRC1 members in 8 week old mice (*n*=1). e) *Cbx7* in 8 week old mice (*n*=3). All data except (d) are mean ± SD. * p<0.05 using 2-tailed Student’s t-test.
4.5. Discussion:

4.5.1. Liver function in Bmi-1 knockout mice:

The results from serum analysis of liver function markers hint at some liver damage in 8 week old Bmi-1 knockout mice, based on increased levels of AST. However, as noted earlier, AST is also expressed in other tissues such as cardiac muscle, so it is not necessarily an exclusive marker of liver dysfunction. ALT levels, which are a better indicator of liver damage, did appear slightly increased in Bmi-1 knockout mice, however the change was not statistically significant, suggesting that any damage is mild. There were also no obvious histological signs of liver disease which could account for the elevated levels of these markers in Bmi-1 knockout livers. Serum AP levels were elevated in Bmi-1 knockout mice, which suggests that there may be some impairment in bile flow, possibly due to bile duct damage. However, as noted earlier, serum AP can also be induced in cases of bone disease, and it is known that Bmi-1 knockout mice have defects in skeletal formation (van der Lugt et al, 1994). Levels of serum bilirubin, which can also be increased in bile duct obstruction and cholestasis (Fox et al, 2006) were normal in Bmi-1 knockout mice, suggesting against any biliary damage. There was also no histological evidence of bile duct damage. The potential for impaired bile flow could be assessed in the future using further serum biomarkers, such as gamma-glutamyltransferase (GGT), which can also be elevated in biliary obstruction.

4.5.2. Liver structure and polyploidisation in Bmi-1 knockout mice:

Assessment of blindly labelled H&E stained sections from newborn Bmi-1 knockout mice revealed no obvious defects in hepatic histology. This is unsurprising given that other organs in the Bmi-1 knockout mouse tend to exhibit defects that get progressively worse with ageing (van der Lugt et al, 1994). For example, the knockout mice are born with a normal thymus, which then becomes depleted of mature
thymocytes in adult mice. They also have a progressive haematopoietic defect which only becomes apparent postnatally, and only exhibit an ataxic gait from 2-4 weeks of age. These factors have led to the conclusion that Bmi-1 function is more important in adult tissues than during development; this appears to fit with the lack of obvious phenotype in the livers of newborn Bmi-1 knockout mice.

Despite the fact that Bmi-1 was strongly expressed in cholangiocytes in wild type mice, biliary structures examined appeared to be morphologically normal in both newborn and adult Bmi-1 knockout mice. This was based on expression of the bile duct marker CK19 and histological analysis of blindly labelled H&E sections. This result suggests that Bmi-1 is not essential for normal bile duct development or homeostasis. Instead, the phenotypic alterations in adult Bmi-1 knockout mice were found in hepatocytes. This is interesting given that Bmi-1 expression in wild type hepatocytes appeared much weaker than in cholangiocytes, and was not clearly observed in all cells. Examination of H&E stained sections revealed abnormal organisation within the parenchyma, with hepatocytes appearing smaller and more uniform in size. Image analysis of individual hepatocytes isolated using touch preparations demonstrated that the hepatocyte nuclei in Bmi-1 knockout mice are on average smaller in area than the wild types. Given that nuclear size is correlated with polyploidy, this result infers that Bmi-1 knockout mice suffer from an impaired liver polyploidisation process. This hypothesis was supported by data demonstrating that Bmi-1 knockout livers have on average fewer centromeres per hepatocyte, suggesting reduced DNA content. Bmi-1 knockout livers also expressed lower levels of Igfbp2, a marker associated with polyploid cells. This ploidy phenotype appears to be due to a lack of accumulation of larger polyploid nuclei as opposed to a reduction in hepatocyte binucleation, as binucleate cell numbers were unchanged. A more comprehensive analysis of the different distribution of 2n, 4n, 8n etc. hepatocytes could be achieved in future through FACS analysis, although this is unable to distinguish between some
types of mononuclear and binuclear hepatocytes (for example 4n mononuclear vs. 2x2n binuclear cells) (Funk-Keenan et al, 2008).

The function of polyploidy in hepatocytes has been the subject of debate and is not well understood (see introduction section 1.5). It is therefore difficult to draw conclusions, both in terms of the reasons for the reduced polyploidisation and the functional consequences of it for the Bmi-1 knockout mice. The signalling pathways that control the generation of polyploid cells have not been well defined, however one factor that does appear to be involved is insulin signalling. Rats treated with streptozotocin to destroy pancreatic β-cells have decreased numbers of binucleated tetraploid hepatocytes, as do ob/ob mice with impaired insulin signalling (Celton-Morizur et al, 2009). Furthermore, injection of insulin into rats increases the number of tetraploid hepatocytes, providing strong evidence for the involvement of insulin signalling in the tetraploidisation process. Bmi-1 knockout mice do suffer from reduced pancreatic β-cell mass, and their serum insulin levels fail to increase in response to glucose challenge (Dhawan et al, 2009). It was therefore considered possible that reduced insulin levels were responsible for the impaired polyploidisation process in Bmi-1 knockout mice. Moreover, Celton-Morizur et al also report that the PI3K/Akt pathway lies downstream of insulin signalling to regulate the generation of tetraploid cells. This was based on data showing a correlation between ploidy and levels of phospho-Akt (Ser473) in rodents with either impaired or increased insulin signalling, and that inhibition of Akt signalling in cultured hepatocytes reduces the number of cytokinesis failure events that lead to polyploidisation. Depletion of BMI-1 in human cell lines also leads to a reduction in phospho-Akt levels (Guo et al, 2007b), suggesting that impaired Akt signalling could be a factor in the ploidy defects in Bmi-1 knockout mice. However, Bmi-1 knockout mice did not exhibit any changes in phospho-Akt levels, based on western blotting analysis. This appears to show that reduced activity of the Akt signalling pathway is not responsible for the impaired
polyploidisation process in Bmi-1 knockout hepatocytes, and that if insulin signalling is involved then it must act via other pathways. It cannot be ruled out that Akt signalling is only impaired in a small number of Bmi-1 knockout hepatocytes, and that phospho-Akt levels would be normal in most liver cells. Western blotting may not have been sensitive enough to detect small variations of this type, and the antibody failed to work for immunohistochemistry.

It is possible there is a link between the mass and growth rates of Bmi-1 knockout mice and their reduced polyploidisation. Mice strains selected for low body weight gain (both between birth and 10 days, and 28 and 56 days of age) appear to show increased numbers of 2C cells (C = DNA content), and reduced 4C, at 56 days of age (Funk-Keenan et al, 2008). One study has also found a strong correlation between the postnatal growth rate of animals and mean cell ploidy (Vinogradov et al, 2001). However, their analysis was based on comparing growth rates and ploidy levels across different mammalian species, so the relevance of these results within a single species is unclear. Nevertheless, growth rates of Bmi-1 knockout mice were reduced relative to wild type littermates, so it would appear that this could be a factor in the impaired polyploidisation phenotype. The smaller size of Bmi-1 knockout mice means that the mice have smaller livers, as the liver to body weight ratio remained constant. Given that polyploidisation is a means of increasing mass through hypertrophic growth, it is possible that there is a reduced requirement for this process in the Bmi-1 knockout mice. As discussed in the introduction, there have also been some suggestions that polyploid hepatocytes have improved function compared to diploid cells, based on upregulation of some key liver-specific genes (Anatskaya & Vinogradov, 2010). If this was the case, then the reduced levels of polyploid hepatocytes in Bmi-1 knockout mice could be associated with impaired liver function. However, this link remains tenuous at present, as the studies are not entirely convincing, being based on comparisons between livers of different species. Diploid
hepatocytes also synthesise higher levels of proteins such as albumin than polyploid ones (Rajvanshi et al, 1998), while other work has shown a negative correlation between hepatic ploidy and metabolic scope when comparing across different species (Vinogradov et al, 2001), so the link between ploidy and cell function is not conclusive.

4.5.3. Similarities to mice with inactivated c-myc:

The hepatic phenotype in Bmi-1 knockout mice, particularly in terms of abnormal cell size and ploidy, bears similarities with that described by Baena et al in mice with inactivated c-myc (Baena et al, 2005). The authors bred c-mycfl/fl mice with inducible mx-cre transgenic mice to create a system capable of c-myc deletion in the liver, although it should be noted that c-myc did not appear to be deleted in all hepatocytes. In addition, the mice also showed c-myc deletion in the bone marrow, so it is possible that secondary effects could be contributing to the hepatic phenotype. Following postnatal c-myc deletion, the authors found that 10 week old mice exhibit a large reduction in total body weight, and a small but significant increase in liver to body weight ratio. The mice also have abnormalities in parenchymal organisation, reduced hepatocyte size and a reduced population of polyploid cells.

The results described above show similarities with the phenotype observed in Bmi-1 knockout mouse livers, in particular in terms of the reduced mouse body weight, reduced hepatocyte size and polyploidy. These similarities are interesting given the long established relationship between c-myc and Bmi-1. Bmi-1 was initially discovered as an oncogene cooperating with c-myc in the generation of mouse lymphomas (van Lohuizen et al, 1991b). It has since been shown that the human BMI-1 promoter contains a c-myc binding site and that c-myc can positively regulate BMI-1 expression (Guo et al, 2007a), demonstrating that c-myc can act as an upstream regulator of Bmi-1. It therefore appears plausible that c-myc and Bmi-1 could be collaborating in the regulation of some processes in murine liver, which leads to similar phenotypic
abnormalities when either is deleted. It would be interesting in future to examine Bmi-1 expression in c-myc deficient mice to assess this hypothesis further. It is also notable that the mice with c-myc deletion have severe anaemia and die after 3 months, similar to Bmi-1 knockout mice. This is likely to be due to deletion of c-myc in the bone marrow rather than liver, however again illustrates similarities between the two mouse strains.

4.5.4. Expression of other Polycomb genes in Bmi-1 knockout livers:

As described earlier, the livers of newborn Bmi-1 knockout mice appeared phenotypically normal, as in other tissues. There were also no obvious abnormalities in biliary structures in adult Bmi-1 knockout mice, despite the fact that Bmi-1 was highly expressed in cholangiocytes in wild type animals. These observations suggest that other pathways can compensate for the deletion of Bmi-1. It is possible that this includes compensation by other members of the Polycomb group family. The most likely candidate is the partial Bmi-1 homologue, Mel-18, which is structurally very similar and therefore would seem likely to have similar functions. There is some debate over the function of Mel-18, with studies suggesting it may act as an oncogene or tumour suppressor in different contexts (see introduction section 1.10.1). However, the fact that Mel-18 and Bmi-1 knockout mice show many similar defects, and that double knockout mice exhibit more severe versions of them, suggests the proteins can act in synergy. In addition to this, some human cell lines (such as WI-38 lung fibroblasts) only show proliferative defects when both BMI-1 and MEL-18 are depleted (Wiederschain et al, 2007). These data suggest that functional redundancy between the two proteins exists in some contexts. Q-RT-PCR analysis of wild type mice showed high Mel-18 mRNA expression in newborn animals, and much lower expression in adult mice. This result suggests that Mel-18 may be more important in fetal development, which could possibly be a factor in the lack of noticeable phenotypic abnormalities observed in newborn Bmi-1 knockout mice. Upregulation of
Mel-18 mRNA was not found in newborn Bmi-1 knockout mice relative to wild types. In contrast, adult knockout mice (8 and 12 weeks) did have higher levels of Mel-18 mRNA expression than wild types. This hints that Mel-18 could be upregulated in older Bmi-1 knockout mice to compensate for the loss of Bmi-1. However, this was only found at the protein level in 12 week old mice, based on western blotting analysis. The physiological importance of this upregulation is therefore unclear, especially given that the mice become generally unwell by this age. Of more interest is the discovery that the Mel-18 expression pattern in murine liver is the same as for Bmi-1, with strong expression in cholangiocytes. This finding advances the possibility that Mel-18 may be able to compensate for the loss of Bmi-1 in cholangiocytes. Future in vitro work could further this work by examining whether knockdown of either Bmi-1 and/or Mel-18 leads to similar phenotypic effects in cultured hepatic cell populations, as this has not specifically been done in liver cells before. We have recently established a collaboration (with Vasanta Subramanian, University of Bath) which will provide access to Mel-18 knockout mice, generating future opportunities to assess whether these mice suffer similar hepatic abnormalities, which would suggest cooperation between the two proteins in liver function.

It is also possible that other Polycomb proteins may be involved in compensating for the loss of Bmi-1. Two other Bmi-1 homologues, MBLR and Nspc1, were expressed at the mRNA level in murine livers, although these were not upregulated in Bmi-1 knockout mice. Functional redundancy between these proteins and Bmi-1 has not previously been investigated. The majority of other Polycomb genes examined by Q-RT-PCR were also expressed in liver, but were not upregulated in Bmi-1 knockout mice. The exception was the PRC1 component Cbx7, which showed increased expression in 8 week old Bmi-1 knockout mouse livers compared to wild types. Cbx7 is able immortalise mouse fibroblasts via repression of the Ink4a/Arf locus, and Cbx7 overexpression can also bypass the premature senescence observed in Bmi-1 null
fibroblasts in culture (Gil et al, 2004). This suggests that Cbx7- and Bmi-1-containing complexes may act independently, and that Cbx7 upregulation could be part of a compensatory mechanism in Bmi-1 knockout mice. This could be examined in more detail in future by looking at the localisation of Cbx7 protein in Bmi-1 knockout liver tissue.

