TOWARDS THE UNDERSTANDING OF CCNB1IP1 AS A CO-REGULATOR OF MEIOTIC CROSSING-OVER IN THE MOUSE

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Edward Remco Strong

January 2014

© 2014 Edward Remco Strong

TOWARDS THE UNDERSTANDING OF CCNB1IP1 AS A CO-REGULATOR OF MEIOTIC CROSSING-OVER IN THE MOUSE

Edward Remco Strong, Ph. D.

Cornell University 2014

It is clear that there are many genes required for meiosis in mammals that are not present in the more tractable model organisms. To identify such genes, our lab has performed forward genetic chemical (ENU) mutagenesis screens for alleles conferring infertility in mice. A novel allele, *Ccnb1ip1*^{mei4}, generated in these screens is of interest because it is defective in a form of recombination called crossing-over. *Ccnb1ip1*$^{mei4/mei4}$ results in male and female infertility of otherwise normal-appearing animals.

CCNB1IP1 is finely regulated in both timing and localization to the events of meiotic crossover formations. Towards understanding the molecular functions of CCNB1IP1 and how the defect in *Ccnb1ip1*$^{me4i/mei4}$ animals leads to meiotic arrest, studies of CCNB1IP1 within meiocytes implicate a role for CCNB1IP1 in SUMOylation. Remarkably little is understood about SUMO-modification consequences to meiosis. Protein-protein interactions with CCNB1IP1 identify a number of putative targets of SUMOylation, and subsequent *in vivo* biochemical interrogations reveal the CCNB1IP1-interacting proteins 4930455F23RIK and GGN as targets of posttranslational modification dependent upon a putative SUMO E3 ligase. In totality, these studies support the hypothesis that CCNB1IP1 performs as a meiotic co-regulator, mediating the SUMO-modification of proteins essential to the stabilization and maturation of crossover intermediates. These studies of CCNB1IP1 point towards a better understanding of meiosis, with emphasis upon new targets and roles of SUMOylation.

BIOGRAPHICAL SKETCH

Edward Strong is the second son of Lytze Nauta and Gary Eldon Strong. His only sibling, Michael Strong, was born seven years earlier and took more than his fair share of the genes for creative and musical talent. Thankfully Edward and his brother fairly share the genes for intelligence and Edward has been reasonably successful at academics, more so than he was with music or athletics. As is appropriate for someone from Southern California who enjoys the warmth of unobstructed sunshine and breezes off the ocean, Edward is fortunate to have studied at Cornell University in Upstate New York- where the long, grey winters are the expense paid for momentary, but remarkably beautiful, summers. Cornell could not have been a better institution for Edward as it has afforded him with world-class resources and education, as well as the latitude to diversity his educational and professional experiences. The resources and the many people who have helped, educated, and trained Edward while at Cornell have allowed him to build a non-traditional graduate education aligned with his passions in both the life sciences as well as how to translate life science discovery towards the broader public's benefit. Edward believes that bridging the divide between scientific research and the businesses that supply life science technologies to the public is a space rightly occupied by a scientist and he will forever be grateful to the University and its faculty who trained him for his future roles, wherever it may take him.

I must recognize my parents, who through their own [mostly error-free] meiosis gave me this opportunity. And my grandparents, whom through their own [absolutely error-free] meiosis likewise gave me wonderful parents. Of course, I must also recognize my older brother- for not bringing a premature end to these life opportunities when I was a pesky young kid. I've been granted a wonderful family and I dedicate this thesis to them.

ACKNOWLEDGMENTS

National Institutes of Health grant R01 GM45415 to John C. Schimenti

National Institutes of Health grant T32 HD052471 (training slot to E.R.S.)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PREFACE

ATGATTAACTCCTGTATAGAAAATTGTGAAGCCAGGGAGACCCATGAAGC
AAATTCATGGGAGAGATCGTGA

CHAPTER 1

Introduction

The process of germ cell development was first proposed by August Weismann in 1887. In the 126 years since the initial descriptions of reductional and equational cell divisions, significant progress has been made in understanding the processes underlying those specialized divisions leading to haploid germ cells. Despite our understanding of the major initiation and DNA repair events of meiosis that are essential to germ cell development, the finer detail regulations of these processes remain largely unknown. Furthermore, while meiosis is highly conserved throughout eukaryotes, technical difficulties and time to mammalian sexual maturity have historically hindered the identifications of more complex regulations of meiosis related to our multicellular and sexually dimorphic nature. While the selection of genes for targeted mutagenesis in the mouse is often based upon information from the more tractable model organisms, it has become clear that there are many genes required for meiosis in mammals that are not present in the other models. To identify such genes, our lab has performed forward genetic chemical (ENU) mutagenesis screens for infertility genes in mice.

A novel allele, *Ccnb1ip1*^{mei4}, generated in these screens is of particular interest because it is defective in a form of recombination called crossing-over. *Ccnb1ip1*$^{mei4/mei4}$ results in male and female infertility of otherwise normal-appearing animals. Male azoospermia occurs upon meiocyte arrest and apoptosis at metaphase I, with complete homologous chromosome desynapsis. Studies into the function of CCNB1IP1 within meiocytes implicate a role for CCNB1IP1 in SUMOylation. Remarkably little is understood about SUMO-modification consequences to meiosis. CCNB1IP1 performs as a meiotic co-regulator mediating the SUMO-modification of

proteins essential to the stabilization and maturation of crossover intermediates. These studies of CCNB1IP1 point towards a better understanding of meiosis, with emphasis upon new targets and roles of SUMOylation.

1. *Meiosis: a specialized and highly conserved cell division*

Meiosis is the defining event of sexual reproduction. The meiotic process of producing haploid cells towards reproduction is most generically defined as "one round of DNA replication, followed by two rounds of cell division". While this statement is accurate, it grossly oversimplifies a fundamental process of sexual reproduction that is both *similar to* and at the same time *highly specialized from* somatic cell division. The meiotic machinery is conserved in eukaryotes and much has been learned from the study of simpler eukaryotes such as *Saccharomyces cerevisiae*. This especially high level of conservation between meiosis of distant species is believed to be attributed to a single evolutionary event, likely evolved from mitosis. Indeed, much of what is understand about meiosis owes itself to these two principles: mitotic DNA repair is the evolutionary father of meiotic DNA repair, and meiotic events in simpler species are likely conserved and inform discovery of more complex processes in high order species.

Mitotic and Meiotic DNA repair

While the fate of mitotic and meiotic cells are completely distinct, both experience DNA lesions and must repair these genomic insults prior to cellular division. Possibly owing to the invading homolog's sequence polymorphisms, and thus nucleotide mispairing in meiotic recombination intermediates, meiotic Double Strand Break (DSB) repair owes at least part of its origins to mitotic DNA *Mismatch Repair* (MMR). In recombination-mediated MMR, MutS proteins form a dimer that

bind single nucleotide mispairs and the MutL protein dimer is then recruited through interaction with the MutS-DNA complex to coordinate repair [1]. These MMR proteins (MutS and MutL homologs) play essential roles in mitotic recombination-mediated DNA repair [2,3]. In mammals, defects in MMR lead to mutations and oncogenesis [4]. While mitotic cells will preferentially repair post-replication genetic lesions off the sister chromatid, in meiotic cells repair off the sister is less efficient and thus promotes crossing-over between the homologous chromosomes and the resulting recombination-driven synapsis. In meiotic, as in mitotic cells, this crossing-over is at least partially dependent upon the RecA homolog Rad51 to promote single-stranded DNA (ssDNA) invasion of the template [5].

While the purpose for, and the ordering of, events in mitotic and meiotic recombination may differ, their intermediates share informative similarity. Mitotic MMR recognizes DNA replication errors to initiate DNA damage repair correcting the error, whereas meiotic cells initiate DNA damage to force interhomolog recombination-dependent repair of the lesions. From these mitotic origins, much has been inferred and subsequently validated towards the understanding of meiotic recombination intermediates, chromatin remodeling, and regulation of protein functions.

Meiosis Overview

In the phase of meiosis, cells undergo DNA replication followed by an elaborate prophase (meiotic prophase I) in which the chromosomes align, recombine, and divide in a manner that halves the number of homologs but retains the sister chromatids (the “reductional division”). Prophase I is cytologically classified into stages based upon chromosome behavior and appearance of a proteinaceous structure known as the Synaptonemal Complex (SC) which tethers together aligned

chromosomes. In the first stage of prophase I, *leptonema*, a protein scaffold known as the Axial Element (AE) forms between the sisters chromatids that functions to bind the chromatids together until the second meiotic division (the equational division of meiosis II). Once the AE are fully formed along the length of sister chromatids, meiocytes enter *zygonema* in which the AEs of homologous chromosomes align and begin to physically tether together through a third protein scaffold (the first two scaffolds are the AEs belonging to opposing homologs) known as the Central Element (CE). As the CE begins to "zipper" the homologs together, the AEs become known as lateral elements (LEs) of the tripartite SC. Complete formation of the SC core along chromosome axis, full synapsis, is the defining feature of *pachynema*. During pachynema chromosomes will mature the intermediate physical exchanges of DNA which, as crossovers, will tether homologs together as the SC begins to disassemble at entry into *diplonema*. The physical tethering of DNA, manifested as chiasmata, represent sites of meiotic homologous recombination that both ensures genetic diversity and maintains homolog association for reductional division of Meiosis I (MI). Chromosomes that fail to undergo crossing-over are at great risk of missegregation as the cells enter the first meiotic division, resulting in aneuploidy. Aneuploidy of a developing embryo/fetus is causal of early lethality or developmental defects and, as such, meiocytes have evolved "checkpoint" surveillance mechanisms through which to halt meiosis upon errant prophase I. As homologous chromosomes separate in the reduction division of MI, cells enter into the cycle of the second meiotic division (MII). In MII, the cells bypass DNA replication and enter into the equational division in which sister chromatids are separated in a manner analogous to mitotic division.

The stages, DNA processing, and much of the fundamental genetics of meiosis are shared between sexes of sexual dimorphic animals such as mouse and human.

However, the timing and checkpoint mechanisms in mammals can differ. Whereas males will initiate meiosis postnatally, followed immediately by spermiogenesis (maturation of haploid cells in sperm); their female counterparts will initiate meiosis during embryonic development. However, the development of female haploid germ cells will arrest and restart on two distinct time points and only complete meiotic divisions following fertilization by the sperm.

In sexually reproducing animals, the end product of meiosis are the gametes which function to contribute genetic material into the offspring. The classical "one round of DNA replication followed by two rounds of cell division" is more accurately described as two sequential, but fundamentally differing, specialized cell divisions: Meiosis I and Meiosis II. Following DNA replication, the homologous chromosomes separate in MI. This MI division nets two haploid cells (secondary spermatocytes in the male; oocyte and 1st polar body in the female). In MII, DNA replication is bypassed and the second meiotic division separates the sister chromatids that co-segregated in MI. Many of the fundamental meiotic process have been characterized in fungi and other genetically tractable organisms but much remains to be understood about the fine-detail molecular actions in each step.

In mammals, although the principal processes and proteins of this meiotic progression remain conserved between the sexes, there are notable differences. First among this sexual dimorphism is the timing of meiotic program initiation [6]. During fetal development, the female will initiate meiosis in the primitive gonad, and during embryogenesis the oocytes will progress through prophase of MI but arrest during diplotene, prior to the first meiotic division. This arrest in the female, dictyate arrest, will hold the oocytes developmentally stationary until resumption of meiosis is triggered in the sexually mature adult ovary. Upon release from arrest, oocytes will complete the first meiotic division, releasing the first polar body and the developing

oocyte completes diakinesis. The oocyte will arrest again, awaiting fertilization to trigger completion of MII with extrusion of the second polar body and forming a true gamete to receive the haploid genome supplied by the fertilizing spermatid. Although meiosis in the male will be delayed from the initiation in the female, beginning postnatally, males are not subject to the arrests seen in females.

2. *Discovery of relevant genes*

Likely due to a single evolutionary event, the meiotic machinery is highly conserved in eukaryotes and much has been learned from the study of simpler eukaryotes such as *Saccharomyces cerevisiae*. Many of the major events of meiosis identified in lower eukaryotes (yeast, plants, flies, and worms) have been later confirmed or shown consistent with events in higher order animals. However, the molecular events principal to meiosis have evolved additional complexities particular to the sexual reproduction in organisms such as mammals. While we have a fundamental understanding of mammalian reproduction, it is through forward genetics that we can gain knowledge and understanding of these unknown levels of complexity and order relevant to our own species.

Genomic and proteomic analysis have offered new tools toward the unbiased discovery of essential genes/proteins in meiosis. Forward genetics was once the tool of discovery, but "-omics" has in many ways taken over this role. While genomic and proteomic studies can offer candidates to cytologically verify location and expression patters as consistent with meiotic functions, their ability to validate function is fundamentally limited. Ultimately, reverse genetics and rigorous biochemistry is necessary to validate functions.

To the benefit of those interested in the genomic profile of meiocytes, the coordinated first wave of spermatogenesis has been well characterized in the male

mouse: 10-11dpp, leptonema; 12-13 zygonema; 15-16dpp, pachynema; 17-18dpp, diplonema [7,8]. Using samples enriched for these cellular populations, studies have profiled the RNA transcripts and built expression patterns that have been mined for candidate genes for further study [9,10].

The technological innovations in proteomics have led to increased sensitivity and accuracy. These advancements, as well as convergence with more traditional techniques such as gel separation and co-immunoprecipitation (co-IP), are driving interest in new proteomic screens built from principles utilized in genomic screens. Increased resolution in proteomic profiling will present the opportunity to finely identify posttranslational modifications of the proteome. Posttranslational modifications of acetylation, methylation, and phosphorylation have been extensively studied for their effects on mitotic chromatin structure and DNA repair. However, through new sensitive proteomic tools, we can assess the wider array of posttranslational modification as well as their roles in meiosis whereas previously limited materials reached the detection thresholds of proteomic assays.

3. *The events of prophase I*

Prophase I of meiosis has been said to be “where all the action takes place”. To accommodate “all the action”, prophase I accounts for over 90% of the total meiotic duration [11]. It is in Prophase I that homologs synapse, recombine, and resolve though progression into the reductional division. Fidelity of prophase I is critical to assure genetic diversity and avoid aneuploidy resultant from homolog mis-segregation. Whereas MII is the *somatic-like* equational cell division (albeit lacking in the DNA replication cycle), MI has attracted the most study with particular interest on the intricate steps of prophase I.

Initiation and homolog synapse:

Programmed Double Strand Breaks are the initiating event of meiotic recombination. Meiotic cells initiate autonomous DNA damage and it is the repair of this damage that consequently drives what we define as the events of meiotic recombination. This DNA damage is preferentially repaired through use of the homologous chromosome template and thus homologs must pair at the many sites of DNA damage. Indeed, observations in many organisms confirm that homologs associate at many nucleating sites from which longer stretches of physical attachment polymerize. This alignment of chromosomes coincides with Rapid meiotic Prophase chromosome Movements (RPMs) in fungi and in mammals [12,13]. These chromosome movements are hypothesized to search the nucleus and facilitate homolog associations. While it remains formally unverified, current hypotheses suggest RPMs function as a physical test of the DSB repair mediated homology search; non-homologous associations will disperse with mechanical RPM force while true homology will endure [14,15].

Initiation:

RA stimulation of meiosis is followed by the initiating meiotic event of cell-autonomous programed DNA damage. Meiosis has been purposefully evolved to provide for haploid gamete production and genetic mixture. The programmed DNA damage, by way of DSBs, is a genomic insult catalyzed by the topoisomerase Spo11 [16]. Homologs of Spo11 can be found in all eukaryotic models of meiosis, owing to the inciting role of this enzyme and the conservation in primary meiotic machinery. In the mouse, as in yeast, homolog alignment is driven through search for a homologous repair template, necessary because of the DSB insults generated by SPO11. In yeast, where Spo11 has been best characterized, there are no less than 9 accessory proteins

(Mre11, Rad50, Xrs2, Mei4, Rec102, Rec104, Mer2, Ski8, and Rec114) [17] that are essential for Spo11 catalyzed DSBs. Following the initiating events of DSB insults, repair processes are signaled through which a complex synchronization of transcription, translation, and post-translational modifications function to repair the DNA while simultaneously providing the mechanisms through which homologs will properly segregate in anaphase.

In order to repair these genomic insults, the SPO11 catalyzed DSBs are resected to leave a 3' overhang to provide for search of a repair template and will thus facilitate homologous chromosome associations. Defects in DSB formation or processing lead to inefficient and precocious homolog pairing, followed by arrest or aneuploidy at the first meiotic division [16]. The invasion of the homolog by the 3' ssDNA overhang forms what is called the D-loop. The system of homology search is surely more complex than the conceptual ssDNA homology search pushed towards the homologous chromosome, and is still largely unknown. In mice, as in yeast, the homologs of ancient DNA repair protein RecA (RAD51 and DMC1) associate with the ssDNA [19,5]. DMC1 and RAD51 facilitate ssDNA invasion and D-loop formation [20]. The SC protein SYCE1 is known to physically interact with RAD51 and it may be through this interaction that RAD51 association with ssDNA initiates homologous synapse [21]. As a diagnostic tool, RAD51 localization in the mouse can be identified at about 250 chromatid loci per meiocyte and can serve as proxy to identify the number of DSBs and thus D-loop formations. Many other proteins play a role in meiotic DNA repair, including the co-regulators ATM and ATR kinases which impart altered functions upon the diverse proteins they phosphorylate. Recently, it has been shown that ATM is activated in response to SPO11 catalyzed DSBs, and ATM kinase will initiate a cascade that will negatively feedback upon SPO11 to regulate DSB formation. Mutants defective for ATM show a severe increase in DSB formation

due to a lack of negative feedback regulation [22]. ATR kinase has been shown to localize at unsynapsed regions of chromosomes and plays an important role in regulating epigenetic marks which repress gene expression from unsynapsed meiotic chromosomes [23].

The mechanisms directing selection of those sites to receive DSBs remain incompletely characterized. Nevertheless, it is clear that some *hotspot* locations in the genome are more susceptible to DSBs. In the mouse, the most well characterized determinant of hotspot location is the *trans*-acting regulator, H3K4 methyltransferase PRDM9 [24]. PRDM9 is given DNA-binding specificity though an array of 12 zinc fingers [25]. Hotspot sites for DSBs have been correlated with H3K4 trimethylation (H3K4me3) [26]. Genomic studies to map hotspot locations have found that genomic loci with the PRDM9 predicted binding motif are 180 times more likely to receive a DSB [27].

Testing true homology:

RPMs are hypothesized to act as mechanical tests against precocious homologous pairing, and/or disentangle chromatid fibers as cells prepare for homologous synapse. In the mouse, dynamic movements of telomeres coalesce into a singular nuclear envelope domain, telomere bouquet, during the leptotene/zygotene transition. The bouquet formation in mammals is facilitated by SUN1, SUN2 and the meiosis-specific KASH5 which localize to the telomeres at leptotene and tether the chromatids to the cytoplasmic microtubules providing the mechanical force for RPMs. Mice deficient for the nuclear envelope associated SUN1 lose telomere-envelope attachment, thus RPMs, and are defective for chromosome synapse [28]. Additionally, loss of the cohesin protein SMC1β also results in failed telomere-envelope attachment [29], illustrating the dependence of sister-chromatid cohesin complex upon RPMs and

further strengthening the observed relationships between cohesin complex, synaptonemal complex formation, and homologous alignment.

Stabilization of intermediates:

Repair mediated by D-loop formation can proceed through non-crossover and crossover pathways, synthesis dependent strand annealing (SDSA) and reciprocal DNA exchange, respectively. The reciprocal DNA exchange of crossing-over forms the classical double Holliday Junction (dHJ). The joint molecule of the dHJ is resolved as either Class I (interference dependent recombination), or Class II (gene conversion) events. The decision for DNA repair resolution is believed to be made very early, with the majority of lesions repaired through SDSA and the bulk of the remaining DSBs repaired as Class I events [30]. In the yeast, this early repair pathway decision is mediated by the Dmc1 protein. The Rad51 protein is dispensable for the specification of crossovers, however crossing-over is lost in the absence of the Dmc1 protein [31]. Interestingly, the role of RAD51 may not be limited to its interaction with SYCE1 in promoting D-loop formation; yeast two-hybrid experiments have found that RAD51 interacts with the SUMO-conjugating enzyme UBC9, suggesting a role for RAD51 in recruiting posttranslational co-regulation at the sites of DNA repair [32]. Indeed, from yeast we understand that the AE protein Red1 binds Smt3/SUMO-conjugates [33] possibly facilitating Red1 coordination of meiotic recombination and synapsis. It is suggested in yeast that Smt3/SUMO-conjugated Zip3 (an essential SC protein) complexes with Red1, and that Red1 promotes phosphorylation of Hop1 which in turn promotes inter-homolog recombination. These observations build a Smt3/SUMO-dependent coordination between SC elongation and meiotic recombination [33,34].

<u>Synaptonemal Complex Formation</u>

The SC is a dense scaffolding of protein-protein interactions that physically tether homologous chromosomes together during meiosis. Since the SC's initial description in 1956 [35,36], much research has been focused on this central structure. Unfortunately, while we have discerned the major cytological events and identified a number of constituent proteins, it is clear that the SC is incompletely characterized and much is still being learned about its assembly and disassembly.

In preparation for SC formation, the chromatin itself reorganizes as a coordinated event receptive to the initiating events of recombination. Notably, epigenetic status of histones is a common mechanism of somatic cells to govern chromatin remodeling and the principle holds true in meiotic cells. Changes in epigenetic modifications to histones promote chromatin compaction that facilitates homolog pairing and installation of the SC. Ubiquitination of histone 2B (H2b) protein by Rad6 and methylation of Histone 3 (H3) by Set1 together promote nucleosome architecture that remodels the yeast meiotic chromatin [37,38]. Indeed, the well-known histone variant H2AX which mediates DSB detection and recruitment of DNA damage response factors has recently been shown to be posttranslational modified by ubiquitin, and SUMO [39].

Leptonema follows shortly after the recombination initiating events of SPO11 catalyzed DSBs. As the homologs remodel, condense, and begin to congress, proteinaceous AEs begin to form in short stretches along the chromosome core. The best understood building blocks of the AE are heterodimeric partners SYCP2 and SYCP3. It has been postulated that these short AE segments originate at D-loop locations and RAD51 facilitates the seeding of this AE formation [40]. SYCP2 contains a coiled-coil domain that is essential for its interaction with SYCP3 [41]. Mice defective for either SYCP2 or SYCP3 fail to form AEs, and thus fail to assemble the SC. Likewise, these meiocytes experience significant aneuploidy from homolog

disassociations. Later research implicates the coiled-coil protein SYCP2 as the master-link building the AE and assembling the AE into the final SC structure [42].

As the cells enter into zygonema, the AEs have formed along the lengths of homologs and the third piece of the tripartite SC, the Central Element, begins to "zipper" the homologs together. The CE assembly is dependent upon Transverse Filament (TF) protein SYCP1. The TF constituent SYCP1 is itself also a coiled-coil domain containing protein and builds the CE through its own homo-dimerization; the C-terminus of SYCP1 associates with the LE to create the TF, and the N-terminus of each homolog TF associates with the other to physically join the LEs and build what is together known as the CE [43]. The physical association between LE and TF is through SYCP2 which interacts with the C-terminus of SYCP1 [42]. As zygonema progresses, the SC forms longer stretches and the homologs near full alignment.

As the homologs fully synapse and CE forms along the full length of the chromosome cores, the meiocytes enter pachynema. In pachynema the autosomes have fully assembled the SC, and the XY have synapsed along the pseudoautosomal region. Due to the high level of asynapsis along the larger X chromosome, the XY is sequestered into a distinct *Sex Body* domain and transcriptionally silenced in a process called Meiotic Sex Chromosome Inactivation (MSCI). The sex body is commonly defined as a readily observed phosphorylated-H2AX (ɣH2AX) enriched domain. More recent fine cytology has observed that SUMO-conjugates proceed ɣH2AX localization at the sex body domain [44]. Presumably the sex body is sequestered to protect it from the autosomal asynapsis surveillance mechanisms of prophase I, but the coincidence of SUMO and ɣH2AX at the sex body as well as DNA lesions and asynapsis remains poorly understood [45]. Asynapsed autosomes, from either errors in SC assembly or highly divergent genetic backgrounds, trigger transcriptional silencing similar to MSCI called Meiotic Silencing of Unpaired DNA (MSUD). Together MSCI and

MSUD fall under a general principle called Meiotic Silencing of Unsynapsed Chromatin (MSUC) [46].

While we know the identities and biochemistry behind SC-forming SYCP1, SYCP2, and SYCP3 we know that many other proteins are either intrinsic building blocks of the SC or localize with the SC to coordinate DSB repair**.** Genomic profiling identified candidate genes *Syce1*, *Syce2*, and *Syce3* (Synaptonemal Complex Central Element Protein 1/2/3) as essential to meiosis. Studies of these proteins hypothesized their localization to the SC in part due to their large coiled-coil domains. Indeed, this family localizes to the SC and mice mutated for each member display synapsis defects [47-49]. Furthermore, in yeast a Smt3/SUMO mutant carrying an allele in which all nine lysine residues in the Smt3 protein are mutated, and thus unable to form polymeric Smt3 chains, are defective in longer stretches of SC assembly [50]. In the Smt3-allR yeast cells, SUMOylation is at least partially functional and SUMO-conjugates can receive Smt3 monomers sufficient to recover the cell lethality seen in *smt3* null cells. The Smt3-allR results suggest that polymeric Smt3/SUMO chains prior to synapse are needed for extensive SC formation. The inability of Smt3-allR mutants to propagate SC formation into longer stretches is similar to the short SCs observed in *zip3* null yeast cells. Yeast mutated for the AE protein and SUMO E3 ligase Zip3 fail to properly extend SCs to full synapse, further suggesting essential function of Smt3/SUMO-modified AE proteins in SC polymerization [51,52]. Although loss of Zip3 results in loss of AE SUMOylation in pachynema, extensive Smt3-conjugates remain in early prophase indicating that the Smt3/SUMO circuitry is more robust than a single E3 ligase. Additionally, these studies with Smt3 found that when SC formation was otherwise disrupted, independent of Zip3 protein, Smt3 co-localized with the precocious Zip1 polycomplexes [50]. Taken together, these

interrogations of SC constituents reveal a level of SC complexity previously underappreciated despite its long history as a focus of research.

Repair of Meiotic Double Strand Breaks

Recombination-mediated repair of DBS proceeds through formation of homologous DNA exchange, otherwise known as crossing-over. While this crossing over is beneficial towards promoting genetic diversity, within the context of meiosis the recombination is secondary to the essential function of maintaining homologous chromosome alignment through physical attachment of the joint molecules in crossing over.

