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Functional analysis of the KDM5B demethylase
In vitro and in vivo studies

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Functional analysis of the KDM5B demethylase:

*In vitro* and *in vivo* studies

Steven Catchpole

August 2013

A thesis submitted in accordance with the requirements for examination for the degree of Doctor of Philosophy

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Declaration

All of the experimental work, results and ideas presented in this thesis, except where acknowledged accordingly, are entirely my own.

None of the work contained within this thesis has previously been submitted for examination by the University of London or any other degree awarding body.

The work was carried out under the supervision of Professor Joyce Taylor-Papadimitriou and Professor Joy Burchell at King’s College London, Guy’s Hospital.
Abstract
Thesis abstract

The KDM5 family consists of four proteins, namely KDM5A, KDM5B, KDM5C and KDM5D. The KDM5 family of demethylase proteins counteract the tri-methylation of H3K4 and can function as transcriptional repressors. They can form a number of different multi-protein complexes that contain other chromatin-modifying factors and co-factors that aid in the recruitment of the protein complex to the genome, whilst also possessing the ability to bind to DNA directly through the ARID domain. Furthermore the KDM5 family has also been implicated in mediating nuclear receptor signalling. The KDM5 family are highly homologous and where co-expressed may act redundantly. A picture is emerging where the KDM5 family may dictate the shift in balance between cellular proliferation and differentiation and are therefore important during development. Although the KDM5 demethylase activity has attracted the greatest attention, it is apparent that this family is an important multi-faceted group of proteins.

To study the function of KDM5B two knockout murine lines were generated. The Kdm5b null murine line resulted in early embryonic lethality, whereas a second murine line expressing a KDM5B protein lacking the ARID domain (ΔARID) was viable and fertile but displayed a mammary gland phenotype, where terminal end bud development and side branching were delayed at puberty. Since intact oestrogen receptor (ER alpha) signaling is a prerequisite for nulliparous mammary gland development we examined the expression of the progesterone receptor in the adult nulliparous KDM5B ΔARID murine mammary gland and found levels to be reduced as compared to the wild-type. Furthermore, co-immunoprecipition of tagged human KDM5B and ER alpha proteins demonstrated an in vivo interaction. These studies suggest that KDM5B is required during embryonic development and may be involved in the ER alpha signaling pathway during nulliparous mammary gland development. Our analysis of the C57BL/6J-Kdm5b ΔARID pregnant mammary gland showed a delay in mammary gland morphogenesis and the female could nurse her pups at parturition. Investigation into the molecular mechanisms that may relate to this showed that KDM5B expression is required to positively regulate the PRLR/JAK2/STAT5 signalling pathway associated with mammary gland development during gestation.
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I would like to thank all those who have contributed to this work. To Dr Samantha Santangelo who designed and constructed the targeting constructs used to generate the B6;129-\textit{Kdm5b Exon 1} knock out and the C57BL/6-\textit{Kdm5b ΔARID} murine lines. To Dr Ian Rosewell and colleagues at Cancer Research UK for their participation in the generation of the B6;129-\textit{Kdm5b Exon 1} knock out and the C57BL/6-\textit{Kdm5b ΔARID} murine lines and Janet Macdonald who carried out the breeding programs and always went beyond the call of duty – thanks Janet. To Debbie Hall for her help with the screening of the mice and for sharing both good times and bad! I would also like to give my special thanks to Dr Bradley Spencer-Dene for being a great guy who helped me with the studies on the murine mammary gland. Finally, I would like to thank Professor Gordon Stamp who provided the pathology reports and Dr. Richard Poulson and colleagues for help with the \textit{in situ} hybridisation data.
Chapter 1. Introduction: Section 1A: The regulation of chromatin: Methylation of histone lysine residues.

1A Prologue

1A.1 The regulation of chromatin: Methylation of histone lysine residues

1A.1.1 The nucleosome and histone tail

1A.1.2 It takes a PHD to be a reader in H3K4 methylation

1A.2 Histone marks and murine mammary development

1A.3 The KDM5 family and gene regulation

1A.3.1 The conserved domains of the KDM5 family of proteins

1A.3.2 The KDM5 family as H3K4 demethylase enzymes

1A.3.3 The KDM5 family of transcriptional repressors

1A.3.4 A function for the KDM5 family of proteins in the activation of transcription

1A.3.5 Direct Recruitment of the KDM5 family to the genome

1A.3.6 Recruitment of the KDM5 family to the genome as part of a multi-protein complex

1A.3.7 Lessons from the past: Studies in C. Elegans and D. Melangaster

1A.4 The expression patterns of the KDM5 family of proteins

1A.4.1 The expression profile of KDM5A and KDM5B in human adult tissue

1A.4.2 The expression profile of KDM5B in murine adult tissue

1A.4.3 KDM5B expression during murine embryonic development

1A.4.4 A function for KDM5B during ES cell commitment

1A.4.5 The expression profile of KDM5C and KDM5D

1A.4.6 A role for the KDM5 family of proteins during neurogenesis

1A.5 A role for KDM5A and KDM5B during senescence and differentiation

1A.5.1 A function for KDM5B during the G1 to S phase transition of the cell cycle

1A.5.2 KDM5A and KDM5B link H3K4 demethylation to retinoblastoma
binding protein mediated senescence

1A.5.3 A function for KDM5A during differentiation 22

1A.6 KDM5B and cancer 23

1A.6.1 KDM5B expression and melanoma 24

1A.6.2 KDM5B expression and prostate cancer 24

1A.6.3 KDM5B and breast cancer 25

1A.7 Clinical sub-types of breast cancer 26

1A.8 The relevance of murine models to study mammary gland morphogenesis 26

Chapter 1. Introduction: Section 1B: Development, differentiation and involution of the murine mammary gland 28

1B Overview 29

1B.1 Murine mammary gland stem cells, the epithelial cell hierarchy 31

1B.1.1 The existence of a murine multi-potent adult mammary gland stem cell 31

1B.1.2 Murine postnatal mammary gland development is defined in the embryonic mammary bud and completed after birth by uni-potent stem cells 34

1B.1.3 Both multi-potent and uni-potent murine mammary gland stem cells contribute to postnatal mammary gland development 35

1B.1.4 Ovarian hormone control of mammary epithelial cell hierarchy organisation during postnatal mammary gland development 35

1B.1.5 Cell fate and histone marks in the mammary gland 37

(A) The chromatin effector protein, Pygo-2 37

(B) The EZH2 histone methyltransferase 38

1B.2 Studies on pubertal mammary gland development 39

1B.2.1 Oestrogen receptor signalling 39

1B.2.2 Progesterone receptor signalling in the adult mammary gland 41

1B.2.3 Growth factor regulation of ductal morphogenesis 42

1B.3 Morphogenesis and Differentiation of the mammary gland at Pregnancy and Lactation 44

1B.3.1 Progesterone and prolactin in alveologenesis and terminal differentiation 45

1B.3.2 The function of the Ets transcription factor ELF5 during cell fate determination in the pregnant mammary gland 46

1B.4 Post weaning and Involution of the mammary gland 47

1B.5 The JAK/STAT pathways during mammary gland development 48
1B.5.1 STAT6 in luminal cell development 50
1B.5.2 The role of STAT5 in alveologenesis and expression of milk proteins 52
1B.5.3 STAT3 function during involution 53

1C Aims 56

Chapter 2. Materials and methods 59
2.1 Solutions and buffer 60
2.2 General statement 61
2.3 Cell culture 61
2.4 Preparation and analysis of DNA 62
  2.4.1 Phenol:chloroform extraction and precipitation of tail-snip genomic DNA 62
  2.4.2 Determining the concentration of DNA 62
  2.4.3 Restriction endonuclease digest of (A) B6;129-Kdm5b-Exon 1 knock out and (B) B6;129-Kdm5b ΔARID genomic DNA for Southern blot analysis 62
  2.4.4 Agarose gel electrophoresis of DNA 63
  2.4.5 Genotyping by Southern transfer 63
  2.4.6 End point PCR on C57BL/6J mammary gland cDNA and sequencing 64
  2.4.7 B6;129-Kdm5b-Exon 1 knock out PCR screen to identify homologous recombination 65
  2.4.8 PCR screen used to detect heterozygous B6;129-Kdm5b ΔARID offspring during backcross to the C57BL/6J genetic background 65
  2.4.9 PCR screen used to detect the polyoma virus middle T antigen transgene (PyV-mT) 65
  2.4.10 Generation of chemically competent Escherichia coli (E.coli) 65
  2.4.11 Transformation of competent E. Coli 66
  2.4.12 Large-scale preparation of plasmid DNA 66
  2.4.13 Plasmid constructs 67
2.5 Preparation and analysis of RNA 67
  2.5.1 Extraction of total RNA 67
  2.5.2 Determining the concentration of RNA 67
  2.5.3 cDNA synthesis 67
  2.5.4 Quantitative PCR and PCR primers 68
2.6 Preparation and analysis of protein 69
  2.6.1 Nuclear and cytoplasmic protein extraction 69
  2.6.2 SDS-PAGE electrophoresis and Western blot transfer 69
2.6.3  Primary antibodies used for Western blot  71
2.6.4  Secondary antibodies used for Western blot  72
2.6.5  Transient transfection of plasmid DNA  72
2.6.6  Immunoprecipitation  73
2.6.7  Antibodies used for immunoprecipitation  73

2.7  Murine studies  74
2.7.1  Development of the B6;129-Kdm5b ΔARID mouse strain and backcross to the C57BL/6J genetic background  74
2.7.2  Development of the B6;129-Kdm5b-Exon 1 knock out murine line  74
2.7.3  Breeding program between the C57BL/6-Kdm5b ΔARID +/- and the FVB-MMTV-PyV-mT +/- murine lines  74
2.7.4  Staging pregnancy and synchronising oestrous cycle  75

2.8  Cell biology techniques  75
2.8.1  Histology  75
2.8.2  Mammary gland whole mount  76
2.8.3  Immunohistochemistry  76
2.8.4  Primary antibody used for immunohistochemistry  77
2.8.5  Secondary antibody used for immunohistochemistry  77
2.8.6  Microscopy and data analysis  78

Chapter 3. Studies on the function of KDM5B in vivo  79
3.1  Aim  80
3.2  Introduction: A role for KDM5B during development  80
3.3  Results  81
3.3.1  Ablation of murine KDM5B results in embryonic lethality  81
3.3.2  Development, screening and preliminary characterisation of the chimeric B6;129-Kdm5b ΔARID murine line  87
  3.3.2.1  The B6;129-Kdm5b ΔARID murine line are viable and fertile  87
  3.3.2.2  Expression of a less abundant KDM5B protein lacking the entire ARID domain and part of the JmjN domain  89
       (A) KDM5B protein expression  89
       (B) Processing of the KDM5B transcript  90
  3.3.2.3  Development of adult tissues in the B6;129-Kdm5b ΔARID murine line  93
       (A) Histological analysis of male and female adult tissue  93
       (B) A possible defect in mammary gland ductal morphogenesis  94
3.4  Discussion  98
  3.4.1  Assessing the developmental role of KDM5B  98
Chapter 4. KDM5B is expressed during mammary gland ductal morphogenesis and is required for normal mammary gland development

4.1 Aims

4.2 Introduction: Murine mammary gland ductal morphogenesis

4.3 Results

4.3.1 Documentation of KDM5B expression during C57BL/6J murine mammary gland development

4.3.1.1 Cellular localisation of the Kdm5b transcript expressed during murine C57BL/6J mammary gland development

4.3.1.2 Quantification of the Kdm5b transcript during murine C57BL/6J mammary gland development

4.3.1.3 KDM5B protein expression during murine C57BL/6J mammary gland development

4.3.1.4 Backcross of the chimeric homozygous B6;129-Kdm5b ΔARID murine line onto the inbred C57BL/6 genetic strain

4.3.1.5 Generation of the homozygous C57BL/6-Kdm5b ΔARID murine line

4.3.1.6 Kdm5b transcript levels in the C57BL/6J-Kdm5b ΔARID female mammary gland do not reflect KDM5B protein levels

4.3.2 Mammary gland development in the C57BL/6-Kdm5b ΔARID nulliparous female

4.3.2.1 The C57BL/6-Kdm5b ΔARID 4-week old mammary gland presents with fewer terminal end buds and a defect in ductal elongation and side branching

4.3.2.2 The C57BL/6-Kdm5b ΔARID 12-week old mammary gland presents with fewer bifurcation points, a defect in ductal elongation and side branching

4.3.2.3 Defective mammary gland development in the C57BL/6-Kdm5b ΔARID pregnant female

4.4 Discussion

4.4.1 KDM5B is expressed during mammary gland morphogenesis
and may suggest a role in epithelial cell proliferation, differentiation and survival

4.4.2 An increase in Kdm5b transcript level translates to a less abundant KDM5B protein in the C57BL/6J-Kdm5b ΔARID female at pd 18.5

4.4.3 A function for KDM5B during nulliparous mammary gland development

4.4.4 A function for KDM5B during pregnancy associated mammary gland morphogenesis

4.5 Supplemental material

Chapter 5. Investigation into the effect of KDM5B ΔARID expression on gene regulation and breast cancer development

5.1 Aims

5.2 Introduction

5.2.1 KDM5B and gene regulation

5.3 Results

5.3.1 Studies on a possible role for KDM5B in ER alpha signalling

5.3.1.1 Analysis of transcription levels in the C57BL/6J-Kdm5b ΔARID nulliparous mammary gland

5.3.1.2 KDM5B interacts with ER alpha in vivo in an ARID independent manner

5.3.2 Studies on KDM5B repressor function in the adult C57BL/6J-Kdm5b ΔARID mammary gland

5.3.3 Molecular assessment of the role of KDM5B during development of the C57BL/6J-Kdm5b ΔARID pregnant mammary gland

5.3.3.1 Molecular analysis in the C57BL/6J-Kdm5b ΔARID mammary gland at mid-pregnancy

(A) Steroid receptor expression at mid pregnancy

(B) Changes in the JAK/STAT and AKT signaling axis at mid pregnancy

5.3.3.2 Molecular analysis in the C57BL/6J-Kdm5b ΔARID mammary gland at late pregnancy

(A) Steroid receptor expression at late pregnancy

(B) Changes in the JAK/STAT and AKT signaling axis at late pregnancy

(C) Analysis of Kdm5a transcript expression in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at late pregnancy
5.4 Discussion

5.4.1 A potential role for KDM5B during oestrogen receptor signaling in the pubertal mammary gland

5.4.2 Analysis of KDM5B target genes in the adult C57BL/6J-Kdm5b ΔARID mammary gland

5.4.3 Importance of epigenetic factors in mammary gland development

5.4.4 KDM5B function during pregnancy associated mammary gland development
   (A) Early to mid-pregnancy
   (B) Late pregnancy

5.4.5 The relative ratio of phosphorylated to un-phosphorylated STAT5

5.4.6 The regulation of STAT5 activation

Chapter 6. Investigation into the effect of KDM5B ΔARID expression on mammary gland tumour development

6.1 Aims
6.2 Introduction
6.3 Results
   6.3.1 The expression of a KDM5B ΔARID results in a delay in mammary gland tumour development
6.4 Discussion
   6.4.1 KDM5B ΔARID expression and breast cancer
6.5 Supplemental material

Chapter 7 Discussion

7.1 Summary
7.2 The KDM5B null phenotype
7.3 Deciphering the role of the KDM5 family of proteins during development
7.4 KDM5B function during pubertal mammary gland
7.5 KDM5B, stem cells and progenitor cells
7.6 KDM5B, pregnancy and involution
   7.6.1 KDM5B and the STAT signalling pathway
7.7 KDM5B, signalling pathways and the development of the mammary gland
7.8 Demethylase independent function of the KDM5 family of proteins

Chapter 8 References
Index of figures
Index of figures

Figure 1. Chromatin, the nucleosome and the histone tail cooperate to control gene regulation 3

Figure 1.1. Schematic representation of the protein domain position for the KDM5 family of H3K4 demethylase enzymes 8

Figure 1.2. Model for KDM5 mediated repression 10

Figure 1.3. Model for C-MYC/KDM5 target gene activation 11

Figure 1.4. Model for PcG/KDM5B binding to the bivalent promoters of developmental genes 14

Figure 1.5. Model for KDM5/NOTCH signalling 15

Figure 1.6. Homology between the human KDM5B and the murine KDM5B proteins 17

Figure 1.7. A model 'switch' in repression during differentiation 23

Figure 1.8. Murine postnatal mammary gland development 30

Figure 1.9. Nulliparous mammary gland development is completed by adulthood through the bifurcation of TEB structures and the generation of ductal side-branches 31

Figure 1.10. Murine mammary gland epithelial cell hierarchy 32

Figure 1.11. The JAK/STAT signalling pathway in the mammary gland 49

Figure 1.12 Schematic representation of mammary gland development In the virgin, at pregnancy, lactation and involution showing the expression of different phosphorylated STAT proteins 49
**Figure 3** Schematic representation of B6;129-Kdm5b-Exon1 knock out strategy detailing an outline for the production of gene targeted mice by manipulation and injection of ES cells

**Figure 3.1** Schematic representation showing the KDM5B protein domains with genomic sequence detailing the positions of the neomycin targeting construct (neo) used to replace exon1 (which codes for part of the JmjN domain) and homologous regions HR1 and HR2

**Figure 3.2** Schematic representation of the Kdm5b-WT allele and the Kdm5b-Exon1 targeting construct together with the screening strategy used to detect homologous recombination in transfected embryonic stem cells

**Figure 3.3** PCR screen on genomic DNA isolated from the off-spring of the heterozygous B6;129-Kdm5b-Exon1 knock out breeding programs

**Figure 3.4** Normal mammary gland development in the heterozygous B6;129-Kdm5b-Exon 1 knock out murine line

**Figure 3.5** Schematic representation showing the KDM5B protein domains with genomic sequence detailing the positions of the neo targeting construct used to replace part of exon 2 and exons 3 to 4

**Figure 3.6** Schematic representation of Kdm5b ΔARID targeting construct and the screening strategy used to detect homologous recombination in murine tail snip DNA

**Figure 3.7** Expression of a of a smaller less abundant KDM5B protein in the B6;129-Kdm5b ΔARID mammary gland and testis

**Figure 3.8** Processing of the Kdm5b primary transcript in the (A) B6;129-Kdm5b WT and (B) B6;129-Kdm5b ΔARID murine line

**Figure 3.9** DNA trace from the B6;129-Kdm5b ΔARID mammary gland transcript at pd 18.5

**Figure 3.10** Abnormal mammary gland development in the adult
**Figure 3.11** Abnormal mammary gland development in the B6;129-Kdm5b ΔARID murine line at pd12.5

**Figure 3.12** Mammary gland development at pd18.5 in the B6;129-Kdm5b ΔARID murine line

**Figure 3S1.** DNA trace from the B6;129-Kdm5b WT mammary gland transcript at pd 18.5

**Figure 3S2.** Murine Kdm5b coding sequence

**Figure 4.** Schematic representation of mammary gland ductal morphogenesis detailing the techniques employed to examine KDM5B expression levels

**Figure 4.1.** In situ hybridization showing the Kdm5b transcript is restricted to epithelial cells in the C57BL/6J mammary gland

**Figure 4.2.** The Kdm5b transcript is differentially expressed in the C57BL/6J WT murine mammary gland.

**Figure 4.3.** KDM5B protein expression in the C57BL/6J murine mammary gland

**Figure 4.4.** Representative neomycin specific PCR reaction on genomic DNA isolationed from the ear-snips of a heterozygous B6;129-Kdm5b ΔARID and C57BL/6J-Kdm5b WT breeding program

**Figure 4.5.** Representative Southern blot on tail-snip genomic DNA isolated from a heterozygous C57BL/6J-Kdm5b ΔARID breeding program

**Figure 4.6.** Representative Southern blot on tail-snip genomic DNA isolated from a homozygous C57BL/6J-Kdm5b ΔARID breeding program

**Figure 4.7.** Elevated Kdm5b transcript level in the C57BL/6J-Kdm5b ΔARID mammary gland at pd18.5 results in the translation of a less abundant KDM5B ΔARID protein
Figure 4.8. Fewer TEB structures in the 4-week old C57BL/6J-Kdm5b ΔARID nulliparous female

Figure 4.9. Abnormal mammary gland ductal development in the 4-week old C57BL/6J-Kdm5b ΔARID nulliparous female

Figure 4.10. Abnormal mammary gland development in the 12 weeks old (adult) C57BL/6J-Kdm5b ΔARID nulliparous female

Figure 4.11. The relatively quiescent state of the adult C57BL/6J-Kdm5b WT nulliparous mammary gland is not mirrored in the adult C57BL/6J-Kdm5b ΔARID female

Figure 4.12. Defective mammary gland development in the C57BL/6J-Kdm5b ΔARID female at pd12.5

Figure 4.13. Representative immunohistochemistry showing Ki67 staining in the C57BL/6J-kdm5b WT and C57BL/6J-Kdm5b ΔARID thoracic mammary glands at day 12.5 of pregnancy

Figure 4.14. Ki67 levels in the (A) alveoli and (B) ductal epithelia of C57BL/6J-kdm5b WT and C57BL/6J-Kdm5b ΔARID thoracic mammary glands at day 12.5 of pregnancy

Figure 4.15. A defect in mammary gland development at mid-pregnancy is overcome at late pregnancy.

Figure 4.16. The number of alveoli are significant reduced in the C57BL/6J-Kdm5b ΔARID mammary gland compared to the wild type counterpart at late pregnancy

Figure 4.17. Representative immunohistochemistry showing Ki67 staining in the C57BL/6J-kdm5b WT and C57BL/6J-Kdm5b ΔARID thoracic mammary glands at day 18.5 of pregnancy

Figure 4.18. Ki67 levels in the C57BL/6J-kdm5b WT and C57BL/6J-Kdm5b ΔARID thoracic mammary glands at day 12.5 of pregnancy
Figure 4.19 C57BL/6J-Kdm5b WT and ΔARID alveoli perimeters are comparable and fat globules are secreted into the lumen

Figure 4S1 Screening strategy used to backcross the chimeric heterozygous B6;129-Kdm5b ΔARID murine line onto the inbred C57BL/6J genetic strain

Figure 5. Relative transcript levels of Esr1 in the C57BL/6J-Kdm5b WT and C57BL/6J-Kdm5b ΔARID nulliparous mammary gland at 4 weeks of age

Figure 5.1. Relative transcript levels of Esr1, Pgr, and Wnt4 in the C57BL/6J-Kdm5b WT and C57BL/6J-Kdm5b ΔARID nulliparous mammary gland at 12 weeks of age

Figure 5.2. KDM5B interacts with ER alpha in vivo in an ARID independent manner

Figure 5.3. Relative transcript levels of Bub1, Brca1 and Mt1F in the C57BL/6J-Kdm5b WT and C57BL/6J-Kdm5b ΔARID nulliparous mammary gland at 12 weeks of age

Figure 5.4. Expression of ER alpha in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at mid-pregnancy

Figure 5.5. Transcript levels of Pgr and Wnt4 in the C57BL/6J-Kdm5b WT and C57BL/6J-Kdm5b ΔARID mammary gland at pd12.5

Figure 5.6. Expression of STAT6 in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at mid-pregnancy

Figure 5.7. The relative ratio of pSTAT5 to STAT5 is reduced in the C57BL/6J-Kdm5b ΔARID mammary gland at mid pregnancy

Figure 5.8. AKT expression is reduced in the C57BL/6J-Kdm5b ΔARID mammary gland at mid pregnancy

Figure 5.9. A delay in mammary gland differentiation is suggested by the reduced beta casein expression level in the C57BL/6J-Kdm5b
Figure 5.10. Expression of ER alpha in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at late pregnancy

Figure 5.11. Progesterone receptor expression level is reduced in the C57BL/6J-Kdm5b ΔARID mammary gland at late-pregnancy

Figure 5.12. Expression of STAT6 in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at late-pregnancy

Figure 5.13. The relative ratio of pSTAT5 to STAT5 is markedly reduced in the C57BL/6J-Kdm5b ΔARID mammary gland at late pregnancy

Figure 5.14. Phosphorylated STAT5 expression level is reduced in the C57BL/6J-Kdm5b ΔARID mammary gland at late-pregnancy

Figure 5.15. AKT expression in the C57BL/6J-Kdm5b ΔARID mammary gland at late pregnancy is comparable with the wild type counterpart

Figure 5.16. Beta casein and WAP expression level in the C57BL/6J-Kdm5b ΔARID mammary gland at late pregnancy indicative of full differentiation

Figure 5.17. Expression of Kdm5a in the in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at late pregnancy

Figure 5.18. Summary of molecular analysis in the C57BL/6J-Kdm5b ΔARID mammary gland

Figure 6. Schematic representation of the B6;FVB-Kdm5b ΔARID +/- : PyV-mT +/- breeding program

Figure 6.1 PCR generated genotype results of a litter from the C57BL/6J-Kdm5b ΔARID +/- female : FVB-MMTV-PyV-mT +/- male breeding program (B6FVBF1)

Figure 6.2 Genotype results of a litter from the B6;FVB-Kdm5b ΔARID +/- : PyV-mT +/- male and a C57BL/6J-Kdm5b ΔARID +/- female breeding program (B6FVBF2)
Figure 6.3 Genotype results of a litter from the B6;FVB-Kdm5b
\[\Delta ARID \text{ }/-: \text{PyV-mT }+/−\] male and a C57BL/6J-Kdm5b \[\Delta ARID \text{ }/-\] female
breeding program (B6FVBF3) 173

Figure 6.4. Box plot showing the distribution of time (in days) taken
to develop mammary gland tumours in the B6;FVB-Kdm5b \[WT:PyV-mT +/−\]
and B6;FVB-Kdm5b \[\Delta ARID \text{ }/-: \text{PyV-mT }+/−\] females 174

Figure 6.5. Kaplan-Meier plot showing time to tumour development in the
B6;FVB-Kdm5b \[\Delta ARID \text{ }/-: \text{PyV-mT }+/−\] and B6;FVB- \[Kdm5b \text{ }WT:PyV-mT +/−\]
female mammary gland 174
Index of tables
Index of tables

Table 2 Table listing primer sequences used in quantitative PCR 68
Table 2.1 Table listing primary antibodies used for Western blot 71
Table 2.2 Table listing secondary antibodies used for Western blot 72
Table 2.3 Table listing antibodies used for immunoprecipitation 73
Table 2.4 Table listing primary antibody used for immunohistochemistry 77
Table 2.5 Table listing secondary antibody used for immunohistochemistry 77
Table 3 Genotype of progeny from a heterozygous B6;129-Kdm5b-Exon1 knock out breeding program 84
Table 3.1 The Kdm5b knock out line is embryonic lethal 85
Table 3.2 Genotyping from two B6;129-Kdm5b ΔARID heterozygous breeding programs demonstrates that the B6;129-Kdm5b ΔARID offspring are born according to Mendelian ratio. 89
Table 3.3 Derived sequence from the DNA trace of B6;129-Kdm5b ΔARID cDNA showing exon 1 to 5 boundaries 93
Table 3.4 Histological analysis of organs from the male and female B6;129-Kdm5b ΔARID murine line at 9 weeks of age (adult) suggesting normal development 94
Table 3S1 Heterozygous offspring of the B6;129 Kdm5b-Exon1 knock out murine line are not born according to Mendelian frequencies 104
Table 3S2 Genotyping results from B6;129-Kdm5b ΔARID heterozygous breeding program demonstrates that the B6;129-Kdm5b ΔARID offspring are born according to Mendelian frequency 104
Table 3S3: Calculations for Chi Squared test (Table 3S2) 105

Table 3S4. Derived sequence from the DNA trace (Fig 3S1) of
B6;129-Kdm5b WT cDNA showing exon 4 to 5 boundaries 105

Table 4. PCR generated genotyping results from the backcross of the
B6;129-Kdm5b ΔARID chimeric line onto the C57BL/6J genetic strain 117

Table 6S1: Table showing time to tumour development (in days from birth)
in the B6;FVB-Kdm5b ΔARID +/- : PyV-mT +/- and
B6;FVB-Kdm5b WT : PyV-mT +/--female mammary gland 177
Abbreviations
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>adenosine</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ALPL</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>AP2 gamma</td>
<td>transcription factor AP-2 gamma (protein)</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>AREG</td>
<td>amphiregulin</td>
</tr>
<tr>
<td>ARID</td>
<td>A-T rich interactive domain</td>
</tr>
<tr>
<td>ARNTL</td>
<td>aryl hydrocarbon receptor nuclear translocator-like</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>B</td>
<td>Bacillus amyloki</td>
</tr>
<tr>
<td>BciVI</td>
<td>Bacillus circulans</td>
</tr>
<tr>
<td>BGLAP</td>
<td>bone gamma-carboxyglutamate protein</td>
</tr>
<tr>
<td>BMAL1</td>
<td>brain and muscle Arnt-like protein-1</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BPTF</td>
<td>bromodomain PHD finger transcription factor</td>
</tr>
<tr>
<td>BRCA1</td>
<td>breast cancer 1</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>BRD2</td>
<td>bromodomain containing 2</td>
</tr>
<tr>
<td>BUB</td>
<td>budding uninhibited by benzimidazole</td>
</tr>
<tr>
<td>B6;129</td>
<td>A mixed strain derived from C57BL/6J and a 129 ES cell line</td>
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<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
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<td>CCL14</td>
<td>chemokine ligand 14</td>
</tr>
<tr>
<td>CCNA1</td>
<td>cyclin A</td>
</tr>
<tr>
<td>CCND1</td>
<td>cyclin D1</td>
</tr>
<tr>
<td>CDKN1B</td>
<td>cyclin-dependent kinase inhibitor 1B</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>c-ErbB2</td>
<td>receptor tyrosine-protein kinase erbB2</td>
</tr>
<tr>
<td>Chlp</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>Chlp-Seq</td>
<td>chromatin immunoprecipitation coupled with sequencing</td>
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<tr>
<td>Chi3Li</td>
<td>Chitinase 3-like 1</td>
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<td>CITED</td>
<td>cbp/p300-interacting transactivator with Glu/Asp-rich carboxyl-terminal domain 1</td>
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<td>CLOCK</td>
<td>circadian locomotor output cycles kaput</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>Cre</td>
<td>cis-regulatory element</td>
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<td>CSN2</td>
<td>beta casein</td>
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<tr>
<td>C-terminus</td>
<td>carboxyl terminus</td>
</tr>
<tr>
<td>c-MYC</td>
<td>myelocytomatosis viral oncogene</td>
</tr>
<tr>
<td>D</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytidine triphosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ds</td>
<td>double stranded</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DTX1</td>
<td>E3 ubiquitin-protein ligase DTX1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>e-box</td>
<td>enhancer box</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
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<td>EED</td>
<td>embryonic ectoderm development</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EHMT2</td>
<td>euchromatic histone-lysine N-methyltransferase 2</td>
</tr>
<tr>
<td>ELF5</td>
<td>Ets domain transcription factor</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ER alpha</td>
<td>oestrogen receptor alpha (protein)</td>
</tr>
<tr>
<td>ER beta</td>
<td>oestrogen receptor beta</td>
</tr>
<tr>
<td>ERE-luc</td>
<td>oestrogen receptor element luciferase reporter</td>
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<tr>
<td>ES</td>
<td>embryonic stem cells</td>
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<td>ESR1</td>
<td>oestrogen receptor alpha (gene)</td>
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<td>EZH2</td>
<td>enhancer of zeste homologue 2</td>
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<td>E2F</td>
<td>E2 promoter binding factor</td>
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<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>FGFR</td>
<td>fibroblast growth factor receptor</td>
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<td>FOXA1</td>
<td>forkhead box A1</td>
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<td>friend virus B</td>
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<td>F1</td>
<td>filial 1</td>
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<td>GATA3</td>
<td>trans-acting T-cell specific transcription factor 3</td>
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<td>green fluorescent protein</td>
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<td>glutamine</td>
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<td>glucocorticoid receptor</td>
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<td>Glutathione S-transferase</td>
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<td>gap 1 phase</td>
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<td>geneticin</td>
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<td>HA</td>
<td>hemagglutinin</td>
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<td>H and E</td>
<td>hematoxylin and eosin</td>
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<td>HCL</td>
<td>hydrochloric acid</td>
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<td>histone deacetylase</td>
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<td>homeobox</td>
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<td>heterochromatin protein 1 alpha</td>
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<td>HR</td>
<td>homologous region</td>
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<tr>
<td>H3K4</td>
<td>histone 3 lysine 4</td>
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<tr>
<td>ICM</td>
<td>inner cell mass</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<td>IMS</td>
<td>industrial methylated spirit</td>
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<td>ING1</td>
<td>inhibitor of growth 1</td>
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<tr>
<td>iNOS</td>
<td>nitric oxide synthetase</td>
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<tr>
<td>Ip</td>
<td>immunoprecipitation</td>
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J
Janus kinase (JAK)
Jarid1 jumonji AT-rich interactive domain 1
JMJC jumonji C
JMJDN jumonji N
JMJD2 jumonji domain 2

K
Kb kilo base
KDa kilo dalton
KDM5 lysine demethylase 5
KMT lysine methyltransferase
KRT18 cytokeratin 18

L
LB lysogeny broth
lid little imaginal disc
LIF Leukemia inhibitory factor
LMP lysosomal membrane permeabilisation
LN lymph node
loxP locus of crossover in P1
LRP5 low density lipoprotein receptor-related protein 5

M
MaSC mammary gland stem cell
MEF mouse embryonic fibroblast
MET mesenchymal to epithelial transition
min minutes
MLL mixed-lineage leukemia
M molar
miRNA micro-RNA
mM millimolar
MMTV mouse mammary tumour virus
MT1F metallothionein 1F
MW Molecular weight

N
NaCl sodium chloride
NaOH sodium hydroxide
Neo neomycin gene
NOP60B nucleolar protein at 60B
N-terminus amino terminus
NURD nucleosome remodeling and histone deacetylase
NURF nucleosome-remodelling factor subunit

O
OCT4 octamer 4
OSM Oncostatin M

P
PAK1 p21-activated kinase 1
PBS phosphate buffered saline
PCR polymerase chain reaction
PcG polycomb group
PCNA proliferating cell nuclear antigen
pd pregnancy day
PGR  progesterone receptor (gene)
PHD  plant homeo domain
PH3  phospho-histone 3 (ser 10)
PIKE-A  PI3-kinase enhancer A
PI3K  phosphoinositide-3-kinase
PKB  protein kinase B
PolII Ser5  phosphorylation of serine 5 in the C-terminus of polymerase II
POU5F1  POU class 5 homeobox 1
PR  progesterone receptor (protein)
pRb  retinoblastoma-binding protein
PRC2  polycomb repressive complex 2
Prl  prolactin
Prr  prolactin receptor
Pro  proline
PSA-luc  prostate specific antigen luciferase reporter
PTEN  phosphatase and tensin homologue
PTP1B  protein tyrosine phosphatase 1B
Pygo  Pygopus
PyV-mT  polyoma middle T

Q
qPCR  quantitative PCR

R
RAS  rat sarcoma
RAR  retinoic acid receptor
RBP-J  recombination signal-binding protein 1 for J-kappa
RBP2  retinoblastoma binding protein 2
RBP2-H1  retinoblastoma binding protein 2 homology 1
Rbr-2  retinoblastoma binding protein related
RING1B  ring finger protein 1B
RNA  ribonucleic acid
RNF2  ring finger protein 2
RT  reverse transcription

S
SAHF  senescence-associated heterochromatic foci
Sca1  stem cell antigen 1
SDS  sodium dodecyl sulphate
Serpin2A  Spi2A
SET  su(var)3-9 and enhancer of zeste
siRNA  short interfering RNA
SMA  smooth muscle actin
SMCX  selected mouse cDNA on X
SMCY  selected mouse cDNA on Y
SOCS2  Suppressor of cytokine signaling-2
S-phase  synthesis phase
SSC  sodium chloride and sodium citrate
s-SHIP  SH2-containing inositol 5'-phosphatase (104kd isoform)
STAT  Signal transducer and activator of transcription
Su(h)  suppressor of hairless
SUZ12  suppressor of zeste 12 homolog
SV40  simian virus 40

T
T  thymidine
TAE  tris acetate EDTA
<table>
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<th>Symbol</th>
<th>Name</th>
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<tr>
<td>TAF3</td>
<td>transcription initiation factor TFIID subunit 3 (gene)</td>
</tr>
<tr>
<td>TEB</td>
<td>terminal end bud</td>
</tr>
<tr>
<td>TFAP2C</td>
<td>transcription factor AP-2 gamma (gene)</td>
</tr>
<tr>
<td>TFIID</td>
<td>transcription factor II D</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TNF</td>
<td>tissue necrosis factor</td>
</tr>
<tr>
<td>TPA</td>
<td>tissue plasminogen activator</td>
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<tr>
<td>Try</td>
<td>tryptophan</td>
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<td>U</td>
<td>ultraviolet B</td>
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<td>UVB</td>
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<td>V</td>
<td>vitamin D receptor</td>
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<tr>
<td>VDR</td>
<td></td>
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<tr>
<td>W</td>
<td>Whey acidic protein</td>
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<td>Wnt4</td>
<td>wingless-related MMTV integration site</td>
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<tr>
<td>WRT</td>
<td>with respect to</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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<td>X</td>
<td>x-linked mental retardation</td>
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<tr>
<td>XLMR</td>
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<td>Y</td>
<td>yellow fluorescent protein</td>
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<tr>
<td>YFP</td>
<td></td>
</tr>
<tr>
<td>Z</td>
<td>zinc finger</td>
</tr>
<tr>
<td>ZF</td>
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Chapter 1: Introduction

Section 1A: The regulation of chromatin: Methylation of histone lysine residues.
Chapter 1. Section 1A: Introduction

1A Prologue

This thesis aims to gain an understanding into the function of a specific histone demethylase, KDM5B (a.k.a PLU-1, JARID1B), by exploring the effect on the murine mammary gland of knocking out the \textit{Kdm5b} gene or introducing a partially defective gene into the \textit{Kdm5b} loci. It is therefore appropriate to introduce the field of histone methylation, and the enzymes which regulate the epigenetic control of transcription (Section 1A) and furthermore, to discuss published work that is relevant to mammary gland biology (Section 1B).

1A.1 The regulation of chromatin: Methylation of histone lysine residues.

1A.1.1 The nucleosome and histone tail

Every cell within the same organism contains the same genes and therefore the same genetic information. Not only during embryonic development but also during the postnatal development of organs such as the mammary gland, extrinsic cues are incorporated which permit cell fate determination. How cell type-specific chromatin organization at specific genomic loci allows access to and the regulation of genes controlling cellular phenotype is governed in part by the interaction of DNA with histone proteins.

The nucleosome – the basic unit of chromatin – contains 146 bp of DNA, wrapped twice around a histone octamer consisting of two copies of histones H3, H4, H2A and H2B (Kornberg, 1974). The nucleosome is repeated throughout the genome with chromatin being further condensed through its association with linker histone H1. Histone proteins contain a hydrophobic internal region and a flexible amino-terminal tail that protrudes outward from the nucleosome (Luger \textit{et al}., 1997, Figure 1).

A major contributor to the regulation of cellular function governed by chromatin structure is the posttranslational modification of histone tails (Bhaumik \textit{et al}., 2007). These post-translational modifications include acetylation, phosphorylation, methylation, monoubiquitination, sumoylation, and ADP ribosylation (Campos and Reinberg, 2009). For almost every modification identified, there are also machineries involved in their removal (Bhaumik \textit{et al}., 2007). The result of histone post-translational modifications is
to affect protein-DNA, protein-protein, and nucleosome-nucleosome interactions, as well as providing docking sites for chromatin effector proteins (Campos and Reinberg, 2009). Therefore the type and degree of histone modification at any particular genomic site can affect cellular function.

All histones can be methylated on any of their basic amino acid groups. Specific histone lysine methylation marks are commonly associated with either transcriptional activation or repression (Pokholok et al., 2005). In general, methylation of Histone 3 Lysine 9 (H3K9), H3K27 or H3K20 is linked to repression while methylation on H3K4, H3K36 and H3K79 is linked to activation. An additional level of specificity that may serve to influence the recruitment of effector proteins to chromatin, and therefore functional interpretation of these methyl marks, is whether a particular lysine residue is mono (me1), di (me2) or tri (me3) methylated. Of the lysine residues methylated on the amino acid ‘tail’ of histone 3, methylation of H3K4, and H3K27 are of particular interest.

Figure 1. Chromatin, the nucleosome and the histone tail cooperate to control gene regulation. The KDM5 family of demethylase enzymes can erase H3K4me3/2. H3K4 trimethylation can function as a docking site for PHD finger containing proteins (see below).
Although H3K4me3 is a modification generally associated with transcription (Pokholok et al., 2005, Vermeulen et al., 2007), and H3K27me3 is widely associated with repression of transcription, H3K4me3 and the H3K27me3 marks have been simultaneously localized to the promoter region of developmental genes (Bernstein et al., 2006, Mikkelsen et al., 2007) and are uniquely associated with cell fate plasticity and with the regulation of developmental genes (Bernstein et al., 2006, Heintzman et al., 2009).

Whilst H3K4me3/2 is found at the promoters of actively transcribed genes (Barski et al., 2007), H3K4me1 is localized to the enhancer regions of tissue-specific genes (Heintzman et al., 2009) suggesting a role during differentiation. Although methylated H3K4 marks areas of the genome that are being transcribed (Pokholok et al., 2005) and of developmental importance (Bernstein et al., 2006, Heintzman et al., 2009), exactly how the methylated H3K4 mark is interpreted or 'read' (Ruthenburg et al., 2007) and to what functional end, is determined by the methyl lysine recognition domain of proteins and executed as part of the associated multi-protein complex in response to environmental signals.

1A.1.2 It takes a PHD to be a reader in H3K4 methylation

One such lysine recognition domain found in proteins is the PHD finger (Wysocka et al., 2006, Shi et al., 2006). The PHD finger is a protein domain that can interact with trimethylated H3K4 to influence transcription positively by promoting the formation of the pre-initiation complex (Vermeulen et al., 2007) and by altering chromatin structure (Barak et al., 2003, Wysocka et al., 2006). Specifically, the PHD finger of TAF(II)140 (a.k.a TAF3) facilitates in the recruitment of the multi-protein complex, TFIID during promoter activation (Vermeulen et al., 2007). Moreover, the PHD finger of BPTF is required for the recruitment of the chromatin-remodelling complex NuRF to H3K4 trimethyl marks and subsequent gene activation (Barak et al., 2003, Wysocka et al., 2006).

The importance of the PYGO-2 PHD sequence in binding to H3K4me3/2 has been shown to be of particular importance in the mouse mammary gland (Gu et al., 2009, 2013. See introduction: Section 1B). The prototype gene, Pygopus (Pygo) was identified in D. melanogaster as a specific downstream component of wingless (Wg)/Wnt signalling pathway, and in association with BCL9/Legless, transduces the Wnt signal by promoting Armadillo/beta-catenin mediated transcription (Belenkaya et
al., 2002, Kramps et al., 2002, Thompson et al., 2002). The association of Pygo with BCL9/Legless leads to binding to H3K4me2/3 (Fiedler et al., 2008, Kessler et al., 2009).

Of the two mammalian orthologs, Pygo2 has been shown to be involved in murine mammary gland development and cell fate determination (Gu et al., 2009, 2013. See introduction: Section1B). In mammals, PYGO2 can bind, through the PHD sequence, directly to H3K4me3/2 (Kessler et al., 2009, Gu et al., 2009), identifying a potential mechanism that directs the recruitment of the beta-catenin/BCL9 complex to the promoters of beta-catenin target genes.

Interestingly, even though H3K4 trimethylation functions to recruit classic factors that are required to promote transcription (Barak et al., 2003, Wysocka et al., 2006), during situations of cellular stress, H3K4 trimethylation also functions to interpret signalling pathways in response to DNA damage (Shi et al., 2006) and cellular senescence (Abad et al., 2011.). Specifically, ING2 binding to H3K4 trimethylation via the PHD finger is increased in response to Doxorubicin (Shi et al., 2006). Similarly, the ING1 protein is required for oncogene-induced senescence through an interaction of the PHD finger with H3K4 trimethylation (Abad et al., 2011).

