CHARACTERIZATION OF INO80 CHROMATIN-REMODELING ACTIVITY DURING GERM CELL DEVELOPMENT

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum in Genetics and Molecular Biology.

Chapel Hill
2015

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ABSTRACT

Daniel Winn Serber: Characterization of INO80 Chromatin-Remodeling Activity During Germ Cell Development
(Under the direction of Dr. Terry Magnuson)

The ability to faithfully transmit genetic information across generations via the germ cells is a critical aspect of mammalian reproduction. The process of germ cell development requires a number of large-scale chromatin modifications within the nucleus. One such occasion arises during meiotic recombination, when hundreds of DNA double-strand breaks are induced and subsequently repaired, enabling the transfer of genetic information between homologous chromosomes. The inability to properly repair DNA damage is known to lead to an arrest in the developing germ cells and sterility within the animal. Chromatin-remodeling activity, and in particular the BRG1 subunit of the SWI/SNF complex, has been shown to be required for successful completion of meiosis. In contrast, remodeling complexes of the ISWI and CHD families are required for post-meiotic processes. Little is known regarding the contribution of the INO80 family of chromatin-remodeling complexes, which is a particularly interesting candidate due to its well-described functions during DNA double-strand break repair. Here we show that INO80 is expressed in developing spermatocytes during the early stages of meiotic prophase I. Based on this information, we used a conditional allele to delete the INO80 core ATPase subunit, thereby eliminating INO80 chromatin-remodeling activity in this lineage. The loss of INO80 resulted in sterility of the animal due to the failure to repair DNA damage during meiotic recombination. Specifically, we observed a disruption in the Fanconi Anemia repair pathway, where early

elements of the pathway were present on the chromosomal axes while BRCA1 remained absent. From these observations, we propose a model where INO80 activity is required to prepare the chromatin landscape local to the break site, creating the physical space necessary for the localization of downstream DNA repair proteins.

In conclusion, this work provides deeper insight in to the critical nature of chromatin-remodeling activity for spermatogenesis, particularly during meiotic recombination, and a foundation for future studies into the genomic functions of the INO80 complex.

ACKNOWLEDGEMENTS

I would like to begin by thanking my advisor, Dr. Terry Magnuson for his continuous support during my graduate training. You have challenged me to think creatively and ask questions, and I have grown immensely from the example you set. I am also extremely grateful to the members of the Magnuson Lab, current and former, who so graciously gave of their time and energy to help me hone my research questions and make me a better scientist.

I would also like to thank the members of my thesis committee, Drs. Scott Bultman, Fernando Pardo-Manuel de Villena, Praveen Sethupathy, and Buddy Weissman. I am especially grateful for your guidance and encouragement. Special thanks are owed to my committee chair, Dr. Scott Bultman, who has always been available to impart advice, reassurance, and clarity.

I am also extremely grateful to the scientists and mentors in my past who have set me on the path into the adventure that is biological research. Dr. Andrew Shaw for instilling in me a love for the field of biology. Dr. Reina Villareal for welcoming me into your lab as a high school student and offering such a foundational experience of what it means to participate in the research community. Drs. Brent Bucker and Diane Janick-Buckner, your example of what it looks like to truly be scientist-educators will forever be the model that I strive to emulate throughout my career.

And last, but certainly not least, I could not have done any of this without the support of my parents, Joe and Jane, and brother, Bill. You have always been there to encourage me to meet each new challenge and rise to the occasion. Finally I would like to thank my wife

and best friend, Katherine, who has been there every step of the way. You have helped me stay sane through the highs and lows of graduate school, edited everything I've written, and know the value of getting a good breakfast together the morning before a presentation. You mean the world to me.

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LIST OF ABBREVIATIONS AND SYMBOLS

ATAC-Seq – assay for transposase-accessible chromatin using sequencing

BRG1 – brahma related gene 1

CHD – chromodomain helicase DNA-binding chromatin-remodeling complex

ChIP-seq – chromatin immunoprecipitation followed by sequencing

CO – crossover

DNMT – DNA methyl-transferase

DSB – DNA double-strand break

E – embryonic day

ES – embryonic stem cells

EUCOMM – European Conditional Mouse Mutagenesis Project

FA – Fanconi Anemia

Fl – floxed allele

GT – gene trap allele

H & E – hematoxylin and eosin stain

H2AX – histone variant H2AX

H2A.Z – histone variant H2A.Z

HR – homologous recombination

ICM – inner cell mass

IF – immunofluorescence

INO80 – inositol-requiring 80 chromatin-remodeling complex

ISWI – imitation switch chromatin-remodeling complex

MEF – mouse embryonic fibroblast

MSCI – meiotic sex chromosome inactivation

MSUC – meiotic silencing of unpaired chromatin

NCO – non-crossover

NHEJ – non-homologous end joining

P – postnatal day

PAS – periodic acid and Schiff's stain

PCR – polymerase chain reaction

PGC – primordial germ cell

qRT-PCR – quantitative reverse transcription polymerase chain reaction

RNA-seq – mRNA isolation followed by sequencing

RPA – replication protein A

RPKM – reads per kilobase per million of reads

SCP1 – synaptonemal complex protein 1

SCP3 – synaptonemal complex protein 3

SSC – spermatogonial stem cell

SWI/SNF – mating type switching/sucrose non-fermenting chromatin-remodeling complex

γ – denotes post-translational phosphorylation event

CHAPTER 1 – GENERAL INTRODUCTION

Chromatin Modification is Critical for Development

Introduction

In order to fit the entirety of the genetic information into the nucleus, eukaryotes have evolved a system whereby the DNA is tightly compacted with proteins to form a structure known as chromatin. The basic unit of chromatin is the nucleosome, an octamer of four canonical histone proteins: H2A, H2B, H3 and H4 (Luger, 2001). The double-stranded DNA helix winds around the nucleosome wrapped at 147 base pairs per turn, in what is called the "beads on a string" configuration (Kornberg and Lorch, 1999). This structure is stabilized by histone H1 which binds the entry and exit points of the DNA (Thoma et al., 1979). Higher order chromatin structures form packaging the DNA more densely, although the exact nature of these structures remains controversial (Luger et al., 2012). The multiple orders of chromatin structure are necessary for packaging, but the benefits come at the expense of reduced accessibility to the underlying DNA sequence information. In order to overcome this repression, there exist several types of chromatin modifications that modulate the DNA-protein interaction, allowing localized access to the DNA template.

Types of Chromatin Modification

The ability to change the chromatin state at sites within the nucleus is critical for many genomic processes. While the dense packaging of chromatin is necessary to fit the

totality of the genome into the nucleus, the cell must modulate the DNA-nucleosome interaction to access the DNA template for a variety of genomic processes. There are a number of epigenetic mechanisms that include both direct modifications to the DNA and changes to the histone proteins. The most basic modification is direct methylation of CpG dinucleotides on DNA, which is catalyzed by DNA methyltransferase (DNMT) enzymes (Moore et al., 2013). DNA methylation has been shown to be involved in DNA repair in multiple ways. For example, hypermethylation of DNA in *Arabidopsis* prevents crossover events associated with homologous recombination (Mirouze et al., 2012). Methylation affecting the expression of DNA repair factors is also important for carcinogenesis (Julsing and Peters, 2014).

In addition to DNA methylation, there are other chromatin modifications that directly affect the nucleosomes. Each histone includes a highly structured core domain and an unstructured amino-terminal "tail" region (Luger et al., 1997; Mersfelder and Parthun, 2006). There are a wide variety of covalent post-translational modifications of the histone tails that can be placed on specific residues, including methylation, acetylation, phosphorylation, etc. (Bannister and Kouzarides, 2011). Acetylation changes the overall charge of the histone, which reduces the strength of the DNA-protein interaction (Hong et al., 1993). Histone modifications can also act as signals, triggering specific genomic events. According to the histone code hypothesis, first proposed by Strahl and Allis, certain combinations of these modifications of different histone tail residues precipitate specific nuclear events by recruiting downstream factors to the chromatin (Strahl and Allis, 2000). Enzymes that catalyze these modifications are classified as "writers", while "readers" are effector proteins containing domains that recognize specific combinations of modifications

on the histone tail and initiate downstream events (Musselman et al., 2012; Rothbart and Strahl, 2014; Yun et al., 2011). Major functions that are regulated in this manner include gene expression, DNA replication, and DNA repair (Kouzarides, 2007).

In addition to the post-translational modification of histones, it is possible to modulate chromatin by replacing the canonical histones with alternative versions within a nucleosome. In mammals, a number of histone variants exist throughout the genome. As with histone modifications, the incorporation of variant histones within a nucleosome can change the DNA-protein interactions or signal downstream events (Skene and Henikoff, 2013). For example, canonical histone H3 is only expressed during S-phase, therefore during the rest of the cell cycle the variant H3.3 is incorporated into nucleosomes instead (Ahmad and Henikoff, 2002). While H3.3 differs by only four to five amino acids, this small difference allows for separate phosphorylation events of the histone variant, which has implications for the local chromatin state (Hake et al., 2005). Recent data has determined that H3.3 is required for development, and mutant embryos lacking H3.3 die at peri-implantation stages. Mouse embryonic fibroblasts (MEFs) cultured from these animals demonstrate critical defects in the maintenance of heterochromatin at sites where H3.3 is normally deposited (Jang et al., 2015).

Histone variant H2AX is especially relevant for this study due to its role in chromatin modification during DNA repair. In contrast to the canonical histone H2A, this particular variant contains additional carboxy-terminal residues, including serine 139, which can be phosphorylated in response to unrepaired DNA breaks (Rogakou et al., 1998). Mice harboring a loss of H2AX are immune deficient and sensitive to ionizing radiation (Celeste et

al., 2002). These observations demonstrate an intriguing connection between chromatin modification through the incorporation of histone variants and specific cellular processes.

Finally, the chromatin landscape within the genome can be modulated through active methods such as ATP-dependent chromatin remodeling. These chromatin-remodeling complexes use energy from ATP hydrolysis to physically affect the interaction between the DNA and the nucleosome. Depending on the mechanism of action for a particular remodeling complex, this could involve sliding the nucleosome to a different position on the DNA, loosening the DNA around the nucleosome, or removing the octamer partially or completely (Narlikar et al., 2013). This is an early event in the repair process, following the deposition of γH2AX at the break site (Fernandez-Capetillo et al., 2003). In addition to remodeling activity, several complexes are able to catalyze replacement of canonical histones with histone variants. For example, chromatin-remodeling complexes of the INO80 family are responsible for modulating the incorporating H2A.Z at sites of DNA damage. In systems from yeast to mammals, the SRCAP (Swr1 in yeast) and INO80 complexes work antagonistically to regulate H2A.Z (Htz1) by depositing and subsequently removing the histone variant (Alatwi and Downs, 2015; Papamichos-Chronakis et al., 2006; Ruhl et al., 2006). This exchange of H2A.Z affects the chromatin compaction around break sites and influences the method of repair employed (Xu et al., 2012). Such functions make ATP-dependent chromatin-remodeling complexes particularly potent chromatin modifiers.

Chromatin-Remodeling Complexes Have Diverse Roles in Development

ATP-dependent chromatin-remodeling complexes have traditionally been grouped into four major classes, which are defined by the incorporation of particular core ATPase

subunits. These complexes include SWI/SNF (BRG1 or BRM ATPase subunits), ISWI (SNF2H or SNF2L), CHD (CHD1-9) and INO80 (INO80, SWR1 or TIP60). The ATPase subunit hydrolyzes ATP to obtain the energy necessary to exchange or evict nucleosomes. In addition to the core, each complex is composed of a number of accessory subunits that provide additional functionalities. Chromatin-remodeling complexes of all families play important cellular and genomic functions and are involved in a wide range of biological processes, from embryonic development to cancer (Ho and Crabtree, 2010; Lusser and Kadonaga, 2003; Müller and Leutz, 2001; Nair and Kumar, 2012).

SWI/SNF Complexes

Chromatin remodelers of the SWI/SNF family were first identified in screens for yeast genes involved in the process of mating type switching and sucrose fermentation (Neigeborn and Carlson, 1984; Stern et al., 1984). SWI/SNF is a large 8-14 protein subunit complex responsible for sliding or evicting target nucleosomes (Nair and Kumar, 2012). While in lower eukaryotes SWI/SNF has a single ATPase subunit, the mammalian complex incorporates two mutually exclusive core subunits, brahma (BRM) or brahma related gene-1 (BRG1) (Sudarsanam and Winston, 2000). *In vitro* assays have demonstrated that BRG1 and BRM are capable of mobilizing nucleosomes in the presence of ATP and a chromatinized template. The addition of the accessory subunits improves the efficiency of the remodeling activity, suggesting that these subunits play important roles, potentially by increasing the affinity of the complex for its substrate (Phelan et al., 1999). The accessory subunits of SWI/SNF contribute a variety of functions, including DNA, histone or actin binding (Cairns et al., 1998; Harata et al., 1999; Patsialou et al., 2005; Wilsker et al., 2004). SWI/SNF has a

potent role in the regulation of gene expression, as recent studies have discovered that BRG1 binds to approximately 4% of the genome in embryonic stem (ES) cells, and is primarily found at gene promoters (Ho et al., 2009). SWI/SNF activity is also involved in activating the DNA repair response, and a number of subunits are highly mutated in cancer, suggesting that the complex functions as a potent tumor suppressor (Lu and Roberts, 2013; Smith-Roe et al., 2015; Weissman and Knudsen, 2009).

In addition to its roles as a tumor suppressor, the SWI/SNF complex is critically important for early developmental processes. Mice harboring a homozygous null allele of the BRG1 subunit of SWI/SNF exhibit peri-implantation lethality prior to embryonic day 6.5 (E6.5). While the majority of Brg1-heterozygous animals progress to adulthood, approximately 15-30% of E17.5 embryos display exencephaly, indicating a dosage sensitivity for *Brg1* in certain developmental processes (Bultman et al., 2000). Additionally, an ENU generated point mutant allele of *Brg1* results in a later mid-gestational lethality. This allele maintains normal ATPase activity, but demonstrates a reduced ability to remodel nucleosomes *in vitro* as a likely result of conformational changes to the protein (Chandler et al., 2014). The affected chromatin-remodeling activity of the hypomorphic point mutant leads to a failure of erythropoiesis in the fetal liver and lethality beginning at E11.5, much later than *Brg1*-null embryos (Bultman et al., 2005). The range of phenotypes observed in embryos harboring *Brg1*-null, *Brg1*-heterozygous or point mutant alleles highlight the variety of roles played by SWI/SNF complexes containing this subunit throughout development.

In contrast to the critical developmental role played by BRG1, whole animal knockout of BRM does not demonstrate the same lethality. *Brm*-null embryos develop normally and live into adulthood (Reyes et al., 1998). It is interesting to note, however, that

recent data has called into question the completeness of the knockout resultant from the *Brm*-null allele, suggesting that a full ablation of Brm may yield an interesting result (Thompson et al., 2015). In addition to the core ATPases, several of the accessory subunits are also required for embryonic development including SNF5 and ARID1A (Chandler et al., 2013; Gao et al., 2008; Klochendler-Yeivin et al., 2000). These results serve to further illuminate the critical involvement of multiple SWI/SNF subunits for proper functionality of the complex.

ISWI Complexes

Complexes of the ISWI family are also required for proper embryonic development. The ISWI ATPase was initially characterized in *Drosophila* in an attempt to identify remodelers similar to the Brm subunit of SWI/SNF (Elfring et al., 1994). *Drosophila* ISWI was determined to function as both a nucleosome spacing and chromatin assembly factor (Corona et al., 1999). In mammals, the ISWI complex incorporates one of two alternative ATPase subunits, SNF2H or SNF2L (Lazzaro and Picketts, 2001). These homologous subunits function antagonistically in certain biological contexts, where SNF2H is associated with proliferation, while SNF2L suppresses it (Alvarez-Saavedra et al., 2014; Eckey et al., 2012). Like SWI/SNF the core ATPase forms complexes with various accessory subunits that can confer different functional roles. These include BPTF, RSF1 and CECR2 (NURF, RSF, CERF complexes), which are involved in transcription, and WSTF, ACF1, CHRAC15 and CHRAC17 (WICH, ACF, CHRAC complexes) which also have roles in replication and repair processes (Erdel and Rippe, 2011).

As with SWI/SNF, ISWI activity is required for early embryonic development. In the developing *Drosophila* embryo the loss of ISWI resulted in lethality at the larval or pupal stages (Deuring et al., 2000). Similar observations have been made during *Xenopus* development. ISWI is expressed throughout embryonic development, but is not maintained in the adult animal. However, the depletion of ISWI did not affect chromatin formation in egg extracts (Demeret et al., 2002). In mouse, ISWI is critical for early embryogenesis. Animals heterozygous for *Snf2h* develop normally, while *Snf2h*-null embryos arrest around embryonic day 7.5 (E7.5). In addition, ES cells could not be cultured from *Snf2h* mutant embryos, suggesting that ISWI complexes are required for cell proliferation and survival (Stopka and Skoultchi, 2003).

CHD Complexes

The CHD family of chromatin remodelers is a particularly diverse group of complexes that also have important functions in development. There are nine different CHD ATPase subunits from which a large variety of complexes are formed. In addition to the differences in ATPase subunits, the CHD chromatin-remodeling complexes are unique in that they include both nucleosome remodeling and histone deacetylatase activity (Tong et al., 1998). Within the CHD family, three subfamilies have been categorized based on the domain structures of the CHD proteins (Hall and Georgel, 2007; Marfella and Imbalzano, 2007). The first subfamily consists of complexes containing the CHD1 and CHD2 ATPase subunits. Proteins of this subfamily contain a DNA binding domain in addition to the ATPase and chromodomains (Delmas et al., 1993; Stokes and Perry, 1995). This family of chromatin remodelers has also been implicated in later aspects of embryonic development.

Murine embryos ablated for CHD2 activity develop normally until E18.5, when they begin to display significant growth retardation. These animals are perinatal lethal, and no mutant pups can be recovered at weaning. The direct cause of the lethality remains unclear, although experiments in ES cells suggest that the particular mutation affects the DNA binding ability of CHD2 (Marfella et al., 2006).

CHD3 and CHD4 are incorporated into remodeling complexes forming the second CHD subfamily. Complexes formed with these core subunits are known as Mi-2α and Mi-2β. The proteins differentiate themselves from the other CHDs by the inclusion of PHD zinc finger domains (Woodage et al., 1997). The Mi-2 remodelers are also potent developmental factors. A conditional deletion of CHD4 in the embryonic vasculature reduces blood vessel integrity and results in mid-gestation lethality (Ingram et al., 2013). Interestingly this model reveals a genetic interaction between CHD4 and a BRG1-containing SWI/SNF complex, where a double knockout of these chromatin remodelers is able to rescue the phenotypes observed in the single mutant embryos through an antagonistic relationship at Wnt target genes (Curtis and Griffin, 2012).

