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The role of Chl1 DNA helicase in cohesion deposition and establishment

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ABSTRACT

Sister chromatid cohesion is crucial for the accurate transmission of genetic material during cell division. The conserved family of cohesin proteins that mediate sister chromatid tethering reactions require Scc2, Scc4 for chromatin association and Eco1 for conversion to a tethering competent state. The mechanism by which cohesin proteins mediate cohesion establishment between newly replicated sister chromatids remains elusive. Popular models posit that cohesins loaded in front of the replication fork is modified by Eco1 and conformational changes in the cohesin complex allows the replication fork to pass through cohesin barriers thereby converting cohesins into a cohesion competent state to enable capture of replicated sister chromatids. This study provides new evidence that challenges previous notions of cohesion establishment.

I use genetic and biochemical studies that link Eco1 with the Okazaki fragment maturation endonuclease Fen1. Furthermore I show genetic and physical interactions between Fen1 and the DNA helicase Chl1, which was previously identified to interact with Eco1 and play a role in sister chromatid cohesion. A detailed investigation of the Chl1 DNA helicase and its role in sister chromatid cohesion revealed its role in regulating cohesin and Scc2 deposition specifically during the S phase. Taken together, my studies suggest a new model of cohesion establishment wherein cohesins loaded in the S phase is modified by Eco1 behind the replication fork and mediates cohesion establishment. Further analysis of Chl1 also revealed the role of Chl1 in the deposition of condensin proteins. My results identify a novel link between the molecular mechanisms of sister chromatid cohesion with DNA condensation and suggests that these cellular processes are linked temporally and mechanistically. Detailed analysis of the Chl1 helicase reveals important novel functions in DNA metabolism and facilitates a better understanding of its clinically important human homologues, hChlR1 and BACH1.

Chapter 1

Introduction

Introduction

The continuity of life though cell division must ensure that each progeny daughter cell receives a complete parental genome. Eukaryotic DNA replication and sister chromatid separation are temporally separated. DNA replication occurs in S phase and replication checkpoints ensure that DNA replication occurs relatively error free and continues onto G2 phase. The replicated DNA must be kept together and then segregated to opposite poles during the process of cell division. The process that identifies sister chromatids, tethering them together from early S phase until anaphase onset is termed “sister chromatid cohesion.” The feat of identifying and organizing sister chromatids to achieve an amphitelic attachment (where microtubules from opposite poles attach to each sister chromatid) before cell division is a remarkable one and occurs through a carefully regulated process. Microtubules attach to the kinetochores presumably through a process of search and capture (Nicklas, 1997). The activity of the Ipl1/Aurora B kinase destabilizes syntelic attachment (attachment of each sister chromatids to microtubules from the same pole) enabling the kinetochores to capture a new microtubule (Biggins et al., 1999; Hauf, 2003; Tanaka et al., 2002). This process continues until bipolar attachment occurs. Sister chromatid cohesion resists the poleward forces on chromosomes and creates a state of tension which helps stabilize microtubule attachment to kinetochores (Miyazaki and Orr-Weaver, 1994) . Tension across the kinetochore also makes kinetochore-microtubule attachments refractory to the destabilizing action of Aurora B kinase. When all the chromosomes have attained a proper bipolar attachment and attain a state in which all the sister kinetochores are under tension, the cohesin complex tethering them together is degraded and the chromosomes segregate to opposite poles during cell division.

Cohesin complex structure

Sister chromatid cohesion is mediated by a large heteromeric complex consisting of 3 main structural components, Smc1, Smc3 (members of a large family of proteins called the Structural Maintenance of Chromosomes proteins), and Scc1/Mcd1 (Guacci et al., 1997; Losada et al., 1998; Michaelis et al., 1997; Toth et al., 1999) (Figure 1). Electron microscopic analyses coupled with genetic and biochemical studies suggest that the cohesin complex exists as a ring like complex that somehow identifies and attaches together the sister chromatids formed during DNA replication (Anderson et al., 2002; Haering et al., 2004; Ivanov and Nasmyth, 2005; Melby et al., 1998). The mechanism by which the cohesin complex associates with the sister chromatids remain controversial and several models have been posited to address this question (Campbell and Cohen-Fix, 2002; Gruber et al., 2003; Haering et al., 2002; Huang et al., 2005; Ivanov and Nasmyth, 2005; Nasmyth, 2005; Nasmyth and Haering, 2005; Skibbens, 2008; Stead et al., 2003; Surcel et al., 2008; Zhang et al., 2008b). Below, I discuss each cohesion subunit and summarize the evidence on which the different models of cohesion-chromosome interactions are based.

The structure of SMC complexes is extremely conserved. All SMC proteins are elongated proteins that fold back on themselves in an anti-parallel fashion so that the N- and C-termini associate on one end to form an ATPase globular head, while the fold site forms the hinge region. Smc1 and Smc3 associate with each other through hinge-to-hinge and head-to-head interactions. A ring like complex is formed with the recruitment of Mcd1/Scc1. Mcd1 in turn recruits Scc3/Irr1 forming the core cohesion holo-complex (Gruber et al., 2003; Haering et al., 2002; Ivanov and Nasmyth, 2005). While Scc3/Irr1 and Mcd1/Scc1 are essential to maintain sister chromatid pairing, they may not be part of the ring but they appear to play a definite role in cohesion ring stabilization.

Possible models of SMC molecules are discussed in this excerpt from Rudra and Skibbens, 2013a: “The highly conserved nature of SMC complexes provides crucial insight into the structure of cohesion but, thus far, remain largely underutilized. For instance, analyses of

interactions of cohesin subunits and their release upon linearization of circular DNA (Farcas et al., 2011; Gruber et al., 2003; Haering et al., 2008; Haering et al., 2002; Ivanov and Nasmyth, 2005) led to a model in which huge cohesin rings encircle DNA (For alternative models see McNairn and Gerton, 2008; Skibbens, 2008; Onn et al., 2008; Dı´az-Martı´nez et al., 2008; Nasmyth and Haering, 2009). The presumption that DNA is embraced by SMC arms, however, is speculative, lacks support from DNA–protein mapping studies and is confounded by findings that Smc1 and Smc3 heads remain closely apposed during anaphase (Mc Intyre et al., 2007). The crystal structure of the Rad50-containing MRN complex, suggests instead that DNA resides between Mre11 dimers (analogous to Mcd1) and closely apposed SMC-like Rad50 heads (Hopfner and Tainer, 2003; Mockel et al., 2012; Rupnik et al., 2010; Schiller et al., 2012; Williams et al., 2009; Williams et al., 2008; Zhang et al., 2008b). Additional evidence suggests that SMC heads reside near SMC hinges (Mc Intyre et al., 2007; Sakai et al., 2003) . Several models high-light DNA positioning to the Mcd1–Smc1–Smc3 interface and allow for cohesin dimerization (oligomerization) to promote sister chromatid tethering." (Rudra and Skibbens, 2013b) (Figure 1).

In addition to the core cohesion subunits, several additional auxiliary or regulatory subunits associate with this cohesin complex, including Pds5, Rad61/WAPL, and Sororin (for vertebrate cell systems). Pds5 is also critical to stabilize cohesin's association with DNA, but neither Pds5 nor Rad61/WAPL are essential in all organisms. Similarly, Sororin is present only in vertebrate cells, suggesting that Pds5, Rad61/WAPL and Sororin provide additional levels of complexity and regulation to a core set of cohesin subunits (Nishiyama et al., 2010; Rankin, 2005; Rankin et al., 2005; Shintomi and Hirano, 2009). Intriguingly, Pds5 appears to associate both near the SMC head (near Mcd1/Scc1) and also at the hinge. While SMC1, 3 rings may encompass DNA as previously suggested, Pds5 may drive ring-ring associations (hinge-to-head) or hinge-head associations that drive a cohesin ring to fold over. This latter model is supported by Atomic Force Microscopy which detected cohesin complexes ½ the length predicted by Smc1 and Smc3 coiled-coil domain lengths. Presently, numerous cohesin structure models exist in the literature: rings that embrace two sisters, rings that embrace a single sister

and subsequently become tethered together (handcuff model), cohesin oligomeric structures that spiral around DNA similar to a bracelet and folded over structures that clamp onto DNA. “In turn, recruited factors, which may exhibit enzymatic activities, reside on top the closely apposed SMC heads. For cohesion, Mcd1 recruits Scc3, Pds5, Rad61 and sororin. Pds5 and Rad61 are expendable in some model systems, whereas sororin is present only in vertebrate cells (Rankin et al., 2005; Dı´az-Martı´nez et al., 2007; Shintomi and Hirano, 2009; Nishiyama et al., 2010) (Rudra and Skibbens, 2013a). Thus, Pds5, Rad61 and sororin are unlikely to be structural components but regulators of potential therapeutic value.”

The cohesin cycle

***Cohesin loading*:**

In budding yeast, cohesin associates with DNA at the end of G1 phase (Guacci et al., 1997; Michaelis et al., 1997), whereas in vertebrate cells loading is initiated in telophase following reformation of the nuclear envelope (Darwiche et al., 1999; Gerlich et al., 2006; Losada et al., 1998). This difference may be attributed to the difference in the mitotic regulation of cohesins in different organisms. Cohesin binding sites were first identified by ChIP experiments. These studies revealed that cohesin binds to discrete Cohesin Attachment Regions (termed CAR’s) on chromosome arms and to larger domains surrounding centromeres (Blat and Kleckner, 1999; Laloraya et al., 2000; Megee et al., 1999; Tanaka et al., 1999). The CAR regions on chromosome arms are on average about 0.8 Kb in size and about 10kb apart. The CAR regions do not possess any specific conserved sequence but have an increased A/T content (Blat and Kleckner, 1999, Laloraya et al., 2000). 80% of all CAR’s in budding yeast, are found in transgenic regions at sites of convergent transcription (Glynn et al., 2004; Lengronne et al., 2004).

In all organisms studied, cohesin loading depends on a complex of two proteins, Scc2 and Scc4 (Bernard et al., 2006; Ciosk et al., 2000; Gillespie and Hirano, 2004; Rollins et al., 2004; Takahashi et al., 2004). In yeast, cohesin binding to DNA is presumably dependent on the

ability of SMC molecules to bind ATP (Arumugam et al., 2003; Weitzer et al., 2003). The hinge domain of the SMC heterodimer has also been implicated to play an important role in DNA binding (Hirano, 2006). The Scc2/4 complex promotes loading of cohesins by stimulating the ATPase activity of SMC proteins which might allow a conformational change in the form of hinge opening, allowing cohesins to load onto DNA. The actual mechanism of cohesion loading is still unclear. One model suggests that Scc2/4 loader complex associate with a chromatin substrate and facilitates interaction of closed Smc1/3 hinges with engaged NBD's (Nucleotide binding domains). The hinges are opened by the disengagement of NBD's driven by ATP hydrolysis thereby allowing the entry of chromatin fibers. Hinge re-association then takes place through free energy of hinge dimerization thereby entrapping chromatin fibers (Hu et al., 2011). The Scc2/4 loader complex may regulate SMC mediated ATPase activity or may facilitate local chromatin remodeling to allow for cohesion deposition. Cohesin deposition at double stranded breaks on DNA mediated by phosphorylated Histone 2A (Ser 129) indicates that epigenetic markers may also contribute to cohesion targeting and loading.

Some reports indicate that cohesin distribution differs from the DNA binding pattern of Scc2 and that cohesin deposited at centromeric DNA spreads to is neighboring regions (Ciosk et al., 2000; Hu et al., 2011; Lengronne et al., 2004). A recent study, however, indicates that Scc2/Scc4 resides at previously mapped CAR's in pericentromeric and arm regions and that the co-localization persists during the G1/S transition (Kogut et al., 2009). Centromeres, which are directly subjected to the pulling forces of the spindle, function as enhancers of cohesin deposition and the kinetochore proteins play a role in cohesin enrichment in these areas (Eckert et al., 2007; Kogut et al., 2009; Tanaka et al., 1999; Tanaka et al., 2002; Weber et al., 2004). Cohesin binds to a large pericentric domain that extends to about 20-50Kb (Kiburz et al., 2005; Weber et al., 2004). Compared to centromeric regions, telomeres show a greater separation of sister chromatids when cohesin proteins are inactivated, suggesting that cohesin enrichment depends on its location on the chromosome (Antoniacci et al., 2004). Cohesins also reside in transcriptionally silent domains like sub-telomeric regions and mating type loci (Dubey and Gartenberg, 2007; Laloraya et al., 2000). This raises the possibility of cohesin complexes

translocating/sliding along the DNA to distal sites after their initial deposition (Ocampo-Hafalla and Uhlmann, 2011).

Cohesion establishment

Cohesin loading is necessary but not sufficient to tether replicated sister chromatids together in a stable manner. Photobleaching studies in mammalian cells provide a direct observation of cohesin dynamics and revealed that approximately one third of all cohesin becomes stably linked to chromosomes during the course of S phase (Gerlich et al., 2006). Cohesin deposition was observed to take place from S phase until anaphase onset with the presence of two measurable pools of cohesins with different binding stabilities (McNairn and Gerton, 2009). Studies in budding yeast indicate that there is no measurable exchange of the functional pool of cohesin that mediates sister chromatid cohesion (Haering et al., 2004). These studies together indicate that there exists a mechanism that regulates the dynamic association of cohesins with the chromosome. The ideal candidate for an establishment factor is Ctf7/Eco1 (henceforth referred to as Eco1), which plays no role in the cohesion deposition and its subsequent maintenance, but is critical for the mechanism of sister chromatid cohesion (Milutinovich et al., 2007; Skibbens et al., 1999; Toth et al., 1999).

Models of cohesion establishment

Several models of cohesion establishment exist in the current literature and are subjects of intense debate. The first one is the replication through the ring model (Gruber et al., 2003). Electron micrograph analyses and biochemical studies that suggest that Smc1 and Smc3 form a large heteromeric ring like structure which is bridged by Mcd1/Scc1. This gigantic proteinaceous ring structure has a diameter of approximately 35nm which is enough to accommodate a 10nm chromatin fibre (Nasmyth and Haering, 2009). Based on this ring structure, the Nasmyth lab posited that pre-loaded cohesins allow the replication fork to pass through the cohesin ring thus entrapping the newly formed sister chromatids (Gruber et al., 2003). This model although simple in its concept, disregards certain important observations. *ECO1* mutants display a unique

“cohesin without cohesion” phenotype wherein the cohesins are loaded onto the chromosomes and DNA replication proceeds unperturbed, but the cells display high levels of sister chromatid cohesion defects (Skibbens et al., 1999; Toth et al., 1999). These reports clearly indicate that cohesion deposition and DNA replication are essential but not sufficient for cohesion establishment. In addition to this, the sheer size of the replisome complex makes it difficult to envision the replication through a ring model. A variation of the replication through a ring model suggests that the looped lagging strand relaxes to allow for a linearized replisome which would pass through the cohesion ring (Bylund and Burgers, 2005). Another corollary to the replication through a ring model suggests that transient ring opening and closing reactions on pre-deposited cohesion rings allow for replication fork passage (Nasmyth, 2011; Terret et al., 2009). However limited evidence supports the notion of cohesion ring opening and closing reactions during cohesion establishment.

An alternative model of cohesion establishment is the replication coupled cohesion model (Skibbens, 2000; Skibbens et al., 2007). The first clue to try and elucidate the mechanism of sister chromatid cohesion came from the observation that *ECO1* interacts genetically with both *POL30* and *CTF18* and that *POL30* was a high copy suppressor of *eco1* mutant cells (Skibbens et al., 1999). Subsequently reports document that Ctf18 and Elg1-RFC complexes are required for sister chromatid cohesion and physically interact with Eco1 (Kenna and Skibbens, 2003; Maradeo and Skibbens, 2009; Mayer et al., 2001). PCNA physically interacts with Eco1 and *pol30-104* mutant cells exhibit cohesion defects (Moldovan et al., 2006). These observations that Eco1 interacts with several replication fork components led to the replication coupled cohesion model which predicts that Eco1 associates with the replicative machinery during DNA replication and establishes cohesion between two newly formed sister chromatids by activating the cohesins that reside on each chromatid (Skibbens, 2000; Skibbens et al., 2007). This model supports the observation that cohesins must be loaded onto chromatin during S phase to participate in sister chromatid cohesion (Ciosk et al., 2000; Uhlmann and Nasmyth, 1998). Also in support of the replication coupled cohesion is the discovery that several DNA helicases perform a role in sister chromatid cohesion (Mayer et al., 2004; Petronczki et al., 2004;

Skibbens, 2004; Skibbens, 2005; Warren et al., 2004). Arguments against the replication coupled cohesion model are based on the fact that the existence of paired rings is still controversial and Ctf7 translocation with the DNA replication fork remains unproven (Ocampo-Hafalla and Uhlmann, 2011; Skibbens et al., 2007).

Molecular mechanism of sister chromatid cohesion establishment

Deeper understanding of the molecular mechanism of Eco1 was heralded with the knowledge that Eco1 was an acetyl-transferase. The acetyl-transferase activity of Eco1 is conserved among its homologs across several species, suggesting the importance of this enzymatic activity (Ivanov et al., 2002). Several in vitro substrates of Eco1 were identified through biochemical analyses which included several lysines within Eco1 itself, Scc3, Pds5, and Mcd1/Scc1 (K210) (Ivanov et al., 2002). Later the actual significance of the acetyltransferase activity of Eco1 in cell fitness and chromosome segregation came under scrutiny when it was demonstrated that a mutant Scc1/Mcd1 with its lysine 210 substituted for arginine showed no obvious growth or morphological defects (Ivanov et al., 2002). Another report showed that a *ECO1* allele with diminished acetyltransferase activity displayed robust cell growth and normal chromosome segregation (Brands and Skibbens, 2005). These reports suggest that either the acetylation activity of Eco1 was dispensable for its specific alleles or that Eco1 had alternate in vivo substrates that remained undetermined.

In a search for the in vivo substrate/s of the acetyltransferase activity of Eco1, genetic and biochemical studies from two independent groups identified two lysine residues of Smc3 (K112 and K113) to be the primary targets (Rolef Ben-Shahar et al., 2008; Unal et al., 2008). These lysines, when mutated to arginine, led to cell death with massive defects in sister chromatid cohesion. The cells displayed the familiar “cohesin without cohesion” phenotype, characteristic of cells lacking Eco1. Mutations of these Lysines to Asparagines, which mimics acetylated lysines, bypassed the need for the acetyltransferase activity of Eco1. The importance of acetyltransferase activity of Eco1 and the conservation of the lysine residues in Smc3 was extended to the human cell system when Zhang and colleagues showed that two lysine residues

(K105 and K106, that correspond to the yeast K112 and K113 of Smc3) of hSMC3 is acetylated by EFO1/ESCO1 (homolog of the yeast Ctf7) (Zhang et al., 2008a). All of the above reports map the essential function of Eco1to S phase under normal conditions. Acetylation shows a marked increase during S phase and persists into anaphase onset (Rolef Ben-Shahar et al., 2008; Unal et al., 2008; Zhang et al., 2008a). This was in perfect agreement with previous reports that Eco1 functions during S phase (Skibbens et al., 1999; Toth et al., 1999).

Sister chromatid cohesion establishment and anti-establishment

The next logical question regards the mechanism behind cohesion establishment. How does Eco1 mediated Smc3 acetylation lead to a cohesion competent state? A popular model posits that Eco1 mediated Smc3 acetylation displaces or inactivates cohesin associated factors (Rad61, Pds5) that would otherwise drive cohesion dissociation from DNA (Chan et al., 2012; Rowland et al., 2009; Sutani et al., 2009; Terret et al., 2009). This hypothesis was based on previous observations that specific alleles of *PDS5* and *RAD61* deletion bypass the essential function of Eco1 and that cohesion association to chromatin is sensitive to the levels of Rad61 in the cell (Chan et al., 2012; Gandhi et al., 2006; Hartman et al., 2000; Kueng et al., 2006; Panizza et al., 2000; Sutani et al., 2009). This early model is predicated on the assumption that anti-establishment factors maintain cohesins in a highly dynamic state and that Eco1 mediated acetylation of specific Smc3 Lysine residues sequesters the positive charge on Lysines and converts the cohesin complex to a “locked state” thereby mediating cohesion establishment.

In the mammalian system, the mechanism of sister chromatid cohesion has an additional player known as sororin. It is posited that the catalytic activities of the establishment factor Esco2 might be stimulated by the replication machinery or replication fork passage and subsequent post fork activities might engender a suitable conformation for the cohesin complex to be modified by Eco1. Sororin recognizes and binds acetylated cohesins, competing with the destabilizing influence of Wapl and/or Pds5 thereby mediating efficient cohesion establishment (Lafont et al., 2010; Song et al., 2012; Zhang and Pati, 2012). In addition to Rad61 and Pds5, other anti-establishment factors such as the alternate RFC clamp loader Elg1 also bypasses the

function of Eco1. The function of Elg1 appears to be directly through Eco1 function however the molecular mechanisms of Elg1 function remain elusive (Maradeo and Skibbens, 2009; Maradeo and Skibbens, 2010; Parnas et al., 2009).

Cohesion Establishment beyond S phase

Although the function of Eco1 is essential during S phase, it is only true for unchallenged cells. Cells challenged by DNA damage during G2/M phase also require the activity of Eco1 to re-establish cohesion. In this case, Eco1 is not only required for establishment of cohesion at damaged induced sites, but also in a global fashion on all chromosomes (Strom et al., 2007; Strom et al., 2004; Unal et al., 2004; Unal et al., 2007). Thus, Eco1 undergoes numerous levels of regulation. For instance, in unchallenged cells, Eco1 is negatively regulated by the protein kinase Cdk1. Phosphorylation after S phase targets Eco1 for SCF-Cdc4-dependent ubiquitination and subsequent degradation (Lyons and Morgan, 2011). This reduces the amount of Eco1 acetyltransferase activity below some threshold required to establish cohesion, which, together with reduced *ECO1* transcription in G1, prevents the establishment of new cohesion until DNA replication occurs in S phase of the next cell cycle. The mechanism of damage induced cohesion establishment during G2/M appears to be different from that of S phase. In response to double stranded breaks, Eco1 acetylates a different substrate, Mcd1/Scc1. In fact, acetylation of Smc3 during damage induced establishment is completely dispensable for cohesion establishment at DNA repair sites (Heidinger-Pauli et al., 2009). DNA damage activates the DNA damage checkpoint leading to the phosphorylation and activation of Mec1 kinase. Mec1 in turn phosphorylates and activates Chk1 which finally phosphorylates Mcd1/Scc1 at a Serine residue (S83) (Heidinger-Pauli et al., 2008). Chk1 also stabilizes Eco1 and makes it refractory to degradation during DNA damage. Phosphorylation of Mcd1/Scc1 is critical for Eco1 re-activation which now acetylates Mcd1/Scc1 at two lysine residues, K84 and K210 (Heidinger-Pauli et al., 2008; Heidinger-Pauli et al., 2009). This clearly shows that the process of cohesion establishment is complex and involves various regulated processes depending on the state of a cell.

Cohesin removal

Following cohesion establishment during DNA replication, replicated chromosomes are maintained stably by cohesin complexes until the transition from Metaphase to Anaphase during mitosis. After the degradation of the inhibitory chaperones Securin and cyclin B, Separase, an endopeptidase that cleaves the cohesin subunit Scc1/Mcd1 (Rad21 in humans), is activated (Ciosk et al., 1998; Cohen-Fix et al., 1996; Uhlmann et al., 1999; Uhlmann et al., 2000). In *Saccharomyces cerevisiae,* all cohesin complexes dissociate from the chromosomes simultaneously when the cohesin subunit Scc1/Mcd1 is cleaved by Separase before the sister chromatids are separated into daughter cells. In Metazoa, however, cohesins are removed from the chromatids in two steps (Waizenegger et al., 2000). At prophase, Plk1 phosphorylates SA2 leading to the dissociation of cohesin complexes from the chromosome arms (Sumara et al., 2000; Sumara et al., 2002). This step relies on Plk1 and Aurora B to phosphorylate cohesin SA2 and Wapl to remove the modified cohesins (Gandhi et al., 2006; Kueng et al., 2006). The centromeric cohesins and leftover cohesins along the chromosome arms, which are protected by Shugoshin, which prevents phosphorylation of SA2 by Plk1(McGuinness et al., 2005), are removed by Separase at the transition from metaphase to anaphase (Haering and Nasmyth, 2003; Hauf et al., 2001)).

The cohesin acetylation cycle comes to a close with the action of the cohesin deacetylase Hos1/HDAC8. Smc3 acetylation by Eco1 during S phase persists till cohesin cleavage during Anaphase onset (Beckouet et al., 2010; Borges et al., 2010; Deardorff et al., 2012a; Xiong et al., 2010). Pds5 protects Smc3 from deacetylation independent of Mcd1 degradation (Chan et al., 2013) . Separase mediated cleavage engenders Smc3 susceptible to Hos1 mediated de-acetylation and regenerates non-acetylated Smc3 to participate in another round of the cohesin cycle (Beckouet et al., 2010; Borges et al., 2010; Deardorff et al., 2012a; Xiong et al., 2010).

Non-essential proteins and sister chromatid cohesion

Over the years, several non-essential proteins have been identified which perform a role in sister chromatid cohesion. Mutations of essential structural cohesin subunits produce approximately 50% cohesion defects at centromere proximal sites (Guacci et al., 1997; Michaelis et al., 1997). Except for telomere proximal sites, where there is a 100% separation of sister chromatids (Antoniacci and Skibbens, 2004), there appears to be additional proteins required to play a role in sister chromatid cohesion near centromeres other than the major structural cohesins, the deposition complex Scc2 and Scc4, and the establishment factor Ctf7/Eco1. Budding yeast mutants lacking these proteins are viable, despite having high rates of chromosomal missegregation. Their patterns of synthetic lethality and the characteristic cohesion defects identified by cohesion assays have helped in their identification and characterization.

Various groups of proteins including those required for DNA replication, chromatin remodeling, DNA helicases all contribute to efficient sister chromatid cohesion. Proteins required for DNA replication, including alternate RFC complexes (Ctf18 and Elg1), Ctf4, Csm3 and Tof1 all play a role in efficient sister chromatid cohesion (Mayer et al., 2001; Kenna and Skibbens 2003; Maradeo and Skibbens 2009; Parnas et al., 2009). Apart from the above, chromatin remodelers like Rsc1, Rsc2, Ino80, Sth1, Hst3 and Hst4 play a role on promoting sister chromatid cohesion (Chang et al., 2005; Ogiwara et al., 2007a; Thaminy et al., 2007). In addition to this, DNA helicases Sgs1, Srs2, Rrm3 and Chl1 perform roles in sister chromatid cohesion (Mayer et al., 2004; Petronczki et al., 2004; Skibbens, 2004; Warren et al., 2004). It is possible that these proteins contribute to sister chromatid cohesion in a redundant manner under various cellular conditions and chromosomal locations. It is interesting to note that proteins involved in replication, like RFC complexes, PCNA, Ctf4, Csm3 and Tof1, play a role in sister chromatid cohesion suggesting a possible link between DNA replication and cohesion establishment (Skibbens, 2000; Moldovan et al., 2006; Xu et al., 2007). Careful investigation of these auxillary

proteins involved in sister chromatid cohesion are required to test the current hypotheses regarding the process of sister chromatid cohesion or to posit new models.