4.6. Summary of Chapter:

In summary, Bmi-1 knockout mice did not exhibit obvious histological abnormalities in biliary structure. This could potentially be due to compensatory effects of Mel-18, which has a similar expression pattern. Instead, the major structural defect was within the hepatic parenchyma, with increased numbers of hepatocytes per microscope field found in Bmi-1 knockout livers. Analysis of hepatocyte cell and nuclear size and centromere numbers suggested that the Bmi-1 knockout mice suffer from impaired polyploidisation. It is unclear if this phenotype is directly caused by the loss of hepatocyte Bmi-1 expression, or is a secondary effect arising from other defects in the mice. The apparently low levels of Bmi-1 in normal hepatocytes, together with the fact that parameters such as reduced growth rate and insulin levels have been linked with reduced ploidy, suggests that it could be a secondary effect. However, the fact that mice with hepatic deletion of c-myc exhibit similar defects is interesting given the established link between c-myc and Bmi-1, and suggests that Bmi-1 could play a direct role in hepatocyte polyploidisation. This link could be further assessed in future by examining Bmi-1 expression levels in mice following c-myc deletion, or by attempting to rescue defects in the c-myc deficient mice by overexpressing Bmi-1. In the longer term, the creation of a liver specific Bmi-1 knockout mouse could further examine these issues without the complication of defects in other organs. In vitro experiments involving knockdown of Bmi-1 in primary hepatocytes which exhibit different ploidy classes would also help address whether Bmi-1 has a direct role in polyploidisation.
Chapter 5

The effect of *Bmi-1* deletion on the cell cycle in murine liver
5. The effect of Bmi-1 deletion on the cell cycle in murine liver:

5.1. Hepatocyte proliferation in Bmi-1 knockout mice:

The loss of Bmi-1 in cultured cells tends to result in a reduction in self-renewal and proliferation. The same effect is seen in vivo, for example Bmi-1 knockout mice exhibit reduced proliferation of pancreatic β-cells (Dhawan et al, 2009) and granule cell precursors in the cerebellum (Leung et al, 2004). There have been no in vitro or in vivo studies examining the effects of Bmi-1 depletion on normal hepatocytes or cholangiocytes, however Bmi-1 knockdown does lead to reduced proliferation in HCC (Sasaki et al, 2008a) and CC (Sasaki et al, 2009) cell lines. To investigate differences in the proliferation of liver cells following Bmi-1 deletion in vivo, Bmi-1 wild type and knockout mice were injected with BrdU one hour prior to sacrifice, and tissue sections were subsequently labelled with an antibody against BrdU to identify cells undergoing DNA synthesis (examples in Figure 30a,b). The slides were labelled blindly to prevent bias, and results were quantified by counting the number of BrdU positive hepatocyte nuclei across five randomly selected 400x microscope fields per mouse. The results were adjusted for the increased number of nuclei per field observed in the Bmi-1 knockout mice (see chapter 4). There were no qualitative differences in proliferation in both newborn and p7 Bmi-1 knockout mice, although it was not possible to quantify this in hepatocytes due to the large number of BrdU positive cells in regions of haematopoiesis. At 8 weeks of age, Bmi-1 knockout mice showed a small but significant increase in hepatocyte BrdU incorporation compared to wild types, indicating increased hepatocyte proliferation, although there was some heterogeneity among different knockout animals (Figure 30c). However, by 12 weeks of age the knockout mice had decreased hepatocyte proliferation compared to the wild types (Figure 30d). To provide validation for these data, tissue sections were also labelled with an antibody against the proliferation marker Ki67, a protein present in all phases of the cell cycle (Scholzen & Gerdes, 2000). The Ki67 immunolabelling data confirmed
an increase in hepatocyte proliferation in 8 week old $Bmi-1$ knockout mice, and a
decrease in 12 week old mice (Figure 30e,f).

These results demonstrate that the loss of $Bmi-1$ does not lead to a decrease in
hepatocyte proliferation in mice up to 8 weeks of age. In contrast, there was actually a
slight increase in hepatocyte proliferation in 8 week old $Bmi-1$ knockout mice. By 12
weeks of age, a decrease in hepatocyte proliferation was observed in $Bmi-1$ knockout
mice. It is possible that this may be secondary to the physiological consequences of
$Bmi-1$ deletion in other organs, as the health of the mice progressively declines with
age, leading them to become visibly unwell by 12 weeks of age.
Figure 30: *Bmi-1* knockout mice show altered hepatocyte proliferation.

a,b) Examples of immunolabelling to detect BrdU in *Bmi-1* wild type (a) and knockout (b) paraffin-embedded liver sections. Antibody visualised using DAB and counterstained with haematoxylin. Scale bars 50 µm. c-d) Average percentages of hepatocyte nuclei positive for BrdU in a 400x microscope field for wild type (Wt) and *Bmi-1* knockout (KO) mice. Hepatocytes were counted in 5 randomly selected microscope fields per section at 8 weeks (c, n=10) and 12 weeks (d, n=6) of age. Data are means ± SD. e,f) Incorporation of Ki67 in hepatocyte nuclei. Results were obtained using the same method as for BrdU in 8 week (e, n=7) and 12 week (f, n=6) old mice. * p<0.05 using 2-tailed Student’s t-test.
5.2. Cholangiocyte proliferation in *Bmi-1* knockout mice:

Due to the high expression in cholangiocytes, it was postulated that the loss of Bmi-1 could lead to impairment in the proliferation of normal cholangiocytes, as occurs in CC lines depleted of Bmi-1 (Sasaki et al, 2009). To investigate this hypothesis, BrdU and Ki67 labelled liver sections from *Bmi-1* wild type and knockout mice were examined. However, the proliferation rate of wild type cholangiocytes is very low *in vivo* under resting conditions, having been previously reported as 0.4% (Glaser et al, 2007). Because of this, and the relatively low number of visible cholangiocytes per tissue section, there were insufficient numbers of cholangiocytes which incorporated BrdU or Ki67 to perform any meaningful quantification, as many sections even in wild type mice did not contain any proliferating cholangiocytes. It was however observed that sporadic cholangiocytes in *Bmi-1* knockout mice expressed Ki67 (Figure 31), although these were not seen on the majority of sections. This observation suggests that *Bmi-1* deficient cholangiocytes retain some capacity to proliferate, however more work would be required to address this issue.

![Image](image_url)

**Figure 31: Cholangiocytes in *Bmi-1* knockout mice sporadically express Ki67.**

Indirect immunolabelling of paraffin-embedded liver sections to detect Ki67. Antibody visualised with DAB and sections counterstained with haematoxylin. Example of tissues from a) a *Bmi-1* wild type mouse and b) a *Bmi-1* knockout mouse, showing Ki67 positive cholangiocytes (black arrow). Scale bars 50 µm.
5.3. Expression changes of cyclin D1 and c-myc in Bmi-1 knockout mice:

5.3.1. Cyclin D1 protein is overexpressed in Bmi-1 knockout mouse livers:

The increased hepatocyte proliferation observed in livers of 8 week old Bmi-1 knockout mice raised the question of whether any proteins involved in facilitating progression of the cell cycle were upregulated. To examine this, the expression of cyclin D1 was assessed by western blotting and immunohistochemistry. Cyclin D1 forms complexes with Cdk4 or Cdk6, which are required for progression through the G1 phase of the cell cycle. Its expression has previously been found to be decreased in pancreatic cancer cell lines depleted of Bmi-1 (Song et al, 2010). In contrast to this result, liver extracts from 8 week old Bmi-1 knockout mice contained increased levels of cyclin D1 protein in comparison to wild type mice, as measured using western blotting (Figure 32a,b). Immunohistochemical analysis revealed that the majority of hepatocytes in 8 week old Bmi-1 wild type mice were negative for cyclin D1, with only occasional hepatocytes displaying weak immunoreactivity (Figure 32e,g). In Bmi-1 knockout mice, the number of positive hepatocytes was greatly increased (Figure 32f,h,i), confirming the western blotting data. These results show that cyclin D1 expression is increased in 8 week old Bmi-1 knockout mouse livers, which is consistent with the increased hepatocyte proliferation.
**Figure 32 (overleaf): Cyclin D1 is overexpressed in Bmi-1 knockout mice.**
a) Western blotting of whole liver protein extracts from 8 week old Bmi-1 wild type and knockout mice for cyclin D1. Total Erk1/2 was used as a housekeeping gene. b) Densitometric analysis of blot photograph using ImageJ software. Data expressed as mean ± SD of the integrated density of cyclin D1 band divided by Erk1/2 band. Significance assessed using 2-tailed Student’s t-test, * p<0.05. c-h) Indirect immunohistochemistry on paraffin-embedded liver sections to detect cyclin D1. Antibody visualised with DAB and counterstained with haematoxylin. c,e,g) Sections from wild type mouse. d,f,h) Sections from Bmi-1 knockout mouse. c,d) Sections incubated with isotype control antibody instead of cyclin D1. All scale bars 50 µm. i) Quantification of percentage of cyclin D1 positive nuclei in Bmi-1 wild type (Wt) and knockout (KO) mice. n=4 mice, 10 images per mice. Significance assessed using 2-tailed Student’s t-test, * p<0.05.
5.3.2. *c-myc* is overexpressed in *Bmi-1* knockout mouse livers:

Preliminary analysis of expression changes in the pro-proliferative transcription factor *c-myc* were also carried out using Q-RT-PCR. This revealed a significant upregulation of *c-myc* mRNA expression in *Bmi-1* knockout livers from 8 week old mice (Figure 33). Future work will assess whether this upregulation is maintained at the protein level. *C-myc* has an important role in cell cycle progression, so its upregulation would again be consistent with increased hepatocyte proliferation in 8 week old *Bmi-1* knockout mice.

![Figure 33: *c-myc* mRNA is overexpressed in *Bmi-1* knockout mice.](image)

Q-RT-PCR data on cDNA made from mRNA extracted from *Bmi-1* wild type (Wt) and knockout (KO) whole mouse livers at 8 weeks of age (n=5 mice per group). Values expressed as molecules of gene of interest per molecule of reference gene (*eEF2*). Data are means ± SD from 3 animals per group. * p<0.05 using 2-tailed Student’s t-test.
5.4. Expression of cell cycle inhibitors in Bmi-1 knockout livers:

5.4.1. $p16^{ink4a}$, $p19^{Arf}$ and $p21$ are upregulated in Bmi-1 knockout mouse liver:

The absence of any decrease in proliferation in hepatocytes in 8 week old Bmi-1 knockout mice appeared to be at odds with the well characterised role of Bmi-1 in repressing the expression of cell cycle inhibitors. These include the Ink4a/Arf locus, which encodes $p16^{ink4a}$ and $p19^{Arf}$ (Jacobs et al, 1999a), and $p21$ (Fasano et al, 2007). It was therefore expected that expression of these would increase in Bmi-1 knockout mice. To determine whether this was the case, Q-RT-PCR was used to assess RNA expression of $p16^{ink4a}$, $p19^{Arf}$, and $p21$ at three time points (Figure 34). In wild type mouse livers, $p16^{ink4a}$ expression was negligible in newborn and adult mice. In the Bmi-1 knockout livers, significant upregulation of $p16^{ink4a}$ was observed at all three time points (Figure 34a). This result agrees with published data that Bmi-1 represses the Ink4a/Arf locus (Jacobs et al, 1999a). A similar result was seen for $p19^{Arf}$ in mice of 8 and 12 weeks of age, although there was no upregulation of this gene in newborn Bmi-1 knockout mice (Figure 34b). Low expression of $p21$ was observed in both Bmi-1 wild type and knockout newborn mice, and levels remained low in the wild type in older mice (Figure 34c). However, there was a small but significant upregulation of $p21$ mRNA in 8 week old Bmi-1 knockout mice, and then a much more dramatic upregulation at 12 weeks of age. Therefore, deletion of Bmi-1 results in increased expression of several cell cycle inhibitors at the mRNA level.
Figure 34: *p16*<sup>ink4a</sup>, *p19*<sup>Arf</sup> and *p21* are upregulated in *Bmi-1* knockout mice.

Q-RT-PCR data on cDNA made from mRNA extracted from *Bmi-1* wild type (Wt) and knockout (KO) whole mouse livers for a) *p16*<sup>ink4a</sup>, b) *p19*<sup>Arf</sup> and c) *p21*. All genes were assessed in livers from newborn (n=5 animals per group), 8 week (n=7) and 12 week (n=3) old mice. Values expressed as molecules of gene of interest per molecule of reference gene (*eEF1α*). Data are means ± SD from n animals per group. * p<0.05 using 2-tailed Student’s t-test.
5.4.2. Expression of p16\(^{\text{ink4a}}\) and p21 proteins are increased in Bmi-1 knockout livers:

Western blotting was then used to assess whether these results were also relevant at the protein level. Expression of p16\(^{\text{ink4a}}\) protein was strongly upregulated in Bmi-1 knockout liver protein extracts at both 8 and 12 weeks of age (Figure 35a,b), in agreement with the Q-RT-PCR results. The same result was observed with p21 protein (Figure 35c,d), although in this case the degree of upregulation appeared similar in knockout mice at both 8 and 12 weeks of age. This was confirmed by densitometric analysis of the p21 blot (Figure 35e). This result was not completely in agreement with the Q-RT-PCR results, where the p21 upregulation was much more dramatic in 12 week old Bmi-1 knockout livers, suggesting the possibility of post-transcriptional regulation. Attempts to detect p19\(^{\text{Arf}}\) protein by western blotting were unsuccessful.
Figure 35: p16^{Ink4a} and p21 protein are overexpressed in Bmi-1 knockout mice. 

a-d) Western blotting on whole liver protein extracts from Bmi-1 wild type (Wt) and knockout (KO) for p16^{Ink4a} (a,b) and p21 (c,d). Erk1/2 was used as a housekeeping gene. a,c) 8 week old mice. b,d) 12 week old mice. e) Densitometric analysis of p21 blot photographs using ImageJ software (n=3). Data expressed as mean ± SD of the integrated density of the p21 band divided by Erk1/2 band. Significance assessed using 2-tailed Student’s t-test, *p<0.05. N.B. Densitometric analysis of the p16^{Ink4a} band was not robust due to the close proximity to a non-specific band (data not shown).
5.4.3. Localisation of p16\textsuperscript{\textit{Ink4a}} and p21 in \textit{Bmi-1} knockout livers:

The Q-RT-PCR and western blotting results both confirmed the upregulation of various cell cycle inhibitors in \textit{Bmi-1} knockout mouse livers. To investigate the relevance of these data, especially with regard to the increased proliferation observed in \textit{Bmi-1} knockout hepatocytes, immunohistochemistry was used to examine the localisation of p16\textsuperscript{\textit{Ink4a}} and p21 within mouse livers. It was again not possible to assess p19\textsuperscript{\textit{Arf}} localisation due to a lack of robust antibodies.

