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The Assembly and Dynamics of Hop1 and Red1 at the Meiotic Chromosome Axis

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The Assembly and Dynamics of Hop1 and Red1 at the Meiotic Chromosome Axis

A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Biomedical Sciences

By

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2017
This Dissertation of Alan West is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego
2017
DEDICATION

To my Mum and Dad. From spelling tests and multiplication tables, to college exams and PhD dissertations, your love and support saw me through every milestone of my education, and gave me strength when they seemed daunting. I wouldn’t be where I am today without you, and words cannot say how grateful I am.

Also, to my friends, teammates, and brothers-in-arms of These Are Not My Pants, The Order of Rebel Knights, and The Scholars of Alcalá. Thank you for making my life in San Diego so joyous and for helping me become a better, happier, more well-rounded person.
EPIGRAPH

“To be a scientist — it is not just a different job, so that a man should choose between being a scientist and being an explorer or a bond-salesman or a physician or a king or a farmer. It is a tangle of very obscure emotions, like mysticism, or wanting to write poetry; it makes its victim all different from the good normal man. The normal man, he does not care much what he does except that he should eat and sleep and make love. But the scientist is intensely religious — he is so religious that he will not accept quarter-truths, because they are an insult to his faith.”

~ Professor Max Gottlieb in Arrowsmith by Sinclair Lewis
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VITA

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ABSTRACT OF THE DISSERTATION

The Assembly and Dynamics of Hop1 and Red1 at the Meiotic Chromosome Axis

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The generation of viable gametes requires the proper pairing and physical association of homologous chromosomes prior to the first meiotic division. The extended prophase of meiosis I is dedicated to this process, and is regulated by cellular signals that delay cell division until the proper paring of homologs is achieved. In humans, misregulation of homolog pairing and segregation can lead to fertility issues, miscarriages, or developmental diseases such as Down Syndrome. Homologs identify and physically associate with one another through a modified homologous recombination pathway, governed by a conserved meiosis-specific protein assembly called the chromosome axis. The axis facilitates the introduction of programmed DNA
double-strand breaks, and their repair via the homologous chromosome, thereby promoting crossover recombination that physically links homologs together. The axis also forms the foundation for the synaptonemal complex, which assembles between the paired homologs, joining them together during recombination. In the budding yeast *S. cerevisiae*, the chromosomes axis consists primarily of DNA-binding cohesin complexes and two meiosis-specific proteins, Hop1 and Red1. Here we present a biochemical and biophysical characterization of Hop1 and Red1 to understand the mechanisms governing chromosome axis assembly and function. We identify specific interactions between the Hop1 HORMA domain and conserved “closure motifs” in Red1 and the Hop1 C-terminus, which are responsible for the localization and self-assembly of Hop1 at the chromosome axis. We find that HORMA domain-closure motif interactions depend upon the conformational dynamics of the Hop1 HORMA domain in a manner reminiscent of a related HORMA domain protein, Mad2. We also find that Red1 contributes to the structure of the chromosome axis through the formation of high molecular weight oligomers assembled through its coiled-coil C-terminus. We find that previously-characterized mutations within this coiled-coil region that prevent formation of the chromosome axis and the synaptonemal complex, disrupt the oligomerization of the Red1 C-terminal domain, suggesting that Red1 oligomerization is essential for axis assembly and function. Together, the findings of this dissertation provide new mechanistic and functional insight into the assembly and dynamics of the meiotic chromosome axis and the means by which it coordinates the events of prophase I.
CHAPTER 1

INTRODUCTION

Meiosis is a fundamental feature of all sexually reproducing organisms. It is the means by which the genetic complement of a parent cell is reduced yielding four haploid gametes cells, each genetically distinct from the parental cell that gave rise to it. The fusion of two haploid gametes via fertilization creates a diploid zygote that contains two copies of each chromosome, one from each parent. The alternation between diploid and haploid generations of cells contributes to the genetic diversity and evolutionary fitness of a species by creating new combinations of alleles with each successive generation. Like mitosis, meiosis begins with the replication of each chromosome to yield a pair of sister chromatids. In contrast to mitosis however, the generation of viable gametes requires that a single copy of each chromosome must be accurately distributed to each of four different nuclei. This is accomplished via two successive rounds of division, namely Meiosis I in which maternal and paternal homologous chromosomes are segregated, followed by the more mitotic-like Meiosis II in which the sister chromatids are segregated (Figure 1.1).
Figure 1.1: Overview of Meiosis
Diploid parent cells generate four haploid gametes by means of two rounds of cellular division: Meiosis I, in which homologous pairs of chromosomes are paired and segregated, and Meiosis II in which sister chromatids are separated.

In both mitosis and meiosis, sister chromatids are held together by sister chromatid cohesion that is established when the diploid genome is replicated prior to division. No such linkage exists between homologous chromosomes, however, so the accurate pairing and bi-orientation of homologs in the first meiotic division presents a complicated set of steric and topological challenges. Much of the extended meiotic prophase is dedicated to overcoming these challenges through the formation and subsequent repair of DNA double-strand breaks (DSBs) along each chromosome by means of homologous recombination, to generate inter-homolog crossovers that physically link each pair of homologs. The reciprocal exchange of genetic information between homologs brought about by crossovers has two important genetic benefits. From an evolutionary perspective, it is the major means by which meiosis increases
genetic variability. Mechanistically, crossovers mediate highly specific physical linkages between homologous chromosomes. When combined with sister-chromatid cohesion mediated by cohesin complexes, crossovers provide the specific association between homologs required for their accurate segregation from one another in meiosis I.

Homologous recombination requires that two regions of homologous DNA identify each other amongst a vast array of surrounding non-homologous sequences. In meiosis, this process is initiated by the formation of DSBs in one of the sister chromatids by the conserved Spo11 endonuclease (1). The 5’ ends of these DSBs are resected, leaving a 3’ single-stranded overhang of approximately 500 nucleotides. The search for a repair template is facilitated by the meiosis-specific recombination proteins Dmc1 and Rad51, which bind to the ssDNA overhangs to mediate strand invasion and exchange with the homologous duplex (2-4) (Figure 1.2A). In contrast to recombination in somatic cells, which mainly utilizes the sister chromatid as the repair template, meiotic cells bias DSB repair toward Inter-homolog (IH) recombination using the intact homolog as the template (5, 6).

The stages of meiotic prophase are defined by cytologically-observable transitions in the structure of the chromosomes as they undergo DSB formation and repair (Figure 1.2A). After DNA replication in S-phase, the cells enter leptotene in which meiotic chromosomes condense into long thin strands of chromatin loops emanating out of a rod-like central axis, which consists of cohesin complexes as well
as meiosis-specific structural and regulatory proteins. In the budding yeast \textit{S. cerevisiae}, these proteins include Hop1 and its principal binding partner Red1. In leptotene, Red1 associates with the chromosomes along their entire length and recruits Hop1 to the axis, completing its assembly (7-10).

\textbf{Figure 1.2: DSB repair and crossover through meiotic prophase I}

(A) Schematic representation of chromosomes through the stages of Prophase I. (B) DSBs form in early leptotene and are resected yielding 3’ overhangs. In zygotene, these single stranded overhangs invade the duplex of the adjacent homolog in search for homologous sequence. Double Holliday junctions appear in pachytene after second strand invasion. A subset of double Holliday junctions are resolved yielding crossovers and chiasmata, while the rest are resolved as non-crossovers. Arrows indicate sites where strands are cut to yield both crossover and non-crossover products.
After DSBs form, the cells begin the zygotene stage, in which the completed axes of each homolog begin to align to facilitate the repair of DSBs. Repair is initiated by the invasion of the resected 5’ overhangs of the break into the strands of the homolog in search of a homologous repair template. While most DSBs interact with the homolog in this manner, only a small subset become designated for crossover repair, with the remainder resolved as non-crossovers, which restores the broken DNA molecule without reciprocal exchange of flanking DNA sequences (11, 12). At sites designated to become a crossover, initial strand exchange is followed by the capture of the second broken DNA end, followed by DNA synthesis at both captured strands, eventually generating a double Holliday junction intermediate (Figure 1.2B).

The formation of the double Holliday junction occurs in the pachytene stage, concomitant with assembly of the synaptonemal complex, which brings the paired homologs’ chromosome axes into close juxtaposition along their entire lengths. The synaptonemal complex (SC) is a tripartite proteinaceous scaffold assembled from transverse filaments, primarily consisting of Zip1 in *S. cerevisiae*, which link the paired axes of the homologous chromosomes, now referred to as the axial or lateral elements of the SC (13-16). It has been suggested that synapsis initiation occurs at DSB sites that have been designated for repair via crossover, in addition to other sites such as centromeres (in many species) and specialized “pairing centers” (in *C. elegans*) (17, 18). In the subsequent diplotene stage, the SC disassembles, leaving homolog pairs physically linked via chiasmata, the cytological manifestation of crossovers, which hold the homologs together until their segregation (Figure 1.2A,B).
To avoid the creation of aberrant chromosomes and defective gametes, meiotic cells have evolved specialized chromosomal architecture and regulatory mechanisms that comprise the Meiotic Checkpoint Network (MCN) (19). This network coordinates the events of recombination, synapsis, and segregation to establish the correct timing of meiotic prophase events and to avoid deleterious interactions between different processes. Generally speaking, the MCN ensures that the homologous chromosomes have been paired properly by delaying meiotic progression until at least one crossover has been formed between each homolog pair. Misregulation of the meiotic processes often leads to aneuploid gametes and subsequent chromosomal abnormalities in conceived embryos. In humans, it is estimated that 10-30% of all fertilized eggs are either trisomic or monosomic for one or more chromosomes (20). Most often this leads to spontaneous miscarriages, however ~0.3% of liveborns are aneuploid (21), most commonly trisomy 21 (Down Syndrome) and sex chromosome aneuploidies such as XXX (Triple X syndrome), XXY (Klinefelter syndrome), and XO (Turner Syndrome). Meiotic defects are also a leading genetic cause of infertility in males. Germ cells in infertile males show impaired progression through prophase, with most cells arresting in the leptotene and zygotene stages (22). This is linked with significantly lower rates of meiotic recombination, a decreased number of crossover events, and impaired synapsis of homologous chromosomes (22, 23). Given its profound impact on human health and fertility, a detailed understanding of the mechanisms by which meiotic
recombination is controlled will be valuable in understanding, preventing, and developing clinical interventions for congenital defects and fertility issues.

The work of this dissertation strives to increase the understanding of how the events of meiotic prophase are regulated by focusing on the structure, mechanism, and dynamics of the meiotic chromosome axis in the budding yeast S. cerevisiae. In both yeast and mammals, the meiotic chromosome axis ensures proper pairing and synapsis of the homologs by providing the basis for the chromosomal architecture necessary for DSB formation, recombination, and the establishment of inter-homolog bias (24-27). This work will focus on the structural and biochemical properties of the S. cerevisiae chromosome axis proteins Hop1 and Red1. Although this dissertation will primarily focus on the chromosome axis in budding yeast, the proteins involved possess readily identifiable orthologs in mammals, and the overall mechanisms involved appear to be largely conserved through eukaryotic evolution (28).

**Cohesins are essential for meiotic axis assembly:**

Formation of the meiotic chromosome axis is closely linked with sister chromatid cohesion, in which the newly replicated sister chromatids are held together by multi-protein complexes known as cohesin complexes. In both mitosis and meiosis, cohesins are deposited onto chromosomes in conjunction with S-phase DNA replication to ensure their accurate alignment and segregation during cellular division, as well as facilitate DNA repair by homologous recombination (29). The two major components of the cohesin complex are Smc1 and Smc3, which form a
heterodimer in which the proteins are joined by two long stretches of antiparallel coiled coil separated by a hinge region forming a V-shaped molecule (30). The Smc1/Smc3 heterodimer is thought to topologically entrap DNA from both sister chromatids by means of a third subunit called kleisin, which in mitotic cells is Scc1. In meiosis, a meiosis-specific kleisin subunit, Rec8, replaces Scc1 and is required for proper meiotic chromosome segregation (31).

In mitosis, anaphase onset occurs when Scc1 is cleaved by Separase along the entire length of the chromosome, allowing sister chromatids to separate and segregate to opposite spindle poles (32). In meiosis, however, Rec8 cleavage occurs in a stepwise manner in the two meiotic divisions. In anaphase I, Rec8 along the chromosome arms is degraded to enable homolog segregation, but centromeric Rec8 is protected by the action of the shugoshin protein, thereby maintaining sister chromatid cohesion through meiosis I (33, 34). Centromeric cohesion persists until anaphase II, at which point Rec8 is cleaved to enable the bipolar segregation of the sister chromatids (35).

Prior to the meiotic divisions, meiosis-specific cohesin complexes are key mediators of the chromosomal architecture necessary for proper axis formation and recombination, acting with other axis proteins to organize the chromosome as a linear array of chromatin loops (27, 36). In both yeast in mammals, rec8 mutants fail to assemble discernable linear chromosome axes and develop abnormal SC structures. Additionally, while meiotic recombination is initiated in rec8 mutants, it
fails to progress beyond the initial steps (37-39). Much of this is due Rec8 interaction with Hop1 and Red1, which depend upon Rec8 cohesin for proper localization and distribution along the axis (24). Rec8 is thought to recruit Red1 to the axis, which in turn recruits Hop1 (40) (Figure 1.3). In yeast rec8 mutants, Hop1 and Red1 still associate along the chromosome although their localization is abnormal, forming only short and disjointed regions of the axis (24, 31). While Hop1 has inherent DNA binding ability and can localize to chromosomes in the absence of Red1 (41, 42), its distribution along chromosomes and proper function requires its interaction with Red1 (7).
After DNA replication, sister chromatids are bound together by meiosis specific Rec8 cohesins. Red1 and Hop1 are subsequently recruited to the axis and the chromatin condenses as a linear array of loops extending out form the axis.

