BRWD1, A UBIQUITOUSLY EXPRESSED GENE, HAS NON-REDUNDANT, SEXUALLY DIMORPHIC FUNCTIONS IN THE MAMMALIAN GERM-LINE.

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BRWD1, A UBIQUITOUSLY EXPRESSED GENE, HAS NON-REDUNDANT, SEXUALLY DIMORPHIC FUNCTIONS IN THE MAMMALIAN GERM-LINE.

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Normal spermiogenesis requires the transcription of many genes that occurs exclusively after meiosis. This wave of transcriptional changes in haploid spermatids is well-documented, but the underlying mechanisms of regulation are not. Deficiency of the dual bromodomain-containing protein BRWD1 has already been shown to be important for both male and female fertility. Here I show that nearly 300 transcripts, most of which are spermatid specific, were downregulated in *Brwd1*$^{-/-}$mutant testes. There was nearly complete elimination of transcripts encoding proteins important for spermiogenesis, such as the protamines and transition proteins. However, the misregulation was not associated with global epigenetic changes in chromatin, suggesting that BRWD1 acts selectively upon certain haploid-expressed genes. Our collaborators showed that *Brwd1* deficient oocytes, on the other hand, do not have any differences in transcript levels but display a two-fold increase in LINE1 expression. Despite BRWD1's expression in development, infertility is the only clear phenotype in *Brwd1* mutants. I found that mice doubly mutant for *Brwd1* and its paralog *Phip* were phenotypically indistinguishable from *Phip*$^{-/-}$ mice, indicating perhaps that another paralog, the X-linked *Brwd3*, may be functionally redundant to them in the soma.

BIOGRAPHICAL SKETCH

Shrivatsav Pattabiraman was born in Chennai, India and spent the first 21 years of his life there. During this time, he completed his schooling under the Central Board of Secondary Education. He then cleared the competitive All India Joint Entrance Examination and gained admission into the prestigious Indian Institute of Technology in Madras. He graduated with a Bachelor's of Technology in Biotechnology from IIT Madras in 2006. During this time he decided to pursue higher education in a field that he was interested in – Molecular Biology. After gaining admission into the PhD program in the department of Biochemistry, Molecular and Cell Biology at Cornell University, he moved to Ithaca, New York and started his graduate degree work under the supervision of Dr. John Schimenti.

DEDICATION

I would like to dedicate this dissertation to my parents, Mrs. Krishnakumari Pattabiraman and Mr. Pattabiraman Ranganathan, who have provided tremendous support through the years for my education and well being. I would also like to dedicate my dissertation to my spiritual Guru, H.H. Sri Sri Ravi Shankar, for providing moral and spiritual guidance in my life.

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CHAPTER 1

INTRODUCTION

Background and Significance

In spite of the fact that the world's population is projected to hit nine billion by 2050, 15% of couples worldwide remain childless due to fertility defects (Matzuk and Lamb 2002). In women the major causes of infertility are ovulation disorder or tubal damage accounting for 50% of affected individuals, whereas in men the major causes include oligospermia, asthenospermia, teratozoospermia and azoospermia which account for 20-25% of infertile men (Hargreave 2000). It is now widely accepted that there is a huge genetic component to fertility and as many as 2000 genes are known to be involved, for example, exclusively in spermatogenesis in humans (Hargreave 2000). The genetic nature of fertility is just beginning to be understood. Scientists have, however, found it increasingly difficult to study the genetics of (in-)fertility in human populations (by linkage analysis, for instance) due to the inability of probands to transmit mutant alleles. To that extent, mouse models have become very useful for studying the genetic causes of male and female infertility.

After recognizing these advantages of forward genetics, some labs have attempted to screen for phenotypes in mice after subjecting the animals to genome-wide mutagenesis (Schimenti and Bucan 1998). ENU (N-ethyl N-

nitrosourea) was, by then, identified as a potent germline mutagen in mouse and was the popular choice for carrying out such large-scale mutagenesis screens in many labs (Hitotsumachi, Carpenter et al. 1985, Schimenti and Bucan 1998, Kennedy, O'Connor et al. 2005). It is a powerful allkylating agent and causes point mutations throughout the genome. The Schimenti lab, in collaboration with others at the Jackson lab, started a genome-wide forward mutagenesis screen for infertility mutants using ENU as part of the reprogenomics program (Handel, Lessard et al. 2006) (Fig 1.1) . One of the novel mutations that arose from this infertility screen was called *repro5,* which was later mapped to the gene *Brwd1*. As will be discussed in detail later, the phenotype of these mutants is unique in that seemingly disparate processes of spermiogenesis and oocyte development are affected (Philipps, Wigglesworth et al. 2008).

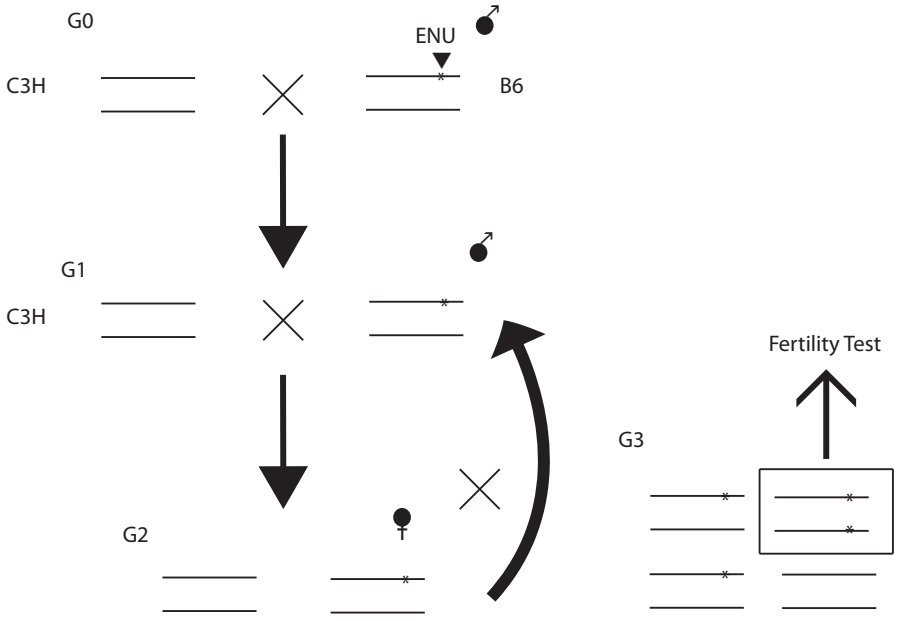


Fig 1.1 Reprogenomics Program. Point Mutations were introduced by ENU Mutagenesis in spermatogonia of C57B6/J male mice . Mice were then mated to WT C3H females. Through this mating scheme, homozygous mutant mice were produced and screened for infertility

I later showed that *Brwd1* is in fact important for the expression of a number of post-meiotic genes in the male. This loss of gene expression is not the result of changes in global chromatin architecture of round spermatids. To provide context to these results, I will describe in depth the molecules that are known to be important for the timely expression of post-meiotic genes as well as the global epigenetic changes that occur during spermatogenesis in this chapter. Our collaboraters showed that in the female, however, BRWD1 does not seem to be important for the expression of genes in the oocyte. Instead the retrotransposon element LINE1 seems to be consistently over-expressed. Chromosome configuration during metaphase and anaphase of meiosis I is also affected. A brief review of the process of oocyte development with emphasis on the transcriptional regulation of mRNAs and silencing of retrotransposons during oocyte growth is provided to better appreciate their results. I will also describe BRWD1 and another dual bromo-domain containing protein BRDT as two examples of dual bromo-domain containing proteins that are important for male fertility. *Brwd1* is expressed all over the mouse, and many of its somatic functions seem to be redundant with its paralogs *Phip* and *Brwd3*. In the final part of this chapter, I will describe to you what is known about these paralogs.

Transcriptional Regulation in Spermiogenesis

Mammalian spermatogenesis is a tightly regulated, well-defined process in which the male germ line stem cells or spermatogonia differentiate to form sperm

in the seminiferous tubules of the testes (Hermo, Pelletier et al. 2010). It can be broadly divided into three phases –

a. An initial proliferation phase, in which the spermatogonia undergo a few mitotic divisions preparing for meiosis (Hermo, Pelletier et al. 2010).

b. A meiotic phase, in which the cells undergo two rounds of cell-division with only one round of DNA replication; thereby becoming haploid (Hermo, Pelletier et al. 2010) and

c. A differentiation phase, in which the radially symmetrical haploid cells (known as round spermatids) undergo massive nuclear and cytoplasmic changes, develop polarity and become highly structured gametes. This phase is also known as spermiogenesis (Hermo, Pelletier et al. 2010).

The processes that occur during spermiogenesis are unique and do not occur anywhere else in the organism. During spermiogenesis, the Golgi secretes and stores enzymes that are important for fertilization and eventually transforms into an organelle called the acrosome. On the other end, the spermatid develops a thickened “mid-piece” surrounded by mitochondria. One of the centrioles in the cell, assisted by a temporary structure called the manchette, elongates to form a tail. Most of the cytoplasm is eventually lost in the form of residual bodies as the spermatids mature. In the nucleus, the chromatin undergoes a significant degree of compaction as histones are replaced by transition proteins and eventually by more basic protamines. At this stage, all transcriptional activity ceases and the

nucleus remains quiescent. The result of spermiogenesis is a sperm - a highly structured cell comprising of a head, a mid-piece and a tail.

The incredible morphological changes that occur during spermiogenesis are made possible by the expression of unique proteins in the spermatid. The expression of these proteins, in turn, is tightly regulated by a wave of post-meiotic transcription that takes place in the spermatid. (Fimia, Morlon et al. 2001, Kimmins, Kotaja et al. 2004, White-Cooper and Davidson 2011) These genes are sometimes referred to as the “haploid genome” as the majority of them are exclusively transcribed in the haploid round-spermatid stage (Dadoune, Siffroi et al. 2004). The importance of the timely expression of these genes is underlined by the fact that their absence or untimely expression can cause male sterility. For example, haploinsufficiency of the genes encoding protamines, *Prm1* and *Prm2,* causes male sterility (Cho, Willis et al. 2001). Similarly male mice deficient for ODF1, the main protein of the sperm tail outer dense fibers, are infertile due to a variety of defects in sperm (Yang, Meinhardt et al. 2012). Notably, both protamines and ODF1 are expressed in the wave of post-meiotic transcription that occurs in spermatids. In spite of the importance of post-meiotic transcriptional regulation, little is known about the key-players of this process in mice, as their study has been hampered by the absence of an *in-vitro* germ-cell differentiation model. Therefore almost all of the genes important for post-meiotic transcription have been discovered through the study of genetic mouse mutant

models (White-Cooper and Davidson 2011). Here I will briefly review some of the literature of what is known about post-meiotic transcriptional regulation in mice.

CREM/ACT System of Transcriptional Regulation

cAMP Response Element Modulator (CREM) and cAMP Response Element Binding Protein (CREB) are related proteins that bring about transcriptional regulation in response to stresses, metabolic and developmental signals in a variety of tissues. They usually respond to signaling pathways affected by the secondary messenger cAMP (Hummler, Cole et al. 1994, White-Cooper and Davidson 2011). Both CREB and CREM function by binding the cis-element CRE (cAMP Response Element), which is characterized by the sequence 5'-TGACGTCA-3' or the half-CRE 5'-TGACG-3' and 5'-CGTCA-3'. While CREB is ubiquitously expressed at similar levels across cell-types, CREM displays cell-specific expression (Foulkes, Borrelli et al. 1991). CREM has many isoforms, the majority of which are transcriptional repressors as opposed to CREB, which is usually an activator of transcription. One of the most well known isoforms of CREM is the repressor ICER (Inducible cAMP early repressor) (Daniel, Rohrbach et al. 2000). Activating isoforms of CREM are expressed in some tissues. The most well studied of these is CREM-τ expressed exclusively in the testis (Foulkes, Mellstrom et al. 1992, Daniel, Rohrbach et al. 2000). CREM-τ was suspected to play a role in post-meiotic transcription as it was expressed only in spermatids and many post-meiotic genes carry a CRE element in their promoter (Delmas, van der Hoorn et al. 1993, Behr and Weinbauer 2001). The importance

of the role of CREM in spermatogenesis, however, only became clear when a *Crem* knock out mouse was created. Male mice deficient in CREM were infertile and displayed spermiogenic defects (Blendy, Kaestner et al. 1996, Nantel, Monaco et al. 1996). As expected, post-meiotic gene expression was affected in these mice (Nantel, Monaco et al. 1996, Beissbarth, Borisevich et al. 2003). Many important post-meiotic genes such as protamines, transition proteins, ODF1, a few AKAPs, etc. were shown to be de-regulated in the mutant, explaining the arrest in spermiogenesis.

Further insight into post-meiotic transcription regulation via CREM came with the identification of its co-activator ACT (Activator of CREM in Testis) – a LIM domain containing protein that is expressed exclusively in round spermatids (Fimia, De Cesare et al. 1999, Fimia, De Cesare et al. 2000)(Fig 1.2). This led to the discovery of a novel pathway of CREM activation that was independent of the phosphorylation status of CREM, as ACT was able to bind unphosphorylated CREM. Interestingly ACT knockout mice were fertile, in spite of some abnormalities in their sperm, the ACT knockout did not phenocopy the CREM knockout. This indicated that ACT is not required for all of CREM's functions and that other pathways may be involved or other proteins are present in the testis that may function redundantly with ACT (Kotaja, De Cesare et al. 2004, Lardenois, Chalmel et al. 2009). ACT itself is regulated by the kinesin KIF17b, an isoform of the ubiquitously expressed kinesin KIF17 found exclusively in brain and testis (Macho, Brancorsini et al. 2002). KIF17b is able to shuttle ACT into

and out of the nucleus in a microtubule independent manner (Macho, Brancorsini et al. 2002, Kotaja, Macho et al. 2005)(Fig 1.2). KIF17b interaction with ACT is dependent upon its phosphorylation status brought about in an as yet unidentified pathway that is regulated by Protein Kinase A (PKA). cAMP may, in turn, regulate the activity of PKA and this is supported by the fact that administration of an adenylate-cyclase inhibitor inhibited KIF17b-ACT interaction *in-vitro*. (Kotaja, Macho et al. 2005). This presents an interesting pathway where cAMP can regulate the function of CREM through its action on KIF17b.

KIF17b also interacts with Testis Brain RNA Binding Protein (TB-RBP) and the post-meiotic RNAs expressed as a result of CREM activation (Chennathukuzhi, Morales et al. 2003). TB-RBP has been shown to be involved in mRNA transport and stability. Interestingly, KIF17b was also found to be associated with MIWI in the chromatoid body (Kotaja, Lin et al. 2006). MIWI is another protein known to be involved in stabilizing mRNPs in spermatids and helping regulate translation of these mRNAs, apart from its potential role in piRNA biogenesis (Deng et al, 2002). Thus KIF17b may be involved in the transportation of post-meiotic RNAs from the nucleus to the cytoplasm bringing them to the well-known RNA processing center chromatoid body. Finally, CREM can interact with TFIIA, but not with its testis-specific homolog ALF (TFIIA-Like Factor), and bring about transcriptional activation (De Cesare, Fimia et al. 2003).

Hormones regulate the expression of the activating isoform of CREM in testis. Follicle Stimulating Hormone (FSH) brings about the shift in expression from a repressive isoform to an activating isoform of CREM in pachytene spermatocytes (Foulkes, Schlotter et al. 1993). Higher CREM-τ expression, however, is not a result of transcriptional activation; instead it is the result of stabilization of CREM-τ mRNA in response to FSH. FSH, through its action on Sertoli cells, is thought to bring about these changes in round spermatids through a series of paracrine signals. These signals, by an as yet unidentified mechanism, modulate the usage of an alternative poly-A adenylation site that is more 5' in the 3'-UTR of the CREM-τ isoform. Usage of this poly-A site eliminates several destabilizer elements found in the 3'-UTR, thereby stabilizing the activating isoform CREM-τ (Foulkes, Mellstrom et al. 1992, Foulkes, Schlotter et al. 1993) The mRNAs are stored in spermatocytes and are not translated until the round spermatid stage.

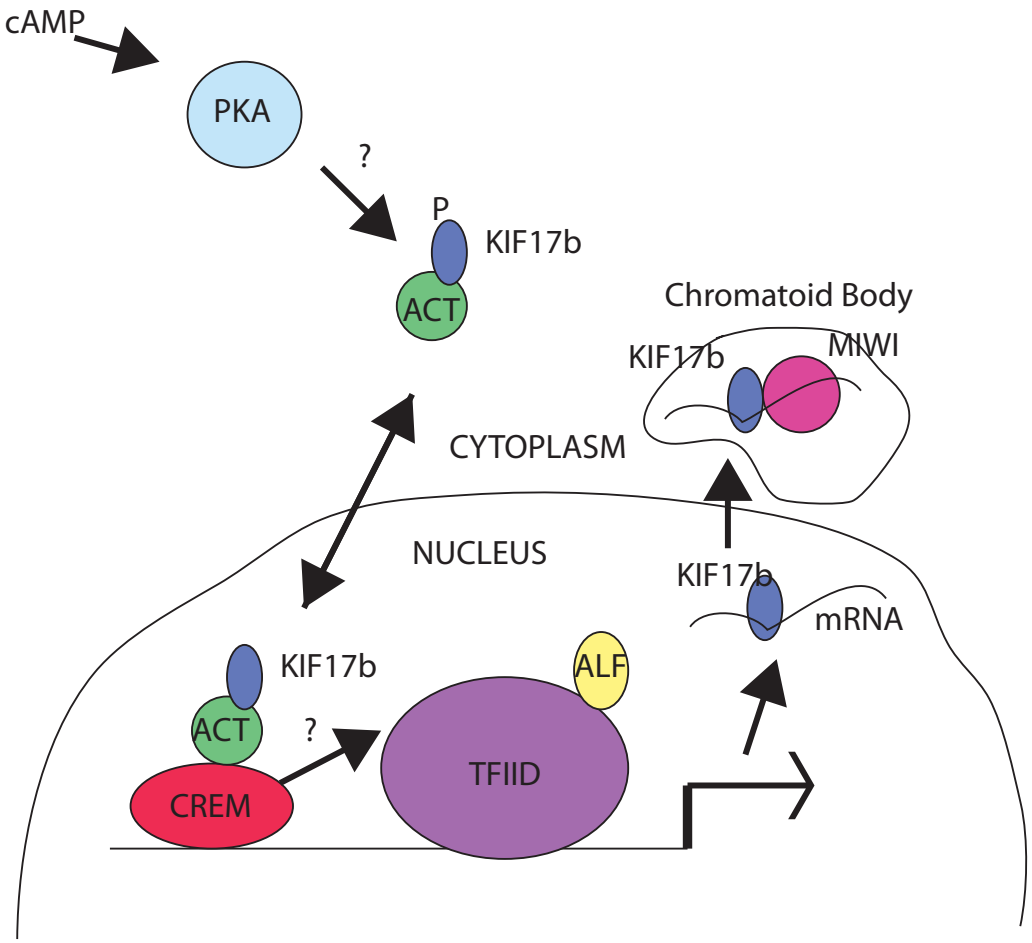


Fig 1.2 The CREM-ACT Pathway of Post-meiotic Transcriptional Regulation. CREM interacts with ACT and activates transcription of post-meiotic genes by interacting with proteins within the TFIID complex and ALF. Kif17b is a protein that further regulates this system by shuttling ACT in and out of the nucleus. Kif17b itself is regulated by phosphorylation perhaps by PKA. Kif17b also shuttles the transcribed mRNA out of the nucleus into the chromatoid body, where it interacts with MIWI.

Unique Homologs of Basal Transcription Factors in Spermatogenesis

Three RNA polymerases transcribe genes in eukaryotic cells – pol I transcribes ribosomal RNAs, pol II transcribes protein coding genes and non-coding genes and pol III transcribes small RNAs (Juven-Gershon and Kadonaga 2010, Akhtar and Veenstra 2011). These polymerases cannot initiate transcription by themselves, and they require basal transcription factors for this. Basal transcriptional factors recognize the core promoter around the transcription start site and initiate transcription. For example, the basal transcription factors important for RNA polymerase II function include TFIIA, TFIIB, TFIID, TFIIE and TFIIF. Together with pol II form they form the pre-initiation complex (PIC) (Thomas and Chiang 2006). TFIID itself is a complex that contains TBP (TATA-Binding Protein) associated with 13-14 polypeptides known as TAFs (TBP – associated factors) (Thomas and Chiang 2006) TBP binds to a TATA box – a specific sequence motif in the promoter of genes and this binding is one of the first steps, which initiates the formation of the PIC (Kim, Nikolov et al. 1993, Kim, Geiger et al. 1993, Kim and Burley 1994).

Some basal transcriptional factors have been shown to have testis-specific isoforms or unique germ-cell specific paralogs both in *Drosophila* and mice (White-Cooper and Davidson 2011). Here I will provide a brief summary of three such factors that are important for murine spermatogenesis and that have been implicated in post-meiotic transcription – TBP-like Factor (TLF) (Also called TBP-

Related factor 2, TRF2); ALF (TFIIA –like Factor) and TAF7L – a testis specific homolog of the more ubiquitously expressed TAF7.

