Unravelling the Role of the Transcription Factor *Plag1* in the Mouse Epididymis

Joanne Wong

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Department of Physiology, Anatomy and Microbiology

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List of abbreviations

BSA	Bovine serum albumin
DEG	Differentially expressed gene
DIG	Digoxigenin
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
FDR	False detection rate
FSH	Follicle-stimulating hormone
GO	Gene ontology
HET	Heterozygote
HPG	Hypothalamo-pituitary-gonadal
IGF	Insulin-like growth factor
KO	Knockout
LH	Luteinising hormone
MMP	Matrix metalloproteinase
PBS	Phosphate-buffered saline
PCD	Primary ciliary dyskinesia
PCR	Polymerase chain reaction
PLAG1	Pleomorphic adenoma gene 1
qPCR	Quantitative polymerase chain reaction (PCR)
SNP	Single-nucleotide polymorphism
SRS	Silver-Russell syndrome
TSS	Transcriptional start site
TIMP	Tissue inhibitors of metalloproteinases
VNTR	Variable-number tandem repeat
WT	Wild-type
X-gal	5-bromo-4-chloro-3-indolyl-D-galactopyranoside

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Abstract

Pleomorphic adenoma gene 1 (PLAG1) is a transcription factor required for normal sperm motility and male fertility as shown by *Plag1* knockout (KO) mice; however, the underlying cause of the sperm motility issues seen in *Plag1* KO mice is unknown. Since sperm gain their motility during sperm maturation, which occurs during transit through the metre-long epididymis tubule, it was hypothesised that PLAG1 deficiency affects epididymis structure and function. This research aimed to investigate the role of *Plag1* in the mouse epididymis by characterising the expression pattern of PLAG1 in the mouse epididymis, the effect of PLAG1-deficiency on epididymal morphology and identifying the target genes of PLAG1 in the epididymis. Widespread PLAG1 expression was detected in all regions of the mouse epididymis, and the staining pattern indicated that it may be secreted or degraded in the cauda epididymidis. *Plag1* KO mice exhibited significantly reduced epididymis weight relative to body weight, a reduction in epididymal tubule elongation and coiling, and the absence of the characteristic bulb shape of the cauda epididymidis. These abnormalities were not a result of delayed puberty, as the same morphology was observed in older mice. Transcriptome analysis revealed that a number of sperm maturation genes were dysregulated in the epididymis of *Plag1* KO mice, and several of these genes are known to be involved in the prevention of premature sperm capacitation. Interestingly, there was also an overall upregulation of genes encoding extracellular matrix (ECM) components and the overall downregulation of the metalloproteinases responsible for degrading ECM components in the epididymis of *Plag1* KO mice. Indeed, protein quantification of dysregulated ECM components, including collagen and laminin, indicated that in the absence of PLAG1, these ECM components are aberrantly expressed in the epididymis. Taken together, this research indicates that PLAG1 is required for normal epididymis development, and that PLAG1 may be a regulator of both sperm maturation and maintenance of epididymal tissue homeostasis.

Statement of authorship

This thesis includes work by the author that has been published or accepted for publication as described in the text. Except where reference is made in the text of the thesis, this thesis contains no other material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma. No other person's work has been used without due acknowledgement in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

Signature:

Date:

05/10/2020

Acknowledgements

As I sit down to write my acknowledgements, I cannot help but chuckle at the how crazy the last few years have been (or perhaps I am just delirious, at this point!). If someone had told me 3 and a half years ago, that my PhD journey would involve changing supervisors, a worldwide pandemic and spending half of 2020 in lockdown, amongst the other curve-balls that life brings in general, I may have turned and ran the other way. Yet here I am at the end of it. It has been a rollercoaster few years, but I am proud (and very relieved) to have made it to the end.

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Chapter 1. General introduction

Pleomorphic adenoma gene 1 (PLAG1) is a zinc finger transcription factor and proto-oncogene initially studied for its role in tumourigenesis. However, subsequent research using a *Plag1* knockout (KO) mouse model revealed significant growth and fertility impairments. The mechanisms behind PLAG1-related cancers are relatively well characterised and *PLAG1* mutations continue to be implicated in tumour formation in a range of cancer types, including three novel mutations most recently identified in lipoblastomas (Wang, Guzman & Batanian 2019). However, the role of PLAG1 in normal physiology is less well understood. Substantial developmental and fertility abnormalities are observed in mice deficient in PLAG1, providing indications of the biological and physiological processes that PLAG1 may be important for.

Early research showed that KO of *Plag1* results in severe growth retardation, which begins in embryonic development and persists into adulthood (Hensen et al. 2004). Although initial studies reported that relative organ weight was normal for all organs aside from the ventral prostrate and seminal vesicles (Hensen et al. 2004), subsequent analysis showed a significant reduction in testis weight in *Plag1* KO mice, relative to body weight, and that daily sperm production was significantly reduced in the absence of PLAG1 (Juma et al. 2017). Furthermore, PLAG1 is required for normal male fertility; initial studies showed that *Plag1* KO males were able to fertilise only 7% of the wild-type (WT) females they were mated with, and matings between KO males and females produced small and infrequent litters (Hensen et al. 2004). However, in our experience, we did not see a single instance of litters being generated from *Plag1* KO males, despite over 100 mating pairs and groups housed together for extended periods of time. This disparity in results indicated a need for a more in-depth breeding analysis with clear parameters to ensure accurate analysis of the fertility status of *Plag1* KO mice and to confirm whether *Plag1* KO male mice are sub-fertile or fully infertile.

Interestingly, in addition to reduced daily sperm production, *Plag1* KO mice exhibited a decreased percentage of motile sperm in the cauda epididymidis and a dramatic reduction in the percentage of sperm showing progressive motility (Juma et al. 2017). A reduction in the fraction of

rapidly motile sperm and an increased fraction of static sperm was also reported (Juma et al. 2017). Additionally, premature sloughing of the germinal epithelium, increased numbers of dying cells and the dysregulation of spermatogenesis genes was found in the testes from *Plag1* KO mice (Juma et al. 2017). These results, together with the decreased daily sperm production, are likely to be significant factors in the fertility problems observed in *Plag1* KO males. However, as sperm motility is gained during epididymal sperm maturation, which occurs during the transit of the spermatozoa through the epididymis, the aberrant sperm motility in *Plag1* KO mice suggests that epididymal dysfunction may also be involved in the fertility problems observed in *Plag1* KO male mice. Furthermore, while spermatogenesis is affected in *Plag1* KO mice, there is apparently still a proportion of spermatozoa undergoing normal spermatogenesis, which would ostensibly result in the production of some viable sperm. However, in our colony of *Plag1* KO mice, males appear to be completely infertile, indicating that there may be dysfunctional post-testicular sperm maturation that impairs fertility in these mice. Therefore, this thesis investigates the role of *Plag1* in the mouse epididymis to further uncover the fertility defects in *Plag1* KO mice and the role that *Plag1* may play in epididymis structure and function.

The aim of this research was to investigate the epididymis as the underlying cause of the sperm motility abnormalities observed in *Plag1* KO mice. The epididymis is a an understudied component of the male reproductive system. In animal models of infertility, studies are generally focused on testicular function, which may be partly due to the fact that epididymis function, namely sperm maturation, remains somewhat of an enigma. As the cause of 40–50% of clinical male infertility cases cannot be determined (Jungwirth et al. 2012), research on epididymis structure and function in rodent models of male infertility could potentially be beneficial for understanding clinical male infertility.

This thesis begins with a literature review in **Chapter 2** on the current knowledge of the role of *Plag1* in tumourigenesis, growth and reproduction, with a focus on male fertility. I discuss recently published data that support a potential role for PLAG1 in the mouse epididymis and provide an overview of epididymis structure, development and function. In the following chapters, I aim to determine whether *Plag1* does indeed play a role in the mouse epididymis that may contribute to

the reduction in fertility and sperm motility defects in *Plag1* KO male mice, and to characterise impairments in epididymal structure, morphology and function when PLAG1 is deficient.

In **Chapter 3** the expression of PLAG1 in the mouse epididymis was characterised for the first time and the morphology of the epididymis in *Plag1* KO mice is investigated. Additionally, given there are contradicting reports and observations of the fertility status of *Plag1* KO male mice, a comprehensive breeding analysis was conducted. I also report the fascinating finding that the *Plag1* KO genotype is significantly associated with the complete absence of one testis and epididymis, a condition termed unilateral anorchidism. This chapter was published in the Asian Journal of Andrology (Wong et al. 2019).

Epididymal sperm maturation, the process by which spermatozoa gain their motility and fertilisation capacity, is facilitated by a multitude of proteins and factors encoded by epididymal genes expressed in a specific pattern (Dacheux & Dacheux 2014). Given that spermatozoa themselves are transcriptionally inactive, the biochemical and structural modifications that encompass sperm maturation are dependent on the tightly regulated expression of epididymal genes, and changes to gene expression can have detrimental effects on sperm maturation. Therefore, in **Chapter 4** the epididymal transcriptome in *Plag1* KO and WT mice was compared using RNA sequencing. I determined if gene expression is altered in the absence of PLAG1 and identified PLAG1 target genes in the mouse epididymis. In addition, using bioinformatics I identified biological pathways or processes that are affected when PLAG1 is absent. Lastly, to determine if PLAG1 may directly regulate differentially expressed genes in the epididymis of *Plag1* KO mice, enrichment of PLAG1 binding sites was analysed. This chapter has been accepted for publication in Developmental Dynamics (Wong et al. 2020).

In **Chapter 5**, the interesting finding from the transcriptome analysis that extracellular matrix (ECM) genes are significantly upregulated and metalloproteinases are significantly downregulated in the epididymis of *Plag1* KO mice was further investigated. The ECM is generally not investigated in mouse models of male infertility; therefore, in this chapter I aimed to characterise the ECM in the epididymis of *Plag1* KO mice. The dysregulation of several key ECM components

identified by the RNA sequencing analysis (Chapter 4) was confirmed at the protein level using immunofluorescent staining and image analysis. Also, the mRNA expression patterns of selected sperm maturation genes, previously identified by RNA sequencing as being up- or downregulated in the epididymis of *Plag1* KO mice (Chapter 4), were characterised using *in situ* hybridisation to determine if the expression patterns of these target genes is altered in the absence of PLAG1.

Finally, in **Chapter 6**, the main findings in the aforementioned chapters are summarised and placed into the context of the current understanding of *Plag1* in male fertility, and suggestions for further studies are discussed.

The following chapters (excluding the general introduction and discussion) were written as individual manuscripts for publication. As such, there is some repetition of background information and materials and methods. For consistency in formatting, all chapters follow the La Trobe University version of Harvard-style referencing. Where a manuscript has been published or accepted for publication in a peer-reviewed journal, the text appears as published; however, it is re-formatted for consistency of referencing, presentation, spelling, copyright purposes and continuity of page and figure numbering.

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Chapter 2. *Plag1* in reproduction: a focus on the role of PLAG1 in male fertility

Abstract

Pleomorphic adenoma gene 1 (PLAG1) is a transcription factor that has a well-established role in the formation of several tumour types, including pleomorphic adenomas and lipoblastomas. The action of *PLAG1* in cancer and adenomas has been linked to its regulation of cell proliferation and growth factors, including insulin-like growth factor 2. The phenotype of *Plag1* knockout (KO) mice includes striking growth retardation, demonstrating a crucial role for PLAG1 in development and growth. While the role of PLAG1 in normal physiology is not well understood, there is increasing evidence that PLAG1 is important for fertility and reproduction, particularly in males. Genome-wide association studies show that *PLAG1* is associated with reproductive traits, such as onset of puberty in cattle and chickens, and male *Plag1* KO mice exhibit remarkable fertility defects, including impaired spermatogenesis and decreased sperm motility. Further, in the absence of PLAG1, testicular transcription of important steroidogenic and spermatogenesis genes is dysregulated and ectopic expression of epididymal genes in the testes is observed. Here, I review the known roles of *Plag1* in tumourigenesis and growth and the emerging role of *Plag1* in reproduction, with a focus on male fertility. I also examine recent sperm analyses in the *Plag1* KO mouse model and propose how these results may be indicative of a role for PLAG1 in the epididymis.

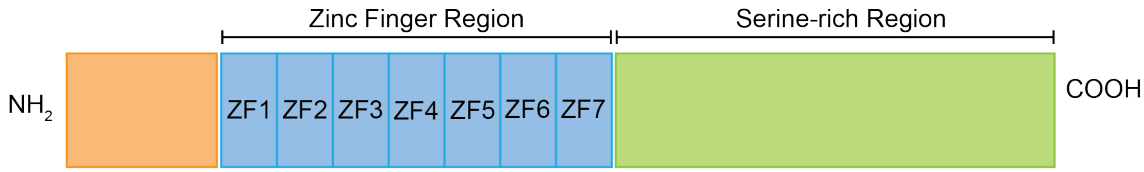
2.1 Introduction

PLAG1 is a highly conserved zinc-finger transcription factor and proto-oncogene that exhibits 96% protein sequence similarity between mice and humans (Juma et al. 2016). The PLAG family of transcription factors includes PLAG-like 1 (PLAGL1; also called LOT1 and ZAC1) and PLAG-like 2 (PLAGL2), and the function of these transcription factors varies. PLAGL1 is a tumour suppressor

that triggers cell cycle arrest and apoptosis, ultimately supressing tumour cell proliferation (Abdollahi 2007). Human *PLAGL1* is mapped to the 6q24-q25 chromosomal region (Varrault et al. 1998), which is deleted in several tumour types including melanomas (Walker et al. 1994), ovarian carcinomas (Foulkes et al. 1993) and breast cancers (Theile et al. 1996). In contrast, PLAGL2 is a proto-oncogene capable of inducing myeloid leukemia (Landrette et al. 2005). However, there is evidence that PLAGL2 can also act as a tumour suppressor in some cell types. In fibroblast and neuroblastoma cells, PLAGL2 activates expression of the proapoptotic Nip3 protein, ultimately leading to the induction of apoptosis (Mizutani et al. 2002).

PLAG1 too has been extensively studied for its role in tumourigenesis, which is largely caused by its function as a transcription factor that controls several cell proliferation- and growth-related genes (Voz et al. 2000). PLAG1 binding to the promoter region of target genes is facilitated by the seven canonical C2H2 zinc finger domains present in the N-terminus, while transcription activation ability is facilitated by the serine-rich C-terminus region (Kas et al. 1998). The consensus PLAG1 binding motif consists of a GRGGC core sequence followed by 7 non-conserved nucleotides and a G-Cluster, broadly taking the form G(A/G)GGCNNNNNNN(A/G)GG(G/T), where N is a random nucleotide and the underlined nucleotides are largely invariant (Voz et al. 2000). Transcriptional regulation is facilitated by the binding of zinc fingers 6 and 7 to the GRGGC core, and of zinc finger 3 to the G cluster (Voz et al. 2000). PLAG1 target genes include insulin-like growth factor 2 (IGF2), bone-derived growth factor 1, vascular endothelial growth factor, cytokine-like factor 1 and placental growth factor (Voz et al. 2004). Indeed, the *Plag1* knockout (KO) mouse line, generated via targeted disruption by homologous recombination in R1 embryonic stem cells and consequent crossing into Swiss Webster mice, exhibits growth retardation that begins in embryonic development and persists into adulthood (Hensen et al. 2004), which provides further indication that PLAG1 is required for normal growth.

