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Condensin, Cohesin and Cdc5 function as choreographers of chromosome condensation.

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Committee in charge:

Professor Douglas Koshland, Chair
Professor Barbara Meyer
Professor Jasper Rine
Professor Michael Freeling

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Professor Douglas E Koshland, Chair

During cellular division, chromosomes undergo a series of cytological events that allow for the maintenance and inheritance of genetic information from one generation to the next. While the necessity of these morphological changes has been appreciated for over a hundred years, we still have but a rudimentary understanding of the basic principles underlying how chromosomes are formed.

Over the past two decades, we have gained an appreciation for the integral role of two related, multi-subunit Structural Maintenance of Chromosomes (SMC) protein complexes, cohesin and condensin, play in chromosome condensation. The necessity of both protein complexes is particularly evident in the budding yeast, *Saccharomyces cerevisiae*, where loss of either cohesin or condensin leads to cytologically indistinguishable loss of chromosome condensation.

In this dissertation, I address some of the gaps in our knowledge of the mechanisms governing mitotic chromosome condensation. This body of work was sparked by the fundamental question of how condensin and cohesin orchestrate their functions to ensure that chromosome condensation occurs in a tightly regulated spatio-temporal manner. To that end, we used a combination of yeast genetics, cytology, sequencing and chromosome conformation capture methodologies to dissect the relative contributions of cohesin and condensin to mitotic chromosome condensation.

First, we describe the role of a kinase, Polo (Cdc5), as part of an interactome of early condensation factors along with cohesin and condensin. In particular, we describe how cohesin modulation of this kinase may impact condensin function. Next, we utilize a chromosome conformation capture technique, Micro-C, to elucidate features of yeast mitotic structures at nucleosome resolution. There, we also begin to dissect the contributions of mitotic factors in these structures. Lastly, we investigate the molecular defects of a unique cohesin mutant that allow us to dissect cohesin’s role in condensation.

Altogether, these findings reshape our understanding how higher order chromosome structures are formed and inspire us to reevaluate some of our

fundamental assumptions about how SMCs coordinate their function. Additionally, this work highlights how yeast can serve as a genetically-tractable model for studying the formation of chromosome loops.

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To my family

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Chapter 1- An Introduction to Chromosome Structure

1.1 The enigma of chromosome condensation and an overview of the cell cycle

The reshaping of genomes from interphase chromatin to rigid, highly condensed mitotic chromosomes has fascinated scientists from early cytologists to modern molecular biologists. During mitosis, chromosomes undergo a series of cytologically-distinct events that allow for the shaping, maintenance and inheritance of chromosomes from one generation to the next. The necessity of these morphological changes is highlighted by observations that cells defective in the machinery coordinating these events have severe defects in chromosome segregation, an event necessary for life and whose defects are associated with diseases such as cancer. A staggering amount of work over the past hundred plus years has sought to understand the fundamental underpinnings of how chromosomes are contorted and reorganized during mitosis. However, despite the scope and depth of these studies, and tremendous technical advances, we still have a rudimentary understanding of even the most basic principles of how chromosomes are formed.

Over the course of the cell cycle, chromosomes are duplicated in S phase. Concurrently, the resulting sister chromatids are linked to each other through a process termed sister chromatid cohesion. Cohesion is necessary both to ensure that sister chromatids are in close proximity and to allow for effective congression of chromosomes at the metaphase plate. This process effectively engage microtubules and orients the microtubule attachment to the kinetochores of the two sister chromatids ensuring that sister chromatids always segregate away from each other to the two daughter cells. Once cohesion is established, chromatids are laterally and/or axially shortened through a process termed condensation. This condensation process is necessary to prevent aberrant cutting of chromosomes across the plane of cytokinesis. Once established, cohesion is maintained until the onset of anaphase, when the linkage between sister chromatids is severed, allowing for the migration of chromatids to opposite spindle poles. In contrast, the process of chromosome condensation is maintained through cytokinesis until the end of mitosis (Figure 1.1).

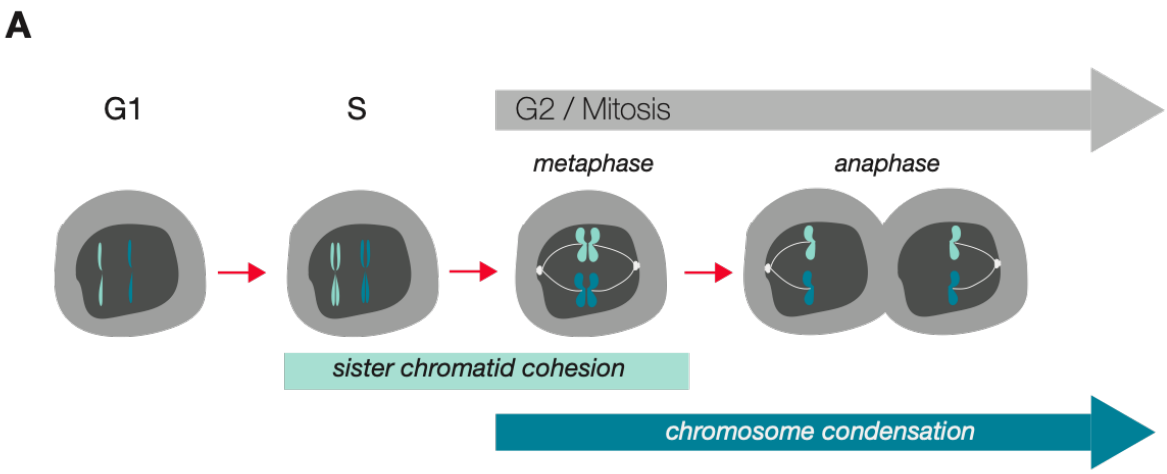

Figure 1.1: Overview of the cell cycle

A. Chromosomes are replicated in S phase, tethered to each other through the process of sister chromatid cohesion and compacted through a process of chromosome condensation. Sister chromatid cohesion is established in S phase and maintained until dissolution of cohesin at the onset of anaphase. In contrast, chromosome condensation is established in mitotic prophase and maintained through anaphase until cytokinesis.

1.2 SMC complexes: genome architects with unusual structures

A large body of work has focused on investigating the factors governing chromosome architecture. Work over the past two decades has elicited that an evolutionarily ancient family of proteins lie at the heart of these processes. SMC (for structural maintenance of chromosomes) proteins constitute the core subunits of a small family of multimeric protein complexes that serve as genome architects with unusual features (Figure 1.2A). These SMC complexes have been implicated in all aspects of chromosome functions, including sister chromatid cohesion, chromosome condensation, modulation of gene expression and DNA damage repair (Figure 1.2B).

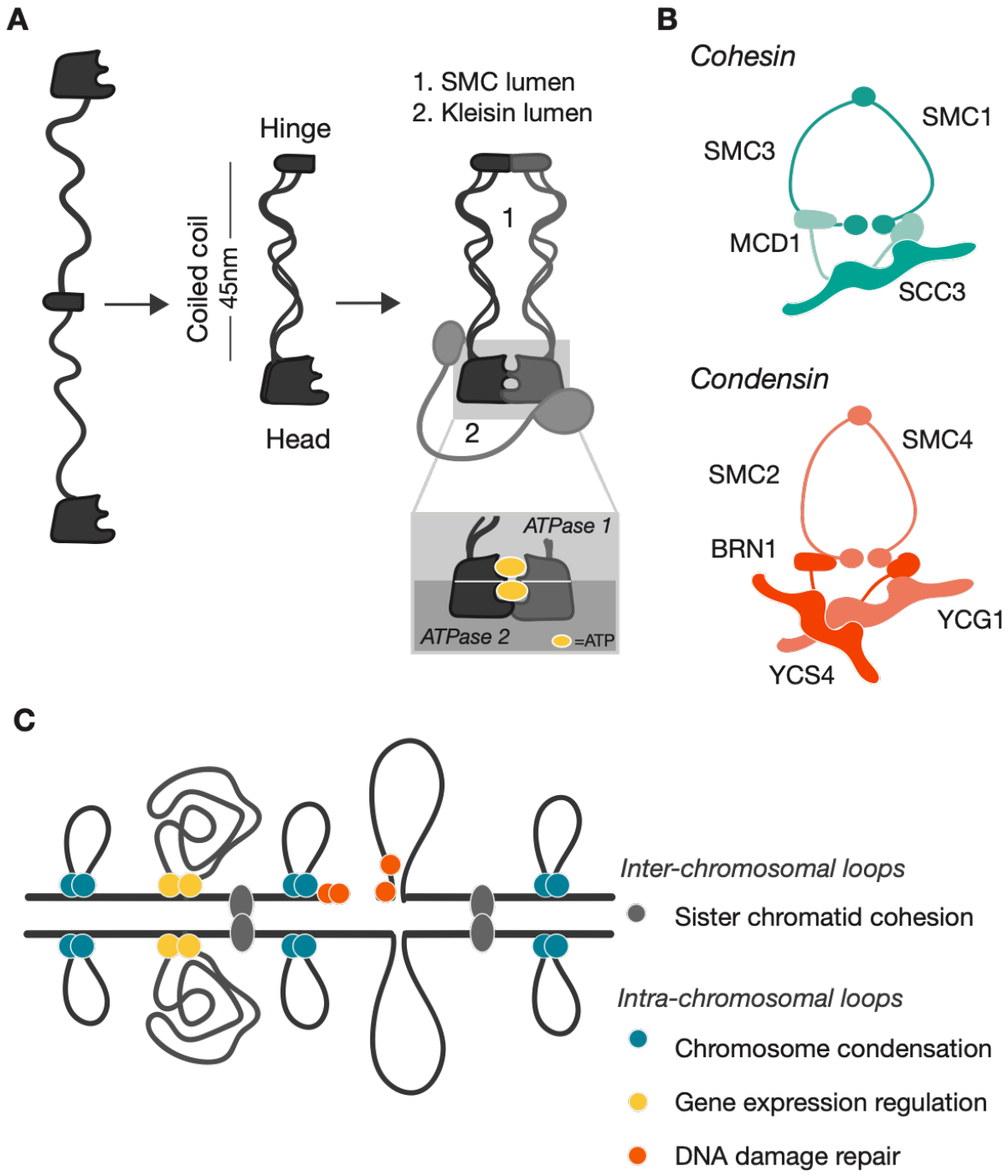

Figure 1.2: Topology of SMC complexes

A. SMC proteins contain globular head and hinge domains separated by long, antiparallel coiled coil region. SMC proteins are dimerized through interactions at their head and hinge domains. These interactions result in the formation of a large lumen “SMC lumen”. A kleisin subunit bridges the heads of the SMC dimers and results in the formation of a smaller lumen “kleisin lumen”.

B. Schematic of the SMC complexes, cohesin and condensin. Cohesin is comprised of a heterodimer of Smc3p and Smc1p, Mcd1p and Scc3p. Condensin is comprised of the Smc2p an Smc4p heterodimer and Brn1p (kleisin), Ycg1p and Ycs4p.

C. SMC complexes are involved in an array of chromosome processes, including sister chromatid cohesion, chromosome condensation, gene expression regulation and DNA damage repair. These activities are generated by the ability of these complexes to bridge together two regions of DNA either intra- or inter-molecularly (gray). This activity may occur through a loop extrusion model whereby DNA is entrapped by cohesin and condensin and processively enlarged until a boundary is reached (see Gandi et al. 2018).

While SMC complexes play a diverse arsenal of roles, they are structurally linked through a shared architecture. (Figure 1.2) Typically, SMC complexes comprise of a dimer of a conserved family of Smc proteins from which the family gets their name. Each individual SMC protein is ~1200 amino acids in length. This primary sequence folds back on itself at the hinge region, resulting in two highly globular domains called the head and the hinge domains that are connected by a long (45 nm) antiparallel coiled coil (Figure 1.2A) (Strunnikov et al. 1993; Haering et al. 2002, Kurze et al. 2011). SMC proteins dimerize through interactions between their heads and between their hinges generating a large potential lumen within the dimer. The globular head contains a number of essential regulatory domains, including key motifs of an ATPase active site. Dimerization of SMC proteins is essential for several reasons. For one, each SMC protein contains only part of the ATPase motifs necessary to hydrolyze ATP (Melby et al., 1998; Hirano, 2001). Dimerization of the SMC heads is necessary to generate the two complete active ATPase sites, similar to the ABC ATPases (Lowe et al. 2001). Interestingly, these ATP sites are asymmetric. Moreover, in the case of two SMC complexes, cohesin and condensin, this asymmetry is fundamental to function (Melby et al. 1998, Hirano et al. 2001, Murayama and Uhlmann 2015, Çamdere et al. 2015, Elbatsh et al. 2016, Palou et al. 2018).

SMC complexes also have several associated regulatory factors (Figure 1.2B). The two SMC head domains are bridged by a so-called kleisin subunit, allowing for the formation of a second potential lumen within the SMC complex. While the formation of this tripartite ring is essential for DNA binding, it is not sufficient for culmination of the architectural function of SMC complexes. The kleisin subunit is also necessary to recruit other components of the complex- typically HEAT or WHD repeat proteins that play essential roles in stability and regulation of SMC complex (Hartman et al. 2000, Neuwald and Hirano 2000, Panizza et al. 2000, Tonkin et al. 2004). Some SMC complexes, such as the SMC5/6 complex and the dosage-compensation complex also recruit other DNA modifying proteins that add additional functions (Lieb et al. 2000, Yonker et al. 2003, Zhao and Blobel 2005, Meyer 2005, Duan et al. 2009, Ercan and Lieb 2009, Gómez et al. 2013, Aragón 2018).

While we have learned a great deal about the composition of SMC complexes, many fundamental questions remain. For example, while crystal and EM structures have been made of the terminal regions, the flexibility and heterogeneity of the coiled-coil regions

render most structural analyses of the holo complexes difficult to interpret. This leaves a large blindspot in our understanding of the structure of SMC complexes. At a more fundamental level, we are still unsure about where within a given SMC complex DNA binds. SMC proteins topologically entrap DNA (Haering et al. 2002, Ivanov and Nasmyth 2005, Cuylen et al. 2011). This entrapment could occur in either of the two lumens (a larger one between the SMC dimers and a smaller one between the dimer and kleisin subunit) within the SMC complexes (Eng et al. 2014, 2015). Furthermore, it is unknown how topological entrapment is exploited to generate activities of tethering of two DNA molecules or loop extrusion. Answering these mechanistic questions is one of the most active areas of research in the SMC field. In my thesis, I will focus on an equally important but largely ignored question. How are SMC complexes controlled spatially and temporarily to ensure their proper biological function? As a paradigm, I will be focusing on two related complexes: cohesin and condensin, which play overlapping yet distinct roles in mitotic chromosome structure, most notably in chromosome condensation in budding yeast.

1.3 Cohesin: master mediator of cohesion

Cohesin is comprised of a heterodimer of Smc1p and Smc3p linked by a kleisen subunit named Mcd1p (also called Scc1p, Rad21p) and an associated subunit, Scc3p. Cohesin was initially discovered over two decades ago and initially characterized for its fundamental role in establishing and maintaining sister chromatid cohesion from S phase to anaphase onset in mitosis (Guacci et al. 1997; Michaelis et al. 1997). A large body of work has subsequently found that cohesin plays critical roles in a number of chromosome functions including chromosome condensation (Blat and Kleckner, 1999; Laloraya et al., 2000; Lengronne et al., 2004; Kogut et al., 2009). How exactly cohesin performs these biologically diverse functions is still the subject of active investigation, but interaction with accessory factors and chromosomal context are essential.

Cohesin is loaded onto chromosomes at the beginning of S phase, concurrent with DNA replication (Figure 1.3A). This loading event requires the activity of the dedicated cohesin loader complex, a dimer comprised of Scc2p and Scc4p (Ciosk et al. 2000). The precise mechanism of loading is still under investigation. In yeast, cohesin binding is enriched at pericentric regions, discrete interspaced cohesin associated regions along chromosome arms (CARs), telomeres and the highly repetitive rDNA (RDN) locus (Laloraya et al. 2000, Lengronne et al. 2004). While CARs do not have a consensus sequence motif, they tend to be AT-rich and are often located between convergent genes (Lengronne et al. 2004, Parelho et al. 2008, Kagey et al. 2010). Additionally, by chromatin immunoprecipitation (ChIP) methodologies, each CAR encompasses a region of around 1kb, suggestive of multiple cohesins bound at these regions (Laloraya et al. 2000).

During S phase, chromatin-bound cohesin is converted to a cohesive state capable of tethering sister chromatids to each other. This conversion is largely accomplished by the essential acetyltransferase Eco1p (Skibbens et al. 1999, Toth et al. 1999, Onn et al. 2009). While Eco1p acetylates many lysines both within and across the subunits of

cohesin, acetylation of the lysine at 113 of Smc3p is essential for the establishment of cohesion and condensation (Ünal et al. 2008; BenShahar et al. 2008; Zhang et al. 2008). The impact of Eco1p acetylation of cohesin's biochemical and biological functions is complex (Rowland et al. 2009; Guacci et al. 2015; Çamdere et al. 2015).

Eco1p acetylation of cohesin appears to regulate cohesion and condensation by distinct mechanisms. Deletion of *WPL1,* which encodes an inhibitor of cohesin, restores viability and condensation but not cohesion to cells lacking Eco1p (Sutani et al. 2009; Rowland et al. 2009). Additionally, insertion and point mutations within Pds5p, Smc3p and Scc3p that phenocopy *wpl1Δ* also allow for cohesin function in the absence of Eco1 (Guacci and Koshland 2012). How acetylation differentially controls cohesion and condensation remains unknown.

Acetylation of cohesin has been tied to two biochemical activities of cohesin, regulating cohesin's ATPase and stabilizing cohesin binding to DNA. Un-acetylated binds to DNA; however, that binding is unstable. In contrast, deletion of *WLP1* stabilizes cohesin binding to DNA. Thus, Wlp1p promotes cohesin dissociation from DNA and this activity is inhibited by cohesin acetylation. However, since the deletion of *WLP1* is not sufficient to restore cohesion to *eco1Δ* cells, acetylation must perform an additional function in cohesion establishment. Indeed, a mutation in cohesin's Smc1p subunit (D1164E) reduces the rate of cohesin ATP hydrolysis, and restores cohesion as well as condensation and viability in cells lacking *eco1Δ* or *eco1 wpl1Δ*. Since the latter bind cohesin stably, restoration of cohesion by the D1164E mutation occurs by modulating a step necessary for cohesion after its stable binding to DNA (Çamdere et al. 2015, Beckouet et al. 2016). Furthermore, one key function of cohesin acetylation is the down regulation of cohesin ATPase.

The maintenance of cohesion until its dissolution at anaphase onset requires additional regulators beyond acetylation. For one, Pds5p is recruited to cohesin after establishment and this binding is maintained through metaphase. The mechanism(s) by which Pds5 performs its role in sister chromatid cohesion maintenance remains fairly mysterious even to this day and is the subject of active research. Nonetheless, studies across many model systems, and particularly in yeast, have highlighted the crucial nature of this "accessory" factor.

In recent studies of budding yeast, two models addressing Pds5p's putative role in cohesion maintenance have emerged. In the first, Pds5p protects Mcd1p from SUMOylation-mediated degradation until anaphase onset (Noble et al. 2006; D'Ambrosio et al. 2014). In the second, Pds5p reinforces the Eco1p-initiated acetylation. The first model arose from the observation that Mcd1p is degraded through a SUMO-dependent pathway in cells lacking Pds5p. In support of this model, the inviability of some Pds5p temperature-sensitive alleles can be partially suppressed by deleting the agents of SUMOylation in yeast (Slx5 and Slx8). However, cells that completely lack Pds5p, either through deletion or conditional depletion (*pds5Δ* and *PDS5-AID*) do not become viable with the depletion of Slx5 or Slx8. The second model is based on the observation that cells deprived of functional Pds5p have decreased levels of Smc3p

K112 and K113 acetylation. Conversely, cells in which Eco1p activity is impaired exhibit decreased Pds5p binding (Chan et al. 2013). Analysis of Pds5p's role is further complicated by the fact that it crosslinks to every cohesin subunit, suggesting that its function may be more complex than previously thought (Huis in t Veld et al. 2014).

Lastly, at the onset of anaphase, cohesin's Mcd1p subunit is rapidly destroyed, resulting in the rapid release of cohesin from DNA (Yamamoto et al. 1996, Ciosk et al. 1998, Uhlmann et al. 1999, Waizenegger et al. 2000, Alexandru et al. 2001, Nakajima et al. 2007). This destruction is mediated by the activity of Esp1p (separase), whose activity is otherwise restricted by binding to Pds1p (securin) (Yamamoto et al. 1996a; 1996b, Guacci et al. 1997, Ciosk et al. 1998, Uhlmann et al. 1999). How precisely chromatin-bound Mcd1p is recognized for degradation is still the subject of active query. Historically, this recognition was thought to be achieved by the phosphorylation of Mcd1p by the polo kinase, Cdc5p (Alexandru et al., 2001). While Mcd1p is phosphorylated in a cell cycle-dependent manner by Cdc5, the functional ramifications of these phosphorylation events remained a mystery. A caveat to this detrimental role for phosphorylation of Cdc5p is the observation that in higher eukaryotes, Mcd1p phosphorylation by polo protects centromere-bound cohesin from being removed by the prophase removal pathway. Interestingly, inactivation of polo kinase in larger eukaryotes is associated with under-condensed chromosomes in metaphase and a propensity for the formation of anaphase bridges.