As expected given the western blotting results, p16\textsuperscript{\textit{Ink4a}} labelling was absent in paraffin-embedded liver sections from wild type mice at 8 weeks of age (Figure 36c). Immunolabelling of liver sections from \textit{Bmi-1} knockout mice revealed that p16\textsuperscript{\textit{Ink4a}} was predominantly expressed in non-parenchymal cells within the liver parenchyma (Figure 36d). There was also sporadic expression of p16 in individual cholangiocytes (Figure 36e), although many bile ducts were completely negative (Figure 36f). There was no p16\textsuperscript{\textit{Ink4a}} immunolabelling observed in hepatocytes, suggesting that the overexpression of p16\textsuperscript{\textit{Ink4a}} would not affect hepatocyte proliferation in \textit{Bmi-1} knockout livers.

Expression of p21 was virtually absent in liver sections from wild type mice at 8 weeks of age, with only occasional hepatocytes within the parenchyma showing detectable nuclear expression (Figure 37c,e). In \textit{Bmi-1} knockout mouse livers, p21 was predominantly expressed in hepatocytes. There appeared to be greater heterogeneity in the hepatocyte labelling, with the parenchyma consisting of a mixture of positive and negative nuclei, and some of the positive cells showing stronger p21 expression than others (Figure 37d,f). This work has confirmed the p21 upregulation seen in the Q-RT-PCR and western blotting.
Figure 36: Localisation of p16^{ink4a} protein in Bmi-1 knockout mouse livers.

Paraffin-embedded 8 week old mouse liver tissue sections labelled to detect p16^{ink4a} using indirect immunohistochemistry. Antibody visualised with DAB and sections counterstained with haematoxylin. a) Absence of p16^{ink4a} labelling in wild type mouse. b) p16^{ink4a} expression in non-parenchymal cells (blue arrow) in Bmi-1 knockout mouse. c) p16^{ink4a} positive (red arrow) and negative (black arrow) cholangiocytes in Bmi-1 knockout liver. d) Bmi-1 knockout bile duct with complete absence of p16^{ink4a} expression. All scale bars 50 µm.
Figure 37: Localisation of p21 protein in *Bmi-1* knockout livers.

See next page for figure legend.
Figure 37 (overleaf): Localisation of p21 protein in Bmi-1 knockout livers.

Paraffin-embedded 8 week old mouse liver tissue sections labelled using indirect immunohistochemistry to detect p21. Antibody visualised with DAB and sections counterstained with haematoxylin. a-b) Sections incubated with isotype control antibody instead of p21. c-f) Sections from 8 week old mice at low (a,b) and high (c,d) magnification. c,e) p21 labelling was generally absent in hepatocyte nuclei wild type livers. d,f) p21 expression in numerous hepatocyte nuclei (black arrows) in 8 week old Bmi-1 knockout mouse livers. All scale bars 50 µm.
5.5. Discussion:

5.5.1. Changes in proliferative frequency in \textit{Bmi-1} knockout livers:

The analysis of BrdU incorporation and Ki67 expression revealed a small but significant increase in hepatocyte proliferation in 8 week old \textit{Bmi-1} knockout mice. It should however be noted that this was not a dramatic increase, and that the level of hepatocyte proliferation in wild type mice was already very low. It is therefore uncertain whether the small increase in hepatocyte proliferation in \textit{Bmi-1} knockout mice would have significant physiological consequences. There was also a degree of heterogeneity in hepatocyte proliferation between different individual \textit{Bmi-1} knockout mice, reflected by the large standard deviation. It is however clear that the loss of \textit{Bmi-1} did not lead to a reduction in hepatocyte proliferation in 8 week old livers. This appears at odds with several \textit{in vitro} and \textit{in vivo} studies demonstrating that \textit{Bmi-1} depletion leads to reduced proliferation, both in hepatic cells (Chiba et al, 2007) and cells from other tissues (e.g. external granular layer cells in the cerebellum (Leung et al, 2004)). However, it is worth noting that proliferation in the exocrine pancreas is also unchanged following \textit{Bmi-1} deletion (Dhawan et al, 2009). \textit{Bmi-1} did appear to be expressed at low levels in hepatocytes, so it is unclear how much influence it has on hepatocyte proliferation. It is therefore possible that any proliferative increase in \textit{Bmi-1} knockout mice is not directly due to the loss of \textit{Bmi-1}, and is a secondary effect. It is however worth considering that most \textit{in vitro} studies on the effects of \textit{Bmi-1} depletion or overexpression have been in putative liver stem cell lines or tumour cells, so its function may be slightly different under physiological conditions, or in the context of a more differentiated cell such as the hepatocyte. Indeed, Chiba et al report that transduction of \textit{Bmi-1} only affects proliferation and colony forming potential in foetal liver cell fractions which give rise to hepatic colony forming units in culture (H-CFU-C), a characteristic of stem cell populations (Chiba et al, 2007). This suggests that \textit{Bmi-1} knockdown may have less consequence for the proliferation of liver cells with more
limited growth potential. In 12 week old mice, there was a decrease in hepatocyte proliferation in Bmi-1 knockout animals compared to wild types. The reasons for the difference between this and 8 weeks are unclear, however it may be secondary to the fact that the knockout mice develop increasingly abnormal physiological consequences during ageing.

The precise reasons for the small increase in hepatocyte proliferation in 8 week old Bmi-1 knockout mice are unclear, although it is possible to speculate on some potential causes. A number of hepatocytes in Bmi-1 knockout mice expressed the cell cycle inhibitor p21, so their proliferation may be inhibited. In this case, it is possible that the proliferation of non-p21 expressing hepatocytes may increase as a compensatory response. There is also some lineage tracing evidence that a portal stem/progenitor cell contributes to hepatocyte turnover in resting liver (see introduction section 1.3.5). Bmi-1 has an important role in the maintenance of hepatic stem cell populations in vitro, so it is possible that the putative portal stem cell population is reduced or defective in Bmi-1 knockout mice. This could be demonstrated experimentally in the future using lineage tracing techniques, based on the stable genetic labelling of cells expressing putative hepatic stem/progenitor cell markers, such as Sox9 (Furuyama et al, 2011). Initial attempts to assess defects in portal stem cell populations using lineage tracing of mitochondrial mutations were unsuccessful (see appendix section A1). If the stem cell response was in fact compromised, then the increased hepatocyte proliferation in Bmi-1 knockout mice may compensate for the loss of the putative portal stem cell contribution to hepatocyte turnover.

It is also possible that there is a link between the reduction in polyploid cell numbers observed in Bmi-1 knockout mice (Chapter 4) and the increased hepatocyte proliferation at 8 weeks of age. Polyploidy has often been considered to represent a shift towards reduced proliferative potential and terminal differentiation (Gupta, 2000),
and diploid rat hepatocytes have higher proliferative potential than normal hepatocytes in culture (Rajvanshi et al, 1998) (Gerlyng et al, 1993). However, hepatocytes of all ploidy classes can contribute equally to repopulate damaged mouse livers (Duncan et al, 2009a). It is possible there are differences between the proliferative potential of polyploid cells in rats and mice, although in general, the published data seem to suggest that hepatocytes of all ploidy classes maintain the ability to proliferate, but the proliferative capacity of diploid hepatocytes is higher. Therefore, it is possible that reduced polyploidy in Bmi-1 knockout mice results in hepatocytes with higher proliferative potential. The onset of polyploidy at weaning is also correlated with a shift from hyperplastic to hypertrophic liver growth in rodents (Funk-Keenan et al, 2008). Given the reduced polyploidy in Bmi-1 knockout mice, it is possible that they have a greater requirement for hyperplastic growth (represented by increased proliferation) to compensate for the reduced hypertrophic growth (represented by polyploidisation). It is also unclear what proportion of hepatocytes usually express Bmi-1 under resting conditions due to the limitations of the immunohistochemical analysis, so it cannot be determined whether the hepatocytes able to proliferate are those that do not normally express Bmi-1.

The points above have discussed some speculative reasons for an increase in hepatocyte proliferation in Bmi-1 knockout mice. Given that previous studies in hepatic cell lines and other organs have associated loss of Bmi-1 with reduced proliferation, it seems more likely that the alterations in hepatocyte proliferation in Bmi-1 knockout mice are not cell-autonomous. With regards to cholangiocyte proliferation, conclusions cannot be drawn due to the low levels of proliferation in wild type mice. Given the high levels of Bmi-1 expression in cholangiocytes, the expectation is that cholangiocyte proliferation should decrease in Bmi-1 knockout mice. However, occasional Ki67 positive cholangiocytes were observed in Bmi-1 knockout mice, suggesting that some may maintain the capacity to proliferate. This
will have to be investigated in future work, potentially through performing bile duct ligations on *Bmi-1* knockout mice to induce cholangiocyte proliferation. It would also be interesting to use FACS analysis to further examine the cell cycle status of *Bmi-1* deficient hepatic cells, to provide support for the *in vivo* data based on BrdU incorporation. One very recent study has noted that cultured *Bmi-1* null haematopoietic stem cells (HSCs) exhibit an increase in cell cycling (decreased number of cells in G₀) compared to control cells (Chagraoui et al, 2011). The *Bmi-1* deficient HSCs then accumulate in G₂, suggesting a defective G₂/M checkpoint, and suffer from chromosome instability. It would be interesting to assess whether something similar is occurring in *Bmi-1* knockout hepatocytes, given that the BrdU and Ki67 data suggests an increase in the numbers of cycling hepatocytes in *Bmi-1* knockout mice.

5.5.2. Upregulation of cyclin D1 and c-myc in *Bmi-1* knockout livers:

The livers of 8 week old *Bmi-1* knockout mice exhibited increased levels of c-myc mRNA and cyclin D1 protein. These findings are consistent with the increase in hepatocyte proliferation observed in 8 week old knockout mice. As detailed in the introduction, the most well characterised Bmi-1 targets have generally been associated with inhibition of the cell cycle. It therefore would appear unlikely that the upregulation of cyclin D1 and c-myc is directly due to the loss of Bmi-1 mediated repression. There is no direct evidence in the literature that cyclin D1 is a direct target of Bmi-1, although a recent study has found a putative Bmi-1 response element (BRE) in the promoter of the related cyclin D3 gene (Meng et al, 2010), and suggests that a few other genes involved in promotion of the cell cycle, such as Cdk6, may be Bmi-1 targets. However, the majority of cell cycle related genes identified in this study are inhibitory, supporting the consensus view that Bmi-1 represses cell cycle inhibitors. Cooperation between c-myc and Bmi-1 has been previously characterised (see introduction section 1.8), however it is thought that c-myc acts upstream of Bmi-1, as
the human \textit{BMI-1} promoter contains a binding site for c-myc (Guo et al, 2007a). The upregulation of c-myc mRNA in the \textit{Bmi-1} knockout mice hints at the possibility that c-myc may also be a Bmi-1 target, although this cannot be confirmed. It would be interesting in future to perform ChIP studies to assess whether c-myc and cyclin D1 are targets of Bmi-1, or whether their upregulation in \textit{Bmi-1} knockout mice is secondary to the effects of other pathways.

5.5.3. Upregulation of $p16^{\text{ink4a}}$ and $p19^{\text{Arf}}$ in \textit{Bmi-1} knockout livers:

The cell cycle inhibitors $p16^{\text{ink4a}}$ and $p19^{\text{Arf}}$ were both upregulated at the mRNA level in \textit{Bmi-1} knockout livers, with $p16^{\text{ink4a}}$ protein also increased. Examination of $p19^{\text{Arf}}$ protein levels was not possible due to the lack of antibodies which gave robust labelling patterns. Immunolabelling studies revealed that the localisation of $p16^{\text{ink4a}}$ protein is restricted mainly to non-parenchymal cells. These appeared to include Kupffer cells based on morphology, however this would need to be confirmed in future by immunohistochemistry using the F4/80 antibody. The lack of $p16^{\text{ink4a}}$ expression in hepatocytes means that it would not have any impact on hepatocyte proliferation, hence the increased $p16^{\text{ink4a}}$ levels are not incompatible with the slight increase in hepatocyte proliferation observed in the \textit{Bmi-1} knockout mice. It is also notable that $p16^{\text{ink4a}}$ labelling was only sporadically observed in cholangiocytes in \textit{Bmi-1} knockout mice, despite the fact that Bmi-1 was strongly expressed in cholangiocytes in wild type mice (see Chapter 3), and that Bmi-1 is commonly regarded as a repressor of $p16^{\text{ink4a}}$ expression. There is some published work suggesting that the function of Bmi-1 in hepatocytes and HCC cells is independent of \textit{Ink4a/Arf} repression, due to the lack of \textit{Ink4a/Arf} expression changes in cultured cells with altered Bmi-1 expression (Xu et al, 2009). These data would agree with the lack of any $p16^{\text{ink4a}}$ overexpression observed in hepatocytes in \textit{Bmi-1} knockout mice, despite apparent low level Bmi-1 expression in wild type cells. It could be hypothesised that the function of Bmi-1 in cholangiocytes may also be \textit{Ink4a/Arf} independent. However, Sasaki et al report
increased p16$^{\text{Ink4a}}$ expression in cholangiocytes from primary biliary cirrhosis patients, which express lower levels of Bmi-1 than normal cholangiocytes, suggesting that p16$^{\text{Ink4a}}$ is a Bmi-1 target in cholangiocytes. One possibility is that the repression of p16$^{\text{Ink4a}}$ is maintained by another protein or polycomb complex in the absence of Bmi-1, potentially involving Mel-18 due to its similar expression pattern to Bmi-1 (see Chapter 4). Mel-18 is able to bind to and repress the INK4A/ARF locus in human fibroblasts (Maertens et al, 2009), so it is possible that Mel-18 could carry out this function in mouse cholangiocytes in the absence of Bmi-1. It is also possible that other growth stimuli are able to override the effects of Bmi-1 deletion. This occurs in the mammary gland, where pregnancy rescues the mammary outgrowth deficiencies in Bmi-1 knockout mice, despite the fact that Ink4a/Arf levels remain high (Pietersen et al, 2008a). The apparent lack of p16$^{\text{Ink4a}}$ overexpression in two major hepatic cell populations (hepatocytes and cholangiocytes) may also explain why the liver phenotype in the Bmi-1 knockout mouse is relatively mild compared to other tissues.