**Figure 1.3: Chromosome Axis Assembly**

The distribution of DSBs along meiotic chromosomes is largely dependent upon chromosomal architecture of collinear chromatin loops joined at their base by the axis defined by Rec8 cohesins, Hop1 and Red1 (10, 27, 43, 44). This chromosomal arrangement helps define a number of ‘hotspots’ that are 100-1000 times more likely
to receive a DSB than other sites (45-48). Interestingly, ChIP-seq data indicates that DSB hotspots largely map between binding sites of axis components, suggesting that while axis proteins are necessary for DSBs they preferentially occur within the loops rather than at the axis (24, 40). DSB formation is mediated by the Spo11 endonuclease, and also requires several Spo11-accessory protein complexes which form the pre-DSB recombinosomes. Three of these factors - Rec102, Rec104, and Ski8 - are directly required for Spo11 DNA binding and endonuclease activity (49, 50) (51). A separate complex consisting of Rec114, Mer2, and Mei4 (termed the RMM complex) partially colocalizes with Spo11-containing complexes onto chromatin, and is required for Spo11’s recruitment to future DSB sites (51, 52). The RMM complex closely associates with the chromosome axis and “tethers” the pre-DSB recombinosome in the chromatin loop to the axis, whereupon Spo11 is able to cleave the DNA (51-56) (Figure 1.4). Although the nature of the interaction is unclear, the association of the RMM complex with the axis is dependent upon Red1 and Hop1 (24). Due to their role in maintaining axis structure and recruiting DSB machinery to the axis, cells in which either Rec8, Hop1 or Red1 are deleted exhibit a reduced number, as well as an altered distribution, of DSBs (26, 57, 58).
Figure 1.4: Tethered Loop Axis Model

DNA hotspots preferentially occur in regions corresponding the chromatin loops, while DSB proteins are associated with the chromosome axis [1], which in turn recruits Pre-DSB recombinases [2]. This interaction tethers DSB sites to the axis (and promotes cleavage by Spo11 [3], resulting in completed DSBs at the axis [4].

Establishment of Interhomolog Bias:

In mitotic cells, DSBs are preferentially repaired using the sister chromatid as the template. Inter-sister recombination, however, does not contribute to homolog pairing or the formation of chiasmata. As such, meiotic cells bias DSB repair toward interhomolog recombination by a factor of five-fold in *S. cerevisiae* (59-62). This “homolog bias” is achieved through the active suppression of inter-sister recombination and the concomitant promotion of interhomolog interactions (63).

Much of this is achieved by the combined action of combined action of Hop1 and the Mek1 kinase. In response to DSBs, Hop1 is phosphorylated at several sites (most
notably S298 and T318) by the DSB-response kinases Mec1 and Tel1 (ATM and ATR in mammals)(64, 65), which promotes recruitment of Mek1 via its FHA domain, a conserved phospho-threonine binding domain. Mek1 in turn phosphorylates several factors, including a regulator of Rad51, to locally suppress DSB repair, thereby forcing the broken DNA end to seek out homologous sequences outside this zone of inhibition (25, 66-68). Hop1’s role in “tethering” DSB sites to the axis may further impede inter-sister recombination, by separating the search for homology at the axis, from the homologous sequences on the sister-chromatid within the chromatin loops (24, 58, 63). Those DSBs that do form in the absence of Hop1, are repaired preferentially using the sister chromatid as template, strongly exacerbating the recombination defect caused by subnormal levels of DSBs.

**Pch2 and Chromosome Axis Remodeling:**

Pch2 (TRIP13 in mammals) is an evolutionarily conserved AAA+ ATPase (ATPases Associated with diverse cellular Activities) required for checkpoint-induced meiotic prophase arrest in response to defects in chromosome organization and synapsis (69). In both mammals and yeast, homolog synapsis is tightly linked to, and depends on, meiotic recombination. *PCH2* was first identified in a genetic screen for mutants that alleviate checkpoint-mediated arrest in pachytene in yeast cells lacking Zip1, the major structural component of the central region of the SC (69). In yeast cells, *zip1* mutants arrest before pachytene due to failed SC formation and consequently unable to complete recombination (14, 70). Deletion of *pch2*
completely removes the meiotic arrest of zip1, and zip1/pch2 double mutants display wild-type levels of sporulation, however, spore viability is greatly reduced due to incomplete recombination (69). Pch2/TRIP13 similarly responds to chromosome organization and synapsis defects in C. elegans and D. melanogaster (71-73).

The primary meiotic function of Pch2 seems to be to negatively regulate the localization of Hop1 at the chromosome axis. In pachytene nuclei, Hop1 and Zip1 display reciprocal patterns of localization in which Hop1 is excluded from synapsed regions of the chromosomes (10, 74, 75). Similar localization patterns are observed with the mammalian Hop1 orthologs HORMAD1/2 and the mammalian SC transverse-filament protein SYCP1, which are likewise dependent upon TRIP13 AAA+ ATPase activity (76, 77). In each case, deletion of Pch2/TRIP13 results in aberrant localization of axis proteins to synapsed regions of the chromosomes. In yeast, pch2 mutants also exhibit delayed pachytene exit, delayed repair of DSBs, and an increase in non-crossover versus crossover recombination (74).

While Pch2’s most visible role is the removal of Hop1 from the chromosome axis in pachytene, there are hints that it may also contribute to Hop1’s initial localization to the chromosome axis and its proper function there. Recent work has shown that Hop1 is not properly phosphorylated in the absence of Pch2; as Hop1 phosphorylation requires that it be localized to the chromosome axis, a reasonable interpretation of this finding is that Pch2 may contribute to Hop1’s localization (78). Additionally, the rice Pch2 ortholog CRC1 is required for the initial localization of the
HORMAD protein PAIR2 to the chromosome axis (79). Recent work with mammalian TRIP13 and its other substrate, the spindle assembly checkpoint protein Mad2, hints at a possible mechanism for these counterintuitive findings. Work in our own lab has shown that TRIP13 partially unfolds MAD2, promoting either the assembly or disassembly of its complexes, depending on the precise molecular circumstances (80, 81). These findings and their implications for Pch2 and Hop1’s functional mechanism are discussed below.

**HORMA Domain and Meiotic HORMAD Proteins:**

Currently much of what is known about Hop1’s function in the regulation of the chromosome axis and the events of recombination has been derived via genetic experiments. The work of this dissertation sets out to gain structural and biochemical insight into the mechanisms by which Hop1 is recruited and relays signals to coordinate the events of prophase by focusing primarily on its well-conserved N-terminal HORMA domain.

Named after the three functionally unrelated *S. cerevisiae* proteins in which it was first identified by sequence similarity, Hop1, Rev7 and Mad2 (82), HORMA domains possess a highly conserved role acting as signal-responsive adaptors mediating protein-protein interactions. Rev7 (also referred to as Mad2B/Mad2L2) is a subunit of the translesion DNA polymerase-ζ, and has also been implicated in mitotic cell cycle control and regulation of recombination pathway choice in DSB repair (83). Mad2 is an essential mediator of the spindle assembly checkpoint (SAC),
which monitors the status of kinetochore-microtubule attachments in both mitosis and meiosis (84, 85). Additional HORMA domains have since been identified in autophagy-signaling proteins Atg13 and Atg101 (86, 87), as well as p31comet, a protein that regulates SAC signaling in conjunction with Mad2 (88-90).

Meiotic HORMA domain proteins, collectively referred to as HORMADs, are an evolutionarily conserved feature of the chromosome axis and play similar roles in coordinating chromosome dynamics and recombination during the first meiotic prophase. Mammals possesses two HORMAD proteins, HORMAD1 and HORMAD2, while the nematode C. elegans possesses four structurally related HORMADs: HIM-3, HTP-1, HTP-2 and HTP-3 (91). Although meiotic HORMADs from fungi as well as plants contain additional domains that may bind DNA, chromatin, or other proteins, the HORMA domain is the best conserved across multiple species, and possesses well characterized paralogs upon which functional models can be based (92).

Despite the diverse range of cellular pathways in which they are involved, an understanding of common structural and functional features of HORMA domain proteins can inform studies of the Hop1 HORMA domain’s role in meiotic chromosome axis architecture and meiotic recombination.

**Conserved HORMA domain structure:**

The HORMA domain is a 200-250 residue, compact fold, with a folded core of ~150 residues and a dynamic C-terminal region known the safety belt (93). The HORMA domain core comprises three \( \alpha \)-helices (\( \alpha \)A, \( \alpha \)B, and \( \alpha \)C) packed against
three β-sheets (β4, β5, β6), and often includes an additional pair of beta-strands (β2-β3) in a hairpin configuration at the “back” of the three alpha-helices (92). The core structure is stabilized by a hydrogen bond network involving an arginine on αA and a glutamate on β4; these are the only residues conserved across all HORMA domains (82). The C-terminal safety belt can adopt one of two conformations, defining the so-called open and closed states of the domain (93-95) (Figure 1.5A). In the open state, the safety belt is folded into two β-strands, β7 and β8, that pack against the β6 strand of the domain’s core. In the closed state, the safety belt is dissociated from the β6 strand, and instead rearranges into two new strands, β8'/β8'', that pack against β5 on the opposite side of the core. This conformation opens a ligand binding pocket adjacent to the β6 strand in which short interacting peptides within binding partners, known as closure motifs, are embraced under the safety belt, forming a topological link between the domain and its partner (92, 96). Because of the topology of this interaction, partner binding or dissociation requires a dramatic conformational rearrangement of the safety belt, potentially also requiring energy input. Thus far, Mad2 is the only HORMA domain protein known to be able to convert between the open and closed states (referred to as O-Mad2, and C-Mad2, respectively) (Figure 1.5A) (85, 93, 94). All other previously-characterized HORMA domain proteins have only ever been observed in a single conformation (92). The mechanism of Mad2 in the SAC therefore serves as a useful model to understand
how the two conformations, and the interconversion between them, may contribute to the overall functioning of this domain in the meiotic HORMADs.

Figure 1.5: Mad2 HORMA domain conformational change
(A) Schematic representation (left), secondary structure (center), and structure (right) of Mad2 in the open (top; PDB ID 1DUJ (97)), and ligand bound, closed conformation (bottom; PDB ID 4AEZ (98)). In the open state, the safety belt occupies the peptide-interaction site. The intermediate state enables an interacting peptide to bind and subsequently become locked into position once the safety belt binds the opposite side of the domain.
(B) Schematic representation of the Mad2 HORMA domain remodeling by TRIP13. Mad2 in the MCC is bound in the closed conformation around the Cdc20 closure motif (yellow). The safety belt (blue) is stabilized in the closed position against the core by a hydrogen bonds [1]. The N-terminus of Mad2 is loaded into the central pore of the TRIP13 hexamer (green), disrupting the hydrogen bond network stabilizing the safety belt [4]. The bound closure motif is released from the HORMA domain [3], allowing the safety belt to re-associate with the core of the domain in the open conformation [4].
The Mad2 Conformational Switching Regulates SAC:

In mitosis, the SAC detects kinetochores that are not attached to spindle microtubules and delays the cell cycle until proper attachment has been achieved on all chromosomes (99-101). This process is regulated by the assembly of the mitotic checkpoint complex (MCC) which delays the cell cycle by directly binding and inhibiting the Anaphase-Promoting Complex/Cyclosome (APC/C) (102, 103). The MCC consists of Mad2 in the closed state (C-Mad2) bound to a closure motif in Cdc20, a coactivator of the APC/C, as well as BubR1 which interacts with both Cdc20 and Mad2 to stabilize the complex (84, 98, 102, 104, 105). While most of the free Mad2 in HeLa cells exists in the open conformation, O-Mad2 by itself is not amenable to Cdc20 binding because the closure motif binding site is occupied by the β7/β8 safety belt strands (97). Relaying the checkpoint signal and assembly of the MCC therefore requires a conformational switch in Mad2 from the inactive open state to the Cdc20-bound closed state. This conformational change occurs at unattached kinetochores, which initially recruit a complex of Mad2 in its closed conformation, bound to Mad1. The Mad1:C-Mad2 complex at the kinetochore recruits cytosolic O-Mad2 and facilitates its conformational rearrangement to the closed state, which occurs concomitantly with Cdc20 binding. The recruited O-Mad2 forms an asymmetric dimer with the Mad1-bound C-Mad2 through a common interface involving helix αC and the β2-β3 hairpin (84, 93). This interaction causes the core of O-Mad2 to undergo a slight rearrangement into an intermediate state known as I-
Mad2 (84, 106, 107). Only recently characterized, I-Mad2 is a hybrid between the two conformations with the same fold as O-Mad2, but with a core structure more closely resembling that of C-Mad2 (107). In this state, the safety belt is thought to more readily access a fleeting transition state in which the β7/β8 strands are unfolded and more easily displaced from the core of the domain (Figure 1.4A). Cdc20 can then be recruited to the kinetochores to bind this transition state, which is then stabilized upon refolding of the Mad2 safety belt into the closed conformation (107). The resulting C-Mad2:Cdc20 complex is then bound by BubR1, completing the assembly of MCC (102).

**TRIP13/Pch2 dissociates MCC by partially unfolding Mad2:**

As the kinetochores become attached to the spindle microtubules, the Mad1:C-Mad2 complexes are stripped from the kinetochore, halting the assembly of new MCC. In order for the SAC to be silenced, however, existing MCC must also be disassembled. This is accomplished by the joint action of TRIP13 and the HORMA domain adapter protein p31comet.