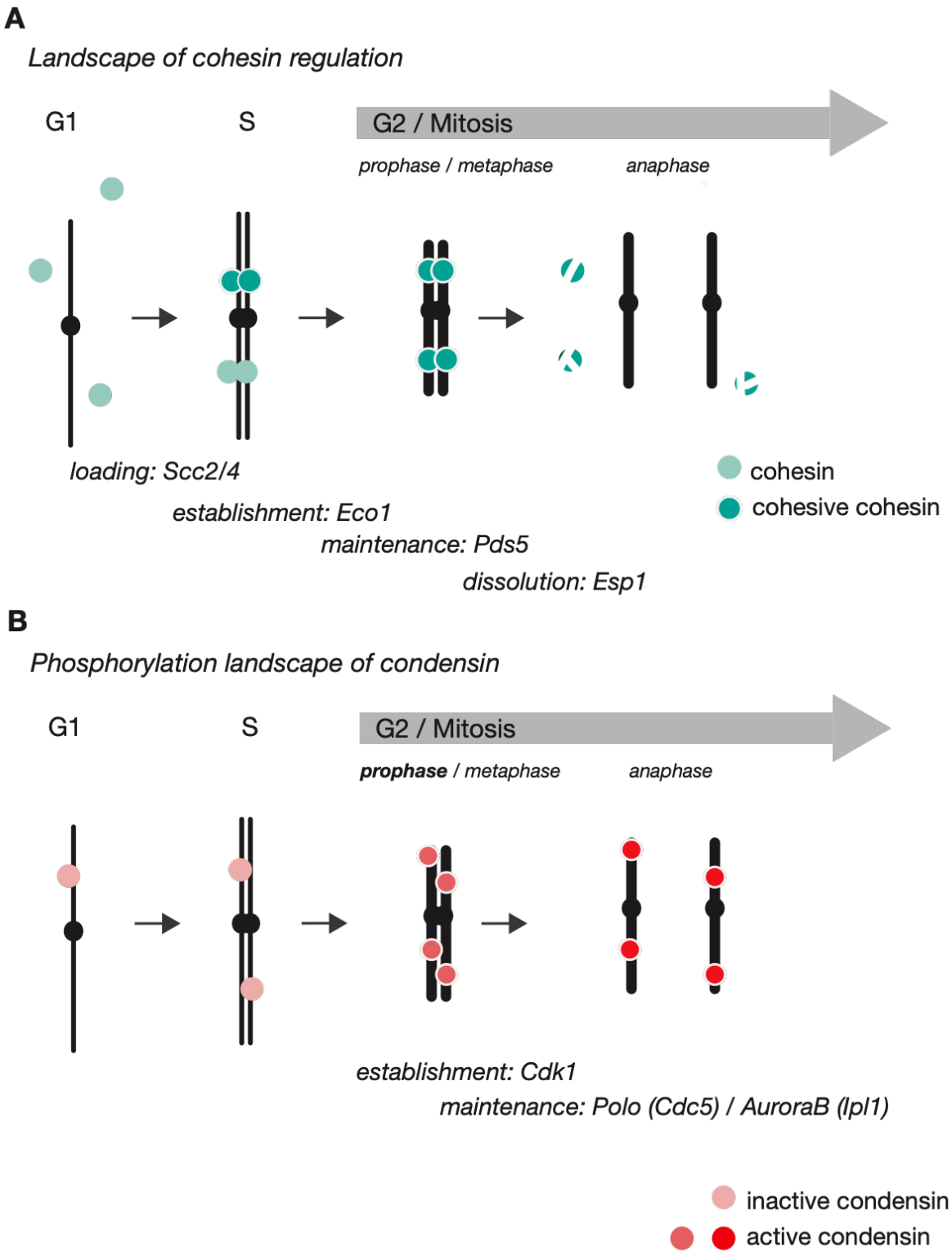

Figure 1.3: Regulatory landscape of cohesin and condensin

Figure 1.3: Regulatory landscape of cohesin and condensin

A. In budding yeast, Mcd1p is resynthesized at the end of G1, allowing for the assembly of a soluble cohesin holocomplex. Cohesin is loaded onto chromosomes in late G1/early S phase by the Scc2p/Scc4p loader complex. The acetyltransferase Eco1p acetylates Smc3p at one essential residue, lysine 113 (K113), during DNA replication. This acetylation converts chromosome-bound cohesin into a cohesive state, resulting in cohesion establishment. Interactions between the core cohesin complex and Pds5p ensure stable maintenance of cohesion until mitotic metaphase. At the onset of anaphase, Mcd1p is subjected to rapid proteolytic degradation by the activity of Esp1p.

B. At least one condensin complex has been shown to be present on chromosomes on chromosomes in G1/interphase. What prevents condensin from being active during that time is still being investigated. However, some data suggests that the CKII kinase phosphorylates condensin to render it inactive. During mitotic prophase, CDK I phosphorylates SMC4p at its N-terminus, resulting in condensin activation. Condensin's regulatory subunits: Brn1p, Ycg1p, and Ycs4p are subsequently phosphorylated in mid-M anaphase by polo kinase (Cdc5p) and in anaphase by Aurora B (Ipl1p). These events are thought to maintain condensation until the end of mitosis.

1.4 Condensin: organizer of chromosome condensation

Like cohesin, condensin is comprised of an SMC heterodimer made of Smc2p and Smc4p along with regulatory subunits. However, in contrast to cohesin, condensin is a constitutive pentamer. Smc2p and Smc4p are joined by a kleisin subunit, Brn1, and two HEAT-repeat proteins: Ycg1 and Ycs4 (Strunnikov et al. 1995, Freeman et al. 2000, Lavoie et al. 2000, 2002, Ouspenski et al. 2000, Bhalla et al. 2002). While condensin was initially characterized for its role in mitotic chromosome condensation in all eukaryotes, this complex has been shown to play crucial roles in sister chromatid cohesion at some genomic loci in budding yeast, DNA repair in *S. pombe* and the repression of gene expression on the X chromosome in XX worms and in quiescent budding yeast (Lam et al. 2006, Kruesi et al. 2013, Xu et al. 2015, Swygert et al. 2019). While condensin is essential in all eukaryotes, finding a universal role for condensin is muddled by the observations that inactivation of condensin affects chromosome structure and condensation kinetics differently across different organisms.

Metazoans possess two essential, non-compensatory condensin complexes: Condensin I and Condensin II, which have dedicated roles in axial and lateral compaction of chromosomes respectively (Hirano and Mitchinson 1994, Neuwald and Hirano 2000, Ono et al. 2003, Schleiffer et al. 2003, Trimborn et al. 2006, Onn et al. 2007). While these two complexes share common Smc2 and Smc4 subunits, there are differences in their regulatory subunits. Interestingly, these same reports showed that the relative ratio of Condensin I and II is critical for mitotic structures to form effectively (Shintomi and Hirano 2011). A confounding factor in studying the role of condensin in larger eukaryotes is the observation that absence of condensin in prophase only results in temporary loss of condensation. Through a condensin-independent mechanism, condensation is eventually recovered as cells progress through metaphase. However,

two caveats reshape our interpretation of these observations. For one, as with cohesin, only a minor fraction of condensin is sufficient to generate effective condensation. Additionally, the superficially condensed state obtained in the absence of condensin is not functional. Chromosomes lacking condensin do not substantially withstand mechanical stress and are defective in subsequent steps of chromosome segregation.

In contrast to larger eukaryotes, budding yeast has only a single condensin complex that shares functional similarity with Condensin I, although it remains nuclear even in interphase. It is essential for viability and condensation. In budding yeast, the non-repetitive parts of the genome do not undergo visible condensation by cytological staining. However, studies that mark specific loci with FISH and lacO or tet two-color fluorescent arrays reveal a two fold compaction in mitosis that is condensin dependent (Guacci et al, 1994). The repetitive RDN locus (75 rDNA repeats) does exhibit cytological condensation in mitosis, and this condensation is also condensin dependent. The presence of only a single condensin complex in budding yeast and its profound effect on RDN condensation, make this organism amenable to study condensin's function and regulation in mitosis.

How does condensin perform its function? Biochemically, we know that ATP is necessary for condensin to perform its function in higher-order chromosome structure (Kimura and Hirano, 1997). Like cohesin, condensin has also been shown to topologically entrap DNA. Early biochemical assays showed that condensin has the capacity to convert complementary ssDNAs into dsDNA *in vitro* (Sakai et al., 2003). It is important to note that this activity does not necessitate the formation of a full complex. However, condensin's most well-characterized biochemical function is its involvement in the formation of positive superhelical tension (supercoils) along dsDNA (Kimura and Hirano, 1997; 2000, Kimura et al., 2001, Hagstrom et al., 2002, St-Pierre et al., 2009). This activity has been particularly demonstrated in closed circular DNA and is dependent on ATP hydrolysis as well as the formation of a condensin holocomplex. Functionally, DNA containing these positive supercoils are more susceptible to Topoisomera-II mediated decatenation than those lacking supercoils. There is strong evidence that this positive supercoiling occurs on topo II-decatenated DNA, resulting in the formation of chiral loops necessary for condensation (Kimura and Hirano 2000, Lavoie et al. 2002). Additionally, genetic and biochemical studies indicate that these functions of condensin may be the result of oligomeric, cooperative interactions among condensin complexes (Swedlow and Hirano 2003, Hudson et al. 2007, Graumann and Knust 2009, Keenholtz et al. 2017, Terakawa et al. 2017).

More recently, condensin has been conclusively shown to perform ATP-dependent loop extrusion (Ganji et al. 2018). These dramatic live imaging assays were performed on DNA curtains and demonstrated that condensin can perform as a single complex that asymmetrically processes DNA through a lumen at a rate of 0.6kbp/s. The integration of this exciting new result with condensin's more established biochemical functions is still the subject of active research. Analysis of different condensin mutants in this context will be very informative.

1.5 Condensin function is regulated by post-translational modifications

The fact that at least one condensin complex (Condensin II) is continually present on chromosomes, yet is only active during specific cell cycle stages implies that its activity must be tightly regulated. Since all subunits of the condensin pentamer are necessary for the establishment and maintenance of condensation, they represent potential targets for regulating condensin activities. Indeed, condensin's activity is regulated through post-translational modifications, particularly: phosphorylation. Condensin phosphorylation is highly dynamic because it is a target of several kinases over the course of the cell cycle (Figure 1.3B) (reviewed in Bazile et al. 2010). In mitotic prophase, condensin's Smc4p subunit is highly phosphorylated in its N-terminus by the cyclin-dependent kinase (Cdk1) (Robellet et al. 2015). This phosphorylation event is thought to serve as a universal trigger in inducing prophase condensation. It is interesting to note that while there are many Cdk sites in this region, the phosphorylation of a few are necessary to convert condensin into a functional state.

While these Cdk phosphorylation events are important for condensin functions, they only comprise one of the multiple layers of kinase-dependent regulation experienced by condensin throughout the cell cycle. Canonically, condensin phosphorylation by the additional kinases described below is thought to be required to maintain condensation from anaphase through the end of mitosis. Polo kinase (Cdc5 in yeast) phosphorylates three condensin subunits (Brn1, Ycg1 and Ycs4) from the onset of mitotic prophase and through anaphase (Lavoie et al. 2004, St-Pierre et al. 2009). These Cdc5-mediated phosphorylation events occur *in vitro* and *in vivo* (St-Pierre et al. 2009). Earlier work had shown that Cdc5 phosphorylation is critical in anaphase and that mutating all 44 annotated phosphorylation sites across all three subunits results in chromosome segregation defects. Condensin phosphorylation by Aurora B (Ipl1 in yeast) phosphorylation has also been shown to be important in anaphase (Lavoie et al. 2004). While few direct tests have been performed to determine precisely how these phosphorylation events affect condensin function, the consensus is that these condensin phosphorylation events promote or enhance condensin's ability to supercoil DNA. Other hypotheses propose that these events reconfigure condensin structure to allow for modulation of ATPase activity or oligomerization.

Work on mammalian Condensin I shows that not all phosphorylation events experienced by condensin are positive. Namely, most Condensin I subunits are phosphorylated by the Casein kinase in interphase as a mechanism to inhibit condensin function (Takemoto et al. 2006). How exactly these different phosphorylative states of condensin are coordinated remains unknown.

While these phosphorylation events are the main investigated branch of condensin function over the cell cycle, other modes of regulation exist. Large-scale studies have shown that several condensin subunits are sumoylated (reviewed in Dasso 2008). However, the functional relevance of this sumoylation is still under investigation. Additionally, a recent paper has shown that condensin's Ycg1 subunit is dynamically

regulated to ensure effective condensin binding to chromosomes. While the rest of condensin subunits are not actively degraded, Ycg1p has been shown to be dramatically degraded until S phase (Doughty et al. 2016). Lastly, in budding yeast, cohesin has been shown to be essential for proper condensin function (Guacci et al. 1997, Lavoie et al. 2004).

1.6 Of loops and rods: how SMC complexes perform their functions

Despite over two decades of study, we have only recently begun to uncover exactly how cohesin and condensin perform their functions. A large part of these recent studies have been a series of advances in biophysical, biochemical, genetics, sequencing and molecular tools that have allowed for more fine-tuned analysis of cohesin and condensin function. These tools have allowed the field to address several fundamental question. For example: do cohesin and condensin act as monomers or multimers? What are the functional implications of conformational changes in cohesin and condensin structure? How do cohesin and condensin reshape genomes? And, lastly, do cohesin and condensin interact with each other to determine their function.

Recent studies have uncovered that cohesin and condensin perform their functions through topological entrapment of DNA either intramolecularly (condensin and cohesin) or intermolecularly (cohesin) to allow for function. Historically, cohesin and condensin were thought to perform their entrapment of DNA through a simple embrace model (Haering et al. 2002). However, recently, a body of work has shown conclusive genetic evidence that cohesin performs its functions by binding DNA through at least two channels. The strongest evidence that cohesin acts as a multimer is the fact that two cohesin mutants can undergo interallelic complementation, thus allowing for the effective performance of cohesin's functions (Eng et al. 2015). Other biochemical assays have also implicated that a detectable fraction of cohesin is present in at least a dimerized state. More recently, some evidence has been presented that condensin is likely to also be functional in an oligomerization state. These recent developments are not surprising in light of the fact that bacterial SMC-like complexes have been long postulated to function as oligomers. Nonetheless, this paradigm shift in how we think SMC complexes raises several questions regarding how these complexes are oligomerize and how the regulators discussed above may play a role in this fascinating process.

One of the prevalent models in the field is that cohesin and condensin accomplish their unique functions on DNA through a loop extrusion mechanism (Fudenberg et al. 2016, Ganji et al. 2018). In brief, by this process, cohesin binds to DNA and processively extrudes DNA through a lumen until a stopping cue has been encountered. In the case of higher eukaryotes, this cue is thought to be a factor named CTCF. More recently, elegant studies using DNA curtains and purified condensin holocomplex have shown that a similar mechanism may be at play in condensin. In particular, this study highlighted the highly dynamic nature of the loops form by condensin. This observation led to the question of if/how these loops are stabilized in a cellular context. More

fundamentally, these studies pose the question about the biological underpinnings and physiological significance of these loops.

1.7 Dissertation Aims

In this dissertation, I address some of the gaps in our knowledge of the mechanisms governing mitotic chromosome condensation. This body of work was sparked by a fundamental question of how condensin and cohesin orchestrate their functions to ensure that chromosome condensation occurs in a tightly regulated spatio-temporal manner. To that end, we used a combination of yeast genetics, cytology, sequencing and chromosome-conformation-capture methodologies to dissect the relative contributions of cohesin and condensin to mitotic chromosome condensation. In Chapter 2, we describe the role of the polo kinase Cdc5p in the interactome of early condensation factors along with cohesin and condensin. In particular, we describe how cohesin modulation of this kinase may impact condensin function. In Chapter 3, we utilized micro-C to begin to characterize mitotic structures at nucleosome resolution. There, we also begin to dissect the fundamental roles of mitotic factors in these structures. Lastly, in Chapter 4, we further investigate the molecular defects a unique cohesin mutant that elegantly separates cohesin's roles in the establishment of cohesion and condensation.

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Chapter 2- Polo kinase Cdc5 as a novel component of establishment of chromosome condensation

2.1 Introduction

Mitotic chromosomes have two structural features, sister chromatid cohesion and chromosome condensation, that are essential for their proper inheritance during cell division. Sister chromatid cohesion is established concurrently with DNA replication and is maintained until the onset of anaphase. In contrast, condensation is established in mitotic prophase and persists through anaphase until the end of mitotic division. As a result, cohesion and condensation coexist from prophase until the onset of anaphase, a period henceforth referred to as mid-M. Perturbing proper condensation or cohesion leads to aneuploidy and chromosome rearrangements, hallmarks of cancer, birth defects and several genetic disorders (Hassler et al. 2018).

Cohesion and condensation are mediated respectively by cohesin and condensin, two related protein complexes in the SMC (structural maintenance of chromosomes) family (Figure 2.1A). Work over the past two decades has shown that these SMC complexes perform their functions through tethering of DNA either intermolecularly, resulting in cohesion, or intramolecularly, resulting in condensation. Condensin also likely promotes condensation thought its ability to induce supercoils (Kimura and Hirano, 1997; 2000, Kimura et al., 2001, Hagstrom et al., 2002, St-Pierre et al., 2009) and more recently to extrude a loop of DNA (Ganji et al. 2018). To establish and maintain condensation in this window of the cell cycle, condensin's activities (DNA binding, tethering, supercoiling or loop extrusion) must be regulated temporally. Furthermore, the amount of one or more of these activities must also be regulated to prevent chromosomes from being hypo or hyper condensed. Important aspects of the mechanisms that underlie condensin's temporal and quantitative regulation remain mysterious.

The first step in solving this mystery came with the identification of several protein kinases that regulate condensin's activity *in vitro* and *in vivo*. Cyclin-dependent kinase (Cdk) is required for condensation in prophase while polo kinase (Cdc5) and Aurora kinase (Ipl1) are important for condensation after the onset of anaphase (Lavoie et al. 2002; 2004, Robellet et al. 2015). The need for this switch likely reflects the need to maintain condensation after the onset of anaphase when Cdk activity is destroyed. These enzymes have been demonstrated to stimulate condensin's supercoiling activity *in vitro*. Conversely, Casein kinase (CK) can inhibit condensin's supercoiling activity *in vitro* and CK overexpression inhibits condensation *in vivo* (Takemoto et al. 2006). Thus CK may act as a buffer to prevent condensin hyperactivation.

Several observations suggest that additional elements likely contribute to condensin regulation in Mid-M. First, the notion that polo kinase promotes condensation only in anaphase came from the observation that inactivation of temperature sensitive polo kinase alleles led to defects in condensation only in anaphase (St-Pierre et al. 2009). However, polo kinase is active in mid-M, leaving open the possibility that it may

contribute to condensin activity prior to anaphase (St-Pierre et al. 2009, Walters et al. 2014, Archambault et al., 2015, Pakchuen et al. 2016). Second, in yeast, cohesin also regulates condensation. In this context, cohesin is thought to guide condensin to proper fold chromatin, thus preventing irreversible, condensin-dependent misfolding of chromosomes (Lavoie et al. 2002). While chromosomes condense in the absence of cohesin in other organisms, this potential role of cohesin in condensin-dependent condensation in mid-M may have been obscured by condensin-independent mechanisms of condensation. In light of these results, we used budding yeast to investigate further the *in vivo* regulation of condensin and condensation by yeast polo kinase (Cdc5p) and cohesin in mid-M. Here, we identify a novel role for Cdc5p in mid-M chromosome condensation and reveal a complex functional interactome of Cdc5p, cohesin and condensin that prevents condensin from misfolding chromosomes.

2.2 Results

The polo kinase Cdc5p is essential for mid-M chromosome condensation

The absence of cohesion and condensation defects in mid-M for *cdc5* temperature sensitive alleles suggested that Cdc5p was not required for mid-M chromosome structure. However, we postulated that these temperature-sensitive mutations may not have sufficiently abrogated Cdc5p function. With this in mind, we took advantage of the auxin-induced degron (AID) system to generate a more stringent *cdc5* allele. Briefly, in this system, fusions of essential proteins, such as Cdc5p, and an AID tag allow for near wild type function in the absence of auxin. However, in the presence of auxin, tagged proteins are targeted by the E3 ligase TIR1 for rapid ubiquitin-dependent degradation (Figure 2.1B, top). This conditional degradation allows for functional analysis of Cdc5p-AID or any similarly AID tagged protein in a tightly regulated time frame.