Peptide-binding studies have shown that KDM5A and KDM5B family can also be recruited to H3K4 trimethylation marks via the C-terminal PHD finger, a feature that is evolutionarily conserved (Wang et al., 2009a, Li et al., 2010, Figure 1). This is of significant interest given that the C-terminal PHD finger of the single KDM5 orthologue in Drosophila melanogaster (lid) is essential for viability (Li et al., 2010). Furthermore, ChIp-Seq data from embryonic stem cells (ES) has shown that KDM5B is recruited to developmental genes enriched in H3K4 and H3K27 trimethylation ((bivalent domain (Bernstein et al., 2006)) which maybe crucial in regulating the temporal expression pattern of developmental genes (Schmitz et al., 2011, Albert et al., 2013).

1A.2 Histone marks and murine mammary development.

The importance of epigenetics in controlling gene expression during the differentiation of the developing mammary gland is now being investigated, and the methylation status of histone lysine marks is found to play an important role in proliferation, differentiation, and cell fate determination. Binding of signal transducer and activator of transcription 5 (STAT5) to mammary gland specific regulatory sequences can precede the establishment of H3K4me3 marks, suggesting that STAT5 binding is required and responsible for gene expression (Kang et al., 2014). Moreover, acquisition of the
H3K4me2 mark together with development of a more open chromatin organisation are also reported to be associated with activation of milk protein genes (Rijnkels et al., 2013). A comprehensive analysis of the global maps of H3K4me3 and H3K27me3 marks during the development and differentiation of the murine mammary gland demonstrates that a clear correlation exists between histone modification maps with that of gene expression signatures in the different epithelial cell subpopulations (Pal et al., 2013), thus demonstrating that the epigenome plays a central role in directing cell fate determination.

In considering the enzymes that are responsible for defining the methylation status of histone lysine residues, studies in the murine mammary gland have focused on the methyltransferase protein, enhancer of zeste homologue 2 (EZH2, a.k.a KMT6) and a demethylase that removes methyl groups from H3K9 (JMJD2B/KDM4B). The H3K27 specific histone methyltransferase, EZH2, is a component of the polycomb repressor complex 2 (PRC2), and is responsible for the di and trimethylation of lysine 27 on histone 3 (Cao et al., 2002). As genetic ablation of Ezh2 results in embryonic lethality (O’Carroll et al., 2001), studies utilizing an inducible system to knock down Ezh2 expression (Michalak et al., 2013) or through conditional deletion of Ezh2 (Pal et al., 2013) have shown that EZH2 plays an important role in cell fate determination during mammary gland development and differentiation (see introduction: 1B1). Furthermore, MMTV (mouse mammary tumour virus) promoter-driven targeted deletion of the H3K9 specific lysine demethylase KDM4B, from mammary gland epithelia results in a reduction in the number of terminal end bud and ductal side branch structures presented in the nulliparous mammary gland (Kawazu et al 2011). Interpretation of external signals in response to the hormonal environment during mammary gland development is regulated at the level of the gene by the epigenetic landscape. It is therefore not surprising that several demethylase enzymes have also been implicated as co-factors in signalling by the androgen and oestrogen receptors (Kawazu et al., 2011, Sventelis et al., 2011, Coffey et al., 2013, Chan and Hong, 2001).

1A.3 The KDM5 family and gene regulation

The KDM5 (lysine demethylase 5) family of demethylase proteins counteract the trimethylation of histone 3 lysine 4 (H3K4) and can function as transcriptional repressors (Christensen et al., 2007, Yamane et al., 2007, Lopez-Bigas et al., 2008, Van Oevelen et al., 2008, Beshiri et al., 2012, Chicas et al., 2012). This family of so-called ‘erasers’ (Ruthenburg et al., 2007) of H3K4 methylation can also ‘read’ methylated lysine residues (Wang et al., 2009a, Li et al., 2010). They can form a number of different
multi-protein complexes that contain other chromatin-modifying factors (Barrett et al., 2007, Hayakawa et al., 2007, Pasini et al., 2008, Banck et al., 2009, Moshkin et al., 2009, Schmitz et al., 2011) and co-factors that aid in the recruitment of the protein complex to DNA (Pasini et al., 2008, Schmitz et al., 2011), whilst also possessing the intrinsic ability to bind to DNA directly (Scibetta et al., 2007, Yao et al., 2010). The KDM5 family are highly homologous and where co-expressed may act redundantly. A picture is emerging where the KDM5 family may dictate the shift in balance between cellular proliferation (Lopez-Bigas et al., 2008, Van Oevelen et al., 2008, Beshiri et al., 2012) and differentiation (Flowers et al., 2010, Flowers et al., 2013) and are therefore important during development (Gildea et al., 2000, Iwase et al., 2007, Klose et al., 2007, Albert et al., 2013). Although the KDM5 demethylase activity has attracted the greatest attention, it is apparent that the KDM5 family is an important multi-faceted group of proteins with additional functions.

1A.3.1 The conserved domains of the KDM5 family of proteins

As shown in figure 1.1, the mammalian KDM5 family consists of four proteins, namely KDM5A (a.k.a RBP2, JARID1A), KDM5B (a.k.a PLU-1, JARID1B), and 2 homologous proteins encoded by the sex-chromosome-specific genes, KDM5C (a.k.a SMCX, JARID1C) located on the X-chromosome but not inactivated (Agulnik et al., 1994a, Wu et al., 1994) and KDM5D (a.k.a SMCY, JARID1D) located on the Y-chromosome (Agulnik et al., 1994b). The KDM5 family expressed in D. melanogaster (lid) and Caenorhabditis elegans (rbr-2) are expressed as single orthologues with the D. melanogaster lid sharing the greatest homology with that of the mammalian KDM5 family.

All the KDM5 family described possess an N-terminal Jumonji N (JMJN), ARID, PHD, catalytic Jumonji C (JMJC) and C5HC2-zinc finger motifs. The greatest divergence in sequence is seen at the C-terminal region of the KDM5 family. Whilst mammalian KDM5A and KDM5B, D. melanogaster lid and C. elegans rbr-2 possess two PHD fingers, KDM5C and KDM5D possess only one PHD finger in this region.

KDM5A (a.k.a RBP2 Retinoblastoma binding protein 2) was initially identified in a yeast-2-hybrid screen for retinoblastoma binding proteins (pRb) (Defeo-Jones et al., 1991). Further studies demonstrated that KDM5A interacts with pRb via a non-T/E1A binding region and p107 via the LXCXE motif (Kim et al., 1994), a motif that is unique amongst the KDM5 family of proteins to KDM5A. (Vogt et al., 1999).
KDM5B was first reported in the same year by independent labs using different signalling pathways to identify novel transcripts (Lu et al., 1999, Vogt et al., 1999). PLU-1 as it was entitled (Lu et al., 1999) was identified as a transcript that was positively regulated via the c-ErbB2 signalling pathway in a human mammary epithelial cell line (Lu et al., 1999). A splice variant form of KDM5B, named RBP2-H1 (named due to the homology with the founding family member, RBP2) was identified as a transcript whose expression was down regulated in melanocytes upon UVB induced DNA damage (Vogt et al. 1999). The difference in the two KDM5B splice variants is that the variant RBP2-H1 contains an additional 36 amino acids inserted after amino acid residue 237. Of the two KDM5B splice variants, PLU-1 expression is the most abundant in a panel of breast cancer and melanoma cell lines examined (Barrett et al., 2002). Currently a function for the additional sequence is unknown, and subsequent reports do not always distinguish between the two isoforms.

Figure 1.1. Schematic representation of the protein domain position for the KDM5 family of H3K4 demethylase enzymes. KDM5B shares the greatest homology with KDM5A – 57% (See black type with dashed line to the right hand side of the figure. Overall homology for the four KDM5 family members is compared to KDM5B. Red type gives size of proteins in amino acid (aa) and details position of domains in relation to the initiating methionine. Yellow type details homology of domains in relation to KDM5B.
The demonstration that methylation is also a reversible histone modification (Shi et al., 2004) led to a renewed interest in the KDM5 family of proteins (Trewick et al., 2005). Incubation of recombinant KDM5 proteins with synthetic histone peptides or bulk histones followed by analysis by Western blot and by mass spectrometry revealed that the KDM5 family of proteins are demethylase enzymes specific for H3K4.

KDM5A, KDM5C and KDM5D can demethylate H3K4me3 and H3K4me2 but not H3K4me1 in vitro (Christensen et al., 2007, Eissenberg et al., 2007, Iwase et al., 2007, Klose et al., 2007, Lee et al., 2007, Tahiliani et al., 2007). However, KDM5B not only demethylates H3K4me3 and H3K4me2 (Christensen et al., 2007, Iwase et al., 2007, Lee et al., 2007) but can also demethylate H3K4me1 to the unmethylated form both in vitro (Iwase et al., 2007), and in vivo (Xiang et al., 2007). These data demonstrate that amongst the KDM5 family of proteins, KDM5B may possess unique enzyme specificity.

Immunostaining demonstrated that over-expression of KDM5A, KDM5B or KDM5C in vivo resulted in a loss of H3K4me3 or H3K4me2 which was dependent upon a catalytically active JMJC domain (Christensen et al., 2007, Iwase et al., 2007, Klose et al., 2007, Seward et al., 2007, Tahiliani et al., 2007). For KDM5B, in addition to the catalytic JMJC domain the JMJD, ARID and ZF sequences are also required for demethylase activity in vivo (Xiang et al., 2007, Yamane et al., 2007). Interestingly, structural studies have shown that the JMJD domain of another demethylase, KDM4A (a.k.a JMJD2) (Chen et al., 2006) is also required for demethylase activity by contributing to the structural integrity of JMJC catalytic core. (Klose et al 2006). Of interest is the observation that point mutations within the KDM5B ARID sequence that ablate binding also negate the demethylase activity of KDM5B when transfected in cell lines (Tu et al., 2008). This suggests that recruitment to DNA via the ARID sequence (Scibetta et al., 2007) could be required for H3K4me3 demethylase activity.

1A.3.3 The KDM5 family of transcriptional repressors

Consistent with the reported catalytic activity of the KDM5 family (Christensen et al., 2007, Eissenberg et al., 2007, Iwase et al., 2007, Klose et al., 2007, Lee et al., 2007, Tahiliani et al., 2007, Yamane et al., 2007) and the localisation of H3K4 trimethylation around transcription start sites (Pokholok et al., 2005) the KDM5 family of proteins have been characterised as transcriptional repressors (Christensen et al., 2007, Scibetta et al., 2007, Yamane et al., 2007, Lopez-Bigas et al., 2008, Van Oevelen et al., 2008).
2008, Beshiri et al., 2012, Chicas et al., 2012) (Figure 1.2). Functional data also suggest that the demethylase activity of the KDM5 family of proteins is required for the repression of genes involved in development (Schmitz et al., 2011, Albert et al., 2013), cell cycle (Van Oevelen et al., 2008, Beshiri et al., 2012), differentiation (Flowers et al., 2010, Flowers et al., 2013), and senescence (Nijwening et al., 2011, Chicas et al., 2012).

**Figure 1.2. Model for KDM5 mediated repression.** The KDM5 family may be recruited to the genome via the ARID sequence and/or other co-factors. KDM5 demethylase activity is dependent on the catalytic JMJC domain and the ARID sequence.

_Hox_ (Homeobox) gene expression determines correct patterning during development (Akasaka et al., 1996, Del Mar Lorente et al., 2000). Both KDM5A and KDM5B have been found bound to the promoter region of _Hox_ genes (Pasini et al., 2008). While ChIp-seq data on ES cells has localised KDM5B to the promoter region of _Hoxa10_, _Hoxb4_ and _Hoxb8_, in the breast cancer cell line MCF7, KDM5B can bind the promoter of _Hoxa5_ and repress transcription in a demethylase-dependent manner (Yamane et al., 2007). Likewise, ChIp-PCR has show that KDM5A is bound to the promoter region of _Hoxa1_, _Hoxa5_, _Hoxa7_ (Christensen et al., 2007), _Hoxa4_, _Hoxa10_, _Hoxd9_, _Hoxd11_ (Pasini et al., 2008) in embryonic stem (ES) cells and is lost upon all-trans retinoic acid induced differentiation from the promoters of _Hoxa1_, _Hoxa5_, _Hoxa7_, concomitantly with an increase in H3K4 trimethylation (Christensen et al., 2007).

A function for KDM5A in the control of the cell cycle is also suggest by the demonstration that KDM5A can silence the expression of cell cycle dependent genes such as _PCNA_ during TPA-induced differentiation of the monocytic U937 cell line (Beshiri et al., 2012).
KDM5A and KDM5B both play a role during RAS-induced cellular senescence (Chicas et al., 2012). Furthermore, using KDM5A as the representative KDM5 family member has shown that during senescence KDM5A can function to repress genes such as CCNA1 (a.k.a Cyclin A1) (Nijwening et al., 2012) that is required for cell cycle progression (Resnitzky et al., 1995).

1A.3.4 A function for the KDM5 family of proteins in the activation of transcription

The function of the KDM5 family appears context-dependent, determined by protein-protein interactions (Secombe et al., 2007, Xiang et al., 2007) and genome-recruitment mechanisms (Li et al., 2010). With this in mind, data suggests that the KDM5 family may also function as activators of transcription (Xiang et al., 2007, Secombe et al., 2007).

In vitro binding studies have also shown that D. melanogaster proteins lid and dMYC can interact via the JMJC domain, the active domain required for demethylase activity (Secombe et al., 2007). This functional interaction is likely to be conserved given that KDM5B and C-MYC can interact in vivo via the JMJC domain (SC, JTP unpublished observation). Given that transgenic D. melanogaster imaginal wing discs that over-expressed lid and dMYC maintain H3K4 trimethylation levels, suggests that the interaction of lid and dMYC may negate the demethylase activity of lid (Secombe et al., 2007). Interestingly, the functional relevance of this interaction is that lid is required for dMYC dependent activation of the MYC target gene Nop60B (Secombe et al., 2007), a function that could be mediated via the C-terminal PHD domain (Li et al., 2010) (Figure1.3).

Figure 1.3. Model for C-MYC/KDM5 target gene activation. An interaction of C-MYC with the JMJC domain of KDM5 may negate demethylase activity. The C-terminal PHD finger of the KDM5 family may stabilize a C-MYC complex at E-box target genes.
Although studies implicate KDM5A and KDM5B in functioning as nuclear receptor co-activators (Chan and Hong 2001, Xiang et al., 2007), more recent studies demonstrate that KDM5A and KDM5B can function to repress hormone inducible genes prior to activation (Stratmann et al., 2011, Vincent et al., 2011, Vincent et al., 2013).

Initial studies using transiently transfected expression constructs demonstrated that KDM5A can interact with a number of nuclear receptors and using oestrogen receptor alpha (ER alpha) as a co-activator model, that ER alpha and KDM5A can potentiate transcription in a hormone-dependent manner from an ERE-Luc reporter construct (Chan and Hong 2001). Similarly, KDM5B can interact with the androgen receptor (AR) and potentiate transcription in a ligand dependent manner from a PSA-Luc reporter construction (Xiang et al., 2007). However, given that the regulation of chromatin architecture is of central importance to the control of gene expression, the use of transiently transfected DNA templates may not adequately represent the endogenous chromatin structure (Smith et al., 1997, Hebbar et al., 2008).

With this in mind, studies are beginning to suggest that KDM5B may play a central role in the repression of the progesterone receptor (PR) signalling pathway. Of the four KDM5 family members, KDM5B is the most abundant family member expressed in MCF7 cells (Krishnakumar et al., 2010. SC, JTP unpublished observation). RNAi-mediated silencing of KDM5B suggested a partial role for KDM5B in PGR repression but only when MCF7 cells were cultured under hormone-deprived culture conditions (Stratmann et al., 2011). In addition to the repression of the PGR transcript itself, KDM5B can form a repressive complex with unliganded PR and repress some 20% of hormone regulated genes that are de-repressed upon progestin treatment (Vincent et al 2011, 2013).

1A.3.5 Direct Recruitment of the KDM5 family to the genome

A number of observations suggest that two domains, the ARID and the C-terminal PHD finger may work in conjunction to recruit the KDM5 family to the genome of developmental genes (Scibetta et al., 2007, Wang et al., 2009a, Li et al., 2010, Yao et al., 2010, Schmitz et al., 2011).

In the first instance, genome-wide binding studies in ES cells have shown that the binding of KDM5B correlates with promoter regions that are both GC-rich and enriched for the bivalent histone marks H3K4me3 and H3K27me3 (Schmitz et al., 2011). Additionally, using electrophoretic mobility shift assays (EMSA) have demonstrated
that the ARID domain of KDM5B can interact with the derived GC-rich sequence, GCACA. (Scibetta et al., 2007, Yao et al., 2010) and that mutation of the ARID sequence also ablates the demethylase activity of KDM5B (Yao et al., 2010). Finally, the KDM5 family can bind H3K4me3 marks via the C-terminal PHD sequence (Wang et al., 2009a, Li et al., 2010).

The only study to compare directly KDM5A and KDM5B expression levels suggests that in the main KDM5A and KDM5B display mutually exclusive expression patterns (Islam et al., 2011a). However, in the hematopoietic system and ES cells where both KDM5A and KDM5B proteins are expressed, a degree of specificity may be seen at the level of DNA binding via the ARID sequence. Whereas the DNA binding sequence for KDM5B is GCACA (Scibetta et al., 2007, Yao et al., 2010) the ARID sequence of KDM5A can bind to a CCGCCC sequence motif found in the KDM5A target gene promoter of BRD2 (Tu et al., 2008).

1A.3.6 Recruitment of the KDM5 family to the genome as part of a multi-protein complex

KDM5A and KDM5B are found in protein complexes that also function to modify the chromatin environment or aid in the recruitment to DNA (Barrett et al., 2007, Pasini et al., 2008, Banck et al., 2009, Schmitz et al., 2011). For example, KDM5B can interact with the histone deacetylase protein, HDAC1 (Barrett et al., 2007), and is also found in a complex with the H3K9 methyltransferase, EHMT2 (a.k.a G9a) (Banck et al., 2009). Both KDM5A and KDM5B have been associated with the polycomb group (PcG) protein complex that, as described below, aid in their recruitment to the genome (Pasini et al., 2008, Schmitz et al., 2011).

The correlation of KDM5B binding with H3K4me3 and H3K27me3 bivalents marks suggests a possible functional relationship between KDM5B and the polycomb group of proteins. In fact, KDM5A and KDM5B can bind to a significant number of PcG target genes in mouse ES cells (Boyer et al., 2006, Pasini et al., 2008, Schmitz et al., 2011), and KDM5A expression has been shown to be necessary for repression (Pasini et al., 2008). The potential for KDM5A and KDM5B to regulate PcG target genes in ES cells (Pasini et al., 2008, Schmitz et al., 2011) does suggest the potential for functional redundancy (Islam et al., 2011b). However, the data also suggest that assembly of a KDM5/PcG protein complex and the mechanisms required for recruitment of a KDM5/PcG to the genome may differ between KDM5A and KDM5B-containing complexes (Pasini et al., 2008, Schmitz et al., 2011). For example, whereas Kdm5a
knock down has no affect on SUZ12 DNA binding (Pasini et al., 2008), knockdown of Kdm5b results in a reduction in the binding of SUZ12 to some PcG-regulated genes (Schmitz et al., 2011). This suggests that KDM5B may have more of an influential role in the recruitment to DNA of a PcG complex. Conversely, PcG proteins are required for the recruitment of KDM5A and KDM5B to the genome (Pasini et al., 2008, Schmitz et al., 2011). KDM5B recruitment is reduced in Eed and Rnf2 (a.k.a Ring1b) knockout ES cell lines (Schmitz et al., 2011) whereas the recruitment of KDM5A is reduced in Suz12 knock out ES cell lines (Pasini et al., 2008). Although the studies for KDM5A are limited, the recruitment of KDM5A to PcG target genes maybe dependent upon PcG complex-components. Furthermore, these findings demonstrate that although KDM5B and PcG proteins can affect the binding of each other to a subset of target genes, neither KDM5B nor PcG proteins, at least individually, are essential for each other's recruitment to the genome. Furthermore, KDM5A can interact with PcG proteins EZH2 and SUZ12 in vivo (Pasini et al., 2008), whereas no endogenous interaction with KDM5B and SUZ12 was observed (Schmitz et al., 2011). It is therefore plausible that KDM5B may only interact with PcG proteins at PcG-target genes (Figure1.4).

![Figure 1.4. Model for PcG/KDM5B binding to the bivalent promoters of developmental genes. The ARID and C-terminal PHD sequence of KDM5B maybe required for complex recruitment to DNA. To date the KDM5B and PcG interacting proteins have not been described.](image)

**1A.3.7 Lessons from the past: Studies in C. Elegans and D. Melangaster**

Studies on the single orthologues lid and rbr-2 have confirmed that the function of the KDM5 family as demethylase enzymes is evolutionarily conserved (Christensen et al., 2007, Eissenberg et al., 2007, Secombe et al., 2007). Furthermore, biochemical and functional studies have shown that a role during NOTCH signalling may also be
conserved (Hayakawa et al., 2007, Moshkin et al., 2009, Liefke et al., 2010 Xie et al., 2011). KDM5A (Hayakawa et al., 2007), KDM5B (Xie et al., 2011) and lid (Moshkin et al., 2009) have been identified either as a complex (Hayakawa et al., 2007, Moshkin et al., 2009) or associated by immunoprecipitation (Xie et al., 2011) with the core proteins components of a SIN3-complex, SIN3A and HDAC2. In addition to the knock down of either lid or sin3a resulting in the de-repression of NOTCH target genes (Moshkin et al., 2009), an interaction between Su(H) and lid as well as the mammalian homologues RBP-J and KDM5A has been described (Liefke et al., 2010). Furthermore KDM5A and RBP-J have been localised to the RBP-J binding site of the NOTCH target gene DTX1 (a.k.a Deltex-1), and are required for repression (Liefke et al., 2010) (Figure 1.5).

![Figure 1.5. Model for KDM5/NOTCH signalling](image)

Although a great deal of interest in the H3K4 demethylase activity of the KDM5 group has thrust this family into the limelight, the demethylase-independent activity is undoubtedly important (Christensen et al., 2007, Catchpole et al., 2011, Li et al., 2010). Whereas disruption of lid expression results in early pupae lethality, presumably due to a defect in optical brain lobe and imaginal disc development (Gildea et al., 2000), viability is not dependent upon the demethylase activity of lid (Li et al., 2010). Similarly, both a KDM5B murine line (Catchpole et al., 2011 and this thesis) and a C.elegans rbr-2 (Christensen et al., 2007) strain ((rbr-2(tm1231)) that express KDM5 proteins that are presumed to lack demethylase activity are both viable. However the rbr-2 (tm1231) strain present with a defect in vulvalmorphogenesis, suggestive of a function for rbr-2 in vulva-precursor cell fate specification.
1A.4 The expression patterns of the KDM5 family of proteins

It is apparent from the studies detailed above that some functional aspects of the KDM5 family of proteins are evolutionarily conserved (Hayakawa et al., 2007, Moshkin et al., 2009, Liefke et al., 2010, Xie et al., 2011). KDM5B can also be recruited directly or indirectly to KDM5A binding sites post KDM5A-targetted RNAi-mediated silencing, suggesting functional compensation (Islam et al., 2011b). Knowledge of the expression profile of the KDM5 family during development and in adult tissue would therefore help our understanding of the function of this group of proteins during development, organogenesis and cancer.

1A.4.1 The expression profile of KDM5A and KDM5B in human adult tissue

Studies on the expression and in vivo function of the KDM5 family during development as well as in the adult have been limited with the exception of KDM5B (Lu et al., 1999, Barrett et al., 2002, Madsen et al., 2002, McGraw et al., 2007, Frankenberg et al., 2007, Islam et al., 2011a, Albert et al., 2013). However, as described below there are data that suggest the potential for functional compensation (Islam et al., 2011b) as well as unique functions during developmental (Klose et al., 2007, Albert et al., 2013).

Only one publication has addressed the pattern of KDM5A expression in normal adult human tissue. Strikingly, expression array analysis revealed that KDM5A expression is restricted to those cells derived from the bone marrow and peripheral blood as well as the pineal gland (Islam et al., 2011a).

The expression of KDM5B in human tissue is also restricted. Northern blot analysis of RNA from a panel of 24 normal adult tissues demonstrated that high levels of KDM5B expression are restricted to the testis (Lu et al., 1999). However, Islam and colleagues (2011a) using microarray analysis on an expanded array of whole human tissue, detected robust levels of the KDM5B transcript in the kidney and the hematopoietic system.

1A.4.2 The expression profile of KDM5B in murine adult tissue

The murine KDM5B protein shares 94% homology with that of human KDM5B protein with almost 100% identity within the conserved domains, suggesting functional conservation (Figure1.6).
As seen with human tissue, the murine \( Kdm5b \) transcript is also detected at high levels in the testis (Madsen et al., 2002). However in addition, the murine KDM5B protein is also detected at moderate levels (compared to the testis), in the colon, prostate, thymus, spleen, and low but detectable levels of KDM5B expression are detected in the mesenteric lymph node, brain, lung, heart and skin (Schmitz et al., 2011).

KDM5B is also expressed in the mammary gland (Madsen et al., 2002, Barrett et al., 2007) as well as the testis (Madsen et al 2002, Madsen et al 2003), organs that undergo cyclic regeneration and are responsive to hormonal stimuli. \( Kdm5b \) is expressed in the luminal epithelial cells of the nulliparous mammary gland with expression levels increasing through pregnancy associated mammary gland development (Madsen et al., 2002, Barrett et al., 2007 and this thesis). In the testis, the expression of the \( Kdm5b \) transcript is restricted to the mitotic spermatogonia and the pachytene/diplotene stage of prophase I during meiosis. This data suggests a potential proliferative function for \( Kdm5b \) during mitosis and synapsis/homologous recombination during prophase I of meiosis (Madsen et al., 2002, Madsen et al., 2003).

1A.4.3 KDM5B expression during murine embryonic development

In contrast to KDM5A (McGraw et al., 2007), a number of studies have examined the expression profile of \( Kdm5b \) during embryonic development (Madsen et al., 2002, McGraw et al., 2007, Frankenberg et al., 2007, Schmitz et al., 2011). As described
below, the expression pattern of KDM5B highlights the potential functional importance of KDM5B during organogenesis.

Use of an *in vitro* bovine embryo development system to examine the expression profile of a number of histone modification factors demonstrated that KDM5A and KDM5B display two different patterns of expression throughout pre-implantation of the developing embryo. Whereas only trace levels of the *Kdm5b* transcript are detected prior to the maternal to embryonic transition (one to two cell stage in the mouse; e0.5-e1.5), the *Kdm5a* transcript is expressed at more robust levels that are maintained thereafter through to development of the blastocyst (McGraw *et al.*, 2007). The expression of the *Kdm5b* transcript is markedly up-regulated at the maternal to embryonic transition (MET) suggestive of a function for KDM5B in the activation of the embryonic genome (McGraw *et al.*, 2007).

The pluripotent inner cell mass (ICM) of the implanting blastocysts (mouse: e4-e4.25) gives rise to all future cell lineages. The epiblast is derived from the pluripotent ICM, and is the site for the future lineage restricted endoderm (the epithelium), mesoderm (e.g. the vasculature and lymphatic system including the blood and the urogenital organs) and ectoderm (e.g. the epidermis of the skin and its derivatives including the mammary gland). The expression of *Kdm5b* is restricted to the epiblast at e5.5 suggesting a function for KDM5B during cell fate determination. Furthermore, by gastrulation (e6.5) the expression of *Kdm5b* is ubiquitous and weaker than the levels detected in the epiblast at e5.5 (Frankenberg *et al.*, 2007). Significantly, gastrulation is defined by the formation of the primitive streak and subsequent germ cell layer specification further implicating the function of KDM5B during cell fate decision.

The expression of *Kdm5b* is also detected at robust levels in whole embryonic tissue extracts at e7.5 (Madsen *et al.*, 2002, Frankenberg *et al.*, 2007) before the levels increase dramatically at e11.5 and subsequently fall by e15.5 and e17.5 (prior to parturition) to levels similar to that detected at e11.5 (Madsen *et al.*, 2002). Whole mount *in situ* hybridisation on e12.5 to e15.5 embryos localised the *Kdm5b* transcript to a number of developing organs including the whisker follicle, thymus, limbs, teeth, eye (neural retina, optic nerve, olfactory epithelium) and stomach. The *Kdm5b* transcript is also expressed in the invaginating epithelium of the mammary bud at e14.5 (Madsen *et al.*, 2002). Furthermore extensive expression of the *Kdm5b* transcript was observed in the developing brain, for example in the telencephalon, the precursor cells to the formation in the adult brain of the cerebral hemispheres.
1A.4.4 A function for KDM5B during ES cell commitment

In keeping with a function for KDM5B during development, the Wynder lab, in 2008 were the first group to describe a role for KDM5B during ES cell commitment (Dey et al., 2008). Inducing ES cells over-expressing KDM5B to undergo neuronal differentiation demonstrated that whilst no effect on neurosphere formation was noted, delineation using POU5F1 (a.k.a OCT4) and phospho-histone H3 (PH3) as markers suggested that the ES cell/progenitor population failed to commit at the same rate as the control population. However, the majority of neurospheres over-expressing KDM5B failed to adhere to poly-D-lysine plates and differentiate toward a neuronal cell fate, further suggesting a function for KDM5B in the maintaining the balance between pluripotency and a differentiated phenotype. The defect in ES cell/progenitor cell commitment may be due to a KDM5B-dependent increase in proliferation (Dey et al., 2008), which upon Kdm5b knock down results in an accumulation of ES cells in the G1 phase of the cell cycle and ultimately morphological differentiation (Xie et al., 2011).

In contrast to these authors, upon Kdm5b knock down Schmitz and colleagues (2011) did not describe a function for KDM5B during ES cell proliferation, within the ES cell cell-cycle profile, on ES cell morphology nor on the expression levels of the pluripotency markers, Pou5f1 and Nanog. However, as described by Dey and colleagues (2008), knock down of Kdm5b was permissive for neurospheres formation but not terminal neuronal differentiation. Schmitz and colleagues (2011) attributed this defect upon neural differentiation on the inability of KDM5B deficient neural progenitor cells to incompletely silence stem and germ cell specific genes.

1A.4.5 Expression profile of KDM5C and KDM5D

Based on the limited number of tissues examined the transcripts of KDM5C and KDM5D (in males) are ubiquitously expressed in both human and murine adult tissue. Northern blot hybridisation with KDM5C and KDM5D specific probes detected transcripts from RNA isolated from human heart, pancreas, skeletal muscle and lung tissue (Jensen et al., 2005). Including those human adult tissues detailed above, murine Kdm5c, and Kdm5d are also expressed in the adult murine kidney, liver and spleen. Similarly, the Kdm5c and Kdm5d transcripts are also detected in the developing embryonic brain (Sheardown et al., 1996, Xu et al., 2002). Taking Kdm5c expression analysis further demonstrated that the transcript was also detected in haematopoietic stem cells (Sheardown et al., 1996), embryonic stem cells and neuronal progenitor cells (Outchkourov et al., 2013).
The $Kdm5d$ gene is mapped to the azoospermia factor region (AZF) (Agulnik et al., 2004b), a hot spot for genes involved in spermatogenesis (Ma et al., 1992, Reijo et al., 1995). Examination of the $Kdm5d$ expression pattern during postnatal development of murine spermatogenesis, from germ cell renewal (mitosis) through differentiation to mature sperm cells (spermatid) demonstrated that $Kdm5d$ is not expressed during mitosis but is expressed throughout meiosis being maximally expressed at the pachytene stage of prophase I with high levels of expression being maintained during the diplotene stage of meiosis as well as in the differentiating spermatid (Akimoto et al., 2008).

1A.4.6 A role for the KDM5 family of proteins during neurogenesis

There is significant evidence for a function for the KDM5 family during neurogenesis. Disruption of $D.\ melanogaster\ lid$ expression results in a lethal defect in optical brain lobe development (Gildea et al., 2000). KDM5C expression is required for dendritic development of rat neuronal cells (Iwase et al., 2007) and mutations of the KDM5C gene have been linked with the neurological disorder X-linked mental retardation (XLMR) (Tzschach et al., 2006, Santos-Reboucas et al., 2011). KDM5A knockout mice displayed abnormal behaviour when held up-side down (Klose et al., 2007) suggesting mental dysfunction. At the time of writing this thesis a report was published describing a neurological function for KDM5B during murine development (Albert et al., 2013) and will be discussed later.

1A.5 A role for KDM5A and KDM5B during senescence and differentiation

1A.5.1 A function for KDM5B during the G1 to S phase transition of the cell cycle

A function for the KDM5 family in the control of the cell cycle is beginning to emerge. In ES cells the expression of CDKN1B (a.k.a p27) promotes cells cycle arrest in the G1-phase of the cell cycle (Menchon et al., 2011). KDM5B can repress the transcription of $CDKN1B$ and KDM5B expression is required for the G1 to S phase transition of the cell cycle (Dey et al., 2008, Xie et al., 2011).

The role of KDM5B in the progression from the G1 to the S-phase of the cell cycle is not limited to ES cells (Yamane et al., 2007, Mitra et al., 2011, Wong et al., 2012). For example, co-transfection of KDM5B together with $TFAP2C$ (a.k.a. AP-2γ) and c-MYC, into HepG2 cells lead to a significant shift from the G1 to S phase of the cell cycle.
(Wong et al., 2012). In keeping with this, Yamane and colleagues (2007) have reported that KDM5B knock down in the breast cancer cell line, MCF7 cells results in reduced cell proliferation, an accumulation of cells in the G1 phase of the cell cycle and a delay in cell cycle exit. In addition to repressing CDKN1B transcription, as described above (Dey et al., 2008), a function for KDM5B downstream of the cyclin-dependent kinase inhibitor protein family has been described. In MCF7 cells, KDM5B represses the expression of Let-7e, a negative regulator of CCND1 (a.k.a Cyclin D1) thereby promoting G1 to S phase transition (Mitra et al., 2011).

As described below the repression of E2F-target genes by KDM5A and KDM5B is required during senescence and differentiation.

1A.5.2 KDM5A and KDM5B link H3K4 demethylation to retinoblastoma binding protein mediated cellular senescence

RAS-induced senescence of the human foetal lung cell line IMR90 is associated with a global reduction in H3K4me3/2 levels, of which many of the gene categories affected are enriched for cell cycle progression and DNA replication (Chicas et al., 2012). The pRb-pathway mediates cellular senescence through the ability to repress E2F target genes, the promoter regions of which become concomitantly depleted of H3K4me3 (Narita et al., 2003, Chicas et al., 2012). Both KDM5A and KDM5B contribute to the global reduction in H3K4me3/2 levels seen during RAS-induced senescence (Chicas et al., 2012). Over-expression of KDM5A in IMR90 cells induced cell cycle arrest and the formation of senescence associated heterochromatin foci (SAHF), which was dependent upon the demethylase activity of KDM5A and the expression of pRb (Chicas et al., 2012).

Whereas both KDM5A and KDM5B can contribute to RAS-induced cellular senescence, using a system in which murine neuronal cells undergo temperature induced senescence, identified KDM5B as the sole member of the KDM5 family that upon knock down was capable of by-passing senescence. Furthermore, KDM5A is not required for replication-induced senescence of mouse embryonic fibroblasts (MEF) (Lin et al., 2011). Genome binding studies on late passage MEF cells that bore the hallmarks of replication-induced senescence, demonstrated that KDM5B was recruited to senescence-associated E2F-target genes concomitantly with a decrease in transcriptional expression and H3K4me3 levels (Nijwening et al., 2011).
1A.5.3 A function for KDM5A during differentiation

Cellular differentiation requires that the cell exits its cycle and enters quiescence when cell cycle genes are repressed and the expression of tissue-specific differentiation markers are initiated. In quiescent cells E2F4, p130, SIN3B, and HDAC1 are the predominant complex bound to the E2F-regulated promoter of cell cycle genes (Catchpole et al., 2002, Rayman et al., 2002).

During the monocytic differentiation of U937 cells, concomitantly with cell cycle arrest, KDM5A functions as a transcriptional repressor of cell cycle genes (Lopez-Bigas et al., 2008). Using the same monocytic differentiation system, ChIP-PCR demonstrated that those genes bound by KDM5A could potentially do so as a complex with E2F4/p130, and further that KDM5A and E2F4 co-operate during transcriptional repression (Beshiri et al., 2012). Similarly, promoter array studies on C2C12 myoblast cells induced to undergo differentiation revealed that SIN3, E2F4, and KDM5A significantly enrich, and repress the expression of cell cycle genes (Van Oevelen et al., 2008). These finding taken together, link a potential E2F4, p130, SIN3, HDAC1, and KDM5A complex to the repression of cell-cycle dependent genes during the differentiation of monocytes and myocytes.

In addition to a role for KDM5A during the differentiation-associated repression of cell-cycle dependent genes, data to date suggests that KDM5A functions to repress the expression of tissue-specific lineage markers prior to the on-set of differentiation. During osteoblast proliferation p130 together with E2F4/5 occupy the promoters, and repress the inappropriate expression of two markers of differentiation namely, alkaline phosphatase (Alpl) and osteocalcin (Bglap), (Flowers et al., 2010). However, upon a signal to differentiation there is a shift in binding of the repressor p130, E2F4, KDM5A complex to an activating pRb, E2F1 complex that promotes polymerase II binding to the promoters of Alpl and Bglap and subsequent transcription (Flowers et al., 2013). It is certainly intriguing to imagine that the 'switch' from proliferation toward differentiation that is intimately linked with KDM5A, is conserved amongst the family members during organogenesis (Figure1.7).
**Figure 1.7.** A model ‘switch’ in repression during differentiation. A KDM5A-repressor complex silences E2F-target gene expression during differentiation. A KDM5A repressor complex is displaced from the promoter of differentiation associated genes leading to gene activation.

### 1A.6 KDM5B and cancer

A function during ES cell commitment and cell cycle control suggests that aberrant expression of KDM5B may contribute to disease development. Indeed KDM5B is over expressed in a number of solid tumours (Lu et al., 1999, Yamane et al., 2007, Xiang et al., 2007, Hayami et al., 2010, Catchpole et al., 2011, Coleman et al., 2011, Islam et al., 2011a, Kuzbicki et al., 2012, Radberger et al., 2013).

In a comprehensive study using a cDNA array containing 337 samples from 17 primary cancers of different tissue types revealed that *KDM5B* is expressed at high levels in cancers of the breast tissue, adrenal glands and the cervix. *KDM5B* expression also correlated with lung tumour stages, increasing in stages IIB, IIIA and IV (Islam et al., 2011a). In addition, immunohistochemistry has confirmed that the KDM5B protein is expressed in lung cancer but also bladder cancer (Hayami et al., 2010) as well as melanoma (Roesch et al., 2005, Kuzbicki et al., 2012, Radberger et al., 2013), tissues not included in the array data published by Islam and colleagues (2011a).
1A.6.1 KDM5B expression and melanoma

Immunohistochemical studies used to examine the expression of KDM5B during the transformation of melanocytes have yielded contrary findings. Roesch and colleagues (2005) reported that whilst 70% of nevi express KDM5B, a lack of KDM5B expression was detected in 90% of primary melanoma and 70% of melanoma metastases. This lack in KDM5B expression was represented as KDM5B being expressed in single melanoma cells that represented approximately 5 to 10% of the tumour bulk (Roesch et al., 2005). However more recent immunohistochemistry studies, report that 90% of nevi, and all melanomas as well as melanoma metastases expressed KDM5B (Kuzbicki et al., 2012), as well as 55% of uveal melanomas (Radberger et al., 2013). This discrepancy in KDM5B protein levels expressed between nevi and advanced melanoma maybe due to antibody specificity. The antibody used by Roesch and colleagues (2005) was specific to the splice variant of KDM5B in contrast to the studies conducted by Kuzbicki and colleagues (2012) as well as by Radberger and colleagues (2013) which would detect both isoforms of KDM5B.

In order to examine the function of KDM5B during the pathogenesis of melanoma, KDM5B-promoter driven EGFP (enhanced green fluorescent protein) positive melanoma cells were FACS sorted and xenotransplanted into NOD/LtScidIL2Rγ null mice. Subsequent oral administration of BrdU, suggested that those tumour cells that express KDM5B represented a slow cycling population. Interestingly, regardless of whether KDM5B positive or KDM5B negative melanoma cells were used for xenotransplantation, the resulting tumour was heterogeneous for KDM5B expression. Further studies suggested that KDM5B is not required for the initiation of tumours but for the maintenance of continuous tumour growth (Roesch et al., 2010).

1A.6.2 KDM5B expression and prostate cancer

The androgen receptor plays an important role in prostate cancer development and progression (Chen et al., 2004). Analysis of a set of data in the Oncomine cancer-profiling database revealed that KDM5B expression is limited in benign prostate but is up regulated in prostate cancer, with highest levels of KDM5B expressed in metastatic prostate tissue suggesting a potential link with KDM5B and patient survival (Xiang et al., 2007). Consistent with Xiang and colleagues (2007), analysis of a data set from the GeneSapiens database identified KDM5B as over expressed in prostate adenocarcinoma compared to normal prostate tissue (Bjorkman et al., 2011). Significantly KDM5B and the AR can interact in vivo (Xiang et al., 2007) further
supporting a causal link between KDM5B expression and the pathogenesis of prostate cancer.

1A.6.3 KDM5B and breast cancer

*KDM5B* is expressed in breast tumours (Lu *et al*., 1999, Islam *et al*., 2011a) and correlates positively with breast cancer grade (Coleman *et al*., 2011). Furthermore, KDM5B maybe required for breast cancer cell proliferation (Yamane *et al*., 2007) and as demonstrated using both a syngenic (Yamane *et al*., 2007) and an ER alpha positive xenograft tumour murine model (Catchpole *et al*., 2011) that KDM5B is required for mammary tumour growth.

Other KDM5B expression data also suggests a role for KDM5B in the progression of breast cancer (Lu *et al*., 1999, Coleman *et al*., 2011, Yoshida *et al*., 2011). A positive effect of KDM5B on breast cancer progression is consistent with transwell migration assays using two breast cancer cell lines MDA-MB-231, BT549 and a prostate cancer cell line DU145 (Yoshida *et al*., 2011). However, a tumour suppressive function has also been suggested during the progression of advanced breast cancer, through the ability of the KDM5B/KDM1/NURD complex to repress the CCL14 promoter resulting in the suppression of MDA-MB-231 transwell invasion. Furthermore KDM5B expression suppressed angiogenesis both *in vitro* and *in vivo* (Li *et al*., 2011). To clarify the function of KDM5B during breast cancer metastasis, *in vivo* models are required.

Human KDM5B was initially identified as a gene whose expression is positively regulated through the c-ErbB2-signalling pathway in luminal epithelial cells (Lu *et al*., 1999). Subsequently, KDM5B was identified as being expressed in breast tumour biopsies (Lu *et al*., 1999, Coleman *et al*., 2011, Islam *et al*., 2011a) the expression of which positively correlates with breast cancer grade (Coleman *et al*., 2011). From a clinical perspective breast cancer can be broadly classified into 3 therapeutic groups; ER alpha positive, c-ErbB2 positive (ER alpha positive or ER alpha negative), and triple-negative.
1A.7 Clinical sub-types of breast cancer

Over half of breast cancer patients are categorized as oestrogen receptor positive and are therefore the most numerous of the clinical sub-groups (Perou et al., 2000, Sorlie et al., 2001). Sustained exposure to the ovarian hormones progesterone and oestrogen are established risk factors with early menarche and late menopause increasing the risk of developing breast cancer (Trichopoulos et al., 1972, Apter et al., 1989, Bernstein et al., 2002). Clinically these patients respond well to endocrine therapy. In fact the use of tamoxifen and aromatase inhibitors on hormone receptor positive breast cancer patients has almost halved the rate of disease recurrence (EBCTCG. 2005, Cuzick et al., 2010).

Amplification of the \textit{ERBB2} gene occurs for some 25% of breast cancer patients (Slamon et al., 1987). Amplification and over-expression of c-ErbB2 is associated with a poorer prognosis (Slamon et al., 1987, Perou et al., 2000, Sorlie et al., 2001). However the use of treatments, such as trastuzumab have significantly improved the survival rates of this category of breast cancer patient (Cobleigh et al., 1998).