A



B

```
PLAG1_HUMAN           1   MATVIPGDLSEVRDTQKVPSGKRKRGETKPRKNFPCQLCDKAFNSVEKLKVHSYSHTGER        60
PLAG1_MOUSE           1   MATVIPGDLSEVRDTQKAPSGKRKRGESKPRKNFPCQLCDKAFNSVEKLKVHSFSHTGER        60
                          *****************.*********:*************************:******

PLAG1_HUMAN          61   PYKCIQQDCTKAFVSKYKLQRHMATHSPEKTHKCNYCEKMFHRKDHLKNHLHTHDPNKET       120
PLAG1_MOUSE          61   PYKCTHQDCTKAFVSKYKLQRHMATHSPEKTHKCNYCEKMFHRKDHLKNHLHTHDPNKET       120
                          **** :************************************************************

PLAG1_HUMAN         121   FKCEECGKNYNTKLGFKRHLALHAATSGDLTCKVCLQTFESTGVLLEHLKSHAGKSSGGV       180
PLAG1_MOUSE         121   FKCEECGKSYNTKLGFKRHLALHAATSGDLTCKVCLQNFESTGVLLEHLKSHAGKSSGGV       180
                          ********.****************************.**********************

PLAG1_HUMAN         181   KEKKHQCEHCDRRFYTRKDVRRHMVVHTGRKDFLCQYCAQRFGRKDHLTRHMKKSHNQEL       240
PLAG1_MOUSE         181   KEKKHQCEHCERRFYTRKDVRRHMVVHTGRKDFLCQYCAQRFGRKDHLTRHMKKSHNQEL       240
                          **********:*************************************************

PLAG1_HUMAN         241   LKVKTEPVDFLDPFTCNVSVPIKDELLPVMSLPSSELLSKPFTNTLQLNLYNTPFQSMQS       300
PLAG1_MOUSE         241   LKVKTEPVDFLDPFTCNMSVPIKDELLPVMSLPSSELLSKPFTNTLQLNLYNTPFQSMQS       300
                          *****************:*******************************************

PLAG1_HUMAN         301   SGSAHQMITTLPLGMTCPIDMDTVHPSHHLSFKYPFSSTSYAISIPEKEQPLKGEIESYL       360
PLAG1_MOUSE         301   SGSAHQMITTLPLGMTCPIDMDAVHPSHHLAFKCPFSSTSYAISIPEKEQPLKGEIESYL       360
                          **********************:********:** **************************

PLAG1_HUMAN         361   MELQGGVPSSSQDSQASSSSKLGLDPQIGSLDDGAGDLSLSKSSISISDPLNTPALDFSQ       420
PLAG1_MOUSE         361   MELQGGAPSSSQDSPA-SSSKLGLEPQSGSPDDGAGDLSLSKSSISISDPLSTPALDFSQ       419
                          ******.******* * *******:** ** ********************.********

PLAG1_HUMAN         421   LFNFIPLNGPPYNPLSVGSLGMSYSQEEAHSSVSQLPPQTQDLQDPANTIGLGSLHSLSA       480
PLAG1_MOUSE         420   LFNFIPLNGPPYNPLSVGSLGMSYSQEEAHSSVSQLPTQTQDLQDPANTVGLSSLHSLSA       479
                          ************************************ ***********:**.*******

PLAG1_HUMAN         481   AFTSSLSTSTTLPRFHQAFQ                                          500
PLAG1_MOUSE         480   AFTSSLSSSTTLPRFHQAFQ                                          499
                          *******:************
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Figure 2.1. PLAG1 protein structure and amino acid sequence alignment. (A) Schematic representation of the PLAG1 protein structure. (B) Amino acid sequence alignment of human PLAG1 and mouse PLAG1 proteins aligned using the Clustal Omega program of UniProt (www.uniprot.org) showing 95.4% similarity and 477 identical positions. Asterisks indicate single, fully conserved amino acid residues, colons indicate amino acid residues with strongly similar properties and periods indicate amino acid residues with weakly similar properties. ZF, zinc finger.

Mutations or polymorphisms of *PLAG1* are not only associated with body stature but also with a range of reproductive traits in production animals, such as the onset of puberty in cattle (Fortes et al. 2012a; 2012b; 2013c) and egg-laying in chickens (Chen C-F et al. 2007; Chen L-R et al. 2007). The role of PLAG1 in male fertility in particular is being uncovered using the *Plag1* KO mouse model. Most recently, analysis of mature sperm from the epididymis of *Plag1* KO mice revealed significant sperm motility defects (Juma et al. 2017), leading to the possibility that PLAG1 may play a role in epididymis function. Taken together with earlier findings that fertility is reduced in *Plag1* KO

male mice (Hensen et al. 2004), these studies highlight the need for further investigation of *Plag1* and male fertility, particularly as such research may be useful for understanding clinical male infertility.

Clinical infertility is defined as the inability to achieve pregnancy after 12 months of regular, unprotected intercourse (Lindsay & Vitrikas 2015). Infertility affects 15% of couples globally and there are an estimated 30 million cases of male infertility worldwide (Agarwal et al. 2015; Jarow et al. 2002). Sperm motility defects are a common diagnosis in clinical male infertility. In a study of sperm samples from 1085 infertile men, 63% of samples exhibited reduced sperm motility (asthenozoospermia) in conjunction with low sperm count (oligozoospermia) and/or abnormal sperm morphology (teratozoospermia), and 19% of samples were asthenozoospermic (Curi et al. 2003). In total, 82% of sperm samples exhibited asthenozoospermia. However, the exact underlying mechanisms behind these sperm defects are largely unidentifiable and as a result, 40–50% of clinical male infertility cases are classified as idiopathic (De Kretser & Baker 1999; Jose-Miller, Boyden & Frey 2007; Lindsay & Vitrikas 2015). This lack of understanding of the causes of human male infertility highlights the need for further research on all aspects of male fertility, including epididymis development and epididymal sperm maturation.

In this review, I first briefly summarise the well-established role of PLAG1 in tumourigenesis and growth. I then review what is currently known about *Plag1* in reproduction and the association of *PLAG1* polymorphisms with fertility traits in production animals. The current understanding of the role of *Plag1* in male fertility is examined in detail. Finally, I discuss how the recent findings on sperm motility defects in *Plag1* KO mice suggest that *Plag1* may play a role in the epididymis.

2.2 PLAG1 in tumourigenesis and growth

PLAG1 was first discovered in pleomorphic adenomas of the salivary glands (Kas et al. 1997) and has subsequently been implicated in a wide array of cancer types, including hepatoblastomas, lipoblastomas, acute myeloid leukemia, myoepitheliomas and uterine myxoid leiomyosarcomas

(Antonescu et al. 2013; Arias-Stella et al. 2019; Åström et al. 2000; Landrette et al. 2005; Wang, Guzman & Batanian 2019; Zatkova et al. 2004). One of the main mechanisms underlying PLAG1-induced tumour formation is chromosomal rearrangement and translocation events that result in swapping of the *PLAG1* promoter with the strong promoters of constitutively expressed genes such as the β-catenin gene *CTBNN1* (Kas et al. 1998), the leukemia inhibitory factor receptor gene *LIFR* (Voz et al. 1998) and the transcription elongation factor SII gene *SII* (Åström et al. 1999). This leads to overexpression or ectopic expression of *PLAG1*, dysregulation of PLAG1 target genes and, ultimately, tumour formation (Voz et al. 2000). The strong oncogenic effect of PLAG1 was demonstrated by targeted overexpression in murine salivary glands and mammary glands, which resulted in the formation of malignant tumours (Declercq et al. 2005). More recently, three novel mutations of *PLAG1* (*PLAG1* amplification, partial deletion and complex rearrangement of chromosome 8q) have been discovered in lipoblastomas of human patients (Wang, Guzman & Batanian 2019). These data indicate that numerous *PLAG1* genetic aberrations can lead to tumourigenesis.

The role of PLAG1 in tumourigenesis is thought to be closely related to its regulation of cell proliferation genes, as PLAG1 overexpression has been associated with increased IGF2 expression and subsequent tumour formation. For example, in salivary gland adenomas in which PLAG1 is overexpressed, IGF2 transcribed from the P3 promoter is also upregulated (Voz et al. 2000). However, in adenomas with normal PLAG1 expression or in normal salivary gland tissues, IGF2 derived from the P3 promoter is undetectable (Voz et al. 2000). The transformation effect of PLAG1 was confirmed to be mediated by IGF2 using *in vitro* transformation studies; PLAG1 overexpression is unable to transform fibroblast cells when the insulin-like growth factor 1 receptor (IGF1R), which is required for IGF2 function, is knocked out (Hensen et al. 2002). It can be inferred from these findings that PLAG1 plays a role in tumourigenesis by activating the mitogenic IGF2 pathway and consequent stimulation of tumour cell proliferation (Hensen et al. 2002).

Another clue regarding the role of PLAG1 in cancer and growth is the effect of maternally provided PLAG1 on timely zygotic genome activation. In heterozygous (HET) mouse embryos lacking the maternal *Plag1* allele, the activation of zygotic genome activation genes, which occurs during the two-cell stage of embryonic development, was delayed (Madissoon et al. 2019). In these genes, a *de*

novo PLAG1 binding motif was significantly enriched, and gene ontology analysis indicated that the genes containing the *de novo* motif are involved in regulation of ribosome biogenesis and protein synthesis. Ribosome biogenesis genes in particular are known to be affected by oncogenes, resulting in increased protein synthesis and thus facilitating the rapid proliferation of cancer cells. This led to the suggestion that, in addition to a functional role in zygotic genome activation, PLAG1 may affect protein synthesis, which correlates to the known roles of PLAG1 in tumourigenesis, growth and cell proliferation (Madissoon et al. 2019). The potential role of PLAG1 in protein synthesis and ribosome biogenesis has not yet been further studied; however, this may assist in uncovering the precise mechanism behind PLAG1 control of growth.

Clear evidence for an important role for PLAG1 in growth is demonstrated by the striking growth retardation seen in *Plag1* KO mice (Hensen et al. 2004). *Plag1* KO embryos were significantly smaller than their WT littermates, with size differences detectable by embryonic day 11.5 (E11.5) (Hensen et al. 2004). The action of *Plag1* appears to be temporally dynamic; at the end of gestation, *Plag1* KO pups were approximately 30% smaller, but this size difference was increased to 50% by postnatal day 21 (P21). After weaning, the growth rate of *Plag1* KO mice increased, so that by P60, they were again 30% smaller than their WT counterparts (Hensen et al. 2004). The underlying mechanisms by which PLAG1 regulates growth is not fully understood; however, it is again likely that it stems from the ability of PLAG1 to regulate IGF2 expression, as IGF2 is crucial for normal embryonic growth (Baker et al. 1993; Chao & D'Amore 2008; Constância et al. 2002) and *Igf2* KO mice exhibit concurrent placental and foetal growth restriction (Coan et al. 2008). Similarly to *Plag1* KO mice, *Igf2*$^{+/p-}$ mice lacking the paternal *Igf2* allele exhibit an ~40% reduction in body weight in comparison to WT littermate controls, with size differences first detected at E11.5. Unlike *Plag1* KO mice however, *Igf2* mutant mice are not reported to display any fertility defects. The similarity in growth phenotype of *Plag1* KO mice and *Igf2*$^{+/p-}$ mice indicate that these genes may be involved in similar biological pathways.

PLAG1 regulation of *IGF2* has also been linked to foetal growth defects. Overexpression of miR-141 in the human placenta suppresses *PLAG1* expression at the transcriptional and translational level, and *PLAG1* expression was correlated with *IGF2* expression in the placenta of foetal growth

restriction patients (Tang et al. 2013). It was therefore proposed that the miR-141–PLAG1–IGF2 pathway is implicated in the pathogenesis of foetal growth restriction (Tang et al. 2013). miR-141 acts as a tumour suppressor or an oncogene depending on tissue and cancer type (Xiong et al. 2017). In chronic lymphocytic leukemias, PLAG1 is overexpressed via the downregulation of a number of microRNAs, including miR-141 (Pallasch et al. 2009). In essence, excessive miR-141 suppression of PLAG1 can have detrimental effects on growth during development, while the aberrant silencing of miRNA-141 results in overexpression of PLAG1 that can subsequently lead to cancer progression.

Further evidence that PLAG1 is involved in foetal growth disorders is demonstrated in a recent clinical study which indicated that *PLAG1* and its upstream regulator *HMGA2* (high-mobility group AT-hook 2) may be implicated in Silver-Russell syndrome (SRS), a syndromic form of foetal growth restriction associated with low IGF2 expression (Abi Habib et al. 2018). Genetic screening of 192 patients resulted in the identification of two different *de novo* single-nucleotide deletion mutations in the *PLAG1* gene in suspected SRS patients; both of these mutations lead to frameshifts and ultimately to the production of truncated PLAG1 proteins (Abi Habib et al. 2018). Gene silencing and overexpression studies in cell cultures indicated that IGF2 expression levels are regulated by HMGA2 in both PLAG1-dependent and -independent pathways (Abi Habib et al. 2018). Adult mice with inactive *Hmga2* present with defective spermatogenesis and are infertile (Chieffi et al. 2002). In addition, they exhibit a 55% reduction in body weight in comparison to WT littermates (Zhou et al. 1995), which is more severe than the 30% reduction seen in *Plag1* KO mice. This may indicate that in *Plag1* KO mice, HMGA2 is still able to stimulate some IGF2 expression via the PLAG1-independent pathway, resulting in growth retardation that is not as severe as in HMGA2-deficient mice.

The role of HMGA2 appears to be conserved across some mammalian species, such as pigs, as *HMGA2* KO boars exhibit infertility due to cryptorchidism (undescended testis), in addition to a 21% reduction in testis weight at 15 weeks of age and a 35–80% reduction in body weight depending on age, in comparison to WT boars (Chung et al. 2018). Interestingly, there is some indication that the reproductive system is affected in human SRS patients as well; genital abnormalities, most notably cryptorchidism, anorchia (absence of the testis) and hypospadias (abnormality where the opening of urethra occurs on the ventral surface the penis) are frequently observed in male patients

(Bruce et al. 2009; Wakeling et al. 2010). Recently, a *de novo IGF2* mutation of the paternal allele was detected in a SRS patient who, in addition to typical SRS characteristics, also exhibited under-masculinised genitalia (Yamoto et al. 2017). The interplay between IGF2, its regulators (including PLAG1), and SRS requires further study and it may be worthwhile to investigate PLAG1 expression and mutations in SRS patients. Further, the occurrence of reproductive abnormalities in male SRS patients may be associated with the emerging data that indicates PLAG1 is important for male fertility.

2.3 *Plag1* in reproduction

Evidence of a role for *Plag1* in fertility was revealed when a *Plag1* KO mouse line was generated **(Figure 2.1)**. Hensen et al. (2004) described reduced conception rates when *Plag1* KO male mice were mated with WT females, with KO males able to fertilise only 7% of female WT mice compared to 20% fertilisation from WT males (Hensen et al. 2004). *Plag1* KO×KO matings resulted in infrequent conception and small litter sizes. Conception rates in *Plag1* KO female mice mated with WT males were reported to be normal, although average litter size was reduced by 50% in comparison to WT females (Hensen et al. 2004). A more recent study showed that reduced litter size is apparent by 7.5–8.5 days *post coitum*, as evidenced by fewer implantation marks in the uteri of *Plag1* KO mothers compared to WT and HET mice (Madissoon et al. 2019). In this study, oocyte yield in response to gonadotropin-induced superovulation, ovarian follicle counts and uterine weight relative to body weight did not differ in *Plag1* KO mice compared to WT controls, altogether indicating that ovarian function was normal and therefore not a contributing factor to the reduction in average litter size (Madissoon et al. 2019). Instead, it was suggested that delayed zygotic genome activation in the absence of maternally provided *Plag1* may lead to reduced numbers of successfully implanted embryos in *Plag1* KO females, because these genes are crucial for the development of implantation-competent blastocysts (Madissoon et al. 2019). These results suggest that *Plag1* may be important for embryo implantation.

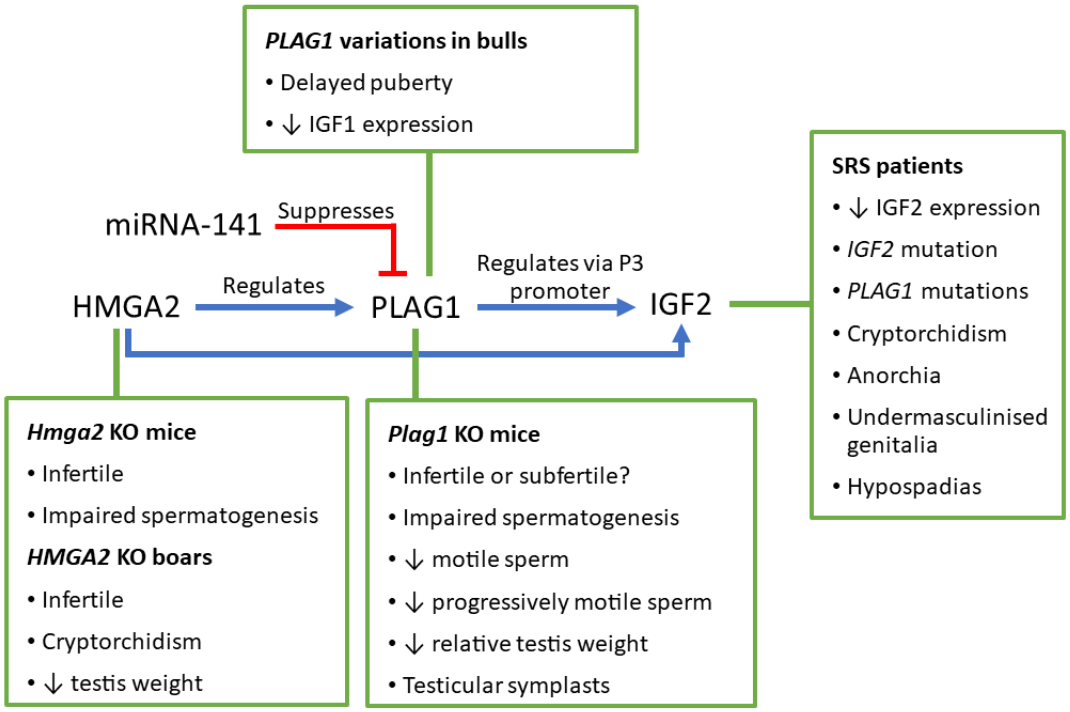


Figure 2.2. Interactions between PLAG1, its regulators, and the associated male reproduction phenotypes. Downward arrows represent a decrease.

PLAG1 expression has been detected in the reproductive organs, suggesting a role for this transcription factor in these tissues, and genome-wide association studies have identified *PLAG1* variations associated with reproduction traits in cattle (Juma et al. 2016). Most recently, striking spermatogenesis and sperm motility aberrations have been revealed in *Plag1* KO male mice, indicating that PLAG1 plays a critical role in male fertility (Juma et al. 2017) **(Figure 2.1)**. Given that sperm motility is gained during epididymal sperm maturation, we speculate that PLAG1 may play a role in the epididymis.

2.3.1 *Plag1* expression in reproductive organs

PLAG1 expression was initially thought to be largely restricted to embryonic/foetal development. Using *in situ* hybridisation, *Plag1* mRNA expression was detected in the urogenital ridge of mouse embryos, suggesting that *Plag1* plays a role in early sexual development (Hensen et

al. 2004). However, other studies subsequently demonstrated *Plag1* expression in adult tissues, including the reproductive organs. Using northern blot analysis, human *PLAG1* was detected in the adult testes, prostate and ovaries (Queimado et al. 1999). *Plag1* mRNA was also detected in the testis, epididymis and vas deferens of adult mice using *in situ* hybridisation and quantitative PCR (Hensen et al. 2004). It should be noted that there have been some inconsistencies in the detection of *Plag1* expression in other tissues; *Plag1* was not detected by northern blot in the adult mouse liver or brain (Hensen et al. 2004), but was detected in mouse liver by quantitative PCR (Zheng & Yang 2005) and in mouse hypothalamus by X-gal staining (Juma et al. 2018). These discrepancies in detection of *Plag1* expression may due to the difference in sensitivity of the techniques used; however, the most recent studies show that X-gal staining produces robust PLAG1 signal in tissue sections (Juma et al. 2017; 2018).

In the *Plag1* KO mouse model, the *Plag1* coding sequence is replaced with a *LacZ* reporter gene (Hensen et al. 2004), which encodes the β-galactosidase enzyme. This enzyme breaks down the galactose in X-gal staining solution and produces a blue precipitate, which allows the use of X-gal staining to localise PLAG1 expression. Given that the *LacZ* reporter gene retains the *Plag1* 5' and 3' untranslated regions, which generally contain mRNA localisation signals that direct the translation of proteins to occur at or near the site of their function (Hervé, Mickleburgh & Hesketh 2004; Mignone et al. 2002), it is likely that X-gal signal is representative of PLAG1 localisation. Recently, X-gal staining has shown that PLAG1 is expressed in all cell types of the seminiferous tubules in the murine testes, namely in small numbers of Sertoli cells and in some spermatogonia, spermatocytes and spermatids (Juma et al. 2017). The function of PLAG1 in germ cells, if any, is currently unknown, and whether PLAG1 is still present in mature spermatozoa following epididymal sperm maturation remains to be investigated. Currently, there are no anti-mouse PLAG1 antibodies that result in specific staining, hence the use of X-gal staining and mRNA expression analyses. In future, generation of an anti-PLAG1 antibody effective in mouse tissues would allow for more direct confirmation of protein expression.

2.3.2 *PLAG1* polymorphisms associated with reproductive traits in production animals

While *PLAG1* has not yet been studied in human male infertility, genetic variants of *PLAG1* have been linked to several reproduction and fertility traits in cattle. Many genes and hormones are conserved between species; however, without functional studies in mice or humans, genome-wide association studies can only provide indications to the role that *PLAG1* may play in fertility. In cattle, the region on chromosome 14 containing *PLAG1* is a known quantitative trait locus containing several genetic markers for the onset of puberty and growth traits in cattle (Fortes et al. 2012a; Karim et al. 2011; Utsunomiya et al. 2014). At this locus, two sequence polymorphisms of the *PLAG1* promoter have been identified: the *Q* and *q* variants resulting from a variable-number tandem repeat (VNTR) and a single-nucleotide polymorphism (SNP) (Karim et al. 2011). The VNTR is a $(CCG)_n$ trinucleotide repeat located immediately upstream of the presumed *PLAG1* transcription start site, with nine copies in the *q* variant and 11 copies in the *Q* variant. The SNP, located 12 bp upstream from the VNTR, is an 'A' in the *q* variant and a 'G' in the *Q* variant. Genome-wide association studies have identified that the *Q* allele of *PLAG1* is associated with increased PLAG1 expression in various tissues; cattle with the *QQ* genotype are associated with increased body size, later onset of puberty and also lower blood IGF1 levels (Fortes et al. 2012a; 2012b; 2013c).

The IGF1 pathway is known to affect the onset of puberty and growth traits in cattle, such as body length and height (Fortes et al. 2013b; Gui et al. 2018; Lirón et al. 2012). It is currently unknown why the *QQ* genotype in cattle is associated with both decreased IGF1 and increased body size, a seemingly paradoxical relationship, given that IGF1 is a growth factor. It has previously been suggested that perhaps the increased expression of *PLAG1* leads to increased IGF1R expression, resulting in increased IGF1 action, despite actual levels of IGF1 being lower (Fortes et al. 2013a). In this scenario, there would presumably be a feedback mechanism that suppresses IGF1 expression when IGF1R expression or activity is increased. Evidently, IGF1 is in some way connected to PLAG1; however, unlike IGF2, so far no experimental evidence exists that indicates *IGF1* may be a direct target of PLAG1. Further, whether *PLAG1* is a major regulator of puberty onset in cattle, or if polymorphisms of chromosome 14 affect other neighbouring genes at this locus remains to be determined (Juma et al. 2016).

It was previously thought that the role of PLAG1 in the onset of puberty may be due to a role in the hypothalamic-pituitary-gonadal (HPG) axis, particularly due to the association of *PLAG1* polymorphisms in the chicken hypothalamo-pituitary with high and low egg laying chicken strains. High *PLAG1* mRNA expression is associated with increased egg laying and decreased time between egg laying (Chen C-F et al. 2007; Chen L-R et al. 2007; Shiue et al. 2006). Furthermore, high levels of *Plag1* mRNA expression were found in the anterior pituitary gland in embryonic and adult mice (Hensen et al. 2004), as well as the aforementioned expression of PLAG1 in the reproductive organs. More recently, expression of PLAG1 was characterised in the hypothalamus and pituitary gland of adult male mice using X-gal staining (Juma et al. 2018); PLAG1 is expressed in gonadotropin-releasing hormone (GnRH) neurones in the hypothalamus and widespread expression is found in gonadotropes and somatotropes in the pituitary gland. GnRH stimulates the gonadotropes to release hormones crucial for reproduction and function of the reproductive organs in both males and females, including luteinising hormone (LH) and follicle-stimulating hormone (FSH) (Stamatiades & Kaiser 2018). Despite the known association of hypothalamic-pituitary PLAG1 and egg laying traits in chickens, the HPG axis appears to be unaffected in *Plag1*-deficient male mice at the hypothalamic and pituitary level, since in these mice, mRNA expression of key reproductive genes, including *Fshb*, *Lhb*, *Gh* and *Gnrh,* were not altered and the circulating levels of the hormones FSH, LH and growth hormone were similarly unaffected (Juma et al. 2018). Therefore, it can be speculated that the role of PLAG1 in the HPG axis may be species- or sex-specific or that PLAG1 affects the HPG axis at the gonadal level only in male mice.

2.3.3 Abnormal spermatogenesis and sperm motility in *Plag1* KO mice

While it appears unlikely that PLAG1 directly regulates reproduction at the hypothalamic-pituitary level in male mice, there is evidence that PLAG1 does regulate male fertility at the gonadal level. Preliminary organ weight analysis in an early study indicated that the ventral prostate and seminal vesicles from *Plag1* KO mice are reduced in relative weight compared to WT controls, while the testes were reported to be proportional to body weight (Hensen et al. 2004). However, it was recently shown that in *Plag1* mice 5–6 weeks of age, testis weight was reduced by 42% in *Plag1* KO

mice compared to WT controls, and seminal vesicle weight was reduced by 67% in *Plag1* KO mice compared to WT controls. Further, in adult mice, daily sperm production was reduced by 48% in *Plag1* KO mice compared to WT controls (Juma et al. 2017). Reduced seminal vesicle and testicular weight and sperm production are generally considered hallmark indications of impaired androgen function, and indeed, transcriptome analysis revealed the dysregulation of major steroidogenesis genes in the testes of adult *Plag1* KO mice. *Hsd17b3* and *Sult1e1* were both significantly downregulated in the testes of 5-week-old *Plag1* KO mice compared to WT controls (Juma et al. 2017); *Hsd17b3* encodes 17β-hydroxysteroid dehydrogenase type 3, an enzyme required for the conversion of androstenedione to testosterone (Geissler et al. 1994; George et al. 2010), while *Sult1e1* encodes an oestrogen sulfotransferase, an enzyme that inactivates oestrogens by catalysing sulfate conjugation (Tong, Christenson & Song 2004). Oestrogen sulfotransferase is required for normal steroidogenesis in Leydig cells (Tong, Christenson & Song 2004) and for sperm motility, and also appears to protect Leydig cells from oestrogen-induced lesions (Qian et al. 2001). The dysregulation of steroidogenesis genes in the testes of *Plag1* KO mice, taken together with the reduction in relative seminal vesicle and testicular weight, suggests that gonadal steroidogenesis is dysregulated in *Plag1* KO male mice.

In addition to the dysregulation of steroidogenesis genes, transcriptome analysis also showed that several spermatogenesis genes were downregulated in the testes of *Plag1* KO mice. *Dazl*, *Stra8* and *Piwil1*—genes known to play a role in spermatogenesis—were downregulated in the testes of *Plag1* KO mice, which is likely related to the spermatogenic abnormalities observed in the testicular epithelium (Juma et al. 2017). In the testes of *Plag1* KO mice, excessive sloughing of the germinal epithelium and the presence of spermatids and spermatocytes in the epididymis were observed (Juma et al. 2017). In addition, the germinal epithelium contained more apoptotic cells and in the seminiferous tubules symplasts were identified, which are multinucleated cells formed when the intercellular bridges between round spermatids collapse and are associated with abnormal spermatogenesis (Juma et al. 2017). Intriguingly, the transcriptome analysis also revealed the ectopic expression of epididymis-specific genes in the testes from *Plag1* KO mice (Juma et al. 2017). These

findings indicate that PLAG1 regulates key spermatogenesis genes in the mouse testes and that PLAG1 may regulate epididymal genes.

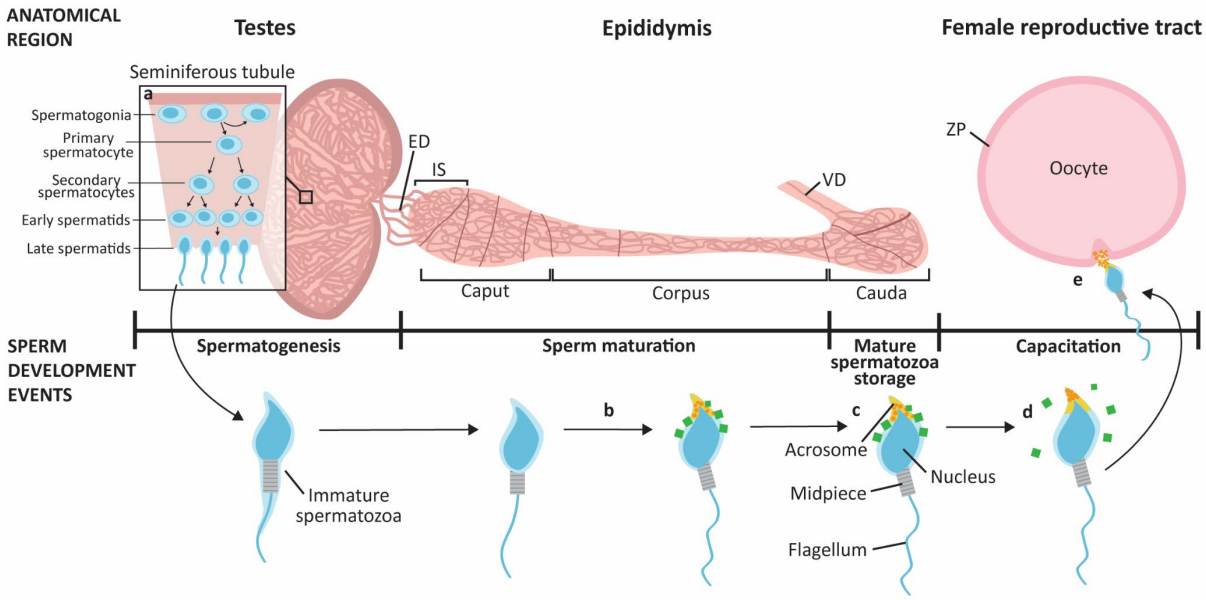
Further evidence for a role for PLAG1 in the epididymis can be inferred from findings that poor sperm motility is associated with PLAG1 deficiency. Sperm abnormalities were discovered when mature-mouse spermatozoa collected from the cauda epididymidis were analysed using computer-assisted sperm analysis (Juma et al. 2017). There was an 80% reduction in overall sperm motility, a 49% reduction in progressive sperm motility, a significantly decreased fraction of rapidly motile spermatozoa and a significantly higher fraction of immotile spermatozoa in *Plag1* KO mice compared to WT controls. These static spermatozoa are either nonviable spermatozoa or completely immotile spermatozoa, which may be indicative of a ciliary disorder such as primary ciliary dyskinesia (immotile-cilia syndrome), a genetic disorder in which patients are typically infertile (Knowles et al. 2013; Leigh et al. 2009). Since sperm motility is acquired during epididymal sperm maturation, these results warrant investigation of the epididymis in *Plag1*-deficient mice.

2.4 A role for *Plag1* in the epididymis?

The striking sperm motility defects observed in *Plag1* KO mice indicate that epididymal function may be impaired. Spermatozoa leaving the testes must transit through the highly convoluted tubule that comprises the mammalian epididymis in order to become fully mature spermatozoa capable of motility and fertilisation; spermatozoa taken directly from the testes are not motile (Cornwall 2009; Dacheux & Dacheux 2014; Robaire, Hinton & Orgebin-Crist 2015) **(Figure 2.2)**. During epididymal sperm maturation, immature spermatozoa are biochemically and structurally modified to produce functionally mature spermatozoa capable of motility and fertilisation of the ovum. Proteins secreted by the epididymal epithelium are largely responsible for these modifications, as spermatozoa themselves are transcriptionally inactive (Guyonnet et al. 2011; Sullivan & Mieusset 2016; Zhou et al. 2018). The series of modifications that comprise sperm maturation are facilitated by the structure of the epididymis, which establishes a strict sequence of secreted factors that spermatozoa encounter as they transit through the tubule. The complex