AAA+ ATPases are a functionally diverse family of proteins, which includes a number of ‘classic remodelers’ known to disaggregate protein complexes (108, 109). The structure of TRIP13/Pch2 consists of an N-terminal domain (NTD) likely involved in substrate recognition, followed by the AAA+ ATPase domain, which assemble into a hexameric ring with a central pore, into which loops from each AAA domain extend (80, 81). Deactivation of the SAC requires BubR1 on the MCC to be replaced by
p31\textsuperscript{comet}, which delivers the C-Mad2:Cdc20 complex to TRIP13/Pch2 (110, 111).
Interaction between the NTD of TRIP13/Pch2 and p31\textsuperscript{comet} brings Mad2 in close proximity to the central pore of the hexamer, whereupon the pore loops engage its unstructured N-terminus (81). Successive rounds of ATP binding, hydrolysis, and release within the ATPase domains cause sequential conformational changes in the pore loops which generate tension in the Mad2 N-terminus (80, 81). This tension causes partial unfolding in the N-terminus and is thought disrupt a network of hydrogen bonds which stabilizes the \(\beta8'/\beta8''\) strands of the safety belt against the \(\alpha A\) helix in the closed conformation (81) (Figure 1.4B). With the safety belt no longer secured in the closed position, Mad2 resumes the open conformation and releases Cdc20, resulting in MCC disassembly.

Given the similarities in structure and mode of binding between HORMA domains, it is reasonable to assume that TRIP13/Pch2 remolds HORMADs on the chromosome axis via a similar mechanism. Upon synopsis of the chromosomes, TRIP13/Pch2 likely facilitates the removal of Hop1 and the other meiotic HORMADs by inducing conformational changes within their HORMA domains, releasing them from their binding partners at the axis. Although TRIP13/Pch2’s ability to affect the conformation of meiotic HORMADs has yet to be determined, recent work demonstrates that the N-terminus of mammalian HORMAD1 is necessary for its proper removal from the chromosome axis, suggesting that its interaction with TRIP13 likely parallels that of Mad2 (81).
The *C. elegans* HORMAD hierarchical assembly model:

Sharing similar functions to Hop1, the four *C. elegans* HORMADs, HIM-3, HTP-1, HTP-2, and HTP-3, coordinate chromatin loop formation, promote DSBs and interhomolog recombination (112-115). Recent work in our lab on the structure and function of the *C. elegans* HORMADs has provided valuable insight into the mechanism of HORMA domain recruitment and assembly along the chromosome axis in all species (96).

Each of the *C. elegans* HORMADs possess a common domain structure: an N-terminal HORMA domain followed by an unstructured C-terminal tail of variable length, ranging from ~50 residues in HIM-3 and ~100 residues in HTP1/2, to over 500 residues in HTP-3 (96). The structures of these proteins’ HORMA domains is similar to that of the Mad2 closed conformation, with the exception of an extended and more structured β5-αC loop, which drapes over the β8'/β8” sheets of the safety belt (96) (Figure 1.5A). This extended loop, unique to the meiotic HORMADs, may stabilize the domain in the closed conformation or otherwise regulate the association of the safety belt to core of the domain. In the first crystal structure of HIM-3, the ligand binding pocket was unexpectedly found to occupied by a beta-strand peptide corresponding to residues 278-286 within HIM-3’s own C-terminal tail (Figure 1.5A). This closure motif contains a highly conserved Pro-Tyr-Gly motif that closely associates with the safety-belt of the HORMA domain (96) (Figure 1.5B). Similar closure motifs were found in the C-terminal tails of each of the other three
HORMADs. Those of HTP-1 and HTP-2 contain nearly invariant Pro-Tyr-Ser motifs, whereas the elongated C-terminal tail of HTP-3 contains six motifs resembling the HIM-3 closure motif; four nearly identical to each other (motifs 2-5), and two variable motifs (motifs 1 and 6; **Figure 1.5B**). Specific interactions between individual HORMADs and closure motifs define assembly of a hierarchical complex of meiotic HORMADs at the *C. elegans* chromosome axis (**Figure 1.5B**). Mammalian HORMAD1/2 also possess conserved motifs within their C-termini that interact directly with their HORMA domains, suggesting that axis assembly of HORMADs via closure motif binding is conserved across all eukaryotes (96). It is not yet known whether the highly conserved C-terminus of Hop1 is also a closure motif, but this region is known to be critical for proper DSB and crossover levels (25, 116).

While prior work presents an elegant mechanism of HORMAD assembly at the chromosome axis, many questions still remain. In each of these cases the HORMA domain has only been observed the in the closed conformation (96, 117). Whether or not meiotic HORMADs can access the open conformation, or if their binding to closure motifs requires a conformational rearrangement similar to that of Mad2, has yet to be determined. Additionally, meiotic HORMAD closure motifs have only been identified within the C-termini of the HORMADs themselves. In the case of Mad2, however the HORMA domain is capable of binding closure motifs in two unrelated partner proteins: Cdc20 and Mad1. What, if any, additional binding partners the meiotic HORMADs bind via the closure motif mechanism have yet to be identified. It
is also unclear how the HORMADs are initially recruited to the chromosome axis, or if their recruitment is likewise dependent upon closure motif interactions at the HORMA domain. In the following chapters, I address these questions through biochemical and biophysical characterization of *S. cerevisiae* Hop1 and its binding partner at the meiotic chromosome axis, Red1.
Figure 1.6: *C. elegans* HORMAD structure and assembly

(A) Left: Generic secondary structure diagram of the *C. elegans* HORMADs showing the HORMA domain ‘safety belt’ (blue), the bound and C-terminal closure motifs (yellow), and the extended β5-αC loop (purple). Right: Crystal structure of *C. elegans* HIM-3 (PDB ID 4TRJ, (96))

(B) Left: Schematic representation of the *C. elegans* HORMADs, with squares representing N-terminal HORMA domains and ovals representing closure motifs in their C-terminal tails. Arrows represent the specific affinities of each HORMA domain to their respective closure motifs. Right: Sequences and alignment of the closure motifs from all four *C. elegans* HORMADs. Below: Schematic representation of the hierarchical assembly of HORMA domain to closure motif binding that define HORMAD assembly at the *C. elegans* meiotic chromosome axis.
References:


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Chapter 2

Conformational dynamics of the Hop1 HORMA domain reveal a common mechanism with the spindle checkpoint protein Mad2

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Abstract

The HORMA domain is a highly-conserved protein-protein interaction module found in eukaryotic signaling proteins including the spindle assembly checkpoint protein Mad2 and the meiotic HORMAD proteins. HORMA domain proteins interact with short “closure motifs” in partner proteins by wrapping their C-terminal “safety belt” region entirely around these motifs, forming topologically-closed complexes. Closure motif binding and release requires large-scale conformational changes in the HORMA domain, but such changes have only been observed in Mad2. Here, we show that \textit{S. cerevisiae} Hop1, a master regulator of meiotic recombination, possesses conformational dynamics similar to Mad2. We identify closure motifs in the Hop1 binding partner Red1 and in Hop1 itself, revealing that HORMA domain-closure motif interactions underlie both Hop1’s initial recruitment to the chromosome axis and its
self-assembly on the axis. We further show that Hop1 adopts two distinct folded states in solution, one corresponding to the previously-observed “closed” conformation, and a second more extended state in which the safety belt region has disengaged from the HORMA domain core. These data reveal strong mechanistic similarities between meiotic HORMADs and Mad2, and provide a mechanistic basis for understanding both meiotic chromosome axis assembly and its remodeling by the AAA+ ATPase Pch2/TRIP13.

Introduction

Meiosis is a specialized two-stage cell division program that gives rise to haploid gametes in sexually reproducing organisms. After a single round of DNA replication, homologous chromosomes segregate from one another in meiosis I, and sister chromosomes subsequently segregate in meiosis II. The extended prophase of meiosis I, in which homologs identify and physically associate with one another through a modified homologous recombination pathway, is governed by a conserved meiosis-specific protein assembly called the chromosome axis. The axis organizes each pair of sister chromosomes as a linear array of chromatin loops and promotes DNA double-strand break (DSB) formation by the conserved Spo11 endonuclease (1, 2). After DSB formation, axis proteins suppress repair of these DSBs via the nearby sister chromosome, thereby promoting repair via the homolog (3-8). This preference is key for the formation of crossovers (COs) that enable homologs to bi-orient on the meiosis I spindle, then properly segregate from one another to reduce ploidy by half.
In late prophase, the synaptonemal complex assembles along the length of paired homologs’ chromosome axes, bringing the homologs into close juxtaposition and promoting the final steps of crossover formation (reviewed in (9, 10)). In many organisms including the budding yeast *S. cerevisiae*, plants, and mammals, synaptonemal complex assembly is coordinated with remodeling of the chromosome axis (11-14). By depleting CO-promoting factors from homolog pairs that have properly associated, this axis remodeling process constitutes a feedback mechanism governing CO levels on a per-chromosome basis (15, 16).

The chromosome axis is highly conserved in eukaryotes. Major axis components include cohesin complexes containing at least one meiosis-specific subunit, the kleisin Rec8 (17-21); one or more proteins of the meiotic HORMA-domain containing (HORMAD) protein family (discussed further below); and in most organisms a coiled-coil domain-containing “linker” protein (*S. cerevisiae* Red1, *S. pombe* Rec10, mammalian SYCP2/SYCP3, and plant ASY3) required for localization of HORMADs (22-28). In the budding yeast *S. cerevisiae*, the axis comprises Rec8-containing cohesin complexes and the HORMAD protein Hop1, plus the linker protein Red1 (28-32). In wild-type cells, Red1’s distribution on chromosomes largely mirrors that of meiotic cohesin complexes (31), suggesting that Red1 may associate directly with cohesins. Red1 has also been shown to bind directly to Hop1 both *in vitro* and in cells (24, 33), and is required for normal chromosome localization of Hop1 (28, 31), suggesting a hierarchical cohesin→Red1→Hop1 axis assembly mechanism. Hop1,
Red1, and Rec8 are all required for normal numbers and spatial distribution of meiotic DSBs (34-36), acting at least in part to recruit the Rec14:Mei4:Mer2 complex to the chromosome axis (29). These proteins are, in turn, required for Spo11 recruitment to the axis and for DSB formation (37-42). After DSBs have formed, Hop1 is phosphorylated by the DNA damage-response kinases Mec1 and Tel1 (homologs of mammalian ATM and ATR) in its SCD region (SQ/TQ Cluster Domain), particularly residues S298 and T318 (5, 43). This phosphorylation promotes the recruitment and activation of the Mek1 kinase, which biases repair of DSBs toward the homolog instead of the sister chromosome, thereby generating COs (6, 44-46). After CO designation and the initiation of synaptonemal complex assembly, Hop1 is thought to be removed from the chromosome axis through the action of the conserved AAA+ ATPase Pch2 (13, 47, 48). As chromosome-localized Hop1 promotes both DSB and CO formation, its removal constitutes a feedback mechanism suppressing further recombination on chromosomes/regions that have already obtained COs (13, 49).

Many of Hop1’s regulatory functions are shared by its orthologs in other eukaryotes, including promoting DSBs and biasing their repair toward the homolog, and removal from the chromosome axis by Pch2 (TRIP13 in mammals) in coordination with synaptonemal complex assembly (11, 14, 50-52).

Hop1 is the founding member of the meiotic HORMAD protein family, which also includes *S. pombe* Hop1, plant ASY1/ASY2, mammalian HORMAD1/HORMAD2, and *C. elegans* HIM-3/HTP-1/HTP-2/HTP-3 (11, 27, 50, 53-56). The meiotic HORMADs
share a peptide-binding domain termed the HORMA domain, named for three functionally-diverse protein families originally shown to share it: Hop1, Rev7, and Mad2 (57, 58). The best-understood HORMA domain protein is Mad2, a key regulator of the mitotic spindle assembly checkpoint. This checkpoint monitors kinetochore-microtubule attachments in mitosis and meiosis, and delays anaphase onset until all kinetochores are properly attached to spindle microtubules (59-61). The Mad2 HORMA domain can adopt two distinct stably-folded conformations, termed “open” (O-Mad2) and “closed” (C-Mad2) (62-64). In the closed conformation, Mad2 binds short peptides called “Mad2-interacting motifs” (MIMs) or, more generally, “closure motifs” (65-67). The two conformations of Mad2 differ mainly in the location and conformation of its C-terminal “safety belt” region: in O-Mad2, this region is stably folded against the closure motif binding site (β-strand 6), preventing closure motif binding (68). In C-Mad2, the safety belt is disengaged from this site, allowing a closure motif to bind strand β6. The safety belt in turn translocates to the opposite side of the HORMA domain (strand β5), wrapping around the ligand to create a topological link between HORMA domain and closure motif (66, 67, 69). Coupled to safety belt motions are changes in the domain’s N-terminus: in O-Mad2, the N-terminus associates with strand β5 to stabilize the open conformation. In C-Mad2, however, this region dissociates from the HORMA domain core and is mostly disordered. In the spindle assembly checkpoint, Mad2 is recruited to unattached kinetochores via a closure motif on its binding partner Mad1 (67, 70). This C-Mad2 in
turn recruits soluble O-Mad2, forming a pseudo-symmetric C-Mad2:O-Mad2 dimer via a conserved surface opposite the closure motif binding site (69, 71). Mad2 dimerization promotes conversion of O-Mad2 to the closed state and binding to a closure motif in Cdc20, to form the core of the mitotic checkpoint complex (72-74). Conformational conversion of Mad2 is believed to involve a transient high-energy state in which the safety belt has dissociated from the HORMA domain core, which allows closure motif binding followed by safety belt re-association to produce the C-Mad2:Cdc20 complex (62, 63, 75). Recently, we and others have shown that Pch2/TRIP13 also functions in this pathway, mediating disassembly of Mad2:Cdc20 complexes with the help of an adapter protein, p31 [81](76-82).