Patients that present with a triple-negative breast tumour; so called because the tumours lack the expression of ER alpha, PR and c-ErbB2 (Reis-Filho et al., 2008), represent some 10-20% of breast cancers (Perou et al., 2000, Sorlie et al., 2001). Patients with triple-negative tumours have a worse prognosis than patients with ER alpha positive tumours (Sorlie et al., 2001), with a higher relapse rate within the first three years (Dent et al., 2007).

Significantly, immunohistochemistry on a cohort of over 300 breast cancers demonstrated that KDM5B is widely expressed in all breast cancer sub-types.

1A.8 The relevance of murine models to study mammary gland morphogenesis

One question that remains relevant to scientific research on normal mammary gland development and breast cancer is: What are the most suitable experimental models to use that can best represent the signalling pathways that drive human mammary gland morphogenesis and disease development? In the first instance and from an \textit{in vivo} stand point, the murine gestation period is approximately 20 days and postnatal adult development is completed by approximately 8 weeks. This timeframe provides a suitable window in which to study the signalling pathways involved in mammary gland morphogenesis. It is well documented that exposure to ovarian hormones can increase
a woman’s chance of developing breast cancer (Trichopoulos et al., 1972, Apter et al., 1989, Bernstein et al., 2002). Murine models of mammary gland development provide a suitable model due to the similarities between the mouse oestrous cycle and the human menstrual cycle. The human follicular phase has a surge of circulating oestrogen that is reflected in the murine pro-oestrus/oestrus, whereas there is a surge of circulating progesterone during the human luteal phase that is reflected in the murine dioestrus. Further supporting the use of murine models to study normal mammary gland morphogenesis and breast cancer are the similarities between the human and murine mammary hierarchical organisation (Shehata et al., 2012) and the signalling pathways involved (Lim et al., 2010, Shehata et al., 2012).

Using murine models to understand the factors that are important to normal mammary gland epithelial cell hierarchy determination will hopefully provide essential information with respect to the development of breast cancer. The next section of the introduction therefore highlights some of those factors that are crucial for the development of the murine mammary gland.
Chapter 1: Introduction

Section 1B: Development, differentiation, and involution of the murine mammary gland
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1B Overview Although the mammary gland is established during embryonic development, the majority of development occurs after birth. In response to the endocrine signal emanating from the hypothalamus-pituitary-axis at puberty the epithelial cells at the distal ends of the mammary duct proliferate to form bulbous structures known as terminal end buds. Ramification of the fat pad through ductal elongation and bifurcation at TEB structures is completed by adulthood. This is followed by cycles of cell proliferation, differentiation and apoptosis during the oestrous cycle and at each recurring pregnancy. From the onset of puberty and throughout the reproductive period of the female, the mammary gland will undergo extensive cyclic regeneration and remodelling suggesting the presence of mammary gland stem cells and an epithelial cell hierarchy. The balance between mammary progenitor cell proliferation and mammary cell fate specification is therefore critical for normal mammary morphogenesis.

The adult mammary gland structure is comprised of two main lineages that are derived from multi-potent (Shackleton et al., 2006, Stingl et al., 2006) and/or uni-potent stem cells (Van Keymeulen et al 2011). The luminal cell lineage line the ducts and differentiate into alveolar units and the basal/myoepithelial cells are localised between the luminal cells and the basement membrane. In response to the hormonal influx, at approximately 4 weeks of age bulbous TEB structures form at the distal tip of the rudimentary ductal structure (Nandi, 1958). TEB structures - the unit of elongation, are a putative niche for mammary stem cells, with evidence from murine studies suggesting that although the majority of actively proliferating stem cells reside in the cap cell layer, actively proliferating stem cells have also been detected in the inner body cells during puberty (Bai et al., 2010). The TEB structure, is comprised of many layers of highly proliferative yet relatively unpolarized luminal body cells surrounded by cap cells, that via collective cell migration result in the ramification of the fat pad (Ewald et al., 2008). Figure 1.8
Figure 1.8 Murine postnatal mammary gland development. The adult mammary gland is comprised of two main lineages. The luminal cell lineage line the ducts and the basal/myoepithelial cells are localised between the luminal cells and the basement membrane. At the onset of pubertal mammary gland development, TEB structures are formed resulting in and the ramification of the fat pad. Ovarian hormones dictate nulliparous mammary epithelial cell hierarchy determination. (Amalgamated from: Shackleton et al., 2007, Stingl et al., 2007, Ewald et al., 2008, Bai et al., 2010, Asselin-Labat et al., 2010, Joshi et al., 2010, Van Keymeulen et al., 2011, Shehata et al., 2012). s-SHIP is the acronym for SH2-containing inositol 5'-phosphatase (104kd isoform), and GFP is green fluorescent protein.

Mammary gland ductal patterning through the bifurcation of TEB structures and subsequent side branching at stem/progenitor rich sites, occurs at regions of little myoepithelial coverage. The completion of ductal morphogenesis occurs once the luminal epithelial cells attain full apicobasal polarity and simple epithelial organisation by approximately 8 weeks of age (Ewald et al., 2008). Coincidentally, as the ductal tree
approaches the outer limits of the fat pad so the mouse reaches sexual maturity (Figure 1.9).

**Figure 1.9** Nulliparous mammary gland development is completed by adulthood through the bifurcation of TEB structures and the generation of ductal side-branches.

During pubertal mammary gland development mammary epithelia are exposed to cyclic rounds of oestrogen and progesterone exposure. The mouse estrous cycle is divided into proestrus, estrus, metestrus and diestrus and lasts for 4-5 days. Although estradiol and progesterone serum levels are detected throughout the estrous cycle, levels do fluctuate. Estradiol levels peak at estrus, and progesterone serum levels peak at diestrus (Fata *et al*., 2001, Joshi *et al*., 2010). Mammary gland stem cells are responsive to steroid hormones (Asselin-Labat *et al*., 2010, Joshi *et al*., 2010) despite lacking oestrogen and progesterone receptors (Asselin-Labat *et al*., 2006, Sleeman *et al*., 2007). Whereas oestrogen primes mammary gland stem cells (Asselin-Labat *et al*., 2010) and is required for ductal elongation (Daniel *et al*., 1987), a rise in serum progesterone levels at diestrous is concurrent with an increase in mammary gland cell number and mammary ductal side-branching (Cole *et al*., 1933, Fata *et al*., 2001)

**1B.1 Murine mammary gland stem cells, the epithelial cell hierarchy**

*1B.1.1 The existence of a murine multi-potent adult mammary gland stem cell*

In order to generate an adult mammary gland from a rudimentary ductal structure, mammary gland stem cells need to undergo self-renewal and progenitor cell expansion toward a luminal or myoepithelial cell fate. Defining the signalling pathways responsible for progenitor cell expansion and mammary cell fate specification requires the identification and isolation of specific mammary gland cell types.
Using a FACS-based approach has enabled the isolation and characterisation of the mammary gland stem cell (Shackleton et al., 2006, Stingl et al., 2006). The isolation of the putative mammary gland stem cell was based upon the cell surface expression of CD29 (β1-integrin), which is a skin stem cell marker (Jones et al., 1995), CD24 (heat-stable antigen) a neural stem cell marker (Rietze et al., 2001), and CD49f (α6-integrin) an epidermal stem cell marker (Li et al., 1998). Confirmation of the existence of a multi-potent adult mammary gland stem cell came with the demonstration that a single cell from either the CD24+CD29 hi (Shackleton et al., 2006) or CD24+CD49f hi (Stingl et al., 2006) epithelial population isolated from an adult nulliparous mammary gland could self renew and generate a functional mammary gland when serially transplanted into the cleared fat pad of recipient mice (Figure 1.10).

**Figure 1.10 Murine mammary gland epithelial cell hierarchy.** Lineage trace experiments have identified the existence of a multi-potent embryonic mammary epithelial stem cell. Adult mammary gland maybe generated from multi-potent and/or uni-potent mammary stem cells. Red arrow indicates bi-potent potential of the uni-potent myoepithelial stem cell upon transplantation. (Amalgamated from: Asselin-Labat et al., 2007, Shackleton et al., 2007, Stingl et al., 2007, Van Keymeulen et al., 2011, Shehata et al., 2012). KRT is the acronym for cytokeratin.
In order to further delineate a mammary gland epithelial cell hierarchy, Shackleton and colleagues (2006) compared the growth of the CD24+CD29\textsuperscript{hi} stem cell-enriched population with that of the CD24+CD29\textsuperscript{lo} population in Matrigel under lactogenic conditions. Under these conditions, the CD24+CD29\textsuperscript{lo} population formed mainly alveolar like structures (85%) demonstrating a population that is enriched for cells that have a restricted luminal cell fate. In contrast the CD24+CD29\textsuperscript{hi} stem cell-enriched population presented with a more expanded repertoire of distinguishable structures including ducts, multicellular spheroid bodies, as well as the occasional alveolar structure (3%).

Leading on from this, the CD24+CD29\textsuperscript{lo} luminal enriched population of mammary cells can be further subdivided based upon the expression of the cell surface marker CD61 (\(\beta^3\)-integrin). The CD61+ sub-population when grown in Matrigel could generate colonies that exclusively expressed KRT18 (cytokeratin 18), indicative of luminal progenitors. In keeping with this the greatest proportion of mammary epithelial cells at the onset of puberty are of the luminal enriched progenitor subtype, CD61+ cells (~60%). As the female reaches adulthood, the mammary gland becomes more differentiated and is composed of fewer luminal progenitor cells (~38% CD61+ epithelial cells). By day 18.5 of pregnancy (pd18.5), the mammary gland is fully differentiated in preparation for parturition. Consistent with this, the mammary gland is comprised of few luminal progenitor cells (~3% CD61+ epithelial cells) (Asselin-Labat \textit{et al.}, 2007).

The cell surface markers, stem cell antigen (Sca1) and CD49b (\(\alpha^2\)-integrin) can also be used to isolate luminal progenitor cells (Li \textit{et al.}, 2009). Using these cell surface markers has demonstrated that the adult mammary gland consists of two luminal progenitor cell types based on their genotypic and phenotypic characteristics, with the Sca1+CD49b+ population representing the luminal progenitors restricted to a ductal luminal cell fate and the Sca1-CD49b+ population representing the luminal progenitors restricted to an alveolar cell fate (Shehata \textit{et al.}, 2012). Although both luminal progenitor populations are clonogenic, the Sca1+CD49b+ enriched population express higher levels of \textit{Esr1}, \textit{Foxa1} and \textit{Gata3} (Shehata \textit{et al.}, 2012). Given that ER alpha (Feng \textit{et al.}, 2007), FOXA1 (Bernado \textit{et al.}, 2010) and GATA3 (Asselin-Labat \textit{et al.}, 2007) are required for ductal development may suggest that the Sca1+CD49b+ enriched population represent a ductal progenitor cell type. In contrast the Sca1-CD49b+ enriched population may represent the alveolar progenitor population. The Sca1-CD49b+ enriched population express no to low detectable levels of \textit{Esr1} but do
express milk protein transcripts and markers of alveolar differentiation (Shehata et al., 2012)

As researchers attempt to delineate the mammary gland epithelial cell hierarchy and the controlling factors involved, a number of issues require redress. Firstly, what is the contribution of the embryonic mammary bud to adult mammary gland morphogenesis? Secondly, what effect, if any, do mammary epithelial cell transplantation assays have on cell behaviour and thirdly, what influence do steroid hormones have on stem cell and progenitor cell numbers and differentiation potential?

1B.1.2 Murine postnatal mammary gland development is defined in the embryonic mammary bud and completed after birth by uni-potent stem cells

In order to address these first two issues, Blanpain and colleagues (2011) used a lineage trace approach to decipher the cellular hierarchy of mammary epithelia under the physiological setting of embryonic, pubertal and pregnancy-associated mammary gland development (Van Keymeulen et al., 2011). Monitoring yellow fluorescent protein (YFP) expression, driven indirectly from lineage specific cytokeratin promoters has demonstrated that a multi-potent stem cell population resides in the embryonic mammary bud and that pubertal and pregnancy-associated mammary gland development is supported by a uni-potent stem cell population as detailed below.

Using an inducible approach to trace KRT14-specific YFP protein expression in the embryonic mammary bud through to birth demonstrated that mammary epithelial cells of the rudimentary ductal system presented at birth were all positive for YFP protein expression. Furthermore, the YFP positive mammary epithelial cells from the rudimentary ductal structure could be divided into 2 populations; a CD24+CD29^{hi} population enriched for myoepithelial cells and a CD24+CD29^{lo} population enriched for luminal epithelial cells.

Transcription profiling identified Krt14 and Krt5 as transcripts enriched in the CD24+CD29^{hi} stem cell enriched population (Shackleton et al., 2006, Stingl et al., 2006). To determine under physiological conditions the contribution of a multi-potent stem cell (Shackleton et al., 2006, Stingl et al., 2006) to mammary gland morphogenesis Van Keymeulen and colleagues (2011) again used an inducible approach to trace KRT14-specific and KRT5-specific YFP protein expression in the pubertal and adult mammary gland. Significantly, these data demonstrated that KRT14
or KRT5 specific YFP protein expression marked only myoepithelial cells. Similarly, KRT8-luminal specific YFP protein expression traced only luminal cells, demonstrating that post-natal mammary epithelial cell fate is determination by uni-potent stem cells specific to the myoepithelial or luminal lineage (Figure 1.10).

1B.1.3 Both multi-potent and uni-potent murine mammary gland stem cells contribute to postnatal mammary gland development

In order to establish whether mammary gland transplantation assays can unlock a differentiation potential not seen under physiological conditions, chimeric mammary glands were generated. Whether the mixing experiments used YFP-protein marked luminal or YFP-protein marked myoepithelial cells, the resulting mammary glands were generated from uni-potent stem cells. Significantly, when YFP positive myoepithelial cells are co-transplanted with a 10x fold reduction in the number of luminal cells normally present during physiological conditions, the KRT14-specific YFP-traced myoepithelial cells can adopt a bi-potent phenotype and can differentiate along both the myoepithelial and the luminal cell lineage. These data suggest that a stem cell hierarchy exist in the adult mammary gland that includes stem cells with uni-potent and bi-potent activity that may perform unique functions during the morphogenesis and maintenance of the murine mammary gland (Van Keymeulen et al., 2011, Figure 1.10).

Given that postnatal murine mammary gland development is absolutely dependent on ovarian hormones (Nandi, 1958) would indicate a role for ovarian hormones in dictating murine stem cell function and mammary epithelial cell hierarchy determination. It is therefore essential to understand the role of ovarian hormones on mammary epithelial cell hierarchy determination during postnatal mammary gland development.

1B.1.4 Ovarian hormone control of mammary epithelial cell hierarchy organisation during postnatal mammary gland development

Studies of the effects of the ovarian hormones oestrogen and progesterone on postnatal mammary gland stem cell stasis have certainly added to the current understanding of mammary gland development. In the first instance, although ovarian hormone deprivation as a result of ovariectomy, had no effect on the total number of the mammary gland stem cell-enriched population (CD24+CD29hi), transplantation of an ovarian hormone deprived CD24+CD29hi stem cell enriched population into the clear fat pad of recipient adult females presented as a delay in ductal morphogenesis. This mammary gland phenotype maybe due to an effect on the cell cycle given that the
hormone deprived CD24+CD29<sup>hi</sup> stem cell enriched population accumulated in the G0/G1-phase of the cell cycle (Asselin-Labat et al., 2010). Therefore, these results demonstrate that the exposure of mammary epithelial cells to a normal hormonal milieu is essential for proper mammary gland stem cell function and that mammary epithelial cells appear to retain a memory of their hormone environment (Asselin-Labat et al., 2010).

Of the ovarian hormones, oestrogen is a potent mitogen in the pubertal mammary gland where it promotes ductal elongation (Daniel et al., 1987), but acts merely permissively in the adult mammary gland where it induces progesterone receptor expression (Haslam et al., 1979, Beleut et al., 2010). Furthermore, data thus far implicates oestrogen in mediating stem cell function and epithelial cell hierarchy determination (Asselin-Labat et al., 2010). Treatment of young adult mice with the aromatase inhibitor Letrozole, which inhibits the production of oestrogen, not only reduced the repopulating frequency of the CD24+CD29<sup>hi</sup> stem cell enriched population, but also the ductal outgrowth potential of transplants. Furthermore, fewer of the CD24+CD29<sup>lo</sup> luminal cell enriched population were detected in Letrozole treated mice. Oestrogen may therefore be required for the survival of the luminal-enriched population (Asselin-Labat et al., 2006, Asselin-Labat et al., 2010, Shehata et al., 2012).

During post-natal mammary gland development, mammary epithelial cells are exposed to cyclic rounds of ovarian hormones. Whilst estradiol serum levels peak at oestrus, progesterone serum levels peak at dioestrus, the stage of the oestrus cycle that is associated with tertiary side branching and lobuloalveolar development (Fata et al., 2001, Joshi et al., 2010). These observed changes in the gross morphology of the mammary gland would therefore suggest that the hormonal changes imposed on the mammary gland during the oestrus cycle could therefore influence mammary epithelial cell hierarchy determination. In contrast to the number of mammary epithelial cells at oestrus; during dioestrus the CD24+CD49<sup>hi</sup> stem cell enriched population had expanded some six-fold whilst the CD24+CD49<sup>lo</sup> luminal cell enriched population had expanded some three-fold. A rise in progesterone therefore drives not only the proliferation of the CD24+CD49<sup>hi</sup> stem cell enriched population but also the proliferation and differentiation of the CD24+CD49<sup>lo</sup> luminal cell enriched population as the expanded luminal population were CD61-.
1B.1.5 Cell fate and histone marks in the mammary gland

(A) The chromatin effector protein, PYGO-2

Genetic studies in mice have shown an effect of the H3K4me3/2 reader, PYGO-2 (see Section 1A) on stem and progenitor cells during embryonic and postnatal murine mammary gland development (Gu et al., 2009). In an epithelial specific Pygo2 null murine line, reduced formation of the rudimentary ductal tree was observed, due to a reduction in embryonic progenitor cell proliferation, whilst in the adult mammary gland, fewer terminal end bud structures are present, ductal elongation is delayed and the female presents with fewer ductal side branches (Gu et al., 2009).

Examination of the differentiation potential of the Pygo2 deficient stem cell enriched population ex-vivo using a 3D Matrigel assay demonstrated a propensity for acinar-like structures and an increase in the transcript levels of Csn2 (beta-casein) and Stat5a, indicating that the expression of PYGO2 is required to suppress the luminal/alveolar differentiation potential of the mammary gland stem cell enriched population. At a molecular level, Pygo2 deficient mammary gland stem cells exhibited a transcriptional expression profile consistent with that of a mature luminal gene set including elevated members of the NOTCH signalling pathway such as Notch3.

The NOTCH signalling pathway promotes the commitment/differentiation of mammary gland stem cells to the luminal cell fate (Buono et al., 2006, Bouras et al., 2008). In contrast, the WNT/beta-catenin signalling pathway promotes the self-renewal of mammary gland stem cells (Zeng and Nusse 2010). Interestingly, the activation of the WNT/beta-catenin signalling pathway suppressed luminal/alveolar differentiation, and Notch3 expression in a PYGO2 dependent manner (Gu et al., 2013). To elucidate the mechanism of action responsible for the suggested PYGO2 dependent cross-talk between the NOTCH-signalling and WNT/beta-catenin signalling pathways these authors demonstrated that beta-catenin binding to the promoter region of the Notch3 gene was reduced in Pygo2 deficient mammary gland stem cells (Gu et al., 2013). Importantly, the nucleosomes surrounding the PYGO2/beta-catenin enriched binding site at the Notch3 locus was enriched with the ‘bivalent’ H3K27me3 and H3K4me3 mark. This chromatin configuration is reminiscent of the ‘bivalent domain’ found at the promoter region of developmental genes whose silence is required in embryonic stem cells yet are poised for activation upon lineage differentiation (Bernstein et al., 2006, Mikkelsen et al., 2007). In Pygo2 deficient mammary gland stem cells an increase in the H3K4me3 mark was observed at the Notch3 locus with a concomitant decrease in the H3K27me3 mark (Gu et al., 2013). These data indicate that in the absence of
Pygo2 the ‘bivalent domain’ at the Notch3 locus was resolved to an active chromatin configuration.

(B) The EZH2 histone methyltransferase

Development of the mammary gland requires interpretation of upstream hormonal signalling at the level of the genome resulting in the alteration of chromatin structure that permits gene regulation, progenitor cell expansion and lineage determination. This results in milk protein synthesis and therefore the ultimate aim of the developing mammary gland - the nourishment of the offspring. Although studies have identified a number of signalling pathways and downstream transcription factors that control mammary gland epithelial cell differentiation, knowledge on the epigenetic factors that regulate chromatin structure during mammary gland development is now only coming to the fore.

Changes in histone methylation are associated with gene expression changes during lineage restriction and is therefore a likely mediator of the mammary epithelial cell hierarchy (Pal et al., 2013). Genes differentially expressed in the mammary gland stem cell enriched population or the luminal progenitor enriched population display an increase in the H3K4me3 mark and a decrease in the H3K27me3 mark upon increased expression, whilst those gene that are silenced display the opposite epigenetic change (Pal et al., 2013).

The hormone environment influences the dynamics of the mammary gland epithelial cell hierarchy (Asselin-Labat et al., 2010, Joshi et al., 2010). Cell cycle genes expressed in the mammary gland stem cell and luminal enriched populations of epithelia of ovariectomized mice are enriched in the H3K27me3 mark (Pal et al., 2013) accounting for the delay in ductal morphogenesis (Asselin-Labat et al., 2010) and linking the hormonal environment with chromatin modifications, gene expression and cell fate determination (Asselin-Labat et al., 2010, Joshi et al., 2010, Pal et al., 2013). As could be predicted therefore, during pregnancy, key luminal-specific genes such as whey acidic protein (Wap), the Ets domain transcription factor (Elf5) and beta casein, whose expression increases during pregnancy and are required for differentiation and milk protein synthesis, display reduced levels of the H3K27me3 mark (Pal et al., 2013).

The histone methyltransferase EZH2, is a component of the polycomb repressor complex 2 (PRC2), and is responsible for the di and trimethylation of lysine 27 on histone 3 (Cao et al., 2002). Of the major pregnancy hormones, progesterone but not
prolactin regulates the increase in the phosphorylation of EZH2 on threonine 487 detected from early to mid pregnancy (Pal et al., 2013) linking further the relationship between the hormonal environment and the function of epigenetic factors during the development of the mammary gland.

Ablation of Ezh2 expression from mammary gland epithelia not only delays ductal elongation during puberty, but also alveoli development during pregnancy due to defective cell cycle control (Pal et al., 2013). Furthermore, using fat pad reconstitution assays, and limited dilutions of FAC-sorted stem cell enriched (CD24+CD29hi) epithelia the frequency of repopulating cells was reduced by 14 fold in the absence of Ezh2 expression. Moreover, the stem cell enriched and the luminal enriched population of epithelia isolated from the Ezh2-deficient mammary gland had poor colony forming ability in vitro (Pal et al., 2013). These observations suggest a critical role for EZH2 in regulating the activity of multiple progenitor subtypes during mammary gland development.

Of interest, one of the top de-repressed gene sets in the stem cell enriched population of Ezh2 deficient mammary epithelia other than those related to cell cycle regulation, was keratinocyte differentiation (Pal et al., 2013), suggesting that a function of EZH2 during mammary gland morphogenesis is to silence inappropriate gene expression. This finding is consistent with observations in other biological systems in which mis-expression of non-myogenic lineage specific genes has been observed in Ezh2 null skeletal muscle (Juan et al., 2011).

1B.2 Studies on pubertal mammary gland development

1B.2.1 Oestrogen receptor signalling

Oestrogen elicits some of its effects on pubertal mammary gland development through the oestrogen receptor. Of the two oestrogen receptors (ER alpha and ER beta) only epithelial ER alpha expression is required for TEB formation and development of the mammary gland beyond the rudimentary ductal system presented at birth (Krege et al., 1998, Forster et al. 2002, Mallepell et al., 2006, Feng et al., 2007). Factors central to the oestrogen receptor signalling-cascade are therefore also important for maintaining pubertal mammary gland development (Asselin-Labat et al., 2007, Bernado et al., 2010, Kawazu et al., 2011). For example, the expression of factors such as FOXA1 (Bernado et al., 2010) and GATA3 (Asselin-Labat et al., 2007) - whose expression is
required for ER alpha expression in the normal mammary gland - are also required for development beyond the rudimentary ductal structure (Asselin-Labat et al., 2007, Bernado et al., 2010). Furthermore, ER alpha can elicit transcriptional control as part of a dynamic multi-protein complex (Metivier et al., 2003). Members of an ER alpha complex can include histone-remodelling proteins such as the histone acetyl-transferase protein p300/CBP (Metivier et al., 2003) and the H3K9me3 demethylase KDM4B (a.k.a JMJD2B) (Kawazu et al., 2011, Gaughan et al., 2013). Depending on how significant these co-factors are to ER alpha function determines the degree to which deletion studies phenocopy the mammary gland defect presented in a Esr1 epithelial knock out murine line (Feng et al., 2007).

GATA3 is a critical regulator of lineage determination and differentiation in a number of cellular systems (Ting et al., 1996, Grote et al., 2006), including the murine mammary gland (Asselin-Labat et al., 2007). Although not exclusively, the expression of ER alpha and GATA3 are also intimately linked during normal murine mammary gland development. GATA3 positively regulates the expression of Esr1 with co-expression being more prevalent in the more mature luminal CD61- epithelial population (Asselin-Labat et al., 2007). Therefore as does the ablation of epithelial ESR1 expression halt pubertal mammary gland development at the rudimentary ductal stage (Feng et al., 2007), similarly, employment of either Krt-14-cre or MMTV-cre to mediate Gata3 deletion during pubertal mammary gland development also results in a severely stunted ductal tree (Asselin-Labat et al., 2007). Interestingly, expression of Gata3 from one allele resulted in an expansion of the luminal-enriched progenitor (CD61+) population in the adult mammary gland, highlighting the importance of GATA3 expression, and potentially ER alpha in the drive toward a mature luminal mammary phenotype.

As described ER alpha expression is a prerequisite for pubertal mammary gland development (Feng et al., 2007). However, deletion of the transcriptional co-activator CITED1 which stabilises the oestrogen dependent interaction between p300/CBP and ER alpha (Yahata et al., 2001) only affected the expression of a sub-set of oestrogen responsive genes in the developing pubertal mammary gland (McBryan et al., 2007). The resulting Cited1 knock out mammary gland presented with fewer terminal end bud structures, a delay in ductal tree development, together with a disorganised lateral branching structure (Howlin et al., 2006).

A number of observations place the expression of the H3K9 demethylase, KDM4B centrally within the oestrogen receptor signalling pathway (Kawazu et al., 2011, Shi et
Using human breast cancer cell lines, oestrogen can induce KDM4B expression (Kawazu et al., 2011) and KDM4B can also cooperate with GATA3 to regulate ESR1 expression itself (Gaughan et al., 2013). KDM4B expression is also required for breast cancer cell proliferation both in vitro and in vivo (Kawazu et al., 2011, Shi et al., 2011). In addition KDM4B expression is required for the proper recruitment of ER alpha to the genome (Kawazu et al., 2011). Furthermore, MMTV-Cre driven deletion of KDM4B from pubertal mammary epithelial cells results in a delay in ductal morphogenesis (Kawazu et al., 2011).

By acting through the epidermal growth factor receptor (EGFR), Amphiregulin (AREG) is a major paracrine mediator of ER alpha function during pubertal mammary gland development (Kenney et al., 1995, Luetteke et al., 1999, Ciarloni et al., 2007). The expression of AREG, the most abundant EGF-family member expressed during pubertal mammary gland development (Kenney et al., 1995) is regulated by ER alpha (Ciarloni et al., 2007). The expression of AREG is essential for pubertal mammary gland development (Luetteke et al., 1999, Ciarloni et al., 2007) and recombinant AREG expression can promote TEB formation and ductal development in ovariectomised mice (Kenney et al., 1996).

1B.2.2 Progesterone receptor signalling in the adult mammary gland

As described above, serum progesterone influences stem cell expansion and luminal progenitor cell differentiation in the adult mammary gland (Joshi et al., 2010). As is the case with the oestrogen receptor (Feng et al., 2007), only the epithelial expression of the progesterone receptor (PR) is required for mammary gland development (Brisken et al., 1998). Furthermore, engraftment of progesterone receptor null epithelial cells into the clear fat pad of the recipient host has shown that the epithelial expression of the progesterone receptor is required for lateral side branching but not the initial ramification of the pubertal mammary fat pad (Brisken et al., 1998). Mammary gland stem cells are oestrogen and progesterone receptor negative (Asselin-labat et al., 2006, Sleeman et al., 2007) and are instructed via paracrine mechanisms (Joshi et al., 2010). The canonical WNT-signalling pathway is one pathway that can mediate the effects of progesterone and is therefore of central importance to mammary gland development (Brisken et al., 2000, Joshi et al., 2010, Van Amerongen et al 2012). Use of an oestrogen and progesterone replacement regime on ovariectomised mice demonstrated that the Wnt4 transcript is induced in the FACS-sorted luminal enriched epithelial cell population (CD24+CD49f+), whilst a canonical WNT-signalling co-
receptor Lrp5 is induced in the CD24+CD49f$^{hi}$ stem cell enriched population (Joshi et al., 2010).

Hormonal cues together with locally produced growth factors stimulate the formation of TEB structures, which as a consequence of mammary epithelial proliferation results in the ramification of the fat pad through the bifurcation of TEB structures and the production of side-branches. This results in an ordered and amply spaced pubertal tree-like structure in anticipation for infilling by alveolar structures. Described below are some of those factors that function downstream or in parallel to ER alpha to mediate the development of the pubertal mammary gland.

1B.2.3 Growth factor regulation of ductal morphogenesis

Of the epidermal growth factor receptor family members, EGFR (c-ErbB1), c-ErbB2 and c-ErbB3 are required for pubertal mammary ductal development (Sebastian et al 1998, Jackson-Fisher et al., 2004, Jackson-Fisher et al., 2008).

AREG is regulated by ER alpha (Ciarloni et al., 2007) and is the major ligand for EGFR (Sternlicht et al., 2005). EGFR therefore mediates ER alpha signaling during pubertal mammary gland development (Sebastian et al., 1998). Egfr null neonatal mammary glands fail to undergo ductal morphogenesis when grafted under the renal capsule of recipient female mice (Sebastian et al., 1998) and therefore phenocopy mammary gland development in an Esr1 null murine line (Feng et al., 2007).

In contrast to the mammary gland phenotype presented in the Egfr knockout line, studies have shown that c-ErbB2 signalling is not absolutely required for pubertal development and is dispensable during pregnancy-associated differentiation (Jackson-Fisher et al., 2004, Andrechek et al., 2005). Whether the approach to examining the role of c-ErbB2 during mammary gland ductal morphogenesis utilized transplanted Erbb2 null epithelial (Jackson-Fisher et al., 2004) or a murine line harbouring a mammary epithelia specific Erbb2 deletion, these females presented with a delay in pubertal ductal morphogenesis yet eventually recovered to ramify the fat pad fully. Moreover, the Erbb2 null female could support her pups at parturition with no difference in the size of the offspring noted (Andrechek et al., 2005).

Similar to the Erbb2 null pubertal mammary gland phenotype (Jackson-Fisher et al., 2004, Andrechek et al., 2005), the Erbb3 null pubertal mammary gland also present with a delay in ductal morphogenesis (Jackson-Fisher et al., 2008). Gene expression
analysis on murine mammary epithelial sub-populations revealed that high levels of ERbb3 are expressed in committed luminal progenitors and mature luminal epithelial cells (Balko et al., 2012). Immunohistochemical staining of TEB structures demonstrates that ablation of ERbb3 results in an increase in the cap cell number (SMA+) and a decrease in body cell number (increased cleaved caspase 3) (Jackson-Fisher et al., 2008). In keeping with this, using an organoid system to replicate mammary gland development demonstrated that in the absence of c-ErbB3 expression the gene expression signature switched from that of a luminal signature to one of basal epithelial in origin (Balko et al., 2012)

In addition to ER alpha and EGFR acting synergistically to promote pubertal mammary gland development (Kenney et al., 1995, Luetteke et al., 1999, Sternlicht et al., 2005, Ciarloni et al., 2007), FGF signalling may also act in parallel to, or in concert with EGFR signalling. Specifically, the FGF2 ligand can induce branching in Egfr null mammary organoids grown in 3-D matrigel (Sternlicht et al., 2005). With respect to mammary gland development both FGFR1 and FGFR2 are expressed in the developing mammary bud, and are the primary FGF receptors expressed in the mouse mammary epithelium during ductal morphogenesis suggestive of a function during pre- and postnatal mammary gland development (Chodosh et al., 2000, Lu et al., 2008). Both FGFR1 and FGFR2 are expressed in the basal and luminal mammary epithelial suggesting that together they may function during the determination of the mammary epithelial hierarchy. In order to study the function of FGFR2 during adult mammary gland development, Lu and colleagues (2008) utilized the inefficiency of the MMTV-Cre transgene to generate a murine line whose mammary glands are chimeric, that is a combination of mammary gland epithelial cells that are Fgfr2 null and Fgfr2 heterozygous. These chimeric females presented with a delay in pubertal mammary gland development and had completely ramified the fad pad some 3 weeks later than the control counterpart. In addition to a delay in ductal elongation these chimeric females also presented with fewer bifurcation points, a phenomenon which was more pronounced during the earlier stages of pubertal development. The mammary gland epithelial cells of these chimeric females were able to undergo pregnancy-associated differentiation with milk secretion during lactation, despite having fewer alveolar units. These authors demonstrated that the pre-pubertal mammary gland had a substantial number of Fgfr2 null cells distributed evenly throughout the rudimentary ductal structure. However, during puberty as the mammary gland undergoes proliferation at TEB structures and consequently ductal elongation and bifurcation, the Fgfr2 null epithelial cells, being at a proliferative disadvantage compared to the Fgfr2 heterozygous epithelial cells are diluted out of the TEB structure. These authors
conclude that the delay in ductal elongation could be due to the substantial number of \textit{Fgfr2} null epithelial cells at the onset of puberty, that due to their defective proliferative capability manifest in a delay in ductal elongation. As ductal development proceeds the proliferative-defective \textit{Fgfr2} null epithelial cells are out competed by the proliferative-able \textit{Fgfr2} heterozygous epithelial cells allowing completion of pubertal ductal development (Lu et al., 2008).

A delay in pubertal development was also displayed using a conditional deletion of \textit{Fgfr1} through \textit{Krt14} mediated Cre expression. The \textit{Fgfr1} null mammary gland presented with fewer terminal end buds as well as ductal branch points and a defect in ductal elongation. The \textit{Fgfr1} null phenotype presented from the on-set of puberty at 3 weeks of age until 5 weeks, after which the mammary gland resembled that of the wild type female. Immunohistochemical analysis post BrdU incorporation demonstrated that during the delay in mammary gland development (between weeks 3 and 5), fewer TEB epithelia were positive for BrdU suggesting that FGFR1 may function to drive the initial stages of pubertal mammary gland development. Additionally, adenoviral-cre mediated deletion of both \textit{Fgfr1} and \textit{Fgfr2} from mammary epithelial cells ablated mammary gland development upon transplantation into cleared fat pads. Furthermore, subsequent FACS-sorted isolation of the CD24+CD29\textsuperscript{hi} stem cell enriched population, revealed that the \textit{Fgfr1/2} null transplanted mammary gland contained significantly fewer CD24+CD29\textsuperscript{hi} stem cell enriched epithelia (Pond et al., 2013).

1B.3 Morphogenesis and differentiation of the mammary gland at pregnancy and lactation

The morphological changes which the mammary gland has to undergo during pregnancy to proceed to lactation involves a large increase in secondary and tertiary branching leading to the development of alveoli, a process known as alveologenesis. Alveoli buds that develop from the proliferating alveolar progenitor cells cleave and differentiate into the alveoli that will form the milk secreting lobules at lactation. Two factors known to play major roles in the development of alveoli structures, and in the production of milk, are progesterone from the ovaries and prolactin secreted by the pituitary gland and - as has been recently demonstrated – prolactin is produced by the mammary gland epithelial cells themselves (Chen et al., 2012. See below). However, in addition to ovarian hormones and prolactin, a role for immune cell cytokines operating through the STAT6 signalling pathway have also been shown to be involved in regulating luminal cell fate (see below and reviews by Watson and Khaled 2008, Macias and Hinck 2012, Oliver and Watson 2013).
1B.3.1 Progesterone and prolactin in alveologenesis and terminal differentiation.

With a rise in circulating progesterone serum levels during pregnancy the mammary gland undergoes morphogenesis. Combined hormone replacement regimes have demonstrated that progesterone and 17-beta estradiol can drive the expansion of the mammary gland stem cell enriched (CD24+ CD61+ CD49f^{hi}) and the differentiated luminal (CD24+ CD61- CD49f^{lo}) populations of epithelial cells (Joshi et al., 2010). From a physiological perspective the isolation of FAC-sorted epithelial cells has demonstrated that there is an increase in the number of the mammary gland stem cell enriched population (CD24+CD29^{hi}) during pregnancy associated mammary gland development with a marked increase in the frequency of repopulating units at mid pregnancy (day 12.5) compared to late pregnancy (day 18.5) (Asselin-Labat et al., 2010) consistent with the physiological drive toward the development of the pregnant mammary gland.

The mitogenic effects of progesterone are elicited via the progesterone receptor, the deletion of which results in a failure of the mammary gland to instigate side branching and alveolar development during pregnancy (Lydon et al., 1995, Brisken et al., 1998). Progesterone, acting through the progesterone receptor functions in a paracrine manner (Brisken et al., 1998, Beleut et al., 2010). Of the two isoforms of the progesterone receptor expressed, progesterone receptor alpha (PR-A) and progesterone receptor beta (PR-B), only PR-B is required for side branching and alveologenesis (Mulac-Jericevic et al., 2003).

Both WNT4 and TNFSF11 (a.k.a RANKL) have been implicated in mediating the mitogenic effects of progesterone (Brisken et al., 2002, Asselin-Labat et al., 2010, Beleut et al., 2010, Joshi et al., 2010). Progesterone induces WNT4 expression (Brisken et al., 2002, Joshi et al., 2010) and gene ablation studies have shown that WNT4 expression is required for ductal side branching at mid-pregnancy (pd12.5) but not at late pregnancy (Brisken et al., 2000). The expression of TNFSF11 is induced by progesterone in PR positive cells and mediates its function in a paracrine fashion to neighboring cells expressing tumor necrosis factor receptor superfamily, member 11a (TNFRSF11A, a.k.a RANK) (Fernandez-Valdivia et al., 2008). Deletion of Tnfsf11 or Tnfrsf11a results in a failure in alveologenesis during pregnancy (Fata et al., 2000) reminiscent of the phenotype presented in the mammary gland of the Pr-b knock out model (Mulac-Jericevic et al., 2003).
Until recently the pituitary gland was considered the primary source of prolactin required for alveologenesis (Vomachka et al 2000). However, a recent study (Chen et al., 2012) has demonstrated that the phosphoinositide-3-kinase - protein kinase B/AKT (PI3K-PKB/AKT) signalling pathway mediates autocrine prolactin production by the mammary epithelia, downstream STAT5 activation and mammary gland secretory differentiation. The importance of this pathway was demonstrated by conditional activation of the PI3K-AKT signalling pathway by doxycyclin-inducible expression of activated Akt1 in mammary epithelia and by conditional ablation of the inhibitor of this pathway, phosphatase and tensin homologue (Pten). These authors demonstrated that epithelial cells in the adult virgin mammary gland could be induced to differentiate, producing milk proteins, when the AKT signalling pathway was activated, in spite the absence of alveoli development that is normally seen during pregnancy. AKT1 induced mammary gland differentiation was abrogated in mice that harbour prolactin (Prl) null, prolactin receptor (Prlr) null or Stat5 null genetic backgrounds (Chen et al., 2012), demonstrating the requirement for the PRLR/JAK2/STAT5 signalling pathway (JAK, Janus kinase 2).

The PI3K-AKT signalling pathway is essential for the synthesis of milk components such as lipids and lactose (Anderson et al., 2007). Significantly, following Akt1 induction the Stat5 or Prlr null epithelia failed to accumulate cytoplasmic lipid droplets or induce lactose synthesis, demonstrating that the major components of milk require a functional PRLR/JAK2/STAT5 signalling pathway (Chen et al., 2012).

1B.3.2 The function of the Ets transcription factor ELF5 during cell fate determination in the pregnant mammary gland.

Genetic ablation studies have demonstrated that mediators of progesterone and prolactin signalling are required for alveologenesis (Lydon et al., 1995, Lui et al., 1997, Ormandy et al., 1997, Brisken et al., 1998, Teglund et al., 1998, Mulac-Jericevic et al., 2003, Cui et al., 2004, Wagner et al., 2004, Lee et al., 2013). Expression of the transcription factor ELF5 is dispensable for pubertal mammary gland development but required for alveoli development, implicating ELF5 as a mediator of pregnancy hormone function (Oakes et al., 2008, Choi et al., 2009). Progesterone induces ELF5 expression in vivo and these two factors act synergistically to promote alveoli development (Lee et al., 2013). Not only does ELF5 transcriptionally activate Stat5 expression but also indirectly regulates the phosphorylation of STAT5 (Choi et al., 2009). Furthermore, STAT5 activates Elf5 transcription (Yamaji et al., 2013) suggesting a positive regulatory feedback loop. Although autocrine prolactin is induced by the PI3K-AKT signalling pathway and mediates alveoli development and secretory
activation via the PRLR/JAK2/STAT5 signalling pathway (Chen et al., 2012), AKT1 can induce Elf5 expression in the mammary gland of nulliparous Stat5 null and Prlr null mice (Chen et al., 2013). This indicates that AKT1 is an upstream transcriptional regulator of Elf5 in a manner that is independent of PRLR/JAK2/STAT5 signalling and that ELF5 expression alone is insufficient to substitute for the differentiation effects of prolactin receptor signalling. However, ELF5 is necessary for mammary gland development since conditional deletion of Elf5 during pregnancy results in a block in alveologenesis and lactational failure (Choi et al., 2009) and over expression of Elf5 in the nulliparous mammary gland results in precocious alveolar development and secretory differentiation (Oakes et al., 2008). This data suggests a function for ELF5 in mammary gland epithelial cell hierarchy determination. Consistent with this, an increase in the FAC-sorted CD61+ subpopulation of luminal progenitor cells has been reported to be present in the pregnant mammary gland of Elf5 null mice, suggestive of a block in differentiation (Oakes et al., 2008, Chakrabarti et al., 2012).

1B.4 Post weaning and Involution of the mammary gland

Following the lack of demand for milk, which can be induced by the removal of the pups, the process of involution is initiated in order to remodel the mammary gland back to its pre-pregnant architecture. This process takes place in two phases, the first is reversible, and no obvious morphological change occurs, but alveolar cells detach and are shed into the lumen. The cell death that occurs during the first phase of involution has been attributed to both intrinsic mitochondrial and extrinsic - death receptor - apoptotic pathways (Marti et al., 2001, Baxter et al., 2006). However, more recent data show that lysosomal membrane permeabilisation (LMP) is responsible for the earliest cell death (Kreuzaler et al., 2011). After 48 hours this phase is followed by a second phase, which is irreversible, when the alveoli begin to collapse, and activation of proteases induce a second wave of cell death that is accompanied by an influx of neutrophils and macrophages leading to the remodelled mammary gland.

The first phase of involution is orchestrated by local cues. This was shown using models where milk release was disrupted in the presence of systemic lactogenic hormones by sealing of the teats, transplanting mammary glands unable to release milk due to no teat connection, or by inactivation of the oxytocin gene (Li et al., 1997). Two groups have performed a detailed transcript analysis during the two phases of involution, and have documented the genes that are expressed (Clarkson et al., 2003, Stein et al., 2003). Along with the signal transducer and activator of transcription 3 (Stat3), which was know to be a crucial factor during involution (Chapman et al., 1999,
Humphreys et al., 2002), genes encoding for death receptors, immune mediators and factors involved in the acute phase response were found to be elevated. The specific immune response resembles a wound healing process involving macrophage infiltration of the mammary gland concurrent with an increase in the expression of several acute phase response genes. The importance of STAT3 in the regulation of several acute phase response genes has recently been reported by specifically documenting the effects of deletion of STAT3 expression in the mammary gland epithelia during involution (Hughes et al., 2012).