Helicases and sister chromatid cohesion

In 2004, helicases were identified to play a role in sister chromatid cohesion (Mayer et al., 2004; Petronczki et al., 2004; Skibbens, 2004; Warren et al., 2004). In a screen to identify proteins required to preserve genomic integrity in the S phase, three helicases (Sgs1, Srs2 and Rrm3) were identified. All the helicases discussed are non essential and cells lacking them are viable but exhibit common phenotypes of hyper-recombination, requirement for functional DNA repair checkpoints and sister chromatid cohesion defects (Torres et al., 2004b; Warren et al., 2004). In combination however, deletion of all three helicases render cells either inviable or severely growth defective (Ooi et al., 2003; Torres et al., 2004a). At the same time, three independent groups identified Chl1 DNA helicase as performing a role in sister chromatid cohesion (Skibbens, 2004, Mayer et al., 2004; Petronczki et al., 2004). Cells lacking Chl1, however, is not lethal in combination with either Sgs1, Srs2 or Rrm3 null cells suggesting that Chl1 performs a role in cohesion that is non-redundant with other helicases (Ooi et al., 2004).

The Chl1 helicase

In 1974, a mutant called *her1* was identified on the basis of a bi-mating phenotype in budding yeast (Haber, 1974). The bi-mating phenotype was found to be a consequence of a significant frequency of loss of one or the other homolog of chromosome III carrying the mating type locus and the mutant allele was thus named *chl1* (Chromosome loss 1). Chl1 mutation also led to an increase in the frequency of spontaneous recombination in all the linkage groups tested. Diploids homozygous for Chl1 mutations yielded spores with reduced viability (Liras et al., 1978). In 1990, 136 independent mutations were isolated in haploid yeast strains that exhibited decreased chromosome transmission fidelity (*CTF*) in mitosis. 11 complementation groups were identified (*CTF1-16*)(Gerring et al., 1990). The largest complementation group, *ctf1*

was found identical to the previously characterized mutation *chl1* (Gerring et al., 1990). The *CHL1/CTF1* gene, henceforth called *CHL1,* was cloned and mapped to chromosome XVI. Nucleotide analysis of *CHL1* revealed a 2.6kb open reading frame with a 99kd predicted protein sequence that contained 2 PEST sequences and was 23% identical to Rad3, which is a nucleotide excision repair protein. Three short domains of homology were identified between these two predicted protein sequences included a HTH (helix turn helix) motif and an A box and B box consensus found in ATP binding proteins. In addition to this, Chl1 sequence contains all seven consensus motifs found in helicases (Gerring et al., 1990; Koonin, 1993) (Figure 2). Chl1 knockouts display extreme chromosome instability and a G2/M delay that was independent of Rad9 (Gerring et al., 1990). Chl1 localized predominantly in the nuclear fraction of *S.cerevisiae* and possess an essential ATP binding site that was critical for the segregation of preferably smaller chromosomes over larger ones (S, 2000).

In 2004, three independent studies identified a role for Chl1 in sister chromatid cohesion (Mayer et al., 2004; Petronczki et al., 2004; Skibbens, 2004) (Figure 3). Chl1 was found to be a nuclear protein that physically interacts with the establishment factor Ctf7/Eco1 (Skibbens, 2004). This association appears physiologically relevant in that *chl1* deletion renders *ctf7/eco1* mutant cells inviable (Skibbens, 2004). *CHL1* was identified as a high copy suppressor of *CTF8Δ* cells and found to be important for chromosomal segregation during meiosis and cohesion establishment in the vicinity of centromeres (Petronczki et al., 2004). Apart from its role in sister chromatid cohesion, Chl1 was shown to have a role in transcriptional silencing, rDNA recombination and aging (Das and Sinha, 2005). Studies also reveal that Chl1 is required for viability when DNA replication is stressed, thus suggesting a role for Chl1 in preserving the genomic integrity of the cell upon DNA damage during replication (Laha et al., 2006). Studies in fission yeast showed that *CHL1* was synthetically lethal with *CTF18* deletion mutants and that a dosage increase of Chl1 rescue the sensitivities of *swi1*Δ cells to S phase stress (Ansbach et al., 2008).

The Chl1 helicases are members of the XPD family of helicases. The XPD helicase family members are believed to be 5'-3' DNA helicases with a structure that includes an

essential iron-sulfur binding domain that is believed to be an evolutionary marker for the XPD helicases. The unique Fe-S domain in XPD helicases is located between motifs I and II contains four conserved cysteine residues (White, 2009; Wu and Brosh, 2009). Two highly related human genes , hChlR1(DDX11) and hChlR2(DDX12) present on chromosome 12 which appear to be homologues (> 90% identical) of the budding yeast *CHL1* were cloned and shown to be expressed only in highly proliferating cell lines (Amann et al., 1997). Although the enzymatic activity of Chl1p is not characterized in budding yeast, the human homolog hChlR1 possesses both ATPase and DNA helicase activities. The hChlR1 protein can unwind both DNA/DNA and RNA/DNA substrates with a preference in the 5'->3' direction on short ss DNA templates (Hirota and Lahti, 2000). Human Chlr1 also interacts with hCtf18-RFC complex, hPCNA and hFen1 (the Flap endonuclease that functions in Okazaki fragment maturation). The interaction between hChlR1 and hFen1 stimulates the flap endonuclease activity of Fen1 suggesting that hChlR1 may have a role in the lagging strand processing events (Farina et al., 2008).

The human ATP dependent helicase hChlR1 is required for sister chromatid cohesion in mammalian cells (Parish et al., 2006). Mice embryos with homozygous null *ddx11-/-* resulted in embryonic lethality at E10.5 due to an accumulation of aneuploid cells generated by cohesion defects and placental malformation (Inoue et al., 2007). In addition, hChlr1 is essential for arm and centromeric cohesion in mice cells (Inoue et al., 2007). Budding yeast *CHL1* exhibits the highest level of homology with the human BACH1 gene (Skibbens, 2004). BACH1 helicase is a well-known helicase like protein that interacts with the tumor suppressor BRCA1 (Cantor et al., 2001). Recent studies reveal that the Chl1 helicase homologs bind to and help resolve secondary structures and G4 quaruplexes arising from replication intermediates (Wu et al., 2012). Investigating the biochemical characteristics of Chl1 and its homologs *in vivo* will be crucial to understand its function and the mechanism through which it exerts its role in various cellular processes.

Helicases and disease states

Why are helicases important from a medical perspective? Recent evidence implicates helicases in several roles required for maintaining genome maintenance. For instance, helicases play a role in various types of DNA repair pathways including nucleotide excision repair, mismatch repair, base excision repair and cross link repair (Gupta and Brosh, 2008). Several groups of helicases map to human disease states. Genetic defects in three human RecQ helicases, BLM, WRN and RECQ4 give rise to defined syndromes: Blooms syndrome (BLM helicase), the Werner's syndrome (WRN helicase) and the Rothmund Thomson syndrome (RECQ4). These disease states share a common predisposition to cancer, chromosome instability and premature ageing (Liu, 2010; Suhasini and Brosh, 2013). The mutations in the XPD group of helicases give rise to three human disease states including, XP (Xeroderma Pigmentosum), TTD (trichothiodystrophy) and CS (Cockayne's Syndrome) all showing a characteristic reduction in DNA repair ability (White, 2009). The XPD paralog, FANCJ mutations have been identified in early onset breast cancer patients and Fanconi anemia (FA) group J patients implicates FANCJ as a tumor suppressor that ensures genomic stability (Cantor et al., 2001). The Chl1 homolog, hChlR1 mutations have been recently implicated in Warsaw Breakage Syndrome patients, harboring characteristic developmental disorders and sister chromatid cohesion defects (van der Lelij et al., 2010a).

BACH1, tumorigenesis and sister chromatid cohesion

The BACH1 helicase binds to the Breast Cancer Associated gene (BRCA1) (Boulton, 2006; Deng, 2006). One study shows that a small number of breast cancer patients were found with a mutation in BACH1 but not BRCA1 or BRCA2. This might suggest that the loss of BACH1 helicase activity can itself suffice to predispose individuals to tumorigenesis (Cantor et al., 2004; Cantor et al., 2001). Diminished BACH1 activity leads to localized cohesion defects (Bridge et al., 2005; Deming et al., 2001). Human cells lacking BACH1 homologs (Chl1 in yeast and hChlR1 in humans) display sister cohesion defects and aneuploidy (Inoue et al.,2007; Petronczki et al.,2004; Skibbens,2004). The above evidence suggests that helicases play a role

in tumorigenesis. Adding to the lines of evidence is the fact that knockout mice homozygous null for RecQL4 exhibit cohesion defects and display phenotypes mirroring the Rothmund-Thomson syndrome including skin abnormalities, skeletal defects ,aneuploidy and predisposition to cancer (Mann et al., 2005).

The Breast cancer associated gene 1, BRCA1 physically and functionally interacts with the human DNA repair complex MRN (Kobayashi, 2004; Wang et al., 2000). Mutations in any one of the yeast homologs of the MRN complex produced cohesion defects (Grenon et al., 2001; Warren et al., 2004). BRCA1 is a part of a CBP/p300 and SW1/SNF complex that exhibits acetyltransferase activity (Cui et al., 1998; Fan et al., 2002; Pao et al., 2000). In yeast the sister chromatid cohesion establishment factor Ctf7/Eco1 has an essential acetyltransferase activity (Ben-Shahar et al., 2007; Heidinger-Pauli et al., 2008; Unal et al., 2007). Also, mutations in BRCA1 or EFO/ESCO (human homologs of *CTF7/ECO1*) related pathways exhibit sister chromatid defects (Skibbens et al., 2005; Skibbens et al., 2007). In lieu of the evidence at hand, it is compelling to speculate a role of sister chromatid cohesion in tumorigenesis.

Recently, helicases have been identified as new prospective targets for anti-cancer therapy. Inactivation of DNA repair pathways may enhance the efficacy of anti-cancer therapies that eliminate cancerous cells through the cytotoxic effects of DNA damaging agents or radiation (Gupta and Brosh, 2008). On one hand, there is enough evidence to link sister chromatid cohesion (and therefore cohesins and their regulators) to tumorigenesis, on the other hand, there is substantial information regarding helicases and their role in cancer. Coming back in a full circle, the role of helicases in genome maintenance; which includes the process of sister chromatid cohesion might play an important role in tumorigenesis.

Figure 1:

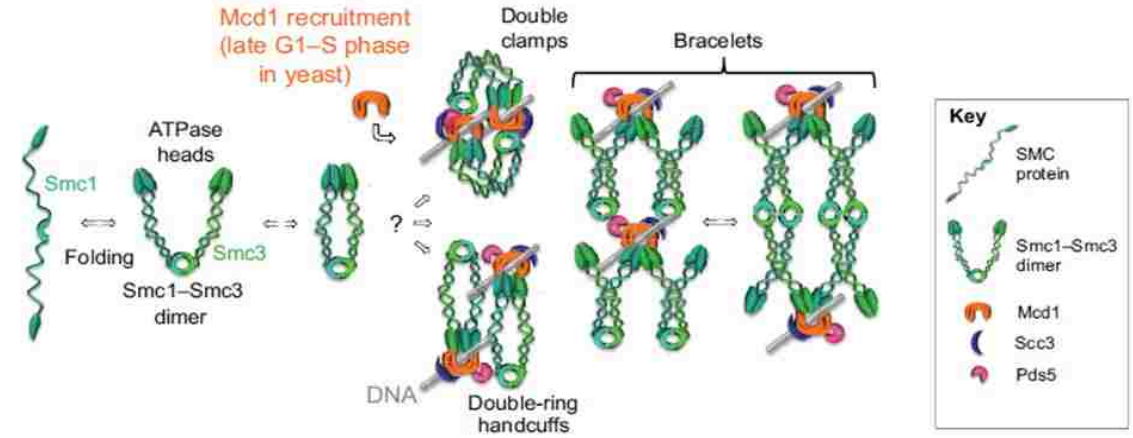

Figure 1: Structure of the Cohesin complex and different models of how Cohesins interact With DNA (Rudra and Skibbens, 2013a)

Figure 2:

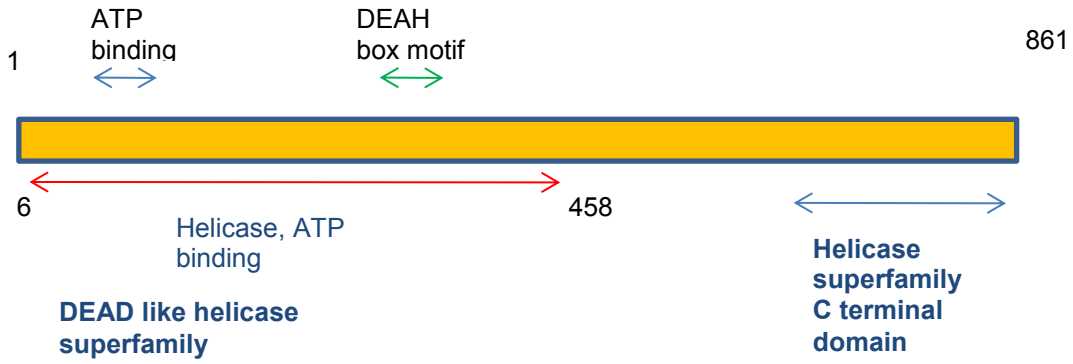

Figure 2 : Diagrammatic representation of the Chl1 helicase

Figure 3

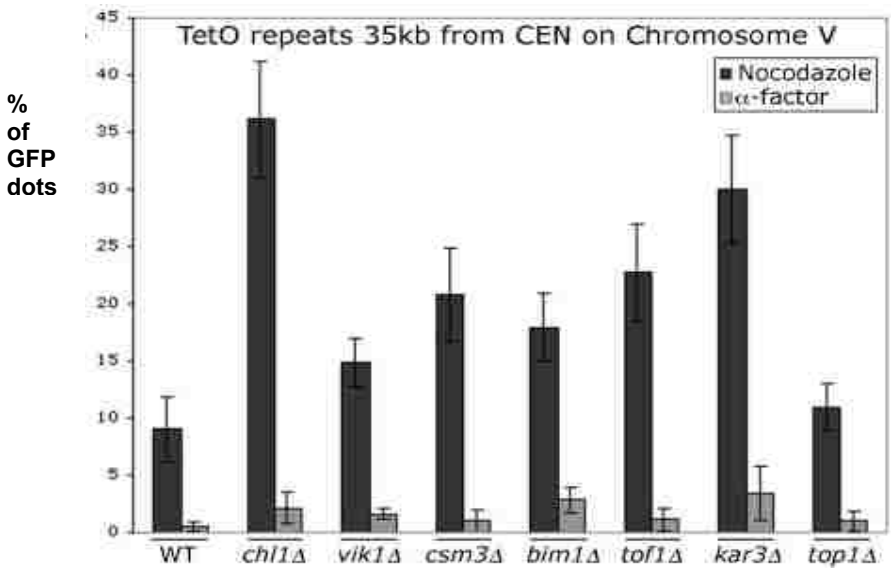

Figure 3 : Cohesion defect of various proteins at an arm locus, 35kb from the centromere on Chromosome V. Chl1 shows a 35% cohesion defect (Mayer et al., 2004).

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Chapter 2

Sister Chromatid cohesion establishment occurs in concert with lagging strand synthesis

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Abstract

Cohesion establishment is central to sister chromatid tethering reactions and requires Ctf7/Eco1-dependent acetylation of the cohesin subunit Smc3. Ctf7/Eco1 interacts with a number of replication proteins (RFC complexes, PCNA) and the DNA helicase Chl1, all of which play individual roles in sister chromatid cohesion. While the mechanism of cohesion establishment is largely unknown, a popular model is that Ctf7/Eco1 acetylates cohesins encountered by and located in front of the fork. In turn, acetylation is posited both to allow fork passage past cohesin barriers and convert cohesins to a state competent to capture subsequent production of sister chromatids. Here, we report evidence that challenges this pre-replicative cohesion establishment model. Our genetic and biochemical studies link Ctf7/Eco1 to the Okazaki fragment flap endonuclease, Fen1. We further report genetic and biochemical interactions between Fen1 and the cohesion associated DNA helicase, Chl1. These results raise a new model wherein cohesin deposition and establishment occur in concert with lagging strand-processing events and in the presence of both sister chromatids.

Introduction:

The continuity of life requires the careful co-ordination of DNA replication and chromosome segregation during cell division. The temporal separation of these two independent events demands a regulated mechanism which maintains the identity of the replicated sister chromatids from the time of replication till anaphase onset. The identity between the two sister chromatids is maintained by molecular complexes known as cohesins that physically link the two sister chromatids together. During Mitosis, microtubules are attached to the kinetochores of the chromosomes in a process of trial and error. Once the bipolar arrangement of all the chromosomes has been established and the kinetochores sense the necessary amount of tension, the spindle checkpoint is relaxed allowing for anaphase onset. A protease called separase cleaves the cohesion complex and the sister chromatids are now free to segregate to the opposite poles with the help of microtubules. Apart from its canonical role in tethering the sister chromatids together, cohesion play key roles in double stranded break repair (Strom et al., 2007; Unal et al., 2007), transcriptional regulation(Dorsett, 2011) and heterochromatin regulation (Gartenberg, 2009).

Sister chromatid cohesion is mediated by a large heteromeric complex consisting of 4 main structural components, that include Smc1, Smc3 (belong to a large family of proteins called the Structural Maintenance of Chromosomes proteins), Mcd1/Scc1 and Scc3 that maintains a topological contact with the DNA molecule (Onn et al., 2008; Skibbens, 2008). In budding yeast, cohesin associates with DNA at the end of G1, whereas in vertebrate cells loading is initiated in telophase following reformation of the nuclear envelope. In all organisms studied, cohesin loading depends on a complex of two proteins, Scc2 and Scc4 (Skibbens, 2008). Cohesin loading is not sequence specific and localizes to poorly defined regions known as CAR (cohesin attachment regions). Cohesin loading is necessary but not sufficient to keep the replicated sister chromatids together. In fact, the initial association of cohesins with chromosomes is dynamic and it is only during S phase that chromatid cohesins are converted into a pairing competent state also known as cohesion “establishment”(Skibbens, 2009).

The mechanism of cohesion establishment is hitherto unknown. The ideal candidate for an establishment factor is the Ctf7/Eco1 which plays no role in cohesin deposition and the subsequent maintenance of cohesion but is critical for the mechanism of sister chromatid cohesion (Skibbens et al., 1999; Toth et al., 1999). Ctf7/Eco1 is an acetyl-transferase which acetylates two critical Lysine residues on the Smc3 subunit during DNA replication in S phase. This acetylation event is essential for cell viability as well as for maintaining efficient sister chromatid cohesion (Rolef Ben-Shahar et al., 2008; Unal et al., 2008; Zhang et al., 2008a).

In order to understand the mechanism of cohesion, it is important to consider how the cohesion complex interacts with DNA during its encounter with the replication machinery. One popular model suggests that Ctf7/Eco1 must acetylate pre-loaded cohesins- those that reside in front of the DNA replication fork. This pre-replicative cohesin acetylation is posited to both allow for fork progression and produce a cohesin state that promotes sister chromatid tethering (Ciosk et al., 2000; Sherwood et al., 2010; Terret et al., 2009). In contrast to this model, little evidence supports the notion that Ctf7/Eco1 binds replication fork components, translocates with the replication fork or functionally alters cohesins prior to fork passage (Lengronne et al., 2006; Skibbens, 2011). Instead, a growing body of findings indicate that fork passage is required for establishment and that cohesins that associate with chromatin prior to fork passage are most likely not relevant to establishment (Bernard et al., 2008; Gerlich et al., 2006; Skibbens, 2011).

The above controversies highlight the importance of pinpointing the location and timing of cohesion establishment with respect to the replication fork. To address this issue, we exploited the very well characterized *RAD27/FEN1* flap endonuclease (herein termed *FEN1)* that is critical for processing Okazaki fragments during DNA replication (Gary et al., 1999; Sommers et al., 1995). Previous studies reveal that *fen1* mutant cells exhibit cohesion defects (Warren et al., 2004), suggesting that cohesion establishment might be linked to Okazaki fragment processing events. Also strengthening the evidence is the fact that hFen1 endonuclease activity is enhanced by hChlR1 (Farina et al., 2008). Chl1 is a DNA helicase like protein which has been previously identified in playing a role in cohesion establishment (Mayer et al., 2004; Petronczki et al., 2004; Skibbens, 2004). We predict that Fen1 forms a genetic and physical interaction network with

Ctf7/Eco1 and Chl1 thereby linking cohesion establishment to Okazaki fragment processing events for the first time in budding yeast.

Results

Inter-related synthetic lethalities support coordination of cohesion establishment to lagging-strand processing.

In contrast to the popular notion that Ctf7/Eco1 acts on cohesins positioned in front of the DNA replication fork, several studies point to factors that reside behind the fork such as the flap endonuclease Fen1 as critical for sister chromatid pairing, formally raising a model that establishment occurs as sister chromatids emerge from behind the fork (Bylund and Burgers, 2005; Farina et al., 2008). To test this alternate model, yeast cells harboring *ctf7*$^{eco1\text{-}1:\ ADE}$ were crossed with *fen1::KAN*^{r} mutant cells and the resulting diploids placed in sporulation medium. Notably, heterozygous *ctf7*$^{eco1\text{-}1:\ ADE}$*/CTF7 FEN1/ fen1* cells exhibited extremely poor sporulation efficiency (<2%). When diploid cells were first transformed with *CEN-URA3-CTF7*^{ECO1} plasmid, however, the resulting transformants sporulated with high efficiency (approximately 85%). Similar haplo-insuffiency in sporulation was previously observed in crosses involving *ctf7/eco1* (Brands and Skibbens, unpublished observation), revealing that Ctf7/Eco1 performs an essential but dosage-dependent role during meiosis. Of the 112 spores obtained from sporulated transformed diploids, we recovered the expected number of wild type cells and both *fen1* and *ctf7/eco1* single mutant cells (Table 1). In contrast, only 7 double mutant *fen1 ctf7/eco1* double mutant cells were recovered at 23°C, 6 of which harbored *CTF7/ECO1* plasmid. Upon plating onto media supplemented with 5'FOA (Boeke et al., 1987), all 6 plasmid-bearing double mutant strains were inviable, revealing a synthetic lethal interaction between *ctf7/eco1* and *fen1* mutations (Figure 1). The single double mutant spore exhibited robust growth at all temperatures and thus likely results from meiotic gene conversion or incorporation of an extragenic suppressor (Figure S1).

Chl1 (and the human homolog hChlR1) is a DNA helicase that promotes sister chromatid cohesion and hChlR1 stimulates the flap endonuclease activity of hFen1 (Farina et al., 2008; Mayer et al., 2004; Petronczki et al., 2004; Skibbens, 2004). Prior evidence that *ctf7/eco1* interacts genetically with *chl1*(Skibbens, 2004), coupled with the synthetic lethal *ctf7/eco1* and *fen1* interaction described above, predicts that *fen1* might also interact genetically with *chl1*. To

test this, *chl1::HIS3* cells were crossed to *fen1::KAN*^{r} cells and the resulting diploids sporulated. High efficiency sporulation was observed. Tetrad analyses recovered the expected number of wild type and both *chl1* and *fen1* single mutant strains (Table 2). In contrast, no *chl1 fen1* double mutant spores were recovered (Table 2). This synthetic lethality extends findings obtained from a high through-put assay that suggested that *chl1* and *fen1* interact genetically (Loeillet et al., 2005; Tong et al., 2004). The inter-related synthetic lethal network (*ctf7/eco1-fen1; fen1-chl1; chl1-ctf7/eco1* - current study and Skibbens(Skibbens, 2004)) supports the model that cohesion establishment is temporally coupled to lagging strand processing.

Ctf7/Eco1 and Chl1 associate with lagging strand-processing factor Fen1

If the above lagging strand-coupled establishment model is correct, then each of these proteins may physically interact. To test this prediction, we transformed cells expressing Fen1-13Myc with either a construct directing elevated expression of Ctf7/Eco1-3HA or 3HA alone as a control. Logarithmically growing cultures of the resulting transformants were lysed and cell extracts incubated with anti-MYC beads. After several washes, bound protein complexes were eluted and assayed by Western blot. As expected, Fen1-13MYC was efficiently immunoprecipitated through this procedure (Figure 2A). Probing duplicate membranes with HA-directed antibodies revealed that Ctf7/Eco1-3HA co-immunoprecipitated with Fen1-13MYC but was not pulled down from lysates obtained from cells expressing untagged Fen1 (Figure 2A). The role of Fen1 in DNA modification raised the possibility that the Fen1-Ctf7/Eco1 association might be mediated through DNA. To address this possibility, we repeated the co-immunoprecipitation but included DNaseI in the cell lysate. The results show that Ctf7-3HA continues to efficiently co-immunoprecipitate with Fen1-13MYC in the absence of DNA (Figure 2B). Complete degradation of lambda DNA that was spiked into the co-immunoprecipitation reaction confirmed the efficacy of the DNAseI treatment (Figure 2C).

Ctf7/Eco1 binding to Fen1 was confirmed using a reciprocal immunoprecipitation strategy. Briefly, logarithmically growing cells co-expressing Ctf7/Eco1-3HA and Fen-13MYC

were lysed and cell extracts incubated with anti-HA affinity beads. After several washes, bound proteins were eluted and analyzed by Western blot using HA-directed antibodies to reveal efficient Ctf7/Eco1 immunoprecipitation. Probing duplicate membranes with anti-MYC antibodies revealed Fen1-13MYC co-immunoprecipitated with Ctf7/Eco1-3HA (Figure 2D). Importantly, Fen1-13MYC was not pulled down in lysates obtained from cells expressing untagged Ctf7/Eco1 or cells expressing only 3HA tags (Figure 2D). Taken together, the reciprocal immunoprecipitation studies uncover a physical in vivo association between Fen1 and Ctf7/Eco1 that occurs independent of DNA.