The final subfamily of CHD chromatin remodelers includes CHD5-9. Several complexes containing these ATPases have interesting developmental roles. A zebrafish morpholino knockdown of CHD5 results in craniofacial and neural development defects (Bishop et al., 2015). Likewise the human CHARGE syndrome, which includes craniofacial and cardiovascular defects, is associated with mutation or deletion of CHD7 (Vissers et al., 2004; Vuorela et al., 2007). Finally, CHD9 deletion affects osteoprogenitor cells, indicating that this complex plays a role in embryonic skeletal formation (Shur et al., 2006). As a

diverse family of chromatin remodelers, CHD containing complexes display a remarkable range of phenotypes, implicating its activity in a wide variety of developmental processes.

INO80 Complexes

Finally, the INO80 family of chromatin remodelers is also critical for development. Remodelers of this family have been described to facilitate DNA repair at sites of damage by altering the local chromatin to allow repair factors access to the underlying template. The complex itself is a multi-subunit complex, which, like most chromatin remodelers, contains a core ATPase subunit. In addition, the INO80 family is unique amongst the chromatin remodelers because it incorporates two additional AAA+ ATPase-containing subunits (Huen et al., 2010). While its functional roles appear to be relatively well conserved from yeast to humans, the complex composition differs significantly, adding several metazoan-specific subunits not found in yeast (Chen et al., 2011). The roles of these subunits remain largely undefined. In addition to its composition, INO80 is unique from the other remodelers in the structure of the core subunit itself. It harbors a spacer region splitting the ATPase domain in half, which has been shown in yeast to be required for assembly of the complex (Morrison and Shen, 2009).

The INO80 complex functions primarily in DNA repair and transcription. Cells that have been depleted for INO80 have an increased sensitivity to DNA-damaging agents and an inability to repair double-strand breaks. INO80 itself is localized to a DSB site by the deposition of γH2AX and can participate in both HR and NHEJ mechanisms. A separate role for the INO80 complex involves an association with the transcription factor YY1 (Cai et al., 2007). YY1 regulated genes are traditionally involved in development, indicating a potential

role for INO80 in the embryo (Affar et al., 2006). In addition, INO80 has been shown to localize at the promoters of several genes involved in DNA repair pathways under conditions where these factors would be necessary (Park et al., 2010). Taken together, these data indicate that INO80 may play dual roles in the repair response, aiding in the expression of repair factors and direct interaction at the break site itself, creating the conditions that permit repair to occur. Additional roles for the complex include involvement in sister chromatid cohesion, telomere maintenance and replication fork progression (Ogiwara et al., 2007; Shimada et al., 2008; Yu et al., 2007).

A number of recent studies have determined that a whole-animal ablation of INO80 is embryonically lethal. Initial work describing the developmental role for the INO80 complex comes from *Drosophila* where embryonic deletion of the core INO80 subunit was lethal at late stages of embryogenesis and displayed severe patterning defects due to misexpression of the Hox genes (Bhatia et al., 2010). In addition, several recent studies have shown the murine INO80 complex to be a potent factor for early development and stem cell maintenance. Through the use of a female germ line specific Cre driver, *Ino80*-null embryos were created. The mutant embryos began to show defects at E7.5 and were resorbed by E14.5. The lethality in *Ino80*-null embryos was not rescued on a *p53*-null background. The authors created an *Ino80*-null MEF line, which is sensitive to replication stress and prone to genomic instability. They speculate that these defects may contribute to the lethality in *Ino80*-null embryos (Min et al., 2013). A second study where $Ino80^{+/-}$ animals are intercrossed demonstrated similar embryonic defects. Blastocyst stage mutant embryos were flushed and cultured, but quickly lost their proliferative ability. This result suggests that INO80 is required for proliferation in the inner cell mass (ICM). Further experiments

determined that defective replication fork progression contributed to a failure of the *Ino80*-null embryos (Lee et al., 2014). Additional evidence connecting the embryonic lethality to a defect in the cells of the ICM was provided by experiments in ES cells. When *Ino80* is silenced in ES cells, expression of several important pluripotency markers is downregulated. The authors determined that the INO80 complex is recruited to the promoter of these genes by OCT4 and WRD5 and is responsible for maintaining the open chromatin necessary for expression. These observations were recapitulated in culture, where embryos treated with *Ino80*-siRNAs displayed diminished expression of the same pluripotency factors (Wang et al., 2014).

The phenotypes associated with the loss of the INO80 core ATPase are mirrored by several other complex members, as homozygous mutations affecting INO80 accessory subunits have been shown to result in embryonic lethality as well. Individual ablation of Ruvbl1 or Ruvbl2 activities are embryonically lethal, with the Ruvbl1 animals dying around the early blastocyst stages (Arnold et al., 2012; Bereshchenko et al., 2012). The phenotypes associated with several discrete members of the INO80 highlight the critical involvement for the complex in development. For the most part, the developmental roles for INO80 have focused on its function as a transcriptional activator. In the study described presently, we instead sought to elucidate the requirement for INO80 DNA repair activity in a developmental system. To this end we chose to study the involvement of INO80 in germ cell development.

Germ Cell Development

Introduction

As discussed previously, a diverse group of chromatin-remodeling complexes plays important roles in different aspects of embryonic development. Germ cell development or gametogenesis is another aspect of development that requires large-scale regulation of chromatin. While the overall picture of ATP-dependent chromatin-remodeling activity during gametogenesis is beginning to take shape, our knowledge regarding the roles of individual remodeling complexes is incomplete. This is particularly true for the INO80 family of chromatin remodelers. As the flagship complex of this family, INO80 is a particularly interesting candidate for meiotic recombination based on its described roles in multiple DNA repair pathways. While methods of chromatin modification are important for both male and female gametogenesis, for the purpose of this study we focused specifically on the role of the INO80 complex during spermatogenesis.

Chromatin Reorganization During Spermatogenesis

The ability to reorganize chromatin is an important feature of spermatogenesis. There are many steps during this process that require the cell to modulate access to the genome. These include spermatogonial stem cell maintenance, meiotic recombination, and the histone-to-protamine transition.

Early in murine embryonic development, the primordial germ cells (PGC) proliferate to colonize the developing gonads. Eventually male PGCs arrest in G1 of the cell cycle and remain mitotically inactive until postnatal day 10 (Coucouvanis and Jones, 1993). In adult testes, the progenitor germ cells are the spermatogonial stem cells (SSC). As these cells

proliferate, they are capable of both self-renewal, maintaining the SSC population, as well as producing spermatogonia, which are able to differentiate to eventually form spermatozoa (de Rooij, 2001). There are a number of chromatin events that take place in the pre-meiotic germ cells that are important for development.

Once spermatogonia begin to differentiate, they undergo a large scale change in chromatin state, generally becoming more heterochromatic as development progresses (Phillips et al., 2010). These events include silencing transposable elements and paternally-imprinted genes though DNA methylation (Zamudio et al., 2008). Also at the spermatogonial stage a chromatin landscape is set up to prepare the genome for embryonic development following fertilization. Promoters of genes that will be expressed in the embryo are marked with histone modifications H3K4me3 and H3K27me3, which are normally associated with active and repressive chromatin landscapes respectively. These modifications are accompanied by DNA hypomethylation, creating a chromatin dynamic amenable to activate the genes necessary for totipotency in the early embryo (Hammoud et al., 2009; Hammoud et al., 2014).

Chromatin modification is also critical for meiosis, the process whereby mature germ cells are formed through a set of reductional divisions (Cheng and Mruk, 2010; Sasaki and Matsui, 2008a). Recombination occurs during prophase I of meiosis I, which can be further broken down into four separate stages. During the leptotene stage, the chromosomes condense within the nucleus. Double-strand DNA breaks are created across the chromosomes by the SPO11 enzyme (Keeney et al., 1997). The DNA breaks are marked by histone variant γH2AX and are required for synapsis of homologous chromosomes (Mahadevaiah et al., 2001; Romanienko and Camerini-Otero, 2000). As a developing germ

cell enters the zygotene stage, the homologous chromosomes are condensed and synapsed. This formation allows for the exchange of genetic material between them in a process called meiotic recombination. By the pachytene stage, repair and recombination has begun. In order for pachytene to complete, DNA repair must be completed on the autosomes. This is required to maintain the genomic integrity of the germ cells. Successful recombination events are required on each chromosome not only to exponentially increase genetic diversity in the progeny, but for proper segregation of the homologs to the daughter cells, which occurs during diplotene stage (Checchi and Engebrecht, 2011).

Following the completion of meiosis, additional chromatin regulation is required. H2A.Z remains associated with the chromatin, especially on the sex chromosomes. This is thought to help maintain the heterochromatic state of X and Y, maintaining their transcriptional silence into spermiogenesis (Greaves et al., 2006). In addition, paternally imprinted genes and transposable elements must remain silenced (Zamudio et al., 2008).

Finally, during the post-meiotic process of spermiogenesis, the genome undergoes a histone-to-protamine transition. Male germ cell development is unique from oocyte development in females in that the genetic material in a mature spermatozoon must be compacted more tightly to fit in the small sperm head. During this process, approximately 85% of histones are replaced with protamines, which allows for tighter packaging of the genome (Oliva, 2006). This is possible due the unique properties of the protamines, which are positively charged proteins that are rich in arginine and cysteine (Brewer et al., 2002). When associated with protamines, the chromatin is able to take on a toroid shape, a dense conformation protecting the DNA from external forces (Ward, 2010). In addition to the role of protamines in packaging DNA into the sperm head, it is thought that the additional

compaction is important for silencing the paternal genome in the embryo post-fertilization (Carrell, 2012). In order to execute this transition, the histones proteins are first hyperacetylated, loosening the DNA-protein connection (Sonnack et al., 2002). The modified histones are then replaced, first with transition proteins and finally protamines, thus executing a major chromatin regulatory mechanism unique to this lineage (Carrell, 2012).

DNA Repair Mechanisms During Meiotic Recombination

Much of the time, DNA damage is unintentional and is detrimental to the cell, but there are a few processes that require the deliberate creation and subsequent handling of breaks during the course of normal cellular functions. The repair of SPO11-created DSBs during meiotic recombination is such a process. During meiosis, the repair of damaged DNA is critical for the ability of an organism to correctly pass on its genetic material to its offspring. Within the cell, a variety of mechanisms are available to detect and repair DNA damage, whether it arises from external agents or internal processes. Improper repair of damaged DNA can lead to a diverse set of problems, from carcinogenesis to infertility (Marchetti and Wyrobek, 2008; Mukherjee et al., 2010).

Diverse mechanisms exist for restoring DNA after a DSB event, depending on the fidelity required. In response to double-stranded breaks in a chromosome, the cell has two options. One option, homologous recombination (HR), involves localizing the repair machinery to the site of the damage, resecting back the ends of the fragment, and finding the identical sequence on the homologous chromosome. Once this segment is found, the damaged strand invades the intact strand, using it as a template to re-derive the sequence lost (San Filippo et al., 2008). While this method is especially important for maintaining the

integrity of the sequence, it is much less efficient than the other method, non-homologous end joining (NHEJ), and is not able to be performed if the sequence around the break is non-unique or repetitive. On the other hand, NHEJ is more efficient but does not maintain the fidelity of sequence. After the repair machinery is localized to the break site, the damaged DNA is stabilized and the two ends are joined back together. The intervening sequence is therefore lost (Mao et al., 2008; van Gent and van der Burg, 2007).

During meiosis, one of the first events that occurs is the induction of DNA double-strand breaks, some of which will be resolved into crossovers. The chromatin landscape within meiotic spermatocytes is involved in directing the sites where the breaks occur. One of the major factors in determining recombination crossover site localization is the H3K4 methyltransferase PRDM9. The PRDM9 protein contains a zinc finger array that targets it to motifs associated with recombination hotspots (Baudat et al., 2010). It has been suggested that PRDM9 binding creates a chromatin dynamic appropriate for recruiting other chromatin modifying factors and SPO11 (Grey et al., 2011). Recombination events are also directed away from regions of highly repetitive sequences such as centromeres, telomeres and transposable elements by high levels of DNA methylation at these genomic features (Law and Jacobsen, 2010).

Once meiotic DSBs are created, the break site must be identified and the repair machinery localized in order for repair to occur. In the context of a chromatinized template, this configuration prevents the repair machinery from gaining the necessary access to the break site. In the case of homologous recombination, the chromatin landscape at the break site and the corresponding region on its homolog must be modified for repair to proceed. Multiple studies focused primarily on DNA repair in yeast, have described important roles

for chromatin modifiers in preparing for HR (Brachet et al., 2012). Once DNA DSBs are induced, γH2AX is deposited around the break site, as well as histone modifications H3K79me and H3K20me2. This pattern helps recruit downstream DNA repair factors and checkpoint proteins (Celeste et al., 2002; Sanders et al., 2004; Wysocki et al., 2005). The surrounding histones are also acetylated, destabilizing the interaction between the nucleosome and the DNA (Downs et al., 2004). Finally chromatin-remodeling complexes, particularly RSC, INO80 and SWR1, remove the local nucleosomes allowing resection and downstream HR processes to proceed (Chai et al., 2005; Van Attikum et al., 2004).

Additional studies on UV induced DNA repair in yeast have suggested that INO80 chromatin remodeling is involved in restoring nucleosomes to the newly synthesized DNA following repair, completing the cycle (Sarkar et al., 2010). While many of the histone modification events also take place during murine meiotic recombination (Rô Me Buard et al., 2009), it remains to be determined whether these chromatin-remodeling factors, particularly the complexes of the INO80 family, perform similar functions during HR associated with mammalian meiotic recombination.

Disruption of Meiotic Factors Has Severe Consequences

Mutations in a wide variety of meiotic factors lead to spermatogenic defects. The timing and phenotypes associated with these defects are useful for illuminating the specific roles that these factors play during meiosis. Amongst the factors that affect meiotic progression when ablated are SPO11, ATM, H2AX and SCP3.

SPO11 is a topoisomerase that is responsible for generating hundreds of DNA double-strand breaks throughout the genome at the outset of meiosis. When SPO11 is

ablated in spermatocytes, the DSBs are not created. During meiosis, synapsis of homologous chromosomes is reliant DSBs and DNA repair. Thus meiocytes lacking SPO11 display extensive asynapsis and arrest at the zygotene stage. Interestingly, synapsis is partially rescued in these cells by treating them with the DNA-damaging reagent cisplatin, further underscoring the importance of DNA damage and repair for synapsis (Romanienko and Camerini-Otero, 2000).

Likewise, the disruption of factors related to DNA repair leads to severe meiotic defects. Histone variant H2AX is phosphorylated in response to DNA damage. During prophase I of meiosis, γH2AX localizes extensively to the chromatin due to DNA breaks created by SPO11. As repair progresses, it regresses to the region around the X and Y chromosomes referred to as the sex body (Chicheportiche et al., 2007; Mahadevaiah et al., 2001; Turner et al., 2004). When *H2AX*-null animals were created, not only were the adult mice sensitive to DNA damage, the males were infertile. Developing spermatocytes arrested at the pachytene stage of prophase I, exhibiting synapsis and repair defects, particularly on the sex chromosomes (Celeste et al., 2002).

In addition to factors related to DNA repair, members of the synaptonemal complex are also critical for meiosis. The synaptonemal complex is a proteinaceous structure that forms a connection between synapsed homologous chromosomes. During the leptotene stage of meiotic prophase I, SCP3 coats the axial elements of the chromosomes as they begin to condense. As synapsis is achieved between the homologs, SCP1 forms a transverse element between them, holding the structure together (Dobson et al., 1994). When SCP is ablated in developing spermatocytes, homologous chromosomes are unable to synapse, and the cells undergo apoptosis at the zygotene stage. In addition to rampant asynapsis, RPA and RAD51

are mislocalized, indicating that DNA repair is inhibited and providing further evidence for the interconnectedness of these two processes in meiosis (Yuan et al., 2000).

Chromatin modifying factors are also among the critical factors required for different stages of spermatogenesis. For example MLL2, which catalyzes H3K4me3, is required early in this process. When its activity is ablated, the differentiated pre-meiotic spermatogonial population is lost, likely due to an inability to transition from the self-renewal program into a more differentiated state (Glaser et al., 2009). A number of other chromatin modifiers are critical during the meiotic stages. These include PRDM9, G9a, and EED, all of which when lost result in pachytene arrest due to synapsis and recombination defects (Hayashi and Matsui, 2006; Mu et al., 2014; Tachibana et al., 2007). Still others are expressed later and are involved in regulating processes such as spermiogenesis. When BRWD1, a bromodomain containing protein that interacts with SWI/SNF is knocked out, sperm counts are reduced and sperm head morphology is abnormal (Philipps et al., 2008). Functionally, BRWD1 is responsible for initiating transcription in the haploid spermatid and is involved in the expression of genes required for spermiogenesis (Pattabiraman et al., 2014). In addition to the aforementioned chromatin modifying factors, the ATPase dependent chromatin-remodeling complexes also play important roles during spermatogenesis, and their functions will be explored presently.

Chromatin Remodeling is Critical for Murine Spermatogenesis

Chromatin modification is integral to spermatogenesis, and ATP-dependent chromatin remodeling is required in addition to the histone modifiers mentioned in the

previous section. Mouse knockouts ablating subunits of CHD, ISWI and SWI/SNF have helped illuminate the specific roles of these complexes.

The CHD family of chromatin remodelers has a large repertoire of core subunits with which to form individual complexes. CHD3 and CHD4 are expressed during the early stages of spermatogenesis up through meiosis, when expression diminishes and is replaced by CHD5 (Bergs et al., 2014). Loss of CHD5 in the male germ line results in a post-meiotic defect, preventing the transition between histone and protamine proteins and proper compaction of the genome (Li et al., 2014; Zhuang et al., 2014). Germ cell related-phenotypes associated with other CHD family chromatin remodeler complexes have yet to be fully explored.

The activities of the ISWI family of chromatin remodelers seem to be restricted to later stages of spermatogenesis. While the two mutually-exclusive core ATPase subunits have yet to be studied in the context of spermatogenesis, the functionality of a number of associated subunits are known to convey specific phenotypes for ISWI. One complex containing the Williams Syndrome transcription factor (WSTF) is dispensable for fertility, although some apoptosis of meiocytes is observed (Broering et al., 2015). The BAZ1A subunit, which defines the ACF and CHRAC complexes, mediates spermiogenesis, and its depletion leads to defects in both head and tail of developing sperm (Dowdle et al., 2013). Motile sperm are formed in the context of a knockout for *Cecr2*, although there is a defect in their ability to fertilize an ovum (Thompson et al., 2012). These results suggest that the role of ISWI lies in the later processes of sperm formation and fertility.

Beyond CHD and ISWI, the SWI/SNF complex has been studied extensively in the context of meiosis. Previous studies have determined that *Brm*-null animals are fertile,

indicating that it is dispensable for spermatogenesis (Reyes et al., 1998). As the alternative ATPase for SWI/SNF, it is also possible that BRG1 is capable of compensating in the absence of BRM. On the other hand, BRG1 activity is required for meiotic progression in spermatocytes. Using a Cre driver active in the PGC population, *Brg1*-null spermatocytes arrest at pachytene stage of prophase I. Synapsis of homologous chromosomes and recombination are impaired, causing the spermatocytes to undergo apoptosis at this stage. In addition, the overall chromatin dynamic is affected in these cells. Heterochromatin-associated mark H3K9me2 and heterochromatin protein HP1γ were maintained into later stages of meiosis, indicating a more closed chromatin state in *Brg*-null spermatocytes (Kim et al., 2012; Wang et al., 2012). Taken together these data suggest that BRG1-containing SWI/SNF complexes are essential meiotic factors, acting earlier in meiosis than previously described complexes of the CHD or ISWI families. Each of these three remodeler families has aspects of their function that are non-redundant.