1B.5 The JAK/STAT signalling pathways during mammary gland development

The JAK/STAT signalling pathways, which transduce external stimuli to regulate gene transcription, involve two JAK proteins (Janus kinase, JAK1 and JAK2), and seven signal transducers and activators of transcription (STAT - STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6). The basic signalling mechanism is illustrated diagrammatically in Figure 1.11. A signal is generated by interaction of a ligand with its surface receptor triggering the activation of an associated JAK protein which phosphorylates tyrosine residues on the intracellular domain of the receptor to which the specific STAT then binds and is itself phosphorylated. The phosphorylated STAT protein dimerises and moves to the nucleus where it can associate with specific promoters to activate transcription. The JAK/STAT signalling pathway has proved to be crucial to the determination of cell fate and in directing the changes in gene expression and morphology occurring in the development of the mammary gland. Evaluating the role of the different STAT proteins has largely been achieved by gene deletion or overexpression studies using transgenic murine lines, in many cases using mammary gland specific promoters to achieve genetic change at specific stages of mammary gland development. Different STAT proteins are expressed in association with JAK1/2, and are activated at different stages of mammary gland development, as is illustrated in Figure 1.12 for the expression of activated STAT proteins 1,3,5 and 6 (For review see Hughes and Watson 2012).
Figure 1.11 The JAK/STAT signalling pathway in the mammary gland. (A and B) Binding of cytokines (red triangle) to their receptor (green rectangle) results in receptor dimerization and activation of JAK (yellow circle). (C and D) The downstream consequence of JAK activation is the phosphorylation and activation of the STAT protein, STAT translocation to the nucleus, DNA binding and gene regulation.

Figure 1.12 Schematic representation of mammary gland development in the virgin, at pregnancy, lactation and involution, showing the expression of the different phosphorylated STAT proteins (Amended from Hughes and Watson 2012).
With the exception of STAT2, all other STAT family members are expressed during mammary gland development (Philp et al., 1996, Khaled et al., 2007). Furthermore, whilst the Stat4 transcript could be detected in the nulliparous mammary gland, Stat4 expression was not detected in a mammary gland cell line suggesting that Stat4 expression could be limited to non-epithelial cells (Philp et al., 1996). Phosphorylation of STAT proteins is the readout for functionality and activated STAT1 is detected in the nulliparous mammary gland and during late involution (Philp et al., 1996). However, studies using MMTV-Cre and Wap-Cre transgenic murine lines to delete Stat1 expression from mammary gland epithelia demonstrated that STAT1 expression is dispensable for development of the nulliparous mammary gland and at lactation (Klover et al., 2010). Whilst activated STAT5 and activated STAT6 proteins are detected in the nulliparous mammary gland (Liu et al., 1996, Khaled et al., 2007) no apparent defect in development was observed in the absence of STAT6 (Khaled et al., 2007). However STAT5 expression is required for the generation of the alveolar progenitor pool required during pregnancy (Yamaji et al., 2009), and is required to mediate secondary side branching during oestrus (Santos et al., 2010). The function for STAT5 and STAT6 during pregnancy associated mammary gland development and the function for STAT3 during involution of the mammary gland will be discussed in more detail below.

1B.5.1 STAT6 in luminal cell development

In the immune system, it has been demonstrated that the differentiation of CD4+ T helper (Th) cells to the T helper 1 (Th1) lineage is induced by interleukin 12 (IL12) signalling through STAT4 (Jacobson et al., 1995, Thierfelder et al., 1996), whereas differentiation toward the T helper 2 (Th2) lineage is mediated through IL4/IL13 activation of STAT6 (Kaplan et al., 1996). Phosphorylated STAT6 is also expressed in the mammary gland being limited to a minority of virgin epithelia, induced at day 5 of gestation where expression is then seen in the majority of ductal/luminal epithelia and remains up regulated at day 15 of pregnancy (Khaled et al., 2007). The importance of the IL4/IL13/STAT6 signalling pathway during pregnancy associated mammary gland development was demonstrated by the observation that in a Stat6 null murine line, or in mice with the genes for both IL4 and IL13 deleted, mammary gland morphogenesis was delayed (Khaled et al., 2007). Examination of the Stat6 null female mammary gland revealed a striking reduction in the number of side-branches and alveolar structures (70%) at pregnancy day 5. This is a potential consequence of reduced proliferation as seen in the reduced number of Ki67 positive alveolar cells and the reduction in cyclin B1/B2 and AKT1 expression levels. As suggested by the ability of
null female to nurse her pups at parturition, pregnancy associated mammary gland development is not arrested at pregnancy day 5. In fact, by day 15 of pregnancy, although the Stat6 null mammary gland presents with fewer alveolar structures, alveolar progenitor cells are proliferating at a significantly greater rate, than the alveolar cells in the wild type counterpart (Khaled et al., 2007).

The delay in Stat6 null mammary gland development during pregnancy, and the ability of the Stat6 null female to nurse her offspring is reflected in the delayed induction of beta-casein expression (Kahled et al., 2007). Interestingly, Khaled and colleagues (2007) demonstrated that whilst IL4 and IL13 transcript levels were reduced at day 5 of pregnancy, expression levels recovered by day 15 of pregnancy and in fact the expression of IL4 was increased above that expressed in the wild type counterpart commensurate with the recovery of the Stat6 null mammary gland. Significantly, in the Stat6 null murine background, IL12 alpha transcript levels were detected at increased levels at day 5 of pregnancy and were still elevated by day 15 of pregnancy. Using opposing systems, these authors (Khaled et al., 2007) proceeded to demonstrate a potential role for IL4/IL13 signalling through STAT6 activation by examining pregnancy associated mammary gland morphology in a double IL4/IL13 null genetic background as well as in Socs5 null genetic background that results in activated IL4/IL13 signalling (Seki et al., 2002).

The IL4/IL13 null double mutant strain also presented with a delay in alveologenesis similar to the mammary gland phenotype presented in the Stat6 null genetic background, whilst the deletion of Socs5 resulted in precocious alveolar development (Kahled et al., 2007). The KIM-2 cell line can be induced to undergo differentiation upon stimulation with lactogenic hormones (prolactin, dexamethasone and insulin) a process that mimics the differentiation of alveolar cells during pregnancy (Gordon et al., 2000). Elegantly, the cytokine expression profile of KIM-2 cells switches from a type-1 to a type-2 cytokine expression profile upon induction of differentiation (Khaled et al., 2007). The type-1 cytokines IL12 alpha, interferon (IFN) gamma and tissue necrosis factor (TNF) alpha are expressed by undifferentiated KIM-2 cells at day 2 of differentiation. When the milk protein beta-casein is first expressed, the expression of the type-1 cytokines is markedly down-regulated and the expression of the type-2 cytokines IL4, IL5 and IL13 is induced (Khaled et al., 2007). By analogy with the T-helper lymphocyte differentiation system in which Th2 cells secrete IL4 and IL13 that promotes the differentiation of naïve T-helper cells toward the Th2 lineage, Khaled and colleagues (2007) proposed that in the mammary gland, the differentiated luminal population secrete IL4 and IL13 that acts upon the luminal progenitor population to
promote proliferation and differentiation. These data suggest that the elevated expression of \textit{IL12 alpha} expressed in the \textit{Stat6} null genetic background could indicate an imbalance in epithelial lineage determination. The involvement of \textit{STAT6} in the regulation of luminal progenitor cell proliferation and commitment to an alveolar lineage demonstrates how cell fate determination during early pregnancy is linked to IL4/IL13 mediated activation of \textit{STAT6} (Khaled et al., 2007).

1B.5.2 The role of \textit{STAT5} in alveologenesis and expression of milk proteins.

\textit{STAT5} has a well defined role in the regulation of stem and progenitor cells in the hematopoietic system (Yao et al., 2006, Wang et al., 2009b). In the mammary gland, binding of prolactin to the prolactin receptor results in receptor dimerization and activation of JAK2 (Darnell et al., 1994, Schindler and Darnell 1995). The downstream consequence of JAK2 activation is the phosphorylation and activation of \textit{STAT5} (Ihle and Kerr 1995, Schindler and Darnell 1995). Two \textit{STAT5} isoforms exist (\textit{STAT5A} and \textit{STAT5B}, collectively known as \textit{STAT5}), sharing 96% homology (Liu et al., 1995). Activated \textit{STAT5A} is expressed in the nulliparous mammary gland at 5 weeks of age (Santos et al., 2008) and whilst \textit{STAT5A} expression is not required for ductal elongation (Yamaji et al., 2009), side branching is impaired in the \textit{Stat5a} null genetic background (Santos et al., 2010).

Early studies demonstrated the importance of \textit{STAT5} in the regulation of the milk protein genes such as beta-lactoglobulin (BLG) (Watson et al., 1991, Burdon et al., 1994), whey acidic protein (Li and Rosen, 1995) and beta casein (Happ and Groner 1993) an observation later confirmed by genome wide interrogation of \textit{STAT5} binding and gene expression analysis in the murine mammary gland of the pregnant female (Yamaji et al., 2013).

During the oestrus cycle the murine mammary gland can develop alveoli structures (Fata et al., 2001), which consist of epithelial cells that bare the gene signature of an alveolar progenitor population (Shehata et al., 2012). Significantly, \textit{STAT5A} expression in the nulliparous mammary gland is required for the generation and proliferation of alveolar progenitor cells the precursor cells required during pregnancy (Yamaji et al., 2009). Expression levels of phosphorylated activated \textit{STAT5} increase in the mammary gland during pregnancy (Liu et al., 1996) suggesting that the level of \textit{STAT5} can control distinct biological programs at different stages of mammary gland development (Cui et al., 2004, Yamaji et al., 2013). In fact studies have shown that \textit{STAT5} expression is required for luminal progenitor cell proliferation and differentiation.
toward alveolar buds and the survival of mammary gland epithelia (Liu et al., 1997, Liu et al., 1998, Cui et al., 2004, Yamaji et al., 2009 and 2013).

The contribution of the two isoforms of STAT5 (STAT5A and STAT5B) to the development of the pregnant mammary gland most likely reflects expression levels as STAT5A is more abundant than STAT5B and constitutes approximately 70% of STAT5 expression levels (Liu et al., 1995). Thus whilst STAT5A expression is required for lobuloalveolar development and terminal differentiation (Liu et al., 1997), a phenotype that can be rescued upon STAT5A re-expression (Yamaji et al., 2009), an increase in the expression of STAT5B in the Stat5a null genetic background can partially compensate for the absence of STAT5A after several rounds of pregnancy (Liu et al., 1998).

Interestingly, Yamaji and colleagues (2013) have proposed a two-stage model of STAT5 dose-dependent mammary gland development whereby expression from either a single Stat5a or Stat5b allele is sufficient for the generation of histologically undifferentiated alveolar units, expression from two alleles is required for the initiation of mammary gland differentiation and the expression of the lactation gene signature, similar to that found with all four alleles (Yamaji et al., 2013).

1B.5.3 STAT3 function during involution

During the later stages of pregnancy pSTAT5 expression is required for the survival of the mammary gland epithelia (Cui et al., 2004) by positively regulating the expression of pro-survival genes such as AKT (Creamer et al., 2010). After the young are weaned, the differentiated mammary gland epithelial cells are redundant and activated STAT5 levels are reduced as STAT3 is activated (Liu et al., 1996, Philp et al., 1996). This switch between survival and the involution of the mammary is exemplified by the demonstration that inducing the over expression of STAT5 (Creamer et al., 2010) or the expression of a constitutive active form of AKT1 (Schwertfeger et al., 2001) delays involution of the mammary gland. In keeping with this, mammary gland specific conditional deletion of Stat3 results in a delay of the first phase of involution and is therefore essential for the initiation of cell death and remodeling of the mammary gland (Chapman et al., 1999, Humphreys et al., 2002).

Accumulation of milk in the mammary gland lumen post weaning results in the production of leukemia inhibitory factor (LIF) and activation of STAT3 via the glycoprotein 130/JAK complex (glycoprotein 130, gp130) (Kritikou et al., 2003, Schere-Levy et al., 2003, Zhao et al., 2004). Thus, activated STAT3 is absent in LIF null
(Kritikou et al., 2003) and gp130 null (Zhao et al., 2004) genetic backgrounds and involution of the mammary gland is delayed (Kritikou et al., 2003, Zhao et al., 2004). Implantation of LIF containing pellets in lactating mammary glands induced STAT3 phosphorylation resulting in an increase in apoptosis (Schere-Levy et al., 2003). Significantly, the use of Stat3 null and LIF null murine lines suggested that the regulatory subunits of phosphoinositide-3-OH kinase (PI3K), p55-alpha and p50-alpha are positively regulated downstream of the LIF/JAK/STAT3 signaling pathway, an observation further substantiated by the demonstration that STAT3 can bind the promoter of p55-alpha and p50-alpha in vivo. Significantly, in the Stat3 null background up-regulation of p55-alpha and p50-alpha is abrogated, activated levels of AKT are sustained and apoptosis is prevented (Abell et al., 2005). Whilst molecular profiling (Clarkson et al., 2004) and studies in Stat3 null genetic background (Chapman et al., 1999) suggested that cell death during the initial, reversible phase of involution was mediated by the classical apoptotic pathway, involution occurs in the absence of executioner caspase 3 and 6 (Kreuzaler et al., 2011). In fact classic signatures of apoptosis such as membrane blebbing and chromatin fragmentation are not observed during the first phase of involution (Kreuzaler et al., 2011). These studies demonstrated that during the first stage of mammary gland involution, epithelia undergo lysosomal membrane permeabilization (LMP) concomitant with an increase in lysosomal protease cathepsin B and L expression. In the absence of STAT3, cathepsin B and L expression is diminished and the expression of the endogenous inhibitor Serpin2A (Spi2A) is up-regulated. Importantly, chemical inhibition (CA-074Me) of cathepsin B delayed involution in wild type mice, and resulted in a reduction in cell death when applied to the murine epithelial cell line EpH4. Furthermore, using the same mammary epithelial cell line EpH4, Kreuzaler and colleagues (2011) further substantiated STAT3 in mediating LMP by demonstrating that chemical inhibition (S3I-201) of STAT3 activation decreased cathepsins B and L protein expression levels and significantly reduced cell death.

During involution there is a marked up-regulation of genes involved in the acute phase response and inflammation (Clarkson et al., 2004, Stein et al., 2004, Hughes et al., 2012). STAT3 expression mediates cell death (Kreuzaler et al., 2011) and regression, post-weaning (Chapman et al., 1999). Hughes and colleagues (2012) therefore proposed a potential role for STAT3 in influencing the innate immune environment and the influx of inflammatory cells into the mammary gland. Using the BLG-Cre system to produce a mammary gland specific Stat3 null murine line, the acute phase response genes were analysed 24 and 48 hours after forced weaning. The ablation of Stat3 resulted in the down-regulation of several acute phase response genes, as compared
to the wild type counterpart. Expression of CD14 on the apical surface of the mammary luminal epithelia was also reduced at 24 hours but not later, suggesting that the proposed acquisition of a phagocytic phenotype by mammary gland epithelia (Stein et al., 2003) is part of the involution program dependent upon STAT3 expression. In the second phase of involution – after 48 hours, the expression of chitinase 3-like 1 (Chi3Li) a factor associated with inflammatory conditions and wound healing (Lee et al., 2011), was decreased in the Stat3 null murine line. In this second phase of involution STAT3 transcriptional activation of Chi3Li expression is induced by oncostatin M (OSM) operating through the OSM receptor, indicating a causal link between STAT3 expression, the regulation of Chi3Li and therefore wound healing response. The increase in the number of macrophages and mast cells normally seen in the second phase of involution is also reduced in the Stat3 null mammary gland, a phenomenon that may be indirectly related to STAT3 and thus reflect a delay in involution and therefore a reduction in the number of dead epithelia. Interestingly during involution macrophages are polarized towards an alternatively activated (M2) phenotype (O’Brien et al., 2010), yet in the absence of STAT3 the macrophage phenotype is also altered, favouring the M1 phenotype, evidenced by an increase expression of inducible nitric oxide synthetase (iNOS) (Hughes et al., 2012). This is a significant observation given that macrophage M1 activity results in tissue damage whereas macrophage M2 activity promotes tissue repair (Mills, 2012).
Thesis aims
1C Aims:

Chapter 3

In order to study the function of KDM5B in vivo, the Kdm5b locus was targeted. The aims were to:

(1) Assess the effect of ablating KDM5B in vivo (B6;129-Kdm5b-Exon 1 knockout)
(2) To develop and characterise the B6;129-Kdm5b ΔARID murine line

Chapter 4

In Chapter 3 preliminary studies on the chimeric B6;129-Kdm5b ΔARID murine line have indicated that KDM5B is required for normal mammary gland ductal morphogenesis. However the influence of genetic background on mammary gland morphogenesis has complicated the interpretation of the presenting phenotype in the chimeric B6;129-Kdm5b ΔARID female. In addition, studies on WT KDM5B expression in the mammary gland are limited. Therefore, understanding the role of KDM5B during mammary gland development could be advanced by the following aims:

(1) To examine the pattern and level of KDM5B expression during mammary gland morphogenesis in the C57BL/6J female.
(2) To backcross the chimeric B6;129-Kdm5b ΔARID murine line onto the inbred C57BL/6J genetic background.
(3) To characterise the mammary gland phenotype presented in the C57BL/6J-Kdm5b ΔARID female

Chapter 5

Using the C57BL/6J-Kdm5b ΔARID murine line we have investigated the effect of KDM5B ΔARID expression on gene expression by:

(1) Examining the effect of KDM5B ΔARID expression on ER alpha target gene regulation during mammary gland development.
(2) Investigating the ability of KDM5B and KDM5B ΔARID to interact with ER alpha.
(3) Examining in the mammary gland, the effect of KDM5B ΔARID expression on the repression of demethylase-dependent KDM5B target genes.
(4) Examining in the mammary gland, the effect of KDM5B ΔARID expression on the AKT and STAT5 signaling pathways.
Chapter 6

Using the C57BL/6J-Kdm5b ΔARID murine line we have investigated the effect of KDMB ΔARID expression on mammary gland tumour development.
Chapter 2: Materials and methods
Chapter 2 Materials and methods

All chemicals and solvents were supplied by Merck Ltd (unless otherwise stated)

2.1 Solutions and buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carmine solution</td>
<td>0.5% carmine dye w/v, 0.2% aluminum potassium sulfate</td>
</tr>
<tr>
<td>Carnoy's fixative</td>
<td>75% ethanol, 25% glacial acetic acid</td>
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<tr>
<td>Lysis buffer</td>
<td>50mM Tris.HCl (pH 8.0), 150mM NaCl, 10% glycerol, 0.5% Triton X-100</td>
</tr>
<tr>
<td>TE</td>
<td>10mM Tris.HCl (pH 8.0), 1mM EDTA</td>
</tr>
<tr>
<td>Tissue lysis buffer</td>
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<td>1% HCL, 70% IMS</td>
</tr>
<tr>
<td>6x DNA loading buffer</td>
<td>30% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF</td>
</tr>
<tr>
<td>1x TAE</td>
<td>40mM Tris, 20mM acetic acid, 1mM EDTA</td>
</tr>
<tr>
<td>20x SSC</td>
<td>3M sodium chloride, 0.3M sodium citrate</td>
</tr>
<tr>
<td>2x Laemmli sample buffer</td>
<td>120mM Tris-HCL (pH 6.8), 4% SDS, 20% (w/v) glycerol, 0.02% (w/v) bromophenol blue</td>
</tr>
</tbody>
</table>
1x SDS-PAGE running buffer 25mM Tris (pH 8.3), 192mM glycine, 0.1% SDS

1x Towbin buffer (PAGE transfer buffer) 25mM Tris (pH 8.3), 192mM glycine, 20% methanol

### 2.2 General statement

Targeting construct used to generate the B6;129-Kdm5b-Exon 1 knock out and the C57BL/6J-Kdm5b ΔARID murine lines was designed and constructed by Dr Samantha Santangelo, KCL.

Transfection of targeting vector into ES cells and subsequent injection of positive clones into the blastocysts or 8-cell embryos and pseudopregnant female was carried out by the Cancer Research UK Transgenic Mouse Service Laboratory, headed by Dr Ian Rosewell.

Southern blot of transfected embryonic stem (ES) cells and screening of the B6;129-Kdm5b-Exon 1 knock out line was done with the help of Debbie Hall, KCL.

I would like to thank Dr. Bradley Spencer-Dene at the London Research Institute, Cancer Research UK, for his patience in teaching me how to dissect inguinal mammary glands (4th from the neck) and the thoracic mammary glands (3rd from the neck) from the B6;129-Kdm5b WT and B6;129-Kdm5b ΔARID females (as well as on the C57BL/6J genetic background).

Staged pregnancies were arranged with Janet Macdonald at the Biological Service Unit, Cancer Research UK.

Pathology on the B6;129-Kdm5b ΔARID murine line was performed by Professor Gordon Stamp, Cancer Research UK. *In situ* hybridization on the C57BL/6J-Kdm5b WT mammary gland series was performed by Dr Richard Poulson at Cancer Research Services.

### 2.3 Cell culture

COS-7 (CV-1 in origin, carrying SV40), an African green monkey kidney fibroblast-like cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life
technologies), supplemented with 10% FCS (Fisher scientific), 2mM L-glutamine and 100U/ml Penicillin-Streptomycin (Life technologies). COS-7 cells were maintained in a humidified atmosphere of 5% CO₂ and 37°C. A sub-cultivation ratio of 1:5 was used to maintain the cell line.

2.4 Preparation and analysis of DNA

2.4.1 Phenol:chloroform extraction and precipitation of tail-snip genomic DNA

5mm of tail-snip tissue was incubated overnight at 55°C in 180 µl of tissue lysis buffer (Qiagen) supplemented with 2mg/ml of proteinase K (Qiagen). The DNA was extracted from the aqueous phase following addition of an equal volume of 25:24:1 phenol:chloroform:isoamylalcohol (Sigma-Aldrich), emulsification and centrifugation at 16,000g for 5 minutes at room temperature. Extraction and centrifugation were repeated with 25:24:1 phenol:chloroform:isoamylalcohol and water saturated chloroform. The DNA was precipitated from the aqueous phase at room temperature for 1 minute with an equal volume of isopropyl alcohol and 1/10th volume of 3M sodium acetate (pH4.8, Sigma-Aldrich). The DNA was pelleted by centrifugation at 16,000g for 1 minute at room temperature. The DNA pellet was washed twice with 70% ethanol, air-dried for 5 minutes at room temperature and re-suspended in 40µl TE (pH8.0)

2.4.2 Determining the concentration of DNA

The concentration of dsDNA was determined using a spectrophotometer where the absorbance reading of 1 unit at 260nm is equivalent to 50µg/ml of dsDNA

2.4.3 Restriction endonuclease digest of (A) B6;129-Kdm5b-Exon 1 knock out and (B) B6;129-Kdm5b ΔARID genomic DNA for Southern blot analysis

(A) B6;129-Kdm5b-Exon 1 knock out

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x buffer 4 (NEB)</td>
<td>1x</td>
</tr>
<tr>
<td>10,000U/ml BciVI (NEB)</td>
<td>200U</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>10µg</td>
</tr>
<tr>
<td>Water</td>
<td>upto100µl</td>
</tr>
</tbody>
</table>
(B) **B6;129-Kdm5b ΔARID**

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x BamH1 buffer (NEB)</td>
<td>1x</td>
</tr>
<tr>
<td>20,000U/ml BamH1(NEB)</td>
<td>200U</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>10µg</td>
</tr>
<tr>
<td>10mg/ml BSA (100x)</td>
<td>10µg (1x)</td>
</tr>
<tr>
<td>Water</td>
<td>upto 100µl</td>
</tr>
</tbody>
</table>

Genomic DNA was incubated over night at 37°C and the DNA precipitated at -20 °C in 2.5 volumes of ethanol (v/v) and 1/10th volume of 3M sodium acetate (pH4.8) for 60 minutes. The DNA was pelleted by centrifugation at 14,000g for 5 minutes, washed twice in 70% ethanol, air dried at room temperature and re-suspended in 20µl of TE (pH8.0)

### 2.4.4 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was performed using a 2% (w/v) agarose gel for quantitative PCR, a 1.0% (w/v) or a 1.5% (w/v) agarose gel for end-point PCR and 1% (w/v) agarose for Southern transfer containing 0.5 µg/ml ethidium bromide in 1x TAE buffer.

### 2.4.5 Genotype screening by Southern transfer

Digested genomic DNA separated by agarose gel electrophoresis (1% w/v) over night at 1V/cm was partially depurinated in 10 volumes of 0.25M HCL for 10 minutes followed by incubation in 10 volumes of 0.4M NaOH for 20 minutes at room temperature. DNA was covalently linked to positively charged nylon membrane (GE Healthcare Life Sciences) by downward alkaline capillary transfer for 60 minutes. After transfer the nylon membrane was neutralised by a briefly wash in 2x SSC.

To screen the B6;129-Kdm5b-Exon 1 knock out murine line for integration of the targeting construct a 999bp fragment was amplified with *Kdm5b* specific primers (sense: 5’-GTATGCCCCTCCTAAGGG-3’ and anti-sense: 5’-GCGCGTTAGACACTATCAG-3’) using 100µg of the targeting construct plasmid DNA, and the following PCR cycling conditions: 94°C for 5 minutes, then 30 cycles at, 94°C for 1 minutes, 60°C for 1 minutes, 72°C for 1 minutes.
To screen the B6;129-Kdm5b ΔARID murine line for integration of the targeting construct a 1.2Kb fragment was amplified with Kdm5b specific primers (sense: 5'-TCCAGCGTAAAGTCTGTCTCC-3' and anti-sense 5'-CTTTTACTAGCAACGGCAACACG-3') using 100ng of the targeting construct plasmid DNA, and the following PCR cycling conditions: 94°C for 5 minutes, then 30 cycles at, 94°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute.

PCR products were resolved on a 1% agarose gel, excised and purified using Wizard SV gel and PCR clean up systems (Promega) according to manufacture’s instructions. Incorporation of [α-32P]-dCTP (PerkinElmer) was achieved by internal random primer extension using 50μg of template DNA and unincorporated [α-32P]-dCTP removed using Sephadex S-300 spin columns according to manufacture’s instructions (GE Healthcare Life Sciences).

Prior to the addition of the probe, the nylon membrane was blocked for 3hrs at 42°C in Ultrahyb (Ambion). Hybridisation was overnight in Ultrahyb at 42°C followed by increasingly stringent washes as follows. The membranes were washed twice using an excess volume of 2xSSC/0.1%SDS at 42°C for 10 minutes. Non-specific binding of the probe to the nylon membrane was checked using a Geiger counter with a beta probe. The nylon membrane was then washed twice using an excess volume of 0.1xSSC/0.1%SDS at 65°C for 10 minutes or until the non-specific signal was reduced to background levels. The nylon membrane was then exposed to autoradiography film (GE Healthcare) and the cassette stored overnight at -80°C. The cassette was warmed on the bench to room temperature and signal developed using a X-ray film developer.

2.4.6 End point PCR on C57BL/6J mammary gland cDNA and sequencing

PCR primers specific to Kdm5b exon 1 and exon 5 (sense: 5’-CGCTTTTATCCACAAGATCC-3’ and anti-sense 5’-CAGACTGCTCTGGGGAATA-3’ respectively), were used to amplify cDNA generated from RNA isolated from mammary gland tissue or testis, using the following PCR cycling conditions: 94°C for 5 minutes, then 30 cycles at, 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute. PCR products were resolved on a 1.5% (w/v) agarose gel, excised and purified using Wizard SV gel and PCR clean up systems (Promega) according to manufacture’s instructions. DNA sequencing reactions were performed using BigDye terminator v3.1
Cycle Sequencing kit and the 3730 DNA Analyzer (Life Technologies) at Cancer Research UK.

2.4.7 B6;129-Kdm5b-Exon 1 knock out PCR screen to identify homologous recombination

PCR amplification reactions were performed using the following PCR cycling conditions: 94°C for 5 minutes, then 30 cycles at, 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute.

To amplify the WT allele the following primer pairs were used:
Sense 5’-TGGATTGTAACTCTGTCTCCCTAC-3’
Anti-sense 5’-TTCTACTAGCAACGGCAACACCTAG-3’

To amplify the integrated targeting vector the following primer pairs were used:
Sense 5’-CATCTGTGACGCCCTTAGTACGTA-3’
Anti-sense 5’-GCTACCGGTGGATGTGGAATGTGTG-3’

2.4.8 PCR screen used to detect heterozygous B6;129-Kdm5b ΔARID offspring during backcross to the C57BL/6J genetic background

PCR amplification reactions were performed using neomycin specific PCR primers:
Sense 5’-TGCTCCTGCGAGAAAGTATC-3’
Antisense 5’-CCACCCCCCAGAATAGAATGAC-3 and the following PCR cycling conditions: 94°C for 5 minutes, then 30 cycles at, 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute.

2.4.9 PCR screen used to detect the polyoma virus middle T antigen transgene (PyV-mT)

PCR amplification reactions were performed using PyV-mT specific PCR primers:
Sense 5’-CCAGAACCCAGGCCGTCTCCAGAAGC-3’
Anti-sense 5’-GGATGAGCTGGGGTACTTGTTCCC-3’ and the following PCR cycling conditions: 94°C for 5 minutes, then 30 cycles at, 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute.

2.4.10 Generation of chemically competent Escherichia coli (E.Coli)

The E.Coli stain DH5 alpha were inoculated in 5ml of LB broth and grown overnight at 37°C with shaking. 100ml of LB broth was inoculated with 1ml of overnight culture,
incubated at 37°C and grow until mid-log phase (OD@550nm = 0.2-0.5). E.Coli where chilled on ice, pelleted by centrifugation at 4000g for 5 minutes at 4°C and re-suspended in ice-cold sterile 60mM calcium chloride. E.Coli were Incubated on ice for 30 minutes, pelleted by centrifugation at 4000g for 5 minutes at 4°C, re-suspended in 5ml of ice-cold sterile 60mM calcium chloride and stored in 100μl aliquots at -80°C.

2.4.11 Transformation of competent E. Coli

A 100μl aliquot of competent DH5 alpha bacterial cells were thawed on ice spiked with 100μg of plasmid DNA, mixed gently and then incubated on ice for 30 minutes followed by heat-shock at 42°C for 30 seconds. The transformed DH5 alpha bacterial cells were incubated in 1ml of LB broth at 37°C for 60 minutes, centrifuged at 500g for 5 minutes and re-suspended in 20μl of LB broth. Re-suspended DH5 alpha bacterial cells were spread on LB-agar plates under selection with 100μg/ml ampicillin (Life technologies). Following incubation of LB-agar plates overnight at 37°C, an ampicillin resistant colony was picked and used to inoculate 5mls of LB broth containing 100μg/ml ampicillin. Inoculated LB broth was incubated overnight at 37°C with agitation in a shaking incubator (300rpm).

2.4.12 Large-scale preparation of plasmid DNA

Large-scale preparation and purification of plasmid DNA was achieved using Qiagen maxi-prep kit as detailed in manufacture’s instructions. Briefly, 1ml of the overnight culture (detailed in 2.4.11) was used to inoculate 500ml of LB broth containing 100μg/ml ampicillin and incubated overnight at 37°C with agitation in a shaking incubator (300rpm). The bacterial culture was centrifuged at 6000g for 15 minutes at 4°C and the bacterial pellet lysed under alkaline conditions. The solution was neutralized and cleared by centrifugation at 20,000g for 30 minutes at 4°C. The plasmid DNA was bound to the column resin under low-salt and low-pH conditions. RNA and proteins were removed by a medium salt wash, followed by elution of plasmid DNA in a high-salt buffer and subsequent isopropanol precipitation. Contaminating salts were removed by a 70% ethanol wash, the pellet air-dried for 10 minutes at room temperature and the DNA re-suspended in 500μl TE (pH8.0).
2.3.13 *Plasmid constructs*

The human expression constructs, pcDNA3.1 Myc-HisA KDM5B were generated in house (Lu, et al., 1999). pcDNA3.1 Myc-HisA KDM5B ΔARID was a kind gift from Dr C.D.Chen (Xiang, et al., 2007) and pXJ41neo-Ha-ER alpha was a kind gift from Dr W. Hong (Chan et al., 2001).

2.5 Preparation and analysis of RNA

2.5.1 Extraction of total RNA

Inguinal mammary glands including the lymph node (4th from the neck) and the adult testis were collected, snap frozen in liquid nitrogen and total RNA isolated using the RNeasy Lipid tissue kit (Qiagen) according to manufacture's instructions. The extraction procedure couples the guanidine thiocyanate with phenol-chloroform total RNA extraction method (Chomczynski and Sacchi, 1987) with silica gel membrane purification. In brief, tissue was homogenised in QIAzol lysis reagent (guanidine thiocyanate, phenol mix) and mixed vigorously after the addition of chloroform. The RNA containing aqueous phase was separated from the organic phase by centrifugation at 12,000g for 15 minutes at 4°C. The RNA containing upper aqueous phase was removed and the silica-gel binding conditions optimized by the addition of an equal volume of 70% ethanol. The RNA was bound to the silica-gel containing columns by centrifugation at 12,000g for 15 seconds at room temperature and contaminants removed by wash steps and subsequent centrifugation. The RNA was eluted from the silica-gel containing columns in 30µl of RNase-free water by centrifugation at 12,000g for 1 minute at room temperature. The RNA was stored at -80°C.

2.5.2 Determining the concentration of RNA

The concentration of RNA was determined using a spectrophotometer where the absorbance reading of 1 unit at 260nm is equivalent to 40µg/ml of RNA.

2.5.3 cDNA synthesis

cDNA was generated from total RNA isolated from the mammary gland or testis using the SuperScript III First-Strand Synthesis System for RT-PCR kit (Life Technologies)
according to manufacturer's instructions. In brief, 0.5µg of total RNA was incubated with random hexamers and a dNTP mix at 65°C for 5 minutes to denature the RNA and then placed on ice for 1 minute. To generate cDNA, a cDNA synthesis cocktail consisting of the M-MLV reverse transcriptase was added to the RNA/random hexamer mix and the sample incubated at room temperature for 10 minutes, followed by incubation at 50°C for 50 minutes. The reaction was terminated at 85°C for 5 minutes and the RNA digested in the presence of RNase H at 37°C for 20 minutes. Samples were stored at -80°C.

2.5.4 Quantitative PCR and PCR primers

Relative quantification of target gene levels was achieved using SYBR Green JumpStart ReadyMix (Sigma-Aldrich) according to manufacturer’s instructions and the Opticom 2 continuous fluorescence detector. Normalisation was against 18s ribosomal RNA in separate tube reactions. To prevent amplification of genomic DNA PCR primers were designed to span intronic regions. The reactions were visualised by agarose gel electrophoresis to confirm target amplicon size. The $2^{-\Delta\Delta CT}$ method (Applied Biosystems; User Bulletin No. 2) was used to calculate the target gene expression level relative to the mammary gland identified as expressing the highest level of the target gene. Target gene and control gene amplification rates were comparable. Each group is represented as an average. For all the primers listed in table 2 the PCR cycle parameters used are: 94°C for 5 minutes, then 40 cycles at 94°C for 15 seconds, 60°C for 30 seconds, 72°C for 40 seconds.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer (5'–3')</th>
<th>Anti-sense primer (5'–3')</th>
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<tbody>
<tr>
<td>$Kdm5b$</td>
<td>CGATAAACTTCATTTCCACCCCG</td>
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<td>18s</td>
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<td>$Wnt4$</td>
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<td>$Pgr$</td>
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<td>$Brca1$</td>
<td>TCTTGTGAGGCTTTGAGGG</td>
<td>AGGTTGGGGTCTGGGGTGC</td>
</tr>
<tr>
<td>$Kdm5a$</td>
<td>GATTGACTCTGGCGTCAGTTGC</td>
<td>GGACAAACACATTGGGTAGAAGGG</td>
</tr>
</tbody>
</table>

Table 2 Table listing primer sequences used in quantitative PCR.
2.6 Preparation and analysis of protein

2.6.1 Nuclear and cytoplasmic protein extraction

Inguinal mammary glands (4th from the neck) and testis were collected, snap frozen in liquid nitrogen and nuclear protein extracts isolated using NE-PER nuclear and cytoplasmic extraction reagents (Thermo scientific) as detailed in manufacturer’s instruction. All steps were carried out at 4°C. In brief, tissue was cut into small pieces, homogenised in phosphate buffer solution (PBS) and cells pelleted by centrifugation at 500g for 3 minutes at 4°C. The supernatant was removed and a 10-fold packed cell volume of reagent 1 added to the cell pellet. The sample was vortexed at the highest setting for 15 seconds and the sample incubated on ice for 10 minutes. Reagent 2 was then added, the sample vortexed at the highest setting for 5 seconds and the sample incubated on ice for 1 minute. The sample was centrifuged at 16,000g for 5 minutes at 4°C and the cytoplasmic fraction (supernatant) removed. The pelleted nuclear fraction was re-suspended in reagent 3 and vortexed on the highest setting for 15 seconds. The nuclear fraction was returned to ice and vortexed for 15 seconds every 10 minutes for a total of 40 minutes. The nuclear fraction was centrifuged at 16,000g for 10 minutes and the supernatant (nuclear extract) removed and stored at -80°C.

For the detection of KDM5B, ER alpha, STAT6, STAT5 and phosphorylated STAT5 expression, nuclear extracts were subject to SDS-PAGE electrophoresis and Western blot transfer. For the detection of WAP, beta-casein, AKT and phosphorylated AKT expression, cytoplasmic extracts were subject to SDS-PAGE electrophoresis and Western blot transfer.

2.6.2 SDS-PAGE electrophoresis and Western blot transfer

For nuclear or whole cell extracts protein concentration was determined based on the Bradford method (Bradford, 1976, Bio-Rad) according to manufacture’s instructions. Either 20 µg of nuclear protein extract or 30 µg of whole cell extract were used. Laemmli sample buffer was added to give a final concentration of 1x Laemmli sample buffer, 0.1M DTT. Samples were heated at 100°C for 5 minutes. Protein samples were separated on a 6% polyacrylamide gel (30% acrylamide:bis-acrylamide, Bio-Rad) using SDS-PAGE running buffer. Samples were run on a Mini-PROTEAN (Bio-Rad) electrophoresis system at 20V/cm for 120 minutes. Proteins were transferred in 1x Towbin buffer to Hybond C extra nitrocellulose membrane (Life technologies) using the
Mini Trans-Blot system (Bio-Rad) at 30V/90mA overnight at 4°C. Non-specific protein binding sites were blocked by incubating the membranes in an excess volume of blocking buffer ((5% non-fat milk powder (w/v), 0.2% Tween-20 in PBS)) at room temperature for 60 minutes. The membranes were incubated with the appropriate primary antibody (see table 2.1) diluted in binding buffer (2% non-fat milk powder, 0.1% Tween-20 in PBS) for 60 minutes at room temperature. The membranes were washed three times in an excess volume of PBS plus 0.2% Tween-20 for 10 minutes at room temperature and incubated with the appropriate secondary antibody at room temperature in binding buffer for 60 minutes (table 2.2). The membranes were washed three times in an excess volume of PBS plus 0.2% Tween-20 for 10 minutes and once in PBS only. Membrane bound immuno-complexes were detected by enhanced chemiluminescence on autoradiography film (GE Healthcare). The signal was developed using a X-ray film developer. To remove membrane bound immuno-complexes, membranes were submerged in stripping buffer (62.5mM Tris-HCL, pH6.7, 2% SDS, 100mM 2-Mercaptoethanol) for 30 minutes at 50°C with occasional agitation. The membranes were washed in copious amounts of PBS, and the removal of membrane bound immuno-complexes confirmed by enhanced chemiluminescence on autoradiography film (GE Healthcare). The signal was developed using a X-ray film developer.
### 2.6.3 Primary antibodies used for Western blot

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunogen</th>
<th>Dilution/concentration</th>
<th>Reference/supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT (rabbit polyclonal)</td>
<td>Raised against a peptide mapping at the C-terminus of AKT of mouse origin</td>
<td>1:500&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>Beta-casein (goat polyclonal M-14)</td>
<td>Raised against a peptide mapping at the C-terminus of beta-casein of mouse origin</td>
<td>0.4 µg/ml</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>ER alpha (rabbit polyclonal MC-20)</td>
<td>Raised against a peptide mapping at the C-terminus of ER alpha of mouse origin</td>
<td>0.4 µg/ml</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Hemagglutinin (mouse monoclonal – clone 16B12)</td>
<td>Raised against the peptide CYPYDVPDYASL</td>
<td>1 µg/ml</td>
<td>Covance</td>
</tr>
<tr>
<td>KDM5B (rabbit polyclonal)</td>
<td>Raised against the C-terminus domain of KDM5B corresponding to aa 1283-1473 of human origin</td>
<td>1:1500</td>
<td>Barrett et al; 2002</td>
</tr>
<tr>
<td>Lamin B (goat polyclonal)</td>
<td>Raised against a peptide mapped to the C-terminus domain of Lamin B1 of human origin</td>
<td>1.3 µg/ml</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Phospho-AKT (rabbit monoclonal 193H12)</td>
<td>Raised against a synthetic phospho-peptide corresponding to residues surrounding Ser473 of mouse AKT</td>
<td>1:500</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Phospho-STAT5 (rabbit monoclonal C11C5)</td>
<td>Raised against a synthetic phospho-peptide of Stat5a corresponding to residues surrounding Tyr694</td>
<td>1:500</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>STAT5 (rabbit polyclonal C-17)</td>
<td>Raised against a peptide mapping at the C-terminus of STAT5 of mouse origin</td>
<td>0.4 µg/ml</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>STAT6 (rabbit polyclonal M-20)</td>
<td>Raised against a peptide mapping at the C-terminus of STAT6 of mouse origin</td>
<td>0.4 µg/ml</td>
<td>Santa Cruz</td>
</tr>
</tbody>
</table>
2.6.3 Primary antibodies used for Western blot continued

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunogen</th>
<th>Dilution/concentration(^a)</th>
<th>Reference/supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAP (goat polyclonal M-16)</td>
<td>Raised against a peptide mapping at the C-terminus of whey acidic protein of mouse origin</td>
<td>0.4µg/ml</td>
<td>Santa Cruz</td>
</tr>
</tbody>
</table>

Table 2.1 Table listing primary antibodies used for Western blot (\(^a\)Dilution factor is given where concentration is not know).

2.6.4 Secondary antibodies used for Western blot

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti mouse HRP conjugate</td>
<td>1.3µg/ml</td>
<td>Dako</td>
</tr>
<tr>
<td>Goat anti rabbit HRP conjugate</td>
<td>1.3µg/ml</td>
<td>Dako</td>
</tr>
<tr>
<td>Rabbit anti goat HRP conjugate</td>
<td>1.3µg/ml</td>
<td>Dako</td>
</tr>
</tbody>
</table>

Table 2.2 Table listing secondary antibodies used for Western blot

2.6.5 Transient transfection of plasmid DNA

COS-7 cells were transfected with plasmid DNA using Lipofectamine LTX according to manufacturers instruction (Life technologies). Briefly, COS-7 were seeded at 1x10\(^6\) per 10cm plate (Gibco) in 10% FCS DMEM media (with supplements as described in 2.3) and allowed to establish overnight (as described in 2.3). To 2ml of serum free, antibiotic free DMEM media, a total of 16µg of plasmid DNA was incubated at room temperature with an equal volume of PLUS Reagent (equal volume of PLUS Reagent to [DNA]) for 15 minutes. 35µl of Lipofectamine LTX was added to the above DNA:PLUS solution, mixed and incubated at room temperature for 25 minutes. The 10% FCS DMEM media was removed from the COS-7 cells and replaced with 8ml of 10% FCS DMEM (with supplements) plus the DNA:liposome complex and incubated at 37°C in a 5% CO\(_2\) incubator overnight. The transfection media was then replaced with 10% FCS DMEM media (with supplements) and the COS-7 cells incubated at 37°C in a 5% CO\(_2\) incubator for 48 hours.
2.6.6 Immunoprecipitation

Forty-eight hours post-transfection, COS-7 cells were washed twice with PBS, then lysed for 15 minutes on ice in lysis buffer, plus EDTA-free complete protease inhibitor cocktail (Roche). Cells were passed ten times on ice through a fine gauge needle, and centrifuged at 16,000g for 5 minutes at 4°C to pellet cell debris. The supernatant was pre-cleared with protein A/G agarose beads (Pierce) with rotation for 60 minutes at 4°C. Samples were centrifuged at 16,000g for 1 minute at 4°C and supernatant removed to a pre-chilled tube. To 300µg of supernatant, antibody conjugated agarose was incubated over-night with rotation at 4°C (Table 2.3). The samples were centrifuged at 16,000g for 1 minute at 4°C to pellet the agarose, the supernatant discarded and the agarose washed three times in ice cold PBS plus 0.1% Nonidet-P40 with rotation at 4°C for 10 minutes. The samples were centrifuged at 16,000g for 1 minute at 4°C and the agarose re-suspended in an equal volume of 2x Laemmli sample buffer (final [DTT] 0.1M). The samples were heated at 100°C for 5 minutes, centrifuged at 16,000g for 1 minute at room temperature and the supernatant removed and stored at -80°C.