Recombination intermediates can be resolved through the interference-sensitive Class I pathway of homologous recombination that forms physical crossing over, or alternatively though the interference-independent Class II gene conversion. Class I recombination is subject to interference promotion of crossing over distribution, thus ensuring distribution and the singular obligate crossover required per homolog pair, a process together known as *crossover homeostasis* that ensures the frequency of crossing-over remains controlled [53].

As has been discussed, there is apparent coordination between the SC forming factors (SYCP1, SYCP2, and SYCP3), the SC associated factors (SYCE1, SYCE2, SYCE3, ZIP3, Smt3/SUMO), and the early recombination intermediates (RAD51, DMC1, D-loop formation). Whereas the majority of RAD51 marked DSBs will be repaired though non-crossover SDSA, some subset will be specified towards those essential sites of crossing-over. Those sites of crossing-over manifest as protein dense nodules, Meiotic Nodules (MNs), which are testament to the complex protein-protein interactions stabilizing and resolving DNA exchange [54, 55]. Early MNs will be largely resolved as gene conversions rather than reciprocal crossing-over [56, 57]. It

remains an area of great interest to identify the co-regulators and enzymes involved in this crossover fate determination.

While early MNs demarcate initial recombination events, late MNs are considered more protein dense and are believed to represent sites of physical DNA crossing-over called *chiasmata* [58]. The relatively numerous early MNs mature down to a few in number late MNs by late pachynema. As the SC dissembles in diplonema, these remaining chiasmata will be resolved as reciprocal crossovers [56,57].

In the selection from the hundreds of early MNs and to the final 20-25 late MNs in the mouse, the mismatch repair proteins (MMR) MSH4 and MSH5 are known to function in stabilization of sites destined as crossovers. MSH4 and MSH5 are meiosis-specific MMR proteins which, as a family, are homologs of the ancient bacterial MutS DNA repair proteins. Through hydrolysis of ATP, the MSH4-MSH5 heterodimer recruits further MSH4-MSH5 complexes to form a sliding clamp and stabilize the dHJ of recombination-mediated DNA repair [59,60]. As MNs mature, intermediate MNs are marked by the MSH4-MSH5 heterodimer and immunolocalized foci decrease in numbers from approximately 150 in zygonema, to about 30 foci in pachynema as recombination intermediates mature towards the 20-25 reciprocal crossover events. In the mouse, one current hypothesis for co-regulator selection of intermediate MNs as sites of future crossing-over involves SUMOylation of unknown conjugates. It is postulated that putative SUMO E3 ligase RNF212/Zip3 stabilizes those MSH4-MSH5 heterodimers destined towards crossing-over [61].

From studies in the yeast, we know that the meiosis-specific MutS homologs recruit the eukaryotic MutL homologs, MLH1 and MLH3, to the sites of late MNs [62]. In pachynema, MLH1 and MLH3 localizations mark the sites of crossing-over [63,64]. In the mouse, potential resolvase factors have been localized to these terminal sites of crossing-over but it is still unknown which factors mediate resolution of the

dHJ into reciprocal crossing-over. It remains a factor of great interest to better understand these resolving events in meiotic recombination: final stabilization of sites for Class I crossing-over, and resolution of the dHJ joint molecules.

Removal of the Synaptonemal Complex

With crossovers specified and homologs physically tethered at the points of chiasmata, the SC has served its function to maintain scaffolding while recombinogenic events progress. In diplonema, the SC begins to disassemble; TF protein SYCP1 is lost and the homologs repel each other. The sites of reciprocal DNA exchange are the final strong link associating homologs as they align for completion of MI. Animals homozygous for mutations in *Mlh1* and *Mlh3* fail to maintain sites of crossing-over and thus homologs fully repel into univalents during diplonema, leading to total infertility (64,65). By diakinesis, crossovers have resolved and the homologs remain paired only at the centromeres, align in metaphase, and the first meiotic division occurs.

4. *Posttranslational modifications in meiosis*

Posttranslational modifications regulate protein activity, localization, stability, and protein-protein interactions. In mouse meiocytes, it is interesting to note that the bulk of observed posttranslational modifications have functional relationships towards transcriptionally repressed heterochromatic regions. Posttranslational modifications in the spermatocyte localize/exclude at the domains of MSUC, and pericentromeric chromatin. The prominence of posttranslational modifications at regions of MSUC may indicate their role in genome defense. Other observations of posttranslational modifications have occurred at the sites of DNA damage, suggesting their role in signaling DDR. The most well-known meiotic posttranslational modification is the

ATR-dependent phosphorylation of H2AX (ɣH2AX) in response to both DNA damage and MSUC. While ɣH2AX is the most well recognized mark of heterochromatic domains in spermatocytes, the transcriptionally repressive mark, H3K9me2, is found at the sex body and likewise activating marks of acetylation, and H3K27-methylation are excluded from the sex body (66,67). While the functions are not understood, pericentromeric heterochromatic regions of spermatocytes display H3S10p from pachynema followed by H4K5ac and H4K16ac in diplonema [68]. And of course, ubiquitin-like modifications have been observed, with ubiquitination of H2A localizing to the sex body and involved in MSCI [69], and the previously discussed SUMOylation of H2AX [70].

Modification of proteins by the Small Ubiquitin-like MOdifier (SUMO) peptide is a poorly understood influence upon meiotic DNA repair. Where the more well-defined modifications of ubiquitination, phosphorylation, and acetylation have been identified and studied in somatic and meiotic cells to varying degrees for decades, SUMO-conjugates are more recent discoveries. SUMOylation is a covalent modification that alters fate or activity of proteins. The growing body of research in the last decade has shown SUMO to be influential in many cell types, including those of germ cells at all stages. Proteomic studies using available resources and material of somatic cells has identified hundreds of targets for SUMO-conjugation [71]. However, in the testis, the most experimentally tractable of the gonads, SUMO-conjugates remain largely uncharacterized but work has identified SC proteins SYCP1 and SYCP2 as SUMOylated in human males [72], and SUMO-conjugates at DSB sites of both mitotic and meiotic cells in the mouse [73]. In somatic cells, SUMOylation of topoisomerases TOPI and TOPII are seen in response to DNA damage, suggesting a larger link between SUMO and topoisomerase catalyzed damage [74]. Cytological studies in the mouse testis implicate SUMO involvement in meiotic recombination and

MSCI, with localization to both sites of DSBs and the sex body. Such localizations make SUMOylation interesting for its presumed roles in regulation of crossing-over, and MSUC. As of yet, few targets of SUMOylation are known, fewer are understood, and even less is known about the co-regulators that specify proteins for SUMOylation.

While the first substrate for SUMO was identified in vertebrates, RanGAP1, it has been through yeast that the understanding of SUMO-activating and –conjugating enzymes was described [75]. SUMO conjugation is analogous to ubiquitin conjugation: the C-terminal carboxyl group of Smt3/SUMO is activated by adenylation through the E1-like enzyme. While SUMO is then tightly bound to the E1 through a thioester bond, activated SUMO is transferred to the only known E2, Ubc9/UBE2I; the E2 enzyme can now transfer SUMO to a lysine residue of the substrate. Analogous to ubiquitination, the transfer from E2 enzyme to substrate is specified through an E3 ligase which functions to bridge the substrate to E2 and provide favorable specificity and kinetics. Since SUMO and ubiquitin can compete for the same lysine residues, it has been canonically proposed that SUMOylation functions to protect from ubiquitin-mediated degradation [76]. Identification of SUMO Interacting Motif (SIM) domains broadened the understanding of SUMO to offering an additional surface onto substrates through which to build protein-protein interactions or alter localizations [77].

In yeast, the often studied SC promoting Zip3 protein was identified as an *SP-RING* (Siz/PIAS-type RING) containing protein and subsequently verified as a SUMO E3 ligase [78]. Those studies further found that *zip3* mutants retain Smt3/SUMO-conjugates that are SC-, recombination-, and cell cycle-dependent, thus implicating additional complexity in E3 ligase co-regulation. An additional level of complexity, with possible implications to meiotic DSB repair, found that in somatic cells the DSB repair protein BRCA1 is recruited to DSBs though hybrid SUMO-Ub chains which are

specified by a new class of *SUMO-targeted Ubiquitin E3 ligases* (STUbL) [79]. These newly appreciated hybrid SUMO-Ub chains and the class of STUbLs have already been proposed as a more general signal mediating recruitment of DSB repair factors to damage sites (80,81).

5. ***Check point control of Meiosis I***

Meiosis I defects trigger checkpoints which either delay the cell and accommodate repair, or arrest the cell. In sexually dimorphic mammals, defects in meiosis I commonly result in infertility due to failure to properly segregate homologous chromosomes. In the females of these animals, oogenesis checkpoints are not considered to be as stringent as those of spermatogenesis and phenotypes vary from infertility due to meiotic arrest and aneuploidy leading to embryonic lethality or birth defects [6]. Notably, many such spermatogenesis defects arrest at the entry into pachynema with chromosome pairing defects, and thus lack any progress into metaphase. Mutations in *Spo11*, *Mei1*, *Dmc1*, *Msh4*, *Msh5* each display pre-pachytene arrest, albeit due to varying deficiencies in the initiation or progression of DSB repair (18,82-85). This arrest at pachynema is triggered by two surveillance mechanisms: the synapsis checkpoint and the recombination checkpoint. In mutants with failure to initiate synapse, chromosome cores localize BRCA1 which recruits the ATR kinase that phosphorylates H2AX thus leading to MSUC (86,87). This silencing of autosomal domains may lead to repression of meiotic genes and thus arrest of development [88]. Until recently, studies of the recombination checkpoint in mice were not possible. The report of mutation of *Trip13* in the mouse has uncovered the separation mutant which displays accurate synapsis while still activating the recombination checkpoint [89].

Future work with *Trip13* will lead towards understanding the recombination checkpoint in mammals.

Mutations affecting events post-pachynema are seen to trigger the spindle checkpoint, which delays meiosis I at metaphase and prevents segregation of the homologous chromosomes. In the mouse, the crossing-over defects observed in alleles of *Mlh1*, *Mlh3*, and *Ccnb1ip1* result in asynapsis at diplonema, thus spindle tension defect, and arrest prior to entry into Meiosis II. The spindle checkpoint is highly conserved between meiosis I, II, and mitosis.

6. *Significance of this work*

Meiosis is a specialized, highly developed, ancient process that enabled sexual reproduction towards the spread of genetic diversity, facilitating species robustness. Likely owing its derivation to mitotic division and DNA repair, it displays both striking mechanistic similarity and fundamental divergence. The apparently singular evolution and subsequent high conservation within meiosis across the species has led to great insights on the defining events, and their responsible proteins that are shared across species. But the additional complexity in species relevant to our own, as well as the co-regulators and fine understanding of checkpoints remain much of a mystery in the 126 years since the original cytological observations described by August Weismann.

Prophase I is of particular interest due to the complexity and defining events that take place. Beyond current understanding of the proteins involved in recombination initiation, stabilization of intermediates, and those proteins which ultimately mark the sites of essential reciprocal crossing-over, little is known about the co-regulators which aid in the selections of these recombinogenic sites. Furthermore, even less is understood about the effects of posttranslational modifications on both the

chromatin structure, and the enzymes which access the chromatin and provide for crossover events. It is both this higher order complexity, and meiotic regulation events in mammals that these efforts here hope to build insight towards and inform future research.

In this thesis I will interrogate the biochemistry and cytology of CCNB1IP1. The meiotic relevance of CCNB1IP1 was revealed through a forward genetic screen for fertility mutants in the mouse [90]. The *mei4* allele was mapped to *Ccnb1ip1* and the resultant CCNB1IP1^{mei4} is at least partially defective. *Ccnb1ip1*$^{mei4/mei4}$ animals fail to specify terminal class I crossing-over, display asynapsis, and thus male and female infertility [91].

As discussed, from other works we know that specification of crossing-over involves localization of at least one SUMO E3 ligase, RNF212/Zip3. Sites of DNA repair localize SUMO-conjugates and synapsed cores contain SUMOylated structural elements. More broadly, regions of MSUC display large amounts of SUMO-conjugation presumably reflective of heterochromatic regulation. The pathways and proteins modified by SUMO-regulations are divergent and expansive, but converge upon the relatively few E3 ligases, a class of co-regulators, that directly specify SUMO-conjugation upon many other proteins and subsequently alter their functions. Clearly SUMO is involved in regulation of meiosis, but its effect and the co-regulators which drive its conjugations remain an unexplored frontier in the biology of meiosis.

Hypothesis: CCNB1IP1 is a meiotic co-regulator; a SUMO E3 ligase functioning to specify SUMO-conjugates and aid to establish crossover fate specification in the resolution of DSB repair intermediates.

REFERENCES

1. Kolodner, Richard D., and Gerald T. Marsischky. "Eukaryotic DNA mismatch repair." *Current opinion in genetics & development* 9.1 (1999): 89-96.
2. Datta, Abhijit, et al. "Mitotic crossovers between diverged sequences are regulated by mismatch repair proteins in Saccharomyces cerevisiae." *Molecular and cellular biology* 16.3 (1996): 1085-1093.
3. Rizki, Aylin, and Victoria Lundblad. "Defects in mismatch repair promote telomerase-independent proliferation." *Nature* 411.6838 (2001): 713-716.
4. Papadopoulos, Nickolas, et al. "Mutation of a mutL homolog in hereditary colon cancer." *Science* 263.5153 (1994): 1625-1629.
5. Golub, Efim I., et al. "Interaction of human rad51 recombination protein with single-stranded DNA binding protein, RPA." *Nucleic acids research* 26.23 (1998): 5388-5393.
6. Morelli, Meisha A., and Paula E. Cohen. "Not all germ cells are created equal: aspects of sexual dimorphism in mammalian meiosis." *Reproduction* 130.6 (2005): 761-781.
7. Nebel, Bernard R., Anthony P. Amarose, and Elizabeth M. Hackett. "Calendar of gametogenic development in the prepuberal male mouse." *Science* 134.3482 (1961): 832-833.
8. Bellvé, Anthony R., et al. "Spermatogenic cells of the prepuberal mouse: isolation and morphological characterization." *The Journal of Cell Biology* 74.1 (1977): 68-85.
9. Chalmel, Frédéric, et al. "The conserved transcriptome in human and rodent male gametogenesis." *Proceedings of the National Academy of Sciences*104.20 (2007): 8346-8351.Schultz
10. Schultz, Nikolaus, F. Kent Hamra, and David L. Garbers. "A multitude of genes expressed solely in meiotic or postmeiotic spermatogenic cells offers a myriad of contraceptive targets." *Proceedings of the National Academy of Sciences*100.21 (2003): 12201-12206.
11. Cobb, John, and Mary Ann Handel. "Dynamics of meiotic prophase I during spermatogenesis: from pairing to division." *Seminars in Cell & Developmental Biology*. Vol. 9. No. 4. Academic Press, 1998.
12. Scherthan, Harry, et al. "Chromosome mobility during meiotic prophase in Saccharomyces cerevisiae." *Proceedings of the National Academy of Sciences*104.43 (2007): 16934-16939.
13. Cremer, Thomas. "Centromere and telomere movements during early meiotic prophase of mouse and man are associated with the onset of chromosome pairing." *The Journal of cell biology* 134.5 (1996): 1109-1125.
14. Bass, Hank W., et al. "Evidence for the coincident initiation of homolog pairing and synapsis during the telomere-clustering (bouquet) stage of meiotic prophase." *Journal of cell science* 113.6 (2000): 1033-1042.

15. Jin, Quan-wen, et al. "Yeast nuclei display prominent centromere clustering that is reduced in nondividing cells and in meiotic prophase." *The Journal of cell biology* 141.1 (1998): 21-29.
16. Keeney, Scott, Craig N. Giroux, and Nancy Kleckner. "Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family." *Cell* 88.3 (1997): 375-384.
17. Li, Jing, Gillian W. Hooker, and G. Shirleen Roeder. "Saccharomyces cerevisiae Mer2, Mei4 and Rec114 form a complex required for meiotic double-strand break formation." *Genetics* 173.4 (2006): 1969-1981.
18. Baudat, Frédéric, et al. "Chromosome synapsis defects and sexually dimorphic meiotic progression in mice lacking Spo11." *Molecular cell* 6.5 (2000): 989-998.
19. Moens, Peter B., et al. "The time course and chromosomal localization of recombination-related proteins at meiosis in the mouse are compatible with models that can resolve the early DNA-DNA interactions without reciprocal recombination." *Journal of cell science* 115.8 (2002): 1611-1622.
20. Camerini-Otero, RD and Peggy Hsieh. "Homologous recombination proteins in prokaryotes and eukaryotes." *Annual review of genetics* 29.1 (1995): 509-552.
21. Bolcun-Filas, Ewelina, et al. "Mutation of the mouse Syce1 gene disrupts synapsis and suggests a link between synaptonemal complex structural components and DNA repair." *PLoS genetics* 5.2 (2009): e1000393.
22. Lange, Julian, et al. "ATM controls meiotic double-strand-break formation." *Nature* 479.7372 (2011): 237-240.
23. Royo, Hélène, et al. "ATR acts stage specifically to regulate multiple aspects of mammalian meiotic silencing." *Genes & development* 27.13 (2013): 1484-1494.
24. Parvanov, Emil D., Petko M. Petkov, and Kenneth Paigen. "Prdm9 controls activation of mammalian recombination hotspots." *Science* 327.5967 (2010): 835-835.
25. Baudat, F., et al. "PRDM9 is a major determinant of meiotic recombination hotspots in humans and mice." *Science* 327.5967 (2010): 836-840.
26. Buard, Jérôme, et al. "Distinct histone modifications define initiation and repair of meiotic recombination in the mouse." *The EMBO journal* 28.17 (2009): 2616-2624.
27. Smagulova, Fatima, et al. "Genome-wide analysis reveals novel molecular features of mouse recombination hotspots." *Nature* 472.7343 (2011): 375-378.
28. Ding, Xu, et al. "SUN1 is required for telomere attachment to nuclear envelope and gametogenesis in mice." *Developmental cell* 12.6 (2007): 863-872.
29. Revenkova, Ekaterina, et al. "Cohesin SMC1β is required for meiotic chromosome dynamics, sister chromatid cohesion and DNA recombination." *Nature cell biology* 6.6 (2004): 555-562.
30. Terasawa, Masahiro, et al. "Meiotic recombination-related DNA synthesis and its implications for cross-over and non-cross-over recombinant formation." *Proceedings of the National Academy of Sciences* 104.14 (2007): 5965-5970.

31. Cloud, Veronica, et al. "Rad51 is an accessory factor for Dmc1-mediated joint molecule formation during meiosis." *Science* 337.6099 (2012): 1222-1225.
32. Kovalenko, Oleg V., et al. "Mammalian ubiquitin-conjugating enzyme Ubc9 interacts with Rad51 recombination protein and localizes in synaptonemal complexes." *Proceedings of the National Academy of Sciences* 93.7 (1996): 2958-2963.
33. Lin, Feng-Ming, et al. "Yeast axial-element protein, Red1, binds SUMO chains to promote meiotic interhomologue recombination and chromosome synapsis." *The EMBO journal* 29.3 (2009): 586-596.
34. Carballo, Jesús A., et al. "Phosphorylation of the axial element protein Hop1 by Mec1/Tel1 ensures meiotic interhomolog recombination." *Cell* 132.5 (2008): 758-770.
35. Fawcett, Don W. "The fine structure of chromosomes in the meiotic prophase of vertebrate spermatocytes." *The Journal of biophysical and biochemical cytology* 2.4 (1956): 403-406.
36. Moses, Montrose J. "Chromosomal structures in crayfish spermatocytes." *The Journal of biophysical and biochemical cytology* 2.2 (1956): 215-218.
37. Rousseaux, Sophie, et al. "Establishment of male-specific epigenetic information." *Gene* 345.2 (2005): 139-153.
38. Dehé, Pierre-Marie, et al. "Protein interactions within the Set1 complex and their roles in the regulation of histone 3 lysine 4 methylation." *Journal of biological chemistry* 281.46 (2006): 35404-35412.
39. Chen, Wei-Ta, et al. "Systematic identification of functional residues in mammalian histone H2AX." *Molecular and cellular biology* 33.1 (2013): 111-126.
40. Mahadevaiah, Shantha K., et al. "Recombinational DNA double-strand breaks in mice precede synapsis." *Nature genetics* 27.3 (2001): 271-276.
41. Yang, Fang, et al. "Mouse SYCP2 is required for synaptonemal complex assembly and chromosomal synapsis during male meiosis." *The Journal of cell biology* 173.4 (2006): 497-507.
42. Winkel, Karoline, et al. "Protein SYCP2 provides a link between transverse filaments and lateral elements of mammalian synaptonemal complexes." *Chromosoma* 118.2 (2009): 259-267.
43. Liu, Jian-Guo, et al. "Localization of the N-terminus of SCP1 to the central element of the synaptonemal complex and evidence for direct interactions between the N-termini of SCP1 molecules organized head-to-head." *Experimental cell research* 226.1 (1996): 11-19.
44. Vigodner, Margarita. "Sumoylation precedes accumulation of phosphorylated H2AX on sex chromosomes during their meiotic inactivation." *Chromosome Research* 17.1 (2009): 37-45.
45. Turner, James MA, et al. "Silencing of unsynapsed meiotic chromosomes in the mouse." *Nature genetics* 37.1 (2004): 41-47.
46. Schimenti, John. "Synapsis or silence." *Nature genetics* 37.1 (2005): 11-13.

47. Schramm, Sabine, et al. "A novel mouse synaptonemal complex protein is essential for loading of central element proteins, recombination, and fertility."*PLoS genetics* 7.5 (2011): e1002088.
48. Bolcun-Filas, Ewelina, et al. "SYCE2 is required for synaptonemal complex assembly, double strand break repair, and homologous recombination." *The Journal of cell biology* 176.6 (2007): 741-747.
49. Bolcun-Filas, Ewelina, et al. "Mutation of the mouse Syce1 gene disrupts synapsis and suggests a link between synaptonemal complex structural components and DNA repair." *PLoS genetics* 5.2 (2009): e1000393.
50. Cheng, Chung-Hsu, et al. "SUMO modifications control assembly of synaptonemal complex and polycomplex in meiosis of Saccharomyces cerevisiae." *Genes & development* 20.15 (2006): 2067-2081.
51. Agarwal, Seema, and G. Shirleen Roeder. "Zip3 provides a link between recombination enzymes and synaptonemal complex proteins." *Cell* 102.2 (2000): 245-255.
52. Hooker, Gillian W., and G. Shirleen Roeder. "A Role for SUMO in meiotic chromosome synapsis." *Current biology* 16.12 (2006): 1238-1243.
53. Martini, Emmanuelle, et al. "Crossover homeostasis in yeast meiosis." *Cell*126.2 (2006): 285-295.
54. Ashley, Terry, and Annemieke Plug. "Caught in the Act: Deducing Meiotic Function from Protein Immunolocalization." *Current topics in developmental biology* 37 (1997): 201-239.
55. Cohen, P. E., S. E. Pollack, and J. W. Pollard. "Genetic analysis of chromosome pairing, recombination, and cell cycle control during first meiotic prophase in mammals." *Endocrine reviews* 27.4 (2006): 398-426.
56. Plug, Annemieke W., et al. "Changes in protein composition of meiotic nodules during mammalian meiosis." *Journal of Cell Science* 111.4 (1998): 413-423.
57. Moens, Peter B., et al. "The time course and chromosomal localization of recombination-related proteins at meiosis in the mouse are compatible with models that can resolve the early DNA-DNA interactions without reciprocal recombination." *Journal of cell science* 115.8 (2002): 1611-1622.
58. Fung, Jennifer C., et al. "Imposition of crossover interference through the nonrandom distribution of synapsis initiation complexes." *Cell* 116.6 (2004): 795-802.
59. Kneitz, Burkhard, et al. "MutS homolog 4 localization to meiotic chromosomes is required for chromosome pairing during meiosis in male and female mice." *Genes & Development* 14.9 (2000): 1085-1097.
60. Edelmann, Winfried, et al. "Mammalian MutS homologue 5 is required for chromosome pairing in meiosis." *Nature genetics* 21.1 (1999): 123-127.
61. Reynolds, April, et al. "RNF212 is a dosage-sensitive regulator of crossing-over during mammalian meiosis." *Nature genetics* 45.3 (2013): 269-278.

62. Santucci-Darmanin, S, et al. "MSH4 acts in conjunction with MLH1 during mammalian meiosis." *The FASEB Journal* 14.11 (2000): 1539-1547.
63. Baker, Sean M., et al. "Involvement of mouse Mlh1 in DNA mismatch repair and meiotic crossing over." *Nature genetics* 13.3 (1996): 336-342.
64. Lipkin, Steven M., et al. "Meiotic arrest and aneuploidy in MLH3-deficient mice." *Nature genetics* 31.4 (2002): 385-390.
65. Edelmann, Winfried, et al. "Meiotic pachytene arrest in MLH1-deficient mice." *Cell* 85.7 (1996): 1125-1134.
66. Khalil, Ahmad M., Fatih Z. Boyar, and Daniel J. Driscoll. "Dynamic histone modifications mark sex chromosome inactivation and reactivation during mammalian spermatogenesis." *Proceedings of the National Academy of Sciences of the United States of America* 101.47 (2004): 16583-16587.
67. Namekawa, Satoshi H., et al. "Postmeiotic sex chromatin in the male germline of mice." *Current biology* 16.7 (2006): 660-667.
68. Khalil, A. M., and D. J. Driscoll. "Epigenetic regulation of pericentromeric heterochromatin during mammalian meiosis." *Cytogenetic and genome research* 129.4 (2010): 280-289.
69. Baarends, Willy M., et al. "Histone ubiquitination and chromatin remodeling in mouse spermatogenesis." *Developmental biology* 207.2 (1999): 322-333.
70. Chen, Wei-Ta, et al. "Systematic identification of functional residues in mammalian histone H2AX." *Molecular and cellular biology* 33.1 (2013): 111-126.
71. Golebiowski, Filip, et al. "System-wide changes to SUMO modifications in response to heat shock." *Science signaling* 2.72 (2009): ra24.
72. Brown, Petrice W., et al. "Small ubiquitin-related modifier (SUMO)-1, SUMO-2/3 and SUMOylation are involved with centromeric heterochromatin of chromosomes 9 and 1 and proteins of the synaptonemal complex during meiosis in men." *Human reproduction* 23.12 (2008): 2850-2857.
73. Shrivastava, Vibha, et al. "SUMO proteins are involved in the stress response during spermatogenesis and are localized to DNA double-strand breaks in germ cells." *Reproduction* 139.6 (2010): 999-1010.
74. Mao, Yong, et al. "SUMO-1 conjugation to topoisomerase I: a possible repair response to topoisomerase-mediated DNA damage." *Proceedings of the National Academy of Sciences* 97.8 (2000): 4046-4051.
75. Melchior, Frauke. "SUMO-nonclassical ubiquitin." *Annual review of cell and developmental biology* 16.1 (2000): 591-626.
76. Müller, Stefan, et al. "SUMO, ubiquitin's mysterious cousin." *Nature reviews Molecular cell biology* 2.3 (2001): 202-213.
77. Hecker, Christina-Maria, et al. "Specification of SUMO1-and SUMO2-interacting motifs." *Journal of Biological Chemistry* 281.23 (2006): 16117-16127.
78. Cheng, Chung-Hsu, et al. "SUMO modifications control assembly of synaptonemal complex and polycomplex in meiosis of Saccharomyces cerevisiae." *Genes & development* 20.15 (2006): 2067-2081.