Fen1 flap endonuclease activity is stimulated by hChlR1 (Farina et al., 2008) and both participate in cohesion (Mayer et al., 2004; Petronczki et al., 2004; Skibbens, 2004; Warren et al., 2004). To test the possibility that Chl1 physically associates with Fen1 and thus position Chl1 function relative to the DNA replication fork, cell lysates obtained from logarithmically growing cells co-expressing Chl1-13MYC and Fen1-3HA were incubated with anti-MYC beads. As before, bound proteins were eluted from washed beads and assayed by Western blot. The results show efficient immunoprecipitation of Chl1-13MYC (Figure 3A). Duplicate membranes probed for anti-HA antibodies reveals that Fen1-3HA co-immunoprecipitates with Chl1-13MYC but not with untagged Chl1 (Figure 3A). We next tested whether Chl1 binding to Fen1 depended on DNA by including DNAseI in the cell lysate prior to pull-down. The results show that Fen1-3HA efficiently co-immunoprecipitated with Chl1-13MYC even in the absence of DNA (Figure 3B). Complete degradation of lambda DNA spiked into the co-immunoprecipitation reaction confirmed the efficacy of the DNAseI treatment (Figure 3C).

The interaction between Chl1 and Fen1 was confirmed using a reciprocal co-immunoprecipitation procedure. Briefly, cells co-expressing Chl1-13Myc and Fen1-3HA were lysed and incubated with anti-HA beads. Bound protein complexes were eluted from the washed beads and analyzed by Western blotting. The results show that Fen1-3HA is efficiently immunoprecipitated (Figure 3D) and that Chl1-13MYC co-immunoprecipitates specifically with Fen1-3HA but does not pull down with untagged Fen1 (Figure 3D).

Discussion

Two key features of current cohesion establishment models are that 1) cohesins are loaded in front of the DNA replication fork and 2) Ctf7/Eco1 acetylates pre-replicative cohesins to both allow for fork progression and engender sister chromatid tethering upon subsequent fork passage and synthesis of sister chromatids. One notion that appeared to confound this pre-replicative establishment model was that Ctf7/Eco1 is recruited to chromatin by PCNA (Moldovan et al., 2006). However, this interpretation is complicated in that Ctf7/Eco1 mutated within the PCNA binding PIP box appears to persist in binding chromatin at normal levels in yeast (Skibbens, 2011). Nor does this region appear necessary for ESCO2 (Ctf7/Eco1 homolog) chromatin-recruitment in human cells (Hou and Zou, 2005). Finally, mutation of PCNA produces only modest cohesion defects – inconsistent with a central role for PCNA in establishment. In fact, there is no convincing evidence to support the notion that Ctf7/Eco1 stably associates with the DNA replication fork at all, despite claims to the contrary (Bernard et al., 2008; Gause et al., 2010; Lengronne et al., 2006; Skibbens, 2011). Thus, the placement of Ctf7/Eco1 relative to the DNA replication fork had yet to be ascertained.

Here, we exploited the well-characterized lagging strand processing factor Fen1 to uncover novel Fen1-Ctf7/Eco1 binding and thus position Ctf7/Eco1 relative to the DNA replication fork. Findings from multiple genetic and biochemical studies indeed support a model in which cohesion establishment occurs immediately behind the fork and possibly coupled to lagging strand processing (Bylund and Burgers, 2005; Skibbens, 2000; Skibbens, 2011). The genetic and physical interactions identified between Fen1, Ctf7/Eco1 and Chl1 (current study and Skibbens (Skibbens, 2004)) does not necessarily link Ctf7/Eco1 recruitment to Okazaki fragment maturation. For instance, neither Fen1 nor Chl1 are essential for cell viability (Gary et al., 1999; Gerring et al., 1990; Reagan et al., 1995; Sommers et al., 1995) and thus are unlikely as platforms critical for Ctf7/Eco1 recruitment. Currently, we cannot exclude the possibility that both cooperatively contribute to recruitment – a model consistent with the lethality of cells harboring deletions of both *fen1* and *chl1* (this study). Instead, we favor a model of cohesion establishment

in which Ctf7/Eco1 transiently cycles in associating with cohesins loaded behind the DNA replication fork. We currently view these transient associations as occurring independent of replication fork factors per se, but that establishment occurs in a post-fork chromatin context that exists in the vicinity of Okazaki fragment maturation as well as on leading strand synthesis. This post-fork establishment model benefits from several accompanying features (Figure 4). First, it posits that establishment occurs when sister chromatids are actually present, in contrast to current models that posit establishment requires acetylation of cohesins that reside in front of the fork and before sister chromatids are synthesized (Sherwood et al., 2010). Second, our model places Ctf7/Eco1 proximal to PCNA but does not require PCNA-dependent chromatin recruitment of Ctf7/Eco1. Nor do we envision Ctf7/Eco1 recruitment requiring Fen1 or Chl1. Instead, we favor a model that Ctf7/Eco1 transiently functions in the local environment that exists immediately behind the replication fork to convert cohesins to a pairing-competent state. Third, this model posits that the cohesins that participate in sister chromatid tethering reactions are most likely loaded behind the DNA replication fork. This is consistent with early reports in which the essential activity of Scc2 (and Ctf7/Eco1) was mapped to S-phase (Ciosk et al., 2000). Thus, both cohesin deposition and Ctf7/Eco1-dependent cohesion establishment likely occur in this post-fork context – cohesin 'association' prior to the replication fork being irrelevant to cohesion. This latter point is supported by the highly dynamic nature of cohesins during G1 (Gause et al., 2010; Gerlich et al., 2006; McNairn and Gerton, 2009). Our current view of establishment parallels that of chromatinization in which newly synthesized histone complexes are deposited onto nascent sister chromatid strands and subsequently post-translationally modified to engender epigenetic states (Jasencakova and Groth, 2010). Cohesin deposition/modifications that engender sister chromatin pairing and transcription regulation may be similarly temporally coupled. Toward this end, we note several chromatin-modifying complexes (INO80, RSC and SWI/SNF components) that promote efficient cohesion establishment (Skibbens, 2008). The physical link reported here between Chl1 and Fen1, coupled with recent findings that Chl1 exhibits 5'-3' unwindase capabilities, removes chromatin-bound proteins (possibly resolving chromatin structures such as G-quads) and is critical for cohesin deposition or stabilization,

provide additional motivation in considering a chromatin-based post-fork model of cohesion establishment (Brosh, 2011; Wu and Brosh, 2012a; Wu et al., 2012). Future research focused on testing cohesion establishment in the context of chromatinization is likely to provide significant insights applicable to multiple fields of inquiry and, given the role of cohesion pathway mutations in tumorigenesis and developmental maladies, be of clinical interest (Dorsett, 2011; Mannini et al., 2010).

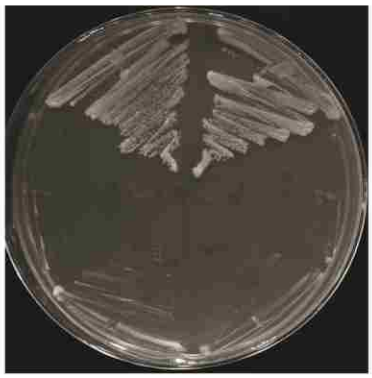
A

YPD 23°C

B

FOA 23°C

C



FOA 30°C

D

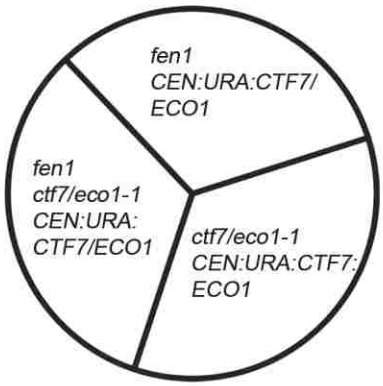
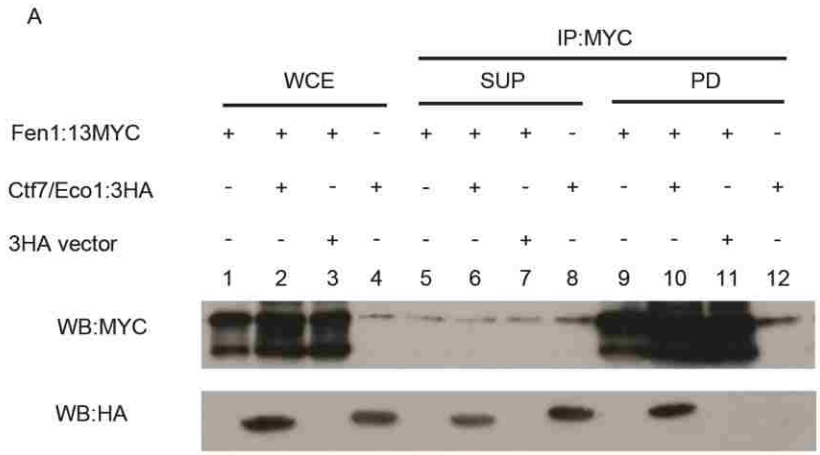
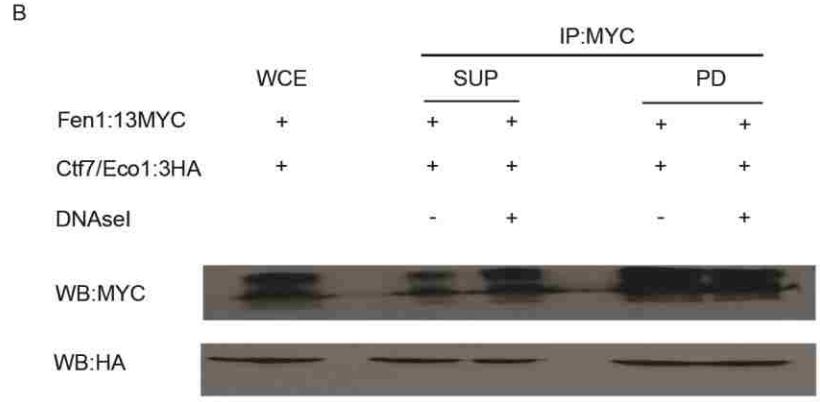


Figure 1


A
IP:MYC
WCE
SUP
PD
Fen1:13MYC + + + - + + + - + + + -
Ctf7/Eco1:3HA - + - + - + - + - + - +
3HA vector - - + - - - + - - - + -
1 2 3 4 5 6 7 8 9 10 11 12
WB:MYC
WB:HA


B
IP:MYC
WCE
SUP
PD
Fen1:13MYC + + + + +
Ctf7/Eco1:3HA + + + + +
DNAseI - + - +
WB:MYC
WB:HA


C
DNAseI - +

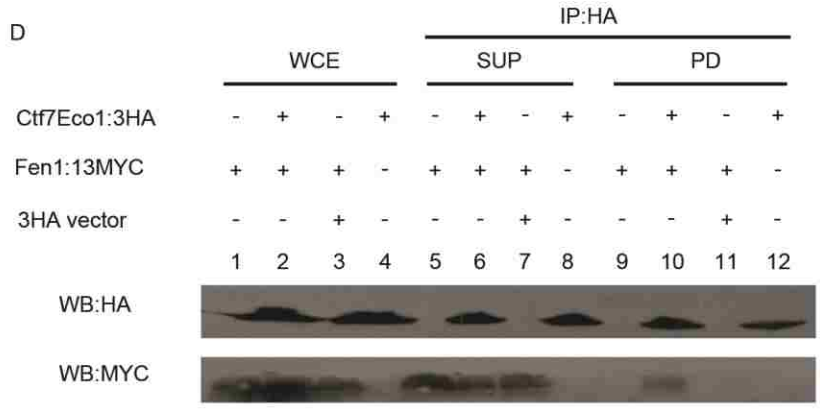
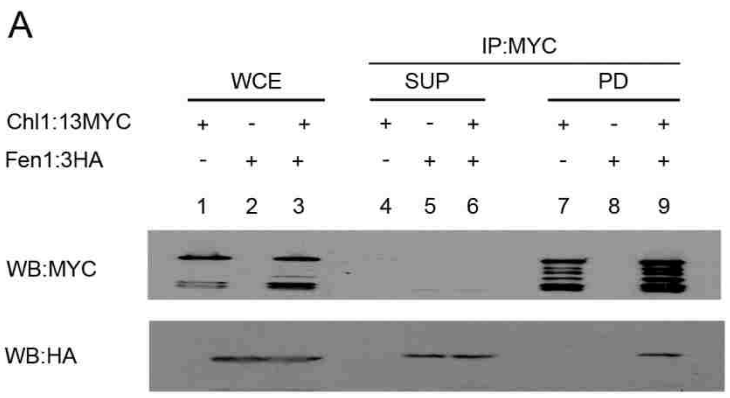

D
IP:HA
WCE
SUP
PD
Ctf7Eco1:3HA - + - + - + - + - + - +
Fen1:13MYC + + + - + + + - + + + -
3HA vector - - + - - - + - - - + -
1 2 3 4 5 6 7 8 9 10 11 12
WB:HA
WB:MYC

Figure 2


A
IP:MYC
WCE
SUP
PD
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Fen1:3HA - + + - + + - + +
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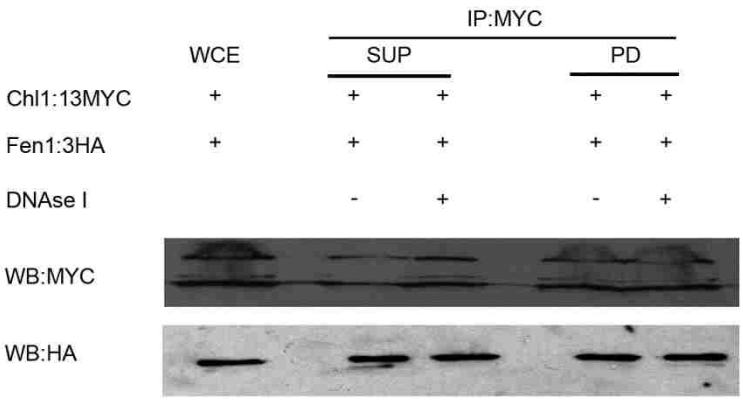
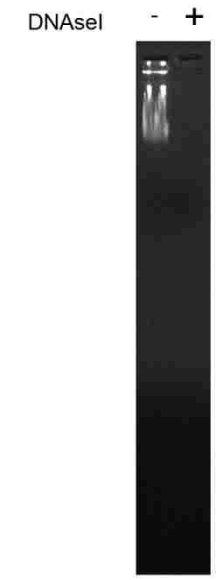
WB:MYC
WB:HA


B
IP:MYC
WCE
SUP
PD
Chl1:13MYC + + + + +
Fen1:3HA + + + + +
DNAse I - + - +
WB:MYC
WB:HA


C
DNAseI - +

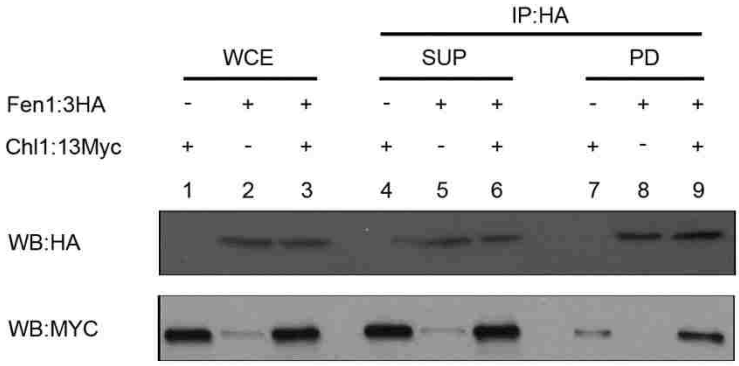

D
IP:HA
WCE
SUP
PD
Fen1:3HA - + + - + + - + +
Chl1:13Myc + - + + - + + - +
1 2 3 4 5 6 7 8 9
WB:HA
WB:MYC

Figure 3

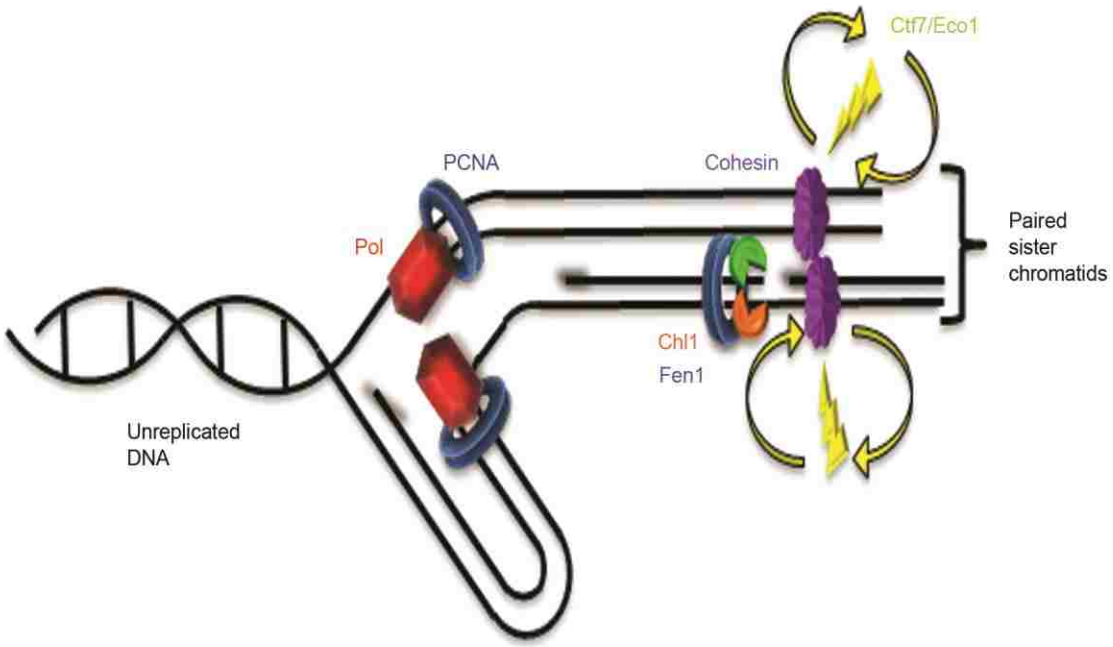

Ctf7/Eco1
PCNA
Cohesin
Pol
Paired
sister
chromatids
Chl1
Fen1
Unreplicated
DNA

figure 4

A

YPD 23°C

B

YPD 30°C

C

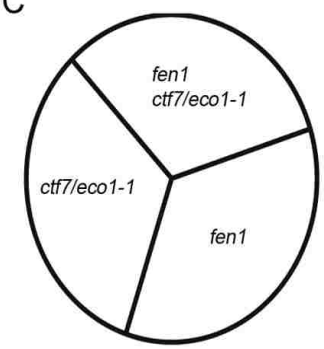


Figure S1

FIGURE LEGENDS

Figure 1: ***ctf7^{eco1-1}*** **is synthetically lethal in combination with *fen1***

Yeast cells harboring *ctf7$^{eco1-1: ADE}$* were crossed with *fen1::KAN^{r}* mutant cells and the resulting diploids were transformed with a *CEN: URA3:CTF7^{ECO1}* plasmid and sporulated. The resulting *fen1* and *ctf7$^{eco1-1: ADE}$* single mutants and *fen1, ctf7$^{eco1-1: ADE}$* double mutants were plated on media with or without FOA (See also Table 1). Two independent isolates are shown for each strain.

A) Growth of *fen1, ctf7^{eco1-1}* single mutants and *fen1::KAN ctf7^{eco1-1} CTF7: URA* double mutants strains at 23^{o}C on YPD.

B) Growth of *fen1::KAN*, *ctf7^{eco1-1}* single mutants and *fen1 ctf7$^{eco1-1: ADE}$ CTF7: URA* double mutants on FOA plates at 23^{o}C.

C) Growth of *fen1::KAN*, *ctf7^{eco1-1}* single mutants and *fen1 ctf7$^{eco1-1: ADE}$ CTF7: URA* double mutants on FOA plates at 30^{o}C (See also figure S1).

D) Schematic representation of *fen1::KAN*, *ctf7^{eco1-1}* single mutants and *fen1 ctf7$^{eco1-1: ADE}$ CTF7: URA* double mutant strains.

Figure 2: **Fen1 and Ctf7/Eco1 physically associate *in vivo***

Cells expressing Fen1:13Myc and Ctf7/Eco1:3HA were mechanically lysed and clarified by centrifugation, the clarified whole cell extract was co-immunoprecipitated using anti-Myc beads (2A) and anti HA Beads (2D) and analyzed by immunoblotting for Fen1:13Myc and Ctf7/Eco1:3HA. Whole cell extracts (WCE, lanes 1-4), Supernatants (SUP, lanes 5-8) and pull down fractions (PD, 9-12) are shown.

A) Co-immunoprecipitation of Fen1:13Myc and Ctf7:3HA with anti-MYC beads. Cells expressing only 3HA tag (Lane 11) and cells expressing Ctf7:3HA but untagged Fen1 (Lane 12) were used to determine the specificity of the co-immunoprecipitation.

B) Clarified whole cell extracts of cells co-expressing Fen1:13Myc and Ctf7:/Eco1:3HA were treated with and without DNAseI before immunoprecipitation with anti-MYC beads.

C) 1µg of λ DNA added in the clarified whole cell extract, with and without DNAseI treatment, run on a 1% agarose gel.

D) Reciprocal co-immunoprecipitation of cells expressing Fen1:13Myc and Ctf7:3HA using Anti-HA affinity beads. Cell expressing untagged Ctf7/Eco1 (Lane 9) and cells expressing 3HA tags alone (Lane 11) were used as a control to determine the specificity of the co-immunoprecipitation reaction.

Figure 3: Chl1:13Myc physically associate with Fen1:3HA *in vivo*:

Cells expressing Chl1:13Myc and Fen1:3HA were mechanically lysed and clarified by centrifugation. The clarified whole cell extract was co-immunoprecipitated using anti-Myc beads (3A) and anti HA beads (3B) and analyzed by immunoblotting for Chl1:13Myc and Fen1:3HA. Whole cell extracts (WCE, lanes 1-3), Supernatants (SUP, lanes 4-6) and pull down fractions (PD, 7-9) are shown.

A) Co-immunoprecipitation of Chl1:13Myc and Fen1:3HA using anti-MYC beads. Cells expressing untagged Fen1 cell extracts were used to determine the specificity of the co-immunoprecipitation reaction (Lane 8).

B) Clarified whole cell extracts of cells co-expressing Chl1:13Myc and Fen1:3HA were treated with or without DNAseI treatment before co-immunoprecipitation with anti-MYC beads.

C) 1µg of λ DNA added in the clarified whole cell extract with and without DNAseI treatment, run on a 1% agarose gel.

D) Reciprocal immunoprecipitation of cells co-expressing Chl1:13Myc and Fen1:3HA with Anti-HA beads. Cells expressing untagged Fen1 were used to determine the specificity of the co-immunoprecipitation reaction (Lane 7).

Figure 4: Cohesion establishment coupled to lagging strand processing:

Replication fork (Pol = DNA polymerase coupled to PCNA) moves to the left: leading strand replication on the top and lagging strand replication on the bottom (RNA primers shadowed). Immediately behind the fork, PCNA associates with Fen1 (green) and Chl1 (orange). Ctf7/Eco1 (yellow) is not stably recruited to chromatin by any factor, but transiently interacts with chromatin to establish cohesion. Therefore, both cohesin deposition and subsequent cohesion establishment occur behind the replication fork. Cohesins (purple) depicted as unstructured to highlight the many models currently posited in the literature(Nasmyth, 2005; Onn et al., 2008; Surcel et al., 2008). MCM helicase, primase and RPA not shown (based on Burgers(Burgers, 2009)).

Figure S1: Test for *ctf7*$^{eco1\text{-}1:\ ADE}$ *fen1* mutant spore

The single *ctf7*$^{eco1\text{-}1:\ ADE}$ *fen1*double mutant obtained from the cross between *fen1* and *ctf7*$^{eco1\text{-}1:\ ADE}$ was grown on YPD at 23^{o}C and 30^{o}C.

A) Growth of *ctf7*$^{eco1\text{-}1:\ ADE,}$ *fen1* single mutants and *ctf7*$^{eco1\text{-}1:\ ADE}$ *fen1* double mutant on YPD at 23^{o}C.

B) Growth of *ctf7*$^{eco1\text{-}1:\ ADE,}$ *fen1* single mutants and *ctf7*$^{eco1\text{-}1:\ ADE}$ *fen1* double mutant on YPD at 30^{o}C.

C) Schematic representation of *ctf7*$^{eco1\text{-}1:\ ADE,}$ *fen1* single mutants and *ctf7*$^{eco1\text{-}1:\ ADE}$ *fen1* double mutant cells.

Table 1: Genetic interaction between *fen1* and *ctf7^{eco1-1}* *mutations*

Tetrad analyses of *fen1::KAN^{r}* cells crossed with *ctf7/eco1-1: ADE* cells.

Genotype	Observed	Expected
fen1	25	28
ctf7/eco1-1	21	28
fen1, ctf7/eco1-1	7 (6 with *CTF7/ECO1:URA*)	28
Wild Type	34	28
Total	87	112

Table 2: **Genetic interaction between *chl1* and *fen1* mutations**

Tetrad analyses of *chl1::HIS3* cells crossed with *fen1::KAN^{r}* cells.

Genotype	Observed	Expected
chl1	15	15
fen1	16	15
chl1, fen1	0	15
Wild type	15	15
Total	46	60

Table3: All strains are of S288C background except those marked with * are W303 background strains

Strain	Genotype
YBS 1019	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52* [4]
YBS 1020	*MATα ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52* [4]
*YBS 1039	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1* [4]
*YMM360	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 ctf7eco1-1::ADE2* [4]
*YSR005	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 fen1::KAN*^{r}
*YSR004	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 chl1::HIS3*
YSR010	*MATα ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 FEN1:3HA:TRP1*
YBS1129	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 CHL1:13Myc:URA3* [6]
YBS040	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 FEN1:3HA:TRP1 CHL1:13Myc:URA3*
YSR051	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 FEN1:13MYC:*TRP1
YSR052	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 FEN1:13MYC:TRP1 (YBS 1070, CTF7:3HA:LEU2)*
YSR054	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 FEN1:13MYC:TRP1 (YBS 1074, 3HA:LEU2)*
YSR071	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 (YBS 1070, CTF7:3HA:LEU2)*

Experimental Methods

Media and Yeast Strains

Saccharomyces cerevisiae strains, plasmids, growth and sporulation media are as described previously(Skibbens, 2004) and as listed in Table 3. To construct *fen1::KAN*^{r} cells, PCR fragments were generated using primers 5'-CGA TGA AAA GCG TTG ACA GCA TAC ATT GGA AAG AAA TAG CGG ATC CCC GGG TTA ATT AA-3' and 5'- CAA GGT GAA GGA CCA AAA GAA GAA AGT GAA AAA AGA ACC CCC GAA TTC GAG CTC GTT TAA AC-3' and pFA6a-kanMX6(Longtine et al., 1998b). The resulting PCR product was transformed into YBS 1039 (Table 3). *FEN1::KAN*^{r} was confirmed by PCR using primers 5'-GGT GAC TTT CGT TAA TGG GGA-3' and 5'-GCA AAC GAA TTA CAG CCA GTG-3'.