2.6.7 Antibodies used for immunoprecipitation

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunogen</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA.11</td>
<td>Raised against the peptide epitope YPYDVPDYA</td>
<td>40µg&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Covance</td>
</tr>
<tr>
<td>(Clone 16B12-mouse monoclonal)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>Raised against poly-Histidine tagged fusion protein</td>
<td>40µg&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Sigma</td>
</tr>
<tr>
<td>(mouse monoclonal)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3 Table listing antibodies used for immunoprecipitation

<sup>b</sup> Purified antibody is coupled to cyanogen bromide activated agarose at 2-2.4 mg antibody per ml bed volume.
2.7 Murine studies

2.7.1 Development of the B6;129-Kdm5b ΔARID mouse strain and backcross to the C57BL/6J genetic background

A *Kdm5b* targeting vector was designed to replace exon 2 to 4. The targeting vector was generated by flanking the loxP-neomycin-LoxP expression cassette with genomic sequence amplified from the sv129/ola murine strain. The 5’ 7.3Kb homologous region comprised of intron 2 and 48bp of exon 2 and the 3’ homologous region comprised of 1.2Kb of intron 5. After injection of the construct into male 129/ola ES cells, screening of G418-selected (200µg/ml) ES clones was performed by Southern hybridization using a PCR generated probe (1.2kb) within the 3’ homologous region. A positive clone (2F9) carrying the integrated targeting vector was injected into C57BL/6J blastocyst to generate a single mouse lineage homozygous for the transgene. The B6;129 chimeric strain was backcrossed over six generations onto the C57BL/6J genetic background to give the C57BL/6J-*Kdm5b* ΔARID murine strain.

2.7.2 Development of the B6;129-Kdm5b-Exon 1 knock out murine line

A *Kdm5b* targeting vector designed to replace exon 1 was generated by flanking the loxP-neomycin-LoxP expression cassette with genomic sequence amplified from the 129/ola murine strain. The 5’ 2.4 kb homologous region (HR1) and the 3’ 5.7 kb homologous region (HR2) comprised of intronic 1 and 2 sequences respectively. After injection of the construct into male B6;129 ES cells, screening of G418-selected (200µg/ml) ES clones was performed by Southern hybridization using a PCR generated probe within the 3’ homologous region. Two positive clones (1A4, strain 1 and 1A8, strain 2) carrying the integrated targeting vector were injected into either C57BL/6J blastocysts (1A8) or C57BL/6J embryos at the 8 cell stage (1A4) of development.

2.7.3 Breeding program between the C57BL/6-Kdm5b ΔARID +/- and the FVB-MMTV-PyV-mT +/- murine lines

Homozygous *Kdm5b* ΔARID female mice on a C57BL/6J background (C57BL/6J-*Kdm5b* ΔARID -/-) were mated with FVB male mice heterozygous for the Polyomavirus middle T driven by the mouse mammary tumour virus promoter (FVB-MMTV-PyV-mT +/-). Males from the progeny were screened for the polyoma middle T integration by PCR on DNA prepared from tail snips and neomycin integration (ΔARID) by Southern
blot. Male off-spring (B6FVB1) heterozygous for PyV-mT (+/-) and Kdm5b ΔARID (+/-) integration were crossed with homozygous C57BL/6J-Kdm5b ΔARID (-/-) female mice to obtain male progeny homozygous for Kdm5b ΔARID (-/-) integration and heterozygous for PyV-mT integration (+/-) (B6FVB2). The Kdm5b ΔARID (-/-) : PyV-mT (-/+) male mice were bred with C57BL/6J-Kdm5b ΔARID (-/-) female mice and the Kdm5b ΔARID (-/-) : PyV-mT (-/+/-) female progeny monitored for tumour development (B6FVB3). Control mice (Control/PyV-mT mice) were derived by breeding FVB-MMTV-PyMT+/- male mice with C57BL/6J female mice for 3 generations, the equivalent number of crosses required to obtain the Kdm5b ΔARID (-/-) : PyV-mT (-/+) test murine line. Test and control mice were examined three times per week for the development of tumours, and the time of first appearance noted (see figure 5.6).

2.7.4 Staging pregnancy and synchronising oestrous cycle

For staged pregnancies, observation of vaginal plug was designated as day zero. To ensure accuracy of staged pregnancies, embryos were assessed for stage of embryonic development at the point of mammary gland harvest. To study the development of the nulliparous mammary gland the female mice were caged together from birth in order to synchronize the estrous cycle, although cytological smear examinations were not performed to unequivocally stage the cycle. For the collection of mammary gland tissue females were culled by carbon dioxide inhalation.

2.8 Cell biology techniques

2.8.1 Histology

The left-sided thoracic mammary glands (3rd from the neck) were formalin-fixed over night at room temperature in an excess volume of 10% neutral buffered formalin (Sigma-Aldrich) and paraffin embedded. Paraffin-embedded sections (4µm) were mounted on slides and incubated for 2 minutes in two changes of xylene to de-wax the sections. To serially hydrate the sections the slides were incubated for 2 minutes with two changes of 100% ethanol and incubated for 2 minutes with one change of 70% ethanol. Slides were placed in a tray of running tap water for 2 minutes and stained in Mayer’s haematoxylin (Sigma-Aldrich) for 5 minutes. The slides were placed in a tray of running tap water for 1 minute and incubated in 1% acid alcohol for 5 second. Slides were placed in a tray of running tap water for 3 minutes and incubated in 1% Eosin for 4 minutes. The slides were placed in a tray of running tap water for a few seconds and
serially dehydrated in 70% ethanol for 5 seconds and incubated for 1 minute with two changes of 100% ethanol. Slides were incubated for 2 minutes in xylene to remove the alcohol and mounted under cover slip. Sections were viewed using an Olympus BX50 microscope.

2.8.2 Mammary gland whole mount

The right-sided thoracic and inguinal mammary glands (3rd and 4th from the neck respectively) were dissected onto glass slides and fixed flat overnight at room temperature in Carnoy’s fixative. The slides were rinsed in copious amounts of distilled water and stained overnight at room temperature in Carmine solution. The mammary gland whole mounts were dehydrated by incubation for 15 minutes in increasing concentrations of ethanol (70%, 95% and 100%). Whole mounts were viewed using a Nikon stereomicroscope SMZ1500

2.8.3 Immunohistochemistry

The left-sided thoracic mammary glands (3rd from the neck) were formalin-fixed over night at room temperature in an excess volume of 10% neutral buffered formalin (Sigma-Aldrich) and paraffin embedded. Paraffin-embedded sections (4µm) were mounted on slides and incubated for 2 minutes in two changes of xylene to de-wax the sections. To serially hydrate the sections the slides were incubated for 2 minutes with two changes of 100% IMS and incubated for 2 minutes with one change of 70% ethanol. The sections were washed in PBS and the tissue subject to antigen retrieval by boiling the sections for 10 minutes in 10mM sodium citrate buffer (pH6.0). The slides were placed in PBS and allowed to cool for 20 minutes. Endogenous peroxidase activity was blocked by incubation in 1.6% hydrogen peroxide for 10 minutes. The sections were washed for 5 minutes with distilled water, rinsed in PBS and the sections incubated in blocking buffer ((10% normal rabbit serum (Sigma-Aldrich), 1%BSA in PBS) for 30 minutes. The blocking buffer was blotted from the section and the section incubated with the appropriate primary antibody (see table 2.4) diluted in binding buffer (1%BSA in PBS) for 60 minutes at room temperature. The slides were washed in three changes of PBS for 3 minutes each and incubated with the appropriate biotinylated secondary antibody (see table 2.5) diluted in binding buffer (1%BSA in PBS) for 60 minutes at room temperature. The slides were washed in three changes of PBS for 3 minutes each and incubated with an Avidin-Biotin Complex (ABC, Thermo scientific) for 30 minutes. The slides were washed in three changes of PBS for 3 minutes each and incubated in DAB solution (Sigma-Aldrich) for 3 minutes. The sections were
counterstained with Mayer’s haematoxylin (Sigma-Aldrich), dehydrated and mounted under cover slips as detailed above (2.8.1). Sections were viewed using an Olympus BX50 microscope.

2.8.4 Primary antibody used for immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunogen</th>
<th>Dilution/ concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67 (rat monoclonal Clone: TEC-3)</td>
<td>Not detailed</td>
<td>1:125&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Dako</td>
</tr>
<tr>
<td>KDM5B (rabbit polyclonal)</td>
<td>Raised against the C-terminus domain of KDM5B corresponding to aa 1283-1473 of human origin</td>
<td>1:700</td>
<td>In house</td>
</tr>
<tr>
<td>PR (rabbit polyclonal)</td>
<td>Raised against the N-terminus region of PR corresponding to aa 533-547 of human origin.</td>
<td>1:30</td>
<td>Dako</td>
</tr>
<tr>
<td>Phospho-STAT5 (rabbit monoclonal C11C5)</td>
<td>Synthetic peptide of Stat5a. corresponding to residues surrounding Tyr694</td>
<td>1:25</td>
<td>Cell signalling</td>
</tr>
</tbody>
</table>

Table 2.4 Table listing primary antibody used for immunohistochemistry (<sup>a</sup>Dilution factor is given where concentration is not known).

2.8.5 Secondary antibody used for immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution/ concentration</th>
<th>supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotinylated rabbit anti-rat</td>
<td>2&lt;sub&gt;µ&lt;/sub&gt;g/ml</td>
<td>Vector labs</td>
</tr>
<tr>
<td>Swine anti rabbit HRP conjugate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For use with the following primary antibodies:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution/ concentration</th>
<th>supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>KDM5B</td>
<td>1.3&lt;sub&gt;µ&lt;/sub&gt;g/ml</td>
<td>Dako</td>
</tr>
<tr>
<td>pSTAT5</td>
<td>6.5&lt;sub&gt;µ&lt;/sub&gt;g/ml</td>
<td>Dako</td>
</tr>
<tr>
<td>PR</td>
<td>1.3&lt;sub&gt;µ&lt;/sub&gt;g/ml</td>
<td>Dako</td>
</tr>
</tbody>
</table>

Table 2.5 Table listing secondary antibody used for immunohistochemistry

2.8.6 Microscopy and data analysis
For each of the C57BL/6J-Kdm5b WT and Kdm5b ΔARID mammary gland whole mounts, the total number of TEB structures (at 4 weeks of age, n=6) or Y-branch points (at 12 weeks of age. WT, n=3. ΔARID, n=4) from the whole mammary gland were counted and expressed as an average.

From each of the C57BL/6J-Kdm5b WT and Kdm5b ΔARID embedded sections at days 12.5 and 18.5 of pregnancy (n=3) approximately 3000 ductal and alveolar epithelial cells were counted. Those cells within the field of vision positive for the cell proliferation marker Ki67 were expressed as a percentage of total epithelial content. Data is represented as an average.

Murine mammary gland whole mounts were visualized using a Nikon stereomicroscope SMZ1500. Slides stained with heamatoxylin and eosin (H and E) or used for immunohistochemistry were scanned with the Hamamatsu digital slide scanner, NanoZoomer-XR C12000. The slides were visualized with the NanoZoomer Digital Pathology Software viewing platform. Six randomly chosen 1mm² areas were selected and the number of alveoli at day 18.5 of pregnancy in the C57BL/6J-Kdm5b WT and Kdm5b ΔARID mammary glands were scored. Three randomly chosen 1mm² areas were selected and the perimeter of the alveoli at day 18.5 of pregnancy in the C57BL/6J-Kdm5b WT and Kdm5b ΔARID mammary glands were determined using NanoZoomer Digital Pathology Software. Quantification of immuno-blots was performed using ImageJ software. Student T-tests were performed using GraphPad software.
Chapter 3: Results

Studies on the function of KDM5B *in vivo*
Chapter 3. Studies on the function of KDM5B in vivo

3.1 Aim:
In order to study the function of KDM5B in vivo, the Kdm5b locus was targeted. The specific aims in this chapter were to:

1. Assess the effect of ablating KDM5B in vivo (B6;129-Kdm5b-Exon 1 knock out)
2. To develop and characterise the B6;129-Kdm5b ΔARID murine line

3.2 Introduction: A role for KDM5B during development

KDM5B is expressed in embryonic stem cells (Dey et al., 2008, Schmitz et al., 2011, Xie et al., 2011) and throughout development of the embryo (Madsen et al., 2002, Frankenberg et al., 2007, McGraw et al., 2007). In adult tissue, the Kdm5b transcript is expressed in the testis during spermatogenesis (Madsen et al., 2003) and differentially during mammary gland morphogenesis (Barrett et al., 2007, Catchpole et al., 2011 and this thesis), two organs containing cells that undergo rounds of proliferation and differentiation. The amino acid sequence of murine KDM5B is 94% homologous with that of human KDM5B, with a high degree of homology within the conserved domains\(^\Delta\) (Madsen et al., 2002 and Figure 1.6), suggesting functional conservation and the relevance of murine systems for the study of KDM5B function. The embryonic expression profile of Kdm5b raises the important issue as to the function and requirement of KDM5B during development. In an attempt to address these issues two candidate approaches to study the function of KDM5B in vivo were used.

\(^\Delta\) % homology within the conserved domains of human and murine KDM5B: JmjN 100%; ARID 100%; JmjC 100%; PHD1 98%; PHD2 88%; PHD3 98% (Murine NCBI reference sequence NP_690855.2 and human Swiss-Prot sequence Q9UGL1)
3.3 Results

3.3.1 Ablation of murine KDM5B results in embryonic lethality

As outlined in the schematic for the production of gene targeted mice (Fig.3), in one approach (referred to hereafter as Kdm5b-Exon 1 knock out), exon 1 was replaced with a floxed neomycin targeting construct (Fig 3.1), which was expected to result in no KDM5B translation. Successful integration of the targeting vector into male ES cells derived from the ICM of the B6;129 chimeric murine line was determined by Southern blotting (Fig 3.2) and the resulting positive ES cells were injected into C57BL/6J blastocysts or 8-cell embryos. The resulting chimeric offspring where ES cells have contributed to germ line transmission (black/brown coat colour) were screened by Southern blot for homologous recombination. Several chimeric heterozygous B6;129-Kdm5b-Exon1 knock out males were identified by Southern hybridisation, and two murine strains (1A4; derived from 8-cell embryo and 1A8; derived from blastocyst) were propagated with wild type C57BL/6J females. In order to streamline the screening for heterozygous B6;129-Kdm5b-Exon1 knock out offspring PCR primers were designed, tested and optimal amplification conditions chosen to distinguish the wild type Kdm5b allele from the engineered allele.

The expression of the Kdm5b transcript at the MET (McGraw et al., 2007), within the epiblast (Frankenberg et al., 2007) and later during embryonic development (Madsen et al., 2002) suggests a role for KDM5B throughout the life of the embryo. In order to explore a function for KDM5B during embryonic development and further whether homozygous B6;129-Kdm5b-Exon 1 knock out offspring were viable, two heterozygous B6;129-Kdm5b-Exon1 knock out breeding programs (derived from strains 1A4 and 1A8) were initiated. The genotype of the offspring, as detailed above and shown in figure 3.3, was determined by PCR. From a total of 139 and 106 offspring derived from strains 1A4 and 1A8 respectively, no homozygous B6;129-Kdm5b-Exon 1 knock out offspring were detected.

* Targeting construct was designed and generated by Dr Samantha Santangelo, KCL.
* Southern blot of transfected ES cells and screening of the breeding program was done with the help of Miss Debbie Hall, KCL.
* Transfection of targeting vector into ES cells and subsequent injection of positive clones into the blastocysts or 8-cell embryos and pseudopregnant female was carried out by Cancer Research UK Transgenic Mouse Service Laboratory, headed by Dr Ian Rosewell
Furthermore, significantly fewer (approx. 50%. Table 3 and 3S1. 1A4: p=0.0002. 1A8: p=0.0001) heterozygous B6;129-Kdm5b-Exon 1 knock out offspring from both murine strains were detected at birth suggesting that the expression level of KDM5B is important for viability, at some stage of embryonic development, with only a proportion of embryos developing to full term.

Figure 3 Schematic representation of B6;129-Kdm5b-Exon1 knock out strategy detailing an outline for the production of gene targeted mice by manipulation and injection of ES cells. (A similar approach was used for the generation of the B6;129-Kdm5b ΔARID murine line with the exception that only blastocysts were used).
Figure 3.1 Schematic representation showing the KDM5B protein domains with genomic sequence detailing the positions of the neomycin targeting construct (neo) used to replace exon1 (which codes for part of the JmjN domain) and homologous regions HR1 and HR2.

Figure 3.2 Schematic representation of the Kdm5b-WT allele and the Kdm5b-Exon1 targeting construct together with the screening strategy used to detect homologous recombination in transfected embryonic stem cells.

(A) Schematic representation depicting the Kdm5b-WT allele with exon 1 (white box 1) and homologous regions (HR1 and HR2) used for the design of the targeting construct.

(B) The targeting construct depicting homologous regions (HR1 and HR2), loxP sites (LP) and the neomycin sequence (arrow) with the screening strategy used to distinguish the WT allele from the engineered allele (BciV1 restriction sites).

(C) Southern blot on genomic DNA isolated from transfected ES cells showing homologous recombination after BciV1 digest.
Figure 3.3 PCR screen on genomic DNA isolated from the offspring of the heterozygous B6;129-Kdm5b-Exon1 knock out breeding programs (A) Schematic representation depicting Kdm5b-WT allele with exon 1 (white box) and homologous regions (HR1 and HR2) used for the design of the targeting construct. Small white rectangles depict PCR primer location. (B) Schematic representation of the targeting construct depicting homologous regions (HR1 and HR2), loxP sites (LP) and the neomycin sequence (arrow). Small white rectangles depict PCR primer location. (C) PCR product (271bp) generated using PCR primers specific to the Kdm5b WT allele. (D) PCR product (440bp) generated using PCR primers designed to amplify the engineered allele.

<table>
<thead>
<tr>
<th>Strain</th>
<th>total</th>
<th>Wild Type</th>
<th>Heterozygous</th>
<th>Homozygous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain 1A4 (14 litters)</td>
<td>139</td>
<td>67</td>
<td>72*</td>
<td>0</td>
</tr>
<tr>
<td>Strain 1A8 (18 litters)</td>
<td>106</td>
<td>57</td>
<td>49**</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3 Genotype of progeny from a heterozygous B6;129-Kdm5b-Exon1 knock out breeding program (*, 1A4: p=0.0002. ***, 1A8: p=0.0001).

In order to determine the stage of development at which KDM5B expression is essential for embryonic development and the development of heterozygous embryos, embryos from a heterozygous B6;129-Kdm5b-Exon1 knock out breeding program (1A4 and 1A8) were harvested at e7.5, e8-8.5 and e9.5-10.5 and genotyped (Table 3.1). For genotyping of earlier embryos, a collaboration with the Curie Institute was instigated. The data obtained from the mice transferred to the Curie Institute showed that homozygous embryos were detected up to e4.5, which
is suggestive of a function for KDM5B at or around the time of implantation. Surprisingly, although the offspring born consisted of fewer Kdm5b heterozygotes than would be expected according to Mendelian genetics (strain 1A4: \( p=0.0002 \), strain 1A8: \( p=0.0001 \)), the embryonic analysis suggested that embryos, heterozygous for Kdm5, had a survival advantage at e7.5 (Table 3.1; strain 1A8: \( p<0.05 \)). As the number of embryos analysed at each stage for each strain were small we combined the genotype data. As can be seen from Table 3.1C, the Kdm5b heterozygote embryos appear to have a survival advantage from e7.5 to 8-8.5, (e7.5, \( p<0.01 \)), which is lost by e9.5-10.5. Even though the combined number of embryos is still small the data suggest a change in the number of heterozygotes with time. Taken together with the data shown in Table 3 suggests that post e10.5 the heterozygous embryos are disadvantaged leading to the non-Mendelian ratios of the live offspring. Thus, the data suggest that the lower expression of KDM5B in the early embryos may be advantageous to development, while higher levels of KDM5B expression are required for viability in the later embryos.

<table>
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<th>Homozygous</th>
</tr>
</thead>
<tbody>
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<td>4</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>e8-8.5</td>
<td>2</td>
<td>9</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>e7.5</td>
<td>0</td>
<td>7</td>
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<table>
<thead>
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<th>Wild Type</th>
<th>Heterozygous</th>
<th>Homozygous</th>
</tr>
</thead>
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<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>e8-8.5</td>
<td>2</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>e7.5</td>
<td>1</td>
<td>13*</td>
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</tbody>
</table>

<table>
<thead>
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<th>C</th>
<th>Strain 1A4 and 1A8</th>
<th>Wild Type</th>
<th>Heterozygous</th>
<th>Homozygous</th>
</tr>
</thead>
<tbody>
<tr>
<td>e9.5-10.5</td>
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<td>0</td>
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<tr>
<td>e7.5</td>
<td>1</td>
<td>20**</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1 The Kdm5b knock out line is embryonic lethal. Table showing genotyping results from embryos isolated from 2 heterozygous B6;129 Kdm5b-Exon1 knock out breeding programs (\* \( p<0.05 \), \** \( p<0.01 \)).
Comparison of the offspring born to the B6;129-Kdm5b WT murine line with that of the offspring born to the heterozygous B6;129-Kdm5b-Exon 1 knock out murine line, indicated that the surviving offspring developed normally, with no difference in the size of the pups noted.

To examine whether the expression levels of KDM5B are important for post-natal development we examine the gross morphology of the mammary gland, an organ known to express KDM5B (Madsen et al., 2002, Barrett et al., 2007, Catchpole et al., 2011 and this thesis). To achieve this, thoracic mammary glands taken from B6;129-Kdm5b WT and heterozygous B6;129-Kdm5b-Exon 1 knock out litter-mates were whole mounted and stained with carmine. As shown in Figure 3.4 development of the B6;129-Kdm5b WT and heterozygous B6;129-Kdm5b-Exon 1 knock out mammary glands appear comparable. This suggests that expression of KDM5B from one allele in the surviving heterozygous B6;129-Kdm5b-Exon 1 knock out females is sufficient for normal post-natal development of the mammary gland.

**Figure 3.4** Normal mammary gland development in the heterozygous B6;129-Kdm5b-Exon 1 knock out murine line. Representative carmine on (A) B6;129-Kdm5b WT and (B) heterozygous B6;129-Kdm5b-Exon 1 knock out adult (12 weeks of age) nulliparous mammary gland (n=2 per group).
3.3.2 Development, screening and preliminary characterisation of the chimeric B6;129-Kdm5b ΔARID murine line

3.3.2.1 The B6;129-Kdm5b ΔARID murine line are viable and fertile

Data from the B6;129-Kdm5b-Exon 1 knock out murine line suggested that KDM5B expression is essential for early murine embryonic development. As an alternative strategy, part of exon 2 (48bp from a total of 78bp) and the entirety of exons 3 and 4 (13.8KDa in total) of the Kdm5b gene was replaced with a floxed neomycin-targeting construct (Fig 3.5). The region of the Kdm5b gene targeted codes for the ARID sequence that is required for demethylase activity (Yamane et al., 2007) and has been shown to bind GC rich sequences (Scibetta et al., 2007, Yao et al., 2010). Successful integration of the targeting vector into male ES cells derived from the ICM of the 129/Ola mouse strain was determined by Southern blotting and the resulting positive ES cells from the only clone identified was injected into C57BL/6J blastocysts followed by injection into a C57BL/6J pseudo-pregnant female. The resulting chimera offspring where ES cells have contributed to germ line transmission (black/brown coat colour) were screened by Southern blot for homologous recombination. The resulting chimeric mouse line generated from injecting 129/Ola derived ES cells into C57BL/6J blastocysts was termed the B6;129-Kdm5b ΔARID murine line (See Fig. 3 for an outline for the production of gene targeted mice).

In order to establish whether:

1. The heterozygous B6;129-Kdm5b ΔARID murine line was fertile.
2. The heterozygous B6;129-Kdm5b ΔARID offspring were born according to Mendelian frequencies.
3. The homozygous B6;129-Kdm5b ΔARID offspring were viable, a heterozygous B6;129-Kdm5b ΔARID breeding program was initiated and the genotype of the offspring examined by Southern blot. As shown in Figure 3.6, BamH1 restriction digest on genomic DNA isolated from the mice in the heterozygous breeding program was used to distinguish the wild type Kdm5b allele from the engineered allele for the identification of wild type, heterozygous and homozygous offspring. Furthermore the genotyping of the offspring from 2 heterozygous B6;129-Kdm5b ΔARID breeding pairs demonstrated that the offspring were born according to Mendelian frequencies (Table 3.2 and Table 3S2 and 3S3 in the supplemental material) and subsequently that offspring from the homozygous
B6;129-Kdm5b ΔARID breeding program were fertile and viable. Moreover the females could support their pups at parturition with no obvious difference in the size of the pups noted.

Figure 3.5 Schematic representation showing the KDM5B protein domains with genomic sequence detailing the positions of the neo targeting construct used to replace part of exon 2 and exons 3 to 4 (codes for part of the JmjN domain and the entire ARID domain) and homologous regions HR1 and HR2

Figure 3.6 Schematic representation of Kdm5b ΔARID targeting construct and the screening strategy used to distinguish the wild type allele from the engineered allele in murine tail snip DNA. (A) Schematic representation depicting the WT Kdm5b allele showing exons 2 to 5 (grey box), and the targeting construct depicting homologous regions (HR1 and HR2), truncated exon 2 (grey box with serrated edge), loxP sites (LP) and the neomycin targeting construct (arrow) with the screening strategy used to distinguish the WT allele from the engineered allele (BamH1 restriction sites). (B) A Southern blot on genomic DNA isolated from B6;129 murine tail-snips showing the WT allele and homologous recombination after BamH1 digest.
Table 3.2 Genotyping from two B6;129-Kdm5b ΔARID heterozygous breeding programs demonstrates that the B6;129-Kdm5b ΔARID offspring are born according to Mendelian ratio. The Chi squared value for 2 degrees of freedom and p=0.05 (5%) is 5.99. As the observed value is lower than 5.99 we accept our hypothesis, that there is no deviation from mendelian ratio.0.1<p<0.5 (See supplemental materials Table 3S3)

3.3.2.2 Expression of a less abundant KDM5B protein lacking the entire ARID domain and part of the JmjN domain

(A) KDM5B protein expression

Given the viability and fertility of the B6;129-Kdm5b ΔARID murine line in contrast to the embryonic lethality of the homozygous B6;129-Kdm5b Exon1 knock out line, we sought to examine whether KDM5B was being expressed by examining tissue known to express high levels of KDM5B, i.e. the testis and the mammary gland (Madsen et al., 2002, Barrett et al., 2007). To achieve this, Western blot transfer of nuclear protein extracts isolated from the B6;129-Kdm5b WT and ΔARID murine adult testis and the pregnant mammary gland (pd18.5) were probed with a KDM5B specific antibody. A similar profile of bands was observed in the mammary gland and testis (Fig. 3.7). In nuclear protein extracts isolated from wild type tissue, two high molecular weight bands were seen, one at 170KDa - which is the predicted molecular weight (MW) of KDM5B (bands - a and i), the other at around 150KDa (bands - b and j). These bands are likely to be a KDM5B splice variant as Western blot transfer using protein extracts isolated from breast cancer cell lines expressing KDM5B can show this pattern or a single 170KDa band depending on growth conditions. Western blot transfer on KDM5B ΔARID nuclear protein extracts also resolved two bands, but these were smaller and less abundant than seen in the wild type tissue (f and g; l and m). As will be shown below, these bands (f and g; l and m) may correspond to a splicing event that has occurred in the B6;129-Kdm5b
△ARID murine line. Furthermore, Western transfer of nuclear proteins from the B6;129-Kdm5b △ARID mammary gland and testis resolved bands h and n respectively that do not appear to be expressed in the wild type counterpart tissue.

The lower molecular weight band (approx. 130KDa), detected in extracts isolated from the B6;129-Kdm5b WT mammary gland and testis (e and k respectively) are not detected in the extracts isolated from the B6;129-Kdm5b △ARID tissue. If this is another splice variant it is clearly not formed in the B6;129-Kdm5b △ARID murine tissue examined. The two faint bands (c and d) observed from wild type murine mammary gland nuclear extracts appear not to be present in the wild type testis and could therefore be tissue specific.

![Western blot images](image)

**Figure 3.7 Expression of a smaller less abundant KDM5B protein in the B6;129-Kdm5b △ARID mammary gland and testis**. Western transfer showing translation of a smaller, less abundant KDM5B △ARID protein product in (A) the mammary gland at pd18.5 and (B) the adult testis.

**(B) Processing of the KDM5B primary transcript**

The neomycin selection cassette used to select for recombinant ES cells during gene targeting experiments contains a number of predicted splice sites (Jin et al.,
During processing of the primary transcript, utilisation of donor and acceptor sites within the targeted gene can result in the translation of fusion proteins that can consist of elements of both the neomycin cassette and the targeted gene (Kos et al., 2002, Shearman et al., 2000). Of note is the possibility that integration of the neomycin selection cassette within exon sequences together with the maintenance of naturally occurring splice donor and acceptor splice could lead to splicing out of the neomycin selection cassette (Gawenis et al., 2005). The design of the targeting construct detailed here maintained the exon 1 splice donor and exon 5 splice acceptor sites. It was therefore possible that the resulting smaller KDM5B protein seen in the mammary gland and testis is as a consequence of the splicing together of exon 1 and exon 5 resulting in the removal of the neomycin gene. To examine whether the expression of a smaller KDM5B protein in the B6;129-Kdm5b ΔARID murine line was as a consequence of:

1. Aberrant splicing of the primary transcript due to the identification of splice sites within the neomycin construct; or due to the

2. Splicing together of exon 1 to exon 5 using naturally occurring splice sites within the Kdm5b gene. To test this possibility, PCR primers were designed to flank the neomycin construct within exons 1 and 5.

Figure 3.8A shows a schematic representation of the processing of the WT Kdm5b primary transcript with the depiction of exon 1 through to exon 5 highlighting the JmJN (grey box) and ARID (red box) domains with the size of the expected PCR product shown (primers: green arrow). In contrast, Figure 3.8B is a schematic representation of one of the predicted splicing events (detailed as 2 above) that would lead to the splicing together of exon 1 to exon 5 with the expected PCR product size shown.

As figure 3.8C shows, the subsequent PCR on the cDNA, that was generated from the RNA, isolated from the B6;129-Kdm5b WT and B6;129-Kdm5b ΔARID murine testis and mammary gland at pd18.5 results in the amplification of a smaller product in the B6;129-Kdm5b ΔARID murine line. (data shown was generated from RNA isolated from the pd18.5 mammary gland). In order to determine the splicing event that lead to the generation of a smaller KDM5B protein product and further to establish whether the neomycin targeting construct had been spliced out during processing of the primary transcript those PCR primers directed to exon 1 and 5 of Kdm5b (as detailed above) were used to generate a DNA sequencing trace using the cDNA transcribed from RNA isolated from the B6;129-Kdm5b WT and ΔARID.
testis and pd18.5 mammary gland. Examination of the WT Kdm5b DNA sequencing trace confirmed the presence of the exon 4 to exon 5 boundary (See Fig. 3S1 and Table 3S4 in the supplemental material). In contrast, examination of the DNA trace from the B6;129-Kdm5b ΔARID at pd18.5 revealed in frame splicing of the primary transcript from exons 1 to 5 resulting in the truncation of the JmjN domain (deletion of amino acids, Asp69, Trp70, Gln71, Pro72, 73) and deletion of the entire ARID domain (Fig 3.9 and Table 3.3) (Exon 1 through to 5 of the WT Kdm5b transcript is shown in figure 3S2 of the supplemental material).

Figure 3.8 Processing of the Kdm5b primary transcript in the B6;129-Kdm5b WT and B6;129-Kdm5b ΔARID murine line. (A) Schematic representation depicting the splicing events of the WT Kdm5b primary transcript (boxes labelled 1 to 5 represent exon sequence). Narrow rectangles represent intronic sequence. Grey area in exon 1 and grey area with horizontal line in exon 2 represent the Jumonji N domain (JmjN). Red area in exon 3 and red area with horizontal line in exon 4 represent the ARID sequence. Green arrows represent the 5' and 3' PCR primers specific to exon 1 and exon 5 showing the expected product size. (B) Schematic representation depicting a predicted RNA splicing event of the engineered Kdm5b primary transcript resulting in the splicing together of exon 1 to exon 5 and the removal of the neomycin construct (large white arrow). (C) PCR on cDNA generated from RNA isolated from the B6;129-Kdm5b WT (540bp) and B6;129-Kdm5b ΔARID (140bp) murine mammary glands at pd18.5.
**Figure 3.9** DNA trace from the B6;129-Kdm5b ΔARID mammary gland transcript at pd 18.5. DNA trace showing anti-sense sequencing data obtained from cDNA reverse transcribed from RNA isolated from B6;129-Kdm5b ΔARID mammary gland (schematic representation of PCR primer location within exon 1 and 5 see Fig. 3S2 in the supplemental material).

<table>
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<tr>
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<th>Exon 2 - 4</th>
<th>Exon 5</th>
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<td>CCG</td>
<td>CCG</td>
</tr>
<tr>
<td>cDNA sequence (Anti-sense)</td>
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<td>GGC</td>
<td>GGC</td>
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<tr>
<td>Translated sequence</td>
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<td>p</td>
<td>Exons 1 and 5 are spliced together removing exons 2 to 4</td>
</tr>
<tr>
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</tr>
<tr>
<td>Exon 5</td>
<td>C</td>
<td>L</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3 Derived sequence from DNA trace (Figure 3.9) of B6;129-Kdm5b ΔARID cDNA showing exon 1 to 5 boundaries. Table showing sequencing data obtained from cDNA reverse transcribed from RNA isolated from B6;129 Kdm5b ΔARID mammary gland tissue at pd18.5.

### 3.3.2.3 Development of adult tissues in the B6;129-Kdm5b ΔARID murine line

(A) **Histological analysis of male and female adult tissues**

In order to examine the gross effect of KDM5B ΔARID expression on adult development, necropsy was performed on litter-matched B6;129-Kdm5b WT and B6;129-Kdm5b ΔARID male and female mice at 9 weeks of age. However even though Kdm5b is expressed during embryonic development and in the adult tissue (Madsen et al., 2002, Barrett et al., 2007, Frankenberg et al., 2007, Schmitz et al., 2011), no gross histological differences were noted between the adult B6;129-Kdm5b WT and B6;129-Kdm5b ΔARID line in any of the tissues examined (Table 3.4)
Table 3.4 Histological examination of organs from the male and female B6;129-Kdm5b ΔARID murine line at 9 weeks of age (adult) suggesting normal development. Those organs highlighted in red represent the organs where KDM5B expression has been reported in the adult (n=4 per group) (Madsen et al., 2002, Barrett et al., 2007, Frankenberg et al., 2007, Schmitz et al., 2011).

(B) A possible defect in mammary gland ductal morphogenesis

Kdm5b is expressed during mammary gland development (Madsen et al., 2002, Barrett et al., 2007, Catchpole et al., 2011 and this thesis), and a function for KDM5B in breast cancer development *in vivo* has been described (Yamane et al., 2007, Catchpole et al., 2011). Histological examination is not sufficient to identify

<table>
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<th>genotype</th>
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<th>Histology</th>
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<td>mammary gland</td>
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<tr>
<td></td>
<td>ovaries and oviduct</td>
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<td>penis and urethra</td>
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<td>epididymis and prostate</td>
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</tr>
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<tr>
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<td></td>
<td>tongue, nasal passages, tooth development</td>
<td>normal</td>
</tr>
<tr>
<td></td>
<td>spinal cord, vertebral bodies</td>
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</table>
any potential defect in the gross morphology of the B6;129-Kdm5b ΔARID female mammary gland. We therefore sought to clarify if an effect on development of the post-natal mammary gland could be seen in the B6;129-Kdm5b ΔARID line murine strain. To achieve this thoracic mammary glands from adult B6;129-Kdm5b WT and B6;129-Kdm5b ΔARID females (> 9 weeks of age) were whole mounted and stained with carmine. Figure 3.10A shows that by adulthood the B6;129-Kdm5b WT nulliparous mammary gland had fully ramified the fat pad by ductal elongation, bifurcation at TEB structures and through ductal side branching. In contrast some adult B6;129-Kdm5b ΔARID females presented with a lack of secondary ductal side branches, although the mammary gland tree had ramified the fat pad (Fig 3.10B). However, this defect in mammary gland ductal morphogenesis is variable between individual mice, as seen in Figures 3.10B and C.

KDM5B is expressed during pregnancy associated mammary gland development (Madsen et al., 2002, Barrett et al., 2007, Catchpole et al., 2011 and this thesis). To examine whether the expression of the KDM5B ΔARID protein affects development of the mammary gland through pregnancy, the thoracic mammary glands of the
B6;129-\textit{Kdm5b} \textit{WT} and B6;129-\textit{Kdm5b} \textit{\Delta ARID} females at mid and late pregnancy (pd12.5 and pd18.5 respectively) were whole mounted and stained with carmine. In order to ensure the accuracy of staged pregnancies, embryos were assessed for stage of embryonic development at the point of mammary gland harvest. Figure 3.11A shows that the B6;129-\textit{Kdm5b} \textit{WT} mammary gland at pd12.5 had undergone further side branching and alveolar development. On the other hand, although the mammary gland of some B6;129-\textit{Kdm5b} \textit{\Delta ARID} female mice at pd12.5 had ramified the fat pad further, a defect in ductal side branching and alveolar development appeared to be manifest. (Figure 3.11B). As noted for the B6;129-\textit{Kdm5b} \textit{\Delta ARID} nulliparous mammary gland, the degree of severity in mammary gland development present in the B6;129-\textit{Kdm5b} \textit{\Delta ARID} female at pd12.5 was also variable (compare Fig. 3.11B and Fig. 3.11C). As shown in Figure 3.11C, the B6;129-\textit{Kdm5b} \textit{\Delta ARID} mammary gland at pd12.5 had developed only a rudimentary ductal system, had failed to ramify the fat pad and further, presented with few ductal side branches, and an absence of alveolar development.

The B6;129-\textit{Kdm5b} \textit{\Delta ARID} females can support their pups at parturition with no obvious difference in the size of the pups noted. This observation suggests that mammary gland ductal morphogenesis recovers sufficiently to support alveolar proliferation and full differentiation. Consistent with this, mammary gland gross morphology, from both the B6;129-\textit{Kdm5b} \textit{WT} (Fig 3.12A) and B6;129-\textit{Kdm5b} \textit{\Delta ARID} (Fig 3.12B) females at pd18.5 were comparable.
Figure 3.11 Abnormal mammary gland development in the B6;129-Kdm5b ΔARID murine line at pd12.5. Representative carmine on (A) B6;129-Kdm5b WT and (B and C) B6;129-Kdm5b ΔARID mammary gland at pd12.5 (n=4 per group).

Figure 3.12 Mammary gland development at pd 18.5 in the B6;129-Kdm5b WT and ΔARID murine line. Representative carmine on (A) B6;129-Kdm5b WT and (B) B6;129-Kdm5b ΔARID mammary gland at pd18.5 suggests that development of the B6;129-Kdm5b ΔARID mammary gland recovered by late pregnancy (n=4 per group).
3.4 Discussion

At the time of writing this thesis a number of outstanding issues remained regarding the function of KDM5B. These include: What function does KDM5B perform during development? Does KDM5B possess a demethylase independent function? How is KDM5B recruited to the genome? Here we attempt to address some of these issues by drawing on the results from our own studies targeting the Kdm5b locus.

3.4.1 Assessing the developmental role of KDM5B

The data from the Kdm5b-Exon 1 knock out line suggests an essential role for KDM5B during embryonic development between e4.5 and e7.5; a role at the time of writing this which is unique amongst the other mammalian KDM5 family members (Klose et al., 2007, Catchpole et al., 2011 and this thesis). The Kdm5b-Exon 1 knock out line is viable up to e4.5 (Catchpole et al., 2011), even though KDM5B expression is required, through the regulation of H3K4 methylation, to silence pluripotent gene expression as ES cells differentiate (Kidder et al., 2013, 2014). An important role for KDM5B expression during post implantation is highlighted by the restricted expression of KDM5B to the epiblast at e5.5 (Frankenberg et al., 2007) and the requirement of KDM5B for embryoid body (EB) formation (Kidder et al., 2013). In fact, gene categories associated with ectoderm, mesoderm and endoderm formation are under-expressed during EB formation in the absence of KDM5B (Kidder et al., 2013) suggesting a requirement for KDM5B during the determination of all future lineages during the early stages of post-implantation. We have also noted that heterozygosity at e7.5 exceeds the numbers expected according to Mendelian frequencies although contamination from the maternal decidua at this early time point cannot be excluded.

However, as described in more detail below, Albert and colleagues (2013) recently reported on the phenotype of a Kdm5b null line in which integrated flipspase recognition target sites were used to remove the neomycin gene. The Kdm5b null line resulted in major neonatal lethality due to respiratory failure. Whilst 95 percent of wild type mice were viable, 50 percent of the Kdm5b null offspring died within the first two hours of delivery and another 20 percent died 12 to 24 hours after birth. Our data suggest that the expression level of KDM5B could be important for embryonic viability post e10.5 given that the number of heterozygous Kdm5b-Exon
1 knock out offspring born is significantly reduced. These findings are contrary to those of Albert and colleagues (2013) who report that although the viability of the heterozygote offspring born is reduced, this reduction is not significant. In addition, these authors examined whether KDM5B expression is important for embryonic development and observed no significant difference in the expected genotype ratios at mid (e9.5-e14.5) and late (e16.5-e18.5) stages of embryonic development. Furthermore, in keeping with Kdm5b expression in the developing embryonic brain, eye, and ganglionic nerve centres (Madsen et al., 2002), Albert and colleagues (2013) also showed that caesarean recovered embryos presented with neurological defects, such as defects in cranial and spinal nerve development, eye development and exencephaly. The Kdm5b null embryos presented with microphthalmic eyes, a disorganised neural retina structure and a significantly smaller lens (Albert et al., 2013). Interestingly, KDM5B has also been linked to the signalling pathway that determines rod versus cone photoreceptor cell fate determination (Hao et al., 2012). Deregulated Hox gene expression in murine polycomb mutant lines results in defective skeletal development (Akasaka et al., 1996, Del Mar Lorente et al., 2000) and KDM5B has been shown to bind and regulate developmental genes (Schmitz et al., 2011, Xie et al., 2011) including Hox genes (Yamane et al., 2007, Pasini et al., 2008). With this in mind, null embryos also present with a transformation of the last lumber vertebra (L6), into the first sacral vertebrae (Albert et al., 2013).