interactions of factors involved in sperm maturation means that *in vitro* culture systems are often not a viable method for investigations of epididymis development and function, and the severely limited availability of human epididymal tissues for research has restricted the number of studies using human tissue. Thus, what is currently known about the epididymis is largely discovered through rodent models. Although the human epididymis differs from that of rodents in regard to segmentation and function of the cauda region (see further), epididymal gene and protein expression are similarly spatially regulated in both humans and rodents and most genes are conserved between species. Therefore, rodents are valuable models for studying the epididymis, but care must be taken when extrapolating observations from rodent studies.

Figure 2.3. Schematic representation of the main sperm development events leading to fertilisation in mice.(a) Spermatogenesis occurs in the seminiferous tubules of the testes.



Spermatogonial stem cells undergo spermatogonial differentiation in which six transit-amplifying mitotic divisions give rise to meiotic primary spermatocytes. Primary spermatocytes undergo two consecutive cell divisions; meiosis I gives rise to secondary spermatocytes, which undergo meiosis II to give rise to haploid round spermatids. During spermiogenesis, round spermatids undergo differentiation to become spermatozoa. Immature, immotile spermatozoa are released into the seminiferous tubule lumen and exit the testes via the efferent ducts which connect the testes to the initial segment of the epididymis. **(b)** Immature spermatozoa gain their motility and fertilisation capacity during epididymal sperm maturation. During sperm maturation, which takes place primarily in the caput and corpus epididymidis, spermatozoa undergo structural alterations, biochemical modifications and sperm surface changes. Decapacitation factors (green) that prevent premature sperm capacitation are bound to the sperm membrane and proteins required for zona-pellucida binding (orange) are taken up from the epididymal luminal fluid. **(c)** Mature spermatozoa are stored in the cauda epididymidis. Spermatozoa are transported from the vas deferens to the ejaculatory

ducts in preparation for ejaculation. **(d)** Entry into the female reproductive tract triggers sperm capacitation, which comprises a series of cellular and molecular changes crucial for fertilisation of the oocyte, including removal of decapacitation factors from the sperm membrane, destabilisation of the sperm membrane, migration of zona pellucida binding proteins to the apical ridge of the sperm head and the activation of sperm hypermotility. **(e)** Upon binding to the zona pellucida the acrosome reaction occurs, in which acrosomal enzymes are released to degrade the zona pellucida, allowing the spermatozoon to penetrate the oocyte. ED, efferent ducts; IS, initial segment; VD, vas deferens; ZP, zona pellucida.

The epididymis remains somewhat of an enigmatic aspect of male fertility research, perhaps in part due to the difficulties in studying human epididymis samples, and in part due to the complex nature of this organ. While epididymis research is gaining increasing interest, it is notable that the testes are often the primary focus of studies on rodent models of male infertility, and that epididymal function, though crucial for male fertility, is often not investigated (Elbashir et al. 2020). The epididymis of *Plag1*-deficient mice has not yet been studied; however, the sperm motility defects suggest that epididymis function may be affected in *Plag1* KO mice, warranting further research into the role of PLAG1 in the epididymis. Ultimately, understanding epididymis development and function using genetic deletion rodent models such as *Plag1* KOs will potentially contribute to a better understanding of the human epididymis and of clinical male infertility.

2.4.1 Epididymis structure and function in rodents and humans

Although their primary function in sperm maturation is the same, the rodent and human epididymides are notably different in some regards. The epididymal duct is just over 1 m long in mice, 3 m in rats and 6 m long in humans (Hinton et al. 2011), and sperm transit through the duct takes approximately 5–5.8, 8–10 and 5.5 days in mice, rats and humans, respectively (França, Avelar & Almeida 2005). Following puberty, the fully developed rodent epididymis comprises a differentiated and segmented duct consisting of four regions: the initial segment, the caput, the corpus and the cauda (**Figure 2.3**). It is only fairly recently that the initial segment has been considered a region, distinct from the caput (Joseph, Yao & Hinton 2009). The caput and corpus are thought to be the main sites of sperm maturation, while the cauda functions primarily in the storage of mature sperm (Cornwall 2009). In humans, there is no initial segment and the cauda does not have

the characteristic bulb shape seen in mice, nor does it function in sperm storage as it does in mice; instead, mature sperm are stored in the proximal vas deferens (Cornwall 2009). Another difference between the human and rodent epididymis is the segmentation of the rodent epididymis by connective tissue septa.

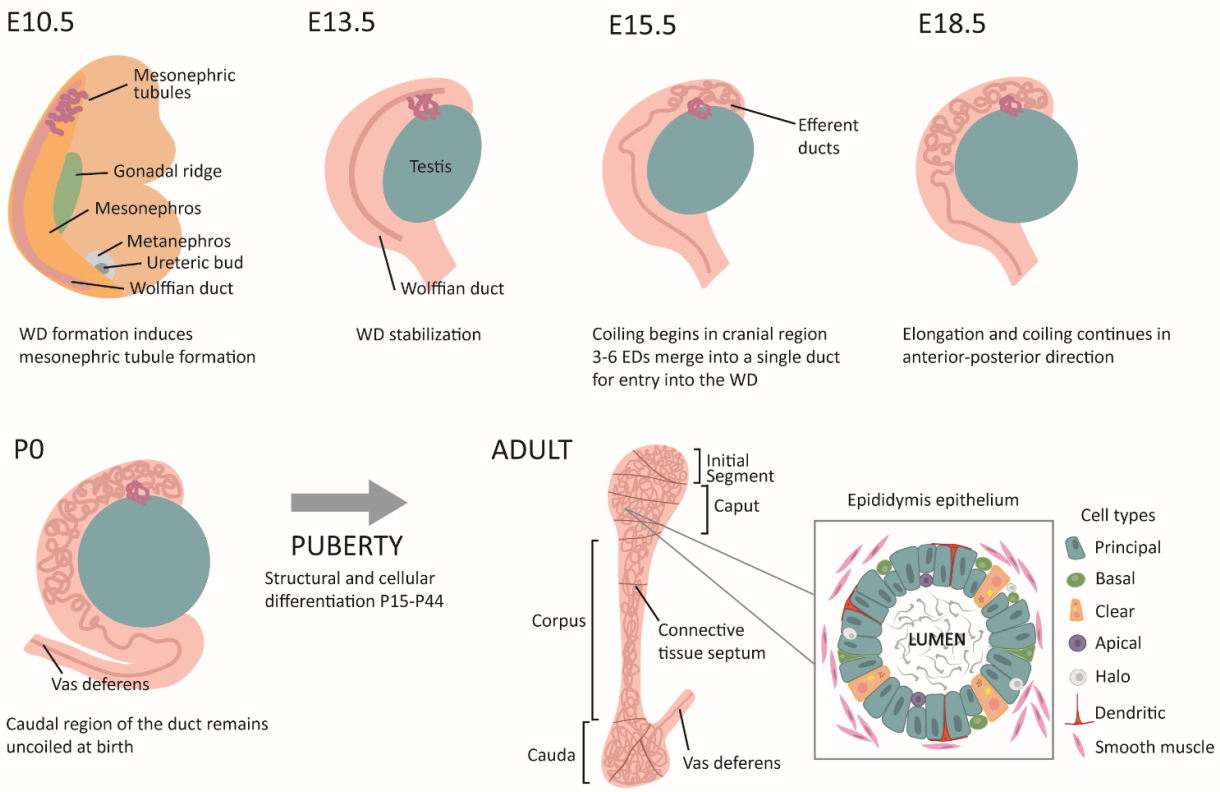


Figure 2.4. Schematic diagram of mouse Wolffian and epididymis development. ED: efferent ducts; WD: Wolffian duct.

While gene and protein expression and the secretion of factors are segment-specific in both humans and rodents, the rodent epididymis is also anatomically segmented due to the presence of connective tissue septa (Turner et al. 2003) (**Figure 2.4**), which begin to form as early as E18 (Domeniconi et al. 2016). The various segments are histologically and functionally distinct, having distinctive luminal microenvironments due to unique but overlapping transcriptomes, and therefore

a distinct pattern of secreted factors (Jelinsky et al. 2007; Jervis & Robaire 2001; Johnston et al. 2007; Thimon et al. 2007; Turner et al. 2007). Indeed, the main epididymal regions can be further divided into 10 transcriptionally-distinct segments in mice (Johnston et al. 2005) and 19 in rats (Jelinsky et al. 2007). The extent to which septa restrict the movement of factors secreted in the interstitium remains to be determined, it has so far been shown that the septa can limit the diffusion of tracer dyes injected into the interstitium (Stammler et al. 2015; Turner et al. 2003) and prevent the ascent of bacteria injected into the vas deferens lumen (Stammler et al. 2015), and that the stimulatory effect of growth factors microperfused into the interstitium are restricted to the segment they were administered to (Tomsig, Usanovic & Turner 2006). The identities of the segments are so different that some researchers have suggested that the epididymis should be considered as a series of distinct organs (Domeniconi et al. 2016).

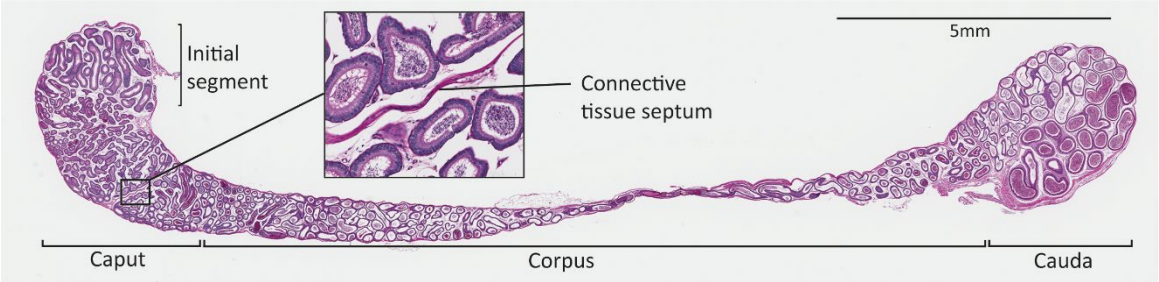


Figure 2.5 Longitudinal tissue section of an adult mouse epididymis. Tissue section is stained with period-acid Schiff and counterstained with hematoxylin. Main regions are indicated and a close-up of a connective-tissue septum is shown. Image courtesy of Prof. Moira O'Bryan (Monash University, Australia)

Proteomic technologies such as mass spectrometry have allowed the identification of luminal proteins secreted by the epididymis. Several hundred have been characterised so far and while the regionalised expression of some, such as glutathione peroxidase and gelsolin, is similar across species, other proteins appear to be secreted in different regions depending on the species (Dacheux & Dacheux 2014). The segment-specific secretion of proteins results in the interaction of spermatozoa with proteins in a precise order and therefore, spermatozoon modification and maturation events occur in a specific sequence during transit through the epididymis. Evidently,

region and segment identity are crucial for establishing the proper expression pattern of the secreted proteins that facilitate sperm maturation. As such, epididymal morphology should be investigated in *Plag1* KO mice, since defective sperm maturation may be implicated in the sperm motility issues exhibited by these mice.

2.4.2 Morphogenesis and development of the mouse epididymis

The Wolffian duct—the epididymis anlagen—begins to form at approximately E8.5 in mice (Hannema & Hughes 2007; Murashima, Xu & Hinton 2015), and derives from the intermediate mesoderm. In early embryonic development, the Wolffian duct extends caudally within the anterior intermediate mesoderm, stimulating the formation of the three primitive kidneys: pronephros, mesonephros and metanephros. The mesonephros ultimately gives rise to part of the male reproductive system (Murashima, Xu & Hinton 2015; Obara-Ishihara et al. 1999). The proximal mesenchymal tissue of the Wolffian duct develops into the epididymis, undergoing extensive remodelling and coiling during embryonic and postnatal growth to become a fully differentiated epididymis by puberty (Joseph, Yao & Hinton 2009; Robaire, Hinton & Orgebin-Crist 2015). In the mouse, Wolffian duct coiling begins at E14 in the proximal region, and coiling progresses caudally as development continues. Initially, coiling occurs in only one plane; then three-dimensional coiling and folding begins to occur from E16 in the initial segment and caput (Joseph, Yao & Hinton 2009).

During morphogenesis of the Wolffian duct and epididymis, coiling of the duct is one of the tubulogenetic mechanisms required and the ability to coil such an extensive duct to fit into the diminutive space of the epididymis is a puzzling phenomenon (Hinton et al. 2011). While processes behind the formation of tubes are well researched, the precise cellular mechanisms behind the intensive and highly coordinated tubule coiling in the epididymis are not fully understood. However, several mechanisms, including convergent extension and apical constriction, are thought to be implicated (**Figure 2.5**). It has also been suggested that it is the space restriction itself that encourages coiling of the elongating epididymal duct, and that the relative lack of proliferation of the mesenchyme in comparison to rapidly proliferating areas of the tubule essentially creates mechanical pressure that forces the duct to coil (Joseph, Yao & Hinton 2009). It is also reasonable to speculate

that the extensive proliferation of the tubule epithelium results in paracrine signalling to the mesenchymal cells that stimulates the surrounding mesenchyme tissue to remodel and expand, accommodating for the elongating tubule.

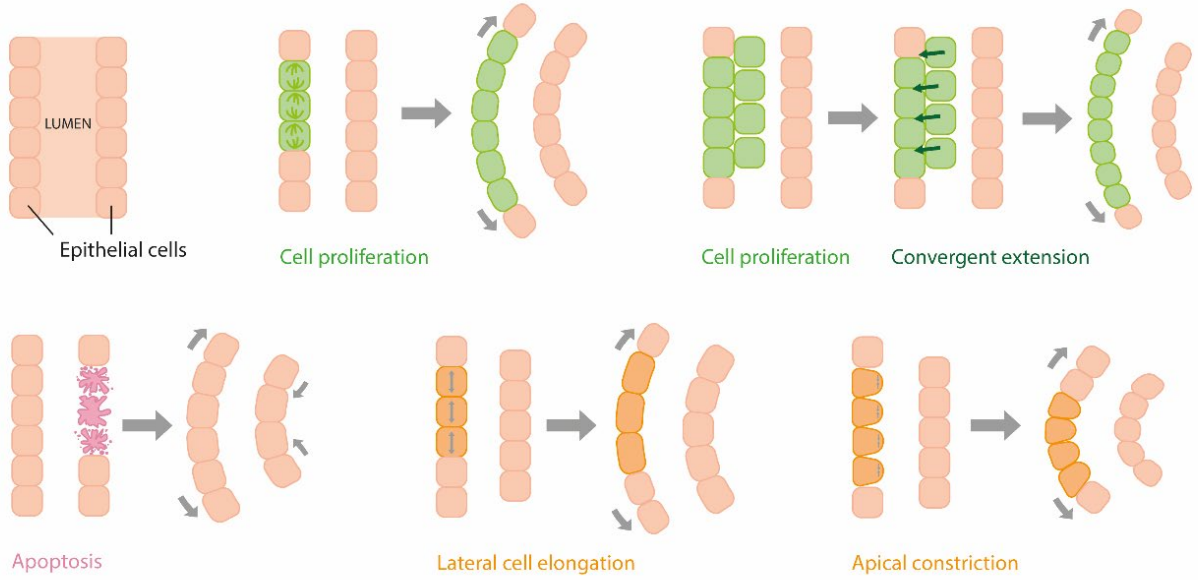


Figure 2.6 Hypothetical mechanisms of Wolffian duct and epididymal tubule coiling. Combinations of these mechanisms are also possible.

The epididymis is not fully developed at birth. After birth, the epididymal duct goes through a significant transformation involving duct elongation and convolution in the cauda region. Then at the onset of puberty, the epididymal epithelium undergoes structural and cellular differentiation to form the fully developed adult epididymis (Joseph, Yao & Hinton 2009; Murashima, Xu & Hinton 2015; Robaire, Hinton & Orgebin-Crist 2015), in which the epithelium is pseudostratified and composed of several cell types: principal, basal, clear, narrow, apical and halo cells. More recently, a dense population of dendritic cells was identified in the base of the epididymal epithelium (Da Silva et al. 2011). These dendritic cells project extensive networks of processes that interact with the epithelium, and within the proximal epididymis, they also project between epithelial cells to the lumen, indicating a potential role in establishing immune tolerance to spermatozoa (Da Silva et al.

2011). Overall, Wolffian duct and epididymis development is a complex process spanning both embryonic and post-natal development.

The role of extracellular matrix (ECM) remodelling and cell–ECM interactions has been increasingly demonstrated to be crucial for the morphogenesis of tubular organ structures (Bonnans, Chou & Werb 2014; Daley & Yamada 2013; Scarpa & Mayor 2016). Interactions between epithelial and endothelial cells and the ECM, and the degradation of ECM components by proteases such as matrix metalloproteinases (MMPs), facilitate cell proliferation, differentiation, migration, adhesion, and survival. The activity of these enzymes is strictly regulated by endogenous inhibitors of ECM proteinases, *e.g.* tissue inhibitors of metalloproteinases and cystatin C (Bonnans, Chou & Werb 2014). The ECM also acts as a reservoir of growth factors, including epithelial growth factor and fibroblast growth factor, which are released via the proteolytic activity of MMPs (Bonnans, Chou & Werb 2014). The proteolysis of ECM constituents is critical for ensuring proper structure and composition of the matrix and to maintain the balance between degradation and deposition of ECM components (Bonnans, Chou & Werb 2014). Dysregulation of ECM deposition results in ECM stiffness, as seen in fibrosis (Karsdal et al. 2015; Long, Artlett & Blankenhorn 2014; Wight & Potter-Perigo 2011), while excessive degradation of ECM components results in tissue destruction as observed in osteoarthritis (Dai et al. 2015; Liu et al. 2014; Troeberg & Nagase 2012). Presumably, as with other tubular organs, ECM remodelling in the developing epididymis is a crucial part of duct morphogenesis.

There are limited studies of defective ECM structure associated with epididymal or fertility defects. Notably, in hypomorphic *Lgr4* (a gene encoding a G protein-coupled receptor) mutant mice, which present with 10% of normal LGR4 transcripts, adult mice exhibit defective sperm motility in addition to shorter, less coiled epididymides, multilamination of the basement membranes, and an accumulation of laminin in the mesenchyme layers of the caput region, indicating that defective ECM remodelling may contribute to the epididymis phenotype (Hoshii et al. 2007). *Lgr4* KO mice exhibit a similar epididymal phenotype; the tubule is less elongated and coiled, in addition to dilation of the duct and a thick condensation of mesenchymal cells surrounding the tubule (Mendive et al. 2006). Interestingly, the epididymides in *Lgr4* KO mice appear normal at birth and the epididymal defects

are only observed from postnatal day 3, indicating that LGR4 specifically plays a role in early postnatal development (Mendive et al. 2006). As previously mentioned, elongation and coiling of the epididymal tubule is normally completed in early postnatal development, particularly in the cauda region (Joseph, Yao & Hinton 2009). These observations in LGR4 mutants allude to a role for LGR4 in epithelial-mesenchymal interactions in epididymal coiling and elongation.

In many other rodent models of impaired epididymis coiling, ECM defects typically have not been mentioned, presenting the possibility that the observed impairments in tubulogenesis are caused by unidentified abnormal ECM remodelling. Therefore, investigations of impaired epididymis elongation and coiling would benefit from consideration of the role of ECM remodelling in epididymis morphogenesis.

2.4.3 Mouse models of aberrant Wolffian duct and epididymal morphology

Aberrant development and morphological abnormalities of the epididymis are commonly observed in conjunction with infertility or subfertility in rodent models, which is not surprising since epididymal maturation of sperm is vital for normal fertility (Dacheux & Dacheux 2014; Hinton et al. 2011). Defective Wolffian duct and early epididymal development and differentiation has been characterised in a number of transgenic mouse models (for a review of these models, see Murashima, Xu & Hinton 2015). The range of defects in these various transgenic models include regression and dysgenesis of the Wolffian duct, and failure of the duct to elongate and coil. It should be noted that in most models of aberrant Wolffian duct and epididymis development, only early stages of development have been investigated. Therefore, for most models, it is unknown whether epididymal morphology is normal by the time the rodents reach adulthood and defects are a result of delayed development or delayed puberty. This is seen in *Inhba* mutant mice (*Inhba*$^{BK/BK}$ mice), which lack expression of activin A (a transforming growth factor-β superfamily cytokine) and exhibit reduced elongation and coiling of the epididymal tubule in earlier stages of development (Wijayarathna et al. 2018). However, the epididymides are comparable to WT animals by 8 weeks of age, indicating that the initial epididymal defects are due to delayed epididymis development in the absence of activin A (Wijayarathna et al. 2018). As such, in rodent models of abnormal Wolffian duct

or epididymis morphology, it may be important to include analysis of older ages to ensure that any observed abnormalities are still present in adulthood, allowing the determination of whether aberrant adult epididymis function is implicated in cases of male infertility.

The epididymal morphology in *Plag1* KO mice has not yet been characterised. It therefore remains unknown whether epididymis development is affected in these mice and whether abnormal epididymis morphology may be a contributing factor to the impaired fertility exhibited by *Plag1* KO males. Analysis of epididymis morphology in adult mice (perhaps including adults more than 10 weeks old) will be particularly important since polymorphisms of the *PLAG1* region are associated with delayed puberty in cattle (Fortes et al. 2013a). This will ensure that any abnormalities in epididymis morphology in *Plag1* KO mice are not repaired during a delayed puberty.

2.5 Conclusion and future perspectives

Epididymal maturation of spermatozoa is critical for sperm motility, and consequently critical for fertility, yet much remains unknown or unclear regarding the development and function of this male reproductive organ. A greater understanding of the epididymis may aid in the identification of druggable targets and the development of non-hormonal male contraceptive options that, without affecting sperm production itself, result in the production of non-functional sperm (Cornwall 2009). So far, research on PLAG1 in male fertility has revealed remarkable sperm motility defects and the ectopic expression of epididymal genes in the testes from *Plag1* KO mice (Juma et al. 2017), indicating that PLAG1 may play a role in epididymal sperm maturation and that PLAG1 may regulate epididymal genes. The role of PLAG1 in the epididymis has not yet been studied; however, it would be worthwhile to characterise PLAG1 expression in the mouse epididymis and to investigate epididymal structure and function in *Plag1* KO mice. Further, since gene pattern expression is crucial for sperm maturation and epididymal genes were ectopically expression in the testes from *Plag1* KO mice, it may be insightful to analyse the transcriptome of the epididymis of *Plag1* KO mice to determine target genes of PLAG1 and whether any important sperm maturation genes are dysregulated in the absence of PLAG1. Ultimately, in order to understand the underlying causes of

the reduced fertility and impaired sperm motility in *Plag1* KO male mice, the role of *Plag1* in the mouse epididymis requires further research.

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Chapter 3. Deficiency of the transcription factor PLAG1 results in aberrant coiling and morphology of the epididymis

Abstract

Mice deficient in the transcription factor pleomorphic adenoma gene 1 (PLAG1) exhibit reproductive issues that are characterised, in part, by decreased progressive sperm motility in the male. However, the underlying cause of this impairment is unknown. As epididymal transit is critical for sperm maturation and motility, the morphology of the epididymis of *Plag1*-deficient mice was investigated and the spatial expression patterns of PLAG1 protein and mRNA were identified. By using X-gal staining and *in situ* hybridisation, PLAG1 was shown to be widely expressed in both the epithelium and stroma in all regions of the mouse epididymis. Interestingly, the X-gal staining pattern was markedly different in the cauda, where it could be suggestive of PLAG1 secretion into the epididymal lumen. At all ages investigated, the morphology of epididymides from *Plag1* knockout (KO) mice was aberrant; the tubule failed to elongate and coil, particularly in the corpus and cauda, and the cauda was malformed, lacking its usual bulbous shape. Moreover, the epididymides from *Plag1* KO mice were significantly reduced in size relative to body weight. In 20% of PLAG1-deficient mice, the left testicle and epididymis were lacking. The impaired morphogenesis of the epididymal tubule is likely to be a major contributing factor to the fertility problems observed seen in male *Plag1*-deficient mice. These results also establish PLAG1 as an important regulator of male reproduction, not only through its involvement in testicular sperm production, but also via its role in the development and function of the epididymis.

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3.1 Introduction

The zinc finger transcription factor pleomorphic adenoma gene 1 (PLAG1) was initially discovered as being involved in pleomorphic adenomas of the salivary gland (Åström et al. 1999), and is now known to play a role in the development of a number of tumour and cancer types (Åström et al. 1999; Juma et al. 2016; Van Dyck et al. 2004). The normal biological role of PLAG1 appears to be largely associated with growth and reproduction (Juma et al. 2016). Embryonic and postnatal growth analysis has shown that *Plag1* knockout (KO; *Plag1*$^{-/-}$) mice are significantly smaller than wild-type (WT; *Plag1*$^{+/+}$) littermates (Hensen et al. 2004). Many of the target genes of PLAG1, as determined by microarray analyses, are involved in growth and cell proliferation, including insulin-like growth factor 2, bone-derived growth factor, cytokine-like factor 1 and placental growth factor (Van Dyck et al. 2004). In addition to reduced growth in mice, Hensen *et al.* (2004) also reported that fertility is reduced in *Plag1* KO mice; compared with WT males, *Plag1* KO males exhibited decreased conception rates when mated with WT females (Hensen et al. 2004). *Plag1* mRNA was detected in the embryonic urogenital ridge and the adult testis (Hensen et al. 2004).

More recently, it has been shown that PLAG1 is required for normal sperm production and sperm motility in mice (Juma et al. 2017). Mice deficient in PLAG1 exhibit decreased daily sperm production compared with age-matched WT littermates and cauda fluid from mature KO males show a 49% reduction in motile spermatozoa, an 80% reduction in progressively motile spermatozoa and a decreased number of spermatozoa exhibiting rapid motility (Juma et al. 2017). This reduced sperm motility is likely to be a major reason for the decreased fertility of *Plag1*-deficient mice. The underlying cause of the reduced sperm motility observed in *Plag1* KO mice has not yet been identified. As spermatozoa gain their motility during transit through the epididymis, it is likely that the function of the epididymis of *Plag1* KO mice is defective. The rodent epididymis is generally divided into four major regions (the initial segment, caput, corpus and cauda), and further divided into 10 transcriptionally unique segments in mice (Johnston et al. 2005) and 19 in rats (Jervis & Robaire 2001). As they transit through the epididymal tubule, spermatozoa undergo a multitude of structural and biochemical modifications that are crucial for proper maturation and the production of spermatozoa capable of both motility and fertilisation of an egg (Dacheux & Dacheux 2014;

Sullivan & Mieusset 2016). In rodents, the cauda epididymidis also functions for storage of mature spermatozoa (Sullivan & Mieusset 2016).

In this study, we investigated the epididymis as the underlying cause of the sperm abnormalities in *Plag1*-deficient mice. We show that PLAG1 is widely expressed throughout the entire epididymal tubule and stroma in mature male mice, and demonstrate that the morphology of the epididymis from *Plag1*-deficient mice exhibits decreased tubule elongation and coiling and an overall reduction in relative size.

3.2 Materials and methods

3.2.1 Animals

All animal procedures undertaken in this study were approved by the Animal Ethics Committee of La Trobe University (AEC17-54, AEC17-27) and the La Trobe Institutional Biosafety Committee (GMSC17-15), Bundoora, Victoria, Australia. All animal care and experimental procedures were conducted in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th edition, 2013) of the National Health and Medical Research Council. Founder mice were a kind gift from Prof. Wim Van de Ven (Laboratory for Molecular Oncology, Center for Human Genetics, Catholic University of Leuven, Leuven, Belgium); the generation of the *Plag1* KO mouse line has been previously outlined (Hensen et al. 2004). Genotypes of mice were determined by PCR with genomic DNA isolated from ear clips, with the following primers: 5'-ATGGCCACTGTCATTCCTGGTGATTTGTCA-3' and 5'-CCTGTGTGTACCACCATGTGTCTCCGGACA-3' to detect the WT *Plag1* allele and 5'-GCATCGAGCTGGGTAATAAGCGTTGGCAAT-3' and 5'-GACACCAGACCAACTGGTAATGGTAGCGAC-3' to detect the *lacZ* reporter gene.

3.2.2 Breeding analysis

Three *Plag1* KO, heterozygous (HET; *Plag1*$^{+/-}$) and WT males aged 10–12 weeks were each housed with two 10–16-week-old WT females for a period of 5 months. Each mouse was genotyped

twice, by two different researchers, to ensure accuracy of genetic status. The number of litters born and litter size were recorded. At the conclusion of the breeding analysis, the male mice were dissected to ensure both testes and epididymides were present, which was the case for all animals.

3.2.3 X-gal staining

Seven-week-old KO, HET and WT male mice (*n*=5 per genotype) were killed by CO_2 asphyxiation. Epididymides were dissected and immediately immersed in 4% (*w/v*) paraformaldehyde in phosphate-buffered saline (PBS) for 2 h at 4°C. The tissues were cryoprotected in 10% (*w/v*) sucrose in PBS for 2 h, then 30% (*w/v*) sucrose in PBS until the tissues sank. The tissues were quick-frozen in cooled isopentane and cryosectioned into 5-µm serial sections. Slides were incubated in X-gal staining solution [0.5 mg ml^{-1} X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PanReac Applichem, Darmstadt, Germany), 5 mmol l^{-1} $K_3Fe(CN)_6$, 5 mmol l^{-1} $K_4Fe(CN)_6$, 2 mmol l^{-1} $MgCl_2$, 0.01% (*w/v*) sodium deoxycholate, 50 mmol l^{-1} EGTA, and 0.02% (*v/v*) IGEPAL CA-630 (Sigma-Aldrich, St. Louis, MO, USA)] in PBS in the dark at 37°C overnight. The sections were then rinsed in PBS and counterstained for 30 s with Nuclear Fast Red (Sigma-Aldrich) before coverslipping. Tissue sections were imaged with an Olympus BX41 microscope with an Olympus DP25 camera (Olympus Scientific Solutions Americas Inc., Waltham, MA, USA).

3.2.4 *In situ* hybridisation

Epididymal sections from 7-week-old *Plag1* WT and KO male mice (*n*=5 per genotype) were made as described above for X-gal staining. *In situ* hybridisation was performed as previously described (Grommen et al. 2017) with the following alterations: riboprobes were transcribed from 1 µg of linearised pCRII-TOPO plasmid (Thermo Fisher Scientific, Waltham, MA, USA) containing a 752-bp insert (bp 503 to 1254 of the mouse *Plag1* coding sequence, GenBank accession number NM_019969.3) in digoxigenin (DIG) RNA Labelling Mix and 40 U SP6 or T7 RNA polymerase (Roche Diagnostics, Risch-RotKreuz, Switzerland). Slides were incubated overnight in the hybridisation buffer containing 800 ng/ml antisense or sense DIG-labelled probe at 66°C. Coverslips were removed and slides were washed two times for 5 min in 0.2× saline sodium citrate at room temperature, followed

by three 5-min washes in PBS with 0.1% (*v/v*) Triton X-100. The protocol continued as described before (Grommen et al. 2017). As an additional negative control, epididymis sections from a *Plag1* KO male were included to ensure there was no non-specific probe binding. Sections were imaged as described above.

3.2.5 Epididymal histology and morphology

Male mice aged 3, 5, 28–33 and 50–55 weeks (*n*=5 per genotype and age) were killed by CO_2 asphyxiation. Epididymides were dissected, immediately immersed in 4% paraformaldehyde in PBS and kept overnight at 4°C. The tissues were cryoprotected in 30% sucrose in PBS until the tissues sank. Tissues were quick-frozen in cooled isopentane and cyrosectioned into 5-μm serial sections and mounted onto glass slides. The sections were thawed at room temperature for 30 min, then stained with Mayer's haematoxylin (Trajan Scientific and Medical, Ringwood, Victoria, Australia) for 2 min and counterstained with eosin (Thermo Fisher Scientific) for 20 s. Tubule cross sections in serial epididymal sections from 7-week-old *Plag1* KO, HET and WT mice (*n*=5 per genotype) were manually counted (**Supplementary Figure 3.1**) and compared between genotypes. A minimum of five longitudinal epididymal sections were analysed per animal. Crosswise sections were made from the caudae epididymidis from 7-week-old *Plag1* KO and WT mice (*n*=5 per genotype). Three to six cross sections in the XY plane per animal were measured for luminal diameter using Olympus cellSens software (Olympus Scientific Solutions Americas Inc.), by drawing a straight line across the maximum luminal width and recording the line length (**Supplementary Figure 3.1**). The mean luminal diameter was calculated for each animal and compared between genotypes. Epididymal weight and body weight were measured in five KO and five WT males aged three weeks, and in five KO and five WT adult males aged at least 11-weeks-old. Epididymal weights relative to body weight were calculated and compared between genotypes.