Research Question

Preliminary Observations

While recent studies have determined that INO80 is essential for the most basic aspects of embryonic development, the timing and severity of *Ino80*-null embryos prevent this system from being fully informative for assessing the *in vivo* contribution of INO80. On the other hand, the process of meiotic recombination presents an ideal context for observing the involvement of INO80 in a system naturally predisposed to requiring the repair-associated chromatin-remodeling activity supplied by INO80.

Chromatin accessibility is critical to proper progression through spermatogenesis, particularly during the complex process of meiosis. A variety of studies which were reviewed previously have shown that a wide variety of chromatin modifiers are required during spermatogenesis. In particular, the BRG1 subunit of SWI/SNF is known to be expressed early in prophase I, and in its absence, double-strand breaks are not efficiently repaired (Kim et al., 2012; Wang et al., 2012).

While studies have shown that INO80 is distinct in the mechanism by which it interacts with and remodels nucleosomes, several of its known functional roles overlap with SWI/SNF. A major question that has yet to be addressed is to what extent are these complexes functionally redundant. Preliminary expression analysis within the seminiferous tubule suggests that both BRG1 and INO80 are expressed in the meiotic population of developing spermatocytes (Figure 1-1). This timing is concordant with meiotic recombination, potentially implicating INO80 in the DNA repair process active at this time.

The staining patterns in seminiferous tubules correspond closely with transcriptome analysis of these stages. A recent study completed by Margolin and colleagues performed RNA-seq on whole testis from postnatal mice, a time period that spans the first wave of spermatogenesis (Margolin et al., 2014). We mined their data for the expression profiles of several ATPase subunits of the SWI/SNF, CHD and INO80 families (Figure 1-2). The expression patterns for BRG1, CHD3 and CHD5 matched patterns known from previous studies. BRG1 and CHD3 are active in spermatogonia and meiosis, which is represented in the RNA-seq by high levels of expression in early postnatal samples that begins to diminish as the animals age. Additionally, the steep increase seen at P20 for CHD5 aligns well with its known post-meiotic expression pattern and function. While INO80 displays lower

expression in terms of RPKM than BRG1 or CHD3, it displays relatively consistent expression until the latest stages of spermatogenesis, suggesting that it may have roles in the spermatogonia or meiosis. An increased role for INO80 chromatin remodeling in meiocytes was expected based on the known activity of the complex in DNA repair, which is an inherent and critical property of this population.

Hypothesis

Based on the expression of INO80 in the testis and the known molecular roles of the INO80 complex, we hypothesized that the ablation of INO80 during spermatogenesis would result in defective repair of DNA double-strand breaks associated with recombination and a concomitant failure in meiotic progression. A defect of this nature would leave sites of unrepaired damage littered throughout the genome, demonstrated by a broad nuclear localization of integral components of the repair machinery. In addition, we speculated that the outcome associated with INO80 loss might be phenotypically similar to that of SWI/SNF, but that differences in function and expression would indicate non-redundant functions for these remodeling complexes.

Experimental Design

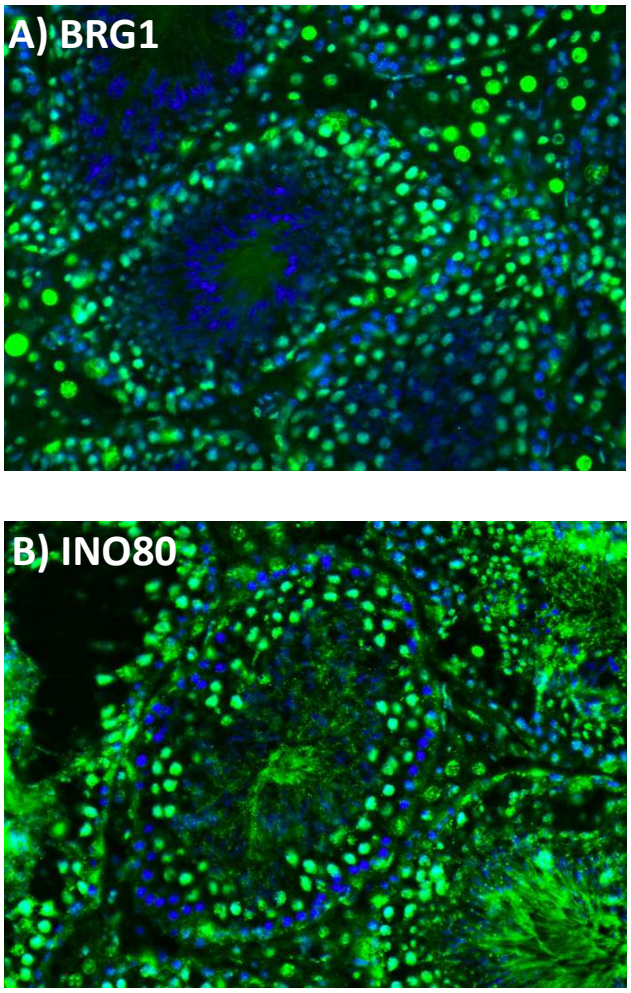
As previously discussed, the timing of *Ino80* expression and the known roles for the complex suggest gametogenesis to be an obvious system to interrogate the *in vivo* functionality of INO80. To this end, we set out to use an *Ino80* conditional allele to ablate the core ATPase subunit in developing germ cells. As this was a newly developed and untested conditional allele, we validated the deletion efficiency by first creating INO80 mutant

heterozygotes using Cre expression driven by the Sox2 promoter (Hayashi et al., 2002). These animals were used to produce *Ino80*^{cKO} embryos that recapitulated the lethality reported in the literature (Lee et al., 2014; Min et al., 2013). Based on this result, we were confident in using the allele to address the role of INO80 in the germ line.

In coordination with the INO80 allele, there are a variety of germ cell specific Cre drivers that were available for use in ablating INO80 function at different stages of spermatogenesis. *Tnap-Cre* and *Vasa-Cre* are expressed in the primordial germ cells, and deletion in the derived tissues exists over the life of the animal (Gallardo et al., 2007; Lomelí et al., 2000). Based on previous data observing the BRG1 subunit of SWI/SNF, *Vasa-Cre* yields a compelling meiotic phenotype in the conditional deletion (Kim et al., 2012). However, the early timing of the deletion had the potential to obfuscate our conclusions. The PGC population, where *Vasa-Cre* is expressed, undergoes a variety of events prior to spermatogenesis that may also require INO80 activity, potentially preventing the germ cell population from reaching the stages that we were interested in observing. Therefore the best choice for these experiments was *Stra8-Cre* (Sadate-Ngatchou et al., 2008). This Cre driver is active postnatally in the spermatogonial population. Following the expression of *Stra8-Cre*, the INO80 core ATPase will be ablated just prior to meiotic entry, thereby suspending the activity of the complex at this critical juncture. As INO80 functions in multiple DNA repair pathways, we observed the localization patterns of factors associated with both HR and NHEJ in the context of *Ino80*-null spermatocytes.

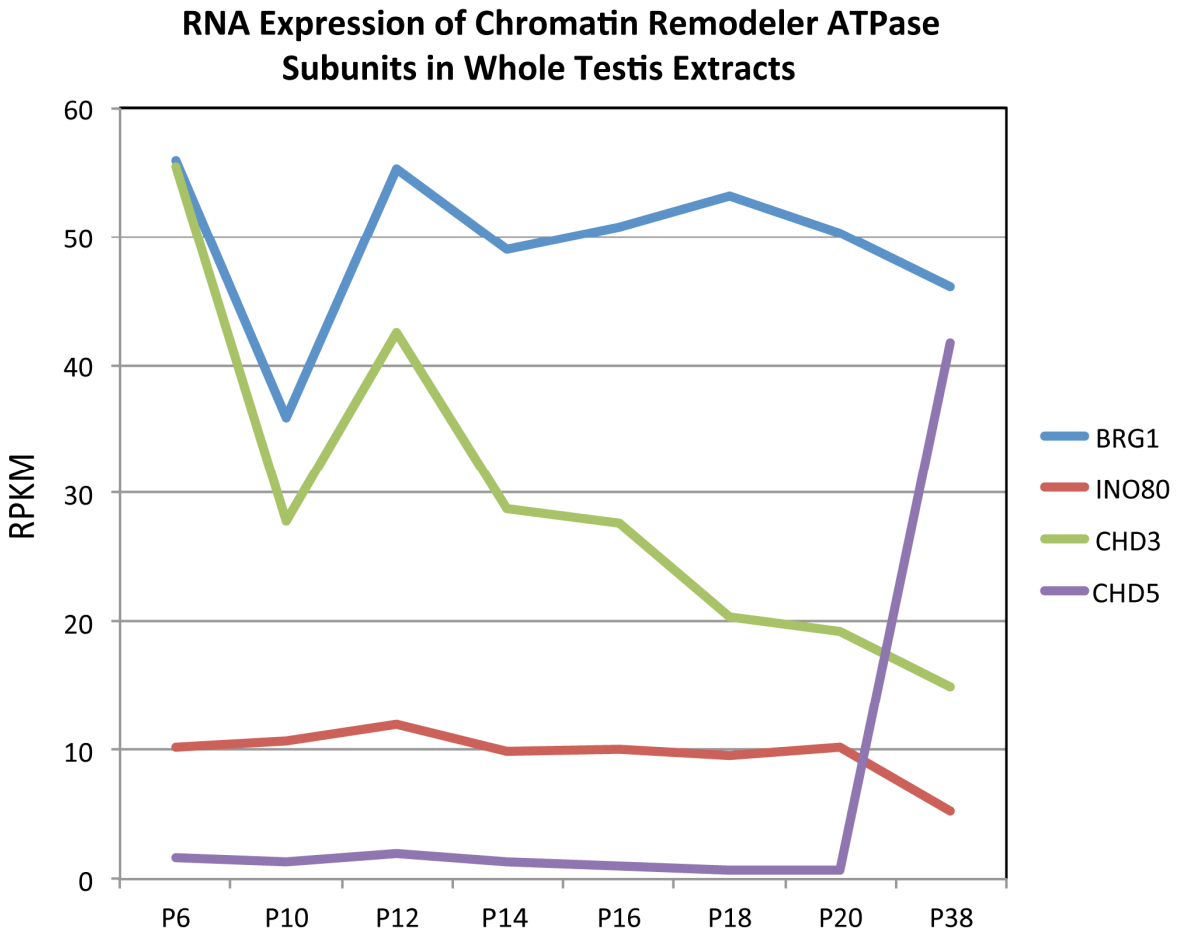
Figures

Figure 1.1 – Localization of BRG1 and INO80 Within Developing Germ Cells.



(**A**) Immunostaining of seminiferous tubules within adult murine testis for co-localization chromatin-remodeling ATPase BRG1 and (**B**) INO80 respectively.

Figure 1.2 – Whole Testis Expression of Remodeling Complex ATPase Subunits



Expression data for core subunits of SWI/SNF, CHD, and INO80 chromatin-remodeling complex families during early postnatal mouse development. Data mined from whole testis mRNA-seq experiments reported in (Margolin et al., 2014).

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CHAPTER 2 – MATERIALS AND METHODS[1]

Introduction

For the *in vivo* portions of this study, we used a newly developed conditional allele for INO80 to create a tissue-specific deletion. The allele, created by EUCOMM, follows their typical design structure for targeting conditional alleles. INO80 is a relatively large gene with 36 coding exons. A domain search predicts two functional domains for the protein, as has been previously described. These are the ATPase domain, which provides the activity for physically remodeling nucleosomes, as well as an additional helicase domain. For the specific targeted allele, the 6th exon is flanked by loxP sites, or floxed, which when exposed to Cre will recombine out this portion, causing a frameshift and premature stop codon to be encountered within the next exon, halting transcription. For INO80, this occurs upstream of the main functional domains of the protein, and any prematurely truncated transcript should be subject to nonsense mediated decay (Figure 2-1).

Initially, the allele exists in a state with a large cassette from the gene targeting steps upstream of the floxed exon 6, which includes both lacZ and Neo genes (Figure 2-1). The lacZ is attached to a splice acceptor, creating a potential gene trap if the splicing occurs into it. This cassette is flanked by Frt sites, therefore in order to remove it, the mice must first be

[1] Portions of this chapter were adapted from **Serber, D. W., Runge, J. S., Menon, D. U. and Magnuson, T.** (2015). The Mouse INO80 Chromatin Remodeling Complex Is an Essential Meiotic Factor for Spermatogenesis. *Biol Reprod.*

mated to an animal expressing a Flp transgene. The removal of the lacZ and Neo cassette leaves a traditional floxed allele around exon 6. Once this allele is obtained it can be bred to a variety of Cre driver lines to create a conditional null in the associated tissue. The breeding strategy that we undertook is included (Figure 2-2).

Description of Methods

INO80 conditional deletion mouse model

Mice harboring an INO80 conditional allele were obtained from the EUCOMM site at Institut Clinique de la Souris (IKMC Project ID: 35678). The allele features a floxed sixth exon inserted with a LacZ gene trap cassette upstream (Figure 2-1). The gene trap was flanked by *Frt* sites and removed through breeding to mice containing a constitutive *Flpe* transgene. Proper targeting of the construct was determined through long range PCR assay. Primers used for this assay were a gene-specific forward primer 5'-GTGCCATCTTGCCTGACTCCTTAGATTATG and cassette-universal reverse primer 5'-CACAACGGGTTCTTCTGTTAGTCC.

Ino80 floxed and *Stra8Cre* (Sadate-Ngatchou et al., 2008) mice were maintained on C57BL/6Tac and C57BL/6J backgrounds respectively and were intercrossed to obtain $Ino80^{\Delta/+}$; $Stra8Cre^{+}$ animals. Males of this genotype were crossed to $Ino80^{fl/fl}$ females to obtain wild-type and $Ino80^{cKO}$ animals for this study. Genotyping primers for *Ino80* used in this study were: Forward 5'-TGGCACCTTTCCAGTCTTTG and Reverse 5'-GCTGTGTGTAGTGGTACATA. *Stra8Cre* specific genotyping primers were: Forward 5'-GTGCAAGCTGAACAACAGGA and Reverse 5'- AGGGACACAGCATTGGAGTC. All

animal work was performed in accordance with IACUC protocols at the University of North Carolina-Chapel Hill.

RNA isolation and RT-PCR

Total RNA from whole individual tissues was extracted from an adult wild-type animal using the TRIzol Reagent method (Invitrogen) followed by cleanup using the RNeasy column (QIAGEN) and DNase1 (Ambion) treatment. To analyze transcript levels of *Ino80* in individual tissues, quantitative RT-PCR was performed using SsoFast EvaGreen supermix (Biorad) and analyzed on a CFX96 thermal cycler using CFX Manager Software (Biorad). INO80 qPCR primers used for this assay were: Forward 5'- GAAGATGGTGGCTGTAAG and Reverse 5'- GATGTCCTGCTGATTGAG.

Testis Histology

Whole testes from adult (8 week old) wild-type and $Ino80^{cKO}$ animals were dissected and fixed in pre-prepared Bouins fixative solution (Fisher Scientific Ricca Chemical 11-201) at 4°C overnight. Tissues were dehydrated through a graded ethanol series containing lithium carbonate to quickly remove the yellow staining. Following paraffin embedding, 7□m sections were obtained on a Leica RM2165 microtome. Stains including Hematoxylin and Eosin and Periodic Acid-Schiff were performed by the Animal Histopathology Core at UNC. Staging of spermatogonial and spermatocyte cells were identified based on morphology as outlined in (de Rooij, 2001) and (Meistrich and Hess, 2013) respectively.

Tissue and spermatocyte spread preparation

Whole testes from adult (5 or 8 week old) wild-type and *Ino80*^{cKO} animals were dissected and sections obtained as previously reported (Kim et al., 2012). In short, after dissection, testes were fixed in 4% paraformaldehyde in 1x PBS (Cellgro 46-013-CM) at 4°C overnight. Samples were washed and dehydrated through a sucrose series and embedded in OCT (Sakura Rinetek, Torrence, CA, USA). Tissue sections were obtained at 8□m thickness on a Leica CM3050S cryostat. Prior to immunofluorescence staining, slides were washed in 1x PBS and permeabilized in 0.1% Triton in 1x PBS.

Alternatively, some testes were dissected and immediately embedded in OCT. Following sectioning, slides were washed in 1x PBS and then fixed for 10 minutes in ice cold methanol, before continuing with immunofluorescence staining protocols.

Spermatocyte nuclear spreads were prepared as described previously (Peters et al., 1997). In short, testes were dissected and the tunica albuiginea removed before the tubules were placed in hypotonic extraction buffer (30 mM Tris pH 8.2; 50 mM sucrose; 17 mM citrate; 5 mM EDTA, 0.5 mM DTT; 0.1 mM PMSF) for 10 minutes. Tubules were then minced in 100mM sucrose solution and spun down to remove large tissue fragments. The supernatant was spread 1:1 with a 1% PFA solution on slides, and incubated overnight in a humidified chamber. Dried slides were washed in 1x PBS/Photo-flo and stored at -80°C prior to immunofluorescence staining.

Spermatocyte spreads were staged for prophase I of meiosis I by the immunofluorescent co-staining patterns of several conventional meiotic factors. These include monitoring the condensation of the synaptonemal complex proteins SCP1 and SCP3, marking transverse and axial elements respectively. These patterns were contrasted with the

localization of phosphorylated H2AX, a marker of unrepaired DNA breaks. Representative images for the staging of meiocytes can be seen in Figure 2-3.

Immunofluorescence Staining

Tissue section or nuclear spread slides were blocked in 5% BSA for 1-3 hours and incubated overnight in predetermined concentration of primary antibody at 4°C. The following day, slides were washed in PBS/Tween-20 and incubated for 1 hour in Alexa-conjugated secondary antibody. Slides were mounted in Prolong Gold antifade medium (Life Technologies, P-36931). Details on primary antibodies used appear in Table 2-2. Secondary antibodies were highly cross-absorbed goat IgG conjugated with fluorescent dyes Alexa Fluor 488, 568, or 647 (1:500 Invitrogen). All imaging in this study was completed using a Zeiss AxioImager-M2 (Carl Zeiss).

