CHARACTERIZATION OF THE COHESIN ACETYLTRANSFERASE ECO1 AND ITS ROLE IN NUCLEAR FUNCTIONS

By

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FUNCTIONAL CHARACTERIZATION OF COHESIN ACETYLTRANSFERASE ECO1 AND ITS ROLE IN CELL PHYSIOLOGY

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ABSTRACT

Sister-chromatid cohesion plays a vital role in precise chromosome segregation and genome stability. This process is carried out by the cohesin complex and its associated proteins. In addition, a growing body of evidence suggests that the cohesin complex affects other processes, including gene transcription, DNA damage repair, and DNA replication. One piece of evidence for cohesin's role in other processes is that individuals with mutations in the cohesin complex can survive, albeit with congenital abnormalities. For example, mutations in ESCO2 cause Roberts syndrome. ESCO2 is the human homolog of Eco1 in budding yeast. Eco1 is a critical acetyltransferase for establishing cohesion during S phase and also re-establishing cohesion in G2/M phase in response to DNA damage. To identify the roles of the cohesin complex beyond chromosome segregation, we have constructed mutations in Eco1 which do not cause gross chromosome separation defect. We have shown that DNA damage repair is strongly affected in the *eco1* mutants and that a specific DNA recombination pathway is affected. Further investigation of *eco1* mutants showed transcription and DNA replication defects genome-wide. Interestingly, deletion of Fob1, a nucleolar protein required for replication fork blocking in rDNA region, corrects the genome-wide replication defects, nucleolar structure and chromosome segregation in an *eco1* mutant by allowing bidirectional replication of the rDNA. This highlights cohesin's central role at the rDNA for global control of DNA replication and gene expression. Our results demonstrate the diversity of cohesin functions and have direct implications for the etiology of human cohesinopathies.

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CHAPTER 1

INTRODUCTION

A. THE COHESIN COMPLEX

Sister-chromatid Cohesion

Robustness and accuracy of cell division requires not only faithful replication of DNA, but also precise segregation of the replicated product, the sister chromatids. Walther Fleming's observations of chromosome segregation in 1878 pointed researchers to the importance of precise chromosome segregation. Twenty-five years later, Walter Sulton and Theodore Boveri proposed the chromosome theory of inheritance, suggesting that genes segregate during chromosome segregation because chromosomes physically contain genes. However, the mechanism by which sister chromatids perfectly segregate into two daughter cells has intrigued scientists for a long time. In this chapter, I will first introduce the cohesin complex, then discuss its functions and regulation, and finally discuss how mutations of the genes in the cohesin complex contribute to human diseases.

Sister-chromatid cohesion refers to the adherence of replicated sister-chromatids. This important physical connection between two sister chromatids ensures their precise segregation into two daughter cells. Cohesion begins during S phase of the cell cycle, persists through G2 phase, and dissolves during the metaphase-

to-anaphase transition. An early hypothesis proposed that sister-chromatid cohesion was established through DNA catenation with DNA molecules topologically interlocked. However, this hypothesis was challenged by the discovery that minichromosomes that had completed replication could be separated by electrophoresis (Koshland and Hartwell, 1987). This result strongly argued that DNA catenation was removed before the onset of anaphase and therefore other forces must hold the two sister-chromatids together and regulate sister-chromatid cohesion.

Extensive research, including screening for mutations in yeast that lead to a cohesion defect, identified several genes that were involved in the mechanism of cohesion. The first gene identified was Mcd1 (mitotic chromosome determinant) (Guacci et al., 1997). Missense mutation of coding region of this gene caused sister-chromatid cohesion defects and impaired chromosome segregation. This result suggested that cohesion might be maintained by a protein. Further screening for mutants that affect cohesion indicated that multiple factors influence sister-chromatid cohesion (Losada et al., 2000; Michaelis et al., 1997; Panizza et al., 2000). It is now well recognized that sister-chromatid cohesion is regulated by a protein complex called “cohesin”, which mediates diverse chromosomal functions.

Cohesin Structure and Function

The cohesin complex is conserved from prokaryotes to humans (Schleiffer et al., 2003). In budding yeast, *Saccharomyces cerevisiae*, the cohesin complex contains four subunits: Smc1 and Smc3 (structural maintenance of chromosomes), Mcd1 (mitotic chromosome determinant) and Scc3 (sister chromatid cohesion) (Guacci et al., 1997; Haering et al., 2004). These four subunits form a ring-like structure that embraces the DNA molecules (Xiong and Gerton, 2010) (**Figure 1.1**).

Smc1 and Smc3 are two homologous ATP binding proteins that belong to the Structural Maintenance of Chromosomes family (SMC), which also includes condensin subunits (Smc2 and Smc4) and Smc5/6 complex components (Smc5 and Smc6). All SMC proteins help modulate chromosome structure and topology. SMC proteins are characterized by ~50 nanometer anti-parallel coiled coils flanked by a globular hinge domain and N and C-terminal domains. The N and C-terminal domains of SMC proteins contain conserved Walker A and Walker B ATPase, respectively. The coiled coil and hinge domains are less conserved between species compared to the N and C-terminal domain of the SMC proteins. The ATPase activity of SMC proteins is important for association of the cohesin complex with the chromosome and relocation of the cohesin ring along DNA (Hu et al., 2011).

In bacteria, a homodimer of SMC proteins forms rather than the heterodimer formed in eukaryotes (Hirano, 2005). Null mutations in SMC genes impairs the growth of bacteria at temperatures above 23^{o}C and many bacteria cells are anucleate, suggesting that the SMC complex in bacteria also play an important role in chromosome organization and segregation (Britton et al., 1998; Jensen and Shapiro, 1999). In fact, the conservation of the functions of SMC complexes is not limited to chromosome segregation. For example, SMC complexes are recruited to the DNA replication origin in bacteria and play a central role in organizing origin regions and promoting chromosome replication (Gruber and Errington, 2009; Sullivan et al., 2009). This role of SMC complexes resembles the function of SMC complexes in eukaryotes. More interestingly, the SMC complexes bind to the ribosome DNA (rDNA) locus and sites of active transcription, suggesting the conservation of additional functions from prokaryotes to eukaryotes. All of these findings are consistent with the results of my experiments on DNA replication and transcription addressed in chapter 3.

Figure 1.1

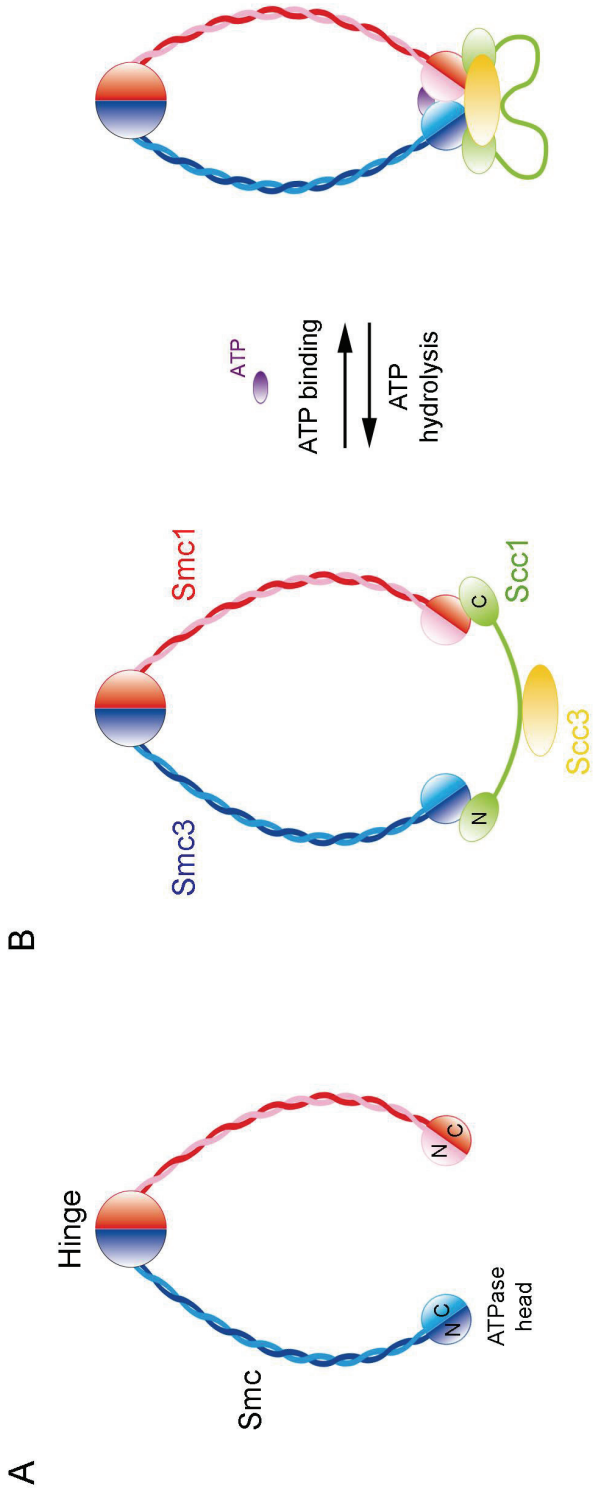

A
Hinge
Smc
N
C
N
C
ATPase
head
B
Smc1
Smc3
C
N
Scc1
Scc3
ATP
ATP binding
ATP
hydrolysis

Figure 1.1 Schematic illustration of the cohesin

(A) Schematic illustration of the cohesin SMC heterodimer. An SMC heterodimer possesses the hinge domain at one end and ATPase head domain at the other connected by a long anti-parallel coiled coil. The heterodimer forms a V-shaped structure which is about 50 nm in length. (B) The Kleisin subunit Mcd1 links the ATPase containing head domains of Smc1 and Smc3 forming a tripartite ring structure. Hydrolysis of ATP is required for cohesion and is important for opening and closing the ring from the head.

(From Xiong & Gerton, 2010)

The Mcd1 (also known as Scc1 or Rad21) subunit is a conserved protein, which belongs to the kleisin family. Kleisins are a superfamily of bacterial and eukaryotic SMC protein partners (Schleiffer et al., 2003). The N terminus of Mcd1 binds to the head domain of Smc3 while the C terminus binds to the head domain of Smc1 (Haering et al., 2002), which links the head of the SMC proteins. Electron microscopy reveals that the Smc1-Smc3 heterodimer forms a V-shaped structure (Anderson et al., 2002; Haering et al., 2002). Because Mcd1 bridges the heads of Smc1 and Smc3, the three proteins are thought to form a circular complex (Gruber et al., 2003; Haering et al., 2002; Haering et al., 2004). Mcd1 is cleaved by a protease prior to the onset of anaphase, allowing the cohesin ring to open and the chromosome to segregate (Hauf et al., 2001; Uhlmann et al., 2000). The fourth cohesin subunit, Scc3 (also known as Irr1) binds to Mcd1. Besides containing protein-protein interaction HEAT repeats motifs (Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast kinase TOR1), little is known about this subunit.

In addition to the core cohesin complex, several other proteins or protein complexes are essential to the function of cohesin complexes. First, accessory proteins, which include Pds5 and Wapl (also known as Rad61), are important for the maintenance of sister-chromatid cohesion. Second, the cohesin loading complex, which contains Scc2 (known as NIPBL in mammalian cells) and Scc4, are important for loading of the core cohesin complex onto chromosomes. Finally, an acetyltransferase named Eco1 (ESCO1 and ESCO2 in mammalian cells) is

important for establishing cohesion in S phase. All these proteins will be described in the next chapter for the detailed mechanism of sister-chromatid cohesion regulation. Cohesion regulation includes sister-chromatid cohesion establishment and maintenance. Establishment of sister-chromatid cohesion is the process of making the core cohesin complex competent to physically bind sister chromatids together. Maintenance of sister-chromatid cohesion is the process of keeping sister chromatids together by cohesin complex prior to the onset of anaphase.

SMC complexes

Several SMC complexes, including the condensin complex and the SMC5/6 complex, are structurally related to cohesin but serve different functions. Since the sequences these complexes are closely related to cohesin, studying these complexes helps further understand the function of cohesin complex.

The condensin complex is a conserved cohesin-related complex, which contributes to chromosome condensation and segregation. In contrast to cohesin, where research suggests that the complex encircles sister-chromatids until the cleavage of Scc1 prior to the onset of anaphase, little is known about how condensin interacts with chromosomes (Thadani et al., 2012). Higher eukaryotes have two condensin complexes: condensin I and condensin II. Condensin I is conserved from budding yeast to human, while condensin II is only found in higher eukaryotes (Hirano, 2012). The two condensin complexes have non-

overlapping functions due to different locations of the complexes. Condensin II is present in the cell nucleus during interphase and it is important for early stages of chromosome condensation in prophase, while condensin I enters the nucleus only after the nuclear envelope breaks down at the end of prophase to further organize chromosomes (Hirano, 2012).

The Smc5/6 complex plays a central role in the maintenance of genome stability. The Smc5/6 complex is required for sister chromatid recombination, but it has not been studied as extensively as the cohesin and condensing complexes (De Piccoli et al., 2006). The Smc5/6 complex is enriched at regulatory region of ribosomal DNA (rDNA) and plays a central role in segregation of repetitive sequences by promoting the resolution of recombination structures in the ribosomal rDNA region (Torres-Rosell et al., 2005).

In addition to condensin complex and Smc5/6 complex mentioned above, there are other cohesin related complexes that are comprised of SMC proteins, such as the MRN complex which is important for initial processing of DNA double-strand breaks and the condensin-like dosage compensation complex which is involved in dosage compensation, a genetic regulatory mechanism that operates to equalize the phenotypic expression. In general, all these complexes share SMC subunits and function in chromosome regulation.

B. REGULATION OF COHESIN

As mentioned above, besides four core cohesin subunits, several accessory factors are needed throughout the cell cycle for regulation of sister-chromatid cohesion. Proper cohesion function includes four elements: cohesin loading onto chromosomes in G1 phase, cohesion establishment during S phase, maintenance of cohesion during G2/M phase, and dissolution of the cohesin ring during the transition from G2/M phase to anaphase (**Figure 1.2**). These functions are essential for precise chromosome segregation. Besides contributing to chromosome segregation, cohesin serves other important functions, which will be discussed in the next section of this chapter.

Cohesin loading

The cohesin complex traps DNA molecules in a process known as cohesin loading. A conserved Scc2-Scc4 complex is required to load the cohesin complex onto chromosomes during the G1 phase in budding yeast and telophase in higher eukaryotes, prior to DNA replication (Ciosk et al., 2000; Gillespie and Hirano, 2004; Takahashi et al., 2004). In budding yeast, Scc2 co-localizes with the cohesin complex on chromosomes, especially in the pericentromeric region (Kogut et al., 2009), which is also the case in the fly genome (Misulovin et al., 2008). Although the Scc2-Scc4 complex loads cohesin rings onto the DNA, the interaction between DNA and the Scc2-Scc4 complex also requires association with the cohesin complex (Fernius et al., 2013). After cohesin loads onto the

chromosomes, cohesin complexes are relocated from the sites of chromosomal loading to the other parts of the genome in an ATP hydrolysis dependent mechanism (Hu et al., 2011). However, the detailed mechanism of relocation of cohesin complexes on chromosomes is still unclear.

Figure 1.2

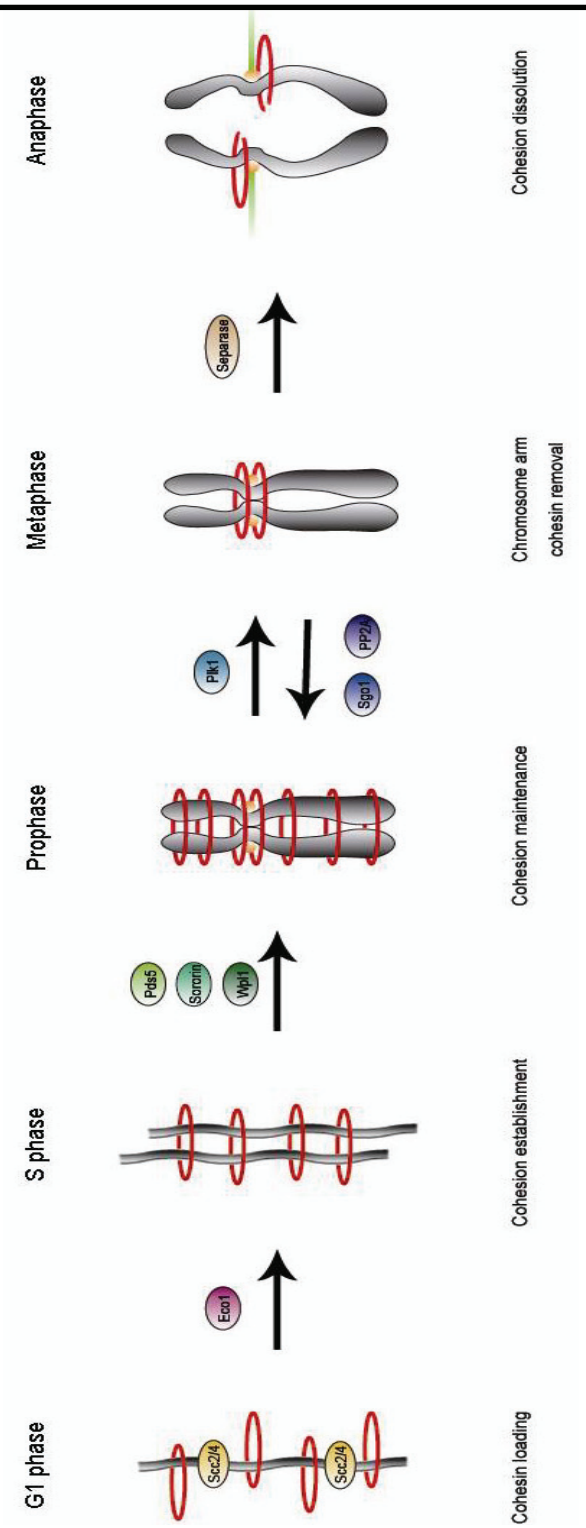

Anaphase
Cohesion dissolution
Separase
Metaphase
Chromosome arm
cohesin removal
Plk1
PP2A
Spo1
Prophase
Cohesion maintenance
Pds5
Scc3
Wpl1
S phase
Cohesion establishment
Eco1
G1 phase
Cohesin loading
Scc2/4
Scc2/4

Figure 1.2 Regulation of cohesin.

The cohesin complex is loaded onto chromatin in G1 phase by the loading complex Scc2-Scc4. During DNA replication, cohesion is established between sister chromatids in an acetyltransferase Eco1 dependent mechanism. Cohesion is maintained by the proteins Pds5 and Wpl1, during G2/M phase. In mammalian cells, arm cohesin is removed during prophase through phosphorylation of Mcd1. During the transition from metaphase to anaphase, a protease called separase cleaves the Mcd1 subunit, which opens the cohesin ring and allows the separation of sister chromatids.

(From Xiong & Gerton, 2010)

Cohesion establishment

Two of the proteins that regulate human cohesion are ESCO1 and ESCO2. Yeast Eco1, the ortholog of human ESCO1 and ESCO2, is an essential gene required to establish cohesion in yeast during S phase (Skibbens et al., 1999). The N-terminus of Eco1 contains a zinc finger domain, which helps substrate recognition (Onn et al., 2009) and the C-terminus of Eco1 contains an acetyltransferase domain which is crucial for its acetyltransferase activity (Ivanov et al., 2002).

The essential substrate of Eco1 is the cohesin subunit Smc3, which has been shown to be acetylated by Eco1 on K112 and K113 (Ben-Shahar et al., 2008; Rowland et al., 2009; Zhang et al., 2008). Mutations on residues K112 and K113 of Smc3 cause precocious sister-chromatid separation (Ben-Shahar et al., 2008), while mutations of acetyltransferase domain of Eco1 do not strongly affect sister-chromatid separation (Gard et al., 2009). It is possible that mutations with reduced acetyltransferase activity of Eco1 measured *in vitro* may retain enough catalytic activity *in vivo* to acetylate a fraction of the Smc3 pool to maintain the chromosome segregation activity (Lu et al., 2010). In fact, chromosome segregation is affected only if total cohesin levels are decreased to 13% of the wild-type cell levels (Heidinger-Pauli et al., 2010). Another possibility is that the K112 and K113 mutations in Smc3 affect more functions than establishment of sister-chromatid cohesion. Acetylation of Smc3 has been reported to promote replication fork progression and regulate the interaction between Smc3, Pds5

and Wapl (**Figure 1.2**) (Terret et al., 2009). In addition to Smc3, Eco1 has other substrates such as Mcd1 and Mps3, acetylation of which promote DNA damage repair or nuclear organization, respectively (Ghosh et al., 2012; Heidinger-Pauli et al., 2008).

Since Eco1 mostly functions in S phase, the activity of Eco1 must be controlled during the cell cycle. Eco1 is phosphorylated by Cdk1 after S phase. The phosphorylation targets Eco1 to SCF complex (Skp1, Cullin, F-box containing complex) for increased ubiquitination and subsequent degradation, thus preventing cohesion establishment after S phase (Lyons and Morgan, 2011). Eco1 also appears to bind with components of the replication factor C (RFC) complex, which enables it to travel with the replication fork in order to promote cohesion by acetylation of Smc3 (Kenna and Skibbens, 2003).

Cohesion maintenance

"Cohesion maintenance" refers to maintaining the cohesive state during G2/M phase prior to cleavage of the cohesin complex, which triggers anaphase. Several proteins play a central role in maintaining cohesion following S phase, including Pds5 and Wapl. Cohesion maintenance during G2/M requires Pds5 (Hartman et al., 2000; Panizza et al., 2000). Pds5 contains tandem HEAT repeats that function as a protein-protein interaction scaffold (Panizza et al., 2000). Pds5 binds with Mcd1 adjacent to Smc3 which promotes acetylation of Smc3 by Eco1 and prevents deacetylation of Smc3 by Hos1/HDAC8 (Chan et al.,

2013). Higher eukaryotes contain two Pds5 homologs, Pds5A and Pds5B. In contrast to the essential role of Pds5 in budding yeast, depletion of Pds5A or Pds5B only has a minor effect on sister-chromatid cohesion. This suggests that Pds5A and Pds5B have redundancy in their function in sister-chromatid cohesion (Zhang et al., 2007).

In addition to Pds5, another cohesin binding protein Wapl (also known as Wpl1 or Rad61) plays an important role in cohesion maintenance. Wapl is conserved from budding yeast to human. In budding yeast, Wapl appears to be a cohesion inhibitor since deletion of Wapl suppresses the temperature sensitivity of Eco1 acetyltransferase mutants (Ben-Shahar et al., 2008; Lu et al., 2010; Rowland et al., 2009; Sutani et al., 2009). Overexpression of Wapl has the opposite effect, leading to premature separation of sisters. In vertebrates, Wapl also negatively regulates sister-chromatid cohesion.

Overall, Pds5 and Wapl work together for the precise regulation of cohesion during the cell cycle. In budding yeast, Pds5 and Wapl form a complex to bind cohesin complex and inhibit cohesion establishment prior to S phase. Eco1 establishes cohesion in S phase by temporarily hindering the interaction of the Pds5-Wapl complex with cohesin complex through Smc3 acetylation (Sutani et al., 2009). In mammalian cells, Wapl is able to bind with Pds5 and antagonize cohesion establishment (Kueng et al., 2006). However, mammalian Wapl also forms a complex with Mcd1 and Scc3 and is important for preventing

reassociation of the proteins with the cohesin complex following their release (Gandhi et al., 2006).

Cohesion dissolution

The cohesin ring cleaves apart during the transition from G2/M phase to anaphase in a process known as “cohesion dissolution”. During this process, the protease separase Esp1 splits the kleisin subunit Mcd1. Cleavage of Mcd1 opens the cohesin ring, which allows chromosome separation (Ciosk et al., 1998; Funabiki et al., 1996a). Normally, Pds1 (also known as securin) inhibits the protease activity of Esp1. Prior to anaphase, however, Pds1 is ubiquitinated and degraded through a Cdc20 dependent pathway and Esp1 is activated (Cohen-Fix et al., 1996; Funabiki et al., 1996b; Yamamoto et al., 1996a, b). Thus, Esp1 activation at anaphase onset leads to cleavage of Mcd1, triggering the metaphase to anaphase transition. Overall, cohesion dissolution is precisely controlled during the cell cycle.

Centromeric cohesin is cleaved by a similar mechanism in mammalian cells and budding yeast cells, but the removal of cohesin on chromosome arms is different. The removal of arm cohesin in mammalian cells happens during prophase in a separase-independent mechanism which depends on polo kinase. Polo kinase phosphorylates the kleisin subunit Mcd1 and allows cohesion to dissociate. Centromeric cohesion is not dissociate at prophase but remains until the onset of anaphase because it is protected from premature dissociation by shugoshin, a

protein family crucial for centromeric cohesin protection, and the PP2A phosphatase (Kateneva and Higgins, 2009). The difference of cohesion dissocation mechanism on chromosome arms suggests a diversified cohesin regulation system.

Post-translational modifications and cohesin regulation

During the cell cycle, multiple post-translational modifications (PTMs) regulate chromosome cohesion. PTMs of the cohesin complex and its related proteins include: acetylation, phosphorylation, sumoylation, and ubiquitination. As mentioned above, acetylation promotes establishment of cohesion. Phosphorylation is critical for positive and negative regulation of cohesion. Sumoylation and ubiquitination are important for dissolution of cohesion. In this section, I will introduce the relationship between post-translational modifications and functions of cohesin complex in detail.

Acetylation and deacetylation of the cohesin complex play a crucial role in regulating cohesion establishment and cohesin removal. As mentioned above, Eco1-dependent acetylation on Smc3 disrupts the association with Pds5 and Wapl, allowing cohesion to be established in S phase (Ben-Shahar et al., 2008; Terret et al., 2009). Deacetylation of acetylated Smc3 by Hos1 (known as HDAC8 in human) is important for recycling Smc3 for the next cell cycle (Beckouet et al., 2010; Borges et al., 2010; Xiong et al., 2010). In addition to cohesion establishment, acetylation plays an important role in non-essential

cohesin functions, such as DNA damage repair. Instead of Smc3, Eco1 acetylates the kleisin subunit Mcd1 in response to a DNA double-strand break signal (Heidinger-Pauli et al., 2009).

Phosphorylation of cohesin subunits is important for the precise regulation of cohesion. As mentioned above, phosphorylation of Mcd1 by Polo kinase in mammalian cells helps remove arm cohesin. In fact, phosphorylation has a broad impact on cohesion regulation. For example, phosphorylation of cohesin subunits also regulates the function of cohesion beyond holding sister-chromatids together. In response to DNA damage, Mcd1 is phosphorylated on S83, which promotes its acetylation at S84 by Eco1, inducing cohesion establishment during G2/M phase (Heidinger-Pauli et al., 2008). In mammalian cells, Smc1 and Smc3 are phosphorylated in response to DNA damage as targets of the ATM kinase (Kim et al., 2002; Luo et al., 2008; Yazdi et al., 2002). In general, phosphorylation of cohesin subunits appears to be an important cellular DNA damage response. However, the response of the cohesin network to DNA damage is not limited to its ability to establish cohesion between sisters. Phosphorylation of Smc1 and Smc3 is important for the intra-S checkpoint activation independent of their role in cohesion establishment (Watrin and Peters, 2009).

In addition to acetylation and phosphorylation, ubiquitylation plays an important role in cohesin regulation. Besides the ubiquitylation of securin and Eco1 in budding yeast mentioned above, ubiquitin-dependent degradation regulates

ESCO2 in human cells. The expression level of ESCO2 peaks in S phase and levels are undetectable by mitosis. By treating with proteasome inhibitor MG132, van der Lelij and colleagues showed that ESCO2 was stabilized in mitotic human cells (van der Lelij et al., 2009). The proteins involved in the ESCO2 ubiquitylation have not been identified, nor have the ubiquitylated residues. Nonetheless, these results suggest that ubiquitylation of Eco1 or ESCO2 may be a general mechanism of regulating the cohesin network.

Sumoylation is another emerging field of cohesin regulation. Sumoylation of Pds5 might promote the disruption of cohesion (Stead et al., 2003). In addition, the cohesin complex itself is sumoylated. Sumoylation of the cohesin complex is an important step for cohesion establishment, in addition to acetylation of cohesin complex by Eco1 (Almedawar et al., 2012). In response to DNA damage, the kleisin family member Mcd1 is sumoylated by Mms21. The sumoylation of Mcd1 will counteract the negative regulator Wapl and help re-establish cohesion in G2/M phase (McAleenan et al., 2012; Wu et al., 2012). Overall, sumoylation of cohesin complex seems to promote cohesion and be complementary to acetylation and phosphorylation.

Combinations of post-translational modifications are often required for multiple functions of the cohesin complex. For example, acetylation and phosphorylation are associated with the S phase cohesion establishment, while phosphorylation and ubiquitination are coupled with cleavage of cohesin complex prior to

anaphase. In higher eukaryotes, the combinations of post-translational modifications are even more important for precise control of the functions of cohesin complex. There is evidence that specific modifications of cohesin play an additional role in checkpoint activation in response to DNA damage, distinct from its role in cohesion at DSB sites. This suggests that post-translational modifications of cohesin are even more precisely controlled in higher eukaryotes than in budding yeast.

C. FUNCTION OF COHESIN BEYOND SISTER-CHROMATID COHESION

The function of the Cohesin complex is not limited to holding sister-chromatids together. Several pieces of evidences suggest it has additional roles. In the remaining sections, I will discuss other functions of cohesin in cell physiology. These functions include chromosome condensation, gene transcription, DNA damage repair, and DNA replication. Impairment of these functions usually does not cause cell lethality. Importantly, disruption of these cohesin functions are thought to be the underlying causes for cohesinopathies, a group of human diseases that will be discussed in the next chapter.