To construct FEN1-3HA:TRP cells, PCR fragments were generated using primers 5'-AGA GCA CAA GAA AAT AAA AAA TTG AAC AAA AAT AAG AAT AAA GTC ACA AAG GGA AGA AGA CGG ATC CCC GGG TTA ATT AA-3' and 5'- CAA GGT GAA GGA CCA AAA GAA GAA AGT GAA AAA AGA ACC CCC GAA TTC GAG CTC GTT TAA AC-3' on pFA6a-3HA-TRP1(Longtine et al., 1998b) and transformed into YBS 1020 (Table 3). FEN1:3HA: TRP was confirmed by western blotting and PCR analysis.

To construct *chl1::HIS3* cells, PCR fragments were generated using the primers 5'- GTA GAA AAC CAG GCT AAA AAC AGT CAC ACT AGT CCA AAA AAC GGA TCC CCG GGT TAA TTA A-3' and 5'-ATA TAG TAG TAA TCA CAG TAT ACA GGT AAA CGT ATT CCT TGA ATT CGA GCT CGT TTA AAA C-3' on p-FA5a-His3MX6(Longtine et al., 1998b) and transformed into YBS 1019 (Table 3). *CHL1::HIS3* was confirmed using primers 5'-TGC CTG GCT GAC TTC TTA GAC-3' and 5'-CGT GAG CAA ACA ACG GGT AAT-3'.

Co-Immunoprecipitations and Western blot analyses

10^9 cells in YPD medium were harvested, washed with sterile water and resuspended in 500ml, IPH150 buffer (150mM NaCl, 50mM TRIS pH8, 5mM EDTA, 0.5% IGEPAL-CA 630

(Sigma), 1mM DTT, 10mM Sodium Butyrate, Roche protease inhibitor cocktail). 0.5mm glass beads were added and the cells were frozen immediately in liquid Nitrogen and stored at -80^{o}C. The frozen cells were then thawed on ice and mechanically lysed by bead beating (Biospec mini bead beater). The soluble fraction was separated from the insoluble fraction by centrifugation at 10,500 rpm (TOMY TX-160, TMA 24 rotor). To test for DNA based interactions, IPH150 was supplemented to 10mM $MnCl_2$ and 500ng of DNAseI (Roche) per ml and incubated for 1 hour at 4^{o} C. The clarified cell extract was then incubated with EZ view anti-C-MYC affinity gel (Sigma) or Anti-HA affinity matrix (Roche) overnight at 4^{o}C. The beads were harvested by centrifugation and washed extensively with IPH50 buffer (50mM NaCl, 50mM TRIS pH8, 5mM EDTA, 0.5% IGEPAL-CA 630 (Sigma), 1mM DTT, 10mM Sodium Butyrate, Roche protease inhibitor cocktail) and bound proteins solubilized with 2x Lamelli buffer (Sigma). Proteins were then separated by SDS PAGE and analyzed by immunoblotting. Immunodetections were performed using anti-MYC 9E10 (1:1000) (Santa Cruz) and anti-HA (1:500) (Santa Cruz) in combination with goat anti mouse HRP (1:10,000) (Bio-Rad) and ECL plus (GE healthcare) for visualization.

Acknowledgements

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Chapter 3

Chl1 DNA helicase regulates Scc2 deposition specifically during DNA-replication in *Saccharomyces cerevisiae*

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ABSTRACT

The conserved family of cohesin proteins that mediate sister chromatid cohesion requires Scc2, Scc4 for chromatin-association and Eco1/Ctf7 for conversion to a tethering competent state. A popular model, based on the notion that cohesins form huge ring-like structures, is that Scc2,Scc4 function is essential only during G1 such that sister chromatid cohesion results simply from DNA replisome passage through pre-loaded cohesin rings. In such a scenario, cohesin deposition during G1 is temporally uncoupled from Eco1-dependent establishment reactions that occur during S-phase. Chl1 DNA helicase (homolog of human ChlR1/DDX11 and BACH1/BRIP1/FANCJ helicases implicated in Fanconi anemia, breast and ovarian cancer and Warsaw Breakage Syndrome) plays a critical role in sister chromatid cohesion, however, the mechanism through which Chl1 promotes cohesion remains poorly understood. Here, we report that Chl1 promotes Scc2 loading unto DNA such that both Scc2 and cohesin enrichment to chromatin are defective in *chl1* mutant cells. The results further show that both Chl1 expression and chromatin-recruitment are tightly regulated through the cell cycle, peaking during S-phase. Importantly, kinetic ChIP studies reveal that Chl1 is required for Scc2 chromatin-association specifically during S-phase, but not during G1. Despite normal chromatin enrichment of both Scc2 and cohesin during G1, *chl1* mutant cells exhibit severe chromosome segregation and cohesion defects – revealing that G1-loaded cohesins is insufficient to promote cohesion. Based on these findings, we propose a new model wherein S-phase cohesin loading occurs during DNA replication and in concert with both cohesion establishment and chromatin assembly reactions - challenging the notion that DNA replication fork navigates through or around pre-loaded cohesin rings.

INTRODUCTION

The generation of viable progeny cells requires the faithful replication of each parental chromosome, producing identical sister chromatids, and faithful segregation of sister chromatids into daughter cells. Since the two cellular events, DNA replication (S phase) and chromosome segregation (M phase) are temporally separated, cells must maintain the identity of sister chromatids over time, in some cases decades. Cells achieve this feat with the help of a conserved multimeric protein complex known as cohesins that consist of Smc3, Smc1, Mcd1/Scc1 and Scc3/Irr1 (Onn et al., 2008; Skibbens, 2008).In addition to their canonical role in sister chromatid tethering, cohesin complexes also function in a multitude of cellular processes including DNA repair, chromatin condensation, transcriptional regulation and rDNA metabolism (Rudra and Skibbens, 2013b; Xiong and Gerton, 2010). The transcription regulatory role may be especially relevant given that mutation in human cohesin subunits (SMC1A/Smc1, SMC3, RAD21/Mcd1/Scc1) and cohesin-regulatory factors (ESCO2/Eco1/Ctf7, HDAC8/Hos1, NPBL/Scc2, APRIN/Pds5, ChlR1/DDX11/Chl1 and BACH1/BRIP/FANCJ/Chl1) result in severe developmental maladies that include Cornelia de Lange Syndrome, Roberts Syndrome, Warsaw Breakage Syndrome and Fanconi Anemia (Deardorff et al., 2012a; Deardorff et al., 2007; Deardorff et al., 2012b; Gillis et al., 2004; Krantz et al., 2004; Levitus et al., 2005; Litman et al., 2005; Musio et al., 2006; Schule et al., 2005; Tonkin et al., 2004; van der Lelij et al., 2010a; Vega et al., 2005; Zhang et al., 2009). The structure through which cohesins tether together sister chromatids or evoke transcription regulation remains undefined, but models include that DNA is embraced within an SMC lumen, clamped by the folding over of SMC arms to bring head and hinge domains into registration, or sandwiched between SMC head domains capped by Mcd1 (the latter based on crystal structure analyses of the SMC-like Mre11,Rad50,Nbs1 complex (Mockel et al., 2012; Rudra and Skibbens, 2013b; Schiller et al., 2012), any of which may assemble into higher order structures (Haering et al., 2008; Huang et al., 2005; Nasmyth, 2011; Ocampo-Hafalla and Uhlmann, 2011; Onn et al., 2008; Rudra and Skibbens, 2013b; Skibbens, 2010).

Cohesin binding to chromatin is not sufficient to tether together sister chromatids. Instead, budding yeast Eco1/Ctf7 (herein Eco1), the founding member of a highly conserved family (EFO1/ESCO1 and EFO2/ESCO2 in vertebrates, DECO in flies) of acetyltransferases, is required to convert chromatin-bound cohesins to a tethering competent state (Bellows et al., 2003; Hou and Zou, 2005; Ivanov et al., 2002; Skibbens et al., 1999; Toth et al., 1999; Williams et al., 2003). To date, cohesin Smc3 is the only known essential Eco1 substrate (Rolef Ben-Shahar et al., 2008; Unal et al., 2008; Zhang et al., 2008a). Eco1 function is essential specifically during S-phase (Skibbens et al., 1999; Toth et al., 1999). In fact, multiple interactions between Eco1, PCNA (the DNA polymerase 'sliding clamp') and Replication Factor C (RFC) complexes that regulate PCNA support the model that Eco1 acetylates Smc3 as sister chromatids emerge from the DNA replication fork (Kenna and Skibbens, 2003; Maradeo and Skibbens, 2009; Maradeo and Skibbens, 2010; Milutinovich et al., 2007; Moldovan et al., 2006; Rolef Ben-Shahar et al., 2008; Skibbens et al., 1999; Song et al., 2012; Unal et al., 2008; Zhang et al., 2008a). Contrary to an early report (Moldovan et al., 2006), it is now clear that Eco1 binding to PCNA is neither required for Smc3 acetylation nor Eco1 recruitment to DNA (Hou and Zou, 2005; Skibbens et al., 2007; Song et al., 2012). Thus, critical gaps remain in our understanding of DNA replication-coupled cohesion establishment.

The timing of cohesin association with chromatin appears to profoundly impact the ability of Eco1 to establish cohesion. Supporting DNA replication-coupled cohesion establishment are findings that the essential function of Eco1 maps to S-phase and that Mcd1 expressed after S-phase fails to participate in sister chromatid pairing, although cohesins also associate with DNA before S-phase in both yeast and vertebrate cells (Bernard et al., 2006; Ciosk et al., 2000; Lengronne et al., 2006; Skibbens et al., 1999; Toth et al., 1999; Uhlmann and Nasmyth, 1998; Watrin et al., 2006). Early cell cycle studies mapped the essential role of the Scc2, Scc4 cohesin deposition complex to S-phase, similar to both Eco1 function and Mcd1 expression (Ciosk et al., 2000; Guacci et al., 1997; Michaelis et al., 1997; Skibbens et al., 1999; Toth et al., 1999). Biochemical analysis of cohesins as huge ring-like structures, however, led to a popular model that Scc2, Scc4 complex is essential only during G1, enabling replication forks to establish

cohesion simply by passing through pre-loaded cohesin rings (Gruber et al., 2003; Haering et al., 2002). Subsequent studies support the notion that Scc2, Scc4-dependent cohesin deposition may be required during the G1 portion of the cell cycle, but remain actively debated (Bernard et al., 2006; Lengronne et al., 2006; Nasmyth, 2011; Skibbens, 2010; Uhlmann, 2011; Watrin et al., 2006). Resolving the important issue regarding which temporally-deposited cohesin population in fact participates in cohesion will likely entail analyses of auxiliary factors that promote efficient cohesion establishment.

The DNA helicase Chl1 (and homologs) is of particular interest in that it is crucial for efficient sister chromatid cohesion: *chl1* mutant cells exhibit significant cohesion defects that exceed even essential gene mutations involved in cohesion such as *pol30* (PCNA) and Chl1 is the only helicase thus far shown to associate with Eco1 (Mayer et al., 2004; Moldovan et al., 2006; Petronczki et al., 2004; Skibbens, 2004). Chl1 is of further import because it is the homolog of both ChlR1/DDX11 and BACH1/BRIP/FANCJ, mutations in which result in Warsaw Breakage Syndrome and both Fanconi anemia and breast and ovarian cancers, respectively (Amann et al., 1997; Cantor et al., 2004; Cantor and Andreassen, 2006; Cantor et al., 2001; Gupta et al., 2007; Inoue et al., 2007; Levitus et al., 2005; Litman et al., 2005; Parish et al., 2006; Peng et al., 2006; Rafnar et al., 2011; Skibbens, 2004; van der Lelij et al., 2010a; van der Lelij et al., 2010b). In the current study, we show that Chl1 plays a critical role in Scc2 recruitment to chromatin, linking for the first time Scc2 regulation to helicase-dependent alterations of DNA. As important, Chl1 is required for both Scc2 and cohesin recruitment specifically during S-phase, but not G1. Despite normal Scc2 (and cohesin) recruitment to DNA during G1, *chl1* mutant cells exhibit significant cohesion defects. These findings significantly impact current models regarding the temporal coupling of cohesin deposition and cohesin establishment.

RESULTS

Chl1 expression and chromatin binding are cell cycle regulated

Results from this lab and others suggest that Chl1 is critical for cohesion establishment during S-phase: Chl1 binds Eco1, PCNA and Fen1 and the human homolog ChlR1 stimulates the flap endonuclease FEN-1 involved in both maturation of replication lagging strands and cohesion establishment (Farina et al., 2008; Moldovan et al., 2006; Rudra and Skibbens, 2013a; Skibbens, 2004). Chl1 binding to chromatin thus far, however, has been demonstrated only in response to DNA damage (Ogiwara et al., 2007b), leaving unresolved fundamental issues of Chl1 expression and chromatin recruitment throughout the cell cycle. To address these deficits of knowledge regarding this homolog of clinically-relevant DNA helicases, we first tested the extent to which Chl1 expression is regulated throughout the cell cycle. Logarithmically growing cells expressing epitope-tagged Chl1 were synchronized in G1 (alpha-factor), released into fresh medium to allow for cell cycle progression and samples harvested at 15 minute intervals to assess both cell cycle progression and changes in Chl1 protein levels. The results show that Chl1 is diminished during G1, rises as cells enter S-phase and remains elevated during mitosis [Chl1 typically runs as two bands, most likely due to C-terminal PEST sites (Gerring et al., 1990; Skibbens, 2004)]. In contrast, constitutively expressed Swi6 protein remains unchanged throughout the cell cycle (Figure 1B, D).

If Chl1 promotes cohesion establishment specifically during S-phase, then Chl1 recruitment to chromatin might be similarly reflected in a cell cycle-dependent fashion. To carefully analyze Chl1 recruitment to chromatin, we exploited Triton X-100 cell fractionation assays previously used to demonstrate chromatin-association of a spectrum of factors including Eco1, cohesin, DNA replication initiators and fork stabilization proteins (Leman et al., 2010; Mendez and Stillman, 2000; Moldovan et al., 2006; Toth et al., 1999). We validated the cell fractionation procedure using Phosphoglycerokinase (PGK) and Histone 2B (H2B) as cytosolic and chromatin fiduciary markers, respectively. The results show an enrichment of chromatin-associated H2B, and absence of cytosolic component PGK, in the Triton-X-100 insoluble fraction

(Figure 1A). Similar to H2B, the bulk of Chl1 resides in the chromatin fraction, although modest levels of both Chl1 and H2B remain soluble. We further tested whether Chl1 chromatin-association might increase in response to DNA damage. Log growth cultures expressing epitope-tagged Chl1 were split and one of the two cultures exposed to 0.1% MMS for 2 hours prior to processing for chromatin binding. The results show that the level of Chl1 chromatin-enrichment is not increased upon MMS treatment, consistent with a model that Chl1 binds chromatin and functions each and every cell cycle (Figure 1A).

Given the critical role for Chl1 in cohesion, it became important to determine whether Chl1 recruitment to chromatin is regulated through the cell cycle. We returned to the validated chromatin-fractionation method described above. The results show that Chl1 recruitment to chromatin increases significantly (>3 fold) as cells enter S-phase (Figure 1C, D). Thus, both Chl1 expression and chromatin recruitment are tightly regulated in normal cycling cells. Notably, while Chl1 protein levels remain elevated during mitosis, Chl1 binding to chromatin decreases as cells exit S-phase - revealing for the first time a post-translational mechanism that drives Chl1 release from DNA. In combination, these results demonstrate that Chl1 expression and DNA binding are tightly regulated through the cell cycle, in support of a model that Chl1 promotes sister chromatid cohesion specifically during S phase and, based on protein interaction studies, in close proximity to the DNA replication fork.

Chl1 regulates cohesion acetylation, but not Eco1 auto-acetylation

Presently, the mechanism through which Chl1 promotes efficient sister chromatin cohesion remains unknown. Given that Chl1 is the only helicase shown to interact with the establishment factor Eco1 (Skibbens, 2004), we hypothesized that Chl1 might regulate Eco1-mediated acetylation of Smc3. We included *fen1* mutant cells in our analysis given that Fen1 also associates with both Eco1 and Chl1 and that both human and yeast cells diminished in Fen1 exhibit cohesion defects (Farina et al., 2008; Rudra and Skibbens, 2012a; Warren et al., 2004). Logarithmically growing wild type cells and *chl1* and *fen1* single mutant cells, all expressing Smc3-3HA as the sole source of Smc3, were lysed, resulting extracts clarified by centrifugation

and incubated with anti-HA-coupled beads. After washing to remove unbound or weakly associated proteins, bead-bound proteins were eluted and analyzed by Western blot. The results show that total levels of Smc3 protein remain unchanged regardless of the presence or absence of either Chl1 or Fen1 (Figure 2A). No signal was obtained for strains expressing untagged Smc3. Querying blots with anti-acetylated lysine antibodies further revealed nearly identical levels of Smc3 modification present in both wildtype and *fen1* mutant cells. In contrast, Smc3 acetylation is markedly decreased in *chl1* mutant cells (Figure 2A and 2B). Thus, Chl1 is critical for Eco1-depedent Smc3 acetylation.

We realized that reduced Smc3 acetylation in *chl1* mutant cells can be explained by at least one of two models: that Chl1 promotes Eco1 acetyltransferase activity or that Chl1 promotes cohesin binding to DNA which in turn becomes a suitable substrate for Eco1. To test the first of these possibilities, we exploited the fact that auto-acetylation is readily detected in Eco1/ESCO proteins (Bellows et al., 2003; Gordillo et al., 2008; Hou and Zou, 2005; Ivanov et al., 2002). Logarithmically growing wild type and *chl1* mutant cells expressing Eco1-18MYC as the sole source of Eco1 were lysed and the resulting extracts incubated with anti-MYC-coupled beads. After washing to remove unbound or weakly associated proteins, bead-bound proteins were eluted and analyzed by Western blot. Results reveal that total Eco1 protein levels remain unchanged despite the presence or absence of Chl1 (Figure 2C). No signal was obtained for strains expressing untagged Eco1. We also found nearly identical levels of modified Eco1 in wildtype and *chl1* mutant cells using antibody directed against acetylated lysine. These results reveal that Chl1 does not directly regulate Eco1 activity: that decreased Smc3 acetylation in *chl1* mutant cells is not predicated on reduced Eco1 acetyltransferase activity or protein levels.

We next tested the possibility that Chl1 functions in cohesin binding to DNA: that the reduced cohesin acetylation in *chl1* mutant cells is based on loss of cohesin chromatin-association. Log phase wild type, *chl1* and *fen1* single mutant cells expressing Mcd1-6HA as the sole source of this cohesin subunit were lysed and the fraction of chromatin-bound Mcd1 assessed using Triton- X-100 fractionation as described above. Wildtype and *fen1* mutant cells contained nearly identical levels of chromatin-bound Mcd1 (Figure 3C, D). In contrast, *chl1* cells

contained a marked reduction (~50% of wildtype levels) of chromatin-bound Mcd1. Importantly, *chl1* mutant cells contained Mcd1 protein levels equivalent to wildtype cells (Supplementary Figure S1), confirming that the observed reduction of chromatin-associated Mcd1 is due to loss of Chl1 helicase and not reduced Mcd1 expression (Figure 3A,B). To confirm that Chl1 participates in the stable binding of cohesin to DNA, we turned to a chromatin immunoprecipitation (ChIP) strategy that allows for quantification of cohesin enrichment on well-documented cohesin association regions (CARs) (Glynn et al., 2004; Kogut et al., 2009; Laloraya et al., 2000). We chose 5 independent CAR sites along the arm of chromosome III. Protein-DNA complexes in logarithmically growing wild type and *chl1* mutant cells expressing Mcd1-6HA were cross-linked using formaldehyde, lysed and sonicated to shear the DNA. Chromatin complexes containing Mcd1 were immunoprecipitated, cross-links reversed and DNA amplified using CAR-specific primers. The results show that Mcd1 levels are significantly decreased (about 50% of wildtype levels) for each of the 5 CAR sites (Figure 3E), documenting that Chl1 is critical for stable cohesin binding along chromosome arms.

Cohesin binding at centromeres is uniquely regulated compared to cohesins that associate along chromosome arms: centromeric cohesins occur at elevated levels, along extended regions of DNA and are differentially sensitive to perturbation of cohesin regulators – especially in vertebrate cells (Heidinger-Pauli et al., 2010; Kitajima et al., 2004; Megee et al., 1999; Shintomi and Hirano, 2009; Tanaka et al., 1999). Given these unique features, it became crucial to test whether Chl1 also promotes stable binding of cohesin to centromeres. We repeated the ChIP analysis, but this time using 5 centromere (*CEN*) sites within chromosome III, regions well-established as enriched in cohesin binding (Glynn et al., 2004; Kogut et al., 2009; Megee et al., 1999; Tanaka et al., 1999). The results show that *chl1* mutant cells exhibit a significant reduction (<40% of wildtype levels) of cohesin association to centromeres (Figure 3E, F). Further analyses discount the possibility that Mcd1 protein levels are decreased in *chl1* mutant cells (Supplemental Figure 1A). These findings extend those of prior studies that cohesins are only loosely chromatin-associated in the absence of Chl1/ChlR1 (Inoue et al., 2007; Laha et al., 2011). We conclude that Chl1 is critical for stable cohesin-DNA interactions at all

CAR sites and that the decrease in cohesin binding in *chl1* mutant cells in part accounts for the reduced levels of Smc3 acetylation (this study).

Chl1 regulates S phase cohesin binding to chromosomes

A presumptive but popular model is that cohesin deposition during G1 is required for subsequent sister chromatid tethering reactions that occur during S-phase (Nasmyth, 2011; Ocampo-Hafalla and Uhlmann, 2011). In contrast is a preceding model that cohesins deposited during S-phase predominantly participate in cohesion establishment (Skibbens, 2000; Skibbens, 2010). Resolving this discrepancy is complicated because *SCC2* mutation abolishes deposition in all phases of the cells cycle such that mapping studies produced conflicting interpretations (Bernard et al., 2006; Ciosk et al., 2000; Kogut et al., 2009; Lengronne et al., 2004; Song et al., 2012). We realized that Chl1, which promotes cohesion specifically during S-phase and is required for cohesin enrichment onto chromatin, might provide a unique venue from which to address these models. To test whether Chl1 impacts cohesin chromatin-association in a cell cycle-regulated fashion, we repeated the chromatin immunoprecipitations described above on cycling cells released from G1 and harvested at 10 minute intervals so that we could clearly differentiate between G1 versus S-phase cohesin deposition. We queried cohesin enrichment at 5 independent CAR sites: three along chromosome arms and two within the centromere of chromosome III (*CEN3.1* and *CEN3.4*). As expected, cohesin binding to DNA increased during G1 in wildtype cells and this elevated cohesin chromatin-association remained through S-phase, declining only as cells exited mitosis. Notably, cohesin enrichment onto DNA during G1 in *chl1* mutant cells was indistinguishable from that of wildtype cells. Thus, Chl1 is not required to promote stable cohesin association during G1. Importantly, however, cohesin enrichment onto CAR sites dropped precipitously as *chl1* mutant cells entered S-phase (Figure 4A-4E). The decrease in cohesin enrichment failed to recover to wild type levels throughout the remainder of the cell cycle. These findings reveal that cohesin-association with replicated sister chromatids depends on the S-phase activity of Chl1 DNA helicase.

Chl1 regulates Scc2 chromatin binding to DNA specifically during S-phase

The above studies reveal that *chl1* mutant cells are deficient in cohesin enrichment onto DNA specifically during S-phase, a deficit that leads to cohesion loss. However, the mechanism through which Chl1 promotes cohesin enrichment remains unknown. We hypothesized that Chl1 might be required for efficient recruitment of the Scc2,Scc4 cohesin-deposition complex to DNA. To test this possibility, log phase wild type and *chl1* mutant cells expressing Scc2-3HA as the sole source of Scc2 function were lysed and the fraction of chromatin-bound Scc2 assessed using Triton X-100 fractionation as described above. Compared to wildtype cells, *chl1* mutant cells contained a marked reduction (~40% of wildtype levels) of chromatin-bound Scc2. Whole cell extracts from wildtype and *chl1* mutant strains contained identical Scc2 levels, confirming that the reduction of Scc2 binding was due to loss of Chl1 helicase and not altered Scc2 expression (Figure 5A,B and Supplemental Figure 1B). Is Scc2 binding to DNA reduced specifically at CAR sites? We investigated whether Chl1 participates in the stable binding of Scc2 at five independent CAR sites that reside along the arm of chromosome III and also five CAR sites within *CEN3* using chromatin immunoprecipitations. The results show that Scc2 levels are significantly decreased (50-60% of wildtype levels) for each of the ten CAR sites queried, revealing for the first time that Chl1 is a critical regulator of Scc2 enrichment onto DNA (Figure 5C,D).

If we are correct that Chl1 is critical for the enrichment of cohesin to DNA specifically during S-phase, then *chl1* mutant cells should similarly exhibit loss of Scc2 binding to DNA as cells enter S-phase, but not before. To test this prediction, we performed chromatin immunoprecipitation assays taking samples at 10 minute intervals from synchronized wild type and *chl1* mutant cells expressing Scc2-3HA. The results show that Scc2 enrichment at all five CAR sites (three arm and two centromere sites) prior to S-phase is identical in both wildtype and *chl1* mutant cells. Wild type cells continued to recruit Scc2 throughout S-phase and maintained this level into mitosis. As soon as *chl1* mutant cells entered S-phase, however, the level of Scc2 binding to DNA dropped significantly at all CAR sites tested and remained low even after S-phase (Figure 6). In combination, these results document that Chl1 is critical for the stable association

of Scc2 with DNA during S-phase and that this DNA replication-coupled deposition is critical for sister chromatid cohesion establishment.