The close evolutionary relationship between Mad2 and the meiotic HORMADs, plus the involvement of a shared ATPase regulator, Pch2/TRIP13, strongly suggests mechanistic commonalities between these two protein families. Recently, we showed that the C. elegans meiotic HORMADs interact through association of their N-terminal HORMA domains with conserved closure motifs at their C-termini, and that the resulting hierarchical complex is essential for proper regulation of CO formation and chromosome segregation (83). We further identified closure motifs in the C-termini of the mammalian meiotic HORMADs, and demonstrated binding of these motifs to the HORMAD1 HORMA domain (83). In S. cerevisiae, prior genetic evidence has demonstrated the importance of both its N-terminal HORMA domain and a short, highly-conserved C-terminal region (46, 84, 85). These features, plus
additional evidence of Hop1 self-association in vitro (86), suggests that Hop1 may
also self-assemble through HORMA domain-closure motif interactions. Importantly,
there is currently no evidence for homodimerization of meiotic HORMADs (58),
suggesting that these proteins’ assembly mechanisms are distinct from those of
Mad2. Thus, the question of how meiotic HORMADs’ HORMA domain conformation
and closure motif binding activities are controlled remains an important question.
Specifically, as meiotic HORMADs have only been observed in their closed, closure-
motif bound conformation, it is not known whether they adopt an “open”
conformation similar to Mad2 during closure motif binding and dissociation. Indeed,
no HORMA domain protein other than Mad2 has been shown to possess the
conformational dynamics that would seem to be necessary for assembly and
disassembly of HORMA domain-closure motif complexes. Another major question is
how meiotic HORMADs are initially recruited to the chromosome axis by coiled-coil
“linker” proteins such as Red1, and whether this recruitment also involves HORMA
domain-closure motif interactions.

Here, we present a detailed biophysical characterization of the S. cerevisiae
Hop1 HORMA domain. We identify related motifs in the Hop1 C-terminus and in
Red1 that bind the Hop1 HORMA domain, indicating that HORMA domain-closure
motif interactions are responsible for both initial localization and self-assembly of
Hop1 on the chromosome axis. We further find that the Hop1 HORMA domain
displays conformational dynamics reminiscent of Mad2, and identify a new
conformation of Hop1, termed “unbuckled”, that likely functions analogously to open Mad2. These results outline the assembly mechanisms of the meiotic chromosome axis and reveal a close structural and functional relationship between the meiotic HORMADs and Mad2.

**Materials and Methods**

**Structural Modeling**

An initial structural model of the Hop1 HORMA domain was generated using the One-to-One Threading mode of the PHYRE2 server (http://www.sbg.bio.ic.ac.uk/~phyre2/) using *C. elegans* HTP-1 (PDB ID 4TZO, chain C) as a template (83), then this model was manually adjusted. Side chain rotamers were not modeled. Sequence similarity between closure motifs in *S. cerevisiae* Hop1/Red1 and *C. elegans* HORMADs is not high enough to accurately model the register of the closure motif peptide in this model.

**Protein Purification**

Hop1\(^{2-255}\) and Hop1\(^{2-255}\) LL were expressed in *Escherichia coli* strain Rosetta 2 (DE3) pLysS (EMD Millipore, Billerica MA) at 20° C for 16 hours, then cells were harvested by centrifugation and resuspended in buffer A (20 mM Tris pH 8.5, 10% glycerol) plus 300 mM NaCl, 10 mM imidazole, and 2 mM β-mercaptoethanol. Protein was purified by Ni\(^2+\)-affinity (Ni-NTA agarose, Qiagen) and ion-exchange (Hitrap Q HP, GE Life Sciences, Piscataway NJ) chromatography. Tags were cleaved
with TEV protease (87), and cleaved protein was passed over a size exclusion column (Superdex 200, GE Life Sciences) in buffer A plus 300 mM NaCl and 1 mM dithiothreitol (DTT). Full-length Hop1 (wild-type and K593A), Hop1\textsuperscript{Δ584}, and Red1\textsuperscript{2-362} were purified as above, but at pH 7.5 instead of 8.5.

For size-exclusion chromatography coupled multi-angle light scattering (SEC-MALS), proteins were separated on a Superdex 200 Increase 10/300 GL size exclusion column (GE Life Sciences) in a buffer containing 20 mM HEPES pH 7.5, 300 mM NaCl, 10% glycerol, and 1 mM DTT. Absorbance was measured at 280 nm, and light scattering and refractive index profiles collected by miniDAWN TREOS and Optilab T-rEX detectors (Wyatt Technology, Santa Barbara CA), respectively, and their molecular weights calculated using ASTRA v. 6 software (Wyatt Technology). For size-exclusion chromatography +/- Red1\textsuperscript{345-362} peptide, proteins were pre-incubated with a two-fold molar excess of Red1\textsuperscript{345-362} peptide for 30 minutes at 4°C, then separated on a Superdex 200 Increase 10/300 GL size exclusion column in a buffer containing 20 mM MES pH 6.0, 300 mM NaCl, 10% glycerol, and 1 mM DTT.

**Protein Interaction assays**

Putative closure motifs in Hop1 (residues 584-605) and Red1 (residues 330-362), along with point-mutants, were cloned into a pET3a-based vector with an upstream Kozak sequence and N-terminal maltose binding protein (MBP) tag, and translated \textit{in vitro} with a TNT T7 Transcription/Translation kit (Promega) with \textsuperscript{35}S-methionine. Ni\textsuperscript{2+} pulldown assays were performed with C-terminally His\textsubscript{6}-tagged
Hop1\textsuperscript{1-255}. 10 μg purified bait protein was incubated with 10 μL of the translation reaction in binding buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 20 mM imidazole, 5% glycerol, 1 mM dithiothreitol (DTT), 0.1% NP-40) for 90 min at 4°C, then 15 μl Ni-NTA beads were added, and the mixture was incubated a further 45 min. Beads were washed three times with 0.5 ml buffer, then eluted with 25 μl elution buffer (2x SDS-PAGE loading dye plus 400 mM imidazole) and boiled. Samples were run on SDS-PAGE, then the gel was dried and scanned with a phosphorimager. For competition assays, fluorescein isothiocyanate (FITC)-labeled competitor peptides (Red1\textsuperscript{330-362} (FEDEKLGETFFHVNNIPKISEVQNLVLDYIE) and a sequence-scrambled version (NSVENFIVEGLIFNDKLEPLFHYKDETVIQ) were added prior to addition of in vitro-translated protein, in two-fold molar excess over bait protein.

Ni\textsuperscript{2+} pulldown assays were performed with purified His\textsubscript{6}-MBP fused Red1\textsuperscript{2-362} and Hop1\textsuperscript{584-605}. 50 μL reactions with 10 μg of bait protein plus 30 μg of prey protein (untagged full-length Hop1, Hop1 K593A, Hop1\textsuperscript{1-255}, or Hop1\textsuperscript{2-255}LL) in binding buffer (20 mM HEPES pH 7.5, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 1 mM DTT, 0.1% NP-40) were incubated 2 hours at room temperature. Samples were then mixed with 20 ul of magnetic Ni-NTA beads (5% suspension; Qiagen) and incubated with rotation for 30 minutes. Beads were washed three times with 0.5 ml binding buffer, eluted with 25 μl of elution buffer (2x SDS-PAGE loading dye plus 400 mM imidazole) then boiled. Samples were analyzed by SDS-PAGE, and visualized by Coomassie Blue staining.
For fluorescence polarization peptide binding assays, FITC-labeled peptides (BioMatik) at 50 nM were incubated with 12 nM-100 µM Hop1\textsuperscript{2-255} or Hop1\textsuperscript{2-255}LL in 50 µL binding buffer for 30 minutes, then transferred to a 384-well black plate. Fluorescence polarization of triplicate reactions were read using a Tecan Infinite M1000 PRO fluorescence plate reader, and binding data were analyzed with Graphpad Prism v.6 using a single-site binding model.

**Hydrogen-Deuterium Exchange Mass Spectrometry**

HD exchange experiments were conducted with a Waters Synapt G2S system. 5 uL samples in exchange buffer (20 mM HEPES pH 7.5, 300 mM NaCl, 10% Glycerol) were mixed with 55 µL of D\textsubscript{2}O buffer for several deuteration times (10 sec, 30 sec, 1 min, 2 min, 5 min) at 15 °C. The exchange was quenched for 2 min at 1°C with an equal volume of quench buffer (200 mM iodoacetic acid (IAA), 400 mM Tris-HCl pH 8.5, 6.2 M urea, 2 mM EDTA). A portion of the quenched sample (50 µL) was injected onto an inline pepsin column (Applied Biosystems, Poroszyme Immobilized Pepsin cartridge). The resulting peptic peptides were then separated on a C18 column (Waters, Acquity UPLC BEH C18, 1.7 µm, 1.0 mm x 50 mm) fit with a Vanguard trap column using a 3-85% acetonitrile (containing 0.1% formic acid) gradient over 12 min at a flow rate of 40 µL/min. The separated peptides were directed into a Waters SYNAPT G2s quadrupole time-of-flight (qTOF) mass spectrometer. The mass spectrometer was set to collect data in the MSE, ESI+ mode; in a mass acquisition range of m/z 255.00–1950.00; with a scan time of 0.4 s. Continuous lock mass
correction was accomplished with infusion of the LeuEnk peptide every 30 s (mass accuracy of 1 ppm for the calibration standard). Peptides were identified using PLGS version 2.5 (Waters, Inc.). The relative deuterium uptake for each peptide was calculated by comparing the centroids of the mass envelopes of the deuterated samples with the undeuterated controls using DynamX version 2.0 (Waters Corp.).

For analysis of peptides showing bimodal HD exchange behavior, assigned peaks for spectra collected at the 5-minute time-point were exported as mass/charge versus intensity, normalized to a maximum intensity of 1.0 for each spectrum, and triplicate samples were fit in Graphpad Prism (version 6; Graphpad Software) to either a single Gaussian distribution:

\[ Y = \text{Amplitude} \times e^{-\frac{(X-\text{Mean})^2}{2\times\text{StDev}^2}} \]

where Amplitude is the amplitude, Mean is the mean, and StDev is the standard deviation of the function, or a sum of two Gaussian distributions:

\[ Y = \left(\text{Amplitude}_{one} \times e^{-\frac{(X-\text{Mean}_{one})^2}{2\times\text{StDev}_{one}^2}}\right) + \left(\text{Amplitude}_{two} \times e^{-\frac{(X-\text{Mean}_{two})^2}{2\times\text{StDev}_{two}^2}}\right) \]

where Amplitude\textsubscript{one}, Mean\textsubscript{one}, and StDev\textsubscript{one} apply to the first Gaussian function, and Amplitude\textsubscript{two}, Mean\textsubscript{two}, and StDev\textsubscript{two} apply to the second.
Results

Hop1 and Red1 possess similar Hop1 HORMA domain-binding “closure motifs”

To better understand the functional parallels between S. cerevisiae Hop1 and its orthologs in C. elegans and mammals, we first sought to determine if Hop1 self-assembles through interactions between its N-terminal HORMA domain and a C-terminal closure motif (83). Sequence alignments show that fungal Hop1 proteins share a highly conserved C-terminal Domain (CTD) of about 20 amino acids (Figure 2.1A). Prior work has shown that deletion of the C-terminal 20 residues of Hop1, or mutation of the highly-conserved “KIS” motif spanning residues 593-595 (K593A or S595N) dramatically reduces meiotic DSB and CO numbers and causes high spore lethality, indicating an important role for this domain in Hop1 function (46, 85, 88).

We noticed that the Hop1 C-terminus bears limited sequence homology with a region of Red1 previously shown to interact directly with Hop1, notably including a “KIS” motif spanning Red1 residues 348-350 (Figure 2.1A) (24). Further, a Red1 K348E mutant has previously been shown to eliminate its ability to bind Hop1, leading to reduced COs, defective homolog synapsis, and low spore viability (24). Together, the similarity of these motifs and their importance for axis structure and function suggest that both Hop1 and Red1 contain closure motifs that bind the Hop1 HORMA domain.
Figure 2.1: Hop1 and Red1 contain putative “closure motifs” with similar sequence

(A) Domain diagrams of *S. cerevisiae* Hop1 and Red1, with known domains and domain boundaries marked (Zn$^{2+}$: Hop1 domain containing a conserved Cys4-His-Cys3 Zn$^{2+}$-binding motif; CC: coiled-coil). Bottom: sequence alignment of putative closure motifs in Red1 and Hop1. (B) Structural model of the Hop1 HORMA domain, based on the structure of *C. elegans* HTP-1 bound to a closure motif from HIM-3 (PDB ID 4TZO; [83]). For sequence alignment, see Figure 2.5A. The β5-αC loop (residues 135-158) replaced with GSG in Hop1$^{1-255}$ LL, is shown in green. The safety belt region is shown in magenta, and the bound closure motif is shown in yellow.
We defined the HORMA domain of *S. cerevisiae* Hop1 by modeling the structure of this domain onto our prior structure of *C. elegans* HTP-1 (15% identity and 35% similarity to Hop1 in this domain) (Figure 2.1B, S2.1). We expressed and purified an isolated Hop1 HORMA domain construct (Hop1^{2-255}), and showed that this truncated construct as well as full-length Hop1 robustly bind purified Red1^{1-362}, which encompasses the ordered N-terminal region of this protein plus the region previously shown to interact with Hop1 (Figure S2.2). Because of high protease-sensitivity of purified full-length Red1, we were unable to demonstrate a direct interaction between purified full-length Hop1 and Red1 proteins.

We next tested binding of Hop1^{2-255} to fluorescently-labeled peptides encoding residues 345-362 of Red1 and 584-605 of Hop1. We detected robust binding between Hop1^{2-255} and the Red1^{345-362} peptide ($K_d = 340$ nM), and weaker but detectable binding to the Hop1^{584-605} peptide ($K_d = 6$ µM) (Figure 2.2A). In both cases, binding was abolished by point-mutations in the peptides based on previously identified mutations, Hop1 K593A and Red1 K348E (Figure 2.2A) (46). We next used a Ni$^{2+}$ pulldown assay with His6-tagged Hop1^{2-255} and *in vitro*-translated Hop1^{584-605} or Red1^{345-362} (produced as maltose binding protein fusions). As in the prior assays, we observed binding of both peptides that was disrupted by mutation of the conserved lysine residue in the “KIS” motif (Hop1 K593A/Red1 K348E) (Figure 2.2B). Moreover, binding of Hop1^{584-605} to Hop1^{2-255} was disrupted by addition of a two-fold molar excess of unlabeled Red1^{345-362} peptide, revealing that the two putative closure
motifs compete for a common binding site on the Hop1 HORMA domain (Figure 2.2B). Together, these data strongly suggest that Red1 and Hop1 possess similar HORMA domain-binding closure motifs. Further, Hop1 likely shares the propensity to self-associate through HORMA domain-CTD binding with its orthologs in C. elegans and mammals. When combined with prior data showing that the hop1-K593A mutation causes an 11-fold reduction in COs and results in high spore lethality (46), our data argues that Hop1 self-association at the meiotic chromosome axis is critically important for its regulatory functions.