TBP-related Factor-2 (TRF2)

TRF2 is a distant paralog of TBP that is expressed in almost all metazoans (Dantonel, Wurtz et al. 1999). It lacks the ability to bind to a TATA box, and is implicated in the transcription of some TATA-less promoters (Moore, Ozer et al. 1999, Rabenstein, Zhou et al. 1999). TRF2 can interact with TFIIA, TFIIB as well as with the germ-cell specifc paralog of TFIIA, ALF (Teichmann, Wang et al. 1999, Catena, Argentini et al. 2005, Kopytova, Krasnov et al. 2006). The function of TRF2 has been extensively studied in different model organisms using knockdowns, dominant-negative and null mutants (Dantonel, Quintin et al. 2000, Veenstra, Weeks et al. 2000, Muller, Lakatos et al. 2001) In almost all of the organisms, TRF2 seems to be robustly expressed in oocytes, growing embryos and in the testis. However the extent of redundancy with TBP in these processes varies with each organism. In *Xenopus*, for example, TRF2 (and not TBP) is important for the transcription of large number of genes in the developing embryo (Jacobi, Akkers et al. 2007), whereas in *Drosophila*, TRF2 mutants show impaired spermatogenesis and oogenesis apart from some specific embryonic development defects (Kopytova, Krasnov et al. 2006). In mice, on the other hand, TRF2 is dispensible for embryonic development but is required for normal spermatogenesis (Martianov, Fimia et al. 2001).

The *Trf2*-null mouse phenotype deserves further description as it relates to transcriptional regulation in round spermatids. *Trf2*-null mice are viable and healthy, and look morphologically indistinguishable from WT mice, but males are infertile. Further analysis showed that spermatogenesis arrests in these mutants during the process of spermiogenesis at round spermatid stage. (Martianov, Fimia et al. 2001). A few post-meiotically expressed genes including *Act* were down-regulated in the *Trf2*$^{-/-}$ mice. *Crem* expression however was unaffected in these mice. TRF2, thus, may have a role in the timely expression of a few post-meiotic genes. TRF2 was later shown to be important for chromatin organization of the spermatid nucleus (Martianov, Brancorsini et al. 2002). The chromo-center, a structure of dense hetero-chromatin found in round spermatids, appeared fragmented in the *Trf2/-* mice. TRF2, unlike TBP, was shown to be associated with both heterochromatin and euchromatin; and thus may have roles other than transcriptional activation. TRF2 is also capable of acting as a transcriptional repressor by sequestering TFIIA (Chong, Moran et al. 2005, Tanaka, Nanba et al. 2007)

More insight into TRF2's role in round spermatids came after it was shown tha TRF2 interacted with TIPT, a testis specific protein (TRF2-Interacting Protein in Testis) (Brancorsini, Davidson et al. 2008) TIPT in turn, can bind HPIα– an integral component of the spermatid chromocenter. Thus TRF2 maintains global chromatin architecture in round spermatids and may also directly regulate transcription of post-meiotic genes.

TFIIA-Like Factor ALF

Another transcription factor homolog that is expressed exclusively in the testis and the ovary is ALF or TFIIA-Like-Factor (Han, Xie et al. 2004, Xiao, Kim et al. 2006). TFIIA is part of the pre-initiation complex that helps RNA polymerase initiate transcription. It has been shown to stabilize the TBP binding to the promoter and can also recruit various co-activators to facilitate transcription. In the mouse male germ cells, ALF, which is a TFIIA paralog localizes within the nucleus in the round spermatid stage (Han, Xie et al. 2004, Xiao, Kim et al. 2006). Thus, it may play a role in the wave of post-meiotic transcription that occurs at this stage. ALF has also been shown to interact with TRF2 (Catena, Argentini et al. 2005). It can also functionally substitute TFIIA in cells. ALF is regulated by the action of the transcription factor *Rfx2* in male germ-cells, which in turn is regulated by *A-Myb* (Horvath, Kistler et al. 2009)

Testis-specific TAFs

TAFs refer to the TBP-associated Factors that bind TBP and aid in its recognition of the promoter. They can directly interact with transcriptional activators and they are critical for the recognition of the promoter and selectivity of RNA polymerase II. Interestingly, however, there are many testis-specific TAFs that are paralogs of the more ubiquitously expressed counterparts (White-Cooper and Davidson 2011). Extensive studies of null-mutants of these genes in *Drosophila* have shown the specificity as well as the importance of these

paralogs in spermatogenesis. *Can* (paralog of dTAF5), *Nht (*paralog of dTAF4), *Mia* (paralog of dTAF6), *Sa* (paralog of dTAF8) and *Rye* (paralog of dTAF12) are all important for spermatogenesis, and the absence of any of these genes cause male infertility (Hiller, Lin et al. 2001, Hiller, Chen et al. 2004). These tTAFs form a testis-specific TFIID complex and are thought to activate transcription of genes important for spermiogenesis. Alternatively, there is evidence that they may also function by re-localizing the repressive Polycomb group of proteins from the promoters of spermiogenesis-specific genes into the nucleolus of spermatocytes (White-Cooper and Davidson 2011). In mice, TAF4B (a paralog of TAF4) is highly expressed both in testis and in the granulosa cells of the ovary. *Taf4b* deficient mice are initially normal, but progressively lose germ cells and this results in male sterility by 3 months of age. There is evidence to show that TAF4B maybe important for the expression of genes in spermatogonia (Falender, Freiman et al. 2005, Oatley and Brinster 2008).

Taf7l is an X-linked paralog of the more ubiquitous *Taf7* that is expressed highly in testis (Pointud, Mengus et al. 2003); however more recently expression in other tissues such as adipocytes, liver, spleen has been reported (Zhou, Kaplan et al. 2013). *Taf7l* is the more ancient paralog of *Taf7* that has evolved a testis-specific function (Cheng, Buffone et al. 2007). *Taf7l* null male mice are not completely infertile, but give birth to smaller litter sizes. They display a wide variety of abnormal sperm that are morphologically defective and have a lower

sperm count. Microarray analyses have shown that 6 genes are mis-regulated in the mutant (Cheng, Buffone et al. 2007).

Insights from the study of cis-elements

The study of cis-elements has also provided some insight into the transcriptional regulation of post-meiotic genes. Here I will discuss two specific examples that have been studied extensively: the promoter of the *Sp-10* gene, an acrosomal protein and the promoter of the protamine genes.

a. SP-10 gene regulation

Sp-10 is a post-meiotic gene that is expressed in round spermatids (Reddi, Flickinger et al. 1999). It encodes the protein SP-10 that is implicated in sperm-egg interaction and is part of the acrosome. The *Sp-10* promoter has been studied quite intensively and the DNA elements important for transcriptional activation have been well characterized (Reddi, Flickinger et al. 1999, Reddi, Shore et al. 2002, Reddi, Shore et al. 2003, Acharya, Govind et al. 2006, Abhyankar, Urekar et al. 2007, Reddi, Urekar et al. 2007). The -186/-91 region upstream of the transcription start site is essential for activation of transcription of *Sp-10* (Acharya, Govind et al. 2006). An interesting aspect of the *Sp-10* promoter is that a short sequence of DNA is sufficient for transcriptional activation, as well as, for insulating the gene from surrounding chromatin environment. Indeed, the *Sp-10* promoter sequence and insulator sequence overlapped in a unique manner unlike other insulators that are usually separate from the promoter

sequences (Reddi, Urekar et al. 2007). Also, the SP-10 insulator did not contain CpG di-nucleotides, nor did it possess a CTCF-binding site –hallmarks of insulator sequences (Reddi, Urekar et al. 2007). TDP-43 (TAR DNA binding protein of 43 kDa size), which is found in the nuclear matrix of round spermatids, was shown to bind this insulator region and may function by tethering the insulator region to the nuclear matrix (Abhyankar, Urekar et al. 2007). Finally, two DNA-binding factors of the NFAT family (Nuclear factors of activated T-cells) NF45 and NF90, both of which are expressed in the nuclei of spermatocytes and spermatids, was shown to bind to the purine box located in the SP-10 promoter and may be important for its transcriptional regulation (Ranpura, Deshmukh et al. 2008).

b. Protamine Gene Regulation

The protamine genes are the most well studied post-meiotic genes in mice. One of the factors that causes transcriptional activation of the protamine genes is TET-1, which was isolated from testis nuclear extracts (Tamura, Makino et al. 1992). Later, numerous testis-specific and ubiquitous proteins capable of binding the *Prm1* promoter were discovered including the Y-box binding protein MSY1 (Zambrowicz and Palmiter 1994, Nikolajczyk, Murray et al. 1995). MSY1 is the mouse homolog of the *Xenopus* germ cell specific RNA/DNA binding proteins p54/p56 and is expressed both in oocytes and spermatids in mice. Five different protein-binding sites in the protamine promoter – including the CRE element that binds CREM, the CAAT box that binds Y-box binding proteins as well as two

hormone response elements were later characterized (Ha, van Wijnen et al. 1997). Interestingly, in a separate study, it was shown that Germ-cell Nuclear Factor and CREM-tau regulate protamine expression reciprocally – GCNF acts as a repressor whereas CREM-tau acts as an activator (Hummelke and Cooney 2004). An in-depth analysis of how the "protamine domain" containing the genes *Prm2, Tnp1* and *Tnp2* is regulated by potentiation; the opening up of higher order chromatin structures to provide access to transcriptional activators; was undertaken by the Krawetz lab (Martins and Krawetz 2007). The protamine domain becomes DNAseI hypersensitive as early as the pachytene spermatocyte stage, even though transcription does not begin until much later. The 5' and 3' ends of this domain are attached to the nuclear matrix thus looping the chromatin in between them and insulating the region from the surrounding chromatin environment (Martins and Krawetz 2007). Histone modifications that favor transcription do not appear until spermatid stage. Histone H3 and H4 acetylation increase markedly in this domain as the germ cells progress from spermatocyte to spermatid stage. Interestingly, H3K9Me2; a methylation mark important for HP1 recruitment does not seem to change much in this region even when the gene starts transcription (Martins and Krawetz 2007).

Epigenetic Modifications and Transcriptional Regulation

Epigenetic modifications broadly refer to the post-translational modifications of histone residues as well as the DNA methylation patterns that affect chromatin architecture and regulate gene expression. Some of the most common histone

modifications include methylation and acetylation of lysine and arginine residues, phosphorylation of serine and threonine residues as well as ubiquitylation and sumoylation of lysine residues (Godmann, May et al. 2010). These modifications play an important role in spermatogenesis and spermatid chromatin condensation (Rousseaux, Caron et al. 2005, Godmann, Lambrot et al. 2009). Interestingly many histone modification enzymes and histone variants are robustly and specifically expressed in male germ cells (Lahn, Tang et al. 2002, Hayashi, Yoshida et al. 2005, Akimoto, Kitagawa et al. 2008). Indeed, many unique chromatin-remodeling events take place during spermatogenesis including establishment of imprinting in PGCs, meiotic chromosomal recombination and segregation as well as histone-to-protamine transition in spermiogenesis necessitating the use of multiple, testis-specific histone modification enzymes (Govin, Dorsey et al. 2010). Most of the insight that we presently have about the role of epigenetic marks in spermatogenesis is from the study of transgenic mouse models.

H3K4 methylation status of the chromatin changes throughout the genome during spermatogenesis– with high levels in spermatogonia, followed by down-regulation in pachytene spermatocytes and partial reappearance in spermatids (Brykczynska, Hisano et al. 2010). H3K4Me2 and H3K4Me3 mark sites of active transcription (Du, Li et al. 2013). H3K9 methylation, on the other hand, is shown to accumulate in the transcriptionally quiescent chromocenter of round spermatids and is implicated to be important for the chromatin condensation that

follows (Liu, Zhou et al. 2010). The importance and uniqueness of these methylation patterns in male germ cells is underlined by the expression of testis-specific methyl transferases and de-methylases and their stringent regulation. *Prdm9* is an example of a methyl-transferase specifically expressed in male and female germ cells that is important for meiotic gene activation. A *Prdm9* knockout showed decreased trimethylation and an increase in dimethylation causing mis-regulated gene expression (Hayashi, Yoshida et al. 2005). Similarly, an example of a testis specific demethylase is JMJD1A. Male mice deficient in JMJD1A display infertility and an arrest in spermiogenesis. The mutant mice show increased H3K9 mono- and di-methylation in spermatocytes and spermatid that cause diminished recruitment of CREM on the promoters of its target genes – *Tnp2, Prm1* and *Prm2* (Liu, Zhou et al. 2010). The mutants also display decreased ACT expression. Another group showed that JMJD1A binds the promoters of the protamine genes directly and de-methylates H3K9 causing transcriptional activation (Okada, Scott et al. 2007). Thus, JMJD1A-dependent de-methylation of H3K9 may be important for post-meiotic transcription. Little is known about the role of other histone modifications in the regulation of post-meiotic transcription. It is however very likely that epigenetic modifications play an important role in post-meiotic gene expression; either by helping to maintain global chromatin architecture or by recruiting specific transcription factors to promoters of post-meiotically expressed genes. An altered epigenetic landscape in round spermatids may prevent loss of repressors from or recruitment of

activators to the promoters of post-meiotic genes causing mis-regulated expression of genes.

Bromo-and WD-Repeat Containing-1 BRWD1

Efforts to characterize Bromodomain and WD-repeat containing-1 (*Brwd1*) began when it was annotated as one of the genes found in the Down-critical-region-2 of the human chromosome 21, a region that is associated with Down-Syndrome (Huang, Rambaldi et al. 2003). *Brwd1* is expressed in a wide-variety of human adult tissues and has several different transcript sizes that have tissue specific expression patterns (Ramos, Vidal-Taboada et al. 2002). For example, in humans, a shorter 2.6 kb fragment is expressed only in the pancreas, and an intermediate 5 kb fragment is expressed only in the liver, whereas a larger 13 kb fragment in expressed in heart and skeletal muscle (Ramos, Vidal-Taboada et al. 2002). In the mouse however, only one transcript size of about 7.8 kb was found by Ramos et al. This transcript is expressed in mouse embryonic development from E11 to E18 and in all of the murine adult tissues that were tested – brain, kidney, testes, pancreas and muscle. In different human cell-lines, *Brwd1* is expressed as a 12 kb or a 5 kb transcript depending on the cell-line. In general high expression of *Brwd1* was found in tissues or cell-lines with a high rate of proliferation such as embryos, cancerous tissues and tumor-derived cell lines (Ramos, Vidal-Taboada et al. 2002).

Huang et al. further characterized *Brwd1* expression in mouse (Huang, Rambaldi et al. 2003). By performing whole mount in situ hybridizations on 10.5 dpc mouse embryos, they found robust expression of *Brwd1* in the crano-facial area, the first branchial arch, the heart and the limb-bud. By 12.5 dpc, strong expression was seen in the frontonasal region, prevertebral column and most visceral organs like liver, heart, lung and the gastrointestinal tract. However unlike Ramos et al., they did not report a signal in the brain or spinal cord. Overall, they found a 8.3kb transcript that begins at 7.5 dpc, peaks around 10.5-11.5 dpc and decreases by 14.5 dpc. They developed an antibody against a C-terminal epitope of the protein to further characterize its expression. Expression of protein was found in a variety of developing embryonic tissue and cell-lines. In a co-immuno-precipitation study, BRWD1 was found to precipitate with BRG1 a component of the SWI/SNF chromatin-remodeling complex but not with TAF250. The poly-glutamine rich region of BRWD1 was shown to possess transcriptional activation properties providing even more evidence that BRWD1 can act as a transcriptional regulator in some of the tissues (Huang, Rambaldi et al. 2003).

The Schimenti lab discovered *Brwd1* as a gene that is important for fertility through a forward genetics approach. An allele that rendered both male and female mice infertile was discovered through an ENU mutagenesis screen and was named *repro5*. The mutation was later mapped to a donor site on exon 10 of the gene *Brwd1* (Philipps, Wigglesworth et al. 2008). The aberrant splicing of *Brwd1* caused exon 9 to splice directly into exon 11, producing a frame-shift that

resulted in a premature stop-codon. These aberrant transcripts presumably undergo nonsense-mediated decay, and thus the *repro5* allele is a null or a severe hypomorph of *Brwd1*. A separate gene-trap allele of *Brwd1* failed to complement the *repro5* allele further indicating that it was absence of BRWD1 that caused the infertility phenotype.

Brwd1 mutants do not display any other discernable phenotype in spite of previous reports of the gene's wide spread expression in embryonic development and mouse adult tissues. *Brwd1* mutant ovaries and testes were found to be morphologically indistinguishable from the controls (Philipps, Wigglesworth et al. 2008). The ovaries contained follicles and oocytes at various stages of development and corpora lutea were also present. In female mutants, the only phenotype observed was defective oocyte development. Only about half of the mutant oocytes harvested at the GV stage were capable of progressing to metaphase II when subjected to *in vitro* maturation. Among all of the metaphase II arrested mutant oocytes, none were able to cleave to the 2-cell stage or form blastocysts, after *in vitro* fertilization. On the other hand, mutant males had lower sperm count and severely abnormal sperm. Most of the sperm isolated from their epididymides were immotile or dead as determined by Eosin-Negrosin staining (Philipps, Wigglesworth et al. 2008). None were able to fertilize WT eggs. More than 90% of the sperm had morphologically abnormal head shapes and sperm; and the tails were defective. The mid-piece was "ragged". The initial stages of spermiogenesis however seemed to be normal and the numbers of round spermatids were not different from wild type. Malformed sperm heads were

evident only in the elongating spermatid stage and were probably caused by defective chromatin condensation. A normal acrosome also failed to form. Unlike most mutations that cause infertility in both males and females, *Brwd1* mutants did not have any gross perturbations in the meiotic stages of germ cell development. Chromosome synapsis and Double Stand Break processing was found to be normal indicated by SYCP3 and yH2Ax staining (Philipps, Wigglesworth et al. 2008). Metaphase I and metaphase II spreads also displayed normal number and configuration of chromosomes. Thus, absence of BRWD1 caused defects exclusively in spermiogenesis and oocyte to embryo transition.

BRWD1 contains a dual bromodomain and several WD repeats that form a beta propeller structure. Bromodomains are the only interaction modules that specifically recognize ε-N acetylation of lysine residues (Mujtaba, Zeng et al. 2007). All known bromodomains fold into a conserved 120 residue structural four helix motif identified as a conserved domain in the Drosophila *Brahma* gene (Tamkun, Deuring et al. 1992, Mujtaba, Zeng et al. 2007) (Fig 1.3). They are present in a wide variety of proteins including transcriptional co-regulators, chromatin modifying enzymes such as HATs (histone acetyl-transferases), HAT-associated proteins, ATP-dependent chromatin remodeling complexes, helicases, SET domain containing methyl transferases and nuclear scaffolding proteins (Yang, Ogryzko et al. 1996, Dhalluin, Carlson et al. 1999, Venturini, You et al. 1999, Jacobson, Ladurner et al. 2000, Cavellan, Asp et al. 2006, Gregory, Vakoc et al. 2007, Trotter and Archer 2008, Malik and Bhaumik 2010). Although

bromodomains exhibit large sequence variations, they share a conserved fold that is a left-handed helix bundle of 4 α helices (αZ, αA, αB, αC) linked by diverse loop regions of variable length (Fig 1.3). Bromodomains recognize the acetyl lysine residue through a central deep hydrophobic cavity. WD-repeats on the other hand, are defined by the presence of four or more repeating units containing a conserved core of approximately 40 amino acids usually ending with Tryptophan-aspartic acid WD (Liu, Sekito et al. 2001) (Fig 1.3). They are minimally conserved and are initiated by a glycine-histidine (GH) di-peptide about 11-24 residues from the N terminus end. All WD-repeats form a circularized β-structure. WD-repeat containing proteins function in a wide-variety of processes ranging from signal transduction and cell cycle to chromatin assembly, RNA synthesis and processing, vesicular trafficking etc (Liu, Sekito et al. 2001). The underlying common function of all WD-repeat family members is coordinating multi-protein complex assemblies by serving as a scaffold for protein interactions. Often, they can simultaneously bind to several different proteins. Some examples of proteins that contain a WD-repeat structure are the β subunit of G proteins, TAFII transcriptional factor and E3 ubiquitin ligase complexes (Tyers and Willems 1999).