79. Guzzo, Catherine M., et al. "RNF4-dependent hybrid SUMO-ubiquitin chains are signals for RAP80 and thereby mediate the recruitment of BRCA1 to sites of DNA damage." *Science signaling* 5.253 (2012): ra88.
80. Raman, Nithya, Arnab Nayak, and Stefan Muller. "The SUMO system: a master organizer of nuclear protein assemblies." *Chromosoma* (2013): 1-11.
81. Guzzo, Catherine M., and Michael J. Matunis. "Expanding SUMO and ubiquitin-mediated signaling through hybrid SUMO-ubiquitin chains and their receptors." *Cell Cycle* 12.7 (2013): 1015-1017.
82. Munroe, Robert J., et al. "Mouse mutants from chemically mutagenized embryonic stem cells." *Nature genetics* 24.3 (2000): 318-321.
83. Pittman, Douglas L., et al. "Meiotic Prophase Arrest with Failure of Chromosome Synapsis in Mice Deficient for Dmc1, a Germline-Specific RecA Homolog." *Molecular cell* 1.5 (1998): 697-705.
84. Kneitz, Burkhard, et al. "MutS homolog 4 localization to meiotic chromosomes is required for chromosome pairing during meiosis in male and female mice." *Genes & Development* 14.9 (2000): 1085-1097.
85. Edelmann, Winfried, et al. "Mammalian MutS homologue 5 is required for chromosome pairing in meiosis." *Nature genetics* 21.1 (1999): 123-127.
86. Burgoyne, Paul S., Shantha K. Mahadevaiah, and James MA Turner. "The consequences of asynapsis for mammalian meiosis." *Nature reviews Genetics*10.3 (2009): 207-216.
87. Turner, James MA. "Meiotic sex chromosome inactivation." *Development*134.10 (2007): 1823-1831.
88. Turner, James MA, et al. "Silencing of unsynapsed meiotic chromosomes in the mouse." *Nature genetics* 37.1 (2004): 41-47.
89. Li, Xin, and John C. Schimenti. "Mouse pachytene checkpoint 2 (trip13) is required for completing meiotic recombination but not synapsis." *PLoS genetics*3.8 (2007): e130.
90. Ward, Jeremy O., et al. "Toward the genetics of mammalian reproduction: induction and mapping of gametogenesis mutants in mice." *Biology of reproduction* 69.5 (2003): 1615-1625.
91. Ward, Jeremy O., et al. "Mutation in mouse hei10, an e3 ubiquitin ligase, disrupts meiotic crossing over." *PLoS genetics* 3.8 (2007): e139.

CHAPTER 2

Evidence implicating CCNB1IP1, a RING domain-containing protein required for meiotic crossing over in mice, as an E3 SUMO ligase

*Reprinted from: Strong, Edward R., and John C. Schimenti. "Evidence Implicating CCNB1IP1, a RING Domain-Containing Protein Required for Meiotic Crossing Over in Mice, as an E3 SUMO Ligase." *Genes* 1.3 (2010): 440-451. *Creative Commons Attribution License*

Abstract

The RING domain-containing protein CCNB1IP1 (Cyclin B1 Interacting Protein 1) is a putative ubiquitin E3 ligase that is essential for chiasmata formation, and hence fertility, in mice. Previous studies in cultured cells indicated that CCNB1IP1 targets Cyclin B for degradation, thus playing a role in cell cycle regulation. Mice homozygous for a mutant allele (*mei4*) of *Ccnb1ip1* display no detectable phenotype other than meiotic failure from an absence of chiasmata. CCNB1IP1 is not conserved in key model organisms such as yeast and *Drosophila*, and there are no features of the protein that implicate clear mechanisms for a role in recombination. To gain insight into CCNB1IP1's function in meiotic cells, we raised a specific antibody and determined that the protein appears at the onset of pachynema. This indicates that CCNB1IP1 is involved with crossover intermediate maturation, rather than early (leptotene) specification of a subset of SPO11-induced double strand breaks towards the crossover pathway. Additionally, a yeast 2-hybrid (Y2H) screen revealed that CCNB1IP1 interacts with SUMO2 and a set of proteins enriched for consensus sumoylation sites. The Y2H studies, combined with scrutiny of CCNB1IP1 domains, implicate this protein as an E3 ligase of the sumoylation cascade. We

hypothesize CCNB1IP1 represents a novel meiosis-specific SUMO E3 ligase critical to resolution of recombination intermediates into mature chiasmata.

Introduction

In previous work, our lab conducted forward genetic mutagenesis screens to identify novel genes required for meiosis in mice [1, 2]. One of the alleles, *mei4*, presented as a recessive male and female sterile. Histological and cytological analyses revealed abnormal alignment and distribution of chromosomes at metaphase/anaphase at the first meiotic division in spermatocytes and oocytes [3]. Immunocytological analyses revealed no abnormalities in non-crossover (NCO) recombination or chromosome synapsis through early pachynema. However, as the meiocytes entered diplonema, the homologous chromosomes failed to maintain interhomolog associations, suggesting an absence of chiasmata. This suspicion was confirmed by an absence of MLH1 and MLH3 foci on pachytene chromosomes [3]. The mismatch repair proteins are well established markers of chiasmata [4].

Positional cloning revealed that *mei4* is a mutant allele of *Ccnb1ip1* (also called *Hei10*), a gene not previously known to have a role in meiosis. *Ccnb1ip1* encodes a coiled-coil RING domain-containing protein. Studies of CCNB1IP1 in cultured somatic cells implicated a role for this putative ubiquitin E3 ligase in Cyclin B regulation, cell cycle progression, and cell invasion [5-7]. However, the exact function of CCNB1IP1 in meiotic recombination remains is unclear. A model proposed by Ward *et al* posited that CCNB1IP1 disrupts association of CDK2 with CCNB3, possibly *via* ubiquitylation, thus permitting CDK2 to recruit or enable binding of MLH1 and MLH3 (and possibly other proteins) to designated crossover sites [3].

To better understand the role of CCNB1IP1 in recombination, and to gain possible support for the aforementioned model, we conducted a yeast two hybrid (Y2H) screen for interacting proteins in the mouse testis, characterized the temporal appearance of CCNB1IP1 during meiosis, and examined bioinformatically the domain structures of CCNB1IP1. Surprisingly, these studies implicate CCNB1IP1 as a SUMO (Small Ubiquitin-like Modifier) E3 ligase. SUMOylation modulates many behaviors of proteins, including interactions with other proteins, subcellular localization, and stabilization though competition with Ubiquitin for lysine residues [8]. The process of SUMO conjugation to target substrates is analogous to that of the well characterized Ub cascade; involving E1, E2 and E3 type ligases [9]. The role SUMO plays in meiosis remains largely unknown; however, immunolocalization studies in the mouse have detected SUMO at sites of DSBs and at centromeric and heterochromatic regions. Additionally, the singular SUMO E2 ligase, UBC9 (UBE2I in the mouse) localizes along synapsed chromosome cores during pachynema and diplonema [10, 11]. The evidence we present in support of CCNB1IP1 as a potential SUMO E3 ligase has the potential to reveal hitherto unknown mechanisms in mammalian meiotic recombination.

Results and Discussion

Expression of CCNB1IP1 and CCNB1IP1^{mei4} during spermatogenesis

CCNB1IP1 is essential for meiotic crossing-over in mice. In *S. cerevisiae*, although double Holliday junctions characteristic of crossover (CO) recombination appear in early-mid pachynema [12], the partitioning of double strand breaks (DSBs) to either the NCO or CO pathways is made much earlier, in late leptonema [13]. Like yeast, mammals have genetically distinct NCO and CO pathways [14]. Therefore,

CCNB1IP1 may be required either for the specifying a subset of DSBs to the CO fate in leptonema, or subsequent processing of CO recombination intermediates in pachynema.

As a first step towards addressing this question, we generated an affinity-purified rabbit polyclonal antibody against N-terminal amino acids 1-245 of CCNB1IP1. The antibody recognized a protein slightly larger than 30kDa (theoretical MW of CCNB1IP1 = 32kDa) in Western blots of WT protein from 20 dpp and adult mouse testis. Juvenile *Ccnb1ip1*^{mei4}/+ extracts had roughly half the amount of the 32kDa species compared to WT animals. The 32kDa species was completely lacking in homozygous mutants, consistent with it being CCNB1IP1 (Fig. 2.1a). Notably, both heterozygous and homozygous testis extracts showed an additional, slightly smaller band on the Western blots (Fig. 2.1a,b). This species was not as robust as wild-type CCNB1IP1, and it appeared to be more predominant in the homozygous mutants than in heterozygotes. Considering that the *Ccnb1ip1*^{mei4} allele is predicted to encode a protein bearing an internal deletion of 24 amino acids (~2.7 kDa) [3], it is likely that the smaller species in the Western blot is this truncated protein. The mutant CCNB1IP1 allele may retain some function. However, the relatively lower amounts of the smaller species in both hetero- and homozygotes suggests that the CCNB1IP1^{mei4} protein is less stable, more rapidly cleared, or translated at a lower efficiency than WT CCNB1IP1.

To further confirm the specificity of the antibody, we performed Western blot analysis of protein from 20 dpp testis extracted from several meiotic mutants (Fig. 2.1b). The 32kDa product is undetectable in *Ccnb1ip1*$^{mei4/mei4}$ animals, but was present in mice homozygous for a mutant *Mybl1* allele that causes meiotic arrest at a stage of meiosis similar to that of *Ccnb1ip1*^{Mei4} spermatocytes (late pachynema/diplonema; unpublished). This result indicates that the 32kDa species is not a cross-reactive

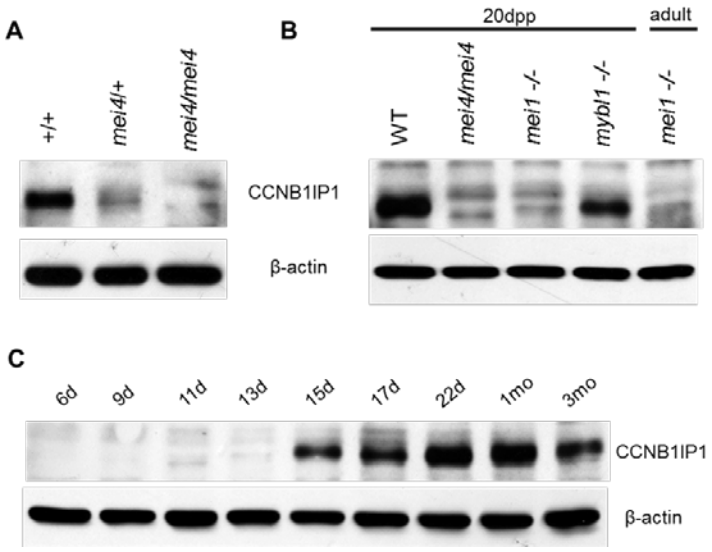

Figure 2.1. Western blot analysis of CCNB1IP1 expression in testis.
(A) Polyclonal anti-CCNB1IP1 recognizes a ~32kDa species in 20dpp testis of WT and heterozygous *Ccnb1ip1*^{mei4} animals. This band is absent in mutants (third lane), but a lower band of ~30kDa is evident that not present in WT. **(B)** CCNB1IP1 is greatly decreased or absent from mutant testes that undergo meiotic arrest prior to pachynema (*Mei1*$^{-/-}$), but not those that progress to approximately diplonema (*Mybl1*$^{-/-}$). **(C)** CCNB1IP1 in testis is first produced between days 13 and 15 dpp, coincident with onset of pachynema.

product from a class of cells that are missing in *Ccnb1ip1*$^{mei4/mei4}$ testes. The product was present at low levels in *Mei1/Mei1* 20 dpp testis, in which meiosis arrests prior to entry intopachynema due to failed DSB formation and extensive asynapsis [15, 16]. This suggests either that *Ccnb1ip1*^{mei4} expression is either dependent upon DSB formation (which occurs in leptonema), or it initiates in pachytene spermatocytes.

To pinpoint the onset of CCNB1IP1 production, we took advantage of the coordinated first wave of spermatogenesis after birth. Leptotene, pachytene, late pachytene and diplotene cells appear *en masse* approximately 10, 14, 18, and >18 dpp, respectively [17, 18]. As shown in Figure 2.1c, CCNB1IP1 is absent in 13 dpp testis and appears at 15 dpp coincident with spermatocyte entry into pachynema. CCNB1IP1 then persists throughout adulthood, although the data does not indicate if it is present in postmeiotic spermatids. These data indicate that CCNB1IP1 is not involved in partitioning DSBs to the CO pathway. Rather, expression after entry into pachynema suggests a requirement for processing CO recombination intermediates.

Identification of CCNB1IP1 interacting proteins

CCNB1IP1 is a coiled-coil RING domain-containing protein shown to have E3 Ubiquitin ligase activity [5]. The RING domain is characteristic of the E3-ligase family of proteins. To identify potential ubiquitylation targets of CCNB1IP1 and other interacting proteins that might illuminate the molecular mechanism by which this protein participates in crossing over, we conducted a yeast two-hybrid (Y2H) screen of a testis library (Fig. 2.2). Full length CCNB1IP1 was found to be auto-activating under the selective growth conditions of the screen. Progressive C-terminal truncations narrowed the autoactivating region to that containing 2 putative Cyclin/Cdk target motifs [5], so these were deleted from the bait vector. Thirty-five interactors were

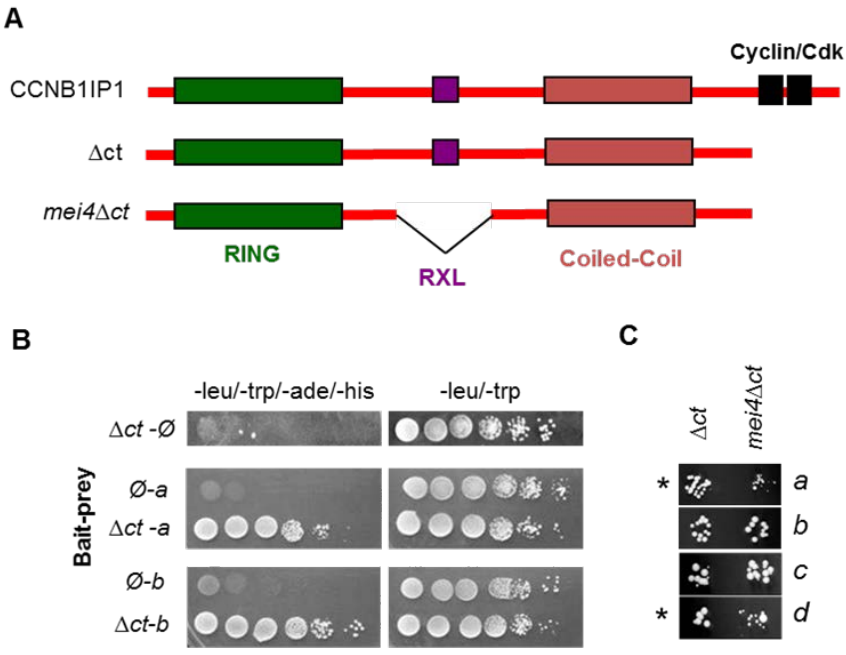

Figure 2.2. Yeast two-hybrid screen for CCNB1IP1 interacting proteins in the testis.

(A) Structure of CCNB1IP1 and known motifs/domains. A C-terminal truncated construct was used as bait (Δct). This lacks two potential phosphorylation sites of cyclin/cdk kinases. A bait construct with the deletion contained in the *Ccnb1ip1*^{mei4} allele (mei4Δct) is shown at the bottom. **(B)** Confirmation of prey clones under maximal selection stringency (ade and his) with leu and trp selection for presence of bait and prey plasmids. **(C)** Growth of yeast containing 4 different prey and either the Δct bait or the mei4Δct bait. A subset of CCNB1IP1 interactors (asterisks) were found to reproducibly display weaker interaction with mei4Δct than Δct, as assessed by vigor of individual colony growth. Ø=empty vector; a = EP400; b = OAZ3; c = 5730469M10Rik; d= 4930455F23Rik.

isolated and validated (Table 2.1; see Methods). Literature review and database searches reveal that none of the CCNB1IP1 interacting genes are known to be essential for meiosis; however, manual survey of the NCBI GEO Profiles database reveals several are transcriptionally up-regulated in the testis, with postnatal testis expression increasing in age and peaking during pachytene of meiosis I (accessions GDS3142, GDS605, GDS401).

In light of the Western blot data indicating that *Ccnb1ip1*$^{mei4/mei4}$ testes produced a deleted version of CCNB1IP1, we hypothesized that the mutant protein might have defective interactions with some subset of the Y2H binding partners, thus potentially explaining the recombination phenotype. We therefore tested the full set of 35 CCNB1IP1 interactors against the mutant allele as bait (signified as *mei4*Δ*ct)*. The *Ccnb1ip1*^{mei4} deletion did not ablate interaction with any of the prey clones, however it did reproducibly lead to a "kinetically" weaker interaction as assessed by colony size under stringent growth selection conditions (Fig. 2.2c). If this is reflective of the *in vivo* situation in mice, it is possible that the weaker interactions, coupled with the decreased level of mutant protein, contributes to the phenotype.

Conspicuous amongst the Y2H interactors was SUMO2. This prompted us to consider a potential role for CCNB1IP1 in the SUMOylation cascade. Inspection of the other CCNB1IP1 interacting proteins revealed one notable common motif: Ψ-K-X-E/D (where Ψ is a hydrophobic amino acid; Table 2.1). This Ψ-K-X-E/D motif is enriched in targets of SUMOylation, and lysine (K) is the residue targeted for SUMO modification in those proteins [19, 20]. Notably, of the 13 proteins with kinetic defects in interaction affinity for *mei4*Δ*ct*, 7 (54%) carry the SUMOylation motif with 5/7 possessing the most common form Y/L-K-X-E. The predicted SUMO-proteome has been estimated to be 38% of all similarly analyzed peptides [21].

Kinetically normal with MEI4Δct	Kinetically defective with MEI4Δct
SUMO2	4930455F23RIK
AKAP9	YPEL2
SPINK10	1700006A11RIK
1700019N19RIK	EP400
POLR2B	POMP
ENAH	MSL1
H3F3B	DDC8
5730469M10RIK	HOOK1
PHF12	GGN
MRRF	FHL5
OCIAD1	4930503B20RIK
B9D1	1700021F07RIK
MORN2	SPATA3
ATOH8	
SRGN	
PENK1	
BRP44	
INSL3	
EMX1	
GSG1	
OAZ3	
MIIP	

Table 2.1. Proteins Identified in Two Hybrid Screen.
Proteins with predicted SUMOylation sites are underlined. All clones for the corresponding proteins were isolated with CCNB1IP1Δct as bait. Later they were individually tested for interaction with the MEI4 deletion version. They were subdivided into two kinetic interaction classes as indicated by the two columns.

Motif analysis of CCNB1IP1 implicates it is a SUMO E3 ligase

PIAS4 and other SUMO E3 ligases have been found to interact with SUMO in Y2H assays [22, 23, 24]. Given that CCNB1IP1 interacts with SUMO2 and other proteins containing consensus SUMOylation sites, we hypothesize that CCNB1IP1 has SUMO E3 ligase function in addition to its reported E3 Ub ligase activity. SUMO E3 ligases often contain a C3H2C3 type RING domain believed to confer interaction specificity to the singular known E2 ligase, UBC9 [25, 26]. Alignment of CCNB1IP1 from mouse and other species as well as known SUMO E3 ligases (ZIP3, SIZ1, SIZ2) reveals the presence and conservation of a C3H2C3 type RING domain. Additionally, non-covalent SUMO interacting motifs (SIMs) have been identified within most SUMO E3 ligases [26, 27, 28]. SIMs are characterized by Ψ-X-Ψ-Ψ where Ψ is V/I or another large hydrophobic residue [27, 29]. CCNB1IP1 indeed has such a sequence conserved across mammals (Fig. 2.3b; non-human mammals not shown).

This putative SIM is just 15 aa residues N terminal to the deletion in the *Ccnb1ip1*^{mei4} allele (Fig. 2.3b). Recently, studies of the Pc2 SUMO E3 ligase showed that pairs of charged and hydrophobic amino acids adjacent to the consensus SIM facilitate E3 function [28]. These residues have been proposed to facilitate interactions with SUMO-conjugated UBC9 [29]. The deletion immediately C-terminal to the putative CCNB1IP1 SIM, if it contains similar facilitating residues, may impair interaction with UBC9-SUMO *in vivo*.

Figure 2.3. SUMO E3 ligase-like domain conservation in CCNB1IP1.
(A) Alignments of C3H2C3-type RING domains in four CCNB1IP1 orthologs and other known SUMO E3 ligases. **(B)** CCNB1IP1 contains a canonical SIM motif (blue) just upstream of the region deleted in the *Ccnb1ip1*^{mei4} allele.

Conclusions

CCNB1IP1 is required for crossing-over, but there is no known mechanistic linkage to recombination. We conducted the Y2H screen in the hope of identifying proteins of known function that might provide such mechanistic linkage. Although none of the identified interacting proteins have reported roles in crossing-over, our analyses of the aggregate Y2H data suggest a function for CCNB1IP1 as an E3 ligase in the SUMO modification pathway. To summarize, we found that CCNB1IP1 interacts with both SUMO2 and proteins containing the consensus SUMOylation motif, properties consistent with computational studies of SUMO E3 ligases [21, 24, 30]. Furthermore, we observed that CCNB1IP1 contains a C3H2C3 type RING domain, conserved in proven SUMO E2 ligases, that constitutes the interaction surface with the SUMO E2, UBC9. Finally, CCNB1IP1 contains a consensus SIM domain found in the majority of characterized E3s. This sequence has been proposed to function in aiding E3 interaction with SUMO-conjugated UBC9 (not unconjugated UBC9) [31, 32]. Whether the putative SIM in CCNB1IP1 actually functions in this manner awaits experimental validation.

There is increasing evidence indicating multiple roles for SUMO modification in regulating DNA repair and meiosis. The *S. cerevisiae* SUMO E3 ligase Zip3 ensures that SC formation is dependent on recombination initiation, and it interacts with a number of recombination proteins including Mre11, Rad51, Msh4 and Msh5 [26, 33, 34]. The Zip3 ortholog in *C. elegans* is required for meiotic crossover formation and is localized to sites of crossing-over in late Prophase I [35]. Variants in the human ortholog *RNF212* have been associated with influencing genome-wide meiotic recombination rates [36, 37]. Furthermore, the synaptonemal complex component protein Zip1 and axial-element protein Red1 have been demonstrated to bind SUMO-conjugated proteins, the latter of which promotes interhomolog

exchange [26, 38]. Finally, the SUMO pathway is involved in regulating ubiquitylation in DNA damage responses in mammalian cells [39, 40].

These results, together with the growing understanding of SUMOylation in higher order eukaryotes, are beginning to shed light on the role for SUMO in DNA damage responses and recombination in meiosis. The defect of *Ccnb1ip1*^{mei4} in meiotic crossing over and its putative role as a SUMO E3 ligase offer us a novel element in our understanding of the mechanisms regulating crossover formation. The result of the Y2H screen, which identified a number of putative SUMOylation target proteins with no known roles in meiosis, suggest that further study of CCNB1IP1 will reveal novel mechanisms of meiotic recombination in mammals.

Materials and Methods

Recombinant expression of CCNB1IP1 and anti-CCNB1IP1 production.

cDNA corresponding to amino acids 1-245 of CCNB1IP1 was subcloned into expression plasmid pQE-30 (Qiagen), so as to add a 6X HIS tag. Bacterially expressed peptide was solubilized using standard procedures with the addition of 5M Urea. The HIS-tagged peptide was purified on a HisPur cobalt resin column according to manufacturer's protocols (Pierce). The peptide purification was verified via SDS-PAGE and concentrated on a Vivaspin 15R column (SartoriusStedim). The purified CCNB1IP1 was used as immunogen for polyclonal antibody production in rabbit, followed by affinity purification over immobilized CCNB1IP1 as per manufacturer's procedures (GenScript). Specificity of the IgG was assessed by dot-blot down to 250pg of recombinant CCNB1IP1.

SDS-PAGE and Western blotting.

SDS-PAGE and Western blotting were performed by standard procedures. Tissues from mice were dounce homogenized in cold lysis buffer (1% SDS, 10mM EDTA, 50mM Tris, pH 8.0) supplemented with protease inhibitors (complete®, Roche) followed by sonication at 1s intervals for 30s. Lysates were boiled for 5min followed by clearing via centrifugation. Protein concentrations of cleared lysates were measured by the BCA protein assay (Thermo). Loading controls were performed with anti-β-actin following SDS/2-MeOH stripping of the PVDF membrane. CCNB1IP1 signal was detected with 1:250 dilution of anti-CCNB1IP1 incubated 3hr at 4C followed by an hour incubation using goat anti-rabbit HRP-conjugated antibody.

Yeast two-hybrid screen for CCNB1IP1 interactors

Full length CCNB1IP1 was found to be auto-activating under the Y2H conditions used. Following analysis of various truncations for loss of auto-activation under screen conditions, cDNA of a C-terminal truncation of mouse *Ccnb1ip1* corresponding to amino acids 1-245 (encoding what we call CCNB1IP1Δct) was expressed as a "bait" fusion protein with the GAL4 DNA-binding domain. This was constructed in plasmid pGBK. A mouse testis cDNA library in pACT2 (Clontech) was used as "prey" for a protein-protein interaction screen with CCNB1IP1Δct. Interactions with CCNB1IP1Δct were selected by colony growth in the absence of histidine on plates supplemented with 7.5mM 3-AT. Strongly growing colonies were confirmed under more stringent interaction selection on plates lacking histidine and adenine. pACT2 cDNA clones were isolated and analyzed by DNA sequencing. The CCNB1IP1Δct interactor clones were then directly tested for interaction affinity with the same c-terminal truncation from *Ccnb1ip1*^{mei4} cDNA (called *mei4*Δct) under the more stringent conditions (Fig. 2.2a). Those clones showing decreased affinity of interaction with *mei4*Δct were confirmed in three independent experiments.