3.2.6 Statistical analyses

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Means were compared using an unpaired Student *t*-test or a one-way ANOVA with

post-hoc Tukey's HSD test for multiple comparisons, with significance set at $P<0.05$. Fisher's exact test was used to assess the association between genotype and presence/absence of the left testis and epididymis.

3.3 Results

3.3.1 *Plag1*-deficient male mice and infertility

There was no statistical difference between HET and WT males in litter size and number of litters born in matings with WT females, but no litters were born from matings with *Plag1* KO males with WT females during the 5-month observation period (**Table 3.1**).

Table 3.1. Breeding analysis of *Plag1* wild-type, heterozygous, and knockout males mated with wild-type females.

Breeding parameter	Genotype			Statistical analysis
	WT	HET	KO	
Litter size (mean ± s.d.)	5.87 ± 1.99	5.37 ± 2.12	0	WT vs. HET *t*-test, $P=0.8960$
Number of litters (mean ± s.d.)	3.33 ± 0.58	2.83 ± 0.29	0	WT vs. HET *t*-test, $P=0.4000$

WT: wild-type; HET: heterozygous; KO: knockout

3.3.2 PLAG1 expression in the mouse epididymis

In *Plag1* KO mice, the *Plag1* gene sequence is replaced with a *lacZ* reporter gene, which encodes β-galactosidase. This allows the use of X-gal staining to identify the spatial expression of PLAG1 in KO and HET animals. X-gal staining was widely found in the epididymis of KO animals; staining was observed in the epithelium, stromal cells and the smooth muscle layer. In the caput and proximal corpus, the X-gal signal was concentrated and in close association with the nuclei of the basal, principal, apical and smooth muscle cells (**Figure 3.1**); however, in the distal corpus and the cauda, the signal in the epithelium became diffuse and localised to the apical cytoplasm (**Figure 3.1**).

Concentrated nuclear expression of PLAG1 was still observed in the basal cells, stromal cells and smooth muscle layer in the distal corpus and cauda. This expression pattern was also seen in HETs, although expression was, as expected, not as strong as in KOs (**Supplementary Figure 3.2**). There was no X-gal signal in the epididymal sections of most WT animals, although a very faint and hazy signal, clearly distinct from that in the HETs and KOs, was seen in some sections of some animals (**Supplementary Figure 3.3**).

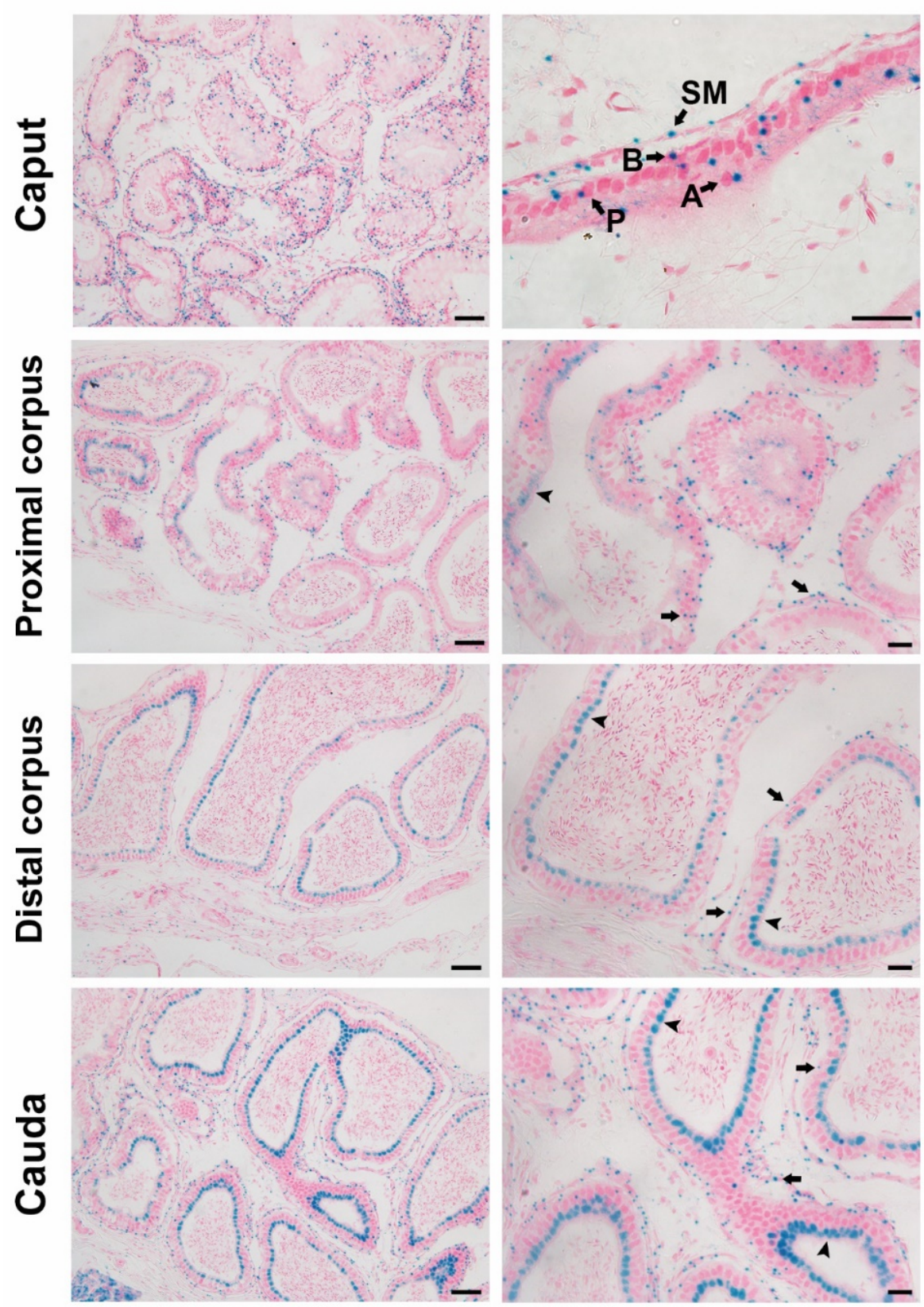

Figure 3.1. Localisation of PLAG1 expression by X-gal staining in the epididymis of 7-week-old *Plag1* knockout mice. Scale bars represent 50 µm in low-magnification images (left column) and 20 µm in high-magnification images (right column). Arrows indicate nucleus-associated PLAG1 signal; arrowheads indicate PLAG1 signal in the apical cytoplasm. P: principal cell; B: basal cell; A: apical cell; SM: smooth muscle cell.

3.3.3 Regional expression of *Plag1* mRNA in the mouse epididymis

Plag1 mRNA was detected by *in situ* hybridisation in the principal, basal and apical cells of the epithelium and the smooth muscle cells and stromal cells, in all regions of the epididymis (**Figure 3.2**). Less abundant expression was observed in the cauda compared with the initial segment, caput and corpus. There was no signal in sections stained with the sense probe and no non-specific staining in the KO control (**Supplementary Figure 3.4**).

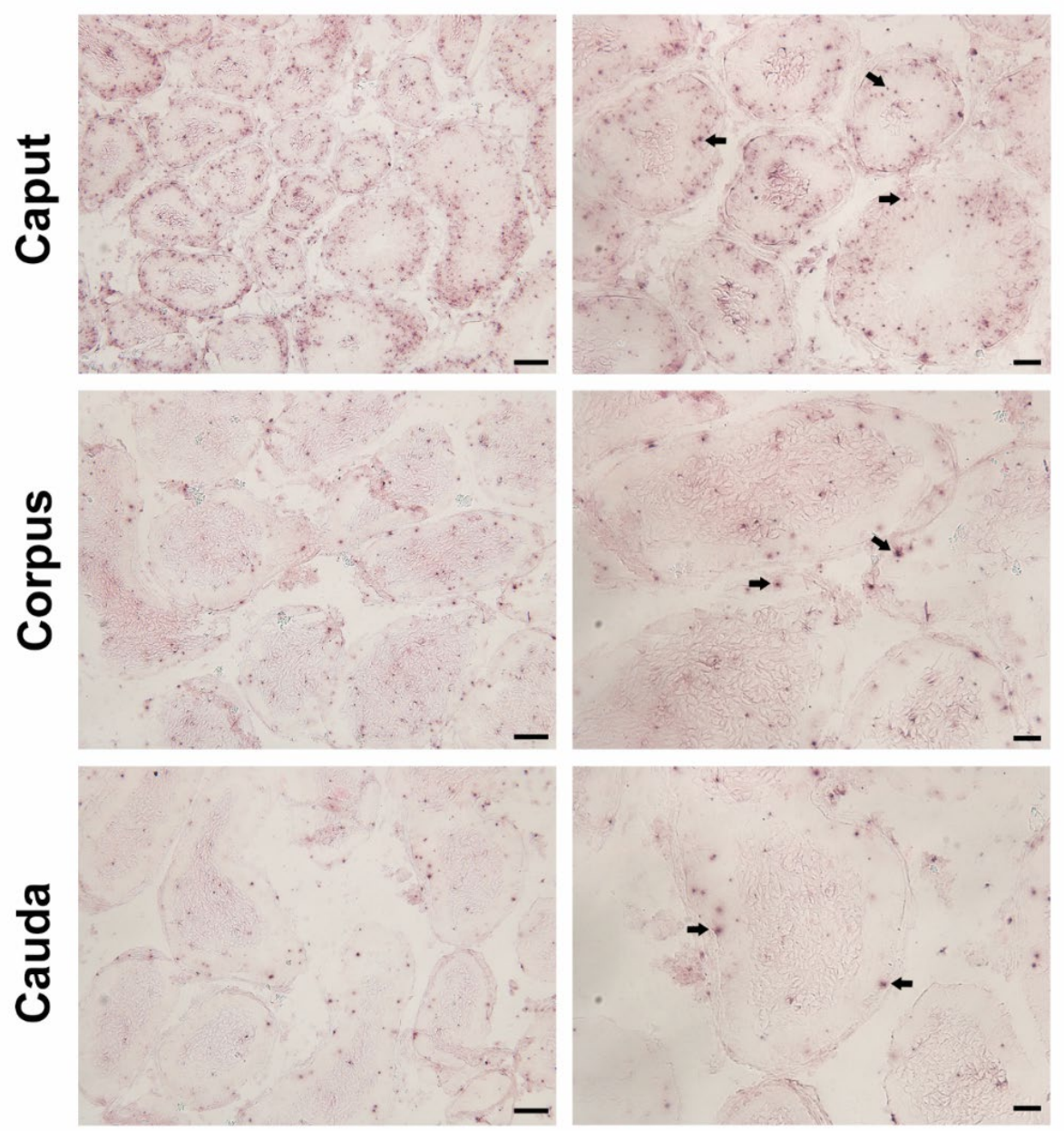

Figure 3.2 Localisation of *Plag1* mRNA in 7-week-old wild-type mice by *in situ* hybridisation. Arrows indicate positive *in situ* hybridisation signal. Scale bars represent 50 µm in low-magnification images (left column) and 20 µm in high-magnification images (right column).

3.3.4 *Plag1*-deficient mice have reduced epididymis weight and aberrant epididymis morphology

Epididymides from 3-, 5-, 28–33- and 50–55-week-old *Plag1* KO and WT mice were examined macroscopically and microscopically. In all mice, testes were descended normally. However, out of 65 male *Plag1* KOs dissected, 13 (20%) were observed with absence of the left testis and epididymis. None of the 49 WTs dissected were lacking the left testis and epididymis. The association of the *Plag1* KO genotype and absence of the left testis and epididymis was analysed using Fisher's exact test, and found to be statistically significant (P=0.0017). At all ages, the cauda in KOs lacked the typical large, bulbous shape (**Figures 3.3–3.4; Supplementary Figures 3.5–3.7**). Epididymal weight relative to body weight was compared between *Plag1* KO and WT mice aged three weeks and in adults. Epididymides from KO mice had a significantly reduced mass relative to body weight, weighing on average (s.d.) 0.025 ± 0.006% compared with 0.060 ± 0.005% in WTs (t-test, P<0.0001) in 3-week-old mice, and weighing on average (s.d.) 0.062 ± 0.019% compared with 0.1105 ± 0.019% in WTs in adult mice aged 11 weeks or older (t-test, P=0.0035).

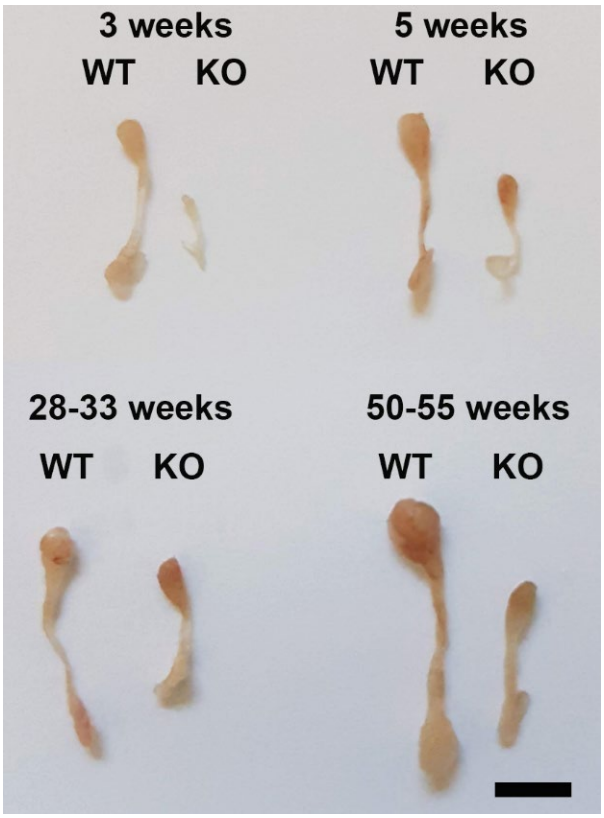

Figure 3.3 Macroscopic images of the epididymides from *Plag1* WT and KO mice aged 3, 5, 28–33 and 50–55 weeks. Scale bar = 5 mm.

The histology of the epithelium was not obviously different between WTs and KOs. At all ages, a normal pseudostratified epithelium was observed, but the epididymal tubule was less coiled and elongated in *Plag1* KOs compared with WTs, particularly in the cauda, as judged by the number of tubule cross-sections and luminal diameter (**Figures 3.4 and 3.5; Supplementary Figures 3.5–3.7**). A decreased number of tubule cross sections was observed in the caput, corpus and cauda of KOs compared with WTs and HETs (ANOVA, $P<0.01$) (**Figure 3.5a**), and the diameter of the epididymal tubule lumen in the cauda was increased in *Plag1* KO mice in comparison with WTs (*t*-test, $P=0.0003$) (**Figure 3.5b**).

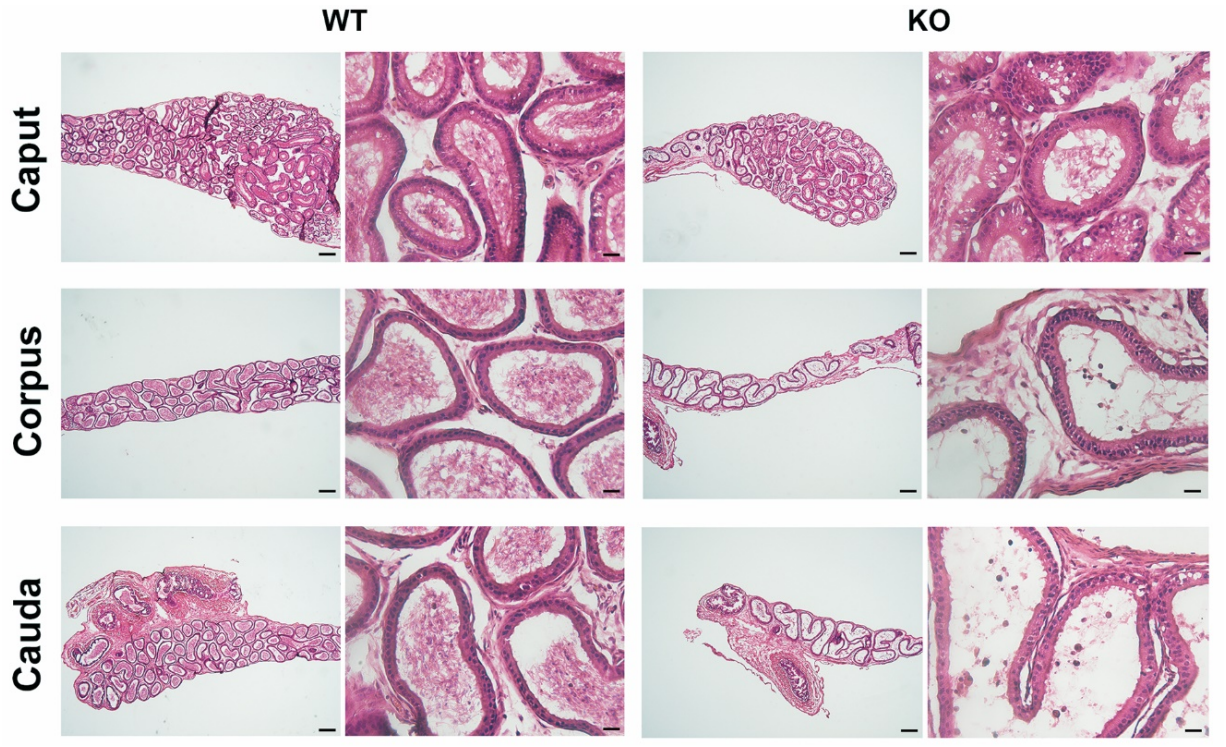

Figure 3.4 Haematoxylin-and-eosin staining of the epididymal sections from 5-week-old *Plag1* WT and KO mice. Scale bars represent 200 µm in low-magnification images (left column) and 20 µm in high-magnification images (right column).

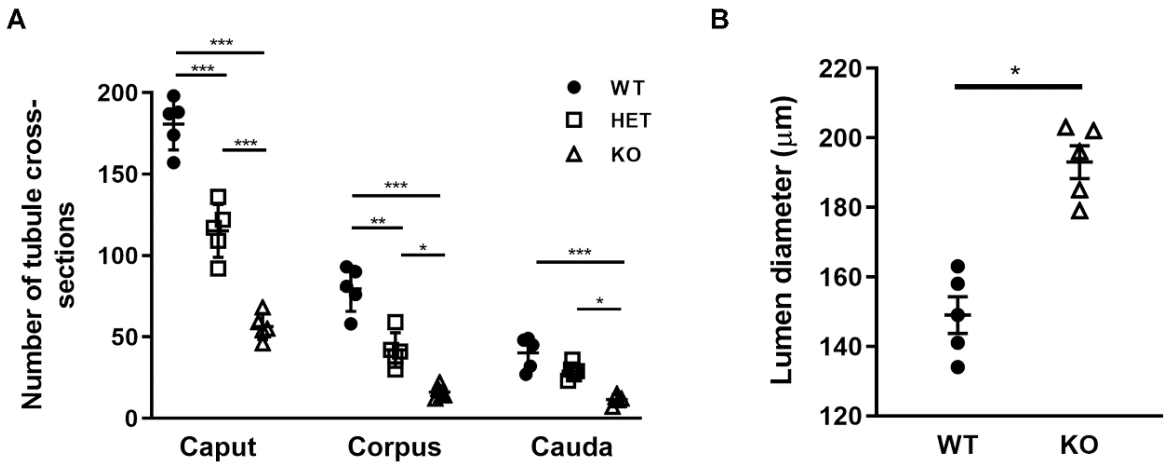

Figure 3.5. (a) Number of XY-plane epididymal tubule cross sections in the caput, corpus, and cauda of 7-week-old *Plag1* WT (circles), HET (squares), and KO (triangles) mice (*n* = 5). Data are presented as mean ± s.d. **P* < 0.01; ***P* < 0.001; ****P* < 0.0001 (one-way ANOVA with Tukey's honest significant difference test). **(b)** Diameter of the tubule lumen in the cauda epididymidis of 7-week-old *Plag1* WT and KO mice (*n* = 5). Data are presented as mean ± s.d.**P* < 0.01 (*t*-test). s.d.: standard deviation.

3.4 Discussion

Plag1-deficient mice are known to have fertility problems (Hensen et al. 2004; Juma et al. 2017). Our breeding analysis indicated that *Plag1* HETs are not sub-fertile, as there was no significant difference in the number of litters born or litter size when compared with WTs, suggesting that one copy of the *Plag1* gene is sufficient for normal fertility. The *Plag1* KO males in our colony were completely infertile, with no litters being born from these matings in the 5-month period of the analysis. These results are contrary to previous findings, in which *Plag1* KO males were observed to successfully impregnate 7% of female WTs they were housed with (Hensen et al. 2004). However, the number of males tested and the length of the mating period was not specified in that study. Plugging behaviour was normal in male KOs, indicating that sexual behaviour was not affected and that the infertility exhibited by *Plag1*-deficient males has other underlying causes.

Apart from reduced sperm production, *Plag1* KO males also show reduced sperm motility (Juma et al. 2017), suggesting a role of PLAG1 in epididymal development or function. The results of this study show for the first time that PLAG1 is widely expressed in the mouse epididymis and that

PLAG1 is required for normal epididymal development and structure. In the caput and proximal corpus, PLAG1 expression was localised to the nucleus of the principal, basal and apical cells. Here, the signal was concentrated and in association with the nuclei of the cells, as is expected of transcription factors, and is the same type of PLAG1 signal observed previously in X-gal staining of the testes (Juma et al. 2017), the pituitary gland (Juma et al. 2018) and the hypothalamus (Juma et al. 2018). However, X-gal staining signal in the distal corpus and cauda epididymis was distinctly different. In the distal segments of the epididymis, X-gal signal became diffuse and localised to the apical cytoplasm. The *lacZ* reporter gene replacing the *Plag1* gene in *Plag1* KO mice is still flanked by the 5' and 3' untranslated regions of the *Plag1* gene (Hensen et al. 2004), which may contain mRNA localisation signals that facilitate the translation of proteins close to the cytoplasmic site of their function (Hervé, Mickleburgh & Hesketh 2004; Mignone et al. 2002). Therefore, we speculate that X-gal signal localisation is representative of the subcellular localisation of PLAG1. The relocation of the transcription factor PLAG1 to the apical cytoplasm could be indicative of it being secreted in the lumen. If PLAG1 were indeed secreted into the epididymal tubule lumen, it may interact with spermatozoa or be involved in maintaining the microenvironment of the luminal fluid. Another possible explanation for the diffuse signal in the apical cytoplasm is the transport of excessive PLAG1 protein to the cytoplasm for degradation; transcription factor levels within a cell can be regulated by proteasome-mediated protein degradation (Desterro, Rodriguez & Hay 2000). Further confirmation of PLAG1 protein secretion with an anti-mouse PLAG1 antibody would be beneficial; however, several commercial antibodies and a custom-made antibody have been tested and have not produced specific staining. Other techniques such as mass spectrometry would require a large amount of epididymal luminal fluid in order to detect low levels of secreted PLAG1.

It has previously been shown that testes from *Plag1*-deficient male mice have a lower relative weight, which presumably contributes to the lower daily sperm production observed in these mice (Juma et al. 2017). In this study, we have shown that the relative epididymal weight in 3-week-old and adult mice is also reduced. The decreased sperm motility and infertility phenotype of *Plag1*-deficient mice indicates that there is an impairment in sperm maturation, which is likely to be a consequence of the defective epididymal elongation and coiling observed in these mice, as the

length of the tubule is well known to be crucial for its function in sperm maturation. As sperm transit the extensive length of the epididymal tubule, they undergo a series of structural and biochemical changes in a very specific sequence (Cornwall 2009; Dacheux & Dacheux 2014; Sullivan & Mieusset 2016; Turner et al. 2003). This precise sequence of maturation events is facilitated by segmentation of the rodent epididymis. Each segment has unique gene and protein expression patterns and is further defined by the presence of connective tissue septa (Turner et al. 2003). As our data show, in *Plag1*-deficient mice, the epididymal tubule fails to elongate to the age-appropriate length. Hensen *et al*. (2004) previously reported that the epididymis from *Plag1* KOs showed no obvious abnormalities; however, the data were not shown and no images were provided. Our results are in direct contrast to this; we observed and show clear morphological abnormalities in the epididymis of *Plag1* KO mice. Presumably, the epididymis in these mice would not be able to facilitate the required sequence of events of sperm maturation and the identity and structure of the epididymal segments may be impaired, which would in turn influence the genes and proteins that should be expressed in each segment. The transformation of the embryonic Wolffian duct into a convoluted tubule over a metre long in mice and over 6 m long in humans (Hinton et al. 2011) is a puzzling process that is not yet well understood. The lack of coiling observed in the epididymis of *Plag1* KO mice is indicative of impaired tubule elongation; however, it is not clear whether the intense tubule coiling of the epididymis is a result of the rapid proliferation of epithelial cells and tubule elongation exerting force on the surrounding tissue, or the mechanical pressure exerted by the mesenchyme surrounding the tubule forcing coiling, or a combination of the two mechanisms (Hirashima 2014; Joseph, Yao & Hinton 2009). The absence of the typical bulbous shape of the cauda epididymidis in *Plag1* KO mice exemplifies this enigma. Does the cauda lack the bulbous shape because the tubule has not elongated properly, or is there a defect in the development or homeostasis of the mesenchyme resulting in a lack of mechanical forces required for tubule development? The epididymal morphology of *Plag1*-deficient mice implies a role for PLAG1 in these dynamics of epididymal development. Furthermore, the absence of the left testis and epididymis was found to be significantly associated with the *Plag1* KO genotype. This condition was not observed in any *Plag1* WT mice. In humans, absence of one testis (monorchidism or unilateral anorchidism) occurs in 3% of males, and is more commonly on the left side (Kogan et al. 1986). As the major cause of anorchidism

are vascular accidents during gestation (Wass & Stewart 2011), PLAG1 deficiency may affect vascular development, resulting in an insufficient blood supply to the developing left testis and epididymis. This would ultimately result in the degeneration of the left testis and epididymis during development.

Several studies have identified a number of genes that are required for normal epididymal development and function, including *Ptk7* (Xu et al. 2016), *Pkd1* (Nie & Arend 2013), *Pkd2* (Nie & Arend 2014) and *Lgr4* (Hoshii et al. 2007; Mendive et al. 2006). Knock-out rodent models for each of these genes exhibit decreased coiling of the Wolffian duct and the epididymis, and decreased fertility. Mesoderm-specific *Ptk7* KO results in a shorter, dilated and significantly less coiled Wolffian duct at E18.5, which testosterone supplementation cannot restore (Xu et al. 2016). *Inhba* is required for anterior Wolffian duct coiling and its activity appears to be dependent of testosterone signalling (Tomaszewski et al. 2007). In both *Pkd1* KO and *Pkd2* KO mice, coiling of the caput epididymidis failed to initiate at E16.5 (Nie & Arend 2013; 2014). Pharmacological inhibition of WNT signalling in mice results in decreased Wolffian duct coiling, while epithelium-specific deletion of the β-catenin (a WNT signalling transducer) gene results in the complete absence of coiling in the epididymis from 1-day-old pups and the constitutive activation of WNT signalling also abolishes any epididymal coiling (Kumar et al. 2016). Furthermore, LGR4, an R-spondin receptor that activates canonical WNT signalling, is crucial for normal postnatal epididymis elongation and coiling (Mendive et al. 2006). *Lgr4* KOs exhibit normal epididymides at birth; however, from postnatal day 3 onwards, epididymides are dilated, the duct fails to continue to elongate and coiling is drastically reduced compared with WT controls (Mendive et al. 2006). The similarities in defective tubule coiling between these gene KO models and *Plag1* KOs suggest that there may be similar pathways and processes affected in the animals, or that PLAG1, as a transcription factor, has downstream control of these genes or pathways, either directly or indirectly. Further, all of these transgenic mouse models, including the *Plag1* KO mice, demonstrate that decreased or defective epididymal tubule coiling is typically associated with decreased fertility. The aforementioned studies focused mainly on the morphology of the embryonic Wolffian duct; however, it would be worthwhile to investigate whether or not the phenotype is restored later in adulthood. Interestingly, mice in which *Inhba* is replaced with an

activin B transgene are found to have a completely normal epididymis morphology by adulthood, despite displaying reduced epididymal weight and delayed epithelial development during earlier postnatal development (Wijayarathna et al. 2018). Evidently, development may be simply delayed in some of the transgenic models and studies would benefit from investigating the adult epididymis.

In summary, PLAG1 is essential for normal epididymal morphology and the establishment of an epididymal tubule that is the appropriate size and length for normal function. Lack of PLAG1 results in reduced coiling of the epididymal tubule, which, as in other KO models, has been associated with reduced fertility. The wide expression of PLAG1 in the epididymis and its potential secretion into the epididymal lumen in adult mice indicates that PLAG1 may also play a role in the epididymal function of sperm maturation and storage. Currently, the target genes of PLAG1 in the epididymis have not been identified. Target genes may include those that have been demonstrated to be crucial for epididymal elongation and coiling, such as the genes described above.

Author contributions

JW carried out all experimental procedures and wrote the manuscript. ARJ assisted with the collection of adult epididymal weights and *in situ* hybridisation. SCT and JGG assisted with genotyping of animals. SVHG and BDG provided advice for experimental procedures, participated in the design of the study and assisted with manuscript preparation. All authors read and approved the final manuscript.

Competing interests

All authors declare no competing interests.

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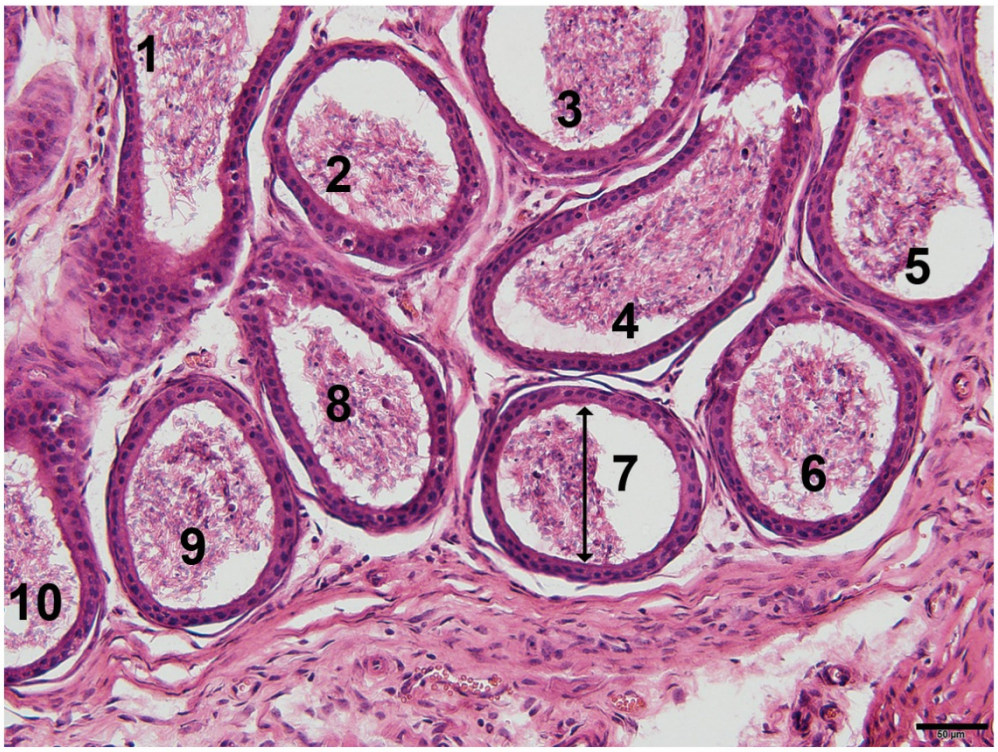
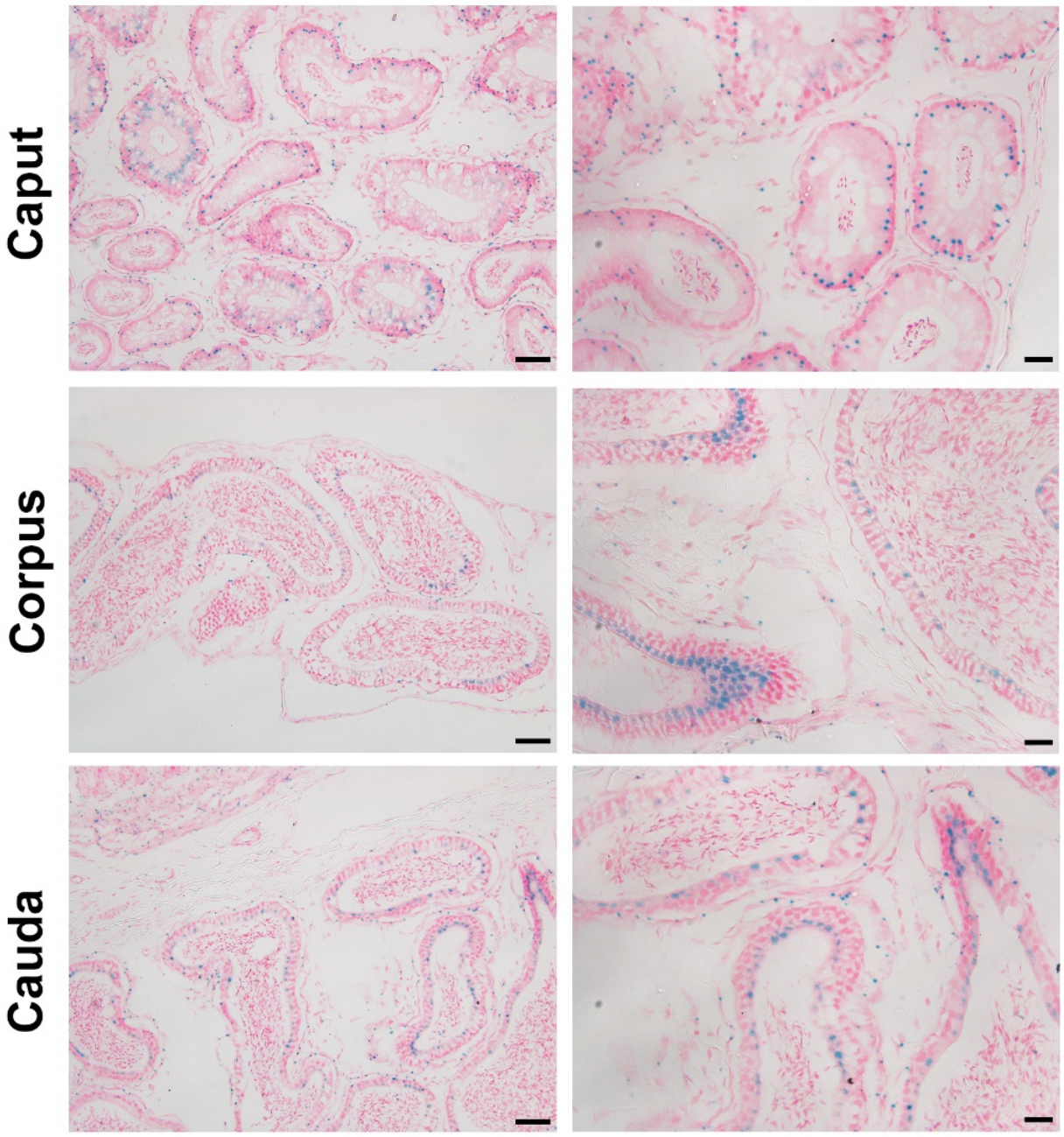
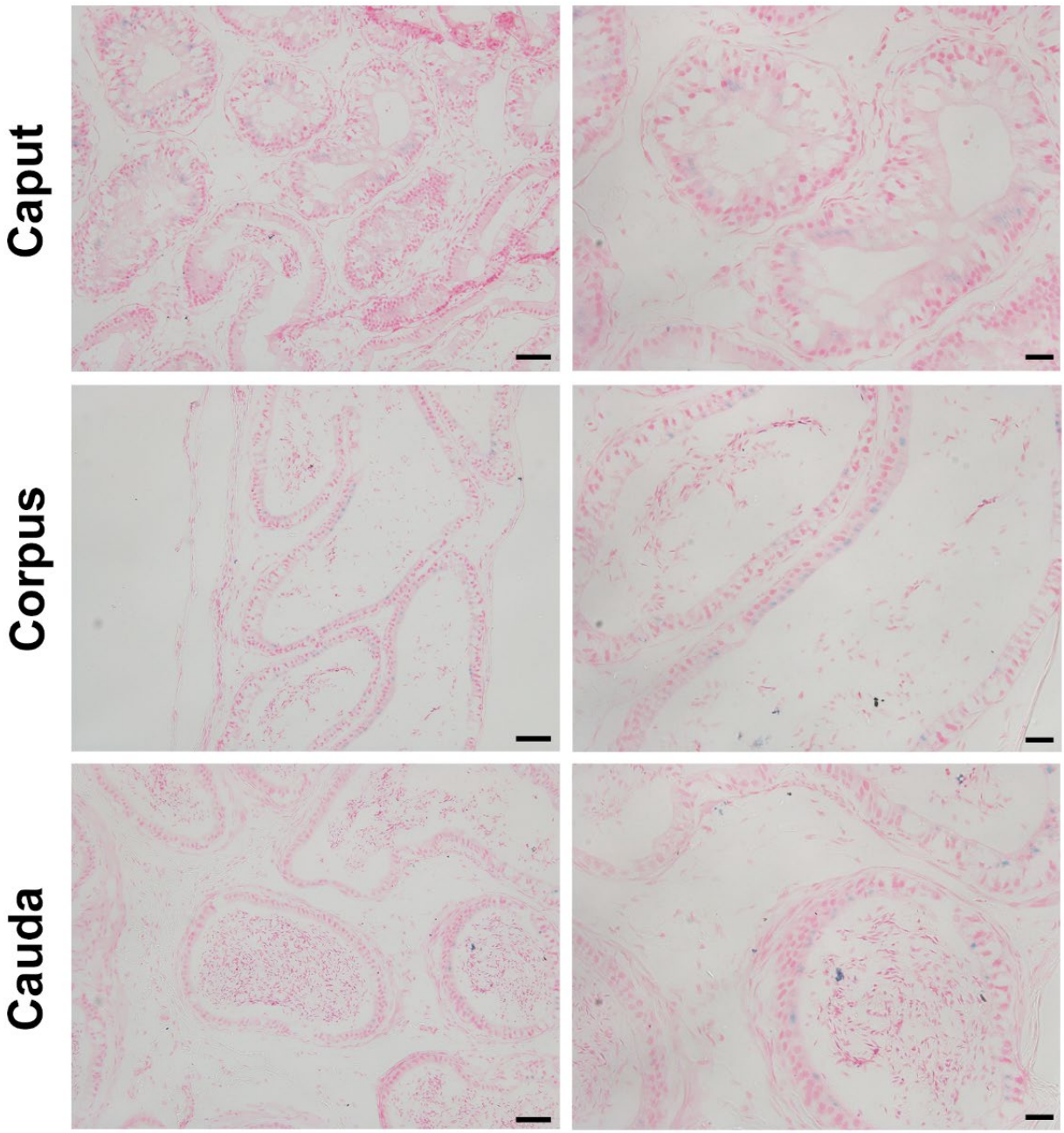
Supplementary data



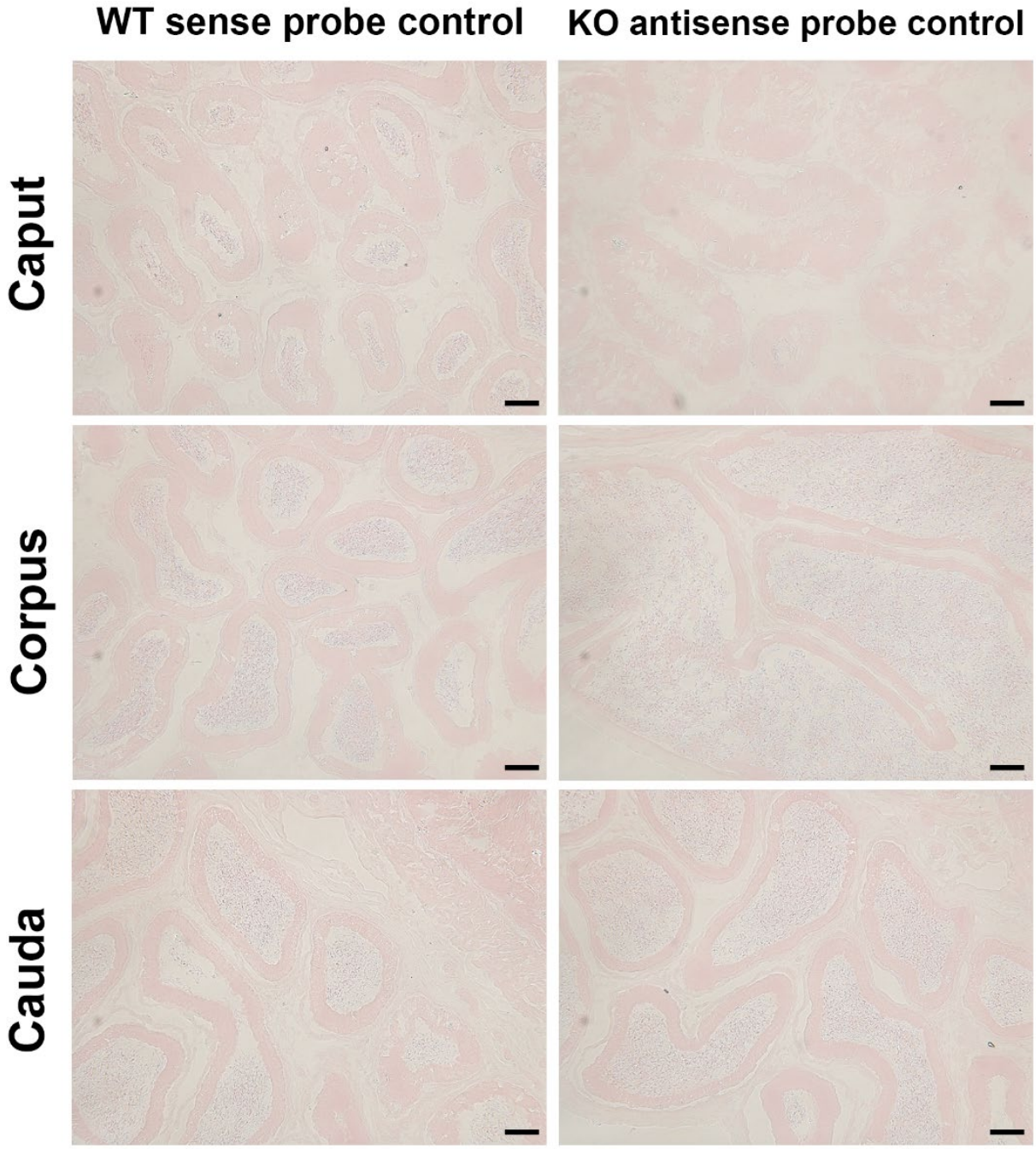
Supplementary Figure 3.1. Representative image to show histological quantification methods. Numbers indicate how tubule cross sections were counted within a field of view. The double-headed arrow indicates a tubule cross section in the XY plane (*i.e.* perpendicular to the tubule's longitudinal axis, resulting in a near circular shape) and how its luminal diameter was measured. The scale bar represents 50 µm.



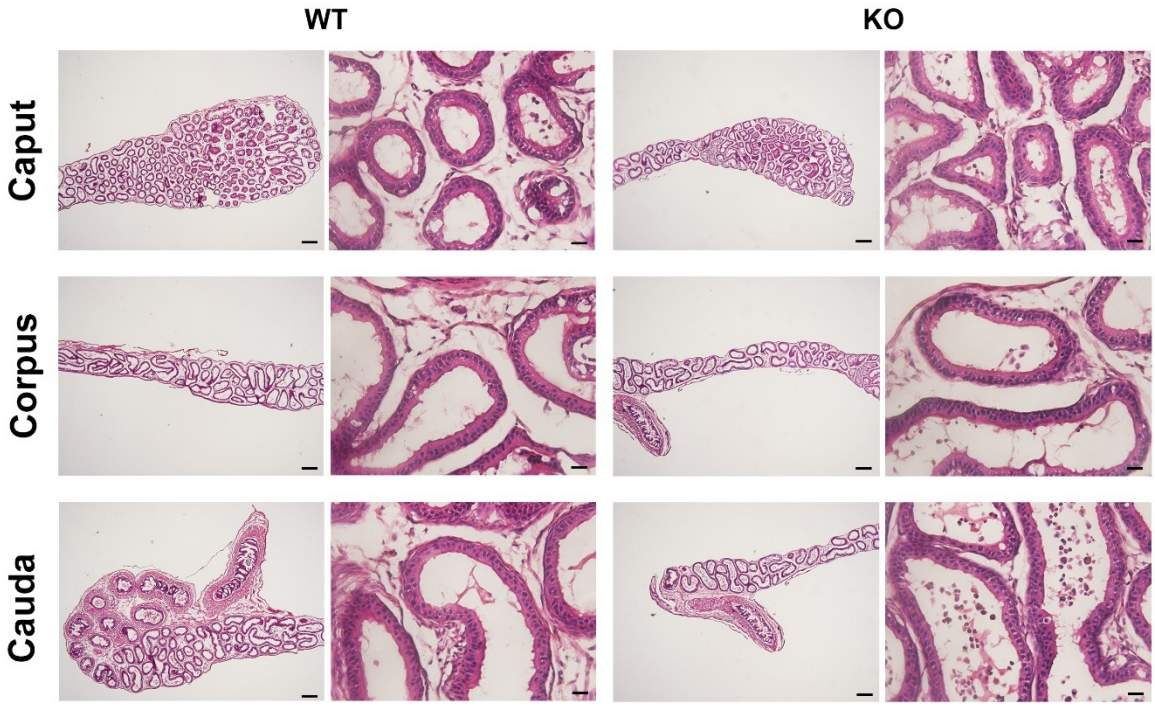
Supplementary Figure 3.2. Localisation of PLAG1 expression by X-gal staining in the epididymis of 7-week-old *Plag1* heterozygous (*Plag1*$^{+/-}$) mice. Scale bars represent 50 µm in low-magnification images (left column) and 20 µm in high-magnification images (right column).



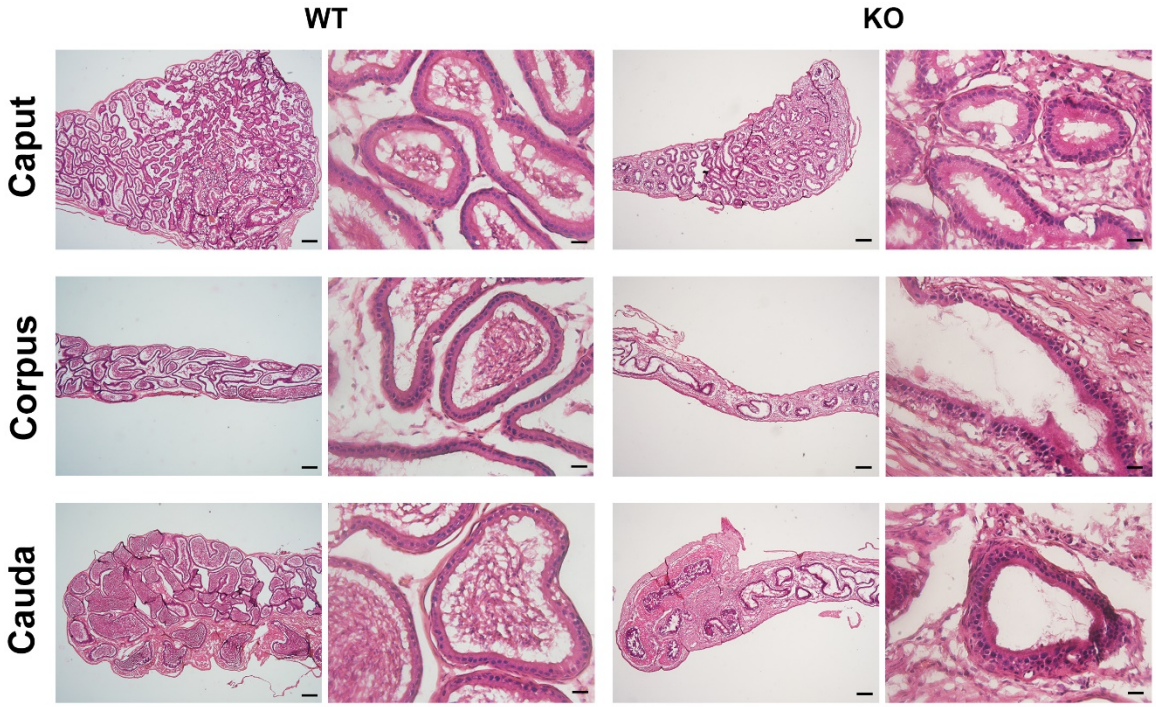
Supplementary Figure 3.3. X-gal staining in 7-week-old wild-type (*Plag1*$^{+/+}$) mice as a negative control. Scale bars represent 50 µm in low-magnification images (left column) and 20 µm in high-magnification images (right column).



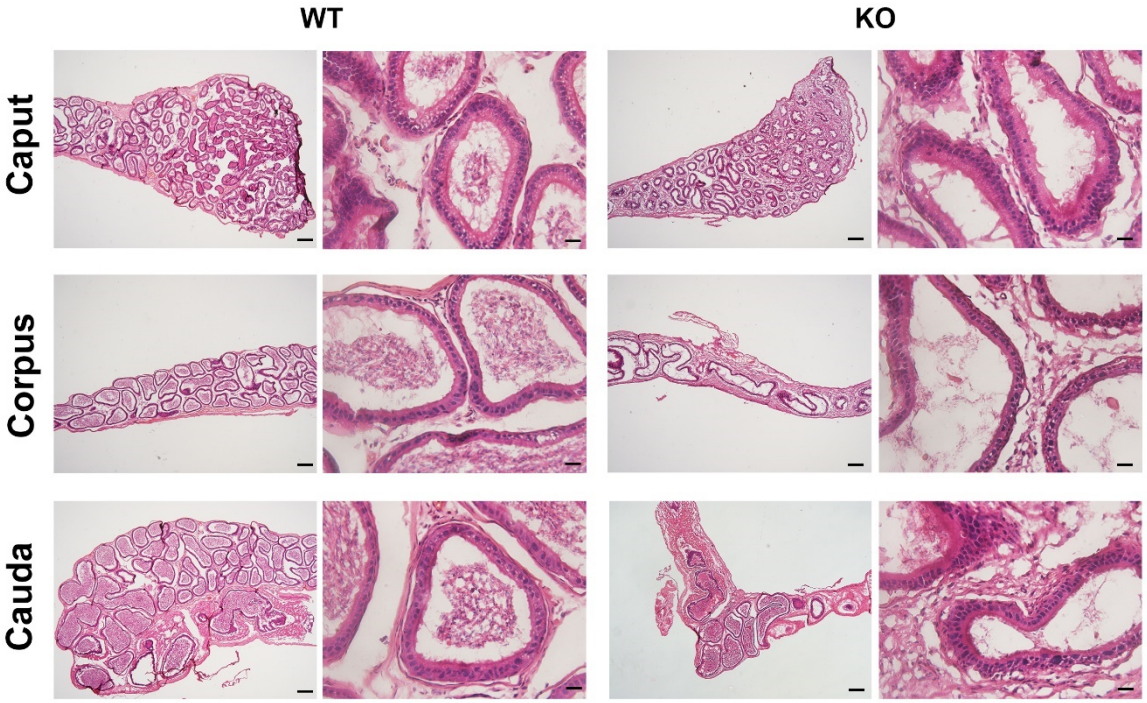
Supplementary Figure 3.4. Negative controls for *in situ* hybridisation staining of *Plag1* mRNA. Epididymal sections from 7-week-old *Plag1* WT mice stained with the sense riboprobe (left column) and epididymal sections from 7-week-old *Plag1* KO mice stained with the antisense riboprobe. Scale bars represent 50 µm.



Supplementary Figure 3.5. Haematoxylin-and-eosin staining of epididymal sections from 3-week-old *Plag1* WT and KO mice. Scale bars represent 200 µm in low-magnification images (left column) and 20 µm in high-magnification images (right column).



Supplementary Figure 3.6. Haematoxylin-and-eosin staining of epididymal sections from 28–33-week-old *Plag1* WT and KO mice. Scale bars represent 200 µm in low-magnification images (left column) and 20 µm in high-magnification images (right column).



Supplementary Figure 3.7. Haematoxylin-and-eosin staining of the epididymal sections from 50–55-week-old *Plag1* WT and KO mice. Scale bars represent 200 µm in low-magnification images (left column) and 20 µm in high-magnification images (right column).

Chapter 4. Transcriptome analysis of the epididymis from *Plag1* deficient mice suggests dysregulation of sperm maturation and extracellular matrix genes

Abstract

Background: The transcription factor pleomorphic adenoma gene 1 (PLAG1) is required for male fertility. Mice deficient in PLAG1 exhibit decreased sperm motility and abnormal epididymal tubule elongation and coiling, indicating impaired sperm maturation during epididymal transit. However, the downstream transcriptomic profile of the *Plag1* knockout (KO; *Plag1*$^{-/-}$) murine epididymis is currently unknown.

Results: In this study, the PLAG1-dependent epididymal transcriptome was characterised using RNA sequencing. Several genes important for the control of sperm maturation, motility, capacitation and the acrosome reaction were dysregulated in *Plag1*$^{-/-}$ mice. Surprisingly, several cell proliferation genes were upregulated, and Ki67 analysis indicated that cell proliferation is aberrantly upregulated in the cauda epididymis stroma of *Plag1*$^{-/-}$ mice. Gene ontology analysis showed an overall upregulation of genes encoding extracellular matrix components, and an overall downregulation of genes encoding metalloendopeptidases in the epididymides from *Plag1*$^{-/-}$ mice.

Conclusion: Together, these results suggest a defect in the epididymal extracellular matrix in *Plag1*$^{-/-}$ mice. These results imply that in addition to maintaining epididymal integrity directly, PLAG1 may also regulate several genes involved in the regulation of sperm maturation and capacitation. Moreover, PLAG1 may also be involved in regulating tissue homeostasis and ensuring proper structure and maintenance of the extracellular matrix in the epididymis.

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4.1 Introduction

Pleomorphic adenoma gene 1 (PLAG1) is a highly-conserved zinc-finger transcription factor required for growth (Hensen et al. 2004), male fertility (Wong et al. 2019), normal sperm production and motility (Juma et al. 2017), and for normal epididymis development and morphology (Wong et al. 2019). *Plag1* knockout (KO; *Plag1*$^{-/-}$) mice exhibit growth retardation during both embryonic and postnatal development, resulting in a ~30% decreased size in adulthood (Hensen et al. 2004). *Plag1*$^{-/-}$ mice also present with substantial fertility defects. Conception rates for male *Plag1*$^{-/-}$ mice are reduced when paired with *Plag1*$^{+/+}$ females (Hensen et al. 2004), while we have previously shown in a 5-month breeding analysis that *Plag1*$^{-/-}$ males are completely infertile, despite normal plugging behaviour (Wong et al. 2019). The number of motile spermatozoa present in the semen from the cauda of KO mice was reduced by 49% compared to their *Plag1*$^{+/+}$ counterparts and progressive motility was reduced by 80% (Juma et al. 2017). As spermatozoa gain motility during epididymal transit, these results indicate that sperm maturation is substantially impaired in *Plag1*$^{-/-}$ mice; however, whether these effects are due to cell-autonomous defects in sperm, or non-autonomous defects due to epididymal dysfunction, is currently unknown.

The epididymis is divided into 10 transcriptionally unique segments in mice (Johnston et al. 2005; Johnston et al. 2007; Turner et al. 2003). During epididymal sperm maturation, spermatozoa undergo a precise sequence of biochemical and structural modifications, which is facilitated by the tightly regulated transcriptomes of these segments (Cornwall 2009; Dacheux & Dacheux 2014; Turner et al. 2003). The epididymides of *Plag1*$^{-/-}$ mice display reduced relative weight, decreased tubule coiling and elongation, and the loss of morphology of the typical bulbous shape of the cauda (Wong et al. 2019). It is therefore possible that the fertility defects in *Plag1*$^{-/-}$ mice are due to loss of epididymal segment identity resulting in altered gene transcription in the epididymis, and ultimately leading to impaired sperm maturation as spermatozoa transit though the duct. Disruption to gene transcription in the epididymis is known to result in dysfunctional, immature or immotile sperm in various rodent models (Jamsai & O'Bryan 2011), and has also been implicated in azoospermatic human male infertility (Dubé et al. 2008).

We hypothesised that the underlying cause of the sperm motility defects observed in *Plag1*$^{-/-}$ mice is due to aberrant transcriptional regulation in the *Plag1*$^{-/-}$ epididymis. To that end, we sought to characterise the transcriptome of the *Plag1*$^{-/-}$ epididymis in order to identify genes and pathways that are dysregulated following deletion of *Plag1.*

4.2 Materials and methods

4.2.1 Animals

All animal procedures undertaken in this study were approved by the Animal Ethics Committee of La Trobe University (AEC17-16) and the La Trobe Institutional Biosafety Committee (GMSC17-15). All animal care and experimental procedures were conducted in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th edition, 2013) of the National Health and Medical Research Council. *Plag1* founder mice were a kind gift from Prof. Wim Van de Ven (Laboratory for Molecular Oncology, Center for Human Genetics, Catholic University of Leuven, Belgium); the generation of the *Plag1*$^{-/-}$ mouse line has been reported previously (Hensen et al. 2004). Genotypes were determined by PCR with genomic DNA isolated from ear clips as previously described (Wong et al. 2019).

4.2.2 RNA sequencing and data analysis

Whole epididymides were used for RNA sequencing (RNA-Seq) in order to characterise the overall transcriptome of the *Plag1*$^{-/-}$ mouse epididymis. Epididymides were harvested from 7-week-old *Plag1*$^{-/-}$, heterozygous (HET; *Plag1*$^{+/-}$) and wild-type (WT; *Plag1*$^{+/+}$) mice (*n*=5 per genotype) euthanised by CO_2 asphyxiation, and teased apart in cold phosphate-buffered saline (PBS). The tissues were rinsed in two changes of sterile PBS to remove spermatozoa. The samples were then homogenised using a mortar and pestle in TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer instructions. Total RNA was treated with DNase using the TURBO DNA-free kit (Invitrogen) and subsequently purified using the Zymo RNA Clean & Concentrator-25 kit (Zymo

Research Corporation, Irvine, CA, USA) following the manufacturer's instructions. RNA concentration was then determined using a Nanodrop (Thermo Fisher, Waltham, MA, USA). RNA-Seq was carried out at the La Trobe Genomics Platform (La Trobe University, Melbourne, Victoria, Australia). Fifteen libraries were constructed using the Illumina TruSeq Stranded mRNA kit (Illumina, San Diego, CA, USA) as per manufacturer instructions and the samples were sequenced by 150-bp, paired-end sequencing using the Illumina NextSeq 500 (Illumina). The raw RNA-Seq data were quality-assessed using fastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapter and low-quality fragments were removed using trimmimatic v0.32 (Bolger, Lohse & Usadel 2014) and the clean reads were then mapped to the *Mus musculus* genome (assembly GRCm38.p6) by HISAT2 v2.0.5 (Kim, Langmead & Salzberg 2015). Alignment quality control was performed using RSeQC v2.6.4 (Wang, Wang & Li 2012) and expression of each gene was determined by the featureCounts function in subread v1.4.6p5 (Liao, Smyth & Shi 2013). Genes with low count values were excluded from analysis; genes with valid expression were defined as genes with log counts per million >0.3 in at least three different replicates. In total, 20,505 genes were identified as meeting this criterion. Differential gene expression was then analysed using edgeR v3.18.1 (Robinson, McCarthy & Smyth 2010) with false-discovery rate <0.05 set as the cut-off. DAVID (Huang, Sherman & Lempicki 2008a; 2008b) was used for functional annotation analysis to identify genes associated with sperm-related gene ontology (GO) terms. The GOrilla analysis tool (Eden et al. 2009) was used to categorise differentially expressed genes into pathways and processes according to functional GO annotations. This allows enriched GO terms and common processes and pathways affected in the data sets to be identified. The RNA-Seq raw data have been deposited to NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE140576.

4.2.3 Quantitative PCR validation of RNA-Seq results

For quantitative PCR (qPCR) validation of the RNA-Seq results, epididymides were collected from different animals to those used for RNA-Seq (*n*=5 for each of WT and KO). RNA was isolated as described above and cDNA was reverse-transcribed using MultiScribe reverse transcriptase (Life Technologies, Carlsbad, CA, USA), 10× M-MuLV buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 10