Figures

Figure 2.1 – *Ino80* Ablation Using Conditional Deletion Strategy

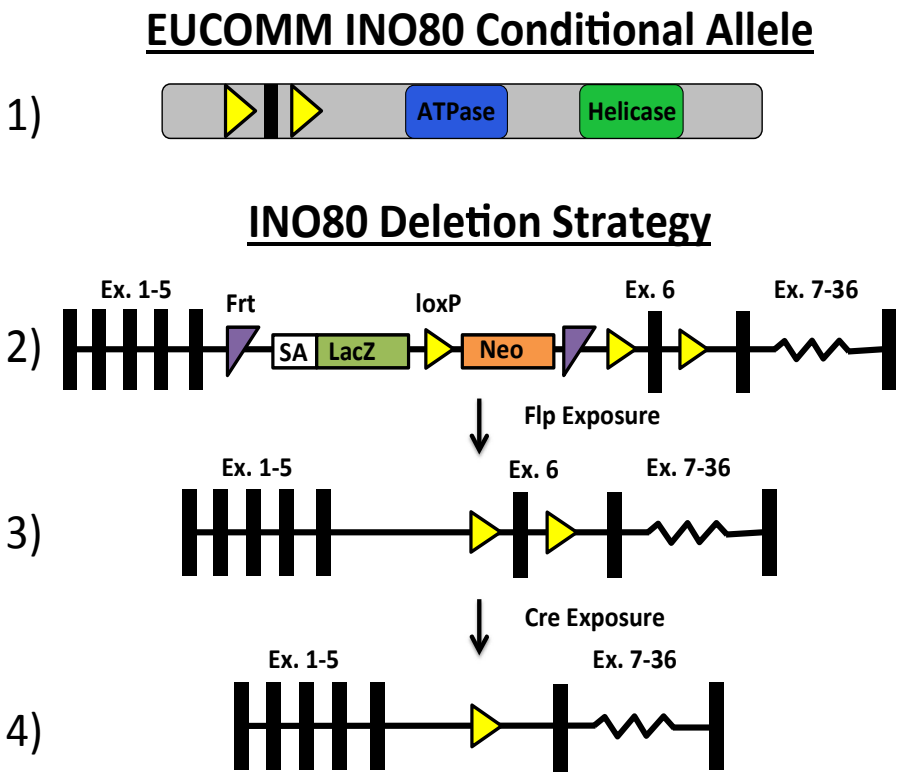


Diagram of *Ino80* conditional allele and strategy for deletion following CRE exposure.

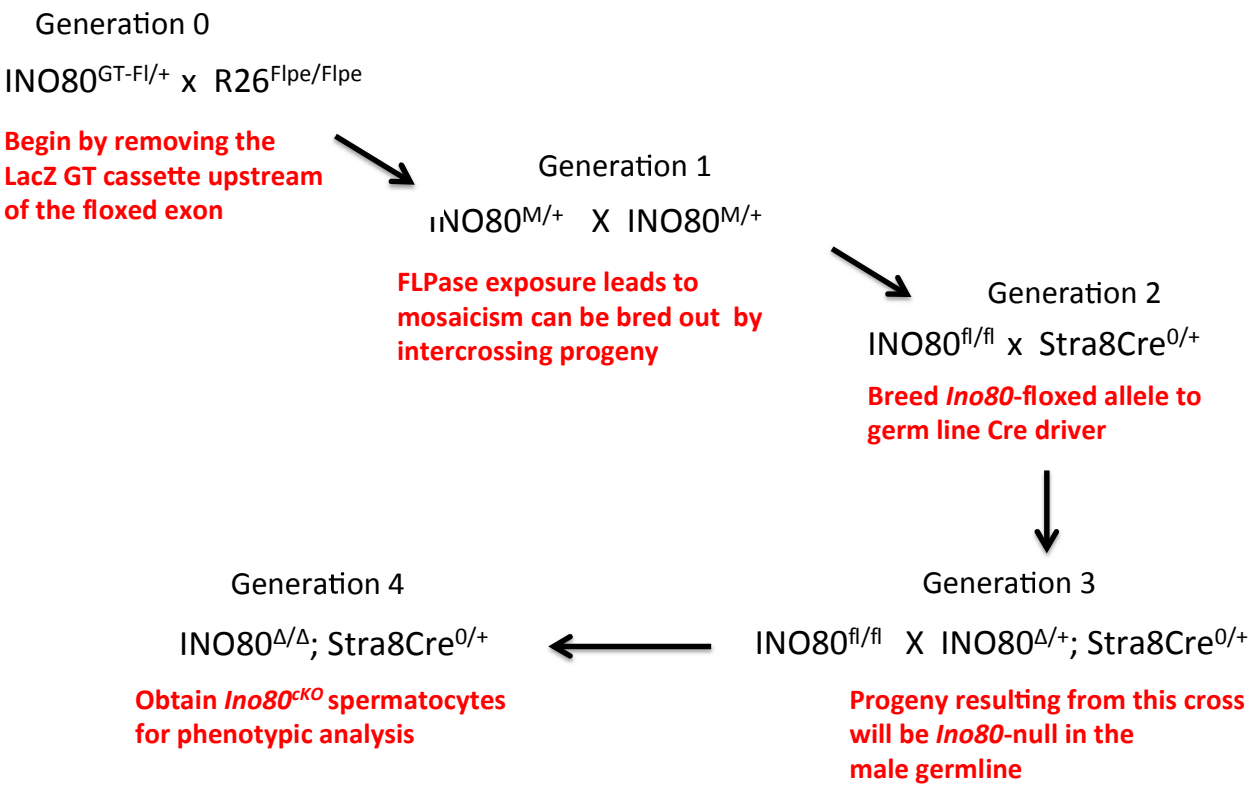
1) INO80 Protein – deletion of Exon 6 early in coding sequence halts transcription ahead of either functional domain in gene product

2) INO80^{tm1a} - full targeting cassette located upstream of Exon 6 – presence of lacZ and splice acceptor creates gene trap allele

3) INO80^{tm1c} - Flp removes targeting cassette, leaving floxed Exon 6

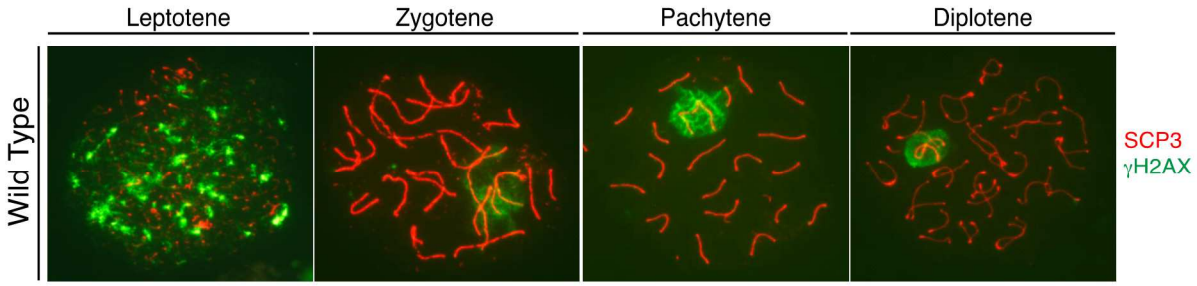
4) INO80^{tm1d} - Cre recombines out Exon 6 creating frameshift in mRNA transcript

Figure 2.2 – *Ino80*^{cKO} Breeding Strategy



Breeding strategy for germline *Ino80*-null using Stra8Cre and EUCOMM *Ino80* conditional allele. Abbreviations: + (wild-type allele), GT-FL (Gene Trapped Floxed Allele), M (mosaic of GT-FL and Fl alleles), Fl (Floxed allele), 0/+ (hemizygous transgene), Δ (Cre-mediated null allele).

Figure 2.3 – Representative Prophase I Staging



Representative staining patterns for meiotic prophase I staging. The staging of spermatocytes was determined by the distribution of SCP3 (red) and γH2AX (green), a marker of unrepaired DNA breaks.

Tables

Table 2.1 – Genotyping and PCR Primers

Gene	Application	Primer	Sequence
Ino80	Long Range PCR	Ino80_LR-Fwd	GTGCCATCTTGCCTGACTCCTTAGATTATG
		Ino80_LR-Rev	CACAACGGGTTCTTCTGTTAGTCC
Ino80	Genotyping	Ino80-Fwd	TGGCACCTTTCCAGTCTTTG
		Ino80-Rev	GCTGTGTGTAGTGGTACATA
Stra8 Cre	Genotyping	Stra8-Fwd	GTGCAAGCTGAACAACAGGA
		Stra8-Rev	AGGGACACAGCATTGGAGTC
Flpe	Genotyping	Flpe-Fwd	CTGTCAATGAAGGGCCTAACGGAGTTG
		Flpe-Rev	TCGTATGCTTCCTTCAGCACTACCCTT
Ino80	qRT-PCR	Ino80_RT-Fwd	GAAGATGGTGGCTGTAAG
		Ino80_RT-Rev	GATGTCCTGCTGATTGAG

Table 2.2 – List of Primary Antibodies For Immunofluorescence

Antibody	Host	Dilution	Source
ATR	Rabbit	1:200	Calbiochem PC538
BRCA1	Rabbit	1:200	Millipore 07-434
FANCD2	Rabbit	1:200	Epitomics 2986-1
HORMAD1	Rabbit	1:200	GeneTex GTX 119236
γH2AX	Mouse	1:800	Millipore #05-636
INO80	Rabbit	1:200	ProteinTech 18810-1-AP
INO80	Rabbit	1:200	Abcam 105451
MLH1	Mouse	1:500	BD BioScience #51-1327GR
RPA	Rabbit	1:100	Bethyl Lab IHC-00409
SCP1	Rabbit	1:500	GeneTex GTX15087
SCP3	Mouse	1:500	Abcam ab97672
SCP3	Rabbit	1:500	Abcam ab15093

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CHAPTER 3 – RESULTS[2]

Introduction

Mammalian spermatogenesis requires chromatin to undergo small and large-scale changes that involve dynamic epigenetic regulation. Homologous chromosomes synapse, double-strand breaks in DNA are created and resolved, chromosomes segregate, and histones are replaced by protamines (Zickler and Kleckner, 2015). In order to accomplish these tasks, developing germ cells take advantage of a combination of chromatin modulations including ATPase-dependent chromatin remodeling (Govin et al., 2004; Sasaki and Matsui, 2008b).

ATP-dependent chromatin remodeling utilizes ATP hydrolysis to selectively mobilize nucleosomes leading to localized areas of nucleosome-depleted chromatin (Yodh, 2013). These events are required for a wide variety of cellular and biological processes including transcription, replication, and DNA repair (Lange et al., 2011). Recent reports have shown that chromatin-remodeling events play critical roles during early meiotic stages of germ cell development (Crichton et al., 2014). As numerous protein complexes harbor ATPase-dependent chromatin-remodeling activity, defining the roles of each is critical to understanding their relevance during gametogenesis.

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[2] Portions of this chapter were adapted from **Serber, D. W., Runge, J. S., Menon, D. U. and Magnuson, T.** (2015). The Mouse INO80 Chromatin-Remodeling Complex Is an Essential Meiotic Factor for Spermatogenesis. *Biol Reprod.*

Chromatin-remodeling complexes have been grouped into four major families (Yodh, 2013). Several are known to have potent roles throughout male germ cell development. The SWI/SNF complex is active early during spermatogenesis. The catalytic subunit, BRG1, is required for homologous recombination, and the ablation of this subunit results in arrest at the pachytene stage of meiosis I (Kim et al., 2012; Wang et al., 2012). The imitation switch (ISWI) and chromodomain helicase DNA-binding (CHD) complexes function comparatively late in this process, with post-meiotic phenotypes associated with spermiogenesis and fertilization (Broering et al., 2015; Dowdle et al., 2013; Li et al., 2014; Thompson et al., 2012; Zhuang et al., 2014). These observations indicate that chromatin-remodeling activity is involved in a wide variety of activities throughout spermatogenesis. One family of chromatin remodelers whose involvement during mammalian germ cell development has yet to be elucidated is INO80. This family includes the INO80, SRCAP and TIP60 complexes (Clapier and Cairns, 2009). Importantly, previously described DNA double-strand break repair functions make the INO80 complex a prime candidate for a critical role early in spermatogenesis.

The INO80 chromatin remodeler is a multi-subunit chromatin-remodeling complex that is active in a variety of cellular processes, most notably the repair of DNA damage (Ebbert et al., 1999; Shen et al., 2000). Yeast INO80 is localized to sites of DNA double-strand breaks by the presence of phosphorylated H2AX (γH2AX) and is responsible for evicting this histone variant (van Attikum et al., 2007). Mammalian cell lines that have been depleted for INO80 demonstrate an increased sensitivity to DNA-damaging agents and an inability to repair double-strand breaks (Gospodinov et al., 2011). Like SWI/SNF, complete loss of INO80 is lethal at the earliest embryonic stages (Lee et al., 2014). Both complexes

share a single subunit in common, BAF53A, an actin-related regulatory protein. In contrast to SWI/SNF, the INO80 complex incorporates three ATPase subunits, INO80 the core ATPase subunit, as well as two additional ATPase-containing helicases, RUVBL1 and RUVBL2 (Chen et al., 2011). As the catalytic subunit of the complex, the INO80 core ATPase presents a potent target for addressing the developmental contributions of the complex.

In this study, we present the first tissue specific knockout of *Ino80*. Our findings indicate that the INO80 complex is involved in coordinating DNA repair via homologous recombination. We show that INO80 expression peaks during early meiosis, and that developing spermatocytes lacking the core ATPase subunit die at these stages due to an inability to fully synapse homologous chromosomes and complete meiotic recombination. These defects suggest a potent involvement for INO80 during meiosis that is not compensated for by the SWI/SNF complex. Therefore, meiotic recombination during spermatogenesis requires the presence of both the SWI/SNF and INO80 complexes.

Results

INO80 is an Essential Meiotic Factor

In order to determine a potential *in vivo* role for the INO80 chromatin-remodeling complex, we analyzed the expression of the core ATPase subunit across a cross-section of male adult mouse tissues. INO80 expression was detectable in a majority of these tissues, with the highest expression in the testis (Figure 3-1). Reported roles for INO80 involvement in DNA repair (Kato et al., 2012; Lee et al., 2014; Min et al., 2013; Sun et al., 2010) made it

important to identity the population within the testis where INO80 expression lies. We detected the presence of INO80 in the spermatocyte population within the seminiferous tubules, co-localizing in developing germ cells with synaptonemal complex protein SCP3 (Figure 3-2A). This stage of meiotic prophase I coincides with timing of the DNA repair associated with the formation of crossovers and recombination.

We obtained a conditional deletion allele for the INO80 core ATPase subunit, which contains a floxed sixth exon. Once exposed to Cre recombinase, the targeted exon will be excised, leading to a frameshift and premature stop codon upstream of the previously described Snf2 or helicase functional domains (Figure 3-3A; (Chen et al., 2011)). In order to convert this allele into a conditional, we utilized FLP-recombinase to excise a gene trap upstream of the floxed exon. Founder animals from this line were tested for correct targeting and orientation of the transgene cassette through a long-range PCR assay. A gene-specific primer binding upstream of the insertion site was paired with a primer internal to the cassette creating a PCR product if the cassette was inserted in the correct location and orientation. This product was obtained in both male founder animals and absent in controls, indicating the correct arrangement of the targeting cassette in the conditional allele (Figure 3-3B).

Initial analysis of the effectiveness of the INO80 conditional allele was performed by intercrossing heterozygous animals carrying the gene trap allele, and no homozygous progeny were obtained from these matings. This was expected based on recently reported results showing *Ino80* nullizygous animals to be lethal at early embryonic stages (Lee et al., 2014). Based on these results, we determined the *Ino80* conditional allele to be an effective tool for eliminating the INO80 gene product in specific biological systems.

In order to evaluate the role for INO80 during spermatogenesis, we crossed the *Ino80* conditional allele with the *Stra8Cre* driver (Sadate-Ngatchou et al., 2008), ablating *Ino80* in the pre-meiotic spermatogonial population. We confirmed the loss of INO80 by immunofluorescence where spermatocytes expressing the meiotic marker SCP3 co-stained negatively for INO80, while expression was maintained in cells from the outer portion of the tubule containing both somatic support cells and pre-meiotic spermatogonia (Figure 3.2B). Quantification of fluorescence indicated that exposure to *Stra8Cre* ablated *Ino80* completely in >80% of seminiferous tubules within mutant testes (Figure 3.4).

To determine the effect of ablating INO80 during spermatogenesis, $Ino80^{\Delta/fl}$; $Stra8Cre^{+}$ (referred to as $Ino80^{cKO}$) testes were analyzed. These animals had dramatically smaller testes than age matched control animals (Figure 3.5A-B). In addition, $Ino80^{cKO}$ males were sterile, siring no litters when placed with wild-type CD1 females. The sterility of $Ino80^{cKO}$ animals was consistent with the lack of mature sperm in the epididymis as compared to the dense populations seen in wild-type animals (Figure 3.5C-D).

Upon closer inspection, $Ino80^{cKO}$ testes exhibited defects in meiosis. Low magnification images of PAS-stained testis sections taken from 8-week-old animals demonstrated that the tubules within the $Ino80^{cKO}$ testes were smaller in size and more sparsely populated with developing germ cells (Figure 3.6A-B). When viewed at higher magnifications, the individual tubules showed significant defects in spermatogenesis. In wild-type testes, all tubules contained the full complement of spermatogenic stages, ranging from spermatogonia to elongating spermatids (Figure 3.6C). In contrast, the tubules within $Ino80^{cKO}$ testes displayed arrested meiotic progression (Figure 3.6D). The majority of tubules (62%) contained a developing germ cell population up to and including meiotic

spermatocytes, with no post-meiotic population, indicating a block during meiosis (Figure 3.6D, left and center panels, quantified in Figure 3.7). Only 22% of tubules contained later stage spermatid populations, however the structure of these tubules appeared compromised (Figure 3.6D, right panel, and Figure 3.7). This spermatogonial failure and loss of cellularity within the *Ino80*^{cKO} tubules did not appear to result from a loss of the pre-meiotic spermatogonial population, as <3% of tubules had lost this population leaving empty tubules devoid of germ cells (Figure 3.7). These observations demonstrate that the loss of INO80 during spermatogenesis causes a meiotic defect leading to sterility.

Synapsis is Impaired in *Ino80*^{cKO} Spermatocytes

Given the failure of spermatogenesis in the *Ino80*^{cKO} mice, we tested how meiotic progression was affected in these animals. The majority of wild-type prophase I meiocytes existed in pachytene stage (64%). This ratio was skewed in the *Ino80*^{cKO} spermatocytes (Figure 3.8). *Ino80*^{cKO} meiocytes displayed a reduced ability to enter the pachytene and diplotene stages (15% and 2% respectively). The bulk of mutant meiocytes were arrested at the zygotene stage (63%). The presence of spermatocytes that were able to complete meiosis normally was likely due to inefficiencies in *Stra8Cre,* allowing residual populations to complete critical meiotic functions unperturbed.

Staging of *Ino80*^{cKO} spermatocytes with the meiotic markers Synaptonemal Complex Proteins 1 and 3 (SCP1, SCP3) emphasized the meiotic defect. As a cell enters meiosis, the chromosomes begin to condense and SCP3 coats the axial elements. Then as homologous chromosomes synapse, SCP1 forms transverse elements between the individual axes. Therefore once a spermatocyte enters pachytene, all of the autosomes should be completely

coated by both SCP1 and SCP3, and the sex chromosomes coated at the pseudoautosomal region (Dobson et al., 1994). In control populations this process was completed uniformly, with synapsis completing on all the autosomes (Figure 3.9A-C).