DISCUSSION

Cohesins that participate in cohesion become chromatin-associated during S-phase

The issue regarding the population of DNA-associated cohesins which both serve as Eco1 substrates and participate in sister chromatin-tethering reactions remains a critical but enigmatic topic of cell biology. One of the major revelations of the current study involving Chl1 is that cohesins that associate with DNA during G1 fail to produce sister chromatid cohesion: *chl1* mutant cells load cohesins onto DNA during G1 to levels identical to wildtype cells and to appropriate CAR sites - yet exhibit significant cohesion defects. While *chl1* mutants are viable, the resulting cohesion defect (35%) nearly rivals that of many cohesin mutants (50-65%) and exceeds that of essential cohesion gene mutations such as *pol30/pcna* (20%) (Skibbens et al., 2007). We also note that loss of cell viability does not necessarily equate to cohesion defects in budding yeast; nor do cohesion defects necessarily impact proper chromosome segregation (Guacci and Koshland, 2012). It is not, however, the level of cohesion defect that occurs in *chl1* mutant cells that is of interest here, but the cell cycle specificity that provides a unique tool from which to assess when cohesin deposition is required for establishment. What then is the basis of the cohesion defect in *chl1* mutant cells? As opposed to the normal cohesin enrichment onto DNA that occurs during G1, *chl1* mutant cells exhibit dramatic defects in cohesin enrichment specifically during S-phase. These findings document that cohesin must associate with DNA during S-phase to both serve as an Eco1 substrate and participate in cohesion (Figure 7). This re-emerging view, which we term *Replication-coupled cohesin deposition*, is supported by numerous findings including that 1) Eco1 can acetylate cohesins prior to S-phase, but that cohesins acetylated during G1 fail to produce sister chromatin cohesion and 2) Scc2 function is essential predominantly during S-phase, even though deposition occurs throughout other portions of the cell cycle (Bernard et al., 2008; Ciosk et al., 2000; Kogut et al., 2009; Lengronne et al., 2006; Skibbens, 2000; Song et al., 2012; Watrin et al., 2006).

Our *Replication-coupled cohesin deposition* model contrasts popular views that cohesin deposition onto DNA is required during G1 – a notion arising not from analysis of deposition complexes but instead from biochemical studies that cohesins might form a huge ring-like complex (Gruber et al., 2003; Haering et al., 2002). The rationale was as follows: if cohesins form huge rings and become loaded prior to DNA replication, then establishment might simply occur by passage of the DNA replication fork through cohesin rings. This *Replication-through-a-ring* model quickly gained popularity and to this day remains widely-discussed (Nasmyth, 2011; Ocampo-Hafalla and Uhlmann, 2011), despite being contrary to prior results that Scc2,Scc4 function is required during S-phase and that *eco1* mutant cells exhibit cohesion defects despite normal cohesin deposition and subsequent DNA replication (Milutinovich et al., 2007; Skibbens et al., 1999; Toth et al., 1999). Partly in response to these challenges, analyses of Scc2,Scc4 (Mis4,Ssl3 in fission yeast) was re-visited, with the results suggesting instead that deposition is critical during G1 (Bernard et al., 2006; Bernard et al., 2008; Lengronne et al., 2006; Watrin et al., 2006). The conflicting interpretations and ongoing debate may be understandable given that i) Scc2, Scc4 is required for all cohesin deposition, ii) deposition occurs throughout the cell cycle, iii) conditional alleles can become refractile to inactivation once complexed with other proteins and iv) very little cohesin (13%) is required to maintain sister chromatid cohesion (Heidinger-Pauli et al., 2010). With regards to this latter point, low levels of cohesins that associate with early-replicating domains may contribute to underestimating the importance of S-phase cohesin deposition in cell cycle mapping studies. Ultimately, size estimates indicating that the DNA replisome (including leading and lagging strand polymerases, helicase/primase, and extruded DNA loops or 'trombones' that occur during lagging strand synthesis) is larger than the lumen posited to form upon Smc1,3 and Mcd1 assembly provided a convincing argument against a simplistic *Replication-through-a-ring (Bylund and Burgers, 2005)*. In fact, the cohesin lumen is likely smaller than first posited given biochemical and FRET studies that Mcd1 sits atop Smc1,3 heads and does not necessarily participate in lumen formation per se (Mc Intyre et al., 2007).

In response, the *Replication-through-a-ring* model evolved in one of two interesting ways. The first of these involved *Replication fork relaxation*. Here, the notion forwarded was that

the replisome partially disassembles upon encountering cohesins such that the extruded DNA loop or 'trombone' that occurs during lagging strand synthesis collapses – allowing for a more streamlined or linearized replisome capable of passing through pre-loaded cohesin rings (Bylund and Burgers, 2005). If true, then *Replication fork relaxation* would occur over a 1,000 times per yeast genome replication to accommodate cohesin-bound loci that appear on average every 12 kb and amidst a million iterations of lagging strand processing (Glynn et al., 2004; Laloraya et al., 2000; Zheng and Shen, 2011). A second branch through which the *Replication-through-a-ring* model evolved included cohesin ring opening/re-closing reactions. In this *Cohesin dynamics* model, opening of G1-loaded cohesins during S-phase was posited to allow for the migration of fully intact replisomes around an open ring – fork relaxation is not required (Lengronne et al., 2006). It remains unclear how cohesin rings, deposited during G1, open and yet remain bound to single-stranded DNA even as the DNA templates for new synthesis. While there is limited evidence that replication fork stability factors associate with cohesins in vitro (Gerlich et al., 2006; Leman and Noguchi, 2013), additional challenges (how does DNA polymerase access ssDNA bound by cohesin; how does the sliding clamp PCNA navigate around cohesin-bound DNA, etc) provide little relief for models that rely on G1-loaded cohesins.

An important feature of the *Replication-coupled cohesin deposition* model posited here is that replisome relaxation and cohesin ring gymnastics, requirements imposed by G1-loaded cohesin rings for replisome progression, are obviated. What happens to these pre-replicative cohesins? The data from the current study suggests that Scc2 and cohesins loaded during G1 are normally bumped off upon cell entry into S-phase (Figure 7). Replisome bump-off (whether through fork progression or formation of secondary DNA structures that arise upon replication fork passage) is consistent with evidence that cohesins that associate with DNA during G1 are highly dynamic and cycle between soluble pools and weakly bound chromatin complexes (Gause et al., 2010; Gerlich et al., 2006; McNairn and Gerton, 2009; Onn and Koshland, 2011). Alternatively, the decrease in Scc2 and cohesin enrichment in *chl1* mutant cells might reflect altered kinetics in which formation of secondary DNA structures precludes only new deposition during S-phase while dissociation rates remain unaffected. Discerning between these two possibilities, or some

combination thereof, awaits further studies. In the meantime, an additional feature of the *Replication-coupled deposition* model is that cohesin enrichment occurs during S-phase when both sister chromatids are present (Figure 7). This model lends support to a dimer/oligomer-based mechanism of sister chromatid tethering and maintenance (Rudra and Skibbens, 2013b).

Chromatin structures that form during DNA replication impact recruitment of Scc2

The timing, and thus position relative to the DNA replication fork, through which Scc2 and cohesin are both recruited to chromatin and participate in establishment is likely one of the most significant informers of cohesin function. Thus, the second major revelation of the current study is that Scc2, and in turn cohesin, binding to chromatin is dependent on Chl1. How does Chl1 promote Scc2 recruitment to chromatin? To date, the only known role for Chl1 (including homologs ChlR1 and BACH/FANCJ) is to bind and resolve secondary DNA structures such as forks and flaps (that arise during Okazaki maturation) and G quadruplex (G4) substrates (Amann et al., 1997; Farina et al., 2008; Hirota and Lahti, 2000; Inoue et al., 2007; Wu et al., 2008; Wu et al., 2012): guanine-rich motifs that form 4-stranded coplanar structures posited to form upon DNA replisome passage (Maizels and Gray, 2013; Wu and Brosh, 2012a). The role of Chl1 homologs in resolving G4s may be particularly informative in that roughly 50% of the predicted 350,000 structures reside at replication origins in humans and likely form immediately behind the DNA replication fork on exposed ssDNA. G4s are capable both of impacting protein recruitment to DNA (including histones) and likely are critical regulators of transcription regulation (Maizels and Gray, 2013; Wu and Brosh, 2012a). These G4 attributes nicely align with findings that Chl1/ChlR1 is critical for maintenance of heterochromatin, tissue development, and regulates protein-associations with DNA including HPV and the epigenetic factor HP1α (Capo-Chichi et al., 2013; Inoue et al., 2011; Inoue et al., 2007; Parish et al., 2006; Rudra and Skibbens, 2013b; van der Lelij et al., 2010b).

The combination of these reports suggests that secondary DNA structures (including forked substrates and G4 structures) that arise during DNA replication are capable of prohibiting

Scc2 enrichment to DNA, which in turn precludes both cohesin deposition and cohesion establishment (Figure 7). The positioning of Chl1 helicase to replicating/maturing sister chromatids, and apparent role of chromatin structure in Scc2/cohesin binding to DNA, supports an emerging view of cohesins in chromosome condensation. Despite early evidence that mutations in either *ECO1, MCD1 or PDS5* produce severe chromosome condensation defects (Guacci et al., 1997; Hartman et al., 2000; Skibbens et al., 1999), the role of cohesins in chromatin architecture remains largely underexplored. More recent evidence that *RAD61/WAPL* mutations, originally thought to rescue cohesin mutation cohesion defects (Rowland et al., 2009; Sutani et al., 2009), instead rescues condensation defects produced by cohesin mutations, dramatically altered the landscape regarding cohesin biology (Skibbens, 2000). While seldom articulated, we note that cohesin mutations also appear to alter chromatin compaction in cells of vertebrate models used to recapitulate phenotypes observed in human developmental disorders such as Cornelia de Lange Syndrome and Roberts Syndrome (Morita et al., 2012; Whelan et al., 2012).

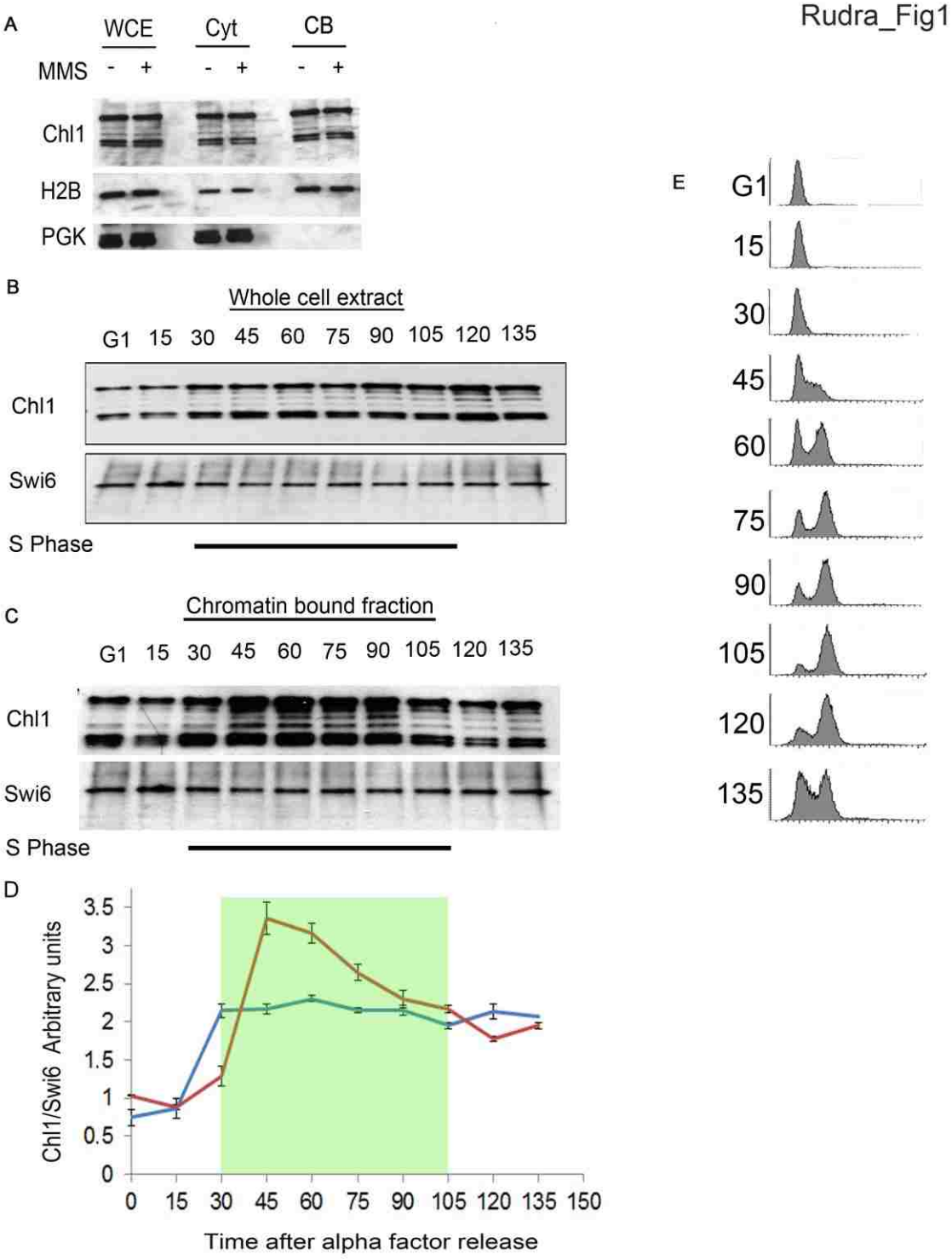
There is wide-spread support for a model that Scc2 and cohesin deposition not only occur during chromatinizing reactions, but that chromatin remodeling complexes play reciprocal roles in cohesin binding to DNA (Chen et al., 2012; Dorsett, 2011; Gartenberg, 2009; Skibbens, 2008). Thus, cohesin deposition and activation not only promote cohesion/condensation, but occur in a context through which other chromatin modifications arise (Rudra and Skibbens, 2013b). Given the role of Chl1 homolog mutations in developmental disorders like Warsaw breakage syndrome, Fanconi anemia, breast and ovarian cancers (Suhasini and Brosh, 2013), assessing the role of Chl1 in chromatin architecture from patient cells may provide important insights regarding the mechanisms through which these maladies arise.

Chl1 DNA helicase expression and chromatin recruitment are tightly regulated throughout the cell cycle

Chl1 is the homolog of human ChlR1/DDX11 and BACH1/BRIP/FANCJ helicases - thus the paucity of information regarding Chl1 expression, chromatin recruitment and regulation is

surprising. During the final stages of manuscript preparation, a report regarding Chl1 was published by the Uhlmann lab (Borges et al., 2013). That study, similar to ours, confirmed a role for Chl1/ChlR1 in stable association of cohesin to chromatin and that cohesin acetylation was reduced in *chl1* mutant cells (Borges et al., 2013; Inoue et al., 2007; Laha et al., 2011). Surprisingly, however, few other similarities with respect to Chl1 regulation exist between that and the current study. For instance, our results clearly reveal that Chl1 binding to chromatin rises dramatically as cells enter S-phase and fall precipitously as cells exit S-phase - even in the continued accumulation of Chl1 protein levels. These results are comparable to those obtained for ChlR1: expression peaks in proliferating cells and mirrors that of PCNA (Farina et al., 2008). In contrast, Borges and colleagues failed to detect cell cycle regulation of Chl1 in terms of either expression or chromatin recruitment (Borges et al., 2013). We have yet to resolve the difference results obtained between the two studies, but note that Chl1 chromatin-association was performed in cells held for hours in an arrested state (Borges et al., 2013) - not in naturally cycling cells as performed in the current study. These findings support our conclusion that Chl1 recruitment to DNA is promoted by active DNA replication. We further note intriguing results that Chl1 is required for Ctf4 [Pol-α-binding factor that promotes cohesion (Hanna et al., 2001; Miles and Formosa, 1992)] to bind DNA specifically during S-phase and not during other portions of the cell cycle (Borges et al., 2013). Thus, at least the fact that Chl1 exhibits S-phase specific activities does not appear to be in dispute. Given our results from chromatin immunoprecipitation studies that Chl1 is critical for Scc2 and cohesin recruitment to DNA specifically during S-phase (but not G1), we conclude that Chl1 expression and recruitment to chromatin are tightly cell cycle regulated.

FIGURES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Cyt
CB
MMS - + - + - +
Chl1
H2B
PGK
B
Whole cell extract
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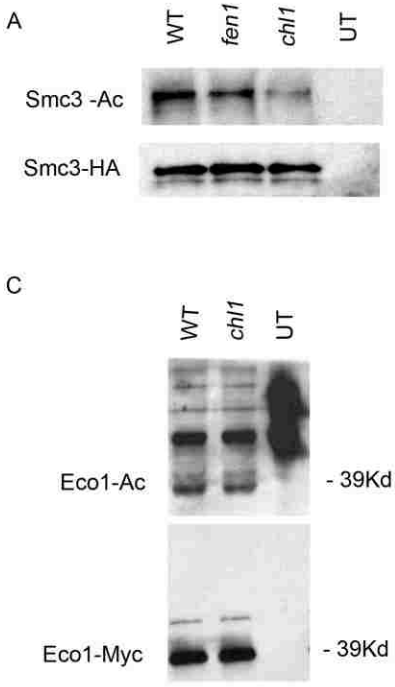
Chl1
Swi6
S Phase
C
Chromatin bound fraction
G1 15 30 45 60 75 90 105 120 135
Chl1
Swi6
S Phase
D
Chl1/Swi6 Arbitrary units
3.5
3
2.5
2
1.5
1
0.5
0
0 15 30 45 60 75 90 105 120 135 150
Time after alpha factor release
E
G1
15
30
45
60
75
90
105
120
135

Rudra_Fig2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WT
fen1
chl1
UT
Smc3 -Ac
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C
WT
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UT
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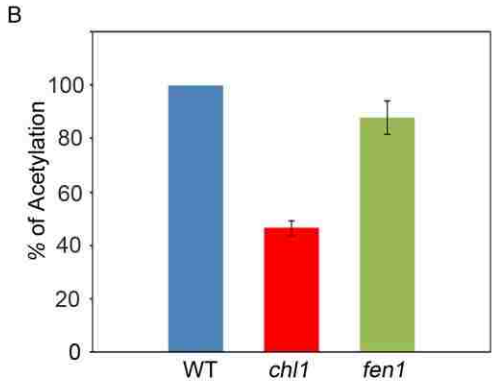
- 39Kd
Eco1-Myc
- 39Kd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% of Acetylation
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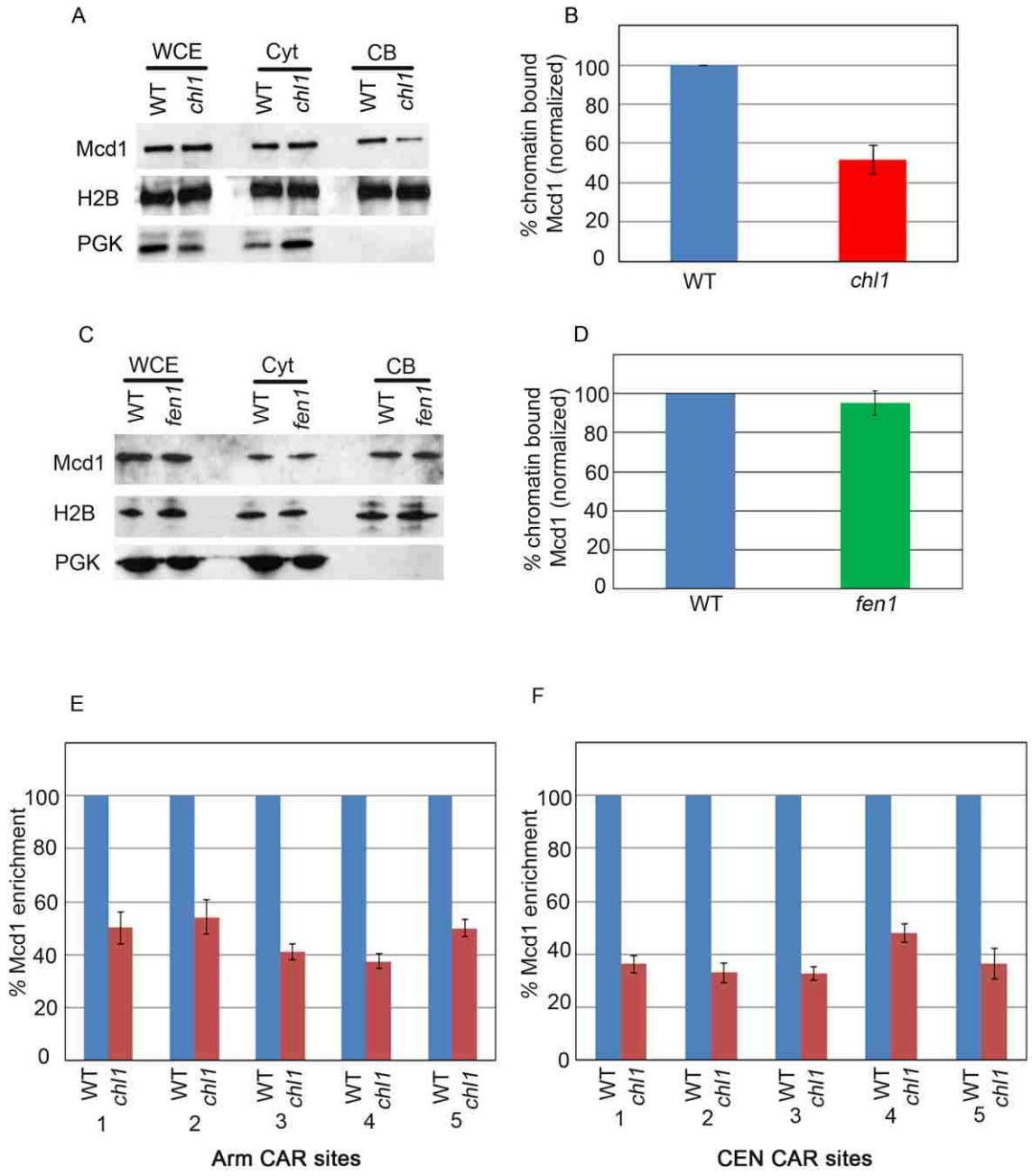
80
60
40
20
0
WT
chl1
fen1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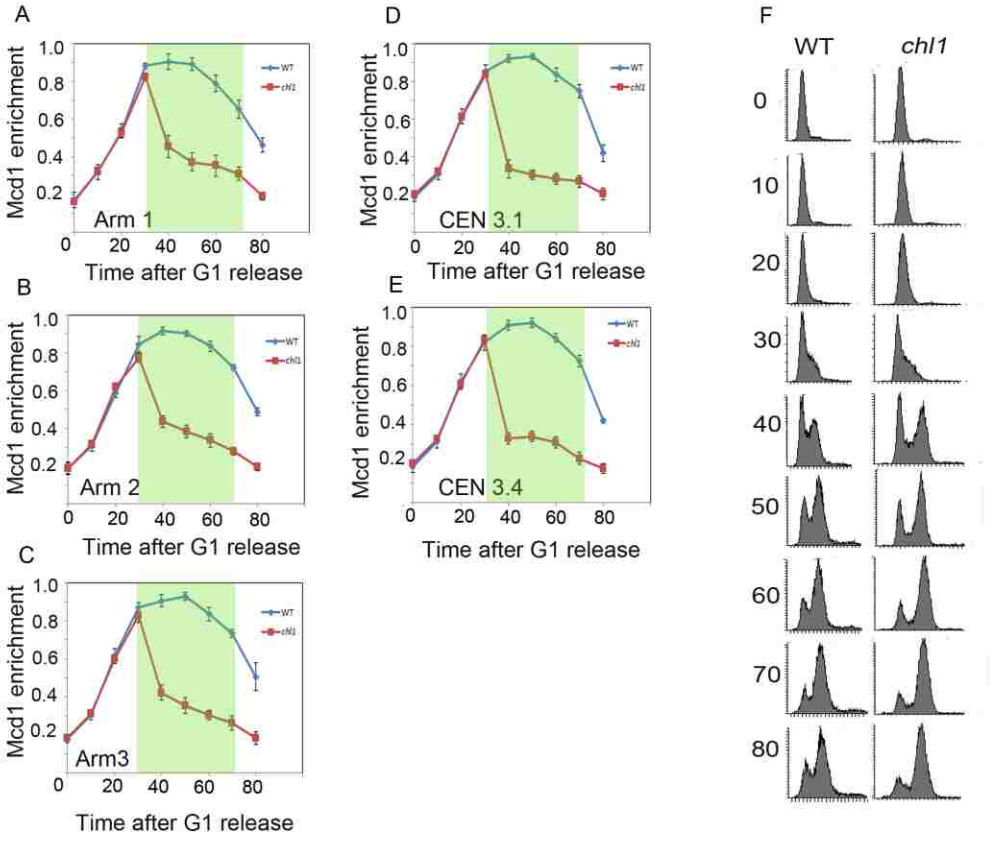
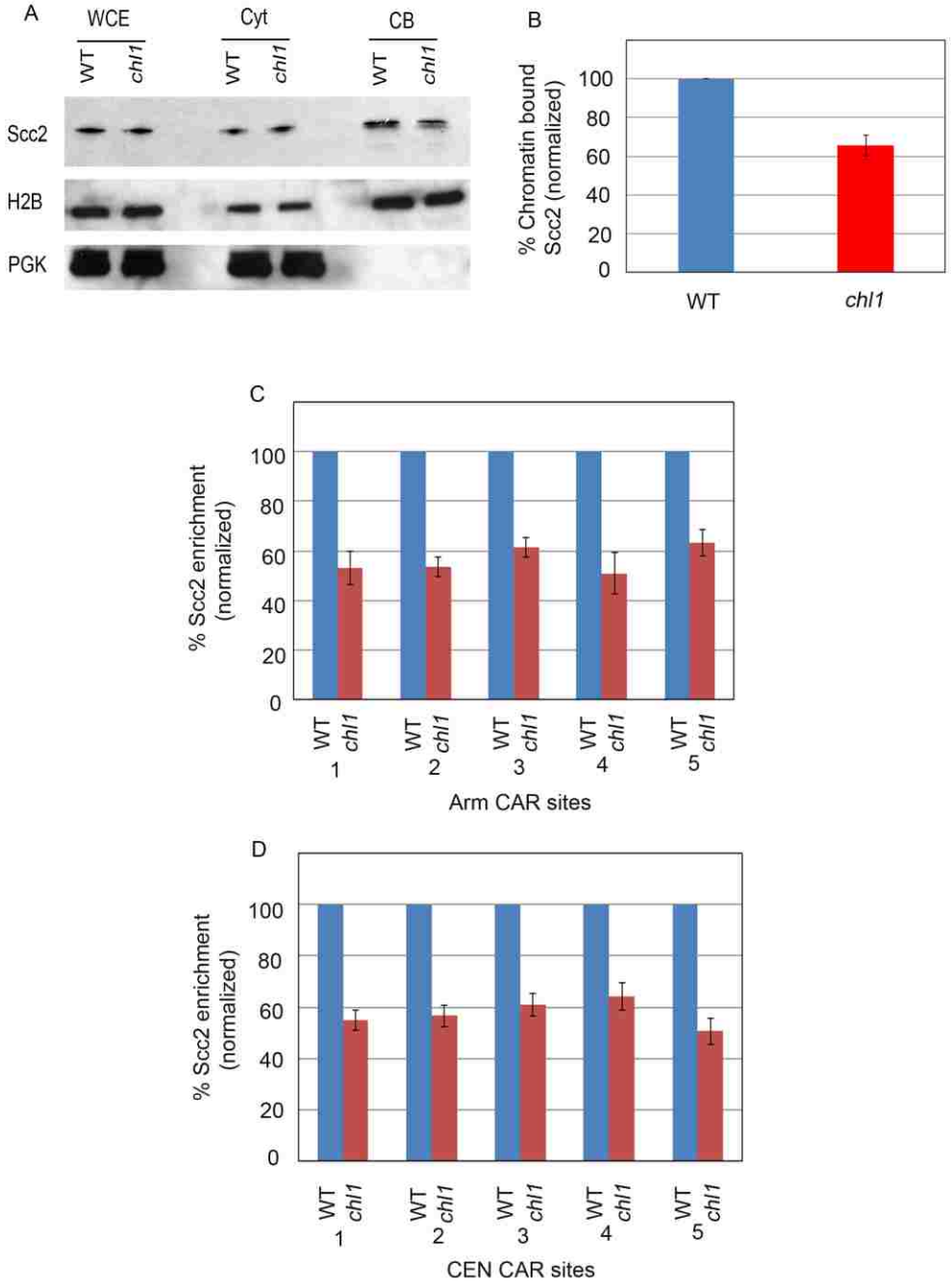
WCE
Cyt
CB
WT
chl1
Mcd1
H2B
PGK
B
% chromatin bound Mcd1 (normalized)
100
80
60
40
20
0
WT
chl1
C
WCE
Cyt
CB
WT
fen1
Mcd1
H2B
PGK
D
% chromatin bound Mcd1 (normalized)
100
80
60
40
20
0
WT
fen1
E
% Mcd1 enrichment
100
80
60
40
20
0
WT
chl1
1
2
3
4
5
Arm CAR sites
F
% Mcd1 enrichment
100
80
60
40
20
0
WT
chl1
1
2
3
4
5
CEN CAR sites