To assess mitotic chromosome structure prior to anaphase, we subjected *CDC5-AID* cells to the following protocol. Yeast strains were first synchronized in G1 with the mating pheromone, αF, then released into media with the microtubule inhibitor nocodazole for 3 hrs (Figure 2.1B, bottom). As a result of this protocol (henceforth referred to as the staged mid-M protocol) cells synchronously arrived at mid-M and spent equal time arrested in this state prior to analysis. To assess Cdc5p function in mid-M chromosome structure, auxin was added from G1 arrest to mid-M to deplete Cdc5p-AID.

We assayed sister chromatid cohesion in wild-type and *CDC5-AID* arrested cells using a well-characterized GFP dot assay. In this system, a lacO array is integrated at a chromosomal locus; elsewhere in the genome, a lacI-GFP capable of binding to this array is integrated. If proper cohesion is present, these GFP foci appear as one. Conversely, when sister chromatid cohesion is absent, two GFP spots are observed. As a positive control, we also subjected a *MCD1-AID* culture to the same regimen. Mcd1p is a core subunit of cohesin. Cells lacking Cdc5p-AID had a three-fold increase in separated sisters in mid-M cells compared compared to wild-type cells (Figure 2.1C). This increase was significantly less than the nine-fold increase in cells depleted for the

cohesin subunit, Mcd1p-AID. Thus, Cdc5p is important, but not essential for sister chromatid cohesion.

To assess whether Cdc5p functions in the establishment or maintenance of cohesion, we repeated the same experiment with cells expressing Cdc5p-AID or Pds5p-AID, and assayed sister chromatid cohesion at 15 minute intervals as cells progressed from G1 to mid-M. Pds5p is a factor known to be required for the maintenance of sister chromatid cohesion after S phase. Under this regimen, the detection of separated sister chromatids upon depletion of Cdc5p-AID occurred even later than depletion of Pds5p-AID (Figure 2.1D). Thus, Cdc5p was needed just for the efficient maintenance of sister chromatid cohesion. This function of Cdc5p is independent of its kinase activity as loss of cohesion was abrogated by the ectopic expression of a kinase-dead allele (Figure 2.1D).

Using the same regimen described above, we also interrogated the role of Cdc5p in mid-M chromosome condensation by assessing the state of the ~1 Mbp RDN locus. This locus contains 75-100 copies of the 9 kb rDNA repeat and undergoes a series of morphological changes depending on cell cycle state (Figure 2.1E, top). In interphase, the RDN locus is a puff that is segregated to the periphery of the bulk chromosomal mass. In contrast, in mid-M, the RDN forms a condensed loop. This dramatic cytological change makes the RDN a much more tractable system for assaying condensation than the rest of the genome that undergoes a limited two fold condensation that is detected only by a laborious assay measuring the change in distance between FISH or GFP probes (Guacci et al. 1994, Vas el al. 2007). Only a small fraction of wild-type cells are not condensed in mid-M. In contrast, the RDN failed to condense in over 80% of Cdc5p-AID depleted cells, to a similar extent as depletion of a core condensin subunit, Brn1p-AID (Figure 2.1E, bottom). Therefore Cdc5p-AID depletion caused a severe condensation defect. Ectopic expression of kinase-dead or polo-box defective alleles did not rescue the condensation defect of cells depleted for Cdc5p-AID. Thus, Cdc5p's kinase activity on one or more of its substrates is crucial for rDNA condensation in mid-M.

To assess the role of Cdc5p in the establishment and/or maintenance of condensation, we first examined RDN condensation in Cdc5p-AID and Brn1p-AID depleted cells at different time intervals as they progressed from G1-M. Both strains exhibited the same low level of condensation at all time intervals (Supplemental Figure 2.1). Therefore, Cdc5p is required for the establishment of condensation. To assess whether Cdc5p also functions in condensation maintenance, we modified the auxin treatment of our cells. We first arrested them in mid-M by adding nocodazole. Then, we added auxin and assessed rDNA condensation in cells prior to and after the addition of auxin (Figure 2.1F, top). Prior to the addition of auxin, the mid-M arrested *CDC5-AID* cells established RDN condensation similar to wild-type. Upon addition of auxin, the *CDC5-AID* cells lost condensation similar to the level of the *BRN1-AID* cells (Figure 2.1F, bottom). Therefore, Cdc5p is an essential regulator of condensation in mid-M, important for both the establishment and maintenance of chromosome condensation.

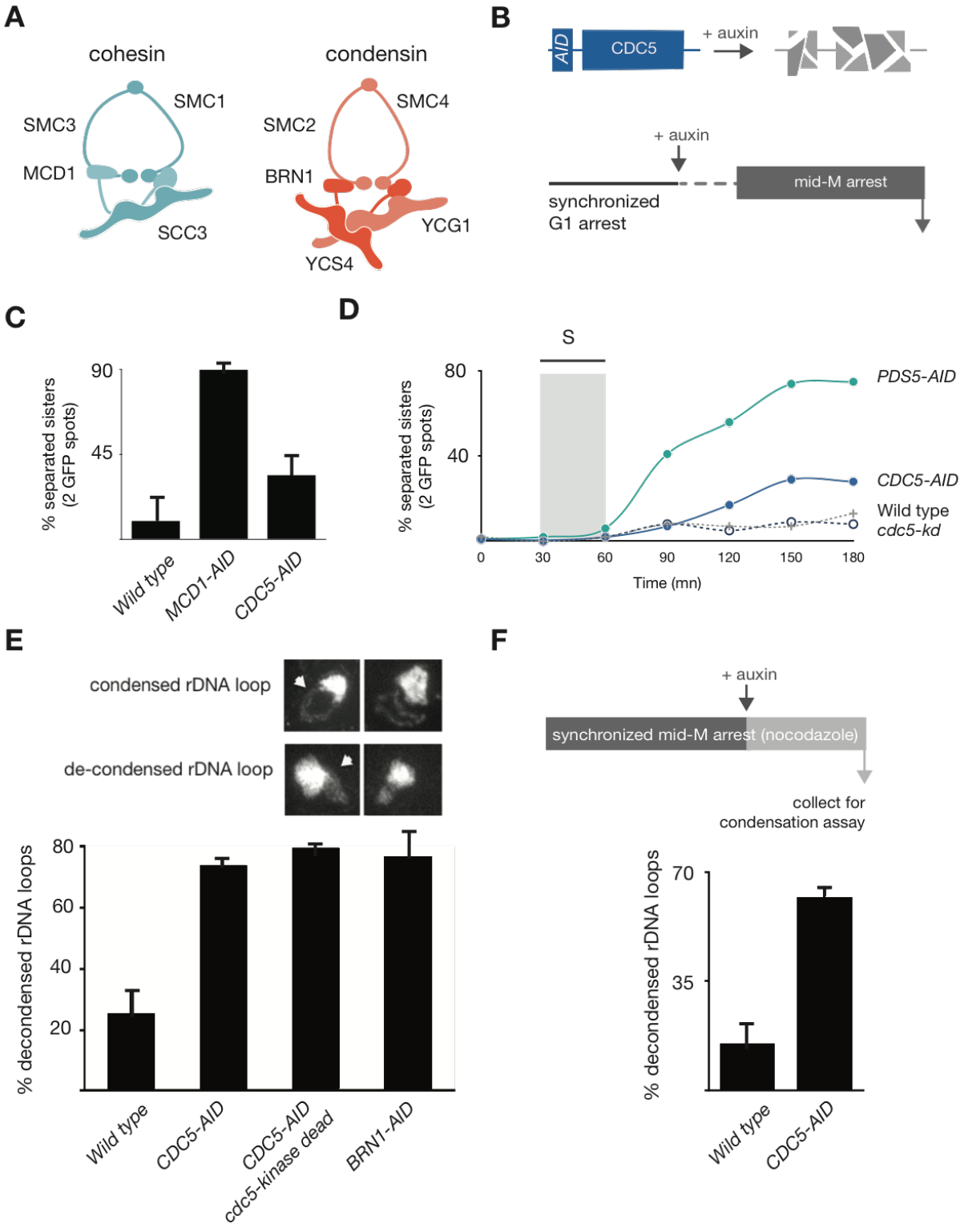

Figure 2.1 The polo kinase Cdc5p is essential for mid-M chromosome condensation

A. Schematic of cohesin and condensin. Cohesin is comprised of a Smc1p/Smc3p heterodimer joined at their head domain through Mcd1p. Mcd1p is likewise bound to Scc3p resulting in the cohesin holocomplex. Condensin is comprised of a Smc2p/Smc4p heterodimer linked by Brn1p.

Brn1p is bound to Ycg1p and Ycs4p. Together, these proteins form the pentameric condensin complex.

B. *Top:* Schematic of the auxin-induced degron (AID) system to study Cdc5 function. Cdc5p is fused to a 3V5-AID2 tag that allows for wild type functions under most conditions. With the addition of auxin, Cdc5p-3V5AID (hence Cdc5p-AID) is rapidly degraded in a proteasome-mediated manner.

Bottom: Regimen used to assess Cdc5 function in mid-M (staged mid-M assay with auxin): Early log phase cultures were grown in YPD, arrested in G1 at 23C using the pheromone alpha factor. Following synchrony in G, Cdc5p-AID was depleted by the addition of auxin for 1hr. G1 arrest was relieved by washes that removed alpha factor. Following washes, cells were resuspended in media with auxin and nocodazole for 3 hours to allow cells to progress through the cell cycle without Cdc5p-AID and then arrest in mid-M.

C. Depletion of Cdc5p results in a moderate cohesion defect. Cells were treated as in B and cohesion assayed in mid-M through a GFP spot assay. In these experiments, LacO arrays are integrated at LYS4, a centromere-distal arm site. The average percentage of separated sister chromatids from three independent experiments are reported. 100-300 cells were scored per sample. Error bars represent SD.

D. Depletion of Cdc5p results in a cohesion maintenance defect. Cells were treated as in B and cohesion assayed at 15mn intervals after release from G1 to mid-M. The average percentage of separated sister chromatids from two independent experiments (100 cells) are reported. S phase was determined by assaying time of DNA replication by flow cytometry.

E. Depletion of Cdc5p results in mid-M condensation loss. *Top:* Example of rDNA morphologies assayed. rDNA are either assayed as condensed “loops” or decondensed “puffs”. Percentage of cells displaying decondensed rDNA loops is quantified. Cells were treated as described in A and processed to make chromosome spreads to score RDN condensation (see Materials and Methods) after arrest in mid-M. Average of three experiments scoring 100-200 cells are shown. SD represents standard deviation.

F. Depletion of Cdc5p-AID in mid-M results in condensation loss. Early-log cells were arrested in mid-M at 23C using nocodazole for two hours. Arrest was confirmed by analysis of bud morphology (>95% large budded cells). Auxin was added for one hour to ensure Cdc5p-AID degradation. Cells were processed to assess condensation prior to and after the addition of auxin as described in E.

Cdc5p stimulates condensin-dependent RDN condensation

How does Cdc5p promote condensation? Previous analyses of cohesin and condensin suggested that Cdc5p could either directly guide the formation of chromosome folds, or alternatively inhibit the formation of a class of misfolded chromatin (Figure 2.2A). These classes were distinguished using a simple genetic test that we will refer to as the “add later” test (Figure 2.2B). If Cdc5p acted like condensin, and directly promoted chromosome folding, eliminating its presence between G1 and mid-M would trap chromosomes in a normal decondensed intermediate state. Addition of the Cdc5p in mid-M would allow normal condensation to proceed, thereby generating properly folded

RDN. In contrast, if Cdc5p acted like cohesin and guided condensin function, its absence between G1 and mid-M would allow condensin to misfold the chromosome. This misfolded state would be incapable of being converted to properly folded RDN even upon later addition of Cdc5p.

We performed this test with *CDC5-AID, BRN1-AID, MCD1-AID* strains. As described previously (Figure 2.1B) these strains were subjected to the staged mid-M protocol in the presence of auxin to deplete these proteins individually and in combination. Aliquots of these cells were assessed for their condensation once they reached mid-M. These cells were then washed and resuspended in auxin-free media while arrested in mid-M to allow the “late” addition of the depleted proteins. RDN condensation and protein levels were reassessed after two hours in auxin-free media when the levels of the proteins had been restored to wild-type levels as compared to no-auxin cultures staged in mid-M. (Figure 2.2C) .

As expected, depletion of any of these three proteins between G1 and mid-M prevented RDN condensation. Mid-M condensation was significantly restored upon addition of either Cdc5p-AID or Brn1p-AID, but not Mcd1p-AID (Figure 2.2D and Figure 2.2E). These results corroborated our previous results that suggested condensin is needed to generate chromosome folds for condensation, while cohesin prevents chromosomes from forming an irreversible disordered state (Lavoie et al. 2004). Furthermore, these results suggest that Cdc5p behaves like condensin and is needed to promote folding rather than guiding a folding activity.

We next asked whether Cdc5p promotes condensation through condensin. We had previously shown that co-inactivation of both Mcd1p and Brn1p between G1 and mid-M prevented condensation. However, this decondensed state became properly folded upon subsequent addition of both of these two factors. Thus, the irreversible misfolding of RDN in the absence of cohesin was prevented by removal of condensin. This result indicated that condensin drove RDN misfolding in the absence of cohesin. In wild-type cells cohesin must guide condensin to ensure it properly folds RDN. We corroborated these findings by comparing mid-M condensation with our “add-later” system in *MCD1-AID and MCD1-AID BRN1-AID* strains (Figure 2.2D).

If Cdc5p did indeed activate condensin in this pathway, then its co-depletion with Mcd1p should also prevent condensin-driven misfolding in the absence of cohesin. Indeed, when the *CDC5-AID MCD1-AID* strain was subjected to the “add later” regimen, the RDN condensed in mid-M upon the addition of Mcd1p and Cdc5p (Figure 2.2E). These results are consistent with a model whereby Cdc5p activates condensin to perform effective RDN condensation. Cohesin guides Cdc5p-activated condensin proper fold the RDN. Without cohesin, Cdc5p-activated condensin misfolds activates condensin, leading to condensin-driven RDN misfolding.

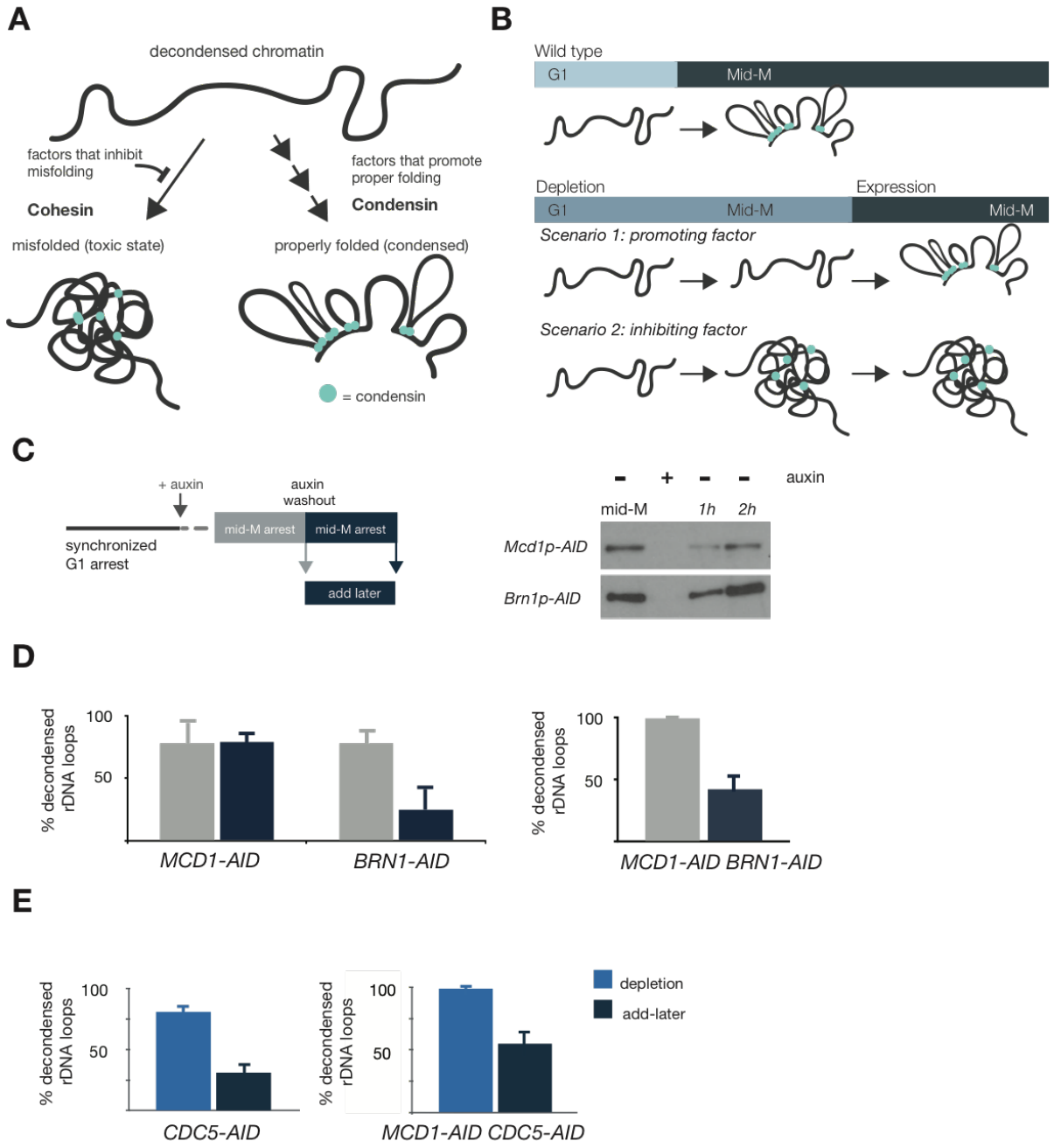

Figure 2.2: Cdc5p stimulates condensin-dependent RDN condensation

A. Schematic of condensation pathways. Decondensed chromatin is converted to condensed chromatin through the actions of factors that promote condensation (condensin) and factors that inhibit toxic decondensed state (cohesin).

B. Schematic of add-later genetic test setup. Regulators can either act as condensation promoters

C. *Left:* Regimen used for add-late genetic test. Early log phase cultures were grown in YPD, arrested in G1 at 23C using the pheromone alpha factor Following synchrony in G1 (>95% schmooed morphology), AID-tagged proteins (Mcd1p-AID, Brn1p-AID, Cdc5p-AID) were

depleted by the addition of auxin for 1 hour. G1 arrest was relieved by washes that removed alpha factor. Following washes, cells were resuspended in media with auxin and nocodazole for 90 minutes to arrest in mid-M. Cells were then resuspended in media containing only nocodazole for two hours to allow the synthesis and function of the AID-tagged proteins. *Right:* Effectiveness of AID-tagged protein depletion and resynthesis confirmed by assessing total protein levels compared to undepleted samples.

D."Add-later" genetic test results in different results from depletion of Mcd1p-AID compared to Brn1p-AID. Percentage of cells displaying decondensed rDNA loops is quantified. Cells were treated as described in 2.3C. Average of two experiments scoring 300-500 cells are shown. Protein levels upon depletion (grey arrow) and resynthesis (dark blue arrow) are shown.

E. "Add-later" genetic test results from depletion and resynthesis of Cdc5p-AID alone (left) or Mcd1p-AID and Cdc5p-AID co-depletion. Percentage of cells displaying decondensed rDNA loops is quantified. Cells were treated as described in 3C. Average of three experiments scoring 300-500 cells are shown. SD represents standard deviation.

To further test whether Cdc5p promotes chromosome folding rather than guiding folding, we compared RDN condensation in cells depleted of Cdc5p-AID, Mcd1p-AID, Pds5p-AID and Brn1p-AID with cells that also lacked the condensation inhibitor Wlp1p. We reasoned that if the RDN condensation defect upon Cdc5p depletion resulted from a failure to stimulate a basal activity of condensin this defect could be suppressed by the removal of Wpl1p, a condensation inhibitor. In contrast, if absence of Cdc5p caused RDN misfolding by condensin, removal of a condensation inhibitor like Wpl1p would only enhance RDN misfolding, thereby failing to suppress the mutant factor's condensation defect.

Guided by this logic, we compared the RDN condensation in *CDC5-AID, BRN1-AID, PDS5-AID, MCD1-AID* cells with double mutants containing a *WPL1* deletion (*wpl1Δ*). These eight strains were subjected to the mid-M staged protocol in the presence of auxin and assayed for condensation. As expected, *wpl1Δ* did not suppress the condensation defect of cells depleted for condensin (*brn1-9* temperature-sensitive inactivation). However, removal of Wpl1p partially suppressed the condensation defects associated with the depletion of Cdc5p-AID and Pds5p-AID (Figure 2.3). These results are consistent with the hypothesis that basal condensin function persists in these cells and the failure to activate this basal activity can be restored by removal of the Wpl1p inhibition. In contrast, removal of Wpl1p did not suppress the condensation defect of Mcd1p-AID depletion as expected if the defect in these cells were malfunctioning condensin rather than under-activated condensin. Taken together, the results from the add later and condensation inhibitor experiments are consistent with the model that Cdc5p acts as promoter of RDN condensation by promoting the ability of condensin to generate chromosome folds.