Chromosome condensation

Besides mediating inter-chromosomal interaction, cohesin complex has also been reported to regulate intra-chromosomal interactions. Chromosome condensation is an example of cohesin controlling the intra-chromosomal interaction. For example, Cohesin can regulate chromosome condensation by influencing the localization of condensin in yeast (Ding et al., 2006; Hartman et al., 2000). In budding yeast, the early step of chromosome condensation may require cohesin, since budding yeast do not have a condensin II complex like higher eukaryotes for early phase chromosome compaction. Because cohesin and condensin are loaded by the same Scc2-Scc4 complex (D'Ambrosio et al., 2008), it is possible that the cohesin and condensin complexes interact with each

other. Another example of cohesin regulating chromosome condensation occurs at the ribosomal DNA (rDNA) locus. The rDNA is a tandemly repeated region of the genome where ribosomal RNA is transcribed. In budding yeast, it forms loop-like structures that are distinct from other regions of the genome. If total cohesin levels are systematically reduced to 30% of the wild-type cell levels, then the rDNA does not condense (Heidinger-Pauli et al., 2010). In mammalian cells, it is reported that deletion of Wapl caused chromosome condensation in interphase by regulating the stability of cohesin-DNA interaction (Tedeschi et al., 2013). However, the detailed mechanism of how cohesin and condensin regulate chromosome condensation differently is unclear.

Gene transcription

A role for cohesin in controlling gene expression has emerged in the past decade. The strongest argument that the cohesin complex regulates gene expression is that many cohesin mutants do not show a chromosome segregation defect, but instead a gene transcription defect. For example, cells from patients with human Cornelia de Lange syndrome (CdLS), a developmental disorder caused by point mutations in cohesin or cohesin-related genes, showed no defect in chromosome segregation (Liu and Krantz, 2008). Both human cell studies and animal models suggest that the pathology is caused by alteration of the transcriptome (Kawauchi et al., 2009; Liu et al., 2009). All cell lines from cohesinopathy patients showed dysregulated gene expression and it is correrated with phenotypic

severity (Liu et al., 2009). Overall, all evidence points to an important role of cohesin in genome-wide transcription.

Although cohesin's regulation of genome-wide transcription is widely-supported, the exact mechanism is still strongly debated. One proposed model predicts that cohesin directly regulates gene transcription. Evidence that cohesin and its associated proteins bind to regions of the genome that are transcriptionally active supports this model (Parelho et al., 2008; Wendt et al., 2008; Yan et al., 2013). These data suggest the existence of a cohesin-dependent transcription mechanism. In further support, cohesin has been reported to bind with paused RNA polymerase to regulate transcription elongation (Fay et al., 2011). Research has also shown that cohesin regulated RNA polymerase II activity across the whole genome (Schaaf et al., 2013). Cohesin is also reported to regulate the chromatin conformation at the imprinted H19-IGF2 locus in a CTCF-dependent (CCCTC binding factor) mechanism. Deletion of the Mcd1 subunit of cohesin led to increased levels of IFG2 mRNA (Nativio et al., 2009). Another study showed that cohesin controlled chromosomal cis-interactions at the IFNG locus, which affected IFN-γ expression and T-cell development (Hadjur et al., 2009). Cohesin is also reported to mediate chromatin interactions at the β-globin locus to regulate β-globin expression (Chien et al., 2011). In addition to controlling individual loci, cohesin has been shown to regulate pluripotency of embryonic stem cells by mediating longer range interactions between distal enhancers and pluripotency genes (Kagey et al., 2010). Overall, these data indicate that cohesin

regulates gene transcription by modulating chromosome conformation. However, a subset of genes are differentially expressed (upregulated or downregulated) in cohesin mutants but do not have cohesin binding (Kawauchi et al., 2009; Liu et al., 2009). It suggests that additional mechanisms by which cohesin regulates global gene transcription might exist besides directly regulating gene expression.

Another model of cohesin's global gene transcription mechanism has cohesin regulating key regulators, which will, in turn, affect the expression of hundreds of genes. One possible key regulator could be a transcription factor such as c-Myc. Cohesin can directly regulate c-Myc expression and affect the expression of c-Myc target genes (Rhodes et al., 2010). Another possible key regulator is the rDNA locus. A budding yeast model for human cohesinopathies revealed that the global gene expression signature indicates translational dysfunction. This suggests that the differential gene expression in cohesin mutants might partially due to the deficiency of translational function. Further analysis showed that the translational dysfunction was caused by deficiency of rDNA transcription (Bose et al., 2012). It is still unclear how cohesin regulates global gene transcription. My study suggests rDNA might be the key regulator for global gene transcription regulation.

DNA damage repair

Sister-chromatid cohesion plays an important role in DNA double-strand break repair. The establishment of cohesion in response to DNA damage outside of the

period of replication is called “damage-induced cohesion” (**Figure 1.3**). Damage-induced cohesion is reported to hold the sister-chromatids together not only at the break sites, but also genome-wide, thereby facilitating repair by homologous recombination between sister-chromatids.

While the phenomenon of damage-induced cohesion has been documented, the mechanism of how cohesion is re-established in response to DNA damaging signal is still an open question. Eco1 acetylates Smc3 for the establishment of cohesion. However, Mcd1 has been proposed as an alternative target for re-establishment of sister-chromatid cohesion during DNA double-strand break repair. When an HO break (an DNA double-strand break by the HO endonuclease) is induced in budding yeast, cohesion is established at the break site (Strom et al., 2004; Unal et al., 2004) and also re-established genome-wide through an Eco1 dependent mechanism (Strom et al., 2007; Unal et al., 2007). Mutation of putative target residues K84 and K210 of Mcd1 causes defect in genome-wide cohesion re-establishment in response to DNA double-strand break (DSB) during G2/M phase (Heidinger-Pauli et al., 2009). An K84Q and K210Q acetylation mimic mutant of Mcd1 bypasses the need for Eco1, indicating Eco1 acetylates Mcd1 on these residues (Heidinger-Pauli et al., 2009). It is interesting to consider how Eco1 functions in G2/M phase in response to a DSB, since Eco1 is normally degraded during G2/M phase. A recent report suggests that sequential phosphorylation events by Cdk1, Cdc7-Dbf4 and GSK-3 prevent degradation of Eco1 in G2/M phase in response to DNA damage (Lyons et al.,

2013). In contrast to the re-established cohesion in G2/M phase, the cohesive cohesin complexes established in S phase are removed to allow the proximity of DNA repair machinery (McAleenan et al., 2013). Overall, these results suggest that cohesin is necessary for the cellular response to DNA damage signal.

While it is clear that cohesin plays a critical role in the cellular response to a mitotic DSB, it is still unclear what effect cohesin has on repair outcome (Dorsett and Strom, 2012). Cohesin might direct the choice of repair template, but experimental data in support of this hypothesis is lacking. I outline experiments in chapter 2 in order to address this question of how cohesin is involved in DSB repair.

Figure 1.3

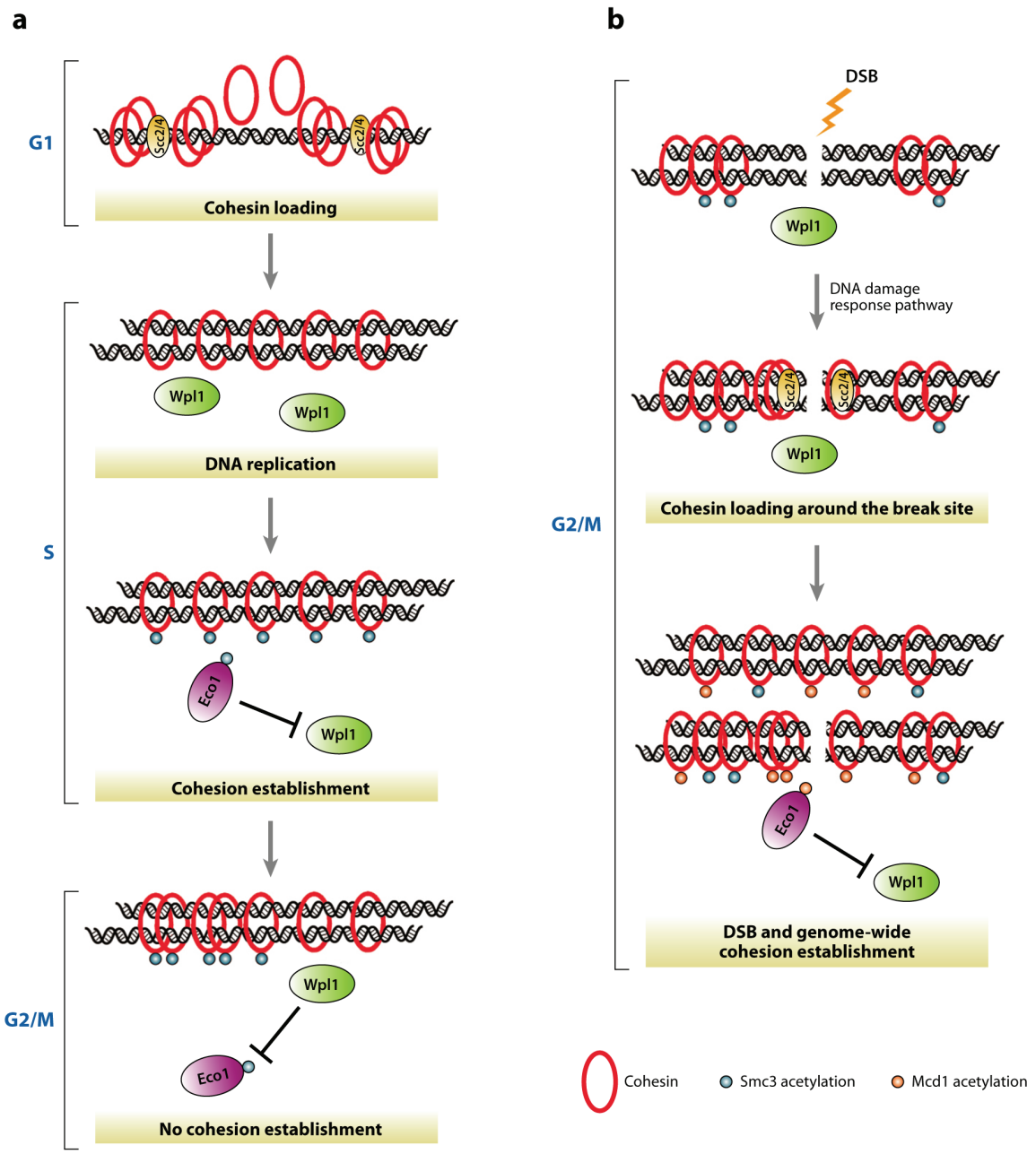

a
G1
Scc2/4
Scc2/4
Cohesin loading
S
Wpl1
Wpl1
DNA replication
Eco1
Wpl1
Cohesion establishment
G2/M
Wpl1
Eco1
No cohesion establishment
b
DSB
Wpl1
DNA damage
response pathway
Scc2/4
Scc2/4
Wpl1
Cohesin loading around the break site
G2/M
Eco1
Wpl1
DSB and genome-wide
cohesion establishment
Cohesin
Smc3 acetylation
Mcd1 acetylation

Figure 1.3 Models of cohesion establishment

Comparison of establishment of cohesion in S phase and damage-induced cohesion in G2/M phase. (a) In G1/S phase, the Scc2-Scc4 complex loads cohesin onto the chromosomes. After DNA replication, Eco1-dependent Smc3 acetylation counteracts the activity of Wpl1 that hinders the establishment of cohesion. At this time, the chromatin-bound cohesin is converted to a cohesive state. Upon entering G2/M phase, cohesion cannot be re-established because the activity of Eco1 decreases and is not high enough to counteract Wpl1. (*b*) When G2/M cells experience a DSB, the DNA damage response pathway induces DSB-proximal cohesin loading and then promotes Eco1-dependent Mcd1/Scc1 acetylation, which in turn allows cohesion establishment in G2/M phase by antagonizing Wpl1. Cohesion is established at the DSB site but is also reinforced genome wide.

(From Xiong & Gerton, 2010)

DNA replication

Cohesin complex is also involved in DNA replication. Cohesion establishment occurs during S phase, when Eco1 acetylates cohesin. The coupling of cohesion establishment with DNA replication is important to prevent premature separation of newly synthesized sister chromosomes. Many results tie cohesion establishment to DNA replication. One mechanism for coupling cohesion establishment with DNA replication is through interaction of Eco1 with the replication fork. Specifically, Eco1 interacts with Replication Factor C (RFC), a complex which acts as a clamp loader to load PCNA (proliferating cell nuclear antigen) onto chromosomes. PCNA is reported to regulate the establishment of sister-chromatid cohesion (Moldovan et al., 2006). In addition, Eco1 is reported to act with PCNA and a DNA helicase Chl1 (Skibbens, 2004, 2009). These interactions might regulate the activity of Eco1 during cohesion establishment in S phase.

The process of cohesion establishment can affect the DNA replication process. For example, cells from human Roberts syndrome patients were more sensitive to drugs that target S phase, which suggests that replication fork progression is affected in cohesin mutant (van der Lelij et al., 2009). Further studies showed that DNA replication fork speed was decreased by knocking down ESCO2 using RNAi or in RBS patient cells (Terret et al., 2009). A detailed analysis of cohesin and replication in budding yeast suggests cohesin has a direct role in recovery of stalled replication forks (Tittel-Elmer et al., 2012). Another study suggested that

cohesin complexes organize DNA loops at DNA replication factories to regulate DNA replication. Supporting this hypothesis, human cells with Rad21 knocked-down showed reduced active origins and lengthened chromatin loops (Guillou et al., 2010). Nonetheless, the mechanism by which cohesin regulates DNA replication is still unclear. More studies are needed to understand how cohesin regulates DNA replication, especially in the region where DNA replicates with difficulty such as rDNA region and how cohesin coordinate DNA replication and transcription (**Figure 1.4**). My experiments in chapter 3 help address the contribution of cohesin to both DNA replication and gene expression.

Figure 1.4

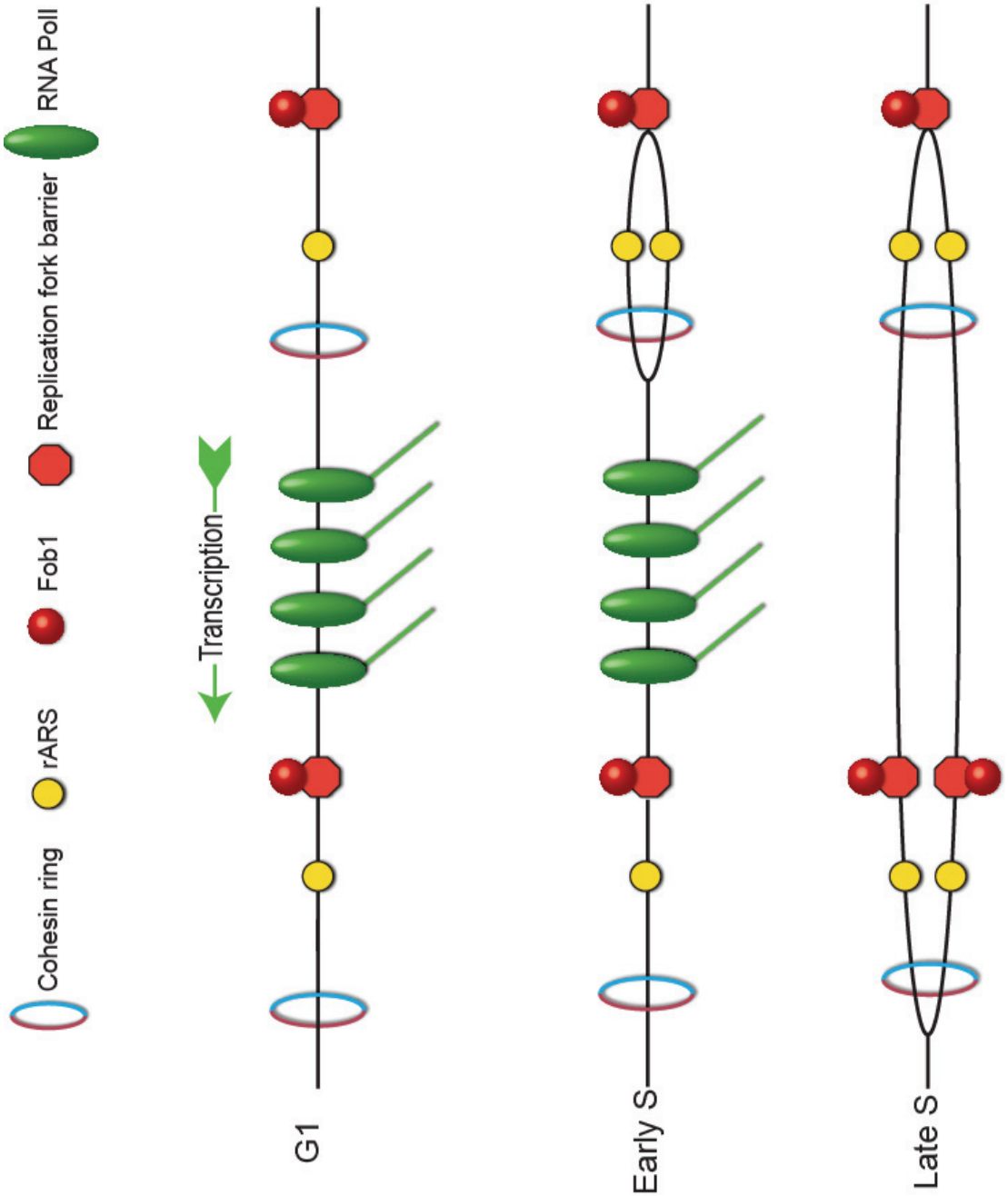

RNA Poll
Replication fork barrier
Fob1
rARS
Cohesin ring
Transcription
G1
Early S
Late S

Figure 1.4 DNA replication and transcription in rDNA locus

Replication fork barrier (RFB) blocks bidirectional replication fork progression and limits DNA replication to the same direction as transcription. Fob1 protein binds at RFB site and is essential for the barrier activity. Cohesin complex is recruited into the rDNA locus. When rDNA replication fork is progressing, transcription in the region cannot take place at the same time.

D. THE COHESINOPATHIES

The cohesinopathies refer to human developmental disorders caused by mutations in cohesin and associated genes. The cohesinopathies include Cornelia de Lange syndrome (CdLS) and Roberts syndrome (RBS).

CdLS is an autosomal dominant genetic disorder caused by mutation of one copy of *NIPBL*, *SMC1*, *SMC3*, or *HDAC8* (Braunholz et al., 2012; Deardorff et al., 2012a; Deardorff et al., 2007; Krantz et al., 2004; Lightfoot et al., 2011; Musio et al., 2006; Tonkin et al., 2004). A related disorder is caused by mutation of *Mcd1* (Deardorff et al., 2012b). CdLS and the related Mcd1 disorder include growth and mental retardation, limb deformities, and craniofacial defects. The severity of CdLS can range from a mild disorder of the nervous system to a severe multisystem disorder. About 50% of CdLS is caused by mutations in *NIPBL*. Mutations in *SMC1*, *SMC3* and *HADC8* only make up a small portion of all people who have CdLS and people with these mutations have relatively mild symptoms. *NIPBL* mutation causes a more severe phenotype than the cohesin ring mutants. This may be due to NIPBL's multiple functions in loading both cohesin and condensin on chromosomes (D'Ambrosio et al., 2008) and responding to DNA damage repair (Lightfoot et al., 2011). About 40% of CdLS patients have mutation unidentified. Discovering new gene mutations that are associated with CdLS will help elucidate the molecular etiology of this disease.

RBS is a rare autosomal recessive disorder caused by mutation of both copies of *ESCO2* (Vega et al., 2005). Patients have growth and mental retardation, limb deformities, and craniofacial defects, and the phenotypes are more severe than those observed in CdLS patients. The mutations in *ESCO2* associated with Roberts syndrome often cause truncation of ESCO2. However, a point mutation in the acetyltransferase domain of *ESCO2* has been identified (Gordillo et al., 2008). This suggests that diminished acetyltransferase activity is sufficient to cause disease. Cells from RBS patients are sensitive to DNA damage materials such as mitomycin C, camptothecin, and etoposide (Gordillo et al., 2008; van der Lelij et al., 2009). There is no evidence that mutations in ESCO1 are associated with human disease. My work, in combination with the literature, suggests ESCO2 may be the primary acetyltransferase for cohesin at the rDNA, while ESCO1 may acetylate cohesin in the rest of the genome. Therefore, mutations in *ESCO1* would be expected to be lethal.

Although mutations in the cohesin network are associated with diseases such as RBS and CdLS, it is still unclear how these mutations cause the syndromes. The consequence of a chromosome segregation defect, aneuploidy, is usually lethal. Consistently, cells from patients with cohesinopathies do not have obvious chromosome segregation defects. With the wide array of processes that cohesin contributes to, it is important to determine which processes are affected in the cohesinopathies. Systematic loss-of-cohesion leads to cessation of cell division after a few cell divisions in budding yeast (Heidinger-Pauli et al., 2010). However,

in RBS and CdLS patients, the cell division process persists long enough for the entire course of development to occur, albeit somewhat disrupted.

So, what is the cause of cohesinopathies? This mystery leads us to sort through possible explanations. One possibility is that besides a mild cohesion defect, the mutations lead to differential gene expression or DNA damage repair defects, which might cause RBS and CdLS. Analysis of cells from RBS patients reveals lack of cohesion in heterochromatin regions (Vega et al., 2005). Because heterochromatic domains are difficult to replicate, it is possible that inefficient replication of these regions is caused by mutation of *ESCO2*. One of these regions, the rDNA region, is highly transcribed. If replication is inhibited, this could affect transcription in the region. If rDNA transcription is affected, this could signal to hundreds of genes and alter the transcription profile of the genome. The lack of understanding of the etiology of the cohesinopathies drives us to explore the basic biology of the cohesin complex. The work presented in this thesis represents an effort to answer the question how cohesin network promotes DNA damage repair, genome-wide transcription and replication. Our hope is that by understanding how cohesin contributes to cellular function we can start to appreciate how mutations in cohesin cause human disease.

CHAPTER 2

ECO1 IS IMPORTANT FOR DNA DAMAGE REPAIR IN *S. CEREVISIAE*

I am the first author and Dr. Matthew Goering is the co-first author of the following manuscript: Eco1 is important for DNA damage repair in S. cerevisiae**.** **Cell Cycle** Volume 9, Issue 16 August 15, 2010. Pages 3315 – 3327.

CONTRIBUTIONS

FIGURE 2.1 Scarlett Gard performed the cohesion assays and Dr. Jennifer Gerton contributed the schematic of the Eco1 protein.

FIGURE 2.2 I performed the *in vitro* acetylation assays and Dr. Bo Xiong and I performed the acetyl-cohesin immunoprecipitation assay.

FIGURE 2.3 Dr. Matthew Goering performed the growth assay.

FIGURE 2.4 I performed the growth assay and the overexpression assay and the immunoprecipitation assay.

FIGURE 2.5 Dr. Matthew Goering performed the growth assay and the western blot.

FIGURE 2.6 I performed the mitotic recombination assay and Dr. Jennifer Gerton contributed the schematic of the Chromosome V construct.

FIGURE 2.7 Dr. Matthew Goering performed the meiotic recombination assay and Dr. Jennifer Gerton contributed the schematic of the Chromosome V construct.

ABSTRACT

The cohesin network has an essential role in chromosome segregation, but also plays a role in DNA damage repair. Eco1 is an acetyltransferase that targets subunits of the cohesin complex and is involved in both the chromosome segregation and DNA damage repair roles of the network. Using budding yeast as a model system, we find that mutations in Eco1, including a genocopy of a human Roberts syndrome allele, do not cause gross defects in chromosome cohesion. We examined how mitotic and meiotic DNA damage repair is affected by mutations in Eco1. Strains containing mutations in Eco1 are sensitive to DNA damaging agents that cause double-strand breaks, such as X-rays and bleomycin. While meiotic crossing over is relatively unaffected in strains containing the Roberts mutation, reciprocal mitotic crossovers occur with extremely low frequency in this mutant background. Our results suggest that Eco1 promotes the reciprocal exchange of chromosome arms and maintenance of heterozygosity during mitosis.

INTRODUCTION

Chromosome cohesion is essential to hold sister chromosomes together from the time they are replicated until the time they must be separated at the metaphase to anaphase transition. Cohesion is essential for the accurate attachment of chromosomes to the metaphase spindle and their subsequent division. Cohesion is mediated by the cohesin complex which consists of four subunits: Smc1, Smc3, Scc3, and Mcd1/Scc1/Rad21 (hereafter Mcd1). There are also several cohesin-associated proteins that are important for chromosome cohesion, including Eco1/Ctf7 (hereafter Eco1), an acetyltransferase important for the establishment of cohesion during S phase (Toth et al., 1999), and Scc2-Scc4, a complex required to load the cohesin complex onto chromatin (Ciosk et al., 2000).

Mutation of human ESCO2, a human homolog of yeast *ECO1*, is associated with Roberts syndrome (RBS) (Vega et al., 2005) and mutation of SCC2/NIPBL, SMC1, and SMC3 have all been associated with Cornelia de Lange syndrome (CdLS) (Deardorff et al., 2007; Krantz et al., 2004; Musio et al., 2006; Tonkin et al., 2004). Together RBS and CdLS syndrome make up the cohesinopathies, which are human developmental disorders (Liu and Krantz, 2008; McNairn and Gerton, 2008b). Chromosome segregation defects are not a feature of these disorders. Instead, the molecular etiology is assumed to be related to non-essential functions of the cohesin network in gene regulation and DNA damage repair (McNairn and Gerton, 2008a).

Along with chromosome cohesion, the cohesin network has been shown to be involved in chromosome condensation (Gard et al., 2009; Guacci et al., 1997), gene regulation (Donze et al., 1999; Gard et al., 2009; Rollins et al., 1999), subnuclear chromatin organization (Adelfalk et al., 2009; Gard et al., 2009), and double-strand break (DSB) repair (Klein et al., 1999; Sjogren and Nasmyth, 2001). DSBs are a significant threat to genome stability and can arise from endogenous and exogenous sources such as collapsed replication forks and exposure to ionizing radiation. In most eukaryotes, DSBs that arise during S-phase or G2/M are repaired by homologous recombination (HR). HR repair of DNA damage requires an intact, homologous DNA sequence to serve as a template, and during mitosis this is often the sister chromatid resulting in genetically silent repair.

When a DSB is induced by the budding yeast site-specific endonuclease HO in G2/M, cohesin binds to the region surrounding the break (Strom et al., 2004; Unal et al., 2004) in an Scc2-dependent manner (Strom et al., 2007). Human cohesin has been shown to interact with Rad50 and accumulate in regions of laser-induced DNA damage during S and G2 phases in a Rad50/Mre11-dependent manner (Kim et al., 2002). Furthermore, cohesion is reinforced genome-wide in G2/M in response to a single DSB. This process is dependent on the acetyltransferase activity of Eco1 and is referred to as damage-induced cohesion (Strom et al., 2007; Unal et al., 2007). Re-inforcement of cohesion in

response to DNA damage has also been shown to occur in human cells (Kim et al., 2010). One target of Eco1 for damage-induced cohesion has been proposed to be Mcd1, although acetylation of Mcd1 has never been directly demonstrated *in vivo* (Heidinger-Pauli et al., 2008; Heidinger-Pauli et al., 2009). Acetylation of the cohesin subunit Smc3 has been shown to be critical for S phase coupled cohesion and is dependent on Eco1 *in vivo* (Ben-Shahar et al., 2008; Unal et al., 2008; Zhang et al., 2008). Acetylation of Smc3 by ESCO1, a human homolog of yeast *ECO1*, has also been shown to be important for the DNA damage response in human cells (Kim et al., 2010). Additional Eco1 targets have been shown *in vitro* (Ivanov et al., 2002) but their biological significance is unknown.

Eco1 has two primary domains, a zinc finger in the N terminus and an acetyltransferase domain in the C terminus. The mutation W539G has been shown to abrogate acetyltransferase activity in the context of ESCO2 (Gordillo et al., 2008), a human ortholog of yeast *ECO1*, and this mutation is associated with RBS (Vega et al., 2005). Cells from RBS patients are sensitive to the DNA damaging agents mitomycin C, camptothecin and etoposide, while no particular sensitivity to UV, ionizing radiation, hydroxyurea or aphidicolin was found (Gordillo et al., 2008; van der Lelij et al., 2009). In budding yeast, mutations in the acetyltransferase domain have been shown to strongly reduce acetyltransferase activity *in vitro* (Ivanov et al., 2002) but do not cause high rates of chromosome loss (Brands and Skibbens, 2005). In contrast, mutations in the zinc finger (C35Y, H53Y) have some acetyltransferase activity *in vitro* but still

have very high rates of chromosome loss (Brands and Skibbens, 2005). The zinc finger enhances the activity of Eco1 for cohesion, but is not essential for interaction with chromatin or protein targets (Onn et al., 2009).

DNA breaks caused by X-ray treatment persist in backgrounds containing mutations in the cohesin network (Sjogren and Nasmyth, 2001; Strom et al., 2004; Unal et al., 2004), suggesting the damage is not efficiently repaired. Somewhat paradoxically, it has also been shown that damage-induced cohesion is not required for intrachromosomal gene conversion of an HO break, or the 5' to 3' resection of this break (Unal et al., 2004). Marker loss at the *RDN1* locus, which is likely due to intrachromosomal or intersister recombination in a haploid, is similarly unaffected by mutation of the acetyltransferase domain of Eco1 (Gard et al., 2009). Thus, the exact molecular role of cohesion in DSB repair remains mysterious. One prevailing idea is that cohesin is involved in the process of selecting the sister as a template for repair, but this is unsupported by experimental evidence. The effect of cohesion on recombination between homologs has never been explored.