A
B
C
D
E
F
Mcd1 enrichment
Time after G1 release
Arm 1
Arm 2
Arm3
CEN 3.1
CEN 3.4
WT
chl1
0
10
20
30
40
50
60
70
80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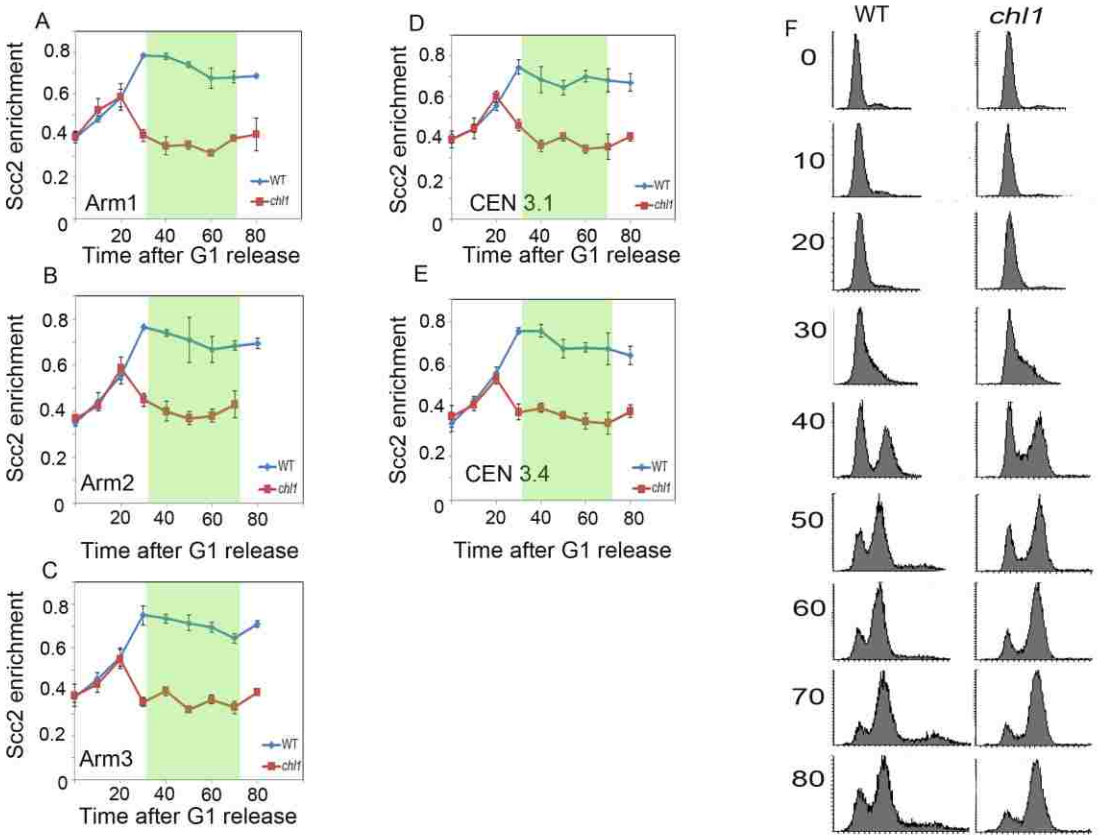
Cyt
CB
WT
chl1
Scc2
H2B
PGK
B
% Chromatin bound Scc2 (normalized)
100
80
60
40
20
0
WT
chl1
C
% Scc2 enrichment (normalized)
Arm CAR sites
D
% Scc2 enrichment (normalized)
CEN CAR sites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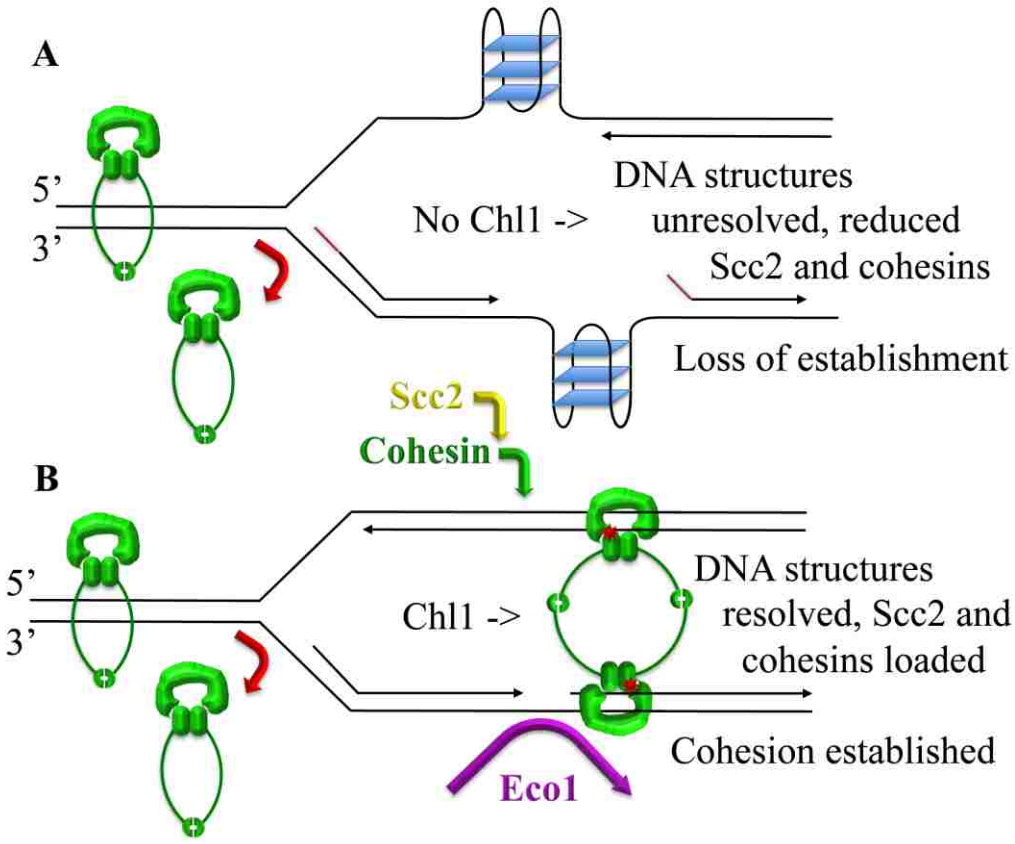
0.8
0.6
0.4
0.2
0
Arm1
WT
chl1
20 40 60 80
Time after G1 release
B
Arm2
C
Arm3
D
CEN 3.1
E
CEN 3.4
F
WT
chl1
0
10
20
30
40
50
60
70
80


A
5’
3’
DNA structures
No Chl1 ->
unresolved, reduced
Scc2 and cohesins
Loss of establishment
Scc2
Cohesin
B
5’
3’
Chl1 ->
DNA structures
resolved, Scc2 and
cohesins loaded
Cohesion established
Eco1

Rudra_Suppl. Fig 1

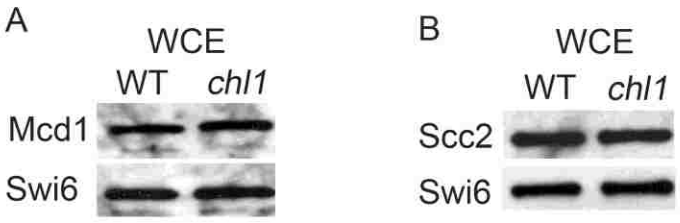


FIGURE LEGENDS

Figure 1. Chl1 expression and chromatin binding are induced during S-phase

A) Logarithmically growing cells expressing Chl1-13Myc (YBS 1129) were harvested and analyzed for chromatin binding with or without MMS exposure. Immunoblots show whole cells extracts (WCE), cytoplasmic fractions (Cyt) and chromatin bound fractions (CB). Histone 2B (H2B) and Phosphoglycerate Kinase (PGK) were probed in parallel as positive controls for chromatin-bound and cytoplasmic proteins, respectively. B) Logarithmically growing cells expressing Chl1-13MYC were synchronized in G1 (alpha factor) and released into fresh medium. Samples were harvested every 15minutes and analyzed by Immunoblotting for Chl1-13MYC and Swi6 as a loading control. Parallel blots were also analyzed for Histone 2B (H2B) and Phosphoglyerinkinase (PGK) to confirm chromatin and cytoplasmic fractions, respectively. C) G1 synchronized cells expressing Chl1-13Myc were released in fresh medium and samples collected every 15 minutes, processed for chromatin binding and probed to detect Chl1-13MYC and Swi6 (loading control). D) Data shown are Chl1 protein levels relative to Swi6 (blue line) and chromatin enrichment relative to Swi6 (red line) over the cell cycle averaged from 3 independent experiments. Shaded portion denotes S-phase. E) Flow cytometric data for cells analyzed in B) and C).

Figure 2. Chl1, but not Fen1, decreases Eco1-mediated Smc3 acetylation without inhibiting Eco1 auto-acetylation

A) Logarithmically growing wild type (YSR 132), *chl1* (YSR 133) and *fen1* (YSR 107) mutant cells expressing Smc3-3HA were harvested and whole cell extracts processed to detect Smc3 and acetylated Smc3. While cell extracts of cells expressing untagged Smc3 also shown. B) Quantification of acetylated Smc3 levels in *chl1* and *fen1* mutant cells compared to that of wildtype cells (normalized to 100%). Data represent the ratio of acetylated Smc3 to total Smc3 levels from three independent experiments. C) Immunoprecipitated Eco1-18MYC from log phase

wildtype (YSR 129) and *chl1* (YSR 131) mutant cells probed for total Eco1 (MYC) and auto-acetylated (Ac) levels. Cells expressing untagged Eco1 (UT) (YSR 130) also shown. Data represent the ratio of acetylated Eco1 to total Eco1 levels from three independent experiments.

Figure 3. Cells lacking Chl1, but not Fen1, exhibit reduced binding of cohesins to chromatin

A) Logarithmically growing wild type (YBS 1157) and *chl1* (YBS 1175) expressing Mcd1-6HA processed for Mcd1 chromatin binding. Whole cell extracts (WCE), Cytoplasmic fractions (Cyt) and Chromatin bound fractions (CB) shown. Histone 2B (H2B) and Phosphoglycerate kinase (PGK) shown as controls for cytoplasmic and chromatin-bound proteins, respectively. B) Quantification of Mcd1 binding to chromatin in *chl1* mutant cells compared to wildtype levels (normalized to 100%). Mcd1 enrichment to DNA based on the ratio of Mcd1-6HA to Histone 2B levels obtained from 3 independent experiments. C and D) Experimental analysis of *fen1* mutant cells (YSR 117) identical to that shown in A and B for *chl1* mutant cells. E) Enrichment of Mcd1-6HA in *chl1* mutant cells at five independent chromosome arm CAR sites along Chromosome III compared to levels obtained from wild type cells (normalized to 100%). F) Enrichment of Mcd1-6HA as shown in E) except for five centromere (*CEN*) sites.

Figure 4. Chl1 regulates cohesin enrichment onto chromosomes during S-phase

A-C) Three panels show Mcd1-6HA binding to three chromosome arm CAR sites in wild type (YBS 1157) (blue lines) and *chl1* mutant cells (YBS 1175) (red lines) progressing through the cell cycle from G1 synchronous release. D, E) Two panels show Mcd1-6HA binding to two unique centromere CAR sites in wild type and *chl1* cells progressing through the cell cycle from G1 synchronous release. F) Flow cytometric data shows DNA content of wild type and *chl1* mutant cells. In all panels, data reflects aliquots harvested at 10-minute intervals. Panels A-E reflect data averaged from three independent experiments. Shaded portion denotes S-phase.

Figure 5. Chl1 regulates the binding of Scc2 to chromatin

A) Logarithmically growing wild type (YSR 135) and *chl1* (YSR 138) expressing Scc2-3HA processed for Scc2 chromatin binding. Whole cell extracts (WCE), Cytoplasmic fractions (Cyt) and Chromatin bound fractions (CB) shown. Histone 2B (H2B) and Phosphoglycerate kinase (PGK) shown as controls for cytoplasmic and chromatin-bound proteins, respectively. B) Quantification of Scc2 binding to chromatin in *chl1* mutant cells compared to wildtype levels (normalized to 100%). Scc2-3HA enrichment calculated from 3 independent experiments. C) Enrichment of Scc3-3HA in *chl1* mutant cells at five independent chromosome arm CAR sites along Chromosome III compared to levels obtained from wild type cells (normalized to 100%). D) Enrichment of Scc2-3HA as shown in C) except for five sites that map across centromere III (*CEN*).

Figure 6. Chl1 regulates Scc2 binding onto chromosomes specifically during S-phase

A-C) Three panels show Scc2-3HA binding to three chromosome arm CAR sites in wild type (YSR 135) (blue lines) and *chl1* mutant cells (YSR 138) (red lines) progressing through the cell cycle from G1 synchronous release. D, E) Two panels show Scc2-3HA binding onto two unique centromere CAR sites in wild type and *chl1* cells progressing through the cell cycle from G1 synchronous release. F) Flow cytometric data shows DNA content of wild type and *chl1* mutant cells. In all panels, data reflect aliquots harvested at 10-minute intervals. Panels A-E reflect data averaged from three independent experiments. Shaded portion denotes S-phase.

Figure 7. Model regarding Chl1 role during cohesion establishment

A) Cohesins (green) that associate with DNA during G1 are highly dynamic. During S-phase, these G1-loaded cohesins are bumped off (red arrow) by the DNA replication fork (not shown) and fail to participate in cohesion establishment. In the absence of Chl1 DNA helicase, secondary DNA structures (forked structures with RNA primers in red; G-quadruplexes or G4 in blue with looped DNA) form immediately behind the DNA replication fork and preclude the stable association to DNA of both Scc2 and cohesin – leading to loss of sister chromatid cohesion. B) In

the presence of Chl1 DNA helicase, secondary DNA structures are resolved and allow for recruitment of both Scc2 (yellow arrow) and cohesin (green arrow). Cohesin recruitment specifically during S-phase, and subsequent acetylation (red) by Eco1 (purple arrow) during S-phase, result in establishment of sister chromatid tethering. Speculative conformation shown of cohesin-association to DNA through Mcd1 capping of SMC complex [reviewed in 4], that differentiates stable cohesin-binding from the highly labile cohesin association that occurs during G1 (see text for details).

Supplemental Figure Legend S1

Whole cells extracts show identical Mcd1 and Scc2 levels in wild type (YBS 1157, YSR 135) and *chl1* cells (YBS 1175, YSR 138). Wild type and *chl1* cells expressing Mcd1-3HA and Scc2-3HA were lysed and a portion whole cell lysates were solubilized with Laemmli buffer for immunoblotting and the rest processed for chromatin binding. A) Immunoblots of whole cells extracts of wild type and *chl1*cells expressing Mcd1-3HA. b) Immunoblots of whole cell extracts of wild type and *chl1* cells expressing Scc2-3HA.

EXPERIMENTAL PROCEDURES

Media and strains: *Saccharomyces cerevisiae* strains and growth media are as described in reference (Skibbens, 2004) and listed in Table 1. Strain constructions and primer sequences are included as Supplemental information.

Chromatin binding assay: Logarithmic growing cells were harvested and processed for chromatin binding assay as previously described (Moldovan et al., 2006) with the following modifications. Briefly, culture densities were normalized (0.4-0.5 OD_{600}, 50ml) and harvested in 25ml CB1 buffer (50mM Sodium citrate, 40mM EDTA, 1.2M sorbitol, pH 7.4), washed with distilled water and 25ml 1.2M sorbitol. The cells were pelleted by centrifugation (1800 rpms for 5 minutes) and resuspended in 1.125ml of CB1 buffer to which was added 125ul of spheroplast mix (125ul CB1, 50ul zymolase, 5ul BME) and then incubated with gentle shaking for 1 hour at room temperature. Spheroplast efficiency was monitored thereafter every 10 minutes until 95% cell lysis was achieved upon exposure to 10% SDS. Spheroplasts were washed 2X with 1.2M sorbitol, resuspended in 425ul of 1.2M sorbitol and snap-frozen in liquid N2. Frozen samples were supplemented with protease inhibitor cocktail (Sigma), thawed on ice and 50ul lysis buffer (500mM Lithium acetate, 20mM MgSO4, 200mM HEPES, pH 7.9) and 20ul of 25% Triton-X-100 added and gently mixed. Cell lysis was monitored by microscopy to achieve 90-95% lysis. WCE fractions were collected and solubilized with 2X Laemmli buffer (Sigma). The remaining lysate was centrifuged at 12,000g for 15 minutes. Supernatant consisting of non-chromatin bound fraction was collected and solubilized with 2X Laemmli buffer (Sigma). The pellet was re-suspended in Lysis buffer + 150mM NaCl and centrifuged at 12,000g for 15 minutes. 2 Units of DNase I (Roche), 5mM MgSO4, and protease inhibitor cocktail was added to the resuspended pellet and incubated at 4°C for 1 hour to release chromatin-bound proteins. The resulting sample was centrifuged at 14,000g for 5 minutes and the supernatant containing released chromatin-bound proteins solubilized with 2X Laemmli buffer (Sigma). Whole cell extract, cytoplasmic and chromatin bound fractions were resolved by SDS-PAGE electrophoresis and analyzed by Western blot using anti-MYC 9E10 (1:1000) (Santa Cruz), anti-HA (1:500) (f7) in combination with

goat anti mouse HRP (1:10,000) (Bio-Rad) or by anti-Histone 2B (1:2000) (Santa Cruz) in combination with goat anti Rabbit HRP (1:10,000) or by anti-phosphoglycerate kinase (Invitrogen) in combination with goat anti mouse HRP (1:10,000) (Bio-Rad) and ECL plus (GE healthcare) for visualization.

Acetylation assay: Cells were processed for acetylation assays as described (Unal et al., 2008) with the following modifications. Briefly, logarithmically growing cells expressing Smc3-3HA were harvested, suspended in IPH150 buffer (50mM TRIS, 150mM NaCl, 5mM EDTA, 0,5% IGEPAL, 10mM Sodium Butyrate, 1mM DTT, pH8) and protease inhibitor cocktails (Sigma). 500µl of glass beads (Biospec) were added and cells snap-frozen in liquid nitrogen. Cells were then thawed on ice, lysed using mechanical lysis (Biospec mini bead Beater), briefly centrifuged and clarified extract incubated overnight with EZ view anti-HA affinity gel (Sigma). The bead-protein complexes were washed with IPH50 buffer (50mM TRIS, 50mM NaCl, 5mM EDTA, 0.5% IGEPAL, 10mM Sodium Butyrate, 1mM DTT, pH8) to remove unbound or weakly-associated proteins prior to centrifugation at 10,500rpms (TOMY). The bead-bound proteins were solubilized with 2X Laemmli buffer (Sigma) and analyzed by SDS-PAGE Western blot using anti-HA (1:2000) (F7, Santa Cruz) in combination with goat anti mouse HRP (1:10,000) (Bio-Rad) or anti-acetylated Lysine antibody (1:2000) (ST1027, Calbiochem) in combination with goat anti Rabbit HRP (1:15,000) and ECL-Prime (GE Healthcare).

Chromatin Immunoprecipitation and CHIP primers: Cells were processed for chromatin immunoprecipitation as described (Kogut et al., 2009) with the following modifications. Cells expressing Scc1-6HA were treated with 1% formaldehyde for 2 hours at room temperature to crosslink protein-DNA complexes. Cells were then harvested by centrifugation and resuspended in HEPES/Sorbitol buffer (20mM HEPES, 1.2M Sorbitol, 0.5mM PMSF, 2mg Zymolase (Seikagaku)) and incubated at 30°C for 30 minutes to spheroplast cells. Spheroplasts were washed several times and resuspended in Lysis buffer (1% SDS, 10mM EDTA, 0.5mM EGTA, 10mM HEPES, protease inhibitor cocktail). Lysed cells were sonicated on ice for 6 cycles of 10 seconds. The suspension was centrifuged at 15000 rpm (TOMY) at 4°C and the supernatant diluted with IP buffer (0.01% SDS, 1.1% Triton-X-100, 1.2mM EDTA, 16.7mM TRIS, pH 8.1,

167mM NaCl). The suspension was then centrifuged at 8400g for 10 minutes and the supernatant collected as the chromatin solution. The chromatin solution was incubated with anti-HA EZ view affinity gel (Sigma) overnight, protein-bound bead complexes washed with TSE 150 buffer (0.1% SDS, 1% Triton-X-100, 2mM EDTA, 20mM TRIS-HCl, 150mM NaCl, pH 8.1) and formaldehyde crosslinks reversed by incubating with 5M NaCl at 65°C for 4 hours. DNA from the resulting sample was extracted using Phenol-Chloroform-Isoamyl alcohol, precipitated with EtOH and resuspended. Extracted DNAs were amplified by PCR prior to analysis by Agarose gel electrophoresis (1.5% agarose). Chromatin enrichment was quantified in the following manner. Band intensities from DNA gels were obtained using Photoshop CS5. To calculate enrichment for each CAR site within each strain, ChiP band intensity (minus background intensity obtained from GST non-specific control) was divided by input intensity (minus background intensity obtained from GST non-specific control) for each time point. The resulting *chl1*/wildtype ChIP ratios shown represent averaged data obtained from three independent experiments. Primer co-ordinates for the chosen CAR sites on chromosome III are as follows: Arm 1 (Primer pairs DK-EU-25 and DK-EU-26, SGD co-ordinates 194137 to 194479), Arm 2 (Primer pairs DK-EU-29 and DK-EU-29 and DK-EU-30, SGD co-ordinates 195996 to 196386), Arm 3 (Primer pairs DK-EU-33 and DK-EU-34, SGD co-ordinates 198380 to 198762), Arm 4 (Primer pairs *MAT36F* and *MAT36R,* SGD co-ordinates 191257 to 191599), Arm 5 (Primer pairs *MAT37F* and *MAT37R*, SGD co-ordinates 191778 to 192108). *CEN1* (Primer pairs *CEN3L5F* and *CEN3L5R*, SGD co-ordinates 99171 to 99460), *CEN2* (Primer pairs *CEN3L3F* and *CEN3L3R*, SGD co-ordinates 108724 to 109020), *CEN3* (Primer pairs *CEN3R7F* and *CEN3R7R*, SGD co-ordinates 139784 to 140099), *CEN4* (Primer pairs PM80 and PM81, SGD co-ordinates 114795 to 115011), CEN5 (Primer pairs PM84 and PM85, SGD co-ordinates 115323 to 115582).

Strain constructions

To construct *fen1*::*KANr* cells, PCR fragments were generated using primers 5'-CGA TGA AAA GCG TTG ACA GCA TAC ATT GGA AAG AAA TAG CGG ATC CCC GGG TTA ATT AA-3' and 5'- CAA GGT GAA GGA CCA AAA GAA GAA AGT GAA AAA AGA ACC CCC GAA

TTC GAG CTC GTT TAA AC-3' and pFA6a-kanMX6 (Longtine et al., 1998b). The resulting PCR product was transformed into YBS1157. *FEN1::KANr* was confirmed by PCR using primers 5'-GGT GAC TTT CGT TAA TGG GGA-3' and 5'-GCA AAC GAA TTA CAG CCA GTG-3'.

To construct *SMC3-HA* cells, PCR fragments were generated using primers 5'- GGT TAT TGA GGT CAA TAG AGA AGA AGC AAT CGG ATT CAT TAG AGG TAG CAA TAA ATT CGC TGA ACG GAT CCC CGG GTT AAT TAA -3' and 5'- TTT AGG TAA GAA GAA GCC AAG TGG TGG ATT TGC ATC ATT AAT AAA AGA TAT TTC AAG AAA AGA ATT CGA GCT CGT TTA AAC -3' on pFA6a-3HA-*TRP1* (Longtine et al., 1998b). The PCR product was transformed into YBS1019. Smc3-HA was confirmed by western blotting and PCR analysis using the primers 5'-GCG GCT CGA GAT TCT TGT TCA ATC GTT GTA ACT CAG C -3' and 5'- AAC TGC ATG GAG ATG AGT GGT-3'.