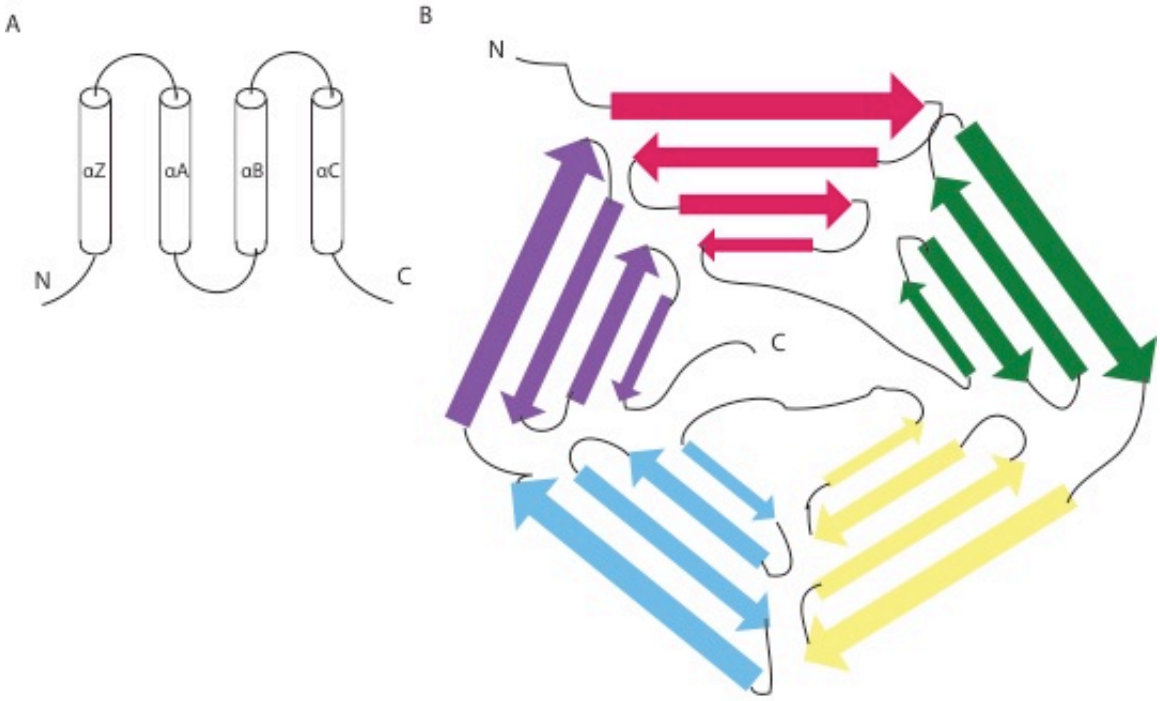


Fig1.3 A. Bromodomains fold into a conserved 120 residue four-helix motif that recognizes acetylated lysine residues on proteins. The helices are linked by diverse loop regions of variable length B. WD repeats have four or more repeating units containing a conserved core of 40 amino acid residues ending with Tryptophan-Aspartic Acid (WD)

BRDT and the BET family of proteins.

Another murine testis-specific protein that is similar to BRWD1 in a number of different ways, including the presence of the dual bromodomains, is BRDT. BRDT is a member of the BET family of proteins characterized by the N terminal pair of tandem bromodomains and a unique C terminal ET domain found only in this family (Berkovits and Wolgemuth 2013). The function of BRDT in mammals has been well studied and has provided insight into the role of a bromodomain-containing protein in spermatogenesis.

The first BET gene discovered was in *Drosophila*; it is called *female sterile 1 homeotic (fs(1)h)*. *Fs(1)h* was discovered in a screen for maternal effect mutants; it is the only BET family gene found in *Drosophila* (Florence and Faller 2008). Yeast have two BET family genes BDF1 and BDF2 – BDF1 was identified in a screen for genes affecting snRNA transcription (Lygerou, Conesa et al. 1994). In mammals there are four BET family genes BRD2, BRD3, BRD4 and BRDT; they are all expressed in testis (Shang, Salazar et al. 2004). However, they are expressed at different time-points, suggesting that they may have non-redundant roles in spermatogenesis. BRD2, BRD3 and BRD4 are also ubiquitously expressed in a variety of mouse tissues. These genes have a number of functions: BRD2 modulates transcription in particular cell cycle induced transcriptional activation (Shang, Salazar et al. 2004). It interacts with the transcription factors, TBP and E2F (Denis, Vaziri et al. 2000, Peng, Dong et al.

2007) and binds to acetylated chromatin in the body of transcriptionally active genes aiding in RNA polymerase progression (LeRoy, Rickards et al. 2008). BRD4 acts as a transcriptional co-activator as it can bind p-TEFb and acetylated histones and can recruit the transcription-complex to promoters of genes poised for transcription (Jang, Mochizuki et al. 2005, Yang, Yik et al. 2005, Yang, He et al. 2008). BRDT on the other hand is the only BET family gene to be uniquely expressed in the testis (Jones, Numata et al. 1997, Taniguchi, Suzuki et al. 2001, Pivot-Pajot, Caron et al. 2003).

The first mutant to be created was *Brdt*$^{\Delta BD1}$ in which the first bromodomain was deleted (Shang, Nickerson et al. 2007). This mutation, when homozygous, rendered all male mice infertile with no other discernable phenotype. Sperm from *Brdt* mutant mice displayed a wide-variety of defects including malformed heads and tails, very similar to the *Brwd1* mutant phenotype. The chromocenter, a structure that contains all peri-centric chromatin in the round spermatids, was fragmented in the mutants and multiple foci were found (Berkovits and Wolgemuth 2011). Interestingly, BRDT protein is not found associated with the chromocenter, but rather is found adjacent to it. The “level of heterochromatin” as assessed by staining for HP1alpha in these mutants was also found increased, and thus it was hypothesized that BRDT plays a role in chromatin architecture by preventing spread of heterochromatin. The impact of the chromocenter fragmentation is seen in the elongation phase of spermatids where the chromatin is elongated with the help of an actin network to form the sperm

head. H1FNT, which is mis-localized in the BRDT mutants, seems to be important for elongation of spermatids (Berkovits and Wolgemuth 2013)

Apart from playing an important role in maintaining chromatin architecture, BRDT plays an important role in regulating gene expression. Microarray studies show that a number of genes are mis-regulated in the mutant. About 1000 genes were shown to be upregulated and greater than 400 genes down-regulated in the mutant (Berkovits, Wang et al. 2012) BRDT also interacts with the spliceosome machinery in the testis and 3' UTR splicing is another mechanism by which BRDT regulates post-meiotic gene expression. BRDT's role in the compaction of chromatin during the histone to protamine transition is still under debate (Berkovits and Wolgemuth 2013).

Oocyte Maturation

Female side of gametogenesis is very different from spermatogenesis in mice. Primordial germ cells in the female genital ridge develop into oogonia or female germ-line stem cells that are pluripotent in nature (Matova and Cooley 2001). Unlike spermatogenesis, where the spermatogonia pool is maintained throughout the life of the mouse, in oogenesis, all of the oogonia replicate their DNA and enter meiosis (Nakatsuji and Chuma 2001). The oocytes however do not complete meiosis and they arrest at prophase I in the diplotene stage. At the same time they become intimately associated with somatic cells known as granulosa cells, thus forming follicles (Matova and Cooley 2001). Hormones in the circulating blood of the female mouse then govern the subsequent

development of the oocyte and its release from this arrest. Follicle Stimulating Hormone (FSH) stimulates batches of follicles to develop – the oocyte starts growing in size and enters the growth phase as the granulosa cells also grow in number and develop (Richards and Pangas 2010). The granulosa cells start secreting large amounts of estradiol into the circulation. Accumulation of estradiol triggers a surge in the level of another hormone in the blood stream– the Luteinizing Hormone (LH) (Shepel, Blashkiv et al. 2012). The LH surge stimulates further growth of the follicle, finally releasing the oocyte from its prophase I arrest – the oocyte completes meiosis I, forms the first polar body and re-arrests at the metaphase II stage (Shepel, Blashkiv et al. 2012). During this process of oocyte meiotic maturation, it is ovulated from the ovary and is released from its surrounding somatic cells (Richards 2005). The oocyte is, however, maintained in this metaphase II arrest until egg activation upon fertilization with sperm.

The term “oocyte maturation” is loosely used to describe the various changes that take place in the primary oocyte during its growth as a result of which it acquires developmental competence for fertilization (Masui and Clarke 1979). A number of changes occur in the oocyte as it matures. It makes specific sperm receptors, accumulates nutritional reserves for the embryo, acquires competency to be activated after fertilization, hence allowing formation of the zygote; for example by equipping itself for mechanisms that prevent polyspermy (Voronina and Wessel 2003). Finally, the nucleus of the oocyte undergoes tremendous

changes – it enters meiosis and re-arrests at metaphase II. All transcription ceases when the oocyte re-enters meiosis, and the chromatin assumes a more condensed configuration during this transition in mammals (Voronina and Wessel 2003). Oocyte maturation thus comprises both nuclear maturation and cytoplasmic maturation.

Changes in Chromatin Configuration and Transcription during Oocyte Maturation

Transcription in the oocyte is tightly regulated in mice; it begins in the enlarged nucleus of the primary oocyte (called germinal vesicle) as it enters the growth phase (Bachvarova and Paynton 1988). At this stage, the chromosomes assume a mostly decondensed and dispersed configuration that allows access to various transcription factors. Transcription ceases as the oocyte begins to mature – this is also correlated with the corresponding condensation of the chromosomes, breakdown of the germinal vesicle and dispersal of nucleoli (Masui and Clarke 1979). Homologous chromosomes then align along the meiotic spindle and separate and the oocyte extrudes the first polar body as it completes maturation. Only after fertilization, at the 2-cell stage, is transcription activated again in mouse.

The changes in chromatin configuration can be observed visually in antral stage follicles. At this time, the GV staged oocytes (oocytes that have not undergone germinal vesicle breakdown (GVBD) i.e. not re-entered meiosis) are

of two possible chromatin configurations as they mature from NSN oocytes (non-surrounded nucleolus stage) and to SN oocytes (Surrounded nucleolus stage). Visibly these oocytes can be differentiated by the presence or absence of a Hoechst-positive rim surrounding the nucleolus. In the SN staged oocytes, this rim is present, whereas in the NSN oocytes it is absent (Tan, Wang et al. 2009). Interestingly these chromatin configurations are associated with different transcriptional states. When Br-UTP was injected into oocytes, in an *in-vitro* transcription run-on assay, only NSN oocytes (and not SN oocytes) showed high levels of fluorescence indicating that transcription was silenced in the SN oocytes (Bouniol-Baly, Hamraoui et al. 1999) (Fig 1D). The visible changes that are seen in this transformation from NSN to SN oocytes can be attributed to the change in localization of transcriptionally silent heterochromatin. In both NSN and SN oocytes, the centromeres co-localize with AT rich heterochromatin; but in the NSN oocytes they are localized in spots near the nucleolus, whereas in the SN oocytes they cluster around the nucleolus to form a compact ring (Longo, Garagna et al. 2003). Attainment of the SN-configuration is important for the oocytes to be able to develop to the metaphase II stage – only 20-30% of the NSN oocytes are able to develop to metaphase II and those that do reach metaphase II, arrest at 2-cells stage after fertilization. Taken together, this means that the NSN oocytes have lower developmental competence than the SN oocytes (Ma, Li et al. 2013). Transcriptional silencing however seems to be independent of the formation of SN chromatin configuration. In mice deficient for nucleoplasmin (*Npm2*$^{-/-}$) the oocytes cannot form the SN-configuration; but

transcriptional silencing occurs at the right time (De La Fuente, Viveiros et al. 2004). Instead, oocyte-somatic cell interaction seems to be important for the timely occurrence of the transcriptional silencing (De La Fuente and Eppig 2001).

Histone modifications also seem to play an important role in the changes of chromatin configuration that occur in GV stage-oocytes (Xia, He et al. 2012). The profile of DNA methylation, histone methylation and acetylation increases quantitatively throughout folliculogenesis until the oocyte becomes fully-grown and has entered the SN stage (De La Fuente, Viveiros et al. 2004). In particular acetylation of H3K9, H3K18, H4K5 and H4K12 increases with oocyte growth (Kageyama, Liu et al. 2007). The fully-grown oocyte now undergoes germinal vesicle breakdown. Coincident with this, a massive wave of deacetylation occurs. Chromatin is re-acetylated only after fertilization (Kim, Cheon et al. 2003, Longo, Garagna et al. 2003). An interesting protein with chromatin remodeling activity called ATRX is found associated with the perinuclear heterochromation in SN oocytes. ATRX belongs to the SNF2 family of helicases and ATPases (De La Fuente, Viveiros et al. 2004). In spite of the genome wide deacetylation that occurs at GVBD, ATRX remains associated with the centromere and may be important for the proper separation of chromosomes in anaphase I (De La Fuente, Viveiros et al. 2004).

All the processes after oocyte growth, including meiotic maturation, fertilization and early embryo development completely depend on the pool of mRNAs that

was transcribed during the growth phase. Oocytes regulate these processes by translational control of the pool of RNAs present in the cell. An important process that occurs during this time is the dramatic degradation of select maternal transcripts (Su, Sugiura et al. 2007). Paradoxical as it may seem, that the oocyte would degrade about 20% of the RNAs that it made during the growth phase, this event is necessary for the oocyte to embryo transition. A few important proteins (such as MSY2) have been implicated in maintaining the stability and the translational regulation of the stored mRNAs (Medvedev, Pan et al. 2011).

Similar to spermatogenesis, oocytes also transcribe many unique genes, different isoforms of ubiquitously expressed genes and paralogs of other somatic genes. For example, oocytes use different paralogs of the basal transcriptional machinery. One such factor is TRF2 that substitutes for the function of TBP in the oocyte nucleus. Even though both TBP and TRF2 can mediate transcription in oocytes, TBP seems to be dispensible for oocyte maturation and remarkably its nuclear concentration seems to decrease with oocyte growth (Gazdag, Santenard et al. 2009). Another TAF paralog that is important for both oocyte maturation and spermatogenesis is TAF4b. While in male mice TAF4b seems to be important for spermatogonial proliferation, in females it seems to play a variety of roles. TAF4b null females have fewer oocytes and they arrest after fertilization. Only 45% of the oocytes are able to re-enter meiosis and they have defective spindles (Falender, Shimada et al. 2005). ALF is a paralog of TFIIA that

was discussed in depth before that is also uniquely expressed in oocytes (DeJong 2006).

Apart from modulating transcription, oocytes also have to maintain genomic integrity and protect their DNA from disruptive factors such as retrotransposon activation (Zamudio and Bourc'his 2010). Indeed several retrotransposons are robustly expressed in mammalian oocytes and active mechanisms to degrade them are present (Piko, Hammons et al. 1984, Park, Shin et al. 2004, Peaston, Evsikov et al. 2004). *Dicer* seems to play an important role in checking the expression of these transposons (Murchison, Stein et al. 2007). *Dicer* knockout mice are unable to complete meiosis and arrest with defects in meiotic spindle organization and chromosome congression. They also have up-regulated expression of many transposons (Murchison, Stein et al. 2007). Another mutant with mis-regulated retrotransposon expression is the *Marf1* knockout. *Marf1* mutant oocytes have a variety of defects including up-regulated expression of transcripts, defective cytoplasmic maturation, meiotic arrest and up-regulated expression of IAP and LINE1 retrotransposons, as a result of which they are ovulated without germinal vesicle breakdown. (Su, Sugiura et al. 2012)

Paralogs of BRWD1

BRWD1 has two highly similar paralogs in mammals – PHIP (Pleckstrin homology Interacting Protein) and BRWD3. Both paralogs contain the WD-40 repeats as well as the dual bromodomains at essentially identical positions and

they share about 60% amino-acid identity and 73% similarity over most of the N-terminal. They are also expressed ubiquitously in tissues similar to *Brwd1* and hence may have redundant functions with BRWD1. Indeed, in spite of the ubiquitous expression of BRWD1, a null mutant of *Brwd1* is only infertile (Philipps, Wigglesworth et al. 2008). Insight into the importance of the *Brwd* family of genes comes from genetic mutants in invertebrates such as *Drosophila* that have only a single *brwd* gene. Several different mutant alleles of this gene have been created in *Drosophila*, the most severe of which, an allele called *ramshackle*, is lethal (D'Costa, Reifegerste et al. 2006). The *brwd* gene in Drosophila seems to have a variety of functions in different tissues and only a few of these functions have been unearthed. Apart from playing important roles in the development of photoreceptor cells in the retina, it was recently shown that the *brwd* gene in *Drosophila* is a DDB1-CUL4 associated factor that recruits a cullin4 RING finger E3 ligase to ubiquitylate the Drosophila CRY gene, an important event for re-setting the photoperiod in flies (Ozturk, VanVickle-Chavez et al. 2013).

Pleckstrin Homology Interacting Protein - Phip

Phip was originally identified as a 100kDa cytoplasmic protein through yeast two-hybrid screening of proteins that interact with the PH domain of the Insulin receptor substrate-1 (IRS-1) (Farhang-Fallah, Randhawa et al. 2002). It was further shown that PHIP is required for insulin receptor mediated mitogenic and metabolic signal transduction. Later another novel isoform of *Phip* of 206kDa size

was isolated from mouse pancreatic islet cells and was called *Phip1*. PHIP1 is nuclear and seems to be involved in the control of beta-cell proliferation and survival and can function both in an IGF-1 dependent and independent manner (Podcheko, Northcott et al. 2007). A deeper insight into the function of the *Phip* gene came from a genetic knockout. *Phip* deficient mice show 40% growth deficit at weaning compared to litter mates. They develop hyperglycemia and live for an average life span of just 4-5 weeks. *Phip* deficient mouse embryonic fibroblasts grow markedly slower than WT fibroblasts, but exhibit normal AKT phosphorylation and increased proliferation in response to IGF1, indicating that this pathway was intact (Li, Francisco et al. 2010).

Brwd3

BRWD3 was first identified as a factor that played a role in STAT92E mediated transcription, as dsRNA that targeted BRWD3 strongly suppressed it (Muller, Kuttenkeuler et al. 2005). Later it was shown that mutations in this gene interacted with the JAK/STAT pathway in Drosophila. Further studies confirmed that BRWD3 is in fact a positive regulator of the Drosophila JAK/STAT pathway (Arbouzova and Zeidler 2006). The human homolog of *Brwd3* is X-linked and is disrupted in a large proportion of individuals with B-cell chronic lymphocyte leukemia (Kalla, Nentwich et al. 2005). It was also shown that mutations in this gene in human causes X-linked mental retardation associated with macrocephaly (Field, Tarpey et al. 2007).

CHAPTER 2

METHODS

RNA Extraction from Testis

RNA was prepared from whole testes of WT and mutant mice using Trizol and purified using RNeasy columns. Briefly, the testes from each mouse were homogenized in 700ul Trizol and total RNA was extracted using 140ul chloroform. The aqueous phase was then separated by centrifugation and 525ul of 100% ethanol was added to it. This phase was then directly loaded onto a Qiagen RNeasy mini column and purified according to the manufacturer's instructions.

Microarray Analysis

RNA extracted from WT and mutant testes were analyzed for quality using BioAnalyzer. They were then converted to biotin labeled cRNA fragments according to the protocols described by Affymetrix. Hybridization was performed using Affymetrix MOE430 2.0 gene chips that represent more than 39000 uniquely expressed murine transcripts. Washing and scanning were also done according to Affymetrix protocols. The signals from scanning were analyzed using Affymetrix GCOS software. The normalized signals were then analyzed using R software using a standard t-test and genes were considered up- or down-regulated if they showed > 2 fold difference in expression with a p value of

< 0.01. Genes were classified as meiotic, post-meiotic or somatic using the BioMart mining tool available at the germonline.org database.

Real Time PCR

cDNA was prepared from freshly extracted RNA from WT and mutant testes using Superscript III Reverse Transcriptase as per manufacturer's instructions. 10ul of a 1:100 dilution of the above reaction was used as template in a 25ul master-mix using 2X SYBR-green. Primers were designed for select genes of the microarray using Integrated DNA Technologies RealTime PCR Tool such that the product sizes were 150bp. Primer sequences are listed in the table below. Non-specific amplification was tested for using dissociation curve analysis in the Real-Time PCR software. Ct values for the different reactions were noted and fold enrichment was calculated as the ratio of $2^{Ctmut}/2^{CtWT}$.

Table 2.1 Real-Time PCR Primer Sequences

	Gene	Forward Primer Sequence	Reverse Primer Sequence
1	*Tnp1*	GGGCGATGATGCAAGTCGCAATTA	TCACGACTGGCATTTACCCACTCT
2	*Tnp2*	AAGTGAGCAAGAGAAAGGCCGTCA	ACATCCTGGAGTGCGTCACTTGTA
3	*Prm1*	AGACTTCAAGAGCATCTCGCCACA	ACAGGTGGCATTGTTCCTTAGCAG
4	*Prm2*	TGCAGGAAATGTAGGAGGCACCAT	AGGGCTCAGACATCGACATGGAAT
5	*Akap4*	ACGGGCCATTTGGATTACCAGAGA	TTGCCACTCCTGAGGGAGAATGTT
6	*Hsp1a*	TTCCTTTATCCAAGCCGTAGGCGA	AGGTGTCATCGCAGGACTCAATGT
7	*Tssk6*	TGCTGTTGGGTTCTGATTCCTCCT	AGGCGCAATGCTTCTCTCTCTTCT
8	*Fscn3*	TGGCCTGCTAATGGCAAATGTCAC	TGGCGACAGGGCAATAGCTGAATA
9	*Oxct2a*	AGGTGCTGCTAAGGATGTCCAGTT	ACCTCAAGCCCATGCAGCAGATTA

Flow Cytometry Analysis

A single cell suspension for flow cytometry analysis was prepared as per the method described by the Fouchet lab (Bastos, Lassalle et al. 2005). Seminiferous tubules were dissociated using enzymatic digestion with collagenase Type I for 25 minutes at 32^{o}C in Hank's Balanced Salt Solution (HBSS) (20 mM HEPES (pH7.2), 1.2 mM $MgSO_4$.7H2O, 1.3 mM $CaCl_2$.2H2O, 6.6mM sodium pyruvate and 0.05% lactate). This was then filtered through a 40micron nylon mesh. Tubules were collected from the mesh and incubated again at 32^{o}C for 25 minutes in HBSS. This was again filtered to remove cell clumps. The cells were then spun down and washed with HBSS. The final pellet was re-suspended in HBSS, and the cell count determined using a hemocytometer. The cells were than fixed in 70% ethanol for 24 hours at 4^{o}C. The fixed cells were then washed with 50ug/ml propidium iodide (PI) and 100 ug/ml Rnase in PBS. Cells were then diluted to a final concentration of 1 million cells/ml and analysis was performed using a FACSCalibur4 flow cytometer (Benton Dickson Biosciences). PI was excited by a 360 nm ultraviolet laser (100mW) and the emitted red fluorescence was detected with a 630nm/30nm band passfilter. The emitted fluorescence was proportional to DNA content and three distinct populations were observed in the fluorescence area vs fluorescence width plot. These populations corresponded to "haploid", "diploid" and "tetraploid" cells in the suspension. The cells in these three distinct populations were counted and this was plotted in a Counts vs Fluorescence graph.