We examined how mutations in Eco1 affect interhomolog recombination in *S. cerevisiae*. We demonstrate that the W216G mutation is a phenocopy of the human RBS mutation in that it eliminates acetyltransferase activity *in vitro*. Using various measures such as in vivo acetylation of Smc3, precocious sister separation, and DNA damage sensitivity, we compare four different mutations in

Eco1. We then use the RBS mutant, which shows limited precocious sister separation but obvious damage sensitivity, to examine the effect on recombination. Following bleomycin treatment, we find a strong deficiency in reciprocal crossing over during mitotic growth, which is needed to maintain heterozygosity. In contrast, we find only subtle defects in meiotic recombination. These results imply that an Eco1-dependent process is needed for specific recombination outcomes, e.g. reciprocal mitotic crossing over, but not for others, such as break-induced replication, gene conversion, or meiotic crossing over.

RESULTS

Replication-coupled cohesion is mildly affected by mutations in Eco1

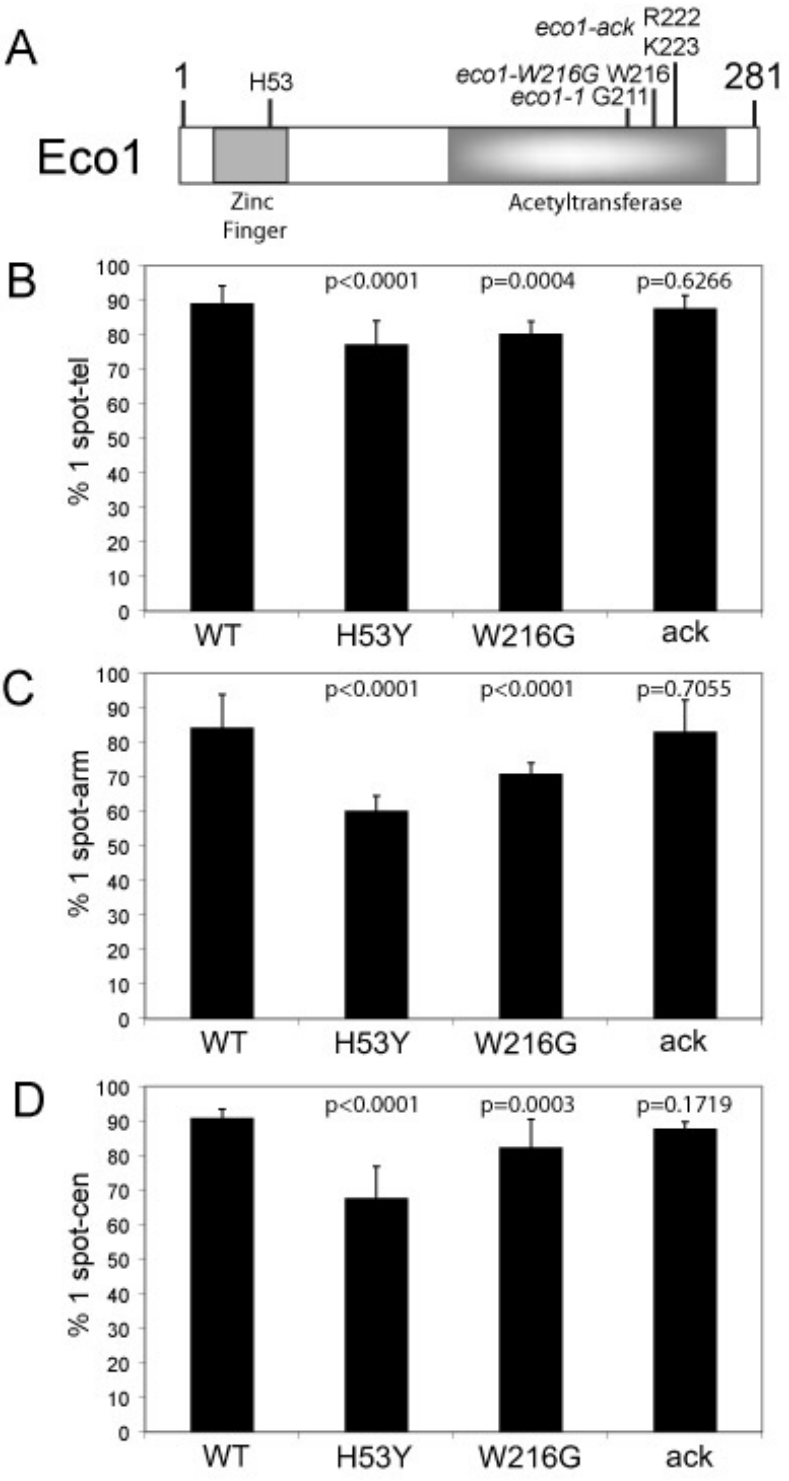
We tested three different mutations in Eco1 for their effect on chromosome cohesion: 1) *eco1-H53Y*, which is expected to disrupt the zinc finger, 2) *eco1-ack* (R222G, K223G), which disrupts acetyltransferase activity, and 3) *eco1-W216G*, which corresponds to the W539G mutation associated with Roberts syndrome (**Figure 2.1A**). Chromosome cohesion was examined in the Eco1 mutant strains in nocodazole-arrested cells using GFP marked telIVR (**Figure 2.1B**), an arm location on ChrIV (**Figure 2.1C**), and cenIV (**Figure 2.1D**) (Straight et al., 1996). Nocodazole arrests were verified by cytometry. While the *eco1-ack* strain shows no defect in cohesion, the *eco1-W216G* mutant has a mild defect in cohesion and the *eco1-H53Y* mutant has a moderate defect in cohesion. The cohesion defect in the *eco1-H53Y* mutant is consistent with the report of elevated rates of chromosome loss in a zinc finger mutant (Brands and Skibbens, 2005). However, this effect (15-20% precocious separation) is not as severe as other mutations that can cause as much as 80-90% loss of cohesion. At 37^{o}C, the *eco1-W216G* strain shows 65% loss of cohesion (Gard et al., 2009). The *eco1-1* allele confers severe cohesion defects at 37^{o}C, but also has cohesion defects (~8% higher than WT at CenV) even at the “permissive” temperature of 22.5^{o}C (Toth et al., 1999). Because this mutant is very temperature sensitive, its phenotype is somewhat difficult to compare with *eco1-ack*, *eco1-W216G*, and *eco1-H53Y*, which have a permissive temperature of 30^{o}C.

Figure 2.1

Cohesion defects in Eco1 mutant strains.

(A) The two domains of Eco1 are shown in a cartoon of the gene, and the mutations characterized are indicated. **(B)** Yeast strains were constructed by replacing the genomic copy of the indicated allele with a cohesinopathy allele, thereby placing the mutant allele under the control of the endogenous promoter at the endogenous locus. Additionally, strains contained lacO repeats on the telomere **(B)**, arm **(C)**, or centromere **(D)** of chromosome IV and a lacI-GFP fusion was inducibly expressed in nocodazole-arrested cells at 30oC. The arrest was confirmed by cytometry (data not shown). The number of cells displaying 1 spot versus 2 spots was counted to determine chromosome cohesion. At least three biological replicates were conducted for each strain and at least 300 total cells were counted. The standard deviation is indicated. Statistical significance was calculated using Fisher's exact test.

Figure 2.1


A
eco1-ack R222
K223
1
H53
eco1-W216G W216
eco1-1 G211
281
Eco1
Zinc
Finger
Acetyltransferase
B
% 1 spot-tel
p<0.0001
p=0.0004
p=0.6266
WT
H53Y
W216G
ack
C
% 1 spot-arm
p<0.0001
p<0.0001
p=0.7055
WT
H53Y
W216G
ack
D
% 1 spot-cen
p<0.0001
p=0.0003
p=0.1719
WT
H53Y
W216G
ack

Acetyltransferase activity is compromised by the eco1-W216G (RBS) mutation

In order to compare the acetyltransferase activity of different Eco1 mutants, each mutant protein was expressed in *E. coli* and purified via a GST tag. Recombinant protein was incubated with ^{3}H-acetyl-Co-A and a recombinant Mcd1 peptide (amino acids 169-337). As had been previously shown, the *eco1-1* (G211D), and *eco1-ack* mutations strongly reduce both autoacetylation of Eco1 and acetylation of an exogenous substrate (Ivanov et al., 2002). The Eco1-W216G mutant protein behaved similarly. However, the H53Y zinc finger mutation results in a protein that retains some auto-acetyltransferase activity, but has a similar deficiency to the other mutants in terms of acetylation of an exogenous substrate (**Figure 2.2A**). This protein might be expected to have low acetyltransferase activity toward its targets *in vivo*. Similar results were obtained when acetylation was detected by Western blotting with an anti-acetyl-lysine antibody (data not shown). Thus, all four mutants have severely compromised acetyltransferase activity toward a target protein *in vitro*.

We next checked the expression of the mutants *in vivo* by adding a 3X FLAG tag to the C terminus and immunoblotting. We find that Eco1-W216G and Eco1-H53Y are present at much lower levels than wild-type protein (12-fold and 6-fold, respectively, **Figure 2.2B**). Regrettably, a strain bearing FLAG tagged Eco1-1 is inviable, so we were unable to measure the level of this mutant protein *in vivo*. The lower levels of the Eco1-W216G and Eco1-H53Y protein *in vivo* combined

with the lack of acetyltransferase activity measured *in vitro* suggest these mutants might have a stronger phenotype than *eco1-ack*, whose levels are only reduced ~2-fold.

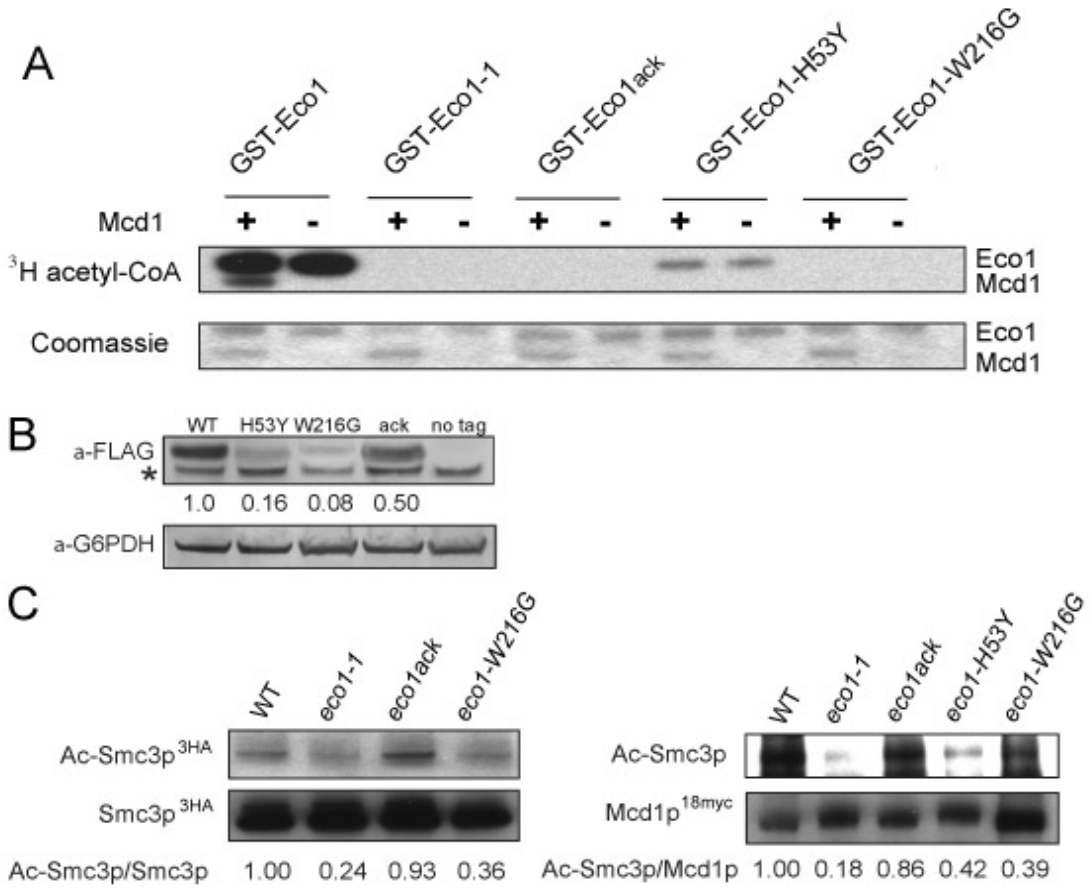
Given the differences in protein levels, we decided to measure the acetylation levels of Smc3 in each mutant background *in vivo* in order to determine their "true" acetylation defect. Acetylation of Smc3 can be detected with an anti-acetylysine antibody when the cohesin complex is immunoprecipitated from whole cell extracts. We examined Smc3 acetylation using immunoprecipitation of either Smc3-HA or Mcd1-18Myc. Smc3-HA in combination with the *eco1-H53Y* mutation is lethal so we could not perform the HA immunoprecipitation in this strain. We found that the level of Smc3 acetylation in *eco1-ack* is nearly wild-type, while the level in the *eco1-1* is the lowest. Acetylation is present at intermediate levels in the *eco1-H53Y* and *eco1-W216G* mutants (**Figure 2.2C**). The level of acetylation measured in either the Mcd1 or Smc3 pull-down is similar. In addition, each of the pull-downs was performed at least twice with similar results. Unfortunately, the level of acetylated Mcd1 cannot be measured *in vivo* since it is not detected with any of the available anti-acetyl-lysine antibodies (Heidinger-Pauli et al., 2009). Although acetylation of an exogenous substrate is undetectable *in vitro*, these mutant Eco1 proteins mediate various levels of acetylation *in vivo*.

Figure 2.2

Acetyltransferase activity associated with Eco1 mutants.

(A) GST-Eco1 and GST-Mcd1$^{169-337}$ fusion proteins were expressed in *E. coli* and purified by glutathione-agarose. Following an *in vitro* acetylation reaction with ^{3}H-acetylCoA in which Mcd1 peptide was either included (+) or omitted (-), the sample was subjected to autoradiography or Coomassie staining following PAGE. Eco1 can autoacetylate (upper band) and can also acetylate Mcd1$^{169-337}$ (lower band). **(B)** The expression of Eco1 was measured by immunoblotting with α-FLAG antibody in whole cell yeast extracts. A 3XFLAG tag was integrated at the C terminus of each protein. 50 µg of total protein was loaded per lane. The expression level of each protein is shown normalized to wild-type. The asterisk indicates a non-specific band. G6PDH serves as a loading control. **(C)** The level of acetylation of Smc3 was measured in WT, *eco1-1*, *eco1-W216G*, *eco1-H53Y*, and *eco1-ack* mutant strains by pulling down either Smc3-HA with anti-HA beads or Mcd1-18Myc with anti-Myc beads from whole cell extracts followed by Western blotting with anti-acetyl-lysine antibody.

Figure 2.2


A
GST-Eco1
GST-Eco1-1
GST-Eco1ack
GST-Eco1-H53Y
GST-Eco1-W216G
Mcd1
+ - + - + - + - + -
3H acetyl-CoA
Eco1
Mcd1
Coomassie
Eco1
Mcd1
B
WT H53Y W216G ack no tag
a-FLAG
*
1.0 0.16 0.08 0.50
a-G6PDH
C
WT eco1-1 eco1ack eco1-W216G
Ac-Smc3p 3HA
Smc3p 3HA
Ac-Smc3p/Smc3p 1.00 0.24 0.93 0.36
WT eco1-1 eco1ack eco1-H53Y eco1-W216G
Ac-Smc3p
Mcd1p 18myc
Ac-Smc3p/Mcd1p 1.00 0.18 0.86 0.42 0.39

DNA damaging agents reduce the growth of strains with mutations in Eco1

Given the role of the cohesin network in DNA repair, we examined the different mutants for damage sensitivity. The *scc2-D730V* mutant strain used in this assay corresponds to a mutation associated with a second cohesinopathy, Cornelia de Lange syndrome. Since both the *eco1-W216G* and *scc2-D730V* mutations have been reported to affect chromosome compaction, the *scc2-D730V* mutant is meant to serve as a control for any effect of chromosome compaction on damage sensitivity. The *scc2-D730V* mutation does not cause a measurable cohesion defect (Gard et al., 2009). A *rad50Δ* mutant serves as a radiation sensitive control. Deletion of *RAD61/WPL1* has previously been shown to rescue the growth of *eco1-1* at 37^{o}C and damage sensitivity associated with the *eco1-1* mutation (Ben-Shahar et al., 2008).

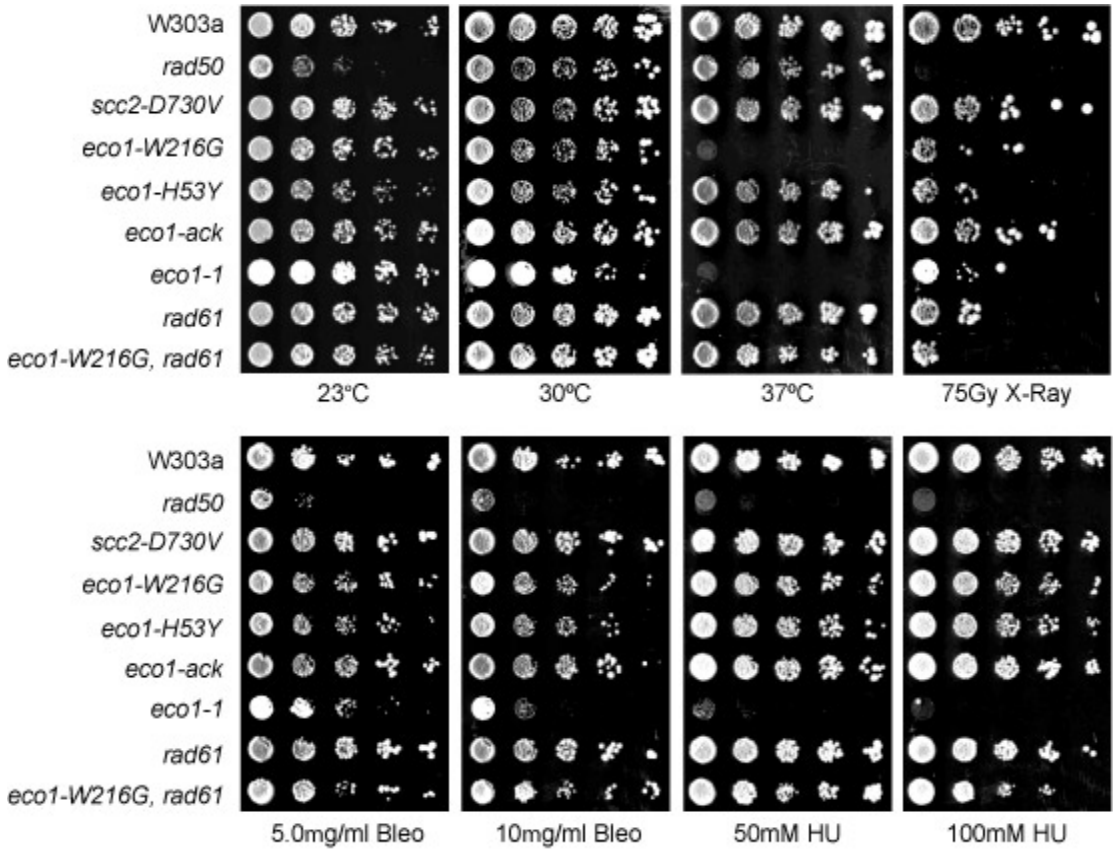
We examined the sensitivity of the *eco1* mutants to different DNA damaging agents. Hydroxyurea (HU) will slow S phase which in turn causes collapsed and stalled replication forks. X-rays and bleomycin will primarily cause DSBs, which can occur at any point in the cell cycle. The *eco1-1* mutant strain is sensitive to HU, bleomycin, and X-rays. We find that none of the other 3 *eco1* mutants nor the *scc2* mutant display sensitivity to HU, but *eco1-H53Y* and *eco1-W216G* are both sensitive to X-rays and bleomycin (**Figure 2.3**). The D730V mutation in *SCC2* does not cause sensitivity to DNA damaging agents. As previously shown, the *eco1-W216G* mutant does not grow at 37^{o}C (Gard et al., 2009), but growth is rescued by deletion of *RAD61*. In contrast to what has been reported for *eco1-1*

(Ben-Shahar et al., 2008), deletion of *RAD61* does not rescue the damage sensitivity of the *eco1-W216G* strain. Deletion of *RAD61* alone causes very mild DNA damage sensitivity, consistent with a previous report (Ben-Shahar et al., 2008). Similar results for all mutants were observed in an S288C background (data not shown).

Figure 2.3

Eco1 mutants are sensitive to agents that cause DNA double-strand breaks. Haploid strains of the indicated genotype were serially diluted, plated, and grown on SD complete medium at 23°C, 30°C or 37°C, subjected to 75 Gray of ionizing radiation followed by growth at 30°C, or plated onto SD complete medium containing 50 or 100 mM hydroxyurea (HU), or 5 or 10 µg/ml bleomycin, followed by growth at 30°C.

Figure 2.3



In order to pinpoint the relevant acetylation target of Eco1 for damage sensitivity, we tried to genetically rescue the damage sensitivity by overexpression of known targets. Acetylation of Mcd1 is thought to be important for cohesion following a DSB in G2/M (Heidinger-Pauli et al., 2008), although this post-translational modification has never been detected *in vivo*. Expression of Mcd1 with mutations that mimic acetylation (K84Q, K210Q) bypassed the need for Eco1 for DSB-induced cohesion (Heidinger-Pauli et al., 2009). Smc3 has also been shown to be acetylated by Eco1 for S phase cohesion on residues K112 and K113 (Ben-Shahar et al., 2008; Rowland et al., 2009; Unal et al., 2008; Zhang et al., 2008). We investigated whether overexpression of *MCD1*, *MCD1*$^{K84Q,\ K210Q}$, *SMC3*, *SMC3*^{K112QK113Q}, *SMC3*^{K112NK113N}, *SMC3*^{K113Q}, or *SMC3*^{K113N} would rescue the damage sensitivity of the *eco1-W216G* mutant strain. We found that overexpression of *MCD1* or *MCD1*$^{K84Q,\ K210Q}$ did not suppress the growth defect caused by X-rays (**Figure 2.4A**). Neither did overexpression of *SMC3* or any of the *SMC3* mutants (**Figure 2.4A**). In conclusion, overexpression of no single known target of Eco1 was able to rescue the damage sensitivity of the *eco1-W216G* mutant strain. This result suggests that the expression of the acetylation mimic mutations may not sufficiently mimic the acetylated state, or that Eco1 may have additional targets for DNA damage repair.

In contrast to the DNA damage sensitivity, overexpression of *SMC3*^{K113Q} or *SMC3*^{K113N}, but not *SMC3*, rescued the temperature sensitivity of the *eco1-W216G* strain. The rescue suggests that the primary reason for lethality at 37^{o}C,

which is correlated with a strong cohesion defect (Gard et al., 2009), is a deficit in acetylation of K113 in Smc3 by Eco1. These findings echo the rescue of the *eco1-1* strain with *SMC3*^{K113N} (Ben-Shahar et al., 2008). Overexpression of *SMC3*^{K112NK113N} or *SMC3*^{K112QK113Q} actually inhibited growth of the *eco1-W216G* strain, suggesting that the constituitive acetylation of both residues is problematic, similar to findings in a previous report (Unal et al., 2008).

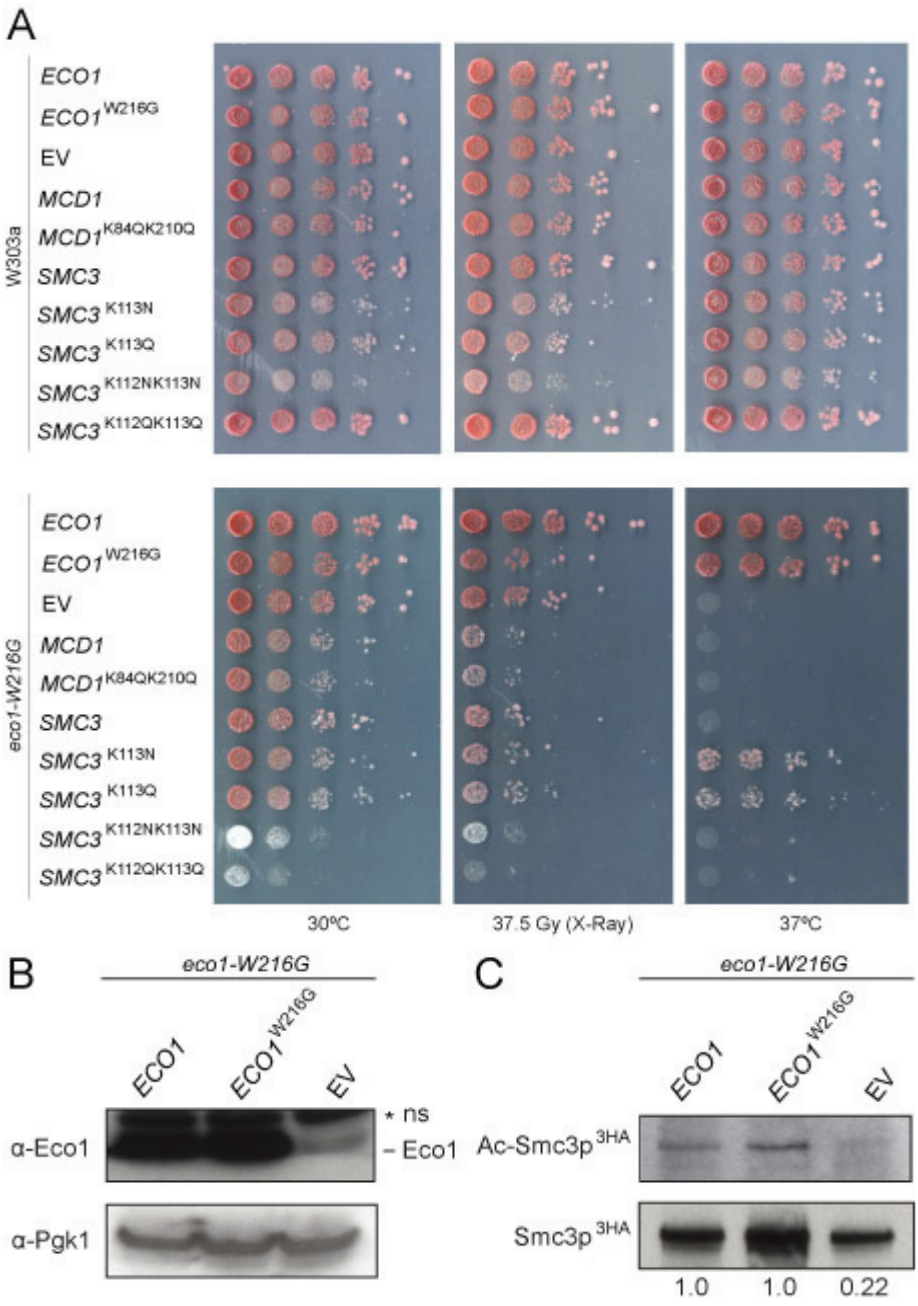
As a control, we overexpressed *ECO1*, which allows robust growth following treatment with X-rays, as expected. Unexpectedly, overexpression of Eco1-W216G rescued the temperature sensitivity of the *eco1-W216G* mutant strain (**Figure 2.4A**), suggesting an abundance of Eco1-W216G is sufficient for its essential function in chromosome segregation, but in contrast, it did not suppress the damage sensitivity. Western blot analysis showed that the *eco1-W216G* mutant protein is expressed at high levels (**Figure 2.4B**), and furthermore, that the acetylation of Smc3 is restored by the overexpression (**Figure 2.4C**). Thus, neither the restoration of acetylated Smc3 nor the expression of the Smc3 acetylation mimic is able to rescue the damage sensitivity of the *eco1-W216G* mutant.

Figure 2.4

Overexpression of known targets of Eco1 does not suppress the damage sensitivity of the eco1-W216G strain.

(A) An eco1-W216G strain was transformed with plasmids containing various genes under the control of a gal promoter or an empty vector (EV). Strains were plated onto gal-ura medium to induce expression and incubated at 30^{o}C, or treated with 37.5 Gray of ionizing radiation followed by incubation at 30^{o}C, or incubated at 37^{o}C. **(B)** Strains with plasmids containing ECO1, *eco1-W216G*, or an empty vector were grown in galactose to examine levels of Eco1 protein using anti-Eco1 antibody by Western blot. Pgk1 serves as a loading control. "ns" indicated a non-specific band. **(C)** From strains containing the plasmids in (B), an IP was performed with anti-HA antibody to pull down Smc3, and subsequently subjected to Western blotting with anti-acetyl-lysine antibody. The level of Smc3 acetylation was quantitated using the level of Smc3 to normalize for IP efficiency, and the numbers are shown below each lane.