5.5.4. p21 expression in Bmi-1 knockout livers:

Both p21 mRNA and protein were upregulated in Bmi-1 knockout livers, and immunohistochemical analysis demonstrated that it was predominantly localised in hepatocytes. p21 has widely been regarded as a cell cycle inhibitor, and has been previously shown to inhibit hepatocyte proliferation when overexpressed in the liver (Wu et al, 1996). Based on this, its upregulation in hepatocytes in Bmi-1 knockout mice would be expected to lead to a reduction in proliferation. However, as described in section 5.1, this was not the case in 8 week old Bmi-1 knockout mice, with a small proliferation increase being observed instead. From the hepatocytic p21 immunolabelling pattern observed in Bmi-1 knockout mice (Figure 37), it appears there is heterogeneity in the degree of expression. Some hepatocytes expressed high levels of p21, while others expressed little or no protein. It is therefore possible that the cells which escape p21 upregulation are those that maintain the ability to
proliferate. This could be tested in the future by double labelling experiments for p21 and a proliferation marker such as Ki67. Based on western blotting data, there was no increase in p21 protein levels in 12 week old Bmi-1 knockout mice compared to 8 week old animals, so this does not provide an explanation for the decrease in hepatocyte proliferation seen at 12 weeks.

As discussed in the introduction (section 1.9.3), it also appears that p21 does not always act as a cell cycle inhibitor. It is in fact required for assembly of Cdk4/cyclin D complexes at basal levels, so the expression level of p21 and stoichiometry relative to other cell cycle regulators is important. Given that p21 expression appeared to be low in some Bmi-1 knockout hepatocytes, it is possible that this would not have an inhibitory effect on the cell cycle. There is also some evidence that cyclin D proteins are able to sequester excess p21 away from Cdk complexes, thus preventing overexpressed p21 from repressing their activity. This is interesting given that Bmi-1 knockout livers overexpressed both p21 and cyclin D1. Elevated levels of cyclin D3 in breast cancer cells leads to both reduced association of p21 with Cdk4- and Cdk2-containing complexes, and an increase in their kinase activity (Russell et al, 1999). The authors suggest elevated cyclin D1 will also be able to perform the same function, although they do not provide specific experimental evidence. It has also been shown that c-myc activation results in increased protein synthesis of cyclin D1 and cyclin D2 in cell lines, which in turn leads to increased binding of the cyclins to p27 and p21 (Perez-Roger et al, 1999). This sequestering effect of cyclins D1 and D2 is required for the pro-proliferative effect of c-myc, as c-myc does not increase proliferation in primary cells deficient for cyclin D1 and D2. This potentially links with what was observed in Bmi-1 knockout livers, as preliminary Q-RT-PCR analysis demonstrated upregulation of c-myc. It is therefore possible that the sequestering effect of overexpressed cyclin D1 allows p21 overexpressing Bmi-1 knockout hepatocytes to retain the ability to proliferate. It would be interesting to investigate in the future.
whether c-myc overexpression may be responsible for this by assessing both its protein localisation, and whether c-myc, cyclin D1 and p21 co-localise, using immunolabelling techniques or co-immunoprecipitation.

There have also been studies demonstrating concurrent overexpression of p21 and cyclin D1 in tumour samples, which is similar to what was seen in Bmi-1 knockout hepatocytes. Rey et al show that p21 overexpression in breast cancers is accompanied by increased protein levels of cyclin D1, and that the p21 overexpressing tumour samples also show an increase in proliferation (Rey et al, 1998). A correlation between p21 and cyclin D1 expression has also been demonstrated by Van Oijen et al in head and neck cancers (van Oijen et al, 1998). Together, these data provide support for the idea that the increase in both p21 and cyclin D1 observed in 8 week old Bmi-1 knockout mouse livers is not necessarily counter-intuitive to the concurrent increase in hepatocyte proliferation.

It is also not clear whether the loss of Bmi-1 in hepatocytes is directly responsible for the altered expression of cell cycle regulatory molecules, or whether the changes are secondary to other signals. It has been demonstrated that p21 is a direct target of Bmi-1 mediated repression in cells isolated from mouse cerebellum (Subkhankulova et al, 2010), so it is possible that the loss of Bmi-1 in hepatocytes results in derepression of p21. However, Bmi-1 expression appeared to be low or negative in many hepatocytes, so it is unclear whether loss of Bmi-1 would directly have this dramatic an effect on p21 levels. It is also possible that the p21 upregulation is indirect, as p21 can be activated by p53 (see introduction section 1.9.3). Activity of p53 could potentially be increased in Bmi-1 knockout mice due to the upregulation of p19Arf, as p19Arf blocks the activity of the p53 inhibitor Mdm2. However, for this to be the case, p19Arf levels would have to be increased in Bmi-1 knockout hepatocytes, and this could not be demonstrated due to lack of a robust antibody. Furthermore, Xu et al have reported that Bmi-1 acts independently of Ink4a/Arf in hepatocytes (Xu et
Expression of p21 protein was also observed in some cholangiocytes, however this was not appreciably stronger than in hepatocytes, despite the fact that all murine cholangiocytes expressed high levels of Bmi-1. This result either suggests that alternative repressive mechanisms of p21 may be maintained in cholangiocytes in Bmi-1 knockout mice, or that p21 is not a direct target of Bmi-1 in these cells. This could be assessed in future by ChIP analysis, to examine whether Bmi-1 directly binds the p21 promoter in hepatic cell populations.

Consequences of p21 overexpression in hepatocytes

The effects of p21 overexpression in hepatocytes have previously been studied using a transgenic mouse model (Wu et al., 1996). It is interesting to compare these observations with the Bmi-1 knockout liver phenotype, given that the Bmi-1 knockout mice also appear to overexpress p21 in hepatocytes. The authors report increased expression of cyclin D1 protein in the transgenic livers, in agreement with the results observed in the Bmi-1 deficient livers. The p21 overexpressing mice themselves exhibit stunted growth and a lifespan of 2-3 months, both of which appear very similar to the Bmi-1 knockout mice. It is therefore interesting to speculate whether the reduced size and lifespan of the Bmi-1 knockout mice is related to the accumulation of p21 in the liver. However, there were also some differences in the phenotypes observed. In particular, the transgenic p21 overexpressing mice have reduced hepatocyte numbers due to a decrease in proliferation, as measured by BrdU incorporation, although this was analysed in younger mice. This is accompanied by the accumulation of large numbers of oval cells, which tend to emerge when hepatocyte proliferation is impaired. These results appear at odds with the phenotype observed in the Bmi-1 knockout livers. However, it is important to note that the work by Wu et al involved driving p21 overexpression using the promoter for transthyretin (TTR), a thyroid hormone carrier produced in hepatocytes. Therefore, more widespread hepatocytic p21 expression would be expected, as opposed to the
heterogeneous p21 expression observed in Bmi-1 knockout mice, although this is not entirely clear from the published p21 immunohistochemistry. In addition to this, the authors report predominantly cytoplasmic p21 expression, whereas p21 appeared to be localised in the nucleus in Bmi-1 knockout hepatocytes. It is also likely that levels of p21 overexpression were much greater in the Ttr-p21 mice than the Bmi-1 knockout. The phenotypic differences can potentially be attributed to these factors, as it is probable that hepatocytes in Bmi-1 knockout mice which do not overexpress p21 retain the ability to proliferate. It is also possible, as mentioned earlier, that upregulated cyclin D1 in Bmi-1 knockout mice can sequester excess p21 and facilitate proliferation. Wu et al do report increased cyclin D1 expression in transgenic p21 overexpressing livers, however they also found that all of the protein is in a complex with p21. It therefore seems that the increased cyclin D1 protein is unable to sequester all of the excess p21, and that remaining unsequestered p21 will inhibit cell cycle progression. The stoichiometry of p21 in relation to other cell cycle regulators is important for its function, and this may be different between the transgenic p21 overexpressing mice and the Bmi-1 knockout mice. This could be studied in future by immunoprecipitating cyclin D1 and p21 containing complexes from Bmi-1 knockout livers to assess their relative stoichiometries.

**Polyploidisation and p21 expression**

The decrease in hepatocyte polyploidy (Chapter 4) and increase in p21 expression observed in Bmi-1 knockout mice initially appear contradictory based on some previous studies linking polyploidisation with increased p21 expression. Partial hepatectomy in rats results in the development of more advanced ploidy in hepatocytes, along with increased p21 levels and decreased proliferative potential of isolated hepatocytes in culture (Sigal et al, 1999). However, the authors do not specifically demonstrate p21 expression in cells of higher ploidy, and propose p21 may be involved in regulating cell cycling after partial hepatectomy. It is therefore
possible that the upregulation of p21 is not linked to the increased polyploidy. Mice deficient for Ercc1, an endonuclease involved in DNA repair pathways, also have increased hepatocyte ploidy and p21 expression at 3 weeks of age, with p21 expression skewed towards nuclei with larger areas (Chipchase et al, 2003). This appears to suggest a link between p21 expression and nuclei of higher ploidy, although p21 was found in some cells with lower ploidy, suggesting p21 overexpression does not cause cells to become polyploid. In addition to this, 2 year old wild type mice, which have similar levels of ploidy to the younger Ercc1 knockout mice, do not exhibit significant upregulation of p21 mRNA. The authors also observed a large degree of heterogeneity in p21 expression in different Ercc1 deficient mice, all of which had highly polyploid livers, with some mice exhibiting virtually no p21 expression. Therefore, these results are not able to demonstrate a simple link between p21 expression and liver polyploidy, and suggest any connection may be limited to Ercc1 deficient mice. Finally, Wu et al report occasional polyploid nuclei in transgenic mice which exclusively overexpress p21 in the liver (Wu et al, 1996). However, these nuclei represent only 2% of total hepatocytes, and no ploidy analysis was performed on the remaining cells. The nuclei are also two to four times the diameter of the largest nuclei in wild type mice, suggesting they are not related to the physiological development of polyploidy. In conclusion, the work discussed above does not demonstrate a direct causative link between p21 expression and increased polyploidy, and thus is not contradictory to the finding of high p21 levels and low polyploidy Bmi-1 knockout mice.

5.5.5. Similarities to mice with inactivated c-myc:

As discussed in chapter 4 (section 4.5.3), there are similarities between the polyploidy phenotype observed in the Bmi-1 knockout mice with that found in transgenic mice with inactivated c-myc (Baena et al, 2005), which are interesting given that Bmi-1 is reported to be a c-myc target. These phenotypic similarities are
also apparent regarding proliferation. Deletion of c-myc did not result in inhibition of hepatocyte proliferation; in contrast BrdU incorporation was increased in 6 week old mice with c-myc deletion compared to wild types. The authors also used PCR analysis of individual laser-dissected cells to confirm that proliferating cells contained the deleted c-myc transgene, thus demonstrating that c-myc is not required for hepatocyte proliferation. It is interesting that both the Bmi-1 and c-myc knockout livers exhibit an increase in hepatocyte proliferation in adult mice at one time point (8 weeks and 6 weeks respectively), despite the fact that c-myc and Bmi-1 are commonly associated with the promotion of cell proliferation. The mice did however show impaired proliferation following partial hepatectomy, suggesting a requirement for c-myc in the regenerative response. It would be interesting to perform partial hepatectomies on Bmi-1 knockout mice in the future to assess whether they show a similar defect. Q-RT-PCR analysis of Bmi-1 knockout mouse livers actually revealed an increase in c-myc mRNA expression. However, given that c-myc appears to function upstream of Bmi-1, this would be not preclude dysregulation of c-myc pathways regulated through promotion of Bmi-1 expression.

5.6. Summary of Chapter:

To summarise this chapter, the deletion of Bmi-1 did not appear to result in any deficiencies in hepatocyte proliferation in 8 week old mice. In contrast, knockout mice actually exhibited a small proliferation increase in hepatocytes relative to wild type mice. The precise reasons for this are unclear, and would be unlikely to result in a dramatic phenotypic effect due to the low hepatocyte proliferation rate in resting liver. The proliferation increase was correlated with increased expression of cyclin D1 protein and c-myc mRNA, both of which are pro-proliferative. This occurred despite the expected upregulation of cell cycle inhibitors p16\(^{ink4a}\), p19\(^{arf}\) and p21, which are established Bmi-1 targets. However, p16\(^{ink4a}\) appeared to be expressed in non-parenchymal cells, so would not affect hepatocyte proliferation. p21 protein was
expressed in some, but not all hepatocytes in Bmi-1 knockout mice. It is possible that those hepatocytes which escape p21 upregulation maintain the ability to proliferate, or that the increased cyclin D1 protein present is able to sequester excess p21. It was not possible to evaluate changes in cholangiocyte proliferation due to the low frequency at which they divide in wild type mice. A summary of the potential effects of Bmi-1 deletion on other cell cycle regulators is provided in Figure 38.

Figure 38 (overleaf): The effects of Bmi-1 deletion on the cell cycle.

Suggested effects of Bmi-1 on cell cycle genes in liver cell populations. a) Kupffer cells: i) Bmi-1 represses p16\textsuperscript{Ink4a} expression in wild type cells. ii) Deletion of Bmi-1 results in upregulation of p16\textsuperscript{Ink4a}. b) Cholangiocytes: i) Bmi-1 is involved in repressing p16\textsuperscript{Ink4a} expression in wild type cells. ii) Repression of p16\textsuperscript{Ink4a} is maintained in the majority of Bmi-1 knockout cholangiocytes, possibly by Mel-18 containing PRC complexes or other unidentified mechanisms. c) Hepatocytes: i) Bmi-1 is involved in repressing p21 expression in some or all wild type hepatocytes. ii) Deletion of Bmi-1 results in p21 upregulation in many hepatocytes, which is likely to inhibit their proliferative capacity. Hepatocyte proliferation is maintained by either: iii) hepatocytes which maintain p21 repression in the absence of Bmi-1 through other unidentified mechanisms, or iv) hepatocytes which also upregulate cyclin D1 (and potentially c-myc), resulting in the sequestering of excess repressive p21 away from cdk complexes.
Figure 38: The effects of Bmi-1 deletion on the cell cycle.

See previous page for figure legend.
Chapter 6

Iron overload in *Bmi-1* knockout mice
6. Iron overload in *Bmi-1* knockout mice:

6.1. Hepatic iron accumulation in *Bmi-1* knockout mice:

A notable observation that arose during analysis of blindly labelled H&E stained sections from *Bmi-1* wild type and knockout mice was the occasional presence of a yellow refractive pigment in Kupffer cells (Figure 39). This was only observed in *Bmi-1* knockout mice, and was particularly prominent at 12 weeks of age. A literature review of basic liver pathology resources raised the possibility that the pigment was haemosiderin, an insoluble iron storage complex found in cells following breakdown of ferritin and haem. To assess whether the *Bmi-1* knockout mice were exhibiting increased hepatic accumulation of iron compounds, paraffin-embedded tissue sections were subjected to a Perls Prussian Blue stain to detect the presence of iron.