Antibody Production

Chicken Anti-sera and antibody-rich yolk from chicken injected with a C-terminal epitope of BRWD1 was provided to me when I joined the lab. I purified antibody from the serum using the AminoLink Plus Immobilization Kit (PIERCE) and from the egg yolk using the EggCellent Chicken IgY Purification Kit (PIERCE). I tested the activity and specificity of these antibodies using a standard dot-blot assay. Briefly the antigenic peptide and an unrelated peptide were blotted at various concentrations on a nitrocellulose membrane. The membrane was then blocked using 15% non-fat dry milk and later probed with the purified antibody at various dilutions (final concentration of 0.1-10ug/ml) . After multiple (3-5) washes with Tween-TBS (TTBS) of 5 minutes each, the blot was probed with a secondary anti-chicken antibody conjugated to HRP (1:1000). This blot was then washed again (3-5 times of 5 minutes each) with TTBS and developed using ECL substrate. The intensity of the “dots” that developed as a result of antigen-antibody interaction was a score of the activity of the antibody. The antibody was termed “specific” if it did not react with the unrelated peptide.

Later, I developed antibodies against epitopes that I designed. Epitopes that were 14-15 amino acids long were used for antibody production against BRWD1. About 15 such epitopes with good antigenic score were designed using the online tool EMBOSS antigenic. The antigenicity of these peptides was further verified by calculating their Surface Probability scores as described by Emini et

al. (Emini, Schleif et al. 1985) using the PROTEAN tool of DNAstar Lasergene software. They were then checked for cross-reactivity with the paralogs PHIP and BRWD3 by a simple BLAST analysis. The following peptides had a high antigenicity score, with low cross-reactivity, and were used for antibody production: CTDNTQTQRKKKRK, RRNNIYELNPHKEPC and DSDGGAVREKSYSNC. These peptides were manufactured and used for raising antibodies in New Zealand White Rabbits by Pacific Immunology. Briefly after injection of the animals with the peptides coupled to an adjuvant, blood serum was collected after 4 months and its peptide-specific reactivity tested by ELISA. The serum was then affinity purified and then re-tested by ELISA. Antibodies that showed peptide specific activity were then shipped to us. I further validated the specificity and reactivity of the antibodies to the antigenic peptides using the dot-blot assay described above.

Preparation of TAP-Tagged BRWD1 using BAC Recombineering

As my efforts to develop a working antibody against BRWD1 were unsuccessful, I decided to epitope tag the gene in the N-terminal and C-terminal and create a transgenic mouse. TAP-tag (Tandem affinity purification tag) was my choice for epitope-tagging. TAP-tagged proteins can be pulled down and eluted in native conditions that allows for the purification and identification of native protein complexes by mass spectrometry (Gloeckner, Boldt et al. 2007). The SF-TAP tag that I used was as described by Gloeckner et. al. 2007, and is a 4.6 kDa tag that combines a Strep-tag II and FLAG tag in tandem.

I attempted to insert the TAP tag into both the N-terminal as well as the C-terminal of *Brwd1* by BAC-recombineering using the modified DH10B strain SW102 that uses a *galK* positive/counterselection cassette as described by Soren Warming (Warming, Costantino et al. 2005).

Cloning of FLAG-BRWD1 into pCAGGS vector

A vector pdream2.1(CMV)FLAGBRWD1 containing the complete BRWD1 sequence with a FLAG tag was purchased from Genscript. FLAGBRWD1 was then amplified from this vector using BIO-X-ACT Long DNA polymerase and cloned into a pCAGGS expression vector using the enzyme XhoI. Clones were verified by sequencing.

Cloning tcp10 promoter into pdream2.1(CMV)FLAGBRWD1

A full length *Tcp10-bt* promoter was amplified from constructs described in the paper by Ewulonu et al. (Ewulonu, Buratynski et al. 1993). The promoter was cloned into the pDREAM2.1(CMV)FLAGBRWD1 using the enzymes FseI and PacI. *Tcp10* promoter was cloned upstream of FLAG BRWD1 and replaced the CMV, p10 and T7 promoters in the original pDream2.1(CMV)FLAGBRWD1. Clones were sequenced and verified.

Preparation of Transgenic Mice from pdream2.1(tcp10)FLAGBRWD1 by Pronuclear Injection

A sequence-verified pdream2.1(tcp10)FLAGBRWD1 clone (3 ug of plasmid DNA) was digested by enzymes XmaI and MluI to create a linear fragment of FLAGBRWD1 driven by *tcp10* promoter. This was then purified from a low-melt agarose gel without using a DNA-chelator dye such as ethidium bromide using the QIAquick gel extraction protocol. The gel-purified DNA was eluted in Injection buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA, 100 mM NaCl). About 2ul of gel-purified DNA was run on an Et-BR stained gel and quantified roughly by running a marker of known concentration in parallel. The DNA was diluted to a concentration of 3 ng/ul for micro-injection. In the meantime, donor females (FVB/n strain) were primed with 2.5 IU PMSG and after 48 hours superovulated with 2.5 IU Human Chorionic Gonadotropin (hCG). On the day of superovulation, about 10 females were set up for mating with males. The next day, all females were sacrificed and embryos collected onto M2 medium (Millipore). After screening embryos for the prescence of pronucleii, DNA was micro-injected into the male pronucleus of each embryo. About 109 embryos were microinjected, but only 72 survived after microinjection. The embryos were cultured overnight to the 2-cell stage. Only about 54 embryos survived to the 2-cell stage, and all surviving embryos were transplanted into 4 recipient pseudo-pregnant female mice (2 CD1 strain and 2 B6D2F1 strain). After pups were born, founder mice were screened by genotyping for the insert. The following primers were used for genotyping: brwd1L: GTCCGATGCTGGATAAGGAG and

brwd1R:AACCATGTACCGCTGAGGTC. Prescence of a 500 bp PCR product confirmed successful integration of the FLAG BRWD1 construct.

Preparation of Transgenic mice from pdream2.1(tcp10)FLAGBRWD1 by ES cell Injection Method

pdream2.1(tcp10)FLAGBRWD1 was digested into a linear fragment containing (tcp10)FLAGBRWD1 with a neomycin resistance marker. This was then used to transfect ES cells (from C57BL/6J albino strain from JAX) by electroporation and stable ES-cell clones were made by growing them under neomycin selection. Transfected ES cells were karyotyped to check for any abnormal chromosome configurations. Briefly cells were grown until they were 50-80% confluent and colcemid stock added at a concentration of 20ng/ml. They were then incubated at 37^{o}C for 30 minutes in an incubator after which they were trypsinized and pelleted. The cells were then resuspended in 100ul PBS, and 0.075 M KCl was added drop by drop until the final volume was 7 ml. This was then incubated for 6 minutes in a 37^{o}C water bath. Cells were spun down, and resuspended in 100ul PBS and 2 ml of freshly made ice-cold fixatve (3:1 Methanol:glacial acetic acid) was added. The resulting suspension was incubated in ice for 1 hour at 4^{o}C. The fixing step was repeated twice with 30-minute incubations on ice and finally the pellet was resupsended in 500 ul fixative. The final suspension was dropped from shoulder's length onto an ic-cold slide placed at a 45^{o} angle and the slides were allowed to dry for 5-10 minutes. Finally the slides were stained in Giemsa (GIBCO Karyomax Cat#`00092-021) dissolved in Gurr's Buffer (0.004M

Phosphate Buffer pH 6.8). The slides were washed three times in water and allowed to dry. These slides were then observed under a light microscope and any abnormalities in chromosome number and configuration was noted. ES cell clones with no abnormalities were then used for injection into blastocysts that were then transferred into the uterus of pseudo-pregnant female mice of C57BL6/J strain. Three rounds of injection were done out of 4 chimeric mice were identified by coat-color. Chimeric pups that contained the insert were mated with WT females (C57Bl6/J background), and their litters genotyped for presence or absence of (tcp10)FLAGBRWD1. The following primers were used for genotyping by PCR: brwd1L: GTCCGATGCTGGATAAGGAG and brwd1R:AACCATGTACCGCTGAGGTC. Presence of a 500 bp PCR product would confirm germline transmission of the construct.

Transient Transfection of HEK cells.

The pCAGGS-FLAG-BRWD1 plasmid was then transfected into HEK cells transiently using TransIT-LT1 reagent from Mirius as per manufacturer's instructions. Briefly about a million freshly grown HEK cells were plated on a 10 cm plate, 24 hours before transfection. 2.5ug of plasmid DNA was added to 250ug Opti-MEM I Reduced Serum and mixed gently. 7.5 ul of TransIT-LT1 Reagent was added to the DNA mixture and the solution was incubated for 15-30 minutes at room temperature. The DNA-*Trans*IT-LT1-Opti-MEM mixture was then added on top of the semi-confluent HEK cells. The cells were then grown at 37^{o}C for 72 hours to allow for expression of protein. Transfection Efficiency was

judged by co-transfecting with vector that expressed mCHERRY protein. Cells expressing mCHERRY were easily identified as those cells that fluoresced red under a fluorescent microscope.

Immuno-staining of Transfected Cells.

HEK cells transiently transfected with pCAGGS-FLAG-BRWD1 were trypsinized, washed and re-suspended in 1% paraformaldehyde for 3 minutes. After pelleting at 360 g for 3 minutes, the cells were fixed again in 1% PFA for 3 minutes. 1-10 million cells were then transferred into a chamber-slide and washed three times with PBS. After the final wash, PBS was replaced with blocking solution (*2.5% goat serum, 2.5% donkey serum, 1% BSA, 2% gelatin, 0.1% Triton X100*), and then treated with 0.1% Triton X 100 separately for an hour for nuclear permeabilization. The blocking solution was then replaced by Rabbit anti-FLAG antibody (Abcam ab124462) at a final concentration of 10ug/ml and incubated overnight at 4^{o}C. Next day, the cells were washed 4 times with PBS for 5 minutes each and then incubated with secondary antibody (Alexa Flour 488 anti Rabbit CY5) at a dilution of 1:80 for 20 minutes in dark at room temperature. After washing 4 times with PBS for 5 minutes each, the cells were mounted on slides with mounting medium that contained DAPI, which stained for the nuclei. These slides were observed under a fluorescent microscope.

Sub-cellular Fractionation and Western Blotting

Transiently transfected HEK cells were harvested and fractionated as per Mendez J and Stillman B et al, 2000 (Mendez and Stillman 2000). Briefly, about 10 million cells were harvested, washed thoroughly with PBS and then suspended in Buffer A (10mM HEPES, pH 7.9, 10mM KCl, 1.5mM MgCl2, 0.34M sucrose, 10% glycerol, 1mM DTT and protease inhibitor cocktail). Triton-X-100 was then added to a final concentration of 0.1% and the cell suspension was incubated on ice for 8 minutes. The suspension was then centrifuged at 1300g to separate the nuclear fraction (P1) from the cytoplasmic fraction (S1). S1 was further centrifuged at 20,000 g at 4^{o}C to collect a clarified supernatant free from debris. P1 on the other hand, was washed thoroughly with buffer A and lysed for 30 minutes using Buffer B (3mM EDTA, 0.2mM EGTA, 1mM DTT and protease inhibitor cocktail). The lysed fraction was then centrifuged at 1,700 g for 5 minutes at 4^{o}C, separating the supernatant from the pellet. The pellet contained the chromatin, whereas the supernatant contained the nucleoplasmic fraction. The chromatin fraction was further washed once with Buffer B and then re-suspended in SDS sample buffer, ready to load in a poly-acrylamide gel.

Protein samples were thus prepared and 15ul loaded onto a 6-15% gradient polyacrylamide gel. After sufficient separation, as judged by the running of a marker in parallel, the gel was dislodged from the electrophoresis apparatus and the proteins were transferred onto a nitrocellulose membrane overnight at 40V. The next day, the blot was blocked using 15% milk for 1 hour and then incubated

overnight again with a FLAG antibody at a final concentration of 2ug/ml (Abcam ab124462). After thorough washing with Tween-TBS (5 times of 5 minutes each), the blot was incubated with an anti-rabbit HRP conjugated secondary antibody that was diluted 1:2500. The blot was washed again thoroughly with Tween-TBS (5 times of 5 minutes each) and then probed with ECL substrate. An X-ray film was exposed to the blot for 1 minute in the dark and developed and scanned. The blot was then later stripped and re-probed for either fibrillarin (a nuclear marker) or GAPDH (cytoplasmic marker) similarly.

In-vitro Transcription of FLAG-BRWD1

I linearized *pdream2.1FlagBrwd1* by digesting with Not1 enzyme. I then purified it using a standard sodium acetate-ethanol precipitation, and the precipitated DNA was resuspended in RNase free water to a final concentration of 1 ug/ul. I used 2 ug of linearized DNA as template for an *in-vitro* transcription reaction using mMESSAGE mMACHINE kit (Ambion). The reactions were set-up and RNA purified as described in the kit. I then performed a poly-A tailing reaction to the transcribed *Flag-Brwd1* mRNA using a poly(A) tailing kit (Applied Biosystems). The reactions were performed and RNA purified and quantitated as described in the kit.

Microinjection of transcribed FLAG-Brwd1 mRNA into oocytes

Female mice of C57B6/J background were primed with an injection of 5 units of PMSG followed by 5 units of hCG 24 hours later. They were then sacrificed

around 12-14 hours later. Microinjection was performed exactly as described in the protocol by Paula Stein 2009, Cold Spring Harbor Protocols. I injected about 6 million molecules of RNA into each oocyte.

Immuno-staining of microinjected oocytes for expressed FLAG-Brwd1

After microinjection of *Flag-Brwd1* mRNA into the oocytes, the oocytes were cultured for 24 hours in an incubator at 37^{o}C to allow for expression. The oocytes were then washed twice for 5 minutes with PBS containing 2% tritonX-100. They were then fixed in fresh fixing solution for 30 minutes at 37^{o}C (0.1 M PIPES, 5 mM $MgCl_2$, 2.5 mM EGTA, 0.5% Triton X-100, 2% Formaldehyde). After a brief wash in 0.1% Goat Serum for 15 minutes, the oocytes were transferred to a blocking solution containing 10% Goat Serum for 1 hour at 37^{o}C. A rabbit Anti-Flag Antibody (Abcam) was then applied to the oocytes at a final dilution of 1:100. The oocytes were then washed in 10% goat serum for 1 hour at 37^{o}C, after which they were transferred into a 5% goat serum solution containing FITC anti rabbit secondary antibody (1:80 dilution) for 1 hour at 37^{o}C. The oocytes were then washed again in 10% goat serum for 1 hour at 37^{o}C, after which they were mounted on slides containing mounting medium with DAPI. They were then observed under a fluorescent microscope.

In-Situ Hybridization

Testes were frozen in Optimal Cutting Temperature Medium (OCT) using dry ice and cryostat sections were cut at a depth of 15 um. They were then fixed with 4% PFA in PBS for 10 minutes at RT and washed 3X with PBS. After fixation, the slides were incubated in 0.1 M triethanol amine “acetylation solution” for 10 minutes at RT followed by three washes with PBS. The probe was applied at a concentration of 200-400ng/ml in hybridization buffer on the slides and incubated at 65^{o}C overnight in a humid chamber. Probe sequences are available on request. The slides were then washed for extensively using 0.2X SSC at 65C. After cooling, the slides were blocked with blocking buffer (10% sheep serum, 0.2% Block Reagent from Roche in TBS). The buffer was then replaced with alkaline-phosphatase conjugated sheep anti-digoxigenin antibody (1:500) in blocking buffer, and incubated overnight at 4 C. This was washed 3X with TBS for 10 minutes and then for 5 minutes using Buffer 3 (0.1 M Tris, 0.1 M NaCl, 50 mM MgCl2, 2 mM levamisole) and finally incubated with 1X NBT/BCIP solution at room temperature for 1 hour. Appearance of a blue color corresponding to the hybridized RNAs was monitored using a dissecting microscope.

Squash Preps and Immuno-staining

Testes were dissected from 2 month-old adult WT and mutant testes and the albuginea was removed. Seminiferous tubules were eased out and cut into little 10micron pieces using microscissors. The tubules were than placed on a Superfrost Plus slide (FisherBrand) and squashed using a glass cover slip,

releasing the germ cells onto the slide. The slides were either frozen at -80^{o}C or used directly for immunostaining.

Blocking solution containing 10% goat serum and 0.1% Tween was added directly on top of the cells for 1 hour. The slides were then incubated overnight with multiple dilutions of primary antibodies (1:50, 1:250 and 1:500) with a PBS negative control. They were then washed next morning 3X with PBS for 10 minutes and then incubated with corresponding secondary at 1:80 for 20 minutes in the dark. This was washed again 3X with PBS and mounted with medium containing DAPI and observed under a fluorescence microscope.

Germ Cell Separation – Isolation of Round Spermatids and Spermatocytes. Round spermatid and Spermatocyte fractions were prepared from a single cell suspension using unit gravity sedimentation exactly as outlined by Wykes et al. 2003 (Wykes and Krawetz 2003). The cell separation steps were done using a CELLSEP apparatus designed by Du Pont Company (SORVALL products).

Dnase I Hypersensitivity Assay.

The method I describe here is based of the unique protocol described by Martins et al. 2007 (Martins, Platts et al. 2007). In this protocol, Martins et al. describe how to track chromatin changes around the protamine domain by treating it with varying concentrations of DNase I and then amplifying the digested products using real-time PCR. Dnase I treatment and whole nuclei of germ cells were

prepared from a single cell suspension (either germ cell suspension prepared from testes or fractionated spermatid or spermatocyte fraction) using the protocol described by Sambrook and Russell Cold Spring Harbor Protocols 2006. Briefly about a million to 100 million cells were resuspended in 1.5 ml of ice cold lysis buffer (0.05% v/v saponin, 200 mM beta mercaptoethanol, 100 mM KCl, 50 mM Tris-CL (pH 7.9), 50% (v/v) glycerol, 5 mM $MgCl_2$) for about 15 minutes on ice. The resulting suspension contained lysed cells but intact nuclei. 10 ul of the nuclei preparation was tested using a Trypan Blue staining. Intact nuclei stained blue. The nuclei were then recovered from the lysed cells by centrifuging at 1300g for 15 min at 4 C. The pellet was then washed in 1.5 ml ice-cold buffer A (50 mM Tris-Cl (pH 7.9), 3 mM $MgCl_2$, 0.2 mM PMSF, 100 mM NaCl, 1 mM Dithiothreitol) and then resuspended in 4 ml of buffer A. DnaseI (Worthington) solution of 10 Units/ul was serially diluted (1:40 to 1:2560) in Dnase I dilution buffer (10 mM HEPES-KOH (pH 7.9), 30 mM $CaCl_2$, 30 mM $MgCl_2$, 50% (v/v) glycerol). 180 ul of the nuclear suspension was aliquoted into single tubes and this was then treated with the serially diluted Dnase I solute for 20 minutes at 37 C. The reactions were terminated by the addition of three separate aliquots of 16.6 ul of 0.5 M EDTA with vortexing. 12 ul of RNase solution (10 mg.ml Rnase A in TE Buffer) was then added to the individual aliquots followed by incubation at 37 C for 30 minutes. Finally 40 ul of proteinase K solution (0.2 mg/ml proteinase K in 50 mM Tris-Cl (pH 7.9) and 100 mM NaCl) was added along with 100 ul of SDS buffer (20 mM Tris-Cl (pH 7.9), 70 mM EDTA (pH 8.0), 100 mM NaCl, 2% (w/v) SDS) and the tubes were incubated overnight at 50 C. The digested DNA

was then extracted using a standard phenol-chloroform method and resuspended in 50 ul TE buffer. The quality of digestion was checked by running samples on a 1% agarose gel. The resulting aliquots were then diluted 1:100 for a real-time PCR with primers designed across the hypersensitive sites on genes. The real-time PCR was done as described previously.