Figure 2.4


A
W303a
ECO1
ECO1 W216G
EV
MCD1
MCD1 K84QK210Q
SMC3
SMC3 K113N
SMC3 K113Q
SMC3 K112NK113N
SMC3 K112QK113Q
eco1-W216G
ECO1
ECO1 W216G
EV
MCD1
MCD1 K84QK210Q
SMC3
SMC3 K113N
SMC3 K113Q
SMC3 K112NK113N
SMC3 K112QK113Q
30ºC
37.5 Gy (X-Ray)
37ºC
B
eco1-W216G
ECO1
ECO1 W216G
EV
α-Eco1
* ns
– Eco1
α-Pgk1
C
eco1-W216G
ECO1
ECO1 W216G
EV
Ac-Smc3p 3HA
Smc3p 3HA
1.0
1.0
0.22

Eco1 mutants activate the DNA damage checkpoint

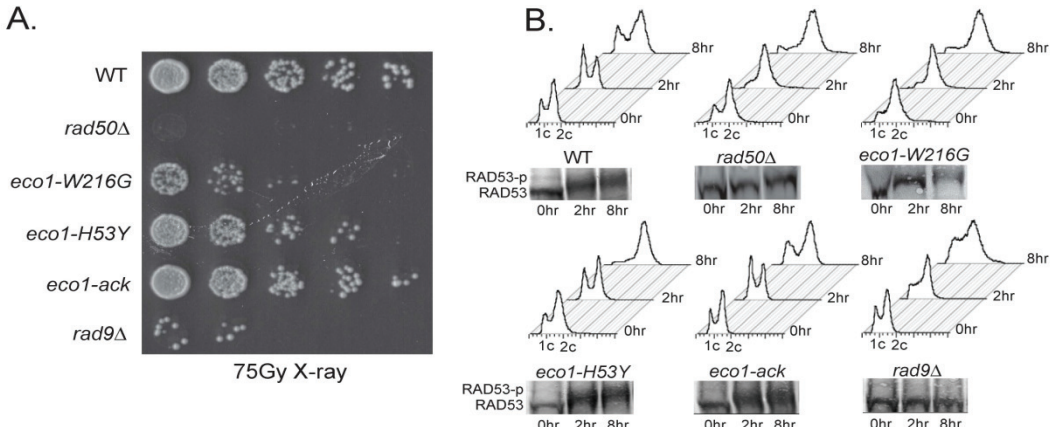
In order to further characterize the DNA damage defect in the Eco1 mutants, we analyzed checkpoint function in the mutants. The sensitivity of *eco1* mutants to DNA damage may result from either a failure activate the DNA damage checkpoint or an inability to repair the damage. In budding yeast the effector kinase, Rad53 (Chk2) is required for checkpoint activity and cell cycle arrest in response to DNA damage (Weinert et al., 1994). Rad53 becomes phosphorylated by Mec1 in a Rad9 dependent manner in response to DNA double strand breaks (Sanchez et al., 1996). We monitored cell cycle progression and the phosphorylation state of Rad53 following exposure of cells to X-rays (**Figure 2.5**).

Figure 2.5

DNA damage checkpoint response in eco1 mutants.

(A) Strains were grown to an OD_{600} of 0.6 and subjected to 75 Gray of ionizing radiation. Following exposure, cells were plated to YPD plates to measure viability. **(B)** Cells were collected for analysis of DNA content by cytometry and phosphorylation of Rad53 by Western blotting prior to exposure (0) and at 2 and 8 hours following exposure.

Figure 2.5



Rad53 was efficiently phosphorylated following treatment with X-rays in the *eco1-H53Y*, *eco1-W216G*, and *eco1-ack* mutants, but only the *eco1-H53Y* and *eco1-W216G* mutants held a persistent arrest in G2/M, similar to a *rad50Δ* mutant (**Figure 2.5**). In contrast, the *eco1-ack* mutant behaved more like WT; it did not arrest, suggesting it could efficiently correct the damage. This observation supports our finding that *eco1-H53Y* and *eco1-W216G* mutant strains are more sensitive to genotoxic stress than *eco1-ack* (**Figure 2.3**). A *rad9Δ* mutant serves as a control; Rad53 is not phosphorylated in this mutant and cells do not arrest (de la Torre-Ruiz et al., 1998; Weinert and Hartwell, 1988). We conclude that the checkpoint is appropriately activated in the mutants and the sensitivity to ionizing radiation is due to a downstream defect. This observation is consistent with damage-induced cohesion being dependent on Mec1 (Unal et al., 2007).

Reciprocal mitotic crossovers require the acetyltransferase activity of Eco1

While mutations that reduce the acetyltransferase activity of Eco1 have little effect on S phase cohesion and chromosome segregation, the same mutations (*eco1-ack*, *eco1-203*) have been demonstrated to reduce DSB-coupled cohesion in G2/M (Unal et al., 2007). Unfortunately the *eco1-W216G* mutation in combination with the *mcd1-1* allele used in the assay developed by the Koshland lab to measure damage-induced cohesion is a lethal combination so we cannot measure damage-induced cohesion in this manner. However, since the Roberts strain is damage sensitive, we decided to study how this mutation affects recombination outcomes in a diploid background.

An elegant system has been developed to analyze mitotic recombination in a 120 kb interval on chromosome V (Barbera and Petes, 2006). This system takes advantage of a variety of heteroallelic selectable markers. One copy of chromosome V contains *HIS3*, *can1-100*, and HYG while the second copy of chromosome V contains *LEU2*, SUP4-o (an ochre suppressor) and KAN at the same positions. The background is homozygous for the *ade2-1* allele, which contains an ochre suppressible mutation. When *ade2-1* is suppressed by SUP4-o, cells are white, but if the cells lack SUP4-o, they will be red (**Figure 2.6A**). This strain can be used to score break induced replication (BIR), local gene conversion (GC), reciprocal crossovers (RCO), and chromosome loss events.

Previously, mitotic recombination following growth on HU was examined (Barbera and Petes, 2006). HU increases the number of recombinants approximately 40-fold over the rate of spontaneous mitotic recombination. When the rate of recombination is high, recombination events do not need to be selected. The number of sectored colonies relative to the total number of colonies is a direct measure of the frequency of recombination. After the two colored sectors are isolated, these colonies can be further scored for the various markers to differentiate types of recombination events.

We grew the diploid WT and *eco1-W216G* mutant strains on plates containing 2.5 µg/ml bleomycin to induce a low level of damage. At this concentration, the

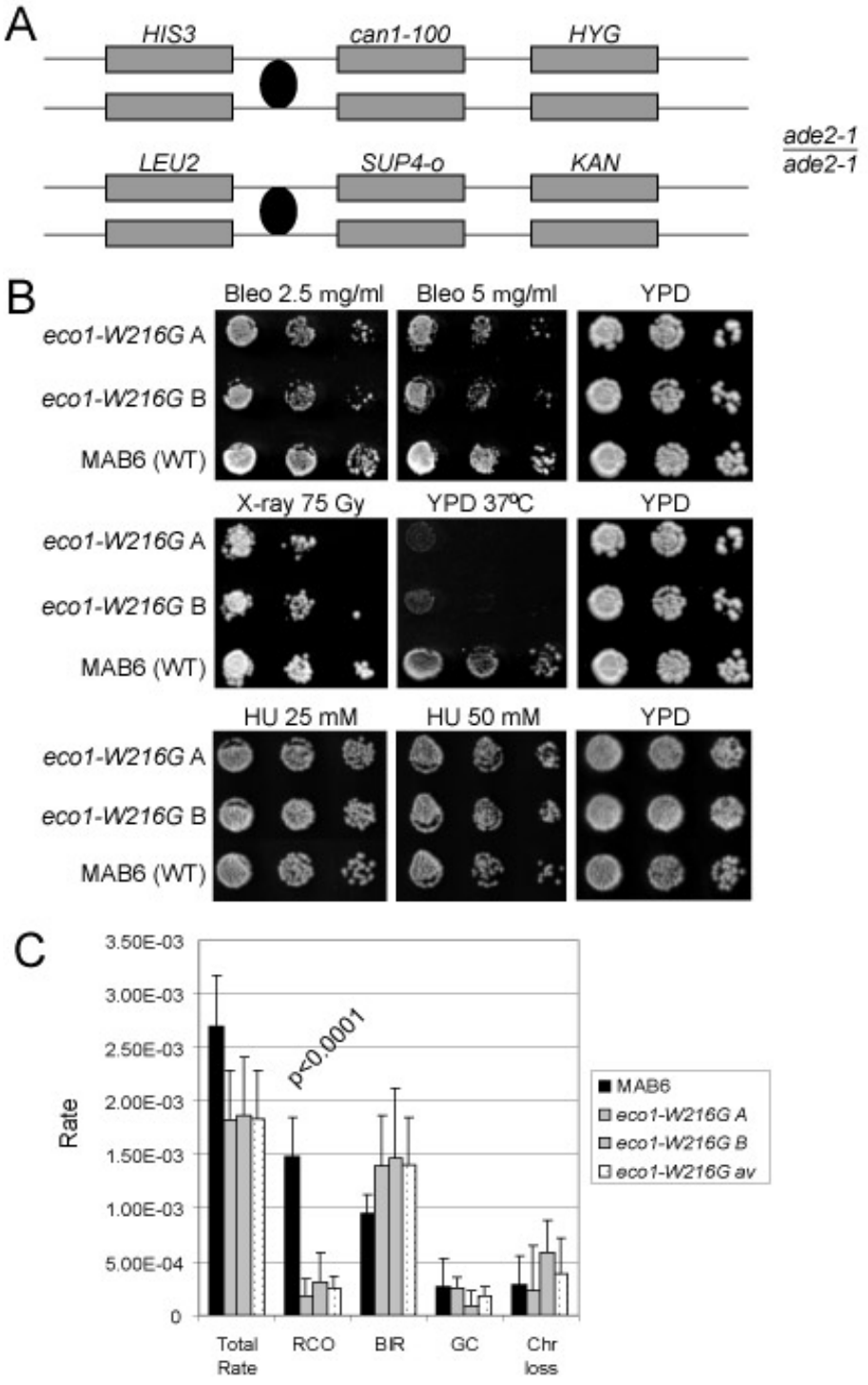
eco1-W216G mutant strain can still grow whereas at higher levels, its growth is significantly impaired (**Figure 2.6B**). Individual colonies were resuspended in water and plated to nonselective medium (SD-arg) and screened for colonies with red/white sectors. We find that growth on 2.5 ug/ml bleomycin elevates the rate of recombination in a WT strain approximately 120-fold compared to no treatment. Both RCO and BIR events are stimulated on bleomycin in a WT strain. However, with HU the ratio of RCO to BIR events is approximately 0.6:1 (Barbera and Petes, 2006) while growth on bleomycin causes RCOs to become more common than BIR events with a shift in the ratio to 1.8:1. This is likely related to the different types of damage caused by HU and bleomycin. The overall rate of recombination in the *eco1-W216G* background appears slightly lower than wild-type (p=0.08, chi square test, **Figure 2.6C**), suggesting the mutation may compromise recombination efficiency.

Figure 2.6

A genetic system to detect mitotic recombination events was used to score recombination in the Roberts mutant.

(A) Schematic of the markers contained on Chromosome V. **(B)** Diploid strains were serially diluted and plated. The *eco1-W216G* diploid strains show similar sensitivity to bleomycin and ionizing radiation as the haploid strain (**Figure 2.3**). **(C)** A wild-type strain and two independent isolates of the Roberts mutant strain were grown at room temperature on plates containing 2.5 μg/ml bleomycin. Individual colonies were picked and plated onto SD-arg medium. Sectored colonies were scored as previously described (Barbera and Petes, 2006). "Total rate" indicates the total number of recombination events. These events were further broken down into reciprocal crossovers (RCO), break induced replication (BIR), local gene conversion (GC), and chromosome loss (Chr loss) based on marker scoring. The results are shown for each of the two mutant strains separately and combined (av). Three independent experiments were performed for each strain and the standard deviation is shown. The p value is derived from a chi square test comparing total recombinants – RCOs and RCOs from the WT strain to the sum of the same values from the *eco1-W216G* mutant strains.

Figure 2.6


A
HIS3
can1-100
HYG
LEU2
SUP4-o
KAN
ade2-1
ade2-1
B
Bleo 2.5 mg/ml
Bleo 5 mg/ml
YPD
eco1-W216G A
eco1-W216G B
MAB6 (WT)
X-ray 75 Gy
YPD 37ºC
YPD
eco1-W216G A
eco1-W216G B
MAB6 (WT)
HU 25 mM
HU 50 mM
YPD
eco1-W216G A
eco1-W216G B
MAB6 (WT)
C
Rate
3.50E-03
3.00E-03
2.50E-03
2.00E-03
1.50E-03
1.00E-03
5.00E-04
0
p<0.0001
Total Rate
RCO
BIR
GC
Chr loss
MAB6
eco1-W216G A
eco1-W216G B
eco1-W216G av

Sectored colonies were scored to assess what type of recombination event had occurred. For the WT strain we examined 11,340 total colonies and found 18 with the phenotype expected for RCO events and 10 with the phenotype expected for BIR events. For the *eco1-W216G* mutant strain, we examined 24,489 total colonies and found 6 with the phenotype expected for an RCO event and 32 with the phenotype expected for a BIR event. The drop in RCO events in the mutant background is statistically significant at the level of $p<0.0001$ using a chi square test with Yates correction. Three independent trials were performed and the standard deviation is shown (**Figure 2.6C**). Chromosome loss events were not significantly elevated in the Roberts mutant background, arguing that chromosome segregation is not defective, and consistent with the 1 spot-2 spot results (**Figure 2.1**). The specific effect of the *eco1-W216G* mutation on reciprocal crossovers implies that Eco1 activity is needed for this particular recombination outcome in mitotic cells. Interestingly RCOs seem to be particularly important for mitotic repair at the rDNA (Casper et al., 2008).

Eco1 activity is not essential for meiotic crossovers

The reduction in reciprocal crossovers during mitosis prompted us to examine the level of crossovers in meiosis in the Roberts mutant background. In meiosis, programmed DSBs elevate the frequency of recombination 1000-fold. In addition to the markers already described, the strain used was heterozygous for *URA3/ura3-1* within the 120 kb interval on ChrV and for *TRP1* inserted at the *TAR1* locus within the rDNA (**Figure 2.7A**). We wanted to monitor recombination

within *RDN1* since this is a cohesin binding site (Glynn et al., 2004; Laloraya et al., 2000). In mitosis, the cohesin complex consists of Smc1, Smc3, Scc3, and Mcd1/Scc1. In meiosis, there is a second cohesin complex in which Rec8 replaces Mcd1 (Klein et al., 1999; Watanabe and Nurse, 1999). In contrast to Mcd1, Rec8 cannot mediate damage-induced cohesion (Heidinger-Pauli et al., 2008). Whether or not Eco1 has essential targets in meiosis is an open question.

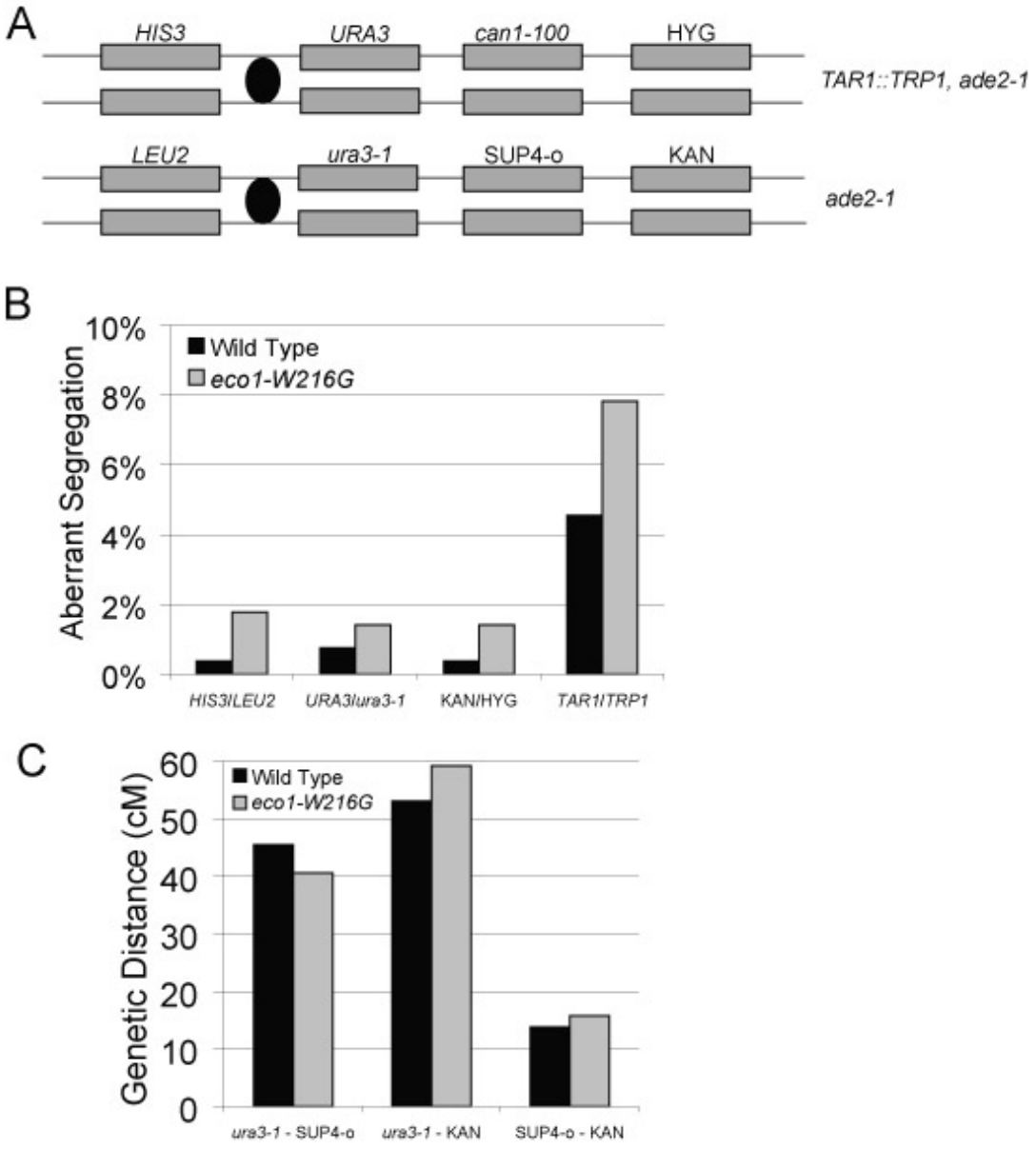
We examined the meiotic products from a WT strain and a Roberts mutant strain. Tetrad formation is reduced in the Roberts mutant relative to the wild-type. Furthermore, spore viability is reduced from 96% to 75%. However, recombination as scored in 4-spore tetrads is indistinguishable from WT. Although the frequency of aberrant segregation for four different markers is elevated in the mutant, these increases are not statistically significant (**Figure 2.7B**). If aberrant segregation is summed for all the loci, the increase becomes statistically significant with a P value of 0.012. Genetic distance, which is a measure of the number of crossovers per kb, is similar for three different intervals (**Figure 2.7C**). Thus, it seems that Eco1 activity is not essential for successful meiotic recombination or crossing over.

Figure 2.7

A genetic system to score meiotic recombination was used to analyze recombination in the Roberts mutant.

(A) Schematic of the markers contained on Chromosome V. In addition, *TRP1* was inserted into the *RDN1* repeat in one parent. **(B)** Aberrant segregation (exceptions to 2:2 segregation) of markers was scored for four different positions in 305 wild-type and 588 mutant tetrads. Two independently isolated strains were used for each genotype. Fisher's exact test indicates no significant differences between mutant and wild-type. **(C)** The genetic distance was calculated for the three intervals indicated, with the caveat that pink/white color was used to score the SUP4-o locus. Fisher's exact test indicates no significant differences between mutant and wild-type.

Figure 2.7


A
HIS3
URA3
can1-100
HYG
TAR1::TRP1, ade2-1
LEU2
ura3-1
SUP4-o
KAN
ade2-1
B
Aberrant Segregation
10%
8%
6%
4%
2%
0%
Wild Type
eco1-W216G
HIS3/LEU2
URA3/ura3-1
KAN/HYG
TAR1/TRP1
C
Genetic Distance (cM)
60
50
40
30
20
10
0
Wild Type
eco1-W216G
ura3-1 - SUP4-o
ura3-1 - KAN
SUP4-o - KAN

DISCUSSION

We have analyzed and compared four mutations in the essential acetyltransferase *ECO1*; each mutant has a distinct phenotype. Together these mutations can be thought of as a series of weak to strong hypomorphic mutations. Three mutations are in the acetyltransferase domain and one is in the zinc finger; all four mutations cause deficiencies in acetyltransferase activity *in vitro*, including a mutation that corresponds to the human Roberts syndrome mutation. Despite similar deficiencies in acetyltransferase activity *in vitro*, strains with the mutations integrated under the control of the endogenous promoter display different levels of Smc3 acetylation *in vivo* and these levels roughly correlate with sensitivity to agents that cause DNA damage, with the *eco1-1* mutant being the most sensitive, *eco1-W216G* and *eco1-H53Y* displaying intermediate sensitivity, and *eco1-ack* showing no detectable sensitivity. Since acetylation of Smc3 is necessary for cohesion, the number of cohesive complexes is likely to be reduced in the mutants. Reducing acetylated complexes may be akin to systematically reducing Mcd1 levels, which has recently been argued to reduce the number of cohesive complexes (Heidinger-Pauli et al., 2010). In this case, accurate chromosome segregation required much less Mcd1 than DNA repair (Heidinger-Pauli et al., 2010), which is consistent with our findings that reduced Smc3 acetylation in the *eco1-W216G* mutant does not affect chromosome segregation but this mutation does affect DNA repair.

Growth at 37^{o}C in the *eco1-W216G* strain was rescued by overexpression of *ECO1-W216G*, *SMC3*^{K113Q} or *SMC3*^{K113N} or deletion of *RAD61,* suggesting these genetic manipulations are sufficient for chromosome segregation. In contrast, none of these genetic manipulations was able to rescue the damage sensitivity, suggesting none of these genetic states are sufficient for an effective response to DNA damage. It is possible that the overexpression of *ECO1-W216G*, *SMC3*^{K113Q} or *SMC3*^{K113N} failed to rescue the damage sensitivity because acetylation is uncoupled with the damage response.

Given the DNA damage sensitivity of the *eco1-W216G* strain, we undertook a detailed characterization of recombination in this mutant background. The mutant displayed a surprising defect in reciprocal crossing over, suggesting that an Eco1-dependent process is required for the exchange of homologous chromosome arms. This defect may be due to a reduction in damage-induced cohesion. The specificity of the effect for mitotic recombination probably reflects the different roles of cohesin in the repair of mitotic DSBs and the programmed DSBs that occur during meiosis.

When DSBs are repaired by non-reciprocal recombination, such as BIR, instead of reciprocal crossovers (RCO), such as in the RBS mutant strain, the net result is a greater chance for loss of heterozygosity (LOH). Although crossing over is a rather rare event during mitosis, mechanisms to ensure reciprocity are quite important since they allow for the maintenance of two copies of a given allele,

rather than a conversion to homozygosity. Retinoblastoma, a human cancer of the retina, provides a compelling example for the importance of maintenance of heterozygosity. When one parent's contribution of the tumor suppressor Rb1 is flawed, this almost invariably results in childhood retinoblastoma due to chance LOH events. Thus, discovering mutations that lead to a bias toward LOH is informative since it identifies a potential mechanism by which LOH can occur. In budding yeast that have been aged by multiple passages, the rate of LOH increases due to a bias toward non-reciprocal recombination (McMurray and Gottschling, 2003), similar to what we observe in the RBS strain.

MATERIALS AND METHODS

GST-fusion protein purification

An *E. coli* culture was grown in 1 L of LB with 100 µg/ml ampicillin at 37°C until OD_{600} was 0.3. Then the culture was shifted to 25°C and continued to grow until OD_{600} was 0.6. IPTG was added to 0.3 mM for 3 hours at 25°C. The cells were subjected to centrifugation at 5K, 4°C for 5 min and washed with cold PBS. The pellet was resuspended in 30 ml metal lysis buffer (25 mM Hepes, pH7.5, 300 mM NaCl, 10% glycerol and 1 mM DTT) with protease inhibitors (Roche). Then 0.5 ml of 500 mg/ml lysozyme was added and mixed by inverting. The mixture was incubated 10 minutes on ice and sonicated on ice 5 times for 30 seconds on 35% power with a 2 minute interval. The lysate was then centrifuged for 20 minutes 16K at 4°C and was then further centrifuged at 50K for 45 minutes. The supernatant was added to glutathione resin and rotated for 2 hours at 4°C. The glutathione resin was rinsed with 5 ml metal lysis buffer and protein was eluted by adding 1 ml of 10 mM reduced glutathione.

In vitro acetylation assay

Purified GST-tagged Eco1p WT, G211D, R222G K223G, W216G and H53Y proteins (0.4 µg) were added to HAT buffer (50 mM Tris [pH 8.0], 5% glycerol, 0.1 mM EDTA, 50 mM KCl, 1 mM DTT, 1mM PMSF and 10 µM ^{3}H-Acetyl-CoA), and incubated with GST-Mcd1$^{169\text{-}337}$ (1 µg) for 60 minutes at 30ºC. Reactions were run on SDS-PAGE and analyzed by both Coomassie staining and exposure to a Phosphor screen.

α-FLAG, α-Eco1, α-Rad53 and α-acetyl-lysine Western blots

The cell pellet from a 200 ml culture grown to OD_{600}=0.8 in YPD was lysed in NP40 Lysis Buffer (50 mM Tris pH 7.5, 150mM NaCl, 10% Glycerol, 1% NP-40) by the addition of glass beads and beadbeating for 60 sec 5 times with 2 min intervals on ice. The supernatant was collected and the protein concentration was determined using the optical density of a standard curve and Bradford reagent. 50 µg of total protein was loaded onto a 4-12% Bis-Tris gel for SDS-PAGE. Proteins were transferred to a nitrocellulose membrane followed by blocking. For detection of Eco1-FLAG, α-FLAG antibody (Sigma #F3165) was used at a 1:2500 concentration followed by α-Mouse HRP (GE Healthcare #NA931V) at a 1:5000 concentration. For detection of overexpressed Eco1, polyclonal α-Eco1 antibody was used at 1:1000 followed by α-rabbit-HRP (GE Healthcare #NA934V) at a 1:5000 concentration. Smc3-HA was pulled down from whole cell extracts using anti-HA beads (Roche) and eluted using SDS buffer (10 mM Tris pH7.5, 1 mM EDTA, 1% SDS). A cocktail of deacetylase inhibitors (100 µM Trichostatin A, 50 mM nicotinamide, 50 mM sodium butyrate) was added during the immunoprecipitation process. The eluate was subjected to SDS-PAGE. Following transfer to a nitrocellulose membrane and blocking, α-acetyl-lysine antibody (Cell Signaling #9441) was used at a 1: 1000 concentration followed by α-Rabbit HRP as above. Detection was achieved with Amersham ECL Plus Western Blotting Detection System (GE Healthcare #RPN2132).

To detect phosphorylated Rad53, 75 µgs of protein were subjected to SDS-PAGE. Following transfer to a nitrocellulose membrane and blocking, α-phosphoRad53 antibody (Santa Cruz, SC-6749) was used at a concentration of 1:1000 followed by α-goat-AP (Promega, V1151) at a 1:5000 concentration. For signal detection, BCIP (5-bromo-4-chloro-3-indolyl-phosphate) was used in conjunction with NBT (nitro blue tetrazolium) for the colorimetric detection of alkaline phosphatase activity in the presence of alkaline phosphatase buffer (100 mM Tris-HCl [pH 9.0], 150 mM NaCl, 1 mM $MgCl_2$).

Yeast strains and tetrad analysis were constructed and analyzed by standard methods. The cohesion assays were carried out as previously described (Gard et al., 2009). The assay to select and analyze reciprocal mitotic crossovers has been extensively described (Barbera and Petes, 2006).

CHAPTER 3

THE COHESIN ACETYLTRANSFERASE ECO1 COODINATES rDNA REPLICATION AND TRANSCRIPTION

I am the first author of the following manuscript: The cohesin acetyltransferase Eco1 coordinates rDNA replication and transcription**,** submitted to **EMBO Report.**

CONTRIBUTIONS

FIGURE 3.1 I performed the GCN4-LacZ assay, FISH assay and Dr. Bo Xiong and Dr. Chris Seidel contributed genome transcription analysis.

FIGURE 3.2 Dr. Jennifer Gerton performed the growth assay. I performed the GCN4-LacZ assay.

FIGURE 3.3 Dr. Bo Xiong and Dr. Chris Seidel contributed genome transcription analysis.

FIGURE 3.4 Bethany Harris performed the growth assay.

FIGURE 3.5 I performed the FACS analysis and PFGE assay.

FIGURE 3.6 Bethany Harris and Dr. Kenneth Lee performed ChIP assays. Dr. Chris Seidel and I performed DNA sequencing assay and analysis. I performed the plasmid transformation assay.

FIGURE 3.7 Dr. Chris Seidel and I performed DNA sequencing analysis.

FIGURE 3.8 I performed the ChIP assay and analysis.

FIGURE 3.9 Dr. Anita Saraf, Gaye Hattem, and Dr. Laurence Florens contributed the proteomic analysis.

FIGURE 3.10 Bethany Harris performed the EM analysis. I performed the western blot and rDNA segregation assay.

ABSTRACT

Eco1 is the acetyltransferase that establishes sister-chromatid cohesion during DNA replication. A budding yeast strain with an *eco1* mutation that genocopies Roberts syndrome displays reduced ribosomal DNA (rDNA) transcription and a disrupted transcriptome. We show that deleting *FOB1*, a gene encoding a specific replication fork blocking protein for the rDNA region, rescues rRNA production and genome-wide transcriptional defects. Further studies show that deletion of *FOB1* corrects the genome-wide replication defects, nucleolar structure and chromosome segregation in an *eco1* mutant by allowing bidirectional replication of the rDNA. Our study highlights cohesin's central role at the rDNA for global control of DNA replication and gene expression.