The Kdm5b-Exon 1 knock out targeting construct was designed to replace exon 1 with a floxed neomycin gene. It has been reported that the presence of the integrated neomycin targeting construct may have unintended consequences on the targeted gene, and or adjacent genes, if not excised from the genome (Olson et al., 1996, Meyers et al., 1998, Nagy et al., 1998, Scacheri et al., 2001). It is therefore a possibility that the embryonic lethality of the Kdm5b knockout and the reduced viability of the Kdm5b heterozygote noted at birth, maybe due to the presence of the neomycin targeting construct. The presence of the endogenous Kdm5b promoter, and the described cryptic splice sites contained within the neomycin gene (Jin et al., 2005) could potentially lead to the expression of hybrid transcripts (Shearman et al., 2000, Kos et al., 2002) consisting of both the neomycin and Kdm5b gene sequences. Furthermore, the presence of the neomycin promoter contained within the neomycin targeting vector could drive transcription that could potentially result in the expression of a truncated Kdm5b transcript lacking exon 1 of Kdm5b. In order to examine this possibility Northern blot transfer of RNA isolated from surviving Kdm5b null embryos at e4.5 and probed with an oligonucleotide sequence directed against
the neomycin gene and the Kdm5b gene sequences should be performed. The translation of any potential truncated KDM5B or hybrid neomycin/KDM5B protein should be explored by Western blot, as expression may result in a dominant negative affect thereby altering protein interactions with endogenous KDM5B. The presence of an integrated neomycin targeting vector into the Kdm5b locus may therefore explain the different phenotype presented in the Kdm5b null murine line reported in this thesis (Catchpole et al., 2011) and that reported by the Helin laboratory (Albert et al., 2013).

However, the results described in this thesis (Catchpole et al., 2011) together with published data (Albert et al., 2013) both demonstrate that mice null for the Kdm5b gene have severe developmental defects although there is disagreement as to the stage at which the defects manifest. Deciphering the role of KDMB during development has been further complicated by a recent report (Zou et al., 2014) and will be discussed at a later point (see Chapter 7 final discussion).

3.4.2 KDM5B protein expression in the adult testis and mammary gland at pd18.5.

The detection of several KDM5B antibody specific bands in the B6;129-Kdm5b WT mammary gland at pd18.5 as well as in the adult testis suggests the existence of isoform variants of KDM5B and could be confirmed by peptide sequencing using mass spectrometry. Although the potential splice variants could not be predicted, KDM5B contains numerous domains required for enzyme activity, including the catalytic JmjC domain, the JmjN domain, ARID sequence and the C5HC2 zinc finger sequence (Xiang et al., 2007, Yamane et al., 2007). The KDM5B protein also contains domains that aid in the recruitment of KDM5B to the genome including the ARID sequence (Scibetta et al., 2007, Yao et al., 2010) and the N- and C-terminus PHD sequences (Li et al., 2011, Klein et al., 2014). Therefore, the different KDM5B isoforms suggested from the Western blot analysis (see Figure 3.7 duplicated below for reference) could result in differences in function, and expression of different isoforms by different tissues could reflect these functional differences. In this context, a specific isoform of KDM5B that is expressed in nevi and melanomas and contains an additional 36 amino acids (Roesch et al., 2005) is only expressed at low levels in primary breast cancers (Barrett et al., 2002).
Figure 3.7 Expression of a smaller less abundant KDM5B protein in the B6;129-Kdm5b ΔARID mammary gland and testis. Western transfer showing translation of a smaller, less abundant KDM5B ΔARID protein product. in (A) the mammary gland at pd18.5 and (B) the adult testis.

3.4.3 A demethylase independent function for KDM5B?

The ARID sequence of KDM5B is required for demethylase activity (Yamane et al., 2007, Xiang et al., 2007) and has been deleted from the Kdm5b ΔARID murine line. The viability of the Kdm5b ΔARID murine line may therefore suggest a crucial demethylase independent function for KDM5B and puts into question which other domains are also important. In fact a demethylase independent function also extends to other members of the KDM5 family of proteins (DiTacchio et al. 2011) and may be conserved across species (Li et al., 2010, DiTacchio et al. 2011). In a demethylase-independent manner KDM5A functions to regulate circadian rhythms by enhancing transcription by CLOCK-BMAL1 (DiTacchio et al. 2011). Also, lid is the only KDM5 homolog expressed in D. Melanogaster and is essential for development, a function that is independent of its demethylase activity (Li et al., 2010). Indeed an enzyme independent function maybe a common mechanism amongst the protein families that regulate the methylation of histone 3 on lysine 4 (Terranova et al., 2006, Yu et al., 1995, Yagi et al., 1998, DiTacchio et al. 2011).
case in point can be made regarding the embryonic lethality of the H3K4 methyltransferase \textit{Kmt2a-null} (a.k.a MLL1) mutant (Yu \textit{et al}., 1995, Yagi \textit{et al}., 1998) in contrast to the viability of the catalytically inactive \textit{Kmt2a SET} domain mutant (Terranova \textit{et al}., 2006). These published data, taken together with our studies certainly highlight the functional importance of the remaining protein domains, which may prove to be as significant as the intrinsic catalytic activity.

### 3.4.4 Recruitment of KDM5B to the genome

In addition to the ARID domain of KDM5B being required for demethylase activity (Xiang \textit{et al}., 2007, Yamane \textit{et al}., 2007), the ARID domain of KDM5B has been shown to bind to DNA (Scibetta \textit{et al}., 2007, Yao \textit{et al}., 2010). Interestingly, deletion of the ARID domain of \textit{Kdm5b} results in a mammary gland phenotype that can differentiate during pregnancy. Certainly ChIP-qPCR studies have demonstrated that in \textit{Kdm5a} null cells, some KDM5A gene targets can be bound by KDM5B (Islam \textit{et al}., 2011b) suggesting the propensity for compensation and therefore demethylase activity. Furthermore KDM5B has been identified in different complexes and can potentially be recruited to the genome via different proteins. For example, in the case of a KDM5B/PcG complex neither KDM5B nor PcG proteins, at least individually, are essential for each other’s recruitment to the genome (Schmitz \textit{et al}., 2011). In addition the N terminus PHD sequence of KDM5B can bind to unmodified histone H3 and H3K9me1/2/3 (Klein \textit{et al}., 2014) whilst the C-terminus PHD sequences of KDM5B can bind H3K4me1/2/3 (Li \textit{et al}., 2011, Klein \textit{et al}., 2014), providing yet another mechanism for the KDM5B \textDelta{ARID} protein to be recruited to the genome, where KDM5B could function in a demethylase independent manner. Therefore any effects on transcription maybe subtle, potentially accounting for the delay in mammary gland development presented in the KDM5B \textDelta{ARID} female.

### 3.4.5 The B6;129-Kdm5b \textDelta{ARID} male is fertile

Although germ cell number and the degree of differentiation were not studied, the reduced protein expression levels of the full length KDM5B protein (heterozygous B6;129-Kdm5b-Exon 1 knock out) or the KDM5B \textDelta{ARID} protein (B6;129-Kdm5b-
\(\Delta ARID\) appear not to grossly affect oogenesis or spermatogenesis. KDM5B and KDM5D expression pattern during spermatogenesis suggests that KDM5D may compensate for KDM5B function during pachytene/diplotene stages of meiosis (Madsen et al., 2003, Akimoto et al., 2008). However, KDM5D was not detected during the mitotic stage of spermatogenesis (Akimoto et al., 2008) suggesting that the reduced expression levels of KDM5B are sufficient and further that alternative H3K4 demethylase activity could be compensated for in the \(Kdm5b \Delta ARID\) murine line.

We have presented here an effect of KDM5B \(\Delta ARID\) expression on mammary gland development. However, variations in mammary gland ductal side branching are known to occur between different strains of mice; the mammary gland architecture in the 129ola murine line being highly branched in contrast to the mammary gland architecture presented in the C57BL/6J murine line. Therefore, the variable genetic contribution of mixed genetic backgrounds can complicate interpretation of the resulting phenotype. This issue of a variable mammary gland architecture is highlighted in a study comparing the mammary gland architecture presented in the F2 generation of 45, B6;129 offspring. Of the 45 mice studied, 12 mice demonstrated the highly side branched structure of the 129 murine strain; 8 mice presented with poor ductal branch structure of the C57BL/6J murine line and the remaining 25 mice presented with an intermediary ductal branch structure between the two extremes (Naylor et al., 2002). This problem in quantitatively and accurately defining a potential mammary phenotype can be overcome by backcrossing the genetically modified mice onto a pure genetic background as detailed in Chapter 4.
3.5 Supplemental materials

### Table 3S1 Heterozygous offspring of the B6;129 Kdm5b-Exon1 knock out murine line are not born according to Mendelian frequencies.
Calculations for Chi Squared test. The Chi squared value for 1 degrees of freedom and \( p = 0.001 \) is 10.83. As the observed value is greater than 10.83 we reject our hypothesis, and state that the heterozygous B6;129 Kdm5b-Exon1 knock out line are not born according to Mendelian frequency (1A4: \( p = 0.0002 \). 1A8: \( p = 0.0001 \))

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**Table 3S2** Genotyping results from B6;129-Kdm5b \( \Delta ARID \) heterozygous breeding program demonstrates that the B6;129-Kdm5b \( \Delta ARID \) offspring are born according to Mendelian frequency. (Pair 1: \( 0.1<p<0.5 \) and Pair 2: \( 0.5<p<0.9 \))
Table 3S3 Calculations for Chi Squared test (data shown in table 3.2 and 3S2). The Chi squared value for 2 degrees of freedom and P=0.05 (5%) is 5.99. As the observed value is lower than 5.99 we accept our hypothesis, that there is no deviation from the expected Mendelian frequency.

Figure 3S1 DNA trace from the B6;129-Kdm5b WT mammary gland transcript at pd 18.5. DNA trace showing anti-sense sequencing data obtained from cDNA reverse transcribed from RNA isolated from B6;129-Kdm5b WT mammary gland tissue at pd18.5 using primers specific to Kdm5b exon 5 (for schematic representation of PCR primer location within exon 1 and 5 see figure 3S2)

Table 3S4 Derived sequence from the DNA trace (Fig 3S1) of B6;129-Kdm5b WT cDNA showing exon 4 to 5 boundaries. Table showing sequencing data obtained from cDNA reverse transcribed from RNA isolated from B6;129-Kdm5b WT mammary gland tissue at pd18.5
Figure 3S2 Murine Kdm5b coding sequence showing translation start site (atg), codon sequence (alternate grey and white highlight), exons 1 to 5 (alternate black (lower case) and red text (upper case)) the JmjN domain (black box), ARID sequence (red box), splice sites (black arrow), exon 1 and exon 5 PCR primer location (horizontal arrow) and amino acid residues removed due to splicing together of exon1 to exon 5 in the ARID sequence. Domain sequence derived from Swiss-Prot:Q80Y84. NCBI Reference Sequence: NM_152895.2
Chapter 4: Results

KDM5B is expressed during mammary gland ductal morphogenesis and is required for normal mammary gland development
Chapter 4. KDM5B is expressed during mammary gland ductal morphogenesis and is required for normal mammary gland development

4.1 Aims:

In Chapter 3 preliminary studies on the chimeric B6;129-Kdm5b \( \Delta \text{ARID} \) murine line have indicated that KDM5B is required for normal mammary gland ductal morphogenesis. However the influence of genetic background on mammary gland morphogenesis has complicated the interpretation of the presenting phenotype in the chimeric B6;129-Kdm5b \( \Delta \text{ARID} \) female. In addition, studies on WT KDM5B expression in the mammary gland are limited. Therefore, understanding the role of KDM5B during mammary gland development could be advanced by the following aims:

1. To examine the pattern and level of KDM5B expression during mammary gland morphogenesis in the C57BL/6J female.
2. To backcross the chimeric B6;129-Kdm5b \( \Delta \text{ARID} \) murine line onto the inbred C57BL/6J genetic background.
3. To characterise the mammary gland phenotype presented in the C57BL/6J-Kdm5b \( \Delta \text{ARID} \) female.

4.2 Introduction: Murine mammary gland ductal morphogenesis

The primary function of the mammary gland is to produce and deliver adequate amounts of milk in order for the female to support her offspring at parturition. Mammary gland ductal morphogenesis occurs in distinct stages with the mammary bud providing all future mammary epithelial cell lineages (Van Keymeulen et al., 2011). Any further mammary gland development is then not fully realized until after birth where further morphogenesis is in synchrony with sexual maturity (puberty) and reproduction. The expansion of mammary epithelium during puberty and pregnancy together with the regenerative capacity of the epithelium during subsequent pregnancies implicates the existence of mammary stem cells. In line with this, the identification of murine and human stem and progenitor populations has provided evidence of a differential hierarchy within mammary epithelium (Shackleton et al., 2006, Stingl et al., 2006, Van Keymeulen et al., 2011, Shehata et al., 2012).
The formation of this extensive tree like network of branched ducts at adulthood provides the foundation for further pregnancy associated alveolar restricted progenitor expansion with differentiation toward milk secreting alveoli lobules in preparation for parturition. At weaning, the milk secreting epithelial cells become redundant and undergo programmed cell death (Kreuzaler et al., 2011), with a morphologically distinct biphasic re-modeling of the mammary gland that ultimately resembles the pre-pregnant mammary gland state (6 days post-weaning) - a process termed involution.

In order to study the molecular mechanisms involved during involution, forced weaning at approximately 7 days promotes synchronous involution, a process that is initiated over 2-3 days and involves a reversible event mediated via local factors rather than systemic hormones (Lund et al., 1996, Li et al., 1997, Marti et al., 1997). This is followed by a second stage that initiates programmed cell death as a consequence of mammary gland remodeling mediated via systemic hormones (Feng et al., 1995 and Lund et al., 1996). Although involution of the mammary gland is marked by two distinct morphological events, these events are under the control of four discrete transcriptional profiles (Clarkson et al., 2004 and Stein et al., 2004).

This process of mammary gland development therefore requires the timely integration of signaling pathways involved in mammary gland epithelial cell proliferation, migration, branching, invasion, differentiation and programmed cell death that are orchestrated at a local level by co-factor interaction and further mediated by reciprocal paracrine interactions between the ductal epithelial cells and the surrounding stroma. Our understanding of KDM5B function during mammary gland morphogenesis would therefore be improved by examining when and in which cells KDM5B is expressed.
4.3 Results

4.3.1 Documentation of KDM5B expression during C57BL/6J murine mammary gland development

To date, studies on KDM5B expression in normal adult mammary gland tissue have been limited (Madsen et al., 2002, Stein et al., 2003). Thus far, Madsen and colleagues (2002) have shown that using in situ hybridisation on sections taken from the adult nulliparous mammary gland, that Kdm5b expression is restricted to ductal epithelial cells (Madsen et al., 2002). Furthermore, these authors also examined Kdm5b expression using Northern hybridization on RNA isolated from mammary gland tissue taken from mid to late pregnancy (day 11, 15 and 18), during lactation (week 1 and 2) and through a period of five days during mammary gland involution (days 1, 2, 3 and 5). This study demonstrated that the expression of the Kdm5b transcript is detected at late pregnancy (day 15 and day 17) with maximal expression seen at pregnancy day 17 and no detectable levels of the Kdm5b transcript being observed during lactation and involution. Using RNA isolated from the mammary gland of Balb/C females, Stein and colleagues (2003), performed microarray analysis to detect the transcript levels expressed during mammary gland morphogenesis. However, the Kdm5b transcript was only detected at low levels throughout mammary gland morphogenesis.

To better understand whether KDM5B may have any potential role during defined stages of murine mammary gland development we embarked on a more detailed examination of the KDM5B cellular localisation and expression profile from puberty through to adulthood (weeks 4 and 12), pregnancy (days 10.5, 12.5, 14.5, 17.5, 18.5) lactation (days 3 and 7) and days 3, 7 and 10 post forced weaning in the C57BL/6J murine line. To achieve this, the thoracic mammary gland was whole mounted and used for in situ hybridisation whilst the inguinal mammary gland was snap frozen for the isolation of protein and RNA and subject to western blot and quantitative RT-PCR (qPCR) (See Fig. 4A, B and C for details of mammary gland developmental stage and technique employed).
Figure 4 Schematic representation of mammary gland ductal morphogenesis detailing the techniques employed to examine KDM5B expression in the C57BL/6J mammary gland (A) nulliparous (B) pregnancy (C) lactation and post weaning.
Development of the post-natal murine mammary gland is initiated at the onset of puberty (4 weeks of age) with the formation of, and proliferation at TEB structures and then fully realised by adulthood (8 weeks of age) at which point the relatively quiescent mammary gland ductal tree is poised for pregnancy associated development. We therefore sought to explore whether KDM5B may have a potential role at these two important stages in the development of the nulliparous murine mammary gland. In order to examine the cellular localisation of the \textit{Kdm5b} transcript by \textit{in situ} hybridisation, 4 and 12 week old C57BL/6J females were culled and the thoracic mammary gland whole mounted. \textit{In situ} hybridisation using an anti-sense riboprobe homologous to the 3' region of the \textit{Kdm5b} transcript (3.9kb to 4.7kb. (Madsen et al., 2002)) has demonstrated that the \textit{Kdm5b} transcript is expressed in a diffuse pattern within epithelial cells of the primary and secondary ductal systems and proliferating TEB structures at 4 weeks of age (Fig 4.1.A). Furthermore, a cross-section through the relatively quiescent adult (12 weeks) mammary gland duct (Fig 4.1.B), demonstrates that the expression of \textit{Kdm5b} is restricted in the main, to the majority of ductal epithelial cells; the expression level being increased compared to that seen in the mammary gland at 4 weeks of age. These data suggest a possible function for KDM5B in the proliferation of mammary gland epithelial cells (at 4 weeks of age) and later the survival and or differentiation of mammary gland epithelial cells in the adult nulliparous mammary gland. We have also demonstrated that at mid-pregnancy (pd12.5) the \textit{Kdm5b} transcript is localised mainly to ductal epithelial cells as well as the epithelium of the alveoli (Fig 4.1.C). Similarly, the \textit{Kdm5b} transcript is detected in the mammary gland at late pregnancy (pd18.5) where the \textit{Kdm5b} transcript is localised to the epithelium of both the duct and alveoli when the murine mammary gland is fully differentiated in preparation for parturition (Fig 4.1.D).
Figure 4.1 *In situ* hybridization showing the *Kdm5b* transcript is restricted in the main to epithelial cells in the C57BL/6J mammary gland. Representative H and E staining on the nulliparous mammary gland at 4 and 12 weeks of age (A and B; top image respectively) and pregnancy at pd12.5 and pd18.5 (C and D; top image respectively). *In situ* hybridization showing the localisation of the *Kdm5b* transcript to mammary gland epithelial cells during C57BL/6J WT mammary gland development (A and B; bottom image at 4 and 12 weeks respectively; C and D; bottom image at pd12.5 and 18.5 respectively). Magnification x20.
4.3.1.2 Quantification of the Kdm5b transcript during murine C57BL/6J mammary gland development

Given the availability of a more sensitive technique – quantitative PCR - for the detection of low-level transcripts, we examined in more detail the level of the Kdm5b transcript during mammary gland morphogenesis. In order to achieve this, cDNA was generated from RNA isolated from inguinal mammary glands taken from the adult virgin (12 weeks), during pregnancy (day 10.5, 12.5 and 17.5) during lactation (day 3 and 7) and through days 3, 7 and 10 post forced weaning. Comparing the Kdm5b transcript level throughout mammary gland development with that expressed in the adult virgin mammary gland, our findings are consistent with our observations using in situ hybridisation, and in part consistent with those findings reported by Madsen and colleagues (2002). We have shown that the Kdm5b transcript is differentially expressed in the developing mammary gland, being detected in the adult nulliparous mammary gland and throughout pregnancy (being maximum at 18.5 days) when epithelial cell proliferation leads to further branching and alveolar expansion followed by differentiation in preparation for parturition. Consistent with Madsen and colleagues (2002), Kdm5b expression is reduced during lactation, however we have shown that the Kdm5b transcript is re-expressed during involution (day 3 post forced weaning) and in the parous mammary gland (Fig 4.2).

![Figure 4.2](image)

Figure 4.2 The Kdm5b transcript is differentially expressed in the C57BL/6J WT murine mammary gland. WRT is an acronym for ‘with respect to’. The Kdm5b amplicon PCR cycle number was normalized against the 18S ribosomal RNA amplicon PCR cycle number. The average expression of 8 adult virgin mice was used to compare the expression of Kdm5b during mammary gland development. RNA was isolated from one mammary gland throughout pregnancy, lactation and post forced weaning. Standard deviation is determined from three RT-PCR experiments. *, p<0.02. **, p<0.05. ***, p< 0.001.
4.3.1.3 **KDM5B protein expression during murine C57BL/6J mammary gland development**

To determine whether the differential \textit{Kdm5b} transcript expression pattern noted during murine mammary gland development correlates with KDM5B protein expression levels, nuclear proteins were extracted from the mammary gland at different stages of development and subject to Western blot analysis (Fig. 4.3). The predicted molecular weight for KDM5B is 170KDa and is expressed in the mammary gland at day 17 of pregnancy (Madsen \textit{et al.}, 2002). Using the same KDM5B specific antibody as used by Madsen and colleagues (2002), we have shown by Western blot transfer that two KDM5B antibody specific bands of approximately equal intensities are expressed in the murine mammary gland at pd18.5; one band at the predicted molecular weight of 170KDa and another band with an approximate molecular weight of 150KDa. Interestingly, Western blot transfer of the nuclear extracts prepared from the mammary gland of the adult virgin through pregnancy, lactation and post forced weaning demonstrates that a 170 KDa KDM5B specific band is expressed throughout mammary gland development, with a noticeable increase at pd18.5. However expression of a KDM5B antibody reactive protein is also detected at approx. 150KDa which is most obvious in the mammary gland at pd18.5, and at days 7 and 10 post forced weaning corresponding to the increased level of the \textit{Kdm5b} transcript detected by quantitative PCR. A faster migrating antibody reactive band is also detected throughout mammary gland morphogenesis, and may represent a KDM5B splice variant.

![Figure 4.3 KDM5B protein expression in the C57BL/6J murine mammary gland. KDM5B is differentially expressed during murine mammary gland development.](image)
4.3.1.4 Backcross of the chimeric homozygous B6;129-Kdm5b ΔARID murine line onto the inbred C57BL/6J genetic strain.

As explained in Chapter 3, the mammary gland morphology presented by chimeric B6;129 off-spring can resemble that of either the parental strain or a intermediate morphology making interpretation of a phenotype difficult (Naylor et al., 2002). Therefore, to establish genetic and phenotypic uniformity in order to better standardize the characterisation of the mammary gland phenotype, the chimeric homozygous B6;129-Kdm5b ΔARID murine line was backcrossed onto the inbred C57BL/6J murine line. A breeding program was instigated between chimeric heterozygous B6;129-Kdm5b ΔARID females and inbred C57BL/6J-Kdm5b WT males to generate F1 progeny. The F1 progeny were genotyped with neomycin specific PCR (Figure 4.4 and Fig. 4S1 in the supplemental material) using tail-snip genomic DNA, and the resulting heterozygous B6;129-Kdm5b ΔARID offspring were paired with the inbred C57BL/6J-Kdm5b WT murine line, repeated over a total of 6 generations (Table 4).

Figure 4.4 Representative neomycin specific PCR reaction on genomic DNA isolation from the ear-snips of a heterozygous B6;129-Kdm5b ΔARID and C57BL/6J-Kdm5b WT breeding program (Data shown from the forth generation offspring of Pair 7 – see Table 4).
Table 4 PCR generated genotyping results from the backcross of the B6;129-Kdm5b ΔARID chimeric line onto the C57BL/6J genetic strain.
Table showing the cumulative genotype results over 6 generations of backcrossing.

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Table showing the cumulative genotype results over 6 generations of backcrossing.
4.3.1.5 Generation of the homozygous C57BL/6J-Kdm5b ΔARID murine line

In order to generate homozygous C57BL/6J-Kdm5b ΔARID offspring, a heterozygous C57BL/6J-Kdm5b ΔARID breeding program was established, and the resulting offspring genotyped by Southern blot analysis (Fig. 4.5) using tail-snip genomic DNA (For schematic representation of screening strategy with probe location used in Southern blot analysis see Chapter 3, Fig. 3.6). Those offspring identified as being homozygous for the Kdm5b ΔARID allele were bred to generate homozygous C57BL/6J-Kdm5b ΔARID females for characterisation of the mammary gland phenotype (For representative Southern blot see Fig. 4.6)

![Figure 4.5 Representative Southern blot on tail-snip genomic DNA isolated from a heterozygous C57BL/6J-Kdm5b ΔARID breeding program showing the Kdm5b WT allele and the engineered allele after BamH1 digest.](image1)

![Figure 4.6 Representative Southern blot on tail-snip genomic DNA isolated from a homozygous C57BL/6J-Kdm5b ΔARID breeding program showing the engineered allele after BamH1 digest.](image2)
4.3.1.6 Kdm5b transcript levels in the C57BL/6J-Kdm5b ΔARID female mammary gland do not reflect KDM5B protein levels

The level of KDM5B protein expression in the chimeric B6;129-Kdm5b ΔARID female mammary gland at pd18.5 is reduced compared to its chimeric B6;129-Kdm5b WT counterpart (Chapter 3, Fig. 3.7). To examine whether this reduced protein expression level is reflected in the C57BL/6J-Kdm5b ΔARID female and correlates with Kdm5b transcript levels, nuclei were extracted for Western blot analysis and the Kdm5b transcript quantified using PCR.

As shown in Fig. 4.7A, the level of the Kdm5b transcript is elevated in the C57BL/6J-Kdm5b ΔARID mammary gland at pd18.5 compared to the C57BL/6J-Kdm5b WT counterpart (p<0.02). Interestingly the elevated level of the Kdm5b transcript expressed in the C57BL/6J-Kdm5b ΔARID mammary gland at pd18.5 does not translate to elevated KDM5B protein levels detected by Western blot analysis (Fig. 4.7B). Furthermore, immunohistochemistry using a KDM5B specific antibody has demonstrated that in comparison with the wild type counterpart, KDM5B specific signal intensity is reduced and slightly fewer KDM5B positive epithelia are detected in the C57BL/6J-Kdm5b ΔARID mammary gland at pd18.5 (Fig.4.7C and Fig.4.7D), and could reflect the reduced KDM5B protein expression level detected by Western blot. In comparison with the C57BL/6J-Kdm5b WT counterpart, the reduced expression level of KDM5B expressed in the mammary gland of the C57BL/6J-Kdm5b ΔARID female at pd18.5 is in keeping with the reduced level of the KDM5B protein documented at the same stage of pregnancy in the mammary gland of the chimeric B6;129-Kdm5b ΔARID female by Western blot analysis.
Figure 4.7 Elevated *Kdm5b* transcript level in the C57BL/6J-*Kdm5b* ΔARID mammary gland at pd18.5 results in the translation of a less abundant KDM5B ΔARID protein (A) Quantitative RT-PCR using primers downstream of the ARID domain showing an increase in the *Kdm5b* ΔARID transcript compared to the *Kdm5b* WT transcript at pd18.5. ( *, P<0.02. WT, n=4. ΔARID, n=5). The *Kdm5b* amplicon PCR cycle number was normalized against the 18S ribosomal RNA amplicon PCR cycle number (B) Western blot showing translation of a smaller, less abundant KDM5B ΔARID protein product in the mammary gland at pd18.5. Immunohistochemistry for KDM5B expression on (C) C57BL/6J-*Kdm5b* WT and (D) C57BL/6J-*Kdm5b* ΔARID mammary gland sections at pd18.5.
4.3.2 Mammary gland development in the C57BL/6J-Kdm5b ΔARID nulliparous female.

4.3.2.1 The C57BL/6J-Kdm5b ΔARID 4-week old mammary gland presents with fewer terminal end buds and a defect in ductal elongation and side branching.

We have shown that the Kdm5b transcript is localised to the luminal epithelial cells at the on-set of puberty (at 4 weeks of age) in C57BL/6J mammary gland (Fig. 4.1) and preliminary data suggest that the chimeric B6;129-Kdm5b ΔARID nulliparous female mammary gland presents with a defect in ductal morphogenesis (Chapter 3). Development at approximately 4 weeks of age of the pubertal nulliparous murine mammary gland ductal tree requires the formation of, and proliferation at TEB structures. We therefore sought to examine whether the expression of a truncated KDM5B protein affects the initial developmental stages of post-natal mammary gland development (TEB formation) by examining the gross morphology of the nulliparous mammary gland at 4 weeks of age. Therefore, at 4 weeks of age the thoracic and inguinal mammary glands were whole mounted and stained with carmine. Figure 4.8A, shows an organised ductal system with TEB formation in the C57BL/6J-Kdm5b WT nulliparous female. However, the C57BL/6J-Kdm5b ΔARID nulliparous gland presents with a less organised ductal system with on average a 2.5 fold reduction in TEB number (Fig. 4.8B and C. *, p<0.0005).
Figure 4.8 Fewer TEB structures in the 4-week old C57BL/6J-Kdm5b ΔARID nulliparous female. Representative carmine on (A) C57BL/6J-Kdm5b WT and (B) C57BL/6J-Kdm5b ΔARID thoracic mammary gland at 4 weeks of age. The C57BL/6J-Kdm5b ΔARID presents with a disorganized ductal structure and a reduction in TEB number. (C) Graph showing a 2.5 fold reduction in TEB number in the C57BL/6J Kdm5b ΔARID mammary gland at 4 weeks of age. (* p<0.0005 n=6 per group).

Whole mount examination of the C57BL/6J-Kdm5b ΔARID inguinal gland demonstrated that abnormal mammary gland development was not specific to the thoracic gland. The use of the lymph node (LN) as a mark for the distance the mammary ductal structures had ramified the fat pad through ductal elongation at TEB structures demonstrated that the C57BL/6J-Kdm5b ΔARID female presented with a defect in ductal elongation as compared to the age matched WT counterpart (Fig 4.9 A, B and C).
Figure 4.9 Abnormal mammary gland ductal development in the 4-week old C57BL/6J-Kdm5b ΔARID nulliparous female. Representative carmine on (A) C57BL/6J-Kdm5b WT and (B) C57BL/6J-Kdm5b ΔARID inguinal mammary gland at 4 weeks of age. The C57BL/6J-Kdm5b ΔARID female presents with a defect in ductal elongation. (C) Graph showing a 3 fold reduction in C57BL/6J-Kdm5b ΔARID mammary gland ductal length at 4 weeks of age. (*, p<0.0005 n=6 per group).

4.3.2.2 The C57BL/6J-Kdm5b ΔARID 12-week old mammary gland presents with fewer bifurcation points, a defect in ductal elongation and side branching.

To determine whether the defect presented in the C57BL/6J-Kdm5b ΔARID at 4 weeks of age is manifest in the C57BL/6J-Kdm5b ΔARID by adulthood (12 weeks) the C57BL/6J-Kdm5b WT and C57BL/6J-Kdm5b ΔARID adult thoracic mammary glands were whole mounted and stained with carmine.
By adulthood the C57BL/6J-Kdm5b WT nulliparous mammary gland had fully ramified the fat pad by ductal elongation, bifurcation at TEB structures and through ductal side branching (Fig. 4.10A). In comparison the mammary gland of the C57BL/6J-Kdm5b ΔARID adult female presented with an extremely disorganised ductal pattern, had failed to ramify the fat pad fully and presented as expected, due to the reduced TEB number, with fewer ductal bifurcation points (Figure 4.10B and C; p<0.005). Additionally, the C57BL/6J-Kdm5b ΔARID adult nulliparous mammary gland presented with a distinct lack of secondary side branches trailing the from the primary ducts (Figure 4.10 B and C).

Figure 4.10 Abnormal mammary gland development in the 12 weeks old (adult) C57BL/6J Kdm5b ΔARID nulliparous female. Representative carmine on (A) C57BL/6J-Kdm5b WT and (B) C57BL/6J-Kdm5b ΔARID thoracic mammary glands at 12 weeks of age. The C57BL/6J-Kdm5b ΔARID presents with a disorganized ductal pattern and a defect in both ductal elongation and ductal side branching. (C) Graph showing reduction in ‘Y’ branch points (bifurcation) in the 12 week old adult (*, p<0.005. WT, n=3. ΔARID, n=4)
The phenotype in the C57BL/6J-Kdm5b ΔARID female mammary at 4 and 12 weeks of age suggests a defect in cell proliferation. However, as detailed in Chapter 3, (Figure 3.12) the mammary gland morphology of the chimeric B6;129-Kdm5b ΔARID female is comparable to that of the chimeric B6;129-Kdm5b WT at pd18.5 and the female can support her pups at parturition. This could suggest that the defect in mammary gland ductal development identified in the nulliparous C57BL/6J-Kdm5b ΔARID female mammary gland at 4 and 12 weeks of age is due to a delay in cell proliferation rather than as a consequence of cell cycle arrest. To examine the proliferative status of the C57BL/6J-Kdm5b WT and C57BL/6J-Kdm5b ΔARID adult mammary gland (12 weeks), sections were stained with the proliferation marker Ki67. By 12 weeks of age the ductal tree of the C57BL/6J-Kdm5b WT adult mammary gland is fully established and therefore relatively quiescent. As expected very few Ki67 positive cells were observed (Figure 4.11A). In contrast, the age matched adult C57BL/6J-Kdm5b ΔARID nulliparous mammary gland presented with areas of Ki67 positive ductal epithelia cells (Figure 4.11B) indicative of proliferation. This suggests that development in the C57BL/6J-Kdm5b ΔARID nulliparous mammary gland is delayed and not arrested, with proliferation signals occurring in the mammary gland ductal epithelia cells of the C57BL/6J-Kdm5b ΔARID female at 12 weeks of age in order to ramify the fat pad.

Figure 4.11 The relatively quiescent state of the adult C57BL/6J-Kdm5b WT nulliparous gland is not mirrored in the adult C57BL/6J-Kdm5b ΔARID female. Representative Ki67 on (A) C57BL/6J-Kdm5b WT and (B) C57BL/6J-Kdm5b ΔARID mammary gland sections at 12 weeks of age. (WT, n=3. ΔARID, n=4).
4.3.2.3 Defective mammary gland development in the C57BL/6J-Kdm5b ΔARID pregnant female.

KDM5B is expressed throughout pregnancy associated epithelial cell proliferation and differentiation (Fig. 4.1, Fig. 4.2 and Fig. 4.3). Therefore, to further examine the affect of KDM5B ΔARID expression on C57BL/6J mammary gland development, we examined the gross morphology of the mammary gland at pd12.5 and pd18.5, mid and late pregnancy respectively. Figures 4.12A and B show the C57BL/6J-Kdm5 WT mammary gland at pd12.5 of pregnancy had undergone further ductal elongation, branching and alveoli development. Interestingly the mammary gland of the C57BL/6J-Kdm5b ΔARID female at the same stage of pregnancy had over come the defect in ductal elongation presented in the adult nulliparous gland and ramified the fat pad further. However, the C57BL/6J-Kdm5b ΔARID female mammary gland at pd12.5 still displayed a defect in ductal side branching and possibly, as a consequence of this, fewer alveoli. (Fig. 4.12 C and D).

Figure 4.12 Defective mammary gland development in the C57BL/6J-Kdm5b ΔARID female at pd12.5. (A, C) Representative carmine and (B, D) Hematoxylin and Eosin staining on (A, B) C57BL/6J-Kdm5 WT and (C, D) C57BL/6J-Kdm5b ΔARID thoracic mammary glands (n=5).
To determine whether the defect presented in the C57BL/6J-Kdm5b ΔARID mammary gland at pd12.5 was due to a possible function of KDM5B in the control of cell proliferation, sections from C57BL/6J-Kdm5b WT and C57BL/6J-Kdm5b ΔARID mammary gland were stained with Ki67, a marker for cell proliferation (Fig.4.13). However, no significant difference in the proliferation of the ductal or alveolar luminal cell population was observed at pd12.5 using the Ki67 antigen as a marker (Fig 4.14). These data suggest that both the C57BL/6J-Kdm5b WT and C57BL/6J-Kdm5b ΔARID ductal/alveolar luminal progenitor cells are expanding at the same rate at this stage of pregnancy.

Figure 4.13 Representative immunohistochemistry showing Ki67 staining in the (A and B) C57BL/6J-kdm5b WT and (C and D) C57BL/6J-Kdm5b ΔARID thoracic mammary glands at day 12.5 of pregnancy (n=4 per group).
Consistent with the phenotype presented on the chimeric B6;129 genetic background, the C57BL/6J-Kdm5b ΔARID females can support their pups at parturition with no obvious difference in the size of the pups noted. This suggested that side branching recovers sufficiently to support alveolar proliferation and full differentiation. Although the gross morphology of the C57BL/6J-Kdm5b ΔARID mammary gland is comparable with the wild type counterpart at late pregnancy (Fig. 4.15A and C), examination of hematoxylin and eosin (H+E) sections demonstrated a significant reduction in the number of alveoli present in the C57BL/6J-Kdm5b ΔARID mammary gland (Fig. 4.15B and D; Fig. 4.16). Even though the numbers of alveoli in the C57BL/6J-Kdm5b ΔARID mammary gland were significantly reduced, the rate of alveolar proliferation as measured by Ki67 was comparable with the wild type counterpart (Fig. 4.17 and Fig. 4.18). Furthermore, whilst the perimeter of the alveoli were comparable between the C57BL/6J-Kdm5b WT and ΔARID females, and fat globules were detected in the lumen of both the C57BL/6J-Kdm5b WT and ΔARID mammary glands, there was a suggestion that secretory activation was more apparent in the C57BL/6J-Kdm5b ΔARID mammary gland (Fig. 4.19), indicative of premature differentiation.
Figure 4.15 Mammary gland development in the C57BL/6J-Kdm5b WT and ΔARID female at late pregnancy. (A and C) Representative carmine and (B and D) hematoxylin and eosin staining on (A and B) C57BL/6J-Kdm5b WT and (C and D) C57BL/6J-Kdm5b ΔARID thoracic mammary glands at pd 18.5 (n=5 per group). Although the C57BL/6J-Kdm5b ΔARID female can support her offspring at parturition fewer alveoli are observed at late pregnancy (pd18.5).

Figure 4.16 The number of alveoli are significantly reduced in the C57BL/6J-Kdm5b ΔARID mammary gland compared to the wild type counterpart at late pregnancy. Six randomly chosen 1mm² areas were selected and the number of alveoli at day 18.5 of pregnancy (pd18.5) in the C57BL/6J-Kdm5b WT and Kdm5b ΔARID mammary gland scored.
Figure 4.17 Representative immunohistochemistry showing Ki67 staining in (A) C57BL/6J-Kdm5b WT and (B) C57BL/6J-Kdm5b ΔARID thoracic mammary glands at day 18.5 of pregnancy (n=4 per group).

Figure 4.18 Percentage of Ki67 positive alveolar cells in the C57BL/6J-Kdm5b WT and C57BL/6J-Kdm5b ΔARID thoracic mammary glands at day 12.5 of pregnancy (n=4 per group).
Figure 4.19 C57BL/6J-Kdm5b WT and ΔARID alveoli perimeters are comparable and fat globules are secreted into the lumen. (A and B) Representative hematoxylin and eosin staining on (A) C57BL/6J-Kdm5b WT and (B) C57BL/6J-Kdm5b ΔARID thoracic mammary glands at pd 18.5 (n=4 per group). Arrow denotes lumen containing fat globules. (C) Three randomly chosen 1mm² areas were selected and the perimeter of the alveoli determined using NanoZoomer Digital Pathology Software (n=3).
4.4 Discussion

In chapter 4 we have used in situ hybridisation, quantitative PCR, Western blot and immunohistochemistry analysis to describe the expression profile of KDM5B throughout mammary gland morphogenesis. Collectively these data suggest a role for KDM5B throughout the development of the mammary gland. Significantly, the nulliparous C57BL/6J-Kdm5b ΔARID female presents with fewer terminal end bud structures and a delay in ductal elongation together with reduced ductal side branching. Furthermore, after a delay in pregnancy associated mammary gland development, by late pregnancy the gross morphology of the mammary gland is comparable with that of the wild-type counterpart and the female can support her pups at parturition.

4.4.1 KDM5B is expressed during postnatal mammary gland morphogenesis and may suggest a role in epithelial cell proliferation, differentiation and survival.

Defining the spatial-temporal expression pattern of KDM5B is important in understanding the function of KDM5B during mammary gland morphogenesis. Examination of the KDM5B transcript level and protein expression profile demonstrated that KDM5B is expressed throughout murine mammary gland morphogenesis in the C57BL/6J mouse. Where an increase in Kdm5b transcription was seen - notably at pd18.5 and day 7 and 10 post weaning – an additional antibody reactive band at approximately 150KDa was also observed. Whether this antibody reactive band represents an alternative KDM5B splice variant or a degradation product has not been investigated. However the possibility of KDM5B splice variant protein products expressed during mammary gland development highlights the possibility of stage and epithelial cell-type specific functions. Furthermore, the KDM5B transcript detected in ductal and alveolar cells by in situ hybridisation used a probe designed to the same region used to study protein expression by Western blot analysis and immunohistochemistry. Interesting the Kdm5b transcript is expressed in only a proportion of epithelial cells in the TEB and ductal region at 4 weeks of age, yet is expressed in the majority of ductal epithelial cells in the adult as well as the ductal and alveolar epithelia during pregnancy. Furthermore, whilst in situ hybridisation and immunohistochemistry has demonstrated that KDM5B is expressed mainly in the epithelia, KDM5B expression is also detected in the stroma and specifically in adipocytes. These studies highlight the potential importance of KDM5B during proliferation, differentiation and survival of mammary gland epithelia and a potential role for KDM5B in a number of signalling pathways.
4.4.2 An increase in Kdm5b transcript level translates to a less abundant KDM5B protein in the C57BL/6J-Kdm5b ΔARID female at pd 18.5.

Examination of KDM5B expression in the C57BL/6J-Kdm5b ΔARID mammary gland female at pd 18.5 confirmed the expression of a smaller less abundant protein but interestingly elevated Kdm5b transcript levels was observed, which may reflect a more stable Kdm5b ΔARID transcript. It is therefore plausible that expression of a ΔARID KDM5B protein may affect expression of the Kdm5b transcript indirectly. However, the increase in Kdm5b transcript levels detected in the C57BL/6J-Kdm5b ΔARID mammary gland female at pd18.5 could also be direct, suggesting the possibility of self-regulation. This hypothesis relies on the ability of KDM5B to function as a transcription repressor, with demethylase activity and therefore repressor function being dependent upon the presence of the ARID and JmJN domains. Alternatively the possible demethylase-independent ability of KDM5B to function as an activator of transcription raises the possibility of a switch in function whereby the ΔARID form of KDM5B can act as an activator of transcription at its own promoter. This interesting question could be addressed by examining Kdm5b promoter-luciferase activity after transfection with KDM5B and KDM5B ΔARID expression constructs. The PCR primers used to quantify the Kdm5b transcript in the C57BL/6J-Kdm5b WT and C57BL/6J-Kdm5b ΔARID mammary gland, do not necessarily detect a full-length transcript whereas the KDM5B specific antibody was raised again the 3′ end of KDM5B (Barrett et al., 2002). This discrepancy between Kdm5b ΔARID transcript and protein level of expression could be due to incomplete translation of the Kdm5b transcript or due to the targeting of the KDM5B ΔARID protein product for degradation.

4.4.3 A function for KDM5B during nulliparous mammary gland development

Significantly the wild type Kdm5b transcript is expressed in the mammary bud at e14.5 (Madsen et al., 2002), a mammary gland developmental stage enriched for multi-potent progenitor cells (Van Keymeulen et al., 2011). This expression data indicates a potential role for KDM5B during lineage specification at the onset of pubertal mammary gland development, and could be addressed using genetic lineage tracing experiments.