In contrast *Ino80*^{cKO} spermatocytes showed aberrations in synapsis. Mutant meiocytes progressed to an abnormal pachytene stage characterized by persistent patterns of asynapsis on either whole or partial portions of chromosomes. These pairing defects were present on the chromosomal axis where SCP3 localized without the presence of transverse element SCP1 (Figure 3.9D-G, closed arrows). While meiosis appeared to initiate properly in *Ino80*^{cKO} spermatocytes (Figure 3.9D), a wide range of synaptic defects quickly became evident. Under normal conditions the X and Y chromosomes exhibited co-localization of SCP1 and SCP3 only at the psuedoautosomal region (Figure 3.9B). Instead, we observed abnormal pachytene spreads where pairing of the sex chromosomes was defective (Figure 3.9E). Incomplete autosomal synapsis was apparent on entire chromosomes (Figure 3.9E-F, closed arrows) or restricted to the chromosomal ends (Figure 3.9G). The synapsis defects that were observed indicated INO80 activity is involved in this process. Importantly, it has been shown that defects in synapsis, particularly of the sex chromosomes, are sufficient to activate a pachytene checkpoint, preventing the continued development of defective spermatocytes (Burgoyne et al., 2009).

Consistent with synaptic defects caused by *Ino80* loss, we observed co-localization of SCP3 with HORMAD1 in mutant spermatocytes. HORMAD1 is a meiotic factor that localizes to asynapsed portions of homologous chromosomes in order to prevent homologous recombination between sister chromatids (Wojtasz et al., 2009). Spermatocytes in prophase I showed localization of HORMAD1 to the unpaired portions of the homologous

chromosomes in a pattern opposite to that of SCP1 (Figure 3.9I-K, closed arrows). Together these data suggest that in the absence of INO80 activity, synapsis of homologous chromosomes is impaired.

Repair of DNA Damage in *Ino80*^{cKO} Spermatocytes is Defective

In addition to a defect in synapsis, developing spermatocytes in *Ino80*^{cKO} animals maintained latent levels of unrepaired DNA breaks. Early in meiosis, SPO11 creates hundreds of DNA double-strand breaks spanning each chromosome (Keeney et al., 1997). As meiocytes progress through prophase I, the phosphorylated form of histone variant H2AX (γH2AX) marks sites of unrepaired breaks throughout the nucleus, and as DNA repair progresses, it regresses to the sex body (Turner et al., 2004). This process occurred normally in wild-type spermatocytes, where a single sex body-specific focus of γH2AX was observed by the pachytene and diplotene stages of prophase I (Figure 3.10A-C). In *Ino80*^{cKO} spermatocytes, aberrant foci of γH2AX persisted into these later stages (Figure 3.10E-G). We observed latent DNA damage on several of the autosomes regardless of their status of synapsis. As expected, spreads showing extensive asynapsis continued to display hallmarks of DNA damage (Figure 3.10E). Alternatively, many *Ino80*^{cKO} spreads showing complete synapsis continued to have γH2AX localized to one or more of the autosomes (Figure 3.10G).

Further analysis of DNA repair factors demonstrated a failure in processing meiotic-associated DNA damage in *Ino80*^{cKO} spermatocytes. MLH1 is a meiotic factor involved in promoting the repair of DNA breaks into crossovers between the synapsed homologous chromosomes (Moens et al., 2007). In wild-type spermatocytes, each pair of homologs

formed at least one crossover site, marked by a focus of MLH1 (Figure 3.11A). This process was affected in *Ino80*^{cKO}, where the abnormal pachytene spermatocytes, specifically those harboring synapsis defects on at least one homologous pair, demonstrated a complete absence of MLH1 foci on any of the chromosomes in the nucleus (Figure 3.11B). Some spermatocytes displayed normal MLH1 foci, likely corresponding to a population maintaining *Ino80* expression. The striking lack of MLH1 foci in the abnormal spermatocyte population is indicative of a failure in the pathway that leads to the formation of meiotic crossovers.

In order to determine the DNA repair pathway affected in *Ino80*^{cKO} spermatocytes, we surveyed the localization of several DNA repair factors. Single stranded DNA binding factor replication protein A (RPA) binds single stranded DNA that is created as a result of DNA damage and is involved in several repair pathways (Zou et al., 2006). During meiosis, RPA binds to ssDNA following SPO11-induced DNA damage. Under normal conditions RPA, like γH2AX, localized ubiquitously on the chromosomal axes and regressed to the sex chromosomes as repair completed during the pachytene stage (Figure 3.12A). However, in many abnormal pachytene-stage spermatocytes from *Ino80*^{cKO} animals, RPA foci were not resolved and lingered on the axes (Figure 3.12B). We observed this pattern on both fully paired chromosomes and those with latent pairing defects, indicating a failure of DNA repair that occurs irrespective of synaptic completion.

The presence of RPA on the chromosomal axes indicated that early processing events occurred properly, but its continued localization suggested that INO80 might be involved in the completion of later stages of DNA damage repair. To test this hypothesis, we analyzed the expression and localization patterns of ATR, FANCD2 and BRCA1, which are all

members of a repair pathway active in homologous recombination (Burdak-Rothkamm et al., 2015). ATR senses DNA double-strand breaks and can activate the Fanconi Anemia DNA repair pathway (Andreassen et al., 2004). FANCD2 is subsequently ubiquitinated and co-localizes to sites of DNA damage with BRCA1 (Garcia-Higuera et al., 2001).

We observed an interruption of the Fanconi Anemia repair pathway. By pachytene stage in wild-type spermatocytes, FA-BRCA1 pathway factors were absent from the autosomes, having regressed to the sex chromosomes (Figure 3.13A, 3.14A, 3.15A). In *Ino80*^{cKO} spermatocytes, ATR and FANCD2 remained on the chromosomal axes of both fully and partially synapsed autosomes (Figure 3.13B, 3.14B). BRCA1 localized infrequently to asynapsed chromosomes where it would be expected (Figure 3.15B). Aberrant localization of repair factors in mutant spermatocytes indicates that INO80 activity is intimately involved in facilitating repair of DNA breaks associated with meiotic recombination.

Discussion

Previous studies have determined that SWI/SNF chromatin-remodeling complexes are essential for meiosis (Kim et al., 2012; Wang et al., 2012) and that CHD and ISWI complexes are required for post-meiotic processes (Broering et al., 2015; Dowdle et al., 2013; Li et al., 2014; Thompson et al., 2012; Zhuang et al., 2014). In this study we demonstrated that, like the SWI/SNF complex, the INO80 chromatin-remodeling complex is a required participant in meiosis. *Ino80*^{cKO} spermatocytes were able to initiate the early stages of meiosis properly but began to arrest shortly thereafter. Mutant spermatocytes displayed a striking phenotype resulting from critical defects in DNA repair and synapsis,

suggesting that INO80 is critical for the coordination of double-strand break repair and synapsis of homologous chromosomes. These two activities are inextricably linked, with one depending on the other. Disruption of these processes can activate mechanisms that prevent spermatocytes from progressing through meiosis until synapsis is completed (Burgoyne et al., 2009; Mahadevaiah et al., 2008; Subramanian and Hochwagen, 2014; Turner, 2007).

Ino80^{cKO} spermatocytes showed severe defects in their ability to complete meiotic recombination. The majority of spermatocytes were arrested at the zygotene stage, while those that made it to later stages displayed disruption of the Fanconi Anemia repair pathway, a mechanism of homologous recombination. Previous studies in yeast and mammalian cell lines support the involvement of INO80 in multiple DNA repair pathways, including homologous recombination (HR), which is involved in forming meiotic crossovers, and non-homologous end joining (NHEJ) pathways (Morrison and Shen, 2009; Morrison et al., 2004; Tsukuda et al., 2005). In particular, INO80 assists in preparing the chromatin landscape for HR through the removal of variant histone H2A.Z at sites of DNA damage resulting from irradiation of cells in culture. INO80 mediated depletion of H2A.Z at break sites is crucial for replacement of RPA by RAD51 following resection (Alatwi and Downs, 2015). Additional support for INO80 involvement in homologous recombination is provided by the lack of MLH1 foci in abnormal *Ino80*^{cKO} spermatocytes, indicating that the specification of crossover sites was disrupted in these cells. Importantly, chromatin architecture contributes to localization of recombination hotspots (Yamada and Ohta, 2013). Therefore loss of INO80 chromatin-remodeling activity may prevent the establishment of a chromatin structure permissive for crossover formation.

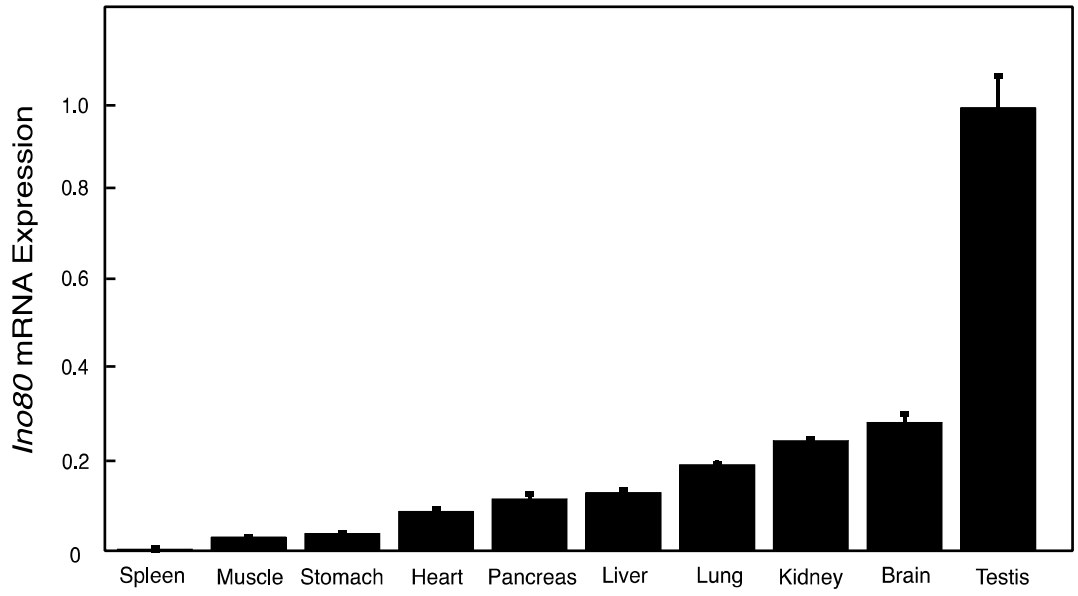
Importantly, the phenotypic consequences of INO80 loss resemble defects caused by experimental manipulation of the SWI/SNF complex. As with INO80, the conditional deletion of the ATPase subunit of the SWI/SNF complex leads to the depletion of spermatocytes during prophase I stages of meiosis, displaying a number of similar defects. These data suggest that these remodelers are unable to compensate for the absence of the other, particularly in their requirement during synapsis and recombination. However, the degree to which these complexes act non-redundantly is unclear. The ablation of both complexes would be required to uncover additional roles that may result in synthetic lethality.

There is precedent for functional interactions between chromatin-remodeling complexes in the regulation of the genome. For example, the yeast ISWI and RSC remodelers act antagonistically to position nucleosomes around the transcription start sites of specific genes. Double mutants for ISWI and RSC suppress the single mutant phenotypes, providing evidence that complexes are in competition (Parnell et al., 2015). In addition, genome-wide localization patterns of ATPase subunits of several remodeling complexes in murine cells suggest that a large portion of genomic targets are shared between complexes (Morris et al., 2014). It is unclear whether these patterns represent direct cooperative or antagonistic interactions between complexes at these loci. During spermatogenesis, interactions between remodelers could provide a mechanism for controlling important processes such as recombination. In this context, INO80 and SWI/SNF may be required for the regulation of a distinct set of meiotic functions, leading to similar phenotypic consequences in their absence. On the other hand, the complexes may act on the same targets, but their relationship, whether cooperative or antagonistic, requires the presence of

both to complete. Therefore, parsing the relationships between INO80 and SWI/SNF will require further investigation into the mechanistic functions of these complexes during meiosis.

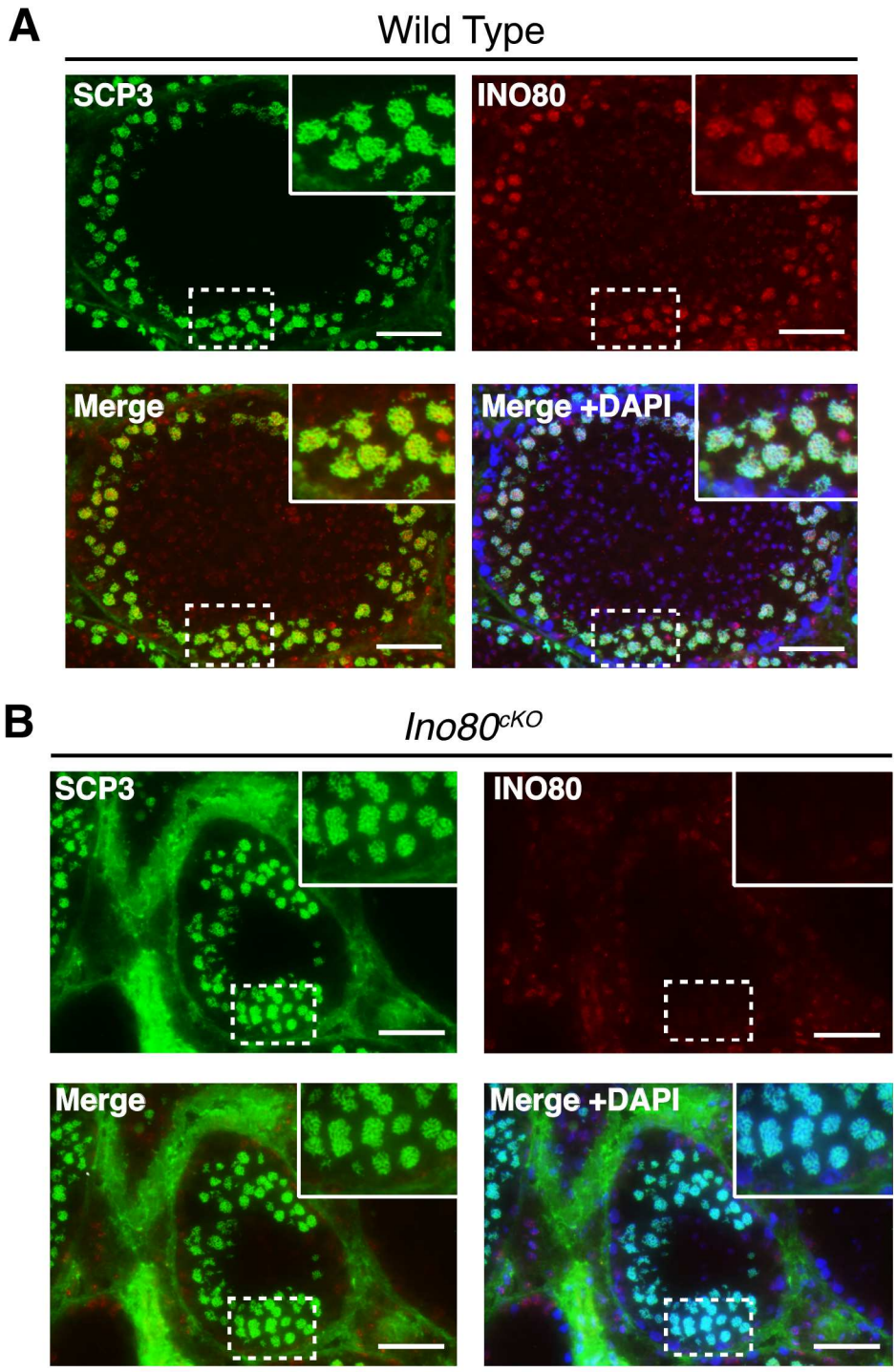
Figures

Figure 3.1 – INO80 mRNA Expression in Adult Mouse Tissues



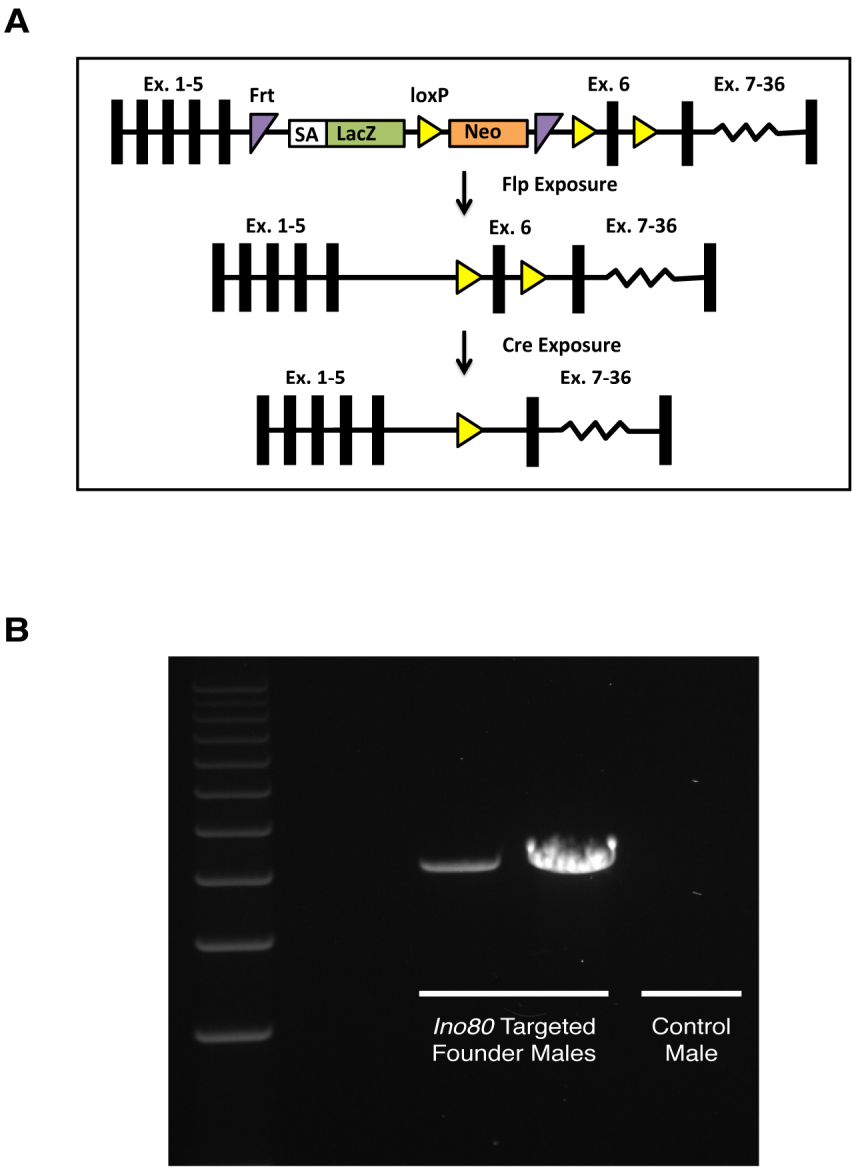
Bar chart quantifying qRT-PCR analysis of *Ino80* mRNA expression from a panel of adult mouse tissues. Error bars: mean ± SEM.