INTRODUCTION

The evolutionarily conserved cohesin complex contributes to chromosome function in many ways. Cohesin contributes to the processes of chromosome segregation, DNA replication, chromosome condensation, and DNA damage repair. A previous study showed that cohesin mutations reduced ribosomal DNA (rDNA) transcription and translation in both budding yeast and human cells (Bose et al., 2012). Roberts syndrome is a human disease caused by mutation of ESCO2, a homolog of the yeast cohesin acetyltransferase *ECO1* gene (Vega et al., 2005). Mutations in cohesin are also associated with a second developmental disorder, Cornelia de Lange syndrome, as well as myeloid neoplasms (Cancer Genome Atlas Research, 2013; Kon et al., 2013; Liu and Krantz, 2008). These diseases are likely to be caused by changes in gene expression, rather than chromosome segregation defects (Bose and Gerton, 2010; Liu and Krantz, 2008; Liu et al., 2009; Wendt et al., 2008). However, the mechanisms by which the cohesin complex influences the whole transcriptome are unclear.

Cohesin binds to the approximately 150 highly transcribed tandem repeats that make up the budding yeast rDNA locus (Laloraya et al., 2000). In fact, cohesin binds to the rDNA regions in every eukaryotic genome in which binding has been examined. Active replication challenges this highly transcribed region. Fob1 controls locus replication in budding yeast, allowing it to occur only in the direction of transcription. The replication fork barrier provided by Fob1 ensures the replication apparatus will not disrupt transcription of the 35S gene (Brewer

and Fangman, 1988; Linskens and Huberman, 1988). A previous genetic interaction screen has shown that cohesin mutants require the function of genes that mediate replication fork progression (McLellan et al., 2012). Indeed, DNA replication forks move more slowly in human ESCO2 mutant cells (Terret et al., 2009). Moreover, the observation of heterochromatic repulsion in Roberts syndrome patient cells suggests that these regions might have cohesion defects due to difficulty with replication (Vega et al., 2005). These regions include centromeres and the nucleolar organizing centers. In budding yeast, the heterochromatic DNA domains include telomeres, the silent mating type loci, and the rDNA repeats. The cohesin complex binds adjacent to the replication fork barrier site (Laloraya et al., 2000) and is important for replication fork restart (Tittel-Elmer et al., 2012). All together, these results indicate an intimate connection between cohesin function and DNA replication, and perhaps a special role for cohesin at the rDNA.

In this study, we observed many defects in DNA replication in an *eco1* mutant. Defects in replication, rRNA production, and gene expression in general, can be rescued by allowing replication at the rDNA to become bidirectional in an *eco1* mutant. While replication defects have also been reported in other cohesin mutants (Crabbe et al., 2010; Guillou et al., 2010; Lengronne et al., 2006; Lopez-Serra et al., 2013; Terret et al., 2009), what has not been appreciated is that the replication defects may interfere with transcription of the rDNA region. We

propose that replication defects associated with mutations in cohesin have a major influence on gene expression.

RESULTS AND DISCUSSION

FOB1 deletion partially rescues the genome-wide expression pattern in an *eco1* mutant

Because Fob1 coordinates transcription and replication at the rDNA locus and because *FOB1* deletion rescues some phenotypes associated with *smc5-smc6* mutants (Torres-Rosell et al., 2007), we wondered how deletion of *FOB1* would affect the phenotypes associated with the *eco1-W216G* mutation. This mutation is associated with decreased acetyltransferase activity in Roberts syndrome (Gordillo et al., 2008; Lu et al., 2010). Gcn4 is a transcriptional activator that is translated when translational activity is poor (Hinnebusch, 2005). We initially employed a Gcn4-lacZ reporter as an indicator for ribosome function. The *eco1-W216G* strain shows a nearly 4 fold increase of *β-galactosidase* activity compared to a wild type strain (Bose et al., 2012). Interestingly, when we deleted the *FOB1* gene in the *eco1* mutant background, *β-galactosidase* levels were reduced (**Figure 3.1A**). The decrease in *β-galactosidase* activity suggested that the poor translational activity in the *eco1-W216G* strain was rescued by *FOB1* deletion. Moreover, when the Fob1 protein was over-expressed, the *β-galactosidase* activity in the *eco1-W216G* mutant increased further (**Figure 3.2**), indicating further impaired translational activity.

Figure 3.1

Differential gene expression in the eco1 mutant was partially rescued by *FOB1* deletion.

(A) WT and indicated mutant strains were transformed with the p180 plasmid that contains Gcn4-lacZ. β-galactosidase activities for each strain were measured in triplicate from mid log phase cells grown in YPD + CSM. All values were normalized to the level of the WT stain and are shown in arbitrary unit (a.u.). The p values were calculated by Student's t test, comparing mutant to wild type. **(B)** Strains with a unique sequence inserted into one rDNA repeat and the indicated mutation were measured for their transcription by FISH. For each strain, at least three independent cultures were monitored and at least 300 cells per culture were quantified. In the plot shown, the dot is the average, the box is the 95% confidence standard error, and the horizontal line within the box is the median. The p values were calculated by Student's t test, comparing mutant to WT. **(C)** Venn diagrams of upregulated genes and downregulated genes with $p < 0.05$ in the indicated strains are shown. **(D)** Genes with Gcn4 or Tbp1 binding sites in their promoters were assessed as a group in each data set by a gene set enrichment test. The resulting p-values are shown as $-\log_{10}$(p-value). The p value is calculated by a hypergeometric test using the number of differentially expressed genes with the binding site versus the number of genes in the genome with the site.

Figure 3.1

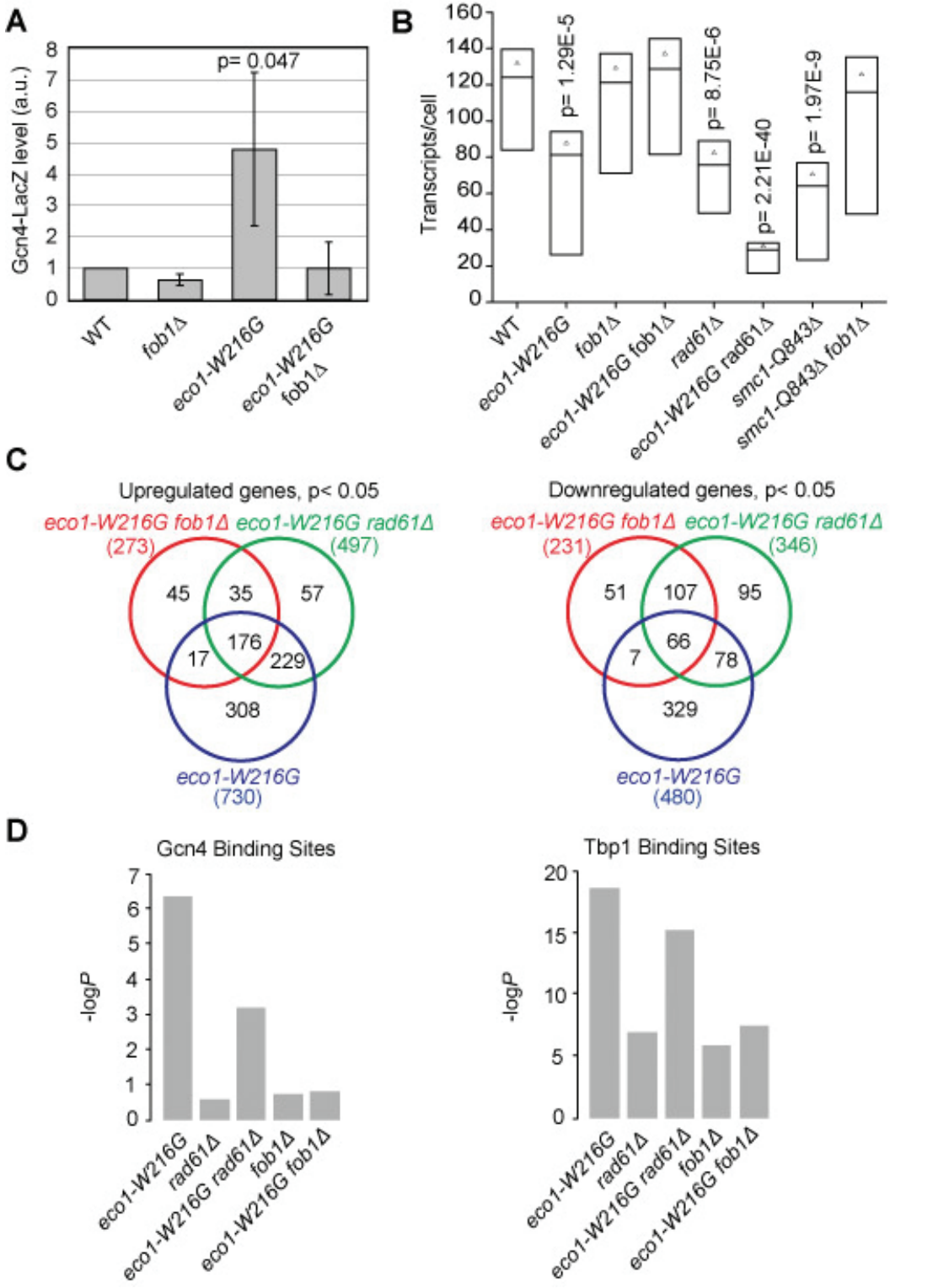
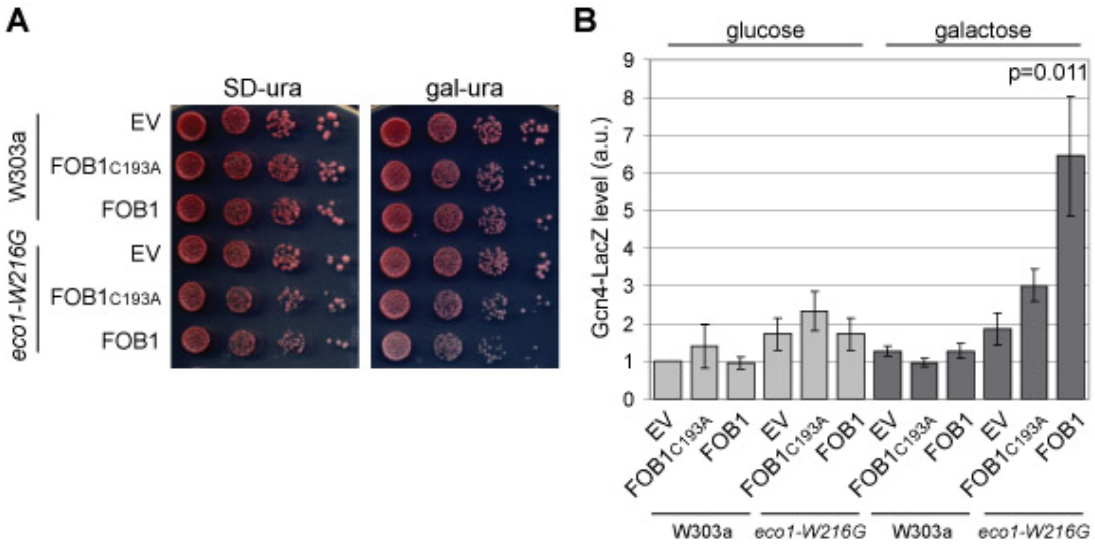

A
Gcn4-LacZ level (a.u.)
p= 0.047
WT
fob1Δ
eco1-W216G
eco1-W216G fob1Δ
B
Transcripts/cell
p= 1.29E-5
p= 8.75E-6
p= 2.21E-40
p= 1.97E-9
WT
eco1-W216G
fob1Δ
eco1-W216G fob1Δ
rad61Δ
eco1-W216G rad61Δ
smc1-Q843Δ
smc1-Q843Δ fob1Δ
C
Upregulated genes, p< 0.05
eco1-W216G fob1Δ
eco1-W216G rad61Δ
(273)
(497)
45
35
57
176
17
229
308
eco1-W216G
(730)
Downregulated genes, p< 0.05
eco1-W216G fob1Δ
eco1-W216G rad61Δ
(231)
(346)
51
107
95
66
7
78
329
eco1-W216G
(480)
D
Gcn4 Binding Sites
-logP
eco1-W216G
rad61Δ
eco1-W216G rad61Δ
fob1Δ
eco1-W216G fob1Δ
Tbp1 Binding Sites
-logP
eco1-W216G
rad61Δ
eco1-W216G rad61Δ
fob1Δ
eco1-W216G fob1Δ

Figure 3.2

Overexpression of *FOB1* impairs growth and increases Gcn4-lacZ expression in the *eco1-W216G* strain.

(A) Growth assay of WT and *eco1-W216G* strains with the indicated galactose-inducible over-expression CEN plasmids or empty vector (EV) on SD-ura and gal-ura plates. **(B)** WT and *eco1-W216G* strains with Gcn4-lacZ integrated into the genome were transformed with the indicated galactose-inducible over-expression plasmids. β-galactosidase activity for each strain was measured in triplicate from mid log phase cells grown in YPD + CSM or YPgal + CSM, as indicated. The C193A point mutation in the zinc finger motif of *FOB1* has been shown to disrupt its association with the RFB. All values were normalized to the level of the WT strain grown in YPD + CSM and are shown in arbitrary units (a.u.). The histogram represents the average of four biological replicates. Error bars indicate standard deviation. The p value was calculated by Student's t test, comparing the level of Gcn4-lacZ with Fob1 over-expression (galactose) to the level of Gcn4-lacZ without Fob1 over-expression (glucose) in the *eco1-W216G* strain.

Figure 3.2


A
SD-ura
gal-ura
W303a
eco1-W216G
EV
FOB1C193A
FOB1
EV
FOB1C193A
FOB1
B
glucose
galactose
p=0.011
Gcn4-LacZ level (a.u.)
0
1
2
3
4
5
6
7
8
9
EV
FOB1C193A
FOB1
EV
FOB1C193A
FOB1
EV
FOB1C193A
FOB1
EV
FOB1C193A
FOB1
W303a
eco1-W216G
W303a
eco1-W216G

Ribosome function depends on rRNAs that are transcribed from the rDNA locus. We speculated that deleting the *FOB1* gene rescued the ribosome function in the *eco1-W216G* mutant by rescuing rDNA transcription. In order to directly measure rDNA transcription, we used fluorescence in situ hybridization (FISH) to detect transcription of a single ribosomal repeat by inserting a unique sequence recognizable by a probe (Tan and van Oudenaarden, 2010). As previously observed, the rRNA transcript level in the *eco1-W216G* strain was approximately half of the observed value in a WT strain (Bose et al., 2012). However, deleting the *FOB1* gene in the *eco1-W216G* strain restored rRNA transcripts to levels comparable to a WT strain (**Figure 3.1B**). For comparison, we measured rRNA transcripts in an *eco1-W216G rad61Δ* double mutant strain. *RAD61* is a negative regulator for cohesion establishment (Gandhi et al., 2006; Kueng et al., 2006; Sutani et al., 2009) and its deletion rescues the temperature sensitivity of the *eco1-W216G* strain, but not the elevated expression from the Gcn4-lacZ reporter (Rolef Ben-Shahar et al., 2008). While *fob1Δ* is expected to have an rDNA-specific effect, *rad61Δ* should produce a more general effect on cohesin. In contrast to *fob1Δ*, *rad61Δ* did not rescue rRNA transcription in the *eco1-W216G* strain.

Eco1 has other targets in addition to the subunits of the cohesin complex (Ghosh et al., 2012; Ivanov et al., 2002). To exclude the possibility that *fob1Δ* might rescue rDNA transcription through a different mechanism, we measured the rRNA level in a *smc1-Q843Δ fob1Δ* double mutant. Smc1 is a subunit of the

cohesin complex. The mutation is a single amino acid deletion that is associated with the human developmental disorder Cornelia de Lange syndrome (Bose et al., 2012). We found that the decreased rRNA level in the *smc1-Q843Δ* strain could also be rescued by *fob1Δ* (**Figure 3.1B**). This further suggests that *fob1Δ* rescues rDNA transcription through a cohesion-related mechanism.

To assess the effect of *fob1Δ* on genome-wide gene expression in the *eco1-W216G* strain, we performed microarray analysis of RNA from the following strains: 1) *eco1-W216G*, 2) *eco1-W216G fob1Δ*, 3) *eco1-W216G rad61Δ*, 4) *fob1Δ*, 5) *rad61Δ*, and 6) WT. Differentially expressed gene sets were selected based on a fold change between mutant and WT of at least 1.4 fold and an adjusted p-value less than 0.05. We found that the number of differentially expressed genes was less in the *eco1-W216G fob1Δ* strain (504) than in the *eco1-W216G* strain (1210) (**Figure 3.3**). The *eco1-W216G fob1Δ* strain also had fewer differentially expressed genes than the *eco1-W216G rad61Δ* strain (843, **Figure 3.1C**, **Figure 3.3**). Since genes containing Gcn4 and Tbp1 binding sites are significantly differentially expressed in the *eco1-W216G* strain (Bose et al., 2012), we asked if these targets were less differentially expressed in the double mutant strains. Gcn4 and Tbp1 are sequence specific transcriptional activators. Tbp1 is a TATA-binding protein that functions with TFIID in the activation of eukaryotic genes transcribed by RNA polymerase II. Interestingly, the number of differentially expressed genes with these sites was decreased in *eco1-W216G fob1Δ* strain compared to the *eco1-W216G* and the *eco1-W216G rad61Δ* strain

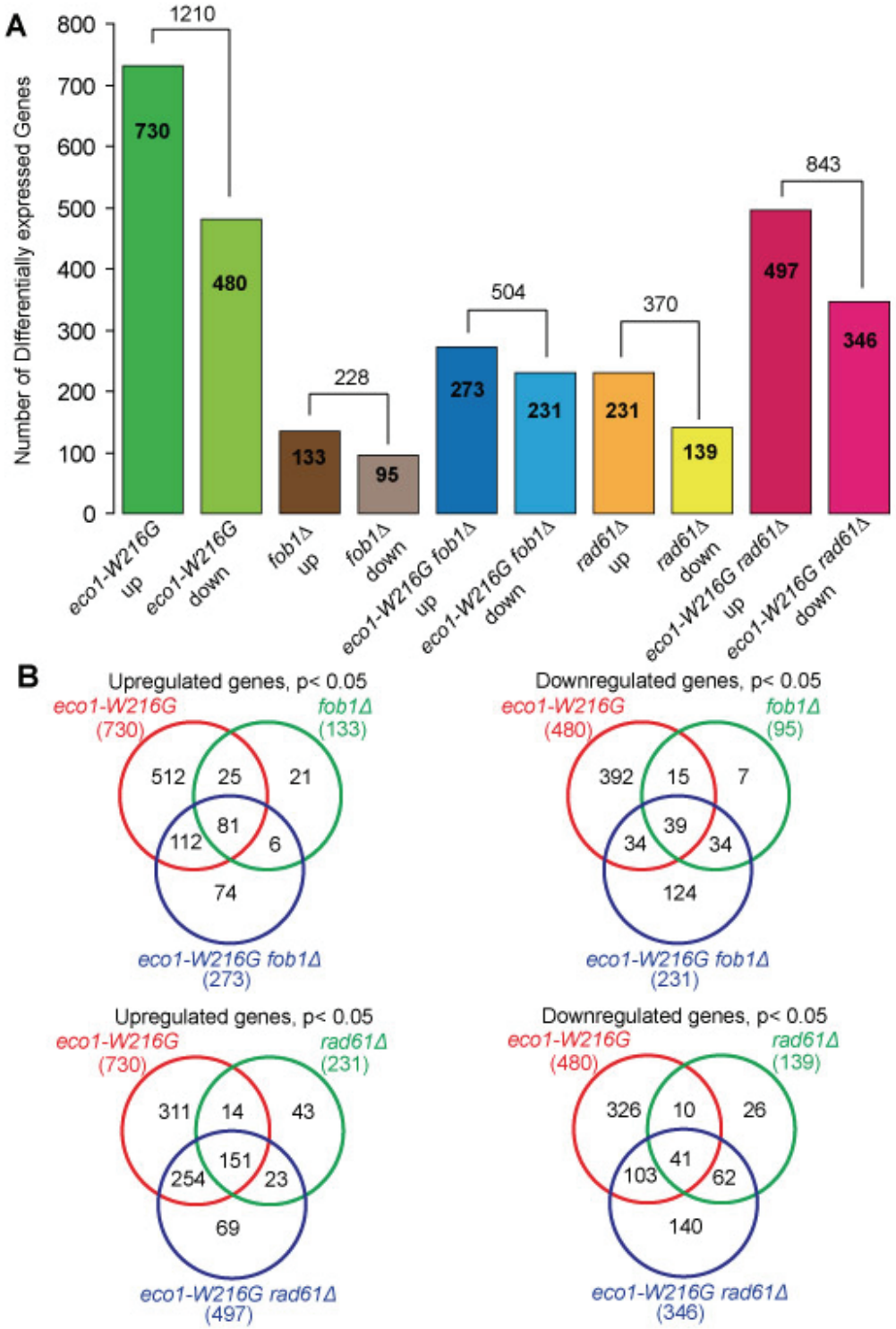
(**Figure 3.1D**). Collectively, these experiments suggest that the differential gene expression in the *eco1-W216G* strain may be due in part to reduced levels of rRNA. Restoration of rRNA levels significantly rescues the transcriptional profile of the mutant.

Figure 3.3

Differentially expressed genes in the indicated strains

(A) Total number of differentially expressed genes in the indicated strains. **(B)** Venn diagrams of differentially expressed genes with $p < 0.05$ in the indicated strains.

Figure 3.3


A
Number of Differentially expressed Genes
800
700
600
500
400
300
200
100
0
1210
730
480
228
133
95
504
273
231
370
231
139
843
497
346
eco1-W216G up
eco1-W216G down
fob1Δ up
fob1Δ down
eco1-W216G fob1Δ up
eco1-W216G fob1Δ down
rad61Δ up
rad61Δ down
eco1-W216G rad61Δ up
eco1-W216G rad61Δ down
B
Upregulated genes, p< 0.05
eco1-W216G (730)
fob1Δ (133)
512
25
21
81
112
6
74
eco1-W216G fob1Δ (273)
Downregulated genes, p< 0.05
eco1-W216G (480)
fob1Δ (95)
392
15
7
39
34
34
124
eco1-W216G fob1Δ (231)
Upregulated genes, p< 0.05
eco1-W216G (730)
rad61Δ (231)
311
14
43
151
254
23
69
eco1-W216G rad61Δ (497)
Downregulated genes, p< 0.05
eco1-W216G (480)
rad61Δ (139)
326
10
26
41
103
62
140
eco1-W216G rad61Δ (346)

FOB1 deletion rescues DNA replication defects associated with the *eco1* mutation

The above results prompted us to ask how *fob1Δ* rescues rDNA transcription in the *eco1* mutant strain. Given the replication fork blocking activity of Fob1 at the rDNA, we speculated that *fob1Δ* would rescue the *eco1-W216G* mutant by means of a DNA replication mechanism.

To explore DNA replication in the *eco1-W216G* mutant strain, we first measured the cell cycle progression by cytometry analysis. We synchronized the cells in G1 by α factor treatment and then released them to pass through S phase. In order to examine the replication timing difference, we released yeast from G1 phase at 33°C. This is still a permissive temperature for growth, but the *eco1-W216G* mutation is lethal at 37°C, so we reasoned 33°C might accentuate any phenotype (**Figure 3.4**). Interestingly, after G1 release, a shift in DNA content was observed at 20 minutes in the *eco1-W216G* mutant, which is earlier than WT, indicating earlier progression to S phase in the *eco1-W216G* strain (**Figure 3.5A**). However, both WT and *eco1-W216G* strains complete the shift to 2N at approximately the same time, suggesting the *eco1-W216G* strain might take longer to complete replication than WT. To assess the effect of *fob1Δ* on cell cycle progression in *eco1-W216G* strain, we also measured cell cycle progression in *fob1Δ* and *eco1-W216G fob1Δ* strains. The earlier initiation of S phase was no longer present in the double mutant, indicating the replication defect might be rescued by *FOB1* deletion (**Figure 3.5A**).

Figure 3.4

Reduced rDNA copy number does not cause a synthetic growth effect in the *eco1-W216G* mutant strain.

Serial dilutions of WT, *eco1-W216G*, *fob1Δ*, *eco1-W216G fob1Δ*, and indicated mutant strains with 20 copies or 40 copies of rDNA were spotted onto YPD plates at 30° C, 33° C and 37° C.

Figure 3.4

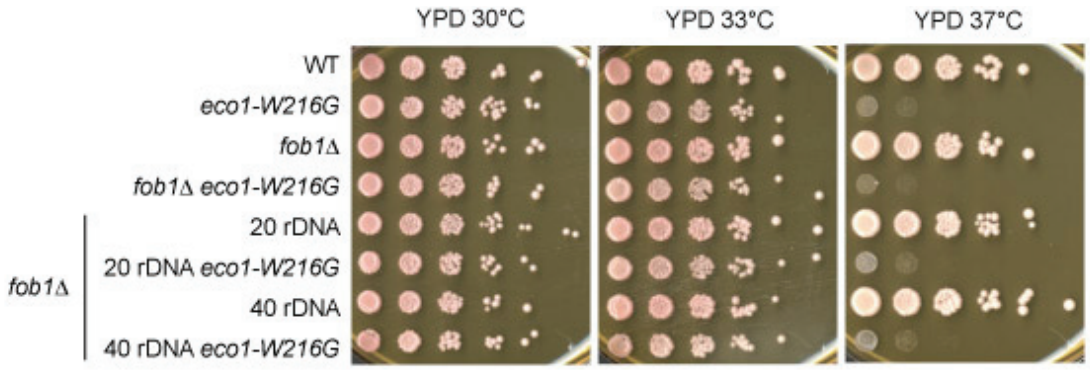
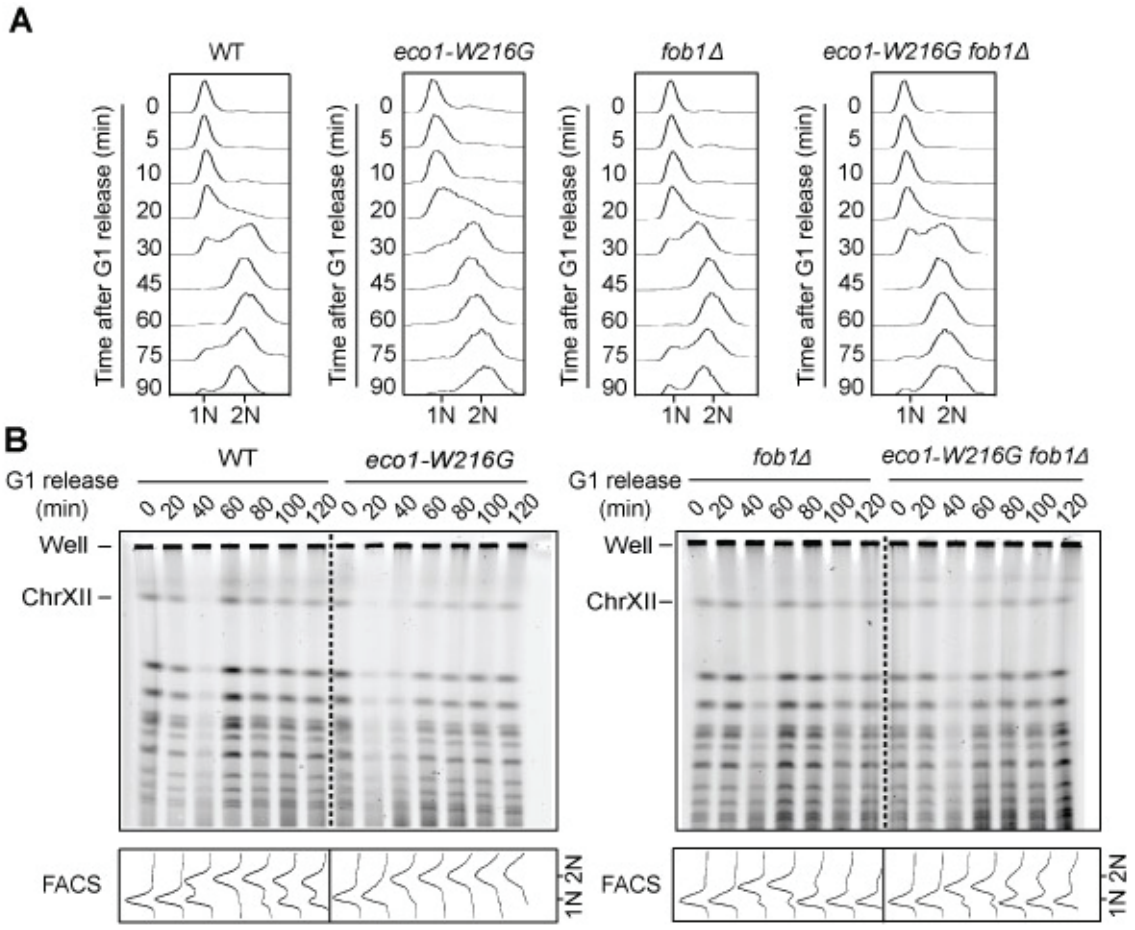


Figure 3.5

DNA replication defects in the *eco1* mutant are rescued by *FOB1* deletion. **(A)** Each strain was synchronized in G1 using α factor at 30°C, released at 33°C and samples were collected at the indicated time points for analysis of DNA content by cytometry. **(B)** Each strain was synchronized in G1 using α factor at 30°C, released at 33°C, and another dose of α factor was added at 60 min to avoid a second round of DNA replication. DNA samples were collected for PFGE at the indicated time points.