Prior to parturition a rudimentary ductal system is established that responds at puberty with the formation of TEB structures. Although not examined here, it is possible, that the defect present in the C57BL/6J-Kdm5b ΔARID female mammary gland at 4 weeks of age is as a consequence of a defect in branching that presents at the embryonic
ductal tree developmental stage, resulting in reduced TEB structure formation in response to oestrogen signalling. Examination of the gross morphology of the newly born rudimentary ductal system would address this issue. However, examination of the extent of ductal ramification in the inguinal mammary gland at 4 weeks of age demonstrates that the C57BL/6J-Kdm5b WT female duct had ramified the fat pad as far as the lymph node, indicating that at 4 weeks of age puberty had already commenced. These data suggest that the C57BL/6J-Kdm5b ΔARID female may have not received the extrinsic stimulus to proliferate. This is of particular importance given that transplantation of ovarian hormone deprived CD24+CD29hi stem cell enriched population into the clear fat pad of recipient adult females resulted in a delay in ductal morphogenesis and furthermore that oestrogen may be required for the survival of the luminal-enriched population (Asselin-Labat et al., 2006, Asselin-Labat et al., 2010, Shehata et al., 2012). Quantification of ovarian hormone serum levels would address these issues. We have not determined the weight of the C57BL/6J-Kdm5b WT and ΔARID murine lines. The size of the C57BL/6J-Kdm5b ΔARID mammary gland fat pad shown in Chapter 3, Figure 3.10B and C (page 95) as well as in Chapter 4, Figure 4.12C (page 126) are smaller than the wild type counterpart. The weight of the C57BL/6J-Kdm5b ΔARID fat pad should be normalised against body weight and compared to the wild type counterpart to accurately determine the extent of fat pad ramification (See final discussion, Chapter 7). Furthermore, images containing lymph nodes should be included to provide a reference point for ductal ramification.

Expression of Kdm5b in the TEB structure of the C57BL/6J-Kdm5b WT female suggests that KDM5B may play a role during ductal morphogenesis. The ability of the C57BL/6J-Kdm5b ΔARID female to generate a functional mammary gland suggests that the mammary gland stem cell population can differentiate toward the ductal lineage, albeit the delay in ductal elongation may represent a reduction in the proliferation of the ductal luminal progenitor population. Although we have not determined whether proliferation is affected in the C57BL/6J-Kdm5b ΔARID mammary gland at 4 weeks of age, studies have indicated a role for KDM5B in the control of the cell cycle (Yamane et al., 2007, Dey et al., 2008, Xie et al., 2011, Wong et al., 2012). It is therefore possible that a defect in proliferation at 4 weeks of age recovers by 12 weeks of age, as seen by the positive Ki67 epithelia, and these studies would therefore benefit from an increase in the number of pubertal stages examined for markers of proliferation. The expression of KDM5B is required for proper G1 to S phase transition of both ES cells (Dey et al., 2008, Xie et al., 2011) and the breast cancer cell line, MCF7 (Yamane et al., 2007, Wong et al., 2012). This requirement for Kdm5b at the G1 to S phase transition may be common to normal murine mammary epithelial cells and
account for the delayed ductal morphogenesis presented in the C57BL/6J-Kdm5b ΔARID nulliparous mammary gland. Mammary gland development in the adult heterozygous B6;129-Kdm5b-Exon1 knock out murine line resembles that of the wild type counterpart. We have not compared the KDM5B expression levels in the heterozygous B6;129-Kdm5b-Exon1 knock out female with the KDM5B ΔARID expression levels in the C57BL/6J-Kdm5b ΔARID female to determine if expressions levels are potentially a factor in effecting mammary gland ductal morphogenesis.

The delay in ductal elongation during puberty and side branching in the adult C57BL/6J-Kdm5b ΔARID mammary gland suggests an involvement of KDM5B in mediating some aspects of the oestrogen and progesterone signaling pathways. KDM5B can interact with the androgen receptor (AR) and potentiate transcription in a ligand dependent manner (Xiang et al., 2007). It is therefore possible that KDM5B may interact with ER alpha and potentiate transcription. In the adult mammary gland, at diestrous an increase in mammary gland cell number and mammary ductal side-branching (Cole et al., 1933, Fata et al., 2001) is attributable to PR expression (Joshi et al., 2010). If KDM5B potentiates transcription in a ligand dependent manner as described for when acting as an AR cofactor, then an interaction of KDM5B with ER alpha may also potentiate Pgr transcription.

The STAT family of proteins are involved in every stage of mammary gland development. STAT5 expression is not required for the generation of mammary gland ductal cells (Yamaji et al., 2009) and therefore ductal elongation during puberty proceeds as seen for the wild type counterpart but side branching is reduced (Santos et al., 2010). The C57BL/6J-Kdm5b ΔARID female presents with a defect in ductal elongation and side branching suggesting that KDM5B may function to mediate both STAT5-dependent and STAT5-independent signalling pathways.

4.4.4 A function for KDM5B during pregnancy associated mammary gland morphogenesis

Three aspects dictate the successful development of the mammary gland during pregnancy and they are proliferation, differentiation and survival. KDM5B is expressed throughout this developmental period and interestingly a KDM5B splice variant is detected late in pregnancy (pd18.5). Of further interest, expression of a KDM5B ΔARID protein results in reduced side branching and alveoli development that recovers sufficiently for the female to support her pups at parturition. These data suggest that although expression of the KDM5B ΔARID protein is sufficient for ductal and alveolar
progenitor expansion and differentiation, a delay in alveolar progenitor cell proliferation is suggested by the presence of fewer alveoli at pd18.5. Although, we have not detected a difference in Ki67 levels compared to the wild type counterpart a delay in cell cycle could account for the ability of the C57BL/6J-Kdm5b ΔARID mammary gland to ‘catch up’ as suggested earlier.

Our analysis on KDM5B expression during mammary gland morphogenesis has extended the current knowledge on the expression profile of KDM5B during mammary gland development. Using Northern blot transfer, a previous study (Madsen et al., 2002) reported that the Kdm5b transcript was not expressed post forced weaning during the first and second phases of involution. However, we cannot confirm the absence of KDM5B expression during the first phase of involution (Madsen et al., 2002), as this stage of mammary gland involution was not included in this study, and this should be addressed. We have however detected KDM5B expression at day 3 post forced weaning suggesting a function for KDM5B during the second phase of involution. These studies would therefore benefit from a detailed analysis of the KDM5B expression profile during both the first and second phases of involution (12hrs, 24hrs, 48hrs, 96hrs and 6 days post forced weaning) and the gross morphology of the C57BL/6J-Kdm5b ΔARID involuting mammary gland examined (see final discussion Chapter 7). KDM5B is also detected at day 7 and day 10 post forced weaning. Interestingly the splice variant seen at day 18.5 of pregnancy when the mammary gland is differentiated is also detected at day 7 and day 10 post forced weaning and could indicate a stage specific function for KDM5B in the parous mammary gland.

We have generated a hypomorphic KDM5B murine line in which both the demethylase activity (Yamane et al., 2007, Xiang et al., 2007) and the DNA binding activity (Scibetta et al., 2007, Yao et al., 2010) have been compromised. The phenotype presented in the nulliparous C57BL/6J-Kdm5b ΔARID female, is suggestive of a function for KDM5B in ER alpha signalling. We will therefore use the C57BL/6J-Kdm5b ΔARID murine line to investigate the effect in the nulliparous mammary gland of KDM5B ΔARID protein expression on ER alpha target genes and those genes reported to be repressed by KDM5B (Scibetta et al., 2007). Mammary gland morphogenesis during pregnancy requires the integration of a number of signalling pathways. The oestrogen and progesterone receptors are required for alveologenesis (Lydon et al., 1998, Brisken et al., 1998, Mulac-Jericevic et al., 2003, Feng et al., 2007) and signalling through these receptors could be affected in the C57BL/6J-Kdm5b ΔARID pregnant mammary gland. Developmental time windows are important during mammary gland morphogenesis. STAT5A is required for the generation of alveolar progenitors as well as their
proliferation, differentiation and survival (Yamaji et al., 2009, Cui et al., 2004), whilst STAT6 mediates proliferation and luminal cell commitment to an alveolar lineage during early pregnancy (Khaled et al., 2007). The phenotype in the pregnant C57BL/6J-Kdm5b ΔARID mammary gland is suggestive of a role for KDM5B in mediating the STAT signalling pathway. We will therefore use the C57BL/6J-Kdm5b ΔARID murine line to explore the effect of KDM5B ΔARID expression on the STAT signalling pathway.
Figure 4S1 Screening strategy used to detect the engineered allele during the backcross of the chimeric heterozygous B6;129-Kdm5b ΔARID murine line onto the inbred C57BL/6J genetic strain. Schematic alignment of KDM5B WT allele and Neo targeting transgene with PCR primer location within the neomycin gene used to distinguish the engineered allele from the wild type allele.
Chapter 5: Results

Investigation into the effect of KDM5B ΔARID expression on gene expression
Chapter 5. Investigation into the effect of KDM5B ΔARID expression on gene regulation

5.1 Aims: Using the C57BL/6J-Kdm5b ΔARID murine line we aim to investigate the effect of KDM5B ΔARID expression on gene expression by:

(1) Examining the effect of KDM5B ΔARID expression on ER alpha target gene regulation during mammary gland development.
(2) Investigating the ability of KDM5B and KDM5B ΔARID to interact with ER alpha.
(3) Examining in the mammary gland, the effect of KDM5B ΔARID expression on the repression of demethylase-dependent KDM5B target genes in the adult mammary gland.
(4) Examining in the mammary gland, the effect of KDM5B ΔARID expression on the AKT and STAT5 signaling pathways.

5.2 Introduction:

5.2.1 KDM5B and gene regulation

Several reports have demonstrated that the function of KDM5B is context dependent, with protein interactions and genome binding location influencing function (Xiang et al., 2007, Secombe et al., 2007, Schmitz et al., 2011 and Xie et al., 2011). Using a differential expression system, approximately 100 genes have been identified as putative KDM5B target genes involved in cell cycle and signal transduction, of which 81% of these were silenced by KDM5B over expression. Of those genes identified, the promoter of the spindle checkpoint gene budding uninhibited by benzimidazole 1 (BUB1) was bound and the gene repressed by KDM5B concomitantly with a decrease in H3K4me3 methylation levels. Metallothionein 1F (MT1F) was also identified as a gene that was bound and repressed in a demethylase dependent manner by KDM5B (Scibetta et al., 2007, Breast Cancer Biology Group unpublished observation). Of interest, MT1F expression positively correlates with the histological grade of breast cancer, being expressed at significantly higher levels in grade 3 tumours (Jin et al., 2001). Furthermore, KDM5B can also bind and repress transcription of the tumour suppressor gene, breast cancer 1 (BRCA1) in a demethylase dependent manner (Yamane et al., 2007).
KDM5B can also function as an activator of transcription (Xiang et al., 2007, Xie et al., 2011), as KDM5B can bind to the androgen receptor and activate transcription from a PSA-luciferase reporter construct (Xiang et al., 2007). The ability of the KDM5 family to interact with nuclear receptors maybe conserved given that KDM5A can also interact with the retinoic acid receptor (RAR), the vitamin D receptor (VDR), the glucocorticoid receptor (GR) and ER alpha (Chan and Hong, 2001).

Signaling through nuclear receptors is of central importance in order to establish a competent mammary gland. Mammary gland ductal morphogenesis at puberty is dependent upon the formation of TEB structures, which in turn requires the expression of ER alpha (Mallepell et al., 2006, Feng et al., 2007). In addition to being required for ductal elongation during puberty, the epithelial expression of ER alpha is essential during pregnancy for alveologenesis and lactation (Feng et al., 2007). Ductal side branching in the adult mammary gland occurs during the diestrous stage of the estrous cycle a process requiring the expression of the progesterone receptor (PR), which results in the up-regulation of Wnt4 expression and expansion of the mammary gland stem cell population (Joshi et al., 2010). Further ductal side branching during pregnancy as well as the development of alveoli structures, is also dependent upon PR expression (Brisken et al., 1998, Mulac-Jericevic et al., 2003) and mediated in a paracrine manner (Brisken et al., 1998, Beleut et al., 2010). One factor, namely WNT4 is implicated in mediating some of the mitogenic effects of progesterone (Brisken et al., 2000, Joshi et al., 2010). Genetic ablation of Wnt4 results in reduced ductal side branching at mid-pregnancy (pd12.5) yet the mammary gland recovers morphologically by late pregnancy to produce a lactation-competent mammary gland (Brisken et al., 2000).

The STAT family of proteins play a central role during gestation (Hughes and Watson 2012). STAT6 expression is required for inducing proliferation and luminal cell commitment to the alveolar lineage (Khaled et al., 2007) and a number of studies have demonstrated the importance of the PRLR/JAK2/STAT5 signalling pathway during the development of the pregnant mammary gland. Mice that harbor genetic alterations in components of the PRLR/JAK2/STAT5 signaling pathway present with an absence of alveolar structures and failed lactation (Lui et al., 1997, Wagner et al., 2004, Horseman et al., 1997, Ormandy et al., 1997). An additional link to the established role of the PRLR/JAK2/STAT5 signalling pathway during pregnancy associated mammary gland development is the compelling evidence that even in the absence of developmental cues associated with pregnancy, AKT signaling is necessary and sufficient to induce
autocrine prolactin production by the mammary gland epithelia, STAT5 activation, and terminal epithelial differentiation (Chen et al., 2012).

We have developed the KDM5B ΔARID murine line that is expected to lack KDM5B demethylase activity – and therefore repressor function (Yamane et al., 2007) - as well as a potential mechanism for the recruitment of KDM5B to DNA (Scibetta et al., 2007, Yao et al., 2010). Given that the mammary gland of the C57BL/6J-Kdm5b ΔARID female presents with a phenotype suggestive of a defect in ER alpha signaling we examined whether KDM5B can interact with ER alpha. In addition we investigated if the levels of ER alpha target genes as well as some of those genes implicated in mammary gland ductal morphogenesis are altered in the C57BL/6J-Kdm5b ΔARID mammary gland. Given that genes involved in cell cycle control (BUB1) and breast cancer progression (BRCA1, MT1F) are repressed in a demethylase dependent manner by KDM5B in a breast cancer cell line, we examined the expression levels of these genes in the C57BL/6J-Kdm5b ΔARID female. The C57BL/6J-Kdm5b ΔARID female mammary gland also presents with a delay in pregnancy associated mammary gland development. We therefore examined whether expression of a KDM5B ΔARID protein affected the expression of a number of key factors whose ablation affects pregnancy associated mammary gland development including ER alpha, PR and WNT4 as well as STAT6 and the AKT and STAT5 signalling pathway.
5.3 Results

5.3.1 Studies on a possible role for KDM5B in ER alpha signalling

5.3.1.1 Analysis of transcription levels of Esr1 and its target genes in the C57BL/6J-Kdm5b ΔARID nulliparous mammary gland

In order to investigate a function for KDM5B in ER alpha signaling we sought to examine the effect of KDM5B ΔARID expression on the ER alpha transcript (Esr1) level of expression and on known ER alpha target genes, namely the progesterone receptor (Pgr) and the downstream transcriptional target of the progesterone receptor (PR), Wnt4. To achieve this, RNA was isolated from the mammary glands of estrous synchronized nulliparous C57BL/6J-Kdm5b WT and C57BL/6J-Kdm5b ΔARID females at the onset of adult mammary gland development (4 weeks) and in the adult (12 weeks). Quantitative PCR on the cDNA generated from the respective C57BL/6J-Kdm5b WT and C57BL/6J-Kdm5b ΔARID mammary glands demonstrated that there is no significant difference in the expression of Esr1 transcript levels at 4 weeks of age (Fig 5). However by 12 weeks of age, although no significant difference in Esr1 transcript levels were detected in the C57BL/6J-Kdm5b ΔARID nulliparous mammary gland compared to the C57BL/6J-Kdm5b WT, a trend indicating a reduction in the level of the Pgr transcript was observed (Fig.5.1) suggesting that KDM5B may function to positively regulate Pgr transcription. To verify an effect on ER alpha signaling in the adult nulliparous C57BL/6J-Kdm5b ΔARID female mammary gland we quantified the transcript levels of a progesterone receptor target gene Wnt4. As shown in Fig. 5.1 Wnt4 transcript expression levels are significantly reduced compared to the C57BL/6J-Kdm5b WT. The reduced Pgr and Wnt4 transcript levels in the C57BL/6J-Kdm5b ΔARID adult nulliparous mammary gland suggests that expression of the KDM5B ΔARID protein may perturb ER alpha signalling, culminating in defective mammary gland morphogenesis.
Figure 5 Relative transcript levels of Esr1 in the C57BL/6J-Kdm5b WT and C57BL/6J-Kdm5b ΔARID nulliparous mammary gland at 4 weeks of age (WT n=3; ΔARID n=2). The acronym WRT is defined as ‘with respect to’. The Esr1 amplicon PCR cycle number was normalized against the 18S ribosomal RNA amplicon PCR cycle number.

Figure 5.1 Relative transcript levels of Esr1, Pgr, and Wnt4 in the C57BL/6J-Kdm5b WT and C57BL/6J-Kdm5b ΔARID nulliparous mammary gland at 12 weeks of age (WT n=3; ΔARID n=4).

5.3.1.2 KDM5B interacts with ER alpha in vivo in an ARID independent manner

To further explore a function for KDM5B in ER alpha signalling we examined whether KDM5B can interact with ER alpha, and whether any interaction is affected by the deletion of the ARID domain. In order to achieve this COS-7 cells were co-transfected with HA-tagged ER alpha and either MYC-HIS-tagged KDM5B or MYC-HIS-tagged KDM5B ΔARID expression constructs. Figure 5.2 demonstrates the ability of the α-HA or α-HIS antibodies to immunoprecipitate ER alpha or KDM5B respectively and the in vivo interaction confirmed by immunoblot detection of KDM5B and ER alpha by α-KDM5B and α-HA antibodies. Of interest, expression of the KDM5B ΔARID protein
does not abrogate an interaction with ER alpha in vivo, suggesting that any effects on ER alpha function are downstream of this interaction.

**Figure 5.2** KDM5B interacts with ER alpha in vivo in an ARID independent manner. COS-7 cells were transfected with HA-ER alpha and MYC-HIS KDM5B WT or MYC-HIS KDM5B ΔARID expression constructs and subject to immunoprecipitation with either HA-beads or HIS-beads and immunblotted with antibodies specific to either KDM5B or HA.

### 5.3.2 Studies on KDM5B repressor function in the adult C57BL/6J-Kdm5b ΔARID mammary gland

The ability of KDM5B to function as a transcriptional repressor is dependent upon its demethylase activity; a function ablated by the deletion of the ARID domain (Yamane et al., 2007, Xiang et al., 2007). As detailed in the introduction, a number of in vitro studies using cell lines have demonstrated that KDM5B can repress transcription from a number of promoters including BUB1 (Breast Cancer Biology Group unpublished observation), BRCA1 (Yamane et al., 2007) and MT1F (Scibetta et al., 2007). However, using quantitative PCR on cDNA generated from RNA isolated from the adult (12 weeks) C57BL/6J-Kdm5b ΔARID and C57BL/6J-Kdm5b WT mammary gland demonstrated that Bub1, Brca1 and Mt1F transcript levels are unaffected in the adult C57BL/6J-Kdm5b ΔARID mammary gland compared with the C57BL/6J-Kdm5b WT counterpart (Fig5.3).
5.3.3 Molecular assessment of the role of KDM5B during development of the C57BL/6J-Kdm5b ΔARID pregnant mammary gland.

The delay in the development of the C57BL/6J-Kdm5b ΔARID pregnant mammary gland would suggest an affect on luminal/alveolar progenitor cell proliferation and differentiation. A number of factors mediate mammary gland epithelial cell hierarchy determination downstream of the ovarian and pituitary axis. To attempt to characterize the molecular pathways affected in the C57BL/6J-Kdm5b ΔARID mammary gland we analyzed the expression of ER alpha, PR, STAT6, STAT5, activated STAT5, AKT, activated AKT, WAP and beta casein at mid and late pregnancy.

5.3.3.1 Molecular analysis in the C57BL/6J-Kdm5b ΔARID mammary gland at mid-pregnancy (pd12.5)

(A) Steroid receptor expression at mid pregnancy

Where ER alpha and its cofactors are required for the orderly development of the pubertal mammary gland, during pregnancy ablation of ER alpha results in a loss in PR expression resulting in reduced side branching, alveolar structures and compromised milk production (Feng et al., 2007). Pregnancy-associated ductal side branching and lobuloalveolar development are markedly reduced in the Prb null murine line due to a decrease in ductal and alveolar epithelial cell proliferation and a decrease in the

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**Figure 5.3** Relative transcript levels of *Bub1*, *Brca1* and *Mt1F* in the C57BL/6J-Kdm5b WT and C57BL/6J-Kdm5b ΔARID nulliparous mammary gland at 12 weeks of age (WT n=3; ΔARID n=4).
survival of alveolar epithelia (Mulac-Jericevic et al., 2003). Furthermore, progesterone can induce WNT4 expression and is required for ductal side branching at pregnancy but is dispensable by late pregnancy (Brisken et al., 2000), reminiscent of the phenotype presented in the C57BL/6J-Kdm5b ΔARID mammary gland. Using the adult C57BL/6J-Kdm5b ΔARID mammary gland, we have shown that whilst the transcript levels of Esr1 are not significantly reduced, a trend indicating a reduction in the level of the Pgr transcript was observed, together with a significant reduction in Wnt4 transcript levels (Fig5.1). These studies presented here suggest that KDM5B could be functioning to mediate some aspects of ER alpha signalling.

Therefore in the first instance we examined the expression of ER alpha, Pgr and Wnt4 at mid pregnancy, a stage of development at which point the C57BL/6J-Kdm5b ΔARID mammary gland presents with a reduction in side branching and alveolar development. Figure 5.4 shows that the expression of ER alpha is not reduced compared to the wild type counterpart. Moreover, whilst a trend indicating a reduction in the expression level of Pgr was observed, Wnt4 expression is not reduced (Fig. 5.5).

![Figure 5.4](image)

**Figure 5.4  Expression of ER alpha in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at mid-pregnancy.**

(A) Western blot showing the expression of ER alpha and HSC70 in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at mid-pregnancy. (B) ImageJ software was used to quantify the band intensity and a histogram plotted showing the expression level of ER alpha normalized against the expression level of HSC70 (n=2 per group).
Figure 5.5 Transcript levels of \textit{Pgr} and \textit{Wnt4} in the C57BL/6J-\textit{Kdm5b WT} and C57BL/6J-\textit{Kdm5b \textDelta ARID} mammary gland at pd12.5 (n=4 per group).

(B) Changes in the JAK/STAT and AKT signaling axis at mid pregnancy

STAT6 expression is required for inducing proliferation and luminal cell commitment to the alveolar lineage, but importantly, proliferation and full differentiation of the mammary gland is achieved by late pregnancy (Khaled et al., 2007). We therefore examined the impact of KDM5B \textDelta ARID expression on STAT6 expression. Figure 5.6 shows that STAT6 expression is comparable between the C57BL/6J-Kdm5b \textDelta ARID and the wild type mammary gland at mid pregnancy indicating that the expression of the KDM5B \textDelta ARID protein has no effect of STAT6 expression.

Figure 5.6 Expression of STAT6 in the C57BL/6J-\textit{Kdm5b WT} and \textDelta ARID mammary gland at mid-pregnancy.

(A) Western blot showing the expression of STAT6 and HSC70 in the C57BL/6J-Kdm5b WT and \textDelta ARID mammary gland at mid-pregnancy. (B) ImageJ software was used to quantify the band intensity and a histogram plotted showing the expression level of STAT6 normalized against the expression level of HSC70 (n=2 per group).
The transcription factor STAT5 plays a pivotal role during mammary gland morphogenesis where it controls the development of alveolar progenitors from stem cells in the nulliparous mammary gland (Yamaji et al., 2009) and during pregnancy where STAT5A is required to promote alveolar proliferation, differentiation and activation of milk proteins as well as the survival of mammary gland epithelia (Cui et al., 2004, Yamaji et al., 2013). STAT5A is required for the development of alveoli structures as early as day 6 of pregnancy (Yamaji et al., 2013), however maximal STAT5 activation does not occur until mid to late pregnancy (Bednorz et al., 2011). Furthermore STAT5A expression is required to induce ductal side-branching in ovariectomised mice supplemented with pregnancy levels of ovarian hormone (Santos et al., 2010). Studies indicate that STAT5 positively regulates the transcription of the pro-survival protein AKT (Creamer et al., 2010), whose activation is required to initiate branch formation in a three-dimensional culture model of murine mammary epithelia (Zhu and Nelson, 2013).

Interestingly, Western blot analysis showed that although the expression level of the STAT5 protein is only marginally increased in the C57BL/6J-Kdm5b ΔARID mammary gland, there is a dramatic reduction in the relative ratio of pSTAT5 to STAT5 (76 percent) and in the level of pSTAT5 expressed in the C57BL/6J-Kdm5b ΔARID mammary gland compared to the wild type counterpart (Figure 5.7). This could account for the reduction in ductal side branching and alveoli development seen in the C57BL/6J-Kdm5b ΔARID mammary gland at mid pregnancy.
Figure 5.7 The relative ratio of pSTAT5 to STAT5 is reduced in the C57BL/6J-Kdm5b \(\Delta\)ARID mammary gland at mid pregnancy.

(A) Western blot showing the expression of STAT5, phosphorylated (p)STAT5 and HSC70 in the C57BL/6J-Kdm5b WT and \(\Delta\)ARID mammary gland at mid-pregnancy. ImageJ software was used to quantify the band intensity and a histogram plotted showing the expression level of (B) STAT5 and (C) pSTAT5 normalized against the expression of HSC70. (D) Histogram showing the relative ratio of pSTAT5 to STAT5 (n=2 per group).

Examination of genes positively regulated by STAT5 shows that in keeping with the reduced relative ratio of pSTAT5 to STAT5 protein levels, there is a marked reduction in AKT expression levels (63 percent) at mid-pregnancy compared to the wild-type mammary gland. Although the relative ratio of pAKT to AKT appears to be increased, the actual level of pAKT is still lower in the C57BL/6J-Kdm5b \(\Delta\)ARID mammary gland compared to the wild type counterpart (Fig. 5.8).
Figure 5.8 AKT expression is reduced in the C57BL/6J-Kdm5b ΔARID mammary gland at mid pregnancy.
(A) Western blot showing the expression of AKT, phosphorylated (p)AKT and HSC70 in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at mid-pregnancy. ImageJ software was used to quantify the band intensity and a histogram plotted showing the expression levels of (B) AKT and (C) pAKT normalized against the expression of HSC70 and (D) Histogram showing the relative ratio of pAKT to AKT (n=2 per group).

The expression of milk proteins, are developmental biomarkers for mammary gland tissue. Therefore, as may have been predicted from the reduction in the levels of pSTAT5, beta casein expression at mid-pregnancy is dramatically reduced in the C57BL/6J-Kdm5b ΔARID mammary gland compared to the wild-type counterpart (89 percent) (Fig. 5.9). Furthermore, whey acidic protein is not expressed in the mammary gland of the C57BL/6J-Kdm5b WT or ΔARID female at mid pregnancy.
Figure 5.9 A delay in mammary gland differentiation is suggested by the reduced beta casein expression level in the C57BL/6J-Kdm5b ΔARID mammary gland at mid pregnancy. (A) Western blot showing the expression of beta casein and whey acid protein in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at mid-pregnancy. ImageJ software was used to quantify the band intensity and a histogram plotted showing the expression level of (B) beta casein normalized against the expression of HSC70. Note that whey acidic protein is not expressed in the C57BL/6J-Kdm5b WT or ΔARID mammary gland at mid pregnancy (n=2 per group).

5.3.3.2 Molecular analysis in the C57BL/6J-Kdm5b ΔARID mammary gland at late pregnancy (pd18.5)

(A) Steroid receptor expression at late pregnancy

The C57BL/6J-Kdm5b ΔARID female can support her pups at parturition and at a gross morphological level the mammary gland resembles that of the wild type counterpart. However, we have observed at late pregnancy a possible increase in fat globules secreted to the lumen in the C57BL/6J-Kdm5b ΔARID mammary gland. Ovariectomy at day 17 of pregnancy triggers premature milk secretion and accumulation of milk proteins in the ductal lumen (Neville et al., 2002), and the reduction in the expression of the progesterone receptor that occurs during late pregnancy contributes to the onset of lactation and terminal differentiation (Ismail et al., 2002). We therefore examined the expression of ER alpha by Western blot analysis and the expression of the progesterone receptor by immunohistochemistry. As seen at mid-pregnancy in the C57BL/6J-Kdm5b ΔARID mammary gland, only a marginal increase was observed in the level of ER alpha expressed in the C57BL/6J-Kdm5b ΔARID mammary gland at pd18.5 compared to the wild type counterpart (Fig. 5.10).
Figure 5.10 Expression of ER alpha in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at late pregnancy.
(A) Western blot showing the expression of ER alpha and HSC70 in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at late pregnancy. (B) ImageJ software was used to quantify the band intensity and a histogram plotted showing the expression level of ER alpha normalized against the expression of HSC70 (n=2 per group).

Of interest, progesterone receptor expression levels and the number of PR positive alveolar epithelia detected in the C57BL/6J-Kdm5b ΔARID female mammary gland is reduced compared to the wild type counterpart (Fig. 5.11). This could indicate that the reduction in PR expression may have accounted for the increase in fat globules secreted to the mammary gland lumen of the C57BL/6J-Kdm5b ΔARID female.
Figure 5.11  Progesterone receptor expression level is reduced in the C57BL/6J-Kdm5b ΔARID mammary gland at late-pregnancy. Immunohistochemistry was performed for progesterone receptor expression on C57BL/6J-Kdm5b WT and C57BL/6J-Kdm5b ΔARID mammary gland sections at late pregnancy. Image of PR expression in the C57BL/6J-Kdm5b WT mammary gland at (A) x10 magnification and (B) x20 magnification. Image of PR expression in the C57BL/6J-Kdm5b ΔARID mammary gland at (C) x10 magnification and (D) x20 magnification. Arrow indicate weakly stained PR positive alveolar epithelia in the C57BL/6J-Kdm5b ΔARID mammary gland (n=2).

(B) Changes in the JAK/STAT and AKT signaling axis at late pregnancy

As seen at mid pregnancy the expression level of STAT6 in the C57BL/6J-Kdm5b ΔARID mammary gland is comparable with the wild type counterpart (Fig.5.6), indicating that expression of a KDM5B ΔARID protein is not overtly affecting STAT6 expression.
Figure 5.12 Expression of STAT6 in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at late pregnancy.
(A) Western blot showing the expression of STAT6 and HSC70 in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at late-pregnancy. (B) ImageJ software was used to quantify the band intensity and a histogram plotted showing the expression level of STAT6 normalized against the expression of HSC70 (n=2 per group).

As the gross morphology of the C57BL/6J-Kdm5b ΔARID mammary gland resembled that of the wild type by late pregnancy and the female can nurse her offspring at parturition we predicted that the relative ratio of pSTAT5 to STAT5 could be equivalent to the wild type counterpart. As shown in Figure 5.13, even though by late pregnancy expression of STAT5 is increased above the level expressed in the wild type counterpart (77 percent), the relative ratio of pSTAT5 to STAT5 is reduced compared to the relative ratio expressed in the wild type mammary gland (50 percent). Also, although western blot analysis on protein isolated from the whole mammary gland, suggests that the expression level of pSTAT5 is comparable with the wild type counterpart, immunohistochemistry analysis demonstrates that pSTAT5 expression is reduced in the alveolar epithelia (Fig.5.14). The discrepancy between the level of pSTAT5 expression seen by immunohistochemistry and Western blot could reflect sample loading. The quantification of pSTAT5 expression from the immunoblot is dependent on the normalization to HSC70. As seen in figure 5.13A, the resolution of the HSC70 bands from the C57BL/6J-Kdm5b ΔARID mammary gland samples is diffuse making accurate quantification less reliable.
Figure 5.13 The relative ratio of pSTAT5 to STAT5 is reduced in the C57BL/6J-Kdm5b ΔARID mammary gland at late pregnancy. (A) Western blot showing the expression of STAT5, phosphorylated (p)STAT5 and HSC70 in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at late pregnancy. ImageJ software was used to quantify the band intensity and a histogram plotted showing the expression level of (B) STAT5 and (C) pSTAT5 normalized against the expression of HSC70 and (D) Histogram showing the relative ratio of pSTAT5 to STAT5 (n=2 per group).
Figure 5.14 Phosphorylated STAT5 expression level is reduced in the C57BL/6J-Kdm5b ΔARID mammary gland at late pregnancy. Immunohistochemistry was performed for phosphorylated STAT5 expression on (A) C57BL/6J-Kdm5b WT and (B) C57BL/6J-Kdm5b ΔARID mammary gland sections at late pregnancy (n=2).

In addition to the proposed role of activated AKT as part of a signalling pathway involved in mammary ductal branch initiation (Zhu et al., 2013), AKT can induce autocrine prolactin secretion from the mammary gland epithelia resulting in the activation of STAT5 and the development of a lactating mammary gland (Chen et al., 2012). Moreover, STAT5 positively regulates the transcription of Akt (Creamer et al., 2010). This indicates that a positive feedback loop exists between STAT5 and AKT. Even though the relative ratio of pSTAT5 to STAT5 in the C57BL/6J-Kdm5b ΔARID mammary gland is 50 percent less at late pregnancy compared to the wild type counterpart, this ratio has increased approximately two fold compared to the relative ratio of pSTAT5 to STAT5 expressed in the C57BL/6J-Kdm5b ΔARID mammary gland at mid-pregnancy. Interestingly, AKT expression increases to near wild type levels (29
percent increase above wild type) and the relative ratio of pAKT to AKT is only slightly reduced compared to the relative ratio expressed in the wild type counterpart (12% percent) (Fig.5.15).

Figure 5.15 AKT expression in the C57BL/6J-\textit{Kdm5b ΔARID} mammary gland at late pregnancy is comparable with the wild type counterpart. (A) Western blot showing the expression of AKT, phosphorylated (p)AKT and HSC70 in the C57BL/6J-\textit{Kdm5b WT} and \textit{ΔARID} mammary gland at late pregnancy. ImageJ software was used to quantify the band intensity and a histogram plotted showing the expression level of (B) AKT and (C) pAKT normalized against the expression of HSC70 and (D) Histogram showing the relative ratio of pAKT to AKT (n=2 per group).

The expression levels of STAT5 are important for gene regulation. For example only 10-15% of total STAT5 expression is sufficient to induce a five-fold increase in beta-casein expression, whereas expression of 30% of the total STAT5 levels resulted in a reduction in the expression levels of WAP by 85% (Yamaji \textit{et al.}, 2013). Given that the
relative ratio of pSTAT5 to STAT5 is reduced by 50 percent in the C57BL/6J-Kdm5b ΔARID mammary gland at late pregnancy compared with the wild type relative ratio, we examined beta casein and whey acidic expression in the C57BL/6J-Kdm5b ΔARID mammary gland at late pregnancy.

Interestingly, compared to the wild type counterpart, beta casein expression increased by 71% and WAP expression increased by 60% in the C57BL/6J-Kdm5b ΔARID mammary gland (Fig. 5.16). These data suggest that the relative ratio of pSTAT to STAT5 is sufficient, or other mechanisms exist that account for the increase in beta casein and WAP expression detected in the C57BL/6J-Kdm5b ΔARID mammary gland at late pregnancy.

Figure 5.16 Beta casein and WAP expression in the C57BL/6J-Kdm5b ΔARID mammary gland at late pregnancy indicative of full differentiation. (A) Western blot showing the expression of beta casein and whey acid protein in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at late pregnancy. ImageJ software was used to quantify the band intensity and a histogram plotted showing the expression level of (B) beta casein and (C) whey acidic protein normalized against the expression of HSC70 (n=2 per group).
Studies suggest that KDM5A and KDM5B may compete for binding, and this competition is related to KDM5 expression levels (Islam et al., 2011b). The delay in differentiation observed in the C57BL/6J-Kdm5b ΔARID mammary gland could suggest that KDM5A compensates for the expression of a less abundant KDM5B ΔARID protein at late pregnancy. We therefore examined the Kdm5a transcript levels in the C57BL/6J-Kdm5b WT and C57BL/6J-Kdm5b ΔARID mammary gland at late pregnancy. Although the expression level of the Kdm5a transcript was not significantly altered in the C57BL/6J-Kdm5b ΔARID mammary gland compared to the wild type counterpart, the expression of KDM5A at pd18.5 could be sufficient for mammary gland recovery; a functional compensation which maybe unique to mammary gland development at late pregnancy (Fig 5.17).

**Figure 5.17** Expression of Kdm5a in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at late pregnancy
Our studies have demonstrated that the expression of a KDM5B ΔARID protein results in a delay in mammary gland development and the female can nurse her pups at parturition. The sample number used for molecular analysis was unavoidably small and is therefore insufficient for statistical analysis. The graphical representation of the immunoblot data is provided solely as a visual tool since loading was not always equal. Therefore the information that it provides is limited. However a couple of notable points are highlighted. These studies would benefit from a larger sample size and further analysis by immunohistochemistry.

As collated in figure 5.18 below, our molecular analysis at mid-pregnancy (pd12.5) demonstrates that whilst the expression of ER alpha, STAT6 and STAT5 are comparable or only slightly increased compared to the wild type counterpart at mid-pregnancy (pd12.5), phosphorylated STAT5 and the relative ratio of phosphorylated to un-phosphorylated STAT5 are dramatically reduced in the C57BL/6J-Kdm5b ΔARID female mammary gland. In keeping with this beta casein, a STAT5 target gene (Yamaji et al., 2013) is also dramatically reduced indicating a less differentiated phenotype. Furthermore AKT, also a STAT5 target gene (Creamer et al., 2010), is markedly reduced and could account for the reduction in the number of side branches observed (Zhu and Nelson, 2013). By late pregnancy (pd18.5), prior to parturition, the level of STAT5 expression increases above the wild type counterpart expression level, and although the relative ratio of phosphorylated to un-phosphorylated STAT5 increases beyond that seen at mid pregnancy (pd12.5), the levels are below that detected in the wild type counterpart at late pregnancy. The expression level of STAT5 is important for gene regulation and may account for the activation of AKT to near wild type levels. In keeping with the ability of the C57BL/6J-Kdm5b ΔARID female to nurse her offspring, the expression level of beta casein and whey acidic protein increases, in fact, above that expressed in the wild type counterpart.
Figure 5.18 Summary of molecular analysis in the C57BL/6J-Kdm5b ΔARID mammary gland. (A) Histogram showing the percentage change in expression of ER alpha, STAT6, STAT5, pSTAT5, AKT, pAKT, beta casein and whey acidic protein in the C57BL/6J-Kdm5b ΔARID mammary gland compared to the wild type counterpart at mid and late pregnancy. (B) Histogram showing the decrease in the relative ratio of pSTAT5 to STAT5 expressed in the C57BL/6J-Kdm5b ΔARID mammary gland compared to the wild type counterpart.
5.4 Discussion

5.4.1 A potential role for KDM5B during oestrogen receptor signaling in the pubertal mammary gland

Development of the mammary gland requires interpretation of upstream hormonal signalling at the level of the genome resulting in the alteration of chromatin structure that permits gene regulation, progenitor cell expansion and lineage determination. Changes in histone methylation patterns are associated with gene expression changes during lineage restriction (Pal et al., 2013) and is therefore a likely mediator of the mammary epithelial cell hierarchy. Expression of a KDM5B protein lacking the ARID sequence would indicate a loss in demethylase activity and DNA binding via the ARID sequence resulting in an alteration in chromatin structure and therefore gene expression. The contribution of the KDM5B ΔARID domain to KDM5B function during mammary gland development will be discussed later in the final discussion.

ER alpha expression is a prerequisite for pubertal mammary gland development (Feng et al., 2007). As Esr1 transcript levels are not affected in the C57BL/6J-Kdm5b ΔARID mammary gland at 4 weeks of age, but the C57BL/6J-Kdm5b ΔARID mammary gland presents with a delay in ductal elongation suggests that KDM5B could function as a ER alpha cofactor and regulate a subset of ER alpha responsive genes. The studies reported here using tagged proteins indicate that KDM5B can form a complex with ER alpha in vivo, independent of the ARID domain. Given that the C57BL/6J-Kdm5b ΔARID adult mammary gland also presents with reduced side branching and we have seen a trend in the reduction in progesterone transcript levels, suggests that KDM5B via the ARID domain recruits ER alpha to the genome and is partially responsible for the activation of progesterone receptor expression. To substantiate this, these studies require further investigation. In the first instance, the expression level of Esr1, Pgr and Wnt4 transcripts would need to be examined on a larger sample size. Furthermore, immunohistochemistry would confirm whether ER alpha and KDM5B are co-expressed, and immunoprecipitation of endogenous ER alpha and KDM5B/KDM5B ΔARID would confirm an in vivo interaction. To determine whether the interaction between ER alpha and KDM5B is direct could be achieved by pull down experiments with glutathione s-transferase (GST) tagged ER alpha and in vitro translated KDM5B or visa versa. Furthermore Chlp for KDM5B/KDM5B ΔARID on the progesterone receptor gene would determine if the ARID domain is responsible for DNA binding and re-Chlp with an ER alpha antibody to establish whether KDM5B and ER alpha are localized to the
same region of the progesterone receptor gene. If proven, this could suggest that KDM5B may function in the context of a luminal epithelial hormone-sensing cell. In this case, KDM5B could potentially regulate mammary gland stem cell expansion and cell fate determination during the development of the nulliparous mammary gland and during gestation (Asselin-Labat et al., 2010, Joshi et al., 2010). One proposed paracrine mediator of progesterone signaling responsible for an increase in mammary gland stem cell number is WNT4, transcript levels of which are reduced in the adult C57BL/6J-Kdm5b ΔARID mammary gland.

5.4.2 Analysis of KDM5B target genes in the adult C57BL/6J-Kdm5b ΔARID mammary gland

Expression of a KDM5B protein without the ARID domain is expected to lack demethylase activity (Yamane et al., 2007, Xiang et al., 2007). Although BUB1, MT1F, and BRCA1 genes are bound and repressed in a demethylase dependent manner by KDM5B in the human breast cancer cell line MCF7, the observation that the levels of these transcripts do not change in the adult nulliparous C57BL/6J-Kdm5b ΔARID mammary gland could be due to a number of considerations. Firstly, a redundant function for KDM5A and KDM5B has been described (Islam et al., 2011b, Chicas et al., 2012). For example, during pRb associated repression of senescence-associated genes, siRNA mediated suppression of either KDM5A or KDM5B expression in senescent cells did not affect the expression of most cell cycle genes tested. However, targeted knocked down of both KDM5A and KDM5B expression cooperated to prevent the repression of these E2F target genes (Chicas et al., 2012). Functional cooperation between KDM5A and KDM5B is not restricted to genes involved in senescence. Similarly, KDM5B was recruited to multiple KDM5A target genes in both KDM5A siRNA targeted cells and Kdm5a null MEFs (Islam et al., 2011b). As KDM5B is the most abundant KDM5 family member expressed in MCF7 cells (Krishnakumar et al., 2010, SC and JTP unpublished observation) the expression level of KDM5A maybe insufficient to compensation for KDM5B mediated gene repression of BUB1, MT1F and BRCA1. Although not examined here, If KDM5A is expressed in the nulliparous adult mammary gland, KDM5A may compensate for the demethylase activity of KDM5B, accounting for the lack of change in Bub1, Mt1F and Brca1 transcript levels expressed in the C57BL/6J-Kdm5b ΔARID mammary gland at 12 weeks of age.
5.4.3 Importance of epigenetic factors in mammary gland development

Given that KDM5B is expressed in embryonic stem cells (Dey et al., 2007, Schmitz et al., 2011, Xie et al., 2011, Kidder et al., 2013) we cannot exclude a possible role for KDM5B in mammary gland stem self-renewal and cell-fate determination. Whilst it is not clear whether KDM5B regulates ES cell self-renewal (Schmitz et al., 2010, Xie et al., 2011, Kidder et al., 2013), the absence of KDM5B impairs differentiation (Schmitz et al., 2011, Kidder et al., 2013) due to the inappropriate expression of pluripotency genes (Schmitz et al., 2011, Kidder et al., 2013) as well as germ cell genes (Schmitz et al., 2011) and the under-expression of developmental genes (Schmitz et al., 2011, Kidder et al., 2013). KDM5B binding is associated with polycomb protein complex target genes enriched in bivalent domains (Schmitz et al., 2010). Interestingly in the absence of EZH2, mammary gland stem cell and luminal activity is reduced and manifests in a delay in pubertal mammary gland development. However although during pregnancy alveoli progenitor cells differentiate, pups die at birth due to a reduction in alveoli structures and therefore insufficient milk production (Pal et al., 2013). Even though alveologenesis proceeds in the C57BL/6J-Kdm5b ΔARID mammary gland, the similar nulliparous mammary gland phenotype to that presented in the mammary gland specific Ezh2 knockout may further implicate KDM5B in mediating gene regulation as part of an EZH2 containing polycomb complex. KDM5A is also implicated in mediating polycomb protein complex target genes (Pasini et al., 2008) and could be responsible for the ‘recovery’ seen in the C57BL/6J-Kdm5b ΔARID mammary gland by late pregnancy.