To construct *SCC2-3HA* cells, PCR fragments were generated using primers 5'-TCA AAT GGC AAG CTT CTT ACA TAT TTT AGA AAA CAG GTG AAG GAT ACG CGG ATC CCC GGG TTA ATT AA-3' and 5'-CAG CAT GGA AAA TGC AAA TGC AAA ATG ATT ATT AAT ACT ATG TAT ATT GAA TTC GAG CTC GTT TAA AC-3' pFA6a-3HA-TRP1 (Longtine et al., 1998b) and transformed into YBS1019. Scc2-HA was confirmed by western blotting and PCR analysis using the primers 5'- TCA AAT GGC AAG CTT CTT ACA TAT TTT AGA AAA CAG GTG-3' and 5'- AAC TGC ATG GAG ATG AGT GGT-3'.

To construct *chl1::HIS3* cells, PCR fragments were generated using the primers 5'- GTA GAA AAC CAG GCT AAA AAC AGT CAC ACT AGT CCA AAA AAC GGA TCC CCG GGT TAA TTA A-3' and 5'-ATA TAG TAG TAA TCA CAG TAT ACA GGT AAA CGT ATT CCT TGA ATT CGA GCT CGT TTA AAA C-3' on p-FA5a-His3MX6 (Longtine et al., 1998a) and transformed into YSR132 and YSR135. *CHL1::HIS3* was confirmed using primers 5'-TGC CTG GCT GAC TTC TTA GAC-3' and 5'-CGT GAG CAA ACA ACG GGT AAT-3'. To construct *fen1::HIS3* cells, PCR fragments were generated using primers 5'-CGA TGA AAA GCG TTG ACA GCA TAC ATT GGA AAG AAA TAG CGG ATC CCC GGG TTA ATT AA-3' and 5'- CAA GGT GAA GGA CCA AAA GAA GAA AGT GAA AAA AGA ACC CCC GAA TTC GAG CTC GTT TAA AC-3' and p-FA5a-His3MX6 (Longtine et al., 1998b). The resulting PCR product was transformed into YBS132.

FEN1::HIS3 was confirmed by PCR using primers 5'-GGT GAC TTT CGT TAA TGG GGA-3' and 5'-GCA AAC GAA TTA CAG CCA GTG-3'.

Primer designations used for CAR sites for centromeres and arm sites along chromosome III

Arm 1 (Primer pairs DK-EU-25 and DK-EU-26, SGD co-ordinates 194137 to 194479), Arm 2 (Primer pairs DK-EU-29 and DK-EU-29 and DK-EU-30, SGD co-ordinates 195996 to 196386), Arm 3 (Primer pairs DK-EU-33 and DK-EU-34, SGD co-ordinates 198380 to 198762), Arm 4 (Primer pairs *MAT36F* and *MAT36R,* SGD co-ordinates 191257 to 191599), Arm 5 (Primer pairs *MAT37F* and *MAT37R*, SGD co-ordinates 191778 to 192108). *CEN1* (Primer pairs *CEN3L5F* and *CEN3L5R*, SGD co-ordinates 99171 to 99460), *CEN2* (Primer pairs *CEN3L3F* and *CEN3L3R*, SGD co-ordinates 108724 to 109020), *CEN3* (Primer pairs *CEN3R7F* and *CEN3R7R*, SGD co-ordinates 139784 to 140099), *CEN4* (Primer pairs PM80 and PM81, SGD co-ordinates 114795 to 115011), CEN5 (Primer pairs PM84 and PM85, SGD co-ordinates 115323 to 115582).

Table 1: Strains used in this study

All strains are of S288C background except where noted (* are W303 strains).

Strains	Genotype
YBS 1019	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52*
YBS 1020	*MATalpha ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52*
YBS 1129	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 CHL1:13Myc:URA3*
YBS 1157	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 MCD1:6HA:TRP1*
YBS 1175	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 SCC1:6HA:TRP1CHL1::KAN^r*
YSR 132	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 SMC3:3HA:TRP1*
YSR 133	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 SMC3:3HA:TRP1 CHL1::HIS3*
YSR 107	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 SMC3:3HA:TRP1 FEN1::HIS3*
YSR 129	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 ECO1::HIS3 ECO1:18MYC:LEU2*
YSR 130	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 ECO1::HIS3 ECO1:LEU2*
YSR 131	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 ECO1::HIS3 ECO1:18MYC:LEU2 CHL1::TRP1*
YSR 117	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 MCD1:6HA:TRP1 FEN1::KAN^r*
*YSR 135	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 SCC2:3HA:TRP1*
*YSR 138	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 SCC2:3HA:TRP1 CHL1::HIS3*

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Chapter 4

Chl1 helicase regulates condensin enrichment to chromatin in *Saccharomyces cerevisiae*

Abstract

Successful transmission of replicated genomes from mother to progeny daughter cells requires newly replicated sister chromatids to be tethered together and re-organized into compact structures that can be segregated by mitotic spindle microtubules. A group of protein complexes called cohesins maintain the identity of sister chromatids from the time of DNA replication during S phase until chromosome segregation at anaphase onset during mitosis. A related set of multi-protein complexes called condensins play a central role in compacting the genetic material, the timing of which parallels that of cohesion. Previous studies reveal that cohesin subunits (Mcd1) and cohesin regulators (Eco1, Pds5, Rad61) are also critical for DNA condensation, however little is known about the mechanisms through which cohesion pathways impact chromosome condensation. Recently, I identified Chl1 as a regulator of both cohesin and Scc2 deposition onto DNA. Importantly, my results show that Chl1 functions specifically during S phase such that *chl1* mutant cells are defective in Scc2 and cohesin enrichment during DNA replication. However, *chl1* mutant cells are fully competent to promote cohesin enrichment onto DNA during G1. It remains unknown whether this early loaded population of cohesin is sufficient to support condensation. The current study investigates the role of Chl1 in DNA condensation to more fully understand the temporal impact of cohesin on condensation. Here, I present data that Scc2 and Chl1 regulate condensin enrichment on chromatin. Although previous studies show that Eco1 and Mcd1 both play a role in DNA condensation, my data reveal that cells harboring mutations in *ECO1* or *MCD1* do not affect the levels of chromatin bound condensins. This suggests a regulatory role for condensin deposition by Scc2 and Chl1 and reveals a novel “condensin without condensation” phenotype for cells harboring mutations in Eco1 and Mcd1.

Introduction:

Structural maintenance of chromosome (SMC) proteins are ubiquitous and conserved from bacteria to higher eukaryotes. The SMC family of proteins plays an integral role in various cellular processes ranging from sister chromatid cohesion, DNA condensation, DNA repair and recombination, gene dosage compensation and transcriptional regulation. SMC molecules show a highly conserved structure. All SMC proteins (Smc1, Smc2, Smc3, Smc4, Smc5, Smc6 and Rad50) are folded in half at a central hinge region forming a coiled coil region and an ATPase head region. SMC molecules associate with each other through a head-head and hinge-hinge interaction. In addition to the core SMC molecules, two other evoolutionary conserved proteins remain closely associated with the head regions of the SMC molecules (Rudra and Skibbens, 2013b).

DNA condensation is mediated by condensin complex consisting of two subunits of the SMC protein family Smc2 and Smc4, the Kleisin component Brm1 (budding yeast)/CAP-H,CAP-H2(vertebrates) and two additional proteins Ycs4 (Budding yeast)/CAP-D2,CAP-D3 (Vertebrates) and Ycg1 (Budding yeast)/ CAP-G, CAP-G2 (vertebrates)(Anderson et al., 2002; Cuylen et al., 2011; Ono et al., 2003; Yeong et al., 2003). Inactivation of any one of the five subunits in yeast or depletion of the Condensin subunits in worms, flies or vertebrate cells cause DNA condensation defects and severe chromosome segregation anomalies leading to a failure in resolving chromosome arms during Anaphase (Hirano, 2012a; Hudson et al., 2009).

Previous studies provide evidence that mutation in *MCD1, ECO1 and PDS5* produce not only sister chromatid cohesion defects but also chromosome condensation defects (D'Ambrosio et al., 2008; Guacci et al., 1994; Guacci et al., 1997; Hartman et al., 2000; Lavoie et al., 2004; Skibbens et al., 1999). In addition, the Cohesin loading complex protein Scc2 promotes condensin association with chromosomes (D'Ambrosio et al. 2008). A recent study reveals that cells lacking the sister chromatid cohesion regulator Rad61/Wapl display increased chromosome condensation (Lopez-Serra et al., 2013). Furthermore cell viability of *eco1*-null cells is rescued by *RAD61* deletion by virtue of a rescue in the condensation defect observed in cells lacking *ECO1*

(Guacci and Koshland, 2012*)*. Taken together these studies reveal that cohesin subunits and their regulators play a crucial role in DNA condensation. Although condensins have been studied extensively over the last decade, current understanding of the molecular mechanisms behind the process of chromosome condensation remains limited. I previously identified an S-phase specific role for Chl1 DNA helicase in both Cohesin and Scc2 chromatin enrichment (Rudra and Skibbens, 2013a). I therefore investigated whether Chl1 also plays a role in Condensin enrichment onto DNA. The data reveal that cells lacking Chl1 are deficient in condensin enrichment onto DNA. However, *chl1 rad61* double mutants failed to rescue condensin enrichment in *chl1* cells. I show further that mutations in *ECO1* and *MCD1* does not perturb condensin enrichment on chromatin revealing a "condensin without condensation" akin to classic phenotypes observed in cohesins. Taken together my research reveals that cohesins and their regulators facilitate DNA condensation through different mechanisms.

Results

Chl1, but not Eco1 or Mcd1, regulate condensin-chromatin enrichment

Previous studies from our lab show that Chl1 plays a crucial role in both Scc2 and cohesin DNA-enrichment during S phase (Rudra and Skibbens, 2013a). We posited that Chl1 helps resolve secondary DNA structures (forks, flaps and/or G-quadruplexes) that arise during Okazaki fragment maturation which otherwise would preclude S phase Scc2 and cohesin deposition. Since Scc2 promotes functional condensin deposition (D'Ambrosio et al., 2008), we decided to test whether Chl1 also is required for efficient condensin enrichment to DNA (Moldovan et al., 2006; Rudra and Skibbens, 2013a; Toth et al., 1999). Briefly, Logarithmically growing wild type, *chl1* and scc2-4 cells expressing HA epitope tagged Smc2 were synchronized in G1 (alpha factor) at 23^0C and released into fresh medium at 37^0C to inactivate *scc2-4*, *eco1-1* and *mcd1-1* temperature sensitive mutant cells. Synchronized G1 cultures were released in fresh medium and cell aliquots harvested after 30 minutes and, then processed for Triton-X-100 insolubility fractionation. Enrichment of Histone 2B was used as a positive control for chromatin bound proteins and to calculate the relative enrichment of Smc2 on chromatin. As expected, condensin enrichment onto DNA was robust in wildtype cells but markedly decreased in *scc2-4* mutant cells. The results show that *chl1* mutant cells are similarly defective in Smc2 enrichment to DNA (~60-65%) (Figure 1A, B).

Cells lacking Rad61 show hypercondensed chromosomes (Serra-Lopez et al., 2013). Furthermore, *RAD61* deletion rescues *ECO1* null cells by virtue of a rescue in DNA condensation defects (Guacci and Koshland, 2012). The remarkable rescue of *rad61 eco1* cells could be the result off *rad61* mediated hypercondensation balancing *eco1* mediated hypo-condensation defects. If this is true, Rad61 could also rescue chl1 mediated decrease in condensin enrichment. To test this possibility, I investigated the effect of Rad61 on condensin enrichment to chromatin by employing previously described triton-X-100 cell fractionation assays. Our results show an increase in condensin enrichment to chromatin in *rad61* cells consistent with previous studies indicating that *rad61* cells show hypercondensed chromosomes, however, *rad61 chl1* cells do not

show a rescue in the decreased levels of chromatin bound condensins in *chl1* cells. (Figure A, B). Whole cell lysates show identical levels of Smc2 in all the cell types used.

Eco1 and Mcd1 both promote chromosome condensation (Guacci et al., 1994; Guacci et al., 1997; Skibbens et al., 1999). I hypothesized that this defect might be a cause of decreased levels of chromatin bound condensins. To test this idea, I analyzed the levels of chromatin bound Condensins in *mcd1-1* and *eco1-1* cells. However, my results reveal that while *chl1* cells show a decrease in chromatin bound condensins, levels of chromatin bound Condensins in *mcd1-1* and *eco1-1* cells remain identical to wild type cells (Figure 2A, B). Taken together, this suggests that condensin loading is not sufficient to engender DNA condensation and complex regulatory mechanisms facilitating chromosome condensation through cohesins and their regulators exist.

Discussion

Sister chromatid cohesion and chromosome condensation are crucial to ensure faithful segregation of the genetic material during cell division. Recent years have witnessed remarkable advances towards a comprehensive understanding of the function of both these processes. For instance, several studies reveal that cohesin and cohesion regulators are critical for DNA condensation. However the molecular basis of functions in condensation remains elusive. Condensins interact with DNA at specific regions possibly through a topological embrace of DNA, akin to cohesin complexes (Piazza et al., 2013). Such an interaction could mediate condensation through long range chromosome interactions , either by the sequential entrapment of two DNA strands by one condensin ring complex or two or several DNA strands entrapped by a dimer or multimers of condensin complexes (Petrova et al., 2013; Piazza et al., 2013). Studies from our lab suggest that sister chromatid cohesion establishment takes place in the wake of the replication fork, temporally and spatially linking cohesion to and DNA condensation reactions (Rudra and Skibbens, 2013a). Early models posit that cohesin subunits like Mcd1 link the core components of cohesin and condensins after DNA replication (Guacci et al., 1997). The cohesin loading complex subunit Scc2 also plays a crucial role in the deposition of functional condensins (Lopez-Serra et al., 2013). These studies allow us to speculate on a mechanistic overlap between the regulatory mechanisms behind cohesion and condensation. In this study, my data shows that Chl1 regulates the levels of chromatin bound condensins, similar to Scc2. Deletion of RAD61 rescues *eco1* mediated condensation defects, but does not rescue the decrease in chromatin bound Condensins in *chl1* cells (Guacci and Koshland, 2012, this study). In fact my data shows that Rad61 deletion increases the levels of chromatin bound condensins, consistent with previous reports that *rad61* cells show hyper-condensed chromosomes.

Previously, both Mcd1 and Eco1 were shown to play a role in DNA condensation (Guacci et al., 1997; Skibbens et al., 1999). However, I show that Mcd1 and Eco1 do not regulate condensin enrichment to chromatin. Mutant *mcd1* and *eco1* appears to display a “condensin without condensation” defect”. It appears that Chl1 and Scc2 regulate Condensin deposition,

whereas Eco1, Mcd1, Rad61 and possibly Pds5 play a separate role in mediating DNA condensation through an unknown mechanism. We posit that Chl1 plays a crucial role in resolving secondary structures which allow both Cohesins and Condensins to bind to DNA substrates, possibly through the role of Scc2. Cohesin subunits and regulators play an undetermined but crucial role in mediating Condensation between DNA loops. When mutated Cohesins and their regulators do not perturb Condensin deposition but somehow interfere with the mechanism of DNA compaction mediated by Condensins (Figure 3). While there is a wealth of information regarding the structural basis of condensins, little is known regarding DNA condensation. A systematic exploration of condesins using genetic, biochemical and single molecule analysis tools will be invaluable to understand the mechanisms behind the function of this conserved class of molecules.

In recent years, mutations in cohesins and cohesion regulators have been mapped to several developmental maladies such as Roberts Syndrome or Cornelia de Lange syndrome characterized by growth and mental retardation, severe limb reduction and numerous cranio facial abnormalities (Hirano, 2012b; Musio and Krantz, 2010; Rudra and Skibbens, 2013b). These disease states are referred to as “cohesinopathies”. A particular subset of these disease states arise due to a defect in transcriptional regulation which is separate from a role in sister chromatid cohesion (Dorsett, 2011). Mutations in the human homolog of CHL1, DDX11 has been recently characterized with Warsaw Breakage Syndrome, a developmental disorder characterized by abnormal limb growth, facial features and cells showing massive chromosome mis-segregation and defects in DNA repair (van der Lelij et al., 2010a; van der Lelij et al., 2010b).

It is evident from recent research that the functions of condensins are not limited to chromosome condensation and chromosome segregation. Subtle changes in condensin functions cause cell or tissue specific defects. Recent screens for essential genes required for the viability of mouse embryonic stem cells (ES) identified Smc2 and Smc4 genes. The same study also showed that condensin depleted ES cells lead to epigenetic modifications and proper condensin levels and functions are crucial for ES cells to maintain their developmental plasticity (Fazzio and Panning, 2010). A mutation in the condensin subunit CAPH2 in mice causes defects in T cell

development and a failure in normal immune response (Gosling et al., 2007). Recent studies show that the ability of MCPH1 (one of the eight gene products leading to human microcephaly) to shape metaphase chromosomes plays a vital role in the prevention of microcephaly (Hirano, 2012b). Condensins have also been linked to genome instability and cancer (Ham et al., 2007). Clearly, both cohesins and condensins play a vital role in regulating not only efficient genome stability and segregation but also the precise regulation of myriad cellular processes. Future research dedicated to understand the regulatory mechanisms behind DNA condensation and sister chromatid cohesion and how these processes overlap will be crucial to understand the etiology of diseases involving these important cellular processes.

Figure 1

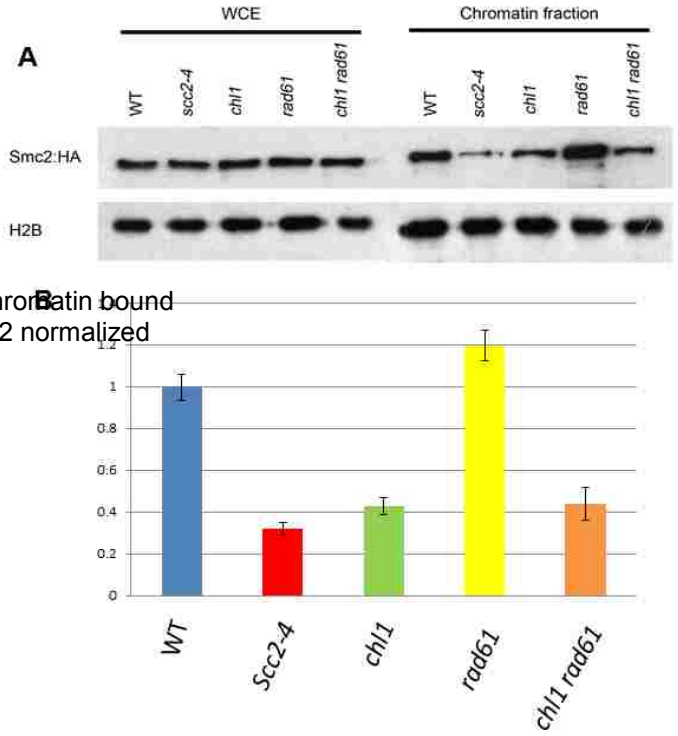
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Figure 2

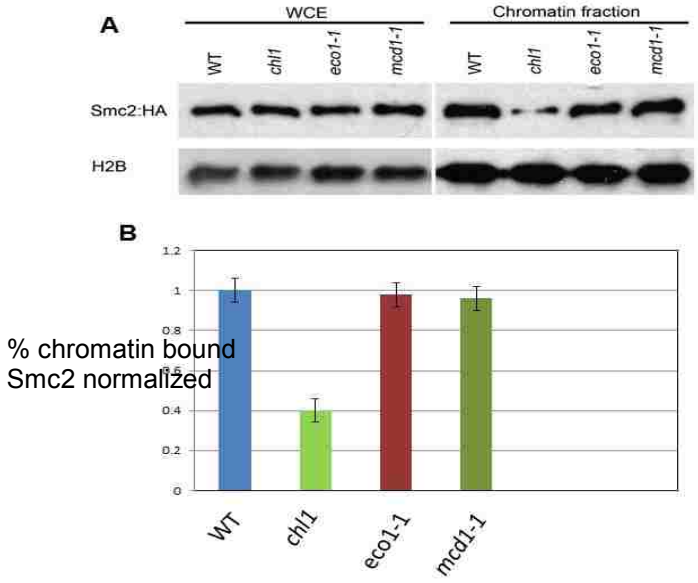
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Figure 3

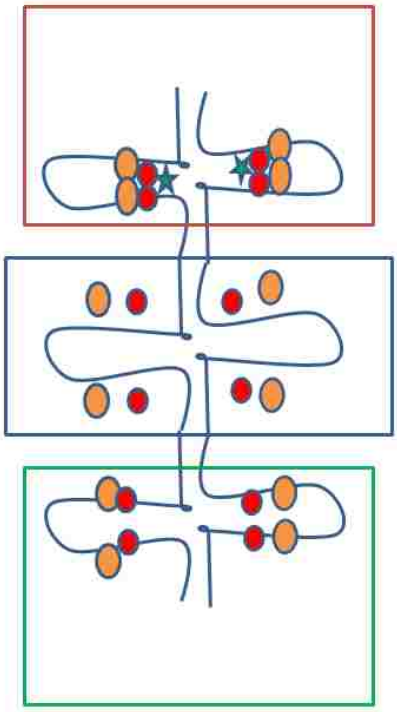

Figure Legends

Figure 1: A) Logartihmically growing wild type, *chl1, scc2-4, rad61,* and *chl1 rad61* cells expressing Smc2-3HA were synchronized at G1 with alpha factor at 23^{o}C and then released into fresh media at 37^{o}C. Samples were harvested after 30minutes of release from Alpha factor arrest and processed for Smc2-3HA chromatin binding. Whole cell extracts (WCE) and Chromatin bound fractions are shown with Histone 2B as a positive control. B) Quantification of Smc2-3HA binding to chromatin in *chl1, scc2-4, rad61,* and *chl1 rad61* cells compared to wild type cells (normalized to 100%). Smc2-3HA chromatin enrichment based on the ratio of Smc2-3HA chromatin enrichment and Histone 2B chromatin enrichment obtained from 2 identical experiments.

Figure 2: A) Logartihmically growing wild type, *chl1, eco1-1* and *mcd1-1* cells expressing Smc2-3HA were synchronized at G1 with alpha factor at 23^{o}C and then released into fresh media at 37^{o}C. Samples were harvested after 30minutes of release from Alpha factor arrest and processed for Smc2-3HA chromatin binding. Whole cell extracts (WCE) and Chromatin bound fractions are shown with Histone 2B as a positive control. B) Quantification of Smc2-3HA binding to chromatin in *chl1, eco1-1 a*nd *mcd1-1* cells compared to wild type cells (normalized to 100%). Smc2-3HA chromatin enrichment based on the ratio of Smc2-3HA chromatin enrichment and Histone 2B chromatin enrichment obtained from 2 identical experiments.

Figure 3: Model of DNA condensation: The red rectangle showing wild type Cohesins and their regulators (red ovals) along with Eco1 (Green star) are loaded properly on DNA sites and mediate DNA condensation by interacting with Condensin subunits (Orange ovals). Blue rectangle showing cells without Chl1 (Not shown), cause Cohesins and Condensins to be improperly loaded onto chromosomes and consequently defective sister chromatid cohesion and possibly condensation. Green rectangle showing cells with mutant Cohesin subunits or regulators where both Cohesins and Condensins occupy sites on DNA but are defective in their function.

MATERIALS AND METHODS

Media and strains: *Saccharomyces cerevisiae* strains and growth media are as described in reference (Skibbens, 2004) and listed in Table 1.

To construct *SMC2-3HA* cells, PCR fragments were generated using primers 5'- GGT TAT TGA GGT CAA TAG AGA AGA AGC AAT CGG ATT CAT TAG AGG TAG CAA TAA ATT CGC TGA ACG GAT CCC CGG GTT AAT TAA -3' and 5'- TTT AGG TAA GAA GAA GCC AAG TGG TGG ATT TGC ATC ATT AAT AAA AGA TAT TTC AAG AAA AGA ATT CGA GCT CGT TTA AAC -3' on pFA6a-3HA-*TRP1* (Longtine et al., 1998b). The PCR product was transformed into YBS1019. Smc2-HA was confirmed by western blotting and PCR analysis using the primers 5'-GCG GCT CGA GAT TCT TGT TCA ATC GTT GTA ACT CAG C -3' and 5'- AAC TGC ATG GAG ATG AGT GGT-3'.

Chromatin binding assay: Logarithmic growing cells were harvested and processed for chromatin binding assay as previously described (Rudra and Skibbens, 2013a) with the following modifications. Briefly, culture densities were normalized (0.4-0.5 OD_{600}, 50ml) and harvested in 25ml CB1 buffer (50mM Sodium citrate, 40mM EDTA, 1.2M sorbitol, pH 7.4), washed with distilled water and 25ml 1.2M sorbitol. The cells were pelleted by centrifugation (1800 rpms for 5 minutes) and resuspended in 1.125ml of CB1 buffer to which was added 125ul of spheroplast mix (125ul CB1, 50ul zymolase, 5ul BME) and then incubated with gentle shaking for 1 hour at room temperature. Spheroplast efficiency was monitored thereafter every 10 minutes until 95% cell lysis was achieved upon exposure to 10% SDS. Spheroplasts were washed 2X with 1.2M sorbitol, resuspended in 425ul of 1.2M sorbitol and snap-frozen in liquid N2. Frozen samples were supplemented with protease inhibitor cocktail (Sigma), thawed on ice and 50ul lysis buffer (500mM Lithium acetate, 20mM MgSO4, 200mM HEPES, pH 7.9) and 20ul of 25% Triton-X-100 added and gently mixed. Cell lysis was monitored by microscopy to achieve 90-95% lysis. WCE fractions were collected and solubilized with 2X Laemmli buffer (Sigma). The remaining lysate was centrifuged at 12,000g for 15 minutes. Supernatant consisting of non-chromatin bound fraction was collected and solubilized with 2X Laemmli buffer (Sigma). The pellet was re-

suspended in Lysis buffer + 150mM NaCl and centrifuged at 12,000g for 15 minutes. 2 Units of DNase I (Roche), 5mM MgSO4, and protease inhibitor cocktail was added to the resuspended pellet and incubated at 4°C for 1 hour to release chromatin-bound proteins. The resulting sample was centrifuged at 14,000g for 5 minutes and the supernatant containing released chromatin-bound proteins solubilized with 2X Laemmli buffer (Sigma). Whole cell extract, cytoplasmic and chromatin bound fractions were resolved by SDS-PAGE electrophoresis and analyzed by Western blot using anti-MYC 9E10 (1:1000) (Santa Cruz), anti-HA (1:500) (f7) in combination with goat anti mouse HRP (1:10,000) (Bio-Rad) or by anti-Histone 2B (1:2000) (Santa Cruz) in combination with goat anti Rabbit HRP (1:10,000) or by anti-phosphoglycerate kinase (Invitrogen) in combination with goat anti mouse HRP (1:10,000) (Bio-Rad) and ECL plus (GE healthcare) for visualization.