Figure 3.5


A
WT
eco1-W216G
fob1Δ
eco1-W216G fob1Δ
Time after G1 release (min)
0
5
10
20
30
45
60
75
90
1N 2N
B
WT
eco1-W216G
fob1Δ
eco1-W216G fob1Δ
G1 release (min)
0 20 40 60 80 100 120
Well
ChrXII
FACS
1N 2N

We next used pulsed field gel electrophoresis (PFGE) to examine DNA replication of the above four strains. PFGE is able to distinguish replication status because chromosomes cannot migrate into the gel while undergoing replication due to replication intermediates. We synchronized the cells in G1 by α factor treatment and then released them, allowing them to pass through S phase, and then added another dose of α factor at 60 minutes to prevent a second round of DNA replication. At the indicated time points, DNA samples were collected for PFGE. Consistent with the above result for cell cycle progression, less chromosome migration was detectable at 20 minutes in *eco1-W216G* strain, which is earlier than the WT strain (**Figure 3.5B**). This result confirmed that DNA replication initiated earlier in the *eco1-W216G* strain, and further demonstrated that all chromosomes were affected. Early initiation of DNA replication was no longer observed in the *eco1-W216G fob1Δ* strain (**Figure 3.5B**), demonstrating that the DNA replication defect could be rescued by *fob1Δ*. These experiments suggested that deletion of the rDNA specific fork blocking factor *FOB1* was rescuing a genome-wide replication defect in the *eco1-W216G* mutant.

While Fob1 has fork blocking activity, it also regulates recombination and copy number at the rDNA. Eco1 plays a role in DNA damage repair and recombination (Lu et al., 2010; Strom et al., 2007; Unal et al., 2007). However, the *eco1* mutation does not affect recombination or copy number at the rDNA locus (Bose et al., 2012; Gard et al., 2009), nor does it have a synthetic growth defect with lower copy number of rDNA (**Figure 3.4**), suggesting that *fob1Δ* is unlikely to

rescue recombination or copy number issues. In addition, deletion of *FOB1* alone does not alter the frequency of origin firing in the rDNA or the fraction of active rDNA genes (Burkhalter and Sogo, 2004). Therefore, the most likely mechanism by which *fob1Δ* rescues the DNA replication defect in the *eco1-W216G* mutant is by allowing bidirectional replication at the rDNA. This may promote the completion of replication of the rDNA, facilitating efficient transcription of the locus. Deletion of *FOB1* has also been shown to partially relieve replication stress in the *smc6-9* mutant at the rDNA locus (Torres-Rosell et al., 2007). Since the Smc5-Smc6 complex is a close relative of the cohesin complex, it suggests that there might be a shared role for SMC complexes in regulating rDNA replication.

Eco1 regulates origin firing activity

In what ways could cohesin be regulating the firing of DNA replication origins? To address this question, we first investigated origin firing activity at the rDNA region of WT and *eco1* mutant cells. Chromatin immunoprecipitation for Cdc45, a replication initiation factor and an indicator for origin firing (Tanaka et al., 2011; Zou et al., 1997), was performed from the WT and *eco1* mutant cells. To measure the kinetics of Cdc45 binding, we released yeast from G1 arrest at 16°C in order to slow down the replication process. As shown in **Figure 3.6A**, the level of Cdc45 binding to the rDNA origin of replication (rARS) in the *eco1* mutant peaked at 90 minutes, which is earlier than the peak at 105 minutes observed in WT cells. This observation indicates that the rARS fires earlier in the *eco1* mutant

compared to WT. This result is consistent with our observation that DNA replication starts earlier in the *eco1* mutant.

To study how the *eco1* mutation affects replication genome-wide, we measured DNA content by deep sequencing of genomic DNA of WT and *eco1* cells (Muller and Nieduszynski, 2012; Natsume et al., 2013). Samples of genomic DNA were collected at 0 and 20 minutes following release from G1 arrest. As shown in **Figure 3.6B** and **Figure 3.7A**, the pattern of the origin firing is different between WT and *eco1* strains. More early origins fire in the WT strain compared to the *eco1* mutant strain, but late origins fire about equally well in the two strains at 20 minutes, indicating the origin firing sequence is disrupted in the *eco1* mutant. In addition, origin firing in the *eco1* mutant occurred not only at origins of replication (ARS) sites, but also at non-ARS sites (**Figure 3.6B**, **Figure 3.7B**). However, the replication from individual sites was generally less pronounced in the *eco1* mutant compared to WT. This might due to the titration of the replication factors by the firing of many additional sites. Replication factors can be limiting for replication progression (Zhong et al., 2013).

To confirm the origin firing defect in the *eco1* mutant, we measured origin activity by transforming WT and *eco1* mutant strains with plasmids containing 1) no ARS sequence, 2) rARS sequence or 3) ARS1 sequence (Kwan et al., 2013). ARS1 is a well-studied highly efficient early ARS located on chromosome IV. These plasmids enable us to assess the ability of these sequences to promote

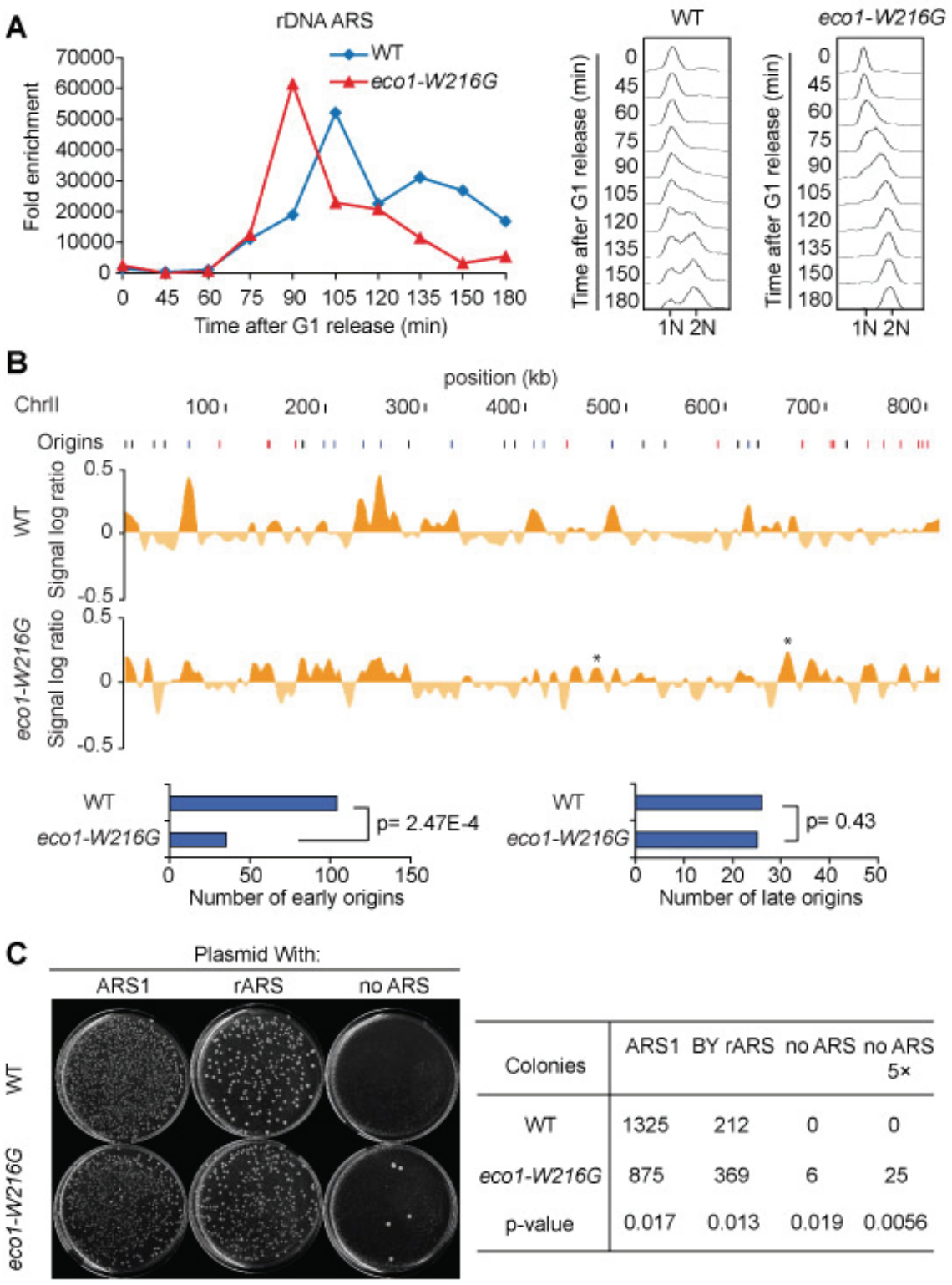
autonomous plasmid maintenance. In the genome each rDNA repeat contains the rARS sequence. However, in a given cell cycle approximately 1 in 5 of these rARSs will fire (Pasero et al., 2002). We observed more transformants for the rARS containing plasmid in the *eco1-W216G* background compared to WT, using the same amount of plasmid DNA (**Figure 3.6C**), suggesting more firing of this ARS in the mutant. There were fewer transformants for the ARS1 containing plasmid in the *eco1-W216G* strain, which is consistent with the result derived from sequencing that ARS1 is firing less efficiently in the *eco1-W216G* mutant than in WT (**Figure 3.7C**). Interestingly, the noARS containing plasmid could be replicated with low efficiency in the mutant (**Figure 3.6C**), further confirming the origin fidelity defect observed in genome-wide sequencing. The above results suggest that origin firing is regulated by cohesin.

Figure 3.6

Replication origin activity was disrupted by the *eco1* mutation.

(A) ChIP of Cdc45-FLAG was performed with α-Flag antibody and analyzed by semiquantitative PCR using primers specific for the rDNA ARS. WT and *eco1-W216G* strains with Cdc45-Flag were synchronized in G1 using α factor at 30°C, released at 16°C, and samples were collected at the indicated time points. **(B)** Strains were cultured as in Figure 2A. Genomic DNA was collected at 20 min and sequenced. The signal intensities relative to a G1 phase strain are shown along ChrII of *S. cerevisiae*. Early and late origins along ChrII are indicated using blue and red color, respectively. Origins shown in black indicate the ARS is either inactive or replication timing data is not available. The asterisks indicate replication at non-ARS sites. The lower panel shows the numbers of early and late origins fired in the indicated strains. The number of fired origin was calculated by counting the peaks on all chromosomes using a 5kb window centered by origin. We observed similar patterns of origin firing in biological replicates. The p values were calculated by Student's t test, comparing mutant to WT. **(C)** DNA origin activity in WT and *eco1-W216G* strains was measured using plasmids. Strains transformed with the indicated plasmid were replica-plated to YPD plates with G418 after a day of growth on YPD medium to assess the efficiency of origin firing. The number of colonies is shown to the right. The p values were calculated by Student's t test, comparing the *eco1-W216G* mutant to WT.

Figure 3.6



Colonies	ARS1	BY rARS	no ARS	no ARS 5×
WT	1325	212	0	0
eco1-W216G	875	369	6	25
p-value	0.017	0.013	0.019	0.0056

Figure 3.7

Replication profiles of the indicated strains

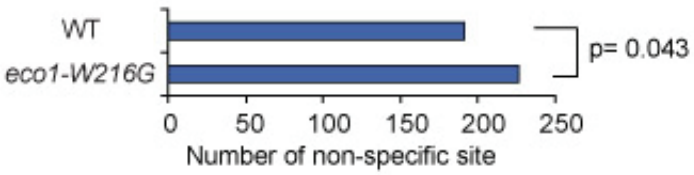
Strains were cultured as in **Figure 3.2A**. Genomic DNA was collected at 0 and 20 min and sequenced. The signal intensities relative to a G1 arrested strain are shown along ChrII of *S.cerevisiae*. Early and late origins along ChrII are indicated using blue and red color, respectively. Origins shown in black indicate the ARS is either inactive or replication timing data is not available. **(A)** Numbers of early and late origin fired in the indicated strains on each chromosome. **(B)** Numbers of replication forks at non-specific sites in the indicated strains. The p values were calculated by Student's t-test, comparing mutant to WT. **(C)** The gray box highlights the replication profile of ARS1 on ChrIV.

Figure 3.7

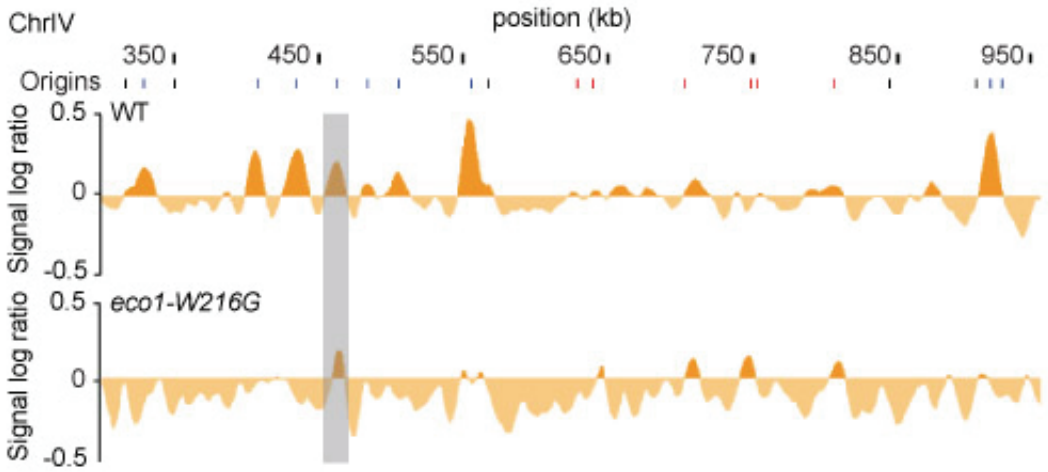
A

	Early		Late	
	WT	*eco1-W216G*	WT	*eco1-W216G*
ChrI	2	1	2	3
ChrII	5	4	2	5
ChrIII	6	3	0	1
ChrIV	7	2	4	2
ChrV	8	3	1	1
ChrVI	3	1	0	0
ChrVII	10	2	0	2
ChrVIII	5	3	2	2
ChrIX	4	2	1	2
ChrX	11	2	0	0
ChrXI	7	3	3	3
ChrXII	8	1	1	1
ChrXIII	5	3	4	0
ChrXIV	4	1	3	3
ChrXV	11	1	0	0
ChrXVI	8	3	3	0

B



C



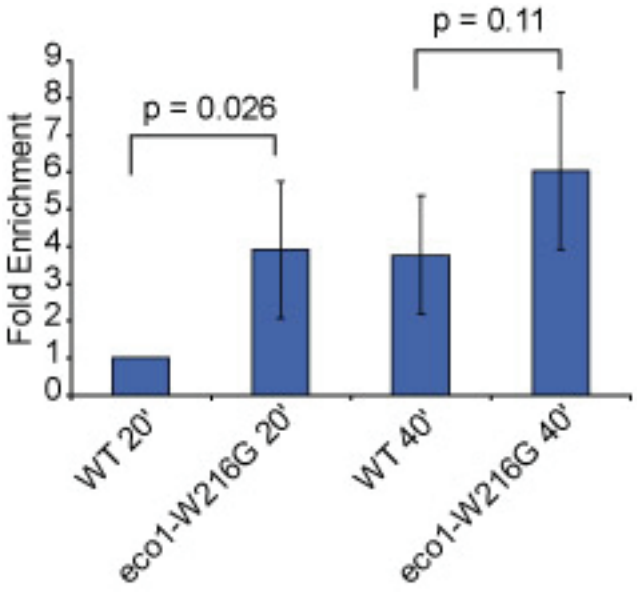
In order to measure firing of the rARS, we decided to use a BrdU incorporation method (Viggiani et al., 2010). The deep sequencing method did not yield information about rARS firing because the data was outside the linear range of the assay. Cells were arrested in G1 with α factor and then released into medium with BrdU. BrdU incorporation was detected using ChIP followed by qPCR. The enrichment for rDNA sequence in the *eco1-W216G* mutant strain was higher than in the WT strain at 20 min, further confirming that the rDNA starts replication earlier in the mutant strain (**Figure 3.8**). However, at the 40 min timepoint, the rDNA enrichment in WT is comparable to that of the *eco1-W216G* strain, suggesting replication fork speed at the rDNA region is slower in the mutant than WT. In budding yeast a replication fork travels an average of 20 kb, but at the rDNA the average distance is closer to 50 kb, making these replication forks some of the longest in the genome (Pasero et al., 2002; Tercero and Diffley, 2001). Furthermore, although these ARSs fire early, the replication of the region continues throughout S phase (Brewer et al., 1980). The observed defects in replication are consistent with the hypothesis that prolonged replication of the rDNA could be interfering with its transcription in the *eco1-W216G* mutant strain.

Figure 3.8

ChIP comparing BrdU incorporation at the rDNA locus in WT and *eco1-W216G* strains

WT and *eco1-W216G* strain were synchronized in G1 and released in the presence of BrdU for 20 and 40 minutes, respectively. Nucleotide incorporation was measured by α-BrdU chromatin immunoprecipitation, followed by qPCR using primers specific for the rDNA region. Enrichment of BrdU incorporation was normalized to the WT strain at 20 min. The histogram represents three biological replicates with PCR for each replicate conducted in duplicate. Error bars indicate standard deviation from three independent biological replicates. The P value is calculated by Student's t-test comparing BrdU incorporation between the WT and *eco1-W216G* strain at 20 minutes and 40 minutes.

Figure 3.8



Cohesin is reported to be enriched at replication origins and to spatially organize replication factories (Guillou et al., 2010). One possibility is that cohesin directly regulates origin firing at ARS sites. Another possible mechanism that has been previously suggested is that mutations in cohesin alter the dNTP pool (Lopez-Serra et al., 2013). It has been shown that increases in the nucleotide pool can modulate origin choice and interorigin spacing (Anglana et al., 2003; Gay et al., 2010). In a genome wide proteomic study of the *eco1-W216G* strain, we found evidence supporting the latter possibility. Many proteins involved in dNTP synthesis were present at higher levels in the *eco1-W216G* mutant, which could increase the dNTP pool (**Figure 3.9**). Once too many regions fire, nucleotide pools and replication factors could be depleted such that replication forks cannot proceed with optimal speed (Bester et al., 2011). Therefore, cohesin may influence origin usage, firing fidelity and timing in part through its effect on gene expression.

Figure 3.9

Proteins involved in purine biosynthetic processes are enriched in the *eco1-W216G* strain compared to a WT strain.

Gene ontology analysis was performed on a filtered list of proteins that showed a significant increase or decrease in protein levels between the *eco1-W216G* mutant and the W303a wild-type. The list of proteins from the plgem analysis were filtered for a signal-to-noise ratio >|0.2| and a p-value<0.05. An additional filter required that the protein be detected in at least two of four wild-type samples for the down-regulated proteins and two of four *eco1-W216G* mutant samples in the up-regulated samples. The filtered list was then annotated with gene ontology terms using the biomaRt. The resulting list of gene ontology terms were summarized using the web-based tool REVIGO by combining terms based on semantic similarity using the SimRel similarity measure.

Figure 3.9

Gene	p-value	Function
CYS3	0.020694	purine nucleotide biosynthetic process
ADE5,7	0.049135	purine base metabolic process, purine nucleotide biosynthetic process
HOM3	0.008462	purine nucleotide biosynthetic process
LYS2	0.000762	purine nucleotide biosynthetic process
AMD1	0.042732	purine base metabolic process, purine nucleotide biosynthetic process
YBT1	0.02437	purine nucleotide biosynthetic process
ARG5,6	0.02276	purine nucleotide biosynthetic process
GAD1	0.027187	purine nucleotide biosynthetic process
MET14	0.045645	purine nucleotide biosynthetic process
POL1	0.035713	nucleobase-containing compound metabolic process

FOB1 deletion rescues nucleolar morphology and chromosome segregation defects associated with the *eco1* mutation

Previous reports used bright field microscopy and indirect immunofluorescence to demonstrate that the nucleolar morphology and subnuclear organization of chromatin was disrupted in cohesin mutants (Bose et al., 2012; Gard et al., 2009). We decided to use a higher resolution method that does not depend on the visualization of any particular protein to analyze nucleoli, the home of the rDNA repeats. Using electron microsopy, the budding yeast nucleolus can be observed as a single dense crescent-shaped structure abutting the nuclear envelop in a WT strain. However, in the *eco1-W216G* strain, the nucleolus is irregularly shaped (**Figure 3.10A**). To assess the effect of *fob1Δ* on nucleolar morphology, we analyzed nucleoli in *fob1Δ* and *eco1-W216G fob1Δ* strains. Interestingly, the irregular nucleolar morphology in the *eco1-W216G* strain was rescued by bidirectional rDNA replication (**Figure 3.10A**). In contrast to *fob1Δ*, *rad61Δ* had less of a rescue effect for nucleolar structure. The lack of rescue with *rad61Δ* correlates with the lack of rescue for rDNA transcription and the global transcriptional profile.

Because cohesion establishment is coupled to DNA replication (Lengronne et al., 2006; Rolef Ben-Shahar et al., 2008; Unal et al., 2008), we wondered whether *fob1Δ* restored nucleolar morphology by improving the levels of acetylated cohesin. To address this question, we measured the acetylation of K112 and K113 of Smc3, the lysines targeted by Eco1 for replication-coupled cohesion

(Rolef Ben-Shahar et al., 2008; Unal et al., 2008). The acetylation level of Smc3 was not rescued by *fob1Δ* (**Figure 3.10B**). This suggests that the recovery of nucleolar morphology in the double mutant is not through restoration of acetylation, but is more likely due to the rescue of the replication and transcription of the rDNA locus.

Replication stress could induce chromosome segregation defects and induce genome instability (Burrell et al., 2013; Chan et al., 2009). To study rDNA segregation, we used tetR-GFP to detect tetO repeats (Torres-Rosell et al., 2007). The operator sequence was inserted in the telomere proximal end of the rDNA (**Figure 3.10C**). We observed that in the *eco1-W216G* strain, ~50% of spots did not segregate correctly. Moreover, DNA was stretched across the bud neck region as detected by DAPI staining (**Figure 3.10C**). This suggests that neither the rDNA nor the rest of the genome is segregated efficiently at anaphase. Next, we addressed whether the chromosome segregation defects in the *eco1-W216G* strain could be rescued by relieving replication defects through *fob1Δ*. We observed that in the *eco1-W216G fob1Δ* double mutant strain, the segregation defect was rescued. This suggests that the replication defect induced by *eco1* mutation could introduce chromosome segregation defects. Replication stress has been reported to cause sister-chromatid bridging, especially at fragile loci (Chan et al., 2009). The rDNA locus is one of the most fragile sites in the eukaryotic genome and could play a “sensor” role for cellular functions (Kobayashi, 2008; Lemoine et al., 2005; Szilard et al., 2010). Our study

suggests cohesin acts as a pivotal factor to regulate gene expression and DNA replication in the rDNA region and genome-wide.

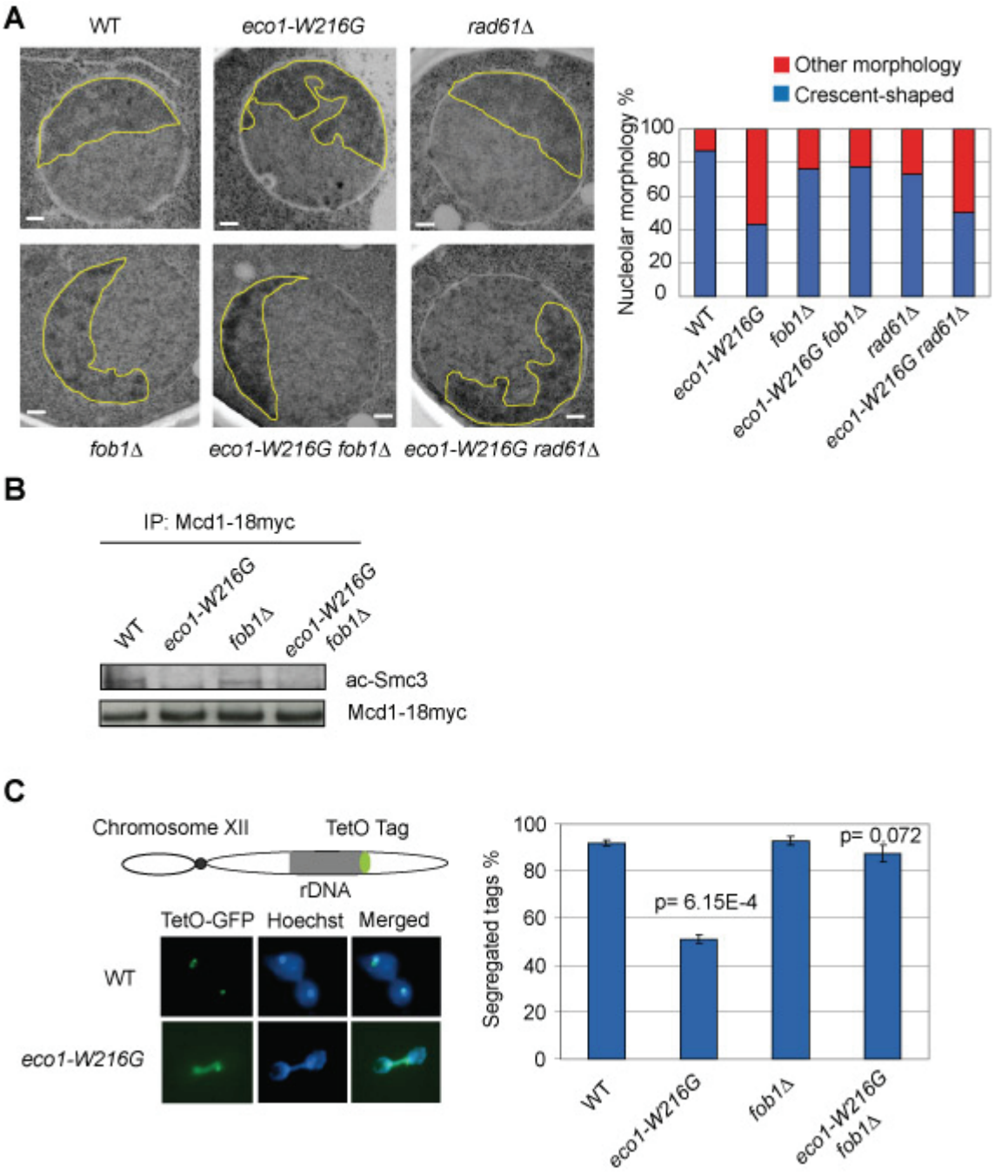
Mutations in cohesin have been associated with the human developmental disorders known as the cohesinopathies (Liu and Krantz, 2008), as well as cancer (Cancer Genome Atlas Research, 2013; Kon et al., 2013; O'Neil et al., 2013; Xu et al., 2011). Both Roberts syndrome (Bose and Gerton, 2010) and cancer are associated with changes in translation (Hsieh et al., 2012). We speculate that the replication defects associated with cohesin mutations interfere with the transcription of rDNA, leading to transcriptional and translational defects that contribute to human disease.

Figure 3.10

***FOB1* deletion rescues nucleolar structure and chromosome segregation in the *eco1* mutant.**

(A) Nucleolar morphology of WT, *eco1-W216G*, *fob1Δ*, and *eco1-W216G fob1Δ* strains was examined by EM. The nucleolus is the electron-dense area. At lease 25 nucleoli were scored per strain. The scale bar represents 0.2 μm. **(B)** Cohesin acetylation in the *eco1-W216G* is not rescued by *FOB1* deletion. The cohesin complex was immunoprecipitated with α-Myc affinity gel and was then analyzed by western blotting with α-acetyl-lysine antibody for Smc3 acetylation level (upper panel). The same immunoprecipitated samples were blotted for α-Myc antibody as a loading control (lower panel). **(C)** Segregation of the tetO:487 tag in WT, *eco1-W216G*, *fob1Δ*, and *eco1-W216G fob1Δ* strains was measured. Each strain was synchronized in G1 using α factor at 30°C, and released at 33°C. Pictures were taken at 80 min after release and all the binucleated were counted to determine the segregation of tetO:487. Blue, DNA; green, tetO:487. Error bars indicate standard deviation from three independent experiments. At least 150 cells per strain were counted per experiment. The p values were calculated by Student's t test, comparing mutant to WT.

Figure 3.10


A
WT
eco1-W216G
rad61Δ
fob1Δ
eco1-W216G fob1Δ
eco1-W216G rad61Δ
Other morphology
Crescent-shaped
Nucleolar morphology %
100
80
60
40
20
0
WT
eco1-W216G
fob1Δ
eco1-W216G fob1Δ
rad61Δ
eco1-W216G rad61Δ
B
IP: Mcd1-18myc
WT
eco1-W216G
fob1Δ
eco1-W216G fob1Δ
ac-Smc3
Mcd1-18myc
C
Chromosome XII
TetO Tag
rDNA
TetO-GFP
Hoechst
Merged
WT
eco1-W216G
Segregated tags %
100
80
60
40
20
0
p= 6.15E-4
p= 0.072
WT
eco1-W216G
fob1Δ
eco1-W216G fob1Δ

METHODS AND MATERIALS

Yeast strains and Cell synchronization

Yeast strains used in this study are listed in Table S1. Exponentially growing cells were arrested in G1 phase by the addition of α-factor (1.5×10^{-8} M final) for 2 hrs. To release cells from α-factor arrest, cells were spun down and washed twice in media containing 0.1 mg/ml Protease (Sigma, P-6911).