Iron deposits were sporadically detectable at very low levels in *Bmi-1* wild type mice at both 8 and 12 weeks of age (Figure 40a,c,g,e). *Bmi-1* knockout mice at 8 weeks of age exhibited accumulation of iron in hepatocytes, predominantly around portal regions (Figure 40b,d) (n=4). There was some heterogeneity in the degree of overload, with some mice appearing more severe than others. However, all knockout mice examined showed evidence of iron deposition. At 12 weeks of age, the *Bmi-1* knockout mice (n=4) showed a similar distribution of hepatocytic iron to the 8 week old mice, however the staining generally appeared more intense (Figure 40f,h). In addition, 12 week old knockout mice exhibited much stronger Perls staining in Kupffer cells (Figure 40f), indicating a greater degree of iron deposition.
Figure 39: Kupffer cell pigmentation in *Bmi-1* knockout mouse livers.

Haematoxylin stained paraffin-embedded liver sections from 12 week old wild type (a) and *Bmi-1* knockout (b) mice, showing presence of yellow/brown refractive pigment in Kupffer cells in *Bmi-1* knockout tissue (black arrow). Scale bars 50 µm.

Figure 40 (overleaf): Iron deposition in *Bmi-1* knockout mouse livers.

Paraffin-embedded liver sections stained with Perls Prussian Blue and counterstained with eosin. PT = portal triad, CV = central vein. a-d) 8 week old mice, e-h) 12 week old mice. Iron deposition was absent in both 8 week (a,c) and 12 week old (e,g) wild type mice. Parenchymal iron overload was evident in 8 week old (b,d) and 12 week old (f,h) *Bmi-1* knockout mice. b) Low magnification showing periportal iron deposition pattern in 8 week old *Bmi-1* knockout mouse liver. f) Extensive iron accumulation in Kupffer cells 12 week old *Bmi-1* knockout mouse liver (black arrows). All scale bars 50 µm.
6.2. Expression of iron regulatory genes in Bmi-1 knockout mice:

A review of literature identified several proteins involved in iron regulatory pathways as potential Bmi-1 and Polycomb targets. Bracken et al performed genome wide mapping of Polycomb target promoters using ChIP-on-chip analysis in human embryonic fibroblasts (Bracken et al, 2006). This study revealed enrichment of one or more of the H3K27me3 mark, Suz12 or Cbx8 on promoters of genes involved in iron homeostasis, namely HAMP (hepcidin antimicrobial peptide), HFE (haemochromatosis protein) and TFR2. ChIP experiments using an anti-Bmi-1 antibody have also identified iron regulatory protein 2 (IRP2) as a potential Bmi-1 target (Meng et al, 2010). These data suggested that the expression of genes involved in iron homeostasis could be directly regulated by Polycomb complexes. To begin to investigate the possibility that impairment of the iron regulatory pathway was responsible for the excessive iron accumulation seen in Bmi-1 knockout mice, the expression levels of several key genes in the pathway were examined using Q-RT-PCR (Figure 41).

Figure 41 (overleaf): Altered expression of iron regulatory genes in Bmi-1 knockout mice.

Q-RT-PCR data on cDNA made from mRNA extracted from Bmi-1 wild type (Wt) and knockout (KO) whole mouse livers. a) Hepcidin antimicrobial peptide (Hamp) in 8 week (n=5 mice per group) and 12 week old (n=4) mice. b) Ferroportin (Fpn), 8 weeks (n=4) and 12 weeks (n=3). c) Transferrin receptor 1 (Tfr1), 8 weeks (n=4) and 12 weeks (n=3). d) Tfr2, 8 weeks (n=3) and 12 weeks (n=3). e) Haemochromatosis protein (Hfe), 8 weeks (n=5) and 12 weeks (n=4). f) Iron regulatory protein 2 (Irp2), 8 weeks (n=5) and 12 weeks (n=3). g) Bone morphogenetic protein 6 (Bmp6), 8 weeks (n=5) and 12 weeks (n=3). Values expressed as transcripts of gene of interest per transcript of reference gene (eEfi2). Data are means ± SD. * p<0.05 using 2-tailed Student’s t-test.
Figure 41: Altered expression of iron regulatory genes in *Bmi-1* knockout mice.
6.2.1. Hepcidin antimicrobial peptide:

Hepcidin, encoded by the *Hamp* gene, is a small peptide which is the major regulator of bodily iron homeostasis. It is synthesised in iron replete conditions to reduce iron uptake via degradation of the iron export protein ferroportin (FPN) (see introduction section 1.6 for further details of iron regulatory pathways). At 8 weeks of age, *Bmi-1* knockout mice exhibited a significant upregulation in *Hamp* expression compared to wild types of the same age (Figure 41a). The increase in *Hamp* levels at 8 weeks is consistent with published studies showing that hepcidin expression reflects body iron stores, and that levels are increased in the livers of iron overloaded mice (Pigeon et al, 2001). Increased hepcidin levels should result in degradation of the FPN protein to reduce both the absorption of iron in the small intestine and the release from stores. Therefore, the iron levels in hepatocyte and macrophage stores should be increased; this is in agreement with the phenotype observed in the *Bmi-1* knockout mice. By 12 weeks of age, the *Bmi-1* knockout mice no longer exhibited upregulation of *Hamp*. This appears contradictory to the hepatic iron overload exhibited on histological sections (Figures 39 & 40), however may reflect the fact that the health of the knockout mice becomes progressively worse by 12 weeks of age. Hepcidin production is suppressed by erythroid regulators, hypoxia and anaemia, so it is possible that secondary factors exert effects in 12 week old *Bmi-1* knockout mice. In support of this, it has been reported that iron overload is less dominant than anaemia and hypoxia in regulating hepcidin expression, as mice with severe iron loading anaemias have reduced hepcidin levels despite hepatic iron overload (Adamsky et al, 2004), and erythropoiesis inhibitors increase hepcidin expression in mice with iron deficiency (Pak et al, 2006).
6.2.2. Ferroportin:

The mRNA levels of the iron exporter ferroportin (*Fpn*) were the same in 8 week old wild type and *Bmi-1* knockout mice (Figure 41b). This is not unexpected, given that hepcidin acts to internalise and degrade FPN at the protein level, so mRNA levels would be unaffected by the increase in *Hamp* expression in 8 week old knockout mice. In contrast, 12 week old *Bmi-1* knockout mice had a small but significant decrease in levels of *Fpn* mRNA relative to wild types. It is unclear why this would be, although it is unlikely to be directly due to the loss of *Bmi-1*, as *Bmi-1* is a transcriptional repressor. If the reduced *Fpn* mRNA translates to a decrease in protein levels at 12 weeks, it would theoretically lead to a reduction in iron export, which is consistent with an iron overload phenotype. A notable difference between the iron overload phenotype in *Bmi-1* knockout mice at 12 weeks compared to 8 weeks was an increase in Kupffer cell iron staining (Figure 40d vs. 40h). Given that Kupffer cells express high levels of *Fpn* (Delaby et al, 2005), it is possible that decreased *Fpn* expression relative to wild types could result in increased iron retention in 12 week old knockout mice. However, this remains speculation without any protein expression or localisation data. The change in *Fpn* expression in 12 week old *Bmi-1* knockout mice was also small, so it seems unlikely that it would have a dramatic effect on the phenotype.

6.2.3. Transferrin Receptor 1:

The 3’ UTR of Transferrin receptor 1 (*Tfr1*), which mediates transferrin uptake into cells, contains an iron regulatory element (IRE). This is bound by iron regulatory proteins (IRPs) in iron starved cells to increase mRNA stability. Therefore, the expectation would be that *Tfr1* mRNA levels would decrease in cells with abundant iron due to increased degradation. The effect of this would be to reduce TfR1 protein levels, and therefore reduce iron uptake to try and prevent overloading the cell. In
agreement with this, Tfr1 mRNA (Adamsky et al, 2004) and protein (Wallace et al, 2009) levels are decreased in mice with iron overload. In contrast to this, iron overloaded livers of 8 week old Bmi-1 knockout mice did not exhibit any change in Tfr1 expression (Figure 41c). By 12 weeks of age, Tfr1 mRNA levels were significantly increased in knockout mice compared to wild types. This result suggests that Tfr1 expression is dysregulated in Bmi-1 knockout mice, although it is possible that Tfr1 is only upregulated in individual hepatocytes or Kupffer cells which are not already iron overloaded. Increased Tfr1 levels are consistent with the iron overloaded liver phenotype observed in Bmi-1 knockout mice, as cells expressing higher levels of Tfr1 should have increased iron uptake. TFR1 was not one of the genes identified as a potential Polycomb/Bmi-1 target in genome wide screens, so it is unclear whether the upregulation in 12 week old knockout mice could be directly due to Bmi-1 loss. The fact that it was not upregulated at 8 weeks of age suggests against this, although it is possible that mRNA degradation due to the IRE-IRP system could negate some transcriptional increase due to lack of Bmi-1 mediated repression. Tfr1 expression is also regulated by other mechanisms, such as hypoxia, which could also be a factor in Bmi-1 knockout mice (Graham et al, 2007). It is also worth considering whether the upregulation of Tfr1 seen in 12 week old knockout mice could be linked to the increased iron deposition relative to 8 weeks, especially in Kupffer cells which are able to obtain iron from transferrin (van Berkel et al, 1987). Immunohistochemical studies to assess localisation of the protein could address this in the future.

6.2.4. Transferrin Receptor 2 and HFE:

Both Tfr2 and HFE are thought to be involved in pathways which regulate hepcidin expression at the transcriptional level in response to iron levels. Increased levels of diferric transferrin lead to the displacement of HFE from Tfr1, and the stabilisation of Tfr2. HFE and Tfr2 subsequently facilitate hepcidin production via pathways which have not been well defined, either as a complex or individually. Both TFR2 and HFE
were identified as putative Polycomb targets in genome wide screens (Bracken et al, 2006), suggesting that their expression may be dysregulated in Bmi-1 knockout mice. In 8 week old knockout mice, mRNA levels of Tfr2 and Hfe were unchanged compared to wild type mice (Figure 41d,e). This indicates that the increased hepcidin production in 8 week old knockouts relative to wild types was not driven by increases in HFE or Tfr2 levels. Expression of both Tfr2 and Hfe were significantly decreased in 12 week old Bmi-1 knockout mice compared to wild types. This would theoretically lead to a decrease in hepcidin expression, as seen in Hfe and Tfr2 null mice (Fleming et al, 2002; Zhou et al, 1998). This is in agreement with the fact that 12 week old knockout mice did not exhibit hepcidin upregulation compared to wild types, although it is unclear if the level of Tfr2 and Hfe downregulation is dramatic enough to be the direct cause. It is also unclear why expression of Tfr2 and Hfe was decreased in 12 week old Bmi-1 knockout mice. It is unlikely to be directly due to the absence of Bmi-1, as Bmi-1 acts as a transcriptional repressor, so any direct targets should be derepressed in Bmi-1 knockout mice.

6.2.5. Iron Regulatory Protein 2:

Iron regulatory protein 2 (IRP2) is synthesised in iron depleted cells, and proteasomally degraded as iron levels increase. It acts by binding to IREs in the 3’ UTR of Tfr1 mRNA to stabilise the transcript, or in the 5’ UTR of Fpn and ferritin mRNA to inhibit translation. High levels of IRP2 therefore promote increased transferrin uptake and reduced iron storage when iron levels are low, and the reverse when iron is plentiful. Irp2 was of particular interest as ChIP studies identified a Bmi-1 responding element (BRE) in its promoter (Meng et al, 2010), suggesting it could be a direct Bmi-1 target. If this was the case, then it may have been aberrantly upregulated in Bmi-1 knockout mice, and could have been responsible for the unexpected increase in Tfr1 levels described earlier. However, mRNA expression of Irp2 was unchanged in both 8 and 12 week old Bmi-1 knockout mice compared to wild types.
(Figure 41f), suggesting that alterations in IRP2 expression are not contributing towards the iron overload phenotype of the mice.

6.2.6. Bone Morphogenetic Protein 6:

Another signalling molecule that may play an important role in hepcidin regulation is bone morphogenetic protein 6 (BMP6), as Bmp6 knockout mice exhibit decreased levels of hepcidin despite severe iron overload (Meynard et al, 2009). Members of the BMP family, including BMP6, have been implicated as potential Polycomb targets based on genome wide mapping (Bracken et al, 2006), so it was considered possible that Bmp6 levels could be altered in Bmi-1 knockout mice. Q-RT-PCR analysis of Bmp6 expression revealed no significant changes in 8 week old Bmi-1 knockout mice (Figure 41g), suggesting altered BMP6 signalling was not responsible for the increased hepcidin expression. In contrast, 12 week old Bmi-1 knockout mice did exhibit a small but significant upregulation of Bmp6 compared to wild types. This agrees with previous work demonstrating that Bmp6 expression increases with iron loading in mouse models (Kautz et al, 2010). However, the significance of this is unclear given that 12 week old knockout mice did not have increased Hamp mRNA levels, again suggesting that alterations in hepcidin levels in Bmi-1 knockout mice do not result from abnormal BMP6 signalling.

6.3. Serum iron analysis:

Iron overload disorders can generally be classed as either primary or secondary. Primary iron overload, often known as haemochromatosis, is usually hereditary and results from mutations in genes involved in iron regulatory pathways. Secondary iron overload characterises disorders where the iron regulatory pathway remains intact, but iron builds up due to other factors such as excessive dietary iron or blood transfusions. The work described above has characterised some abnormalities in the expression of genes involved in iron regulatory pathways in Bmi-1 knockout mice.
However, it is unclear whether these were actually causing the hepatic iron overload phenotype, or resulted from secondary iron overload. To begin to assess potential causes of the iron overload observed in Bmi-1 knockout mice, serum samples from 8 week old female mice were analysed for iron levels. There was no significant change in serum iron levels in 8 week old Bmi-1 knockout mice compared to wild type littermates (Figure 42). This suggests that the liver iron overload was not caused by a dramatic increase in serum iron levels. It also shows that the mice were not suffering from iron deficiency as a result of increased hepcidin expression in 8 week old knockout mice, suggesting that the altered hepcidin levels did not have a dramatic effect. Due to limitations in the amount of serum that could be obtained from the knockout mice due to their small size, only the total serum iron was measured. In future, the measurement of total iron binding capacity (TIBC), transferrin saturation, which is usually increased in haemochromatosis (Batts, 2007) and NTBI, which can be taken up by hepatocytes in an unregulated manner (Chua et al, 2004), will provide a more detailed picture of whether there are abnormalities in plasma iron transport in Bmi-1 knockout mice.
Figure 42: Serum iron levels are unchanged in Bmi-1 knockout mice.