REFERENCES

1. Munroe, R.J.; Bergstrom, R.A.; Zheng, Q.Y.; Libby, B.; Smith, R.; John, S.W.M.; Schimenti, K.J.; Browning, V.L.; Schimenti, J.C. Mouse mutants from chemically mutagenized embryonic stem cells. *Nat. Genet.* **2000**, *24*, 318-321.
2. Ward, J.O.; Reinholdt, L.G.; Hartford, S.A.; Wilson, L.A.; Munroe, R.J.; Schimenti, K.J.; Libby, B.J.; O'Brien, M.; Pendola, J.K.; Eppig, J.; Schimenti, J.C. Toward the genetics of mammalian reproduction: induction and mapping of gametogenesis mutants in mice. *Biol Reprod* **2003**, *69*, 1615-1625.
3. Ward, J.O.; Reinholdt, L.G.; Motley, W.W.; Niswander, L.M.; Deacon, D.C.; Griffin, L.B.; Langlais, K.K.; Backus, V.L.; Schimenti, K.J.; O'Brien, M.J.; Eppig, J.J.; Schimenti, J.C. Mutation in mouse Hei10, an e3 ubiquitin ligase, disrupts meiotic crossing over. *PLoS Genetics* **2007**, *3*, e139.
4. Marcon, E.; Moens, P. MLH1p and MLH3p localize to precociously induced chiasmata of okadaic-acid-treated mouse spermatocytes. *Genetics* **2003**, *165*, 2283-2287.
5. Toby, G.G.; Gherraby, W.; Coleman, T.R.; Golemis, E.A. A novel RING finger protein, human enhancer of invasion 10, alters mitotic progression through regulation of cyclin B levels. *Mol Cell Biol* **2003**, *23*, 2109-2122.
6. Gronholm, M.; Muranen, T.; Toby, G.G.; Utermark, T.; Hanemann, C.O.; Golemis, E.A.; Carpen, O. A functional association between merlin and HEI10, a cell cycle regulator. *Oncogene* **2006**, *25*, 4389-4398.
7. Singh, M.K.; Nicolas, E.; Gherraby, W.; Dadke, D.; Lessin, S.; Golemis, E.A. HEI10 negatively regulates cell invasion by inhibiting cyclin B/Cdk1 and other promotility proteins. *Oncogene* **2007**, *26*, 4825-4832.
8. Geiss-Friedlander, R.; Melchior, F. Concepts in sumoylation: a decade on. *Nature Rev* **2007**, *8*, 947-956.
9. Ulrich, H.D. The SUMO system: an overview. *Methods Mol Biol* **2009**, *497*, 3-16.
10. Shrivastava, V.; Pekar, M.; Grosser, E.; Im, J.; Vigodner, M. SUMO proteins are involved in the stress response during spermatogenesis and are localized to DNA double-strand breaks in germ cells. *Reproduction, 139*, 999-1010.
11. La Salle, S.; Sun, F.; Zhang, X.D.; Matunis, M.J.; Handel, M.A. Developmental control of sumoylation pathway proteins in mouse male germ cells. *Dev Biol* **2008**, *321*, 227-237.
12. Hunter, N.; Kleckner, N. The single-end invasion: an asymmetric intermediate at the double-strand break to double-holliday junction transition of meiotic recombination. *Cell* **2001**, *106*, 59-70.
13. Borner, G.V.; Kleckner, N.; Hunter, N. Crossover/noncrossover differentiation, synaptonemal complex formation, and regulatory surveillance at the leptotene/zygotene transition of meiosis. *Cell* **2004**, *117*, 29-45.

14. Guillon, H.; Baudat, F.; Grey, C.; Liskay, R.M.; de Massy, B. Crossover and noncrossover pathways in mouse meiosis. *Mol Cell* **2005**, *20*, 563-573.
15. Libby, B.J.; De La Fuente, R.; O'Brien, M.J.; Wigglesworth, K.; Cobb, J.; Inselman, A.; Eaker, S.; Handel, M.A.; Eppig, J.J.; Schimenti, J.C. The Mouse Meiotic Mutation mei1 Disrupts Chromosome Synapsis with Sexually Dimorphic Consequences for Meiotic Progression. *Dev Biol* **2002**, *242*, 174-187.
16. Libby, B.J.; Reinholdt, L.G.; Schimenti, J.C. Positional cloning and characterization of Mei1, a vertebrate-specific gene required for normal meiotic chromosome synapsis in mice. *PNAS* **2003**, *100*, 15706-15711.
17. Nebel, B.R.; Amarose, A.P.; Hacket, E.M. Calendar of gametogenic development in the prepuberal male mouse. *Science* **1961**, *134*, 832-833.
18. Bellve, A.; Cavicchia, J.; Millette, C.; O'Brien, D.; Bhatnagar, Y.; Dym, M. Spermatogenic cells of the prepubertal mouse. Isolation and morphological characterization. *J. Cell. Biol.* **1977**, *74*, 68-85.
19. Melchior, F.; Schergaut, M.; Pichler, A. SUMO: ligases, isopeptidases and nuclear pores. *Trends Biochem Sci* **2003**, *28*, 612-618.
20. Denison, C.; Rudner, A.D.; Gerber, S.A.; Bakalarski, C.E.; Moazed, D.; Gygi, S.P. A proteomic strategy for gaining insights into protein sumoylation in yeast. *Mol Cell Proteomics* **2005**, *4*, 246-254.
21. Zhou, F.; Xue, Y.; Lu, H.; Chen, G.; Yao, X. A genome-wide analysis of sumoylation-related biological processes and functions in human nucleus. *FEBS Lett* **2005**, *579*, 3369-3375.
22. Weger, S.; Hammer, E.; Engstler, M. The DNA topoisomerase I binding protein topors as a novel cellular target for SUMO-1 modification: characterization of domains necessary for subcellular localization and sumolation. *Exp Cell Res* **2003**, *290*, 13-27.
23. Kahyo, T.; Nishida, T.; Yasuda, H. Involvement of PIAS1 in the sumoylation of tumor suppressor p53. *Mol Cell* **2001**, *8*, 713-718.
24. Goehler, H.; Lalowski, M.; Stelzl, U.; Waelter, S.; Stroedicke, M.; Worm, U.; Droege, A.; Lindenberg, K.S.; Knoblich, M.; Haenig, C.; Herbst, M.; Suopanki, J.; Scherzinger, E.; Abraham, C.; Bauer, B.; Hasenbank, R.; Fritzsche, A.; Ludewig, A.H.; Bussow, K.; Coleman, S.H.; Gutekunst, C.A.; Landwehrmeyer, B.G.; Lehrach, H.; Wanker, E.E. A protein interaction network links GIT1, an enhancer of huntingtin aggregation, to Huntington's disease. *Mol Cell* **2004**, *15*, 853-865.
25. Johnson, E.S.; Gupta, A.A. An E3-like factor that promotes SUMO conjugation to the yeast septins. *Cell* **2001**, *106*, 735-744.
26. Cheng, C.H.; Lo, Y.H.; Liang, S.S.; Ti, S.C.; Lin, F.M.; Yeh, C.H.; Huang, H.Y.; Wang, T.F. SUMO modifications control assembly of synaptonemal complex and polycomplex in meiosis of Saccharomyces cerevisiae. *Genes Devel* **2006**, *20*, 2067-2081.

27. Song, J.; Durrin, L.K.; Wilkinson, T.A.; Krontiris, T.G.; Chen, Y. Identification of a SUMO-binding motif that recognizes SUMO-modified proteins. *PNAS* **2004**, *101*, 14373-14378.
28. Merrill, J.C.; Melhuish, T.A.; Kagey, M.H.; Yang, S.H.; Sharrocks, A.D.; Wotton, D. A role for non-covalent SUMO interaction motifs in Pc2/CBX4 E3 activity. *PLoS ONE* **2010**, *5*, e8794.
29. Song, J.; Zhang, Z.; Hu, W.; Chen, Y. Small ubiquitin-like modifier (SUMO) recognition of a SUMO binding motif: a reversal of the bound orientation. *J Biol Chem* **2005**, *280*, 40122-40129.
30. Takahashi, Y.; Toh-e, A.; Kikuchi, Y. A novel factor required for the SUMO1/Smt3 conjugation of yeast septins. *Gene* **2001**, *275*, 223-231.
31. Zhu, J.; Zhu, S.; Guzzo, C.M.; Ellis, N.A.; Sung, K.S.; Choi, C.Y.; Matunis, M.J. Small ubiquitin-related modifier (SUMO) binding determines substrate recognition and paralog-selective SUMO modification. *J Biol Chem* **2008**, *283*, 29405-29415.
32. Lin, D.Y.; Huang, Y.S.; Jeng, J.C.; Kuo, H.Y.; Chang, C.C.; Chao, T.T.; Ho, C.C.; Chen, Y.C.; Lin, T.P.; Fang, H.I.; Hung, C.C.; Suen, C.S.; Hwang, M.J.; Chang, K.S.; Maul, G.G.; Shih, H.M. Role of SUMO-interacting motif in Daxx SUMO modification, subnuclear localization, and repression of sumoylated transcription factors. *Mol Cell* **2006**, *24*, 341-354.
33. Macqueen, A.J.; Roeder, G.S. Fpr3 and Zip3 ensure that initiation of meiotic recombination precedes chromosome synapsis in budding yeast. *Curr Biol* **2009**, *19*, 1519-1526.
34. Agarwal, S.; Roeder, G.S. Zip3 provides a link between recombination enzymes and synaptonemal complex proteins. *Cell* **2000**, *102*, 245-255.
35. Bhalla, N.; Wynne, D.J.; Jantsch, V.; Dernburg, A.F. ZHP-3 acts at crossovers to couple meiotic recombination with synaptonemal complex disassembly and bivalent formation in C. elegans. *PLoS Genetics* **2008**, *4*, e1000235.
36. Chowdhury, R.; Bois, P.R.; Feingold, E.; Sherman, S.L.; Cheung, V.G. Genetic analysis of variation in human meiotic recombination. *PLoS Genetics* **2009**, *5*, e1000648.
37. Kong, A.; Thorleifsson, G.; Stefansson, H.; Masson, G.; Helgason, A.; Gudbjartsson, D.F.; Jonsdottir, G.M.; Gudjonsson, S.A.; Sverrisson, S.; Thorlacius, T.; Jonasdottir, A.; Hardarson, G.A.; Palsson, S.T.; Frigge, M.L.; Gulcher, J.R.; Thorsteinsdottir, U.; Stefansson, K. Sequence variants in the RNF212 gene associate with genome-wide recombination rate. *Science* **2008**, *319*, 1398-1401.
38. Lin, F.M.; Lai, Y.J.; Shen, H.J.; Cheng, Y.H.; Wang, T.F. Yeast axial-element protein, Red1, binds SUMO chains to promote meiotic interhomologue recombination and chromosome synapsis. *EMBO J* **2010**, *29*, 586-596.
39. Galanty, Y.; Belotserkovskaya, R.; Coates, J.; Polo, S.; Miller, K.M.; Jackson, S.P. Mammalian SUMO E3-ligases PIAS1 and PIAS4 promote responses to DNA double-strand breaks. *Nature* **2009**, *462*, 935-939.

40. Morris, J.R.; Boutell, C.; Keppler, M.; Densham, R.; Weekes, D.; Alamshah, A.; Butler, L.; Galanty, Y.; Pangon, L.; Kiuchi, T.; Ng, T.; Solomon, E. The SUMO modification pathway is involved in the BRCA1 response to genotoxic stress. *Nature* **2009**, *462*, 886-890.

CHAPTER 3

CCNB1IP1 as a regulator of SUMO conjugation

Abstract

The gene for Cyclin B1 Interacting Protein 1 (*Ccnb1ip1*) is essential in the mouse for meiotic crossing-over. Animals carrying an allele for a defective *Ccnb1ip1* fail to form obligate meiotic crossovers in Prophase I and present as male and female infertile while otherwise appearing grossly normal. I implicated CCNB1IP1 as a SUMO E3 ligase with function as a co-regulator of SUMOylation upon targets more directly involved in crossover formation. Here we present biochemical interrogations of CCNB1IP1 activity both *in vivo* and *in vitro*. *In vitro*, recombinant CCNB1IP1 displays a propensity toward autoSUMOylation in conditions deficient for a subsequent final target of SUMOylation, a characteristic property of E3 ligases. *In vivo*, the previously identified interacting partners of CCNB1IP1, GGN and 4930455F23RIK, are themselves targets of posttranslational modification dependent on interaction with a putative SUMO E3 ligase. Together, these studies support the hypothesis that CCNB1IP1 functions as a SUMO E3 ligase essential to the formation of meiotic crossovers.

Introduction

Genetic mutation in the mouse reveals *Ccnb1ip1* as an essential factor in mammalian spermatogenesis. *Ccnb1ip1*$^{mei4/mei4}$ spermatocytes arrest due to a failure to maintain sites of reciprocal crossing-over [1]. Previous functional studies of CCNB1IP1 activity in somatic cells have shown the protein to function as an E3 adapter ligase within the well-understood Ubiquitin pathway [2]. Subsequent studies

by our group have implicated CCNB1IP1 as being associated with factors in the SUMO (Small Ubiquitin-like MOdifier) pathway. Proteomic analysis of CCNB1IP1 domains, including the C3H2C3-like RING domain, together with its non-covalent binding to SUMO2 have led us towards the hypothesis that CCNB1IP1 orchestrates SUMO-conjugation to target peptides as an E3 adapter ligase in spermatocytes.

As a SUMO E3 ligase, CCNB1IP1 would present itself as a “meiotic co-regulator”, a class of proteins that directly affect altered function upon multiple other proteins. In the case of CCNB1IP1 as a co-regulator, a set of its subjugates would be presumed to more directly impact the observed CCNB1IP1-dependent reciprocal crossing over within meiosis. These CCNB1IP1-regulated factors would be essential to faithful crossing-over, and thus physical tethering together of homologous chromosomes entering into metaphase of meiosis I. Failure in accurate homolog segregation at metaphase I clinically manifests as infertility in the adult or developmental defects of the fetus, underscoring the importance of crossover regulation in mammalian meiosis [3]. The co-regulators of cellular processes, including meiosis, represent single points upon which the diverse functions of many other proteins converge, presumably for timely and/or response-driven regulation of activity. Conjugation with SUMO regulates diverse functions, and the essential E3 adapter ligase co-regulators specify the targets of SUMO-conjugation and thus represent one class of these convergences and coordination. While SUMOylated peptides have been observed in nearly all stages of spermatogenesis, identities, pathways, regulations, and sites of action are poorly understood.

The covalent-modification of proteins with Ubiquitin or SUMO is an enzymatic action in which the modifying peptide is transferred from the respective E2 enzyme onto the target protein. The class of E3 ligase enzymes functions as the adapter between the E2 and target proteins so as to regulate and increase efficiency of

the modification. Hershko and Ciechanover have defined the E3 as “an enzyme that binds, directly or indirectly, specific protein substrates and promotes the transfer of ubiquitin [or similar], directly or indirectly, from a thioester intermediate amide linkages with proteins or polyubiquitin [or similar] chains” [4].

The founding members in the class of SUMO E3 ligases were first uncovered through yeast two-hybrid screens as proteins that interact with SUMO [5,6]. In structural similarity to the ubiquitin cascade, Histidine and Cysteine residues in both Ub and SUMO-specific E3 ligase RING fingers coordinate a pair of Zinc atoms forming a “cross-brace” which, in turn, builds a globular tertiary structure that can directly bind a specific E2 enzyme. In the SUMOylation cascade there is believed to be a single E2 enzyme, Ubc9/UBE2I, responsible for all SUMO-modification. This is thusly coincident with a dominant SP-RING type domain found in known SUMO E3 ligases. Analogous to the ubiquitination cascade, functional tests of SUMO E3 ligases have shown precocious E3 auto-sumoylation, and poly-SUMO chain formation when adequate target protein is not present [7,8].

Due to a similarity of molecular response to analogous insults, particularly double strand breaks (DSBs), much has been previously extrapolated and validated from somatic- and into meiotic- DNA Damage Response (DDR). X-ray induced DSBs in HeLa cells result in increased levels of SUMO2 and presumably signaling DDR repair [9]. Such studies illuminate an alleged role for SUMO in response to genomic insults. Knockout studies of the sole E2 enzyme, *Ubc9/Ube2i*, and presumably ablation of the SUMO proteome underscores the essential role SUMO plays; mice deficient for *Ube2i* undergo early lethality [10]. High tertiary structural similarity amongst SUMO1/2/3/4, and coincident expression of SUMO1/2/3 could indicate functional redundancy, and indeed mice mutated for SUMO1 bear no major developmental or fertility defects [11]. While the SUMO peptides themselves may be

redundant in function, SUMOylation is essential and plays a role in response to genomic insults. Studies of the DDR response in somatic cells are identifying SUMO targets, their roles and the E3s which specify their SUMOylation, little progress has been made in understanding SUMO-mediated meiotic DDR response.

Most of what we know about SUMO, its conjugates, and the E3 ligases that give specificity to the system stem from studies of the PIAS family of E3-like RING domain proteins. In the mouse, PIAS family members represent the most well studied SUMO E3 ligases and have been used to identify many SUMO-substrates. PIAS4 (PIASy) was the first PIAS to be verified as a SUMO E3 ligase of LEF1 *in vitro,* as well as unknown other substrates *in vivo* [12]. Likewise, as the SP-RING domain would suggest, all other PIAS family proteins function as SUMO E3 ligase, notably including PIAS1-dependent SUMOylation of P53 [13]. In yeast, *Siz1* and *Siz2* are the most closely related genes to mammalian PIAS and were identified as SUMO E3s through two-hybrid interaction with Smt3 (yeast homolog of SUMO), as well as *in vitro* activity [14,15]. The SP-RING derives its name from the Siz/Pias family of E3 ligases.

Reliable culture systems are not available for spermatogenesis and consequently little is known about the co-regulators and post-translational modifications in mammalian meiosis. Studies in yeast have identified a role for SUMO in meiotic regulation of SC formation and DSB repair though crossing-over [16]. Interrogations of these events, and discovery of higher order SUMOylation has been hindered by cell type, low detection levels of SUMO-conjugates, and technical differences in antibodies. While gross-detection of SUMO-conjugates has shown selected localization to Prophase I heterochromatin and centromeres, UBC9/UBE2i is seen to localize in a purposeful manner along the length of the SC and studies from the

human have shown SC proteins SYCP1 and SYCP2 are SUMOylated in spermatocytes [17-19].

While not much is understood about the effective nature of SUMO-conjugated proteins in meiotic recombination, a few well-established factors shared between mitotic and meiotic recombination are identified as part of the SUMO proteome. The yeast DNA helicase Sgs1 (commonly called BLM in mammals) is involved in both mitotic and meiotic joint molecule resolution and is SUMO-regulated [20,21]. Two-hybrid studies have shown physical interaction between the marker of early recombination initiation, RAD51, and the SUMO E2 ligase in humans [22]. The potential consequences of this interaction are unknown but may indicate that SUMOylation plays a role in the processing of DSBs in accord with the observed SUMOylation of Lim15/Dmc1 in *Coprinus cinereus* [23]. Concurrent with the observation of SUMO-conjugates at centromeres in mammalian spermatocytes, yeast Smt3/SUMO localizes to meiotic centromeres and is hypothesized to facilitate nucleation and further polymerization of SCs [24-26].

Meiotic recombination is facilitated by the physical tethering together of homologous chromosomes through the proteinaceous Synaptonemal Complex (SC). While research has revealed several essential components of this proteinaceous structure, there is a lack of thorough understanding of the quaternary structure and the nature of the protein-protein associations building the SC. Biochemical studies of known SC constituents have revealed a propensity for these proteins, notably those of the Transverse Filament, under conditions non-suitable for native SC assembly to self-assemble into SC-like aggregates known as polycomplexes [27-30]. The propensity to build SC-like structures and the consistent localization of SUMO peptide to these structures underscores the essential role SUMO is hypothesized to play in meiotic progression. This possibly indicates that SUMO plays a role in facilitating protein-

protein interactions and assembly of proteinaceous structures facilitating DDR. It is interesting that many of the identified Central Element and Transverse Filament proteins contain coiled-coil domains, since such domains are known to facilitate homo- and hetero- protein-protein interactions [31]. Presumably often mediated through coiled-coil domains, under events of native SC assembly failure, polycomplexes may be the manifestation of an intrinsic action for Central Region and Transverse Filament proteins to self-assemble into SC-like structures.

Informed by the secondary and tertiary structural predictions of CCNB1IP1, together with the previously reported two-hybrid data, I set forth to understand the biochemistry of CCNB1IP1 interactions and functions. I hypothesized that understanding the functional biochemistry of domains within CCNB1IP1 would complement the existing hypothesis of meiotic SUMOylation and SC localized proteins as well as builds the foundation upon which to understand the targeted role of CCNB1IP1-dependent SUMOylation upon specification of crossing-over.

Results

CCNB1IP1 ectopic expression forms polycomplex-like structures**.**

CCNB1IP1 has been previously hypothesized to function in cell cycle regulation, mediated though it's C-terminal CDK-like domains. Furthermore, due its essential role in Prophase I progression, formation of crossing-over, and its hypothesized role as SUMO E3 ligase together in context to the known localization of SUMO-conjugates at heterochromatic DNA, and UBE2I localization to condensed meiotic cores of Pachynema, I suspected CCNB1IP1 localization in the nucleus or upon the chromatin itself.

My previously generated custom antibody against CCNB1IP1 proved unsuccessful with immunolocalization on both fixed tissue sections and disassociated

spermatocytes (data not shown). Faithful tissue culture of meiocytes has thus far proved unsuccessful. Due to this, I implemented ectopic expression of fluorescently-tagged CCNB1IP1 in the well-defined CHO and HEK293 cell lines. The complete coding sequence of *Ccnb1ip1* was cloned into pmCherry-N1, and expression was driven though the CMV promoter (fig. 3.1a). In both transient transfections and stably selected lines of CHO and HEK293 cells, CCNB1IP1 was observed to self-interactions into long filamentous structures, as visualized through fluorescent microscopy of the mCherry tag (fig. 3.1b). Clonal selection for stable lines expressing CCNB1IP1^{mCherry} yielded homogenous populations, indicating that these structures were cell-cycle independent.

The formation of CCNB1IP1 filamentous structures is reminiscent of the "polycomplexes" formed by aggregation of SYCP3 and SYCP1 upon similar ectopic expression [28,30]. In both cases, ectopic expression of DNA-directed, synaptonemal complex-associated proteins aggregated into thin fibers which then further associated and appeared to build thicker fibrous structures. These self-assembling arrays have been termed "polycomplexes" and have been thought to be an indicative characteristic of synaptonemal complex associated proteins, possibly reflecting storage of excess SC-associated components [30,32].

Previous studies of mine and other labs found the full length isoform of CCNB1IP1 to be autoactivating to Y2H systems, as well as difficult to express in the soluble fraction of recombinant protein production (discussed later). I considered that these observations may be related to the self-assembly of fibrous networks as seen in the ectopic cell culture expression system. My previous expression attempts in yeast (for two-hybrid) and in bacterial cells (for recombinant protein production) demonstrated that the protein could be faithfully expressed when the c-terminal tail, including putative CDK-like domains, was truncated. My two-hybrid experiments

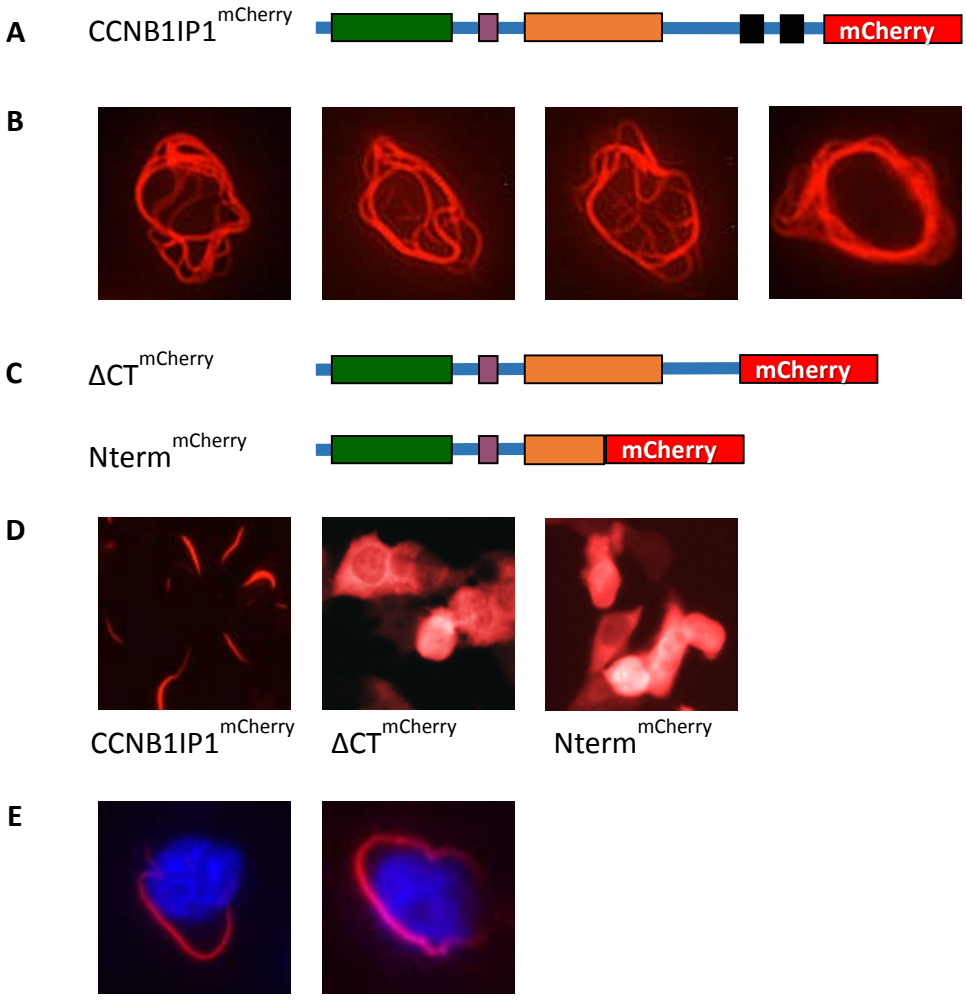

Figure 3.1. CCNB1IP1 ectopic expression in cultured cells.
Towards understanding the localization and biochemistry of CCNB1IP1, fusion alleles of CCNB1IP1 were expressed in CHO and HEK293 under control of the CMV promoter. (A) mCherry is C-terminally tagged to full-length CCNB1IP1 so as to maximize distance from N-terminal RING and SIM domains. (B) Expression of *Ccnb1ip1*^{mCherry} results in dense filament formations as observed through mCHERRY fluorescence in live CHO cells. Filamentous structures are reminiscent of *polycomplex* formations. (C and D) C-terminal truncations of CCNB1IP1 were able to abolish polycomplex formations in HEK293 cells. Abrogation of polycomplex-like filaments may be due to removal of an uncharacterized homodimerization domain on the c-terminal, or resultant from the bulky mCherry tag's proximity/disruption to the known homodimerization domain of coiled-coil domain. (E) CCNB1IP1^{mCherry} polycomplex is cytoplasmic in HEK cells, fixed and nuclei marked with DAPI (blue).

would suggest that the absence of the 31a.a. on the C-terminal of CCNB1IP1 are not catastrophic to protein tertiary structure and the protein retained ability to form protein-protein interactions. Ccnb1ip1Δct, a c-terminal truncation of CCNB1IP1, was cloned into the pmCherry-N1 vector (fig. 3.1c). As suspected, CCNB1IP1Δct^{mCherry} abolished polycomplex-like structure formations (fig 3.1d). Furthermore, deeper truncation of *Cccnb1ip1*, similar to that as used by other groups for two-hybrid studies, likewise abolished polycomplex-like formations (fig. 3.1c/d). These results, along with truncations of CCNB1IP1, illustrate that the extreme C-terminal is at least partially responsible for the protein's self-assembly into continuous filaments.