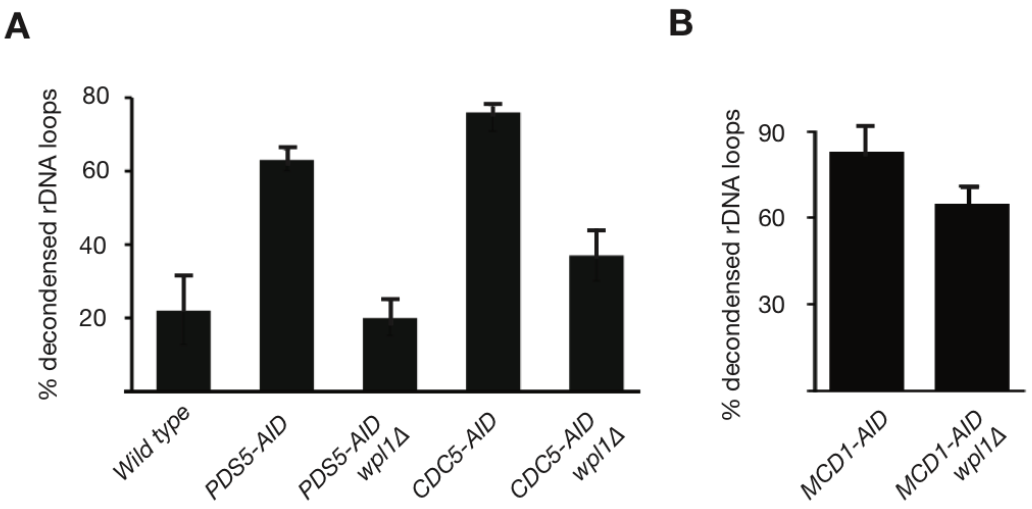

Figure 2.3: Deletion of Wpl1 ameliorates loss of Cdc5

A. Depletion of Wpl1p ameliorates condensation defect of Pds5p and Cdc5p loss. Percentage of cells displaying decondensed rDNA loops is quantified. Cells were treated as described in 1E. Average of three experiments scoring 100-200 cells are shown. SD represents standard deviation.

B. A. Depletion of Wpl1p does not ameliorate condensation defect of cohesin loss. Percentage of cells displaying decondensed rDNA loops is quantified. Cells were treated as described in 1E. Average of three experiments scoring 100-200 cells are shown. SD represents standard deviation.

Cdc5p binding at the RDN is modulated by cohesin but not condensin

From these results, we envisioned two scenarios by which cohesin and Cdc5p could modulate condensin function on chromosomes. In the first scenario, cohesin and Cdc5p could serve to affect condensin binding in DNA. Alternatively, cohesin and Cdc5p could modulate condensin function in a post-binding step. In the first scenario, we would predict that condensin binding would be affected by depleting either cohesin or Cdc5p. Conversely, if these factors affect condensin function through a post-binding step, condensin binding would be unaffected in the absence of these factors. To distinguish between these two scenarios, we performed chromatin-IP (ChIP) followed by sequencing (henceforth: ChIP-seq) to acquire high resolution genome-wide maps of cohesin, condensin and Cdc5 binding.

Using the staged mid-M regimen, we first assessed the wild type binding of cohesin, condensin and Cdc5. Our ChIP-seq results corroborated previous findings regarding Mcd1p and Brn1p binding (Lengronne et al. 2004, Wang et al. 2005). Briefly, Mcd1p binding occurred in large domains of unique DNA surrounding the centromeres (10-20 kb) and in 1-kb regions spaced ~10-15 kb along the length of the chromosome arms. The latter are referred to as CARs (cohesin associated regions) (Laloraya et al. 2000).

Mcd1p ChIP-seq also revealed the cohesin-bound domain within the RDN locus on Chromosome XII. Brn1p was enriched within a sub domain of the cohesin CAR and at pericentric regions, but not at any other unique sequences including the other CARs. The hyper enrichment of condensin at the RDN fits with the fact that the RDN undergoes a readily-visualized cytological change from a puff in interphase to a rod shaped loop in mid-M. As had been observed by ChIP-qPCR, Cdc5p colocalized with Mcd1p in the RDN as well as with Mcd1p in the pericentric and unique CARs genome-wide. This mid-M binding of Cdc5p to CARs genome wide including the RDN is consistent with a function in rDNA condensation and cohesion (Figure 2.4 A and B).

With these reference points, we systematically depleted cohesin (*MCD1-AID*), condensin (*BRN1-AID*) and Cdc5 (*CDC5-AID*) and assessed binding of the other components in our condensation pathway. Brn1p binding did not appreciably change at either the pericentromere or the RDN locus upon depletion of cohesin or Cdc5 (Supplemental Figure 2.3). Likewise, cohesin binding remained unaltered in the absence of either Brn1p-AID and Cdc5p-AID. Together, these results strongly suggest that cohesin and Cdc5p regulate condensin-dependent chromosome folding of the RDN by a mechanism other than promoting its normal pattern of chromosome binding.

Previous work indicated that Cdc5 binding at pericentric regions and unique CARs is largely dependent on cohesin binding. Indeed, upon Mcd1p-AID depletion from G1 to mid-M, Cdc5p binding is abolished at pericentric regions, CARs and the RDN locus and CARs genome-wide. As predicted, Cdc5p binding at pericentric regions and CARs was unaffected by depletion of condensin (Figure 2.4C and Figure 2.4D). Taken together, these data indicate that the inability to localize Cdc5p to the CAR at the RDN locus upon cohesin depletions correlates with RDN misfolding. Thus, cohesin-dependent localization of Cdc5p may be critical for proper condensation and cohesin function in the RDN.

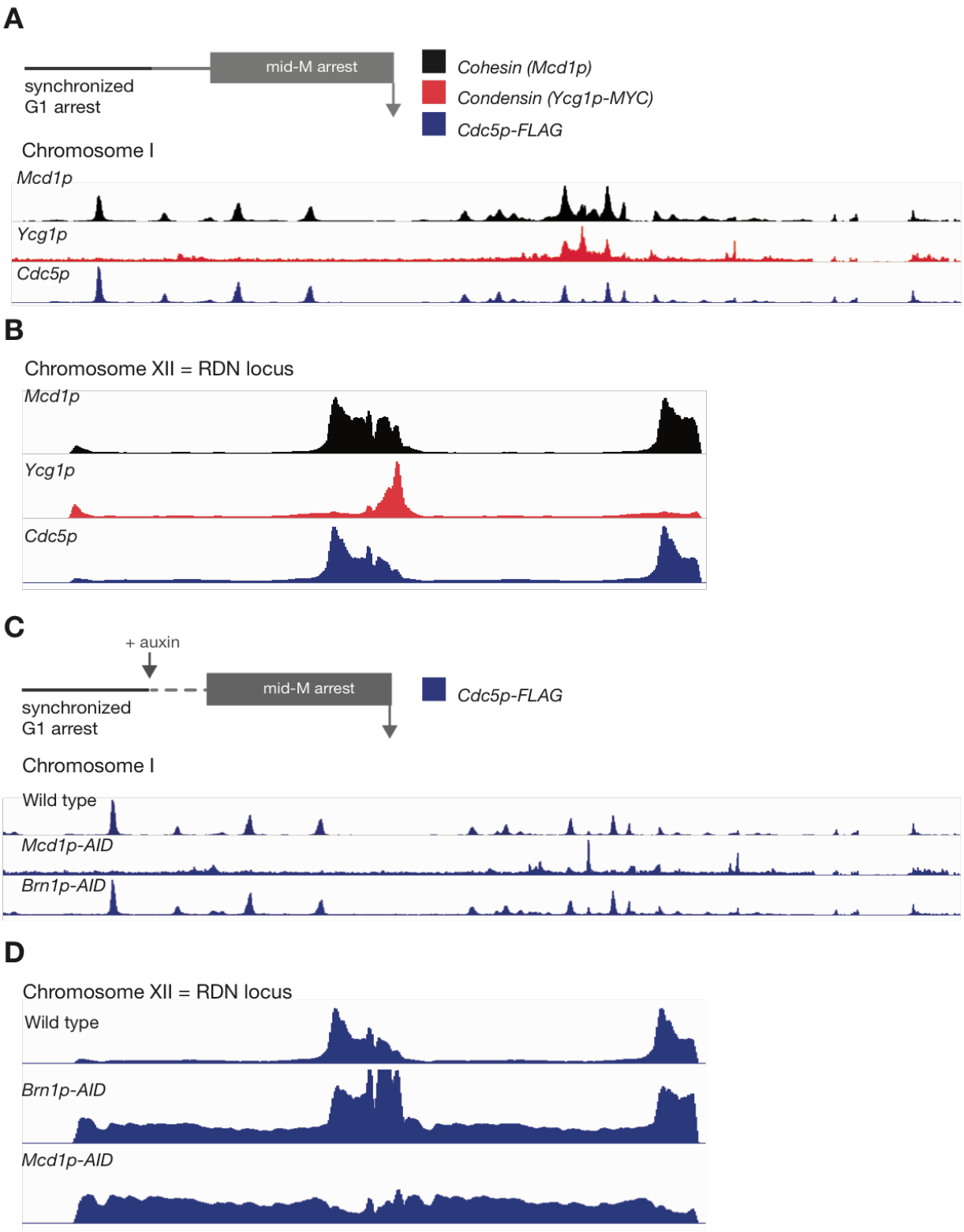

Figure 2.4: Cdc5p binding at the RDN is modulated by cohesin but not condensin

Figure 2.4: Cdc5p binding at the RDN is modulated by cohesin but not condensin

A. *Top:* Regimen for assessing chromosomal binding of Mcd1p, Ycg1p and Cdc5p in mid-M.

Bottom:
Chromosome binding pattern of Mcd1p (black), Ycg1p (red) and Cdc5p (blue) on Chromosome I. Mid-M arrested cells were processed for ChIP-seq as described in the materials and methods.

B. Binding of Mcd1p (black), Ycg1p (red) and Cdc5p (blue) at the RDN locus on Chromosome XII (shown: 440k-480k).

C. *Top:* Regimen for assessing regions of chromosomal binding of Cdc5p in mid-M after auxin depletion of cohesin or condensin.

Bottom: Binding pattern of Cdc5p (blue) on Chromosome I after depletion of either Mcd1p-AID or Brn1p-AID. Chromatin was sheared and immunoprecipitated with anti-Flag (Cdc5p) antibody. Samples were processed for ChIP-seq as described in the materials and methods.

D. Binding of Cdc5p at the RDN locus on Chromosome XII upon depletion of either Mcd1p-AID or Brn1p-AID (shown: 440k-480k).

As a preliminary test of this model, we engineered a strain where Cdc5p could be tethered to the RDN locus. Briefly, in this system, a catalytically-dead Cas9 protein is fused to Cdc5p (*pGAL:dCas9-Cdc5p*). In the presence of the appropriate sgRNA and galactose, Cdc5p can be tethered at sites of cohesin and condensin overlap at the RDN locus. As a proof-of-concept, we introduced this system into a strain where cohesin can be conditionally degraded (*MCD1-AID*). Usually, cohesin depletion results in nearly every cell experiencing a severe condensation defect. In the presence of the *dCas9-Cdc5p*, we observed that a small fraction of cells, 4.5%, generated a partially condensed RDN structure (small puffy loop), that we have not previously observed with only the depletion of *Mcd1p-AID*. If corroborated, these results are consistent with the model that localization of Cdc5p to the RDN can alter RDN structure.

2.3 Discussion

In this study, we have identified an interactome of early condensation factors (comprised of cohesin, condensin and the polo kinase Cdc5p) that control the establishment of chromosome condensation for the 1 megabase RDN locus of budding yeast. Cdc5p actively promotes proper condensation, likely by affecting condensin function, while cohesin prevents Cdc5p-activated condensin from misfolding chromosomes. Cohesin-controlled recruitment of the polo kinase Cdc5 at the repetitive RDN locus does not affect condensin binding. Rather, our data suggest that productive condensin activity is reliant on cohesin's spatio-temporal regulation of Cdc5p. Altogether, our study describes a novel mechanism by which cohesin regulates condensin activity and begins to provide mechanistic insight into how SMC complexes can be spatially and temporally coordinated to achieve their functions.

Cdc5's promotes the establishment and maintenance of condensation prior to anaphase

A key finding of our study is that Cdc5p is required for both the establishment and maintenance of condensation in mid-M of budding yeast. We showed that the depletion of Cdc5p prior to mitosis prevented condensation of the RDN locus and its depletion in mid-M caused the condensed RDN to unfold. Our results suggest that Cdc5p-dependent phosphorylation of condensin or a condensin regulator is necessary for the establishment of condensation and that continued phosphorylation of these sites is necessary to maintain condensation. We favor the idea that condensin is the key Cdc5p substrate since a previous study showed that Cdc5p phosphorylates condensin prior to anaphase (St-Pierre et al. 2009). Identifying the putative Cdc5p-dependent phosphorylation sites in condensin necessary for pre-anaphase condensation will be an important, but challenging future direction given the complexity of condensin phosphorylation in mitosis (Bazile et al. 2010).

The requirement for Cdc5p prior to anaphase expands its role in condensation. A previous study of condensation in yeast had suggested Cdc5p was only required to maintain condensation in anaphase. The failure to observe a pre-anaphase condensation function for Cdc5p was likely due to the use of temperature-sensitive alleles of Cdc5p. At the non-permissive temperature, these alleles likely had sufficient residual kinase activity to establish pre-anaphase condensation, but not enough to maintain it post anaphase. The degron allele in our study likely removes this residual activity. The requirement for less Cdc5p activity for pre-anaphase condensation may reflect an overlapping function with cyclin-dependent kinase which also phosphorylates condensin and is essential for condensation establishment (Robellet et al. 2015). Indeed, both kinases have been shown to stimulate condensin's supercoiling activity *in vitro* (St-Pierre et al. 2009). However, the fact both kinases are required *in vivo* for pre-anaphase condensation suggests that they must also have distinct biochemical roles in this process. These distinct roles may reflect the fact that condensin is a complex machine with multiple DNA binding activities, the ability to supercoil DNA, and the ability to translocate along DNA to extrude loops (Kimura and Hirano, 1997; 2000, Kimura et al., 2001; Hagstrom et al., 2002, Lavoie et al. 2002, St-Pierre et al., 2009, Ganji et al. 2018). The generation of loops may require that the different condensin activities are appropriately turned on and off by Cdc5p and Cdk phosphorylation of distinct sites on condensin or its regulators. Consistent with this idea, Cdk phosphorylation is limited to the Smc4p subunit of condensin while Cdc5p phosphorylates Ycg1, Ycs4 and Brn1 subunits.

Cdc5p and cohesin impact condensin-dependent condensation by distinct mechanisms

Here, we provide evidence that Cdc5p promotes RDN condensation by stimulating condensin-dependent condensation while cohesin guides Cdc5p-activated condensin to prevent RDN misfolding. We show that condensation establishment is blocked by the depletion of either condensin or Cdc5p but subsequent addition of either of these two factors in mid-M allows for the tardy establishment of condensation. These results imply that in the absence of these two factors, the decondensed RDN is trapped as a

condensation-competent state. In stark contrast, depletion of cohesin leads to a decondensed RDN state in mid-M that cannot be condensed upon subsequent cohesin addition (Lavoie et al. 2004). Thus, in the absence of cohesin, the RDN is trapped in an irreversibly decondensed state. Importantly, this misfolded state is prevented when either condensin or Cdc5p are depleted along with cohesin (this study, Lavoie et al. 2004). These results are consistent with the model that Cdc5p activates condensin and that cohesin guides Cdc5p-activated condensin to fold properly the RDN. In the absence of cohesin, condensin-dependent misfolding occurs. This misfolding is prevented by either by removing condensin itself or blocking its activation by Cdc5p.

This model predicted that the condensation defect in Cdc5p-depleted cells likely resulted from under activation of condensin-dependent condensation. Consistent with this model, we observed that the condensation defect of Cdc5-depleted cells could be suppressed by deletion of Wp1p, an established condensation inhibitor. Wlp1p was originally identified as a factor that binds to cohesin to dissociate it from chromosomes. Given this function, its ability to inhibit condensation was assumed to be due to an indirect consequence of its activity on cohesin. However, the Wlp1 ortholog in worms was shown to bind condensin (Hernandez et al. 2018). We suggest that the antagonistic functional relationship between Wlp1 and Cdc5p reflects their ability to bind to and directly modulate condensin function.

Cohesin and Cdc5p regulate condensation likely by a mechanism that affects a post DNA binding function of condensin

Additional genetic and molecular analyses of cohesin, condensin and Cdc5p suggest that the binding of Cdc5p to cohesin in the RDN is critical for proper RDN condensation. Using ChIP-seq, we extended previous studies regarding the relative chromosomal localization of Cdc5p, cohesin and condensin (Blat and Kleckner, 1999, Megee et al., 1999, Tanaka et al., 1999, Laloraya et al. 2000, Rossio et al. 2010, Leonard et al. 2015, Mishra et al. 2016). We showed that while condensin bound within the cohesin associated region of the RDN, its binding was independent of both Cdc5p or cohesin. Similarly cohesin binding to the RDN was independent of Cdc5p and condensin binding. These indicate that cohesin and Cdc5p affect a post DNA binding step of condensin. Altogether, these results lead us to propose a model whereby recruitment of Cdc5p to cohesin leads to the preferential phosphorylation of proximally bound condensin to regulate its function in properly folding the RDN. To begin to test of this model, we used a CRISPR based system to tether Cdc5p at sites of cohesin and condensin overlap within the RDN locus. Preliminary results suggest that this targeting of Cdc5p to the RDN could partially suppress the condensation defect of cells depleted for cohesin (*MCD1-AID*). These preliminary results support the potential importance of Cdc5p localization at the RDN by cohesin as a mechanism to modulate condensin function in condensation.

The fact that only a minor population was able to obtain this intermediate structure illustrates that simply localizing Cdc5p to the RDN is not sufficient for proper condensation. Rather, Cdc5p binding in the context of cohesin provides more

sophisticated spatial and/or temporal regulation of Cdc5p's ability to phosphorylate condensin. For example, the Mdc1p subunit of cohesin may serve as a phosphorylation sink. Since Cdc5p has been shown to phosphorylate Mcd1p at earlier and at a temporally distinct phase from condensin, we posit that saturated Mcd1 phosphorylation may act as a timer for Cdc5p phosphorylation of condensin (Alexandru et al. 2001). Characterization of cohesin mutants will be informative in determining precise mechanisms.

In summary, Cdc5p is a novel promoter of condensin-dependent condensation in mid-M. This Cdc5p function appears to be guided by its ability to bind cohesin, which binds proximal to condensin at the RDN. Could this mechanism serve as a general paradigm for how cohesin and condensin, or more broadly, SMC complexes, communicate to achieve proper chromosome structure? Cohesin is required for condensation during meiosis and mitosis in a number of organisms. It will be of future interest to test the functional relationship between Cdc5p and cohesin in these other contexts. Finally, cohesin also colocalizes with the Smc5/6 complex. It will be intriguing to determine whether their function is also coordinated by Cdc5p or another kinase.