mM dithiothreitol, pH 8.3; New England BioLabs, Ipswich, MA, USA), 10 mM dNTP mix (Bioline, London, UK), oligo(dT)$_{18}$ primer (Bioline) and 40 U/µl RiboSafe RNase Inhibitor (Bioline). Reactions were incubated for 10 min at 25°C, 2 h at 42°C and then 5 min at 85°C in a thermocycler. qPCR was used to validate differential expression of the genes *Defb9*, *Mep1a*, *Spink14* and *Timp2* (primer sequences listed in **Supplementary Table 4.1**). Each qPCR amplification reaction mix contained 5 µl of cDNA, 300 nM of forward and reverse primer and 5 µl Fast SYBR Green Master Mix (Life Technologies). Reactions underwent the following qPCR protocol using the Biorad CFX96 Real Time System (Biorad, Hercules, CA, USA): 20 s at 95°C, 40 cycles of 3 s at 95°C and 30 s at 60°C. All samples were measured in triplicate. *Actb* was used as the reference gene, as expression levels of this gene was not different in the epididymides of *Plag1*$^{-/-}$ compared to *Plag1*$^{+/+}$ mice according to the RNA-Seq data. qPCR data were processed and fold changes presented using LinRegPCR v.2017.0 software (Ruijter et al. 2009) as previously described (Juma et al. 2017). Unpaired *t*-tests were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) to compare average normalised mRNA expression levels in the epididymides of *Plag1*$^{-/-}$ compared to *Plag1*$^{+/+}$ mice, as determined by qPCR.

4.2.4 Ki67 immunofluorescence

Epididymides were collected from adult (7-week-old) mice (*n*=5 for each of WT and KO), fixed for 2 h in 4% (w/v) paraformaldehyde then cryoprotected for 2 h in 10% (w/v) sucrose followed by 30% sucrose until the tissues sank. Tissues were stored at –80°C until sectioned into 5-µm cryosections, and subsequently stored at –80°C. Thawed sections were first treated with boiling 1 M trisodium citrate dihydrate (VWR, Radnor, PA, USA) for 40 min for antigen retrieval. Sections were then washed in phosphate-buffered saline (PBS), incubated for 2 h in PBS containing 4% (w/v) bovine serum albumin (BSA), 0.1% (v/v) Triton X-100, 0.1 M lysine and 5% (v/v) normal goat serum (Antibodies Australia, Melbourne, Australia) (BSA-PBS-TX) at room temperature, then washed in PBS, before overnight incubation at 4°C with anti-Ki67 antibody (ab15580; Abcam, Cambridge, United Kingdom) diluted 1:500 in BSA-PBS-TX. Control slides were incubated in BSA-PBS-TX with the primary antibody omitted. Next, sections were washed in PBS, incubated for 2 h at 4°C with goat anti-rabbit

IgG Alexa Fluor 555 (A27039; Thermo Fisher, Waltham, MA, USA) secondary antibody diluted 1:500 in BSA-PBS-TX. Sections were then washed in PBS with 0.1% Triton X-100 before application of DAPI diluted in PBS according to manufacturer instructions (Thermo Fisher). The sections were washed in PBS and mounted using aqueous mounting medium.

4.2.5 Microscopy and image analysis

Sections were imaged using an Olympus BX41 microscope with an Olympus DP25 camera (Olympus Scientific Solutions Americas Inc., Waltham, MA, USA). 3–6 images were taken (20× magnification) of each region (caput, corpus and cauda) for analysis using Image J software (https://imagej.nih.gov/ij/). Percentages of $Ki67^+$ cells in the epithelium and stroma, and the percentage of stromal cells (identified by cell size, shape and location) out of total cells, were determined using the 'analyse particles' function. Epithelial and stromal areas were isolated using the freehand drawing tool to create 'regions of interest', and sperm cells were excluded from the DAPI total cell counts by blanking out the luminal area using the freehand drawing tool and the 'fill' function. Animal and genotype details were omitted from image files prior to the analysis to exclude bias. Unpaired *t*-tests were performed using GraphPad Prism 7 (GraphPad Software) to compare the mean percentages of $Ki67^+$ or stromal cells between *Plag1*$^{-/-}$ and *Plag1*$^{+/+}$ mice. Means were considered significantly different if $P < 0.05$

4.2.6 PLAG1 binding motif scanning

The TxDb.Mmusculus.UCSC.mm10.knownGene R library was used to obtain all the annotated transcriptional start sites (TSS) in mouse (mm10 genome version). The JASPAR database PLAG1 motif (JASPAR MA0163.1) and the binding sites defined by Madissoon et al. (2019) were used for the analysis. Two different promoter sizes were analysed: the genomic regions spanning 300 bp upstream to 50 bp downstream of the TSS, and between 2 kb upstream to 100 bp downstream of the TSS. First we scanned selected target genes for PLAG1 sites using the Homer suite of sequence analysis tools (Heinz et al. 2010) and performed χ-square test analysis to study differences in the occurrence of the sites compared to all other promoters. Then we extended the analyses to all

differentially regulated genes and subjected the promoter regions to *de novo* motif discovery and general motif scanning using the findMotifs.pl program in Homer. The output of these analyses is a list of significantly enriched motifs in the promoters of the differentially regulated genes over all promoters in the genome, and their similarity to known motifs.

4.3 Results

4.3.1 Transcriptome analysis of *Plag1*$^{-/-}$ and *Plag1*$^{+/+}$ mouse epididymis

RNA-Seq was used to analyse and compare the transcriptomes of epididymides from 7-week-old *Plag1*$^{-/-}$ and *Plag1*$^{+/+}$ mice. Although the epididymis is often divided into 10 transcriptionally distinct segments in mice, the shortened, dysmorphic and aberrantly coiled epididymis in *Plag1*$^{-/-}$ mice made it unfeasible to accurately dissect and sequence "match" transcriptionally-analogous segments or regions from *Plag1*$^{-/-}$ and *Plag1*$^{+/+}$ epididymides. Rather, we reasoned that analysis of the transcriptome from whole *Plag1*$^{-/-}$ epididymides would allow us to accurately determine the overall molecular profile of the *Plag1*$^{-/-}$ epididymis. We found that 1,728 genes were differentially expressed in the epididymis of *Plag1*$^{-/-}$ compared to *Plag1*$^{+/+}$ mice; 1,102 genes were upregulated and 626 genes were downregulated **(Figure 4.1)**. The top-10 up- and downregulated genes in *Plag1*$^{-/-}$ compared to *Plag1*$^{+/+}$ are listed in **Supplementary Table 4.2**. Additionally, we performed RNA-Seq comparing the transcriptome in epididymides from *Plag1*$^{+/-}$ with that of *Plag1*$^{+/+}$ mice, but did not find any differentially regulated genes **(Figure 4.1)**. For standard validation of the RNA-Seq, *Mep1a*, *Spink14* and *Timp2* were arbitrarily chosen from the list of dysregulated genes for confirmation of differential gene expression by qPCR, using samples from animals separate to those that were used in the RNA-Seq. Validation of *Defb9* expression, which was not differentially expressed in the epididymis from *Plag1*$^{-/-}$ mice in the RNA-Seq dataset, was also included. We validated the differential expression of *Mep1a*, *Spink14* and *Timp2* in the epididymides of *Plag1*$^{-/-}$ mice; fold changes in mRNA as determined by qPCR are shown alongside fold changes as determined by RNA-Seq and mRNA expression levels determined by qPCR, confirming the differential

expression of *Mep1a*, *Spink14* and *Timp2* (*t*-test, *P*<0.01) are shown **(Figure 4.1)**. RNA-Seq indicated that *Defb9* was not differentially expressed in the epididymides of *Plag1*$^{-/-}$ mice, and this was confirmed by qPCR (*P*=0.121) **(Figure 4.1)**. Together, these data validate our RNA-Seq analysis as a robust methodology to determine the PLAG1-dependent transcriptome within the murine epididymis.

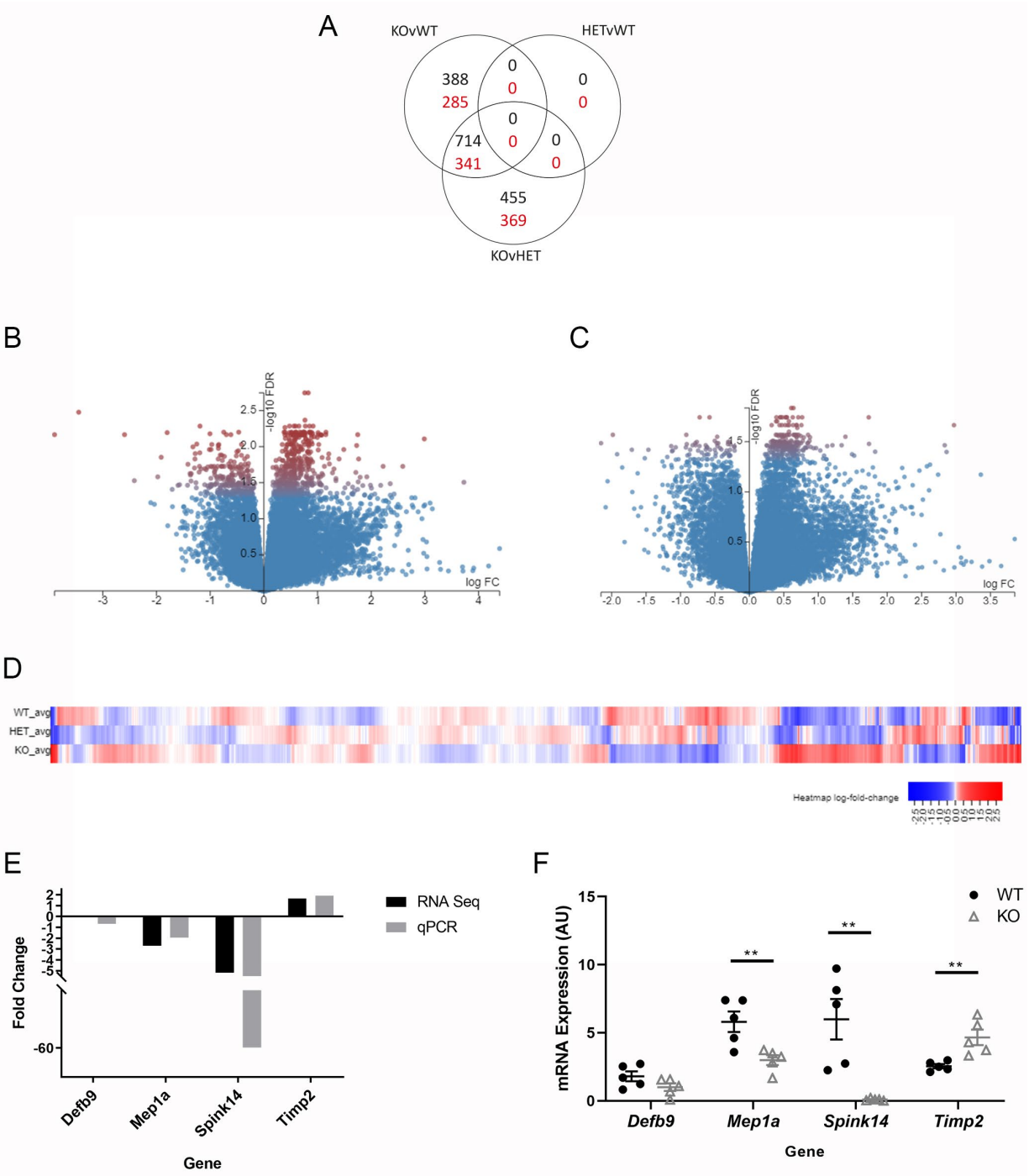

Figure 4.1. Significantly differentially expressed genes (DEGs) between *Plag1* wild-type (WT), heterozygous (HET) and knockout (KO) mouse epididymis (FDR<0.05) (*n*=5 per genotype). (a)

Overlapping DEGs in the epididymides from *Plag1* WT, HET and KO mice. Red represents downregulated genes and black represents upregulated genes. **(b)** Volcano plot showing fold changes of DEGs in the epididymis between WT and KO *Plag1* mice. Red dots indicate significantly differentially expressed genes and blue dots represent non-significantly differentially expressed genes. **(c)** Volcano plot showing fold changes of DEGs in the epididymis between KO and HET *Plag1* mice. **(d)** Heat map of all 21,910 genes identified from RNA sequencing and their expression across the three genotypes, compared to average expression. Each line represents one gene. Red indicates a positive fold change and blue indicates a negative fold change. **(e)** Fold changes in mRNA expression of selected dysregulated genes in the epididymides from *Plag1* knockout compared to wild-type mice (*n*=5 per genotype). **(f)** mRNA expression levels of selected dysregulated genes in the epididymides of *Plag1* WT and KO mice, determined by qPCR (*n*=5 per genotype). Values are normalised to *Actb* mRNA expression, and means were compared by *t*-tests. ***P*<0.01, *Defb9*, defensin beta 9; *Mep1a*, meprin A subunit alpha; *Spink14*, serine peptidase inhibitor Kazal type 14; *Timp2*, tissue inhibitor of metalloproteinase 2

DAVID functional annotation analysis was performed to identify genes associated with sperm maturation that are dysregulated in the epididymis of *Plag1*$^{-/-}$ mice. This analysis revealed that a number of genes involved in sperm maturation, motility, capacitation, the acrosome reaction and the prevention of premature sperm capacitation were dysregulated in *Plag1*$^{-/-}$ mice **(Tables 4.1–4.2)**. Surprisingly, despite the fact that the epididymis of *Plag1*$^{-/-}$ mice is underdeveloped, several cell proliferation genes were significantly upregulated in the epididymis from *Plag1*$^{-/-}$ mice. Indeed, GO analysis revealed that the GO term 'Regulation of cell proliferation' was overrepresented; 18 genes significantly upregulated in the epididymis from *Plag1*$^{-/-}$ mice are categorised under this term **(Table 4.3)**. Next, we performed GO analysis to identify pathways and processes the were affected in the epididymis of *Plag1*$^{-/-}$ mice, which showed an overall downregulation of genes involved in cell migration, cell motility, metalloendopeptidase activity and metallopeptidase activity in *Plag1*$^{-/-}$ mice. Additionally, GO analysis also showed an overall upregulation of genes associated with cell adhesion, molecular binding and extracellular matrix (ECM) components in KOs **(Figure 4.2)**. Taken together, these data indicate a defective maintenance and integrity of the epididymis in *Plag1*$^{-/-}$ mice. Enriched 'biological process', 'molecular function' and 'cellular component' GO terms and dysregulated genes in the epididymis of *Plag1*$^{-/-}$ mice categorised under the GO terms 'Metalloendopeptidase activity' and 'Extracellular matrix component' and are listed in supplementary tables **(Supplementary tables 4.3-4.7)**.

Table 4.1. Selection of significantly upregulated genes with functions related to sperm maturation in the epididymis of *Plag1* knockout mice compared to wild-type mice. FDR: false-discovery rate; HET: heterozygous; KO: knockout; LFC: log-fold change KO compared to WT; TPM: transcripts per kilobase million; WT: wild-type.

Gene symbol	Gene name	FDR	LFC	TPM			Known functions	References
				WT	HET	KO		
Acrv1	Acrosomal vesicle protein 1	0.027	1.10	1.43	1.55	3.05	• Involved in sperm–oolemma binding in humans • Segment-specific expression in the mouse epididymis	(Turunen *et al.* 2011; Margalit *et al.* 2012)
Adam2	A disintegrin and metallopeptidase domain 2	0.040	1.60	0.65	0.65	2.13	• Encodes subunit of the sperm membrane glycoprotein fertilin • Deletion results in male infertility, defective sperm migration from uterus to oviduct, and defective sperm aggregation and zona pellucida binding in mice • Required for ADAM3 function	(Cho *et al.* 1998; Nishimura *et al.* 2004; Han *et al.* 2010)
Adam3	A disintegrin and metallopeptidase domain 3 (cyritestin)	0.031	1.60	1.91	2.68	6.41	• Deletion results in male infertility, defective sperm migration from uterus to oviduct, and defective sperm aggregation and zona pellucida binding in mice	(Shamsadin *et al.* 1999; Nishimura *et al.* 2001; Yamaguchi *et al.* 2009; Han *et al.* 2010)
Akap3	A-Kinase anchoring protein	0.024	1.88	0.99	1.25	3.82	• Plays a role in regulating murine, human and bovine sperm motility, capacitation and acrosome reaction	(Luconi *et al.* 2004; Bajpai *et al.* 2006; Xu and Qi 2014)
Aldoart1	Aldolase 1 A, retrogene 1	0.026	1.22	0.29	0.33	0.72	• Undergoes tyrosine phosphorylation during sperm capacitation in mice, which may regulate glycolysis during capacitation	(Arcelay *et al.* 2004)
Atp1a4	ATPase Na^+/K^+ transporting subunit alpha 4	0.039	1.17	1.65	2.22	3.65	• Deletion in mice results in reduced sperm motility; sperm unable to fertilise egg; abnormal ion regulation in sperm causing bend in sperm flagellum • Regulates sperm capacitation in bulls	(Jimenez *et al.* 2011; Rajamanickam *et al.* 2017)
Dnajb13	DnaJ heat shock protein family member B13	0.012	0.82	3.86	4.20	6.95	• Mutations result in primary ciliary dyskinesia and male infertility. • Localised to sperm flagella in human sperm and murine sperm annulus during flagellar development	(Guan *et al.* 2009; Guan *et al.* 2010; El Khouri *et al.* 2016)
Txndc3 *Txndc2*	Thioredoxin domain-containing protein 3/2	0.023 0.049	1.73 0.61	0.40 2.46	0.49 2.67	1.33 3.88	• *Txndc3/Txndc2*-deficient mice exhibit age-related sperm motility loss. • *Txndc3* is required for sperm flagellar axoneme; mutations implicated in human primary ciliary dyskinesia, which is associated with male infertility.	(Duriez *et al.* 2007; Smith *et al.* 2013)

Table 4.2. Selection of significantly downregulated genes with functions related to sperm maturation in the epididymis of *Plag1* knockout mice compared to wild-type mice. FDR, false-discovery rate; HET, heterozygous; KO, knockout; LFC, log-fold change KO compared to WT; TPM, transcripts per kilobase million; WT, wild-type.

Gene symbol	Gene name	FDR	LFC	TPM			Known functions	References
				WT	HET	KO		
Akt2	Thymoma viral proto-oncogene 2	0.043	–0.20	30.28	27.85	27.17	• Deletion results in decreased fertilisation *in vitro*, sperm motility and concentration, and increased apoptotic sperm.	(Kim *et al.* 2012)
Bsph2	Binder of sperm protein homolog 2	0.013	–0.84	2261.00	2607.40	1758.40	• Murine BSPH2 protein on sperm surface is modified during sperm capacitation.	(Plante *et al.* 2014)
Pebp1	Phosphatidylethanolamine binding protein 1	0.0498	–0.24	1558.94	1394.72	1384.99	• Can act as a decapacitation factor or as a membrane receptor that facilitates the loss of decapacitation factors • Localised to the acrosome and flagellum of murine and human sperm, and to bovine epididymosomes • Deletion results in premature capacitation in murine sperm.	(Gibbons *et al.* 2005; Nixon *et al.* 2006; Moffit *et al.* 2007; Girouard *et al.* 2011)
Rgn	Regucalcin	0.022	–0.45	54.00	51.78	41.76	• Involved in sperm maturation via regulation of epididymal Ca^{2+} levels • Overexpression in rats results in decreased epididymis epithelial cell height, decreased sperm counts and motility, and increased sperm viability and frequency of morphologically normal sperm.	(Correia *et al.* 2013)
Smox	Spermine oxidase	0.045	–0.44	25.49	21.43	20.15	• Encoded enzyme required for spermine to spermidine oxidation • High concentrations of spermine facilitate the inhibition of the acrosome reaction in bovine sperm.	(Rubinstein and Breitbart 1991)
Spink10	Serine peptidase inhibitor 2C Kazal type 10	0.028	–0.42	398.56	367.64	314.47	• Encodes a Kazal family protease inhibitor, which are crucial in regulating sperm membrane protein modifications • Imbalance between proteases and protease inhibitors results in azoospermia, low fertility and impaired sperm functions.	(Jalkanen *et al.* 2006; Gurupriya and Roy 2017)
Spinkl	Serine protease inhibitor 2C Kazal type-like	0.012	–0.74	248.93	205.54	167.47	• Acts as a decapacitation factor, suppressing premature murine sperm capacitation *in vitro* • Can bind to murine sperm and increase motility	(Lin *et al.* 2008; Tseng *et al.* 2013)

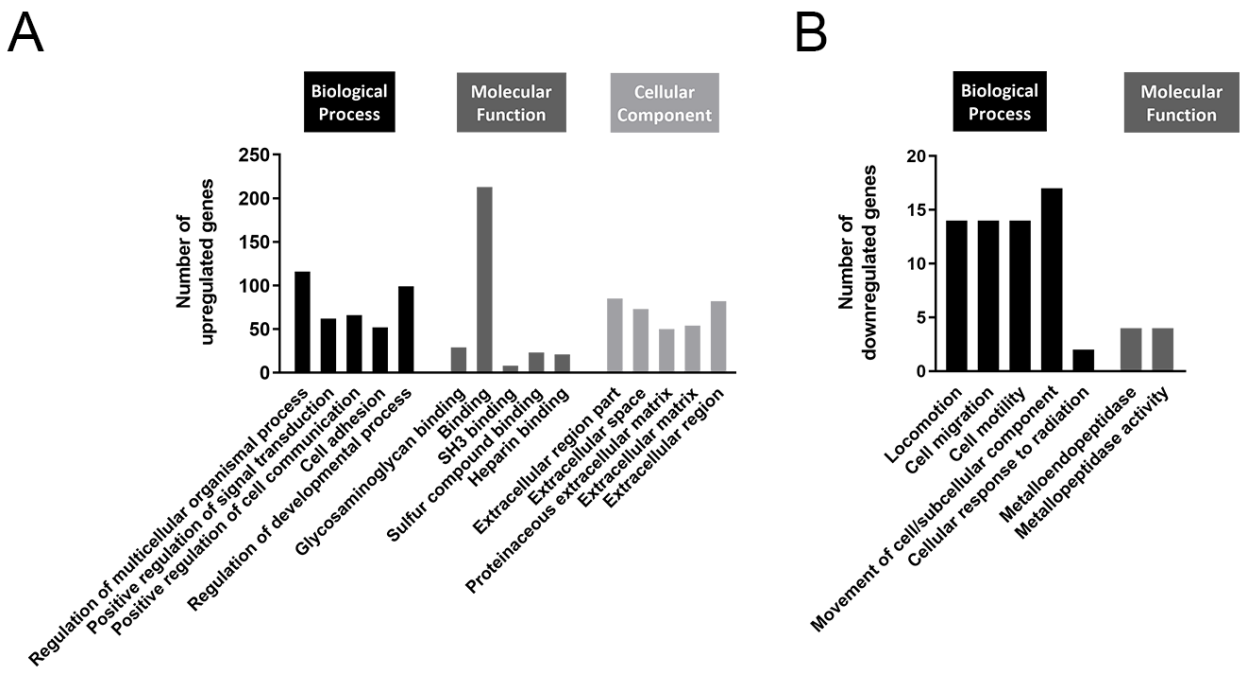

Figure 4.2. Top-5 overrepresented GO terms determined by GOrilla analysis, within the dataset of significantly dysregulated genes in epididymides from *Plag1* knockout mice compared to wild-type mice. (a) Overrepresented GO terms within significantly upregulated genes. **(b)** Overrepresented GO terms within significantly downregulated genes. There was no cellular component GO terms in the list of downregulated genes.

4.3.2 Cell proliferation in the epididymis from *Plag1*$^{-/-}$ mice

Ki67 staining and quantification was used to determine if the overall upregulation of cell proliferation genes **(Table 4.3)** correlates with increased cellular proliferation, in the epididymis of *Plag1*$^{-/-}$ mice. The percentage of Ki67^{+} cells was significantly higher in the stroma of the cauda epididymidis from *Plag1*$^{-/-}$ compared to *Plag1*$^{+/+}$ mice (*t*-test, *P*=0.0007), but not in the epithelium (*P*=0.9889) **(Figure 4.3)**. There was no difference in the percentage of Ki67^{+} cells in the epithelium or stroma in the caput and corpus between genotypes (*P*>0.05). There was a significantly higher percentage of stromal cells (relative to the total number of cells) in the caput (*t*-test, *P*=0.0033), corpus (*P*=0.0028) and cauda (*P*=0.0080) of *Plag1*$^{-/-}$ mice compared to *Plag1*$^{+/+}$ **(Figure 4.3)**. These data indicate that the stroma, albeit not the epididymal epithelium of *Plag1*$^{-/-}$ mice, is hyperproliferative relative to WT controls.

Table 4.3. Upregulated genes categorised under the GO term 'Regulation of cell proliferation (GO: 0042127)' in the epididymis from *Plag1* knockout versus wild-type mice. FDR: false-discovery rate; HET: heterozygous; KO: knockout; LFC: log fold change KO compared to WT; TPM: transcripts per kilobase million; WT: wild-type.

Gene symbol	Gene name	FDR	LFC	TPM		
				WT	HET	KO
Sparc	Secreted acidic cysteine rich glycoprotein	1.40E–3	0.72	431.65	488.10	753.94
Ednrb	Endothelin receptor type B	1.40E–3	0.90	4.65	5.05	8.64
Lgals9	Lectin, galactose binding, soluble 9	1.74E–3	0.71	11.00	12.75	19.00
Trf	Transferrin	1.61E–3	1.13	10.78	12.10	22.71
Scube2	Signal peptide, cub domain, EGF-like 2	1.85E–3	1.82	0.44	0.58	1.59
Hoxa5	Homeobox A5	1.25E–3	0.70	6.44	6.67	10.56
Aldh1a2	Aldehyde dehydrogenase family 1, subfamily A2	9.78E–4	0.87	15.64	17.51	29.55
Gas1	Growth arrest specific 1	1.99E–3	0.56	18.25	19.26	28.71
Efemp1	Epidermal growth factor-containing fibulin-like extracellular matrix protein 1	1.33E–3	0.82	31.22	36.25	56.05
Tgfbr2	Transforming growth factor-beta receptor 2	1.33E–3	0.63	9.58	10.33	15.68
Timp2	Tissue inhibitor of metalloproteinase 2	1.23E–3	0.72	59.02	66.84	102.61
Cxcl12	Chemokine (c-x-c motif) ligand 12	1.40E–3	0.42	18.06	17.39	24.07
Cx3cr1	Chemokine (c-x3-c) receptor 1	1.40E–3	0.71	4.68	5.55	7.52
Ptn	Pleiotrophin	1.96E–3	0.93	8.14	8.96	15.02
Igf1	Insulin-like growth factor 1	9.78E–3	1.23	6.25	7.58	14.79
Htra1	Htra serine peptidase 1	1.40E–3	0.52	38.28	39.28	56.25
Cdh5	Cadherin 5	1.78E–3	0.79	7.04	7.70	13.01
Wt1	Wilms tumor 1 homolog	2.01E–3	1.82	0.86	1.29	3.18

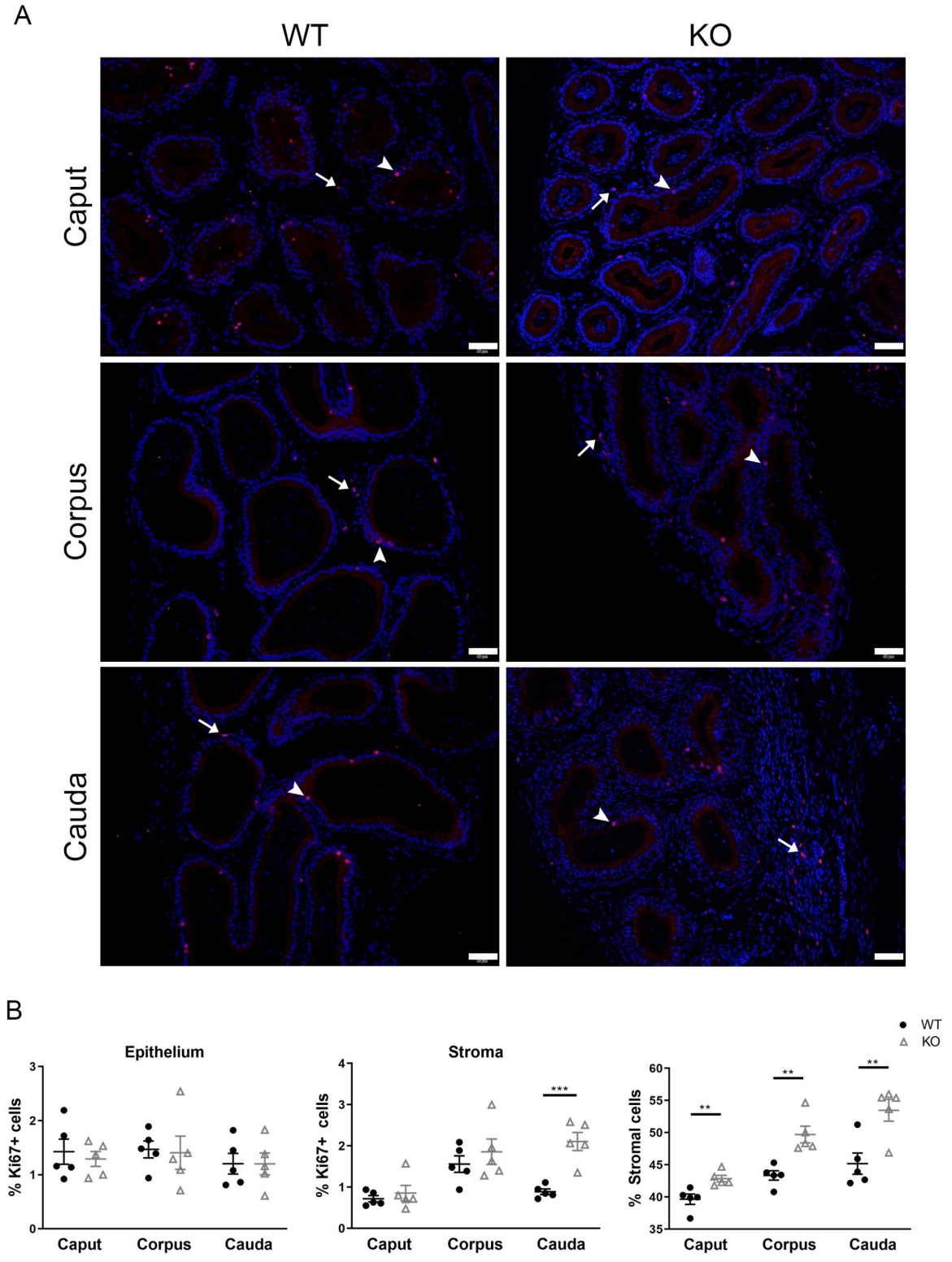

Figure 4.3. Expression of Ki67 in the epididymis of 7-week-old *Plag1* knockout (KO) and wild-type (WT) mice. (a) Immunofluorescence staining of Ki67 (red) and DAPI (nuclei, blue) in the epididymis of WTs and KOs. Arrowheads indicate epithelial Ki67 expression; arrows point to stromal Ki67 expression. Scale bars represent 50 µm. **(b)** Quantification of Ki67-positive cells relative to total cell number in the epithelium and stroma of the epididymis of *Plag1* KO compared to WT mice (*n*=5 per genotype) and quantification of the number of stromal cells relative to total cell number in the epididymis (far right graph) of *Plag1* KO compared to WT mice (*n*=5 per genotype). Values shown are means ± SEM. ***P* < 0.01; ****P* < 0.001 (*t*-test).

4.3.3 PLAG1 binding sites are not enriched in dysregulated genes

To determine if PLAG1 binding sites were enriched in the target genes determined by the RNA-Seq analysis, genes up- or downregulated in the epididymis from *Plag1*$^{-/-}$ mice were analysed for significant enrichment of PLAG1 binding sites using the PLAG1 motif from the JASPAR database and the binding sites defined by Madissoon *et al*. (2019). This motif comprises two G-rich regions (a GRGGC Core and a G-Cluster) separated by 7 non-conserved nucleotides, and broadly takes the form "GA/GGGCNNNNNNNA/GGGG/T", where N is a random nucleotide and the underlined nucleotides are largely invariant. Of all annotated murine genes, 16,163 were found to have at least one PLAG1 binding site in their promoters, whereas 7,390 did not have any PLAG1 binding sites, suggesting that the presence of a putative PLAG1 site is extremely commonplace, and that enrichment analysis may present more accurate indication of PLAG1 binding activity. The results of the Homer analysis, however, indicated that among the genes dysregulated in the epididymis from *Plag1*$^{-/-}$ mice, there was no significant enrichment of PLAG1 binding sites. Furthermore, the motif scanning did not reveal significant enrichment of binding sites of any other transcription factors, suggesting that the PLAG1-dependent transcriptome in the epididymis does not comprise an over-represented hierarchical pathway of any known transcription factor families.

4.4 Discussion

The cause of male infertility is unidentified in 30–45% of clinical cases (Jungwirth et al. 2012), exemplifying the lack of knowledge pertaining to the underlying molecular and genetic mechanisms of sperm dysfunction. Several studies using transgenic mice have identified gene candidates involved in poor or abnormal sperm function, including *Plag1,* required for sperm motility (Juma et al. 2017), normal epididymal morphology and male fertility (Wong et al. 2019). However, the underlying cause of these defects is unknown and the genes that are controlled by PLAG1 in the epididymis have not yet been characterised.

As PLAG1 is expressed in brain and pituitary (as well as elsewhere), we cannot rule out hormonal, metabolic, paracrine signalling or other, non-epididymal contributions to both male infertility and epididymal morphology defects we describe within our study. Nonetheless the abundant widespread expression of PLAG1 in the epithelium and stroma of the adult mouse epididymis (Wong et al. 2019) points to an important role in male fertility. This study aimed to characterise the epididymal target genes of PLAG1, and to identify processes and functions that may be affected in the epididymis when *Plag1* is deficient.

RNA-Seq results showed that there are no significantly differentially expressed genes in the epididymis from *Plag1*$^{+/-}$ mice compared to *Plag1*$^{+/+}$, indicating that one copy of the *Plag1* gene is sufficient for normal gene expression. This is in agreement with previous findings that *Plag1*$^{+/-}$ males have normal fertility, as numbers of litters born and litter size was not affected in the mice (Wong et al. 2019). However, *Plag1*$^{+/-}$ males do exhibit decreased epididymal tubule elongation and coiling in the caput and corpus epididymidis (Wong et al. 2019), suggesting that although gene expression does not appear to be significantly dysregulated, the morphology of the epididymis in these mice is altered. RNA-Seq revealed a number of dysregulated genes in the *Plag1*$^{-/-}$ epididymis compared to *Plag1*$^{+/+}$, that have known sperm maturation and storage-related functions (see refs. in **Tables 4.1–4.2**) and common functions are seen among these PLAG1 target genes. As spermatozoa were removed from the tissue samples, these genes identified by the RNA-Seq experiment are exclusively epididymal genes. Several genes, such as *Txndc3*, *Txndc2*, *Dnajb13*, *Pebp1* and *Rgn*, are known to play a role in sperm motility or sperm flagellar development, or have been localised to the developing sperm flagellum, and several KO models of these genes exhibit defective sperm motility (see refs. in **Tables 4.1–4.2**). *Txndc3* and *Dnajb13* have been implicated in primary ciliary dyskinesia (PCD), a disease in humans resulting from dysfunction of motile cilia in which the majority of male sufferers are infertile (Horani et al. 2016). The role of PLAG1 in PCD has not yet been studied, but may be a worthy genetic candidate for investigation, as the emerging diagnostic approach for this disease is genetic testing (Horani et al. 2016).

Several dysregulated genes in the *Plag1*$^{-/-}$ epididymis play a role in regulating the acrosome reaction or sperm capacitation (see refs. in **Tables 4.1–4.2**). Interestingly, this includes the

downregulation of two genes that encode decapacitation factors (*Pebp1*, *Spinkl*), which bind to the sperm head to prevent premature sperm capacitation (see refs. in **Table 4.2**). It is crucial for capacitation to be preserved for when the spermatozoon reaches the ovum; premature capacitation results in redundant spermatozoa that are incapable of fertilisation (Bailey 2010). In addition, previous research has shown that the most severe morphological abnormalities in the epididymis of *Plag1*$^{-/-}$ mice are observed in the cauda region (lack of bulbous shape, reduced tubule elongation and coiling) and that in *Plag1*$^{+/+}$ mice, PLAG1 is potentially secreted into the lumen in this region (Wong et al. 2019). Altogether, these findings may be indicative of dysfunction of the cauda epididymidis where mature spermatozoa are stored, resulting in premature capacitation and therefore infertility, and future studies should be targeted to examine differential gene expression specifically within this region.

The maintenance of epididymal integrity through the regulation of ion transport also appears to be affected; *Atp1a4* and *Rgn* are dysregulated genes that are related to ion balance within the microenvironment of the epididymal lumen. *Atp1a4* (upregulated in the *Plag1*$^{-/-}$ epididymis) encodes the α4 subunit of the Na,K-ATPase and is required for sperm fertility due to its role in maintaining sperm ion balance (Jimenez et al. 2011), which is crucial for sperm morphology and motility, and fertilisation capacity (Cooper et al. 2004; Darszon et al. 2006; Lishko et al. 2012). The deletion of this gene in mice results in infertility in males, reduced sperm motility and abnormal sperm tail morphology, and spermatozoa are unable to fertilise eggs *in vitro* (Jimenez et al. 2011). *Rgn* (downregulated in *Plag1*$^{-/-}$) encodes a Ca^{2+}-binding protein important for regulating Ca^{2+} balance, and overexpression of this gene results in increased luminal Ca^{2+} and decreased sperm motility and sperm counts (Correia et al. 2013). Dysregulation of these genes may disrupt the movement of ions, sperm ion balance or the epididymal Ca^{2+} gradient and contribute to the decreased sperm motility and infertility observed in *Plag1*$^{-/-}$ mice, or the dysregulation of these genes in the epididymides from *Plag1*$^{-/-}$ mice may be a compensatory mechanism. Interestingly, the expression of several genes such as *Txndc3* and *Dnajb13* have not been reported in the epididymis previously; *Txndc3* expression was thought to be testis-specific and *Dnajb13* expression has been

detected on developing spermatids. However, as spermatozoa were rinsed from the epididymis tissues, it is unlikely that the expression detected was not epididymal expression.

GO analysis revealed several cell proliferation GO terms enriched in the dataset of significantly upregulated genes in the epididymis from *Plag1*$^{-/-}$ mice, which is surprising given that the epididymides in these mice are reduced in weight, the tubule is less developed (Wong et al. 2019), and PLAG1 is known to upregulate cell proliferation genes during early development (Juma et al. 2016). Ki67 quantification showed that cell proliferation was significantly increased in the cauda stroma of the epididymis from *Plag1*$^{-/-}$ mice, but not in the caput or corpus, or in the epithelium of any region. The proportion of stromal cells, out of total cells, was significantly increased in all regions of the epididymis from *Plag1*$^{-/-}$ mice. These results indicate that cell proliferation is upregulated specifically in the cauda region, which correlates with the morphological defects being most severe in this region in *Plag1*$^{-/-}$ mice (Wong et al. 2019) and may correlate with the dysregulation of ECM components shown by the RNA-Seq data, as the ECM regulates several cell behaviours including cell proliferation (Frantz, Stewart & Weaver 2010; Wells 2008). Although the GO term 'positive regulation of epithelial cell proliferation' was also enriched in the upregulated genes in the epididymis from *Plag1*$^{-/-}$ mice, the results showed that cell proliferation was normal in the epithelium. The upregulation of these genes may be a compensatory mechanism for the lack of tubule of elongation, which is a defect that persists into adulthood in *Plag1*$^{-/-}$ mice (Wong et al. 2019).

GO analysis also revealed that within the list of downregulated genes in the epididymis from *Plag1*$^{-/-}$ mice, there was significant enrichment of genes associated with the GO terms cell migration, cell motility, movement of cell or subcellular component and metalloendopeptidase/metallopeptidase activity. The *Mmp1b*, *Mep1a*, *Mme* and *Pappa* genes encoding metallopeptidases or matrix metalloproteinases (MMPs) were downregulated in the *Plag1*$^{-/-}$ epididymis, and are important in degrading proteins in the ECM, thereby playing a crucial role in tissue development, remodelling and repair (Nagase, Visse & Murphy 2006). MMPs are also involved in cell signalling, cell migration and the activation of growth factors (Chang & Werb 2001; Yamamoto, Murphy & Troeberg 2015), and are partly regulated by tissue inhibitors of metalloproteinases (TIMPs) (Nagase, Visse & Murphy 2006). The balance between MMPs and TIMPs is vital for ECM