Co-expression, *in vivo* studies of CCNB1IP1 activity upon targets.

The protein targets for posttranslational modification from Ubiquitin and SUMO vary in expression both developmentally and in a tissue-specific manner. While the E3-like ligase proteins with activity in Ubiquitin and SUMO modifications vary in a similar manner of time and location, it is clear that the general mechanism of Ubiquitylation and SUMOylation is not restricted to developmental stage nor tissue specificity. In both variants of posttranslational modifications, the core machinery (Ub/SUMO, E1-, and E2- enzymes) is present in all cells and should be available in access for suitable E3 enzymes and the matching targets of modification. Due to the lack of spermatocyte cell culture techniques to *in vivo* interrogate CCNB1IP1 biochemical activity, I sought to take advantage of this universally expressed machinery and recapitulate the interactions between posttranslational target and the specifying E3 ligase, CCNB1IP1.

I proceeded to co-express CCNB1IP1^{mCherry} with previously identified putative protein conjugates, *in vivo*, as targets of CCNB1IP1-dependent targets of Ubiquitin or SUMO modification (fig 3.2a). As a putative E3 ligase of the SUMOylation cascade, I

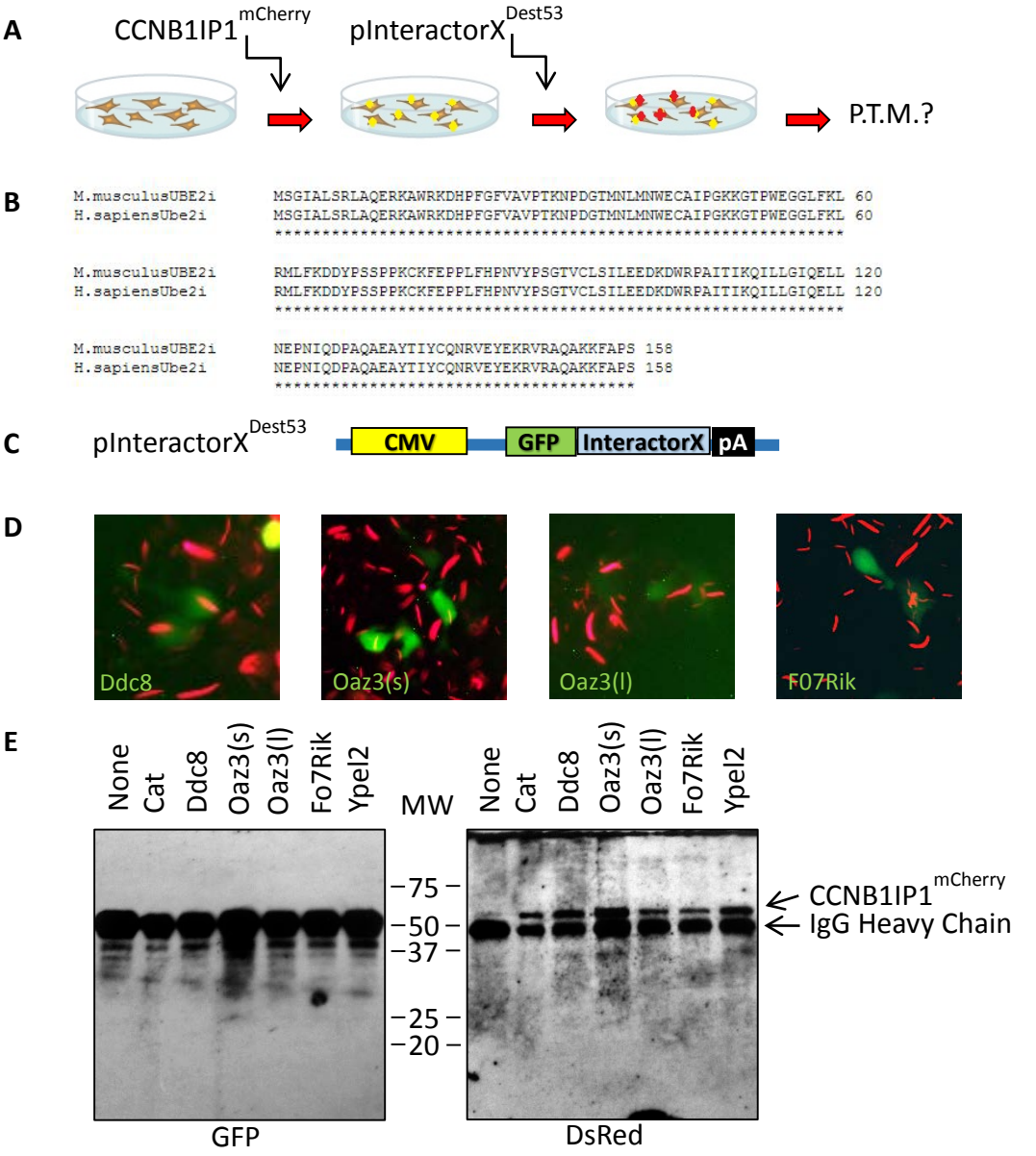

Figure 3.2. *in vivo* co-expressions of CCNB1IP1^{mCherry} and putative interactors. (A) Interactions between CCNB1IP1 and putative partners was attempted in HEK293 cells. CCNB1IP1 function as an E3-type ligase should target peptides to receive posttranslational modification. (B) Alignment of human and mouse SUMO E2 ligases (UBE2I) show exact conservation suitable for interspecies interaction with SUMO E3 ligases. (C) A selection of putative CCNB1IP1 targets were N-terminally fused to GFP and expression driven by the CMV promoter. (D) HEK293 cells with homogenous stable expression of CCNB1IP1^{mCherry} were transfected with the indicated putative targets, (E) however co-immunoprecipitations against CCNB1IP1 were unable to detect interactions with putative targets (as assessed by Western blot for associated fluorescent tag, as indicated at bottom of the blot). Failure to detect interaction likely owing to the polycomplex obscuring domains.

suspected some of the previously reported interacting partners of CCNB1IP1 would represent true targets for SUMO modification in a CCNB1IP1-dependent manner. CMV promoter-driven GFP-labeled putative targets of CCNB1IP1-mediated SUMOylation were transiently transfected into CHO and HEK cells with stable ectopic expression of CCNB1IP1^{mCherry} (fig. 3.2c-d). Total cell lysates were assessed by Western blot to detect levels of protein expression and retarded migration due to an increased size dependent upon posttranslational modification. Unfortunately, likely owing to obscured interaction domains resultant to the *in vivo* aggregations of CCNB1IP1^{mCherry}, interactions between CCNB1IP1 and its previously identified associates of protein-protein interaction were unable to be co-immunoprecipitated (fig. 3.2e).

Reconstituted CCNB1IP1-dependent SUMOylation using recombinant peptides, *in vitro*.

Due to the problems encountered from the lack of spermatocyte culture systems and dense fibrous filament formation of CCNB1IP1 ectopic expression in alternative somatic culture systems, I sought to recapitulate the SUMOylation function of CCNB1IP1 *in vitro*. The core components (SUMO peptides, E1-, and E2- enzymes) are readily obtained as recombinant proteins, and the enzymatic modification of SUMO to peptides can be recapitulated *in vitro* as an ATP-dependent reaction. Previous studies have used similar *in vitro* reconstitutions and shown that E3 ligase members of the SUMO cascade will both autoSUMOylate in high concentrations as well as increase the kinetic efficiency of target protein modification by the SUMO peptide (fig. 3.3)**.** Similarly to the studied processes of ubiquitination, in conditions where upon the SUMO cascade is active and the E2 ligase is staged and loaded with SUMO to be transferred to a target peptide, autoSUMOylation of the interacting E3

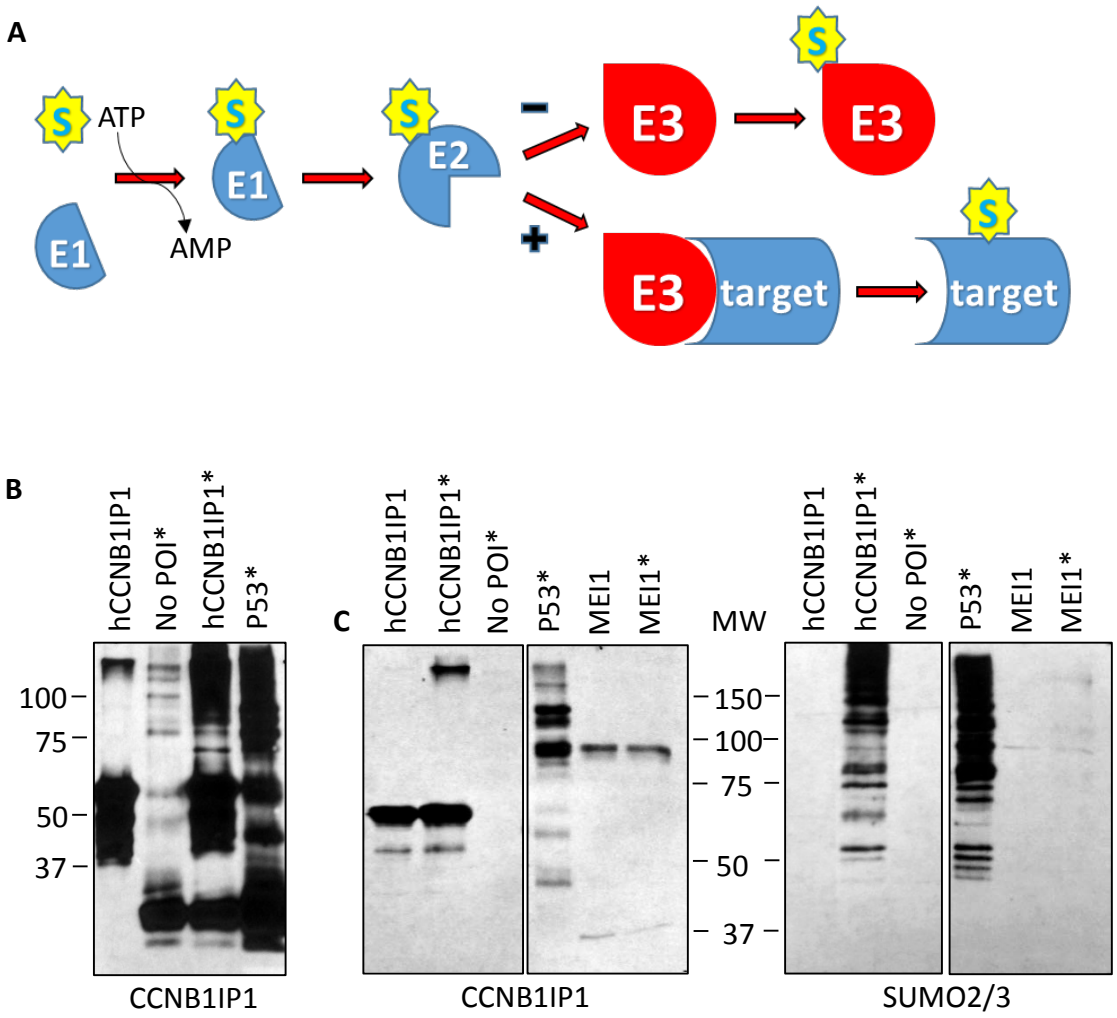

Figure 3.3. Reconstituted *in vitro* SUMOylation.
Reconstituted *in vitro* reactions of SUMOylation were established using recombinant proteins; human CCNB1IP1 (CCNB1IP1, 32kDa; fused with GST, 25kDa) purchased from Abnova. (A) Supplied with the necessary constituents for SUMO-conjugation, terminal targets can be efficiently modified in the presence of the suitable E3 ligase (lower path). When the *in vitro* reaction lacks suitable terminal target of SUMO-conjugation, the E2 enzyme catalyzes transfer of SUMO peptide to the adjacent E3 ligase, leading to *autoSUMOylation* of the E3 (top path). (B) Recombinant human CCNB1IP1 shows MW shifts *in vitro* corresponding to SUMO2 supplementation, lacking appropriate terminal target protein. (C) AutoSUMOylation of recombinant human CCNB1IP1 is not dependent upon GST fusion, as the recombinant MEI1-GST is negative for MW shifting. (P53 is a known positive terminal target of SUMOylation. General anti-CCNB1IP1 cross reactivity in P53 and MEI1 samples, but background reactivity is low in *in vitro* reactions lacking a Protein of Interest, POI. The "*" indicates SUMO2 added to reaction.)

ligase will be observed in the lack of a suitable terminal target. The purpose of this autoSUMOylation is not well understood, but it may demonstrate that the E2 has an intrinsic function in SUMO delivery and the E3 ligase is an adequate, although not ideal, target to receive SUMO. When these similar *in vivo* reactions are established in the presence of a terminal target for SUMO modification, the E2 ligase again eagerly transfers the SUMO peptide to lysine residues of proximal proteins. The presence of the suitable adaptor-like E3 ligase, which functions as a bridge between the SUMO-loaded E2 and the terminal target, increases the kinetic efficiency of SUMO-conjugation to targets.

Taking advantage of the pure reconstituted *in vitro* SUMOylation system, I used both purchased and custom-derived recombinant CCNB1IP1. Because the function of CCNB1IP1 either as an available target of "autoSUMOylation" or as the ligase adapter between E2 and the target requires proper tertiary structure folding of the peptide, GST fusions were used in both cases. Purification of GST-bound proteins reduces harvest of unfolded and aggregate peptides because purification over Glutathione offers the advantage that GST must be folded in its proper tertiary structure so as to facilitate its dimerization, which is essential to binding the Glutathione resin. Likewise, recombinant proteins suspected as terminal targets for SUMO were obtained as GST fusions. Using the complete *in vitro* reconstitution for faithful SUMO modification, the autoSUMOylation of CCNB1IP1 was tested in reactions lacking a canonical terminal target of SUMOylation (fig. 3.3b/c). In these reactions, using an unrelated peptide (MEI1) fused to GST and similarly purified, it was observed that CCNB1IP1 did indeed specifically autoSUMOylate. Similar autoSUMOylation was observed when custom CCNB1IP1-GST was harvested from my own *small batch* bacterial expressions (fig. 3.4a/b). This autoSUMOylation is

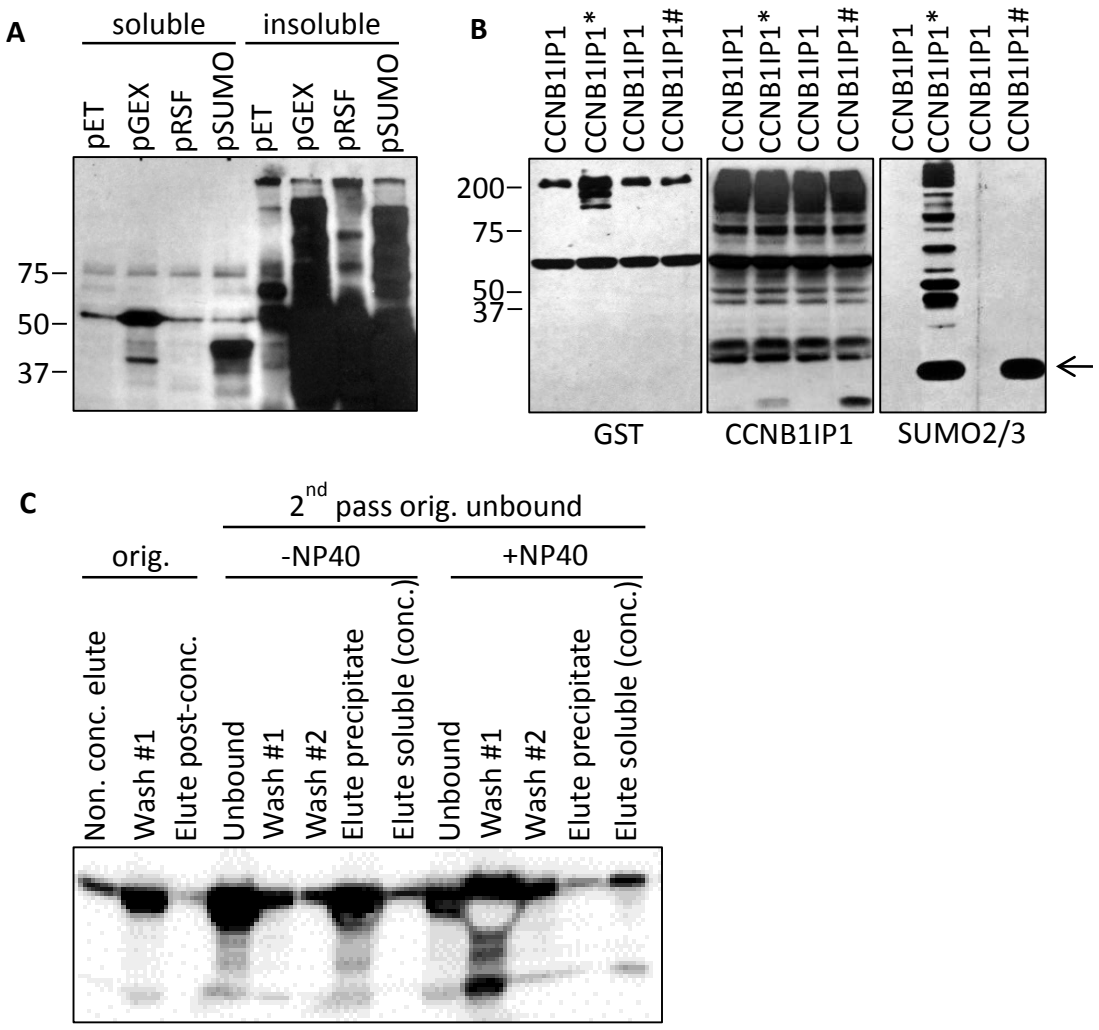

Figure 3.4. Reconstituted *in vitro* SUMOylation with custom CCNB1IP1. (A) Recombinant CCNB1IP1 was expressed though each pET, pGEX, pRSF, and pSUMO in the Rosetta(DE3) strain. Western blot expression qualifications reveal only a fraction of total CCNB1IP1 is found in the soluble supernatants. (B) Western blots of reconstituted *in vitro* SUMOylation with the recombinant CCNB1IP1 reveals MW shifting and autoSUMOylation of CCNB1IP1. The high MW shifting is dependent upon poly-SUMO chain formation and lost in the sample supplied with mtSUMO2 unable to form poly-linkages. ("*" denotes samples receiving SUMO2; "#" denotes mutated mtSUMO2. Arrow marks free SUMO. Extreme high MW band, seen independent of SUMO2, may reflect oligomerization of CCNB1IP1 *in vitro*, as observed previously *in vivo*). (C) Detergents are unable to increase CCNB1IP1-GST purification efficiency in *large batch* preparations. The unbound soluble fraction of pGEX-*Ccnb1ip1* expression was subjected to a second round of purification across Glutathione. Mild detergent, NP40, was unable to increase purification efficiency. Possibly indicating some level of CCNB1IP1 oligomerization, resistant to detergent, precluding GST binding to Glutathione.

consistent with CCNB1IP1's internal lysine residues (K58, K142) predicted as receptive to SUMO modification.

The presence of autoSUMOylation of CCNB1IP1 presents further evidence implicating CCNB1IP1 as a member of the E3 class of SUMO ligase peptides. Having some functional indication of CCNB1IP1 as a SUMO E3 ligase, I investigated the *in vitro* receptivity of proteins previously reported to interact with CCNB1IP1 and suspected as terminal targets for SUMO modifications. Similar *in vitro* reconstitutions were used in the presence of putative terminal targets from SUMO modifications. In these instances, so as to test the efficiency of CCNB1IP1 to specify targets for modification by the SUMO peptide, the addition of recombinant CCNB1IP1 was varied; In reactions receiving CCNB1IP1, the efficiency of SUMO conjugation to the terminal targets should be increased as opposed to those instances lacking CCNB1IP1. This would confirm that CCNB1IP1 is an efficient E3 ligase of the SUMOylation cascade and confirm CCNB1IP1-dependent targets of SUMO modification. Unfortunately, these reactions were inconclusive due to problems detecting low concentrations of peptide, background impurity, inactivity, and solubility (data not shown). Because of these technical difficulties in assaying E3-mediated SUMO-conjugation with the available recombinant peptides, consumption of small batch CCNB1IP1, and failure of *in vitro* activity from later *large batch* CCNB1IP1 purification (likely due to exceptional solubility problems; fig. 3.4c), I abandoned these efforts and looked to validate the targets of SUMOylation through other means.

<u>A synthetic SUMO E3 ligase posttranslationally modifies interactors of CCNB1IP1.</u>

With the goal of more concretely validating CCNB1IP1 function as an E3 ligase of the SUMO posttranslational pathway, and to examine putative targets of SUMO modification, I devised a method for establishing a synthetic *in vivo*

SUMOylation system. The E3 ligase can broadly be considered as two separate units: the domain to recruit the E2 ligase and the domain to recruit terminal targets for SUMOylation. If these unit functions are separated, one can validate an E3 ligase as functional in the SUMO cascade if its E2-interaction domain (commonly the RING) can *in vivo* interact with UBC9/UBE2I so as to bring into proximity an unquestionable target of SUMOylation. Conversely, targets of SUMOylation, as suspected through their interaction with E3 ligase proteins, can be assayed for their receptivity if they can be recruited into the proximity of the SUMO-loaded E2 ligase though an unquestionably effective SUMO E3 ligase domain.

I employed inducible herterodimerization domains towards building chimeric proteins that can *in vivo* separate the functions of E3 ligase members in the SUMOylation pathway. The iDimerize system implements two expression vectors that allow for expression of genes fused to the coding sequence for the DmrA and DmrC peptides. The DmrA and DmrC domains have poor affinity towards each other. However upon addition of the *A/C heterodimerizer* compound, these two domains form a heterodimer, thus creating a physical linkage between their respectively attached proteins. Employing this system, I was able to separate the function of an E3 RING domain from that of the E3's target specifying role. By fusing the PIASI protein (P-RING) to the DmrC domain, a chimeric E3 can thus recruit an otherwise known target of SUMOylation that has been fused to the DmrA domain, in an inducible manner (fig. 3.5a). The P-RING-DmrC chimeric protein is then used to induce recruitment of suspected targets for SUMO modification that have been fused to the DmrA domain. Should these chimeric proteins fused to DmrA be SUMOylated when recruited to a physical linkage with the E2 ligase in this inducible manner, they will be shown to be dependent upon a SUMO E3-like mechanism and true targets for *in vivo* SUMOylation.

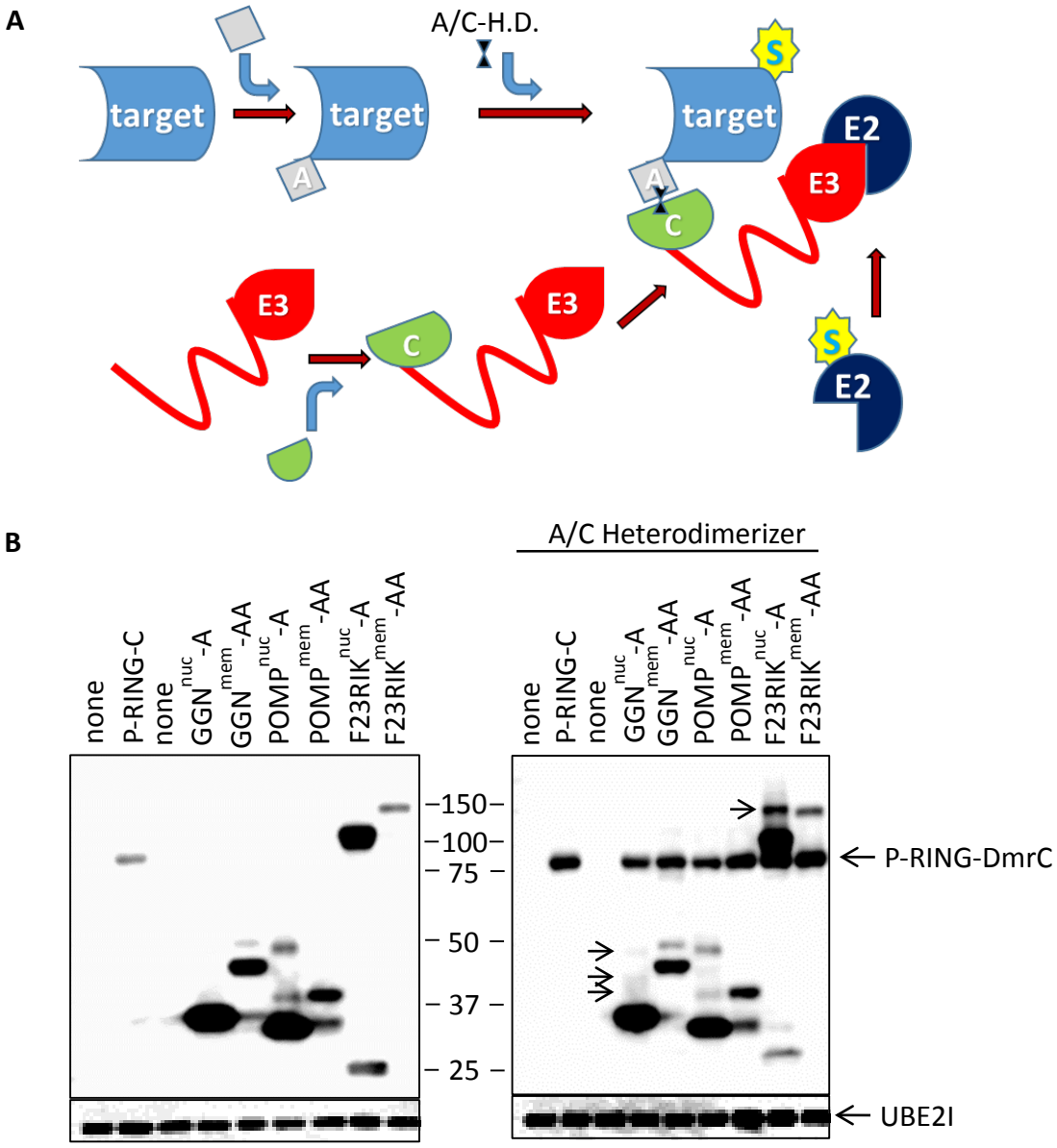

Figure 3.5. iDimerize inducible target specification; a synthetic E3 ligase. (A) Schematic of the iDimerize synthetic P-RING-DmcC mediated sumoylation of a DmrA fused putative target of SUMOylaton. Proteins of interest to be assayed as receptive to SUMOylation are fused to the DmrA peptide (grey square). In the presence of *A/C Heterodimerizer* (A/C-H.D.) the protein of interest is recruited to interact with the DmrC domain (green half circle) that has been fused to the SP-RING domain containing SUMO E3 ligase, creating an inducible recruitment of targets for SUMOylation. (B) Putative targets of SUMOylation, fused to H.D. responsive dimerization domain DmrA, with nuclear or cell membrane directed localization. In response to P-RING interaction induced by A/C-H.D., MW shifting of GGN and 4930455F23Rik (arrows) are observed in Western blots probed for HA-epitope tags.