5.4.4 KDM5B function during pregnancy associated mammary gland development

(A) Early to mid-pregnancy

In the adult nulliparous mammary gland STAT5 expression is required to generate alveolar progenitor cells (Yamaji et al., 2009) that expand and differentiate during pregnancy. Expression from either one allele of Stat5a or Stat5b during pregnancy is sufficient to promote alveoli bud formation, whilst expression from two alleles initiates differentiation similar to expression from all four alleles of Stat5 (Yamaji et al., 2013). Thus, lower levels of activated STAT5 appear to be sufficient to promote alveolar bud formation at mid-pregnancy in the C57BL/6J-Kdm5b ΔARID mammary gland but not differentiation to the level seen in the wild type counterpart. Given that KDM5B expression is required to fully activate STAT5 could suggest that fewer alveolar
progenitor cells reside in the adult nulliparous C57BL/6J-Kdm5b ΔARID mammary gland and/or an effect of alveolar progenitor cell expansion/differentiation during early pregnancy (Cui et al., 2004).

Whist STAT6 expression is not required for the development of the nulliparous mammary gland, during early pregnancy STAT6 expression is required for proliferation and commitment to the alveolar lineage (Khaled et al., 2007). Whilst the studies reported here cannot exclude a function for KDM5B in mediating STAT6 signaling, STAT6 expression levels are relatively unperturbed in the KDM5B ΔARID mammary gland during pregnancy. An attempt was made to examine pSTAT6 expression levels by Western blot but the antibody failed to detect expression. In future the expression of activated STAT6 should be investigated. However activated STAT5, AKT and beta casein levels are reduced at mid-pregnancy indicating a delay in differentiation. Activated STAT5 positively regulates AKT expression (Creamer et al., 2010) and activated AKT is required to initiate branch formation in a three-dimensional culture model of murine mammary epithelia (Zhu et al., 2013). This agrees with the lack of side branches manifest in the C57BL/6J-Kdm5b ΔARID mammary gland at this stage of pregnancy.

The level of ER alpha expression in the C57BL/6J-Kdm5b ΔARID mammary gland at mid pregnancy is comparable with the wild type counterpart. However whilst a trend in the reduction of Pgr transcript expression is observed, Wnt4 transcript level in the C57BL/6J-Kdm5b ΔARID mammary gland is comparable with the wild type counterpart. Given that we have seen a trend in the reduction in Pgr transcript expression and moreover a significant reduction in Wnt4 transcript expression levels in the nulliparous mammary gland, it is possible that KDM5B functions to mediate signaling pathways at distinct mammary gland developmental windows. This observation would be confirmed using immunohistochemical analysis by co-staining for PR and WNT4 expression in the C57BL/6J-Kdm5b WT and ΔARID mammary gland at these stages of development.

Although WNT4 expression is required for ductal side branching at mid-pregnancy (Brisken et al., 2000), other factors such as RANKL and ID4 also mediate side branching and alveologenesis (Fernandez-Valdivia et al., 2009, Dong et al., 2011). Of Interest, ovarian hormone replacement in a Stat5a null genetic background failed to induce Rankl expression (Santos et al., 2010). Furthermore, a PR/STAT5 complex can bind to the ld4 gene in response to prolactin and progesterone to elicit an increase in ld4 expression (Fernandez-Valdivia et al., 2009), highlighting that cross talk between the PRL/JAK2/STAT5 and progesterone signaling pathways is important in mediating
mammary gland morphogenesis. Given the reduction in activated STAT5 levels at mid-pregnancy, RANKL and ID4 are likely candidates in mediating the downstream effects of progesterone and prolactin signaling and could be reduced in the C57BL/6J-Kdm5b ΔARID mammary gland.

(B) Late pregnancy

By late pregnancy, pSTAT5 levels are still reduced compared to the wild type but increased in relation to the activated pSTAT5 levels seen at mid pregnancy in the KDM5B ΔARID mammary gland. The induction of beta casein and whey acidic protein expression would indicate that pSTAT5 levels at this stage of pregnancy are sufficient to induce full differentiation (Yamaji et al., 2013). Interestingly beta casein and WAP are expressed at levels higher in the C57BL/6J-Kdm5b ΔARID mammary gland than in the wild-type counterpart, even though the mammary gland consists of fewer alveoli structures at late pregnancy (See Chapter 4). This suggests that activated AKT may be inducing WAP and beta-casein expression in ductal luminal cells in the absence of alveoli structures (Chen et al., 2012).

The level of ER alpha expression in the C57BL/6J-Kdm5b ΔARID mammary gland is comparable with the wild type counterpart at late pregnancy, yet the expression level of the progesterone receptor is reduced. We attempted to confirm the reduction in progesterone receptor expression seen using immunohistochemistry by Western blot. However the antibodies used failed to detect expression. The progressive reduction in progesterone receptor expression that occurs during late pregnancy has also been suggested to contribute to the onset of lactation and terminal differentiation (Ismail et al., 2002), and may possibly be accelerated in the C57BL/6J-Kdm5b ΔARID female. This could also reflect the suggestion that an increase in fat globules has been observed in the lumen (Chapter 4) and could be examined by immunohistochemistry using a perillipin specific antibody. Furthermore accelerated differentiation is also in keeping with the increase in beta casein and WAP proteins detected in the C57BL/6J-Kdm5b ΔARID mammary gland compared to the wild type at late pregnancy.

5.4.5 The relative ratio of phosphorylated to un-phosphorylated STAT5

The activated STAT5 levels are reduced in the C57BL/6J-Kdm5b ΔARID mammary gland altering the ratio of un-phosphorylated to phosphorylated STAT5 protein levels. Interestingly, it has been reported that un-phosphorylated STAT1 and STAT3 regulate expression of target genes that are distinct from those controlled by activated STAT1.
and STAT3 pathways (Yang and Stark, 2008). Studies in *D. melanogaster* using a leukemia model have demonstrated that the un-phosphorylated form of STAT is localized in heterochromatin in association with HP1 and upon STAT activation results in a loss of heterochromatin formation (Shi *et al.*, 2008). Significantly, un-phosphorylated STAT5 binds to heterochromatin protein 1 alpha (HP1-alpha) and stabilizes heterochromatin formation (Hu *et al.*, 2013). These studies highlight the importance of the non-canonical function of STAT5 and may indicate a function for the increased level of un-phosphorylated STAT5 in the C57BL/6J-Kdm5b ΔARID mammary gland.

5.4.6 The regulation of STAT5 activation

In mammary gland epithelial cells STAT5 can be activated by a number of different signalling pathways that could be affected in the C57BL/6J-Kdm5b ΔARID mammary gland, however JAK2 is a key signaling node for the activation of STAT5 (Wagner *et al.*, 2004), and is mediated by prolactin binding to the prolactin receptor (Horseman *et al.*, 1997. Ormandy *et al.*, 1997). The expression of AKT is essential for the activation of STAT5 via the PRLR/PRL/JAK2 signalling pathway (Chen *et al.*, 2012), yet although AKT expression levels in the C57BL/6J-Kdm5b ΔARID mammary gland at late pregnancy are comparable with the wild type, activated STAT5 is reduced. It would therefore be important to examine prolactin and prolactin receptor expression levels in the C57BL/6J-Kdm5b ΔARID mammary gland. Furthermore STAT5 activation in the mammary gland is also controlled by negative regulators, whose expression could be affected in the C57BL/6J-Kdm5b ΔARID mammary gland including Caveolin-1 (Sotgia *et al.*, 2006), suppressor of cytokine signaling-2 (SOCS2) (Harris *et al.*, 2006), PI3-kinase enhancer A (PIKE-A) (Chan *et al.*, 2010), protein tyrosine phosphatase 1B (PTP1B) (Milani *et al.*, 2013) and p21-activated kinase 1 (PAK1) (Wang *et al.*, 2003).
Chapter 6: Results

Investigation into the effect of KDM5B ΔARID expression on mammary gland tumour development
Chapter 6. Investigation into the effect of KDM5B ΔARID expression on mammary gland tumour development

6.1 Aims: Using the C57BL/6J-Kdm5b ΔARID murine line we aim to investigate the effect of KDMB ΔARID expression on mammary gland tumour development.

6.2 Introduction:

KDM5B was first reported as being expressed in breast cancer (Lu et al., 1999). Since then, KDM5B dys-regulation has been reported in several types of solid tumors, including prostate cancer (Xiang et al., 2007), bladder cancer (Hayami et al., 2010) and melanoma (Roesch et al., 2005, 2006, 2008, 2010), in which KDM5B expression defines a subset of slow cycling melanoma cells that are required for continuous tumour growth (Roesch et al., 2010). Using transplantable tumour models of human (Catchpole et al., 2011) and murine (Yamane et al., 2007) cell lines and by knocking down KDM5B expression using RNA interference, it has been shown by us (Catchpole et al., 2011) and others (Yamane et al., 2007) that KDM5B expression is required for mammary tumour growth. We therefore sought to expand our studies by examining the effect of the KDM5B ΔARID protein on murine mammary gland tumour development.

To examine how expression of a KDM5B ΔARID protein can affect tumour development an endogenous tumour model expressing the KDM5B ΔARID protein is required. This can be achieved by introducing an oncogene, driven by a mammary gland specific promoter, into the C57BL/6J-Kdm5b ΔARID murine line. We therefore made use of the polyoma middle T murine model that expresses the polyoma middle T antigen driven from the mouse mammary tumour virus (MMTV) promoter. This results in the development of spontaneous mammary gland tumours with a 100 percent penetrance.
6.3 Results

6.3.1 The expression of a KDM5B ARID protein results in a delay in mammary gland tumour development

To investigate the role of KDM5B in mammary gland tumour development two parallel breeding programs were initiated; the test group was bred over 3 generations to generate females homozygous for the ARID allele and heterozygous for the polyoma middle T antigen, whilst the control group was bred over the same number of generations in order to control for genetic variability (Fig. 6).

*Figure 6 Schematic representation of the B6;FVB-Kdm5b ΔARID +/- : PyV-mT +/- breeding program. Δ, for the control murine line, the C57BL/6J-Kdm5b WT female was bred with the FVB-MMTV-PyV-mT +/- male over three generations as detailed for the test study. ⭐, ⭐, genotype selected for subsequent breeding programs. B6FVB1, B6FVB2, B6FVB3: F1, F2, F3 generations derived from the breeding program between C57BL/6J and FVB murine strains (See Fig. 6.1, 6.2, 6.3).*

A breeding program was established between a C57BL/6J-Kdm5b ΔARID +/- female and a FVB-MMTV-PyV-mT +/- male. In order to identify and select a breeding male heterozygous for both the Kdm5b ΔARID and polyoma middle T antigen alleles (B6FVB1), genomic DNA was isolated and screened by PCR for the presence of the polyoma middle T antigen allele (Fig 6.1).
Figure 6.1 PCR generated genotype results of a litter from the C57BL/6J-Kdm5b ΔARID +/- female : FVB-MMTV-PyV-mT +/- male breeding program (B6FVBF1). The male identified as positive for the polyoma middle T antigen allele (lane 2, *) was paired with a C57BL/6J-Kdm5b ΔARID +/- female to generate the B6FVBF2 generation.

Once identified, the B6FVBF1-Kdm5b ΔARID +/- : PyV-mT +/- male was paired with a C57BL/6J-Kdm5b ΔARID +/- female and the offspring (B6FVBF2) screened for the presence of the ΔARID allele by Southern blot and the polyoma middle T antigen by PCR (Fig. 6.2 A and B respectively). The final breeding program was then initiated between the C57BL/6J-Kdm5b ΔARID +/- female and the B6FVBF1-Kdm5b ΔARID +/- : PyV-mT +/- male and the ΔARID homozygous status confirmed by Southern blot (Fig 6.3A); the presence of the polyoma middle T antigen confirmed by PCR (Fig 6.3B). Third generation homozygous B6FVBF3-Kdm5b ΔARID or B6FVBF3-Kdm5b WT females heterozygous for the polyoma middle T antigen were monitored from birth and time in days to tumour development noted (See Table 5S1 in the supplemental material).

Figure 6.2 Genotype results of a litter from the B6FVBF3-Kdm5b ΔARID +/- : PyV-mT +/- male and a C57BL/6J-Kdm5b ΔARID +/- female breeding program (B6FVBF2). The offspring were screened for the presence of the (A) KDM5B ΔARID allele by Southern blot and (B) the polyoma middle T antigen by PCR. Lane 3 (*) represents the genotype result for a B6FVBF3-Kdm5b ΔARID +/- male used to pair with a C57BL/6J-Kdm5b ΔARID +/- female to generate the B6FVBF3 generation.
Figure 6.3 Genotype results of a litter from the B6;FVB-Kdm5b ΔARID -/- : PyV-mT +/- male and a C57BL/6J-Kdm5b ΔARID +/- female breeding program (B6FVB3). The offspring were screened for the presence of the (A) KDM5B ΔARID allele by Southern blot and (B) the polyoma middle T antigen by PCR. B6FVB3 offspring were used to monitor tumour development.

The results showed that expression of a KDM5B ΔARID protein, which is expected to lack demethylase activity, results in a delay in spontaneous mammary gland tumour growth (p<0.05). The box-plot in Figure 6.4 demonstrates that the time taken for the B6;FVB- Kdm5b WT:PyV-mT +/- female to present with tumours is varied, ranging from 46 days to 156 days with a median value of 96 days and an interquartile range of 27. In comparison, the time taken for the B6;FVB-Kdm5b ΔARID -/- : PyV-mT +/- female to present with tumours is less varied ranging from 76 days to 130 days with one mammary gland tumour presenting at 162 days being represented as an outlier (See Table 6S1 in the supplemental material). The median value for the time to tumour development in the B6;FVB-Kdm5b ΔARID -/- : PyV-mT +/- female is 108 days with an interquartile range of 21 days. Representing the data as a Kaplan-Meier plot additionally highlights that mammary gland tumours develop earlier in the B6;FVB-Kdm5b WT: PyV-mT +/- female than in the B6;FVB-Kdm5b ΔARID -/- : PyV-mT +/- female. The later appearance of mammary tumours in the B6;FVB-Kdm5b ΔARID -/- : PyV-mT +/- females may reflect the delay in mammary gland development.
Figure 6.4 Box plot showing the distribution of time (in days) taken to develop mammary gland tumours; the median value, 25th and 75th percentile range; sample maximum, minimum and outlier values in the B6;FVB- Kdm5b WT : PyV-mT +/- and B6;FVB- Kdm5b ΔARID -/- : PyV-mT +/- females (n=23 per group).

Figure 6.5 Kaplan-Meier plot showing time to tumour development in the B6;FVB- Kdm5b ΔARID -/- : PyV-mT +/- and B6;FVB- Kdm5b WT : PyV-mT +/- female mammary gland. The B6;FVB- Kdm5b ΔARID -/- : PyV-mT +/- female presents with an initial delay in spontaneous mammary gland tumour development (n=23 per group).
6.4 Discussion

6.4.1 KDM5B ΔARID expression and breast cancer

Studies in this laboratory have shown that on a cohort of over 300 breast cancers that KDM5B is widely expressed in all breast cancer sub-types (unpublished observation). Activated STAT5 is also detected in all sub-types of breast cancer but primarily ER+ tumours and when so, is associated with a more differentiated phenotype, responsiveness to endocrine therapy and an increase in the patient survival rate (Cotarla et al., 2004, Nevalainen et al., 2004, Yamashita et al., 2006, Peck et al., 2011). It would therefore be of interest to correlate expression levels of KDM5B with that of activated STAT5.

Our studies indicate that KDM5B may regulate some aspects of ER alpha signaling and studies suggest that knockdown of KDM5B expression impairs breast cancer cell proliferation in vivo and in vitro (Yamane et al., 2007, Catchpole et al., 2011). Whereas knock down of KDM5B dramatically affects tumour growth in vivo using the ER positive breast cancer cell line MCF7 (Catchpole et al., 2011), reduced expression levels of a KDM5B ΔARID protein in a ER-negative spontaneous mammary gland tumour model (PyV-mT), delays the time to first tumour appearance and the initial growth. This delayed tumour development in the C57BL/6J-Kdm5b ΔARID female possibly reflects the presence of fewer mammary gland epithelial cells. However, breast cancer growth may be dependent upon KDM5B expression levels. This data could suggest that the oncogenic potential of KDM5B may reside in either the demethylase activity of KDM5B, it’s DNA binding ability or both. Interestingly, hemizygous loss of the Stat5 allele also delayed the first appearance of SV40-T antigen mediated tumours (208 days compared with 188 days) (Ren et al., 2002).

Given that polyoma middle T antigen induces tumours that are ER negative whereas the epithelial breast cancer cell line, MCF7 is ER alpha positive and was isolated from a metastatic site, makes comparisons between these two tumour models difficult. The C57BL/6J-Kdm5b ΔARID murine line should therefore be crossed to an ER positive tumour model (Kumar et al., 2007, Chan et al., 2012). However as noted above KDM5B is expressed in all human breast cancer sub-types.

Interestingly, the luminal progenitor gene signature correlates with the gene signature transcribed in the ER-negative polyoma middle T tumour model (Lim et al., 2010). Furthermore, the ER-negative luminal progenitor population most likely represents the alveolar progenitor population (Shehata et al., 2012). By analogy then, given that
STAT5 expression is required for the generation and proliferation of alveolar progenitors suggests that this is the population affected during normal mammary gland development in the C57BL/6J-Kdm5b △ARID murine line.

Whilst activation of STAT5 has been shown to be tumourigenic, with mice expressing a constitutively activated STAT5 protein developing occasional adenocarcinomas after a long latency of between 8 and 12 months (Iavnilovitch et al., 2004, Vafaizadeh et al., 2012), activation of STAT5 via constitutive activation of JAK2 required additional oncogenic insults for tumour development (Caffarel et al., 2012). Activation of JAK2 circumvents apoptosis (Creamer et al., 2010, Caffarel et al., 2012) that is a hallmark of cancer (reviewed Hanahan and Weinberg, 2011). Studies show that activated JAK2 expression and therefore activated STAT5 not only induces proliferation and resistance to cell death but can enhance the expression of differentiation markers in vitro (Caffarel et al., 2012). These authors suggest that activation of the JAK2/STAT5 pathway may have a dual role whereby STAT5 promotes the earlier steps of tumour progression, but maintains the differentiation status of established breast cancers (Caffarel et al., 2012). This may account for the improved prognosis of breast cancer patients expressing activated STAT5 and markers of differentiation.

Studies are currently underway to identify KDM5B specific inhibitors (Sayegh et al., 2013) and significantly, knock down of KDM5B sensitizes cells to genotoxic insults (Li et al., 2014). Collectively this suggests that inhibition of KDM5B in combination with genotoxic drugs, in pSTAT5 positive tumours during the early stages of tumour progression could impinge on two important cellular processes subverted during cancer. (1) inhibition of KDM5B through chemical inhibition would inhibit alveolar hyperplasia by down-regulating the JAK2/STAT5 signaling pathway and (2) upon a second oncogenic insult fail to repair double strand breaks resulting in genomic instability and sensitizing the tumour to genotoxic drugs.
## Supplemental material

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<th>B6;FVB- <em>Kdm5b</em> WT:<em>PyV-mT</em> +/-</th>
<th>Tumour development (Days from birth)</th>
<th>B6;FVB- <em>Kdm5b ΔARID</em> -/-:<em>PyV-mT</em> +/-</th>
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Table 6S1: Table showing time to tumour development (in days from birth) in the B6;FVB- *Kdm5b ΔARID* -/-:*PyV-mT* +/- and B6;FVB- *Kdm5b WT:*PyV-mT* +/- female mammary gland (n=23 per group).
Chapter 7: Discussion
Chapter 7 Discussion

7.1 Summary

In this thesis the attempts to generate a constitutive Kdm5b null murine line is described. Generation of one model, called the B6;129-Kdm5b-Exon1 knock out murine line resulted in constitutive Kdm5b deletion and demonstrated that KDM5B maybe required for embryonic development. A second model, called the C57BL/6J-Kdm5b ΔARID murine line resulted in the expression of a truncated hypomorphic KDM5B protein that lacked 5 of the 42 amino acids that constitute the JMJN domain and the entire ARID domain that would result in a loss of demethylase activity and interfere with direct chromatin interaction. Although otherwise developmentally normal, the C57BL/6J-Kdm5b ΔARID female presented with a delay in ductal morphogenesis at puberty suggestive of a role for KDM5B in mediating oestrogen receptor signalling. In line with this the expression levels of an oestrogen receptor target gene, the progesterone receptor, and a downstream target of the progesterone receptor, Wnt4 are reduced in the adult nulliparous C57BL/6J-Kdm5b ΔARID mammary gland. Furthermore it has been demonstrated that an in vivo interaction between epitope tagged ER alpha and KDM5B is independent of the ARID domain. As the ARID domain is one potential mechanism by which KDM5B can be recruited to the genome we propose that KDM5B may be involved in the recruitment of ER alpha to some regions of the genome. Our analysis of the C57BL/6J-Kdm5b ΔARID pregnant mammary gland showed a delay in mammary gland morphogenesis and the female could nurse her pups at parturition. Investigation into the molecular mechanisms that may relate to this showed that KDM5B expression is required to positively regulate the PRLR/JAK2/STAT5 signalling pathway associated with mammary gland development during gestation. Interestingly, beta casein levels are reduced at mid-pregnancy in the C57BL/6J-Kdm5b ΔARID mammary gland indicating a delay in alveolar differentiation. However, by late pregnancy expression of beta casein exceeds wild type expression level and progesterone receptor expression is reduced indicating premature differentiation.

7.2 The KDM5B null phenotype

The studies presented in this thesis, were the first to report on a Kdm5b null genetic line and these studies demonstrated that KDM5B is essential for embryonic
development (Catchpole et al., 2011). During the writing of this thesis Zou and colleagues (2014) also reported on a \textit{Kdm5b} null genetic line. Whereas the \textit{Kdm5b} null murine line reported in this thesis is on the mixed (B6;129) genetic background, using the C57BL/6J genetic background studies have yielded contrasting phenotypes (Albert et al., 2013, Zou et al., 2014). Albert and colleagues (2013) reported that deletion of the \textit{Kdm5b} gene resulted in major neonatal lethality (Albert et al., 2013), whereas in the \textit{Kdm5b} null murine line reported by Zou and colleagues (2014) the offspring are viable but presented with increased mortality, decreased body weight and the females were infertile beyond three months of age. Furthermore the deletion of \textit{Kdm5b} resulted in the formation of fewer TEB structures and a delay in pubertal mammary gland development. Given that KDM5B expression (Madsen et al., 2002) and molecular mechanisms (Yamane et al., 2007, Pasini et al., 2008, Hao et al., 2012, Kidder et al., 2013) could account for the phenotypes presented in the \textit{Kdm5b} null murine lines reported (Catchpole et al., 2011, Albert et al., 2013, Zou et al., 2014) makes resolving these discrepancies difficult, particularly for those studies on the same genetic background.

However, as discussed in Chapter 3, the neomycin-targeting vector used to generate the \textit{Kdm5b-Exon 1} knock out murine line was designed to replace \textit{Kdm5b} exon 1. The presence of the endogenous \textit{Kdm5b} promoter, and the described cryptic splice sites contained within the neomycin gene (Jin et al., 2005) could potentially lead to the expression of hybrid transcripts consisting of both the neomycin and \textit{Kdm5b} gene sequences (Shearman et al., 2000, Kos et al., 2002). Furthermore, the presence of the neomycin promoter contained within the neomycin targeting vector could drive transcription that could potentially result in the expression of a truncated \textit{Kdm5b} transcript lacking exon 1 of \textit{Kdm5b}. We are currently crossing the B6;129-\textit{Kdm5b-Exon1} knock out murine line with a FVB/N-Tg(ACTB-cre)2MrT/J murine line to remove the neomycin gene in order to address this discrepancy on the role for KDM5B during embryonic development.

### 7.3 Deciphering the role of the KDM5 family of proteins during development

Understanding the function of KDM5B during development is further complicated by the co-expression of the KDM5 family of proteins in a number of tissues and a propensity for compensation. In particular all four of the KDM5 family members are expressed in the embryonic brain (Sheardown et al., 2006, Madsen et al., 2002, Xu et al., 2002, Ouchtkourova et al., 2013) and KDM5B and KDM5D are expressed
during spermatogenesis (Madsen et al., 2003, Akimoto et al., 2008). Furthermore, KDM5A, KDM5B and KDM5C are also expressed in the haematopoietic system (Sheardown et al., 1996, Islam et al., 2011a). Our studies in the murine mammary gland have shown that KDM5B is involved in STAT5 activation and so could potentially affect the development of the lymphocyte system in the C57BL/6J-Kdm5bΔARID murine line. In conclusion, a better understanding of the KDM5 family of proteins during embryonic development and most likely postnatal development of the mammary gland and spermatogenesis awaits combined knock out studies.

However it is worth noting at this stage that the KDM5B ΔARID murine line are fertile beyond three months of age and although the mortality rate was not examined, no obvious survival phenotype was apparent. This suggests that the expression level of a KDM5B ΔARID are sufficient, or other domains of KDM5B are required to rescue some of the phenotypes presented on the Kdm5b null genetic background reported by Zou and colleague (2014). However as discussed below the ARID domain of KDM5B could be required for KDM5B binding to chromatin and the recruitment of cofactors thereby promoting gene expression changes toward the luminal cell lineage.

Despite the reduced body weight in the Kdm5b null murine line reported by Zou and colleagues (2014), when normalised the fat pad was comparable with the wild type counterpart, indicating an intrinsic effect on ductal elongation (Zou et al., 2014). Furthermore oestrogen levels were reduced in the absence of KDM5B (Zou et al., 2014) further highlighting the need to examine hormone levels in the C57BL/6J-Kdm5bΔARID murine line. However, whilst extrinsic effects on pubertal mammary development occur, KDM5B also controls gene regulation as discussed below.

7.4 KDM5B function during pubertal mammary gland

Signalling through the oestrogen receptor regulates pubertal mammary gland ductal elongation and is delayed when a KDM5B protein lacking the ARID domain is expressed (Catchpole et al., 2011 and this thesis), and also in the Kdm5b null murine line (Zou et al., 2014), indicating that the phenotype is attributable to the absence of the ARID domain. In the C57BL/6J-Kdm5bΔARID murine line, DNA binding is compromised and demethylase activity is lost providing an important tool to study the role of the KDM5B ARID domain in gene regulation. Importantly, KDM5B promotes
FoxA1 and Stat5a expression by binding directly to their promoters and facilitating recruitment of the master luminal regulator GATA3. Significantly, an absence of KDM5B results in a decrease in H3K4me3 levels at the activated promoters (Zou et al., 2014) indicating a demethylase independent mechanism for KDM5B-mediated gene activation and is discussed later. It is therefore possible that not only is the ARID domain of KDM5B responsible for the recruitment of cofactors to DNA but it is also the DNA interacting domain that distinguishes between gene activation and repression. Interestingly FoxA1 and Stat5a expression is not entirely dependent upon KDM5B expression and so is only reduced in its absence (Zou et al., 2014). This is in keeping with FoxA1 null mammary epithelia failing to develop beyond the rudimentary ductal structure, whereas FoxA1 haploinsufficiency result in a delay in ductal elongation (Bernado et al., 2010). Furthermore, STAT5A expression is dispensable for ductal elongation but required for ductal side branching during oestrus (Santo et al., 2010), phenotypes affected in the C57BL/6J-Kdm5b ARID mammary gland. To substantiate this, FOXA1 and STAT5A expression levels should therefore be examined in the nulliparous C57BL/6J-Kdm5b ΔARID mammary gland and furthermore ChIp-Seq analysis together with RNA-Seq performed to correlation KDM5B binding with H3K4 methylation levels and gene expression. Whilst further investigation is required, in chapter 4 we propose that KDM5B via the ARID domain recruits ER to the progesterone receptor and is partially responsible for gene activation. In keeping with this, the expression level of the Pgr transcript is also reduced in the Kdm5b null genetic background (Zou et al., 2014) further suggesting a function for the KDM5B ARID domain in gene activation.

If the expression of KDM5B is required for the recruitment of ER alpha to the genome resulting in gene expression as proposed in this thesis, and KDM5B can recruit GATA3 to the promoter of FoxA1 and facilitate expression (Zou et al., 2014), the question is whether there is a causal relationship between ER alpha, GATA3, FOXA1 and KDM5B. Genome wide mapping of ER alpha, GATA3 and FOXA1 in the breast cancer cell line MCF7 has demonstrated that 45 percent of ER alpha binding sites map to GATA3 binding sites, 37 percent of ER alpha binding sites map to FOXA1 binding sites and 30 percent of ER alpha binding sites were co-occupied by GATA3 and FOXA1 (Kong et al., 2011). An important first step would be to correlate KDM5B genome wide binding with that of ER alpha, GATA3 and FOXA1. Genome wide mapping of ER alpha, GATA3 and FOXA1 after RNAi-mediated silencing of KDM5B
expression would go someway in establishing if KDM5B expression is required to recruit these factors to the genome.

The role of KDM5B during nuclear receptor signalling is not clear, with KDM5B being implicated in both the activation and repression of hormone inducible genes. While KDM5B binds to AR and activates transcription from a PSA-Luc reporter construct (Xiang et al., 2007) in a hormone dependent manner, KDM5B forms a repressor complex with progesterone receptor in the absence of hormone (Vincent et al., 2011, 2013). These findings if extrapolated may suggest a function for KDM5B as a gatekeeper to hormone induction. For instance, where the role of KDM5B is to keep genes repressed in the absence of hormones, this would require the demethylase activity of KDM5B. Conversely, in the presence of hormones KDM5B may switch to an activator of transcription and would require DNA interaction via the ARID domain.

7.5 KDM5B, stem cells and progenitor cells

*In vitro* binding studies (EMSA) and expression of KDM5B mutant constructs in cell lines have shown that the KDM5B ARID domain is required for DNA binding (Scibetta et al., 2007, Yao et al., 2010) and demethylase activity (Xiang et al., 2007, Yamane et al., 2007) and therefore repression. We have developed a murine model in which KDM5B mediated activation as well as repression can be studied. In the *Kdm5b* null murine line, gene expression analysis suggests that KDM5B expression not only promotes gene expression changes toward the luminal cell lineage but also represses stem cell associated genes during nulliparous mammary gland development (Zou et al., 2014). This is analogous to the role of KDM5B in embryonic stem cells in which *Kdm5b* depletion results in a delay in the expression of neuronal differentiation markers in part due to the expression of pluripotent genes (Schmitz et al., 2011, Kidder et al., 2013). The phenotype presented in the postnatal C57BL/6J-*Kdm5b* ΔARID mammary gland could be due therefore to continue self-renewal even in the presence of differentiation signals (Kidder et al., 2013) resulting in perturbed postnatal mammary gland development. KDM5B positively regulates *Stat5a* expression in the adult nulliparous mammary gland (Zou et al., 2014) suggesting that the population of alveolar progenitor cells present in the nulliparous C57BL/6J-*Kdm5b* ΔARID mammary gland, and expanded upon pregnancy, is reduced. Whilst the mammary gland cell hierarchy is not alter in the nulliparous mammary gland in
the absence of KDM5B (Zou et al., 2014) an effect on the cell cycle is possible given that KDM5B expression is required for the transition from the G1 phase of the cell cycle (Dey et al., 2007, Yamane et al., 2007).

7.6 KDM5B, pregnancy and involution

7.6.1 KDM5B and the STAT signalling pathway

Our morphological and molecular characterisation of the C57BL/6J-Kdm5b ΔARID mammary gland at mid pregnancy suggests an effect at the very least on luminal ductal progenitor expansion and the expansion/differentiation of the alveolar progenitor pool at mid-pregnancy. The adult mammary gland provides the ductal and alveolar progenitor population that expand during pregnancy providing the mature luminal ductal system and differentiated alveolar structures. The delay in C57BL/6J-Kdm5b ΔARID mammary gland ductal side branching and alveologenesis observed during pregnancy may originally reside in the adult nulliparous mammary gland. Although the morphology of the C57BL/6J-Kdm5b ΔARID mammary gland by late pregnancy resembles the wild type counterpart, significantly fewer alveoli are present, suggesting reduced proliferation. However no difference in Ki67 levels were detected at two stages, mid pregnancy (pd12.5) and late pregnancy (pd18.5). KDM5B expression is required for proper G1 to S phase transition of both ES cells (Dey et al., 2008, Xie et al., 2010) and the breast cancer cell line, MCF7 (Yamane et al., 2007, Wong et al., 2012). This requirement for Kdm5b at the G1 to S phase transition may be common to normal murine mammary epithelial cells during pregnancy. However we have shown a reduction in the pro-survival protein AKT at mid pregnancy, which could indicate an increase in apoptosis at this stage, resulting in fewer alveoli at late pregnancy even in the presence of near normal AKT expression levels. This question could be resolved by examining the expression of cleaved caspase 3 and 6 by immunohistochemistry.

As discussed above KDM5B recruits GATA3 to the FoxA1 and Stat5a promoter resulting in gene expression during nulliparous mammary gland development (Zou et al., 2014). KDM5B most likely mediates transcription of only a subset of GATA3 responsive genes given that GATA3 expression is required for placode formation and postnatal mammary gland ductal and alveolar progenitor differentiation (Asselin-Labat et al., 2007). Therefore an effect on a subset of GATA3 responsive genes in
the C57BL/6J-Kdm5b ΔARID mammary gland during pregnancy-associated development is also possible.

GATA3 expression is required for the expression of Th2 cytokines IL4, IL5, and IL13 during T cell differentiation (Sasaki et al., 2013). The expression of IL4 and IL13 activates STAT6 leading to proliferation and luminal cell commitment to an alveolar lineage (Khaled et al., 2007). It is certainly intriguing to imagine that during early mammary gland differentiation KDM5B is involved in the recruitment of GATA3 to the IL4 and IL13 genes leading to expression, STAT6 activation and mammary gland differentiation, which is delayed in the C57BL/6J-Kdm5b ΔARID mammary gland. To further substantiate this, while total STAT5 protein levels remain comparable, activated STAT5 levels are also reduced in the Stat6 null (Khaled et al., 2007) and C57BL/6J-Kdm5b ΔARID genetic backgrounds throughout gestation. These studies would therefore be greatly enhanced by a morphological and molecular characterisation of early gestation stages in the C57BL/6J-Kdm5b ΔARID mammary gland. GATA3 has also been shown to activate STAT5 in the hematopoietic system (Bhattacharya et al., 2001), via the expression of IL5 and by the same analogy maybe reduced in the C57BL/6J-Kdm5b ΔARID mammary gland.

Given that overexpression and forced activation of STAT5 delays post-lactational involution (Iavnilovitch et al., 2004) could suggest that involution in the C57BL/6J-Kdm5b ΔARID mammary gland is accelerated and therefore the gross morphology of the mammary gland should be examined post forced weaning. Furthermore, as detailed above, the recent evidence that KDM5B is involved in the recruitment of GATA3 to specific genes (Zou et al., 2014) could implicate KDM5B in mediating STAT6 activation. This could suggest that KDM5B is involved in the general activation of STAT family members and raises the question whether KDM5B is involved in the activation of STAT3?

As discussed below KDM5B is likely to be involved in a number of signalling pathways that control mammary gland morphogenesis. In keeping with this, although activated STAT5 is required for alveolar progenitor expansion and differentiation it is dispensable for ductal development (Liu et al., 1997, Cui et al., 2004, Yamaji et al., 2009), suggesting that STAT5 dependent and independent pathways are affected in the C57BL/6J-Kdm5b ΔARID mammary gland. The same analogy applies to STAT6 signalling given that the Stat6 null nulliparous mammary gland presents with no
apparent defect and AKT expression levels are unaffected during pregnancy. To
further these studies and define the function of KDM5B and particularly the role of
the ARID sequence, requires defining those genes that KDM5B directly interacts
with and regulates, together with changes in H3K4 methylation patterns in the
different subpopulations of mammary gland epithelia. In the first instance therefore, it
could prove informative to examine in the C57BL/6J-\textit{Kdm5b} \textit{ΔARID} mammary gland
throughout mammary gland morphogenesis, the effect of KDM5B \textit{ΔARID} expression
on H3K4 methylation patterns by immunohistochemistry.

Multiple signalling pathways are integrated permitting cell proliferation, migration and
differentiation, in order to establish the pattern of the mammary gland. KDM5B can
elicit its function as part of a multi-protein complex that contain other chromatin-
modifying factors (Barrett \textit{et al.}, 2007, Banck \textit{et al.}, 2009) to control transcription
through the modulation of chromatin structure. The function of KDM5B has been
implicated in the control of a number of different signalling pathways including those
involved in cell cycle (Yamane \textit{et al.}, 2007, Dey \textit{et al.}, 2008, Xie \textit{et al.}, 2011, Wong \textit{et al.}, 2012), differentiation (Schmitz \textit{et al.}, 2011, Kidder \textit{et al.}, 2013) and migration (Li \textit{et al.}, 2011, Yoshida \textit{et al.}, 2011), which are important during mammary gland
morphogenesis.

7.7 KDM5B, signalling pathways and the development of the mammary gland.

Most significantly, \textit{KDM5B} was initially identified as a transcript down regulated in
response to inhibition of the c-ErbB2 signalling pathway in a human mammary gland
epithelial cell line (Lu \textit{et al.}, 1999). As seen for the C57BL/6J-\textit{Kdm5b} \textit{ΔARID} murine line, ablation of \textit{Erbb2} expression also result in a delay in pubertal ductal
morphogenesis and the female can support her offspring at parturition (Jackson-
Fisher \textit{et al.}, 2004, Andrechek \textit{et al.}, 2005) Although changes in mammary gland
morphology during pregnancy were not examined in the \textit{Erbb2} null female these
studies may further support a role for KDM5B downstream of the c-ErbB2 signalling
pathway.

The NOTCH signalling pathway plays a central role during mammary gland
morphogenesis by not only directing mammary gland stem cells toward a luminal cell
fate during puberty (Bouras \textit{et al.}, 2008), but by also maintaining the luminal cell fate
during pregnancy and thereby regulating alveolar development (Buono et al., 2006). Although studies have not been conducted in order to examine a function for KDM5B as part of the NOTCH signalling pathway, KDM5A and the *D. Melanogaster* Lid can interact with the NOTCH pathway effector protein, RBP-J in mammals and repress NOTCH target genes (Moshkin et al., 2009, Liefke et al., 2010). This may suggest that the KDM5 family of proteins may function as NOTCH signalling pathway effector proteins. Loss of RBP-J expression in mammary gland stem cells lead to the expansion of basal cells (Bouras et al., 2008) as did the loss of RBP-J expression from luminal cells during pregnancy (Buono et al., 2006).

Reduced TEB number has been reported for a number of cytokine and chemokine deficient murine models. Studies have also implicated KDM5B in the regulation of immune mediators (Li et al., 2011) further highlighting a function for KDM5B during the invasion and ductal patterning of the murine mammary gland. Although the functional consequence of KDM5B promoter binding was not assessed, never-the-less gene ontology analysis has categorized KDM5B as a protein whose binding is enriched at promoter sites of genes encoding members of the chemokine pathway (Li et al., 2011). In addition, in order for myeloid progenitor cells to differentiate to macrophages, expression of the micro-RNA-17-92 cluster is repressed in a demethylase dependent manner by KDM5B (Pospisil et al., 2011). In deed macrophages are recruited to the TEB by CSF1 (colony stimulating factor 1) and mice homozygous null for Csf1 present with a depleted macrophage population, and impaired TEB formation, ductal elongation and side branching (Gouon-Evans et al., 2000, Van Nguyen et al., 2002).

During mammary gland morphogenesis epithelial cells become incompletely polarized, multi-layered and remodel cell junctions to allow cell rearrangement (Ewald et al., 2008). A genome wide transcriptional analysis of TEB structures revealed an increase in the epithelial to mesenchmal-related transcripts including *Snai1* and *Snai2* (Kouros-Mehr et al., 2006). Support for an EMT-like event during mammary gland ductal patterning was further suggested by the observation that green fluorescent protein driven from the vimentin promoter was detected at branch points using an in vitro system to control ductal patterning (Nelson et al., 2006). Interestingly, KDM5B has also been implicated in promoting the epithelial to mesenchyme transition in both cancer cell lines as well as the mouse mammary epithelial cell line NMuMG. The expression of KDM5B can repress the expression of the microRNA-200 (miRNA-200) family resulting in an increase in ZEB1 and ZEB2
levels followed by downregulation of E-cadherin and upregulation of mesenchymal marker genes, $Snai1$ and $Snai2$ (Enkhbaatar et al., 2013).

7.8 Demethylase independent function of the KDM5 family of proteins

The expression of a KDM5B lacking part of the JMJD domain and the entire ARID domain indicates that other demethylase independent aspects of KDM5B function are preserved. This highlights the importance of defining the demethylase independent functions of KDM5B, which to date have not been addressed. However, a demethylase independent function for other members of the KDM5 family have been described, a function that is potentially conserved.

The lower organisms $D.\ Melanogaster$ and $C.\ Elegans$ express only a single orthologue of the KDM5 family namely Lid and rbr-2 respectively. The viability of $D.\ Melanogaster$ is not dependent on the demethylase activity of Lid (Li et al., 2010), and a mutant strain of $C.\ Elegans$ expressing rbr-2 lacking demethylase activity presents with only a defect in vulvamorphogenesis highlighting the functional importance of the other domains of the KDM5 family of proteins (Christensen et al., 2007). Studies have shown that the enzyme activity of histone modifying enzymes can be modulated through the association with other proteins (Lee et al., 2009, DiTacchio et al., 2011). Interestingly, KDM5A and Lid inhibit the deacetylase activity of HDAC1 and Rpd3 (a HDAC homologue) respectively to activate transcription at specific loci (Lee et al., 2009, DiTacchio et al., 2011). The co-expression of Lid and MYC potentiates transcription and a Lid/MYC interaction inhibits Lid demethylase activity (Secombe et al., 2007). Furthermore KDM5B and MYC interact in vivo (SC and JTP, unpublished observation) suggesting functional conservation. Although the mechanism for gene activation was not addressed by these authors (Secombe et al., 2007), deacetylase inhibition by Lid, in a demethylase independent manner is plausible. Taken together this data suggest that the KDM5 family may perform two independent functions. One function is to demethylate H3K4 resulting in gene repression, whereas the other is to antagonize HDAC activity to promote transcription.

A potential model for KDM5B function is summarised below. The studies reported in this thesis together with published data suggest that expression of a KDM5B ΔARID protein potentially negates DNA binding activity and GATA3 recruitment to the genome resulting in reduced $FoxA1$ and $Stat5a$ expression in the nulliparous
mammary gland. In the C57BL/6J-Kdm5b ΔARID mammary gland, KDM5B demethylase activity and repression is also negated resulting in an increase in the stem cell gene signature. The overall effect of KDM5B ΔARID expression is a delay in pubertal mammary gland development. During pregnancy, expression of a KDM5B ΔARID protein could potentially negate KDM5B DNA binding activity and GATA3 recruitment to the IL4, IL5, IL13 genes resulting in reduced expression. The consequence is reduced activation of STAT6 and a delay in pregnancy associated mammary gland development. Reduced IL5 expression results in a lower level of STAT5 activation resulting in a reduction in AKT and beta casein expression at mid-pregnancy. Elevated expression levels of activated STAT5 at late pregnancy are potentially sufficient to induce AKT, beta casein and whey acidic protein expression and thus a lactating mammary gland.

The work reported in this thesis has contributed to our knowledge of the function of KDM5B in the development of the mammary gland. This work has opened the way forward to further investigations.
Chapter 8: References
Chapter 8 References


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