Analysis of serum from 8 week old wild type (Wt) and Bmi-1 knockout (KO) mice for total iron levels. n=4 mice per group, p=0.86 using 2-tailed Student’s t-test.
6.4. Discussion:

6.4.1. Summary of iron overload phenotype:

The results detailed in this chapter have demonstrated a hepatic iron overload phenotype in \textit{Bmi-1} knockout mice at 8 and 12 weeks of age, in both hepatocytes and non-parenchymal cells which were histologically identified as Kupffer cells. This identification could be confirmed in future by double labelling immunohistochemistry using the Kupffer cell specific F4/80 antibody. There was also some preliminary evidence of aberrant regulation of iron regulatory pathways based on altered mRNA expression. However, a detailed picture of the phenotype is not yet clear. It is notable that the iron overload in \textit{Bmi-1} knockout mice does not appear as severe as in several other models, for example in mice with transgenic overexpression of hepcidin in the liver (Nicolas et al, 2002a) or in \textit{Hfe} null mice (Zhou et al, 1998). The changes in expression of some genes involved in iron regulatory pathways, although significant, are also less dramatic than in these models. These expression changes were also only determined for mRNA, so it is not certain that they will also apply at the protein level. This will be addressed in future experiments. There was also no obvious iron related parenchymal damage in the \textit{Bmi-1} knockout mice, such as sideronecrosis, fibrosis or iron-free foci (Turlin & Deugnier, 1998); therefore it seems unlikely that the phenotype has a dramatic effect on the livers of the mice. As such, the phenotype can be described as haemosiderosis (the presence of excess iron in the liver) rather than haemochromatosis (a pathological state of haemosiderin deposition) (Batts, 2007). With these caveats in mind, the following section will speculate on some potential causes of the iron accumulation based on the data available.
6.4.2. Potential causes of iron overload:

Secondary iron overload

As detailed previously, iron overload disorders are either primary (caused by mutations in genes involved in iron regulatory pathways) or secondary (due to factors such as increased dietary iron or iron-loading anaemias). Due to the fact that the \textit{Bmi-1} knockout mouse is not liver specific, it is possible that the hepatic iron overload is a secondary consequence of defects in other organ systems, in particular anaemias. Iron overload is a consequence of anaemias involving ineffective erythropoiesis, such as \(\beta\)-thalassaemia. Ineffective erythropoiesis results in continued production of erythroid regulators such as erythropoietin, increased levels of which indicate high demand for iron. Therefore, erythroid regulators continuously signal to reduce hepcidin expression and thus increase iron absorption (Pak et al, 2006), ultimately leading to iron overload. Adult \textit{Bmi-1} knockout mice have dramatic reductions in HSC numbers (Park et al, 2003), hence their phenotype has been described by others as aplastic anaemia (Chagraoui et al, 2006). Aplastic anaemia is characterised by insufficient production of new blood cells, and usually arises due to the destruction of haematopoietic stem cells (HSC) (Leguit & van den Tweel, 2010). Aplastic anaemias are often accompanied by increased iron deposition, in particular in macrophages (Leguit & van den Tweel, 2010), presumably due to excess iron that is not incorporated into red blood cells. This pattern appears consistent with the iron overload observed in \textit{Bmi-1} knockout mice, particularly in 12 week old mice which have significant iron accumulation in Kupffer cells. It therefore appears possible that anaemia in \textit{Bmi-1} knockout mice is responsible for the iron overload phenotype.

However, a caveat to the hypothesis that anaemia in \textit{Bmi-1} knockout mice is causing the excessive hepatic iron accumulation is that Van der Lugt et al report that erythropoiesis is normal in adult \textit{Bmi-1} knockout mice in the FvB background, and that
haematopoiesis defects occur in lymphopoiesis and myelopoiesis (van der Lugt et al, 1994). They detail that adult (2-3 month old) Bmi-1 knockout mice have normal red blood cell counts, mean red blood cell volume, haemoglobin concentration and haematocrit. If robust, these results suggest that there are no defects within the red blood cell population, which should in turn mean no abnormalities in incorporation of iron into these populations. However, this does not explain why the same authors report anaemia in moribund Bmi-1 knockout mice, which suggests that erythropoiesis defects must occur at some stage. It also appears inconsistent with the findings that adult Bmi-1 knockout mice have significantly reduced numbers of long term repopulating HSCs (Park et al, 2003). Given that erythrocyte lineages are derived from HSCs, it seems surprising that a dramatic reduction in total HSC numbers would have no effect on red blood cell counts or erythropoiesis as the mice age. It may be necessary in the future to repeat the analysis undertaken by Van der Lugt et al on blood cell parameters such as haemoglobin content and cell volume, which are indicative of restricted erythropoiesis. The anaemic phenotype in Bmi-1 knockout mice could also be further defined by examining erythrocyte morphology using Wright-Giemsa stained blood smears, to determine whether erythropoiesis is altered (Nicolas et al, 2002a). It would also be interesting to examine serum levels of erythroid regulators, such as erythropoietin, to assess whether Bmi-1 knockout mice have increased erythropoietic demand.

Iron loading anaemias are also usually characterised by reduced hepcidin expression (Origa et al, 2007), whereas in 8 week old Bmi-1 knockout mice, hepcidin expression was significantly increased. The erythroid regulation of hepcidin dominates over hepcidin regulation by iron overload (Pak et al, 2006), suggesting against the idea that anaemias involving ineffective erythropoiesis cause the iron overload in Bmi-1 knockout mice. However, the regulation of hepcidin is complex, with factors such as hypoxia and inflammation also affecting its expression, so these may be a factor in
Bmi-1 knockout mice. It is also possible that other uncharacterised defects in other tissues in Bmi-1 knockout mice may lead to abnormal iron absorption or release from stores. Further analysis of other organs important in iron homeostasis may shed further light on this possibility, in particular in the duodenum which is the site of dietary iron absorption.

**Primary iron overload**

An alternative possibility to consider is that the iron accumulation in Bmi-1 knockout mouse livers is due to primary defects in iron regulatory pathways within the liver, either directly or indirectly due to the absence of Bmi-1. Some of the findings from the Q-RT-PCR analysis of iron regulatory pathway components were consistent with an iron overload phenotype in Bmi-1 knockout mice. In 8 week old mice, the increased hepcidin expression should result in degradation of the iron exporter FPN, and hence increased iron retention in hepatic stores. The *HAMP* gene has been identified as a putative Polycomb target (Bracken et al, 2006), so it is possible in theory that it is directly derepressed due to loss of Bmi-1. However, regulation of hepcidin is complex, so there are many other secondary factors such as inflammation which could cause fluctuations in its expression. The fact that hepcidin levels are not increased in 12 week old Bmi-1 knockout mice also suggests against the idea that *Hamp* is a direct Bmi-1 target. However, it is also possible that other secondary effects could become dominant, such as increased severity of anaemia or hypoxia due to the worsening health of the mice. The downregulation of *Hfe* and *Tfr2* expression in 12 week old knockout mice also fits with the decreased hepcidin levels, as these proteins are involved in regulating hepcidin synthesis. Future ChIP experiments could assess whether *Hamp* is actually a Bmi-1 target in liver. The expression of these genes has also only been examined at the mRNA level, so it will be important in future to look at protein expression to determine whether the effects are the same.
As discussed above, it is theoretically possible that abnormal *Hamp* upregulation in 8 week old *Bmi-1* knockout mice is directly causing the hepatic iron accumulation. However, this is not fully consistent with the fact that 8 week old *Bmi-1* knockout mice exhibited normal levels of serum iron. Hepcidin also acts to degrade FPN in digestive enterocytes which absorb iron from the diet, so the overall effect of increased hepcidin levels should be a reduction in total body iron levels. In agreement with this, C57BL/6 transgenic mice overexpressing hepcidin in the liver suffer severe iron deficiency anaemia and early death (Nicolas et al, 2002a), although this may be linked to the fact that they express hepcidin during embryonic development, whereas wild type mice do not. If increased hepcidin levels in *Bmi-1* knockout mice were having a significant prolonged effect, the mice should suffer from iron deficiency anaemia due to lack of iron release into the blood. Although anaemia has been reported in older moribund *Bmi-1* knockout mice (van der Lugt et al, 1994), the fact that serum iron levels were unchanged in 8 week old *Bmi-1* knockout mice suggests against this. It could also be that the level of *Hamp* upregulation in 8 week old *Bmi-1* knockout mice is not dramatic enough to have severe effects on bodily iron levels, and only causes some gradual accumulation within the liver.

In 12 week old *Bmi-1* knockout mice, mRNA expression levels of *Fpn* were decreased relative to wild types, while *Tfr1* expression was increased. If these translate to protein levels, increased TfR1 and decreased FPN should lead to an increase in iron uptake and decrease in iron export respectively. This would be consistent with an iron overload phenotype. However, the fact that expression of these genes was unchanged in 8 week old knockout mice suggests these effects were not directly due to *Bmi-1* loss, and were unlikely to be major causative factors of the iron overload. They could potentially explain the increased iron accumulation in Kupffer cells in 12 week old knockout mice relative to animals at 8 weeks, as Kupffer cells should express both proteins, although it has to be noted that the change in *Fpn* expression
in particular was not large. Immunohistochemical assessment of the localisation of these proteins in \textit{Bmi-1} knockout livers could help investigate this in the future. As detailed earlier, \textit{Tfr1} mRNA should be degraded in cells with abundant iron due to the IRP-IRE regulatory system. The fact that \textit{Tfr1} mRNA was not decreased in either 8 or 12 week old \textit{Bmi-1} knockout mice suggests this was not happening as expected. It is therefore possible there is some dysregulation of pathways that control its expression, although it is unclear at this stage what these may be.

Overall, it appears unlikely that the iron overload in \textit{Bmi-1} knockout livers can be completely explained by the expression changes in the iron regulatory genes studied. It seems more probable that adult \textit{Bmi-1} knockout mice also have increased bodily iron levels due to secondary factors, possibly including anaemias as discussed earlier. This could potentially lead to increased hepcidin production (in 8 week old mice), resulting in increased iron retention in hepatic stores to maintain serum concentrations in the normal range. It is also possible that the \textit{Bmi-1} knockout mice have increased levels of non-transferrin-bound iron (NTBI), which can be taken up into cells in a much less regulated manner than iron bound to transferrin.

6.4.3. Histopathology of iron deposition in \textit{Bmi-1} knockout mice:

Another consideration is the localisation of the iron accumulation in \textit{Bmi-1} knockout mice. Primary haemochromatosis is usually associated with iron deposition in hepatocytes and biliary cells, as opposed to Kupffer cells. It tends to begin with iron accumulation in periportal hepatocytes, and progressively spreads towards centrilobular cells as the disease worsens (Batts, 2007). The initial parenchymal iron overload in \textit{Bmi-1} knockout mice does appear to be predominantly periportal. However, loading of macrophages only appears much later in haemochromatosis, when hepatocytes accumulate lethal levels of iron leading to necrosis and iron release (Batts, 2007). In contrast, Kupffer cell iron is more associated with secondary
overload, for example due to haematological disorders. Given that there was no obvious parenchymal damage or necrosis in 12 week old Bmi-1 knockout mice, the excessive iron accumulation in Kupffer cells would appear to suggest a secondary effect. However, it is unclear why 8 week old Bmi-1 knockout mice exhibited much less Kupffer cell iron deposition than at 12 weeks, given that the 8 week old animals all displayed some hepatocyte deposition.

6.4.4. Iron overload and the cell cycle:

It is also worth noting the established link between iron levels and cell cycle progression. Iron depletion causes G1/S phase arrest (Yu et al, 2007), along with reductions in the protein levels of cyclin D1 and p21 through proteasome mediated, ubiquitin independent degradation pathways in cultured cells (Fu & Richardson, 2007; Nurtjahja-Tjendraputra et al, 2007). Moreover, increases in proliferating hepatocytes (measured by incorporation of proliferating cell nuclear antigen (PCNA) and upregulated cyclin D1 protein levels are seen in iron overloaded rat livers (Brown et al, 2006). Increased cyclin D1 mRNA levels are also found in murine livers with iron overload (Troadec et al, 2006), with the authors also observing increases in mitotic index and DNA synthesis in cultured hepatocytes isolated from iron overloaded mice. These data are broadly in agreement with the phenotype observed in the Bmi-1 knockout mice at 8 weeks of age, which exhibited both excessive iron loading in hepatocytes and increased protein levels of cyclin D1 and p21 (see chapter 5). It therefore appears plausible that the increased hepatic iron levels may be a contributory factor to the altered proliferation levels observed in Bmi-1 knockout mice. However, there did not appear to be any correlation between the localisation of the iron overload, which was predominantly around portal regions, and the localisation of the overexpressed p21 and cyclin D1, which did not appear specifically periportal. This suggests against the idea that increased p21 and cyclin D1 levels are caused
directly by reduced proteasomal degradation due to increased iron levels in individual cells.

6.5. Summary of Chapter:

The work presented in this chapter has shown that adult Bmi-1 knockout mice suffer from a degree of iron overload, which appears to worsen slightly as the mice age towards 12 weeks. Based on the fact that serum iron levels were unchanged, and that there was no visible iron-related parenchymal damage, it seems unlikely that this would have a dramatic effect on the health of the mice. It is possible that the iron accumulation would result in tissue damage in the longer term, however the short lifespan of the mice may preclude this from becoming apparent. It is also unclear whether the hepatic iron accumulation is directly due to the deletion of Bmi-1 in the liver, or a secondary effect, as the pathological distinction between primary and secondary iron overload is often difficult (Batts, 2007). The Bmi-1 knockout is not liver specific, and the mice become progressively unwell as they age towards 12 weeks, so may suffer from other pathologies which could lead to iron overload. Regulation of iron homeostasis, and hepcidin expression in particular, is very complex, with factors such bodily iron levels, anaemia, hypoxia and inflammation all having an effect. As such, it is difficult to untangle these competing effects in the Bmi-1 knockout mice, and the potential reasons for the overload discussed previously in this chapter are speculative at this stage. The best way to address these issues in the future would be to create a liver specific Bmi-1 knockout mouse, to assess whether the iron overload phenotype is directly due to Bmi-1 deletion in hepatic cells.