remodelling and maintenance (Nagase, Visse & Murphy 2006), and it has been shown that MMPs and TIMP2 are present in the epididymal fluid in the boar, ram and stallion, suggesting a role in epididymal sperm maturation (Métayer et al. 2002). Notably, *Timp2*, which encodes a TIMP protein, was upregulated in the *Plag1*$^{-/-}$ epididymis. These results suggest that MMP activity may be decreased by both the downregulation of MMP genes and the upregulation of an MMP inhibitor. GO analysis also showed that among upregulated genes in the epididymides from *Plag1*$^{-/-}$ mice, there was significant enrichment of genes mapped to the GO terms cell adhesion, glycosaminoglycan binding, ECM/region and proteinaceous ECM. Notably, a large number of collagen genes was upregulated in *Plag1*$^{-/-}$ epididymides (*Col6a3*, *Col12a1*, *Col4a6*, *Col15a1*, *Col6a1*, *Col23a1*, *Col1a2*, *Col6a5*, *Col4a3*, *Col4a5*, *Col11a1*, *Col3a1*, *Col6a2*, *Col5a2*, *Col6a6*, *Pcolce*, *Col1a1*, *Col9a1*, *Col5a3*, *Col5a1*, *Col4a1*, *Col14a1*, *Cogalt2* and *Col4a4*), and upregulated proteinaceous ECM genes included *Spon1*, *Spon2*, *Lama2*, *Lamb1* and *Ecm2*. Overdeposition of ECM components can result in ECM stiffness or fibrosis in other tissues, leading to diseases such as cancer, cardiovascular disease (Lampi & Reinhart-King 2018), pulmonary fibrosis (Cox & Erler 2011) and kidney sclerosis (Ho et al. 2014). ECM stiffness, however, has not yet been investigated in the epididymis. While ECM-related genes are known to be expressed in the Wolffian duct during embryonic development (Snyder et al. 2010) and ECM remodelling is recognised as a crucial aspect of epididymis morphogenesis (Hinton et al. 2011), the role of ECM genes in the function of the adult epididymis is not well studied and the implication of ECM defects on sperm maturation are not known. As the ECM acts as a harbour for growth factors (Yue 2014), it is reasonable to speculate that defective ECM structure would have an impact on the release of growth factors or other proteins that are important for sperm maturation or epididymal tissue homeostasis. Taken together, the downregulation of cell motility and MMP genes, the upregulation of ECM component genes and *Timp2*, and the increased cell proliferation in the cauda epididymidis stroma of *Plag1*$^{-/-}$ mice suggest a defect in the maintenance and structure of the ECM, or perhaps disruption of normal tissue homeostasis in the epididymis of *Plag1*$^{-/-}$ mice.

PLAG1 binding site analysis on dysregulated genes in the epididymides from *Plag1*$^{-/-}$ mice did not show significant enrichment of PLAG1 binding sites. This could indicate either that PLAG1 control of these particular genes may be upstream or indirect, that PLAG1 may act in conjunction

with other transcription factors, or perhaps that epididymal defects occurred earlier in development than we have analysed during the present study. Moreover, motif scanning did not reveal enrichment of any other transcription factor motifs, providing further indication that the regulation of these target genes may require other transcription factors to act in concert. Some of the effects of PLAG1 deficiency on gene expression may be due to androgen imbalance; *Hsd17b3* and *Sultle1* (genes involved in the testosterone biosynthesis pathway) were downregulated in the testis from *Plag1*$^{-/-}$ mice and the seminal vesicles from *Plag1*$^{-/-}$ mice were disproportionately reduced in size, indicating decreased testosterone levels (Juma et al. 2017). Androgenic control of many epididymal genes has been established (Chauvin & Griswold 2004; Robaire et al. 2007), however, some genes involved in sperm maturation are not controlled (or not entirely controlled) by androgens, suggesting that at the molecular level sperm maturation regulation is more complex than a requirement for normal androgen action. For example, *Acrv1* mRNA expression in the mouse caput epididymidis spikes 8 h after gonadectomy, but normalises to control levels 1 day post gonadectomy, indicating that *Acrv1* is not exclusively regulated by androgens (Turunen et al. 2011).

In conclusion, we investigated the genetic pathways that are regulated by PLAG1 in the mouse epididymis and identified dysregulated genes in *Plag1*$^{-/-}$ mice that may be involved in the facilitation of proper sperm maturation, regulating the acrosome reaction and regulating sperm capacitation. Further, we show that there is an overall dysregulation of genes involved in the maintenance and structure of the ECM in *Plag1*$^{-/-}$ mice, and that cell proliferation is significantly increased in the cauda epididymidis from *Plag1*$^{-/-}$ mice. However, since there was no significant enrichment of PLAG1 binding sites in the differentially expressed genes, PLAG1 control of these genes may be indirect and further investigation is required to further elucidate the precise direct targets that PLAG1 binds to and activate in order to influence proper sperm maturation. While the data presented in this study is suggestive of possible premature sperm capacitation and imbalanced ECM deposition and degradation in the epididymis from *Plag1*$^{-/-}$ mice, further experimental validation is required. Future studies analysing the functional capabilities of mature spermatozoa from *Plag1*$^{-/-}$ mice such as the acrosome reaction, or characterisation of the ECM structure in epididymides from *Plag1*$^{-/-}$ mice may be insightful. Moreover, as global defects in *Plag1*$^{-/-}$ mice may

also contribute to generalised defects in tissue growth and development, targeted, tissue-specific deletion of *Plag1* may ultimately be necessary to determine epididymis-specific function. Although *PLAG1* mutations have not yet been investigated in the context of fertility in human males; as the underlying causes of clinical male infertility are largely unidentified, we suggest *Plag1* as a worthy candidate for investigation.

Author contribution statement

JW designed and conducted the experiments and wrote the manuscript. AD and PD performed the motif scanning analyses. JGG and SCT assisted with experimental work and acquisition of data. SVHG, BDG and SD provided advice for experimental work and assisted with manuscript preparation. All authors have read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Supplementary data

Supplementary Table 4.1. Primers used for qPCR validation of RNA sequencing results.

Gene	Gene ID	Primer sequence (5'→3')
Defb9	246079	Forward: GCTCTCTGCTGCTGATATGCTG
		Reverse: GCACCGTTCCATTTCTGATACACC
Mep1a	17287	Forward: GTCAAGGACTGACCGGGATG
		Reverse: ACGAAAATGGCCCGTAGTGC
Spink14	433178	Forward: TTTGGTGGCCTACACATGGAC
		Reverse: CCGCAGATGGGTTGTTTTAGGTC
Timp2	21858	Forward: ACCCAGAAGAAGAGCCTGAACC
		Reverse: AGCAAGGGATCATGGGACAGC
Actb	11461	Forward: GGCTGTATTCCCCTCCATCG
		Reverse: CCAGTTGGTAACAATGCCATGT

Supplementary Table 4.2. Top-10 up- and downregulated genes in the epididymis of *Plag1* knockout mice compared to wild-type mice. FDR: false-discovery rate; HET: heterozygous; KO: knockout; LFC: log fold change KO compared to WT; TPM: transcripts per kilobase million; WT: wild-type.

Gene symbol	Gene name	FDR	LFC	TPM		
				WT	HET	KO
Upregulated						
Tenm3	Teneurin transmembrane protein 3	3.52E–4	0.87	0.77	0.85	1.40
Col4a4	Collagen, type IV, alpha 4	9.78E–4	0.43	11.46	11.69	15.47
Steap4	STEAP family member 4	9.78E–4	0.77	9.60	10.62	16.74
Spag11b	Sperm associated antigen 11B	9.78E–4	0.68	4571.77	5494.71	7114.11
Aldh1a2	Aldehyde dehydrogenase family 1, subfamily A2	9.78E–4	0.87	15.64	17.51	29.55
Igf1	Insulin-like growth factor 1	9.78E–4	1.23	6.25	7.58	14.79
Rflnb	Refilin B	9.78E–4	0.77	14.02	15.88	24.30
Timp2	Tissue inhibitor of metalloproteinase 2	1.23E–3	0.72	59.02	66.84	102.61
Hoxa5	Homeobox A5	1.25E–3	0.70	6.44	6.67	10.56
Chst15	Carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15	1.33E–3	0.94	2.31	2.58	4.40
Downregulated						
Evx1os	Even skipped homeotic gene 1, opposite strand	9.78E–4	–3.52	0.55	0.17	0.04
Mcoln3	Mucolipin 3	1.33E–3	–1.28	5.23	4.02	2.42
Pappa2	Pappalysin 2	1.40E–3	–1.42	0.84	0.62	0.39
Mep1a	Meprin 1	1.60E–3	–1.43	15.17	13.05	7.03
Sfrp2	Secreted frizzled-related protein 2	1.60E–3	–0.98	14.16	8.88	8.34
Sult1c2	Sulfotransferase family, cytosolic, 1c, member 2	1.65E–3	–0.91	32.04	30.92	19.83
Gm5737	Predicted gene 5737	1.74E–3	–0.98	4.90	4.12	2.56
Gm41125	Predicted gene 41125	2.13E–3	–0.49	21.94	21.21	16.27
Mme	Membrane metalloendopeptidase	2.20E–3	–0.64	82.73	76.16	56.83
Zc3h12d	Zinc finger CCCH type containing 12D	2.20E–3	–0.89	2.24	1.68	1.32

Supplementary Table 4.3. 'Biological process' gene ontology (GO) terms enriched among differentially expressed genes in the epididymis from *Plag1* knockout versus wild-type mice. The top 30 of 107 upregulated terms are shown.

GO Term ID	Term Name	Number of genes
Upregulated		
GO:0051239	Regulation of multicellular organismal process	116
GO:0009967	Positive regulation of signal transduction	62
GO:0010647	Positive regulation of cell communication	66
GO:0007155	Cell adhesion	52
GO:0050793	Regulation of developmental process	99
GO:0023056	Positive regulation of signalling	65
GO:0022610	Biological adhesion	52
GO:0042327	Positive regulation of phosphorylation	46
GO:0048584	Positive regulation of response to stimulus	72
GO:2000026	Regulation of multicellular organismal development	82
GO:0001934	Positive regulation of protein phosphorylation	44
GO:0051094	Positive regulation of developmental process	63
GO:0048518	Positive regulation of biological process	122
GO:0008284	Positive regulation of cell proliferation	42
GO:0031401	Positive regulation of protein modification process	46
GO:0048522	Positive regulation of cellular process	142
GO:0043410	Positive regulation of MAPK cascade	30
GO:0009987	Cellular process	208
GO:1902533	Positive regulation of intracellular signal transduction	38
GO:0045597	Positive regulation of cell differentiation	48
GO:0042325	Regulation of phosphorylation	55
GO:0010562	Positive regulation of phosphorus metabolic process	48
GO:0045937	Positive regulation of phosphate metabolic process	48
GO:0009653	Anatomical structure morphogenesis	64
GO:0045595	Regulation of cell differentiation	67

GO:0051240	Positive regulation of multicellular organismal process	69
GO:0030324	Lung development	6
GO:0032502	Developmental process	148
GO:0001501	Skeletal system development	15
GO:0048519	Negative regulation of biological process	130
Downregulated		
GO:0040011	Locomotion	14
GO:0016477	Cell migration	14
GO:0048870	Cell motility	14
GO:0006928	Movement of cell or subcellular component	17
GO:0071478	Cellular response to radiation	2
GO:0060349	Bone morphogenesis	2

Supplementary Table 4.4. 'Molecular function' gene ontology (GO) terms enriched among differentially expressed genes in the epididymis from *Plag1* knockout versus wild-type mice.

GO Term ID	Term Name	Number of genes
Upregulated		
GO:0005539	Glycosaminoglycan binding	29
GO:0005488	Binding	213
GO:0017124	SH3 domain binding	8
GO:1901681	Sulfur compound binding	23
GO:0008201	Heparin binding	21
GO:0043167	Ion binding	89
Downregulated		
GO:0004222	Metalloendopeptidase activity	4
GO:0008237	Metallopeptidase activity	4
GO:0004175	Endopeptidase activity	4
GO:0070011	Peptidase activity, acting on L-amino acid peptides	4
GO:0008233	Peptidase activity	4

Supplementary Table 4.5. 'Cellular component' gene ontology (GO) terms enriched among differentially expressed genes in the epididymis from *Plag1* knockout versus wild-type mice.

GO Term ID	Term Name	Number of genes
Upregulated		
GO:0044421	Extracellular region part	85
GO:0005615	Extracellular space	73
GO:0005578	Proteinaceous extracellular matrix	50
GO:0031012	Extracellular matrix	54
GO:0005576	Extracellular region	82
GO:0005604	Basement membrane	22
GO:0044420	Extracellular matrix component	26
GO:0009986	Cell surface	49
GO:0005581	Collagen trimer	15

Supplementary Table 4.6. Downregulated genes categorised under the GO term 'Metallo-endopeptidase activity' (GO: 0004222) in the epididymis from *Plag1* knockout versus wild-type mice. FDR: false-discovery rate; HET: heterozygous; KO: knockout; LFC: log fold change KO compared to WT; TPM: transcripts per kilobase million; WT: wild-type.

Gene symbol	Gene name	FDR	LFC	TPM		
				WT	HET	KO
Mmp1b	Matrix metallopeptidase 1b	0.03	0.94	2.30	2.14	1.20
Mep1a	Meprin 1 alpha	1.60E–3	–1.43	15.17	13.05	7.03
Mme	Membrane metalloendopeptidase	2.20E–3	–0.64	82.73	76.16	56.83
Pappa2	Pappalysin 2	1.40E–3	–1.42	0.84	0.62	0.39

Supplementary Table 4.7. Upregulated genes categorised under the GO term 'Extracellular matrix component' (GO:0044420) in the epididymis from *Plag1* knockout versus wild-type mice. FDR: false-discovery rate; HET: heterozygous; KO: knockout; LFC: log fold change KO compared to WT; TPM: transcripts per kilobase million; WT: wild-type.

Gene symbol	Gene name	FDR	LFC	TPM		
				WT	HET	KO
Col12a1	Collagen, type X, alpha 1	1.60E–3	0.70	10.57	10.48	18.50
Fbln5	Fibulin 5	2.20E–3	0.66	5.96	7.15	9.91
Loxl2	Lysyl oxidase-like 2	5.47E–3	0.74	2.31	2.47	4.28
Sparc	Secreted acidic cysteine rich glycoprotein	1.40E–3	0.72	431.65	488.10	753.94
Col15a1	Collagen, type XV, alpha 1	2.37E–3	0.93	4.68	4.80	9.26
Trf	Transferrin	1.61E–3	1.13	10.78	12.10	22.71
Tgfbi	Transforming growth factor, beta induced	3.44E–3	0.46	15.74	16.89	22.91
Lamb1	Laminin b1	4.16E–3	0.50	12.98	13.35	19.00
Col4a4	Collagen, type IV, alpha 4	9.78E–4	0.43	11.46	11.69	15.47
Col4a3	Collagen, type IV, alpha 3	3.44E–3	0.36	8.70	9.00	11.29
Adamts1	A disintegrin-like and metallopeptidase with thrombospondin type 1 motif 1	2.20E–3	0.45	9.49	10.07	13.07
Col4a5	Collagen, type IV, alpha 5	3.58E–3	0.54	14.07	13.60	21.03
Efemp1	Epidermal growth factor-containing fibulin-like extracellular matrix protein 1	1.33E–3	0.82	31.22	36.25	56.05
Lama2	Laminin, alpha 2	2.71E–3	0.60	7.46	11.54	7.77
Timp2	Tissue inhibitor of metalloproteinase 2	1.23E–3	0.72	59.02	66.84	102.61
Lamc3	Laminin gamma 3	7.68E–3	0.69	5.06	5.21	8.35
Mmrn2	Multimerin 2	2.68E–3	0.76	4.97	4.99	8.57
Fbn1	Fibrillin 1	3.58E–3	0.61	5.37	5.54	8.92
Col11a1	Collagen, type XI, alpha 1	6.83E–3	0.68	2.47	2.45	4.17
Ccdc80	Coiled-coil domain containing 80	6.03E–3	0.97	4.73	4.83	10.51
Nid1	Nidogen 1	7.13E–3	0.41	23.97	23.09	33.26
Matn2	Matrilin 2	1.40E–3	0.62	26.72	29.31	41.40
Col1a2	Collagen, type I, alpha 2	2.71E–3	0.86	91.01	98.77	176.67
Ptn	Pleiotrophin	1.96E–3	0.93	8.14	8.96	15.02
Col4a6	Collagen, type IV, alpha 6	1.60E–3	0.62	7.55	7.74	11.69
Egflam	Egf-like, fibronectin type III and laminin g domains	5.46E–3	0.54	5.33	5.87	8.10

Chapter 5. Characterisation of mRNA expression of dysregulated sperm maturation genes and the extracellular matrix in the epididymis of PLAG1-deficient mice

Abstract

Pleomorphic adenoma gene 1 (PLAG1) is a transcription factor required for normal sperm motility and epididymis morphology. RNA sequencing has shown that a number of genes known to play a role in sperm maturation or the prevention of premature sperm capacitation and a large number of extracellular matrix (ECM)-related genes are dysregulated in the epididymis of *Plag1* knockout (KO) mice compared to wild-type (WT) controls. To investigate whether these changes in gene expression affect the spatial mRNA expression of selected dysregulated sperm maturation genes, *in situ* hybridisation was used. Also, to determine if the abundance of selected ECM components is affected in the epididymis of *Plag1* KO mice, as previously suggested by the dysregulation of the encoding genes, semi-quantitative staining techniques were used. Picro-Sirius red staining was used to semi-quantitatively analyse total collagen abundance, and immunofluorescent staining of laminin, membrane metalloendopeptidase (MME), meprin 1α (MEP1α) and tissue inhibitor of metalloproteinase 2 (TIMP2) was used to semi-quantitatively analyse protein expression. The results showed that the mRNA expression pattern of dysregulated sperm maturation genes was altered in *Plag1* KO mice. In addition, collagen, laminin, MME, MEP1α and TIMP2 were all differentially expressed in the epididymis of *Plag1* KO mice compared to WT controls, confirming the dysregulation of the ECM components at the protein level. Altogether, these findings suggest that in the absence of PLAG1, the expression pattern of several important sperm maturation or sperm capacitation genes is altered, and that PLAG1 is required for proper maintenance of the ECM in the mouse epididymis.

5.1 Introduction

Pleomorphic adenoma gene 1 (PLAG1) is a transcription factor required for male fertility. *Plag1* knockout (KO) male mice are infertile (Wong et al. 2019), and exhibit decreased sperm motility (Juma et al. 2017) and aberrant epididymal morphology (Wong et al. 2019). The epididymal tubule is shorter and significantly less coiled, relative epididymis weight is reduced by 44% and the cauda epididymidis lacks the typical bulbous shape in *Plag1* KO mice compared to wild-type (WT) controls (Wong et al. 2019).

The epididymis is grossly divided into four main regions (initial segment, caput, corpus and cauda). The caput and corpus are the sites of sperm maturation, while the cauda is thought to largely function in storage of mature sperm (Cornwall 2009). In rodents the epididymis can be further divided into segments based on unique transcriptomes and the presence of connective tissue septae; in mice, there are 10 distinct segments (Johnston et al. 2005; Turner et al. 2003). Decreased epididymal tubule elongation and coiling presumably impact sperm maturation, as this process encompasses a precise sequence of biochemical and structural modifications controlled by segment-specific gene expression during epididymal transit (Johnston et al. 2007; Turner et al. 2003). Spermatozoa gain their motility during epididymal sperm maturation, and since *Plag1* KO mice exhibit a 49% reduction in sperm motility and an 80% reduction in spermatozoa showing progressive motility (Juma et al. 2017), improper sperm maturation may be a major underlying cause behind the fertility issues seen in these mice.

Transcriptome analysis of the epididymides of *Plag1* KO mice revealed that several sperm maturation-related genes were dysregulated, including *Acvr1*, *Pebp1*, *Sfrp2*, *Bsph2* and *Smox* (Wong et al. 2020). Bioinformatics analysis also showed that these genes have various functions related to sperm maturation or are known to be involved in epididymis development (Wong et al. 2020). The dysregulation of these genes provides an indication of possible defects that may contribute to fertility issues seen in *Plag1* KO male mice. Since the expression of epididymal genes is spatially regulated, tissue expression studies would be useful to show any alterations to the gene expression patterns of these dysregulated genes.

Furthermore, transcriptome analysis also showed that genes relating to the extracellular matrix (ECM) and its maintenance were dysregulated in the epididymis of *Plag1* KO mice compared to WT controls (Wong et al. 2020). Over 20 collagen genes and several ECM component genes were upregulated, and a number of genes encoding the metalloproteinases responsible for degrading ECM components were downregulated (Wong et al. 2020). The dysregulation of ECM genes presents the possibility that ECM homeostasis is affected in *Plag1* KO mice. Importantly, this is an epididymal phenotype that has not previously been reported in mouse models of male infertility.

As the epididymal epithelium is the main source of secreted proteins and other factors that facilitate sperm maturation as immature spermatozoa transit through the epididymis, this epithelium is generally the focus of studies of sperm maturation, epididymal function and mouse models of fertility issues. The epithelium is surrounded by the ECM, which is the non-cellular component of connective tissues that provides physical, biochemical and biomechanical support to the cellular parenchyma (Frantz, Stewart & Weaver 2010). The ECM is generally not mentioned in studies of the adult epididymis in rodent models of male infertility; however, the ECM is known (1) to be involved in the regulation of several cell behaviours, including adhesion, motility, growth and survival (Wells 2008), (2) to act as a harbour of growth factors and also (3) to regulate cell-to-cell signalling and gene transcription via cell surface receptor binding (Frantz, Stewart & Weaver 2010). Furthermore, epithelial tissue homeostasis is dependent on interaction with the surrounding stromal tissue, so ECM dysfunction may in turn impact epithelial integrity. Stiffening of the ECM due to excessive deposition of ECM components is associated with the progression of fibrotic diseases and tumours (Frantz, Stewart & Weaver 2010; Handorf et al. 2015; Lampi & Reinhart-King 2018). Since the ECM plays several important roles as outlined above, and since the underlying cause of male fertility issues cannot be determined in 30–45% of clinical cases (Jungwirth et al. 2012), investigating the role of the ECM in the epididymis may provide new information that can help unravel the enigma of male infertility by providing new insights into epididymis development and function.

The aim of this study was to characterise the mRNA expression of selected sperm maturation-related genes previously shown to be dysregulated in the epididymis from *Plag1* KO mice (namely *Acvr1*, *Pebp1*, *Sfrp2*, *Bsph2* and *Smox)*, and to provide confirmation of alterations to the

structure of the ECM at the protein level. Semi-quantitative staining was used to analyse collagen, laminin, membrane metalloendopeptidase (MME), meprin 1α (MEP1α) and tissue inhibitor of metalloproteinase 2 (TIMP2) as the genes encoding these ECM components were identified by RNA sequencing (RNA-Seq) as dysregulated in the epididymides from *Plag1* KO mice (Wong et al. 2020).

5.2 Methods

5.2.1 Animals

All animal procedures were approved by the Animal Ethics Committee of La Trobe University (AEC17-16, -27, -54) and the La Trobe Institutional Biosafety Committee (GMSC17-15). All animal care and experimental procedures were conducted in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th edition, 2013) of the National Health and Medical Research Council. Founder mice were generously gifted by Prof. Wim Van de Ven (Laboratory for Molecular Oncology, Center for Human Genetics, Catholic University of Leuven, Belgium). The generation of the *Plag1* KO mouse line has previously been described (Hensen et al. 2004). Genotypes of mice were determined by PCR with genomic DNA extracted from ear clips as previously described (Wong et al. 2019).

5.2.2 *In situ* hybridisation

Epididymides were dissected from adult male *Plag1* WT and KO mice (*n*=5 per genotype), killed by CO_2 asphyxiation. The tissues were fixed overnight in 4% (*w/v*) paraformaldehyde in PBS, cryoprotected in 30% (*w/v*) sucrose in PBS until the tissues sank, and then quick-frozen in cooled isopentane. Epididymides were cryosectioned into 5-μm serial sections and stored at –80°C until staining. Riboprobes were synthesised using RNA extracted from adult *Plag1* WT mouse epididymides as previously described (Grommen et al. 2017); primers targeting *Acvr1*, *Pebp1*, *Sfrp2*, *Bsph2* and *Smox* for riboprobe synthesis are listed in **Supplementary Table 5.1**. *In situ* hybridisation staining was performed according to the method previously described (Wong et al. 2019), with an additional stringency wash included after incubation with the probe: slides were incubated in 0.2×

saline sodium citrate at 70°C for 30 min. Slides were incubated in hybridisation buffer containing antisense riboprobes complementary to *Acvr1*, *Pebp1*, *Sfrp2*, *Bsph2* and *Smox* mRNA; hybridisation temperatures are listed in **Supplementary Table 5.1**. For each gene, epididymis sections from a WT mouse were incubated in hybridisation buffer containing the sense riboprobe as a negative control (**Supplementary Figure 5.1**). Tissue sections were imaged using an Olympus BX41 microscope and Olympus DP25 camera (Olympus Scientific Solutions Americas Inc., Waltham, MA, USA). The highest magnification in which the *in situ* hybridisation signal was clearly in focus was used.

5.2.3 Picro-Sirius red staining

Epididymides were collected from 7-week-old mice (*n*=5 for each of WT and KO), fixed for 2 h in 4% (*w/v*) paraformaldehyde in PBS and then cyroprotected for 2 h in 10% (*w/v*) sucrose in PBS, followed by 30% sucrose in PBS until the tissues sank. Tissues were stored at –80°C until sectioned into 5-µm cryosections. Sections were treated with 4% paraformaldehyde for 5 min, washed in distilled water and then stained with 0.05% (*w/v*) Sirius Red (Direct Red 80; Sigma Aldrich, St. Louis, USA) dissolved in a saturated aqueous solution of picric acid (Sigma Aldrich) for 1 h. Sections were washed in two changes of acidified water, dehydrated in 70% (*v/v*) ethanol followed by two changes of 100% ethanol, cleared in xylene and mounted with DPX (Sigma Aldrich). Sections were imaged using an Olympus BX41 microscope with an Olympus DP25 camera (Olympus Scientific Solutions Americas Inc., Waltham, MA, USA). Using 5–8 images (20× magnification) per epididymal region (caput, corpus, cauda) per animal, the percentage area stained red (collagen-positive) was determined using ImageJ software (https://imagej.nih.gov/ij/). Regions of interest (ROIs) encompassing stromal areas were traced using the freehand drawing tool, and the percentage area of collagen was determined using the 'threshold' function. Animal and genotype details were omitted from image files prior to the analysis to exclude bias.

5.2.4 Immunofluorescence

Epididymides were collected from 7-week-old mice (*n*=5 for each of WT and KO) and then fixed, cryoprotected and sectioned as above (section 5.2.2) Thawed sections were first treated with

boiling 1 M trisodium citrate dihydrate (VWR, Radnor, PA, USA) for 40 min for antigen retrieval. Sections were then washed in phosphate-buffered saline (PBS), incubated for 2 h in PBS containing 4% (*w/v*) bovine serum albumin (BSA), 0.1% (*v/v*) Triton X-100, 0.1 M lysine and 5% (*v/v*) normal goat serum (Antibodies Australia, Melbourne, Australia) (BSA-PBS-TX) at room temperature, then washed in PBS, before overnight incubation at 4°C with primary antibody diluted in BSA-PBS-TX (for antibody details and dilutions, see **Supplementary Table 2**). Negative-control slides were incubated in BSA-PBS-TX with the primary antibody omitted. Next, sections were washed in PBS, incubated for 2 h at 4°C with goat anti-rabbit IgG Alexa Fluor 555 (catalogue number A27039, Thermo Fisher, Waltham, MA, USA) or goat anti-rabbit IgG Alexa Fluor 488 (catalogue number A-11008, Thermo Fisher) secondary antibody diluted 1:500 in PBS. Sections were then washed in PBS with 0.1% Triton X-100 before DAPI diluted in PBS (Thermo Fisher) was applied according to manufacturer instructions. The sections were washed in PBS and coverslipped with Fluoro-Gel aqueous mounting medium (ProSciTech, Kirwan, QLD, Australia). Sections were imaged as described above.

5.2.5 Immunofluorescent image analysis

For all antibodies, 3–6 images were taken (20× magnification) per region (caput, corpus and cauda), per animal for Image J analysis. Stromal and epithelial proteins were quantified by tracing areas of epithelium and stroma, respectively, using the freehand drawing tool and creating ROIs in ImageJ. This ensured that results were not skewed by the decreased tubule elongation and coiling or the increased tubule luminal area in epididymides from *Plag1* KO mice. Protein expression was quantified by determining the percentage area with positive staining using the 'threshold' function. Animal and genotype details were omitted from image files prior to the analysis to exclude bias.

5.2.6 PLAG1 motif optimisation

Gene lists of the significantly up- and downregulated genes determined by RNA-Seq (Wong et al. 2020) were used for the *de novo* motif scanning using the findMotifs.pl programme in the Homer suite of sequence analysis tools (Heinz et al. 2010). The '-opt' flag with the JASPAR database PLAG1 motif (JASPAR MA0163.1) (http://jaspar.genereg.net) was used as an input. The analysis was

performed by examining two different promoter sizes: between 300 bp upstream to 50 bp downstream, and between 2 kb upstream to 100 bp downstream from the transcription start site.

5.3 Results

5.3.1 Spatial mRNA expression pattern of selected dysregulated sperm maturation genes in the epididymis of *Plag1* KO and WT mice

mRNA spatial expression patterns of selected dysregulated genes related to sperm maturation in the epididymides from *Plag1* KO mice, previously identified by DAVID analysis, were characterised using *in situ* hybridisation. *Acrv1* mRNA expression decreased from caput to cauda in *Plag1* KO epididymides, while expression increased towards the cauda in WT controls (**Figure 5.1**). In *Plag1* WT mice, *Pebp1* mRNA expression was dramatically lower in the corpus compared to the caput, while in *Plag1* KO mice this decrease was far less drastic, as expression in the corpus of the *Plag1* KO mice appeared stronger than in WT controls. Cauda expression of *Pebp1* was stronger in WT controls compared to *Plag1* KO mice (**Figure 5.2**). *Plag1* KO mice exhibited decreased *Sfrp2* mRNA expression compared to WT controls, and strong expression was observed on the spermatozoa in the cauda of WT controls (**Figure 5.3**). *Bsph2* mRNA expression was stronger in the corpus of WT controls compared to *Plag1* KO mice, and expression appeared similar between genotypes in the caput and cauda (**Figure 5.4**). Minimal *Smox* mRNA expression was observed in the epididymis of both WT controls and *Plag1* KO mice (**Supplementary Figure 5.2**).

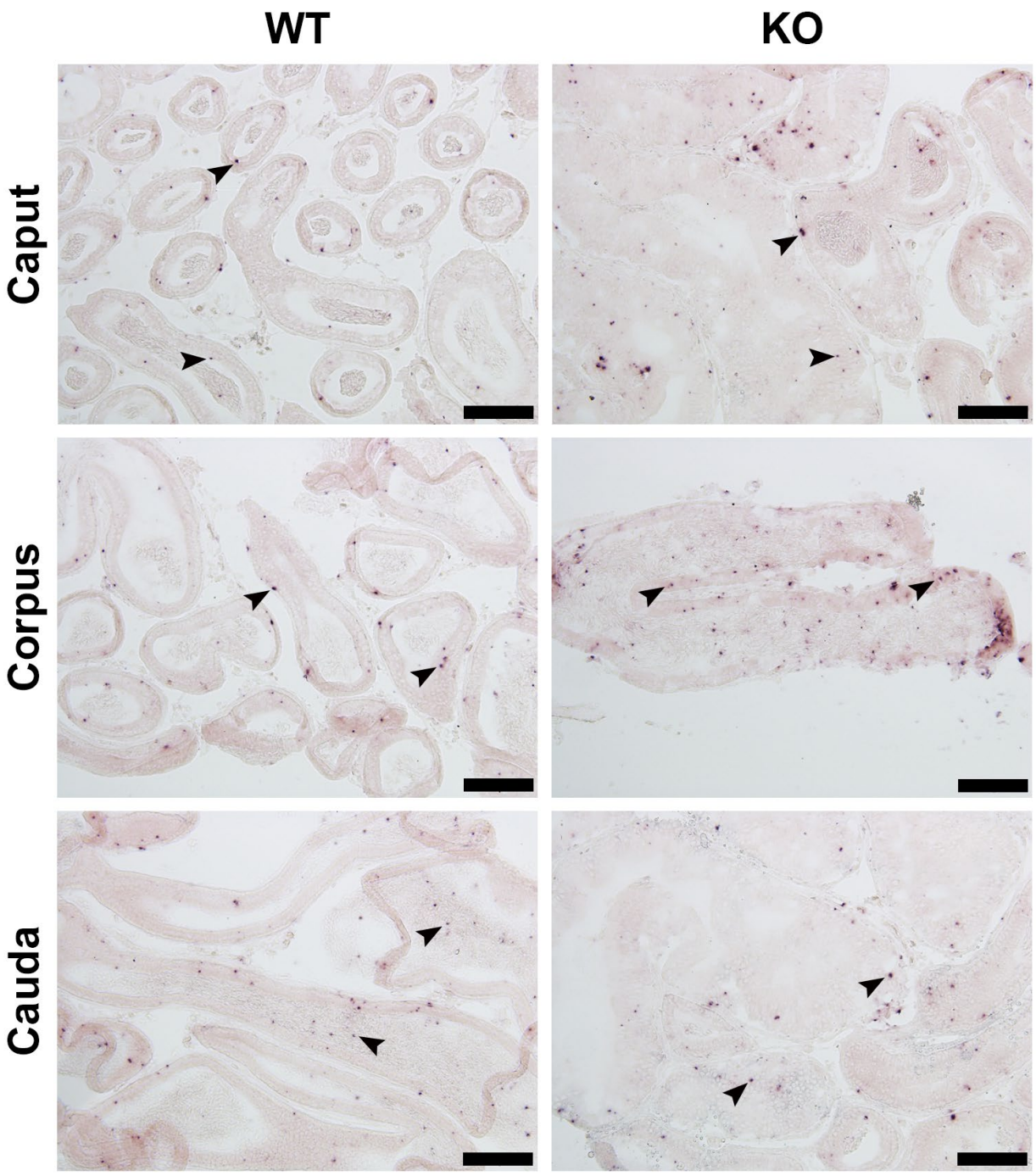