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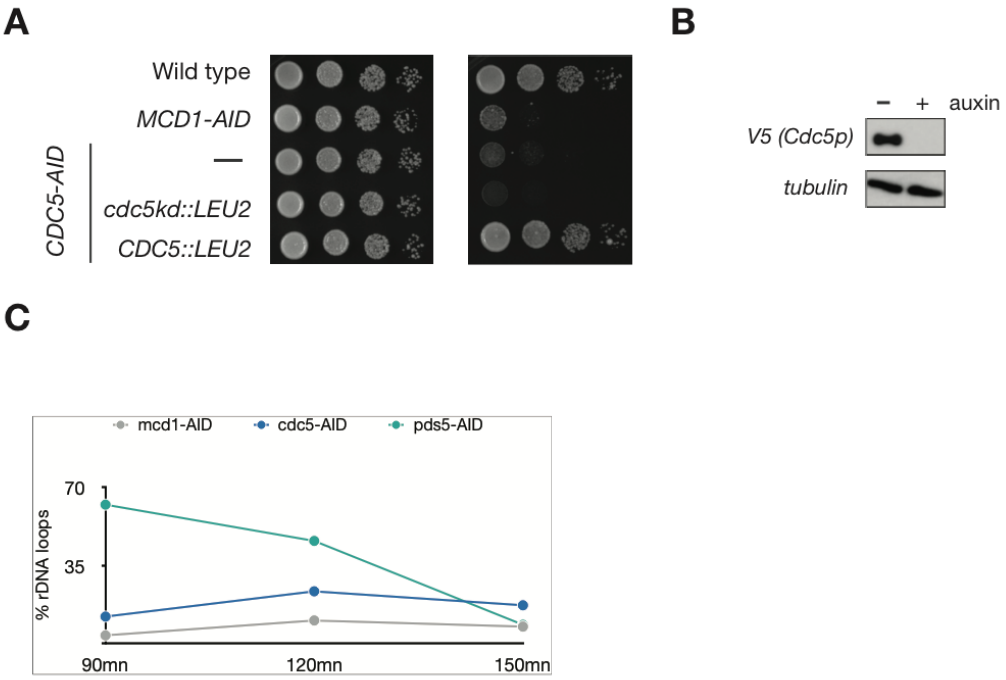
Supplemental Figures

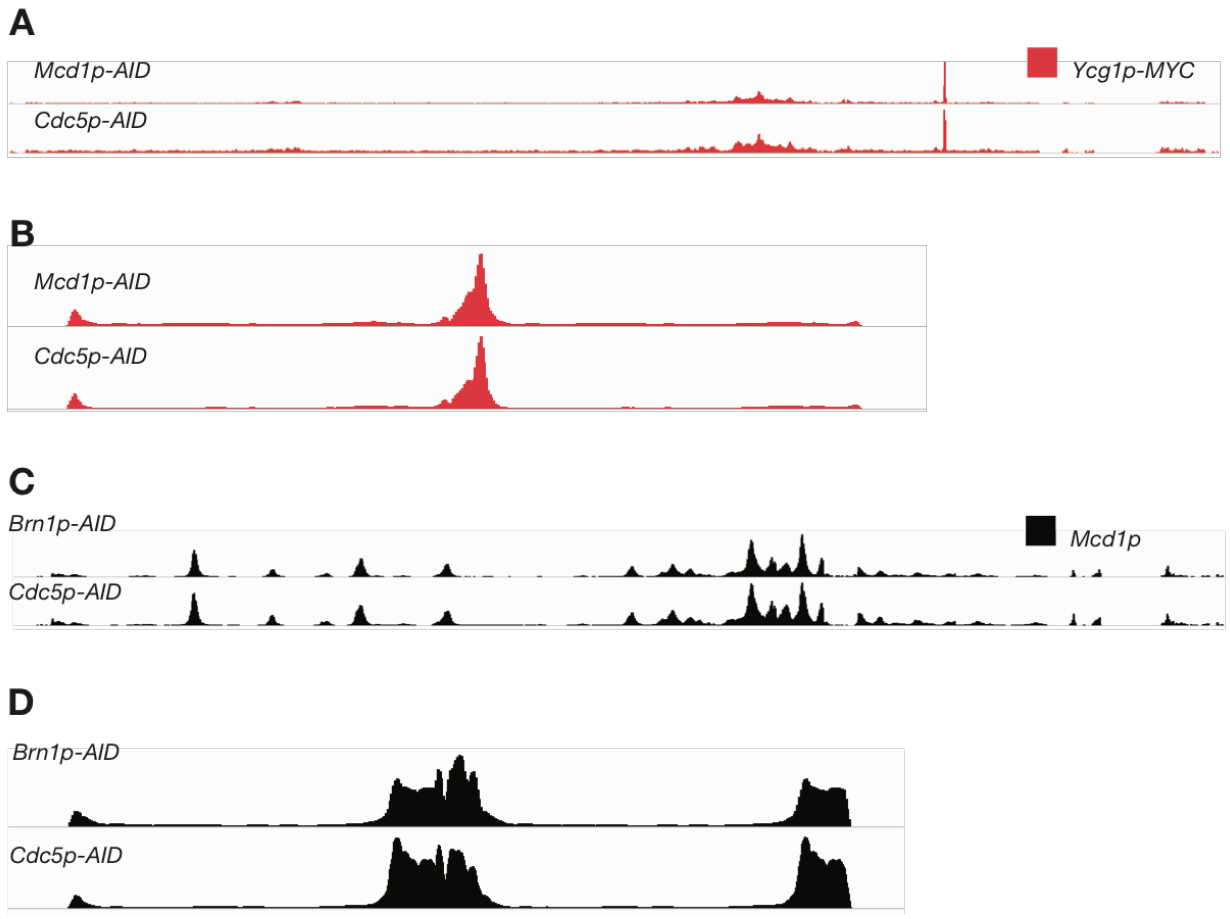


Supplemental 2.1: Characterization of CDC5-AID

A. Cdc5p is essential for viability. Asynchronous cells were serially diluted and plated on YPD (left) or YPD with 750uM auxin (right). Plates were then incubated at 23C for three days and imaged.

B. Depletion of Cdc5p-AID assayed by Western blotting. *CDC5-AID* strains were grown to mid-log overnight in YPD. Cells were then split and grown either in the presence or absence of auxin for 60mn. Cells were then pelleted and protein extracted by TCA as described in the materials and methods.

D. Loss of condensation assayed at the rDNA locus in *MCD1-AID*, *CDC5-AID* and *PDS5-AID* strains. Cells were arrested in G1 using alpha factor and released into YPD media. Samples were taken at the indicated time point. Fraction of cells with condensed rDNA loops is depicted.



Supplemental 2.1: Binding of condensin and Mcd1

A and B. Binding of Ycg1p upon depletion of Mcd1p-AID or Cdc5p-AID. Cells were arrested in mid-M and processed for ChIP-seq as described in materials and methods. Representative ChIP-seq plots of Ycg1p binding on Chromosome I (A) and at the RDN locus (B) are depicted.

C and D. Binding of Mcd1p upon depletion of Brn1p-AID or Cdc5p-AID. Cells were arrested in mid-M and processed for ChIP-seq as described in materials and methods. Representative ChIP-seq plots of Mcd1p binding on Chromosome I (C) and at the RDN locus (D) are depicted.

Chapter 3- Features of yeast mitotic chromosomes revealed by Micro-C

3.1 Introduction

Cytological and genetic studies have been instrumental in providing insights into the formation and regulation of mitotic chromosome structures. However, a detailed molecular understanding of how these changes occur has remained elusive. A major advance in the field came with the development of chromosome conformation capture methods, which function as higher-resolution assays for detecting changes in chromosome structures. This assay measures the contact frequency between any two sequences in the genome (Dekker et al., 2002, Lieberman-Aiden et al. 2009). From these contact frequencies, one can deduce patterns of inter- and intrachromosomal contacts that inform on the folding elements and conformation of chromosomes.

Chromosome conformation capture assays typically begin by treating cells with crosslinkers that covalently preserve the contact between two nucleosomes in close proximity. Chromatin is then digested either by restriction enzymes (HiC) or micrococcal nuclease (Micro-C) (Hsieh et al 2015, Hsieh et al, 2016). The addition of DNA ligase preferentially joins DNA molecules between crosslinked nucleosomes. The coordinates of the paired-end reads are plotted to generate a chromatin interaction map. The vast majority of joint molecule reads come from crosslinked nucleosomes from adjacent DNA sequences which map to the diagonal of the chromosome interaction map. Less abundant reads that lie off the diagonal come from crosslinking more distal nucleosomes on the same or different chromosomes. Off diagonal triangles in the map reflect chromatin interaction domains (CIDs), also called topological associated domains (TADs) that comes from crosslinking distal nucleosomes within a loop. The vertex of the triangle is the base of the loop (Figure 3.1A).

Many studies in mammalian cells have used chromosome conformation capture techniques to characterize the chromosome contacts in interphase and mitotic chromosomes and the relative contributions of cohesin, condensin and their associated regulators to the formation of these structures (Lazar-Stefanita et al. 2017, Schalbetter et al. 2017). From these works, we know that the bases of the loops in mammalian CIDs are marked by the boundary element CTCF which recruits cohesin (Hadjur et al, 2009, Dixon et al 2012, 2015, Nora et al. 2017, Rao et al. 2014, 2017). Both cohesin and CTCF are required for CID formation (Bonev et al. 2017). Additionally, the size and abundance of these CIDs are modulated by the cohesin-associated factors Pds5 and Wpl1, implying that cohesin stabilization and turnover are important for proper CID formation (Busslinger et al, 2017, Haarhuiss et al. 2017, Schwarzer et al. 2017, Wutz et al. 2017). While the role of condensin in these structures has been less studied, a condensin-related complex has an essential role in CID formation during dosage compensation of the X chromosome in *C. elegans*. Condensin is required to form secondary off-diagonal axes that form transiently during mitosis in animal cells (Nagano

et al 2017). Modeling of this secondary axis suggests that condensins organise chromatin into loops that are stacked around a condensin scaffold to form the dense rod-shaped structures of mitotic chromosomes.

While these studies provide key insights into the large-scale structural changes that genomes undergo, three essential questions still remained: How conserved are these structures? What factors determine their formation and maintenance in mitosis? And lastly, what is their functional significance? These questions have the potential to be thoroughly investigated using the budding yeast, *S. cerevisiae*, where cis and trans factors that either directly generate these structures or regulate their formation can be easily manipulated. However, HiC has failed to visualize CIDs or loops in yeast. The conceptualization of loop formation through the cell cycle has been largely model-driven. In fact, the strongest experimental evidence of CIDs in yeast came from analysis of transcriptionally-silent quiescent cells (Swygert et al. 2019). There, it was concluded that condensin is necessary for the formation CIDs. The absence of CIDs and loops in HiC data from dividing yeast may reflect fundamental differences in chromosome structure between yeast and mammals. Alternatively, loops and CIDs formed throughout the yeast genome could be smaller and thus below the detection limit of HiC. This is a likely scenario since the smallest loops and CIDs that can be detected by HiC are tens of kilobases in size because of the relatively rare cutting of crosslinked chromatin by restriction enzymes.

This size limitation of HiC is overcome by Micro-C, which, by using micrococcal nuclease, increases the resolution of chromosome conformation capture to identify nucleosome interactions in the range of hundreds of base pairs (Hsieh et al. 2015, 2016). Previous Micro-C analysis of asynchronously dividing yeast genomes revealed novel CIDs confined to individual genes, a few kilobases in size, but no larger loops or CIDs. These results again suggested that yeast lacks these features of mammalian chromosome structure.

However, we wondered whether larger loops and CID structures may have been missed because these structures are limited to specific cell-cycle stages. If this were the case, the diagnostic contact frequencies for these structures in dividing cells would be diluted below detection by the presence of cells in other parts of the cell cycle. We were motivated to test this potential cell-cycle effect since detection of these structures might provide a powerful assay to interrogate the function of Cdc5p, cohesin and condensin in chromosome structure. In Chapter 2, we showed that mutations in these factors exhibited the same dramatic elevation of decondensed RDN puffs. We wondered whether this common macro scale manifestation of decondensation might result from mutant-specific structural differences that may be revealed by Micro-C. These differences would inform on the function of these three critical factors.

In this chapter, we use Micro-C to provide evidence that the budding yeast, *S. cerevisiae*, forms mitotic loop structures similar to those observed in higher eukaryotes. These loops are acquired as cells progress through the cell cycle. The two sequences at the base of the loops are almost invariably CARs. Furthermore, these loops are

cohesin-dependent and modulated by the cohesin regulator Wpl1. Lastly, we provide early evidence that the RDN locus forms CIDs in interphase that are mostly eliminated in condensed chromosomes through the combined action of cohesin and condensin. No specific features of the chromosome capture map correlate with the cytological differences observed between condensed RDN in wild-type and decondensed RDN in the *cdc5* mutant, suggesting the existence of a yet to be discovered chromosomal structural feature.

3.2 Results

Yeast mitotic cells form loops in the non-repetitive sequences and reduces CIDs in the repetitive RDN

We began our studies by generating a chromosome interaction map for mitotic chromosomes. Wild-type cells were synchronized in mid-M and then processed for Micro-C (Figure 3.1B). The vast majority of reads in the chromosome interaction map lay along the diagonal as expected. In the non-repetitive sequences, we also observed off-diagonal vertices, consistent with the crosslinking of nucleosomes at the base of approximately 10-15 kb loops (Figure 3.1B and 3.1D). These loops appeared regularly along the arms of all chromosomes. The contact frequencies between sequences inside each loop were much lower than the contacts between the sequences at the base of the loop, suggesting that interactions within a loop were disfavored. In contrast, interactionswithin loops is the predominant feature of mammalian genomes.

Because of the established presence of cohesin at the base of the mammalian loops, we asked whether the two sequences at the base of the loops were enriched for cohesin binding. We compared the position of the base of the loops with cohesin-associated regions (CARs) of yeast as determined by ChIP-seq. The two sequences at the base of the loops were almost invariable the product CAR sites pairing, but not every CAR participated in a loop (Figure 3.1B).

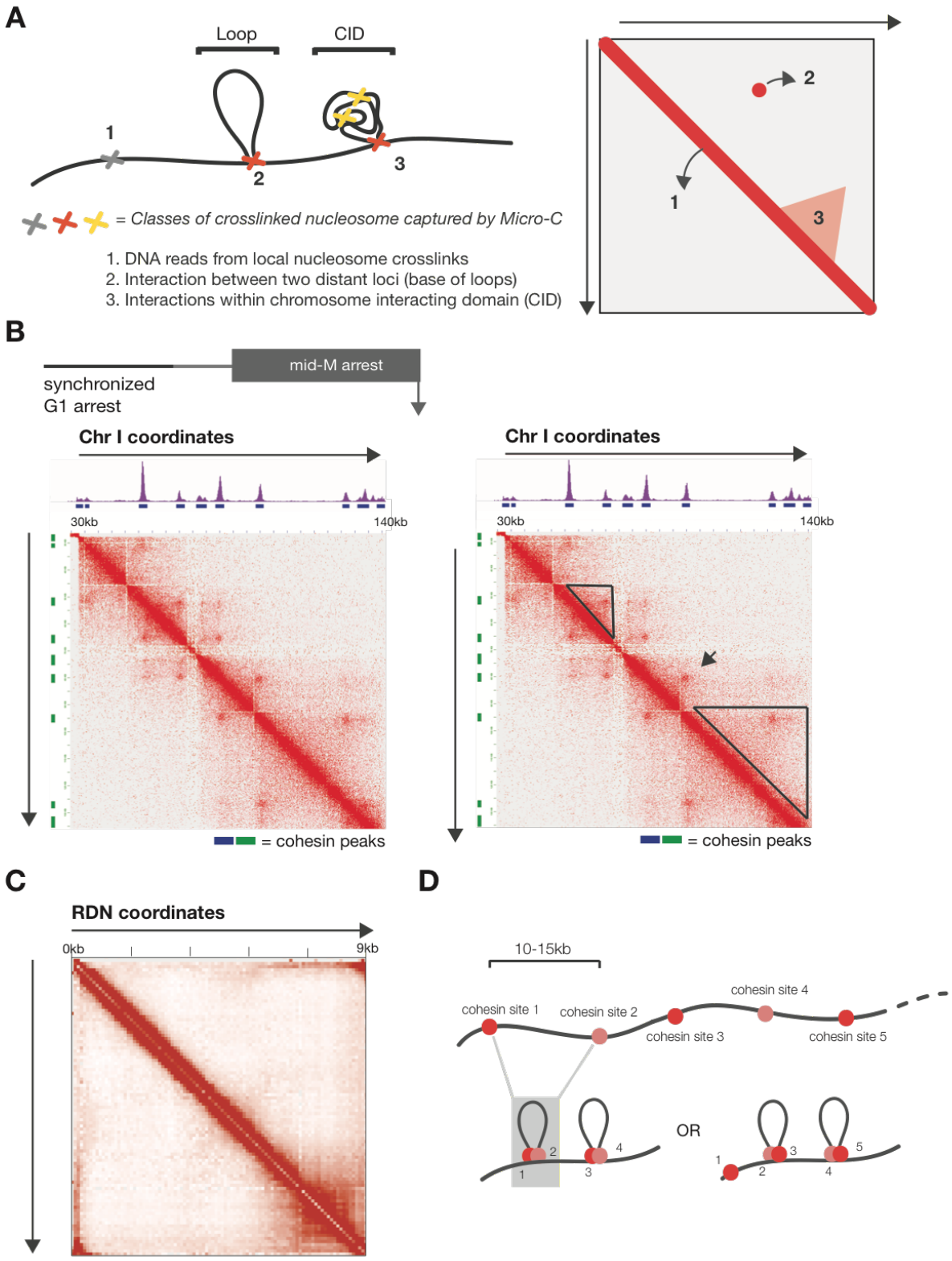


Figure 3.1 Yeast mitotic cells form loops in the non-repetitive sequences and reduces CIDs in the repetitive RDN

Figure 3.1 Yeast mitotic cells form loops in the non-repetitive sequences and reduces CIDs in the repetitive RDN

A. Schematic of crosslink classes captured by micro-C. Gray: DNA reads from neighboring nucleosomes, red: interaction between two distant regions brought together at the base of a loop, yellow: interactions within chromosome interacting domains (CIDs).

Right: Micro-C reads can be visualized on a plot. Coordinates are indicated in kb along top and sides of graphs. Center diagonal intensity indicates local interactions. Spots off diagonal are indicative of base of loops. Triangles indicate CIDs.

B. *Top:* Regimen for arresting cells in mid-M. Early log phase cultures were grown in YPD, arrested in G1 at 30C using the pheromone alpha factor at a concentration of 10-8M for 2.5 hours. Following synchrony in G1, cells were washed three times with media containing pronase and once with YPD. Following washes, cells were resuspended in YPD containing 15ug/mL nocodazole for 2.5 hours to arrest in mid-M. Cells were subsequently fixed and processed for micro-C as described in materials and methods.

Bottom: Left- Representative Micro-C pattern of Chromosome I (30k-140k). Data from ChIP-seq overlaid above with called cohesin peaks indicated in green and blue. Right- features of mitotic chromosomes revealed by micro-c: base of loop (arrow) and 10kb and 30kb CIDs (triangles).

C. Representative Micro-C pattern at the RDN locus (9kb region on Chromosome XII).

D. Schematic model of cohesin interactions.

To distinguish whether these 10-15 Kb loops were specific to mitosis, we carried out a preliminary analysis of contact frequencies as cells progressed through the cell cycle. We arrested wild-type cultures in G1 followed by release into media lacking any additional factors to allow for continuation in an unperturbed cell cycle (Figure 3.2A). Cells were collected at 15 minute intervals to assess cell-cycle stage by flow cytometry and micro-C analysis. G1-arrested cells as well as early time points lacked discrete loops but did contain CIDs (Figure 3.2B). However, time points enriched in G2/M populations (60 to 75 minutes) began to exhibit the loops seen in nocodazole-arrested micro-C contact maps. The loops were less abundant than that seen in arrested cells, likely because the mitotic synchrony of the arrested cells was better. These loops began to disappear by 90 minutes, a time at which cells would normally enter anaphase. Thus, we conclude that loops are a distinguishing feature of yeast mitotic chromosomes.

The non-repetitive DNA from mitotic chromosomes also contained large CIDs that ranged from 10 to 30 Kb in size. Within these CIDs were smaller CIDs from 1-3 Kb.The boundaries of all CIDs correlated with intergenic regions and not CARs. The presence of the large CIDs was unchanged as cells progressed through the cell cycle.

Our analysis of the chromosome conformation map of the repetitive RDN revealed several differences from the map of the non-repetitive DNA. When we mapped the contact frequencies of RDN sequences onto one rDNA repeat, no off diagonal vertices were detected in mitotically arrested cells or in cells as they progressed to mitosis

(Figure 3.1C and Figure 3.2 right panels). This difference from the non-repetitive sequences is likely explained if the bases of the loops in the RDN are confined to CARs as they are in the non-repetitive sequences. Since there is only a single CAR in a rDNA repeat, the association of this CAR from different repeats would bring together identical sequences. The paired-end reads from these inter-repeat contacts would lie along the diagonal and be obscured by the paired end reads from local intra-repeat contacts. Therefore, we cannot conclude from these data that the RDN locus does not have loops.

In addition, the rDNA repeat contained multiple small CIDs in G1-S cells that diminished as cells progressed through the cell cycle. One notable exception to this pattern is a CID associated with the spacer region where both cohesin and condensin are located. In mitotically arrested cells, this CID persisted while the other CIDs became undetectable. These results suggest that the contact between sequences within a rDNA repeat becomes more inhibited in mitotic cells.