As the Hop1 protein possesses a putative closure motif on its own C-terminus, this motif might be expected to compete for binding to peptides added in trans. To test this idea, we expressed and purified full-length Hop1, as well as variants with either the K593A mutation (Hop1^K593A) or with residues 585-605 in the CTD deleted (Hop1^Δ584), and tested their binding to the Red1^345-362 peptide. Compared to Hop1^2-255, we found that full-length Hop1 bound Red1^345-362 with dramatically lower affinity ($K_d = 2 \mu M$ vs. 340 nM for Hop1^2-255). In contrast, both Hop1^K593A ($K_d = 51$ nM) and Hop1^Δ584 ($K_d = 450$ nM) bound Red1^345-362 comparably to Hop1^2-255 (Figure 2.2C). Together, these data suggest that while Hop1’s own CTD does compete with a Red1 peptide added in trans for HORMA domain binding, the higher affinity of Red1 for the Hop1 HORMA domain nonetheless likely enables Red1 to recruit full-length Hop1 directly to the chromosome axis.
Figure 2.2: Closure motif peptide binding by Hop1

(A) Fluorescence polarization binding assays for Hop1<sup>2-255</sup> with four peptides: Hop1<sup>584-605</sup> (blue, \(K_d = 6.1 \pm 1.0 \mu M\)), Hop1<sup>584-605</sup> K593A (dashed blue, \(K_d\) could not be fit), Red1<sup>345-362</sup> (red, \(K_d = 340 \pm 30 \text{nM}\)), and Red1<sup>345-362</sup> K348E (dashed red, \(K_d\) could not be fit). Error bars indicate standard deviation from triplicate measurements. (B) Ni<sup>2+</sup>-pulldown assay with His<sub>6</sub>-tagged bait MBP (maltose binding protein; negative control) or Hop1<sup>2-255</sup> (C-terminal tag) and in vitro-translated MBP-fused Red1<sup>330-362</sup>, Red1<sup>330-362</sup> K348E, Hop1<sup>584-605</sup>, and Hop1<sup>584-605</sup> K593A. Competitor peptides were added at a two-fold molar excess over bait protein. (C) FP Binding assay for Red1<sup>345-362</sup> binding Hop1<sup>2-255</sup> (blue, \(K_d = 340 \pm 30 \text{nM}\)), full-length Hop1 (green, \(K_d = 2.0 \pm 0.1 \mu M\)), K593A (orange, \(K_d = 51 \pm 5 \text{nM}\)), \(\Delta 584\) (black, \(K_d = 450 \pm 50 \text{nM}\)). See Figure 2.S2 for Ni<sup>2+</sup> pulldown assays of Hop1 binding Red1<sup>330-362</sup> and Hop1<sup>584-605</sup>. 
The Hop1 HORMA domain adopts two distinct conformations in solution

During the purification of Hop1^{2-255}, we noticed that the protein elutes from a size-exclusion column as two closely-spaced peaks, one with an elution volume consistent with a compact monomer and the other consistent either with a dimer or an extended monomer (Figure 2.3A). Further analysis by size exclusion chromatography coupled to multi-angle light scattering (Figure 2.3B) showed that both peaks contain monomeric Hop1^{2-255}, demonstrating that the domain can likely adopt two different conformations in solution: one compact, and one more extended. We wondered how these two conformations of Hop1^{2-255} might relate to the previously-described “closed” and “open” states of Mad2. We found that pre-incubation of Hop1^{2-255} with the Red1^{330-362} peptide shifted the equilibrium toward the later-eluting, more-compact size exclusion peak. This finding suggests that the more-compact peak represents “closed” Hop1 HORMA domain, and that the earlier-eluting, less-compact peak represents a second, potentially open-like conformation.

Consistent with the idea that the HORMA domain of full-length Hop1 can associate with its own C-terminal tail, we fund that full-length Hop1 elutes from a size-exclusion column as a single peak (Figure 2.3B).

To further examine the conformational differences between the two states of the Hop1 HORMA domain, we generated several Hop1^{2-255} variants based on mutations known to stabilize either the open or closed conformation of Mad2 (66, 89, 90), and examined their behavior in solution. While most variants destabilized
Hop1^{2-255}, one markedly improved expression and stability of the protein. This mutant, referred to as “loopless” or Hop1 LL (residues 135-158 replaced by a Gly-Ser-Gly linker), is based on the Mad2 “loopless” construct in which the extended β5-αC loop is replaced by a short linker (69). This mutation is thought to inhibit O-Mad2 to C-Mad2 conversion by preventing dissociation of the protein’s N-terminus from the β5 strand, thereby disallowing safety belt movement and conversion to the C-Mad2 state. The structural effect of this mutant in Hop1 is difficult to predict, as the β5-αC loop is longer and more well-ordered in meiotic HORMADs than in Mad2, draping over the safety belt β-strands 8’ and 8” (Figure 2.1B) (83). We found that in contrast to wild-type Hop1^{2-255}, Hop1^{2-255} LL migrated as a single species on a size-exclusion column consistent with a compact monomer, and that its elution profile was mostly unaffected by addition of the Red1^{330-362} peptide (Figure 2.3A, B). We next tested binding of Hop1^{2-255} LL to the putative closure motif peptides Hop1^{584-605} and Red1^{345-362}, and found that the mutated protein robustly binds both peptides; indeed, the loopless mutation seems to subtly increase peptide binding affinity compared to unmutated Hop1^{2-255} (Figure 2.3C). As the putative closure-motif binding site of Hop1 is not altered in this mutant, we interpret this slightly-increased affinity as indicating that the loopless mutation stabilizes the peptide-binding conformation of the Hop1 HORMA domain; namely, the “closed” conformation.
Figure 2.3: The Hop1 HORMA domain shows two-state behavior in solution

(A) Size exclusion chromatography of Hop1^{2-255} alone (blue), Hop1^{2-255} + Red1^{345-362} (yellow), Hop1^{2-255} LL alone (green), and Hop1^{2-255} LL:Red1^{345-362} (orange). (B) Size exclusion chromatography/multi-angle light scattering (SEC-MALS) analysis of full-length Hop1 (absorbance at 280 nm in black, measured molecular weight in grey), Hop1^{2-255} (blue, two peaks defined as P1 and P2) and Hop1^{2-255} LL (green). Below: calculated and measured molecular weights for all constructs. (C) FP Binding assay for Hop1^{2-255} LL binding putative closure motif peptides Hop1^{584-605} (blue), Hop1^{584-605} K593A (dashed blue), Red1^{345-362} (red), and Red1^{345-362} K348E (dashed red). Error bars indicate standard deviation from triplicate measurements. Below: Peptide-binding $K_d$’s for Hop1^{2-255} (from Figure 2.2A) and Hop1^{2-255} LL.
Conformational dynamics of the Hop1 HORMA domain revealed by hydrogen-deuterium exchange mass spectrometry

We next sought further structural insight into the relationship between Hop1 HORMA domain conformation and peptide binding. As we were unable to identify crystallization conditions for the Hop1 HORMA domain or its complex with the Red1 closure motif, and low expression levels prevented examination by NMR, we instead turned to hydrogen-deuterium exchange mass spectrometry (HDX-MS). In HDX-MS, exchange of backbone amide hydrogen atoms with deuterium from D$_2$O-containing buffer provides information on the solvent-accessibility and secondary structure of different regions of a protein. We detected mass spectra for a set of common peptides in Hop1$^{2-255}$ and Hop1$^{2-255}$LL (covering 61% and 70% of the two constructs, respectively), including the C-terminal safety belt region but notably excluding the β5-αC loop (deleted in the Hop1 LL mutant, and not detected in Hop1$^{2-255}$) and the N-terminus (Figure S2.3A-B). The rates of deuterium uptake in different regions largely agreed with predicted secondary structure in our model of the Hop1 HORMA domain based on C. elegans HTP-1 in the “closed” conformation (Figure S2.3C-D) (83). This was especially true for Hop1$^{2-255}$LL, which our size-exclusion chromatography analysis shows adopts exclusively the more-compact conformation.

We next compared HDX-MS profiles of Hop1$^{2-255}$ and Hop1$^{2-255}$LL in the absence and presence of the Red1$^{345-362}$ peptide. We found that all regions of Hop1$^{2-255}$ become more protected from H-D exchange in the presence of Red1$^{345-362}$ (Figure
Supporting our assignment of Red1\textsuperscript{345-362} as a closure motif, Hop1\textsuperscript{2-255} regions adjacent to the putative closure motif binding site, including residues 182-195 and 198-211, are the most protected upon Red1\textsuperscript{345-362} binding (Figure 2.4A, D, E).

Residues 91-99 in helix $\alpha$B, which is positioned just behind the closure motif binding site, as well as residues 74-90 in the $\beta$2-$\beta$3 hairpin adjacent to helix $\alpha$B, also show a high degree of protection upon Red1\textsuperscript{345-362} binding (Figure 2.4B, E). Finally, we also observed significant protection in regions corresponding to the $\beta$8$'$ and $\beta$8$''$ strands of the safety belt (residues 234-248), as well as helix $\alpha$C (residues 164-173), which packs against these $\beta$-strands in the closed HORMA domain conformation (Figure 2.4C, E). Significant protection of the safety belt and adjacent regions upon binding of Red1\textsuperscript{345-362} strongly suggests that the two Hop1 conformations we observe in solution differ largely in the conformation of the safety belt, consistent with the known conformational changes of Mad2.

In Hop1\textsuperscript{2-255}LL, H-D exchange rates for most regions, including peptides within and adjacent to the safety belt (residues 165-173 and 234-248), are much less affected by the addition of Red1\textsuperscript{345-362} than in wild-type Hop1\textsuperscript{2-255}, supporting the idea that this mutant pre-forms the more-compact closed conformation in the absence of closure motif peptides (Figure 2.4C, S4B). Notable exceptions include residues 182-195 and 198-211, which make up the putative closure motif binding site: these regions would be expected to show different H-D exchange rates based on closure motif binding alone (Figure 2.4A, F). Thus, Hop1\textsuperscript{2-255}LL likely adopts an
“empty” closed conformation, to which closure motif peptides can directly associate without HORMA domain conformational conversion.

**Figure 2.4: HD exchange in Hop1^{2-255} and Hop1^{2-255} LL**

(A) Deuterium uptake plots of Hop1 of residues 182-195 and 198-211, corresponding to the β6 strand and putative closure motif binding site, for Hop1^{2-255} (blue, +Red^{345-362} in yellow) and Hop1^{2-255} LL (green, +Red^{345-362} in orange). (B) Uptake of residues 74-90 and 91-99, corresponding to the β2-β3 hairpin and helix α.B. (C) Uptake of residues 164-173 and 234-248, corresponding to portions of the α.C-helix and β8'-β8" of the safety belt. See Figure 2.53 for exchange data on all detected peptides. (Continued on next page)
Figure 2.4: HD exchange in Hop1<sup>2-255</sup> and Hop1<sup>2-255 LL</sup> (Continued)

(D) Structural model of Hop1<sup>2-255</sup> showing regions described in panels (A-C), closure motif binding site (light blue), β2-β3 hairpin and helix αB (light green), safety belt (pink). (E) Structural model of Hop1<sup>2-255</sup> colored according to the difference in relative deuterium uptake between the unbound and Red1<sup>345-362</sup> bound state. Blue indicates no change in HD exchange rate, and magenta indicates significantly less exchange (more protection) upon binding the closure motif peptide. Regions shown in gray were not detected. (F) Structural model of Hop1<sup>2-255 LL</sup>, showing protection upon binding Red1<sup>345-362</sup> as in panel (E). See Figure 2.S4 for exchange differences over time for Hop1<sup>2-255</sup> and Hop1<sup>2-255 LL +/- Red1<sup>345-362</sup></peptide.}
Our size-exclusion chromatography data showed that in the absence of a bound peptide, Hop1<sup>2-255</sup> adopts two different conformations in solution. Consistent with this finding, a number of peptides in the Hop1<sup>2-255</sup> construct show a bimodal distribution of H-D exchange rates (Figure 2.5). Bimodal H-D exchange rate distributions arise when the region in question can stably adopt two differently solvent-exposed conformations, resulting in high-exchanging (more solvent-exposed) and low-exchanging (less solvent-exposed) populations. The regions showing the most pronounced bimodality include residues 234-248, spanning β8’ and β8” in the safety belt (Figure 2.5B), residues 164-173 in the adjacent αC-helix (Figure 2.5C), and residues 40-49 and 74-90 in the β2-β3 hairpin (Figure 2.5D-F). These peptides’ bimodal behavior is nearly eliminated in the presence of the Red1<sup>345-362</sup> peptide, and they all show a monomodal H-D exchange rate distribution in Hop1<sup>2-255</sup>LL regardless of Red1<sup>345-362</sup> binding (Figure 2.5A-C). Taken together, our HDX-MS data as a whole supports our identification of two stable conformations of the Hop1 HORMA domain, and suggest that the two states differ largely in the conformation of the domain’s C-terminal safety belt region. While the strong bimodality in H-D exchange rates in these regions of wild-type Hop1<sup>2-255</sup> shows that this construct populates both states in solution, addition of the Red1<sup>345-362</sup> peptide shifts the equilibrium strongly in favor of the “closed” conformation (Figure 2.5G). Hop1<sup>2-255</sup>LL shows a strong bias toward
Figure 2.5: Bimodal HD exchange in Hop12-255

(A) Graphs showing normalized peak intensity versus mass/charge ratio (m/z) at 5 minutes exposure to D2O for an example monomodal peptide, Hop191-99. Top: Hop191-99, alone (blue) and with Red1345-362 (yellow). Bottom: Hop12-255 LL alone (green), and with Red1345-362 (orange). Each dataset was fit to a single Gaussian distribution. (B) Graphs for Hop1163-173 showing a bimodal distribution of HD exchange. For this peptide, Hop12-255 (+/-Red1345-362) was fit to a sum of two Gaussian distributions, and Hop12-255 LL (+/-Red1345-362) was fit to a single Gaussian distribution. (C) As (B), for Hop1234-248. In this case, only Hop12-255 alone was fit to a sum of two Gaussian distributions. (D) and (E) Graphs for Hop140-49 and Hop174-90, showing only data for Hop12-255 (data for Hop12-255 LL was not annotated well enough to fit). (F) Location of peptides showing bimodal HD exchange behavior (blue) on the modelled structure of Hop12-255 bound to a closure motif (yellow). (G) Schematic of proposed Hop1 HORMA domain equilibrium in four states. Hop1LL (orange) shows a strong bias toward the closed conformation compared to wild-type (blue), while addition of the Red1345-362 peptide to either wild-type (yellow) or loopless Hop1 (orange), further shifts the equilibrium toward the closed conformation.
the closed state on its own, which is further shifted toward this state upon binding the Red1$^{345-362}$ peptide.