Chromatin-Immunoprecipitation Assay

Germ-cell suspension and spermatocyte and spermatid fractions were made as described previously. Cells were fixed with formaldehyde at a final concentration of 1% (v/v) for 10 minutes. After washing twice with PBS, the cells were then lysed with 1 ml SDS-Lysis Buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH8.1) and sonicated such that DNA were broken into fragments that were 500-1000bp in length. After saving 10% of the fixed cells as input, I centrifuged the sonicated suspension at 20,000 g for 15 minutes at 4 C to pellet all the cell debris. Before immunoprecipitation, this suspension containing chromatin was pre-cleared with Protein A beads (Millipore). The pre-cleared chromatin was then incubated with antibodies against the epigenetic marks H4K16Ac, H3K9Me2 and H3K9/14Ac (1-2 ug each/vial) overnight at 4 C. This suspension containing a mixture of chromatin fragments and antibodies was then incubated for 2-4 hours with a 50% solution of protein A agarose bead slurry. This was centrifuged at a speed of 4000 rpm to remove the supernatant, and the pelleted beads were washed with 1 ml of wash buffer (0.1% SDS, 1% TritonX-100, 2mM EDTA pH 8, 150 mM NaCl and 20 mM Tris-HCl pH 8) and 1 ml of final wash buffer (0.1%

SDS, 1% TritonX-100, 2 mM EDTA pH 8, 500 mM NaCl, 20 mM Tris-Hcl pH 8). The bound chromatin was then eluted at 65^{o}C by incubating the beads with 5ul of elution buffer (10 mM Tris pH 8, 1 mM EDTA and 1% SDS) for 10 minutes. The beads were then spun down and discarded. 120 ul of elution buffer was further added to the eluted chromatin to make a final volume of 170 ul, which was then incubated at 65^{o}C overnight. The next day, 150 ul of a proteinase K mix (1 X TE Buffer, ph 8, 10 mg/ml glycogen, 20 mg/ml proteinase K) was added to each tube and incubated for 2 hours at 37^{o}C. The DNA was then extracted using a standard phenol-chloroform extraction method and suspended in 30 ul of RNase TE (0.3 ug/ul RNase A in TE buffer). This solution was then incubated at 37^{o}C for 2 hours and the DNA purified using a standard QIAquick PCR purification kit. The DNA was then amplified using a Whole Genome Amplification Kit (Sigma) according to manufacturer's instructions. After amplification, a PCR was done with primers designed across the protamine domain. The primers are listed in table. The distance from transition start site of *Prm2* gene is listed against each primer set.

Table 2.2 Primer Sequences along Protamine Domain used for ChIP-qPCR

Region	Primer Sequence	Distance from *Prm2* TSS (0kb)
A	AL:CCATGAAGCCTGTATCACAT AR:AAAAGGAGAGGGAGTTGTAGT	-20.4 kb
B	BL:CAAATTGGGACTCTATTGAGG BR:TGTAACTGACGTTTCATGTGAC	-8.2 kb
C	CL: CCACAGACGGCACAACT CR:AGTGAGTAGATATGTGCGGATG	-6.5 kb
D	DL:TCCTGGTCCTCTTTGACTTCATAAT DR:ATCTGCTCCTGCTTTTGCTG	-5.2 kb
E	EL:LGCGGAGGAGGCGAAGTAAGT ER:AGCCCTTCCCATTTGACCAGT	-4.9 kb
F	FL:GAGGCCATCTCACATTCAATA FR:GCTCCTCATTCGGTAGCG	-0.6 kb
G	GL:ATGCAGGTGCAGGAAATGTAGGAG GR:AGCTCAGGGCTCAGACATCGAC	0 kb
H	HL:CCACACCCACCAGTCTCACA HR:CTCTCTTGCTTTTCGGGCTCC	0.7 kb
I	IL:AATCGGCCCAGCTATATAACTAGG IR:CACTGGTTACTGGTGTGACTTTGA	2.9 kb
J	JL:GTGTCCATTTGATCCCAA JR:AAAGCTGCTGACTCAAGACTA	3.6 kb
K	KL:AGGGGAGGAGGCGTGTCAGC KR:TTCTCTTTTCCGGCAAGTCTT	5.8 kb
L	LL:TTAAGTGGGGGGCGCCTTATTA LR:CCTGAGAGGTGGGGATGAGGTC	7.4 kb
M	ML:GCTCTAATTTTCCACCTAACA MR:CCAGACTGTGCTCCATAAG	18 kb

Creation of Phip knock-out mice

Phip knock-out mice were created by micro injecting ES cells containing the ene trap RRJ571 (Baygenomics) into C57BL/6J blastocysts. The chimeric mice male founders were crossed to C57BL6/6J females to generate F1 heterozygous (*Phip+/-*) mice. These F1 heterozygotes were then mated with each other to create *Phip-/-* mutants. All genotyping was done using PCR as described in Li et al. 2010 (Li, Francisco et al. 2010).

Mating Scheme to produce double mutants

For creating mice that were doubly mutant, $Brwd1^{+/-}$ mice of C3H background were mated to $Phip^{+/-}$ in a mixed C57BL/6J-129 background to create doubly heterozygous mice $Phip^{+/-},Brwd1^{+/-}$. The double heterozygotes (now in a mixed C3H-C57BL6/J-129 background) were than mated with each other in order to create doubly mutant $Brwd1^{-/-};Phip^{-/-}$ mice. Pups were collected either at wean-age or 2 days after birth and genotyped by PCR. Timed matings were also performed and embryos collected from plugged females after either 12.5dpc or 18.5dpc. Embryos were fixed with 4% paraformaldehyde and genotyped. Double mutants were homozygous for both *Phip* and *Brwd1* mutant alleles as identified by PCR. *Phip* genotyping was done using PCR as described in Li et al. 2010 (Li, Francisco et al. 2010). *Brwd1* genotyping was done by amplifying the fragment containing the mutation from genomic DNA by PCR and then sequencing the PCR product.

CHAPTER 3

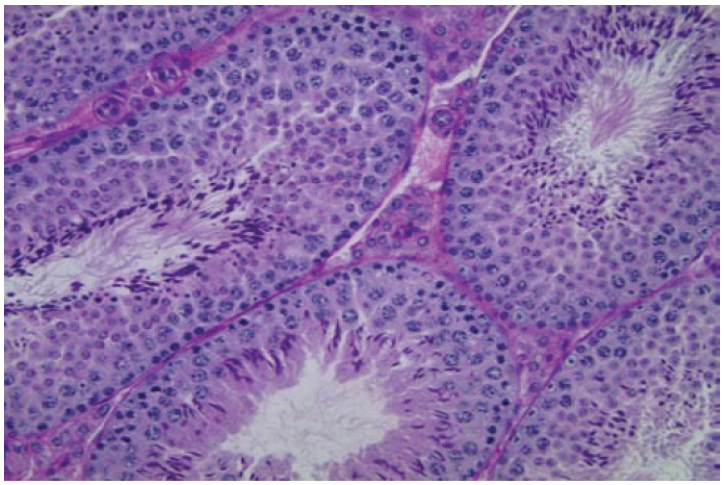
RESULTS

Brwd1^{Gt} fails to complement Brwd1$^{repro5/repro5}$ mutation.

In order to confirm if the observed infertility phenotype was indeed due to the *Brwd1$^{repro5/repro5}$* mutation, I created *Brwd1^{Gt}* mutant animals that contained a mutation in the *Brwd1* gene as outlined in the methods section. *Brwd1$^{Gt/+}$* heterozygotes were crossed to *Brwd1$^{repro5/+}$* heterozygotes to create *Brwd1$^{Gt/repro5}$* compound heterozygotes and their phenotype was evaluated.

Similar to the *Brwd1$^{repro5/repro5}$* homozygotes, the compound heterozygotes were infertile. The seminiferous tubules of these animals contained fewer spermatozoa than controls and the spermatids exhibited a wide variety of abnormally shaped heads and acrosomes (Fig 3.1). The epididymal sperm count was reduced > 5 fold. The sperm displayed poor motility with morphological abnormalities similar to *Brwd1$^{repro5/repro5}$* homozygotes (Table 3.1). Sperm count was ~3 fold lower than controls. The proportion of abnormalities in the *Brwd1^{Gt}* homozygotes however was lower than the *Brwd1^{repro5}* homozygotes. This maybe because the genetrap allele may not be a complete null or that the introduction of the 129-strain background suppresses the phenotype slightly. Overall, however, the *Brwd1^{Gt}* allele does not complement the *Brwd1^{repro5}* mutation, confirming that a mutation in the *Brwd1* gene is responsible for the observed infertility phenotype.

A



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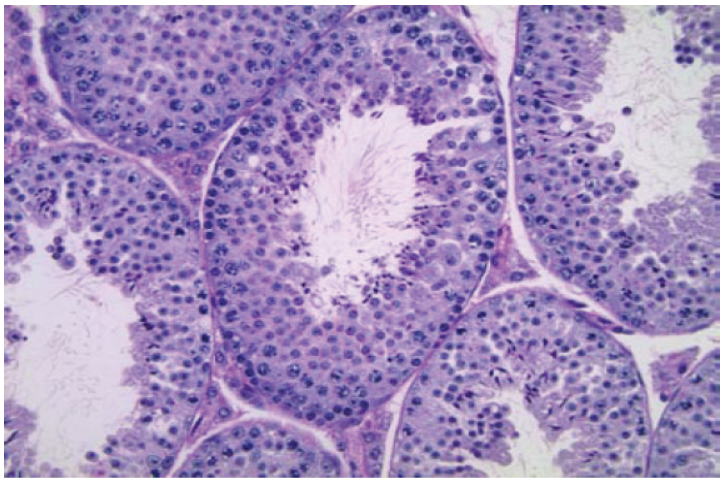

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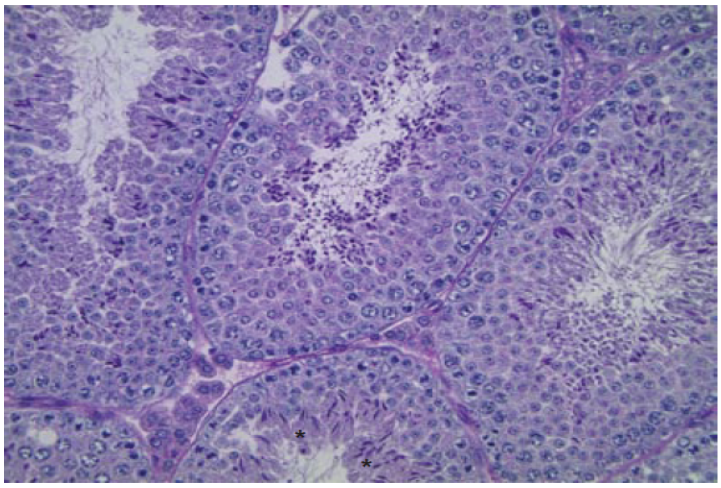

Fig 3.1 Testes Histology of Genetrap mice. Testes were sectioned and stained with PAS. A. Wild-type B. *Brwd1*$^{repro5/Gt}$ C. *Brwd1* $^{Gt/Gt}$ In both panels B and C, a marked decrease in the number of sperm is seen. Sperm heads in panels B and C also look morpholgically defective.

Table 3.1 Sperm count of Genetrap mice and Percentage of Abnormal Sperm

Animal. No	Genotype	Sperm/ml	% Abnormal Sperm
1	*Gt/+*	$1.0x10^6$	4%
2	*+/+*	$1.3x10^6$	8%
3	*Gt/+*	$9.0x10^5$	11%
	Average	**$1.0x10^6$**	**7%**
4	*repro5/Gt*	$2.0x10^5$	98%
5	*repro5/Gt*	$1.7x10^5$	97%
	Average	**$1.85x10^5$**	**81%**
6	*Gt/Gt*	$3.3x10^5$	81%
7	*Gt/Gt*	$1.9x10^5$	86%
8	*Gt/Gt*	$4.5x10^5$	95%
	Average	**$3.2x10^5$**	**87.3%**

Haploid genome transcription is disrupted in Brwd1-deficient testes.

Since BRWD1 contains bromodomains typically associated with acetylated histones, I hypothesized that BRWD1 is involved in chromatin remodeling required for proper transcription in post-meiotic spermatids and maturing oocytes. To test this hypothesis, I performed microarray-based gene expression profiling of 27-day old WT (N=4) and mutant (N=4) testes. At this stage, the first wave of germ cells has progressed to the elongating spermatids stage. In the mutant, 286 transcripts were decreased by at least two fold compared to WT, while 11 transcripts were over-expressed (Fig 3.2). Nine of the most affected transcripts were tested by quantitative real-time RT-PCR (qRT-PCR), validating their mis-expression. In contrast, none of 11 potentially over-expressed genes were validated by real-time PCR. Transcript levels of most of the under-expressed genes were decreased more than four-fold; a few of them such as the protamines and transition proteins were decreased as much as thirty fold. To exclude the possibility that the lower transcript levels observed in mutants might

be due to a decrease in elongated spermatids in *Brwd1*$^{-/-}$ testes, or a delay in meiotic progression, two experiments were performed. First, I isolated germ cells from both WT and mutant testes, stained them with propidium iodide, and analyzed the populations by flow-cytometry. The amount of fluorescence was proportional to the DNA content of the cell, enabling us to quantify the relative proportions of "1C" (haploid spermatids), "2C" (primarily diploid spermatogonia) and "4C" (primarily spermatocytes). In 27-day testes, there was no significant difference in the proportion of haploid "1C" cells between WT and the mutant (Fig 3.2). Second, selected transcripts in 21-day old WT and mutants testes were compared by qPCR analyses (primarily *Prm1, Tnp1* and *Tnp2* – all of which are mix-expressed in the microarray). Significant differences in expression were also apparent at this time point, which is when the first round spermatids normally appear (Fig 3.2). Because there were no differences in % of haploid spermatids between WT and mutant, and the expression differences were apparent immediately at the post-meiotic stage, this further argues that the expression differences were not due to a delay in meiosis or postmeiotic development. These data suggest that BRWD1 is required for proper expression of genes in spermatids.

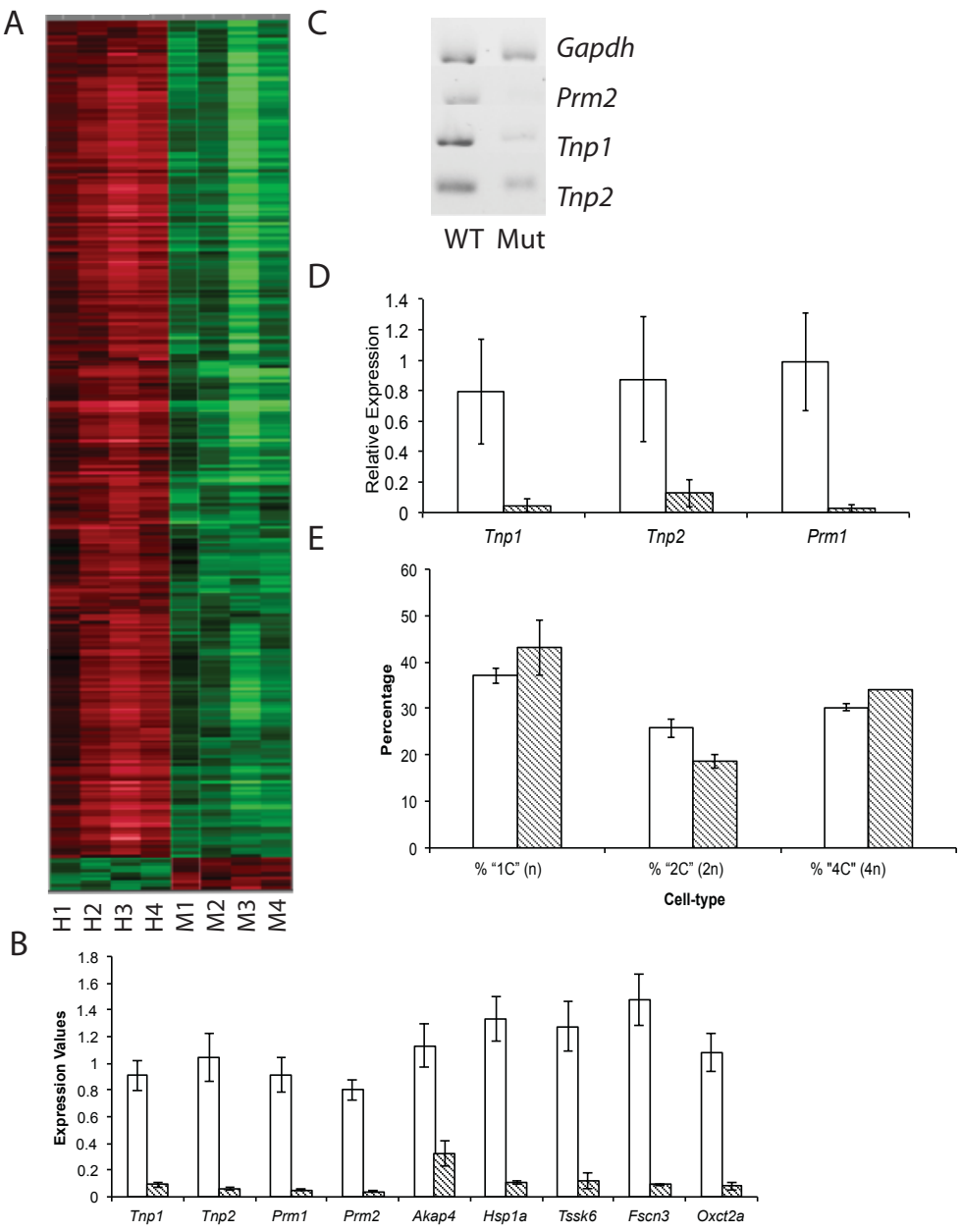

Fig 3.2 Expression of genes involved in spermiogenesis is affected in *Brwd1* mutants. A. Heat Map Comparison of gene expression in 27-day old *Brwd1*$^{+/-}$ heterozygous mice (H1to H4) vs 27-day old *Brwd1*$^{-/-}$ mutant mice (M1 to M4). Expression levels are denoted by color – Bright green indicates no or very low expression to bright red standing for highest expression. B. Differences in Expression Levels are verified by an independent real-time PCR experiment for a few candidate genes. C. Semi-quantitative PCR shows very little expression of the most-down-regulated genes *Prm2, Tnp1* and *Tnp2* in the mutant testes. D. Real-Time PCR performed on RNA extracted from 21-day old testes shows differences in expression already. E. Flow Cytometry Analysis of the Cells based on ploidy shows that there are no differences in the number of "1C", "2C" and "4C" cells in the mutant testes

To test whether BRWD1 is required specifically for haploid gene expression, we classified the mis-expressed genes as being meiotic (spermatocyte specific), post-meiotic (spermatid specific), somatic (Sertoli cell specific) or mitotic (spermatogonia specific) using data from a comprehensive characterization of differentially expressed genes in the testes from the GermOnline database (Chalmel, Rolland et al. 2007, Gattiker, Niederhauser-Wiederkehr et al. 2007). 157 of these genes were found to be differentially expressed in testes (Chalmel, Rolland et al. 2007). 139 of these genes were further clustered as “post-meiotic”, but only 6 as “meiotic”, 6 as “mitotic” and 6 as “somatic”. The rest 140 genes were not found in the dataset of differentially expressed testes specific genes as reported by Chalmel et al. 2007. Thus, 87% of the mis-expressed genes in the mutant that are also considered differentially expressed in testes according to the Germonline database are clustered as post-meiotic. This is clearly an over-representation of post-meiotic genes, as only 20% of all the differentially expressed genes in the testes are clustered as post-meiotic in the Germonline database (Fig 3.3) (Chalmel, Rolland et al. 2007). Further, as depicted in the heat map of the expression data (Fig 3.2) almost all under-expressed genes from our experiment show high expression in the post-meiotic spermatids, and almost no expression in spermatogonia. Transcripts of some of these genes are present in spermatocytes, but most are exclusively post-meiotic. This suggests that BRWD1 predominantly regulates certain aspects of post-meiotic germ cell differentiation.

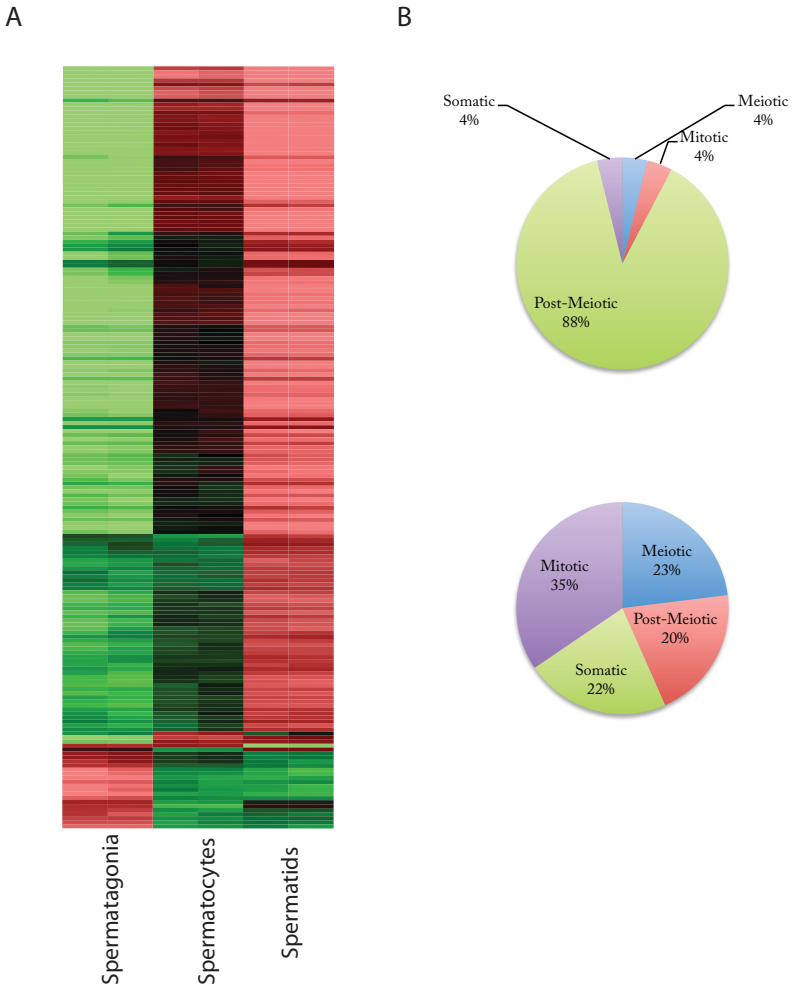

Fig 3.3 Characterization of the Genes Regulated by BRWD1 A. Heat Map shows the expression pattern of the mis-regulated genes across three cell-types: Spermatogonia, Spermatocytes and Spermatids. Level of Expression denoted by color, with bright green indicating no to low level of expression to bright red standing for high levels of expression. B. 1. Classification of the mis-regulated genes as meiotic, mitotic, somatic or post-meiotic based on data from GermOnline database. 2. Classification of all testis-specific transcripts on GermOnline Database for comparison.