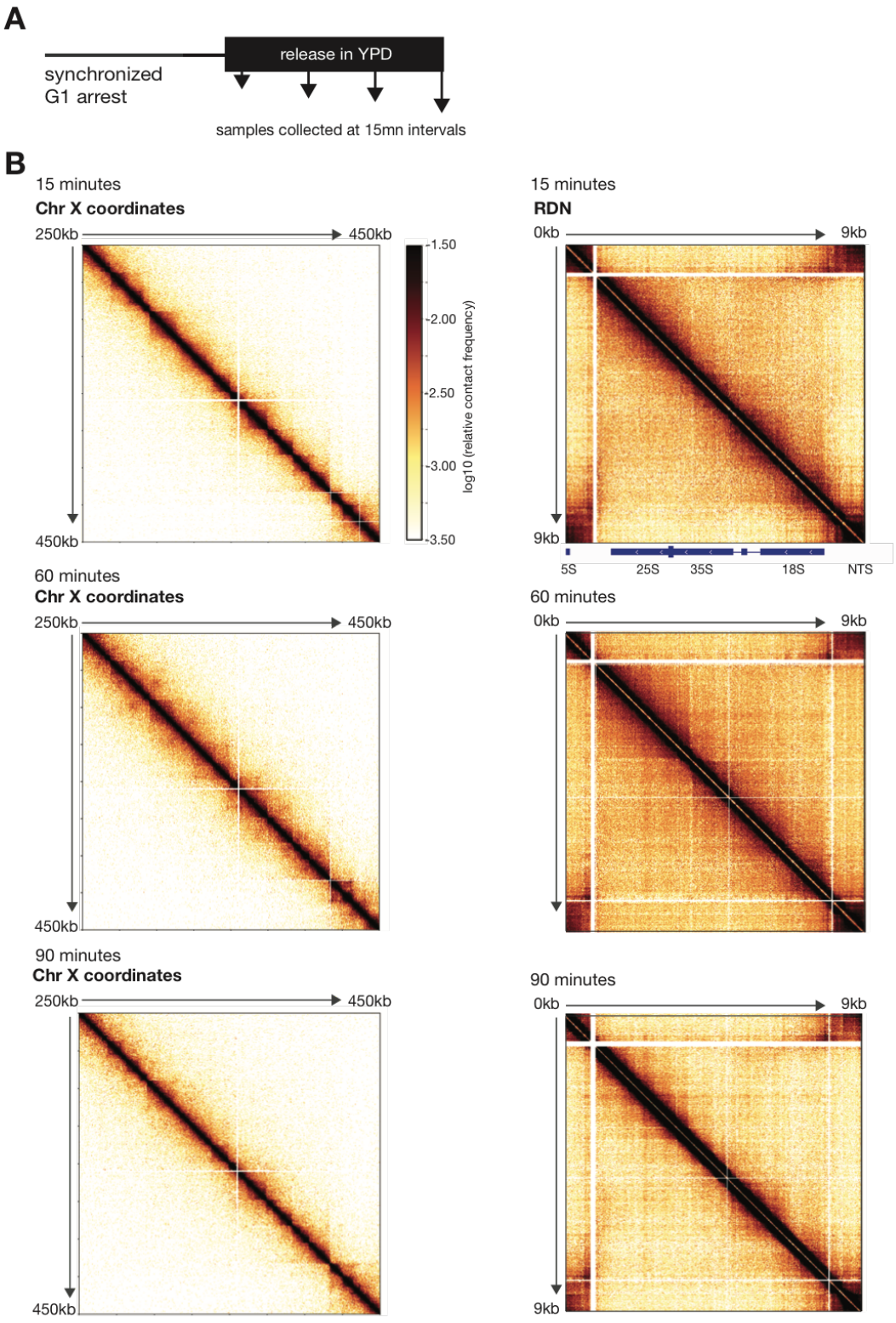


Figure 3.2: Changes in micro-c patterns over the cell cycle

Figure 3.2: Changes in micro-c patterns over the cell cycle

A. Regimen for sampling changes in Micro C as cells progress through the cell cycle. Early log phase cultures were grown in YPD, arrested in G1 at 30C using the pheromone alpha factor at a concentration of 10-8M for 2:30 hours. Following synchrony in G1, cells were washed three times with media containing pronase and once with YPD. Cells were subsequently released into YPD and samples were collected and fixed for Micro-C at 15 min intervals.

B. Micro-C pattern observed for representative region of Chr X and the RDN at indicated time points after release from G1.

Cohesin, condensin and Cdc5p different contributions to chromosome structure revealed by chromosome conformation capture analysis

Our analyses of chromosomes in wild-type cells provided a foundation to study the respective impact of cohesin, condensin and Cdc5p on chromosome structure. Using our staged mid-M assay, we depleted each of these factors from G1 until mid-M with the auxin-induced degron system detailed in Chapter 2 and Figure 3.3A. Depletion of cohesin was associated with the elimination of the discrete off-diagonal spots observed in the non-repetitive sequences in mitotic wild type cells (Figure 3.3B). This cohesin-dependence is consistent with the presence of two CARs at the base of each loop. In contrast, mitotic loops persisted despite depletion of either condensin or Cdc5p (Figure 3.3B and Figure 3.4A). Thus, neither of these factors are necessary for the formation or maintenance of these loops.

To further test the cohesin dependence of these loops, we assessed contact frequencies in cells lacking Wlp1p. In yeast, Wlp1p acts as a condensation inhibitor and has been shown to function by destabilizing cohesin binding to DNA. This activity is thought to generate a dynamic pool of cohesin that is needed for cohesin's distinct functions. To determine the function of Wpl1p on these loop structures, we performed Micro-C on mid-M-arrested *wpl1Δ* cells. Intriguingly, *wpl1Δ* cells exhibited increased interaction frequencies among more distant CARs (Figure 3.4B). Unlike wild-type cells, where interactions were often constrained to neighboring CARs, we observed that any given CAR now had more interaction vertices indicative of interactions with multiple additional CARs. Thus, we conclude that Wlp1p normally constrains the size and number of loops. These results indicate that cohesin is not only essential for loop formation but also that cohesin regulation is essential for controlling loop size and number.

We next turned to examine the impact of cohesin, condensin, Cdc5p and Wpl1p on RDN CIDs. Inactivation of either cohesin or condensin caused CIDs to appear along the length of the rDNA repeat in a pattern similar to what we observed in interphase cells (Figure 3.3 and Figure 3.4 right panels). These results suggested that the repression of CIDs in the mitotic RDN likely occured through a common structure that is dependent upon both cohesin and condensin. This common repressive structure was not

dependent upon Cdc5p as the CIDs did not appear in Cdc5p-depleted cells. The repression of CIDs in Cdc5p-depleted cells but combined with the macro-scale lack of condensation indicates that CID repression is not sufficient for condensation.

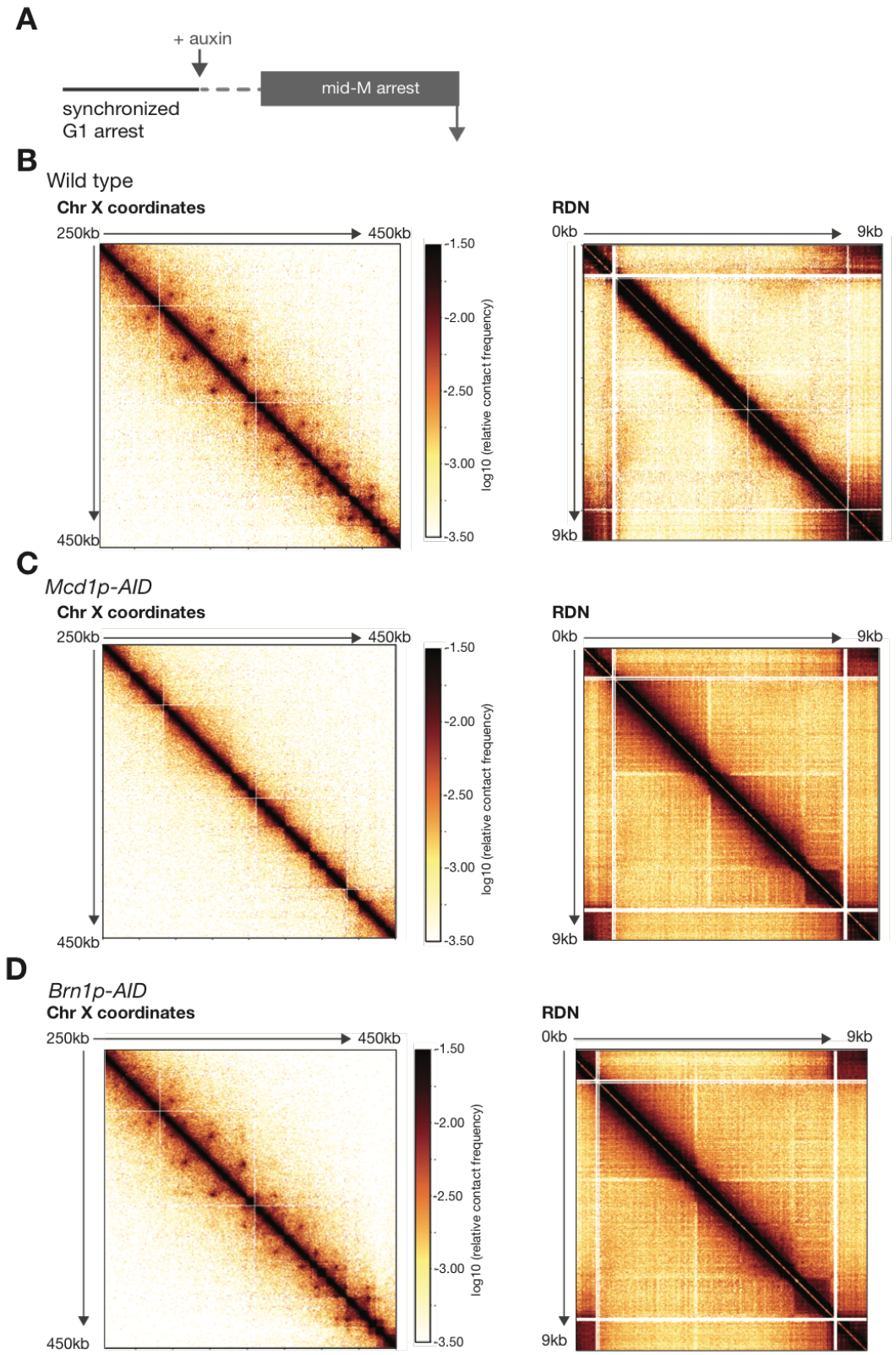


Figure 3.3 Different contributions of cohesin and condensin to chromosome structure revealed by chromosome conformation capture analysis

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A. Regimen for arresting cells in mid-M. Early log phase cultures were grown in YPD, arrested in G1 at 30C using the pheromone alpha factor at a concentration of 10-8M for 2:30 hours. Following synchrony in G1, cells were washed three times with media containing pronase and once with YPD. Following synchrony in G1 (>95% schmooed morphology), AID-tagged proteins were depleted by the addition of 500uM auxin for 1hr. Cells were then washed three times with media containing pronase and once with YPD. Following washes, cells were resuspended in YPD containing 500uM auxin and 15ug/mL nocodazole for 2:30 hours to arrest in mid-M. Cells were subsequently released into YPD and samples were collected and fixed for Micro-C as indicated in materials and methods.

B. Micro-C patterns observed with depletion of cohesin (Mcd1p-AID) or condensin (Brn1p-AID) compared to wild-type. Left: Chromosome X, Right: RDN locus on Chromosome XII.

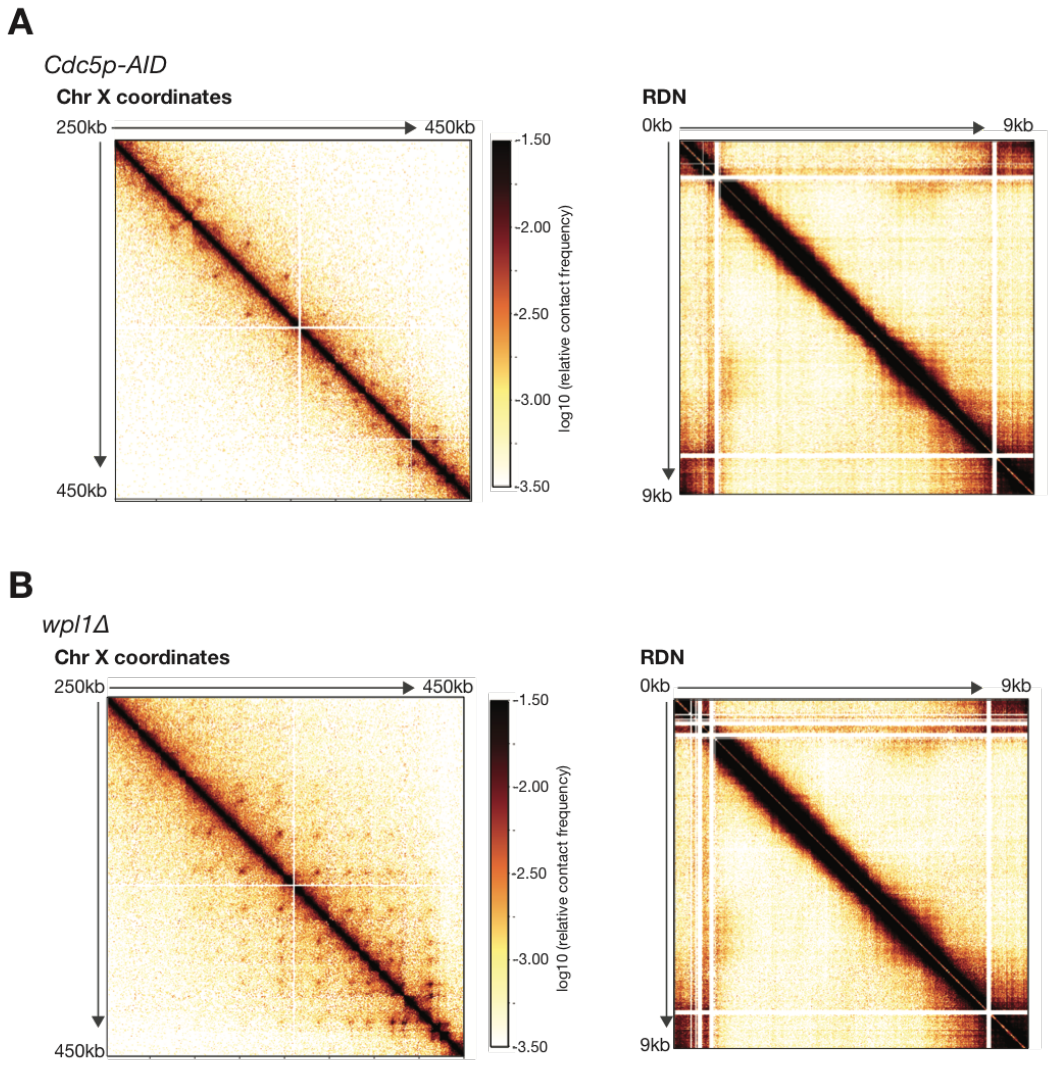

Figure 3.4 Different contributions of Cdc5p and Wpl1p to chromosome structure revealed by chromosome conformation capture analysis

A. Micro-C patterns observed with depletion of Cdc5p (Cdc5p-AID). Cultures were processed as in 3.3A. Left: Chromosome X, Right: RDN locus on Chromosome XII.

B. Micro-C patterns observed with deletion of Wpl1p (*wpl1Δ*). Cultures were processed as in 1A. Left: Chromosome X, Right: RDN locus on Chromosome XII.

3.3 Discussion

Here, we use Micro-C to provide evidence for the formation of cohesion-dependent loops in budding yeast. In mitotic cells, we observed robust off-diagonal vertices on the chromosomes conformation map that are spaced on average every 15 Kb. These vertices are consistent with the joining of distal sequences into a loop. The sequences at the base of the loops are almost always from two neighboring cohesin-associated regions (CARs). These loops are cohesin-dependent since they are abolished when cohesin is inactivated and likely post anaphase when cohesin is destroyed. Additionally, the inactivation of Wpl1p, a cohesin inhibitor, increases the number and size of loops associated with a CAR. The dependence of loop formation on cohesin and its regulation by Wlp1 are strikingly similar to the characteristics of loops in metazoans. Taken together, these results suggest that cohesin-dependent loops are a conserved structural feature of chromosomes from budding yeast to metazoans.

However, a comparison of the loops in yeast and metazoans revealed several important differences. For one, the size of cohesin-dependent loops (as indicated by the spacing of the vertices) is much smaller in yeast than metazoans (this study**)**. This difference in scale is likely due to the increased density of cohesin binding to chromosomes in yeast (on average every 15 Kb) compared to metazoans (average every 40 Kb). This increased density of cohesin likely also leads to more loops per unit length of DNA since cohesin at most CARs in yeast participate in loop formation (this study). Finally, cohesin-depedent loops appear to be constrained to different portions of the cell cycle in yeast and mammalian cells. In yeast, loops are absent in G1, appear in late S and persist in mid-M cells (this study) while in mammalian cells loops exist in G1 but disappear in prophase consistent with the dissociation of cohesin from chromosomes by the prophase removal pathway (Nora et al. 2017, Gibcus et al. 2018). The existence and different distribution of loops between metazoans and yeast provides tools to inform on their function and mechanism of formation.

In metazoans, loops have been proposed to be important for proper gene expression in part because loop structure could help restrict enhancer interactions to their appropriate promoters (Minajigi et al. 2015, Giorgetti et al. 2016, Symmons et al. 2016). However, a recent report appears to uncouple cohesin/CTCF dependent-loops with control of gene expression (Despang et al. 2019). The notion that loops may not control gene expression is supported by our finding of loops in budding yeast during mitosis, when gene expression levels are diminished. In budding yeast, inappropriate enhancer promoter interactions are unlikely because budding yeast enhancers act over very short distances, thus greatly reducing the possibility of inadvertent promoter activation.

Therefore, loops likely facilitate another conserved aspect of DNA metabolism between yeast and metazoans.

Another difference between budding yeast and metazoan loops is that yeast has no CTCF or any other candidate DNA binding proteins that preferentially interact with cohesin and/or sequences neighboring CARs. In metazoans, CTCF binding sites have been proposed to act as determinants of loop bases while cohesin acts as the motor that drives loop formation. In yeast, no other DNA binding factor has been found to co-localize with cohesin at CARs genome wide. Since most CARS and associated cohesin participate in loop formation, cohesin may directly mediate the chromosomal position and the formation of loops. How could cohesin mediate this process? Genetic and biochemical observations support the model that cohesin forms oligomers (Eng et al. 2015). Therefore, we propose that oligomerization of cohesins bound at CARs could provide a cohesin-intrinsic mechanism for the generation of loops.

In addition to loops, we also observed two classes of CIDs in wild type cells: larger CIDs that were approximately 10 kb in size and smaller gene size CIDs. These two CID classes appear throughout the genome. The location and intensity of these CIDs does not change during the cell cycle, suggesting that CIDs are not directly involved in chromosome condensation. Furthermore, these CIDs persist in cells depleted for cohesin or condensin. These results suggest that other factors must exist in budding yeast that regulate higher order chromosome structure.

In contrast, CIDs specifically disappear in the repetitive RDN locus in mitotically arrested cells. This suppression of CID formation in the RDN requires cohesin and condensin. This result suggests that cohesin and condensin have the ability to restrict interactions between different regions of a chromosome over the range of several kilobases. This function of cohesin and condensin may have been missed in Hi-C studies of mammalian that did not have the resolution to observe CIDs at this scale. A recent micro-C study in mammalian cells suggest CIDs at this scale are conserved in mammalian cells. It will be interesting to determine if they are altered by cohesin and condensin. If so, this relatively local impact of these two proteins on chromosome structure may provide an alternative explanation for their ability to modulate gene expression.

Interestingly, we observe that the suppression of CID formation in the RDN, was not dependent from Cdc5p function. This observation may seem at odds with our conclusion from our genetic experiments that Cdc5p likely enhances condensin activity for RDN cytological condensation. One observation that reconciles these two results is that Cdc5p activates basal condensin activity. This conclusion comes from our observation that removal of Wlp1p, a condensation inhibitor, suppresses the condensation phenotype of Cdc5p-depleted cells (see Chapter 2). The basal Cdc5p-independent activity of condensin may suffice for CID repression. Given that all three factors are required for RDN cytological condensation, CID repression is either necessary but not sufficient for RDN condensation or independent of RDN condensation.

The failure to detect a feature of chromosome capture that correlates perfectly with cytological loop formation in the RDN suggests that at least one type of chromosome fold is not detected by this technique. Indeed, while a recent study of mammalian cells has revealed a mitotic specific secondary axis, this axis appears for a very short time during mitosis, well after extensive chromosome condensation during prophase. The failure to observe the contacts diagnostic of these condensin-dependent prophase folds suggests that like yeast that important condensin-dependent folds remain undetected and awaits new technology for their exploration.