Taking advantage of the endogenous SUMOylation present in HEK293 cells, and the transient mammalian expression granted by the iDimerize system, I moved to validate a subset of the CCNB1IP1 physical interactors as true *in vivo* targets of SUMO modification. Full-length clones of *Ggn*, *Pomp*, and *4930455F23Rik* were fused to the DmrA domain and expressed chimeric proteins were directed either to a nucleus or cytoplasm, through a nuclear localization signal or membrane interaction motif, respectively. Upon induced A/C-dimerization, recruitment of the target protein of interest to the chimeric P-RING-DmrC, the synthetic E3 ligase should facilitate the posttranslational modification of the receptive target proteins. Indeed, in this synthetic and inducible test for SUMOylation, Western blot analysis of ectopically expressed proteins shows MW shifting of GGN and 4930455F23RIK peptides, which is indicative of posttranslational modification dependent upon physical interaction with the P-RING SUMO E3 ligase domain (fig. 3.5b).

From the development of a synthetic E3-like SUMO ligase and the induction of target interaction with this E3 ligase, I have shown that GGN and 4930455F23RIK are targets for SUMOylation when localized to the nuclear compartment. The fact that GGN and 4930455F23RIK are posttranslational modified in response to induced interaction with the RING domain of PIASI, a known SUMO E3 ligase, is cause to suspect the MW shifting is due to SUMOylation. Furthermore, the fact that they are modified mutually in the nuclear compartment, but not in the cytoplasm, is consistent with the expectation that this MW shifting is not an artifact of the target peptide under these experimental conditions.

Discussion

My previous results had implicated CCNB1IP1 as a putative E3-like ligase involved in the SUMOylation of a subset of protein-protein interactors. The possibility

of identifying a meiotic co-regulator essential for SUMO-conjugation is exciting for both the immediate implication of understanding the function of CCNB1IP1, as well as in the longer term as a first step towards further understanding the role played by SUMOylation in meiotic DSB repair. While very little is presently known about the role of SUMO in meiosis, there are indications that similar to mitotic DNA repair, SUMO-conjugates are essential to DSB repair and directing repair processes.

CCNB1IP1 contains a highly conserved C3H2C3 RING domain, a type of RING domain previously identified as having affinity towards the single SUMO E2 enzyme UBE2I/UBC9. It was unfortunate that *in vivo* studies with CCNB1IP1 are hindered by want for spermatocyte culture systems, and by dense *polycomplex-like* aggregations of CCNB1IP1 when expressed in the somatic culture systems. Some groups have hypothesized that polycomplex structures are indicative of SC-associated proteins expressed in conditions restrictive to SC formation, but it has not yet been formally established. Another possible explanation for the polycomplex structures may be the intertwining of coiled-coil domains amongst individual peptides which are building polymers of coiled-coil proteins. Indeed, polycomplex-forming SYCP1 and SYCP3 are also coiled-coil domain proteins and the polycomplex may reflect that attribute rather than a generalized biochemical principle of SC relationships.

Excitingly, *in vitro* recapitulation of the E3 biochemical activity was able to function in lieu of *in vivo* experiments. Like the *in vivo* attempts, *in vitro* activity is dependent upon natively folded and thus functionally accessed CCNB1IP1. GST-fusions of CCNB1IP1 were used as a means to isolate that small fraction of recombinant CCNB1IP1 not sequestered to aggregates or otherwise unfolded. Reconstituted *in vitro* reactions proficient in SUMO-conjugation revealed high molecular weight shifting of CCNB1IP1 in the presence of SUMO2 peptide. This MW shifting was observed using both human CCNB1IP1 (produced in wheat germ) and

my own produced mouse CCNB1IP1 (produced in *e. coli*), and is consistent with autoSUMOylation by poly-chains of SUMO2. In support of these poly-SUMO2 chain modifications to CCNB1IP1, the high MW shifting was nullified in reactions utilizing a mutated SUMO2 that was deficient for chain formations. AutoSUMOylation is a previously reported behavior of functional SUMO E3 ligase proteins and this result supports our hypothesis that CCNB1IP1 likewise functions in vivo as SUMO E3 ligase.

The second function of E3-type ligases is to interact with terminal targets for posttranslational modifications and recruit them to the E2 enzyme. Technical and reagent limitations restricted my ability to assay effects of CCNB1IP1 upon putative target SUMOylation *in vitro*, and polycomplex formations prevented CCNB1P1 *in vivo* studies so I set towards building a synthetic SUMO E3 ligase though which to inducible test target receptivity to SUMOylation. Using the DmrC dimerization epitope tag, I built a chimeric E3 ligase from the RING domain of PIASI. In the presence of a bridging compound (A/C Heterodimerizer) the DmrC domain interacts with a similar DmrA domain fused to the putative target of SUMOylation. Initial trials with this inducible system succeeded in posttranslational modifications of GGN and 4930455F23RIK as assessed though an accompanying HA epitope tag. GGN and 4930455F23RIK, as previously identified interacting partners with the putative SUMO E3 ligase CCNB1IP1, are thus themselves targets for SUMOylation.

The identifications of GGN and 4930455F23RIK have interesting implications. CCNB1IP1 functions to regulate formation of intermediate Meiotic Nodules nearing terminal stabilization of Class I crossovers in late pachytene. Through the interaction with GGN there appears to be a protein-protein interaction cascade uncovered in that GGN additionally interacts with GGNBP1 (Gametogenetin binding partner 1) [33] and GGNBP1 interacts with the DSB essential factor MEI1 [Bjarte Furnes, unpublished

communication]. This interaction cascade would tie together the essential role MEI1 plays in the initiation of meiosis through DSB formation, through the yet unstudied GGNBP1 role in meiosis, to GGN which has recently been shown to function in meiotic DSB repair where haploinsufficiency results in moderate persistence of RAD51 in pachytene [34], and finally through to the role of CCNB1IP1 in crossover formation. Indeed, due to the moderate persistence of RAD51 in GGN haploinsufficient mice, it may be that the CCNB1IP1-directed modification of GGN is the mediating factor in crossover-dependent meiotic DSB repair. Likewise, it is intriguing to note that due to an internal coiled-coil domain to 4930455F23RIK, it has also received the designation as *Ccdc181* (Coiled-coil domain containing 181). 4930455F23RIK/CCDC181, like CCNB1IP1, is strongly expressed in the testis with transcription significantly increased between 14-18dpp [GEO profiles, NCBI] and may represent another SC-localized protein with functions yet unappreciated.

Taken together, these biochemical studies of CCNB1IP1 support the hypothesis that it functions as a SUMO E3 ligase regulating the posttranslational modifications of proteins necessary towards the formation of crossing-over. As meiotic nodules mature, and sites of DSB repair through crossing-over are processed, protein density surrounding these crossovers increases in accordance with new and more complex protein-protein interactions. From somatic cell DNA repair, we know SUMO-conjugation effects both repair process choice, and fidelity. This leaves us to suspect that CCNB1IP1-dependant SUMO-conjugates are necessary towards the stabilization of meiotic DSB repair via crossing-over, and deficiency in this co-regulation through posttranslational modification by a yet unknown number of SUMO-conjugates accounts for the lack of crossing over in *Ccnb1ip1*$^{mei4/mei4}$ animals.

Materials and Methods

Western blot analysis

Protein concentrations were assessed with BCA kit (Pierce) as per manufacturer instructions. 30µg protein for cell lysates, or total protein quantity from *in vitro* SUMOylation reactions, was separated through SDS-PAGE and electro-transferred to PVDF membrane (PALL Corporation), and probed with designate antibodies per standard protocols. Detection of HRP-conjugated secondary antibody through chemiluminescence (Pierce ECL and EMD Millipore Luminata Forte). Antibodies used: anti-CCNB1IP1 (GenScript: custom), anti-GFP (Millipore: AB3080), anti-DsRed (Clontech: 632496), anti-SUMO2/3 (Cell Signaling: 4971), anti-GST (Abcam: ab92), anti-HA (Sigma: H3663).

Cloning of mCherry-fused CCNB1IP1 alleles

Full length, ΔCT (bp 1-639), and Nterm (bp 1-507) cDNA sequences for mouse *Ccnb1ip1* were cloned into the BamHI and EcoRI sites of pmCherry-N1 (gift from Paula Cohen's laboratory). Vector pmCherry-N1 is derived from pEGFP-N1 in which the 3' EGFP coding sequence has been exchanged for the mCherry fluorescent peptide. Resultant pCcnb1ip1^{mcherry}, pCcnb1ip1Δct^{mcherry}, and pCcnb1ip1Nterm^{mcherry} were sequence verified.

Cloning of GFP-fused targets of SUMOylation

Full length cDNA amplified from the testis for *Ddc8*, *Ypel2*, *1700021F07Rik*, and both the short (s) and long (s) cDNA for *Oaz3* were cloned in pDONR221 (Invitrogen, Cat. No. 12536-017), and moved into pDEST53 (Invitrogen, Cat. No. 12288-015) using the Gateway system. Final products were sequence verified.

Cell transfections

HEK293 and CHO cells were grown in 6 well plates to approximate 50% confluence. Fresh complete DMEM media was added 3 hours prior to transfection with Transit-LT1 reagent per manufacturer's directions (Mirus: MIR 2300). Briefly, 2.5ug of plasmid DNA was transfect to cells in overnight incubation, followed by changing of media 3 hours prior to visualization or lysis.

Recombinant CCNB1IP1

Recombinant GST-fused human Ccnb1ip1 produced though wheat germ expression was purchased from Abnova (Cat. No. H00057820-P01). Custom GST-fused mouse CCNB1IP1 was expressed from pGEX-6P-1 (GE Healthcare, Cat. No. 28-9546-48) using Rosetta(DE3) *e. coli* cells (gift from Fabio Rinaldi). A starter culture was used to inoculate 500mL (*Small batch* production) of LB with antibiotics Chloramphenicol and Ampicillin. Cultures were grown with shaking overnight at room temperature with 50uM IPTG for expression induction. Cells were lysed though sonication and the soluble fraction purified over GST-Bind resin (Novagen, Cat. No. 70541) and washed with buffer (20mM Tris pH 7.5, 300mM NaCl, 5% glycerol). Elution of the purified products using buffer supplemented with 10uM reduced Glutathione. Protein fractions were concentrated through filters at high speed centrifugation (EMD Millipore, UFC203024). Similar technique for 5L (*large batch* production) expression of CCNB1IP1.

Recapitulated in vitro SUMOylation

SUMOylation reaction in vitro performed as per the manufacturer's suggested protocol (Active Motif, Cat. No. 40220). Briefly total 0.5ug of Protein(s) of Interest were used in the standard reaction, incubated at 30 C water bath for 3hrs, followed by quenching of the reaction with addition of 2x SDS loading buffer and boiling of the

samples. Reaction products we resolved though Western blot analysis. Protein(s) of interest we the previously mentioned recombinant CCNB1IP1 and recombinant GST-fused peptides for SPATA3 (Abnova, Cat. No. H00130560-P01), OAZ3 (Abnova, Cat. No. H00051686-P01), POMP (Abnova, Cat. No. H00051371-P01), and GGN (Abnova, Cat. No. H00199720-P01).

iDimerize inducible heterodimerization

Vectors and mammalian cell culture expressions for inducible heterodimerization followed the manufacture's protocol (Clontech, Cat. No. 635067). Full-length clones of cDNA isolated from 18 day old mouse testis for *Pomp*, *Ggn*, and *4930455F23Rik* were inserted into the XbaI site of pHet-Mem1 and pHet-Nuc1. cDNA isolated from human cells coding for the full-length PIASI was inserted into the SpeI site of pHet-1. Expression vectors were co-transfected as previously described at a 3:1 molar ratio of pHet-1:pHet-Mem/Nuc1. 100nM of A/C Heterodimerizer compound was supplemented to induce dimerization 3hrs prior to harvesting of total proteins.

REFERENCES

1) Ward, Jeremy O., et al. "Mutation in mouse hei10, an e3 ubiquitin ligase, disrupts meiotic crossing over." *PLoS genetics* 3.8 (2007): e139.
2) Singh, Mahendra K., et al. "HEI10 negatively regulates cell invasion by inhibiting cyclin B/Cdk1 and other promotility proteins." *Oncogene* 26.33 (2007): 4825-4832.
3) Hassold, Terry, and Patricia Hunt. "To err (meiotically) is human: the genesis of human aneuploidy." *Nature Reviews Genetics* 2.4 (2001): 280-291.
4) Hershko, Avram, and Aaron Ciechanover. "The ubiquitin system." *Annual review of biochemistry* 67.1 (1998): 425-479.
5) Kahyo, Tomoaki, Tamotsu Nishida, and Hideyo Yasuda. "Involvement of PIAS1 in the sumoylation of tumor suppressor p53." *Molecular cell* 8.3 (2001): 713-718.
6) Takahashi, Yoshimitsu, Akio Toh-e, and Yoshiko Kikuchi. "A novel factor required for the SUMO1/Smt3 conjugation of yeast septins." *Gene* 275.2 (2001): 223-231.
7) Potts, Patrick Ryan, and Hongtao Yu. "Human MMS21/NSE2 is a SUMO ligase required for DNA repair." *Molecular and cellular biology* 25.16 (2005): 7021-7032.
8) Johnson, Erica S., and Aseem A. Gupta. "An E3-like factor that promotes SUMO conjugation to the yeast septins." *Cell* 106.6 (2001): 735-744.
9) Guo, Wen-zhi, et al. "Nm23-H1 is responsible for SUMO-2-involved DNA synthesis induction after X-ray irradiation in human cells." *Archives of biochemistry and biophysics* 486.1 (2009): 81-87.
10) Nacerddine, Karim, et al. "The SUMO pathway is essential for nuclear integrity and chromosome segregation in mice." *Developmental cell* 9.6 (2005): 769-779.
11) Zhang, Fu-Ping, et al. "Sumo-1 function is dispensable in normal mouse development." *Molecular and cellular biology* 28.17 (2008): 5381-5390.
12) Sachdev, Shrikesh, et al. "PIASy, a nuclear matrix–associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies." *Genes & development* 15.23 (2001): 3088-3103.
13) Kahyo, Tomoaki, Tamotsu Nishida, and Hideyo Yasuda. "Involvement of PIAS1 in the sumoylation of tumor suppressor p53." *Molecular cell* 8.3 (2001): 713-718.
14) Takahashi, Yoshimitsu, et al. "Yeast Ull1/Siz1 is a novel SUMO1/Smt3 ligase for septin components and functions as an adaptor between conjugating enzyme and substrates." *Journal of Biological Chemistry* 276.52 (2001): 48973-48977.
15) Takahashi, Yoshimitsu, Akio Toh-e, and Yoshiko Kikuchi. "A novel factor required for the SUMO1/Smt3 conjugation of yeast septins." *Gene* 275.2 (2001): 223-231.
16) Serrentino, Maria-Elisabetta, et al. "Differential Association of the Conserved SUMO Ligase Zip3 with Meiotic Double-Strand Break Sites Reveals Regional Variations in the Outcome of Meiotic Recombination." *PLoS genetics* 9.4 (2013): e1003416.

17) La, Salle S., et al. "Developmental control of sumoylation pathway proteins in mouse male germ cells." (2008): 227.
18) Rogers, Richard S., et al. "SUMO modified proteins localize to the XY body of pachytene spermatocytes." *Chromosoma* 113.5 (2004): 233-243.
19) Brown, Petrice W., et al. "Small ubiquitin-related modifier (SUMO)-1, SUMO-2/3 and SUMOylation are involved with centromeric heterochromatin of chromosomes 9 and 1 and proteins of the synaptonemal complex during meiosis in men." *Human reproduction* 23.12 (2008): 2850-2857.
20) Cromie, Gareth A., et al. "Single Holliday junctions are intermediates of meiotic recombination." *Cell* 127.6 (2006): 1167-1178.
21) Jessop, Lea, et al. "Meiotic chromosome synapsis-promoting proteins antagonize the anti-crossover activity of sgs1." *PLoS genetics* 2.9 (2006): e155.
22) Shen, Zhiyuan, et al. "Associations of UBE2I with RAD52, UBL1, p53, and RAD51 proteins in a yeast two-hybrid system." *Genomics* 37.2 (1996): 183-186.
23) Koshiyama, Akiyo, et al. "Sumoylation of a meiosis-specific RecA homolog, Lim15/Dmc1, via interaction with the small ubiquitin-related modifier (SUMO)-conjugating enzyme Ubc9." *Febs Journal* 273.17 (2006): 4003-4012.
24) Tsubouchi, Tomomi, and G. Shirleen Roeder. "A synaptonemal complex protein promotes homology-independent centromere coupling." *Science* 308.5723 (2005): 870-873.
25) Tsubouchi, Tomomi, Amy J. MacQueen, and G. Shirleen Roeder. "Initiation of meiotic chromosome synapsis at centromeres in budding yeast." *Genes & development* 22.22 (2008): 3217-3226.
26) Lin, Feng-Ming, et al. "Yeast axial-element protein, Red1, binds SUMO chains to promote meiotic interhomologue recombination and chromosome synapsis." *The EMBO journal* 29.3 (2009): 586-596.
27) Sym, Mary, and G. Shirleen Roeder. "Zip1-induced changes in synaptonemal complex structure and polycomplex assembly." *The Journal of cell biology*128.4 (1995): 455-466.
28) Yuan, Li, et al. "The synaptonemal complex protein SCP3 can form multistranded, cross-striated fibers in vivo." *The Journal of cell biology* 142.2 (1998): 331-339.
29) de Carvalho, Carlos Egydio, and Mónica P. Colaiácovo. "SUMO-mediated regulation of synaptonemal complex formation during meiosis." *Genes & development* 20.15 (2006): 1986-1992.
30) Öllinger, Rupert, Manfred Alsheimer, and Ricardo Benavente. "Mammalian protein SCP1 forms synaptonemal complex-like structures in the absence of meiotic chromosomes." *Molecular biology of the cell* 16.1 (2005): 212-217.
31) Ohtaka, Ayami, et al. "Meiosis specific coiled-coil proteins in Shizosaccharomyces pombe." *Cell Div* 2 (2007): 14.
32) Goldstein, Paul. "Multiple synaptonemal complexes (polycomplexes): origin, structure and function." *Cell biology international reports* 11.11 (1987): 759-796.

33) Zhang, Jin, et al. "Yeast two-hybrid screens imply that GGNBP1, GGNBP2 and OAZ3 are potential interaction partners of testicular germ cell-specific protein GGN1." *FEBS letters* 579.2 (2005): 559-566.
34) Jamsai, Duangporn, et al. "Loss of GGN Leads to Pre-Implantation Embryonic Lethality and Compromised Male Meiotic DNA Double Strand Break Repair in the Mouse." *PloS one* 8.2 (2013): e56955.

CHAPTER 4

CCNB1IP1 as a meiotic co-regulator in the regulation of non-crossover vs. crossover resolution of recombination intermediates

Abstract

Through largely unappreciated mechanisms, a small fraction of the hundreds of meiotic programed DNA Double Strand Breaks (DSBs) are selected and resolved as sites of genetic crossing-over. The formation of chiasmata, the physical representations of crossovers, are essential to tether homologous chromosomes and ensure accurate homolog segregation as meiocytes complete the first meiotic division. The mechanisms through which these crossover formations are regulated remain unknown, but failure brings about meiotic arrest or deleterious aneuploidy. CCNB1IP1 is a putative regulator of posttranslational modifications by SUMO and deficiency of CCNB1IP1 results in a failure to specify the obligate crossovers. Here I present evidence that CCNB1IP1 acts locally at the sites of maturing crossover intermediates, and failure of CCNB1IP1-mediated regulations results in the persistence of immature crossover intermediates which eventually fail and result in unrepaired DSBs and asynapsis immediately prior to the first meiotic division.

Introduction

Mammalian CCNB1IP1/HEI10 has been shown to act as an E3 ligase specifying target proteins for posttranslational regulation through ubiquitin [1] and is hypothesized to modify meiotic proteins through SUMOylation [2]. CCNB1IP1 expression predominates in the gonad, and animals carrying the *Ccnb1ip1*^{mei4} allele present as both male and female infertile due to meiotic arrest [3]. In the

Ccnb1ip1$^{mei4/mei4}$ spermatocytes, recombination initiates and achieves full synapse of homologous chromosomes but later events specifying sites of crossing-over fail, which are observed though deficiency in the MutLγ complex (MLH1 and MLH3). CCNB1IP1 is an essential factor of meiosis, presumably targeting proteins for posttranslational modification, and a putative molecular regulator in the coordination of factors involved in the maturation of recombinogenic DNA repair.

The specification of meiotic crossing-over is essential for the accurate segregation of homologous chromosomes at the first meiotic division. Dense proteinaceous structures, called Meiotic Nodules (MNs), form at the sites of selective meiotic DSBs and mature down to a few locations as the MNs grow in size and density to be ultimately resolved as reciprocal crossovers [4,5]. The vast majority of early observed MNs are resolved as gene conversion events of DNA repair but it is still unclear which enzymes are involved in selection of that subset which progresses to mature MNs as sites of DNA damage selected for crossover repair resolution. While the molecular basis of these MN structures are uncertain, studies in yeast have shown the sites of these Class I crossovers are at least partially populated by the meiosis-specific ZMM proteins, Zip1/2/3/4, MutL heterodimer (Msh4/5), and Mer3. *Ccnb1ip1*$^{mei4/mei4}$ failure to form this reciprocal Class I crossing-over may indicate deficiency to mature sites selected for crossing-over or an earlier failure to switch from gene conversion repair to reciprocal recombination.

Although there are many open questions regarding the signaling and enzymes involved in meiotic DSB repair switching between gene conversion and reciprocal crossing-over, looking towards somatic cell repair of DSBs gives some insight into the role SUMOylation might play. In somatic cells insulted by DSBs, both ubiquitin and SUMO are found directly at the sites of DSBs [6,7]. In particular, all three major SUMO isoforms (SUMO1/2/3) are observed immediately following DSB insults [7,8].

The coincident localization of SUMO1/2/3 may be further indication of their putative functional redundancy, and their recruitment to DSBs is consistent with observations that SUMO-conjugation can act as a molecular scaffold from which to promote protein-protein interactions and thus alter functions or recruit additional factors.

As MNs mature and grow in complexity towards resolving as sites of crossing-over, the MutS homologs MSH4 and MSH5 colocalize and function to facilitate homology-driven alignment, synapse and resolution as crossing-over. The MSH4-MSH5 heterodimer stabilizes D-loop intermediates and the maturation of their sites of localization coincides with those sites selected towards crossing-over. Failure in crossover intermediate maturation results in failure of MSH4-MSH5 dependent recruitment of the MLH1-MLH3 dimer and thus failure in crossover mediated repair of DSBs. The phosphorylation of the histone variant H2AX acts as an indirect marker of DSB damage. Phosphorylation of ɣH2AX is readily observed at the sites of DNA damage and persistence of ɣH2AX is indicative of failure to repair meiotic DSBs.

With the observations of SUMO-conjugates localized to mitotic DSB repair as well as meiotic bodies in the human, it is not surprising that closer examination of spermatocytes in the mouse has revealed similar patterns of localizations of SUMO. Detailed examination of surface-spread meiotic nuclei has revealed SUMO1 and SUMO2/3 localizations to the heterochromatic- XY body and chromocenters of pachytene and diplotene spermatocytes, with SUMO2/3 remaining detected at centromeres of metaphase I [9]. Subsequent studies in mouse spermatocytes finely timed SUMO1 localization to precede ɣH2AX localization to the XY in zygotene (SUMO2/3 was not observed in zygotene) suggest a role for SUMO in Meiotic Sex Chromosome Inactivation (MSCI) or possibly the wider Meiotic Silencing of Unsynapsed Chromatin (MSUC) [10]. Further evidence of intersecting roles of SUMOylation in meiosis localized the conjugate to sites of DSBs in leptotene and

zygotene spermatocytes, similar to the patterns of γH2AX and the DNA repair protein BRCA1 [11]. In agreement with the observed localization of SUMO to sites of genomic insult, proteomic efforts to identify SUMO-conjugates have built an "integrated SUMO functional map" in which DDR and chromatin remodeling are the top functional categories for SUMO-conjugates [12].

Ccnb1ip1$^{mei4/mei4}$ was identified in a genetic screen for infertility mutants [13]. Animals homozygous for this allele display meiotic failure in diplotene, as homologous chromosomes fail to remain associated due to lack of physical tethering from reciprocal crossing-over [3]. While originally identified as specific to chordates, subsequent genetic studies of the genes responsible for reproduction in other organisms have identified distant homologues of *Ccnb1ip1/Hei10* in *Arabidopsis* and *Japonica* [14,15]. These studies in plant species seemingly confirm a role for CCNB1IP1/HEI10 in DSB repair fate specification through crossing-over; HEI10 foci localize prior to synapsis but after axial formation, and HEI10 localization eventually decreases to a few foci co-localizing with MLH1 at chiasmatic sites [14,15]. The co-localization of HEI10 and MLH1, together with quantitative analysis of residual crossovers in *hei10*-/- rice, supports the previously reported phenotypes in mouse and CCNB1IP1's essential role in specifying interference-sensitive class I crossing-over [15,14,3]. Genomic analysis of *hei10* in the plant concludes that it is the ortholog of the well-studied yeast SUMO E3 ligase SC-promoting protein Zip3 [14,15]. This genomic analysis leads the authors to hypothesize that *Cccnb1ip1/Hei10* is a member of the *Zip3* family of E3 ligases, including *Rnf212* and *Mer2*. In further support of this Zip3 protein family, studies in c. elegans have shown early ZHP-3/Zip3 localization along the length of the chromosomes is processed down to a few foci marking the sites of crossover recombination, reminiscent of the HEI10 patterns in plants and RNF212 in the mouse [16]. Furthermore, these ZHP-3 isoforms in the worm are able to

properly form crossing-over but fail to resolve and ensure faithful segregation of homologs in MI, a phenotype similar to SMO-1/SUMO mutants in that organism. This is further evidence of the dependent-relationship of SUMO E3s, SUMOylation, and crossing-over [17] A Zip3 family of E3 ligases presents interesting prospects for identifying the meiotic co-regulators responsible for SUMOylation signals observed at DSBs, SCs, and sites of crossing over.