Production of Antibodies to BRWD1

A previous post-doc (Dr. Dana Philipps) in my lab had collected antibody-rich serum and yolk from chicken injected with an antigenic C-terminal epitope of BRWD1. I purified this serum using Eggcellent Chicken IgY Purification kit as described in the methods section. I tested the specificity and activity of the purified antibody by a dot blot assay to the C-terminal peptide also described in the methods section. Reactivity of the purified antibodies was also tested for using a Western Blot assay of testes protein extracts. *Brwd1*$^{-/-}$ testes protein extracts served as negative controls. In spite of specific activity toward the C-terminal epitope in the dot blot assay, the purified antibody failed to recognize BRWD1 specifically in the Western Blot. There was a strong signal in the blot around the expected size of 250 kDa but it was also found in the *Brwd1*$^{-/-}$ lane, indicating that this band was non-specifc. A commercially available BRWD1 antibody from IMGENEX also did not recognize BRWD1 specifically.

As a specific antibody to BRWD1 was vital to our studies, I decided to re-make antibodies to three different antigenic epitopes of BRWD1 as determined by an online software - PROTEAN tool. I tested the activities of these new antibodies using dot blot and the Western Blot assays as described above. Again, all three antibodies did not show specific BRWD1 binding.

BAC recombineering to generate TAP tagged Brwd1 transgenic mice.

I used a well established protocol by Dr. Soren Warming that uses *galK* positive/counter-selection cassette (Warming, Costantino et al. 2005). In this method, the *galK* cassette is first inserted into the position in the gene in the BAC, where the tag must be inserted using a modified bacterial strain (SW102) that does not contain the *galK c*assette. The cassette is inserted at the precise position by flanking it with 400bp homology arms. The bacteria are then grown in media that contain galactose as the only carbon source, as a result of which, only those with a *galK* cassette survive. Furthermore these colonies appear pink in the medium of choice that is described by Dr. Warming. The pink colonies are subjected to another round of recombineering, this time using the TAP-tag with the same exact homology arms. This replaces the *galK* cassette in the bacteria. The bacteria are then grown on DOG medium; DOG is a harmless carbon source, unless phosphorylated by a functional *galK,* which turns into a toxic metabolite. Thus only bacteria that have successfully recombineered the TAP-tag will survive.

In this experiment, I was able to recombineer the *galK* cassette into the SW102 strain containing the BAC. However, many attempts to recombineer the TAP-tag into the galk-SW102 remained unfruitful. I decided to stop trouble-shooting this set of experiments, when a commercial FLAG-tagged pDREAM2.1(CMV)BRWD1 plasmid became available.

Creating Transgenic Mice using pDREAM2.1FLAG-taggedBRWD1

I then used the commercially available construct of FLAG-taggedBRWD1 for preparing transgenic mice. I first cloned the promoter of *tcp10* gene upstream of the *Flag-Brwd1* gene in *pdream2.1* vector replacing the CMV promoter. This promoter has been shown to drive male germ-line specific expression of genes, specifically during the pachytene stage (Ewulonu, Buratynski et al. 1993). I prepared transgenic mice using both pronuclear injection and ES cell injection methods. These methods are described in detail in Chapter 2.

From the pronuclear injection experiments, I obtained one male founder pup that had a successful integration of the transgene (out of 16 pups). This male was later out-bred to WT C3H females. From these females, he sired 26 pups. 12 out of 26 pups were positive for the transgene (~50%). I culled these mice at 35 days post partum (adult stage) and prepared protein extracts from their testes. I checked for expression of FLAG-BRWD1 in these mice by a Western Blot Assay and RT-PCR. None of the mice carrying the transgene expressed the tagged gene in the testes.

For the ES cell transfection method, I linearized the pdream2.1(tcp10)FlagBrwd1 plasmid into a fragment that contained the (tcp10)FlagBRWD1 along with neomycin cassette. I transfected this fragment into ES cells, and grew them under neomycin resistance to select for clones that had successfully integrated the transgene cassette. I was able to isolate 54 positive clones successfully. I

chose 20 clones at random and karyotyped them to check for any visible chromosomal defects. None of the clones had any defects. Out of these 20 clones, I chose 3 clones at random (C1, A8 and B5) and injected them into blastocyst stage embryos. A work-sheet of the number of embryos injected and transferred, as well as the number of pups born from each round of injection is shown in Table 3.2. In the end, I had 5 chimeras, 4 male and 1 female. The percentage chimerism is indicated in Table 3.2. None of the chimeras successfully transmitted the transgene in the germline. The female chimera was infertile and did not sire any offspring.

Table 3.2 Worksheet of Production of Transgenic Mice

Colony Injected	BRWD1C1	BRWD1A8	BRWD1B5
Embryos Transferred	37	35	40
Recipient Animals	3	2	2
Pups Born	3	5	0
Number of Chimeras and % Chimerism	1 chimeric pup with 40% chimerism	4 chimeric with 40-50% chimerism	0
Germline Transmission	No	No	N/A

Expression and Subcellular Localization of FLAG tagged BRWD1

Previous work has shown that *Brwd1* is transcribed in a variety of tissues and cell types, including brain, kidney, testis, pancreas, muscle, ovary, developing embryo and dividing cell lines (Huang, Rambaldi et al. 2003). In order to determine which cell types in the testes express *Brwd1*, RNA *in situ* hybridization was performed to cross-sections of seminiferous tubules. *Brwd1* transcripts were seen in spermatocytes and round spermatids (Fig 3.4)

Attempts to produce specific antibodies to BRWD1 failed, so I generated a plasmid construct containing full-length BRWD1 with an N-terminal FLAG tag. Following transfection of this expression construct into HEK cells, a clear nuclear localization was observed (Fig 3.4). Cytoplasmic, nucleoplasmic and chromatin fractions were prepared from the cells that expressed FLAG-BRWD1 using a subcellular fractionation protocol as described by Mendez and Stillman et al. (Mendez and Stillman 2000). These protein fractions were then run on a polyacrylamide gel and transferred onto a Western Blot. When the blot was probed using an anti-FLAG antibody, FLAG-BRWD1 was found only in the chromatin-bound fraction. Actin served as a negative control for chromatin-bound proteins, and was found only in the cytoplasmic and nucleoplasmic fractions. Fibrillarin on the other hand served as a positive control, and was found in both nucleoplasmic as well as chromatin bound fractions, as expected (Reimer, Pollard et al. 1987). There is the possibility that FLAG-BRWD1 is insoluble and

precipitates along with the chromatin fraction, especially under conditions that over-express the protein causing it to aggregate or mis-fold. I do not see any easily identifiable aggregate formation, however, in our immuno-stained cells that express FLAG-BRWD1.

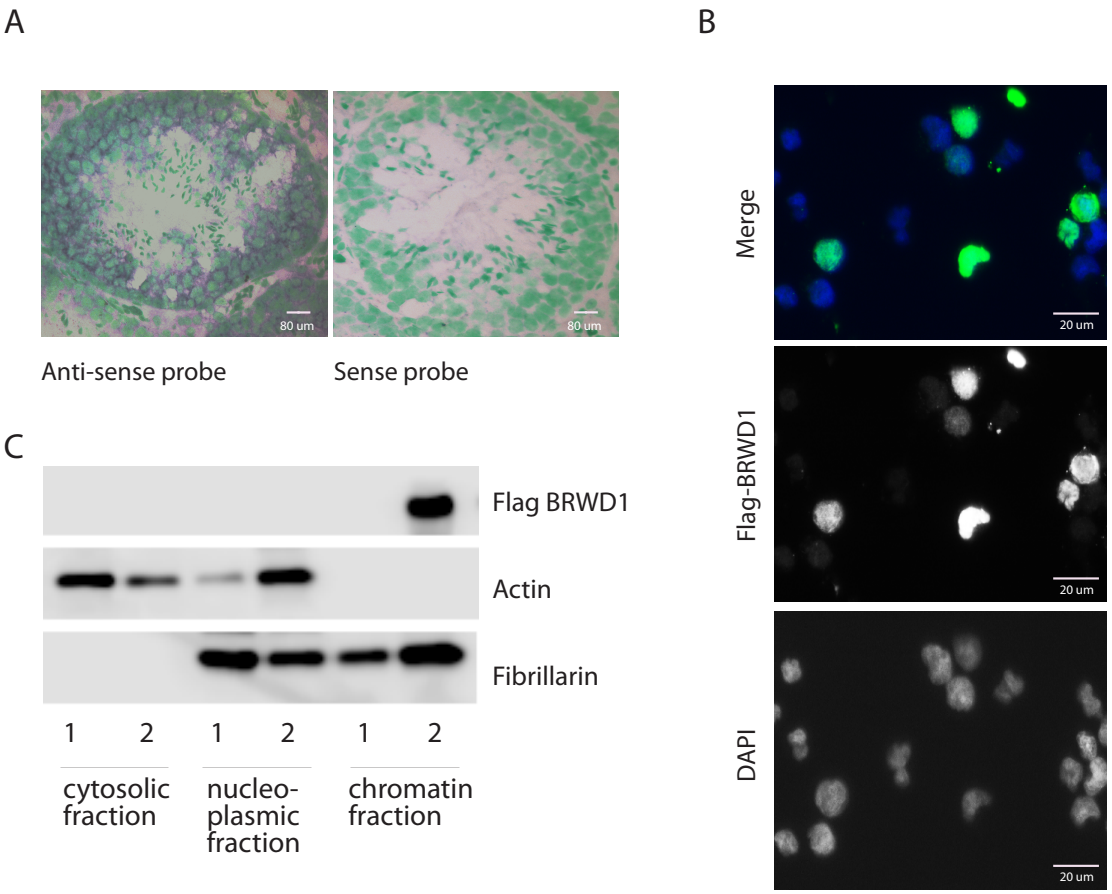

Fig 3.4 A. In-situ hybridization reveals *Brwd1* expression in spermatocytes and spermatids. B FLAG BRWD1 transiently expressed in HEK cells - a predominantly nuclear localization was observed in the nucleus. C. Flag-BRWD1 was also found exclusively in the chromatin fraction in a sub-cellular fractionation experiment. A cytoplasmic marker, actin and a nuclear marker fibrillarin were used as positive controls for the fractionation experiment.

In a separate experiment I tried to express FLAG-BRWD1 protein in oocytes to see where they localized. I expressed *Flag-Brwd1* mRNA, which I then micro-injected into the oocytes. The figure shows mRNA transcribed and poly-adenylated *in-vitro*. I then stained the oocytes with an anti-Flag antibody to look for expression of protein, but I was never able to detect any expression. This maybe because of many reasons, three potential reasons I have listed below:

1. Transcribed *Flag-Brwd1* was not of quality good enough for translation.
2. *Brwd1* is at least 8 kb long, making it more susceptible for degradation by RNAses.
3. Amount of RNA injected into the oocytes was not ideal.

BRWD1 deficiency does not cause gross disruption of the spermatid epigenetic landscape.

Epigenetic modifications of histones are central to transcriptional regulation, and the process of spermatogenesis occurs in the context of dramatic and wholesale chromatin remodeling (Hermo, Pelletier et al. 2010). Different nuclear domains are thought to exist within the round spermatid nucleus that can be distinguished by their unique epigenetic marks. Any defects in the distribution of these marks and formation of these nuclear domains may cause transcriptional mis-regulation in spermatids. Both *Brdt* and *Tlf* mutants show dramatic chromatin-architecture defects in spermatids that are associated with mis-regulated expression of genes

(Martianov, Brancorsini et al. 2002, Shang, Nickerson et al. 2007, Berkovits and Wolgemuth 2011). In both mutants, the chromocenter (the nuclear structure that contains peri-centric heterochromatin in round spermatids) is fragmented and spermatid elongation is affected. When the testis-specific demethylase JMJD1A is deleted, increased H3K9 mono- and di-methylation is seen by immunostaining in spermatids, and recruitment of CREM to the promoters of the protamine genes is affected, causing the spermatids to arrest in spermiogenesis. (Liu, Zhou et al. 2010). Thus, the reduced expression of genes in *Brwd1-/-* mutants may be due to disrupted global chromatin architecture or distribution of epigenetic marks.

To see if the global chromatin architecture is disrupted in spermatids in the *Brwd1*$^{-/-}$ mutants, I stained mutant spermatids with a number of epigenetic marks and chromatin associated proteins and looked for any dramatic differences from WT spermatids. I found that the chromocenter structure was preserved in the mutants as seen by staining for HP1alpha (Fig 3.5). I also found no major differences in the distribution of epigenetic marks between the *Brwd1*$^{-/-}$ spermatids and WT spermatids. No discernable differences were found in the distribution or intensity of staining of the transcription factors TBP and TLF. Thus, the extensive transcriptional mis-regulation observed in the mutants is not a result of gross defects in the chromatin architecture, or due to the altered distribution of epigenetic marks or transcriptional factors within global nuclear domains of spermatids. BRWD1 may however affect chromatin architecture around specific promoter sequences, and further studies are being undertaken to

test this hypothesis. Specifically, any altered epigenetic landscape around specific promoter sequences in the mutants can be discovered using ChIP-seq analyses of histone marks. I will be looking at the well-known epigenetic marks H3K4Me1, H3K4Me2 and H3K27Ac in the mutant mice to being with for any altered distribution around promoter sequences of post-meiotic genes.

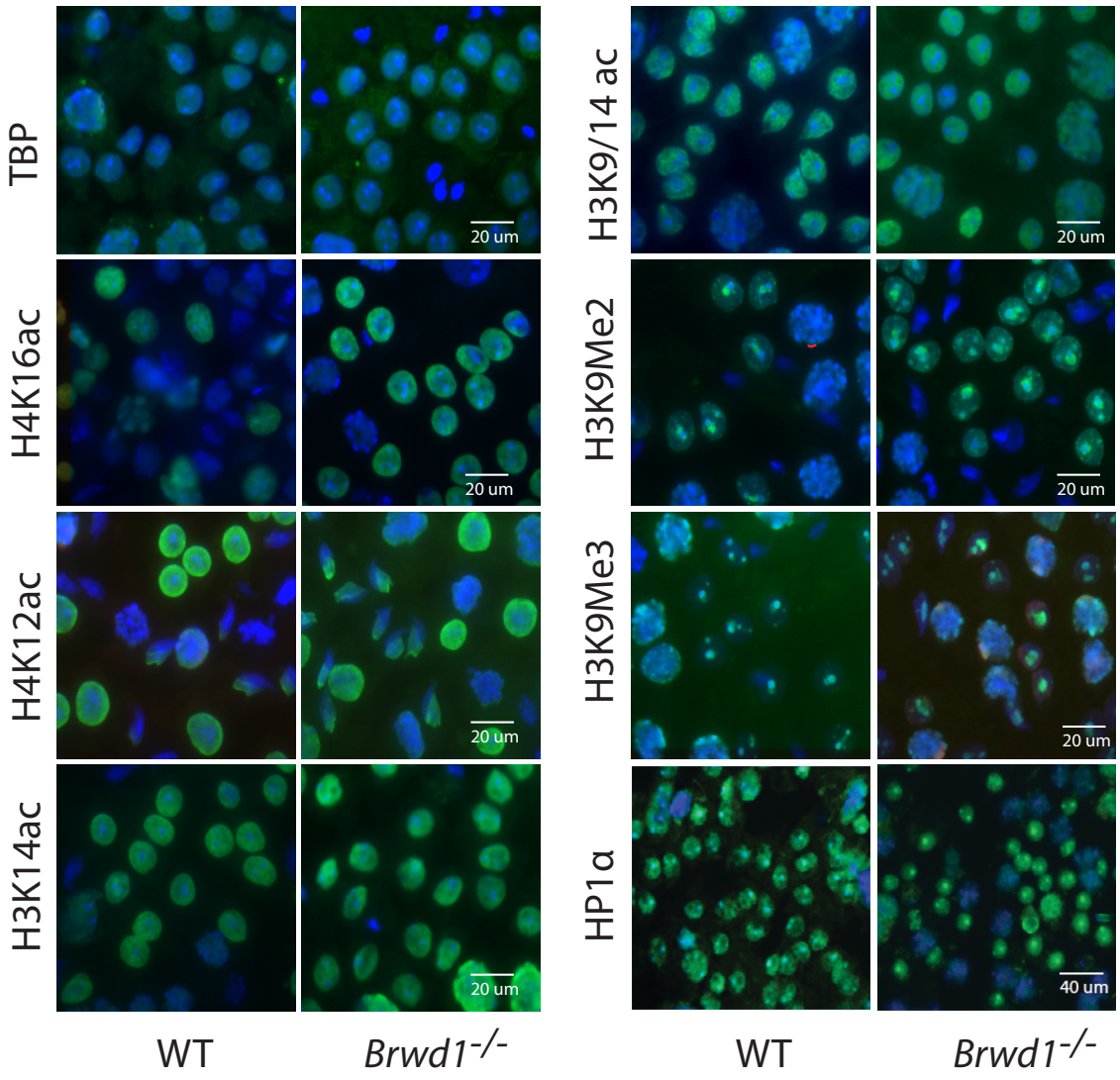

Fig 3.5 Staining for Epigenetic Marks and some chromatin associated proteins do not reveal any differences in their distribution between WT and mutant. This indicates that nuclear domains that are formed in spermatids are unaffected in the mutant. Global chromatin structures remain unaltered.

Preliminary Studies on Local Chromatin Architecture

As *Brwd1* deficiency did not affect global chromatin architecture, I attempted to look at local chromatin architecture around genes that were affected in the mutant. In particular, I chose to work with the "protamine domain" that contained a cluster of genes *Prm1, Prm2, Prm3* and *Tnp2*, all of which were under-expressed in the mutant. The chromatin architecture changes that take place in the protamine domain have been characterized as spermatocytes differentiate into spermatids (Martins and Krawetz 2007). Expression of all four genes in the "protamine domain" was affected in the mutant. One possible reason for this is that the chromatin around these genes has not become "potentiated" or ready for transcription to take place. Two signs of "potentiated" chromatin permissible for active transcription are

i. "open" chromatin architecture with lesser higher order structures; making it "hypersensitive" to Dnase I
ii. gain of epigenetic marks that are permissible to transcriptional activation. (Martins and Krawetz 2007)

In my first set of experiments, I attempted to compare the DNAse I hypersensitivity of chromatin containing the "protamine" domain of *Brwd1* mutants with WT configuration in spermatids. In my second set of experiments I looked for any changes in localized epigenetic marks in *Brwd1* deficient

spermatids that may explain the differences in expression of the genes in protamine domain in the mutant.

After several rounds of trouble-shooting with a single cell suspension prepared from mouse testes, I decided to do the assay using the protocol described by Sambrook and Russell 2006. After digesting the chromatin with DNase I, I attempted to quantify the index of hypersensitivity using a real-time PCR assay. Briefly, I designed primers to amplify across a hypersensitive site in the protamine domain and an actin gene site (which served as control). The more hypersensitive the site is, the more it is digested by Dnase I, thereby reducing template available for amplification. The Ct values of the real-time PCR products, therefore, increase with increasing hypersensitivity. To be able to compare the hypersensitivity of a site, however I need to choose a concentration of Dnase I that is not too high; for otherwise the chromatin maybe completely digested whether the site is hypersensitive or not. To figure out the ideal concentration of Dnase I, I did a serial dilution of Dnase I and digested aliquots of chromatin with it, as described in the methods section. I then did a real-time PCR assay for protamine as well as actin hypersensitive sites across all the different enzyme concentrations. Plotting Ct versus Dnase I concentration would result in a linear graph. I calculated the index of Dnase I sensitivity as $2^{\Delta Ct}$ protamine / $2^{\Delta Ct}$ actin, where ΔCt = Ct at mid-point of linear portion of graph – Ct of untreated chromatin. I was never able to get consistently increasing Ct values for either the actin hypersensitive site or the protamine site. After trying to trouble shoot for a

while, I stopped doing the experiment. The most likely explanation for getting inconsistent Ct values with increasing Dnase I activity is possibly due to variation in DNA preparation. Especially because very small quantities of DNA involved, even small variations in DNA preparation are reflected in real-time PCR.

In order to compare the epigenetic profile of the protamine domain of mutant spermatids vs WT spermatids, I decided to do a ChIP assay for the epigenetic marks H4K16Ac, H3K9Me2 and H3K9/14Ac. I chose these histone marks because the way they change has been well documented for the protamine domain (Martins and Krawetz 2007). I found some differences in the regions that I tested between WT and mutant that I have documented in the table below. However, this experiment has not been replicated mainly because of the nature of germ-cell separation protocol – which is tedious and involves a lot of mice. I decided it maybe easier to do ChIP-seq, instead of individual PCRs. I did a preliminary ChIP-seq trial with these marks, but I was not able to find any discernable differences between WT and mutant in the profiling of these marks.