Figure 3.2 – INO80 Expression During Spermatogenesis



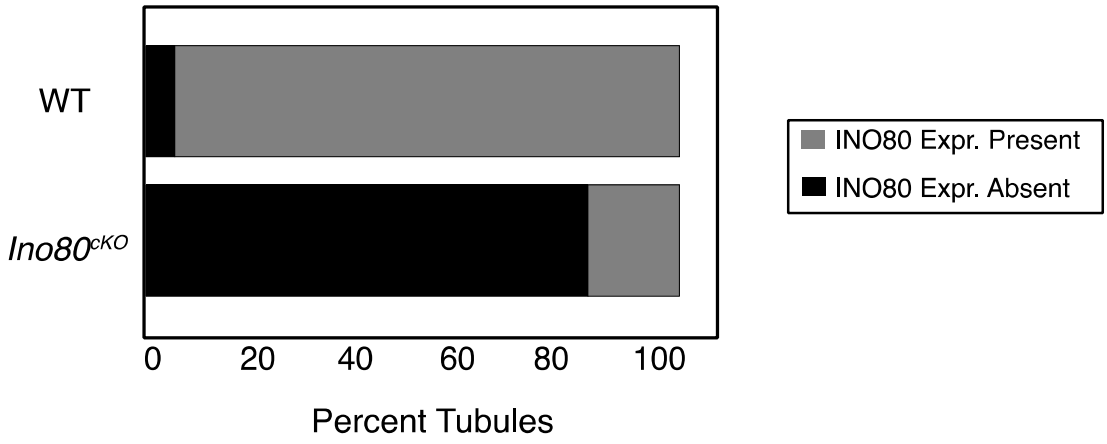
(**A**) Testis sections showing seminiferous tubules co-stained for INO80 and SCP3 in wild-type and (**B**) *Ino80*^{cKO} animals. Inserts show magnified view of the outer edge corresponding to the marked area from the respective section. Scale bars: 50 μm.

Figure 3.3 – Validation of Ino80 Conditional Allele



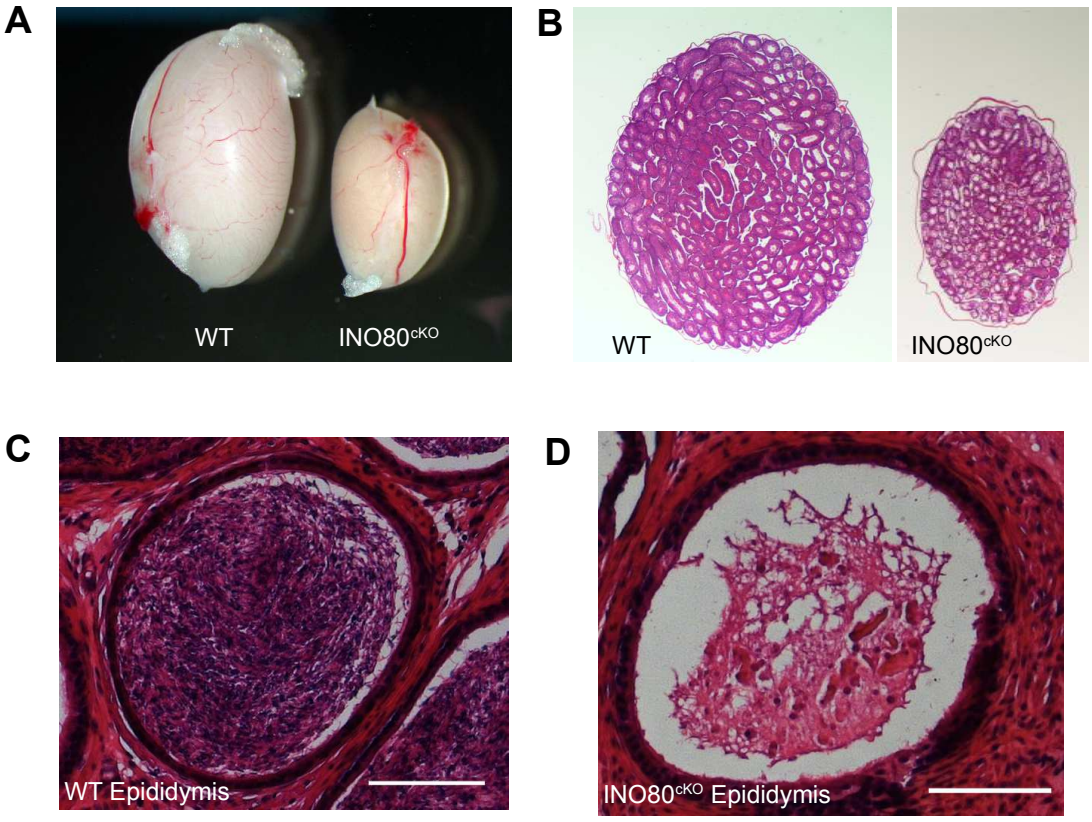
Ino80 ablation using conditional deletion strategy. (**A**) Diagram of *Ino80* conditional allele and strategy for deletion following CRE exposure. (**B**) Long-range PCR confirming proper 5` targeting of cassette for the *Ino80* conditional allele in two founder male animals.

Figure 3.4 – Quantification of Stra8Cre Mediated Ablation of INO80



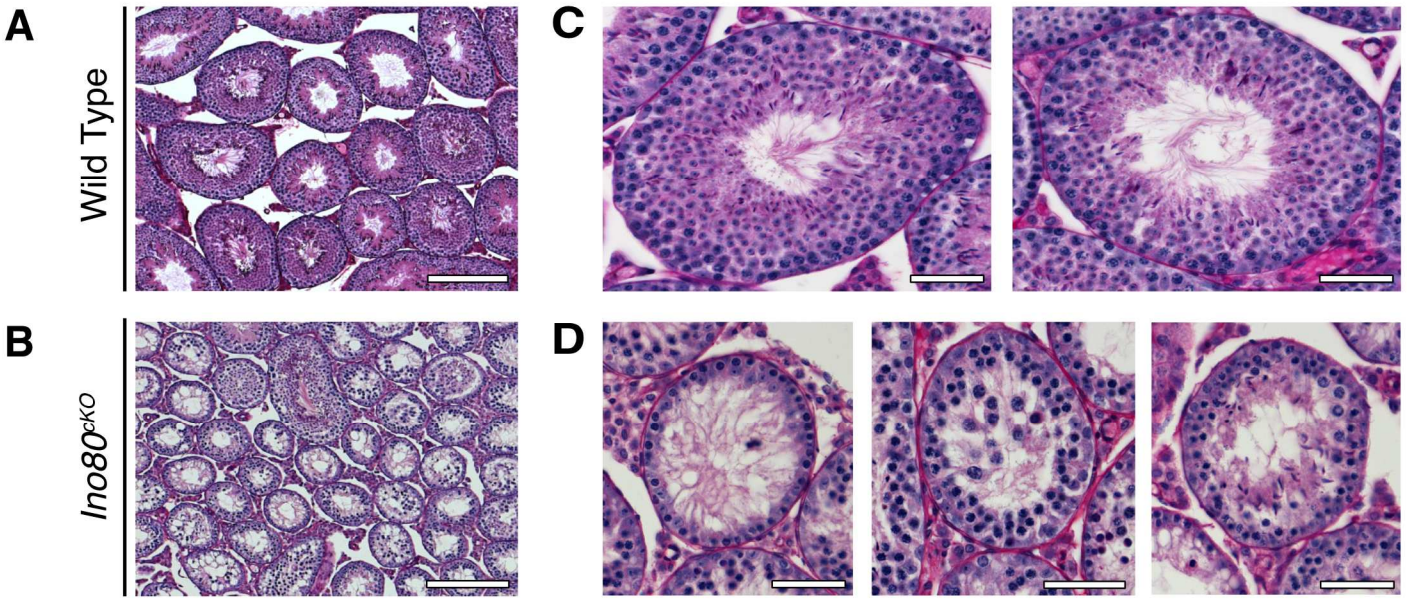
Quantification of the percentage of seminiferous tubules expressing INO80 protein in wild-type and *Ino80*^{cKO} animals. Tubules were scored for the presence of INO80 as defined by immunofluorescent staining co-localizing with SCP3 in developing germ cells (grey) or the absence of INO80 signal in the SCP3 population (black)

Figure 3.5 – *Ino80*^{cKO} Males are Sterile and Unable to Produce Sperm



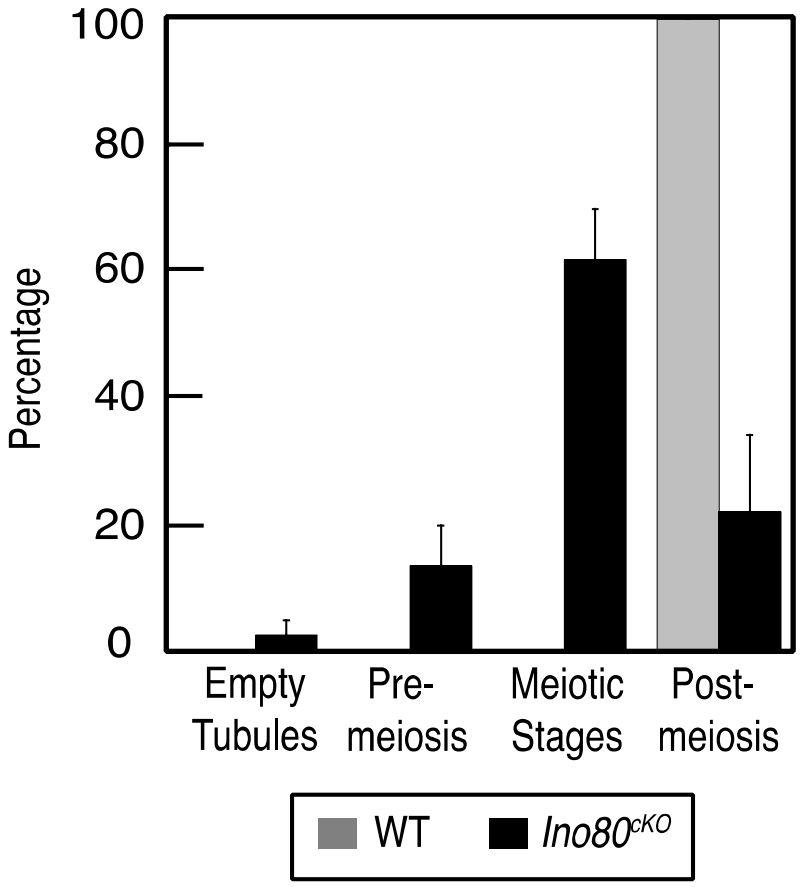
(**A**) Whole mount comparison of testes dissected from wild-type and *Ino80*^{cKO} 8-week-old littermate animals. (**B**) Low magnification comparison of hematoxylin and eosin stained histological sections from WT and *Ino80*^{cKO} testes. (**C**) H&E stained sections of representative tubules within the cauda epididymis of 8-week-old wild-type and (**D**) *Ino80*^{cKO} littermate animals. Scale bars: 100 μm.

Figure 3.6 –*Ino80*^{cKO} Mutant Seminiferous Tubules Show Defects in Meiosis



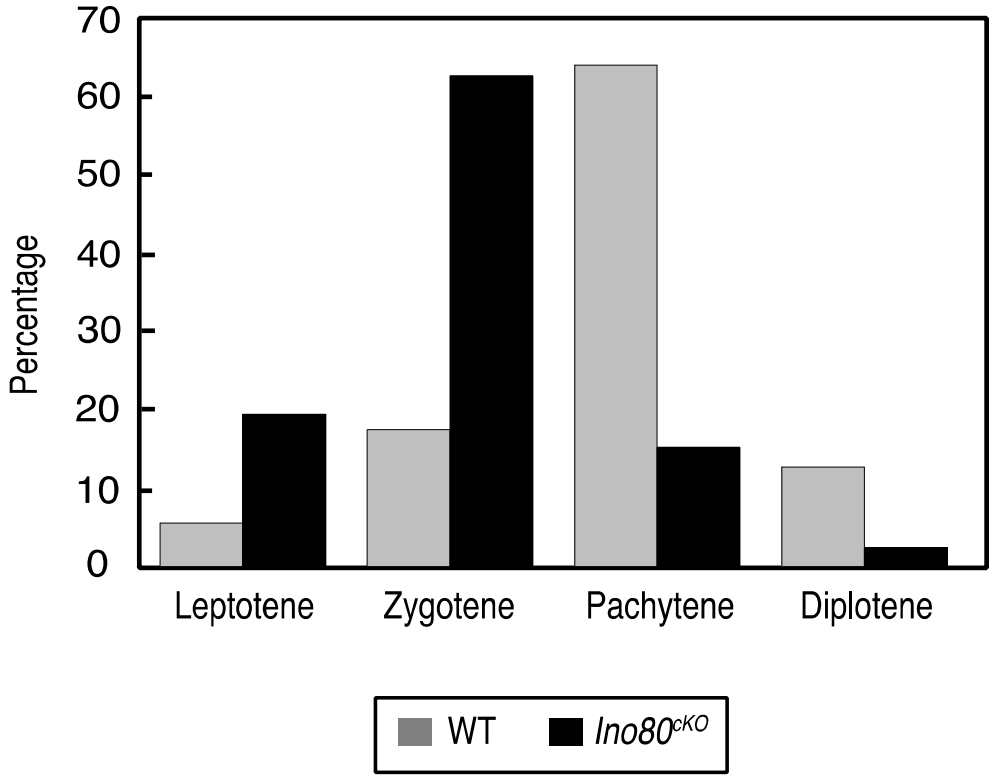
A) Low-magnification PAS stained histological testis sections from 8-week-old wild-type and (**B**) *Ino80*^{cKO} littermate animals. Scale bars: 200 μm. (**C, D**) Representative high-magnification PAS stained tubules from testes sectioned from (A, B). Scale bars: 50μm.

Figure 3.7 – *Ino80*^{cKO} Seminiferous Tubules Stall at Meiotic Stages



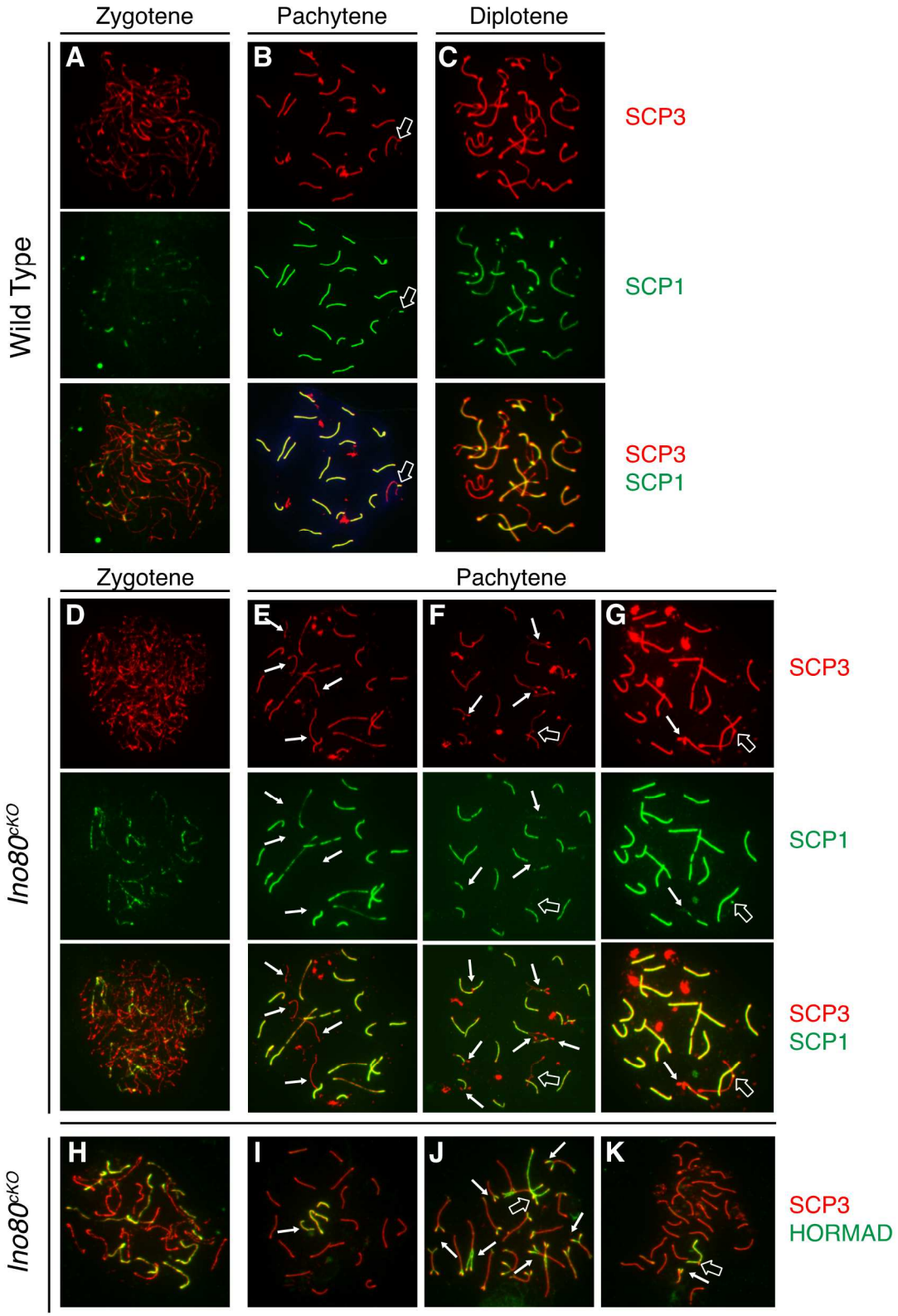
Quantification of seminiferous tubules based on the stage of the most advanced cell type in wild-type (grey) and *Ino80*^{cKO} (black) animals. Error bars: mean ± SD.