It would also be interesting to examine whether other key organs in Bmi-1 knockout mice suffer a similar iron overload phenotype. Iron is absorbed by duodenal enterocytes, so a histological examination of iron loading in the small intestine would help determine whether iron absorption is proceeding normally in Bmi-1 knockout
mice. In addition to this, iron overload disorders can lead to iron accumulation in cardiac tissue. This can lead to cardiomyopathy and heart failure, and is a major determinant of survival in secondary iron overload (Murphy & Oudit, 2010). Examination of iron loading in the hearts of Bmi-1 knockout mice will allow further assessment of whether iron overload is potentially a contributor to the shortened lifespan of the mice.
Chapter 7

Final Discussion
7. Final Discussion:

7.1. Conclusions:

This thesis set out to investigate the role of Bmi-1 in hepatic growth and function \textit{in vivo}. This was achieved firstly by examining the expression of Bmi-1 in normal and regenerating hepatic tissue, and then using a mouse model to assess the effect of \textit{Bmi-1} deletion on liver structure and function. This is one of the first studies investigating the role of a PcG family member in hepatic physiology \textit{in vivo}. The important findings from the thesis have been discussed in the respective chapters, and can be summarised as follows:

1. Bmi-1 protein is expressed in all cholangiocytes and some hepatocytes in normal murine liver.

2. Bmi-1 is strongly expressed in murine oval cells, and in some ductular cells and regenerative nodules in human cirrhotic liver tissue. These results suggest that Bmi-1 may have a function in hepatic repair.

3. Mel-18, a partial Bmi-1 homologue, is also expressed in biliary cells in murine liver, and is upregulated in older \textit{Bmi-1} knockout mice. This suggests that Mel-18 may have a compensatory effect following \textit{Bmi-1} deletion, and may account for the lack of obvious abnormalities in biliary structure observed in \textit{Bmi-1} knockout mice.

4. \textit{Bmi-1} knockout mice have reduced hepatocyte cell and nuclear size, and average centromere numbers which are indicative of impairment in the postnatal hepatocyte polyploidisation process. This suggests a novel function for Bmi-1 in cellular polyploidisation, which has not previously been reported.
5. *Bmi-1* knockout mice exhibit a small increase in hepatocyte proliferation at 8 weeks of age, which is in contrast to findings in other cell types *in vivo* and *in vitro*. This occurs together with an increase in expression of cyclin D1 and *c-myc*, which are positive regulators of cell cycle progression. This shows that Bmi-1 is not essential for the proliferation of mature hepatocytes.

6. The expression of Bmi-1 targets, namely the cell cycle inhibitors *p16Ink4a*, *p19Arf* and *p21* is increased in *Bmi-1* deficient livers, despite the increase in hepatocyte proliferation.

7. *Bmi-1* knockout mice suffer from a hepatic iron overload phenotype, which increases in severity as the mice age. This is coupled with alterations in the expression of some genes involved in iron regulatory pathways. The iron overload is potentially secondary to other defects in *Bmi-1* knockout mice, most notably their anaemic phenotype.

### 7.2. Discussion and Future Perspectives:

The work detailed in this thesis has focused on the *in vivo* effects of *Bmi-1* deletion using a total embryonic knockout mouse model. This has the limitation that the mice suffer from deleterious effects in other organs, especially the haematopoietic and nervous systems, which leads to them becoming progressively unwell as they age towards 3-5 months (van der Lugt et al, 1994). As such it cannot be ruled out that some of the effects observed in the liver are secondary to these issues, and it is possible that the phenotypes observed in *Bmi-1* knockout mice are non-cell autonomous. It also creates the possibility of a complex phenotype involving competing pathways, resulting from both hepatic deletion of *Bmi-1* and secondary
effects, which is difficult to unravel. There are a number of approaches that could address these issues in the future. The most advantageous would be to use a conditional knockout mouse model to facilitate liver specific deletion of \textit{Bmi-1}, thus eliminating the possibility of secondary defects to \textit{Bmi-1} deletion in other tissues. Conditional \textit{Bmi-1} knockout mice have not previously been published, however it would be desirable in future to have a strain with a LoxP flanked \textit{Bmi-1} allele available. These mice could be crossed with mice expressing Cre under the control of promoters expressed in specific liver cell populations (e.g. albumin in hepatocytes) to allow targeted \textit{Bmi-1} deletion. This would allow assessment of whether the parenchymal defects observed in the total \textit{Bmi-1} knockout mouse directly result from the absence of \textit{Bmi-1} in the liver. It would also be possible to examine the effects of targeted \textit{Bmi-1} overexpression in liver in a similar manner, using a Cre/LoxP based mouse model (Yadirgi et al, 2011). As an alternative to conditional knockout mice, hepatocytes from \textit{Bmi-1} knockout livers could be grafted into recipient mice suffering from hepatic injury, such as uPA transgenic mice (Braun et al, 2003), which allow preferential expansion of transplanted cells. This would allow comparison of the proliferative capacity of wild type and \textit{Bmi-1} knockout hepatocytes. It would also help determine whether the phenotypes observed in \textit{Bmi-1} knockout mice, such as the reduced polyploidisation, are cell autonomous, although this method does not offer many advantages over a conditional \textit{Bmi-1} knockout in this respect.

Alternatively, \textit{in vitro} studies could provide additional data on the major phenotypic effects observed in 8 week old \textit{Bmi-1} knockout mice, notably the decreased hepatocyte polyploidisation, the small increase in hepatocyte proliferation, and hepatic iron overload, and help determine whether they are cell autonomous. Stable shRNA mediated \textit{Bmi-1} depletion in suitable cell populations would allow a direct assessment of whether these phenotypes result directly from \textit{Bmi-1} loss. These experiments would include assessment of the proliferative ability of primary hepatocytes and
cholangiocytes following *Bmi-1* knockdown, to determine whether Bmi-1 has a role in the proliferation of mature hepatic cell populations. Cultured primary rodent hepatocytes can be used to examine the development of polyploidy *in vitro* (Duncan et al, 2010), so *Bmi-1* depletion in these cells would determine whether Bmi-1 directly affects this process. Although the balance of evidence suggests that the iron overload in *Bmi-1* knockout mice is secondary to factors such as anaemia, there remains a possibility that Bmi-1 loss affects iron homeostasis in hepatocytes. This could be investigated by examining iron uptake and storage in *Bmi-1* depleted hepatocytes in response to iron loaded transferrin (Gao et al, 2009). However, there are caveats to *in vitro* systems, notably that prolonged culture can lead to changes in gene regulation, including loss of the H3K27me3 repressive mark (Bracken et al, 2007). Isolated cells are also not necessarily relevant to the behaviour of cells in complex *in vivo* systems; for example HepG2 cells lack HFE expression (Gao et al, 2009), so would not be a good model to study iron homeostasis. Finally, the precise function of Mel-18 in hepatic cell populations has not been established, with work in cell lines derived from other tissues suggesting it may have similar or opposing functions to Bmi-1 in different contexts. Depletion of one or both of *Bmi-1* and *Mel-18* would allow assessment of whether their functions are complementary in liver cell populations. We also now have the possibility of obtaining liver tissue samples from *Mel-18* knockout mice (Akasaka et al, 1996). It would be interesting to assess the hepatic phenotype in these to see whether they suffer similar defects to *Bmi-1* knockout mice, which would suggest that the two proteins act in synergy in liver cells.

This thesis has established that a number of genes/proteins are upregulated at some time points following *Bmi-1* deletion in mouse livers, including cyclin D1, *c-myc*, *Tfr1* and *Hamp*. However, it is not clear whether these are direct targets of Bmi-1, or whether their upregulation is secondary to alterations in other pathways. There are also likely to be other important Bmi-1 targets in liver which have not yet been
characterized. These questions could be assessed in the future using ChIP sequencing (ChIP-seq) to map Bmi-1 binding sites in murine liver. This would also help address whether the phenotypic abnormalities observed in Bmi-1 knockout hepatocytes are cell autonomous.

7.2.1. Final discussion of Bmi-1 knockout liver phenotypes:

The hepatic phenotype in the Bmi-1 knockout mice appeared to be less severe than that observed in other organs, such as the neural or haematopoietic systems. On the other hand, it should be noted that not all Bmi-1 knockout organs show any demonstrable phenotype, as lung development and morphology are normal in Bmi-1 knockout mice (Dovey et al, 2008). The milder phenotypes in Bmi-1 knockout livers in comparison to other organs may reflect the fact that the adult liver is generally a quiescent organ. The haematopoietic system, for example, constantly relies on self-renewing haematopoietic stem cells for the generation of new mature blood cells. Foetal livers of Bmi-1 knockout mice have normal numbers of haematopoietic stem cells (HSCs), however the quantities become markedly reduced in postnatal livers (Park et al, 2003). There is little detectable self-renewal of HSCs in Bmi-1 knockout mice, so it is unsurprising that the mice suffer progressive haematopoietic defects. Cell turnover in the adult liver is much lower than in the haematopoietic system, and there is some debate over whether a liver stem cell compartment plays any role in the process (see introduction section 1.3.5). It is also clear from the data described in Chapter 5 that hepatocyte proliferation is unimpaired in all but the oldest Bmi-1 knockout mice. Given these factors, it seems likely that if there are any defects in stem cell populations in Bmi-1 knockout livers, they would not lead to major phenotypic abnormalities under resting conditions. The slow cell turnover in the adult liver, coupled with the 12 week lifespan of Bmi-1 knockout mice, also means that more severe structural defects may not have time to emerge.
One piece of evidence in favour of contribution of portal stem/progenitor cell contribution to human liver turnover is the presence of patches of hepatocytes with identical mitochondrial mutations, suggesting the cells are clonally derived (Fellous et al, 2009a). During the first few months of my PhD, I ascertained that these patches are also present in mice (see appendix section A1). However, attempts to use this mitochondrial lineage tracing technique to examine stem cell defects in Bmi-1 knockout mice were unsuccessful due to a lack of patches, probably due to the shortened lifespan of the mice.

Another point of note is that there appear to be some differences in the phenotypes observed in 8 week old and 12 week old Bmi-1 knockout mice. In particular, 8 week old mice exhibited an increase in hepatocyte proliferation, while 12 week old mice showed a decrease. An increase in expression of the Bmi-1 homologue Mel-18 was found in 12 week old Bmi-1 knockout mice relative to wild types, but not in 8 week old mice. The 12 week old mice also exhibited more severe iron overload, which was coupled with some changes in the expression of genes involved in iron regulatory pathways that were not observed at 8 weeks. The predominant difference between the knockout mice at 12 weeks of age compared to 8 weeks is their worsening health, meaning that all mice were sacrificed by 12 weeks. It is therefore possible that the phenotypic differences between the two time points represent the predominance of worsening secondary effects due to ill health at 12 week of age. As such, the 8 week old mice could be considered more representative of the effects of Bmi-1 deletion in liver. However, studies in other organs have shown more severe defects with age, so it is possible that some of the additional abnormalities in 12 week old knockout mice have physiological relevance. This would become clearer with a liver specific Bmi-1 knockout mouse.

It is also notable that the majority of the phenotypic changes observed in Bmi-1 knockout livers (altered hepatocyte proliferation, polyploidisation, iron overload) affect
hepatic parenchymal cells rather than cholangiocytes. This is interesting, given that Bmi-1 appeared to be most strongly expressed in cholangiocytes. It is possible that Bmi-1 is not required in cholangiocytes under homeostatic conditions, as the proliferation rate of cholangiocytes is very low. Alternatively, the loss of Bmi-1 may be compensated for by proteins such as Mel-18, which is similarly expressed in cholangiocytes. Immunohistochemical analysis showed Bmi-1 expression in some hepatocytes at lower levels, so it is possible that the hepatic phenotypes in Bmi-1 knockout mice directly result from the absence of Bmi-1 in hepatocytes. As mentioned previously, the fact that the Bmi-1 knockout is not liver specific again means that secondary effects cannot be ruled out. As discussed in the relevant chapters, the hepatic iron overload could be secondary to anaemia, while the polyploidisation defects could be linked to a reduction in insulin signalling. The creation of a liver specific Bmi-1 knockout mouse model would again address these issues.

7.2.2. Bmi-1 in hepatic stem cell mediated regeneration:

Much of the previously published work on the liver-specific function of Bmi-1 has centred on its role in maintenance of hepatic stem cells in vitro. Chiba et al have demonstrated some reduced colony forming potential and bilineage differentiation in putative foetal liver stem cell populations following Bmi-1 knockdown, both using shRNA (Chiba et al, 2007) and in cells derived directly from Bmi-1 knockout mice (Chiba et al, 2010). These effects do not appear as dramatic as in some other organs, although they do suggest that Bmi-1 has some function in hepatic stem cell maintenance.

It is established that hepatic stem cells play a role in liver regenerative responses, namely the oval cell response which emerges when hepatocyte mediated regeneration is impaired. Oval cells are generally believed to be derived from resident hepatic stem cells (see introduction section 1.3). Given the in vitro work suggesting
that Bmi-1 is important in liver stem cell maintenance, it was considered possible that Bmi-1 could play a role in the oval cell response. In support of this, cytokeratin 19 positive oval cells from two different mouse models displayed strong immunoreactivity for Bmi-1. It was therefore hypothesised that the loss of Bmi-1 could result in an impaired oval cell response in Bmi-1 knockout mice. One original aim for this thesis was to assess this theory by subjecting Bmi-1 knockout mice to protocols which induce an oval cell response, namely CDE and DDC diets. Since the commencement of this work, Chiba et al have reported an impaired oval cell response in Bmi-1 knockout mice in the C57BL/6 background (Chiba et al, 2010). Although this was not characterised in great detail, the Bmi-1 knockout mice exhibit a significant decrease in the numbers of A6 positive oval cells following DDC treatment. This reduction appears to be approximately 50% based on cell counting studies, demonstrating that the oval cell response is impaired but not abolished. This led the authors to suggest that redundancy among other Polycomb group genes may weaken the phenotype. This would agree with the similar hepatic expression patterns of Bmi-1 and Mel-18 we observed in murine liver. Attempts to assess Mel-18 expression in oval cell reactions were unsuccessful, due to poor immunoreactivity of the anti-Mel-18 antibody with the archival paraffin blocks, and high background staining. This could be assessed in the future on fresh blocks.