Results

CCNB1IP1 dynamics and localization in Prophase I of meiosis.

Having interrogated the biochemical activity of CCNB1IP1 and observed its characteristic to form polycomplex-like structures and the genetic defect to specify sites of crossing over in *Ccnb1ip1*$^{mei4/mei4}$ animals, I suspected CCNB1IP1 would localize to chromosome cores of Prophase I. Having previously been reported essential to the formation of meiotic crossing-cover [3], I was curious to observe if CCNB1IP1 would localize the terminal sites of crossing-over or if localization might reflect a more intermediate role in maturation of DSB repair through crossover. Localization of CCNB1IP1 to the synaptonemal complex itself would confirm that the protein is *in vivo* recruited to the same domains as UBE2I/UBC9, and may play a direct role in stabilizing components of the meiotic nodules though SUMO attachments, presumably facilitating complex protein-protein interactions by offering an additional surface through which to build protein complexes upon.

To obtain fine resolution localization of fixed proteins, I examined surface-spread spermatocyte nuclei. The fixed cells were disassociated, settled onto slides, and hypotonic swelling affords for separation of chromosomes for fine resolution immune-based localization of proteins of interest. Antibodies generated to recognize CCNB1IP1 were used to probe against these chromosome spreads, and fluorescent

conjugated secondary antibodies were used to visualize the localization of CCNB1IP1. In agreement with my expectation of DNA- or SC-directed localization, CCNB1IP1 is observed to form discrete foci along the chromosome cores (fig 4.1). These foci are most numerous during pachytene as chromosomes have paired and remaining DNA damage is being specified for class I vs. class II repair. As meiosis progresses and the final sites for crossing-over are specified, the dynamics of CCNB1IP1 foci localization are observed to coordinately decrease, putatively in accordance with sites of class I crossing over. The dynamics of CCNB1IP1 localization from many foci during pachytene, to relatively few by late pachytene, is reminiscent to the dynamics of meiotic nodule maturation as they select towards and recruit additional factors towards stabilization of intermediates and final formation of crossovers. A role for CCNB1IP1 in maintaining meiotic nodule progression and specification of crossing over would be consistent with the failure to form crossovers in the *Ccnb1ip1*$^{mei4/mei4}$ animals.

While it is known that SUMO-conjugates function in DSB repair of somatic cells, it has still not been determined what roles SUMOylation might play in meiotic DSB repair and crossing over. Localization of a putative SUMO E3 ligase directly to SCs and a role in maturation of MNs offers significant implications towards understanding this role of meiotic-specific SUMO-conjugates at DSBs of meiocytes.

Interrogation of meiotic progression in Ccnb1ip1$^{mei4/mei4}$ spermatocytes.

Prophase I of meiosis represents a complex series of events in which the dominant events include the initiation of recombination through double strand break formation, chromosome pairing through DNA-directed homology search, chromosome condensation and attachment through both DNA interaction and the proteinaceous synaptonemal complex, progressive DNA repair, and finally faithful separation of homologous chromosomes along the metaphase plate facilitated by resolution of the

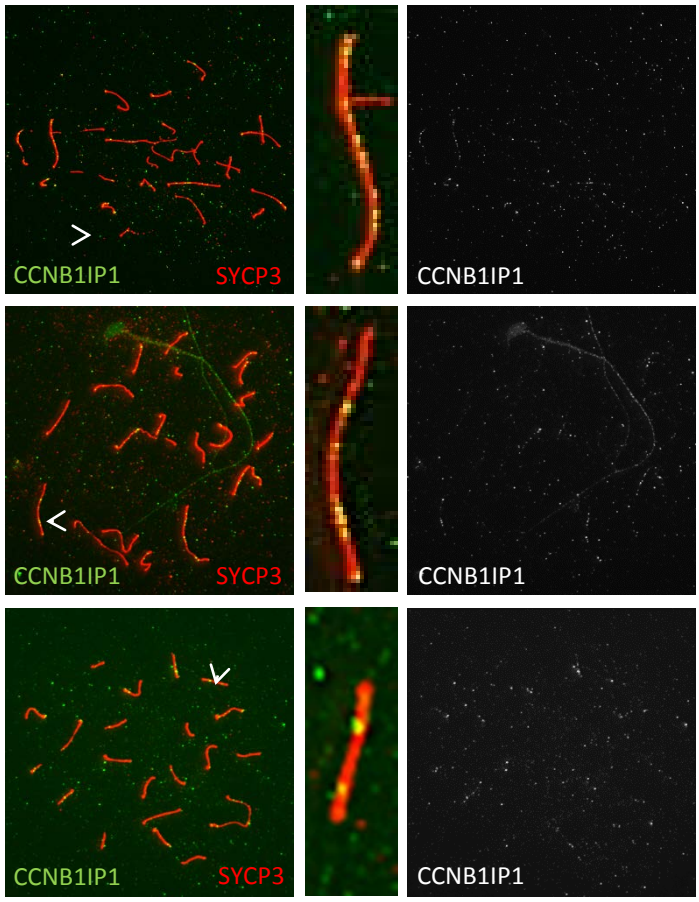

Figure 4.1. CCNB1IP1 localizes to the Synaptonemal Complex of pachytene spermatocytes.
Immunolocalization of CCNB1IP1 to the Synaptonemal Complex cores of spermatocytes during Pachynema. Numerous punctate foci are observed dispersed across chromosome cores. CCNB1IP1 localization decreases in mid- to late-pachytene to a few residual foci per SC core, presumably marking the sites specified as crossovers. (Arrowheads indicate chromosome enlarged for higher resolution)

crossover which acts as the final physical tether between homologues. It has previously been reported that while meiosis fails in the *Ccnb1ip1*$^{mei4/mei4}$ animals, the initial events of initiation, pairing, condensation, attachment and SC formation appear normal. It is at the final events of homologous chromosome segregation facilitated through crossing-over at which failure is observed; MLH1 and MLH3 fail to localize in the effected spermatocytes, indicating a failure to fully mature meiotic nodules and specify final sites of crossing-over.

Because I believe CCNB1IP1 to be a SUMO E3 ligase and these results show its localization to foci on the SCs, putative sites of crossover intermediates, I was initially curious if a defect in CCNB1IP1 would have any grossly observable deficiency in SUMO-conjugate localizations during pachytene. In order to understand the *Ccnb1ip1*$^{mei4/mei4}$ failed progression in intervening events between synapse and final formation of sites of crossing-over, immunolocalization was deployed to assess the proper localization or dysfunction of known proteins involved in DNA damage repair and maturation of meiotic nodules.

I was interested to understand the localization of the previously reported SUMO-modified proteins in spermatocytes [9]. While it is unlikely that CCNB1IP1 represents the dominant SUMO E3 ligase functioning though meiosis, due to the total defect to specify obligate crossing-over it is possible that CCNB1IP1 specifies a major contribution of the SUMO proteome during Prophase I. Antibodies specific to SUMO1 and SUMO2/3 were used as probes against meiotic chromosome spreads as previously described. The gross-localization patterns of SUMO isoforms did not vary from WT and mutant spermatocytes (fig 4.2). SUMO1 was seen to localize to the heterochromatic regions of the Sex Body and centromeric regions of synapsed chromosomes while the localization of SUMO2/3 was likewise observed at the Sex Body and centromeric domains. Noting that gross defects in SUMOylation were not

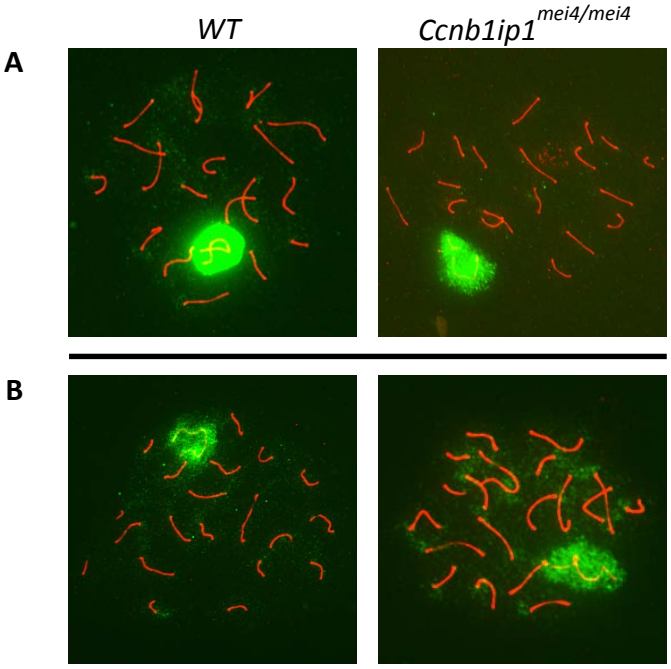

Figure 4.2. Gross SUMOylation remains unperturbed in *Ccnb1ip1*$^{mei4/mei4}$ spermatocytes.
Gross levels of (A) SUMO1 and (B) SUMO2/3 conjugates appear unperturbed in pachytene spermatocytes lacking CCNB1IP1. Major localizations of SUMO-conjugates appear at the heterochromatic regions of the Sex Body and pericentromeric regions. Together with the previously observed CCNB1IP1 localization to foci of similarly staged spermatocytes suggests a directed, rather than global, function of CCNB1IP1-mediated conjugation.

apparent in the affected spermatocytes, it is reasonable to understand that CCNB1IP1 likely targets only a subset of the SUMO-modified proteome and may not be apparent against the otherwise normally modified proteome.

Since the role of crossing-over, and recombination more generally, is to repair the SPO11 catalyzed DNA damage necessary for the homology search, I moved to see if large amounts of DNA damage (as marked by phosphorylated-H2AX; ɣH2AX) remained in the mutant spermatocytes. The major defect resultant from *Ccnb1ip1*$^{mei4/mei4}$ is lack of class I crossovers, and thus it is presumed that the majority of DNA damage is repaired through the dominant gene conversion mechanism. In agreement with prior knowledge, I did not observe large amounts of global ɣH2AX localization. However, pachytene spermatocytes do reveal low level of residual ɣH2AX in domains along chromosome cores (fig. 4.3a). The low signal level at domains along chromosomes of *Ccnb1ip1*$^{mei4/mei4}$ spermatocytes is reflective of failure to repair residual DSBs and it agrees with failure to repair DSBs through crossing over. The particular defect of CCNB1IP1 localization to meiotic nodules does not appear to have an effect on the global process of DNA damage response, but rather remains localized to those meiotic nodule sites that would otherwise be destined as crossovers.

The MHS4-5 complex plays an essential role in meiotic nodule maturation. Understanding that the defect of *Ccnb1ip1*$^{mei4/mei4}$ is presumably finely attenuated to the maturation of meiotic nodules into sites of crossing-over, I examined the localization dynamics of MSH4 as these nodules mature. In WT meiocytes, MSH4 foci will mark intermediately maturing nodules which are progressively selected from approximately 100 sites to roughly 30-35 meiotic nodules that will be terminally marked by MSH4 and later displaced by MLH1/3 at final sites of crossing over. In large part, the constituents of these growing meiotic nodules remain unknown, as do

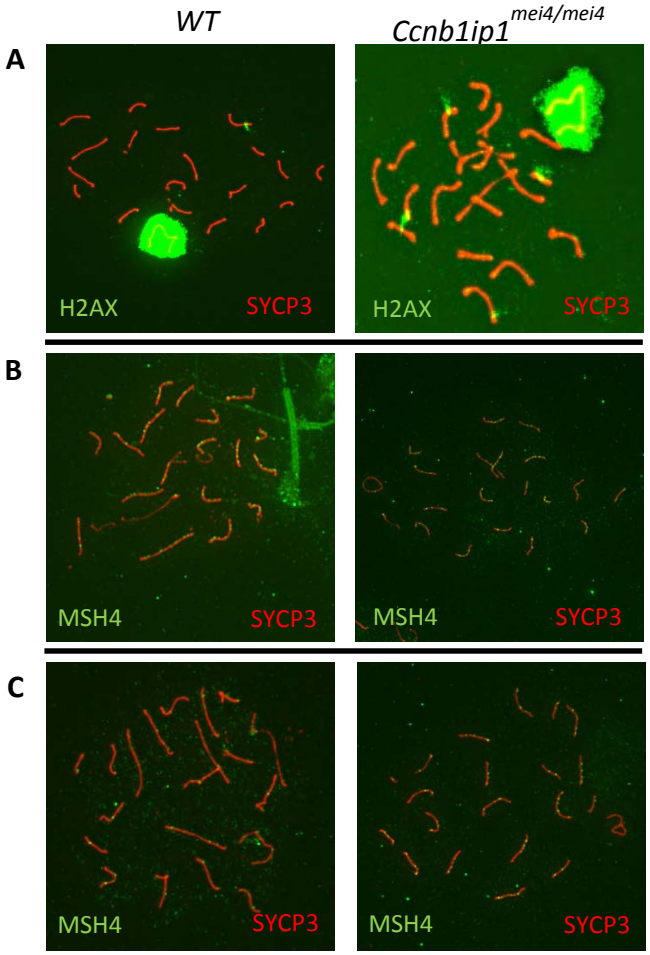

Figure 4.3. Repair of Double Strand Breaks is impaired in *Ccnb1ip1*$^{mei4/mei4}$ spermatocytes.
(A) Low levels of residual ɣH2AX, a mark of unrepaired DSBs, are seen in Pachynema *Ccnb1ip1*$^{mei4/mei4}$ spermatocytes. The low level of residual DSBs reflects that the vast majority of crossover-independent DSB repair is unperturbed in these spermatocytes defective for crossover-dependent DSB repair. DSBs are progressively matured (B; late zygotene/early pachytene) towards sites of repair through crossing-over (C; mid/late pachytene) through stabilization of the MSH4-MSH5 heterodimer. *Ccnb1ip1*$^{mei4/mei4}$ spermatocytes display retained MSH4 localization in mid/late pachytene (C), reflective of regulation failure to mature meiotic nodules towards chiasmata.

the mechanisms that drive their selection, maturation, and processing of crossover choice. Using antibodies directed at MSH4, I am able to identify defect in the maturation of these meiotic nodules in the nuclei of *Ccnb1ip1*$^{mei4/mei4}$ spermatocytes (fig. 4.3b,c). By onset of late pachytene MSH4 localization of WT spermatocytes matures down to those few foci marking late MNs and soon-to-be displaced by MLH1/3 as final crossing over is specified. In the *Ccnb1ip1*$^{mei4/mei4}$ spermatocytes, many MSH4 foci are retained towards late pachytene. These results indicate failure to mature MNs and designate DSB repair towards repair through crossing over.

Observing in vivo expression of CCNB1IP1 using fluorescent tags

Having established CCNB1IP1 localization to SC cores of Prophase, the dynamics of its processing at sites of meiotic nodule maturating, and the dysfunction in localization of essential crossing-over intermediaries such as MSH4, MLH1, and MLH3 I wished to visualize CCNB1IP1 dynamics in the live animal, *in vivo*. The dynamic localization of CCNB1IP1 during Prophase I at presumed sites of crossover fate decisions made me suspect CCNB1IP1 would provide a beautiful *in vivo* protein marker through which to assess faithful progression of Prophase I. Additionally, my previous two-hybrid interaction and *in vitro* studies indicating a role for CCNB1IP1 in SUMOylation essential for maturation of MNs drew my interest in providing an epitope-tagged transgene through which to identify SUMO-conjugates unknown in the MNs. Such an *in vivo* tool would allow for further precise study in totality of CCNB1IP1, SUMO-conjugates, as well as a marker to monitor progression of DNA repair though crossing-over in other mutant animals defective for crossing-over.

Towards developing such a tool for *in vivo* monitoring CCNB1IP1 dynamics, and general marking of formation of meiotic crossing over, a transgenic mouse strategy was developed. Using the constructs previously implemented for expression

in the HEK293 and CHO cells of tissue culture, the CMV promoter driving expression of CCNB1IP1^{mCherry} was replaced with the previously characterized *Tcp10b* promoter [18] (fig. 4.4). TCP10 is restrictively expressed in developing spermatocytes from the onset of Pachynema. The expression driven by the promoter of *Tcp10* closely mimics that of endogenous CCNB1IP1 and thus offered an ideal driver for the chimeric *Tcp10-Ccnb1ip1*^{mCherry} transgene.

ESCs were clonally selected (fig 4.4b) and chimeric animals were set to breeding. The F1 generation animals would be heterozygous for allele and *Tcp10-Ccnb1ip1*^{mCherry} should express the fluorescent tag from the transgene. Unfortunately, in all ESC-derived animals, there was failure to detect any level of CCNB1IP1^{mCherry}. Families of transgenic animals were also derived from random integration of this similarly derived construct though the methods of pronuclear injection of linearized DNA (fig 4.4c). These injections yielded two pronuclear-derived transgenic founders for *Tcp10-Ccnb1ip1*^{mCherry} (fig 4.4d). However, as before, progeny from these *Tcp10-Ccnb1ip1*^{mCherry} founders failed to express the chimeric protein.

Development of a fluorescent marker through which to live monitor *in vivo* specification of sites of crossing-over would be a very useful tool. It is a project worthy of pursuit, but unfortunately was not successful in this case. Additionally, the epitope-tagged chimeric protein of CCNB1IP1^{mCherry} would have offered a clean mechanism through which to purify, *in vivo*, CCNB1IP1 and the proteins through which it acts upon in the spermatocyte. Although this transgenic approach to monitoring and identifying constituents of the MNs and those proteins involved in specification of sites for obligate crossing-over were not pursued further, It should continue to be considered for future efforts, as this would provide a valuable molecular tool.

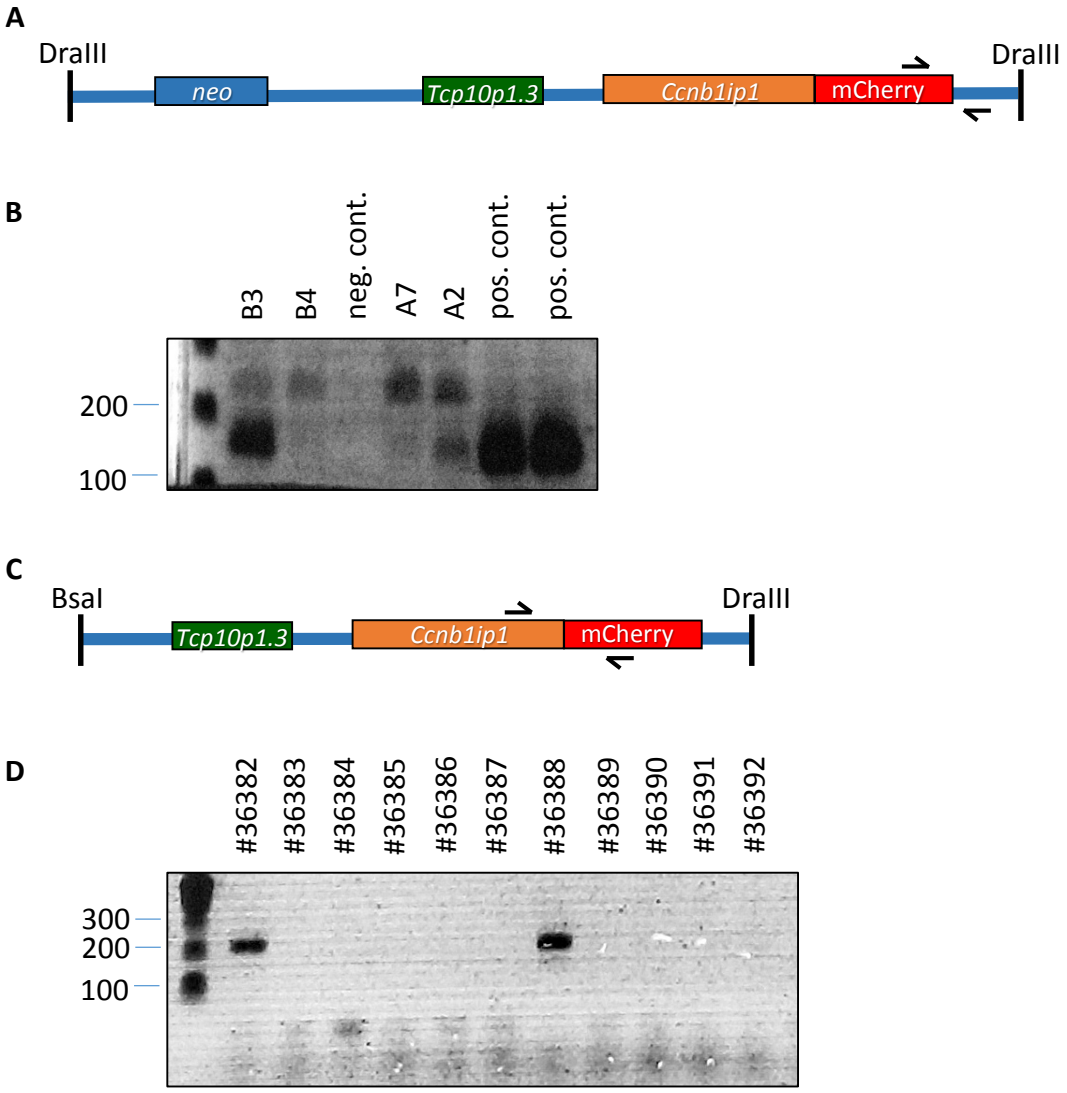

Figure 4.4. Transgenic animals carrying CCNB1IP1 marked with the mCherry fluorescent conjugate.
(A) ESCs were electroporated with DraIII linearized plasmid of Tcp10-*Ccnb1ip1*^{mCherry} under regulation of the pachytene specific promoter from *Tcp10* (arrows indicate approximate location of genotyping primers). (B) Three clonal lines of ESCs (B3, A7, A2) were identified as candidates for blastocyst injections. (C) The allele used for ESC selection, panel A, was liberated of the neo gene through excision of the DraIII-BsaI fragment prior to injection to pronuclear staged embryos (allele-specific genotyping primer indicated by arrows). (D) Two founder animals derived from pronuclear injections of Tcp10-*Ccnb1ip1*^{mCherry}.

Discussion

In these studies, I set forth to understand the localized sites of CCNB1IP1 function. Previous results had uncovered failure to establish obligate crossing-over, pointed towards SC-association, and supported the hypothesis of CCNB1IP1 a meiotic co-regulator designating proteins for posttranslational modification by SUMO. As a primary concern, I observed CCNB1IP1 associates with the SC cores of pachytene spermatocytes. CCNB1IP1 localization dynamics are consistent with those observed for the maturation of MNs and the selection of DSB repair towards crossover vs. alternate non-crossover fates. The persistent few CCNB1IP1 foci in late pachytene spermatocytes suggests a role for CCNB1IP1 in terminal stabilization of crossing-over intermediates. That CCNB1IP1 is neither a putative clamp to stabilize nucleic acid structures nor a catalytic enzyme implicated in processivity of crossing-over is exciting. Rather, CCNB1IP1 presents as a co-regulator acting directly at the sites of crossing-over, from which multiple proteins and pathways are synchronized. Thus CCNB1IP1 dynamically localized to maturing MNs offers entrance into further appreciation of the numerous yet unknown factors involved in stabilization of the obligate crossovers and the unappreciated role SUMO-conjugates may play in meiotic DSB repair.

Significantly, I have observed that the dominant SUMO-conjugates in spermatocytes remain unperturbed in *Ccnb1ip1*$^{mei4/mei4}$ animals. The bulk of the SUMO-proteome in spermatocytes localizes to heterochromatic regions of the Sex Body and centromeres. These heterochromatic SUMO-conjugates appear unaltered in the absence of the CCNB1IP1 SUMO E3 ligase function. This lack of global SUMO alterations is in agreement with the presumed function of CCNB1IP1 as a regulator at those small fractions of DSBs to be repaired through crossing-over. Such a localized and specific effect upon crossing-over is seemingly internally consistent with the lack

in crossing-over, the directed CCNB1IP1 localization putative maturing sites of crossing-over, and lack of a global alteration of the SUMO-proteome. Presumably with sufficiently strong reagents and detection sensitivity, the fraction of the SUMO-proteome altered within sites maturing towards crossing-over would be detected in the *Ccnb1ip1*$^{mei4/mei4}$ spermatocytes.

The failure to stabilize and mature intermediates towards completion of crossover-mediated DSB repair would be expected to result in lingering DNA damage. Such damage would be sequestered within residual ɣH2AX domains. Fitting with this suspicion, small regions of faintly persistent ɣH2AX are detected in the *Ccnb1ip1*$^{mei4/mei4}$ spermatocytes. This unrepaired DNA damage is further proof that the failing spermatocytes lack direction towards final maturation of crossovers. Concurrently to the persistent DNA damage, the failure to form terminally specified crossing-over is reflected in persistence of MSH4 localization late pachytene spermatocytes. MSH4 dynamic localization is a mark of intermediate processing of sites selected towards the few residual repaired though crossing-over. The failure of *Ccnb1ip1*$^{mei4/mei4}$ effected spermatocytes to clear the majority of MSH4-localized intermediates reflects the defect in late stage terminal specifications of crossing-over as regulated by CCNB1IP1 localized action.

Taken together, these results localizing CCNB1IP1 site of function to those specific domains selecting towards the obligate crossovers is quite exciting. Fitting to the originally observed phenotype presenting singular defect in crossover specification, these fine detail molecular phenotyping reveal CCNB1IP1 as a poignantly directed regulator at the sites of crossover. CCNB1IP1 localizes to the discrete foci presumably marking crossover intermediates, readily observed domains of dense SUMO-conjugates are unperturbed by CCNB1IP1 deficiency, and residual DNA damage is congruent with failure to further process MSH4 stabilized

intermediates which thusly go unrepaired. Knowing that CCNB1IP1 does not act directly to resolve intermediates, but rather presents and a posttranslational regulator and point of convergence for coordination of the intricacies involved in crossover maturation, offers CCNB1IP1 as an important candidate to monitor and build towards understanding the finer details of crossing-over.