Redundant Functions of the BRWD family of Proteins

Though *Brwd1* is expressed in a wide variety of tissues both in adult mice as well as developing embryos, *Brwd1* mutants have no obvious defects other than infertility (Ramos, Vidal-Taboada et al. 2002, Huang, Rambaldi et al. 2003, Philipps, Wigglesworth et al. 2008). *Brwd1* has two highly similar paralogs, *Phip* and *Brwd3* that are also expressed in tissues expressing *Brwd1*. These genes are about 62% identical and 73% similar, raising the possibility that there is some level of functional redundancy within the family (Philipps, Wigglesworth et al. 2008). To test this hypothesis, we attempted to generate mice deficient for both *Brwd1* and *Phip*. We were unable to create a *Brwd3* mutant as the gene is X-linked and the chimeric mice that we created were not able to transmit the *Brwd3* gene-trap through the germ line. One possibility maybe that BRWD3 is important for fertility of male mice rendering all germ cells that inherited the mutant allele infertile. The other possibility is that the chimeras that were created did not inherit the *Brwd3* mutant allele in the germline and therefore none of the progeny carried the mutant allele. Mutation of *Phip* alone causes semi-lethality prenatally, and survivors were runted and typically died before puberty (Li et al, 2010).

I was unable to identify any double mutant offspring from 167 pups genotyped at weanage and 354 pups genotyped at 2 days after birth from crosses between double heterozygotes. Surprisingly I found that *Brwd1*$^{-/-}$ mutants became semi-

lethal in the mixed backgrounds contributed by the parental strains (containing C3H,129, and C57BL/6), indicating that BRWD1 had important functions in the soma as well.

To explore when embryos were being lost during development, I genotyped 12.5 day old embryos from timed matings between *Brwd1*$^{+/-}$ *Phip*$^{+/-}$ parents. Embryos of the doubly mutant genotype (*Phip*$^{-/-}$ *Brwd1*$^{-/-}$) are indistinguishable from their siblings at this age. At E18.5, doubly mutant embryos were still alive and indistinguishable in appearance from *Phip*$^{-/-}$ embryos. The *Phip*$^{-/-}$ mutants at this stage were smaller than their WT or *Brwd1*$^{-/-}$ siblings, as expected (Fig 3.6). I also found one doubly mutant pup that was born dead or died shortly after birth.

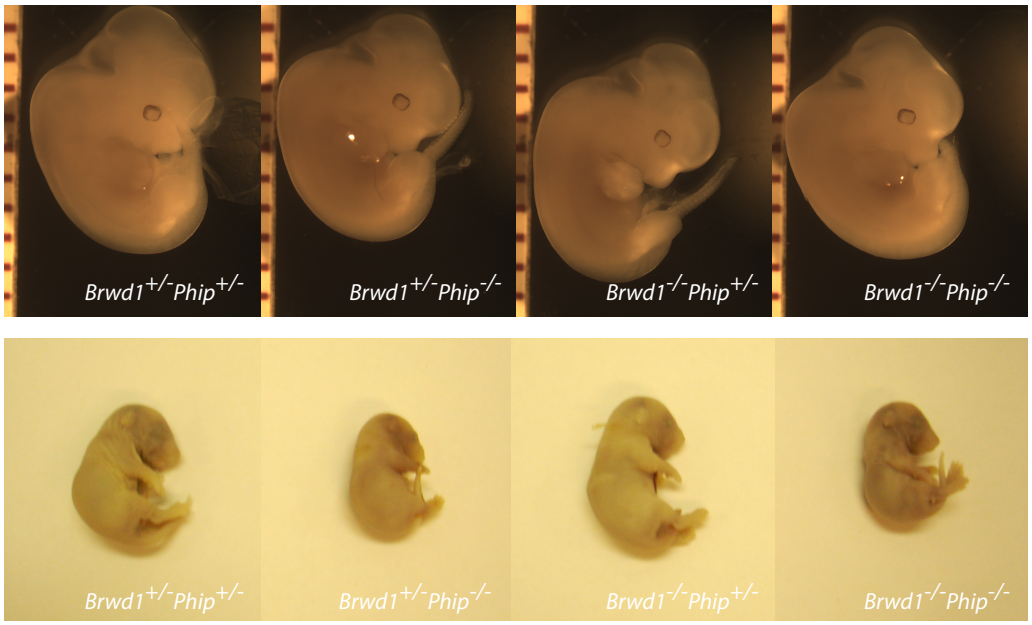

Fig 3.6 Comparison of embryos that are doubly mutant for *Brwd1* and *Phip*. On both 12.5 days and 18.5 days post coitum, the doubly mutant embryos did not display a more severe phenotype than either of the single mutants.

Table 3.3 Results of *Phip+/-;Brwd+/-* X *Phip+/-;Brwd1+/-* (at wean-age)

Brwd1 Genotype	*Phip* Genotype +/+	+/-	-/-
+/+	19	34	2
+/-	38	51	4
-/-	9	10	0

Total = 167 mice genotyped at wean-age.

Table 1.2 *Brwd1* mutants are semi-lethal in a mixed C3H-B6-129 background.

	Brwd1 Genotype +/+	+/-	-/-
Observed	53	89	19
Expected	40.25	80.5	40.25

Total = 161 mice genotyped at wean-age.

Table 1.3 Results of *Phip+/-;Brwd+/-* X *Phip+/-;Brwd1+/-* (2 days after birth)

Brwd1 Genotype	*Phip* Genotype +/+	+/-	-/-
+/+	30	78	4
+/-	64	101	5
-/-	26	45	0

Total = 354 mice genotyped at 2 days of age.

CHAPTER 4

DISCUSSION

Drastic decreases in expression of critical post-meiotic genes are the likely cause of defective spermiogenesis in Brwd1$^{-/-}$ male mice.

Around 300 genes show mis-regulated expression in *Brwd1*$^{-/-}$ mutants. GO (Gene Ontology) analysis of the misexpressed genes using the online tool DAVID shows that the most significant functional cluster (enrichment score = 6) is the process of spermatogenesis. Other significant functional clusters include genes involved in cytoskeletal dynamics, flagellum formation, chromatin organization, sperm motility, protein dynamics, and metabolic pathways. As about 80% of these genes are classified as post-meiotic by GermOnline, they may function in events that occur during spermiogenesis such as nucleosome remodeling, tail and head formation, cytoplasm extrusion, metabolic pathways important for sperm motility and survival, as well as regulation of expression of post-meiotic proteins. Indeed, mouse knock-outs of 15 of these genes have been made and they display male sterility or reduced fertility because of defective spermiogenesis (Table 4.1). Four of the most dramatically under-expressed genes (about 16-30 fold down-regulated) - *Tnp2, Tssk6, Prm2* and *Tnp1* - are important for the process of histone to protamine transition that occurs during spermiogenesis. Mouse mutants of these genes display abnormal sperm head shapes and defective chromatin condensation similar to *Brwd1*$^{-/-}$ sperm (Yu, Zhang et al. 2000, Cho, Willis et al. 2001, Zhao, Shirley et al. 2001, Philipps, Wigglesworth et al. 2008). Mutants of the mis-expressed genes *Akap4,*

Calreticulin 3, Atp1a4, Pldi, Gapdhs and *Herk4* display impaired sperm motility as does *Brwd1*$^{-/-}$ sperm (Miki, Qu et al. 2004, Huang, Somanath et al. 2005, Rodriguez and Stewart 2007, Heinen, Staubach et al. 2009, Ikawa, Tokuhiro et al. 2011, Jimenez, Sanchez et al. 2012). Other genes such as *Odf1* and *Oaz3* are important for head-flagellum attachment (Tokuhiro, Isotani et al. 2009, Yang, Meinhardt et al. 2012). The extent of down-regulation of the above genes is indicated in Table 4.1. Thus a severe shortage of these genes' protein products is probably responsible for the defective spermiogenesis and infertility in the *Brwd1*$^{-/-}$ mutants.

Table 4.1 List of Mis-expressed Genes that have been knocked-out previously

S. No.	Gene	Fold-Misregulated log2(mut/het)	Phenotype	Reference
1	*Tnp2*	-4.26	Reduced fertility and some morphological defects in sperm. KO	(Zhao, Shirley et al. 2001)
2	*Tssk6*	-4.19	KO, male sterility, impairment in motility and sperm morphology, DNA condensation impaired in spermatids.	(Spiridonov, Wong et al. 2005)
3	*Prm2*	-4.18	Male Infertility, variety of abnormal sperm heads, lower motility, haploinsufficiency seen in chimeras transmitting germ cells that were heterozygous for Prm2	(Cho, Willis et al. 2001)
4	*Tnp1*	-3.32	Reduced Fertility and defects in sperm condensation. KO	(Yu, Zhang et al. 2000)
5	*Akap 4*	-2.39	Knockout is infertile. Sperm motility disrupted and fibrous sheath disrupted, knockout	(Huang, Somanath et al. 2005)
6	*Tssk2*	-2.28	Male infertility, Failure to form elongated spermatids, apoptosis of spermatocytes and spermatids, appearance of round cells in the epididymal lumen	(Xu, Hao et al. 2008)
7	*H1fnt*	-2.14	Mutant mice were infertile. Shape and function of sperm were abnormal.	(Tanaka, Matsuoka et al. 2006)
8	*Calreticulin 3*	-2.10	Produced normal looking sperm but were infertile due to defective sperm migration from uterus into oviduct and defective binding to Zona Pellucida, KO	(Ikawa, Tokuhiro et al. 2011)
9	*Oaz3*	-2.07	Detached flagellum impaired acrosome reaction male infertility KO	(Tokuhiro, Isotani et al. 2009)
10	*Atp1a 4*	-2.00	KO is infertile, sperm motility affected.	(Jimenez, Sanchez et al. 2012)
11	*Pidi*	-1.94	Reduced testis weight and lower sperm motility, knockout	(Heinen, Staubach et al. 2009)
12	*Gapd hs*	-1.92	KO is Infertile and sperm motility is sluggish.	(Miki, Qu et al. 2004)
13	*Lipe*	-1.88	Low sperm count	(Osuga, Ishibashi et al. 2000)
14	*Odf1*	-1.78	Infertile, sperm head is detached. KO	(Yang, Meinhardt et al. 2012)
15	*Herc4*	-1.59	KO, reduced male fertility, kinked tail with cytoplasmic droplet, reduced sperm motility.	(Rodriguez and Stewart 2007)

The CREM-tau ACT pathway has been shown to regulate expression of post-meiotic genes important for spermiogenesis (Blendy, Kaestner et al. 1996, Krausz and Sassone-Corsi 2005, Sassone-Corsi 2005, Martianov, Choukrallah et al. 2010, Kosir, Juvan et al. 2012). In *Crem* knock-out mice, close to 5000 genes are deregulated in the testes, 2000 of which were down-regulated and 3000 were up-regulated (Kosir, Juvan et al. 2012). , About 50% of the genes (~111 genes) that are mis-expressed in the *Brwd1*$^{-/-}$ mice are also mis-regulated in the CREM knock-out. *Brwd1*, however, is not mis-regulated in the CREM knock out. CREM-tau and BRWD1 may function in the same pathway to regulate these genes, but we can also imagine scenarios where they may be working independently. More studies are needed to test these possibilities. *Crem* knockouts also have a more severe male infertility phenotype as mutant spermatids arrest before spermiogenesis is complete. This may be attributable to the larger number of misregulated genes in *Crem* mutants. The 50% of the genes down-regulated in the *Brwd1* but not Crem mutants implicate more than one pathway for post-meiotic gene regulation.

My experiments showed that FLAG-BRWD1 tends to associate with chromatin when expressed in HEK cells. If it functions similarly in round spermatids we can think of a scenario where BRWD1, by virtue of its bromodomains, binds acetylated lysine residues of histones found on the nucleosomes of post-meiotic genes, causing transcriptional activation. Indeed, the “protamine domain” comprising the clustered genes *Prm1, Prm2, Prm3* and *Tnp2* (all of which are

down-regulated in the mutant) progressively gain acetylated histone marks as the germ cells differentiate from spermatocytes to spermatids where they are expressed (Martins and Krawetz 2007). BRWD1 also has a poly-Q transcriptional activation domain that has been shown to activate luciferase expression in an in-vitro assay (Huang, Rambaldi et al. 2003). The WD structures that are present in the BRWD1 protein may also enable it to interact with a wide-variety of transcription factors, nucleosome remodelers and other signaling proteins. We can imagine a transcriptional pathway where BRWD1 might be part of unique post-meiotic transcriptional-activator complexes that interact with acetylated histones around post-meiotic genes.

Despite its role in proper expression of many postmeiotic genes, my data shows that BRWD1 is not involved in maintaining or forming the chromocenter or other nuclear domains marked by specific epigenetic marks in round spermatids, unlike BRDT or TRF2,. BRWD1 might function more locally on specific promoter sequences around post-meiotic genes. We are currently pursuing this hypothesis by looking for potential changes in localized epigenetic marks, in particular the well-studied H3K4Me1, H3K4Me3 and H3K27ac, in the mutants using ChIP-seq.

Because of its role as a transcriptional regulator in the male germline, we hypothesized that BRWD1 maybe involved in transcriptional regulation important for oocyte maturation. Indeed, in a transcription run-on assay performed *in-vitro*, *Brwd1* mutant GV oocytes of the SN stage displayed abnormally elevated

transcription levels. A microarray comparing WT and mutant oocytes however did not show any differences in the transcript levels, in direct contradiction with the above observation. One possibility that can explain this contradiction is that the microarray probes only mRNA transcripts; and transcription of other elements such as small RNAs, retrotransposons, etc is unaccounted for. Another striking phenotype of *Brwd1* deficient oocytes was the presence of extremely de-condensed chromosomes during meiosis I especially during metaphase and anaphase stages. The mutant oocytes showed a wide variety of chromosomal defects including univalent chromosomes, anaphase bridges and chromatid breaks including the presence of single chromatids in a large number of mutant oocytes. Taken together, the chromosome condensation defects in meiosis and the potential genomic instability caused by aberrant retrotransposon expression, help explain the infertility phenotype observed in *Brwd1* knockout oocytes.

Non-Germline Functions of BRWD1

Brwd1 is expressed in a wide variety of tissues in the developing mouse embryo as well as in the adult animal, however, mutant animals have no discernable phenotypes other than infertility (Ramos, Vidal-Taboada et al. 2002, Huang, Rambaldi et al. 2003, Philipps, Wigglesworth et al. 2008). This may be explained by the fact that *Brwd1* may have redundant functions with two other highly similar paralogs, *Phip* and *Brwd3,* both of which are expressed in multiple tissues like *Brwd1*.

All of the somatic functions of *Brwd1* in the mouse can only be determined by a complete simultaneous knock out of all three genes. Indeed, invertebrates have only one homolog of the *Brwd* family of genes and a null mutation of it causes early lethality in the embryonic stage in *Drosophila* (D'Costa, Reifegerste et al. 2006). Not surprisingly, the *brwd* gene (*dbrwd3*) in *Drosophila* is expressed in a wide variety of tissues analagous to the mammalian homologs, including germ cells. However, the function of *dbrwd3* in germ cells has not been determined yet, as all mutant alleles identified thus far are lethal. Like the mammalian *brwd* genes, *dbrwd3* is nuclear in many cells and also associates with actively transcribing chromatin in the polytene chromosomes, and may potentially act as a transcriptional activator (D'Costa, Reifegerste et al. 2006). I also found that *Brwd1* mutants become semi-lethal in a C57BL/6J-C3H-129 mixed mouse background, thus alluding to its role in somatic tissue.

I created a *Phip* mutant that had a different, more severe phenotype than *Brwd1* mutants as described in the literature review. I went on to create a double knock-out of *Phip* and *Brwd1,* but the double mutants did not show a more severe phenotype than the *Phip* mutant alone, indicating either that *Brwd3* maybe able to compensate for the loss of both *Brwd1* and *Phip* or there are no redundant functions between *Brwd1* and *Phip*.

How and why a ubiquitously expressed gene such as *Brwd1* has evolved a non-redundant role in the mammalian germline is still unanswered. *Brwd1 i*s not the

only gene that has testis-specific unique functions, *Crem-tau* is also expressed widely in the mouse; but a null mutant is only male-infertile. Also, many transcriptional factor paralogs are exclusively expressed in male or female germ-line, including those of the ubiquitously expressed TBP-associated factors and TBP. Not only does it seem that mice prefer to maintain a unique set of transcription factors and regulators in the germline, but also other ubiquitously expressed metabolic genes have a germ-line specific isoform or paralog that is expressed. The germline is of special importance to the organism, understandably so, as it ensures the continuity of the species into the next generation. Importantly, the development of germline cells has several unique features that are not present in any other somatic tissue. Transcription occurs from haploid cells only in the germ-line. Only the germline undergoes meiosis. Both male and female germ cells undergo genome-wide transcriptional silencing and impressive chromatin re-modeling and packaging – therefore all protein products required for processes that occur after transcriptional silencing have to be expressed before-hand and translationally regulated. Many of the genes expressed in oocytes and spermatids are very unique and must be silenced in all other cell-types. Therefore it seems that the regulation of transcription in the germ-line poses a unique challenge to the organism due to a number of reasons, and it has evolved a separate set of regulators to deal with it. *Brwd1*, a ubiquitously expressed gene, might have co-evolved specific functions in the male and female germ-line along with these specialized factors.

CHAPTER 5

CONCLUSIONS

1. BRWD1 is important for genes expressed post-meiotically in the mammalian male germ-line. The genes that it regulates are important for the process of spermiogenesis by which round spermatids differentiate into sperm and a *Brwd1* mutant is thus infertile due to defective spermiogenesis.
2. *Brwd1* is expressed in spermatocytes and spermatids in the male germ-lin. When a construct containing FLAG-BRWD1 was transiently transfected into HEK cells, BRWD1 was found to be exclusively nuclear and bound to chromatin.
3. *Brwd1* deficiency does not cause fragmentation of the chromocenter, a heterochromatin structure found in spermatids nor does it affect the formation of nuclear domains characterized by unique epigenetic marks.
4. BRWD1 has somatic functions that become non-redundant in certain mouse backgrounds. It also appears that *Brwd1* does not have any redundant functions with *Phip*, as double mutant embryos do not look different from the singly mutant *Phip*.