We now have a Home Office project license in place permitting experiments to induce liver injury in Bmi-1 knockout mice. This opens up the possibility for future studies into the role of Bmi-1 in liver regeneration. Bmi-1 expression was observed in some hepatocyte regenerative nodules in human cirrhotic tissue, suggesting that Bmi-1 may have a role in hepatocyte mediated regeneration. This could be investigated further by subjecting Bmi-1 knockout mice to 2/3 partial hepatectomies, and assessing whether the subsequent regenerative process is impaired. We are also aiming to induce the oval cell response in Bmi-1 knockout mice on the FVB background using a DDC diet.
This will examine whether the reduced oval cell response observed by Chiba et al in C57BL/6 Bmi-1 knockout mice is also seen in FVB mice. Depending on the results, the characterization of expression changes in other Polycomb genes (especially Mel-18) and potential Bmi-1 target genes will allow investigation of the mechanistic reasons behind an impaired oval cell response (if one is observed), or potential compensatory pathways which may prevent Bmi-1 deletion from having a large effect on oval cell proliferation (if an impaired oval cell response is not observed). In addition to this, the effects of Bmi-1 deletion on cholangiocyte mediated repair could be assessed following bile duct ligation. It was not possible to assess the proliferative ability of cholangiocytes in resting Bmi-1 knockout livers, due to the very low proliferation frequencies observed. Performing bile duct ligations on Bmi-1 knockout mice will force cholangiocytes to proliferate (Yoshioka et al, 2005), and hence will uncover any defects in the proliferative response of Bmi-1 deficient cholangiocytes.

Bmi-1-Cre-ER transgenic mice (Sangiorgi & Capecchi, 2008; Sangiorgi & Capecchi, 2009) also provide a useful model to assess the function of Bmi-1 expressing cells in liver regeneration, as they allow stable genetic labelling of cells which express Bmi-1, and their progeny. The induction of both hepatocyte and oval cell mediated regeneration in these mice would allow direct assessment of the contribution of Bmi-1 expressing cells to hepatic regeneration.

7.2.3. Tumourigenesis in Bmi-1 knockout mice:

Finally, another issue worth considering is the fact that aspects of the hepatocyte phenotype in Bmi-1 knockout mice show similarities with tumourigenic environments. Bmi-1 knockout livers appear to exhibit reduced hepatocyte polyploidisation, which will result in increased numbers of smaller diploid cells. Increased numbers of small diploid cells have previously been reported in hyperplastic nodules that characterise rat liver carcinogenesis (Schwarze et al, 1984). At 8 weeks of age, Bmi-1 knockout
livers also exhibit a small increase in hepatocyte proliferation, coupled with upregulation of c-myc mRNA and cyclin D1 protein. C-myc is commonly overexpressed in tumours (Adhikary & Eilers, 2005), while transgenic mice overexpressing cyclin D1 develop HCC (Deane et al, 2001). Furthermore, iron overload has been associated with tumourigenesis, as patients with haemochromatosis have a significantly increased risk of developing HCC (Batts, 2007) (although it should be noted that this is usually following cirrhosis, which was not observed in Bmi-1 knockout mice). HCC has also been reported in some other hepatic iron loading conditions, including anaemias (Kew, 2009), although it is unclear whether lower levels of iron accumulation do actually predispose to HCC. These observations hint at the possibility that the environment in Bmi-1 knockout livers could be a precursor to the development of hepatic tumours. It is possible that tumours would not have time to develop given the short lifespan of the mice. However, this hypothesis would appear contradictory given that most literature suggests Bmi-1 is oncogenic and is overexpressed in tumours. Future experiments to subject Bmi-1 knockout and overexpressing mice to tumour inducing protocols (Alison & Lovell, 2005) could provide further insight into the role of Bmi-1 in hepatocarcinogenesis.

7.3. Concluding paragraph:

Bmi-1 has emerged as a potential molecular target in cancer therapy due to its established oncogenic functions, including promoting self-renewal in cancer stem cell populations in a variety of tissue types. Targeting Bmi-1 is likely to be possible in future through the use of small molecule drugs that reduce its expression, or through siRNA mediated approaches (Cao et al, 2011). It is therefore important to have a thorough understanding of both the function of Bmi-1 and the consequences of its depletion in normal cell populations. There is also a lack of whole human donor livers available for transplant. By understanding and harnessing the proliferative potential of hepatic stem cells, novel transplantation therapies could be developed (Duncan et al,
2009a; Kung & Forbes, 2009). The epigenetic regulation of regenerative responses is not well understood, so a better understanding of the role of epigenetic regulators, including Polycomb genes such as Bmi-1, could possibly improve cell-based treatments or stimulate an endogenous response.

This thesis has furthered our understanding of the role of Bmi-1 in hepatic cells by examining the phenotypic consequences of Bmi-1 deletion on murine liver growth and function. The work undertaken has uncovered potential roles for Bmi-1 in hepatocyte polyploidisation, proliferation and bodily iron homeostasis which have not previously been described in the published literature. In addition, the high levels of Bmi-1 expression in murine oval cells and some regenerative cells within human cirrhotic tissue hint at an important role for Bmi-1 in promoting hepatic tissue regeneration. Together, these observations provide scope for a variety of future studies to further define the mechanisms underlying the Bmi-1 deletion phenotype and the function of Bmi-1 in liver growth and regeneration.
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255


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262


Appendix:

A1. Lineage tracing using mitochondrial DNA mutations:

Recently established cell lineage tracing techniques using naturally occurring, non-pathogenic mitochondrial DNA mutations have been to study clonal cell populations within human tissue samples, including in hepatic tissue (Fellous et al, 2009b). The technique involves using histochemical methods to stain tissue sections for activity of the enzyme cytochrome c oxidase (COX), which is encoded by mitochondrial DNA. In occasional cells, mutations in COX will accumulate to the extent that the majority of mitochondria in the cell contain the same mutation, and the cell will hence stain as COX deficient. Mitochondrial DNA sequencing of laser-dissected tissue samples can then be used to identify the mutation. This technique identifies patches of COX negative cells within various tissues, all of which have the same mutation and are hence clonally derived. It can therefore be applied to investigate the location of stem cell niches within adult tissues, as it is believed that only stem cells will remain in the tissue long enough to accumulate the necessary mutations. In the liver, patches of COX negative hepatocytes appear to be connected to portal regions (Fellous et al, 2009a). This has led the authors to propose the existence of a liver stem/progenitor cell niche within the portal region, which may contribute to the turnover of hepatocytes in normal liver tissue. This is in agreement with other work which suggests that liver stem-cell derived oval cells originate from the canals of Hering, the terminal biliary ducts of the liver (Paku et al, 2001), and that Sox9 positive cells within the biliary tree can give rise to hepatocytes in normal liver (Furuyama et al, 2011). In vitro work in foetal liver derived putative stem/progenitor cells has demonstrated that knockdown of Bmi-1 has a negative effect on the colony forming ability and bilineage capacity of these cells (Chiba et al, 2007). It was therefore hypothesised that the absence of Bmi-1 would have an effect on the function adult liver stem cells in vivo, which would in turn potentially result in either a reduction or absence of the portally-derived clonal
COX deficient patches in Bmi-1 knockout mice. To assess whether the mitochondrial lineage tracing technique used in humans could be equally applied to mice, frozen liver sections from a 9 month old BL6J mouse were stained for COX activity (Figure 43). Sections were also counterstained for the nuclear encoded enzyme, succinate dehydrogenase (SDH), to allow easier visualisation of COX-negative patches. As in humans, small patches of COX negative cells were detected, and many of these appeared to originate from portal regions (Figure 43a). To investigate whether the patches were either reduced in number or absent completely in Bmi-1 knockout mice, thus suggesting defects in the putative portal stem/progenitor cell population, frozen sections from 12 week old Bmi-1 wild type and knockout mice were stained for COX activity. No COX negative patches were detected in any of the wild type or knockout mice examined, so it was not possible to make any comparison between the two (Figure 43b,c) (n=5). A potential reason for this is that the mitochondrial mutations take a long time to become established, hence they have only been observed in human patients above 40 years old (Fellous et al, 2009b). It is therefore likely that mice at 12 weeks of age were not old enough to detect any COX negative cells, whereas they could be detected at 9 months of age. Due to the fact that the Bmi-1 knockout mice become unwell as they age, it was not possible to keep the mice beyond 12 weeks, and hence this work was not pursued further.
Figure 43: Application of mitochondrial lineage tracing techniques to *Bmi-1* knockout mice.

Frozen liver sections were stained for cytochrome c oxidase (COX, brown stain) and counterstained for succinate dehydrogenase (SDH, blue stain). a) COX negative patch in 9 month old mouse (red arrow). b,c) Absence of COX negative patches in 12 week old *Bmi-1* wild type (b) and knockout (c) mice. All scale bars 200 µm.
**A2. Selection of reference gene for Q-RT-PCR experiments:**

The selection of a suitable internal control gene to use to normalise the expression data is a necessary step in designing robust Q-RT-PCR experiments. The transcription of several commonly used reference genes can be altered dramatically by different experimental conditions, for example hypoxia (Caradec et al, 2010), so it is important to find a gene that is expressed relatively consistently across all the conditions to be studied. Preliminary analysis of cDNA from 12 week old *Bmi-1* wild type and knock out mouse livers revealed different relative levels of two commonly used reference genes; β-actin and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). It was therefore decided to determine the most stable reference genes using the Normfinder method (Andersen et al, 2004). Briefly, Normfinder uses mathematical modelling of gene expression data to estimate the variation of candidate normalisation genes, both overall and between different subgroups of samples (in this case, groups of *Bmi-1* wild type and knockout mice at different ages). Four commonly used reference genes in different functional classes were selected for analysis: β-actin, *Gapdh*, β-glucuronidase (*Gus*), and eukaryotic elongation factor 2 (*eEF2*). The expression of each gene was analysed in pairs of *Bmi-1* wild type and knockout mice at 3 different time points: 2 sets of newborn, 4 sets of 8 week old and 2 sets of 12 week old mice. The raw expression data were input into the Normfinder Microsoft Excel add-in program (http://www.mdl.dk/publicationsnormfinder.htm) to generate values for expression stability, with lower values describing more stable genes. The program selected *eEF2* as the most stable gene, with a stability value of 0.098. In cases where there is no optimal gene for normalisation, it is possible to use a normalisation factor based on the average expression of two or more genes to improve accuracy. In this case, addition of the gene predicted by Normfinder as the second most stable (*Gus*) slightly improved the stability value to 0.074. It was decided that this was not a significant enough improvement to warrant use of a normalisation
factor. To provide support for the choice of normalisation factor, the expression data for the four chosen candidate genes were also analysed using another program, geNorm, which examines pairwise variation between candidate genes (Vandesompele et al, 2002). This program also identified eEf2 as the most stable reference gene. Therefore, eEf2 expression was run as a reference gene in all subsequent Q-RT-PCR experiments. A second gene, β-actin, was also run for all subsequent samples to ensure the expression ratio of two genes remained relatively constant. In any cases where there was a significant change in the ratio of the two genes, up to four additional genes (Gapdh, Gus, Ribosomal protein L27 (Rpl27) and Ribosomal 18S) were run to assess which gene showed abnormal expression. In rare cases where multiple reference genes showed abnormal expression in a sample, that sample was discarded. For all results described in this thesis, eEf2 expression alone was considered a suitable normalisation factor, and the addition of another gene did not make any alterations to the significance of any results.

A3. Analysis of hepatocyte nuclear area using touch preparations:

The measurement of nuclear size in H&E stained sections is imperfect, due to the fact that nuclei may not be cut through the same plane and hence may appear to be smaller in diameter. To circumvent this problem, hepatocytes were isolated from excised mouse livers digested in collagenase by touching a freshly cut surface onto a microscope slide. This provided a preparation of whole cells suitable for nuclear size analysis and binuclearity counting, which is also imperfect in H&E stained sections. The touch preparation slides were stained with methylene blue and haematoxylin, and blindly labelled images subjected to analysis using ImageJ software. The image analysis procedure is outlined in Figure 44. The threshold feature of ImageJ was used to highlight the hepatocyte nuclei in the image. Images were taken using the same microscope exposure settings, allowing consistent threshold values to be used for analysis of each image, thus removing the need for subjective judgement of threshold.
settings for each image. The Nucleus Counter of ImageJ was now used to measure the average area of marked nuclei. The touch preparations also contained some populations of non-parenchymal cells, which were not wanted for analysis. To eliminate these, the Nucleus Counter was adjusted to exclude particles below a certain size from the average area measurements. However, it was not possible to define a single size threshold which completely eliminated all non-parenchymal cells without eliminating any of the smallest hepatocytes. Therefore, measurements for each image were taken for two different particle minimum sizes: 75 pixels (7.8 µm²), which counted all hepatocyte nuclei along with the largest non-parenchymal cells, and 100 pixels (10.4 µm²), which eliminated all non-parenchymal nuclei and occasionally a few of the smallest hepatocytes. The same overall result was observed whichever size threshold was used (results using 100 pixel threshold are shown in this thesis). The results shown in this thesis are for female mice only to eliminate any possible sex specific variations, although males examined did not exhibit any differences.

**Figure 44 (overleaf): Analysis of nuclear area in hepatocyte touch preparations.** Freshly excised mouse liver sections were touched onto microscope slides and the cells stained with methylene blue and haematoxylin. a,b) Touch preparation images from 12 week old *Bmi-1* wild type (a) and knockout (b) mice. c-f) Analysis of nuclear area in touch preparation image using ImageJ. c) Image converted to greyscale. d) Nuclei highlighted using threshold feature. e) Thresholded image converted to black and white. f) Numbered nuclei, above minimum size threshold, were included in counting and average area measurements. All scale bars 50 µm.
Figure 44: Analysis of nuclear area in hepatocyte touch preparations.

See previous page for figure legend.