Figure 3.8 – Majority of *Ino80*^{cKO} Spermatocytes Arrest in Zygotene



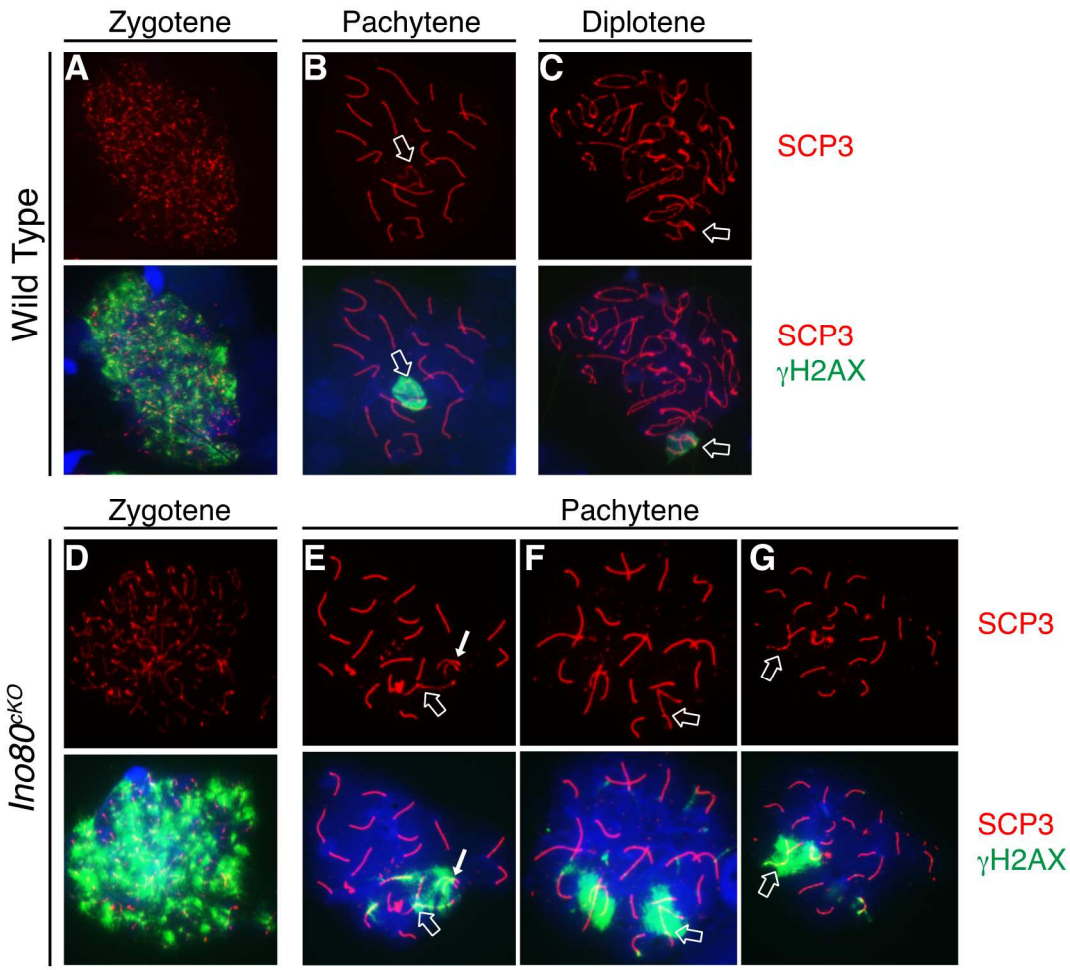
Quantification of the proportion of spermatocytes in individual meiotic prophase I stages from wild-type (grey) and *Ino80*^{cKO} (black) testes. Staging determined by SCP1 and SCP3 immunofluorescent staining of spermatocyte spreads.

Figure 3.9 – *Ino80*^{cKO} Spermatocytes Fail to Complete Synapsis



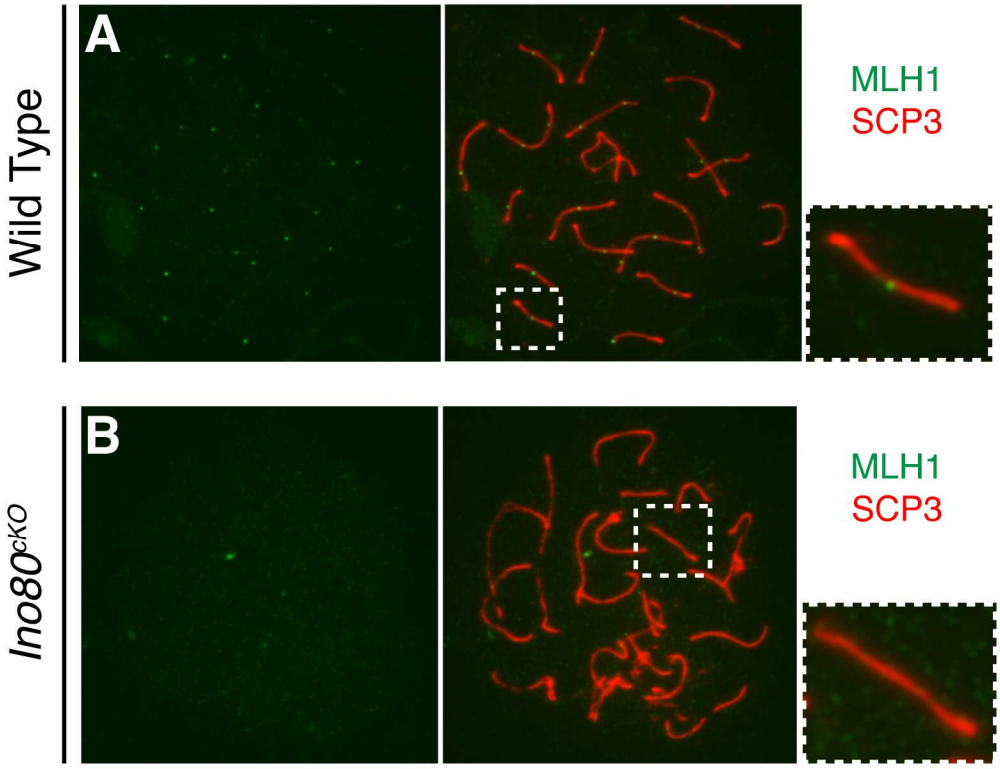
(**A-C**) Comparison of immunofluorsecent staining of SCP3 (lateral elements – red) and SCP1 (transverse elements – green) in wild-type and (**D-G**) *Ino80*^{cKO} 8-week-old spermatocyte spreads. (**H-K**) Overlay of SCP3 with HORMAD1 in *Ino80*^{cKO} spermatocytes. Staging of spermatocytes was determined by the patterns of SCP1 and SCP3 co-staining. Open arrows indicate sex chromosomes; closed arrows indicate areas of incomplete synapsis on autosomes.

Figure 3.10 – Latent Unrepaired DNA Breaks in *Ino80*^{cKO} Spermatocytes.



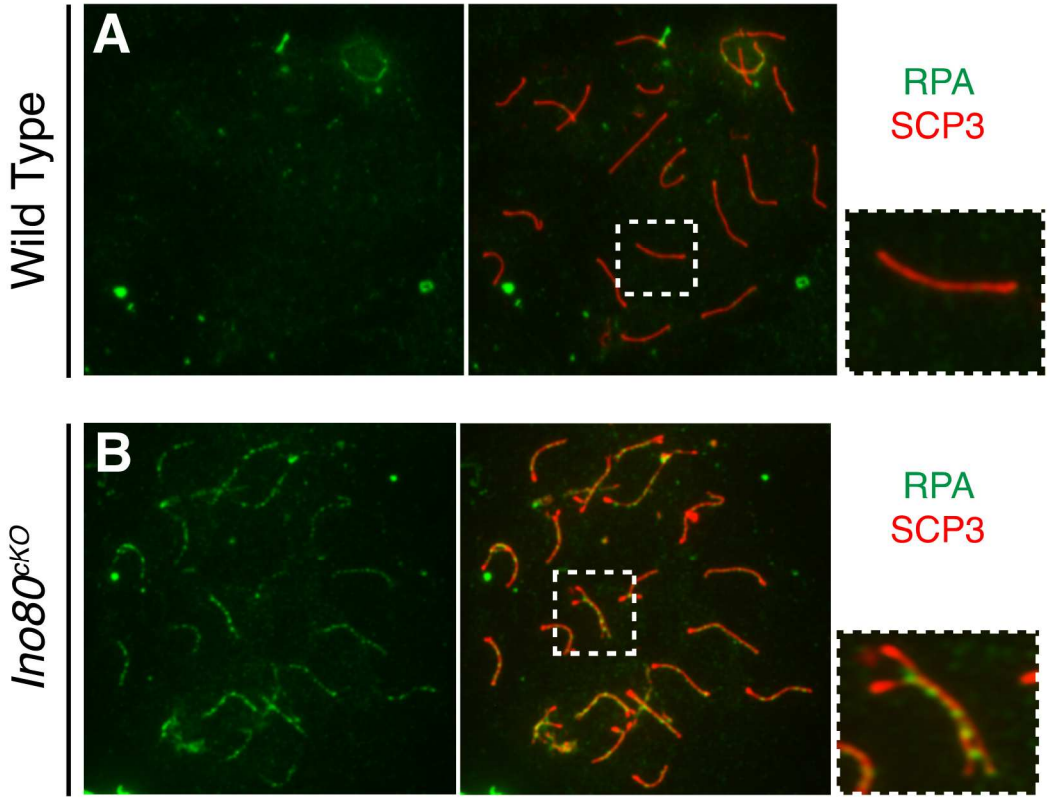
(**A-C**) Distribution of SCP3 (red) with γH2AX (green), a marker of DNA breaks in wild-type and (**D-G**) *Ino80*^{cKO} spermatocyte spreads. Staging of spermatocytes was determined by the pattern of SCP3 staining. Open arrows indicate the sex chromosomes; closed arrows indicate areas of incomplete synapsis on autosomes.

Figure 3.11 – Failure to Form Crossovers in *Ino80*^{cKO} Spermatocytes.



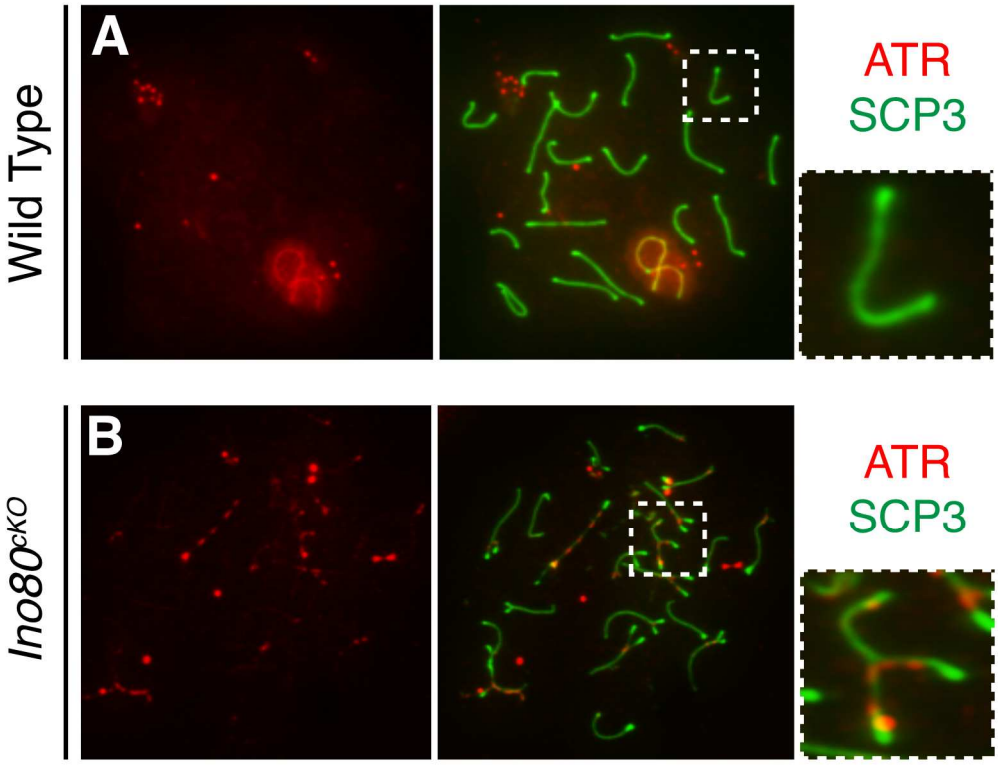
(**A**) Distribution of crossover marker MLH1 (green), co-stained against SCP3 (red) in pachytene-staged spermatocyte spreads from wild-type and (**B**) *Ino80*^{cKO} spermatocytes. Inserts show magnified view of a representative autosomal chromosome corresponding to the marked area from the respective spermatocyte spread.

Figure 3.12 – Failure to Repair DNA Damage in *Ino80*^{cKO} Spermatocytes.



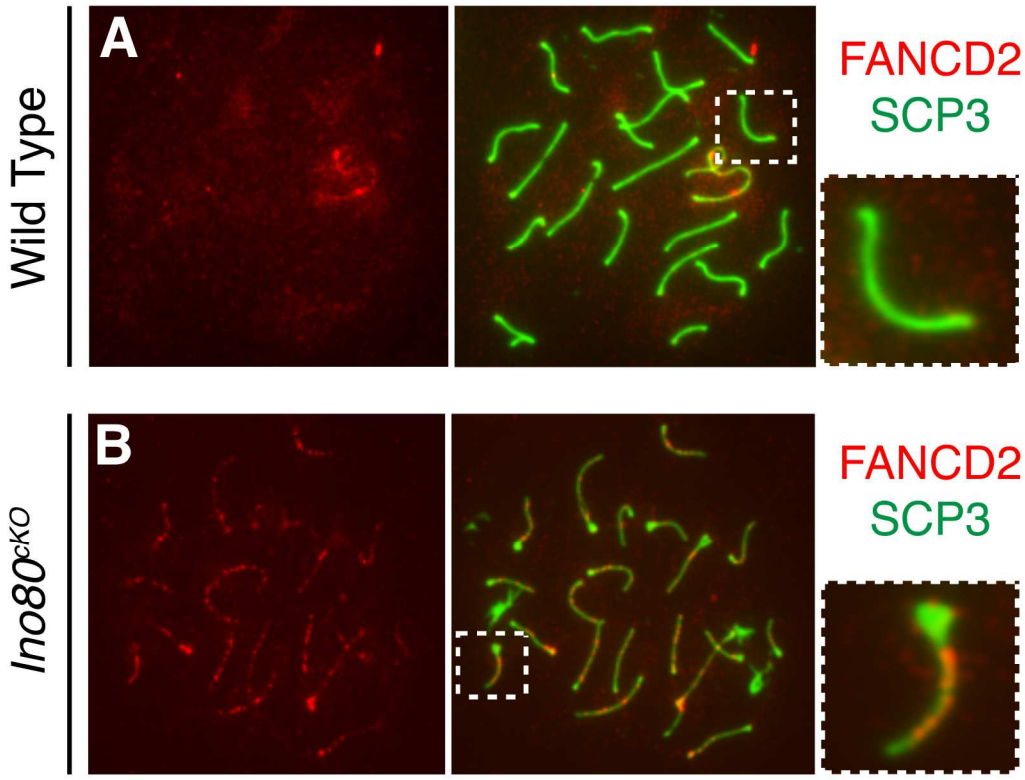
(**A**) Distribution of single-stranded DNA binding factor RPA (green), co-stained against SCP3 (red) in pachytene-staged spermatocyte spreads from wild-type and (**B**) *Ino80*^{cKO} spermatocytes. Inserts show magnified view of a representative autosomal chromosome corresponding to the marked area from the respective spermatocyte spread.

Figure 3.13 – ATR Remains on Axes in *Ino80*^{cKO} Spermatocytes



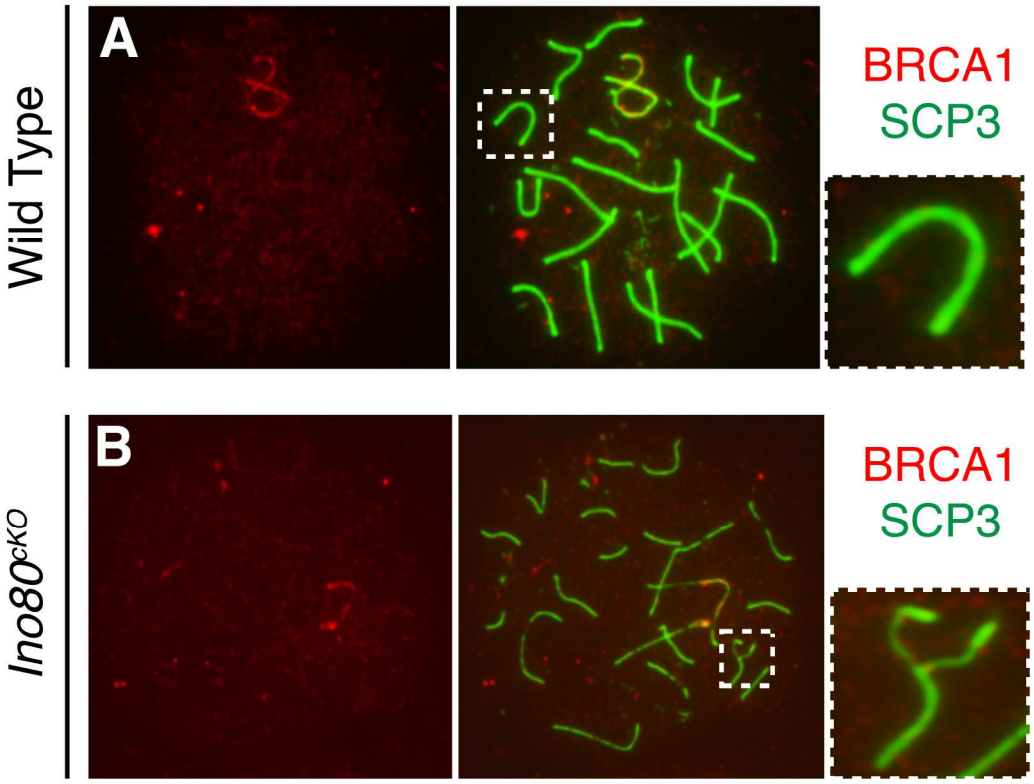
(**A**) Distribution of factors from the FA-BRCA1 DNA repair pathway in pachytene-stage wild-type and (**B**) *Ino80*^{cKO} spermatocytes. Double staining of SCP3 (green) with ATR (red). Inserts show magnified view of a representative autosomal chromosome corresponding to the marked area from the respective spermatocyte spread.

Figure 3.14 – FANCD2 Remains on Axes in *Ino80*^{cKO} Spermatocytes.



(**A**) Distribution of factors from the FA-BRCA1 DNA repair pathway in pachytene-stage wild-type and (**B**) *Ino80*^{cKO} spermatocytes. Double staining of SCP3 (green) with FANCD2 (red). Inserts show magnified view of a representative autosomal chromosome corresponding to the marked area from the respective spermatocyte spread.

Figure 3.15 – BRCA1 Absent From Axes in *Ino80*^{cKO} Spermatocytes



(**A**) Distribution of factors from the FA-BRCA1 DNA repair pathway in pachytene-stage wild-type and (**B**) *Ino80*^{cKO} spermatocytes. Double staining of SCP3 (green) with BRCA1 (red). Inserts show magnified view of a representative autosomal chromosome corresponding to the marked area from the respective spermatocyte spread.

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CHAPTER 4 – CONCLUSIONS

Discussion

Summary of Findings

Chromatin modification in all of its forms plays an important role during the tightly choreographed process of spermatogenesis. Histone marks are written and erased, and remodeling complexes are expressed at all different stages. These events have consequences throughout germ cell development, affecting the pre-meiotic spermatogonial population, meiosis, or spermiogenesis (Crichton et al., 2014). Even within the four major categories of chromatin remodelers, specific complexes defined by the incorporation of alternative members can play roles at multiple stages.