Materials and Methods

Surface spread meiotic nuclei

Testis from adult or 21 day old mice were dissociated in PBS so to liberate the spermatocyte cells from tubules. The washed cellular fraction was suspended hypotonic solution (1.125g Sucrose, 25mL water) to suitable density. Cells were added drop-wise to poly-lysine coated slides (Fisher Sci. Cat. No. 22-034-979), and incubated at room temperature for 1hr. Two drops of 0.05% TritonX-11 were added to settled spermatocyte slides, incubated for 10mins at room temperature, followed by eight drops of 2% paraformaldehyde/0.02% SDS solution with incubation for an additional 1 hour. Fresh slides washed in PBS were then used for immunolocalization studies.

Immunolocalization of proteins in meiotic nuclei

Surface spread nuclei were blocked with 5% donkey serum in PBST (o.1% Tween20 in PBS) followed by incubation with primary antibodies at 4 C, overnight. Slides were washed three times in PBST, drained and incubated for 1hr with a 1:1000 dilution of Alexa Fluor conjugated secondary antibodies (Invitrogen). Washed slides were mounted and DAPI stained with ProLong Gold Antifade (Invitrogen, Cat. No. P36935). Primary antibodies were; anti-HEI10/CCNB1IP1 (abcam, Cat. No. ab118999), anti-SYCP3 (abcam, Cat. No. ab97672 or Cat. No. ab15093), anti-MSH4

(abcam, Cat. No. ab58666), anti-SUMO1 (Cell Signal, Cat. No. 4931), anti-SUMO2/3 (Cell Signal, Cat. No. 4971), anti-ɣH2AX (Upstate, Cat. No. 07-164).

Generation of the TCP10-driven Ccnb1ip1^{mCherry} transgene

The *Ccnb1ip1^{mCherry}* transgene under promoter control of the *Tcp10b* gene was derived from the previously described pCcnb1ip1^{mcherry}. The CMV promoter from pCcnb1ip1^{mcherry} was excised at the AseI and NheI sites and replaced by the 1.3kb promoter fragment from pTcp1.3-Lac-SV, generating the pTcp10-Ccnb1ip1^{mCherry} expression vector.

Selection and screening of ESCs for transgene

Linearized pTcp10-Ccnb1ip1^{mCherry} was electroporated to ESCs and selected for integration through neomycin resistance. Clones were screened for integration with primers spanning the 3' terminus of pTcp10-Ccnb1ip1^{mCherry} (forward primer within mCherry: CACCATCGTGGAACAGTACG; reverse primer in vector backbone: TCAGGTTCAGGGGGAGGT). ESCs integrating the 5' neo and full *Tcp10-Ccnb1ip1^{mCherry}* transgene would amplify the 3' PCR product of 155bp.

Transgenic animals through pronuclear injection

The *Tcp10-Ccnb1ip1^{mCherry}* transgene for pronuclear injection was produced from pTcp10-Ccnb1ip1^{mCherry} linearized by digestion at the DraIII and BsaI sites, excising out the neo-resistance cassette. This linearized DNA was injected into pronuclear staged embryos and founder animals were genotyped across the allele specific *Ccnb1ip1-mCherry* junction (forward primer within *Ccnb1ip1*: GGAAATCAAGGTGGTGGAGA; reverse primer within *mCherry*:

AAGCGCATGAACTCCTTGAT). The allele specific PCR predicts a 252bp product from animals carrying the *Tcp10-Ccnb1ip1*^{mCherry} transgene.

Western blot analysis

Protein concentrations were assessed with BCA kit (Pierce) as per manufacturer instructions. 30µg protein for cell lysates, or total protein quantity from immunoprecipitations and *in vitro* SUMOylation reactions, was separated through SDS-PAGE and electro-transferred to PVDF membrane, and probed with designate antibodies per standard protocols. Detection of HRP-conjugated secondary antibody through chemiluminescence (Pierce ECL and EMD Millipore Luminata Forte). Antibodies used: anti-DsRed (Clontech: 632496), anti-CCNB1IP1 (GenScript: custom).

REFERENCES

1) Toby, Garabet G., et al. "A novel RING finger protein, human enhancer of invasion 10, alters mitotic progression through regulation of cyclin B levels." *Molecular and cellular biology* 23.6 (2003): 2109-2122.
2) Strong, Edward R., and John C. Schimenti. "Evidence Implicating CCNB1IP1, a RING Domain-Containing Protein Required for Meiotic Crossing Over in Mice, as an E3 SUMO Ligase." *Genes* 1.3 (2010): 440-451.
3) Ward, Jeremy O., et al. "Mutation in mouse hei10, an e3 ubiquitin ligase, disrupts meiotic crossing over." *PLoS genetics* 3.8 (2007): e139.
4) Plug, Annemieke W., et al. "Changes in protein composition of meiotic nodules during mammalian meiosis." *Journal of Cell Science* 111.4 (1998): 413-423.
5) Moens, Peter B., et al. "The time course and chromosomal localization of recombination-related proteins at meiosis in the mouse are compatible with models that can resolve the early DNA-DNA interactions without reciprocal recombination." *Journal of cell science* 115.8 (2002): 1611-1622.
6) Morris, Joanna R., et al. "The SUMO modification pathway is involved in the BRCA1 response to genotoxic stress." *Nature* 462.7275 (2009): 886-890.
7) Galanty, Yaron, et al. "Mammalian SUMO E3-ligases PIAS1 and PIAS4 promote responses to DNA double-strand breaks." *Nature* 462.7275 (2009): 935-939.
8) Hu, Xin, Atanu Paul, and Bin Wang. "Rap80 protein recruitment to DNA double-strand breaks requires binding to both small ubiquitin-like modifier (SUMO) and ubiquitin conjugates." *Journal of Biological Chemistry* 287.30 (2012): 25510-25519.
9) La Salle, Sophie, et al. "Developmental control of sumoylation pathway proteins in mouse male germ cells." *Developmental biology* 321.1 (2008): 227-237.
10) Vigodner, Margarita. "Sumoylation precedes accumulation of phosphorylated H2AX on sex chromosomes during their meiotic inactivation." *Chromosome Research* 17.1 (2009): 37-45.
11) Shrivastava, Vibha, et al. "SUMO proteins are involved in the stress response during spermatogenesis and are localized to DNA double-strand breaks in germ cells." *Reproduction* 139.6 (2010): 999-1010.
12) Makhnevych, Taras, et al. "Global map of SUMO function revealed by protein-protein interaction and genetic networks." *Molecular cell* 33.1 (2009): 124-135.
13) Ward, Jeremy O., et al. "Toward the genetics of mammalian reproduction: induction and mapping of gametogenesis mutants in mice." *Biology of reproduction* 69.5 (2003): 1615-1625.
14) Wang, Kejian, et al. "The role of rice HEI10 in the formation of meiotic crossovers." *PLoS genetics* 8.7 (2012): e1002809.
15) Chelysheva, Liudmila, et al. "The Arabidopsis HEI10 is a new ZMM protein related to Zip3." *PLoS genetics* 8.7 (2012): e1002799.
16) Reynolds, April, et al. "RNF212 is a dosage-sensitive regulator of crossing-over during mammalian meiosis." *Nature genetics* 45.3 (2013): 269-278.

17) Bhalla, Needhi, et al. "ZHP-3 acts at crossovers to couple meiotic recombination with synaptonemal complex disassembly and bivalent formation in C. elegans." *PLoS genetics* 4.10 (2008): e1000235.
18) Ewulonu, U. Kevin, et al. "Promoter mapping of the mouse Tcp-10bt gene in transgenic mice identifies essential male germ cell regulatory sequences." *Molecular reproduction and development* 43.3 (1996): 290-297.

CHAPTER 5

Summary of findings

In this thesis I have set forth to further our understanding of the defect causal to the infertility of the *mei4* animals isolated in a forward genetic screen aimed at uncovering and understanding the genetics governing mammalian meiosis. The original identification of *mei4* discovered a lack to specify obligate crossovers, resulting in meiotic arrest of otherwise phenotypically normal animals. The genetic defect in *mei4* was mapped to *Ccnb1ip1*, a gene with unknown functions within the gonads of both sexes. It was my goal to understand the function of CCNB1IP1, and how the defect in *Ccnb1ip1*$^{mei4/mei4}$ animals leads to meiotic arrest.

In agreement with the meiotic specific phenotype, and defect in the pachytene/diplotene transition to form sites of crossing-over, Western blot mediated expression analysis of the mouse testis found CCNB1IP1 restricted to the gonad, and detectable as the first wave of spermatogenesis enters pachynema when crossover intermediates are progressively stabilized and matured towards the obligate crossover. While the *Cccnb1ip1*$^{mei4/mei4}$ animals are infertile, they do produce some low level of a truncated CCNB1IP1^{mei4}. These characterizations agree with *Ccnb1ip1* as essential to meiosis and the locus of the causal *mei4* mutation.

Although the *mei4* animals were otherwise normal in all tissues other than the testis, my original work toward discovering informative protein-protein interactions with CCNB1IP1/HEI10 was predicated on previous studies of CCNB1IP1/HEI10 function in somatic cells; CCNB1IP1 had been implicated as an ubiquitin E3 ligase regulating cell cycle through directed degradation of cyclin B. Informed by those previous studies, I replicated the two-hybrid screen while using a more complete

fragment of the mouse CCNB1IP1 in conjunction with a two-hybrid “prey” library from the adult testis, to discover those protein-protein interactions reflective the tissue expression analysis and phenotyping had restricted CCNB1IP1 within. Through two-hybrid discovery of protein-protein interactions between CCCNB1IP1 and SUMO2 as well as predicted targets of SUMO-conjugation, I developed the hypothesis that CCNB1IP1 functions as a SUMO E3 ligase in regulating the posttranslational modifications of proteins more directly involved in the maturations of crossover sites.

Having implicated CCNB1IP1 as a SUMO E3 ligase based upon both conservation of domain and protein-protein interactions, I next moved to validate this SUMO ligase activity through direct biochemical evaluation. *In vivo* studies of CCNB1IP1 were obstructed due to lack of spermatocyte cell culture techniques and aggregations of CCNB1IP1 resembling polycomplex structures when expressed in cultured somatic cells. Fortunately, the SUMOylation of peptides can be recapitulated *in vitro* with recombinant proteins. Through this recapitulated SUMOylation reaction, soluble recombinant CCNB1IP1 was observed to be autoSUMOylated, a property previously identified for E3 ligases when terminal targets for SUMO-conjugation are lacking. Reagents limited my ability to detect CCNB1IP1-dependent SUMOylation of target proteins through these similar *in vitro* recapitulations, but efforts towards building a synthetic SUMO E3 ligase with inducible associations to suspected targets of SUMOylation show GGN and 4930455F23RIK as targets of posttranslational modification when induced to interact with this synthetic SUMO E3 ligase. CCNB1IP1 thus displays biochemical autoSUMOylation consistent with a SUMO E3 ligase, and proteins to which it interacts are themselves posttranslationally modified dependent upon interaction with a putative SUMO E3 ligase.

Having supported my hypothesis that CCNB1IP1 functions as a SUMO E3 ligase essential to meiotic crossing-over regulation, I moved to understand the fine

detail sites of action for CCNB1IP1 and the defect in the *Ccnb1ip1*$^{mei4/mei4}$ animals. Immunolocalization of CCNB1IP1 confirms previous suspicion as a SC-associated protein acting upon those sites progressing towards crossing-over. The points of CCNB1IP1 localization along SC cores decrease in number as meiocytes approach diplonema, the point at which crossovers are terminally matured. These CCNB1IP1 dynamics, together with low levels of persistent DNA damage and failure to displace MSH4 through maturation of crossover intermediates, implicate CCNB1IP1 as functioning in regulating the processing of crossover intermediates during pachynema. It is thus hypothesized that CCNB1IP1 functions at maturing crossover intermediates to impart SUMO modifications upon other proteins and alter their activity, localizations, and/or facilitating the building of more protein-dense MNs at the sites of those intermediates designated to resolve as the Class I crossovers marked by the MLH1/3 heterodimer (fig 5.1).

Taken together, the results of this thesis reveal CCNB1IP1 and a meiotic co-regulator specifying proteins for posttranslational regulation through SUMOylation. CCNB1IP1's SUMO-dependent regulation of crossing-over is finely localized to crossover intermediates and failure in this SUMO-dependent regulation results in failure to repair DSBs though the obligate crossover.

In the broader sense, these studies with CCNB1IP1 begin to reveal the conserved role SUMO-conjugation plays in the regulations of DNA damage repair (a general principle), and meiotic recombination (uncovered here). While the study of SUMO is a relatively new field, it has become clear from work in somatic cells that SUMO regulated functions are diverse but as discussed earlier, SUMO may be particularly important in DNA damage response and repair regulation. Uncovering a role for SUMO-dependent regulation in the maturation of crossover intermediates is

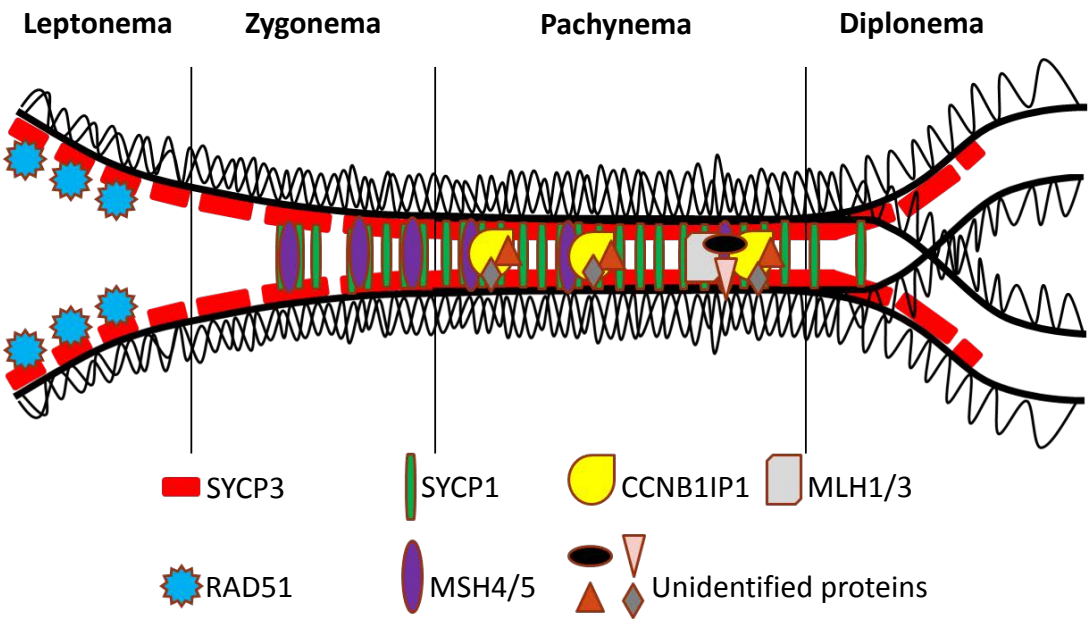

Figure 5.1. Model of localized CCNB1IP1 regulations stabilizing maturing crossover intermediates.
CCNB1IP1 functions locally at the sites of crossover intermediates during Pachynema of prophase I meiocytes. As meiotic DSB repair progresses towards those sites of Class I crossover-mediated repair, CCNB1IP1 localizations begin at early Pachynema and dynamically decrease towards those final crossover as marked by the MLH1/3 heterodimer. CCNB1IP1 imparts SUMO modifications upon some number of unidentified proteins at crossover intermediates and alters their activity, localizations, and/or facilitates the building of more protein-dense MNs at these intermediates designated to resolve as the Class I crossovers.

further evidence of the conserved role SUMO plays in regulations of DNA damage response.

Within the field of meiosis, the processes through which crossovers are regulated remains poorly understood, and the wide roles of SUMOylation within any function of meiosis is largely unknown. It is exciting that CCNB1IP1 implicates SUMO-mediated regulations in the maturation of crossover intermediates.

Future thoughts

CCNB1IP1 presents itself as the entry point to identifying SUMO-conjugates and their more direct roles in crossing-over. Because CCNB1IP1 finely localizes to sites of crossover intermediates, and because CCNB1IP1 directly interacts with proteins regulated through conjugation by SUMO, a primary interest should be the immunoprecipitation of CCNB1IP1 and the identification of those *in vivo* interacting partners modified by SUMO at these particular crossover intermediates. This type of proteomic *discovery* of SUMO-conjugates is complicated by the branch-structure peptides produced from digestion of SUMO-conjugates.

Mass spectrometry relies on computational identification of proteins in mixture based upon peptide sizes retrieved from digestions and matching these retrieved masses to those predicted from *in silico* digestions. While accommodating for the minor mass increases resultant from small mass changes of phosphorylation and acetylation is relatively easy, the branched fragment from SUMO complicates the analysis of digestion fragments and thus the discovery of unknown constituents in the protein isolates. The technical difficulties of this *in silico* analysis of SUMO-conjugates is made nearly insurmountable from anything less than an exceptionally clean and “simple” mixture of peptides. Thus, identifying the CCNB1IP1-interacting proteins from meiocytes would be primarily reliant upon efficient co-

immunoprecipitations. Traditionally, immunoprecipitations towards discovering SUMO-conjugates are best produced through cells in culture, followed by tandem affinity purifications under strong denaturing conditions so as to remove most contaminants and produce an exceptionally clean mixture. Due to the goal of identifying CCNB1IP1 interacting partners and SUMO-conjugates from meiocytes, the protocols of purification traditionally used for SUMO-conjugates are precluded in this experiment. The *in vivo* immunoprecipitation of CCNB1IP1 and its interactors would thus be best produced through highly selective epitope-tagging of CCNB1IP1 in the animal. Because prior attempts at such a transgene were unsuccessful, I would approach this through targeting the endogenous locus. And because another interest would be the *in vivo* live imaging of CCNB1IP1 and more generally the dynamics of intermediate MNs, I would maintain the mCherry fluorescent-tag fusion as both a means for live imaging and efficient immunoprecipitations. Additionally, since the traditional use of denaturing immunoprecipitations is ill-advised in this *in vivo* discovery of CCNB1IP1-interacting proteins, it would further be necessary to "simplify" the proteins for identification through size fractionations across polyacrylamide gel prior to digestion for mass spectrometry. Once a clean (i.e efficient immunoprecipitation) and simple (i.e. size fractioned) mixtures of CCNB1IP1-interacting proteins are isolated, the experiment is approachable and *in silico* tools such as ChopNSpice would identify these SUMO-conjugates at the sites of intermediate MNs. An additional advantage of this *in vivo* identification of CCNB1IP1-interacting proteins is that I believe the SUMO E2 ligase, UBE2I/UBC9, would be identified through Western blot or protein sequencing of the mCherry-mediated co-immunoprecipitation. This *in vivo* interaction between CCNB1IP1 and UBE2I has been difficult because of limitations largely due to the available anti-

CCNB1IP1 antibody and having the mCherry epitope would present an especially nice solution to this technical difficulty.

An additional consideration to this CCNB1IP1-mediated discovery of SUMO-conjugates may be that the steps and conditions used to yield appropriately clean and simplified mixtures, together with the apparent specificity of CCNB1IP1 at those few MN sites may result in samples below the detection thresholds of accurate mass spectrometry analysis, especially considering the complicated computations therein for SUMO-conjugates. This complication can be overcome through isolating a starting sample of pachytene spermatocytes enriched from adult testis by either velocity sedimentation separation with STA-PUT or cell sorting based upon DNA content and size via flow cytometry.

I believe the identification of SUMO-conjugates has the most interesting long-term implications resultant from these CCNB1IP1 studies. Through CCNB1IP1, and recently RNF212, we are appreciating the role SUMO plays in meiosis I. While discovery of CCNB1IP1-interacting partners is a direct approach to offer *in vivo* confirmation of the studies in this thesis, a more global identification of SUMO-conjugates in meiocytes may be both more significant and a more quantifiably accurate method to assess CCNB1IP1-depent SUMOylation and defects in the *Ccnb1ip1*$^{mei4/mei4}$ animals. As a general tool for studies of SUMO in the mouse, transgenic mice expressing a tagged-SUMO regulated from the endogenous locus would prove a valuable resource. Traditionally a direct purification of SUMO and thus the SUMO-conjugates through tandem affinity purification (TAP) has been the most successful methodology for unbiased discovery of SUMO-conjugates. For the specific purpose of understanding the role of SUMO-mediated regulations in meiosis, generation of mice expressing TAP-tagged SUMO1/2/3 alleles would allow for efficient purification of SUMO-conjugates from spermatocytes. Having the

foundational knowledge of proteins SUMOylated during meiosis, and the dynamics of the SUMO proteome within each stage of Prophase I, might prove as informative and important towards future studies as our understanding the transcriptome has been. Furthermore, comparing the dynamic SUMO proteome between SUMO1, SUMO2 and SUMO3 would help understand if SUMO proteins are complementary or to what extant their targets, and possibly functions, differ. The reference database of SUMO in meiosis, and the resources of TAP-tagged SUMO in the mouse would have significant impacts toward advancing the understanding of SUMO-mediated regulation in higher order animals.

The advantage of knowing the SUMO proteome would also serve to more accurately validate and further the CCNB1IP1 studies I have presented in this thesis. As I have already elaborated, discovery of SUMO-conjugates through interactions with CCNB1IP1 is technically very challenging. However, if the ground state of the SUMO-proteome is prior knowledge, then the identification/confirmation of those SUMO-conjugates co-immunoprecipitated with CCNB1IP1 is much more reliable. Because the library of branched peptides to identify would then be *a priori* knowledge in the CCNB1IP1 immunoprecipitation samples, the proteins submitted to mass spectrometric analysis do not need be as clean nor as "simple" as previously described. Furthermore, having the knowledge of the SUMO1 vs. SUMO2 vs SUMO3 proteome would allow for more justified conclusion of the SUMO isoform modified to the CCNB1IP1 interactors, *in vivo*. Having knowledge of those few proteins directly interacting with CCNB1IP1, the SUMO isoform regulating these proteins, and the TAP-tagged transgenic animal for that SUMO isoform would thus present the very nice option to *quantify* the differential SUMOylation levels of those *a priori* targets of SUMOylation in the WT vs *Ccnb1ip1*$^{wt/mei4}$ vs *Ccnb1ip1*$^{mei4/mei4}$ pachytene spermatocytes. Indeed, this objective to comparatively quantify differential

SUMOylation between effected animals is not reliant upon prior co-immunoprecipitation with an epitope-tagged CCNB1IP1, but the additional CCNB1IP1 immunoprecipitation step would serve to reduce the complexity of data analysis. Reducing the complexity is advantageous because it would narrow to a specific SUMO isoform and focus upon those SUMO-conjugates directly influenced by CCNB1IP1 interaction, but indeed the SUMO proteome in its entirety could be differentially assessed between the *Ccnb1ip1* alleles.

Although the above mentioned proteomic studies would identify the CCNB1IP1 interactome as a whole, the directed studies of candidate CCNB1IP1 interacting partners may prove more traditionally straightforward. Because of CCNB1IP1's focal localizations to intermediate MNs, narrowly focused interaction studies of similarly localized candidate proteins could prove informative. In this approach, biased by candidates known to localize to MNs, antibodies suitable for immunoprecipitations are the limiting reagents and in most circumstances the directed yeast two-hybrid assessment for interaction would be the most approachable option. As discussed earlier, the MSH4/5 heterodimer stabilizes intermediate MNs and the MLH1/3 heterodimer marks sites of crossing-over. Because the *Ccnb1ip1*$^{mei4/mei4}$ animals display retained MSH4 localization, and fail to localize MSH1/3, it is reasonable to directly assess potential protein-complex interactions of CCNB1IP1 with MSH4, MSH5, MLH1, and MLH3. Additionally, an interaction with RNF212/Zip3 should be considered- RNF212 localizes to intermediate MNs as well, and in the *Cccnb1ip1*$^{mei4/mei4}$ animals it has been shown that RNF212 foci at intermediate MNs persist and fail to mature similar to the MSH4 dynamics reported here (Neil Hunter, unpublished). Similar to CCNB1IP1, RNF212 is hypothesized to function as a SUMO E3 ligase and contains an internal coiled-coil domain which may mediate an interaction with CCNB1IP1. It should be noticed that these proteins were not isolated

from my previously reported two-hybrid screen, but this may be a function of simply not recovering that interaction, lack of suitable representation of these clones in the library screened, or the necessity of a full-length clone of these partners for the tertiary structure adequate to a CCNB1IP1 interaction.

From the studies I have presented in this thesis, two candidate genes for further explorations on putative roles in meiosis jump out: *Ggn* and *4930455f23Rik/Ccdc181*. Ggn has recently been knocked out in the mouse and found essential to embryogenesis. However, the studies with *Ggn* haploinsufficient mice find that GGN is involved in meiotic DSB repair and those mice with half the GGN levels retain some moderately increased localizations of RAD51 into pachytene. This single report on the role of *Ggn* function in meiosis is incomplete and lacked refined staging of pachytene spermatocytes and thus calls into question the significance of the "moderate" retained/delayed dynamics of RAD51 localizations and the reported DSB repair defect. Nevertheless, my studies here implicate a role for GGN in meiosis, supported by its specific expression during spermatocyte entry into Pachynema and the previously discussed interaction cascade between CCNB1IP1, GGN, GGNBP1, and MEI1 (the latter of which is essential to DSB initiation of meiosis). To explore in careful detail the role of GGN in meiotic DSB repair is especially attractive due to the recent availability of these animals and the evidence for direct interactions linking the initiation of meiosis through to maturing Class I crossovers specified by CCNB1IP1.

Finally, it makes sense to consider *4930455f23Rik/Ccdc181* as a candidate for mutation in the mouse. While CCDC181 is expressed in several tissues, it is relatively strongly expressed in the testis but does not appear to be expressed in the ovary. *Ccdc181* maybe represent that minor class of genes with sexually dimorphic functions in meiotic prophase I- especially interesting if indeed shown to have a role in recombination of in male, but not the female meiocyte. In the testis, CCDC181

expression increases dramatically during 14-18dpp and this expression is consistent with a putative role during prophase I. Furthermore, CCDC181, like CCNB1IP1 and RNF212 as well as other SC-associated proteins, contains an internal coiled-coil domain and may indeed be found to localize to the SC like these other coiled-coil domain containing proteins. Although the mutant animals for *Ccdc181* are not available, mutating the gene would be attractive for its putative role and suspected localization at meiotic recombination sites. CCDC181 and GGN together represent options of further investigations opened up by identification of CCNB1IP1 as a meiotic co-regulator of crossing over. Through the identification of candidate genes *Ggn* and *Ccdc181*, we appreciate CCNB1IP1's role in interacting with many other proteins towards imparting regulatory marks effecting the diverse functions of these yet unappreciated factors in mammalian meiosis. Studies of CCNB1IP1 has presented these candidate genes and their corresponding posttranslational modifications for further studies.