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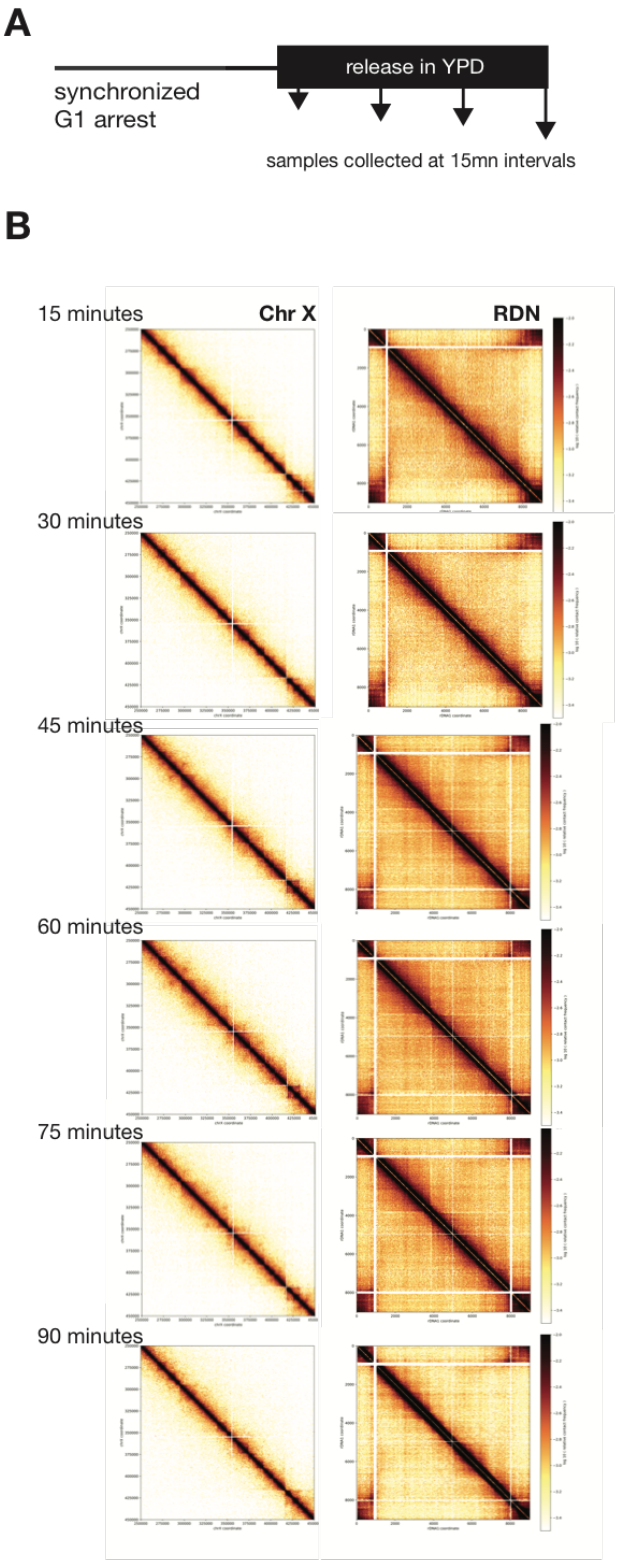
Supplemental Figures



Supplemental 2.1: Micro-C time course

A. Schematic of regimen.

B. Micro-C plots at Chromosome I (left) and at the RDN locus (right). Samples were taken at 15 minute time intervals and processed as described in the materials and methods.

Chapter 4- Cohesin mutant uncovers interactions between cohesin and Cdc5p

4.1 Introduction

Cohesin plays well-established roles in sister chromatid cohesion, chromosome condensation modulation of gene expression and DNA damage repair (Guacci et al. 1997; Michaelis et al. 1997, Blat and Kleckner, 1999; Laloraya et al., 2000; Lengronne et al., 2004; Kogut et al., 2009). In particular, mis-function of any one of cohesin's subunits results in detrimental effects on cellular fitness and chromosome segregation in mitosis and meiosis. The fact that cohesin is involved in such a diverse range of roles suggests that its activity must be heavily regulated and coordinated with cell cycle state and chromosomal context. While a lot of work has been done through depletion of cohesin's individual subunits, complete abolishment does allow for a more nuanced characterization of cohesin function.

An approach to parcel out cohesin's multitude of roles is by genetically isolating mutants that decouple its different functions. Recently, a ten-amino acid cluster in the linker region of Mcd1 was identified and named ROCC (Regulation of Cohesion and Condensation) (Eng et al. 2014, 2015). Mutations in ROCC allow for cohesion establishment but cannot maintain cohesion prior to anaphase. More importantly, cells harboring these mutations are incapable of establishing or maintaining condensation. This result showed that an activity of cohesin is specifically required for the establishment of condensation and not the establishment of cohesion. Additional studies of ROCC mutations revealed that stable cohesin binding to chromosomes is not sufficient for cohesin function and that Wpl1 antagonizes the condensation establishment activity of cohesin.

However, from these studies, a number of unaddressed questions remained. For example, how does ROCC contribute to condensation? Does it impact condensin function? If so, does the ROCC region of Mcd1p impact condensin function directly or indirectly through Cdc5p? Does ROCC-dependent activity of cohesin affect the activity or chromosomal binding of condensin? Here, using a combination of genetics, ChIP-seq and Micro-C, we began to investigate the molecular underpinnings of ROCC function in condensation.

4.2 Results

Synthetic lethality links the function of Mcd1p's ROCC domain to condensin.

We began our studies by addressing whether the condensation phenotype of *mcd1-Q266* cells reflected a previously unappreciated functional interaction between cohesin and condensin. To that end, we crossed a strain with *MCD1-AID* allele at the normal MCD1 locus and the *mcd1-Q266* allele integrated at the *LEU2* gene with a *MCD1* strain

harboring *YCG1-HA.* Half of the spores should inherit the *mcd1-Q266* allele which on its own is lethal. However, all these spores should be viable because they also inherit either *MCD1* or *MCD1-AID*, which masks the lethal phenotype of *mcd1-Q266.* One fourth of all spores from this cross should have both *YCG1-HA* and *mcd1-Q266* along with either *MCD1 or MCD1-AID*. Therefore we expected from 24 dissected tetrads, 24 spores to have both *mcd1-Q266* and *YCG1-HA.* However, none were recovered (Figure 4.1A). We did observe the expected number of spores with the genotype *MCD1-AID YCG1-HA and MCD1 YCG1-HA.* Therefore, *mcd1-Q266* caused a dominant synthetic lethality with *YCG-HA*. We also observed a similar synthetic lethality between *mcd1-Q266*and other tagged regulatory condensin subunits. These results are consistent with a functional link between ROCC and condensin.

Synthetic Lethality links the function of Mcd1p's ROCC domain to Cdc5p

The *ROCC* domain of Mcd1p lies near a consensus phosphorylation site of Cdc5p. Given our discovery of the interplay between Cdc5p and cohesin in condensation regulation (Chapter 2), we hypothesized that mutations in this domain of Mcd1p might have detrimentally affected Cdc5p function which in turn impacted condensin function. An additional hint that deregulation of Cdc5p might be the cause of *mcd1-Q266*'s condensation defect was the discovery *mcd1-Q266* cells had increased levels of Cdc5p.

With this in mind, we decided to investigate the impact of elevating Cdc5p levels in *mcd1-Q266* cells. Since *mcd1-Q266* is lethal, cells with this allele are kept alive by an extra copy of *MCD1-AID*. The phenotypes of *mcd1-Q266* are revealed usually by removal of Mcd1p-AID with auxin. Using this strain as base, we introduced a *CDC5* gene driven by the galactose promoter. The addition of galactose to the *mcd1-Q266 MCD1-AID pGal-CDC5* strain caused inviability even when Mcd1p-AID was present (no auxin addition) (Figure 4.1B). Furthermore, this inviability was restricted to specific cell cycle stages since transient overexpression of Cdc5p in cells arrested in S phase (hydroxyurea) and mid-M (nocodazole), but not G1 (αF) results in death. In contrast galactose-induced overexpression of Cdc5p in wild-type cells did not reduce viability (Figure 4.1C). This synthetic lethality between *mcd1-Q266* mutations and *CDC5* overexpression is consistent with the notion that ROCC regulates Cdc5p function to ensure proper condensin-dependent condensation.

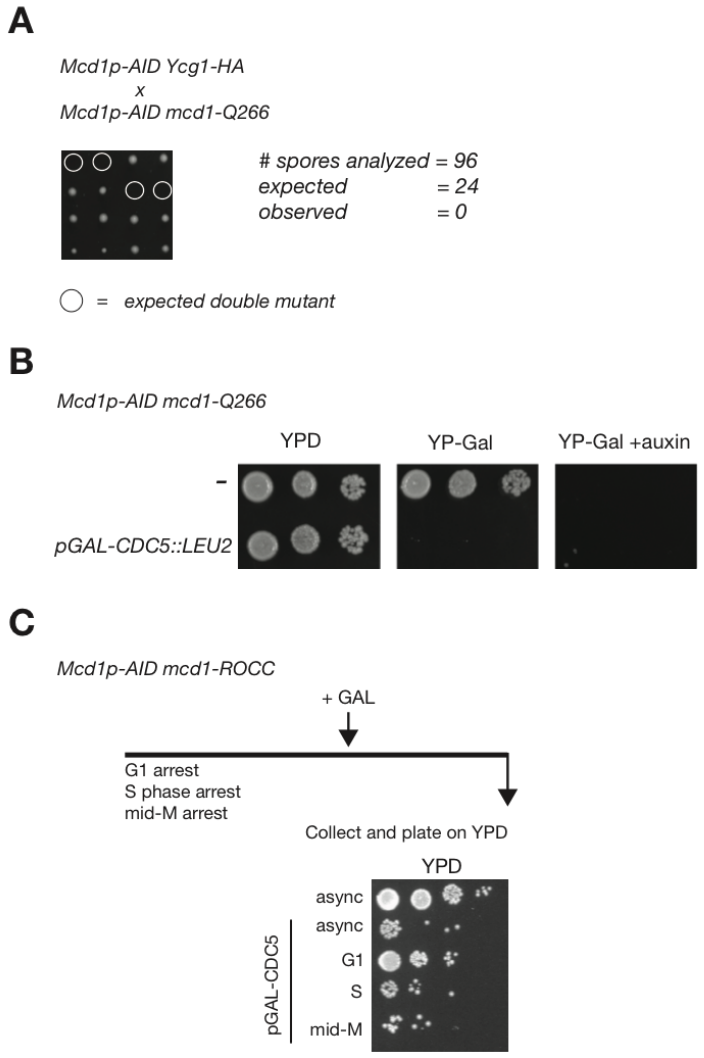


Figure 4.1: Synthetic Lethality links the function of Mcd1p's ROCC domain to Cdc5p and condensin functions.

A. Representative tetrad dissections of crosses between *MCD1-AID mcd1-Q266* and *MCD1AID YCG1-HA* haploid cells. Circles highlight inviable spores from tetrad dissections.

B. Growth phenotypes of *MCD1-AID mcd1-Q266*. *MCD1-AID mcd1-Q266* cells with or without an integrated copy of pGAL-Cdc5 were serially diluted and grown on either nutrient-rich media (YPD), nutrient-rich media with 2% galactose (YP-Gal), or nutrient-rich media with 2% galactose and 750uM auxin (YP-Gal + auxin) at 30°C for 3 days.

C. *Top:* Schematic of transient overexpression of Cdc5p in asynchronously dividing cells and cells staged in G1, S, or M.

Bottom: McCD-AID mcd1-Q266 pGal-Cdc5p cells were arrested either in G1 (alpha factor), S phase (hydroxyurea) or mid-M (nocodazole) in nutrient-rich media containing 2% raffinose for 2:30h. Overexpression of Cdc5 was induced by addition of 2% galactose to media for 90mn. Cells were then washed and serially diluted and grown on YPD at 30°C for 3 days.

mcd1p-Q266 impact condensation at a step independent of cohesin-dependent recruitment of Cdc5p

How could *mcd1-Q266* misregulation impair condensin function? One simple scenario was that *mcd1-Q266* mutations failed to recruit Cdc5p to chromosomes. To address this possibility, we synchronized *mcd1-Q266 MCD1-AID CDC5-FLAG* and wild type *CDC5-FLAG* cultures in G1. Auxin was added to deplete Mcd1p-AID. These cultures were subsequently arrested in mid-M with nocodazole, and cells were subjected to ChIP-seq (Figure 4.2A). The binding patterns of *mcd1-Q266* and Mcd1p to chromosomes were indistinguishable in the two strains indicating that the ROCC mutation didn't alter the level or position of cohesin binding to chromosomes, as reported previously (Figure 4.2B). The binding pattern of Cdc5p to chromosomes was also indistinguishable in the two strains (Figure 4.2C). Therefore, ROCC is not necessary to recruit Cdc5p to chromosomes. Therefore, ROCC may modify the activity of Cdc5p rather than its localization.

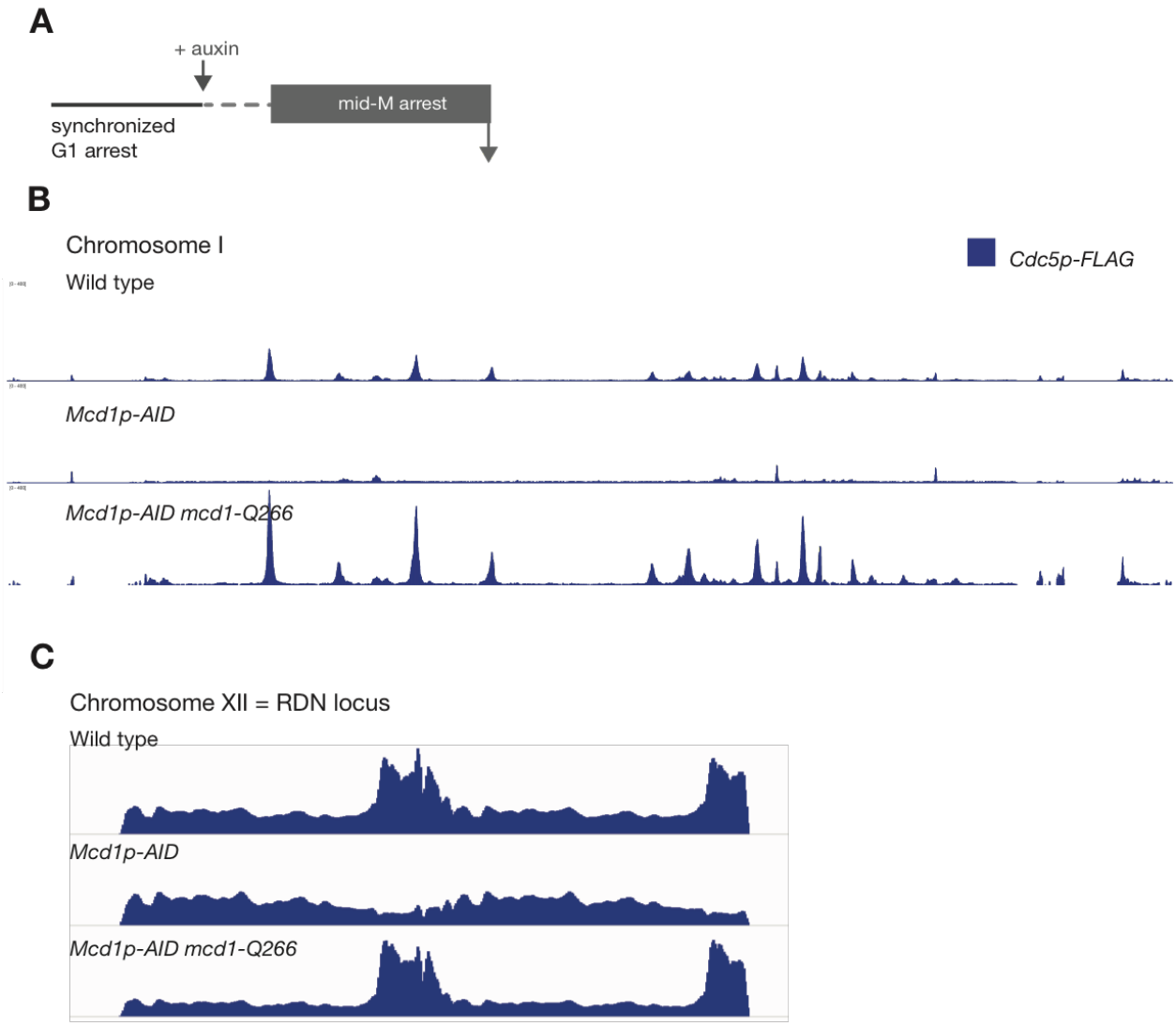


Figure 4.2: Mcd1p-Q266 impact condensation at a step independent of cohesin-dependent recruitment of Cdc5p

A. Regimen for assessing ChIP-seq in mid-M.

B. Binding of Cdc5p-Flag to chromosome I in wild type, *Mcd1p-AID* and *Mcd1p-AID mcd1-Q266* cells. Mid-M arrested cells were processed for ChIP-seq as described in the materials and methods.

C. Binding of Cdc5p-Flag in wild type, *Mcd1p-AID* and *Mcd1p-AID mcd1-Q266* at RDN locus on Chromosome XII. Mcd1p binding (top) is shown for comparison. Mid-M arrested cells were processed for ChIP-seq as described in the materials and methods using anti-Flag antibody to detect Cdc5p-Flag.

The ROCC *domain of Mcd1p controls chromosome structure*

In Chapter 3, we used Micro-C, a recently developed conformation capture methodology, to assess contact interactions throughout the yeast genome. In brief, we found that cohesin-dependent loops form along chromosome arms and that the RDN locus contains a single CID at cohesin-bound regions. While this assay is not fully reflective of the condensation defects cytologically and genetically, it does provide some insight into the structures that are formed and their dependencies on chromosome architecture factors.

With that logic, we sought to ascertain whether *mcd1-Q266* alters chromosome interactions. At unique regions, we observed that while spots consistent with loop formation are observed in the presence of *mcd1-Q266p*, the intensity of these loops is reduced compared to wild type (Figure 4.3A). At the RDN locus, we also now observed many low-frequency interactions (Figure 4.3B). Importantly, this phenotype is distinct from that observed upon depletion of cohesin and condensin, where novel, CIDs were formed.

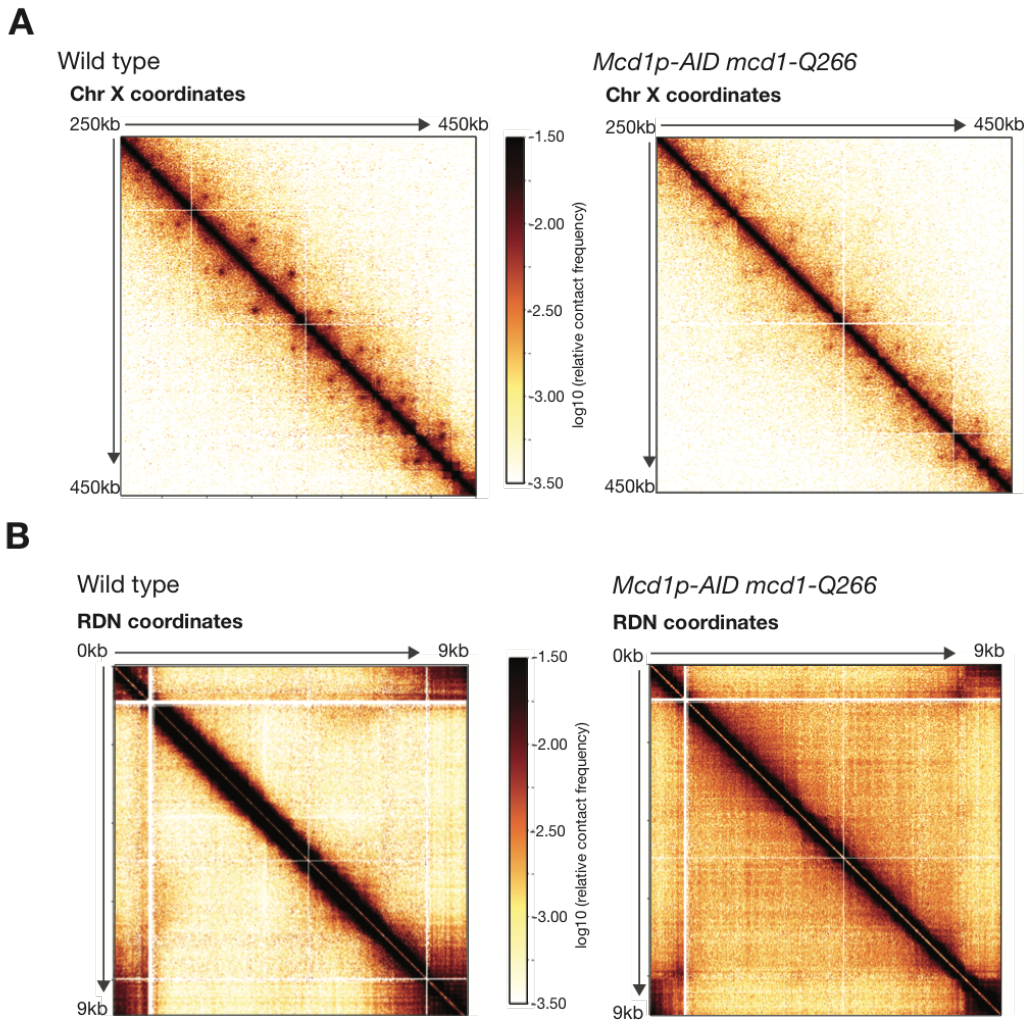


Figure 4.3: The ROCC *domain of Mcd1p controls chromosome structure*

A. Micro-C pattern of wild type and *mcd1-AID mcd1-Q266* strains at Chromosome X.

B. Micro-C pattern of wild type and *mcd1-AID mcd1-Q266* strains at RDN locus on Chromosome XII.

4.3 Discussion

Here, we begin to assess the molecular underpinnings of the molecular defects exhibited by mutations in the ROCC domain of Mcd1p. We observed that a mutation in the ROCC domain of Mcd1p was synthetically lethal with tagged condensin subunits of Brn1p and Ycg1p. This same ROCC mutation was also synthetically lethal with overexpression of Cdc5p. This synthetic lethality was observed when Cdc5p overexpression was transiently induced in S phase or mid-M cells, which are time frames where both cohesin and Cdc5 perform their functions. These genetic studies

show that the ROCC domain of cohesin functionally interacts with condensin and Cdc5p.