FACS and Microscopy

FACS analysis was performed to analyze cell cycle phase on cells fixed in 70% ethanol. Cells were washed with FACS buffer (50 mM Sodium Citrate), treated with RNase, stained with Propidium Iodide (4 μg/ml final), and analyzed by using a MACSQuant analyzer (Miltenyi Biotech). Fluorescence signal was observed using a Zeiss Axiovert 200M microscope (63X objective, NA=1.40). Image acquisition and analysis was performed with Axiovision (Carl Zeiss).

Pulse-field gel electrophoresis

Pulse-field gel electrophoresis was carried out as previously described (Ide and Kobayashi, 2010).

β-galactosidase assay

Yeast cells were grown overnight at 30°C in SD-ura and then diluted to OD_{600} = 0.2 in YPD+CSM. Cells were allowed to grow for two generations and were

collected. Protein extracts were made by bead beating. β-galactosidase activity was measured following standardized protocols, using ONPG (o-nitrophenyl-β-D-galactopyranoside) as the substrate.

Gene expression analysis

Gene expression analysis was carried out using Affymetrix Yeast Genome 2.0 microarrays and following the protocol as previously described (Bose et al., 2012).

FISH

FISH experiments were carried out following the protocol as previously described (Bose et al., 2012).

Chromatin Immunoprecipitation

Cells were grown to an OD_{600} of ~0.6 and fixed in 1% formaldehyde for 15 minutes. Cells were then lysed for 1 hour at 4°C in Lysis buffer (50mM HEPES pH 7.5, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Sodium Deoxycholate). Lysate was then sonicated using Biorupter (Diagenode) for 30 minutes, 30 seconds on/off, medium intensity. 2.5µl of α-FLAG antibody (Sigma F3165) was added to 400µl of chromatin extract and incubated at 4°C overnight. 50µl of Protein G Dynabeads (Invitrogen 100-04D) was added incubated at 4°C for 2 hours then washed with Lysis buffer, twice in Lysis buffer with 500mM NaCl, TEL buffer (0.25M LiCl, 10mM Tris-HCl pH 8.0, 1mM EDTA, 1% NP-40, 1%

Sodium Deoxycholate), then twice with 1x TE. Elutions were done by adding 200µl of Elution Buffer (1% SDS, 250mM NaCl, TE) at 65° with shaking, twice. Elutions were combined and treated with Proteinase K (Sigma) for 1 hour at 55°C. Crosslinking was then reversed and DNA was extracted. Quantitative PCR was performed and analyzed using iCycler iQ real-time PCR detection system (Bio-rad).

Immunoprecipitation and Western blotting analysis

Cells from a 220 ml culture grown to OD_{600}=0.8 were pelleted and quickly frozen in liquid nitrogen. Pellets were suspended in 1 ml Lysis Buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% NP40, 1 mM DTT, 10% glycerol, and protease inhibitor tablet). For the Smc3 acetylation experiment, a cocktail of HDAC inhibitors (10 µM Trichostatin A, 50 mM nicotinamide, and 50 mM sodium butyrate) was contained. Then cells were lysed by adding glass beads followed by beadbeating for 60 sec 5 times with 2 min intervals on ice. The supernatant was separated by centrifugation at 14K rpm at 4°C for 20 min. Immunoprecipitations were performed at 4°C using 30 µl α-HA affinity gel or α-Myc affinity gel (Sigma) overnight, followed by 5 washes with wash buffer (50 mM Tris pH 7.5, 150 mM NaCl, and 1% Triton X-100) and eluted with 2× SDS buffer (10 mM Tris pH 7.5, 1 mM EDTA, and 1% SDS). Eluates were loaded onto a 4-12% Bis-Tris gel for SDS-PAGE. Proteins were transferred from gels to a nitrocellulose membrane (Whatman), followed by blocking. Membranes were blotted with primary antibodies with α-HA (Roche, 3F10, #1867423), α-Myc (Covance, #MMS-150P),

α-acetyl-lysine (CST, #9441) or α-GST (Covance, #MMS-112P). Detection was achieved with Amersham ECL Plus Western Blotting Detection System (GE Healthcare #RPN2132).

Transmission Electron microscopy

Cultures were grown in 200 ml YPD to OD_{600} ~0.8. Samples were frozen on the Leica EM-Pact at ~ 2050 bar, then transferred under liquid nitrogen into 2% Osmium Tetroxide/0.1% Uranyl Acetate/Acetone/2% Water and transferred to the Leica AFS2. The freeze substitution protocol started samples at -90° C for 72 hours, up 5° C an hour, then -20° for 12 hours, up 5° C hourly to 0 ° C for 5 hours. Samples were removed from the AFS2 and allowed to come to room temperature for 1 hour. Samples went through 3 changes of acetone over 1 hour and were then removed from the planchettes. Samples were stepwise embedded in acetone/Epon/Araldite mixtures to final 100% Epon/Araldite over several days as described previously (McDonald, 1999). Samples were cut into 80 nm section on a Leica UC6, stained with Uranyl acetate and Sato's lead, and imaged on a FEI Technai Spirit.

BrdU ChIP

Exponentially growing cells were synchronized in G1 by α-factor. The cells were released from G1 and a final concentration of 800 μg/mL of 5-bromo-2'deoxyurindine (BrdU) was added into culture. Cell pellets of the WT and *eco1* strains were collected at 20 minutes and 40 minutes, respectively. α-BrdU ChIP was performed as described (Viggiani et al., 2010).

Mass Spectrometry for proteomics

TCA precipitated pellets were solubilized in Tris-HCl pH 8.5 and 8M urea; TCEP (Tris(2-Carboxylethyl)-Phosphine Hydrochloride, Pierce) and CAM (Chloroacetamide, Sigma) were added to a final concentration of 5 mM and 10 mM, respectively. Protein suspensions were digested overnight at 37°C using Endoproteinase Lys-C at 1:50 wt/wt (Roche). Samples were brought to a final concentration of 2M urea and 2 mM $CaCl_2$ before performing a second overnight digestion at 37°C using Trypsin (Promega) at 1:100 wt/wt. Formic acid (5% final) was added to stop the reactions. Samples were loaded on split-triple-phase fused-silica micro-capillary columns (McDonald et al., 2002) and placed in-line with linear ion trap mass spectrometers (LTQ, ThermoScientific) coupled with quaternary Agilent 1100 or 1200 series HPLCs. A fully automated 10-step chromatography run (for a total of 20 hours) was carried out for each sample, as described in (Florens and Washburn, 2006), enabling dynamic exclusion for 120 sec. The MS/MS datasets were searched using SEQUEST (Eng et al., 1994) against a database of 11990 sequences, consisting of 5819 S. cerevisiae non-redundant proteins, 177 usual contaminants (such as human keratins, IgGs, and proteolytic enzymes), and, to estimate false discovery rates, 5995 randomized amino acid sequences derived from each non-redundant protein entry. Peptide/spectrum matches were sorted, selected and compared using DTASelect/CONTRAST (Tabb et al., 2002). Combining all runs, proteins had to be detected by at least 2 peptides, leading to FDRs at the protein and spectral levels of 1.14 ± 0.27% and 0.18 ± 0.05%, respectively. To estimate relative

protein levels, Normalized Spectral Abundance Factors (dNSAFs) were calculated for each detected protein, as described in (Zhang et al., 2010).

Deep sequencing and data analysis

Deep sequencing was performed on the Illumina HiSeq 2500 system. Sequencing libraries were made using the Nextera DNA Sample Preparation Kits (Illumina) as advised by the manufacturer. Illumina HiSeq single-end 50 bp sequence reads were aligned to the UCSC SaccCer3 genome using bowtie v0.12.9 and parameters (-k 1 -m1) which report only unique matches. Read counts were summarized on 1kb intervals across the genome using the GenomicRanges library from Bioconductor in R, and then normalized to counts per million. The $\log_2$ ratio of normalized S to G1 phase cells was taken after adding 1 to the intervals from each data set to prevent division by zero errors. The data was then smoothed in R using loess with a span of 25kb and degree of 1. The final ratios were multiplied by 1.5 to aid visualization. Custom Perl scripts following the previous description (Muller and Nieduszynski, 2012; Natsume et al., 2013) were used to calculate this ratio. Early and late firing origins are determined according to the previous publication (Raghuraman et al., 2001; Yabuki et al., 2002).

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Precise chromosome segregation requires the cohesin complex. In addition to holding sister chromatids together, evidence suggests that cohesin functions in gene transcription, DNA damage repair, and DNA replication. However, these additional functions are not fully understood. The interdependence of the functions is also intriguing. We have provided evidence that DNA damage repair, especially homologous recombination, is strongly affected in cohesin mutants. We have also provided evidence that cohesin controls genome-wide transcription through its regulation of transcription and replication at the rDNA locus. This work represents a significant step forward in understanding the role of cohesin in cell physiology and the molecular mechanisms underlying cohesinopathies.

Eco1 and DNA damage repair

We found that *eco1* mutants that do not have gross chromosome segregation defects showed varying degrees of DNA damage sensitivity. All the *eco1* mutants, including the human Roberts syndrome homolog mutant, *eco1-W216G*, showed a decrease in acetyltransferase activity *in vitro*. *In vivo*, the acetylation of Smc3, the Eco1 substrate, displayed different levels. These levels roughly correlated with the sensitivity of the mutants to DNA damaging agents.

We employed an elegant recombination event counting system to further analyze the mechanism by which the DNA damage repair is affected in an *eco1* mutant (Barbera and Petes, 2006). We have shown that a specific DNA recombination event, the reciprocal crossover, was strongly affected in the *eco1-W216G* mutant. This data suggests that the exchange of homologous chromosome arms requires Eco1 (**Figure 4.1**). The result strongly supports the argument that cohesin regulates recombination pathway choice.

Figure 4.1

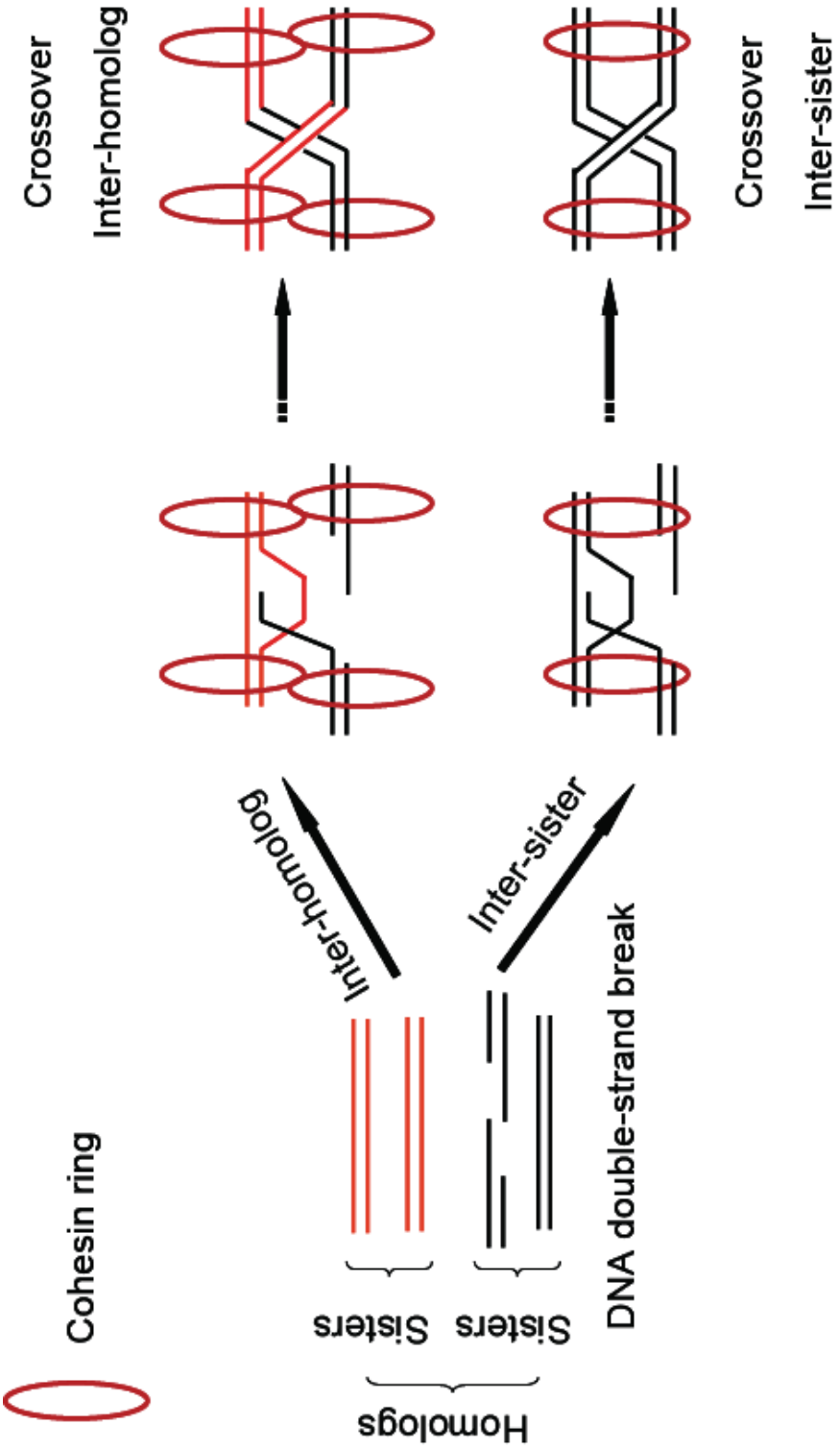

Crossover
Inter-homolog
Crossover
Inter-sister
Inter-homolog
Inter-sister
DNA double-strand break
Sisters
Sisters
Homologs
Cohesin ring

Figure 4.1 Cohesin promotes DNA double-strand break repair

During DNA double-strand break repair, the break site could be repaired by either choosing sister chromatid or homolog chromosome as a template. In our model, cohesin complex facilitates both inter-sister and inter-homolog repair by bringing sister-chromatids or homologous chromosomes together.

The above result suggests that Eco1 is important for DNA damage repair in mitosis. We also assessed its role in DNA damage repair in meiosis, a special type of cell division necessary for sexual reproduction in eukaryotes. In contrast to reduction of crossover formation in mitosis, no difference was observed in an *eco1-W216G* mutant in meiotic recombination, indicating that Eco1 is not required for the crossover formation between homologous chromosomes during meiosis. Because the DNA double-strand break formation and repair mechanisms differ between mitosis and meiosis, it is not surprising that Eco1 affects one and not the other. In fact, the primary cohesin complex in meiosis uses Rec8 as the kleisin subunit rather than Mcd1, further highlighting that meiotic and mitotic recombination require different cohesin functionality. However, reduction in sporulation efficiency and spore viability was observed in the *eco1-W216G* mutant in meiosis, suggesting that Eco1 does play a role during meiosis. Further studies are needed to characterize alternative substrates of Eco1 in mitosis and meiosis. Also, how Eco1 is activated and inactivated in response to DNA damage and after DNA damage repair is an important question to address.

Our results suggest that Eco1's role in DNA damage repair is an important area for future study, especially to understand the etiology of Roberts syndrome. Roberts syndrome is a human developmental disorder caused by mutation of *ESCO2*. Mutation of *ESCO2* is expected to lead to an increase in the pool of cohesin that is not acetylated, and is therefore, not cohesive. Because cohesin

can affect so many different biological processes, it is important to discover which processes are defective in Roberts syndrome.

DNA damage repair deficiency of *ESCO2* mutation might be the cause of Roberts syndrome. Since both Roberts syndrome patient cells and *ESCO2* knockout cells are sensitive to DNA damage materials (van der Lelij et al., 2009; Whelan et al., 2012a) and the functions of ESCO2 and ESCO1 are non-redundant (Kim et al., 2010), it is quite possible that the DNA damage repair function of ESCO2 significantly contributes to Roberts syndrome. The difference of functions of ESCO1 and ESCO2 will be further discussed below.

We speculate that *ESCO2* mutation might act differently in different tissues. DNA damage is prone to occur in tissues that undergo rapid cell proliferation. Therefore, if DNA damage is not repaired efficiently, more apoptosis may occur in those tissues and cause developmental defects. Further studies are needed to understand the role of ESCO2 in DNA damage repair, especially in the tissues that undergo rapid cell proliferation using animal models with an *ESCO2* mutation. *ESCO2* mutant mice could be used to examine developmental defects in different tissues. In addition, DNA damage repair in different *ESCO2* mutant mice cell types could also be assayed to test the hypothesis that DNA damage repair is impaired in rapidly developing cells with an *ESCO2* mutation.

Role of Eco1 in DNA replication and gene expression

A previous study showed that a budding yeast strain with the *eco1-W216G* mutation, which genocopies the human Roberts syndrome disease allele, reduces ribosomal DNA (rDNA) transcription and disrupts the transcriptome (Bose et al., 2012). We found that deletion of *FOB1*, a gene encoding a specific replication fork blocking protein for the rDNA region, can both rescue rDNA expression and genome-wide transcriptional defects in the *eco1-W216G* mutant. This suggests that Eco1 may regulate transcription genome-wide through its effects on a key regulatory locus, the rDNA. Because the rDNA locus is difficult to replicate due to its high levels of transcription and its repetitive nature, we argued that Eco1 may regulate DNA replication and transcription in the rDNA locus. Our studies suggest that the *eco1-W216G* mutant has not only rDNA replication defects but genome-wide replication defects and that the deletion of *FOB1* rescued these defects.

Deletion of *FOB1* allows replication at the rDNA locus to occur bidirectionally, rather than only in the direction of transcription (**Figure 4.2**). In fact, most cells, including human cells, employ a replication fork block to prevent DNA replication forks from running into RNA polymerase I at the rDNA. We found that allowing bidirectional replication of the rDNA corrected the nucleolar structure and chromosome segregation in the *eco1-W216G* mutant. In this mutant background, the benefit of bidirectional replication outweighed its cost. Our study highlights a

possible mechanism for the replication defect reported in Roberts syndrome (Terret et al., 2009). We postulate that these replication defects affect the transcription of the rDNA, leading to translational defects and differential gene expression that contributes to this developmental disorder.

Figure 4.2

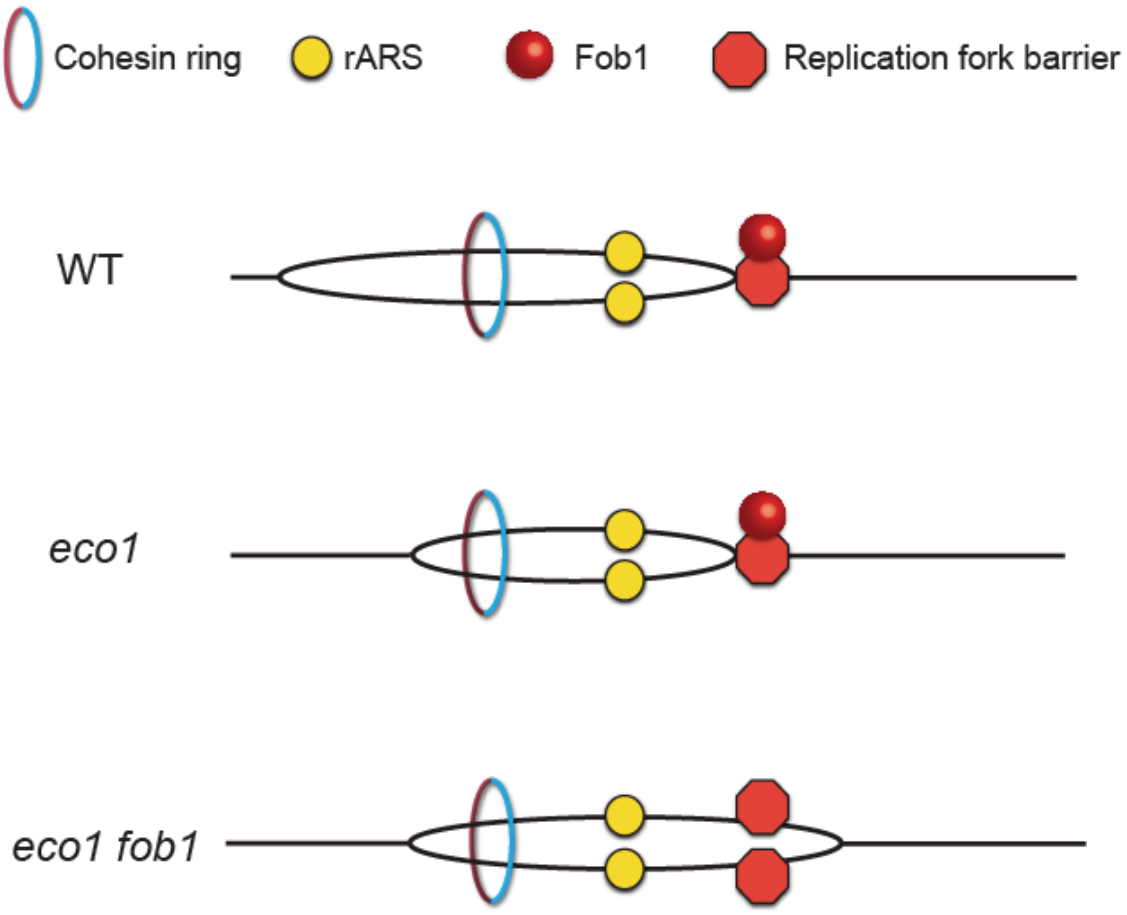

Cohesin ring
rARS
Fob1
Replication fork barrier
WT
eco1
eco1 fob1

Figure 4.2 Fob1 deletion promotes bidirectional replication

Replication fork barrier (RFB) blocks bidirectional replication fork progression. Fob1 protein binds at RFB site and is essential for the barrier activity. In *eco1* mutant, DNA replication fork progression is slower comparing to wild type. Deletion of Fob1 allows bidirectional replication and promotes DNA replication at rDNA locus in *eco1* mutant.

We speculate that cohesin's role in regulating DNA replication and gene transcription in the rDNA region is conserved across the species. Cohesin binds to the rDNA region from bacteria to human (Gruber and Errington, 2009; Kasowski et al., 2013; Laloraya et al., 2000; Losada et al., 2000). Since the rDNA region is highly transcribed and inherently difficult to replicate due to its highly repetitive structure, the coordination of transcription and replication is needed to prevent collision of these events. Our study suggests that cohesin and the replication fork barrier work together to facilitate both transcription and replication in rDNA region. Further studies are needed to understand the detailed mechanism of how cohesin coordinates DNA replication and transcription at the rDNA locus. Further experiments could be designed to knockdown TTF-I (Transcription Termination Factor, RNA Polymerase I) and assay DNA replication and transcription in *ESCO2* mutant cells.

Etiology of cohesinopathies

Budding yeast contain only a single enzyme, Eco1, that is involved in the process of establishment of sister-chromatid cohesion. Why do higher organisms need two enzymes (ESCO1 and ESCO2) to regulate this process? Although ESCO2 is mutated in human Roberts syndrome (RBS), its homolog, ESCO1, has been reported to be the major acetyltransferase to acetylate Smc3 (Zhang et al., 2008). However, the knowledge of how ESCO1 functionally overlaps with ESCO2 is still unknown (Zhang et al., 2008). Because ESCO1 might play a major role in sister-

chromatid cohesion but human mutations of *ESCO1* have never been reported, it is quite possible that mutations in this gene are lethal. Based on this rationale, we propose the central hypothesis that ESCO1, but not ESCO2, is the major acetyltransferase for the establishment of cohesion. Our study suggests that ESCO2 may act more regionally than it was previously thought. ESCO2 may specifically function in the heterochromatin region, especially at the rDNA locus, to acetylate cohesin. Experiments could be designed by knocking down ESCO1 and ESCO2 by siRNA, respectively. ESCO1 knockdown should result in cohesion defects throughout the genome, whereas ESCO2 knockdown might have regional cohesion defect. In support, ESCO2 is enriched in the pericentrometric region in mid-late S phase. In addition, lack of cohesin was only observed in heterochromatic region of human Roberts syndrome cells that is caused by mutation of *ESCO2* (**Figure 4.3**) (Vega et al., 2005; Whelan et al., 2012b). Further studies are needed to test the hypothesis that ESCO2 is a heterochromatin specific acetyltransferase for cohesin. To test this hypothesis, ESCO2 can be knocked down by siRNA and chromosomes from a metaphase spread of ESCO2 knocked down cells can be assayed for heterochromatic repulsion. In addition, the expression of genes near the heterochromatic regions can be analyzed. If cohesion is defective in heterochromatic regions, the expression of the genes near these regions might also be affected.

Figure 4.3

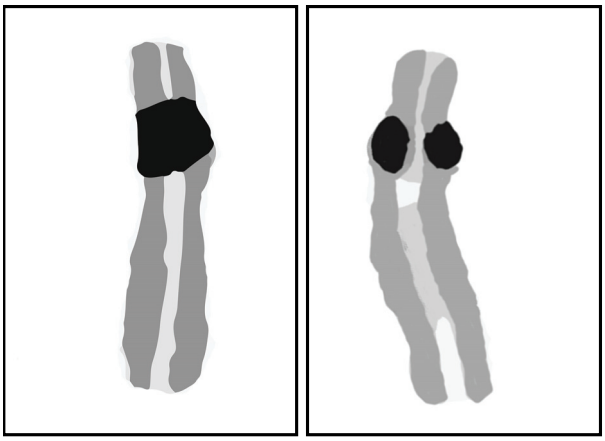


Figure 4.3 Chromosomal repulsion at heterochromatic regions

Chromosomes derived from a metaphase spread of a RBS patient cell show repulsion at heterochromatic regions, which includes pericentric regions and ribosomal DNA. We speculate that the acetylation activity of ESCO2 is necessary for cohesion of these regions. Although both chromosomes are derived from the same metaphase spread presented in Vega et al., 2005, the one on the left appears normal while the one on the right shows repulsion.

(From Xu, Lu and Gerton submitted)

We speculate that mutations in ESCO2 may affect a particular pool of cohesin, the cohesin at heterochromatic regions. When cohesion at the rDNA is reduced, nucleolar structure is disrupted and nucleolar function is less efficient. In contrast, mutations in subunits of the cohesin ring may affect cohesin throughout the genome, unless the mutation compromises protein-protein or protein-DNA interactions that occur at specific cohesin associated regions. These mutations could have anywhere from undetectable to severe effects on the pool of cohesin at the rDNA. In budding yeast, a mutation in SMC1 that genocopies a mutation associated with CdLS causes nucleolar phenotypes that are milder than the *eco1* mutation (Bose et al.; Gard et al., 2009), suggesting some CdLS mutation may be associated with defects in nucleolar function. In zebrafish models for CdLS, some mutations appear to be associated with more growth delay and apoptosis than others (Deardorff et al.; Ghiselli, 2006; Horsfield et al., 2007; Monnich et al.; Muto et al.) and nucleolar function has not been analyzed. However, if nucleolar function is affected by non-ESCO2 mutations, this could potentially contribute to the gene expression changes associated with CdLS.

We found cohesion within the rDNA appears to promote nucleolar structure and function in budding yeast. Our model is that cohesin regulates loops of the rDNA repeats, which are reduced in budding yeast bearing an RBS mutation (**Figure 4.4**). These loops together with DNA replication controls transcription of rDNA and nucleolar integrity. Given these observations in budding yeast, we speculate *ESCO2* mutation in human RBS cells affect rDNA region in a similar mechanism.

Indeed, study in human RBS cells showed defects in transcription of the rDNA (Bose et al., 2012) and highly fragmented nucleoli (**Figure 4.5**) (Xu et al., 2013). Further studies are needed to understand the mechanism of how effects on rDNA by cohesin mutants lead to global changes in gene expression and cell physiology.