APPENDIX

LIST OF GENES DOWN-REGULATED IN BRWD1 MUTANTS

Gene.Symbol	Gene.Title	log2(mut/het)	p value
Tnp2	transition protein 2	-4.26	0.002
MGI:2148775	serine/threonine protein kinase SSTK	-4.19	0.005
Prm2	protamine 2	-4.18	0.001
Prm1	protamine 1	-3.51	0.005
1700011F03Rik	RIKEN cDNA 1700011F03 gene	-3.48	0.004
LOC432552	similar to Hypothetical protein MGC26988	-3.33	0.005
Tnp1	transition protein 1	-3.32	0.005
1700007N14Rik	RIKEN cDNA 1700007N14 gene	-3.28	0.000
Oxct2a	3-oxoacid CoA transferase 2A	-3.28	0.003
Prm1	protamine 1	-3.28	0.005
4922504M18Rik	RIKEN cDNA 4922504M18 gene	-3.26	0.001
Fscn3	fascin homolog 3, actin-bundling protein, testicular (Strongylocentrotus purpuratus)	-3.23	0.003
	Adult male testis cDNA, RIKEN full-length enriched library, clone:4930543A13 product:unknown EST, full insert sequence	-3.22	0.000
Hspa1l	heat shock protein 1-like	-3.21	0.003
1700080E11Rik	RIKEN cDNA 1700080E11 gene	-3.16	0.004
1700009J07Rik	RIKEN cDNA 1700009J07 gene	-3.16	0.000
		-3.16	0.001
1700012A03Rik	RIKEN cDNA 1700012A03 gene	-3.13	0.001
1700015G11Rik	RIKEN cDNA 1700015G11 gene	-3.11	0.002
1700126L10Rik	RIKEN cDNA 1700126L10 gene	-3.09	0.000
Prm1	protamine 1	-3.04	0.009
0610010I15Rik	RIKEN cDNA 0610010I15 gene	-3.01	0.002

1700016C15Rik	RIKEN cDNA 1700016C15 gene	-2.99	0.004
4931440L10Rik	RIKEN cDNA 4931440L10 gene	-2.91	0.000
4930557A04Rik	RIKEN cDNA 4930557A04 gene	-2.89	0.005
1700042G07Rik	RIKEN cDNA 1700042G07 gene	-2.87	0.001
Tuba8	tubulin, alpha 8	-2.86	0.004
1700049K14Rik	RIKEN cDNA 1700049K14 gene	-2.84	0.008
Spata3	spermatogenesis associated 3	-2.83	0.006
1700019I23Rik	RIKEN cDNA 1700019I23 gene	-2.82	0.007
4931407G18Rik	RIKEN cDNA 4931407G18 gene	-2.79	0.004
4933411K16Rik	RIKEN cDNA 4933411K16 gene	-2.79	0.008
Nmnat3	nicotinamide nucleotide adenylyltransferase 3	-2.77	0.000
1700067I02Rik	RIKEN cDNA 1700067I02 gene	-2.76	0.001
Txndc2	thioredoxin domain containing 2 (spermatozoa)	-2.75	0.006
4930503B20Rik	RIKEN cDNA 4930503B20 gene	-2.75	0.009
4921530L21Rik	RIKEN cDNA 4921530L21 gene	-2.73	0.006
Trim42	tripartite motif-containing 42	-2.70	0.001
1700023I07Rik	RIKEN cDNA 1700023I07 gene	-2.69	0.001
1700008I05Rik /// 1700129I15Rik	RIKEN cDNA 1700008I05 gene /// RIKEN cDNA 1700129I15 gene	-2.66	0.002
Chn2	chimerin (chimaerin) 2	-2.64	0.006
Arpm1	Actin related protein M1	-2.63	0.009
1700001F04Rik	RIKEN cDNA 1700001F04 gene	-2.60	0.003
4921507P07Rik	RIKEN cDNA 4921507P07 gene	-2.56	0.005
4921517D22Rik	RIKEN cDNA 4921517D22 gene	-2.55	0.001

MGI:1915176	novel leucine zipper testicular protein	-2.54	0.007
1700063H04Rik	RIKEN cDNA 1700063H04 gene	-2.53	0.001
LOC433674	similar to RIKEN cDNA 1700129C05	-2.50	0.002
1700024P04Rik	RIKEN cDNA 1700024P04 gene	-2.50	0.006
Dscaml1	Down syndrome cell adhesion molecule-like 1	-2.49	0.001
1700095A13Rik	RIKEN cDNA 1700095A13 gene	-2.48	0.000
1700012F11Rik	RIKEN cDNA 1700012F11 gene	-2.44	0.002
LOC435142	similar to hypothetical protein FLJ23584	-2.44	0.005
4933437F05Rik	RIKEN cDNA 4933437F05 gene	-2.43	0.000
Akap4	A kinase (PRKA) anchor protein 4	-2.39	0.009
1700019M22Rik	RIKEN cDNA 1700019M22 gene	-2.37	0.005
BC048507	cDNA sequence BC048507	-2.37	0.003
1700047L15Rik	RIKEN cDNA 1700047L15 gene	-2.36	0.007
Klk8	kallikrein 8	-2.35	0.006
		-2.35	0.001
4930522D07Rik	RIKEN cDNA 4930522D07 gene	-2.34	0.007
Spata3	spermatogenesis associated 3	-2.34	0.008
Ccin	calicin	-2.33	0.008
Spatc1	spermatogenesis and centriole associated 1	-2.33	0.004
Skp2	S-phase kinase-associated protein 2 (p45)	-2.31	0.003
4930405N21Rik	RIKEN cDNA 4930405N21 gene	-2.29	0.000
MGI:1920603	actin related protein M2	-2.28	0.009
Tssk2	testis-specific serine kinase 2	-2.28	0.006
1700007N18Rik	RIKEN cDNA 1700007N18 gene	-2.27	0.008
1700019O17Rik	RIKEN cDNA 1700019O17 gene	-2.27	0.000

1700121K02Rik	RIKEN cDNA 1700121K02 gene	-2.25	0.008
1700029H14Rik	RIKEN cDNA 1700029H14 gene	-2.25	0.008
2810002D13Rik	RIKEN cDNA 2810002D13 gene	-2.23	0.000
1700058C13Rik	RIKEN cDNA 1700058C13 gene	-2.23	0.009
4931431F19Rik	RIKEN cDNA 4931431F19 gene	-2.22	0.007
1700027F06Rik	RIKEN cDNA 1700027F06 gene	-2.21	0.006
Clmn	calmin	-2.21	0.002
Tssk3	testis-specific serine kinase 3	-2.20	0.007
4932443D16Rik	RIKEN cDNA 4932443D16 gene	-2.20	0.000
1700006E09Rik	RIKEN cDNA 1700006E09 gene	-2.19	0.009
1700022A21Rik	RIKEN cDNA 1700022A21 gene	-2.19	0.004
4933401F05Rik	RIKEN cDNA 4933401F05 gene	-2.19	0.007
Dnajb8	DnaJ (Hsp40) homolog, subfamily B, member 8	-2.19	0.002
Ppp2r2b	protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), beta isoform	-2.18	0.000
		-2.16	0.003
BC033606	cDNA sequence BC033606	-2.16	0.007
1700026P10Rik	RIKEN cDNA 1700026P10 gene	-2.14	0.007
4931415M17	4931415M17 mRNA	-2.14	0.008
4930521I23Rik	RIKEN cDNA 4930521I23 gene	-2.13	0.003
1700094E07Rik	RIKEN cDNA 1700094E07 gene	-2.10	0.006
4930406D18Rik	RIKEN cDNA 4930406D18 gene	-2.10	0.006
Calr3	calreticulin 3	-2.10	0.003
4930592I03Rik	RIKEN cDNA 4930592I03 gene	-2.09	0.001

4930404F17Rik	RIKEN cDNA 4930404F17 gene	-2.08	0.000
4933411C14Rik	RIKEN cDNA 4933411C14 gene	-2.08	0.004
Oaz3	ornithine decarboxylase antizyme 3	-2.07	0.010
4933439G12Rik	RIKEN cDNA 4933439G12 gene	-2.06	0.001
4933428D01Rik	RIKEN cDNA 4933428D01 gene	-2.06	0.000
Usp12	ubiquitin specific protease 12	-2.06	0.004
LOC238329	similar to chromosome 14 open reading frame 166B	-2.06	0.003
Sufu	suppressor of fused homolog (Drosophila)	-2.05	0.006
Cabyr	calcium-binding tyrosine-(Y)-phosphorylation regulated (fibrousheathin 2)	-2.02	0.008
	Gene model 614, (NCBI)	-2.01	0.001
4930413P14Rik	RIKEN cDNA 4930413P14 gene	-2.01	0.004
4933406A14Rik	RIKEN cDNA 4933406A14 gene	-2.00	0.007
Atp1a4	ATPase, Na+/K+ transporting, alpha 4 polypeptide	-2.00	0.007
5430432M24Rik	RIKEN cDNA 5430432M24 gene	-2.00	0.006
1700016P04Rik	RIKEN cDNA 1700016P04 gene	-1.98	0.004
1700001D09Rik	RIKEN cDNA 1700001D09 gene	-1.97	0.005
Tuba4	tubulin, alpha 4	-1.97	0.001
Lasp1	LIM and SH3 protein 1	-1.97	0.004
4930549C01Rik	RIKEN cDNA 4930549C01 gene	-1.97	0.001
4933425D22Rik	RIKEN cDNA 4933425D22 gene	-1.95	0.001
Akap1	A kinase (PRKA) anchor protein 1	-1.95	0.004
4930405A07Rik	RIKEN cDNA 4930405A07 gene	-1.95	0.001
1810060J02Rik	RIKEN cDNA 1810060J02 gene	-1.95	0.004
LOC433814	similar to hypothetical protein FLJ36119	-1.94	0.002
1700109K24Rik	RIKEN cDNA 1700109K24 gene	-1.94	0.004

1700125F08Rik	RIKEN cDNA 1700125F08 gene	-1.94	0.004
Gapds	glyceraldehyde-3-phosphate dehydrogenase, spermatogenic	-1.92	0.008
		-1.91	0.003
Tuba4	tubulin, alpha 4	-1.91	0.002
1700020B09Rik	RIKEN cDNA 1700020B09 gene	-1.90	0.002
4931430N09Rik	RIKEN cDNA 4931430N09 gene	-1.88	0.001
4933403G17Rik	RIKEN cDNA 4933403G17 gene	-1.88	0.003
Asb15	ankyrin repeat and SOCS box-containing protein 15	-1.88	0.008
6330505N24Rik	RIKEN cDNA 6330505N24 gene	-1.87	0.001
4933436H12Rik	RIKEN cDNA 4933436H12 gene	-1.86	0.002
Cdyl	chromodomain protein, Y chromosome-like	-1.86	0.008
Oaz3	ornithine decarboxylase antizyme 3	-1.85	0.009
Grin2b	glutamate receptor, ionotropic, NMDA2B (epsilon 2)	-1.85	0.002
5730596K20Rik	RIKEN cDNA 5730596K20 gene	-1.84	0.009
1700001O22Rik	RIKEN cDNA 1700001O22 gene	-1.83	0.003
1810060J02Rik	RIKEN cDNA 1810060J02 gene	-1.83	0.002
4930438O03Rik	RIKEN cDNA 4930438O03 gene	-1.83	0.008
BC048651	cDNA sequence BC048651	-1.83	0.006
Calr3	calreticulin 3	-1.83	0.002
Osbp2	oxysterol binding protein 2	-1.83	0.006
1700023E05Rik	RIKEN cDNA 1700023E05 gene	-1.82	0.000
1700019N19Rik	RIKEN cDNA 1700019N19 gene	-1.79	0.005
4833412E22Rik	RIKEN cDNA 4833412E22 gene	-1.78	0.007
Odf1	outer dense fiber of sperm tails 1	-1.78	0.008
Cd96	CD96 antigen	-1.78	0.002

4833412E22Rik	RIKEN cDNA 4833412E22 gene	-1.77	0.003
Proc	protein C	-1.76	0.010
4933400E14Rik	RIKEN cDNA 4933400E14 gene	-1.73	0.005
Prkcd	protein kinase C, delta	-1.72	0.000
4930562D21Rik	RIKEN cDNA 4930562D21 gene	-1.71	0.000
Arhgef2	rho/rac guanine nucleotide exchange factor (GEF) 2	-1.71	0.008
1700095H12	hypothetical protein 1700095H12	-1.71	0.004
Kcnip2	Kv channel-interacting protein 2	-1.70	0.001
4930533L02Rik	RIKEN cDNA 4930533L02 gene	-1.69	0.002
Pwwp2	PWWP domain containing 2	-1.69	0.004
4933406L23Rik	RIKEN cDNA 4933406L23 gene	-1.68	0.008
4933417D19Rik	RIKEN cDNA 4933417D19 gene	-1.68	0.008
4933430N04Rik	RIKEN cDNA 4933430N04 gene	-1.67	0.000
Gm1267 /// Kctd16	gene model 1267, (NCBI) /// potassium channel tetramerisation domain containing 16	-1.67	0.006
Habp4	hyaluronic acid binding protein 4	-1.67	0.005
4930563D23Rik	RIKEN cDNA 4930563D23 gene	-1.66	0.004
Tes3-ps /// Tes3	testis derived transcript 3, pseudogene /// testis derived transcript 3	-1.65	0.009
Nbr1	neighbor of Brca1 gene 1	-1.63	0.002
4933407I08Rik	RIKEN cDNA 4933407I08 gene	-1.63	0.001
Tm4sf6	transmembrane 4 superfamily member 6	-1.62	0.010
4933425O20Rik	RIKEN cDNA 4933425O20 gene	-1.62	0.001
Wdr13	WD repeat domain 13	-1.61	0.004
1110017D15Rik	RIKEN cDNA 1110017D15 gene	-1.61	0.002
Dmp1	dentin matrix protein 1	-1.61	0.001
Gpd2	glycerol phosphate dehydrogenase 2, mitochondrial	-1.60	0.008
Herc4	RIKEN cDNA D230019N24 gene	-1.59	0.006

Dnaja4	DnaJ (Hsp40) homolog, subfamily A, member 4	-1.59	0.010
Cdv3	carnitine deficiency-associated gene expressed in ventricle 3	-1.58	0.009
4930500A05Rik	RIKEN cDNA 4930500A05 gene	-1.58	0.001
Tle3	transducin-like enhancer of split 3, homolog of Drosophila E(spl)	-1.57	0.002
		-1.57	0.007
Fbxo2	F-box only protein 2	-1.56	0.002
2010001J22Rik	RIKEN cDNA 2010001J22 gene	-1.56	0.000
Rab3il1	RAB3A interacting protein (rabin3)-like 1	-1.55	0.007
Ndst3	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 3	-1.55	0.008
2810408A11Rik	RIKEN cDNA 2810408A11 gene	-1.55	0.001
Klk6	kallikrein 6	-1.54	0.009
Zfyve1	zinc finger, FYVE domain containing 1	-1.53	0.007
		-1.53	0.003
	Adult male testis cDNA, RIKEN full-length enriched library, clone:4930509G02 product:unclassifiable, full insert sequence	-1.53	0.000
2310067L16Rik	RIKEN cDNA 2310067L16 gene	-1.52	0.002
Daf1	decay accelerating factor 1	-1.52	0.004
Srgap1	SLIT-ROBO Rho GTPase activating protein 1	-1.51	0.004
1700061N14Rik	RIKEN cDNA 1700061N14 gene	-1.51	0.009
1700051I12Rik	RIKEN cDNA 1700051I12 gene	-1.50	0.001
Pfn3	profilin 3	-1.50	0.010
1700051I12Rik	RIKEN cDNA 1700051I12 gene	-1.50	0.000
MGC6357	hypothetical protein MGC6357	-1.49	0.002
1700029J11Rik	RIKEN cDNA 1700029J11 gene	-1.49	0.008
2010001J22Rik	RIKEN cDNA 2010001J22 gene	-1.49	0.001
Nmnat3	nicotinamide nucleotide adenylyltransferase 3	-1.49	0.002
Nqo2	NAD(P)H dehydrogenase, quinone 2	-1.49	0.001

Nptxr	neuronal pentraxin receptor	-1.48	0.005
4930463O16Rik	RIKEN cDNA 4930463O16 gene	-1.47	0.000
AF366264	cDNA sequence AF366264	-1.47	0.001
4930483C13Rik	RIKEN cDNA 4930483C13 gene	-1.47	0.007
1700106J12Rik	RIKEN cDNA 1700106J12 gene	-1.46	0.010
Stk22s1	serine/threonine kinase 22 substrate 1	-1.46	0.002
4933429I20Rik	RIKEN cDNA 4933429I20 gene	-1.45	0.003
Klc3	kinesin light chain 3	-1.44	0.001
4933402N03Rik	RIKEN cDNA 4933402N03 gene	-1.44	0.001
Cpeb3	cytoplasmic polyadenylation element binding protein 3	-1.44	0.003
4932411G14Rik	RIKEN cDNA 4932411G14 gene	-1.43	0.007
Cd3d	CD3 antigen, delta polypeptide	-1.42	0.009
4921513I03Rik	RIKEN cDNA 4921513I03 gene	-1.41	0.006
5730410I19Rik	RIKEN cDNA 5730410I19 gene	-1.40	0.008
Gm191 /// A630095E13Rik	gene model 191, (NCBI) /// RIKEN cDNA A630095E13 gene	-1.39	0.006
LOC208231	similar to sentrin 15	-1.39	0.009
4833412E22Rik /// LOC433814	RIKEN cDNA 4833412E22 gene /// similar to hypothetical protein FLJ36119	-1.38	0.006
4633402D15Rik	RIKEN cDNA 4633402D15 gene	-1.37	0.002
4930563J15Rik	RIKEN cDNA 4930563J15 gene	-1.36	0.001
4933423N03Rik	RIKEN cDNA 4933423N03 gene	-1.36	0.007
Ttc7	tetratricopeptide repeat domain 7	-1.35	0.003

4930505O20Rik	RIKEN cDNA 4930505O20 gene	-1.35	0.003
	Adult male testis cDNA, RIKEN full-length enriched library, clone:4930550J05 product:unknown EST, full insert sequence	-1.34	0.005
4930474N09Rik	RIKEN cDNA 4930474N09 gene	-1.33	0.007
4933413G19Rik	RIKEN cDNA 4933413G19 gene	-1.33	0.006
4933407I18Rik	RIKEN cDNA 4933407I18 gene	-1.32	0.007
Rex2	reduced expression 2	-1.32	0.006
Pip5kl1	phosphatidylinositol-4-phosphate 5-kinase-like 1	-1.32	0.008
MGI:1921406	cytosolic acetyl-CoA hydrolase	-1.31	0.005
Abhd7	abhydrolase domain containing 7	-1.31	0.001
D11Wsu47e	DNA segment, Chr 11, Wayne State University 47, expressed	-1.30	0.008
	AYM1 (Aym1)	-1.30	0.000
4931429L15Rik	RIKEN cDNA 4931429L15 gene	-1.30	0.003
4930523O13Rik	RIKEN cDNA 4930523O13 gene	-1.30	0.006
1700026L06Rik	RIKEN cDNA 1700026L06 gene	-1.29	0.007
4933432B09Rik	RIKEN cDNA 4933432B09 gene	-1.28	0.004
A930035J23Rik	RIKEN cDNA A930035J23 gene	-1.28	0.008
1700083M11Rik	RIKEN cDNA 1700083M11 gene	-1.28	0.006
1110038M16Rik	RIKEN cDNA 1110038M16 gene	-1.27	0.009
Catna3	catenin alpha 3	-1.27	0.003
Rasal1	RAS protein activator like 1 (GAP1 like)	-1.26	0.001
1700048F04Rik	RIKEN cDNA 1700048F04 gene	-1.26	0.002
4921506I22Rik	RIKEN cDNA 4921506I22 gene	-1.25	0.003
		-1.25	0.001
4933421H10Rik	RIKEN cDNA 4933421H10 gene	-1.24	0.006

2010001J22Rik	RIKEN cDNA 2010001J22 gene	-1.24	0.003
Gdpd1	glycerophosphodiester phosphodiesterase domain containing 1	-1.22	0.002
	Transcribed locus	-1.21	0.002
Slc36a3	solute carrier family 36 (proton/amino acid symporter), member 3	-1.21	0.008
Rnf4	ring finger protein 4	-1.20	0.005
Csnk1g2	casein kinase 1, gamma 2	-1.20	0.004
Cnr1	cannabinoid receptor 1 (brain)	-1.19	0.006
4933435G04Rik	RIKEN cDNA 4933435G04 gene	-1.19	0.002
4930453O03Rik	RIKEN cDNA 4930453O03 gene	-1.17	0.004
2200002J24Rik	RIKEN cDNA 2200002J24 gene	-1.16	0.010
4921509A06Rik	RIKEN cDNA 4921509A06 gene	-1.15	0.005
1100001H23Rik	RIKEN cDNA 1100001H23 gene	-1.15	0.003
D2Ertd750e	DNA segment, Chr 2, ERATO Doi 750, expressed	-1.15	0.005
Pi4k2b	phosphatidylinositol 4-kinase type 2 beta	-1.14	0.007
	Transcribed locus	-1.14	0.008
4932703K07Rik	RIKEN cDNA 4932703K07 gene	-1.14	0.005
2810405F18Rik	RIKEN cDNA 2810405F18 gene	-1.13	0.001
5031400M07Rik	RIKEN cDNA 5031400M07 gene	-1.13	0.001
6720463E02Rik	RIKEN cDNA 6720463E02 gene	-1.13	0.006
Bcmo1	beta-carotene 15,15'-monooxygenase	-1.12	0.008
Trpm5	transient receptor potential cation channel, subfamily M, member 5	-1.12	0.004
Zfp294	zinc finger protein 294	-1.12	0.004
4921513H07Rik	RIKEN cDNA 4921513H07 gene	-1.11	0.009
Sncaip	synuclein, alpha interacting protein (synphilin)	-1.09	0.007
Etsrp71	ets related protein 71	-1.09	0.001
Skd3	suppressor of K+ transport defect 3	-1.09	0.008
Lemd1	LEM domain containing 1	-1.08	0.005

1700092E16Rik	RIKEN cDNA 1700092E16 gene	-1.08	0.000
Dp1l1	deleted in polyposis 1-like 1	-1.07	0.000
Rnf138	ring finger protein 138	-1.07	0.006
4933434G05Rik	RIKEN cDNA 4933434G05 gene	-1.07	0.004
Rfx3	regulatory factor X, 3 (influences HLA class II expression)	-1.07	0.001
D2Ertd750e	DNA segment, Chr 2, ERATO Doi 750, expressed	-1.06	0.001
Rnf38	ring finger protein 38	-1.06	0.008
Za20d3	zinc finger, A20 domain containing 3	-1.06	0.005
Sntg1	syntrophin, gamma 1	-1.06	0.001
Hipk1	homeodomain interacting protein kinase 1	-1.05	0.002
1700019L13Rik	RIKEN cDNA 1700019L13 gene	-1.05	0.006
Wwp2	WW domain containing E3 ubiquitin protein ligase 2	-1.04	0.002
1700025D03Rik	RIKEN cDNA 1700025D03 gene	-1.04	0.006
4933440N22Rik	RIKEN cDNA 4933440N22 gene	-1.04	0.004
Spata6	spermatogenesis associated 6	-1.04	0.009
4933411E08Rik	RIKEN cDNA 4933411E08 gene	-1.03	0.004
Hemp1	hematopoietic protein 1	-1.03	0.001
		-1.01	0.000
Kif2c	kinesin family member 2C	-1.00	0.006
1810037B05Rik	RIKEN cDNA 1810037B05 gene	1.02	0.003
Six4	sine oculis-related homeobox 4 homolog (Drosophila)	1.11	0.000
		1.12	0.005
6720468P15Rik	RIKEN cDNA 6720468P15 gene	1.18	0.002
Parp3	poly (ADP-ribose) polymerase family, member 3	1.23	0.001
		1.27	0.004
MGI:1333876	G substrate	1.39	0.007
		1.41	0.001
Ltk	leukocyte tyrosine kinase	1.51	0.006

4932441J04Rik	RIKEN cDNA 4932441J04 gene	1.53	0.001
E230013L22Rik	RIKEN cDNA E230013L22 gene	1.58	0.000

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