In this study, we demonstrated that INO80 chromatin-remodeling activity is required for the successful repair of DNA double-strand breaks that are created as part of meiotic recombination. We observed that spermatocytes depleted for the INO80 core ATPase subunit were able to initiate the early stages of meiosis properly. Upon closer inspection, *Ino80*^{cKO} spermatocytes are depleted beginning at early stages of meiosis. These observations suggest that INO80 plays a critical role in the proper progression through meiosis.

The loss of INO80 activity in developing germ cells has a wide range of consequences, leading to defects in major processes occurring during prophase I. Early in spermatogenesis, the cell must contend with a large number of double-strand breaks induced

by SPO11. In order to progress through meiosis, the cell must synapse its homologous chromosome and undergo DNA repair. These processes are interdependent and many defects leading to asynapsis can potentially have severe consequences (Burgoyne et al., 2009). In spermatogenesis, asynapsis is anticipated to occur on the sex chromosomes outside of the pseudoautosomal region, and is compensated for by meiotic sex chromosome inactivation (MSCI) (Turner, 2007). A related process called meiotic silencing of unpaired chromatin (MSUC) is activated in response to asynapsis on one of the autosomes (Mahadevaiah et al., 2008). During spermatogenesis the MSUC response prevents cells with improper aysnapsis from continuing through meiosis until synapsis can complete (Subramanian and Hochwagen, 2014). The prevalence of *Ino80*^{cKO} meiocytes stalled in the zygotene stage indicates that the failure of synapsis is a critical consequence of INO80 loss.

Despite the majority of *Ino80*^{cKO} spermatocytes stalled in zygotene, some are able to progress to the pachytene stage. Many of these cells display DNA repair defects as evidenced by persistent localization of DNA damage makers on the autosomes. Data presented in this study suggest that INO80 interacts with a FANCD2-BRCA1 repair pathway, but that this form of DNA repair is stalled or otherwise disrupted. Previous studies have shown that FANCD2 and BRCA1 act together in a pathway involved in the pairing of homologous chromosomes (Garcia-Higuera et al., 2001), although little data exist describing the role of a FA-BRCA1 pathway in meiosis. The presence of early factors from this pathway on chromosomal axes while BRCA1 remains absent suggests that without INO80 this repair pathway cannot be fully completed.

Relationship Between INO80 and SWI/SNF During Meiosis

Considering the range of functions performed by chromatin-remodeling complexes during spermatogenesis, the phenotypes observed in the *Ino80*^{cKO} and *Brg1*^{cKO} mutant animals show interesting overlap. As previously mentioned, there is precedent to suggest that chromatin-remodeling complexes act at a similar set of targets. In the context of spermatogenesis, this indicates that the INO80 and SWI/SNF complexes may act in a manner that involves the activity of both, either in an antagonistic or cooperative relationship.

There are also aspects of the *Ino80*^{cKO} and *Brg1*^{cKO} associated phenotypes that are suggestive of informative differences between the complexes. Like INO80, the conditional deletion of the BRG1 core subunit of the SWI/SNF complex leads to the depletion of spermatocytes during prophase I stages of meiosis (Kim et al., 2012; Wang et al., 2012). However, despite the use of *Vasa-Cre,* which depletes *Brg1* much earlier than the *Stra8Cre* used in this study (E12.5 vs. P3 respectively), the proportion of *Ino80*^{cKO} meiocytes progressing to the pachytene stage is diminished to 15% of total compared to the approximately 40% with *Brg1*^{cKO} (compare Figure 3.8 and (Wang et al., 2012)).

Although the differences in Cre drivers used limits our ability to draw comparisons between the two conditional mutants, the timing of the arrest suggest that INO80 activity may be involved upstream of SWI/SNF. Additionally, their roles in meiotic recombination appear distinct. While both BRG1 and INO80 loss affect DNA repair ability, the outcome is different. *Brg1*-null meiocytes demonstrate a diminished ability to form recombination crossovers (Kim et al., 2012), while the subset of pachytene spermatocytes in *Ino80*^{cKO} animals lose this function entirely.

Taken together the prevalence of both phenotypic similarities and distinctions may point an important functional relationship between SWI/SNF and INO80, although more difinitive conclusions would require additional experiments. Of particular interest would be a double conditional mutation of *Brg1* and *Ino80*, from which we could explore potential genomic and functional interactions.

Proposed Mechanism for INO80 Activity in Recombination

As previously described, the ablation of histone variant H2AX leads to male sterility, with developing spermatocytes arresting at pachytene. Specifically, these meiocytes show synapsis and recombination defects, with the latter associated with a near total loss of MLH1 foci (Celeste et al., 2002). Interestingly, these phenotypes mimic those associated with the loss of INO80 activity. Previous data in yeast suggest that INO80 is required early during DNA repair, immediately following deposition of γH2AX at the break sites. Once the INO80 complex localizes, it is responsible for remodeling the nucleosomes, opening the local chromatin landscape and providing the physical space for downstream factors to bind and complete DNA repair. Studies on ES cells have determined that INO80 is not required for sensing DNA breaks (Min et al., 2013). This comports with our observations that in $INO80^{cKO}$ spermatocytes, early repair events are able to occur, but the localization of downstream factors is interrupted.

Based on these known roles and our data, we can propose a mechanism for the role of INO80 during meiotic recombination (Figure 4-1). At the outset of meiosis in spermatocytes, SPO11 creates DNA double-strand breaks throughout the genome. These breaks are sensed by ATM or ATR, which is responsible for phosphorylating histone variant

H2AX and activating the Fanconi Anemia repair pathway. SWI/SNF and INO80 are recruited to the break site where they remodel the local nucleosomes to allow access to the DNA template. Additionally, INO80 may also be responsible for maintaining appropriate levels of H2A.Z at these sites. The removal of H2A.Z, catalyzed by INO80, is involved in promoting DNA repair by homologous recombination pathways. From there, published data regarding SWI/SNF and INO80 diverge. The ablation of BRG1 during this process suggests that SWI/SNF is responsible for recruiting RAD51 to the break sites and facilitating DNA repair (Kim et al., 2012). Based on our result, INO80 prepares the local chromatin landscape for the recruitment of repair factors such as the Fanconi Anemia pathway members (particularly BRCA1), a process that is disrupted in *Ino80*^{cKO} spermatocytes. Taken together, the multiple functions of INO80 required for HR would explain the lack of MLH1 sites in *Ino80*^{cKO} meiocytes, which indicate sites that are converted into crossovers.

Based on our observations, INO80 appears to be an early meiotic factor responsible for remodeling chromatin at the break site, preparing space for a repertoire of repair factors to localize. However, INO80 may remain associated with the sites of repair once its nucleosome remodeling function is completed in order to facilitate downstream events. This could involve an active role in synapsis of homologous chromosomes or the recruitment of repair machinery required for HR and crossover formation. Additional evidence of direct protein interactions between INO80 complex members and specific downstream targets, such as the Fanconi Anemia DNA repair pathway, which is disrupted in *Ino80*^{cKO} spermatocytes, would be required to confirm these proposed mechanistic roles for INO80 in spermatogenesis.

Innovation

This is the first study to demonstrate a tissue-specific role for the INO80 complex in an *in vivo* system, making use of a conditional allele to ablate Ino80 during germ cell development. Spermatocytes lacking Ino80 exhibit a range of defects occurring during prophase I of meiosis, in particular increased asynapsis of homologous chromosomes and latent DNA damage. As a result, the process of spermatogenesis fails and mutant male animals are sterile.

The findings presented in this study provide conclusions crucial for understanding the individual role of INO80 within the broader context of chromatin-remodeling complexes acting during spermatogenesis. Surveying reported roles for SWI/SNF, ISWI, and CHD family remodelers, it is clear that INO80 performs essential meiotic functions potentially upstream of the other complexes. These results represent a clear advance in our understanding of chromatin remodeling and its role in germ cell development.

Future Directions

The data presented in this dissertation begin to address the critical role for INO80 chromatin-remodeling activity during spermatogenesis. We were able to report the phenotypic consequences of INO80 loss, including major defects in meiotic recombination, and propose a model for INO80 action during this process. However, our understanding of the mechanism by which INO80 acts during spermatogenesis is incomplete. The following section outlines a series of experiments that would greatly expand our understanding of INO80 functions and the interplay between various chromatin-remodeling complexes.

Analysis of Chromatin Status in *Ino80*^{cKO} Spermatocytes

The phenotype of *Ino80*^{cKO} spermatocytes demonstrates that the INO80 chromatin-remodeling complex is required for meiotic recombination, and that major DNA damage repair pathways are unable to complete in its absence. This conclusion is supported by previously reported roles for INO80 in homologous recombination. However, it is unclear from the data obtained in this study whether INO80 modifies chromatin directly at sites of DNA damage or merely acts to recruit other repair factors. It is know from immunofluorescence experiments on *Brg1*^{cKO} spermatocytes that the overall chromatin dynamic is affected, with mislocalized HP1γ and H3K9me2 (Kim et al., 2012).

This information suggests that it would be insightful to assess the chromatin changes that occur in our system at a genome-wide level. The method of ATAC-seq is ideally suited for these experiments. The ATAC-seq protocol assays for regions of open chromatin based on the accessibility of a region to Tn5 transposase and is accurate with low amounts of starting material (Buenrostro et al., 2013). This is an important feature in order to perform the assay on *Ino80*^{cKO} spermatocytes, which begin to arrest at the spermatocyte stage. Based on the known roles for INO80 in other systems, we would expect the loss of *Ino80* to cause an overall compaction of the chromatin, resulting in less accessibility for meiotic factors. Therefore the comparison of chromatin accessibility patterns between wild-type and mutant meiocytes will shed light on the activity of INO80 at the genomic level.

Transcriptional Effects of INO80 During Spermatogenesis

In addition to the direct involvement of INO80 at sites of DNA damage, other functions of the complex may also be contributing to successful spermatogenesis. This may

include more indirect roles, such as an involvement in transcriptional regulation. Based on the data presented in this study, INO80 is expressed in pre-meiotic spermatogonia as well as the spermatocyte population. It is in the spermatogonia where germ cell-specific transcription begins and continues through the meiotic phase (Kimmins et al., 2004). ChIP experiments done in HeLa cells demonstrate that INO80 localizes to the promoters of genes involved in DNA repair pathways, including Rad54B and XRCC3 (Park et al., 2010). Both of these factors have been previously described as playing critical roles in meiotic recombination. If INO80 is involved in the expression of these proteins, this activity has the potential to be as critical as the meiotic stage involvement demonstrated in this study.

In order to test this hypothesis, a ChIP-seq strategy would be informative. By isolating spermatocytes from early postnatal mice before the first wave of germ cells enter meiosis, it is possible to isolate a relatively pure population of spermatogonia. Once this population is obtained, the ChIP-seq experiment would determine the binding patterns of INO80 at a stage where its transcriptional role would be prominent. Later stages could be tested as well, and the binding patterns could be separated based on alignment with RNA polymerase for transcriptional targets or against known meiotic recombination hotspots for DNA repair roles.

To obtain the full picture for INO80 in transcriptional control, an RNA-seq experiment would be insightful. It would be expected that if INO80 were responsible for the transcription of target genes identified by ChIP-seq, then their expression would decrease in *Ino80*^{cKO} spermatocytes. This change would be revealed by RNA-seq. Currently similar data sets are being generated for the BRG1 subunit of SWI/SNF by colleagues in our lab, which will allow for a direct comparison of the transcriptional regulation provided by these two

chromatin remodelers in the context of spermatogenesis. These data sets will allow us to fully leverage this biological system, uncovering unique observations about the interaction between complexes.

Spermiogenesis is another time point where INO80 may function. Chromatin associated factors such as BRWD are known to be critical for reactivation of haploid genome after meiosis (Pattabiraman et al., 2014). As the *Stra8-Cre*-mediated INO80 deletion results in an arrest at pachytene, a different *Cre* driver is necessary to observe potential roles for INO80 later in spermatogenesis. For this experiment P*rm1-Cre*, a driver associated with the protamine 1 gene would be useful as it is expressed following meiotic exit (O'Gorman et al., 1997). *Prm1-Cre* was used to analyze the involvement of β-catenin during spermiogenesis (Chang et al., 2011). The ability to ablate INO80 in this population would provide the opportunity to observe post-meiotic functions of the complex without the limitation of meiotic arrest related to the spermatogonial knockout.

Combinatorial Ablation of Chromatin-Remodeling Complexes

It is clear from the phenotypes observed in the $Brg1^{cKO}$ and $Ino80^{cKO}$ spermatocytes that SWI/SNF and INO80 act non-redundantly during meiosis. However, the similarity between the phenotypes suggests that there may be functional overlap between the two complexes. In this case, an experiment addressing the genetic interaction between INO80 and SWI/SNF subunits would be beneficial. Possible relationships include a scenario where these complexes function cooperatively or antagonistically at the same targets, and the ablation of both results in a rescue of the single mutant phenotypes. On the other hand, if they act on different pathways, it may result in a synthetic lethality. Testing these hypotheses

would require the creation of *Brg1* and *Ino80* double-null spermatocytes using *Stra8Cre*. From there, phenotyping assays similar to those presented in this study for $Ino80^{cKO}$ spermatocytes would uncover clues regarding the nature of the relationship between the complexes.

In addition to genetic experiments, biochemical assays could also be undertaken to address the functional overlap of SWI/SNF and INO80. Co-immunoprecipitation experiments done on the single mutants for *Brg1* and *Ino80* would further explore their direct role in modulating activity at sites of recombination by showing the existence of direct physical interactions with specific repair factors. Recently our lab has undertaken experiments to identify the interacting partners of BRG1 during meiosis via IP-mass spec. A number of critical meiotic factors were observed to physically interact with BRG1. The completion of similar experiments for INO80 could provide additional insight into the direct roles for INO80. Not only would these experiments reveal the core components of the germ cell specific INO80 complex, but they also would highlight the potential mutual and individual repair factor targets between INO80 and BRG1.

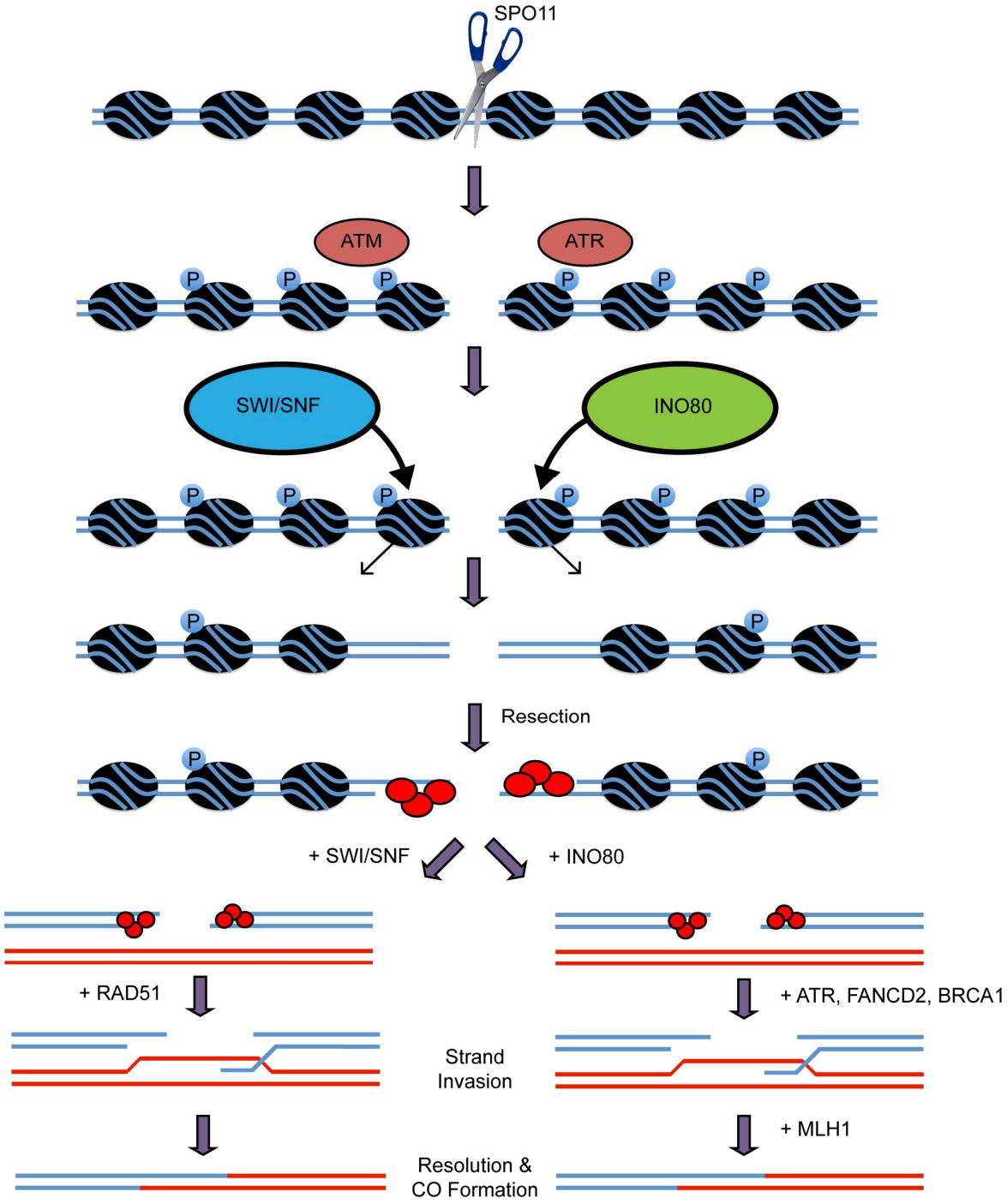
Concluding Remarks

As explored in this study, proper functioning of DNA repair pathways in response to double-strand breaks is critical for meiosis and fertility. Spermatocytes lacking important chromatin-remodeling factors including INO80 fail to complete DNA repair and arrest during development. Spermatogenesis and its SPO11-induced breaks are not the only biological process where INO80 may play an instrumental role. DNA repair is also critically important for the development and treatment of cancer. Unrepaired DNA double-strand

breaks leave cells prone to genomic instability and tumorigenesis. While mutations in a number of SWI/SNF subunits are found at high percentages in human tumors, comparatively little is known regarding the role of INO80 and its DNA repair-related activity in cancer. Ideally the observations described in this dissertation will prompt further examination into the *in vivo* roles of the INO80 chromatin-remodeling complex.

Figures

Figure 4.1 – Proposed Model for SWI/SNF and INO80 During Meiosis



Proposed model incorporating known roles for SWI/SNF and INO80 chromatin-remodeling complexes during meiotic recombination. INO80 and SWI/SNF are involved in remodeling chromatin around the DNA double-strand break site. When INO80 is lost, the Fanconi Anemia DNA repair pathway (ATR, FANCD2, BRCA1, etc.) is disrupted and MLH1 foci do not form, suggesting that INO80 is required for homologous recombination and crossover formation. Previous data from *Brg1*^{cKO} spermatocytes describe a requirement for SWI/SNF for proper localization of RAD51 (Kim et al., 2012).

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