Features of these complex functional interactions between ROCC and condensin and Cdc5p are consistent with our model for the proper regulation of condensin-dependent condensation by cohesin regulation of Cdc5p. The functional interaction between ROCC and condensin is likely indirect as co-immunoprecipitate of cohesin and condensin has not been observed under wild type conditions. Modulation of Cdc5p provides a mechanism for this indirect interaction. In support of this indirect effect, the tagged subunits that exhibit synthetic lethality are also phosphorylated by Cdc5p. Finally, the fact that the synthetic lethality of the ROCC mutation is dominant suggests that the ROCC mutation leads to Cdc5p misregulation. Some obvious potential essential targets of Cdc5p are the condensin subunits.

However Cdc5p has numerous other essential targets. Therefore it will be important to follow up on these exciting observations. For example, Cdc5p overexpression in the *mcd1-Q266 MCD1-AID* strain should lead to defects in chromosome condensation as well as lethality. Similarly, the *mcd1-Q266* allele may be viable with tagged alleles of the condensin subunits Smc2p and Smc4p since they are not Cdc5p substrates. Lastly, we predict that the ROCC mutation may alter the timing or pattern of condensin phosphorylation.

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Chapter 5- Future directions inspired by this thesis

5.1 Overview

This dissertation details three key findings regarding how condensin, cohesin and Cdc5p orchestrate the early steps of chromosome condensation. First and foremost, this work establishes Cdc5p as a novel promoter of condensin-dependent condensation in mid-M (Chapter 2). Second, we provide evidence that *S. cerevisiae* forms cohesin-dependent loops similar to those observed in higher eukaryotes (Chapter 3). Lastly, we begin to link the function of a unique region of Mcd1p to functions of condensin and its regulator Cdc5p (Chapter 4). Altogether, these findings reshape our understanding how higher order chromosome structures are formed and inspired us to reassess some of our fundamental assumptions about how SMCs coordinate their function and how yeast can serve as a model for studying the formation of loops. However, intriguingly, these results leave us with many unanswered questions that have the potential to restructure how we think about higher order chromosome structures.

5.2 A paradigm for communication among SMC complexes

Cdc5p is part of a novel interactome of factors necessary in the establishment condensation. As alluded to in the introduction, Cdc5p was previously thought to only impact condensin in anaphase (St-Pierre et al. 2009, reviewed in Bazile et al. 2010). This shift in when and where we know Cdc5p functions poses the question of what earlier kinase functions may have been previously missed because of the sensitivity of their substrates at different cell cycle stages. Two examples come to mind as possible subjects of analysis. Ipl1p (Aurora B), has also been implicated for its role in maintaining condensin function in anaphase (Lavoie et al. 2002, 2004). Additionally, the casein kinase has been proposed to phosphorylate metazoan condensins as a mechanism to inhibit precocious condensin function during this time frame (Takemoto et al. 2006). Since yeast possesses orthologs of both these kinases, it would be interesting to assess the effect of auxin-mediated degradation of these proteins during early cell cycle stages. This is particularly true for Ipl1p since this kinase functions at much earlier cell cycle stages in the context of chromosome structure in metazoans. Re-analysis of kinase inactivation under more stringent conditions may help resolve some of the disparity in phenotypes observed in yeast and mammalian contexts.

The observation that Cdc5p's function on condensin appears to be guided by cohesin may serve as a paradigm for how SMC complexes communicate with each other to mediate large scale chromosome architecture phenomena. The question of how such strikingly similar proteins can occupy the chromosomal landscape and effectively perform their respective functions has been an unappreciated puzzle in our field. With this new perspective of one complex restricting the spatio and/or temporal regulation of another complex, we can begin to envision ways through which these simple machines could generate properly folded chromosomes rather than cross-linked tangled spaghetti. While our work thus far has focused on the interaction between cohesin and condensin, it is important to note that there is an additional SMC complex, SMC5/6, that also plays

less well-understood roles in chromosome structure. Could cohesin or condensin also interact with this complex? Indeed the binding sites of Smc5/Smc6 and cohesin overlap in S phase similar to the overlap of cohesin and condensin in the RDN in mitosis (Lindroos et al. 2006, Gallego-Paez 2014). This proximity suggests that regulation of cohesin and Smc5/Smc6 complex will also be intertwined. If so, how would these interactions be mediated? Lastly, we have restricted our studies to the mitotic cell division. One interesting avenue of study may be to study the conservation of this method of communication in the context of other cell cycle programs, such as meiosis.

Finally, we have shown that Cdc5p functions as a novel promoter of condensin-dependent condensation in mid-M. This Cdc5p function appears to be guided by its ability to bind cohesin, which, in turn binds proximal to condensin in the RDN. Is this an evolutionarily conserved paradigm for how cohesin and condensin, or, in fact, any two SMC complexes communicate to achieve proper chromosome structure? Cohesin is required for condensation during meiosis and mitosis in a number of organisms. In contrast, chromosomes can achieve a condensed state in the absence of cohesin in mammalian cells, but whether this a properly condensed state that ensures proper chromosome segregation has never been tested. It will be interesting to test the functional relationship between Cdc5p, condensin and cohesin in these other contexts.

5.3 Loops, CIDs and chromosome architecture

Studies in higher eukaryotes using chromosome conformation capture assays uncovered large scale structural changes of chromosomes (Dekker et al., 2002, Lieberman-Aiden et al. 2009, Hsieh et al 2015, Hsieh et al, 2016). Nonetheless, questions regarding the conservation, regulation, and functional significance of these structures still remained. These questions were particularly poignant since these structures had largely remained unobserved in a fundamental model system: *S. cerevisiae*. Our results in this dissertation indicate that yeast forms mitotic loops and CIDs and that condensin, cohesin and Cdc5p make different contributions to the formation of these structures. In sum, these structures are dependent on cohesin throughout most of the genome, and on both cohesin and condensin at the repetitive RDN locus. The observation that these loops can be affected by cohesin regulators, such as Wpl1p, suggests that budding yeast may be a genetically tractable system to thoroughly investigate the cis and trans factors necessary to generate and maintain these structures.

These studies may be particularly fruitful in yeast for two reasons. For one, as described above, discrete cohesin-associated regions (CARs), are located every 10-15kb (Laloraya et al. 2000). We have recently confirmed these with our high-resolution ChIP-seq assays. With the knowledge that neighboring CARs participate in loop formation, one avenue of research could be to systematically delete individual CARs to determine the effect of that deletion on the loop formation of nearby CARs. Secondly, work in our lab and others has generated a plethora of cohesin mutants defective in nearly every cohesin function (Guacci et al. 2012, Eng et al. 2014, Camdere et al. 2015, Robison et al. 2018). This includes a number of mutants that separate out cohesin's roles in

generating cohesion and condensation. More recently, we have acquired genetic and biochemical hints that cohesin molecules oligomerize and have started to test factors necessary for this function (Eng et al. 2015). It will therefore be of great interest to study how these different aspects of cohesin function modulate the patterns we observe by Micro-C.

One key distinction between the formation of cohesin-dependent loops in yeast compared to metazoans is the presence of CTCF, a protein which is thought to serve as a boundary element (Hadjur et al, 2009, Dixon et al 2012, 2015, Nora et al. 2017, Rao et al. 2014, 2017, Bonev et al. 2017). The result that loops occur in yeast despite the absence of this factor suggests that a different mechanism may be at play in establishing these loops. Interestingly, while yeast does not contain the canonical boundary element CTCF, one early study from 2002 attempted to express CTCF under the gal promoter and suggested that this allele could function as insulator at specific genomic loci (Defossez and Gilson 2002). A future avenue of study may be to determine the effects of this ectopic CTCF expression on chromosome structure by Micro-C.

Lastly, a key caveat to our studies in that the mitotic loops we observed were not affected by the depletion of condensin. This result stands in stark contrast to numerous FISH and live imaging studies that revealed that condensin is necessary even for the two-fold compaction that most of the yeast genome undergoes during mitosis (Guacci et al. 1994, Lavoie et al. 2002, 2004, Vas et al. 2007, Thadani et al 2018). Since condensin is not enriched at specific regions along chromosome arms, one possibility is that interactions among condensin complexes is not uniform and thus, abolishing condensin would not be appreciably noticed in our assays. An alternate possibility is that the loops and CIDs that we observe are reflective of a condensation-independent mechanism. One such possibility is that these structures are reflective of a change in gene expression that occurs during mitosis. This is particularly relevant given that the clearest example of CIDs that has been observed in yeast to date was determined from transcriptionally-silent quiescent cells (Swygert et al. 2019). Could our results be reflective of a similar machinery in mitosis? Future studies will need to assess the different transcriptional state of the mutants that we have recently characterized.

5.4 Cohesin mutants as mechanism to study why how Cdc5 function is restricted

Lastly, throughout this dissertation, we have shown that localizing Cdc5p to the RDN is not sufficient for proper condensation. Rather, its binding in the context of cohesin provides a mechanism for regulation of Cdc5p's phosphorylation of condensin. In Chapter 4, we described how a mutant of Mcd1 (*mcd1-Q266*) is especially sensitive to levels of Cdc5p. Importantly, this context renders cohesin synthetic lethal with tagged condensin. This provides us with a unique opportunity to assess how Cdc5p is modified through its binding to cohesin. One avenue of investigation is to analyze how chromosome-bound Cdc5p is modified under wild type conditions, in the absence of cohesin, condensin and in the presence of the *mcd1-Q266* allele of Mcd1. One strategy

to further dissect the difference in these different states of Cdc5p would be to employ IP-mass-spec to analyze differences in Cdc5 modifications in wild type and mutant cells. A similar approach could be used in parallel to determine whether there are cohesin-dependent modification sites on condensin that we may have missed in our bulk phosphorylation assays. Together, these avenues of investigation will potentially uncover how cohesin spatially and temporally affect condensin function.

More fundamentally, our studies reflected a previously unappreciated functional interaction between cohesin and condensin. The observation that *mcd1-q266* and tagged condensin are synthetically lethal with each other imply that cohesin and condensin do indeed function together. As described in the appendix, we have recently uncovered some very preliminary evidence that two cohesin regulators, Pds5p and Wpl1p interact with one condensin subunit, Ycg1p.

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Appendix 1- Materials and Methods

Yeast strains, growth and media

All strains used in this study are derived from the A364A background and their genotypes can be found in Table 1. YEP (yeast extract, peptone, dextrose) media and YPG (yeast extract, peptone and galactose) were prepared as previously described (Guacci 1997). Conditional AID degron strains were grown in YPD or YPG and auxin (3-indoleacetic acid; Sigma Aldrich Cat. I3750) at a final concentration of 500μM to deplete AID-tagged proteins. YPD auxin agar plates were prepared by cooling molten YPD 2% agar to 55°C and supplemented with auxin at a final concentration of 750μM.

Sister chromatid cohesion

Sister chromatid cohesion was assessed as previously described (Robison and Koshland 2017). In brief, in our strains, a lacO array is integrated at the *LYS4* locus on chromosome IV. These arrays can be visualized by binding of GFP-lacI, which is integrated at the *HIS3* locus. To assess cohesion, cells were grown to early-log phase (OD600 0.1-0.2) at 23°C overnight and arrested in G1 using the pheromone alpha factor at a concentration of 10-8M (Sigma-Aldrich T6901-5MG). If indicated, auxin was added to a final concentration of 500μM to deplete AID-tagged proteins after a 2:30h arrest. Cells were released from G1 by washing with YPD containing auxin (if indicated) and 0.15mg/mL pronase E (Sigma Aldrich) three times, and once with YPD plus auxin. After the last wash Cells were resuspended in YPD medium containing auxin and 15 μg/mL nocodazole (Sigma Aldrich). Cultures were then once again incubated at 23°C for 3h. 1mL of these cells was then fixed in 70% ethanol and stored at 4C until ready for visualization. Cohesion was assessed by scoring the number of GFP-lacI foci in each cell on an Axioplan2 microscope (Zeiss, Thornwood, NY) using the 100X objective (numerical aperture 1.40) equipped with a Quantix charge-coupled camera (Photometrics). For time course experiments, 1mL of cells was collected every 15mn after release from alpha factor.

Assessing condensation at the rDNA locus

Cells were grown as described for the sister chromatid cohesion assay. After 3h in nocodazole, cells were fixed, spheroplasted and prepared for FISH as previously described (Guacci et al 1994). DNA masses were then visualized with DAPI (4',6-diamidino-2-phenylindole) and imaged as described above. rDNA morphology was scored as condensed "looped" or decondensed "puffy" as previously described (Guacci et al 1994).

Maintenance Assay

For maintenance assays, cells were grown to early log phase at 23C and arrested in mid-M using 15ug/mL nocodazole. For AID-tagged proteins, auxin was added after

synchronous mid-M arrest, 2h after nocodazole addition. Cells were collected after 1h of auxin addition for condensation assay.

Depletion Add-Back time course

Cells were grown as described in the sister chromatid cohesion section to get synchronous population of cells arrested in Mid-M depleted for the AID-tagged protein(s). To restore the presence of the AID-tagged proteins, these cells were washed 3 times in YPD medium containing nocodazole and grown in media containing nocodazole for 2h. Samples were collected after 2h to assess condensation state and protein levels by TCA extraction followed by Western.

Chromatin immunoprecipitation (ChIP) and sequencing

Cells were grown and arrested at 30oC as described for assessing condensation. 60OD of mid-M cells were collected, fixed for one hour with 1% formaldehyde, quenched with 5mM glycine and processed for ChIP as previously described (Robison and Koshland 2017, with the following modifications). Frozen cell pellets were disrupted 3 times using a FastPrep-24 5G instrument (40 seconds, 6.0m/sec each) (MP Bio, Santa Ana, CA). Chromatin was sheared 10 x 30s ON/30s OFF with a Bioruptor Pico (Diagenode, Denville, NJ). For ChIP-qPCR, 1/20 of sheared and cleared lysate was reserved as input. Chromatin extracts were incubated with 4uL of the following antibodies monoclonal mouse anti-MYC (Roche), monoclonal mouse anti-FLAG (Sigma Aldrich), polyclonal rabbit anti-Pds5p (Covance Biosciences, Princeton, NJ), or polyclonal rabbit anti-Mcd1p (Covance Biosciences, Princeton, NJ) overnight at 4C. Antibody-bound lysates were incubated with 120uL Protein A dynabeads (Invitrogen, Cat#: 10001D) for 1h. In parallel, cells were collected for assessment of protein depletion. Following ChIP, the library was prepared using the Accel-NGS 1S Plus DNA Library Kit (Swift Bioscience) following the manufacturer protocol. Libraries were sequenced using Illumina Hiseq 4000. The sequencing files were aligned to the SacCer3 yeast genome using Bowtie2 tool and identified regions of enrichment were called using MACS, with default settings.

Micro-C

Cells were grown to early log phase and arrested in alpha factor at an OD of 0.12OD/mL for 2:30h at 30C to ensure synchronous G1 arrest. If indicated, strains were depleted of AID-tagged protein as described above, washed and resuspended in medium containing nocodazole (with 500uM auxin if indicated). 60OD of mid-M arrested cells were processed for micro-C protocol as previously described (Ref). In brief, cells were crosslinked with 3% formaldehyde for 10mn at 30C, quenched with 250mM glycine, pelleted and washed with water. Following this, cell pellets were suspended in 10mL buffer Z with 10mM B-ME and permeabilized with 250ug/mL zymolyase for 50mn at 30C. Following washing in cold 1XPBS, spheroplasted cells were resuspended in 3mM DSG crosslinker for 40mn at 30C and quenched with 250mM glycine. Cells were

then washed with PBS, aliquoted and frozen at -80C. Samples were then processed for MNAse treatment and library prep and analysis as described in Swygert et al. 2019.

TCA protein extraction

To assess protein level, cells were collected, pelleted, washed with 1X PBS and frozen at -80. Frozen pellets were resuspended in 200uL 20% TCA and broken for 40s using a FastPrep-24 5G instrument (6.0m/sec each) (MP Bio, Santa Ana, CA). Lysates were diluted with 1mL 5%TCA and spun for 10mn at 14,000rpm at 4C. Pellets were resuspended in 2X Laemmli buffer and boiled for 7mn and spun. Cleared lysates were used for Western blotting on 6% SDS-PAGE gels.

Appendix 1- Table 1 Strains

Strain	Genotype	Reference
DK5542	MATa MCD1-AID-KANMX6 ADH1-OsTIR1-URA3::ura3-52 lys4::LacO(DK)-NAT trp1-1 GFPLacI-HIS3:his3-11,15 bar1 leu2-3,112	Eng et al. 2014
TE228	MATa PDS5-3V5-AID2-KANMX6 lys4::LacO(DK)-NAT pHIS3-GFP-LacIHIS3::his3-11,15 trp1-1 ura3-52	Eng et al. 2014
RL400	MATa mcd1-Q266-6MYC-URA3::ura3-52 MCD1-AID-KANMX trp1∆::pGPD1-TIR1-CaTRP1 lys4::LacO(DK)-NAT leu2-3,112 pHIS3-GFPLacI-HIS3:his3-11,15 bar1	This study
RL06	MATa CDC5-3V5-AID2-HPHMX ADH1-OsTIR1- URA3::ura3-52 lys4::LacO(DK)-NAT trp1-1 GFPLacI-HIS3:his3-11,15 bar1 leu2-3,112	This study
RL401	MATa BRN1-D375-3V5-AID2-HPHMX trp1∆::pGPD1-TIR1-CaTRP1 lys4::LacO(DK)-NAT leu2-3,112 pHIS3-GFPLacI-HIS3:his3-11,15 ura3-52 bar1	This study
VG3620-4C	MATa trp1∆::pGPD1-TIR1-CaTRP1 lys4::LacO(DK)-NAT leu2-3,112 pHIS3-GFPLacI-HIS3:his3-11,15 ura3-52 bar1	Çamdere et al. 2015
RL402	MATa pGAL-CDC5::LEU2 trp1∆::pGPD1-TIR1-CaTRP1 lys4::LacO(DK)-NAT leu2-3,112 pHIS3-GFPLacI-HIS3:his3-11,15 bar1	This study
RL403	MATa pGAL-CDC5::LEU2 mcd1-Q266-6MYC-URA3::ura3-52 MCD1-AID-KANMX trp1∆::pGPD1-TIR1-CaTRP1 lys4::LacO(DK)-NAT leu2-3,112 pHIS3-GFPLacI-HIS3:his3-11,15 bar1	This study

RL404	MATa CDC5-3V5-AID2-HPHMX YCG1-S406-6MYC-KANMX ADH1-OsTIR1-URA3::ura3-52 lys4::LacO(DK)-NAT trp1-1 GFPLacI-HIS3:his3-11,15 bar1 leu2-3,112	This study
RL405	MATa MCD1-AID-KANMX6 YCG1-S406-6MYC-HPHMX ADH1-OsTIR1- URA3::ura3-52 lys4::LacO(DK)-NAT trp1-1 GFPLacI-HIS3:his3-11,15 bar1 leu2-3,112	This study
RL406	MATa MCD1-AID-KANMX6 BRN1-D375-3V5-AID2-HPHMX ADH1-OsTIR1-URA3::ura3-52 lys4::LacO(DK)-NAT trp1-1 GFPLacI-HIS3:his3-11,15 bar1 leu2-3,112	This study
RL407	MATa MCD1-AID-KANMX6 CDC5-3V5-AID2-HPHMX ADH1-OsTIR1- URA3::ura3-52 lys4::LacO(DK)-NAT trp1-1 GFPLacI-HIS3:his3-11,15 bar1 leu2-3,112	This study
RL408	MATa MCD1-AID-KANMX6 wpl1Δ::HPHMX ADH1-OsTIR1- URA3::ura3-52 lys4::LacO(DK)-NAT trp1-1 GFPLacI-HIS3:his3-11,15 bar1 leu2-3,112	This study
RL409	MATa PDS5-AID-KANMX6 wpl1Δ::HPHMX ADH1-OsTIR1- URA3::ura3-52 lys4::LacO(DK)-NAT trp1-1 GFPLacI-HIS3:his3-11,15 bar1 leu2-3,112	This study
	rad61Δ::HPHMX ADH1-OsTIR1-URA3::ura3-52 lys4::LacO(DK)-NAT trp1-1 GFPLacI-HIS3:his3-11,15 bar1 leu2-3,112	Guacci and Koshland 2012
RL410	MATa MCD1-AID-KANMX6 CDC5-3FLAG-HPHMX ADH1-OsTIR1- URA3::ura3-52 lys4::LacO(DK)-NAT trp1-1 GFPLacI-HIS3:his3-11,15 bar1 leu2-3,112	This study
RL411	MATa BRN1-D375-3V5-AID2-HPHMX CDC5-3FLAG-KANMX ADH1-OsTIR1-URA3::ura3-52 lys4::LacO(DK)-NAT trp1-1 GFPLacI-HIS3:his3-11,15 bar1 leu2-3,112	This study

RL412	MATa mcd1-Q266-6MYC-URA3::ura3-52 MCD1-AID-KANMX CDC5-3FLAG-HPHMX trp1Δ::pGPD1-TIR1-CaTRP1 lys4::LacO(DK)-NAT leu2-3,112 pHIS3-GFPLacI-HIS3:his3-11,15 bar1	This study