Figure 5.1. Spatial expression pattern of *Acrv1* mRNA in the epididymis from *Plag1* WT and KO mice as determined by in situ hybridisation. Arrowheads indicate *in situ* hybridisation signal. Scale bars represent 100 μm.

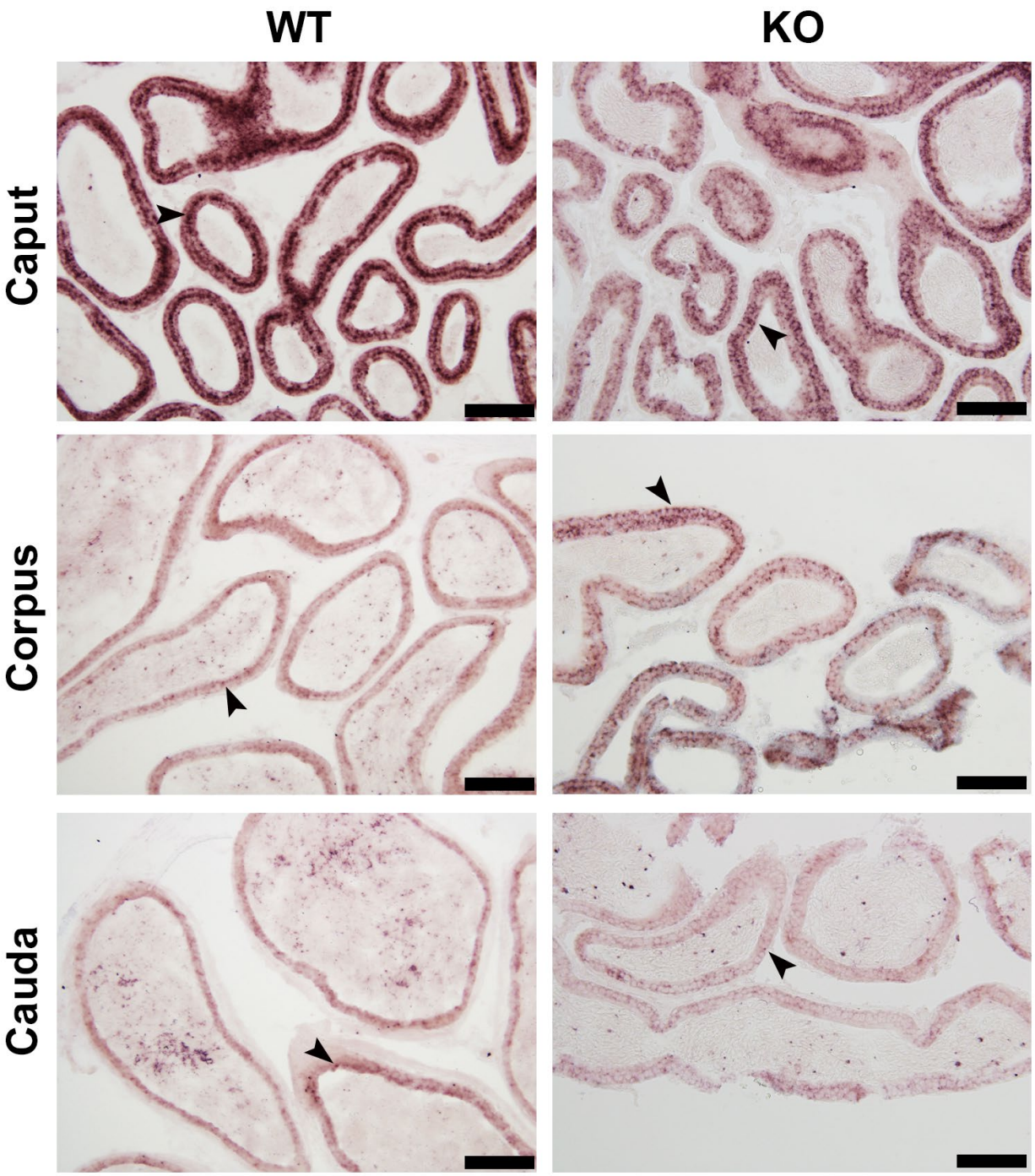

Figure 5.2. Spatial expression pattern of *Pebp1* mRNA in the epididymis from *Plag1* WT and KO mice as determined by *in situ* hybridisation. Arrowheads indicate *in situ* hybridisation signal. Scale bars represent 100 μm.

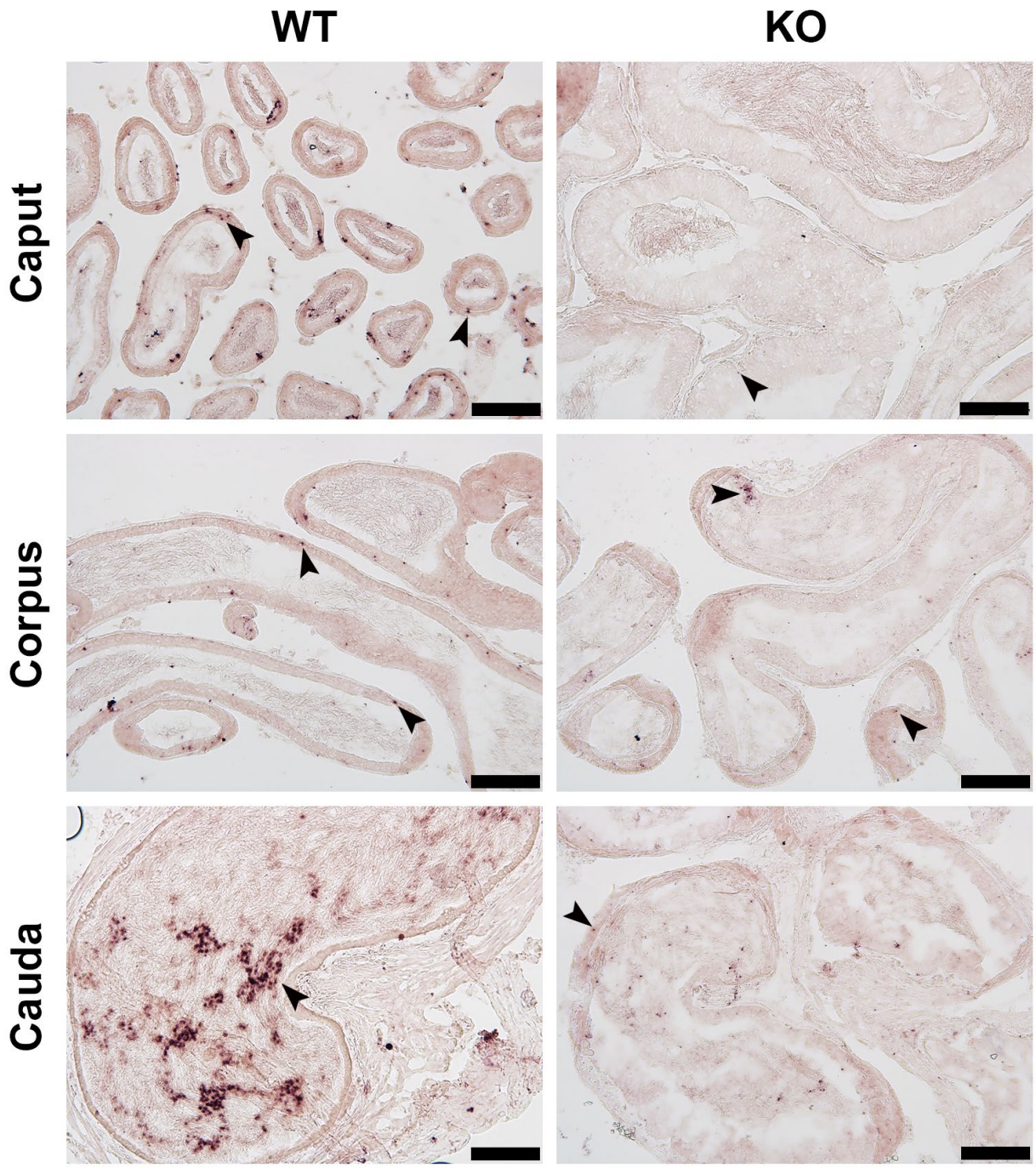

Figure 5.3. Spatial expression pattern of *Sfrp2* mRNA in the epididymis from *Plag1* WT and KO mice as determined by *in situ* hybridisation. Arrowheads indicate *in situ* hybridisation signal. Scale bars represent 100 μm

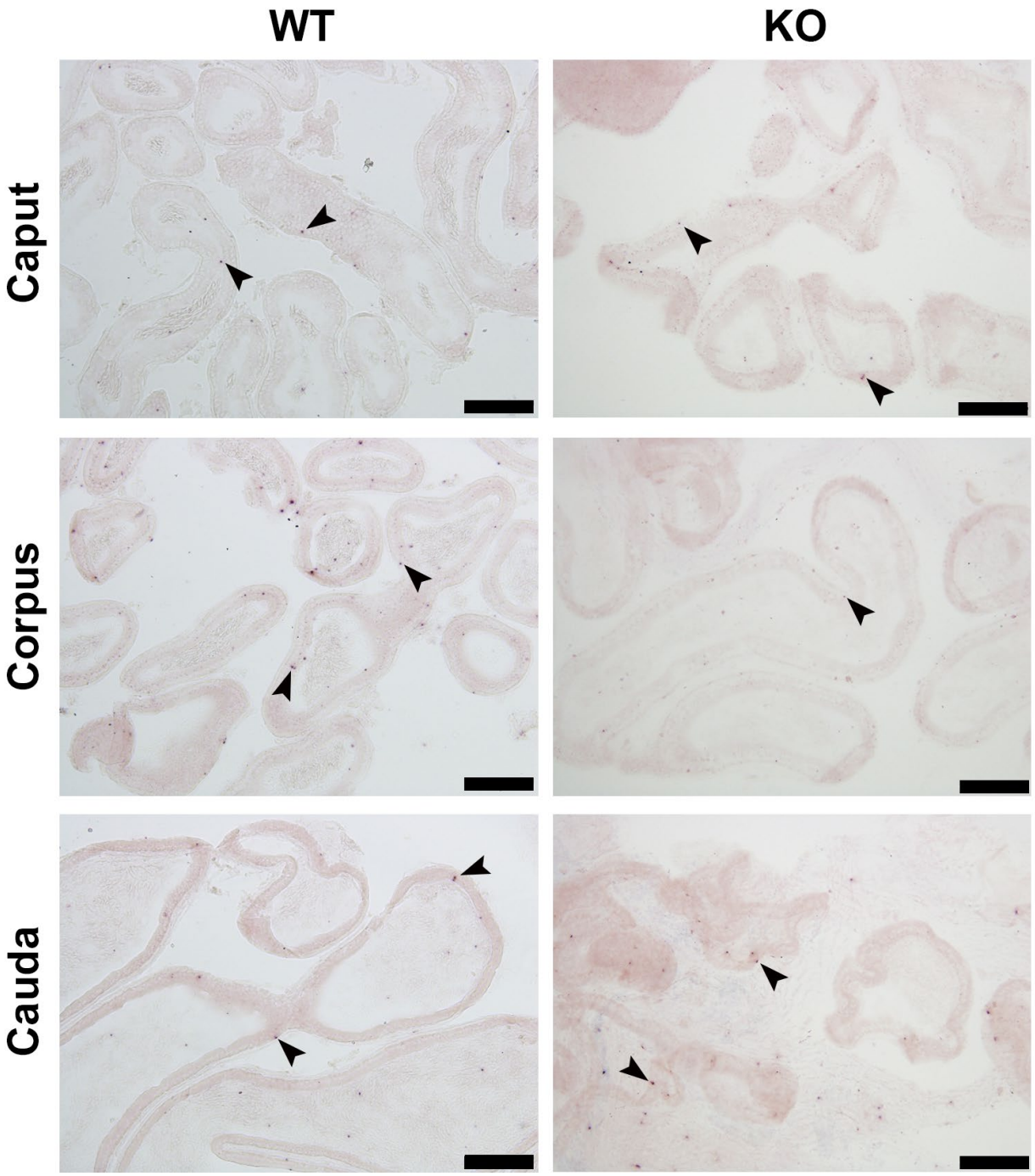

Figure 5.4. Spatial expression pattern of *Bsph2* mRNA in the epididymis from *Plag1* WT and KO mice as determined by *in situ* hybridisation. Arrowheads indicate *in situ* hybridisation signal. Scale bars represent 100 μm

5.3.2 Dysregulated expression of ECM components

In a previous study, RNA-Seq revealed the upregulation of genes encoding components of the ECM and the downregulation of genes encoding regulators of the ECM in the epididymis from *Plag1* KO mice compared to WT controls (Wong et al. 2020). In order to investigate the composition of the ECM and to confirm whether the dysregulation of ECM genes results in altered levels of ECM constituents within the tissue, collagen and the expression of selected ECM proteins were semi-quantified in the epididymis from *Plag1* KO mice. Picro-Sirius red staining was used to compare the percentage of collagen-positive area in the stroma of the epididymis in *Plag1* KO and WT mice. Bright-field microscopy images were used for semi-quantification of total collagen; polarised light distinguishes type I collagen (yellow/orange birefringence) and type III collagen (green birefringence), but cannot be used for collagen quantification as absorption of polarised light by the Sirius Red dye is dependent on the orientation of the collagen fibres (Lattouf et al. 2014). Therefore, not all collagen bundles are visible under polarised light and such images would not be accurate for quantification studies. ImageJ quantification of the Picro-Sirius red staining indicated that the percentage of collagen-positive area was significantly higher in the stroma of the cauda epididymidis of *Plag1* KO mice (*t*-test, P=0.0064) compared to that of *Plag1* WT mice, while there was no difference between *Plag1* KO mice and WT controls in the percentage of collagen-positive area in the caput and corpus regions of the epididymis (**Figure 5.5**). Images taken with polarised light show that type I and III collagen are both present in epididymides of *Plag1* KO and WT mice (**Supplementary Figure 5.3**).

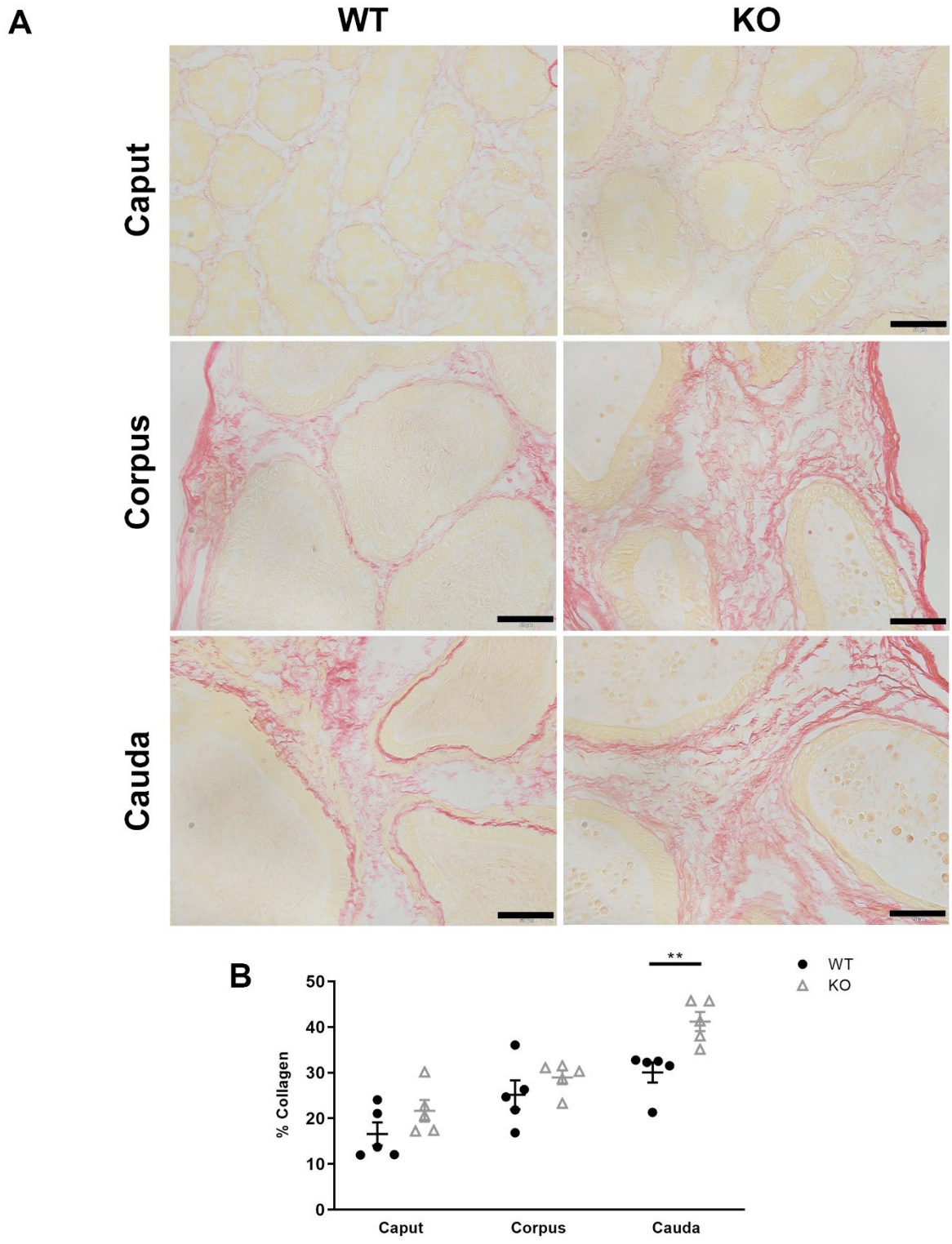

Figure 5.5. Localisation of collagen in the epididymis of *Plag1* WT and KO mice. (A) Picro-Sirius red staining of the caput, corpus and cauda epididymidis in *Plag1* WT and KO mice. Collagen is stained red; scale bars represent 50 µm. **(B)** Percentage collagen-positive area in the epididymis of KOs *vs* WTs (*n*=5 per genotype). Values shown are means ± SEM. ***P* < 0.01 (*t*-test).

Immunofluorescence and ImageJ image analysis were used for semi-quantification of ECM protein expression in the epididymis of *Plag1* KO and WT mice. Laminin, MME, MEP1α and TIMP2 were previously shown to present with dysregulated mRNA expression in the epididymis of *Plag1* KO mice (Wong et al. 2020); therefore, antibodies against these proteins were used to compare protein expression between *Plag1* KO mice and WT controls. The percentage of laminin-positive stromal area was significantly higher in the caput (*t*-test, *P*=0.0001), corpus (*P*=0.0240) and cauda (*P*=0.0084) in the epididymides from *Plag1* KO mice. The cauda of *Plag1* KO mice also showed significantly higher intensity of staining (*t*-test, *P*=0.0417), but this was not the case in the caput and corpus (**Figure 5.6**). Furthermore, in the epididymis of *Plag1* KO mice, the laminin appears to surround a bi-layer of cells, while in *Plag1* WT mice, the laminin surrounds a single layer of cells (**Figure 5.6**). Apart from the ECM, laminin was also observed in DAPI-stained cells in the tubule lumen of the corpus and cauda epididymidis from *Plag1* KO mice.

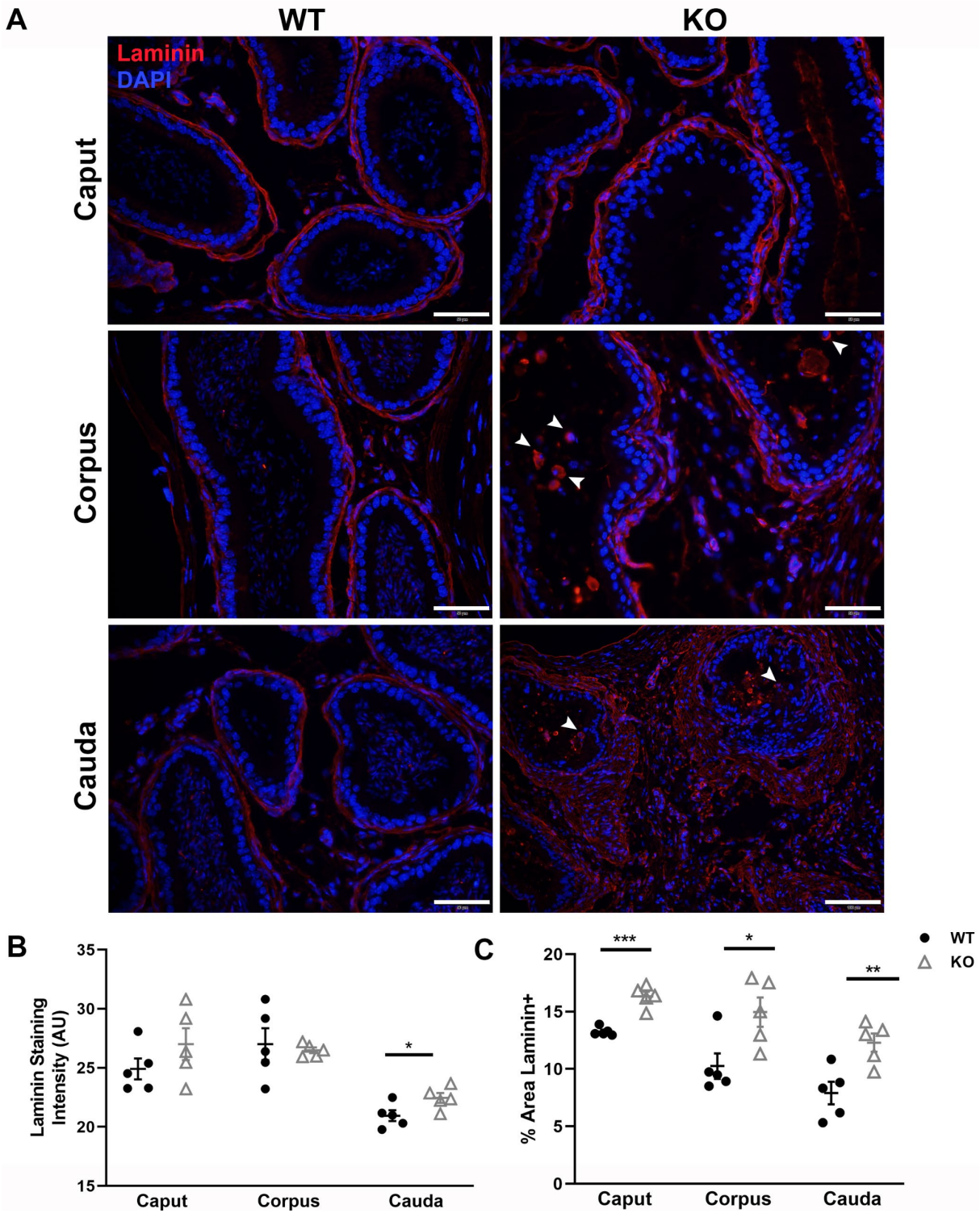

Figure 5.6. Expression of laminin in the epididymis of *Plag1* WT and KO mice. (A) Immunofluorescent staining of laminin (red) and DAPI (nuclei, blue) in the epididymis of *Plag1* KO mice and WT controls. Arrowheads indicate co-localisation of laminin and DAPI in cells within the tubule lumen. Scale bars represent 50 µm. **(B)** Intensity of fluorescent laminin staining in the epididymis from KOs *vs* WTs (*n*=5 per genotype). **(C)** Percentage of laminin-positive area in the epididymis of KOs *vs* WTs (*n*=5 per genotype). Values shown are means ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 (*t*-test). AU, arbitrary units.

MME exhibited a different staining pattern in the caput compared to the corpus and cauda (**Figure 5.7**); caput expression signal appeared as dot-like areas of expression in epithelial cells at the tubule brush border, while expression signal in the corpus and cauda was remarkably more wide-spread across the entire brush border. MME expression was significantly higher in the corpus (*t*-test, *P*=0.0034) and cauda (*P*=0.0006) epididymidis from *Plag1* KO mice compared to WT controls, but not in caput (*P*=0.7512). Staining intensity was significantly lower in the corpus (*t*-test, *P*=0.0236), but was not significantly different in the caput (*P*=0.5332) or cauda (*P*=0.9536) epididymidis from *Plag1* KO mice compared to WT controls.

In order to characterise whether proteolytic MEP1α was different in the epididymis from *Plag1* KO mice, stromal MEP1α was semi-quantified. The mean percentage stromal area expressing MEP1α was significantly lower in the cauda (*t*-test, *P*=0.0009) but was not significantly different in the caput (*P*=0.0506) or corpus (*P*=0.1711) epididymidis from *Plag1* KO mice compared to WT controls (**Figure 5.8**).

TIMP2 expression, which was minimal overall, was significantly higher in the caput (*t*-test, *P*=0.0370), corpus (*P*=0.0363) and cauda (*P*=0.0089) epididymidis from *Plag1* KO mice compared to WT controls TIMP2 staining intensity was not significantly different in the epididymis from *Plag1* KO mice compared to WT controls (**Figure 5.9**).

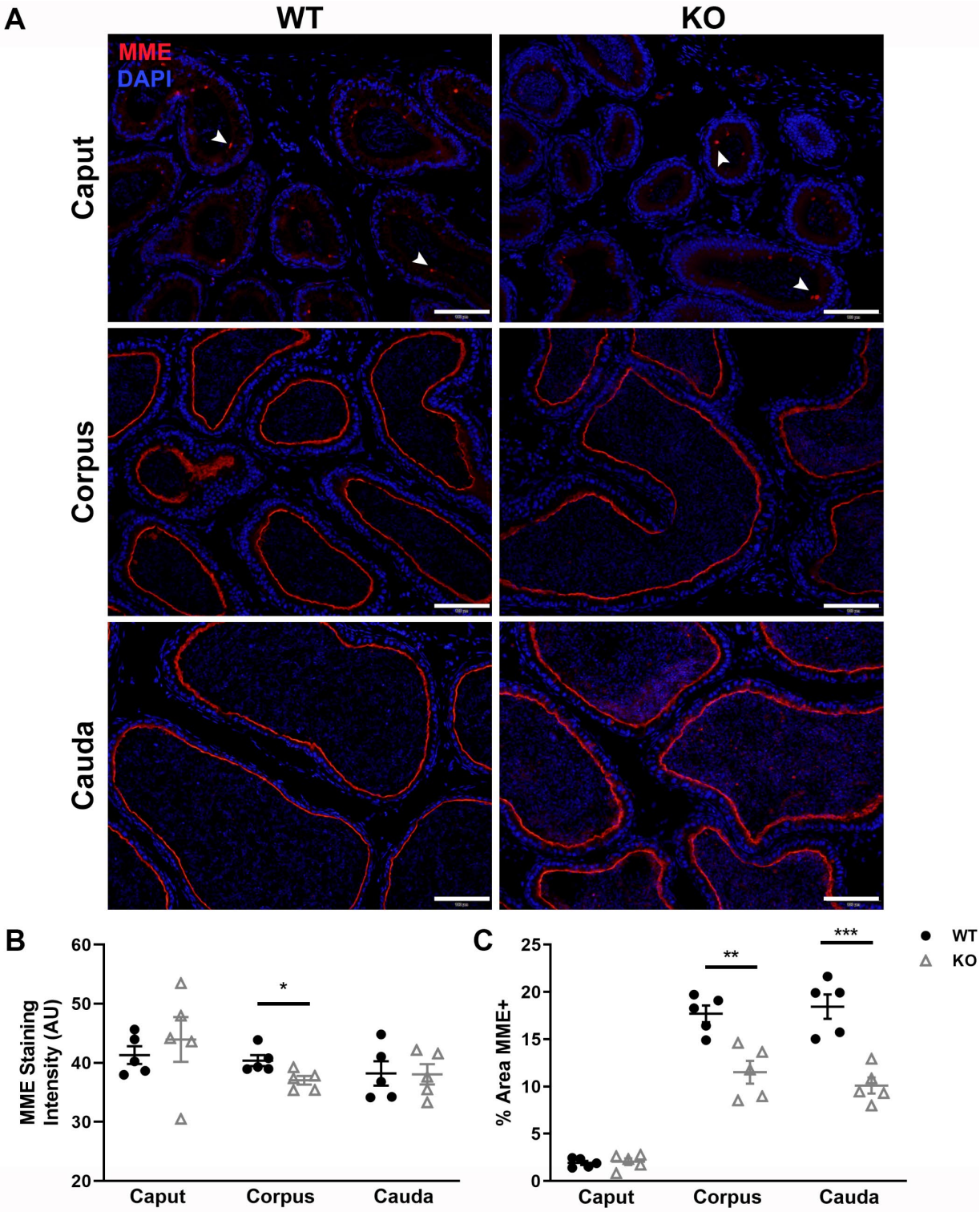

Figure 5.7. Expression of membrane metalloendopeptidase (MME) in the epididymis of *Plag1* WT and KO mice. (A) Immunofluorescent staining of MME (red) and DAPI (nuclei, blue) in the epididymis of *Plag1* KO mice and WT controls. Arrowheads indicate areas of MME expression in the brush border of the caput epididymidis. Scale bars represent 100 µm. **(B)** Intensity of fluorescent MME staining in the epididymis of KOs *vs* WTs (*n*=5 per genotype). **(C)** MME-positive area relative to total area in the epididymis of KOs *vs* WTs (*n*=5 per genotype). Values shown are means ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 (*t*-test). AU, arbitrary units.

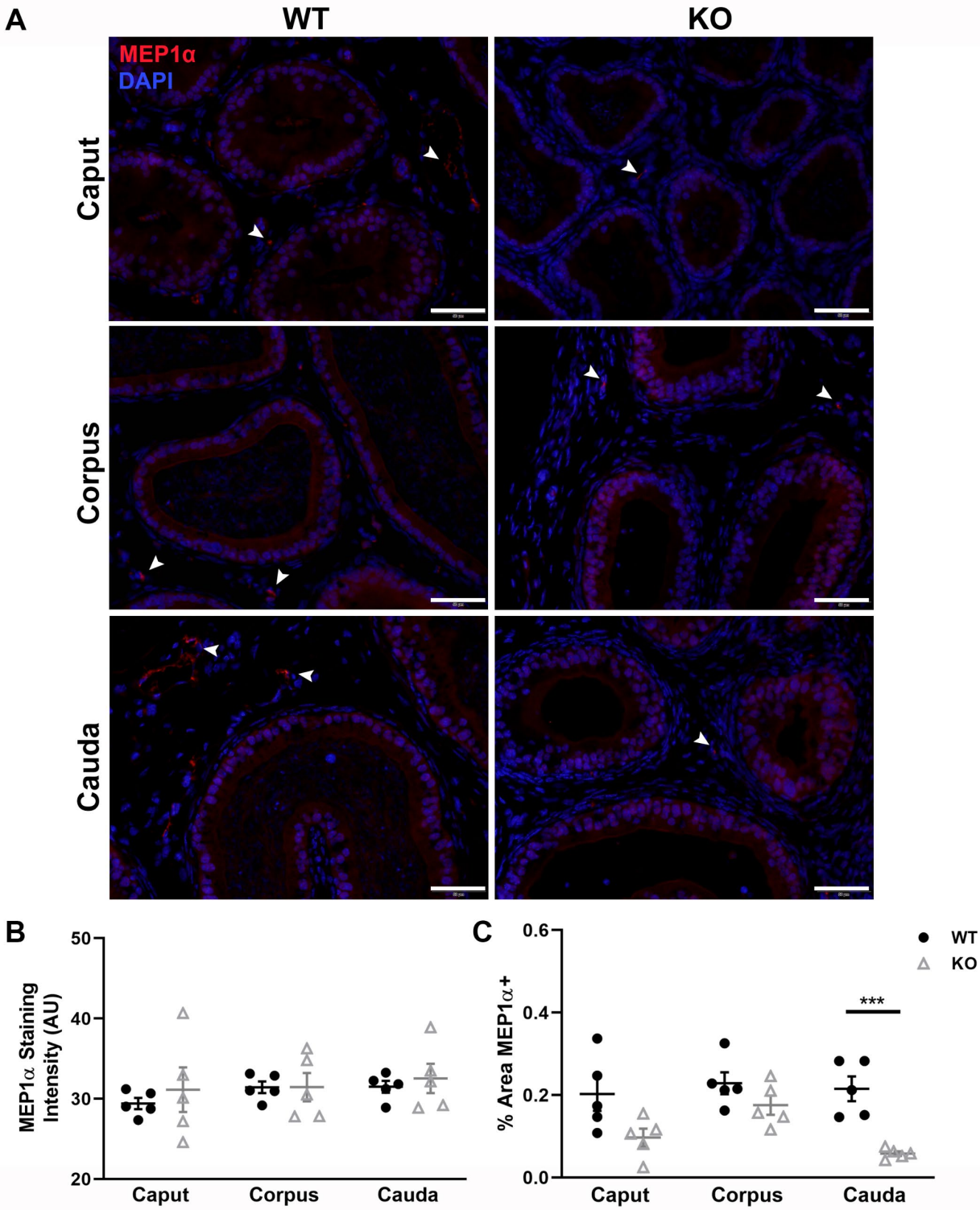

Figure 5.8. Expression of meprin 1α (MEP1α) in the epididymis of *Plag1* WT and KO mice. (A) Immunofluorescent staining of MEP1α (red) and DAPI (nuclei, blue) in the epididymis of *Plag1* KO mice and WT controls. Scale bars represent 50 µm. **(B)** Intensity of fluorescent MEP1α staining in the epididymis of KOs vs WTs (n= 5 per genotype). **(C)** MEP1α-positive area relative to total area in the epididymis of KOs vs WTs (n= 5 per genotype). Values shown are means ± SEM. ***$P < 0.001$ (*t*-test). AU, arbitrary units.

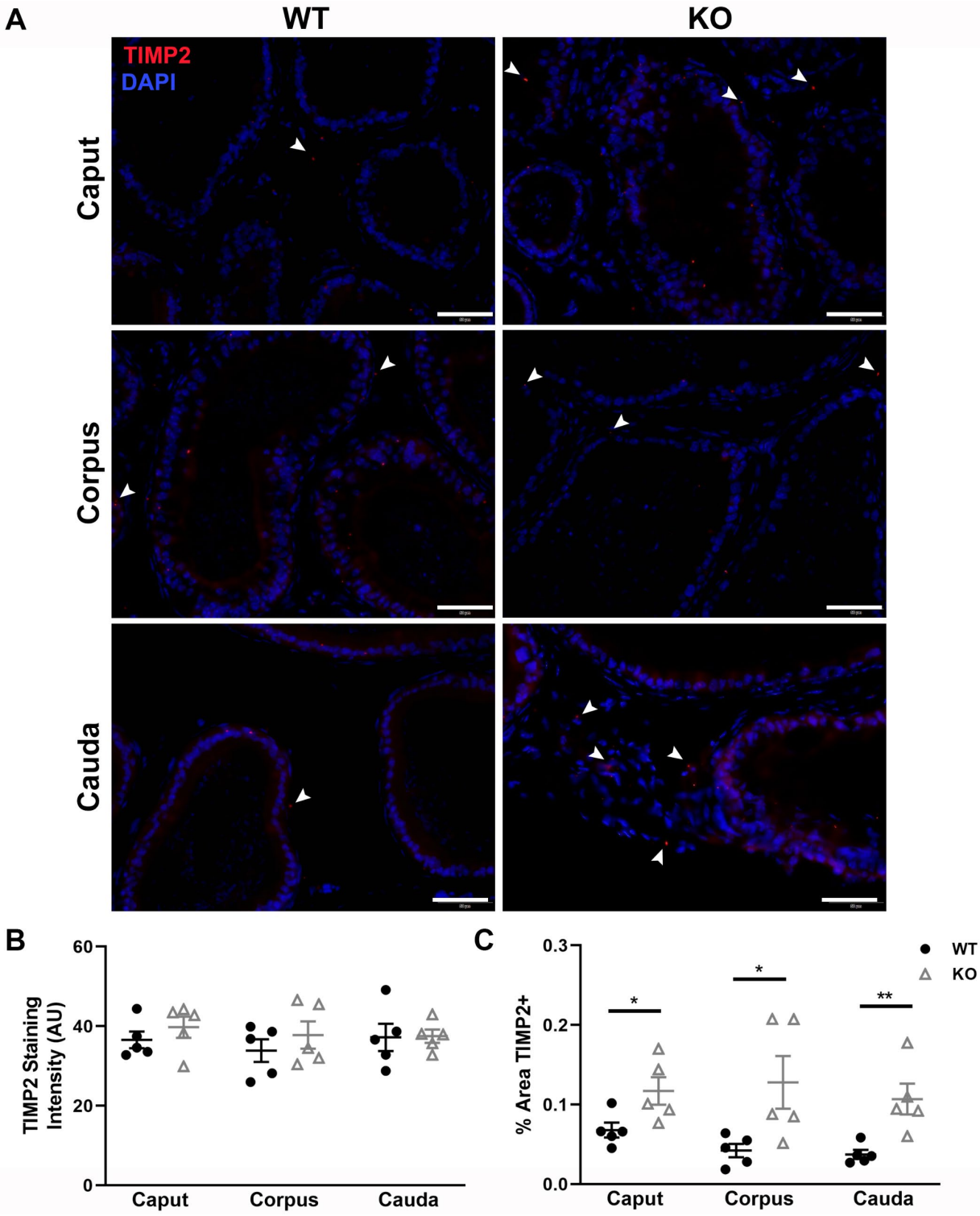

Figure 5.9. Expression of tissue inhibitor of metalloproteinase 2 (TIMP2) in the epididymis of *Plag1* WT and KO mice. (A) Immunofluorescent staining of TIMP2 (red) and DAPI (nuclei, blue) in the epididymis of *Plag1* KO mice and WT controls. Arrowheads indicate areas of TIMP2-positive staining. Scale bars represent 50 µm. **(B)** Intensity of fluorescent TIMP2 staining in the epididymis of KOs *vs* WTs (*n*=5 per genotype). **(C)** TIMP2-positive area relative to total area in the epididymis of KOs *vs* WTs (*n*=5 per genotype). Values shown are means ± SEM. **P* < 0.05; ***P* < 0.01 (*t*-test). AU, arbitrary units.

5.3.3 E-cadherin expression

It was shown previously that there is an increased proportion of stromal cells present in the caput, corpus and cauda epididymidis, and increased cell proliferation in the stroma of the cauda epididymidis in *Plag1* KO mice compared to WT controls (Wong et al. 2020). In order to determine if epithelial-to-mesenchymal transition (EMT) was a contributing factor to this increased density of stromal cells, rather than the proliferation of stromal cells alone, immunofluorescent staining of E-cadherin was performed. The immunofluorescent staining and ImageJ analysis results indicated that E-cadherin expression was not significantly different in any region of the epididymis in *Plag1* KO mice compared to WT controls ($P>0.05$) (**Figure 5.10**).

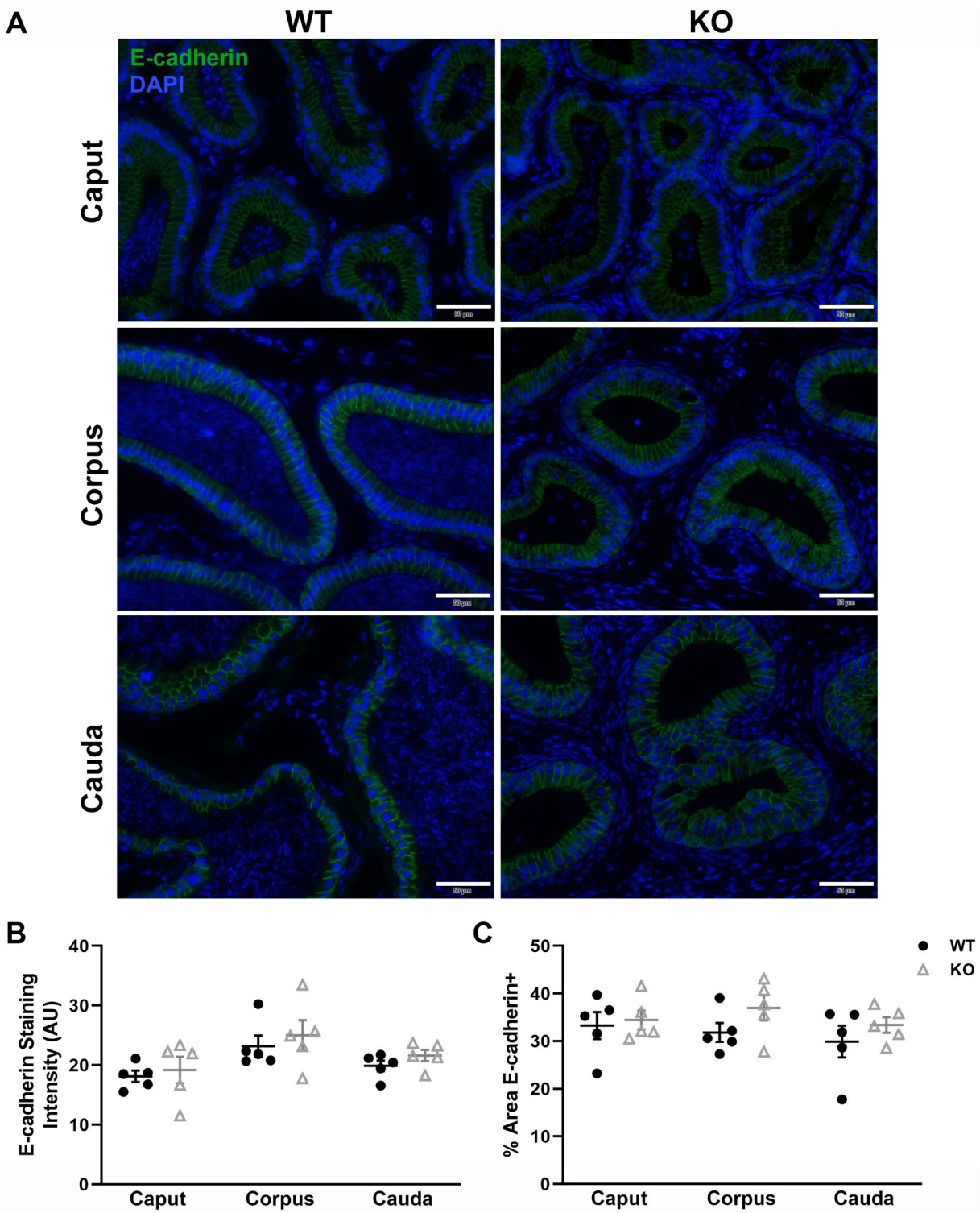

Figure 5.10. Expression of E-cadherin in the epididymis of *Plag1* WT and KO mice. (A) Immunofluorescent staining of E-cadherin (green) and DAPI (nuclei, blue) in the epididymis of *Plag1* KO mice and WT controls. Scale bars represent 50 µm. **(B)** Intensity of fluorescent E-cadherin staining in the epididymis from KOs *vs* WTs (*n*=5 per genotype). **(C)** E-cadherin-positive area relative to total area in the epididymis of KOs *vs* WTs (*n*=5 per genotype). Values shown are means ± SEM. Means are not significantly different (*t*-test, $P > 0.05$). AU, arbitrary units.

5.3.4 PLAG1 motif optimisation

Previous motif scanning analysis did not reveal any significant enrichment of PLAG1 binding motifs in the dysregulated genes in the epididymis of *Plag1* KO mice (Wong et al. 2020). In order to determine if there are any *de novo* PLAG1 binding motifs in the promoter regions of the dysregulated genes in the epididymis, we performed a motif optimisation analysis using the motif discovery programme Homer. This analysis scans the promoter regions of the genes of interest and identifies any motifs that are significantly similar to the PLAG1 binding motif. These *de novo* motifs may have base differences or minor sequence variations from the PLAG1 binding motif, would not be identified in a standard motif scanning analysis, such as that previously reported (Wong et al. 2020). A significance value is provided for any motifs that are similar to the target binding motif; truly significant *de novo* motifs have strongly significant *P* values (*i.e*. $P < e^{-10}$). PLAG1 motif optimisation revealed one significant *de novo* motif in each of the datasets (downregulated and upregulated genes) with some sequence identity to the PLAG1 motif (**Figure 5.11**). However, as these are considered only weakly significant for this type of analysis ($P > e^{-10}$) (Heinz et al. 2010), these are likely to be false-positives and are flagged as such in Homer. Lastly, the analysis did not reveal any convincing *de novo* motifs for any other transcription factors.

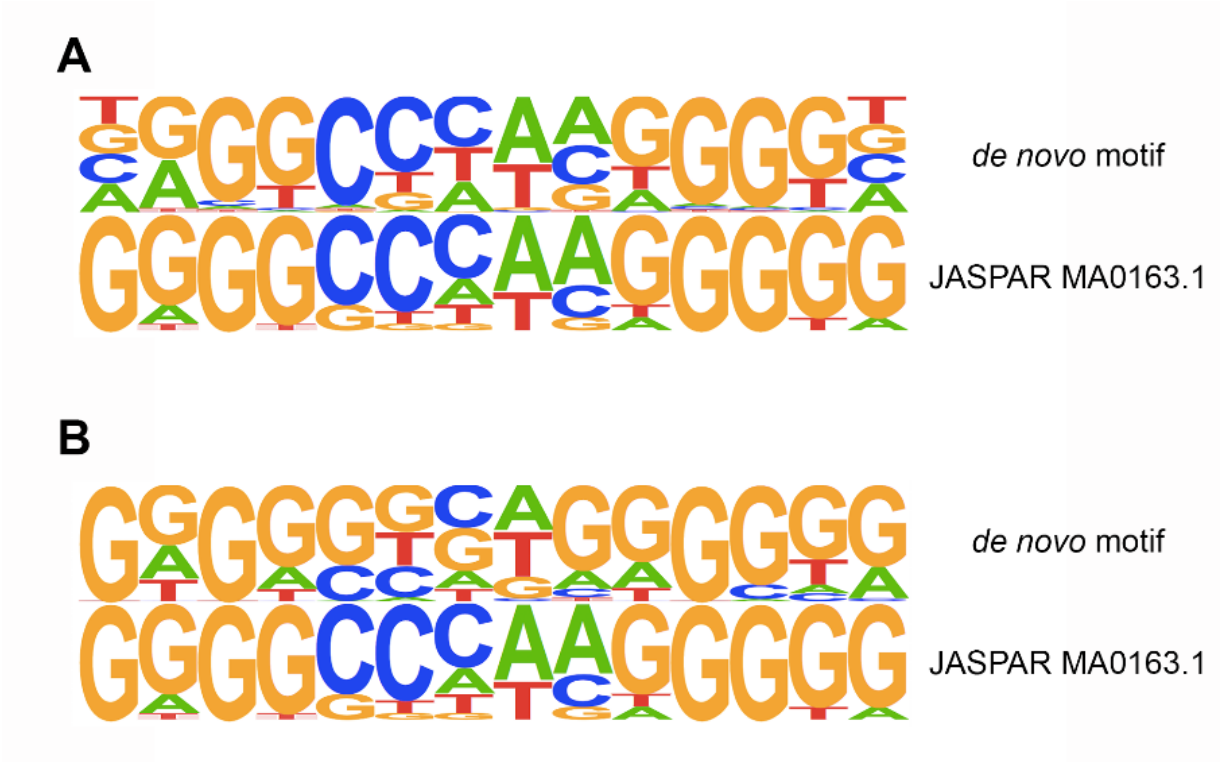

Figure 5.11. Optimised *de novo* PLAG1 binding sites compared to the PLAG1 binding motif from the JASPAR database (JASPAR MA0163.1). (A) *De novo* motif (*P*=0.01) from the dataset of downregulated genes in the epididymis from *Plag1* KO mice and WT controls. **(B)** *De novo* motif (*P*=0.0001) from the dataset of upregulated genes in the epididymis from *Plag1* KO mice compared to WT controls.

5.4 Discussion

As a number of sperm-related genes are dysregulated in the mouse epididymis in the absence of PLAG1, *in situ* hybridisation was used to both confirm the mRNA dysregulation of these genes and to characterise their expression patterns. Epididymal genes are often highly spatially regulated, but for many of the aforementioned genes, their spatial expression patterns in wild-type animals are still unknown. However, the results of this study show that *in situ* hybridisation has significant disadvantages when used in fragile tissue such as epididymal sections. Tissue sections of the epididymis are essentially tubule cross-sections, which are highly fragile. *In situ* hybridisation requires thin tissue sections in order for the probes to effectively penetrate the tissue and bind to the target sequence. Further, overnight incubation at high hybridisation temperatures (generally around 65–75°C) is required to produce specific staining while limiting background staining that can

mask signal. These high temperatures and the post-hybridisation stringency washes can degrade the thin, fragile tissue sections, resulting in poor tissue quality and difficulty obtaining high-quality, high-magnification microscopy images. Despite these limitations, the *in situ* hybridisation experiments produced specific signal that is clearly absent in the negative control tissue sections that were incubated with the sense riboprobe.

Sfrp2 is a gene involved in embryonic Wolffian duct development (Warr et al. 2009), but its role in the adult epididymis has not yet been reported. *In situ* hybridisation showed that *Sfrp2* mRNA was strongly expressed in cauda spermatozoa in *Plag1* WT mice, whereas minimal expression was observed in the cauda spermatozoa of *Plag1* KO mice. *Sfrp2* mRNA expression in cauda spermatozoa has not been reported previously. RNA-Seq showed that *Sfrp2* was downregulated in the *Plag1* KO epididymis (Wong et al. 2020) and indeed, overall decreased expression was observed in the *Plag1* KO epididymis by *in situ* hybridisation. The expression of *Sfrp2* in the WT mouse epididymis suggests this gene plays some role in the adult epididymis, and strong expression on the spermatozoa in the cauda epididymidis suggests a role in the preservation of mature spermatozoa or in oocyte fertilisation. Further studies on the function of *Sfrp2* are required to understand its role, which may then indicate whether the reduction in *Sfrp2* expression in the epididymis of *Plag1* KOs may contribute to the reduction in sperm motility and fertility phenotype of these mice.

Pebp1 is a PLAG1 target gene shown to downregulated in the *Plag1* KO epididymis transcriptome compared to WT controls (Wong et al. 2020). *In situ* hybridisation indicates that *Pebp1* mRNA expression in *Plag1* KO mice was decreased in the caput and cauda but increased in the corpus epididymidis compared to WT controls. *Pebp1* encodes a decapacitation factor, which binds to spermatozoa to prevent premature sperm capacitation, a process that must be preserved for just prior to fertilisation (Gibbons, Adeoya-Osiguwa & Fraser 2005). If premature capacitation occurs, spermatozoa are essentially redundant by the time they reach the ovum. Changes to *Pebp1* expression in the epididymis of *Plag1* KO mice may result in premature sperm capacitation, which would ultimately result in non-functional spermatozoa.

Bsph2 is another PLAG1 target gene previously shown to be downregulated in the *Plag1* KO epididymis transcriptome (Wong et al. 2020). *Bsph2* was decreased in the corpus of the *Plag1* KO epididymis in comparison to WT controls, as shown by *in situ* hybridisation. While the function of BSPH2 is not well understood, it has been demonstrated that the protein is modified during sperm capacitation, indicating that BSPH2 may play a role during capacitation (Plante, Fan & Manjunath 2014). Similarly to *Pebp1*, the altered expression pattern of *Bsph2* may result in premature sperm capacitation. Sperm capacitation analysis on mature sperm from *Plag1* KO mice may indicate whether this process is occurring prematurely in these mice.

In situ hybridisation showed that *Acrv1* mRNA expression was stronger in the caput and corpus of the *Plag1* KO mice compared to WT controls, while expression was similar between genotypes in the cauda. *Acrv1* expression in the *Plag1* WT mice increased from the caput to the cauda, which is in agreement with previously reported findings using reverse transcription PCR (Turunen et al. 2011). The increased expression of *Acrv1* in the caput and corpus epididymidis in *Plag1* KO mice may explain the upregulation of *Acrv1* gene expression previously shown by whole-epididymis RNA-Seq (Wong et al. 2020). In *Plag1* KO mice, expression decreases from caput to cauda. *Acrv1* is known to be involved in sperm–oolemma binding in humans (Margalit et al. 2012). Presumably, *Acrv1* expression is more likely to be required in the distal cauda epididymidis, where mature spermatozoa are stored prior to events that occur once they reach the female reproductive tract, including sperm capacitation and sperm–oocyte binding. Further analysis on mature spermatozoa from *Plag1* KO mice is required to understand whether the altered expression pattern of *Acrv1* in the epididymis of *Plag1* KO mice may affect sperm function.

The *in situ* hybridisation signal for *Smox,* a gene known to be involved in spermine prevention of the acrosome reaction in bovine species (Rubinstein & Breitbart 1991), was minimal and no definitive observations could be made regarding the expression pattern. Indeed, RNA-Seq indicated that *Smox* is expressed at low levels in the mouse epididymis (Wong et al. 2020).

Altered spatial gene expression patterns would presumably result in the loss of the specific order or amount of proteins secreted into the epididymis lumen and therefore change in the order of

modifications to the maturing spermatozoa. This is likely to affect sperm maturation and may contribute to the infertility and sperm motility defects observed in *Plag1* KO mice. In summary, the expression patterns of *Acrv1*, *Pebp1* and *Bsph2* were clearly altered in *Plag1* KO mice compared to WT controls; however, confirmation at the protein level would be worthwhile, given the limitations of *in situ* hybridisation on fragile epididymis tissues.

In addition to the dysregulation of several sperm maturation genes, previous transcriptomic analysis showed that the gene expression of several ECM components and their inhibitors were dysregulated (Wong et al. 2020). In the present study, we confirmed the dysregulation of a selection of these ECM genes at the protein level, as gene expression determined by RNA-Seq does not always reflect actual protein expression. The results show that several important ECM components are dysregulated in the epididymis of *Plag1* KO mice, which suggests that there is an imbalance between ECM deposition and degradation leading to matrix stiffness. The ECM is the molecular structural scaffolding that provides physical and biochemical support to the cells that it surrounds (Frantz, Stewart & Weaver 2010), and is composed of an array of macromolecules, largely categorised into proteoglycans, glycoproteins and fibrous proteins. The proteoglycans (*e.g.* decorin, versican) play a role in the hydration and buffering of the ECM, while fibrous proteins (eg. collagens, laminin) provide tensile strength and regulate cell adhesion, migration and other cellular processes (Kim, Turnbull & Guimond 2011).

Collagen is the most abundant protein and predominant structural element in the ECM (Frantz, Stewart & Weaver 2010), and increased collagen density and collagen turnover are strong indicators of ECM stiffness (Ng & Brugge 2009). Total collagen was significantly increased in the cauda epididymidis of *Plag1* KO mice. Laminin, which is the predominant non-collagenous glycoprotein found in the basement membrane (Mak & Mei 2017), was significantly increased in the caput, corpus and cauda epididymidis of *Plag1* KO mice. Laminin molecules bind to collagen molecules and other basement membrane molecules, as well as other laminin molecules, to help form the architecture of the basement membrane and facilitate basement membrane functions, including the regulation of cell movement and tissue maintenance (Colognato & Yurchenco 2000; Karsdal 2019). The formation of the laminin network in the basement membrane is also crucial for

establishing and maintaining the organisation and polarity of the epithelium (Devergne et al. 2014; Matlin, Myllymäki & Manninen 2017). The polarised architecture of the epithelium allows the formation of the apical and basal membranes, which is required for normal cellular functions and cell signalling. However, to further determine whether polarity is affected, specific markers of cell polarity such as PARD3 and SCRIB should be used for immunofluorescent staining.

Furthermore, inside the lumen of the corpus and cauda tubules in *Plag1* KO mice, laminin appears to be present on some cells. These laminin-positive cells have large, round nuclei, indicating that these cells are not sperm cells, which have a characteristic sickle-shaped nucleus. The laminin-positive cells in the tubule lumen may be immature spermatocytes or spermatids that have not undergone proper sperm maturation, as it was previously shown that spermatocytes were prematurely shed in the testes in *Plag1* KO mice (Juma et al. 2017). However, if this were the case, it is unclear as to why these cells do not appear in the caput epididymidis. Another possibility is that they are epithelial cells that have been shed into the tubule lumen. Further studies, such as double-immunohistochemistry using both laminin and epithelial cell markers (epithelial membrane antigen, E-cadherin, EpCam or pan-cytokeratin) or laminin immunofluorescent staining of sperm smears, are required to confirm the identity of these laminin-positive cells.

While ECM defects are largely not investigated in mouse models of aberrant epididymis development, laminin was analysed in *Lgr4* KO mice (Hoshii et al. 2007). *Lgr4* KO mice are infertile, exhibit increased lamination of the postnatal caput epididymidis basement membrane and decreased epididymal tubule elongation (Hoshii et al. 2007). *Lgr4* was not dysregulated in the epididymis of *Plag1* KO mice compared to WT controls (Wong et al. 2020), indicating that PLAG1 deficiency does not affect *Lgr4* expression and the phenotype of the epididymis from *Plag1* KO mice is unlikely to be a result of *Lgr4* downregulation. Instead, PLAG1 and LGR4 may converge on a common pathway that plays a role in regulating ECM genes (including laminin genes), though this has not been reported in the literature so far. The increase in collagen in the cauda, and the increase in laminin in the epididymis of *Plag1* KO mice, provide confirmation that these ECM components are overexpressed in the epididymides from *Plag1* KO mice, as suggested by RNA-Seq data (Wong et al.

2020). The over-deposition of collagen and laminin indicates that ECM homeostasis may be disrupted, and that the ECM may be excessively stiff in the epididymis of *Plag1* KO mice.

The ECM undergoes constant remodelling in order to maintain the dynamic scaffolding that is capable of resisting intrinsic mechanical forces. The balance between matrix metalloproteinases (MMPs) that degrade components of the ECM and tissue inhibitors of MMPs (TIMPS) that control MMP activity is crucial for maintaining ECM homeostasis (Arpino, Brock & Gill 2015; Bonnans, Chou & Werb 2014). MMPs also release growth factors, since the ECM acts as an enzymatically accessible harbour for growth factors by binding them within its molecular structure (Frantz, Stewart & Weaver 2010; Lu et al. 2011). Proteoglycans play a crucial role in protecting the growth factors from untimely degradation by proteases (Elfenbein & Simons 2010), while MMPs cleave peptide bonds to release them (Yue 2014). By binding growth factors, the ECM controls the availability of growth factors for binding to their respective receptors, contributing to the establishment of growth factor concentration gradients (Lu et al. 2011).

In *Plag1* KO mouse epididymides, the α-subunit of the metalloprotease meprin A, MEP1α, showed decreased expression in the cauda epididymidis compared to WT controls. The two meprin subunits (α and β) form homo- or heterodimers and are able to degrade a diverse range of ECM substrates, including type IV collagen and laminin (Kruse et al. 2004). The α-subunit, but not the β-subunit, contains an I domain insert that enables proteolytic cleavage, facilitating its secretion into the extracellular space (Marchand et al. 1995). MEP1α is constitutively secreted in the extracellular space where as an activated soluble protease, it can cleave ECM substrates (Arnold, Otte & Becker-Pauly 2017). In *Plag1* KO mice, stromal expression of MEP1α was dramatically decreased compared to WT controls. Meprin α is known to be downregulated in the kidneys of diabetic rats; through their role in the proteolytic degradation of ECM collagen and other ECM components, meprins may have a protective role against diabetic nephropathy, in which one of the key pathological changes is excessive ECM deposition (Mathew et al. 2005). However, the role of meprin α in ECM remodelling and maintenance is not yet clear, as studies have shown that meprin α is also involved in collagen fibril assembly via its ability to process procollagens to their mature form, and that increased meprin α expression is associated with a fibrotic phenotype in human skin (Broder et al. 2013; Kronenberg et

al. 2010). The role of meprins may be dynamic, in that they may degrade ECM components to inhibit over-deposition in some conditions, and may facilitate collagen fibre deposition in others, depending on the microenvironment or tissue type. In *Plag1* KO mice, the significant decrease of MEP1α may lead to decreased proteolytic activity and may ultimately contribute to the increase in collagen and laminin in the epididymal ECM.

In the epididymis of *Plag1* KO mice, MME showed increased expression in the corpus and cauda compared to WT controls. In addition to its role as a protease, MME also regulates cell behaviours and remodelling of the microenvironment (Maguer-Satta, Besançon & Bachelard-Cascales 2011). The upregulation of MME in the corpus and cauda epididymidis of *Plag1* KOs mice may be a compensatory mechanism to counteract the decrease in meprin activity and/or the possible increase in matrix stiffness caused by overexpression of collagen and laminin. It is also possible that MME is upregulated due to an increased requirement for one or both of these regulatory roles.

Interestingly, TIMP2, an inhibitor of metalloproteinases, was increased in all main regions of the epididymides of *Plag1* KO mice, indicating that MMP activity may be inappropriately inhibited in the epididymis, leading to decreased MMP degradation of ECM proteins. All TIMPs are able to inhibit all known MMPs, albeit at varying efficacies (Arpino, Brock & Gill 2015). Increased TIMP2 expression has been identified in a number of pathologies where protection of the ECM from proteolysis is key in its progression, including Dupuytren's contracture (Ratajczak-Wielgomas et al. 2012), hepatic fibrosis (Nie et al. 2004) and cardiac fibrosis (Heymans et al. 2005). It is possible that increased TIMP2 activity may result in the inhibition of MMP activity without a decrease in the mRNA or protein expression of the MMP; this would result in decreased MMP activity that would not be indicated by mRNA or protein quantification analysis, in addition to the decrease in genes encoding MMPs previously shown by RNA-Seq. It should also be noted that in some studies, decreased expression of various TIMPs has been associated with increased deposition of ECM components, suggesting that, like meprins, TIMP activity in ECM homeostasis may be dependent on the microenvironment or tissue type (Arpino, Brock & Gill 2015). Altogether, our results suggest that there is an imbalance between MMP and TIMP activity, potentially leading to insufficient

degradation of overly abundant ECM components such as collagen and laminin and ultimately to ECM stiffness, in the epididymides of *Plag1* KO mice.

ECM stiffness resulting from an imbalance between ECM deposition and degradation, and the subsequent mechanical stress, can induce aberrant EMT (Frantz, Stewart & Weaver 2010). Therefore, we sought to determine if EMT was contributing to the increased density of stromal cells in the epididymis from *Plag1* KO mice that was previously reported (Wong et al. 2020). EMT is characterised by the loss of E-cadherin expression in the epithelium; E-cadherin is a crucial component of cell–cell adhesion junctions in epithelia, the expression of which ceases when cells undergo EMT (Kang & Massagué 2004). This allows E-cadherin to be used as a marker for EMT. The E-cadherin analysis indicated that EMT is not increased in the epididymides of *Plag1* KO mice, which may imply that the increase in stromal cells is, at least in the cauda, a result of proliferating stromal cells, as previously indicated by the increase in Ki67-positive cells (Wong et al. 2020), rather than epithelial cells transitioning into stromal cells. Given that the density of stromal cells was increased in the caput and corpus epididymidis in *Plag1* KO mice, but neither Ki67 nor E-cadherin were differentially expressed in these regions, the underlying cause of the increased stromal cellular density remains unclear. It is possible that cellular turnover is aberrant, a process which is in part regulated by the ECM through both physical cues and signalling pathways (Pellettieri & Alvarado 2007), resulting in cell death not occurring as it should.