**Discussion**

In this study, we present a combined biochemical and biophysical characterization of the *S. cerevisiae* meiotic HORMA domain protein Hop1, identifying Hop1-binding closure motifs in both Hop1 and its binding partner Red1, and characterizing two distinct conformations of the Hop1 HORMA domain. When combined with prior genetic data, our results strongly suggest that HORMA domain-closure motif interactions underlie both the initial association and self-assembly of Hop1 on meiotic chromosomes. As both *C. elegans* and mammalian meiotic HORMADs self-assemble through HORMA domain-closure motif interactions (83), and most eukaryotes possess a Red1-like “linker” protein required for axis assembly, our results suggest that initial association of meiotic HORMADs with meiotic chromosomes may also be mediated by HORMA domain-closure motif interactions. In *C. elegans*, our earlier work suggested that HTP-3 may bind directly to a subunit of the cohesin complex (83). Mammalian HORMAD1 and HORMAD2 may also bind cohesin complexes directly, or alternatively bind a closure motif in SYCP2 or SYCP3, in a parallel of the Hop1-Red1 association (22, 91).

Our data suggest that Hop1 initially binds chromosomes through a closure motif in Red1, followed by additional Hop1 recruitment via head-to-tail oligomerization (Figure 2.6A). The competition we observe between Red1 and
Hop1’s own C-terminus for binding the HORMA domain suggests that in solution, Hop1 tends to form a “self-closed” state but that recruitment of Hop1 to the chromosome axis by Red1 displaces the Hop1 CTD from this protein’s HORMA domain. This Hop1 may in turn recruit additional Hop1 monomers from solution, leading to multimeric assembly. In principle, Hop1 oligomerization on the axis would be favored if conformational or other constraints not present in our reconstituted system were to inhibit self-interaction relative to oligomerization. Such constraints could include post-translational modifications and/or partner protein binding in the region between Hop1’s HORMA domain and CTD, that could impair self-interaction and thereby promote oligomer formation. Pch2 may also contribute to Hop1 oligomerization (at least in some systems; see below), but its role is likely to be purely kinetic; that is, Pch2 could generally stimulate Hop1 conformational dynamics but is unlikely to alter the equilibrium Hop1 oligomer length unless it is specifically localized to chromosomes (see below). While we are not yet able to explain how Hop1 oligomers form, the strong effects on DSB and CO levels, homolog synapsis, and overall spore viability observed when mutating or deleting the Hop1 CTD (46, 85) constitute strong indirect evidence that Hop1 oligomerization plays a key role in CO formation. Interestingly, the CO defect caused by a hop1-K593A mutant can be rescued by ectopic dimerization of Mek1, through its fusion to GST (46). This result suggests that one role of Hop1 self-association is to activate Mek1, likely by bringing multiple copies of the kinase into close proximity at the chromosome axis to enable trans-autoactivation (Figure 2.6A). Notably, the defect in both DSB and CO levels in a
hop1-K593A strain is significantly less severe than in hop1Δ, and the Hop1 K593A mutant protein behaves well in vitro (this work) and localizes to meiotic chromosomes (46). These data support the idea that Red1 can recruit Hop1 directly, but that additional Hop1 recruitment through head-to-tail binding is nonetheless required to support wild-type levels of DSB formation and Mek1 kinase activation, which combine to support CO formation.

In addition to the meiotic HORMADs, HORMA domains are found in Mad2, Rev7, and two autophagy-related proteins, Atg13 and Atg101 (57, 65). While Mad2 has been shown to adopt two stable conformations in solution, similar conformational dynamics have not been observed in any other HORMA domain protein family (65). Here, we show for the first time that the S. cerevisiae meiotic HORMAD Hop1 can adopt two different stable states in solution: a closed conformation similar to what we previously observed for the C. elegans meiotic HORMADs (83), and a more extended conformation. Our HDX-MS data indicate that the two states differ mainly in the conformation of the protein’s C-terminal safety belt region, and that this region is probably disengaged from the HORMA domain core in the extended state. This model contrasts with Mad2’s open conformation, in which the safety belt is stably associated with the closure-motif binding site, generating a structure that is similarly compact to the protein’s closed conformation (92) (Figure 2.6B). The extended state of the Hop1 HORMA domain may therefore correspond more closely with the theorized transient Mad2 intermediate state, in
which the safety belt has disengaged from the HORMA domain core to allow Cdc20
closure motif binding (62, 69) (Figure 2.6B). Because the term “intermediate” has
more recently been used to refer to a compact Mad2 conformation adopted just
prior to this conformational change (75), we instead introduce the term “unbuckled”
to refer to a HORMA domain conformation in which the safety belt is stably
disengaged from the HORMA domain (Figure 2.6C).

While we designed the Hop1 loopless mutation based on a Mad2 variant that
stabilizes this protein’s open conformation (69), this mutation appears to stabilize
the closed conformation in Hop1. In Mad2, truncation of the β5-αC loop is thought to
disallow dissociation of the protein’s N-terminal β1 strand from strand β5 in the
HORMA domain core, thereby inhibiting the open-to-closed conformational change
(69). We propose that Hop1 may not possess an N-terminal β1 strand, and that the
“unbuckled” conformation is instead stabilized by the extended β5-αC loop itself. As
this loop is much longer in meiotic HORMADs than in MAD2 (24 residues in Hop1
versus 9 in Mad2), the loop could bind against β5 after safety belt dissociation to
stabilize the unbuckled state. In this scheme, truncation of the β5-αC loop would be
expected to destabilize the unbuckled state, thereby favoring the closed state as we
observe for Hop1^{2-255}LL (Figure 2.6C). It is important to note that, while Hop1^{2-255}LL
binds closure-motif peptides at least as tightly as wild-type Hop1^{2-255}, this mutant is
likely to be strongly defective in vivo. As binding of a closure-motif peptide to an
“empty” closed Hop1 requires that the peptide thread underneath part of the safety
belt region, binding of the Red1 closure motif (more than 300 amino acids from both termini of Red1) would be expected to require a conformational conversion in Hop1, much as in Mad2. Our efforts to introduce the Hop1 LL mutation into *S. cerevisiae* to test this idea have so far been unsuccessful, due to extremely poor expression of the protein. As Hop1<sup>2-255</sup>LL is more highly expressed in *E. coli* and is extremely stable in solution, the low levels of Hop1 LL we observe in *S. cerevisiae* may indicate that the protein is unstable, perhaps due to an inability to assemble on meiotic chromosomes.

Our data indicate that in *S. cerevisiae*, chromosome axis assembly is mediated in large part by binding of the Hop1 HORMA domain to closure motifs in both Hop1 and Red1. In contrast to Mad2, where assembly with Cdc20 occurs specifically at unattached kinetochores through dimerization-catalyzed conformational conversion, we envision meiotic chromosome axis assembly to be largely spontaneous. A major remaining question, however, is how meiotic HORMADs are removed from the chromosome axis by Pch2/TRIP13 in coordination with synaptonemal complex assembly (*Figure 2.6A*). We have recently shown that mammalian TRIP13 binds the disordered N-terminus of Mad2 and then partially unfolds the protein to mediate the disengagement of the Mad2 safety belt from the HORMA domain core (76, 93). We further showed that N-terminal truncation of mouse HORMAD1 inhibits its TRIP13-mediated disassembly from meiotic chromosome axes, highlighting a common remodeling mechanism with Mad2 (93). We propose that Pch2/TRIP13 may be important to drive HORMA domain conformational dynamics and closure motif
association/dissociation by meiotic HORMADs throughout eukaryotes. In this scheme, synaptonemal complex assembly triggers recruitment of Pch2/TRIP13 to the chromosome axis, where it removes HORMADs by disassembling HORMA domain-closure motif interactions. An important question in this regard is whether there exists two pools of meiotic HORMADs on chromosome axes, distinguished by their binding partners: in both *S. cerevisiae* and mammals, not all Hop1/HORMAD1 is removed from the axis upon synaptonemal complex assembly (11-13, 28, 94). This finding, plus the higher affinity of Hop1 for the Red1 closure motif and the location of the Red1 closure motif far from either terminus of this protein, suggests that Pch2 might act differently on Hop1-Hop1 vs. Hop1-Red1 complexes.

In some systems, notably the rice *Oryza sativa*, mutation of Pch2/TRIP13 causes a loss of initial association of meiotic HORMADs with the chromosome axis (95). This observation suggests another potential role of Pch2/TRIP13, that also parallels earlier findings with Mad2. In the spindle assembly checkpoint, a pool of O-Mad2 is required for kinetochore-recruitment and assembly with Cdc20, and TRIP13 is required to maintain this pool of O-Mad2 (76, 96). In a similar vein, if the meiotic HORMADs in a given organism are much more stable in the closed conformation, Pch2/TRIP13 may be required to stimulate their dynamics to promote initial assembly of the chromosome axis, in addition to its later functions in HORMAD disassembly.

Overall, the role of Pch2/TRIP13 in promoting function of meiotic HORMADs, and
especially its detailed mechanism of meiotic HORMAD recognition and remodeling, will be an interesting avenue for future research.

Figure 2.6: Model for Hop1 HORMA domain dynamics and meiotic chromosome axis assembly/disassembly

(A) Cartoon model of Hop1’s recruitment, multimeric assembly, and disassembly along the chromosome axis. Hop1 (dark blue) is recruited to chromosomes by binding the Red1 closure motif (yellow). The displaced CTD closure motif serves to recruit additional copies of Hop1 (light blue) to the chromosome axis through self-association. Self-association of Hop1 facilitates the trans-autoactivation of Mek1, resulting in a suppression of sister chromosome strand invasion and biasing DSB repair toward the homolog. After CO formation, Pch2 facilitates the removal of Hop1 from the axis by disrupting closure-motif interactions as the synaptonemal complex forms. (B) Cartoon representation and energy diagram of the Mad2 safety belt mechanism. The dimerization-stimulated conversion from O-Mad2 to C-Mad2 upon closure motif binding requires dissociation of the N-terminus from the core of the HORMA domain and translocation of the safety belt (magenta). TRIP13 facilitates the conversion back to O-Mad2 by disengaging the safety belt from the HORMA domain core. (C) Cartoon representation and energy diagram of the proposed Hop1 safety belt mechanism. Closure motif binding stabilizes the closed state by promoting binding of the safety belt (magenta) to the HORMA domain core. The β5-αC loop (green) stabilizes the “unbuckled” state through an unknown mechanism. Pch2/Trip13 promotes a closed-to-unbuckled conformational change of Hop1 by disengaging the safety belt from the HORMA domain core.
Chapter 2 Supplementary Material:

Figure 2.S1: Modeling Hop1 HORMA domain structure

Sequence alignment of *S. cerevisiae* Hop1 and *C. elegans* HTP-1 used for modeling Hop1 HORMA domain structure. Insertions in Hop1 are shown in red, and insertions in HTP-1 are shown in orange. Residues 135-158, replaced by “GSG” in Hop1 LL, are shown with a green box.
Figure 2.S2: Ni\textsuperscript{2+} Pulldown assay of Hop1 constructs
Ni\textsuperscript{2+}-pulldown assay with (left to right) no bait, His\textsubscript{6}-MBP (maltose binding protein), His\textsubscript{6}-MBP-Red\textsubscript{1-362}, and His\textsubscript{6}-MBP-Hop1\textsubscript{584-605} as baits (green arrows). Untagged prey proteins include full-length Hop1, Hop1 K593A, Hop1\textsuperscript{1-255}, and Hop1\textsuperscript{2-255}LL (red arrows). Top row show input prior to the addition of Ni-NTA beads, bottom row shows proteins eluted from the beads. Red star indicates a contaminant in purified His\textsubscript{6}-MBP-Hop1\textsubscript{584-605}.
Figure 2.S3: HD Exchange behavior of Hop$^{1-255}$ and Hop$^{2-255}$ LL (Continued on next page)

(A) Schematic of peptide coverage and HD exchange rates for Hop$^{1-255}$, both alone (unbound, upper bars) and bound to a closure motif peptide (+Red1$^{345-362}$, lower bars). Magenta represents relatively little HD exchange (more protection), and blue represents high exchange (less protection). HD exchange is represented as a percentage of deuterium uptake relative to the maximum possible uptake for each peptide (one D per residue). (B) Data from panel (A) mapped onto structural model of the Hop1 HORMA domain.
Figure 2.S3: HD Exchange behavior of Hop1^{2-255} and Hop^{2-255} LL (Continued)

(C) Schematic of peptide coverage and HD exchange rates for Hop1^{2-255} LL, as in panel (A). (D) Data from panel (C) mapped onto structural model of the Hop1 HORMA domain.
Figure 2.54: Protection from HD exchange upon closure motif binding

(Continued on next page)

(A) Change in relative fractional deuterium uptake in Hop1^{2-255} upon binding Red1^{345-362}. Magenta represents no change in HD exchange rate, and blue represents significantly less exchange (more protection) upon binding the closure motif peptide.
Figure 2.S4: Protection from HD exchange upon closure motif binding (Continued)

(B) Change in relative fractional deuterium uptake in Hop1<sup>2-255</sup> LL upon binding Red1<sup>345-362</sup>. Whereas most of Hop1<sup>2-255</sup> is stabilized by Red1<sup>345-362</sup> binding, protection in Hop1<sup>2-255</sup> LL is confined to peptides immediately adjacent to the closure motif binding site.
Acknowledgements:

Chapter 2, in full, is a manuscript submitted and accepted for publication by Nucleic Acids Research and is currently in press as: West, Alan M.V., Komives, Elizabeth A., Corbett, Kevin D. Conformational dynamics of the Hop1 HORMA domain reveal a common mechanism with the spindle checkpoint protein Mad2. The dissertation author is the primary investigator and author of this paper.