Table 1:

Strains	Genotype
YSR 139	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 SMC2:3HA:TRP1*
YBS 140	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 SMC2:3HA:TRP1 chl1::HIS3*
YBS 141	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 SMC2:3HA:TRP1scc2-4*
YBS 142	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 SMC2:3HA:TRP1rad61:URA3*
YBS 143	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 SMC2:3HA:TRP1 chl1::HIS3 rad61::URA3*
*YSR 144	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 SMC2:3HA:TRP1 eco1-1*
*YSR 145	*MATa ade2-101 his3Δ200 leu2 lys2-801 trp1Δ63 ura3-52 SMC2:3HA:TRP1mcd1-1*

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Chapter 5

Conclusions and future directions

Conclusions:

One of the most crucial roles of cell division is to ensure the segregation of replicated sister chromatids formed during S phase into the newly formed daughter cells during mitosis. To accomplish this important task, a conserved protein complex known as cohesins tether the sister chromatids together from the time of DNA replication till anaphase onset. The last decade has witnessed numerous important advances made in the field of sister chromatid cohesion, however crucial questions regarding the molecular mechanisms dictating this important cellular process still remain unanswered. My studies have profoundly influenced the chromosome segregation field by challenging current models that cohesion establishment requires only the DNA replication to pass through pre-loaded cohesins and that Eco1 function is to induce a conformational change in pre-fork cohesins which allows for fork passage (Chan et al., 2013; Sherwood et al., 2010; Terret et al., 2009).

In chapter 1, I provide evidence regarding the exact location where Eco1 acts with respect to the replication fork. Prior to this, no convincing evidence supports the notion that Eco1 translocates with the replication fork (Bernard et al., 2008; Gause et al., 2010; Lengronne et al., 2006; Skibbens, 2011). My research reveals novel genetic and physical interactions between Eco1 and the well characterized Okazaki fragment maturation flap endonuclease Fen1. My data also reveal genetic and physical interactions between Fen1 and the Chl1 helicase, which was previously identified as a protein that interacted with Eco1 and played a role in sister chromatid cohesion. Using Fen1 as a fiduciary marker to which both Eco1 and Chl1 associate, I posited that cohesion establishment takes place in the wake of the replication fork in conjunction with Okazaki fragment maturation events. I further suggested that the function of Eco1 is transient and specific in the local environment behind the replication fork. This model obviates the requirement of Eco1 being stably recruited by any factor and translocating with the replication fork and instead suggests that it interacts with newly deposited cohesin behind the replication fork (Rudra and Skibbens, 2012b). The interactions between Fen1 and Chl1 also raises the possibility that Chl1 promotes Okazaki fragment maturation and/or resolves secondary DNA structures arising behind

the replication fork to facilitate cohesion establishment. This latter function for Chl1 is further explored in Chapter 2.

How does Chl1 promote sister chromatid cohesion? In chapter 2, I carefully analyze Chl1 expression and chromatin binding. The results show that Chl1 is tightly regulated throughout the cell cycle with both protein expression and chromatin binding peaking during S phase. Contrary to previous reports that DNA damage may be necessary for Chl1 recruitment to chromatin (Ogiwara et al. 2007), my results show that Chl1 binds to chromatin irrespective of DNA damage and only requires active DNA replication. Together, these results reveal that Chl1 expression and chromatin enrichment is regulated throughout the cell cycle with peak expression levels and Chl1 chromatin enrichment during S phase. In order to understand the role of Chl1 in cohesion establishment, I tested whether Chl1 regulates Eco1 mediated acetylation. Using the well characterized acetylation assay, I showed that cells lacking Chl1 indeed contain decreased levels of Smc3 acetylation – despite having normal levels of Eco1 enzymatic activity. This suggests that Smc3 availability as an Eco1 substrate is reduced in *chl1* mutant cells. Given prior evidence that Eco1 acetylates only chromatin-bound cohesins, I tested whether cohesin-enrichment to DNA is reduced in the absence of Chl1. Using Triton-X-100 cell fractionation assays and chromatin immunoprecipitation on specific CAR sites, I show that cohesin binding to DNA is reduced in chl1 mutant cells – revealing that Chl1 regulates the enrichment of cohesins on chromatin. Kinetic analysis further revealed that Chl1-depedent cohesin-chromatin enrichment occurs specifically during S-phase. In an attempt to understand how Chl1 regulates cohesin-chromatin binding, I studied the enrichment of Scc2 loading complex in *chl1* mutants. Cell fractionation and chromatin precipitation studies revealed that Chl1 regulates Scc2 chromatin enrichment on specific CAR sites specifically during S phase. In combination, these findings reveal that both cohesin deposition and cohesion establishment takes place during the S phase and in the local environment behind the replication fork. Based on prior studies that Chl1 DNA helicase is capable of displacing proteins from DNA and also resolve secondary DNA structures, I hypothesize that Chl1 helps resolve secondary structures like forks, flaps and G quadruplexes that arise behind the replication fork that, if unresolved, would preclude the loading onto DNA of

Scc2 (and consequently cohesins). Based on the fact that new histone deposition, chromatinization and DNA condensation also takes place behind the replication fork – I further suggest that these processes are functionally and temporally regulated (Figure 1) (Rudra and Skibbens, 2013a).

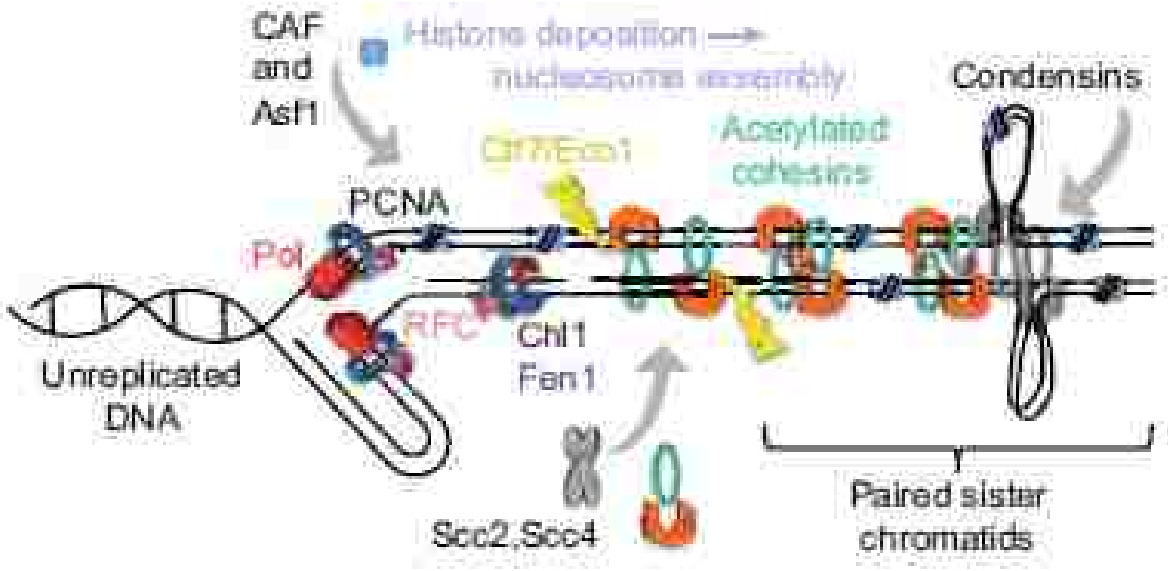

Figure 1: Cohesion establishment probably occurs immediately behind the replication fork. The core enzymes of the replisome (leading and lagging strand polymerases, i.e. DNA polymerases coupled to PCNA) move to the left (lagging strand at the bottom). Histone deposition and chaperone factors (CAF and Asf1) interact with PCNA (Corpet and Almouzni, 2009; Franco et al., 2005) to promote nucleosome assembly (blue barrels) on nascent DNA strands, prviding a context in which cohesion establishment is likely to occur. In the context of continued maturation (RFC, PCNA, Chl1 and Fen1) of the nascent sister chromatids, Eco1 (yellow) acetylates (red star) chromatin bound Smc3. Cohesins, Mcd1 (orange), Smc1 and Smc3 (green) and Eco1 also promote condensation (illustrated by the looping DNA structures at the far right). Histone chaperones or deposition factors interact with PCNA and RFC, which suggests that cohesion establishment and chromosome condensation are co-ordinated with chromatinization (Corpet and Almouzni, 2009; Franco et al., 2005; Rolef Ben-Shahar et al., 2009). Here, cohesin that

participated in cohesion is deposited by Scc2 and Scc4 in the context of replicated sister chromatids (behind DNA polymerase) (Rudra and Skibbens, 2013b).

To what extent does Chl1 DNA helicase impact chromatin metabolism? In the 3rd chapter, I investigated the role of Chl1 in condensin enrichment on chromatin. This endeavor was prompted by previous studies that Chl1 is critical for Scc2 and cohesin enrichment to DNA - both of which are required for proper chromosome condensation (Lopez-Serra et al., 2013; Rudra and Skibbens, 2013a). I next asked to what extent Chl1 is role in chromosome condensation dependent on other chromosome condensation pathways. To explore this question, I included in my analyses *eco1* and *mcd1* mutant cells, both of which were previously shown to play a role in DNA condensation (Guacci et al., 1997; Hartman et al., 2000; Skibbens et al., 1999). I also included *rad61* mutant cells, which display hyper-condensed chromosomes (Serra-Lopez et al. 2012). Intriguingly, *rad61* deletion rescue cell viability in cells lacking *eco1* – showing that this balance in condensation is essential (Guacci and Koshland, 2012). My results show that *rad61* cells have increased levels of chromatin bound condensins compared to wild type. However deletion of *RAD61* in *chl1* cells does not rescue the decreased levels of condensins on chromatin suggesting that the role of Chl1 in regulating condensin enrichment is different from Rad61. I also show that cells with temperature sensitive *mcd1-1* and *eco1-1* do not affect chromatin bound condensins at non-permissible temperatures. These cells therefore show a "condensin without condensation" defect suggesting a novel and discreet step required to regulate DNA condensation which is separate from condensin loading to chromatin. Taken together, my results reveal a novel role for Chl1 in regulating condensin enrichment on chromatin and suggest that cohesin subunits and their regulators play a role in the stepwise recruitment and subsequent activation of condensins in promoting DNA condensation independent of condensin deposition.

My Ph.D. thesis research sheds new light on the crucial process of sister chromatid cohesion establishment. New models proposed based on my research raise the intriguing possibility of a regulatory and mechanistic overlap between sister chromatid cohesion,

chromatinization and DNA condensation. Below I summarize the future directions of my thesis research.

Future directions

Role of Chl1 DNA condensation

My research so far raises the possibility that sister chromatid cohesion is temporally and functionally linked to chromatinization and DNA condensation. I show that Chl1 regulates condensin enrichment on chromatin, possibly through Scc2 recruitment However it remains unknown whether the reduction in condensin enrichment to DNA in chl1 mutant cells falls below the threshold required for proper chromosome condensation. To test whether *chl1* cells have a DNA condensation defect I shall employ previously characterized condensation assays in budding yeast (Lopez-Serra et al. 2013). The condensation status can be analyzed by visualizing the budding yeast rDNA by fusing the rDNA protein Net1 with GFP. The rDNA serves as an excellent marker for the condensation status of chromosomes as it reflects the action of condensins at both repetitive and unique sites (Guacci et al., 1994; Lavoie et al., 2002; Lavoie et al., 2004). Previous studies show that in G1, rDNA regions are maximally separated and adopt a disordered puffy morphology. In contrast the distance between euchromatin regions in rDNA in early M phase is minimized and the rDNA adopts a distinct loop or line like structure (Guacci et al., 1994; Lopez-Serra et al., 2013). Wild type cells and *chl1* cells will be synchronized in G1 with alpha factor and then released into fresh medium containing Nocodazole to arrest the cells in M phase. The rDNA architecture of wild type and *chl1* cells in G1 and M phase will be visualized and the DNA condensation status analyzed. Rad61 has been previously shown to play a role in DNA condensation and deletion of *RAD61* rescues *eco1* mediated condensation defects (Lopez-Serra et al., 2013). If *chl1* cells show a DNA condensation defect, I shall analyze DNA condensation in *chl1 rad61* double mutants to test whether *rad61* can rescue *chl1* mediated DNA condensation defects.

These experiments can have several possibilities: 1) Cells lacking Chl1 might show DNA condensation defects at both the rDNA as well as chromosomal loci. 2) Cells lacking Chl1 might show condensation defects at either the rDNA loci or the chromosomal loci, but not both. This scenario would provide an intriguing possibility of differential function of Chl1 in DNA condensation at specific chromosomal loci. 3) Chl1 might play no detectable role in DNA condensation despite the finding that chl1 mutant cells adversely impacting condensin enrichment on chromatin. This possibility is interesting because it will indicate that merely loading condensins is not enough to mediate DNA condensation and it suggests a separate regulatory role necessary for DNA condensation.

Role of Chl1 in chromatin architecture

My research suggests that Chl1 plays a role in maintaining chromatin architecture and that this architecture is critical for both sister chromatid cohesion and DNA condensation. It is tempting to speculate that Chl1 plays a role in histone deposition, nucleosome assembly or modification. To test this hypothesis, I will use the well-established Micrococcal nuclease digestion assay. Micrococcal nuclease preferentially cleaves the linker DNA between individual nucleosomes and can be used to determine the approximate positions of nucleosomes in a region of DNA. Briefly, isolated nuclei from wild type and chl1 cells will be treated with micrococcal nuclease and the DNA purified and analyzed by Southern blot. Nucleosome positioning in wild type and *chl1* can be analyzed using restriction enzyme end labeling or ligand mediated PCR (LM-PCR) methods.

My research revealed that Chl1 regulates S phase specific chromatin enrichment of Scc2 as well as cohesins in the wake of the replication fork (Rudra and Skibbens, 2012b; Rudra and Skibbens, 2013a). I also show that Chl1 regulates chromatin bound condensins. To test whether Chl1 plays a role in chromatin organization in the CAR regions or at replication origins, Southern blot analysis of DNA purified from nuclease digested chromatin using specific PCR probes to detect the DNA region at CAR sites, specific condensin binding sites and replication origins. I expect a number of possibilities from the above experiments: 1) Cells lacking Chl1

reveal altered nucleosome spacing and chromatin architecture at specific CAR sites, condensin sites and replication origins. This would validate my hypothesis that Chl1 alters chromatin architecture to facilitate cohesin and condensin deposition in the wake of the replication fork. 2) Mutant *chl1* cells show altered nucleosome positioning at CAR sites but not at condensin binding sites. 3) *chl1* cells show altered nucleosome positioning at condensin binding sites but not at CAR sites. Both these possibilities will reveal important regulatory information on how Chl1 regulates cohesin and condensin enrichment to chromatin and whether its role is dependent on its role in chromatin architecture 4) Cells lacking *chl1* do not alter nucleosome positioning or chromatin architecture. This result will indicate that chl1 regulates cohesin and condensin binding without perturbing chromatin architecture.

If Chl1 plays a role in the formation of a conducive chromatin architecture behind the replication fork, which is required to recruit Scc2, cohesins, condensins, it may also regulate the deposition of new histones in the wake of the replication fork. Newly deposited histones during DNA replication are characterized by epigenetic modifications (methylations and acetylations) (Franco and Kaufman, 2004). I shall employ Chromatin immunoprecipitation assays using antibodies specific to histone epigenetic marks and primers targeting histone enriched regions to test for replication coupled histone deposition in *chl1* and wild type cells. These experiments will validate the hypothesis that Chl1 plays a role in the deposition of new histones behind the replication fork.. Synthetic lethality between *CHL1* and the histone deposition factor *ASF1* can also be tested to lend further support to the idea that Chl1 plays a role in histone deposition.

Role of Chl1 in cohesin and condensin complex structure and dynamics

The molecular mechanisms of cohesin and condensin loading are still poorly understood. Cohesins are loaded with the help of the Scc2/4 loading complex during the G1 phase, however functional cohesin deposition and establishment takes place during S phase. In budding yeast, condensins are bound to chromosomes in interphase and their levels and distribution remain relatively unaltered throughought the cell cycle. The mechanisms dictating loading and the dynamics of both the cohesin and condensin complexes throughout the cell cycle

are poorly understood. One popular model of cohesin loading suggests that the opening of the cohesin "ring" at the hinge interface allows the entry of DNA and a separate "exit gate" exists at the SMC-kleisin interface that is blocked by Eco1 mediated acetylation during cohesion establishment (Chan et al., 2012). Condensins are also thought to interact with the DNA through a topological embrace (Piazza et al., 2013). Single molecule FRET experiments on cohesin subunits in wild type and *chl1* cells can be used to test these models and understand the role of Chl1 in cohesin complex dynamics. Using CFP and YFP fluorophore fusions of budding yeast cohesin proteins (at the hinge region and head regions) expressed from their genomic loci, the interaction between cohesin subunits and their dynamics throughout the cell cycle can be ascertained in the presence and absence of Chl1. If cohesin loading entails ring opening reactions at the hinge interface, I should be able to observe a loss of FRET signals between fluorophores at the hinge interface. Since Chl1 regulates cohesin enrichment to chromatin, it is possible, that Chl1 regulates the dynamics of hinge opening reactions. Single molecule FRET analysis on wild type and *chl1* cells will test this hypothesis.

The same experiments using condensin subunits will shed new light on how condensin subunits are loaded on chromatin and how Chl1 plays a role in condensin deposition. These experiments will allow me to investigate the detailed mechanisms of cohesin and condensin loading. If single molecule FRET analyses reveal that cohesin deposition does not require hinge region opening, then loading probably occurs through a separate mechanism. If on the other hand, these experiments detect the role of hinge region opening during cohesin and condensin loading but Chl1 does not alter FRET signals, it suggests that an alternate mechanism of cohesin and condensin loading exists.

Role of Eco1 mediated acetylation on chromatinization

If sister chromatid cohesion establishment is a necessary condition for chromatinization as predicted from my hypothesis, and if sister chromatid cohesion is measured by Eco1 mediated cohesin acetylation, then cohesin mutants refractile to Eco1 mediated acetylation and ECO1

mutants should also have DNA aberrant histone deposition. Based on the behavior of Smc3 mutant alleles in cohesion and DNA condensation, we had posited a cohesin code which dictates various cellular processes according to the sequence and order of cohesin modifications (Rudra and Skibbens, 2013a). Studies from the Koshland lab show that Eco1 mediated Smc3 acetylation on Lysine 113 (K113) are crucial for DNA condensation and acetylation of K112 is not essential for cell viability but essential for efficient cohesion establishment (Guacci and Koshland, 2012). Different Smc3 alleles could similarly affect histone deposition and chromatin assembly. MNase digestion assays using various Smc3 alleles (Smc3 K112R, Smc3112Q, Smc3 K112R, K113Q) would provide important new evidence on the role of Eco1 dependent Smc3 acetylation on histone deposition and chromatin assembly.

Role of Eco1 on Meiosis and sporulation

While studying the genetic interactions between *ECO1* and *FEN1*, I observed that the heterozygous diploid *ECO1*$^{eco1\text{-}1}$*/ECO1 FEN1/fen1* exhibited extremely poor sporulation frequency (<2%). When diploid cells were transformed with plasmids containing a wild type ECO1, the resulting transformants sporulated with high frequency (~85%). Similar haplo-insuffeciency phenotypes in *ECO1* mutant crosses were previously observed in our lab (Brands and Skibbens, unpublished data). This suggests an essential dose dependent role for Eco1 in meiosis. So far, the role of Eco1 in Meiosis is uncharacterized. ESCO2, the mammalian homolog of Eco1 was found to co-localize with a gamma histone 2A family member and regions of double stranded breaks in male mice spermatocytes and female mice ovaries. Taken together these reports suggest that ESCO2 play a vital role in both male and female meiosis in mice (Evans et al., 2012). In Arabidopsis, atCTF7 (Eco1 homolog) is required for sister chromatid cohesion during meiosis (Bolanos-Villegas et al., 2013; Singh et al., 2013).

To study the role of Eco1 in budding yeast meiosis, live cell imaging and genetic analyses can be utilized. Live cell imaging of chromosomes undergoing meiosis in *eco1-1* temperature sensitive mutants using meiotic stage specific markers should help identify the role

of Eco1 in meiosis. If Eco1 plays a role downstream of pre-meiotic DNA replication, co-localization with specific proteins involved in synapse formation, cross over, homologous recombination and chaisma formation in various stages of Prophase I and visualizing chromosome structures will help elucidate the role of Eco1 in meiosis and sporulation in budding yeast. These experiments will be important to understand the role of Eco1 mammalian homologs in meiosis in both male and females.

Role of Chl1 in DNA damage repair

Although previous reports suggest a role for Chl1 in DNA repair, there is no conclusive evidence to support the notion (Ogiwara et al. Das et al.). To test the role of Chl1 in DNA repair, I will use the previously characterized break induced replication (BIR) assay (Lydeard et al., 2007). This assay uses a GAL induced HO endonuclease cut site cloned at a non-essential site on a particular chromosome. Galactose induced HO expression creates a double stranded break at the HO cut site which is then repaired through homologous recombination through the break induced replication repair pathway. DNA repair can then be monitored using primers designed to amplify the region of repaired DNA. Efficiency of DNA repair can be monitored by the percentage of PCR product produced as a function of time after the induced double stranded break. Using this assay I will be able to assess the efficiency of double stranded break repair in *chl1* cells compared to wild type cells. If *chl1* cells reveal impaired double stranded break repair, the mechanisms through which Chl1 facilitates DNA repair can be studied. I will test synthetic lethality between *CHL1* and genes previously characterized in BIR repair (MCM10, CTF4, RAD51, DPB11) (Lydeard et al., 2007). These genetic interactions will uncover the mechanisms through which Chl1 contributes to DNA repair.

The *CHL1* gene consists of 6 conserved helicase domains and a conserved Fe-S cluster domain that are important for the biochemical activities of its human homologues *in vitro* (Wu and Brosh, 2012b). In addition to this *CHL1* contains a PIP box region, crucial to bind PCNA (Bylund and Burgers, 2005). However the biological significance of these conserved regions *in vivo* remain uncharacterized. I shall use site directed mutagenesis to create *chl1* alleles with mutations

in the conserved helicase domains, Fe-S domains and the PIP box regions. Each allele shall be tested for their ability to repair DNA using the above mentioned BIR repair assay and their role in sister chromatid cohesion using the widely used TetR-GFP-TetO based cohesion assay. Mutant *chl1* alleles may be deficient in either DNA repair or sister chromatid cohesion or both. These experiments might unravel a novel separation of function for Chl1. On one hand, I can find that the helicases activity of Chl1 is dispensable for its role in sister chromatid cohesion and it merely acts as a scaffolding protein to mediate protein-protein interactions. Alternatively, Chl1 helicase activity may be indirectly related to the process of cohesion establishment possibly by modifying the DNA template or processing recombination intermediates also exists. There might also be a separation of function for Chl1 in its role in DNA damage repair. In the possibility that all the *chl1* alleles are equally important for sister chromatid cohesion and DNA repair, it suggests a possibility that Chl1 might lie at the interface of sister chromatid cohesion and DNA repair. This possibility also suggests that Chl1 must have a redundant role with another helicase. *CHL1* can be tested for synthetic lethality with other known non-essential DNA helicases (*SGS1*, *SRS2, RRM3*, *PIF1*) to test this possibility. These experiments will provide novel insights to the interplay of DNA helicases in various aspects of DNA metabolism and the process of sister chromatid cohesion.

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1)**Rudra S,** Skibbens RV (2013) **Chl1 DNA helicase regulates Scc2 deposition specifically during DNA-replication in *Saccharomyces cerevisiae.*** Plos One 8(9): e75435.

2) **Rudra S,** Skibbens RV (2013*) **Cohesin codes - interpreting chromatin architecture and the many facets of cohesin function.*** J Cell Sci 126: 31-41.

3)**Rudra S**, Skibbens RV (2012) ***Sister chromatid cohesion establishment occurs in concert with lagging strand synthesis.*** Cell Cycle 11: 2114-2121.

Conference Abstracts

1) **Rudra S,** Skibbens RV (2013)***Chl1 helicase regulates cohesin and Scc2 chromatin binding during S phase.*** Abstract accepted for poster presentation at EMBO workshop on chromosome segregation and aneuploidy, Breukelen, Netherlands, 2013.

2) **Rudra S,** Skibbens RV (2012) ***Cohesion establishment and lagging strand replication.*** Abstract accepted for poster presentation at Cold Spring Harbor cell cycle meeting.

3) **Rudra S** , Skibbens RV (2011) ***The Chl1 helicase, Fen1 flap endonuclease and the establishment factor Ctf7/Eco1 interact together to perform a role in cohesion establishment.*** Abstract accepted for poster presentation at American Assiociation of Cell Biology conference, Denver

Other presentations and talks

1) Poster presentation at Biological sciences open house, Lehigh University, 2008.
2) Poster presentation at Cold spring harbor, yeast cell biology meeting, August 2009.
3) Poster presentation at Biological sciences open house, Lehigh University, 2009.
4) Poster presentation at Biological sciences open house, Lehigh University, 2010.
5) Talk entitled “The case of the helicase, Chl1 and sister chromatid cohesion.” At the graduate student colloquium, October, 2011.
6) Talk entitled, “Role of Chl1 in sister chromatid cohesion”, September 2012, Nemes Fellowship Seminar, Lehigh University.
7) Talk Entitled, Chl1 helicase regulates Cohesin and Scc2 binding in S phase, February 2013. Nemes fellowship seminar, Lehigh University.

Teaching and Mentoring experience

Teaching assistantship, Biochemistry lab (September-December 2009,2011, 2012, September- Present, 2013)
Mentored and taught a class of 10-12 students in Biochemistry lab techniques and guided through short term independent projects.

Honors and Awards

1. Award for academic excellence for the year 2004, Ramaiah College, Bangalore University, India. Awarded for exemplary academic performance.

2. Award for academic excellence for the year 2006, Ramaiah College, Bangalore University, India. Awarded for exemplary academic performance.

3. Lehigh University fellowship Award for the academic year 2007-2008.

4. Estes Fellowship award, Lehigh University, Biological Sciences for summer 2008. For outstanding academic and research performance.

5. Lehigh University Fellowship Award for the academic year 2008-2009.

6. Nemes Fellowship award, Lehigh University, Biological Sciences, Spring- Summer 2012. Awarded for outstanding research merit.

7. Nemes Fellowship award, Lehigh University, Biological Sciences, Spring-Summer 2013. Awarded for outstanding research merit.

8. EMBO travel grant award for EMBO workshop on chromosome segregation and aneuploidy, June 2013.

References:

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