Over-deposition of ECM components and the dysregulation of ECM remodelling leads to increased ECM stiffness and can ultimately lead to fibrosis and fibrotic diseases. For example, pancreatic fibrosis is a fundamental factor in the progression of several pancreatic diseases, including type 2 diabetes mellitus and chronic pancreatitis (Blaine et al. 2009). Tissue injury leading to liver fibrosis is associated with the progression of cirrhosis, liver failure and portal hypertension (Bataller & Brenner 2005). Interestingly, it appears that liver ECM stiffness precedes fibrosis (Georges et al. 2007). Indeed, ECM proteins are an emerging biomarker of fibrosis in the kidney (Genovese et al. 2014), lung (Leeming et al. 2012) and liver (Leeming et al. 2013). ECM stiffness is also a crucial factor in the progression of cancer and tumour invasion (Gkretsi & Stylianopoulos 2018; Najafi, Farhood & Mortezaee 2019; Seewaldt 2014). While ECM defects and imbalances between ECM degradation and

deposition have been identified in the pathology of disease in other organs, it has not yet been characterised in epididymal pathology or in male infertility. In mouse models of male infertility and epididymal defects, the ECM is generally not analysed or discussed. In *Plag1* KO mice, it is currently unclear whether ECM defects are established during embryonic development and contribute to the aberrant morphology of the epididymis, or if these defects begin post puberty and affect epididymis function.

In this chapter, I investigated the expression pattern of selected dysregulated sperm maturation-related genes, showing that PLAG1 influences the spatial expression pattern of some genes in the epididymis and further confirming the dysregulation of those genes. Given that gene expression occurs in a very specific pattern in the epididymis, altered expression patterns of sperm maturation genes may affect sperm maturation. Future studies analysing the functional capabilities of mature spermatozoa from *Plag1* KO mice, such as the acrosome reaction, or further characterisation of the ECM structure in epididymides from *Plag1* KO mice may be insightful. The results of the analyses of collagen and other ECM components indicate that PLAG1 may be involved in the regulation of ECM homeostasis. However, as a previous study showed that there was no significant enrichment of PLAG1 binding sites and this study indicated that there are no *de novo* PLAG1 binding motifs in the dysregulated genes in the epididymis of *Plag1* KO mice, PLAG1 control of these genes may be upstream or PLAG1 may control or act in concert with other transcription factors, as discussed previously (Wong et al. 2020).

In summary, studies involving models of male infertility typically do not consider the ECM when characterising the epididymal phenotype, despite the widespread roles of the ECM in epithelial function and maintenance, in cell behaviours such as cell movement, differentiation and shape, in the regulation of tissue homeostasis and in signalling mechanisms. In *Plag1* KO mice, the alterations to the normal ECM may be correlated to the epididymal dysfunction and infertility exhibited by these mice. ECM defects were generally more severe in the cauda epididymidis of *Plag1* KO mice, which may have implications for the proper storage of mature sperm and is accompanied by the dysregulation of genes involved in the prevention of premature sperm capacitation. Nevertheless, the possibility that the ECM defects observed are simply a remnant from embryonic morphogenesis

that persists throughout adulthood and that they have no effect on the function of the adult epididymis, cannot be discarded. However, as the ECM is highly involved in signalling, the extensive ECM defects are likely to impact adult epididymis function in some way. ECM defects are unlikely to be obvious in basic histology stains such as haematoxylin and eosin staining, so phenotyping of mouse models of male infertility may benefit from instead using histological techniques that also stain collagens, such as Masson's trichrome stain. Further, the ECM of other tissues where PLAG1 is expressed, such as the brain, have not yet been examined; whether the ECM in other organs of *Plag1* KO mice show ECM stiffness or if it is an epididymis-specific defect remains to be investigated. Evidently, the ECM may play a more significant role in epididymal function than previously considered and investigation of potential ECM disruptions in other mouse models of infertility may lead to uncovering an underlying mechanism of clinical male infertility.

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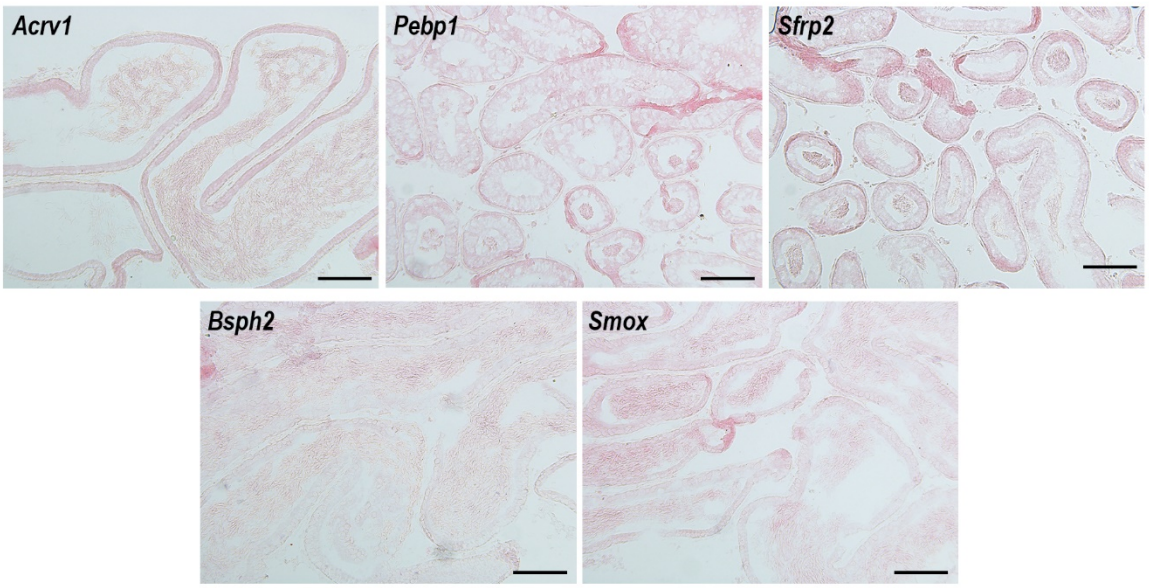
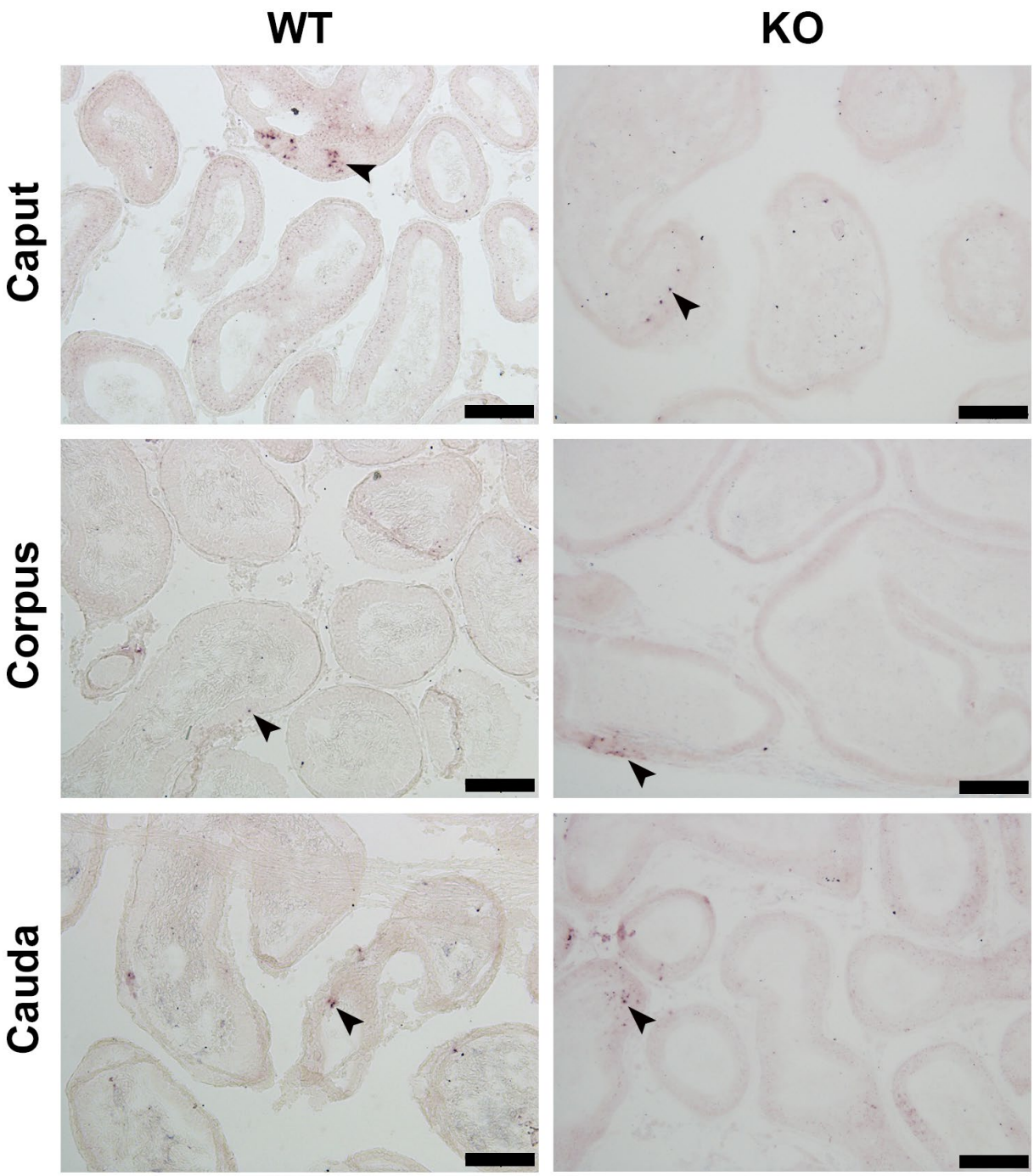
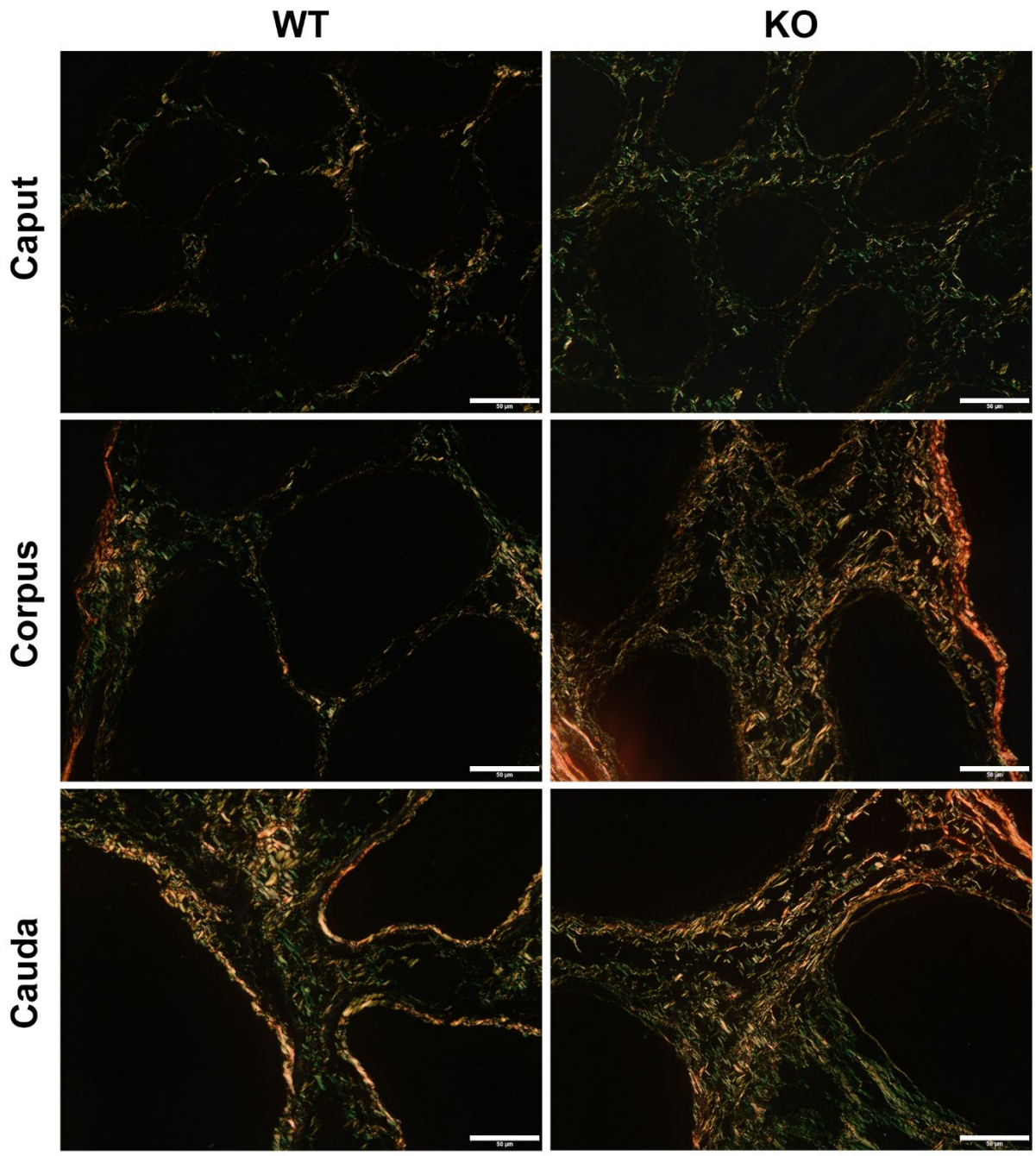
Supplementary data



Supplementary Figure 5.1. Negative control sections for *in situ* hybridisation, incubated in hybridisation buffer containing sense riboprobe. Scale bars represent 100 μm



Supplementary Figure 5.2. Spatial expression pattern of *Smox* mRNA in the epididymis from *Plag1* wild-type (WT) and knockout (KO) mice as determined by *in situ* hybridisation. Arrowheads indicate *in situ* hybridisation signal. Scale bars represent 100 μm.



Supplementary Figure 5.3. Picro-Sirius red staining under polarised light in the epididymis of *Plag1* wild-type (WT) and knockout (KO) mice. Polarised light distinguishes collagen types: type I collagen (yellow/orange birefringence) and type III collagen (green birefringence).

Supplementary Table 5.1. Primers used for riboprobe synthesis and probe incubation temperatures used for *in situ* hybridisation.

Gene	NCBI ascension number	Amplicon length	Primer sequence (5'→3')	Hybridisation temperature
Acrv1	NM_007391	695 bp	Forward: GCTTGGATCTGCCCAAGGAGCA	67°C
			Reverse: GAGAGGTTCATTCCGACAGCAC	
Pebp1	NM_018858	512 bp	Forward: ATGGCCGCCGACATCAGCC	73°C
			Reverse: AGCTGCTCGTACAGCTTGGG	
Sfrp2	NM_009144	805 bp	Forward: CTAGTCCTCGCCTCGCACTG	70°C
			Reverse: TTGCGGATGCTGCGGGAGAT	
Bsph2	NM_001080942	341 bp	Forward: GGAAGTGATGAGCCATCTTGTG	63°C
			Reverse: GTTAGGAGAACATTGCTTCCAT	
Smox	NM_001177835	804 bp	Forward: CTGCAGCTAGAGCCCTTCTG	70°C
			Reverse: AATCCTCGCACTCCACGACTAC	

Supplementary Table 5.2. Antibodies and dilutions used for immunofluorescence.

Antibody target	Antibody type	Manufacturer and catalogue number	Dilution
Ki67	Rabbit polyclonal	Abcam (ab15580)	1:500
Laminin	Rabbit polyclonal	Abcam (ab11575)	1:250
CD10	Rabbit polyclonal	Life Technologies (PA5-85875)	1:100
E-cadherin	Rabbit polyclonal	Abcam (ab15148)	1:25
TIMP2	Rabbit polyclonal	Abcam (ab180630)	1:100
MEP1α	Rabbit polyclonal	Abcam (ab232892)	1:100

Chapter 6. General discussion

Proper development of the epididymis is crucial for the production of viable spermatozoa capable of motility and fertilisation. Mice with a deficiency in pleomorphic adenoma gene 1 (PLAG1) exhibit decreased sperm motility and decreased progressive motility (Juma et al. 2017). These mice also exhibit premature sloughing of spermatocytes and germinal epithelium, and decreased relative testis weight, which is likely to be a major causative factor of the decreased daily sperm production seen in these mice (Juma et al. 2017). However, as sperm motility is gained during epididymal sperm maturation, the dramatically reduced sperm motility in *Plag1* knockout (KO) mice may be a result of defective epididymal sperm maturation. Furthermore, even though there is still a fraction of sperm showing normal motility in *Plag1* KO mice (Juma et al. 2017), our breeding analysis indicated that *Plag1* KO males are completely infertile, suggesting that there may be other aspects of sperm maturation that are affected in the absence of PLAG1. Therefore, it is reasonable to suspect that epididymis function is affected in *Plag1* KO mice. It was hypothesised that the underlying cause of sperm motility issues in *Plag1* KO mice may be in part due to abnormal epididymis structure and function. The work in this thesis aimed to investigate the role of *Plag1* in the mouse epididymis by characterising PLAG1 expression, analysing the effect of *Plag1* deficiency on epididymis morphology and, given that sperm maturation is facilitated by segment-specific gene expression, by comparing the epididymal transcriptome of *Plag1* KO mice to WT controls. We also investigated the extracellular matrix (ECM) in the epididymides from *Plag1* KO mice, since the transcriptome analysis revealed that ECM genes were dysregulated. Key findings from these studies include the aberrant morphology of the epididymis of *Plag1* KO mice, the dysregulation of several sperm maturation genes and genes encoding decapacitation factors, and the potential imbalance between ECM deposition and degradation, which may be indicative of impaired tissue homeostasis **(Figure 6.1)**.

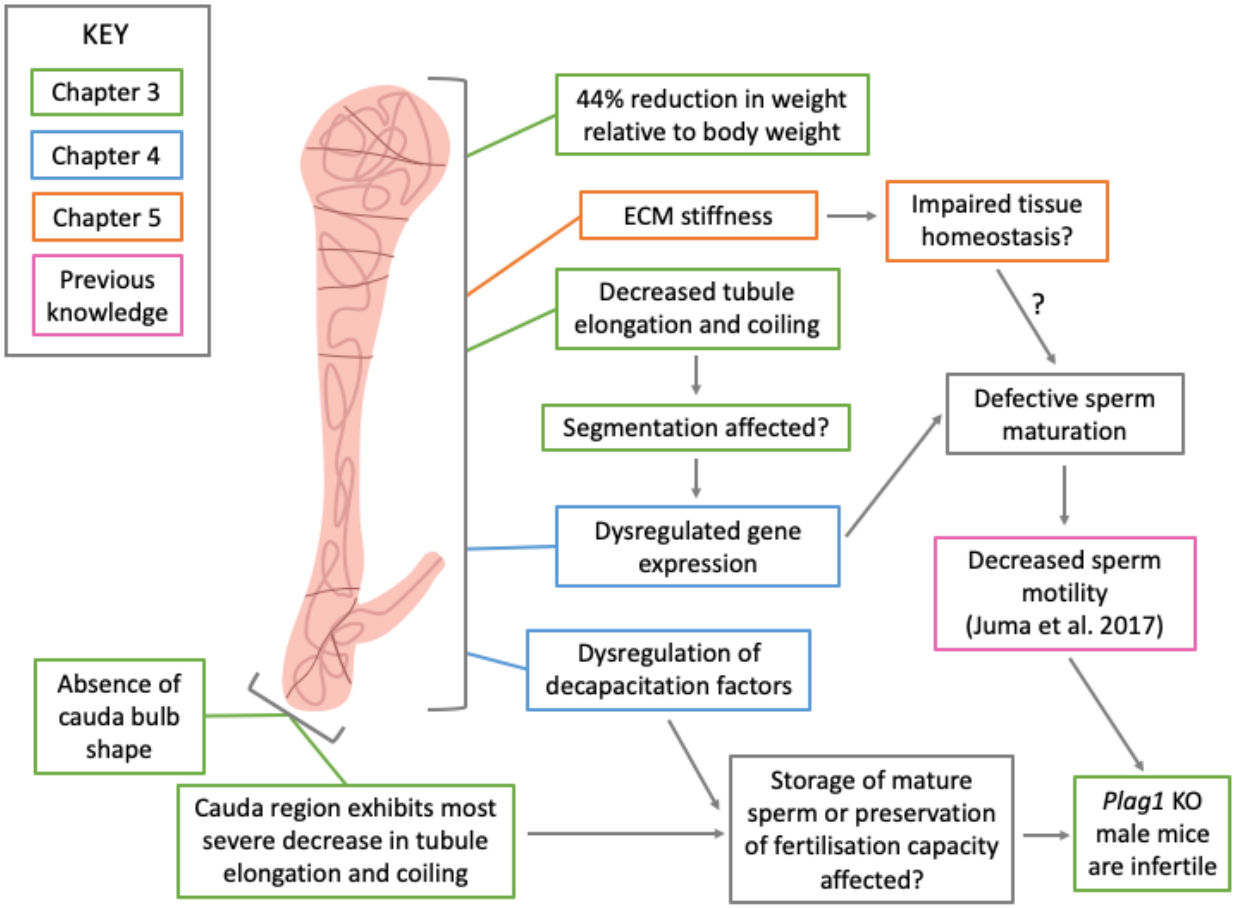


Figure 6.1. Overview of epididymal defects and fertility phenotype of *Plag1* knockout male mice. Schematic diagram of the findings from this thesis and possible interactions, potential consequences for epididymis function, and how epididymis defects in *Plag1* KO mice may theoretically lead to decreased sperm motility and infertility.

6.1 *Plag1* in the mouse epididymis

6.1.1 *Plag1* KO mice are infertile and PLAG1-deficient mice exhibit aberrant epididymal morphology

Hensen et al. (2004) generated a *Plag1* KO mouse line and reported that males were capable of producing litters, albeit small and infrequent. However, the parameters of the breeding analysis conducted were not specified and in our colony of mice, numerous matings over several years using *Plag1* KO males never resulted in any litters. Therefore, it was clear that a breeding analysis with clear parameters including a narrowly defined age range and assessment of copulatory

plugs, as outlined by Borg *et al*. (2010), was required. The results from the breeding analysis conducted in **Chapter 3** were contradictory to those previously reported (Hensen et al. 2004); our results indicate that *Plag1* KO males are fully infertile, while *Plag1* heterozygous (HET) mice are not subfertile. The discrepancy in results could be due to differences in housing and breeding paradigms. The finding that *Plag1* HET male mice have normal fertility correlates with the finding that there are not any significantly differentially expressed genes in the epididymis from *Plag1* HET compared to *Plag1* WT mice **(Chapter 4)**. However, *Plag1* HET mice have an intermediate epididymal phenotype, as they exhibit significantly reduced epididymal coiling compared to WT controls **(Chapter 3)**. It is possible that while gene expression is not significantly different in *Plag1* HET mice compared to WT controls, the statistically non-significant changes in gene expression do have an effect on the morphology of the epididymis in *Plag1* HET mice. However, these structural aberrations are evidently not severe enough to significantly affect fertility in these mice, as shown by the breeding analysis **(Chapter 3)**.

It should be noted that while the same mouse strain is used in our studies and in that of Hensen et al. (2004), the discrepancies in fertility analyses results may be influenced by genetic changes that have occurred after several more generations of breeding in our colony. The *Plag1* KO mouse strain was generated via a *Plag1*-targeting vector used to replace the entire open reading frame of *Plag1* with the lacZ reporter gene in murine R1 embryonic stem cells from 129/Sv x 129/Sv-CP embryos (Hensen et al. 2004). Chimeric offspring were generated by injecting successfully transfected stem cells into blastocysts of Swiss Webster mice, and were subsequently mated with Swiss Webster mice. One chimeric male transmitted the targeted allele, thus generating *Plag1* heterozygous mice which were then used to breed *Plag1* mice for the breeding analyses (Hensen et al. 2004). Mice from our colony used for fertility analysis were inbred for several more years, and in essence, are more inbred than the mice used by *Hensen et al.* (2004).

In addition to contradicting breeding analysis results, there have previously been contradicting data regarding *Plag1* expression in the brain and liver (Hensen et al. 2004; Juma et al. 2018), indicating the need for confirmation of PLAG1 expression in mouse tissues. We have shown that PLAG1 is widely expressed in the adult mouse epididymis using X-gal staining **(Chapter 3)**. This

widespread expression indicates that PLAG1 may play a role in the adult epididymis, and the fact that expression signal becomes hazy and diffuse, and localised to the apical cytoplasm, suggests that PLAG1 may be secreted in the distal epididymis. Alternatively, transport of overabundant transcription factors to the cytoplasm for proteasome-mediated protein degradation is an established mechanism of regulating excessive transcription factor levels within a cell (Desterro, Rodriguez & Hay 2000). As previously mentioned, we have so far been unable to find an effective and specific anti-PLAG1 antibody. Ideally, PLAG1 protein expression can be more accurately determined and quantified when an antibody that produces genuine and specific staining becomes available.

The epididymis of *Plag1* KO mice exhibits aberrant gross morphology, is reduced in weight relative to body size, and the epididymal tubule exhibits significantly reduced coiling and is less elongated **(Chapter 3)**. These findings indicate that PLAG1 is required for normal epididymis development and maintenance. As PLAG1 is known to be a regulator of early development via its action of cell proliferation genes, it is likely that PLAG1 does affect embryonic Wolffian duct development, and possibly even earlier morphogenesis during development of the urogenital ridge. It should be noted, however, that at birth the epididymis is not fully developed (in both WT and *Plag1* KO mice) and undergoes extensive duct elongation and coiling in early postnatal development (Joseph, Yao & Hinton 2009). Furthermore, structural and cellular differentiation also does not occur until puberty. The morphological defects observed in *Plag1 KO* mice are most severe in the cauda, which is region that is undergoes much of its elongation and coiling during early postnatal development. Thus, given that duct elongation and coiling in the cauda occurs largely during postnatal development, that the reduction in coiling and elongation is most severe in the cauda region and that the cauda lacks the characteristic bulbous shape in *Plag1* KO mice, it can be speculated that PLAG1 plays a significant role in early postnatal development of the mouse epididymis. Comparison of Wolffian duct and epididymis morphology at regular time points during embryonic and postnatal stages may provide some insight into which stages of epididymis development are affected by PLAG1 deficiency.

During dissections of epididymides it was unexpectedly found that the left epididymis and testis was absent in a large number of *Plag1* KO mice, and was never observed in any *Plag1* WT or

HET mice. This condition, termed unilateral anorchidism, was found to be significantly associated with the *Plag1* KO genotype **(Chapter 3)**. There have not been any other reports of associations between anorchidism and PLAG1 mutations or variations. However, it may be worth analysing whether PLAG1 mutations or polymorphisms exist in patients with unilateral anorchidism. Since there are no other obvious phenotypic abnormalities relating to left–right asymmetry in *Plag1* KO mice, the unilateral anorchidism is more likely a result of adverse vascular events during gestation, the typical cause of clinical anorchidism, as discussed in **Chapter 3**. Indeed, microarray analyses have previously shown that PLAG1 upregulates vascular endothelial growth factor (VEGF) and placental growth factors (both of which are strong mitogens for endothelial cells) and ephrin B1 (which induces angiogenic sprouting), indicating that PLAG1 may be involved in the regulation of vasculogenesis and angiogenesis (Voz et al. 2004). Furthermore, preliminary studies in our laboratory indicate that there may be vascular abnormalities in the brain of *Plag1* KO mice (J. Wong and G. Gasperoni, unpublished results). The role of PLAG1 in vasculogenesis in the brain and other organs in *Plag1* KO mice requires further investigation.

6.1.2 PLAG1 regulates epididymal target genes indirectly

We hypothesised that altered gene expression in the epididymis may contribute to the sperm motility defects observed in *Plag1* KO mice, since the biochemical and structural modifications that occur during sperm maturation are dependent on the tightly regulated expression of epididymal genes. The transcriptome analysis **(Chapter 4)** revealed the dysregulation of several sperm maturation genes and genes encoding decapacitation factors in the epididymis of *Plag1* KO mice compared to WT controls. Whether or not the dysregulation of these genes has a functional effect on the spermatozoa remains to be confirmed using *in vitro* fertilisation to assess the fertilisation capacity of spermatozoa from these mice. It should be noted that not all genes involved in sperm maturation, the acrosome reaction and sperm capacitation have been identified as yet. RNA sequencing also revealed the dysregulation of several predicted genes (eg. *Gm5737*, *Gm41125*) which are computationally predicted genes, with no known functions. There may be many genes with epididymal or sperm maturation functions that have not been identified thus far. Furthermore,

our PLAG1 motif analyses indicated that PLAG1 regulation of target genes, including sperm maturation genes, in the mouse epididymis is most likely indirect. A more precise understanding of PLAG1 targets upstream from the target genes identified by RNA sequencing is required, or PLAG1 interaction with other transcription factors can be investigated.

In mouse embryos, significant enrichment of PLAG1 binding sites was revealed in zygotic genome activation genes (Madissoon et al. 2019). However, the PLAG1 binding motif scanning analysis performed on dysregulated genes in the epididymis from *Plag1* KO mice compared to WT controls indicated that there was no significant enrichment of PLAG1 binding sites in this case. As discussed in **Chapter 4**, these results imply that in the epididymis, PLAG1 regulation of target genes is upstream or indirect, meaning that PLAG1 may regulate genes or other transcription factors that in turn regulate these target genes. Indeed, the RNA sequencing data showed that several transcription factors are downregulated (for example *Gata3, Zfp13, Hlf, Hoxd9*) or upregulated (for example *Wt1, Ets1, Foxo1, Hoxa5*) in the epididymides from *Plag1* KO mice **(Chapter 4)**, indicating that PLAG1 may indirectly regulate the expression of these transcription factors. Further analysis of PLAG1 binding to target genes using ChIP assays would be valuable for showing direct binding, although this will only be possible when a functional, specific anti-PLAG1 antibody becomes available. The possibility of *de novo* binding motifs in the promoter regions of epididymal target genes that would be identified by known PLAG1 motif scanning studies was also considered (**Chapter 5**). However, motif optimisation analysis looking for *de novo* motifs similar to known PLAG1 motifs did not reveal any convincing *de novo* binding motif candidates, nor were any other transcription factor binding sites significantly enriched within the promoters of the target genes. Taken together, it can be inferred that, unlike mouse embryo zygotic genome activation genes that are thought to be directly regulated PLAG1, PLAG1 regulation of target genes in the mouse epididymis is not likely to be facilitated by direct PLAG1 binding to their promoter regions.

It is also possible that PLAG1 acts in concert with other transcription factors; if this were the case, PLAG1 motifs would not be indicative of PLAG1 action. It is common for transcription factors to form homodimers, heterodimers and oligomers, which results in gene regulation occurring at DNA sites different from the consensus site for a particular transcription factor and in transcription factors

having different (sometimes opposing) roles in different cell and tissue types (Amoutzias et al. 2008; Matthews & Sunde 2012; Toivonen et al. 2018). If PLAG1 did indeed form dimers with itself or other transcription factors, including with family members PLAG2 or PLAGL1, current motif scanning or *de novo* motif discovery analyses such as those performed in this study, would not be able to identify dimeric binding sites that are different from the known PLAG1 binding motifs, nor known binding sites of partner transcription factors. Interactions between transcription factors are complex, but emerging computational tools (Toivonen et al. 2018) may be a useful first step in identifying protentional transcription factor binding partners of PLAG1, which could then be validated using *in vitro* protein interaction assays such as co-immunoprecipitation-mass-spectrometry and pull-down assays.

Further evidence that PLAG1 regulates epididymal genes was provided in **Chapter 5**, in which altered gene expression of sperm maturation related genes (*Acvr1*, *Pebp1*, *Bsph2)* were shown by *in situ* hybridisation. Although this analysis provides an indication of the spatial expression patterns of these genes in the main epididymis regions, segment-specific expression cannot be confirmed due to the aberrant morphology of the epididymis of *Plag1* KO mice. The significantly reduced coiling and reduction in epididymal tubule length is very likely to result in altered segment identity, as previously discussed **(Chapter 3)**. To provide the most accurate possible representation of the main epididymis regions in the *in situ* hybridisation staining in **Chapter 5**, microscopic images were taken in the centre of the region, and the areas likely to be bordering two regions were avoided.

6.1.3 ECM defects in the epididymis of *Plag1* KO mice: a new avenue for epididymis research?

As mentioned in **Chapter 5**, it may be worthwhile for researchers to include histology techniques that stain collagen, such as Picro-Sirius red or Masson's trichrome staining, when phenotyping of rodent models of male infertility, in addition to haematoxylin and eosin staining. This would provide the opportunity to examine ECM abnormalities in addition to epithelium aberrations. The general role of the ECM in acting as a harbour for growth factors and in regulating various cell behaviours are established roles of the ECM across various tissues (Frantz, Stewart & Weaver 2010); however,

additional roles of the ECM specifically in epididymis function have not been investigated. Also, to the best of our knowledge, infertility phenotypes associated with fibrosis in the epididymis have not yet been reported in the literature. The analysis of ECM components in the epididymis of *Plag1* KO mice **(Chapter 5)** indicated that ECM homeostasis may be affected, and the ECM may be excessively stiff or fibrotic in this mouse model of male infertility (**Figure 6.2**). Currently, the ECM is generally not investigated in rodent models of male infertility; doing so may reveal other models with a similar epididymal phenotype and determine whether ECM defects may be a hallmark of epididymis dysfunction. However, it should be acknowledged that it is also possible that this ECM phenotype may be a secondary defect in *Plag1* KO mice, unrelated to fertility issues.

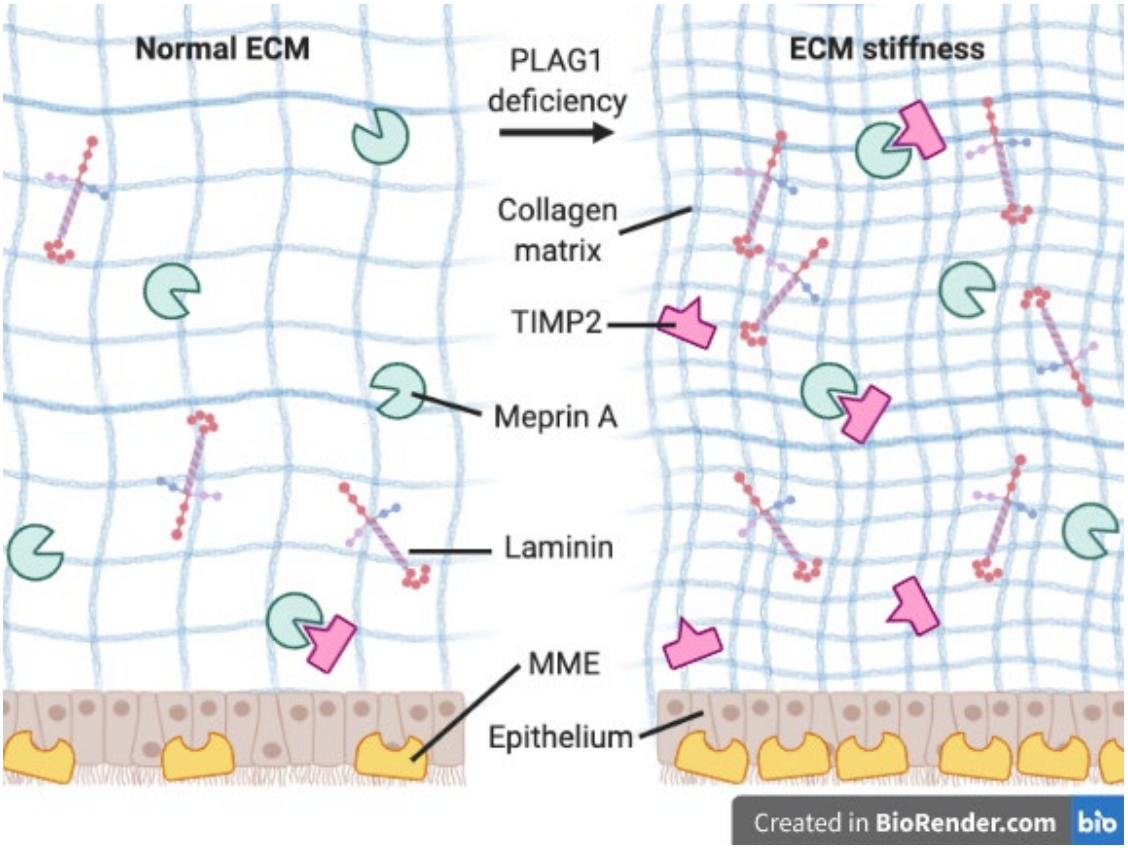


Figure 6.2. Schematic diagram of dysregulated extracellular matrix components in the cauda epididymidis of *Plag1* knockout mice. Increased or decreased expression of proteins in the extracellular matrix (ECM) of the cauda epididymidis of *Plag1* KO mice potentially leading to ECM stiffness, as shown by immunofluorescent staining in Chapter 5.

6.2 Summary and future perspectives

The findings in this thesis present new roles for *Plag1* in proper epididymal morphology and tubule elongation and in the maintenance of the epididymal ECM. The results also imply that PLAG1 may regulate sperm maturation genes and genes involved in the prevention of premature sperm capacitation, although this is indirect or in concert with other transcription factors. Further studies that investigate whether mature sperm from the *Plag1* KO cauda epididymidis are prematurely capacitated are required to directly address this hypothesis. There are several methods used to detect prematurely capacitated sperm, and different aspects of the process can be targeted to ensure broad detection (Ded et al. 2019). Rather than using cauda sperm only, it would perhaps be most useful to analyse sperm samples from several locations along the epididymis, as this would allow for the detection of premature sperm capacitation that could be occurring in the more proximal regions of the epididymis in *Plag1* KO mice. Also, PLAG1 supplementation in cultured epididymides and subsequent analysis of sperm motility to investigate whether the phenotype can be rescued by PLAG1 may be possible. However, given that epididymis function is a complex interaction between secreted testicular factors, secreted epididymal factors and cues from the surrounding ECM, *in vitro* studies may be insufficient in simulating these complex interactions that create the *in situ* microenvironment.

The lack of an effective anti-PLAG1 antibody presents some challenges in allowing further investigation of the possible secretion of PLAG1 into the epididymis tubule lumen in *Plag1* KO mice. Further, it is also possible that the protein structure changes during secretion, which may not allow detection by antibody binding. An alternative technique that may allow the tracking of the PLAG1 protein is fluorescent tagging. Protein labelling using small fluorescent tags would allow for the sub-cellular and extracellular localisation of PLAG1 with minimal interference with protein folding or disruption to its binding action (Lotze et al. 2016; Sahoo 2012; Thorn 2017).

It should also be noted that the dramatically reduced size of the epididymis presents some limitations. It is well established that the transcriptome is unique within individual, discrete segments of the epididymis. Therefore, RNA sequencing on epididymides separated into individual segments

rather than separated into main regions, would be most insightful. However, the epididymides from *Plag1* KO mice are extremely small, making accurate dissection of the organ into the 10 segments an unrealistic option. Single-cell RNA Sequencing (scRNA-seq) may be a more feasible method to analyse gene expression in specific segments. scRNA-seq could also be used to determine how many transcriptionally unique segments there are in the epididymis of *Plag1* KO mice, and whether this differs from the 10 segments found in the WT mouse epididymis. It is highly unlikely that segment identity in *Plag1* KO mice is the same as in WT mice, as suggested by the changes in gene expression patterns **(Chapter 5)**. While proteome sequencing of the epithelium or secretome sequencing of the epididymal luminal content could reveal dysregulation of sperm maturation factors, due to the dramatically reduced epididymis size in *Plag1* KO mice these studies would require a very large number of *Plag1* KO mice, which may not be feasible in most research settings.

Lastly, given that it is currently unknown whether the ECM defects in the epididymis of *Plag1* KO mice are established during embryonic development or if the ECM becomes progressively more stiff over time during early adult life, it would be worthwhile characterising the morphology of the Wolffian duct and epididymis from the early postnatal stages of development in *Plag1* KO mice. This would determine whether ECM defects originate during embryogenesis, or whether they only begin during postnatal development.

In conclusion, the data presented in **Chapters 3 to 5** have revealed new potential roles for PLAG1 in the epididymis. PLAG1 is widely expressed in the mouse epididymis and is required for normal epididymal morphology and tubule elongation and coiling (**Chapter 3**); however, the potential secretion of PLAG1 remains to be confirmed. PLAG1 also appears to indirectly regulate several sperm maturation genes, genes encoding decapacitation factors and a wide array of genes encoding ECM components, or proteins that control ECM content (**Chapter 4**). Lastly, I have characterised the ECM in the epididymis of *Plag1* KO mice and have shown that the ECM is presumably aberrantly stiff because of the dysregulation of key ECM components, implying that there is an imbalance between ECM deposition and degradation (**Chapter 5**). The intriguing finding that the epididymal ECM is affected in *Plag1* KO mice and the fact that the ECM has such widespread

regulatory effects raises the possibility that the ECM should be given more consideration in the phenotyping and tissue analyses of mouse models of male infertility.

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Articles in peer-reviewed journals

Wong, J, Damdimopoulos, A, Damdimopoulou, P, Gasperoni, JG, Tran, SC, Grommen, SV, De Groef, B & Dworkin, S 2020, 'Transcriptome analysis of the epididymis from *Plag1* deficient mice suggests dysregulation of sperm maturation and extracellular matrix genes', *Developmental Dynamics* (Accepted for publication; doi: 10.1002/dvdy.254)

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Conference abstracts

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