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Chapter 3

Structural basis of chromosome axis assembly through oligomerization of the Red1 C-terminus

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Introduction:

As chromosomes condense during early prophase of the first meiotic division, each pair of sister chromatids becomes organized along a proteinaceous structure called the chromosome axis, which orchestrates the introduction and subsequent repair of deliberate double strand breaks (DSBs) via interhomolog crossover recombination, thereby facilitating the proper pairing of homologous chromosomes. Protein interactions within the chromosome axis maintain cohesion between sister chromatids, coordinate the events of homologous recombination and relay cell cycle arrest signals (reviewed in (1)). Proper pairing and recombination between homologous chromosomes culminates in the process of synapsis, in which a large zipper-like proteinaceous structure, known as the synaptonemal complex (SC), is formed between the axes of paired homologs and connects them along their entire lengths.
In addition to facilitating pairing and recombination between homologs, the meiotic chromosome axis is the basis of synapsis and SC formation. Upon synopsis, elements of the chromosome axis are incorporated into the growing SC, whereupon they are referred to as the lateral elements (LEs). Between the LEs of each homolog lies the central region comprised of two distinct substructures: the central element (CE), an electron dense linear structure running parallel to the LEs, and the transverse filaments (TFs) which run perpendicular to the LE and attach them to the CE (2, 3). Although the overall structure of the SC is conserved among different organisms, the lack of sequence homology between SC proteins belonging to different species means that each newly identified SC component must be characterized individually in order to determine its position and role in the higher-order SC assembly and structure. Here we will focus on interactions within the budding yeast meiotic axis/LEs that contribute to SC assembly and function.

In *S. cerevisiae*, Zip1 is the major structural component of the SC transverse filament. Consisting of poorly-ordered N- and C-termini linked by an extensive central coiled-coil region, Zip1 spans the width of the SC with the N-termini interacting with the central element and the C-termini oriented toward the chromosome axes/LE (4-7). The axis/LEs in yeast contains the axis proteins Red1, a major structural component of the chromosome axis (8), Hop1, a master regulator of meiotic recombination (9), as well as cohesin complexes containing the meiosis-specific Rec8 kleisin subunit (10). Genetic and biochemical analysis suggests a model
in which Rec8 either recruits or otherwise controls the chromosome association of Red1, which in turn recruits Hop1 directly (11, 12). Cells deficient in any of these three proteins exhibit severe abnormalities in eventual Zip1 localization and fail to undergo synapsis, suggesting that the hierarchical assembly of the chromosome axis/LEs is essential for SC formation (5, 8, 9, 13, 14). As synapsis occurs concurrently with the dissociation or active removal of most Hop1 from the chromosome axis (8, 15, 16), Red1 is the most likely to facilitate interactions necessary for Zip1 localization and SC formation, however specific details as to the nature of this interaction remain unclear.

Red1 is predicted to possess a conserved globular N-terminal domain immediately followed by a short “closure motif” that binds and recruits Hop1 to the chromosome axis (Chapter 2 of this dissertation, (17, 18)). The closure motif is followed by a long disordered region and a conserved ~100 residue C-terminal domain predicted to form a coiled-coil oligomer (Figure 3.1A) (19). The function of the N-terminal domain is largely unknown, but it likely facilitates the localization of Red1 to chromosomes, through interactions with cohesin complexes or other chromatin-associated proteins. The C-terminal domain has been shown to self-associate via yeast 2-hybrid assays (14, 18) (Figure 3.1B), and an intact Red1 C-terminus is also essential for Red1’s biological functions at the chromosome axis/LE (18, 20, 21). Although highly diverged, the functionally related mammalian axis/LE proteins, SYCP2 and SYCP3, both possess a similar C-terminal alpha-helical domain.
A recent crystal structure of SYCP3 reveals a tetrameric coiled-coil architecture, which is theorized to further polymerize to form the contiguous backbone of the meiotic chromosome axis (22, 23).

Figure 3.1: Conserved structural domains of Red1
(A) Structured domains of S. cerevisiae Red1 shown as rectangles, unstructured regions shown as lines. Hop1 HORMA domain closure motif shown in yellow.

Two conserved hydrophobic residues within the C-terminus of S. cerevisiae Red1, I743 and I758, have been shown through genetic assays to be critical for Red1’s role in crossover formation and SC assembly (20, 21). Mutation of either of these hydrophobic residues to a positively-charged arginine residue results in severe meiotic chromosome segregation defects as measured by spore viability (20, 21). Mutation of I743 was shown via yeast 2-hybrid to disrupt Red1’s association with the
heterotrimeric 9-1-1 complex (the proteins Rad9, Hus1, and Rad1 in mammals; the proteins Ddc1, Mec3, and Rad17 in *S. cerevisiae*), which is loaded onto meiotic DSB sites to recruit downstream effectors involved in meiotic checkpoint arrest (24, 25). The 9-1-1 complex is structurally similar to the homotrimeric DNA polymerase sliding clamp proliferating cell nuclear antigen (PCNA) (20, 26), and as such is thought to interact with partner proteins in a similar manner (20). I743 is thought to facilitate Red1’s binding to the Ddc1 subunit of the complex as part of a hydrophobic QxxΨ motif (with Ψ being the residues L, M, V, and I) motif (Figure 3.1B) in a manner similar to that of PCNA interacting proteins (26).

Red1 residue I758 was originally found to be necessary for Red1’s association with Smt3, the yeast Small Ubiquitin-like Modifier (SUMO) (21), as part of a putative SUMO-interacting motif (ISII, residues 758-761; Figure 3.1B) (21, 27). Previous studies have suggested that the Zip1 C-termini may associate with the axis/LEs through an interaction with SUMOylated (or SUMO-bound) Red1 (20, 21, 28, 29). Mutation of the conserved Red1<sup>I758R</sup> disrupts this protein’s yeast 2-hybrid interaction with Smt3, but was also shown to cause a partial defect in the homo-oligomerization of the Red1 C-terminus (21). Cells in which either I758 or I743 are mutated exhibit limited accumulation of Zip1 at the chromosome axis and fail to form complete SCs (20, 21).

In this study we seek to further understand Red1’s function and role in maintaining the integrity of the chromosome axis and SC by characterizing the
oligomerization and structure of the protein’s conserved C-terminal alpha-helical domain. We find that the Red1 C-terminal domain is capable of forming high molecular weight oligomers, and that the meiotic defects observed in previously-identified mutants of Red1 residues I743 and I758 mutants can be more simply explained by their disruption of Red1 oligomerization and axis/LE structure.

**Results:**

**Red1 C-terminus Forms High Order Oligomers:**

Prior studies have shown that the C-terminal 290 amino acids of Red1 self-associate in yeast 2-hybrid assays (14, 18). This region is predicted to contain a series of highly conserved alpha-helices capable of forming coiled-coil structures ([Figure 3.1B](#)) (30, 31). During the purification of MBP-fused Red1^{731-827}, we noticed that the protein elutes from a size-exclusion column much earlier than would be expected for its molecular weight of 56.7 kDa ([Figure 3.2A](#)). Comparing the elution volume to that of known chromatography standards suggests that this protein is capable of forming high molecular weight oligomers beyond that of a simple dimeric coiled-coil ([Figure 3.2A](#)). Further analysis by size exclusion chromatography coupled to multi-angled light scattering (SEC-MALS) shows that this construct forms a complex with a calculated molecular weight of 354 kDa, just over the expected molecular weight of a hexamer ([Figure 3.2B](#)).
Figure 3.2: Size Exclusion Chromatography of Red1 C-terminal domain
(A) Size exclusion chromatography of wild-type (red) and mutant (I743R, orange; I758R, grey) MBP-Red1^{731-827}
(B) Size exclusion chromatography/multi-angle light scattering (SEC-MALS) of wild-type and mutant MBP-Red1^{731-827}(colored as in panel A). Below: calculated and measured molecular weights for all constructs, as well as approximate oligomeric state.
Figure 3.3: Size Exclusion Chromatography of Fungal Red1 C-terminal domain

(A) Size exclusion chromatography of MBP-tagged Red1 C-terminal constructs from *Z. rouxii* (pink) and *C. glabrata* (purple).

(B) SEC-MALS of *Z. rouxii* and *C. glabrata* MBP-Red1 C-terminal constructs (colored as in panel A). Below: calculated and measured molecular weights for all constructs, as well as approximate oligomeric state.
We obtained broadly similar results using corresponding constructs of Red1 homologs from *Z. rouxii* (ZrRed1\textsuperscript{704-798}) and *C. glabrata* yeast (CgRed1\textsuperscript{696-789}). In both of these cases, the majority of the protein eluted in the void of the size-exclusion column with a calculated molecular weight exceeding 30 times that of the monomer ([Figure 3.3AB]). This suggests that in contrast to the defined oligomer we observe with the *S. cerevisiae* Red1 C-terminal region *in vitro*, Red1 may in fact polymerize to form significantly higher-order structures. These data are reminiscent of ongoing work in our lab with mammalian SYCP2 and SYCP3, and may indicate a previously-unappreciated functional parallel between yeast and mammalian axis proteins (Scott Rosenberg, unpublished data).

**Mutation of Conserved Hydrophobic Residues in the Red1 Coiled-coil Disrupts High Order Oligomerization:**

Mutation of the conserved residues I743 and I758 within Red1’s C-terminal domain result in abnormal localization of Red1 as well as defects in chromosome axis and SC formation (20, 21). While these effects have been attributed to the disruption of Red1’s interactions with the 9-1-1 complex and Smt3, respectively, we hypothesize that their most important effect is instead to disrupt Red1 oligomerization. To test this, we purified MBP-Red1\textsuperscript{731-827}I743R and MBP-Red1\textsuperscript{731-827}I758R and analyzed their oligomeric state. Both mutants migrated similarly on size exclusion column, and their
elution volumes suggest that they are still capable of forming oligomers, however both mutant proteins elute later than wild type MBP-Red1^{731-827} suggesting some disruption of their higher order oligomerization (**Figure 3.2A**). Subsequent analysis via SEC-MALS reveals that MBP-Red1^{731-827}I743R has an average calculated molecular weight of 207 kDa, approximately that expected of tetrameric complex (**Figure 3.2B**). MBP-Red1^{731-827}I758R on the other hand eluted as a more dispersed peak with an average calculated weight approximating that of a monomer (**Figure 3.2B**). The difference in oligomeric state of MBP-Red1^{731-827}I758R observed between the SEC and the SEC-MALS results can likely be attributed to the higher temperature at which SEC-MALS analysis was performed (25° as opposed to 4° in SEC analysis). This suggests that the I758 residue plays a more critical role in the oligomerization interactions within the Red1 C-terminus than I743.

**Structural Characterization of the Red1 C-Terminus:**

In order to confirm the nature of the oligomerization interactions at the Red1 C-terminus, we set out to determine a crystal structure of this region. We were unable to identify crystallization conditions for Red1^{731-827}, however we could crystallize a slightly truncated fragment, Red1^{734-820} more closely conforming to the predicted alpha-helical region. These crystals formed in space group P2₂2₁ and currently diffract anisotropically to ~3.5 Å resolution (**Figure 3.4AB**). Based on the size of the crystallographic asymmetric unit, we estimate that each asymmetric unit likely contains two 4-subunit Red1 oligomers. We used a truncated version of the SYCP3 tetrameric coiled-coil structure as a search model to determine the structure
by molecular replacement. The current best solution shows an R-factor of 43% and free-R factor of 48%, indicating that while the model is incomplete and inaccurate, it nonetheless represents a real solution (Table 3.1).

Our current crystals of Red1\textsuperscript{734-820} are small and diffract weakly to 3.5 Å, and our first priority is to improve crystal size and thereby data quality and resolution. For this, we are varying buffer conditions, protein concentration, and crystallization temperature, and screening for helpful additives. We will also alkylate lysine residues, an established method for altering the protein’s surface chemistry to promote crystallization. Our goal is to collect high-resolution diffraction datasets (better than 3.0 Å in all directions), then determine the structure by either molecular replacement or anomalous-diffraction methods.