Figure 4.4

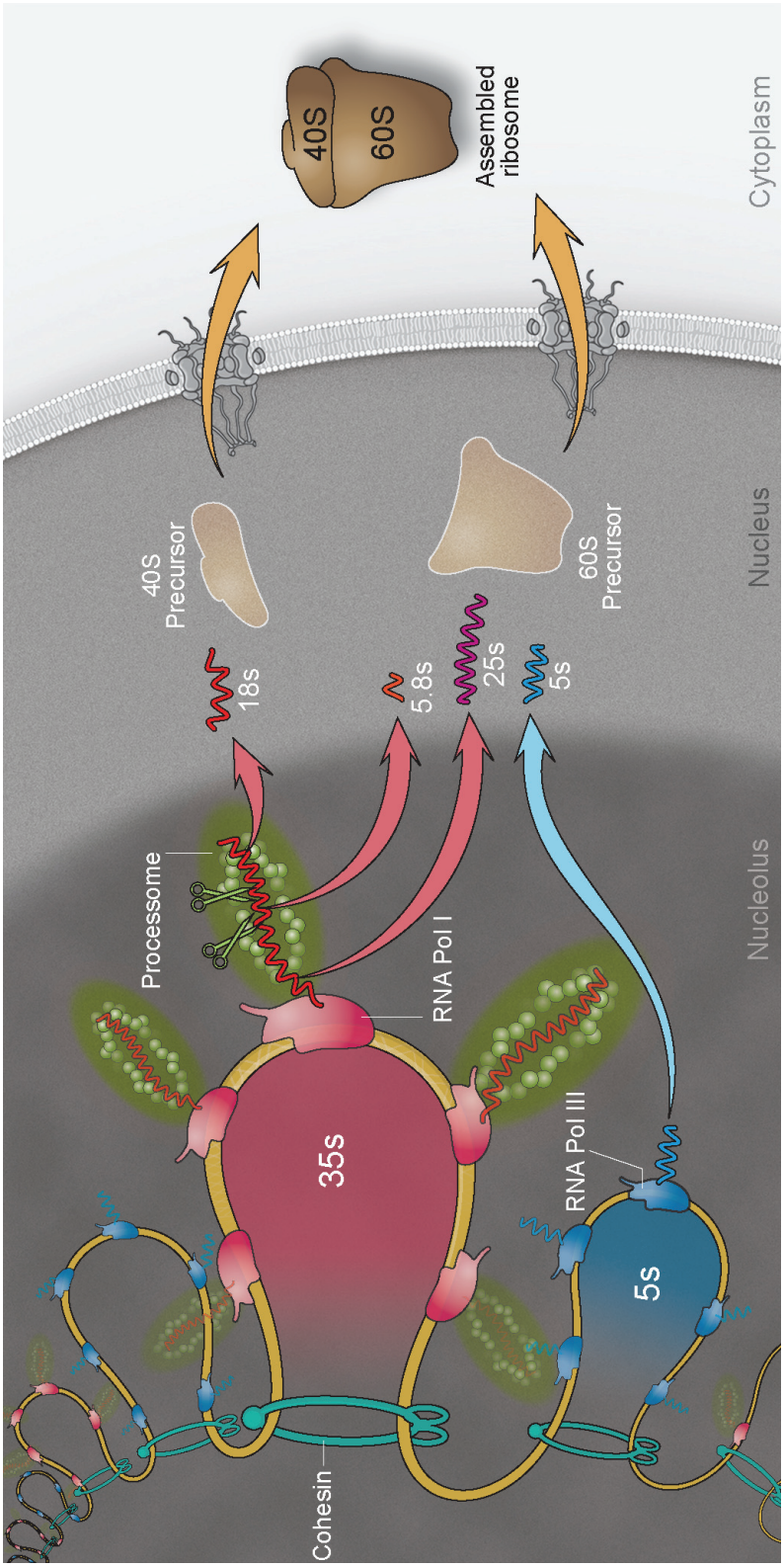

40S
60S
Assembled ribosome
Cytoplasm
Nucleus
Nucleolus
40S Precursor
60S Precursor
18s
5.8s
25s
5s
Processome
RNA Pol I
RNA Pol III
35s
5s
Cohesin

Figure 4.4 Model for the role of cohesion within the nucleolus

Cohesin binding to the rDNA repeats is evolutionarily conserved from bacteria to human cells. In budding yeast, cohesin binds to the non-transcribed region between repeats in such as way that it could trap loops of 35S genes. The 35S gene is transcribed by RNA polymerase I and then cleaved and modified by the processome to make 18S, 5.8S, and 25S rRNAs that are included in the large (60S) and small (40S) ribosomal subunits. RNA polymerase III transcribes the 5S RNA that is included in the large ribosomal subunit. Cohesion at this region could promote transcription by RNA polymerase I as well as the overall structural formation of the nucleolus.

(From Xu, Lu and Gerton submitted)

Figure 4.5

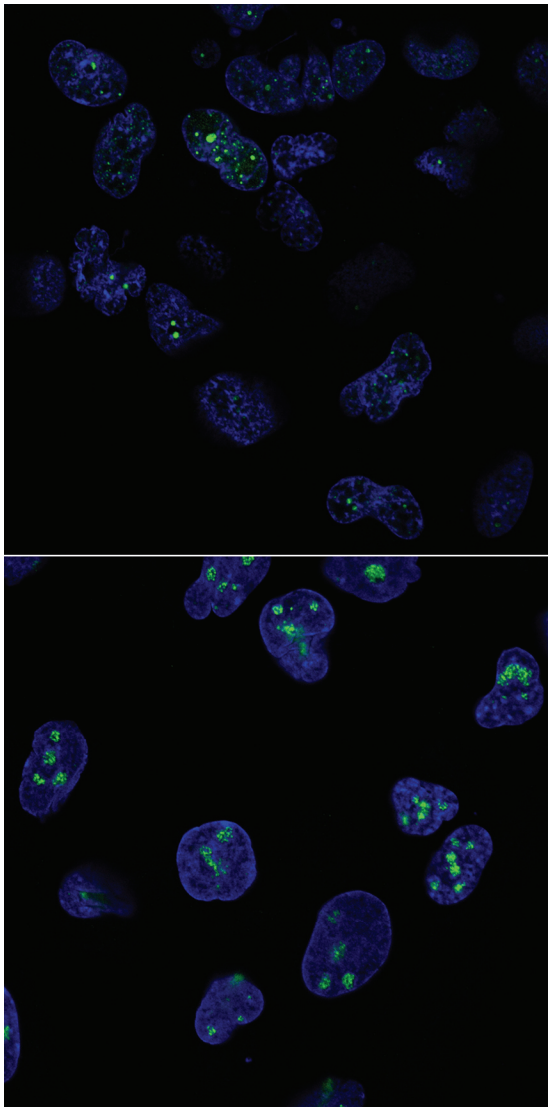


Figure 4.5 RBS patient cells have highly fragmented nucleoli

Skin cells from patients with RBS display defects in the organization of nucleoli, specialized nuclear subdomains dedicated to the production of ribosomes. Fibrillarin, a nucleolar marker stained with an anti-fibrillarin antibody, is shown in green. DNA is stained with DAPI, shown in blue. Colors were adjusted for improved visual presentation. Top: RBS cells, Bottom: Normal cells.

(From Xu, Lu and Gerton submitted)

Our study suggests that cohesin mutants might have translational defects in addition to genome transcription defect, since mutation of cohesin affects rDNA. Translational defects are associated with both human developmental disorders and cancer. A recent study found that 13% of AML (acute myeloid leukemia) cases were caused by mutations in cohesin genes, including *RAD21*, *STAG2*, *SMC1*, and *SMC3* (Ley et al., 2013). In general, AML is not associated with abnormal karyotypes, raising the possibility that the cohesin mutations are causing transcriptional or translational changes that lead to AML, and emphasizing the need to understand the molecular origin of these changes. Deregulation of translational controls can promote cellular transformation, tumor susceptibility and development by activation of several key oncogenic pathways. If we can understand the contribution of translational mechanisms and particular key regulatory pathways, such as p53 and mTOR, in Roberts syndrome and CdLS, we might also understand how cohesin mutations lead to myeloid neoplasms (Kon et al., 2013; Ley et al., 2013).

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APPRENDIX I. YEAST STRAINS

Parent Strain	Strain Name	Genotype
Lu et al, 2010		
W303	SLJ1989 (NBY292)	MATa *bar1- ura3 leu2 trp1 lys2 ade2 his3::pCUP1-GFP12-LacI12::HIS3 tellV::LacO-LEU2*
SLJ1989	JG1592.1, JG1592.2	MATa *bar1- ura3 leu2 trp1 lys2 ade2 his3::pCUP1-GFP12-LacI12::HIS3 tellV::LacO-LEU2 ECO1::eco1-W216G*::HYG
SLJ1989	JG1599	MATa *bar1- ura3 leu2 trp1 lys2 ade2 his3::pCUP1-GFP12-LacI12::HIS3 tellV::LacO-LEU2 ECO1::eco1-ack*::HYG
SLJ1989	JG1596	MATa *bar1- ura3 leu2 trp1 lys2 ade2 his3::pCUP1-GFP12-LacI12::HIS3 tellV::LacO-LEU2 ECO1::eco1-H53Y*::HYG
W303	SLJ1988 (NBY291)	MATa *bar1- ura3 leu2 trp1 lys2 ade2 his3::pCUP1-GFP12-LacI12::HIS3 armIV::LacO-URA3*
SLJ1988	SG363.1, SG363.2	MATa *bar1- ura3 leu2 trp1 lys2 ade2 his3::pCUP1-GFP12-LacI12::HIS3 armIV::LacO-URA3 ECO1::eco1-W216G*::HYG
SLJ1988	SG362.1, SG362.3	MATa *bar1- ura3 leu2 trp1 lys2 ade2 his3::pCUP1-GFP12-LacI12::HIS3 armIV::LacO-URA3 ECO1::eco1-ack*::HYG
SLJ1988	SG361.3 (JG1623)	MATa *bar1- ura3 leu2 trp1 lys2 ade2 his3::pCUP1-GFP12-LacI12::HIS3 armIV::LacO-URA3 ECO1::eco1-H53Y*::HYG
W303	SLJ1773	MATa *bar1- ura3 leu2 trp1 lys2 ade2 his3::pCUP1-GFP12-LacI::HIS3 trp1::LACOR-TRP1*
SLJ1773	SG367.1, SG367.2	*ECO1*::*eco1-W216G*::HYG
SLJ1773	SG366.2, SG366.5	*ECO1*::*eco1-ack*::HYG
SLJ1773	SG365.38, SG365.39	*ECO1*::*eco1-H53Y*::HYG
W303	MAB6	Mat α/a *can1-100*/*can1*Δ::SUP4-o V9229::HYG/V9229::KANMX V261553::*HIS3*/V261553::*LEU2 ade2-1*/*ade2-1* RAD5+/ RAD5+
MAB6	JG493	*eco1-W216G*-nat/ *eco1-W216G*-nat

JG1613		Mat α/a *can1-100*/*can1*Δ::SUP4-o V9229::HYG/V9229::KANMX V261553::*HIS3*/V261553::*LEU2 ade2-1*/*ade2-1 URA3*/*ura3* tar1-*TRP1*/*TAR1 ECO1*-nat/*ECO1*-nat
JG1613	JG1614	*eco1-W216G*-nat/ *eco1-W216G*-nat
W303		MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1*
	SG156.1	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 ECO1*::*eco1-W216G*::HYG
	SG154.2	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 SCC2*::*scc2-D730V*::HYG
	JG1595	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 ECO1*::*eco1-H53Y*::HYG
	JG1598	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 ECO1*::*eco1-ack*::HYG
	MGY152	MATα *ECO1::eco1-1 SCC3-HA3-HISMX4*
	MGY151	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 rad50::*KAN
	SLJ1985	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 rad9::*LEU2
	SG297.1	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 rad61::*KAN
	SG272.7	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 rad61::*KAN *ECO1::eco1-W216G::*HYG
	LS1002	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 pRS316(CEN/ARS, URA3)*
	LS1003	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 pRS316(pGAL-MCD1, CEN/ARS, URA3)*
	LS1004	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 pRS316(pGal-MCD1$^{K84Q,\ K210Q}$, CEN/ARS, URA3)*
	LS1005	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 ECO1*::*eco1-W216G*::HYG *pRS316(CEN/ARS, URA3)*
	LS1006	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 ECO1*::*eco1-W216G*::HYG *pRS316(pGAL-MCD1, CEN/ARS, URA3)*
	LS1007	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 ECO1*::*eco1-W216G*::HYG *pRS316(pGAL-MCD1$^{K84Q,\ K210Q}$, CEN/ARS, URA3)*
	LS1046	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 pRS316(pGAL-ECO1, CEN/ARS, URA3)*

	LS1047	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 pRS316(pGAL-SMC3, CEN/ARS, URA3)*
	LS1048	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 pRS316(pGAL-SMC3^{K113N}, CEN/ARS, URA3)*
	LS1049	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 pRS316(pGAL-SMC3^{K113Q}, CEN/ARS, URA3)*
	LS1050	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 pRS316(pGAL-SMC3$^{K112N, K113N}$, CEN/ARS, URA3)*
	LS1051	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 pRS316(pGAL-SMC3$^{K112Q, K113Q}$, CEN/ARS, URA3)*
	LS1052	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 pRS316(CEN/ARS, URA3)*
	LS1053	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 ECO1*::*eco1-W216G*::HYG *pRS316(pGAL-ECO1, CEN/ARS, URA3)*
	LS1054	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 ECO1*::*eco1-W216G*::HYG *pRS316(pGAL-SMC3, CEN/ARS, URA3)*
	LS1055	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 ECO1*::*eco1-W216G*::HYG *pRS316(pGAL-SMC3^{K113N}, CEN/ARS, URA3)*
	LS1056	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 ECO1*::*eco1-W216G*::HYG *pRS316(pGAL:SMC3^{K113Q}, CEN/ARS, URA3)*
	LS1057	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 ECO1*::*eco1-W216G*::HYG *pRS316(pGAL-SMC3$^{K112N, K113N}$, CEN/ARS, URA3)*
	LS1058	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 ECO1*::*eco1-W216G*::HYG *pRS316(pGAL-SMC3$^{K112Q, K113Q}$, CEN/ARS, URA3)*

	LS1059	MATa *ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 ECO1*::*eco1-W216G*::HYG *pRS316(CEN/ARS, URA3)*
BY4742	AM405.4	MATα *his3Δ1 leu2Δ0* LYS2 *met15Δ0 ura3Δ0* ECO1-FLAG::KAN
BY4742	AM415.1	MATα *his3Δ1 leu2Δ0 lys2Δ0* MET15 *ura3Δ0* ECO1::*eco1-W216G*-FLAG::KAN
BY4742	JG1642	MATα *his3Δ1 leu2Δ0 lys2Δ0* MET15 *ura3Δ0* ECO1::*eco1-ack*-FLAG::KAN
BY4742	JG1643	MATα *his3Δ1 leu2Δ0 lys2Δ0* MET15 *ura3Δ0* ECO1::*eco1-H53Y*-FLAG::KAN

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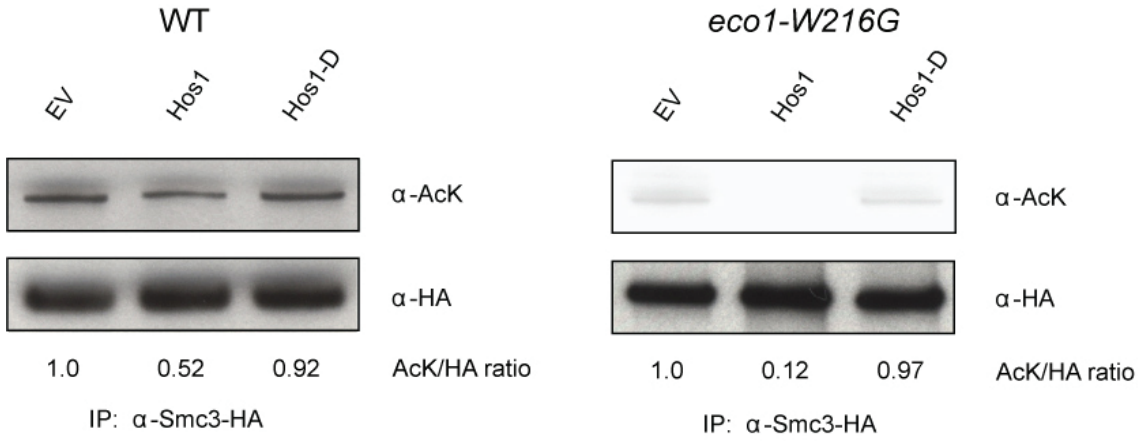
MAB1	JG1720	MATα *ura3-1* leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 V261553::HIS3 GCN4-lacZ::TRP1
MAB1	JG1721	MATα ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 V261553::HIS3 GCN4-lacZ::TRP1 ECO1:: eco1-W216G::NAT
MAB1	JG1736	MATα ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 V261553::HIS3 GCN4-lacZ::TRP1 fob1Δ::KAN
MAB1	JG1737	MATα ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 V261553::HIS3 GCN4-lacZ::TRP1 ECO1:: eco1-W216G::NAT fob1Δ::KAN
W303a	LS1069	MATa ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 RDN1::Probe(32)::URA3
W303a	LS1075	MATa ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 RDN1::Probe(32)::URA3 ECO1:: eco1-W216G::HYG
W303a	LS1091	MATa ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 RDN1::Probe(32)::URA3 fob1Δ::KAN
W303a	LS1092	MATa ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 RDN1::Probe(32)::URA3 ECO1::eco1-W216G::HYG fob1Δ::KAN
W303a	LS1077	MATa ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 RDN1::Probe(32)::URA3 SMC1::smc1-Q843Δ::HYG
W303a	LS1086	MATa ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 RDN1::Probe(32)::URA3 SMC1::smc1-Q843Δ::HYG fob1Δ::KAN

W303a	JG680	MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15
W303a	JG2087.1	MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 eco1-W216G::HYG
W303a	JG2087.2	MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 eco1-W216G::HYG
W303a	SG156	MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 eco1-W216G::HYG
W303a	JG1830	MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 fob1Δ::NAT
W303a	BH664	MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 Eco1::eco1-W216G::HYG fob1Δ::KAN
W303a	SG297.1	MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 rad61Δ::KAN
W303a	SG272.1	MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 Eco1::eco1-W216G::HYG rad61Δ::KAN
MMY027	BH685	bar1::HIS3, CDC45-3FLAG::kanMX
MMY027	BH687.1	bar1::HIS3, CDC45-3FLAG::kanMX Eco1::eco1-W216G::HYG
CG1300	LS1093	TetR-YFP ADE2 TetO(5.6Kb):450Kb-ChrXII URA3
CG1300	LS1094	TetR-YFP ADE2 TetO(5.6Kb):450Kb-ChrXII URA3 ECO1::eco1-W216G::HYG
CG1300	LS1095	TetR-YFP ADE2 TetO(5.6Kb):450Kb-ChrXII URA3 fob1Δ::KAN
CG1300	LS1096	TetR-YFP ADE2 TetO(5.6Kb):450Kb-ChrXII URA3 ECO1::eco1-W216G::HYG fob1Δ::KAN
W303a	LS1064	MATa ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 Mcd1-18myc::TRP1
W303a	LS1065	MATa ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 Mcd1-18myc::TRP1 ECO1::eco1-W216G::HYG
W303a	LS1089	MATa ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 Mcd1-18myc::TRP1 fob1Δ::KAN

W303a	LS1090	MATa ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 Δbar1 Mcd1-18myc::TRP1 ECO1::eco1-W216G::HYG fob1Δ::KAN
W303a	LS1101	MATa ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 psi+ RAD5 URA3::GPD-TK7
W303a	LS1102	MATa ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 psi+ RAD5 URA3::GPD-TK7 ECO1::eco1-W216G::HYG
MAB1	JG1768	MATα ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 V261553::HIS3 GCN4-lacZ::TRP1 pBY011(pGAL-FOB1, CEN/ARS, URA3)
MAB1	JG1769	MATα ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 V261553::HIS3 GCN4-lacZ::TRP1 pRS316(CEN/ARS, URA3)
MAB1	JG1770	MATα ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 V261553::HIS3 GCN4-lacZ::TRP1 ECO1:: eco1-W216G::NAT pBY011(pGAL-FOB1, CEN/ARS, URA3)
MAB1	JG1771	MATα ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 V261553::HIS3 GCN4-lacZ::TRP1 ECO1:: eco1-W216G::NAT pRS316(CEN/ARS, URA3)
MAB1	JG1786	MATα ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 V261553::HIS3 GCN4-lacZ::TRP1 pBY011(pGAL-FOB1^{C193A}, CEN/ARS, URA3)
MAB1	JG1787	MATα ura3-1 leu2,3-112 his3-1 trp1-1 ade2-1 can1-100 V261553::HIS3 GCN4-lacZ::TRP1 ECO1:: eco1-W216G::NAT pBY011(pGAL-FOB1^{C193A}, CEN/ARS, URA3)

APPRENDIX II. HOS1 AS A DEACETYLASE OF SMC3

WT and *eco1-W216G* strains expressing Smc3-3HA and the indicated plasmids were grown to mid-log phase in gal-ura medium and whole cell extracts were used for immunoprecipitation with α-HA affinity gel. The eluates were subjected to Western blotting with either α-AcK antibody (upper) to measure acetylated Smc3 or α-HA antibody (lower) to measure total Smc3. The ratio of signal from the upper panel to the lower panel is shown below the panels.



APPRENDIX III. FISH METHOD

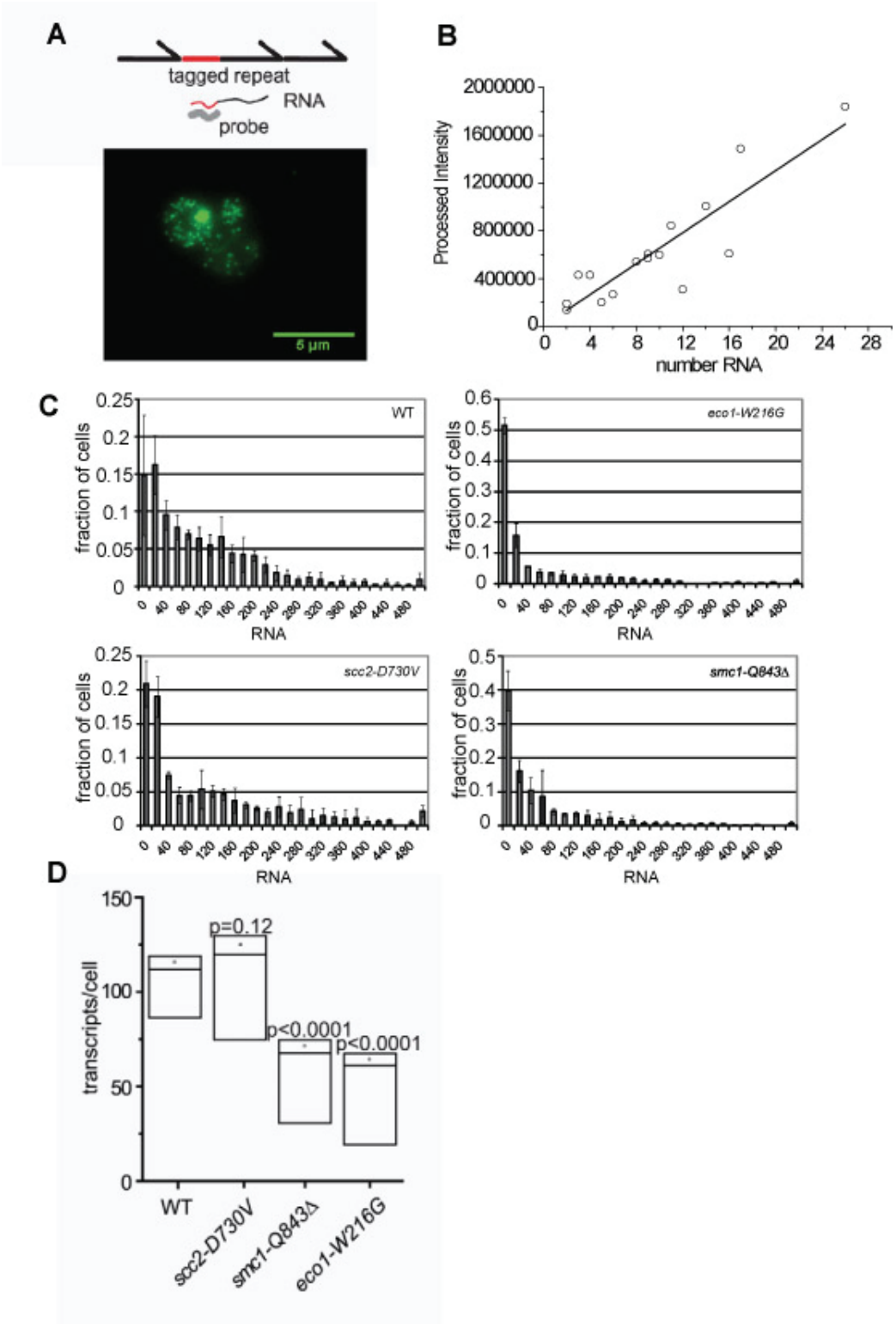
Materials and Methods

Yeast cells were grown in CSM-URA at 30°C to an OD_{600} of 0.4. The cells were then fixed by adding formaldehyde to a final concentration of 4% (v/v) for 45 min at room temperature with shaking. After three washes with wash buffer (1.2 M sorbitol, 0.1 M potassium phosphate, pH 7.5), the cell wall was digested with 0.3 mg/ml zymolase in spheroplast buffer (1.2 M sorbitol, 0.1 M potassium phosphate, 10 mM vanadyl ribonucleoside complex, 0.06 mg/ml PMSF, 28 mM β-mercaptoethanol) at 37°C for 45 min. After digestion, the cells were washed three times with FISH wash buffer (30% formamide, 2×SCC). The cells were then hybridized in 30 µl hybridization solution containing 5 ng/ µl DNA probe in 25% (v/v) formamide, 2×SCC, 1 mg/ml BSA (nuclease free), 10 mM vanadyl ribonucleoside complex, 0.5 mg/ml salmon sperm DNA and 0.1 g/ml dextran sulfate overnight at room temperature. Before imaging, cells were washed twice with FISH wash buffer for 30 min and then added to slides pre-coated with Poly-L lysine. The probe used for the FISH experiment is a synthesized DNA oligonucleotide referring to but modified from previous publication(Tan and van Oudenaarden, 2010). The sequence of the oligonucleotide is 5'-CGGCRGGTAAGGGRTTCCATARAAACTCCTRAGGCCACGA-3'; the 'R's indicates an amino-dT replacing a regular dT where a fluorescein molecule was coupled. The probe was further purified by polyacrylamide gel purification to ensure that each amino-dT was coupled with a fluorescein molecule. For FISH strain construction, a unique sequence was inserted into one rDNA repeat that

can be recognized by FISH probe (**Appendix IIIA**). As shown in **Appendix IIIA**, each rDNA transcript can be detected by the FISH probe. To validate that the number of rDNA transcript is linear related with the fluorescence intensity, fluorescence intensity from different numbers of rDNA transcript is plotted (**Appendix IIIB**). rDNA transcript levels of WT and indicated mutants are shown in **Appendix IIIC** and **Appendix IIID.**

Expanded presentation of the FISH data

(A) A unique sequence has been inserted into one rDNA repeat in order to monitor its transcription by FISH. A representative image from the wild-type strain is shown; the scale bar is 5 μm (B) The standard curve shown was used to determine how fluorescence intensity relates to number of RNAs. The fluorescence for each cell is measured and then binned to show the fraction of the population with each RNA number. The fluorescence for 300 cells from 3 independent cultures for each strain was measured (900 cells total per strain). The error bars indicate the standard deviation. (C) Numbers of rDNA transcripts in a population of WT, scc2-D730V, eco1-W216G, and smc1-Q843Δ cells. Cells were binned based on the number of transcripts per cell. (D) Box plot of rDNA transcription of WT, *scc2-D730V*, *eco1-W216G*, and *smc1*-Q843Δ strains. In the plot shown the dot is the average, the two lines around it are the standard error, and the lowest line is the median. The p value was derived from a two tailed Student's t test.


A
tagged repeat
RNA
probe
5 µm
B
Processed Intensity
number RNA
C
WT
eco1-W216G
scc2-D730V
smc1-Q843Δ
fraction of cells
RNA
D
transcripts/cell
p=0.12
p<0.0001
p<0.0001
WT
scc2-D730V
smc1-Q843Δ
eco1-W216G

APPRENDIX IV. PUBLISHED PAPERS AND MEETING ABSTRACTS

PUBLICATIONS:

Eco1 is Important for DNA Damage Repair in *S. cerevisiae*
Shuai Lu, Matthew Goering, Scarlett Gard, Bo Xiong, Adrian J. McNairn, Sue Jaspersen and Jennifer L. Gerton
Cell Cycle
Volume 9, Issue 16 August 15, 2010
Pages 3315 - 3327
DOI: 10.4161/cc.9.16.12673

The cohesin acetyltransferase Eco1 coordinates rDNA replication and transcription
Shuai Lu, Kenneth Lee, Bethany Harris, Bo Xiong, Tania Bose, Anita Saraf, Gaye Hattem, Laurence Florens, Chris Seidel, Jennifer L. Gerton
(Submitted to EMBO Reports)

Hos1 is a lysine deacetylase for the Smc3 subunit of cohesin
Bo Xiong, Shuai Lu, Jennifer L. Gerton
Current Biology
Volume 20, Issue 18 September 28, 2010
Pages 1660 – 1665
DOI: 10.1016/j.cub.2010.08.019

Cohesin proteins promote ribosomal RNA production and protein translation in yeast and human cells
Tania Bose, Kenneth K. Lee, Shuai Lu, Baoshan Xu, Bethany Harris, Brian Slaughter, Jay Unruh, Alexander Garrett, William McDowell, Andrew Box, Hua Li, Allison Peak, Sree Ramachandran, Jennifer L. Gerton
PLOS GENETICS
Volume 8, Issue 6 Jun 14, 2012
Pages e1002749
DOI: 10.1371/journal.pgen.1002749

Cohesion promotes nucleolar structure and function
Bethany Harris, Tania Bose, Kenneth K. Lee, Fei Wang, Shuai Lu, Rhonda Trimble Ross,Ying Zhang, Sarah L. French, Ann L. Beyer, Brian D. Slaughter, Jay R. Unruh, Jennifer L.Gerton
(Submitted to Molecular Biology of the Cell)

Roberts syndrome-a deficit in acetylated cohesin at heterochromatic regions leads to nucleolar dysfunction
Baoshan Xu, Shuai Lu, Jennifer L. Gerton
(Submitted to Rare Diseases)

MEETING ABSTRACTS AND PRESENTATIONS:

The cohesin acetyltransferase Eco1 promotes DNA damge-induced reciprocal mitotic crossovers in *S. cerevisiae*
FASEB Summer Research Conference - Genetic Recombination and Genome Rearrangements, Snowmass Village, CO (2009).

The cohesin acetyltransferase Eco1 promotes DNA damge-induced reciprocal mitotic crossovers in *S. cerevisiae*
Midwest Yeast Genetics Meeting, Northwestern University (2009).

The acetyltransferase activity of Eco1 is important for DNA damage repair in *S. cerevisiae*
Crossroads Student and Postdoctoral Association, Stowers Institute for Medical Research, Young Investigators Day (2010).

Studying the role of sister-chromatid cohesion in regulating the dynamics of rDNA transcription by transcript counting in single cells
Crossroads Student and Postdoctoral Association, Stowers Institute for Medical Research, Young Investigators Day (2011).

The cohesin acetyltransferase Eco1 regulates replication and segregation of ribosomal DNA
Crossroads Student and Postdoctoral Association, Stowers Institute for Medical Research, Young Investigators Day (2012).