\textbf{Figure 3.4: Crystallography of Red1 C-terminal Domain}
\begin{enumerate}
\item [(A)] Crystals of Red1\textsuperscript{734-820}.
\item [(B)] Red1\textsuperscript{734-820} diffraction. Yellow circle shows a spot at 3.65 Å; box shows the 2\_ screw axis along the c edge.
\end{enumerate}
Discussion:

In this study we show that Red1’s ability to self-associate through the C-terminus forms high molecular weight oligomers beyond a simple coiled-coil dimer. This observation is consistent with what is observed with mammalian SYCP3, which forms fibers along DNA *in vitro* consisting of contains stacked layers of SYCP3 coiled-coil tetramers (23). The biological relevance of this structure, however, is questionable, as SYCP3 cannot localize to chromosomes or function without the additional axis protein SYCP2 (32). Possessing similar patterns of axial localization, secondary structure, as well as a HORMA domain closure motif within its C-terminal domain, SYCP2 is likely to represent a close functional analog of Red1 (33, 34)(Scott Rosenberg, unpublished data). Recent work in our lab has shown that the interaction between SYCP2 and SYCP3 depends upon the predicted coiled-coil regions of each protein (Scott Rosenberg, unpublished data). Additionally, purified complexes of SYCP2 and SYCP3 form long filaments visible by negative-stain electron microscopy (EM), whose width is consistent with a 4- or 6-helix coiled-coil bundle (Scott Rosenberg, unpublished data). Although Red1 forms a homo-oligomer where the analogous mammalian complex is a hetero-oligomer, it is likely that the high molecular weight oligomers we observe with budding yeast Red1 C-terminal constructs are representative of similar filaments, suggesting a conserved mechanism for axis assembly.

In this study, we demonstrate that the Red1 C-terminal region forms high-
molecular weight oligomers, and show that previously-characterized mutations within this region disrupt oligomerization. *In vivo*, both Red1<sup>I743R</sup> and Red1<sup>I758R</sup> mutants fail to form full SCs and exhibit poor spore viability (20, 21), effects that were originally attributed to their failure to bind the 9-1-1 complex and Smt3, respectively. The direct interaction between Red1 and the 9-1-1 complex is questionable, since in immunofluorescent microscopy of chromosome spreads, the 9-1-1 complex only partially localizes with Red1 (24), and their direct binding was not evident in either co-immunoprecipitation or GST-pulldown assays (21). The notion of Red1’s association with Smt3 is similarly questionable. While Smt3 is a necessary component of the SC, a direct functional role for SUMO in SC assembly or structure has not been rigorously demonstrated. In fact, cells expressing an unSUMOylatable Red1 allele did not display any SC defects (20). More recent super-resolution immunofluorescence microscopy analysis has revealed that Smt3 is found at the CE, rather than the axis/LE where Red1 localizes (7). The data in this study would indicate that a more reasonable explanation for the effects of these mutations is to disrupt the formation of Red1 oligomers at the chromosome axis, as either mutation also leads to discontinuous localization of Red1 along the chromosome axis (20, 21).

Both I743 and I758 are contained within the helical region of Red1 predicted to form coiled-coils (31)(**Figure 3.1B**). While both residues are predicted to be in either the ‘a’ or ‘d’ position of the coiled-coil heptad register, I758R is more strongly predicted to lie within hydrophobic center of the coil, as such its mutation to arginine
likely disrupts the coiled-coil interactions, preventing the formation of stable oligomers. While I743 still affects the oligomerization of the domain, it is less strongly predicted to be involved in coiled-coil interactions. It is therefore likely that it helps facilitate self-association interactions independent of the coiled-coil structure. These predictions are consistent with our \textit{in vitro} findings, which indicated that mutation of I758 has a more severe effect on Red1 oligomerization than I743 (Figure 3.2AB).

Overall, our data plus previously-published results indicate that the formation of Red1 oligomers along the chromosome axis is likely critical for SC formation. These oligomers likely not only serve to maintain the proper chromosome topology and organization necessary for DSB formation and interhomolog recombination, but also as a structural scaffold upon which the TFs of the SC can form. Mutations that disrupt the formation of Red1 oligomers likely lead to discontinuities in the axis/LEs, thus preventing proper SC formation and causing subsequent meiotic defects (Figure 3.5). Moreover, the architecture of the \textit{S. cerevisiae} chromosome axis likely closely resembles that of mammals, highlighting the strong functional conservation of axis and SC proteins through evolution.
Figure 3.5: Axis Assembly Model Via Red1 C-terminal Oligomerization
(Left) Red1 is recruited by Red1 via Rec8 cohesins in early leptotene. Homo-oligomerization via the Red1 C-terminal coiled-coils associate distal axis sites forming a contiguous linear axis/lateral element. (Right) Mutation of the conserved isoleucines with in Red1 C-terminus prevents coiled-coil formation and homo-oligomerization leading to improper axis assembly.

Materials and Methods:

Protein purification and molecular weight analysis:

All Red1 constructs were expressed in *Escherichia coli* strain Rosetta 2 (DE3) pLysS (EMD Millipore, Billerica MA) at 20° C for 16 hours, then cells were harvested by centrifugation and resuspended in buffer A (20 mM Tris pH 8.5, 10% glycerol) plus 300 mM NaCl, 10 mM imidazole, and 2 mM β-mercaptoethanol. Protein was purified by Ni²⁺-affinity (Ni-NTA agarose, Qiagen), ion-exchange (Hitrap Q HP, GE Life

Purified proteins were passed over a size exclusion column (Superdex 200, GE Life Sciences) in buffer A plus 300 mM NaCl and 1 mM dithiothreitol (DTT). MPB-Tags were cleaved with TEV protease (35), and cleaved protein was passed over a size exclusion column (Superdex 200, GE Life Sciences) in buffer A plus 300 mM NaCl and 1 mM dithiothreitol (DTT).

For size-exclusion chromatography coupled multi-angle light scattering (SEC-MALS), proteins were separated on a Superose 6 Increase 10/300 size exclusion column (GE Life Sciences) in a buffer containing 20 mM HEPES pH 7.5, 300 mM NaCl, 10% glycerol, and 1 mM DTT. Absorbance was measured at 280 nm, and light scattering and refractive index profiles collected by miniDAWN TREOS and Optilab TrEX detectors (Wyatt Technology, Santa Barbara CA), respectively, and their molecular weights calculated using ASTRA v. 6 software (Wyatt Technology).

**X-Ray Crystallography:**

*S. cerevisiae* Red1<sup>734-820</sup> was purified as above and crystalized 20mM HEPES pH 8.0, 1.5M NaCl, 22.5% Ethanol and cryoprotected using 25% w/v xylitol.
Table 3.1 – Crystallographic Data of ScRed1734-820

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<tr>
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<td>I/σ (last shell)</td>
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</tr>
<tr>
<td>( b R_{\text{meas}} ) (last shell)</td>
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</tr>
<tr>
<td>( ^c CC_{1/2} ), last shell</td>
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</tr>
<tr>
<td>Completeness (last shell) %</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>Number of sites</td>
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</tr>
</tbody>
</table>

\[ b R_{\text{meas}} = \sum_h \left( n/(n-1) \right) \sum_i \left[ l_{ih} - \langle l_{ih} \rangle \right] / \sum_h \langle l_{ih} \rangle \]

where \( l_{ih} \) is a single intensity measurement for reflection \( h \), \( \langle l_{ih} \rangle \) is the average intensity measurement for multiply recorded reflections, and \( n \) is the number of observations of reflection \( h \).

\[ ^c CC_{1/2} \] is the Pearson correlation coefficient between the average measured intensities of two randomly-assigned half-sets of the measurements of each unique reflection (34).

\[ e R_{\text{work, free}} = \sum ||F_{\text{obs}}| - |F_{\text{calc}}|| / |F_{\text{obs}}|, \]

where the working and free \( R \)-factors are calculated using the working and free reflection sets, respectively.
References:


Chapter 4

CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation set out to address key questions regarding the assembly of the meiotic chromosome axis and the coordination of homolog pairing and recombination. By focusing on the biochemical and biophysical characteristics of conserved domains within the major axis components, Hop1 and Red1, we discovered new mechanisms governing their recruitment and assembly along the axis. These findings, in addition to prior genetic studies and complementary work in our own lab on regulators like Pch2/TRIP13, provide a more complete and detailed understanding of the interactions and events governing the assembly and disassembly of the chromosome axis, and how this structure orchestrates meiotic recombination.

In our investigation of Hop1, we identified HORMA domain-binding closure motifs in both Red1 and Hop1’s own C-terminus, revealing that the HORMA domain mediates both initial recruitment and self-assembly of Hop1 on meiotic chromosomes, a requirement for downstream regulation of recombination partner choice via the Mek1 kinase. We further show that partner-protein binding via closure motifs is accompanied by conformational conversion within Hop1’s HORMA domain, drawing strong functional parallels to the mechanisms of the related HORMA domain protein Mad2 in the spindle assembly checkpoint. The Hop1 HORMA domain adopts two distinct conformations in solution, one equivalent to the
peptide bound “closed” state, previously observed in *C. elegans* meiotic HORMADs (1), and a second “unbuckled” state similar to Mad2’s “open” state. This is the first demonstration that a HORMA domain protein other than Mad2 displays conformational dynamics, and this mechanism can explain how meiotic HORMADs localize and self-assemble on the meiotic chromosome axis.

The Hop1 HORMA domain’s structural and mechanistic parallels with Mad2 also provides insight into the mechanism by which HORMADs are removed from the chromosome axis by the Pch2/TRIP13 ATPase. Recently it has been shown that TRIP13 disassembles the Mitotic Checkpoint Complex through partial unfolding of Mad2’s disordered N-terminus, causing the safety belt to dissociate from the closed position, thereby releasing the bound closure motif and converting Mad2 to the open conformation (2, 3). This mechanism is dependent on TRIP13’s recognition and engagement of the Mad2 N-terminus. A similar dependency is found between TRIP13 and the N-terminus of mammalian meiotic axis protein HORMAD1, suggesting that TRIP13 operates via a similar mechanism in both mitosis and meiosis (2). It is therefore likely that this mechanism is conserved in Pch2’s role in remodeling the *S. cerevisiae* meiotic axis and the removal of Hop1 concomitant to synapsis of the homologs. To investigate this, it will be necessary to determine whether Pch2 binding of Hop1 is likewise dependent upon the N-terminus and/or a particular conformation of the HORMA domain. While the “loopless” mutation of Hop1 stabilizes the HORMA domain into a closed conformation, a mutation that stabilizes the open or unbuckled conformation would have to be developed.
A hydrogen-bonding network required to stabilize the closed conformation of Mad2 is conserved in meiotic HORMADs (2, 4). The equivalent residues in Hop1, S22 in the αA helix and H244 in the β8’ sheet, lay at the interface between the HORMA domain core and the safety belt in the closed conformation. Mutation of these residues will likely disrupt the stable association of the safety belt in the closed conformation, causing the domain to favor the unbuckled state. HDX-MS and peptide binding experiments, similar to those used in Chapter 2, can determine whether this is the case. Such mutants, along with wild-type and loopless Hop1, can be used to test whether Pch2 recognizes a specific conformation of Hop1. Similar experiments can likewise be used to determine whether Pch2 is capable of inducing conformational changes in the Hop1 HORMA domain to release bound Red1 or the CTD closure motif.

This line of inquiry may also address an outstanding question arising from the work of this dissertation; namely, how the C-terminal closure motif of Hop1 is initially dissociated from the HORMA domain to allow Red1 binding or Hop1 oligomerization. Depending on circumstances, the action of Pch2 on the HORMA domain may dissociate “self-closed” Hop1 allowing for its association to the axis. The dependence of PAIR2, the meiotic HORMAD in rice (*Oryza sativa*), on the Pch2/TRIP13 ortholog, CRC1, for axis localization suggests that this may be the case (5), however additional factors not addressed in the scope of this dissertation may also contribute. One such factor is a second structured domain in Hop1, C-terminal to the HORMA domain (residues 320-380, approximately). This region contains conserved cysteine residues
and shown to bind zinc ions, and as such it was initially considered a putative zinc-finger DNA binding domain (6, 7). While Hop1 has been shown to associate with DNA and form aggregates with recombination intermediates that resemble higher-order nucleoprotein complexes in vitro (7-10), Hop1’s direct binding to DNA, and the presence of such complexes at the chromosome axis has not been demonstrated in vivo. More detailed sequence and structural homology analysis shows that this region contains a Cys4-His-Cys3 zinc-binding motif that likely folds as a PHD finger domain (11). Therefore, a more likely function for this domain is to facilitate Hop1’s interaction with, or recruitment of, additional partner proteins at the chromosome axis. Further characterization of this domain and any partners that may bind, and what affect it might have on the conformation of the HORMA domain, would provide a more complete understanding of Hop1’s assembly and function on the axis.

In Chapter 3, we further characterized the homo-oligomerization of the Red1 C-terminus and showed that this protein’s conserved C-terminal coiled-coil region forms high molecular weight oligomers. These coiled-coil oligomers likely form the basis of the axial/lateral elements of the SC, and previously-characterized mutations that disrupt this region’s self-assembly show that the Red1 oligomer is necessary for proper localization of Zip1 (12, 13). Thus, our model provides new insight into axis assembly, and provides an alternative explanation for meiotic defects caused by previously characterized mutations within the Red1 C-terminus. As it stands however, the oligomeric state and the precise nature of these interaction cannot be fully established without better crystallographic data. While we present a promising
molecular-replacement solution that may provide enough phasing information to eventually determine the structure, further work optimizing crystal conditions to reduce anisotropy and increase diffraction quality is required.

Further understanding of Red1’s role at the chromosome axis can be achieved by characterizing its globular N-terminal domain, the function of which remains unknown. While Red1 and Hop1 are thought to form a complex with Rec8, and are dependent on it for proper localization and distribution along the axis (14, 15), the nature of this interaction remains unclear. As the only other predicted structured domain in Red1, the N-terminal is a likely candidate to facilitate binding to cohesins or other chromatin-associated proteins as a means of associating of Red1 coiled-coil oligomers to the chromosome axis. The N-terminal domain’s proximity to the HORMA domain-binding closure motif also suggests that it might somehow modulate Red1’s binding with Hop1. Structural and biochemical characterization of this domain and identification of any binding partners would further our understanding of Red1’s role in axis assembly, synapsis, and recombination.

While many details and questions remain to be investigated, this dissertation presents data to outline a new model for the interactions of Hop1 and Red1, and their contributions to axis assembly and meiotic recombination. This model will inform future investigations into chromosome axis function and the regulation of the events of meiotic prophase I in both yeast and mammals.
References:


