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By

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Professor Douglas Koshland, Chair
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Abstract

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The Structural Maintenance of Chromosomes (SMC) family of proteins form a class of protein complexes that mediate chromosome structure through sister chromatid cohesion, chromosome condensation, DNA repair and transcription. Accessory proteins including Wpl1p, Pds5p and Eco1p regulate the SMC complex, cohesin, temporally and spatially to achieve these different functions. The roles and interactions of these three regulators are complicated. The goal of my project is to parse out the physical and genetic interactions between these regulators to understand how they regulate cohesin's various functions.

Pds5p and Wpl1p, known to form a sub-complex, appear to positively and negatively regulate cohesin. However, it is not known which functions of Wpl1p are mediated through its interaction with Pds5p, and which, if any are independent of Pds5p. I have shown that Wpl1p interacts with a non-essential domain in the N-terminus of Pds5p to promote cohesion and inhibit condensation. There is a discrepancy between the N-terminus of Pds5p acting as an inhibitor of condensation, and the full-length protein acting as a promoter or condensation. Thus, I have proposed a model in which Wpl1p inhibits Pds5p function through its interaction with the N-terminal regulatory domain of Pds5p. Additionally, this interaction is necessary but not sufficient for Wpl1p function.

I have also expanded our knowledge of an underappreciated role for Wpl1p in the DNA damage response. I have shown that Wpl1p function is important for efficient repair of S-phase DNA damage. Additionally, I have shown that this role in DNA repair is independent of Pds5p. Thus there are likely two forms of cohesin, a Pds5p-bound form that promotes cohesion and condensation, and a form not bound to Pds5p that mediates DNA damage-induced cohesion.

Eco1p is known to critically acetylate the Smc3p sub-unit of cohesin at K112 K113 to promote cohesion. Aside from inhibiting Wpl1p function, it is unknown how this acetylation promotes cohesion. The *smc3-D1189H* allele located in the head domain of

cohesin is able to compensate for loss of acetylation at K112 K113 to promote cohesion in a Wpl1p-independent manner. This characterization, along with other alleles in the Smc3p ATP binding pocket, show that cohesion activation through this pathway down-regulates ATPase activity, implicating ATPase function in a step past DNA binding for the first time. Additionally, in the absence of Eco1p function, *smc3-D1189H* only modestly promotes cohesion and fails to support viability, indicating that additional targets of Eco1p are needed to fully promote cohesion, viability and condensation.

Together, the work presented here shows how both positive and negative regulation influence cohesin function. Additionally, put together, this work shows that balance between the positive and negative regulation is important for proper cohesin functions in cohesion, condensation and DNA repair. Wpl1p must promote cohesin to be dynamic by destabilizing cohesin's interaction with DNA, while Eco1p and Pds5p promote stabilization of cohesin. Being able to promote a dynamic form of cohesin allows DNA tethering to change under each context. There still remains much to parse out, such as how Pds5p promotes condensation through cohesin, as well as how Wpl1p promotes destabilization of cohesin. Finally, identification of additional targets of Eco1p, and understanding how they modulate cohesin function will help to understand how cohesin-mediated tethering of DNA can structure the genome to perform diverse functions.

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This dissertation is dedicated to my nephews Daniel and Shlomo Bloom, born March 17th, 2014 and April 17th, 2016, for bringing me joy throughout this difficult process.

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CHAPTER 1: INTRODUCTION TO COHESIN REGULATION OF CHROMOSOME STRUCTURE

Functions of Chromosomes

Faithful transmission of genetic information from mother to daughter cells is one of the most fundamental processes in biology. To properly segregate chromosomes, the cell must ensure each chromatid has been replicated and paired properly with its sister. This cohesion between sister chromatids is critical for the bi-polar spindle attachment of sister chromatids to microtubules from opposite poles, thereby ensuring their proper segregation. The sister chromatids also undergo chromosome condensation to ensure the chromatids are packaged tightly enough to be pulled efficiently to the poles without becoming entangled or cut off as the daughter cells are pinched off. The timing and ordering of cohesion and condensation are tightly regulated. Understanding the mechanisms underlying cohesion and condensation, and their regulation has long been an important goal for basic science and for the prevention of genetic diseases and cancers that result from aberrations of their regulation.

A group of protein complexes termed for their function in the Structural Maintenance of Chromosomes (SMCs), are key players in cohesion and condensation (Table 1.1). The founding member of the SMC complexes is called cohesin (Strunnikov et al. 1993). Cohesin has been the most extensively studied of the SMC complexes in eukaryotes. The loss of cohesin function can lead to missegregation of genetic information and the development of diseases or cellular death.

In addition to its roles in chromosome segregation, cohesin has also been implicated in regulation of transcription and the DNA damage response. It is thought that cohesin must act through a common mechanism to mediate its diverse functions, and that temporal and spatial regulation of cohesin allows to it to perform each function distinctly. The keys to understanding how a simple complex can mediate diverse functions are in figuring out the basic mechanism through which cohesin can tether genomic loci and in identifying and understanding the roles of the accessory factors that regulate this tethering. This dissertation focuses on the physical changes to cohesin structure that mediate tethering in the context of cohesion as well has how the regulators Wpl1, Pds5 and Eco1 discern between cohesin function in cohesion establishment, condensation and DNA repair.

To understand the connection between cohesin structure and function in each context there are three basic questions to explore: 1) How does cohesin physically tether chromosomes? 2) How is tethering regulated throughout the cell cycle? 3) How is tethering regulated spatially to achieve its functions?

How does cohesin physically tether chromosomes?

Cohesin structure

Cohesin generates tethers between two DNA molecules or two regions within a single DNA molecule. Cohesin and all SMC complexes have structural similarities that predict a common mechanism in tethering which is then modulated to achieve their specific functions (Table 1.1)(Strunnikov et al. 1995). Cohesin is a four-subunit complex

containing two Smc proteins (Smc1p and Smc3p), a kleisin subunit, Mcd1p and a HEAT repeat subunit Scc3p (Guacci et al. 1997; Michaelis et al. 1997; Tóth et al. 1999).

The general structure of SMC complexes is driven by two Smc subunits, from which these complexes get their name. Smc proteins have a highly conserved structure in which the N- and C- termini interact to form a globular domain called the head. The intervening polypeptide chain between the N- and C-termini folds back on itself to form long coiled-coil region. At the site of the bend, a second globular domain called the hinge is formed (Melby et al. 1998; Haering et al. 2002) (Figure 1.1A).

The first step in the assembly of cohesin involves heterotypic interactions between Smc1p and Smc3p at both the hinge domains and the head, which forms an ATPase. Due to the nature of the Smc proteins having long flexible coiled-coil regions connecting the head and the hinge domains, it is believed that this Smc dimers forms a ring with a ~45nm diameter (Haering et al. 2002). While electron micrographs have shown that cohesin can adopt a ring shape, many other shapes have been seen as well, indicating that there is a high degree of flexibility in cohesin structure. These structures neither implicate nor preclude a ring from being the biologically functional form of cohesin. The dynamic nature of cohesin also suggests that dramatic structural changes may occur to allow cohesin to mediate its functions (Melby et al. 1998; Anderson et al. 2002).

Two other subunits contribute to cohesin's remarkable structure. The kleisin subunit, Mcd1p binds to Smc1p and Smc3p, with its N-terminus binding to the coiled coil just above the head of Smc3p and the C-terminus of Mcd1p binding to the bottom of head of Smc1p (Figure 1.1A). The HEAT repeat-rich protein Scc3p comprises the fourth and final subunit of cohesin. Scc3p does not structurally contribute to the cohesin ring, but critically interacts with Mcd1p to mediate cohesin function (Haering et al. 2002). The unusual architecture of cohesin is critical to the mechanism and regulation of its tethering activity. However, structural changes that are imposed to achieve cohesin function are not well understood.

Structure and function of the cohesin ATPase

A key feature of SMC complexes is the ATPase that is formed through the interactions between the Smc proteins in the head domain (Figure 1.1A). The function of the ATPase is poorly understood in cohesin. However, as it provides the only inherent enzymatic function of the complex, it is likely that the ATP binding and hydrolysis cycle is a key regulator of structure and function of the complex.

The ABC-like ATPases of SMC complexes are similar to those seen in transporters that pump solutes across membranes. The interaction between the two Smc molecules in the head form two ATPase active sites, each of which is comprised of four conserved domains: Walker A, Walker B, signature motif, and D-loop. In cohesin, the Smc3p ATPase active site is defined because the Walker A and Walker B domains are encoded by Smc3p. Smc1p provides the signature motif and D-loop *in trans*, and the opposite is true for the Smc1p ATPase active site (Figure 1.1B) (Hopfner et al. 2000; Lowe et al. 2001; Çamdere et al. 2015).

Understanding the function of the ATPases in cohesin has proved difficult. First, mutations that block the function of either of the two ATPase active sites, eliminates cohesin ATPase activity (Arumugam et al. 2006). Thus like all ABC ATPases, the

activity of the two ATPase active sites are coupled. This coupling complicates the ability to dissect the contributions of each ATPase active site to cohesin function. Second, abrogation of the Walker A or Walker B motifs of either active site abolishes cohesin binding to DNA (Arumugam et al. 2003; Heidinger-Pauli et al. 2010). Thus, it has been difficult to study the role of the ATPase in tethering steps past DNA binding *in vivo*. Additionally, the ATPase of cohesin has been difficult to study *in vitro* as purification of the complex proved difficult until recently.

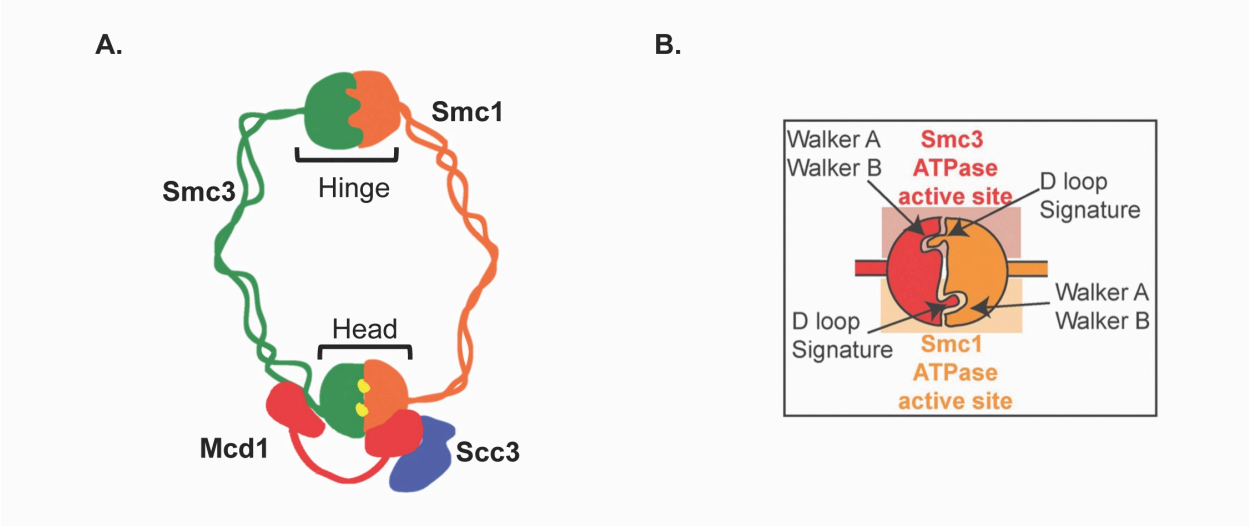

Figure 1.1: Schematic of cohesin structure
(A) Schematic of cohesin complex. Smc1p (orange), Smc3p (green) interact at head and hinge. Mcd1p (red) interacts with Smc1p and Smc3p at head and coiled-coil, respectively. Scc3p (blue) interacts with Mcd1p. **(C)** Schematic of ATPases within the cohesin head from (Çamdere et al. 2015). Smc3p ATPase is made up of Walker A and Walker B from Smc3p (red), and D-loop and Signature motif from Smc1p (orange). Smc1p ATPase is comprised of Walker A and Walker B from Smc1p and D-loop and signature motif from Smc3p.

Important clues about ATPase function have come from studies of the bacterial SMC complex, and the DNA repair complex Rad50 that were more easily purified. Studies of Rad50 showed the importance of an invariant aspartate (D) of the D-loop in mediating ATP hydrolysis. These studies highlighted the importance of cross-talk between the two subunits forming the ATP binding pocket (la Rosa and Nelson 2011). Recent studies have corroborated the functional importance of these aspartates in the D-loops in cohesin's ATPase active sites, showing that they also are critical for both ATPase activity and tethering (Çamdere et al. 2015). Finally, crystal structures of Rad50 indicate that ATP binding mediates long range structural changes of the coiled-coils (Mockel et al. 2012). These long range structural changes are commonly seen in other ABC ATPase proteins, such as transmembrane transporters in which the ATP binding and hydrolysis cycle modulates the opening and closing of the transporters to pump cargo across membranes (Hopfner and Tainer 2003).The long-range changes in Rad50

and ABC transporters suggest that ATPase function in cohesin may similarly cause large conformation changes to mediate tethering.

While these studies have greatly furthered our understanding of the structure/function relationship of SMC ATPase function, Rad50 has some key differences from cohesin. For one, Rad50 forms a homodimer. As a result, the ATP binding pockets of the ATPases are symmetrical (Table 1.1). A feature of the heterotypic interactions of the Smc proteins in cohesin is that it has asymmetrical ATP binding pockets. The Smc1p ATP binding pocket has a consensus signature motif, while the Smc3p ATPase has a non-consensus signature motif (unpublished). This asymmetry is also seen in many SMC complexes and ABC transporters. Analysis of the transporter ATPases showed that the consensus ATPase provides power for the conformation change, whereas the non-consensus ATPase slows down the ATP-hydrolysis cycle to prevent excessive hydrolysis (Procko et al. 2009). The presence of a non-consensus signature motif in the Smc3p ATPase may dampen cohesin ATPase activity, providing an explanation for the slow rate of cohesin ATPase. Additionally, the asymmetrical nature of the cohesin ATPase, suggests that it is a source of regulation of cohesin function. Indeed the Smc3p head is the target of modifications that control cohesin's activities. Advances in the ability to purify cohesin from the yeast, *Schizosaccharomyces pombe*, have allowed for more in depth study of the function of the asymmetrical nature of the ATPase and can hopefully elucidate its role in cohesion and condensation through future studies.

	Cohesin	**Condensin**	**Smc5/6**	**Rad50**	**SMC (bacterial)**
SMC	Smc1 Smc3	Smc2 Smc4	Smc5 Smc6	Rad50	SMC
Kleisin	Mcd1	Brn1	Nse4		ScpA
HEAT	Scc3	Ycg1 Ycs4	Nse5 Nse6		
Other			Nse1 Nse2 Nse3	Mre11 Xrs2/Nbs1	ScpB

Table 1.1: Conservation of SMC complex structure

Models of tethering

The ring structure of cohesin easily lends itself to a few simple models of tethering DNA molecules. The simplest model is the topological entrapment of sister chromatids within the ring. The finding that cleavage of the Smc3p coiled-coil leads to dissociation of cohesin from DNA supports entrapment of DNA within the cohesin ring

(Gruber et al. 2003). Similarly, cleavage of circular DNA molecules leads to dissociation of cohesin from DNA, presumably due to cohesin sliding off the end of a linear DNA molecule (Ivanov and Nasmyth 2005). The entrapment of DNA within the cohesin ring indicates that at least one interface of the ring must open for DNA to enter and exit. *In vivo* crosslinking between the Smc1p and Smc3p hinge domains prevented cohesin's association with DNA. Additionally, this process was lethal to the cell, most likely from an inability to mediate cohesin function (Gruber et al. 2006). In contrast, neither fusion of the Smc3p head to the Mcd1p N-terminus nor fusion of the Smc1p head to the Mcd1p C-terminus compromised cohesin function *in vivo*, suggesting that disengagement of these interfaces were not critical for cohesin association with DNA. These studies suggested that the opening of the Smc1p-Smc3p hinge interface was critical to mediate cohesin loading on to DNA and termed this interface the DNA "entrance gate" (Gruber et al. 2006).

There are a few models for how entrapment of DNA within the cohesin ring could mediate tethering. One variation of this model posits that both sister chromatids are entrapped within a single cohesin molecule (Figure 1.2A). Entrapment of a single sister chromatid is an attractive model for stable association of cohesin with DNA. However, in order to capture the second DNA molecule, the ring would have to open again. This second opening of the ring would make it just as likely for DNA to escape as it would to enter the ring upon each opening.

Alternatively, the handcuff model proposes that each DNA molecule is entrapped in a separate ring, and that the two different rings then oligomerize to mediate tethering (Figure 1.2B). As each ring only has to open once to allow only one sister to enter each ring, entrapment of DNA within the ring is favored in this model. Additionally, the handcuff model suggests that cohesin can be stably bound to DNA without mediating

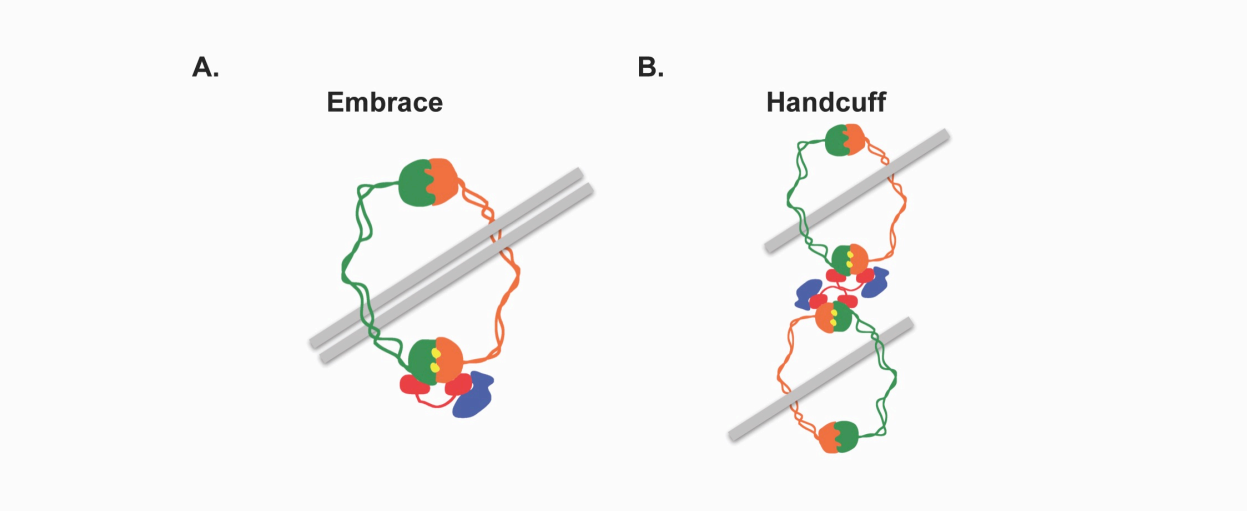

Figure 1.2: Models for cohesin tethering of DNA
(A) Embrace model. A single cohesin molecule captures both DNA molecules. **(B)** Handcuff model. Each ring captures a single DNA molecule. Cohesin rings oligomerize to tether both genomic loci.

cohesion. This model was supported by analysis of two *MCD1* alleles (Q266 and V137K) that showed that cohesin can associate stably with DNA, but cannot establish cohesion (Chan et al. 2013; Eng et al. 2014).

Additional support for tethering through oligomerization of cohesin molecules has been seen through inter-allelic complementation between cohesin subunits. These experiments showed that while two different mutant alleles of either *MCD1* or *SMC3* could not support viability or cohesion when provided as the sole copy in cell, when both alleles of a single gene were provided in *trans* in the same cells, viability, cohesin binding to DNA, and cohesion were all restored (Eng et al. 2015). Biochemical interactions between cohesin subunits that support the handcuff model have been detected in *S. pombe*, however biochemical evidence of interactions are lacking in other organisms (N. Zhang et al. 2008).

A model in which cohesin-cohesin interactions mediate tethering provides a model for regulating where and when genomic loci are tethered. This ability to regulate the spatial tethering of DNA is in contrast to the single ring model, in which tethering is most likely dictated by spatial proximity of the loci.

How is cohesin temporally regulated?

Independent of the specific mechanism of physical tethering of DNA by cohesin, its functions must be highly regulated. Accessory factors regulate the temporal and spatial functioning of cohesin. Their roles in mediating cohesion are best understood, however they have also been implicated in condensation. A complex of Scc2p and Scc4p regulates the first step toward mediating cohesion: loading cohesin onto chromosomes prior to S-phase. The acetyltransferase, Eco1p, then promotes establishment of cohesion concurrent with replication. Pds5p associates with cohesin to maintain cohesion through the remainder of the cell cycle, while chromosome condensation occurs. Eco1p and Pds5p both promote condensation in addition to

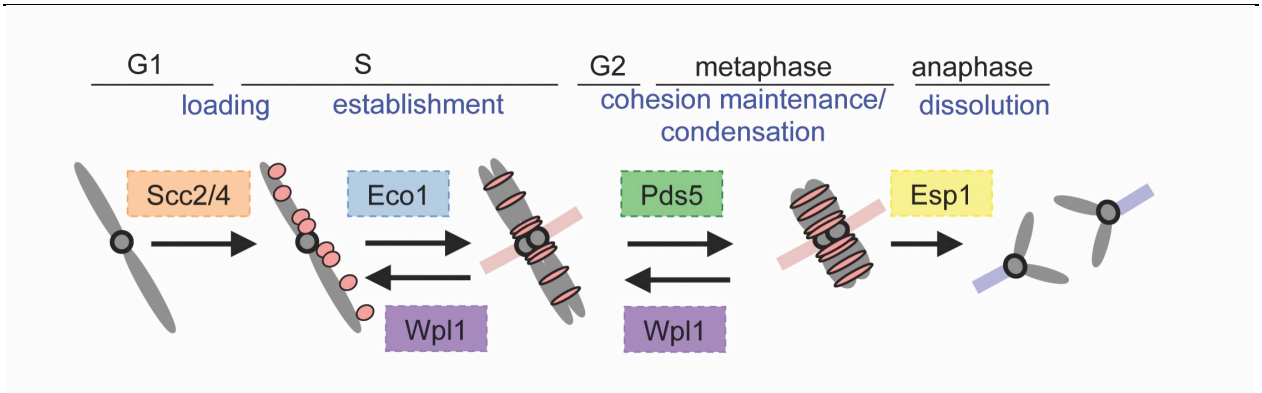

Figure 1.3: Progression of cohesion establishment during cell cycle
Scc2/4p (orange) promote cohesin loading into DNA during G1. Eco1p (blue) promotes cohesion establishment during S-phase. Pds5p (green) promotes cohesion maintenance and condensation during G2 and mitosis. Wpl1p (purple) antagonizes cohesion and condensation. Esp1p (yellow) promotes segregation of chromosomes at anaphase.

cohesion. Finally, in order for chromosomes to segregate, cohesin must be removed. Dissociation of cohesion can occur via two pathways: 1) Non-proteolytic removal of cohesin is mediated by the protein Wpl1p/Wapl, which acts to inhibit both cohesion and condensation throughout the cell cycle or 2) Cleavage of Mcd1p by Esp1p/Separase promotes the irreversible loss of cohesion and transition from metaphase to anaphase (Figure 1.3). As the loading and unloading of cohesin onto chromosomes and the establishment, maintenance and dissolution of tethering, are all critical to my thesis. I will describe each in more detail below.

Cohesin Loading

The life cycle of sister chromatid cohesion begins with the loading of cohesin onto chromosomes by Scc2p/Scc4p (Ciosk et al. 2000). Loading occurs prior to DNA replication, either during telophase of the previous cell cycle, as in vertebrates or during G1, as in yeast. Current evidence supports the model that the Scc2/4p complex acts as a receptor to localize cohesin to specific regions of DNA. Recently, structures of both *Saccharomyces cerevisiae* and *Ashbya gossypii* Scc2/4 complexes were solved, furthering our understanding of the function of the complex. This structural data along with other studies show that the complexes form flexible hook shapes, which can bind DNA independently of cohesin *in vitro*. Along with physical interactions between cohesin and Scc2/4p, these data support the model that Scc2/4p facilitates the interaction between cohesin and DNA (Fernius et al. 2013; Chao et al. 2015; Hinshaw et al. 2015).

While it is known that Scc2/4p stimulates cohesin binding to DNA, how Scc2/4p alters cohesin structure to do mediate binding is unknown. *In vitro* studies with purified Scc2/4p and cohesin from *S. pombe* have shown that cohesin can bind DNA in the absence of Scc2/4p, though only a small fraction of these complexes bind stably. Stable cohesin binding to DNA significantly increases in the presence of Scc2/4p suggesting that the loader can stabilize a conformation change in cohesin that is needed for stable association with DNA (Çamdere et al. 2015). It is thought that this stable association is the result of entrapment of DNA inside the cohesin ring, and that ATPase function promotes opening of the ring. In fact, Scc2/4p can stimulate ATP hydrolysis when combined with DNA *in vitro* (Murayama and Uhlmann 2013). Consistent with this hypothesis, cohesin mutants that are compromised for ATP binding and hydrolysis due to mutations in the Walker A and B motifs fail to localize to chromosomes *in vivo* (Arumugam et al. 2003; Heidinger-Pauli et al. 2010). These data together suggest that Scc2/4p promotes stable cohesin binding to DNA through opening of the cohesin ring by stimulating the ATPase.

Cohesion Establishment

Establishment of cohesion occurs as the sister chromatids are being formed during S-phase. Establishment is mediated by the acetyltransferase, Eco1p (Skibbens et al. 1999; Tóth et al. 1999). Genetic interactions between *ECO1* and replication machinery including, *PCNA, CTF18 and CHL1* suggested that Eco1p establishes cohesion concurrent with DNA replication during S phase (Moldovan et al. 2006; Borges et al. 2013; Samora et al. 2016). The loss of many non-essential replication factors led to mild defects in cohesion establishment, indicating either that cohesion can be established independently of replication, or that Eco1p interacts with many parts of the

replication machinery. Both of these hypotheses have been supported. Cohesion establishment can be uncoupled from replication when *de novo* cohesion establishment is induced in response to double-strand breaks in G2/M (Sjogren and Nasmyth 2001). Additionally, two genetically distinct families of replication factors have been shown to contribute to cohesion establishment: one group containing Ctf4p, Tof1p, Csm3p, Chl1p, and another containing Ctf18-RFC and Mrc1p (Xu et al. 2007). As cohesion can be establishment both concurrent with and independent of replication, it may be possible that DNA replication is a way to ensure that sister chromatids become paired, or tethered in register to one another during S-phase. Thus, it is possible that tethering independent of replication may result in improper pairing and the loss of genetic information during segregation.

Eco1p promotes cohesion establishment by acetylating two conserved tandem lysine resides on the Smc3p subunit. In *S. cerevisiae*, K112 and K113, acetylation of these two residues is critical for viability, cohesion and condensation (Rolef Ben-Shahar et al. 2008; Unal et al. 2008; J. Zhang et al. 2008). The individual contributions of acetylation of either K112 or K113 to establishment are unknown. K113 was initially identified as the critical site that, when mutated, resulted in a loss of viability to yeast cells, whereas mutation of K112 had no effect (Rolef Ben-Shahar et al. 2008). However, K112 is highly conserved, indicating some functional relevance. A recent *in vitro* analysis of the kinetics of acetylation of each residue showed that acetylation of K112 occurs faster than acetylation of K113, suggesting that acetylation of K112 may promote acetylation of K113 (Chao et al. 2017).

The difference in acetylation kinetics between K112 and K113 suggests that there is modularity of acetylation throughout the cell cycle. Consistent with this hypothesis, mutating K112 or K113 to mimic the acetylation modification cannot restore viability and cannot fully restore cohesion (Unal et al. 2008; Guacci and Koshland 2012). These findings suggest that constitutive acetylation, or acetylation at the improper time can be detrimental to the cell.

While acetylation of Smc3p at residues K112 and K113 have been identified as the critical acetylation sites for both cohesion and viability, analysis of Eco1p targets suggests that there may be additional biologically relevant acetylation sites for cohesin function. Eco1p can acetylate each cohesin subunit, and the regulators Scc2p, Scc4p, Pds5p and Wpl1p as well as auto-acetylate itself *in vitro* (Ivanov et al. 2002; Chao et al. 2017). Thus a large task for future studies will be to determine which targets are of biological relevance and to understand the function of these acetylation events.

Though these acetylation events are known to be critical for cohesin functions, how they facilitate these functions is unknown. A crystal structure of the *S. cerevisiae* Smc3p head domain shows that K112 and K113 lie in close proximity to the ATP binding pocket (Gligoris et al. 2014). However, until recently the ability to purify cohesin precluded direct study of how acetylation affects activity of the ATPase.

Aside from a proposed role in modulating the ATPase, a major function of acetylation is to counteract the cohesin antagonizer Wpl1p/Wapl (Rolef Ben-Shahar et al. 2008; Unal et al. 2008). This inhibition has been shown by the fact that cells lacking Eco1p are inviable when Wpl1p is present, but are alive when Wpl1p function is lost (Rolef Ben-Shahar et al. 2008; Guacci and Koshland 2012). Additionally, overexpression of Wpl1p can induce loss of cohesion (Lopez-Serra et al. 2013). As

Wpl1p can mediate cohesin removal from chromosomes, it has been hypothesized that Eco1p acetylation of cohesin makes cohesin refractory to Wpl1p. Thus, acetylation of cohesin may be a way to regulate the timing of Wpl1p function and restrict its action of removal of cohesin from chromosomes until mitosis.

Cohesion Maintenance

While Eco1p has a critical role in mediating critical acetylation marks to promote cohesion, the deacetylase Hos1p has been implicated in the removal of these marks (Borges et al. 2010; Xiong et al. 2010). The loss of these marks through Hos1p deacetylation correlates with the loss of cohesion (Chan et al. 2013). It has been hypothesized that an important biological function of Hos1p may be to remove existing modifications to reset cohesin for the subsequent cell cycle. However prior to segregation of chromosomes it is important to protect these modifications to maintain cohesion after it is established. This maintenance of acetylation marks and cohesion could occur either through constitutive Eco1p function that overcomes Hos1p-mediated deacetylation or through protection of acetylation marks from Hos1p removal activity.

The finding that Eco1p function is limited to S-phase indicates that another factor is needed to maintain acetylation after S-phase (Skibbens et al. 1999; Tóth et al. 1999). The protein Pds5p has been implicated in this role, though its function is more complex than simply protecting acetylation(Chan et al. 2013). Pds5p is a key factor in coordinating both cohesion and condensation as the loss of this protein leads to a loss of cohesion and an inability to condense chromosomes.

The importance of Pds5p has grown recently as it has been found that Pds5p interacts with other key cohesin regulators as well as cohesin subunits. Pds5p was found to interact with the Mcd1p unstructured region around residue V137 (Chan et al. 2013). Though the molecular function of this interaction is unknown, it is important for viability and cohesion maintenance. Additionally, *in vivo* cross-linking studies have suggested that Pds5p interacts with the Smc3p coiled-coil near its interface with Mcd1p, and other *in vitro* and *in vivo* studies have shown that Pds5p interacts with Wpl1p, Eco1p and the vertebrate cohesion maintenance factor sororin (Noble et al. 2006; Chan et al. 2013; Huis in 't Veld et al. 2014; Ouyang and Yu 2017; Goto et al. 2017).

Pds5p is a protein that is comprised of HEAT repeats, which are structural motifs made of up alpha helices. Proteins containing HEAT repeat domains are often involved in mediating protein-protein interactions and not enzymatic function. Thus a simple model for Pds5p function is that it acts to recruit these various factors to cohesin, acting as a scaffold to mediate their interactions with cohesin.

Alternative to this scaffold model, Pds5p may play an active role in modulating cohesin structure to promote cohesion and condensation and to unload cohesin from DNA. The numerous contacts Pds5p makes with other cohesin proteins and regulators implicate Pds5p as a complex regulator of cohesin function. Additionally, a recent unpublished screen from our lab failed to identify point mutants that could suppress the loss of *PDS5*. This result indicates that Pds5p function is vital to the cell and provides a unique function that cannot be compensated for by other proteins. Thus, understanding the function of Pds5p will be invaluable to our understanding of the mechanism of tethering. Additionally, understanding the relationship of Pds5p to the other regulators will further our understanding of positive and negative cohesin regulation.

Removal of cohesin

Cohesin is loaded on to DNA to promote cohesion, but cohesin is also removed from DNA to counteract cohesion. Cohesin can be removed by the cleavage of Mcd1p by Esp1p/Separase to promote segregation of sister chromatids at the onset of anaphase (Ciosk et al. 1998; Uhlmann et al. 2000). Alternatively, cohesin can be removed in a non-proteolytic manner through the protein Wpl1p/Wapl (Kueng et al. 2006).

In vertebrates, most of the cohesin is removed from the arms of chromosomes by Wapl during prophase of mitosis, while cohesin around the centromere is protected by shugoshin and sororin (Gandhi et al. 2006; Nishiyama et al. 2010). The removal of most of the cohesin from chromosome arms by Wapl is thought to promote the synchronous segregation of chromosomes at the onset of anaphase. This Wapl-mediated removal either ensures that all cohesin is removed from chromosomes to mediate the entrance into anaphase. Alternatively, it allows more synchronous cleavage of Mcd1p by Esp1p by reducing the amount of cohesin left on chromosomes to be cleaved.

In *S. cerevisiae* there is no evidence of a sororin homolog and no prophase removal pathway has been detected during mitosis. However, it is thought that Wpl1p acts as an inhibitor of cohesion and condensation by mediating removal of cohesin from chromosomes, as it does in vertebrates. The nature of this inhibitory function of Wpl1p in yeast remains enigmatic, but it is possible that Wpl1p inhibits condensation to ensure that it does not occur too early in the cell cycle. This hypothesis is consistent with the finding that cells lacking Wpl1p have premature compaction of chromosomes, although the cost of premature condensation to the cell is unknown, as Wpl1p is not essential in yeast (Lopez-Serra et al. 2013).

Esp1p cleavage of Mcd1p dissolves cohesion in an irreversible manner because once cleaved, the complex is neither able to mediate cohesion anymore, nor is the complex able to stably form or bind chromosomes (Uhlmann et al. 2000). In contrast, Wpl1p-mediated removal is neither proteolytic nor irreversible. In fact, cohesin is constantly loaded onto chromosomes throughout the cell cycle, but does not become cohesive after S-phase due to lack of Eco1p function (Kueng et al. 2006). Apart from specific removal of cohesin during prophase, it is thought that after S-phase, Wpl1p removes non-cohesive cohesin from chromosomes and mediates turnover of cohesin through this pathway.

The removal of cohesin in a non-proteolytic manner suggests that similar to loading, an interface of the tri-partite ring must be disengaged to allow the DNA to escape the ring. While it is thought that disengagement of the hinge is important for association of cohesin with DNA, this interface is not thought to be important for the unloading of cohesin. Instead, it was shown that Smc3p interaction with the N-terminus of Mcd1p is important for removal. Fusion of the Smc3p head to the N-terminus of Mcd1p reduced cohesin turnover, and prevented the destabilization of cohesin on DNA by overexpression of Wpl1p (Chan et al. 2012).

The interface between Smc3p and Mcd1p has been shown to be dynamic compared to the interaction between Smc1p and Mcd1p. The unstable nature of the Smc3p/Mcd1p interface suggests that it may be a sight of regulation on cohesin function. Indeed, Wpl1p function destabilizes the interaction of Mcd1p and Smc3p suggesting a mechanism for Wpl1p-mediated removal of cohesin (Beckouët et al.

2016). While these studies indicate that one function of the Smc3p/Mcd1p interaction is to act as an “exit gate” that opens and closes the cohesin ring, these data does not preclude the idea that this interface may do more than that.

Conclusion to regulation of cohesin

Cohesin, at its core, is a simple molecule that mediates diverse and complex functions. The complex regulation of cohesin to mediate bi-polar spindle attachments, cohesion, and condensation are difficult to parse, as they are not mutually exclusive functions. Understanding the timing and mechanisms through which regulators affect cohesin structure and function will help elucidate how these chromosomal functions are ordered. Until recently, much of this understanding has come from genetic studies and *in vivo* cell biological studies. The recent advancement in purification of cohesin has allowed *in vitro* assessment of cohesin’s inherent ATPase function and how regulators modulate ATPase function to mediate DNA binding and tethering. Additionally, the advancement in purification has led to crystallization of many cohesin subunits and many of its regulators. Combined, these two techniques have opened up directions for future research to analyze the relationship between cohesin structure and function, and how regulators alter this relationship.

How is cohesin spatially regulated?

Though cohesin function alone is likely a simple mechanism of tethering two genomic loci through entrapment within one or multiple cohesin rings, there are many possible combinations of loci that can be tethered. Tethering of different loci leads to different chromosome structures and, in turn, functions. Cohesion of sister chromatids occurs through inter-molecular interactions, while condensation occurs through intra-molecular interactions. Likewise, cohesin function in mediating DNA repair likely functions similarly to S-phase cohesion establishment, tethering two sister chromatids inter-molecularly to promote repair off of the intact sister. The differences between these functions suggest an ability to discern between inter- and intra-molecular interactions and that the regulatory proteins can help choose between the two.

Localization of cohesin

Cohesin localization within the genome is highly regulated. Though there is no specific cohesin-binding motif, cohesin is enriched at specific regions of the genome. In yeast, these Cohesin Associated Regions (CARS) are A-T rich and primarily intergenic sequences that are spaced about 10kb away from each other (Laloraya et al. 2000). This pattern, however, is not conserved and the DNA properties that define cohesin-binding sites can vary among organisms.

One feature of cohesin binding that is common among most organisms is that cohesin is highly enriched around centromeres (Blat and Kleckner 1999). Centromere DNA is highly structured to reinforce the region where the kinetochore is built, in order to withstand the spindle forces needed to establish bi-polar attachment and segregate chromosomes. In yeast, the centromeres of all sixteen chromosomes associate in a barrel, which forms a rigid body that can withstand the spindle forces (Yeh et al. 2008). Cohesin and condensin are spatially organized at the centromere to form a spindle-axis from inter-molecular interactions between sisters, and intra-molecular interactions via

DNA that loops out radially from this central axis. Interactions between condensin molecules create the central axis, and cohesin molecules tether intra-chromosomally to make radially extruded loops. These interactions serve to structure the chromatin, giving it the ability stretch under the force of the spindle (Stephens et al. 2011).

Cohesin on chromosome arms functions differently from cohesin at the centromere. On chromosome arms cohesin serves to tether sister chromatids. Here cohesin primarily mediates inter-molecular interactions (though this interaction does not preclude intra-sister interactions that serve other functions). Interestingly these inter-sister links are also thought to suppress interaction between homologous chromosomes (Cortés-Ledesma et al. 2007). This distinction between sisters and homologs indicates a role for cohesin in maintaining genome stability.

Cohesin function in DNA Repair

In G2/M cohesion establishment is upregulated in response to double-strand breaks (DSBs) through pathway that is distinct from that of canonical cohesion establishment during S-phase (Strom et al. 2007; Unal et al. 2007). While both cohesion establishment pathways involve many of the same regulators such as Scc2/4p for loading, and Eco1p to mediate establishment, there are distinct differences (Table 1.2). The targets of Eco1p acetylation are different between the two pathways. S-phase cohesion requires acetylation of Smc3p-K112 K113, while DNA damage-induced cohesion requires acetylation of the Mcd1p subunit at K84 and K210 (Heidinger-Pauli et al. 2009). The different acetylation targets indicate a clear distinction between the molecular bases of cohesion in the two pathways, as acetylation of K112 K113 cannot compensate for loss of acetylation at Mcd1p-K84 K210 upon DSB induction. Similarly, acetylation of Mcd1p-K84 K210 cannot facilitate S-phase cohesion in the absence of acetylation at Smc3p-K112 K113. Despite not being able to compensate for each other, acetylation under both contexts is thought to counteract Wpl1p function (Heidinger-Pauli et al. 2009). Thus, these acetylation events do function similarly in some respects. The differences may either indicate different modes of tethering between the two pathways, or that the acetylation marks themselves have distinct molecular functions.

Recent analysis of the mechanism of Eco1p acetylation suggested that the upstream step of Chk1p phosphorylation of Mcd1p-S83 promoted the acetylation of K84 (Chao et al. 2017). This finding suggests that acetylation of K84 is favored in the DNA damage response because of the upstream phosphorylation event. Interestingly, both S83 and K84 are located at the interface of Smc3p and Mcd1p, suggesting that these residues may be protected from modification when Mcd1p is interacting with Smc3p. This observation poses the question of how modification of these residues alters the interaction of Mcd1p with Smc3p, and how this acetylation may be similar or different from the mechanism through which acetylation of K112 K113 modulates cohesin structure. Mcd1p acetylation may destabilize the Smc3p/Mcd1p interface, while Smc3p acetylation may modulate ATPase function. It is unknown if these two structural changes have the similar effects on cohesin tethering. Differentiating the molecular mechanisms of acetylation in both contexts would give great insight into the mechanism for cohesion establishment and how cohesin can discern between cohesion in either context.

While the activation of cohesin in the context of replication of sister chromatids and DNA repair are distinct, it is likely that their tethering functions are similar, in holding sister chromatids in close proximity and in register. Interestingly, as DNA damage-induced cohesion has primarily been studied in G2/M, it is unknown how cohesin is regulated in the event of DNA damage during S-phase. A question that emerges from simultaneous DNA damage and DNA replication in S-phase is whether the DNA damage-induced cohesion pathway is upregulated or whether S-phase cohesion sufficient. Discerning the regulation of cohesion in S-phase DNA damage may highlight the differences between the two cohesion establishment pathways.

Cohesin function in Condensation

Condensation of chromosomes is a critical process that is mediated by two separate SMC complexes: cohesin and condensin. Temporally, cohesin-mediated condensation occurs after cohesion has been established, but it is unknown how cohesin mediates condensation. The condensin complex has been ascribed the function of mediating chromosome compaction and the formation of a chromosomal axis during mitosis by directly mediating long-range chromosomal interactions. However, loss-of-function mutations in cohesin subunits and regulators lead to the loss of condensation as well as cohesion (Hartman et al. 2000). Thus, there are two models for how cohesin may mediate condensation: 1) Directly through tethering of chromosomes intra-molecularly, or 2) Indirectly by communicating with condensin to temporally regulate compaction

A distinguishing feature of these two models is that condensin-mediated condensation is reversible during mitosis in yeast, while cohesion mediated condensation is not. This was shown by the inactivation temperature-sensitive alleles and re-expression of these subunits at the permissive temperature. In mid-M-arrested cells, chromosomes can decondense upon inactivation of condensin and can be restored upon re-activation of condensin. However, the inactivation and re-expression of cohesin subunits does not show this same ability to re-activate condensation (Lavoie et al. 2004).

Recently, my lab uncovered a region of Mcd1p that is important in promoting condensation. Mutants in this region of Mcd1p, termed *ROCC* (regulation of cohesion and condensation), are defective for promotion of condensation and for maintenance of cohesion, like loss of Pds5p function (Eng et al. 2014). This phenotype suggests that there is a connection between maintenance of cohesion and establishment of condensation, and that ordering of these two functions is critical to proper segregation. Thus, performing these functions in the wrong order could be detrimental to the cell. A possible function of cohesin in condensation is to signal condensin once cohesion has been established either through directly interaction with condensin or through common regulators that mediate indirect communication between the two complexes.

Just as both Eco1p and Pds5p promote condensation, Wpl1p is an inhibitor of condensation (Guacci and Koshland 2012). Thus a key function of Wpl1p may be to inhibit condensation until after cohesion has been established. Having a greater understanding of the interplay between cohesin and condensin in mediating condensation will help to elucidate how tethering is regulated differentially between the two complexes and will help to understand how cohesin promotes condensation.

Additionally, understanding the connection between cohesin and condensin will give us insight into communication between other SMC complexes to mediate critical chromosomal functions.

Conclusion

Many regulatory factors, including Wpl1p, Pds5p and Eco1p, have been implicated in multiple cohesin functions (Table 1.2). This overlap corroborates the hypothesis that a common mechanism of tethering occurs in each context. The current knowledge of cohesin regulation in cohesion, condensation and DNA repair, leaves important questions that I will address in this thesis: 1) How are the functions of the regulatory proteins coordinated? 2) How does acetylation control cohesin function? 3) Does cohesin in S-phase have a function beyond establishment of cohesion? 4) How does cohesin mediate multiple functions within a single cell cycle?

	Cohesion	Condensation	DNA repair
Scc2/4	✓	✓	✓
Eco1	✓	✓	✓
Pds5	✓	✓	**?**
Wpl1	✓	✓	✓

Table 1.2: Versatility of cohesin regulators in different functions

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CHAPTER 2: BUDDING YEAST WPL1P REGULATES COHESIN FUNCTIONS IN COHESION, CONDENSATION AND DNA REPAIR THROUGH DISTINCT MECHANISMS

Introduction

Cohesin mediates a myriad of nuclear functions essential for both viability and the accurate transmission of genetic information. These processes include sister chromatid cohesion, condensation of chromosomes and the repair of damage to DNA. Cohesin's interactions with accessory factors ensure that these diverse processes occur in the proper time and place. Understanding the relationships between these accessory factors and the mechanisms through which they regulate cohesin is crucial to furthering our understanding of how cohesin can mediate its variety of functions. Wpl1p is one of these regulators, whose function remains enigmatic.

Wpl1p has long been implicated as a negative regulator of the cohesin complex. Its best understood function is in vertebrates where it participates in the non-proteolytic removal of cohesin from chromosome arms in mitotic prophase (Gandhi et al. 2006). To ensure that this activity of Wpl1p does not inactivate the cohesin that mediates sister chromatid cohesion during S-phase, cohesion is acetylated by Eco1p at two conserved lysine residues on the cohesin sub-unit Smc3p (K112, K113 in the budding yeast *Saccharomyces cerevisiae*) (Rolef Ben-Shahar et al. 2008; Unal et al. 2008). These acetylation marks are essential and one of Pds5p's key functions is to maintain these high levels of acetylation after S phase(Chan et al. 2013).

In addition to their functions in cohesion, Wpl1p, Eco1p, and Pds5p have all been implicated in regulation of chromosome condensation. In budding yeast, Wpl1p was implicated as an inhibitor of condensation because *wpl1*Δ leads to premature condensation and *wpl1*Δ restores viability and condensation to cells lacking Eco1p function (*eco1*Δ) (Guacci and Koshland 2012; Lopez-Serra et al. 2013). In contrast, Eco1p and Pds5p promote condensation and inactivation of either factor results in a dramatic condensation defect (Skibbens et al. 1999; Hartman et al. 2000). Together these results paint a simple picture of Wpl1p as an inhibitor of cohesin functions and Eco1p and Pds5p as counteracting promoters of cohesin function.

However, a number of results suggest a more complicated view of Wpl1p functions and its interaction with Pds5p. In budding yeast *wpl1*Δ cells display a mild, but reproducible cohesion defect implicating Wpl1p as a positive factor required for efficient cohesion (Figure 2.1) (Guacci and Koshland 2012). Furthermore, Wpl1p and Pds5p have been shown to form a sub-complex that, together, is capable of unloading of cohesin from DNA *in vitro* (Kueng et al. 2006; Murayama and Uhlmann 2015). Recent *in vivo* studies also suggest that Pds5p may have inhibitory activity on cohesin as alleles in *PDS5* suppress inviability of cells with an *eco1-ts* allele that reduces cohesin acetylation (Rowland et al. 2009; Sutani et al. 2009). Further analysis of these *pds5* alleles showed a complex relationship with *WPL1*. These alleles appeared to be defective for recruitment of Wpl1p to pericentromeric regions as evidenced by altered Wpl1p-GFP localization, yet were not compromised in the formation of the Wpl1p-Pds5p sub-complex (Rowland et al. 2009; Chan et al. 2012). Together these results suggest that Wpl1p and Pds5p can act both positively and negatively to regulate cohesin functions.

The complexity of Wpl1p and Pds5p functions coupled with the knowledge that they form a complex raises interesting questions: Is Pds5p an inhibitor of condensation like Wpl1p? If so, is the interaction of Wpl1p with Pds5p important for Wpl1p negative and positive functions? How does the complex perform these functions? Finally, does Wpl1p have additional functions and, if so are they always coupled to Pds5p?

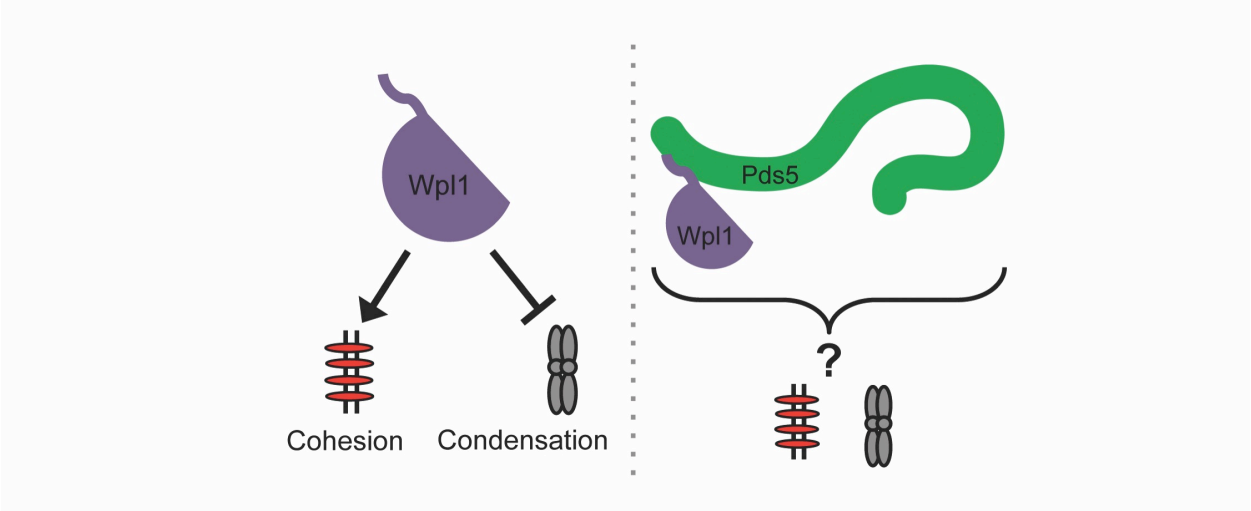

Figure 2.1: Wpl1 positively and negatively regulates cohesin function
Left: Model of Wpl1p function in yeast. Wpl1p promotes cohesion and inhibits condensation. Right: Schematic of Wpl1p-Pds5p interaction at Pds5p N-terminus. This study aims to understand which functions are mediated through Wpl1p-Pds5p interaction.

Results

Wpl1p regulates cohesion and condensation through Pds5p N-terminus

The first hint at potential functional cooperation between Wpl1p and Pds5p came from the phenotypic similarities between *wpl1*Δ and specific alleles of *pds5*, which suppress the inviability of *eco1-ts* mutations (Rowland et al. 2009; Chan et al. 2012). I performed additional experiments to further characterize the extent of these phenotypic similarities between *wpl1*Δ and representative alleles in the N-terminal region of *pds5*: *pds5-S81R, pds5-P89L,* and *pds5-E181K.* I first tested whether these alleles, like *wpl1*Δ could restore viability to *eco1*Δ, when no acetylation function is present. I constructed an *ECO1* shuffle strain, in which either wild-type *PDS5*, or one of the mutant alleles *pds5-S81R*, *-P89L* or *–E181K*, was the sole copy of *PDS5* in the cell. I then knocked out *eco1*Δ at its endogenous locus in the presence of a plasmid containing *ECO1 URA3* to maintain viability. By counter selection against cells with the plasmid on media containing 5-FOA, I could observe whether different alleles of *PDS5* sustain viability in the *eco1*Δ background. As expected, cells containing wild-type *PDS5* were unable to restore viability to *eco1*Δ. In contrast, *pds5-S81R* and *pds5-P89L* restore viability to *eco1*Δ. (Figure 2.2A)

In contrast to *pds5-S81R* and *-P89L*, *pds5-E181K* was unable to support viability

in *eco1*Δ (Figure 2.2A). To test if this inability to restore viability to *eco1*Δ was due to weak suppressor activity, I rebuilt *pds5-E181K* into a strain containing our lab's *eco1-ts* allele (*eco1-203*). At the restrictive temperature, 34˚C, *pds5-E181K* but not *PDS5* was able to restore viability to *eco1-203* (Figure 2.2B). Thus I conclude that *pds5-E181K* is a weak suppressor allele and that *pds5-S81R*, and *pds5-P89L* can bypass *ECO1* function, similar to *wpl1*Δ.

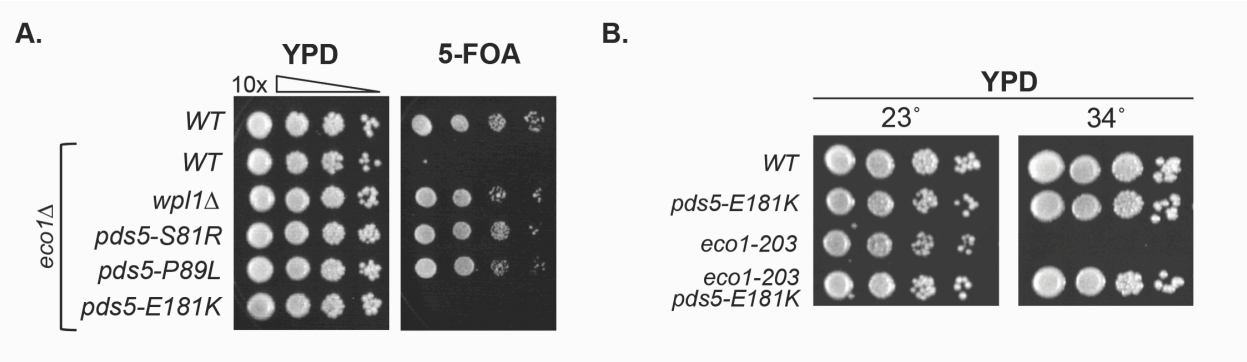

Figure 2.2: *pds5* N-terminal mutants restore viability to cells lacking *ECO1* function
(A) Plasmid shuffle assay to assess viability of *pds5* N-terminal mutants in *eco1*Δ background. Strains contain plasmid pBS1030 *(ECO1 CEN URA3)* transformed into haploid wild-type (VG3349-1B), *eco1*Δ (VG3499-1B), *eco1*Δ *wpl1*Δ (VG3503 #4), *eco1*Δ *pds5-S81R* (MSB138-1K), *eco1*Δ *pds5-P89L* (MSB139-2J), *eco1*Δ *pds5-E181K* (MSB147-1A). Cells were grown in YPD media and plated at 10x dilution on YPD or 5-FOA media at 23˚C for 3 days to select for loss of plasmid. **(B)** *pds5-E181K* can restore viability to *eco-203* temperature-sensitive allele. Strains containing *WT* (VG3349-1B), *pds5-E181K* (MSB101-3C), *eco1-203 PDS5* (VG3223-12B)*, eco1-203 pds5-E181K* (MSB189-2B) were serially diluted 10-fold and plated on YPD media. Plates were incubated at 23˚C and 34˚C for 3 days.

Suppressors of *ECO1* deficiency fall into two classes: those in the cohesin ATPase active sites that restore both cohesion and condensation, or *wpl1*Δ that restore only condensation but not cohesion (Guacci and Koshland 2012; Çamdere et al. 2015). To test whether the *pds5* N-terminal mutants restored viability to *eco1*Δ through the same mechanism as *wpl1*Δ, I examined their condensation and cohesion phenotypes in *ECO1* deficient cells. I assessed cohesion in *eco1*Δ cells containing the *pds5* N-terminal mutants by monitoring the separation of sister chromatids at both CEN-proximal (*TRP1*) and CEN-distal (*LYS4*) loci on chromosome IV. Cohesion is assessed by quantification of cells that contain either a single LacI-GFP focus (indicating cohesion) or two GFP foci (indicating cohesion loss) at these loci where tandem repeats of the *LacO* sequence have been integrated. I allowed cells to progress synchronously through one cell cycle after release from G1, and arrest in mid-M by addition of nocodazole (Figure 2.3A). As expected, in wild-type cells, sister chromatids remained tethered in both regions while *eco1*Δ *wpl1*Δ cells exhibit ~70% of cells with separated sisters. This high level of separated sisters was also observed in *pds5-S81R eco1*Δ and *pds5-P89L eco1*Δ cells

at both CEN-proximal and distal loci (Figure 2.3B). Additionally, *eco1-203* experienced a severe cohesion defect at 34˚C, which *pds5-E181K* failed to ameliorate (Figure 2.3C). Thus, the *pds5* N-terminal mutants behave like *wpl1*Δ, in that they fail to restore cohesion to *eco1* deficient cells.

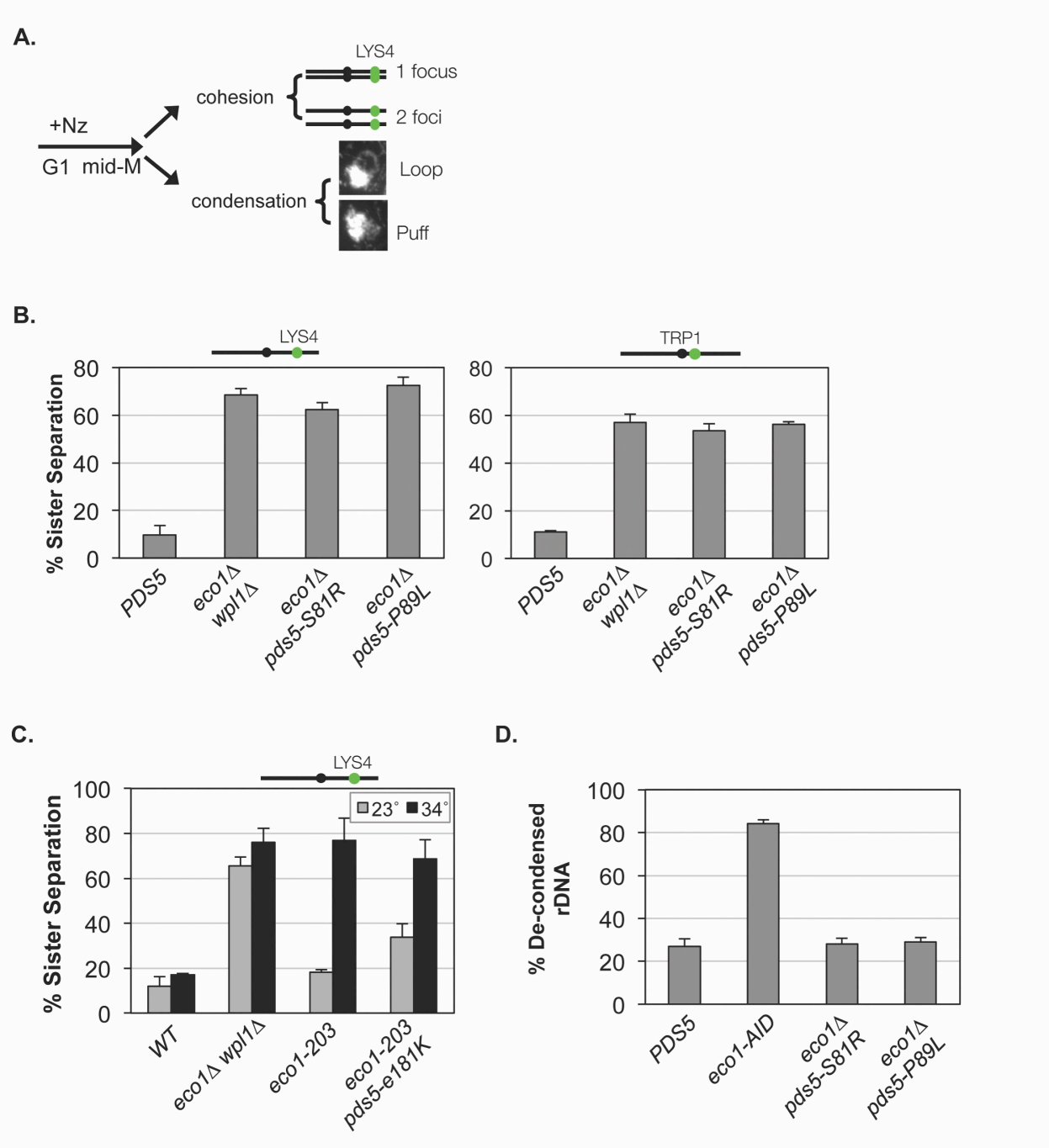

Figure 2.3: *pds5* N-terminal mutants restore condensation but not cohesion to cells lacking *ECO1* function

(A) Schematic of time course for assessment of cohesion loss and condensation. Cohesion loss is assessed by separation of LacI-GFP at CEN-distal *LYS4* locus or CEN-proximal *TRP1.* 1 GFP focus indicates cohesion and 2 GFP foci indicate loss of cohesion. Chromosome condensation is assessed by morphology of the rDNA locus. "Loop" morphology indicates proper condensation while "puff" indicates a decondensed rDNA locus. **(B)** Cohesion loss of *pds5* N-terminal mutants in *eco1*Δ*.* Cells were synchronously arrested in mid-M as described in Materials and Methods. Left: strains assessed for separation of LacI-GFP foci at *LYS4. PDS5* (VG3349-1B), *eco1*Δ *wpl1*Δ (VG3503 #A), *eco1*Δ *pds5-S81R* (MSB138-1K), *eco1*Δ *pds5-P89L* (MSB139-2J) Right: strains assessed for separation of LacI-GFP foci at *TRP1. PDS5* ((MSB185-1A), *eco1*Δ *wpl1*Δ (VG3502 #A), *eco1*Δ *pds5-S81R* (MSB210-2A), *eco1*Δ *pds5-P89L* (MSB211-2J). **(C)** Assessment of ability of *pds5-E181K* to restore cohesion to *eco1-203*. Strains were synchronized in G1 by addition of alpha-factor to media. Strains were split and incubated at 34˚. Strains were then released and re-arrested in mid-M as described in Figure 2.1A. **(D)** Assessment of rDNA condensation through FISH. Cells were synchronously arrested in mid-M through same progression as part A, with addition of Auxin during alpha-factor arrest. Detection of DNA through DAPI stain. Quantitation of "puff"-like structures in *PDS5* (VG3349-1B)*, eco1-AID (VG3633-2D), eco1*Δ *pds5-S81R* (MSB138-1K)*, and eco1*Δ *pds5-P89L* (MSB139-2J)

Through a similar regimen, I assessed the chromosome condensation in *pds5-S81R eco1*Δ and *pds5-P89L eco1*Δ in mid-M phase. Condensation in budding yeast can be easily monitored by the morphology of the rDNA locus, which distinctly protrudes from the main DNA mass in the nucleus. A condensed rDNA locus forms a distinct loop structure, while decondensed rDNA locus form a "puff" morphology (Figure 2.3A)(Guacci et al. 1993). In mid-M arrested wild-type cells, almost all cells exhibited the condensed rDNA loops. Using the auxin-degradation system, I depleted Eco1p by the addition of auxin to cultures containing the *eco1-AID* alleles and observed that over 80% of these cells had decondensed rDNAs. Consistent with what has been reported for *eco1*Δ *wpl1*Δ cells, *pds5-S81R eco1*Δ and *pds5-P89L eco1*Δ cells had ~20-30% of cells with decondensed rDNA loci (Figure 2.3D) (Guacci and Koshland 2012). From these data, I conclude that mutants in the *pds5* N-terminus, like *wpl1*Δ*,* restore condensation but not cohesion to *ECO1* deficient cells. Thus, the N-terminus of Pds5p shares with Wpl1p the ability to inhibit condensation.

Both Wpl1p and Pds5p promote cohesion. However, *wpl1*Δ cells only exhibit a minor cohesion defect (~30%), while cells lacking Pds5p exhibit a severe cohesion defect (~80%) (Hartman et al. 2000; Guacci and Koshland 2012). This quantitative difference suggested that these two factors might promote cohesion by different mechanisms. However, it was possible that Pds5p promotes cohesion by two mechanisms, one dependent on Wpl1p and the other independent of Wpl1p. Given the phenotypic similarity between *wpl1*Δ and the N-terminal alleles of *pds5* in condensation, I wondered whether the N-terminus of Pds5p might be involved in a Wpl1p-dependent pathway for cohesion. To test this pathway I monitored the ability of the *pds5* N-terminal mutants to mediate cohesion both in the presence and absence of *WPL1*.

When cohesion was monitored at both CEN-proximal and distal loci, all three *pds5* N-terminal mutants exhibited cohesion defects of ~20% and 30%, respectively,

similar to that of *wpl1*Δ (Figure 2.4A &B). Additionally, kinetic analysis of the *pds5p* N-terminal mutants showed that they lost cohesion similarly to *wpl1*Δ throughout the course of the cell cycle (Figure 2.4C). These quantitative similarities were consistent with the model that Wpl1p and the Pds5p N-terminal domain acted in a common pathway to promote cohesion. To test this model further, I assessed the cohesion defect of the *pds5* N-terminal mutants in the absence of *WPL1*. If both Wpl1p and the N-terminal domain of Pds5p were required for distinct functions in the promotion of cohesion, I would have expected to see an additive cohesion defect in the double mutants. When combined with *wpl1*Δ each of the *pds5* N-terminal mutants experienced a slight increase in cohesion loss from the single mutants, but not significantly different from *wpl1*Δ cells (Figure 2.4A&B). These results are consistent with the model that Pds5p N-terminal domain and Wpl1p promote cohesion through a common pathway, which is fully inactivated by the *wpl1*Δ and mostly inactivated by the *pds5* N-terminal alleles. These results suggest that Wpl1p interacts functionally with Pds5p both to inhibit condensation and to promote an aspect of cohesion.

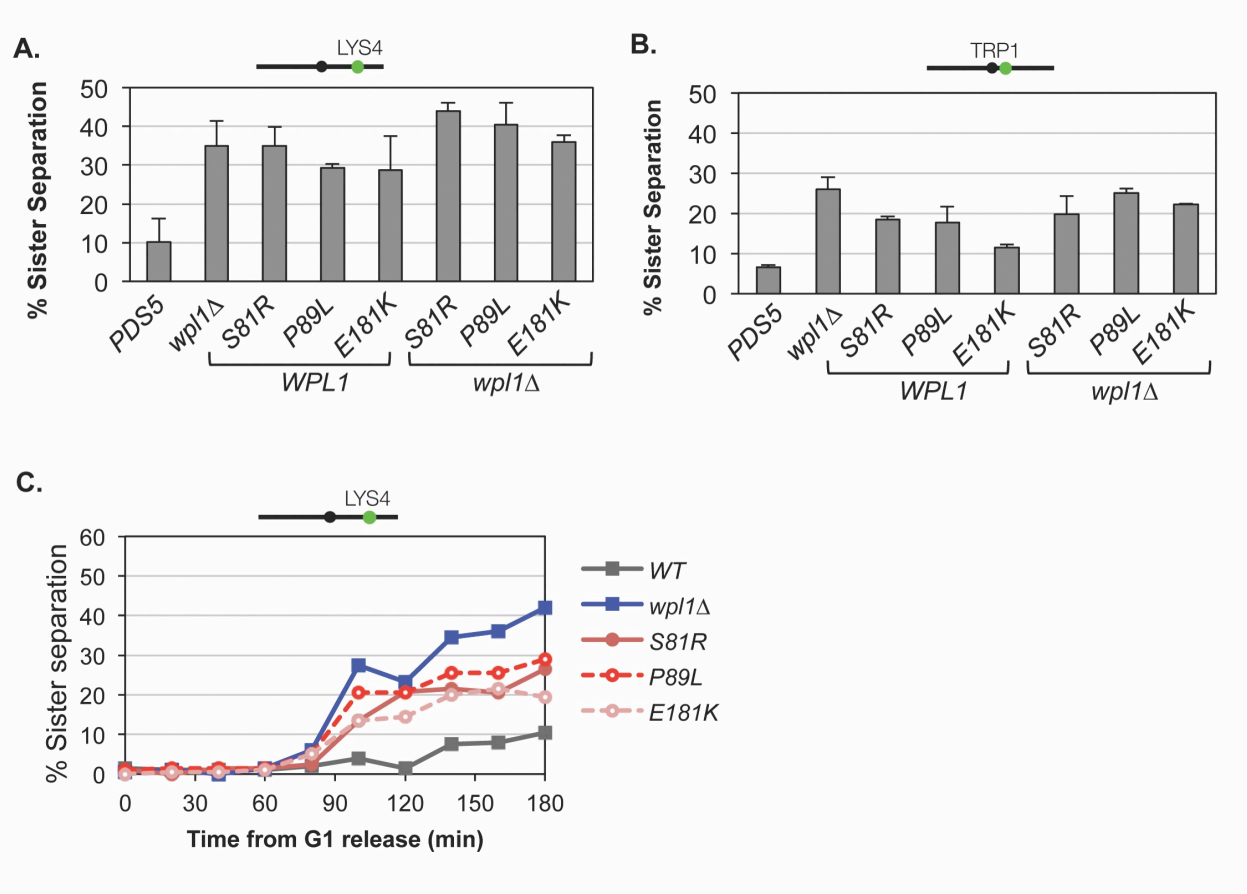

Figure 2.4 *PDS5* N-terminus promotes cohesion through same pathway as *WPL1* **(A)** A&B: Cohesion loss of *pds5* N-terminal mutants in presence or absence of *WPL1.* Cells were synchronously arrested in mid-M as described in Materials and Methods. Strains assessed for separation of LacI-GFP foci at *LYS4: PDS5* (VG3349-1B), *wpl1*Δ (VG3360-3D), *pds5-S81R* (MSB183-1A), *pds5-P89L* (MSB184-3A), *pds5-E181K*

(MSB101-3C), *pds5-S81R wpl1*Δ (MSB133-3C), *pds5-P89L wpl1*Δ (MSB134-1L), *pds5-E181K wpl1* Δ (MSB223-1A). **(B)** Strains assessed for separation of LacI-GFP foci at *TRP1* as in part A: *PDS5* (MSB185-1A), *wpl1*Δ (VG3513-1B), *pds5-S81R* (MSB190-3E), *pds5-P89L* (MSB191-3A), *pds5-E181K* (MSB186-2E), *pds5-S81R wpl1*Δ (MSB204-1B), *pds5-P89L wpl1*Δ (MSB205-4C), *pds5-E181K wpl1* Δ (MSB206-6A). **(C)** Kinetics of cohesion loss in *pds5* N-terminal mutants. Time course performed as described in Materials and Methods. Segregation of LacI-GFP was assessed at *LYS4* every 20 minutes for *PDS5 (VG3349-1B)*, wpl1Δ *(VG3360-3D), pds5-S81R* (MSB183-1A), *pds5-P89L* (MSB184-3A) and *pds5-E181K* (MSB101-3C) cells

Pds5p N-terminus is a regulatory domain that mediates Wpl1p function

A simple model that could explain my conclusions that Wpl1p and the N-terminus of Pds5 functioned together to inhibit condensation and promote cohesion was that Wpl1p directly interacted with Pds5p in this region. Indeed during the course of this work, a crystal structure was obtained of a short peptide of the human Wapl bound to the N-terminus of human Pds5B (Ouyang et al. 2016). As this region of Pds5B was highly conserved with yeast Pds5p, I was able to map the analogous residues of the N-terminal mutations from my analysis on the crystal structure. I found that these residues were located either within or in very close proximity to the Wapl binding site (Figure 2.5A & Sup. Figure 2.11A). Additionally, yeast Wpl1p contains a partial consensus sequence to the conserved [K/R] [S/T] YSR motif important for Wapl interaction with Pds5B in vertebrates, suggesting that Wpl1p and Pds5p may bind in a similar manner in yeast (Ouyang et al. 2016). Given this structural information, I asked whether the Pds5p N-terminal mutations altered the physical interaction between of Pds5p and Wpl1p.

I immunoprecipitated Wpl1p that is C-terminally tagged with the Flag epitope and examined levels of co-immunoprecipitation with wild-type and mutant Pds5p. I observed that Pds5p robustly co-immunoprecipitated with Wpl1p-3FLAG but not with untagged Wpl1p, showing that the presence of Pds5p in the immunoprecipitate reflected a specific interaction with Wpl1p. In contrast, the Wpl1p-3FLAG immunoprecipitates contained very little Pds5p-P89L and significantly reduced Pds5p-E181K (Figure 2.5B). The findings that *pds5-P89L* and *pds5-E181K* phenocopied *wpl1*Δ and that both mutations disrupt binding to Wpl1p suggest that Wpl1p binding to the N-terminal domain of Pds5p is required for Wpl1p's functions as both an inhibitor of condensation and promoter of cohesion. Interestingly, the levels of Pds5p and Pds5p-S81R that co-immunoprecipitate with Wpl1p-3FLAG were not significantly different. *pds5-S81R* also fully phenocopies a *wpl1*Δ in all metrics I measured, suggesting that Wpl1p binding to Pds5p N-terminal domain is necessary but not sufficient to ensure Wpl1p function.

I also assessed the role of the Pds5p N-terminal domain in Wpl1p binding to cohesin by assessing Mcd1p levels that co-immunoprecipitate with Wpl1p-3FLAG. Cells containing wild-type Pds5p and Wpl1p-3FLAG associated with Mcd1p, when Wpl1p-FLAG was immunoprecipitated, while no Mcd1p was pulled down in lysates of the untagged Wpl1p cells. This specificity recapitulated the known interaction of Wpl1p and cohesin. In lysates expressing either Pds5p-S18R, Pds5p-P89L or Pds5p-E181K, co-immunoprecipitation of Mcd1p with Wpl1p-3FLAG was decreased, but not abolished relative to wild-type Pds5p. Additionally, levels of Mcd1p co-immunoprecipitation were similar in Pds5p-S81R, -P89L, and –E181K (Figure 2.5B). Since Pds5p-P89L abrogates

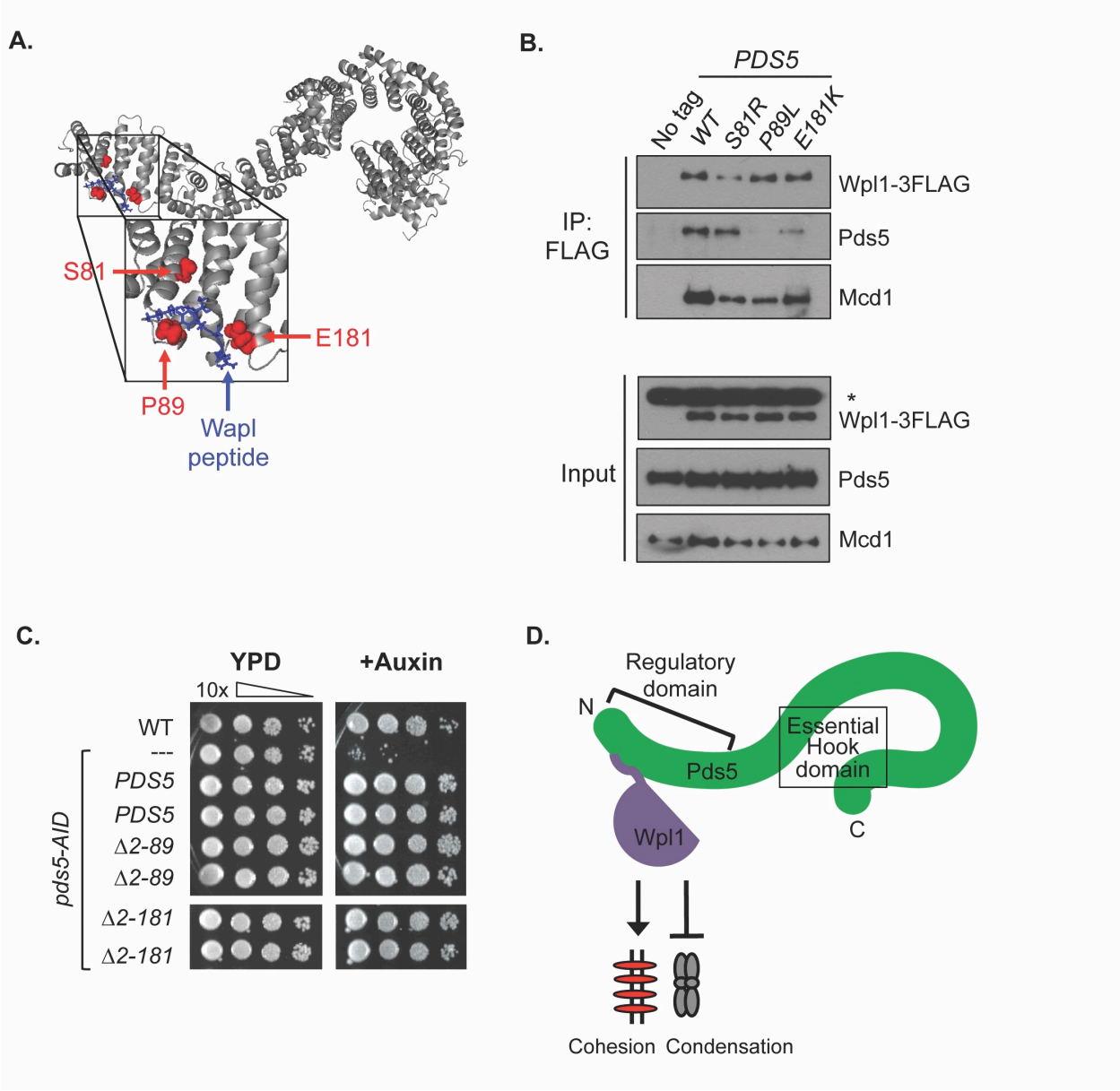

Figure 2.5 Pdsp5 N-terminus is a regulatory domain that mediates Wpl1 function
(A) Crystal structure of Pds5B bound to YSR motif of Wapl from (Ouyang et al. 2016). Gray: Pds5B Blue: Wapl peptide Red: *eco1-ts* suppressors from (Rowland et al. 2009) and (Sutani et al. 2009). Yeast residues were mapped to analogous residues on Pds5B through alignment. **(B)** Assessment of interaction between Wpl1-3FLAG and Pds5 N-terminal mutants through co-immunoprecipitation. Wpl1-3FLAG was immunoprecipitated in asynchronous cultures containing either *PDS5* (MSB192-2A), *pds5-S81R* (MSB193-1B), *pds5-P89L* (MSB194-1C), or *pds5-E181K* (MSB195-2D) as described in materials and methods. “No tag” control contains wild-type *WPL1* and *PDS5* alleles (VG3349-1B). Western blot analysis of protein levels through mouse anti-FLAG, rabbit anti-Mcd1 or rabbit anti-Pds5 antibodies. A non-specific species detected by FLAG is denoted by asterisk and is present in cells lacking epitope tagged Wpl1. **(C)** Assessment of viability of *pds5* N-terminal truncation alleles. Full length *PDS5*

(MSB213-1A&1B) and truncation alleles *PDS5 ∆2-89* (MSB214-2A&2B) and *PDS5 ∆2-181* (MSB215-3A&3B) were integrated at *LEU2* in strain containing second *PDS5-3V5-AID* (TE228) allele. Strains were plated on YPD media either with or without 750uM Auxin and incubated at 23˚C for 3 days. **(D)** Summary model for function of Wpl1-Pds5 interaction. Pds5 (green), and Wpl1 (purple) interact in N-terminal regulatory domain to promote cohesion and inhibit condensation. Regulatory domain (bracket) is distinct from the hook region of Pds5 that promotes its essential function (square).

Wpl1p interaction with Pds5p, these results suggest that Wpl1p can interact with cohesin independent of its association with Pds5p, as is expected given that Wpl1p interacts directly with the Scc3p subunit of cohesin in yeast and other organisms (Rowland et al. 2009; Shintomi and Hirano 2009).

I reasoned that if the sole function of the Pds5p N-terminal domain was to promote Wpl1p functions, then this domain should be nonessential like Wpl1p. To test this, I generated alleles of *pds5* that truncated the N-terminus up to residues 89, 181, or up to 334 (*pds5-∆2-89, -∆2-181, -∆2-201, -∆2-208, -∆2-251, -∆2-289, -∆2-334*) and integrated these alleles into a strain harboring *pds5-AID*. Upon depletion of *pds5-AID* by plating on media containing auxin, these truncations were able to sustain viability (Figure 2.5C, Sup. Figure 2.11B). Thus the N-terminal domain of Pds5p was not critical for Pds5p's essential function. I additionally characterized the cohesion defect in a subset truncation alleles. When measured at CEN-distal *LYS4*, *pds5 ∆2-89* had a ~30% cohesion defect, reminiscent of the *wpl1∆*. *pds5 ∆2-181* had an exacerbated cohesion defect, ~50% (Sup. Figure 2.11C). Thus is it possible that the N-terminus of Pds5p has multiple functions in promotion of cohesion, one that is mediated through Wpl1p and one that is distinct from Wpl1p. These results suggest that Wpl1p binding to the N-terminal domain of Pds5p forms a nonessential regulatory module that promotes cohesion and inhibits condensation (Figure 2.5D).

Wpl1p is necessary for efficient repair of DNA damage induced in S phase by Camptothecin and MMS

Cohesin has previously been implicated in the efficient repair of DNA damage by the *de novo* establishment of cohesion in response to double-strand break (DSB) formation in G2. This process is thought to be regulated by Eco1p and Wpl1p through a pathway similar to cohesion establishment in S-phase where Eco1p promotes cohesion establishment and Wpl1p inhibits it (Sjogren and Nasmyth 2001; Strom et al. 2007; Unal et al. 2007). These roles were corroborated by the finding that *eco1∆ wpl1∆* cells are highly sensitive to the DNA damaging agent camptothecin (CPT), a phenotype thought to be associated with lack of cohesion (Guacci and Koshland 2012). In contrast, Wpl1p has also been implicated as a promoter of DNA repair as *wpl1∆* cells exhibit sensitivity to ionizing radiation (Game et al. 2003). Additionally, Wpl1p was shown to inhibit the induction cohesion in response to double strand break in G2 (Heidinger-Pauli et al. 2009). These findings once again complicate our interpretation of Wpl1p function as it plays both positive and negative roles in the DNA damage response. As further analysis of Wpl1p's role in mitigating DNA damage has not been pursued, it is not clear what its positive role is.

To begin to assess the role of *WPL1* in DNA damage, I first characterized the impact of CPT on *wpl1*Δ cells. I compared the growth of wild-type, *wpl1*Δ and *eco1*Δ *wpl1*Δ cells after plating serial dilutions on media containing 20 µg/ml CPT. The growth of *wpl1*Δ cells was dramatically retarded relative to wild-type cells taking several additional days to form colonies. As expected, the growth of *eco1*Δ *wpl1*Δ cells was significantly more impaired as no growth appeared even after 5 days (Figure 2.6A). These results suggested that Wpl1p function was required for the timely repair of CPT damage. However, loss of Eco1p function is significantly more detrimental to cells, presumably due to the inability to form cohesion.

To further characterize the kinetics of DNA damage repair, I compared the progression of wild-type and *wpl1*Δ cells through the cell cycle in the presence and absence of CPT using the extent of the cell cycle delay is an indirect measure of repair. I synchronized wild-type and *wpl1*Δ cells in G1 by addition of alpha-factor and released them into media containing either DMSO or 20 µg/ml CPT. In order to measure the kinetics of a single division, I arrest the cells in the subsequent G1 by addition of alpha factor to the media. At 30-minute increments throughout the cell cycle I collected cells and analyzed them for bud morphology, DNA content, and kinetics of DNA segregation.

Analysis of DNA content revealed that both wild-type and *wpl1*Δ cells treated with CPT progressed though S-phase with similar kinetics as their DMSO-treated counterparts. However, the 2C DNA peak, indicating replicated DNA, persisted longer in the *wpl1*Δ sample treated with CPT than either the DMSO-treated *wpl1*Δ sample or the wild-type samples (Sup. Figure 2.12A). This indicated that *wpl1*Δ cells are delayed in completing the cell cycle because of persisting CPT damage that activated the G2/M DNA-damage checkpoint.

To test the possibility of a G2/M delay, I examined the bud and DNA morphology of wild-type and *wpl1*Δ cells put through this regimen. During an unperturbed cell division, DNA replication is completed when the bud is small. The nucleus migrates to the bud neck and mitosis ensues quickly resulting in the segregation of chromosomes into mother cell and a medium sized bud. When cells stall in G2/M, the unsegregated chromosomes remain at the bud neck and bud continues to grow giving rise to large budded cells with unsegregated chromosomes at the bud neck (Hartwell 1974). As expected, most cells (both wild-type and *wpl1*Δ) in the absence of CPT did not exhibit a cell cycle delay and divided prior to bud enlargement. 120-minutes after release from G1 arrest, only a small percentage of cells were large-budded with undivided nuclei. At 150-minutes post release, most cells accumulated in telophase (with divided nuclei) and steadily underwent cytokinesis as time progressed, until most had gone through and arrested in G1 by 240-minutes (Figure 2.6B left panels).

When treated with CPT, both wild-type and *wpl1*Δ cultures exhibited a similar increase in the proportion of large-budded cells that were stalled with undivided nuclei at 120-minutes post release (~40% of cells) compared to their DMSO-treated counterparts (Figure 2.6B right panels). This similarity in initial cell cycle arrest was consistent with wild-type and *wpl1*Δ initially experiencing the same level of DNA damage. The wild-type cells overcame this arrest quickly, and began dividing their nuclei by 150-minutes and were mostly divided by 180-minutes. In contrast, the levels of arrested *wpl1*Δ cells did not decrease until after 180-minutes, with division occurring much slower than wild-type (Figure 2.6B bottom-right panel). Eventually all the stalled

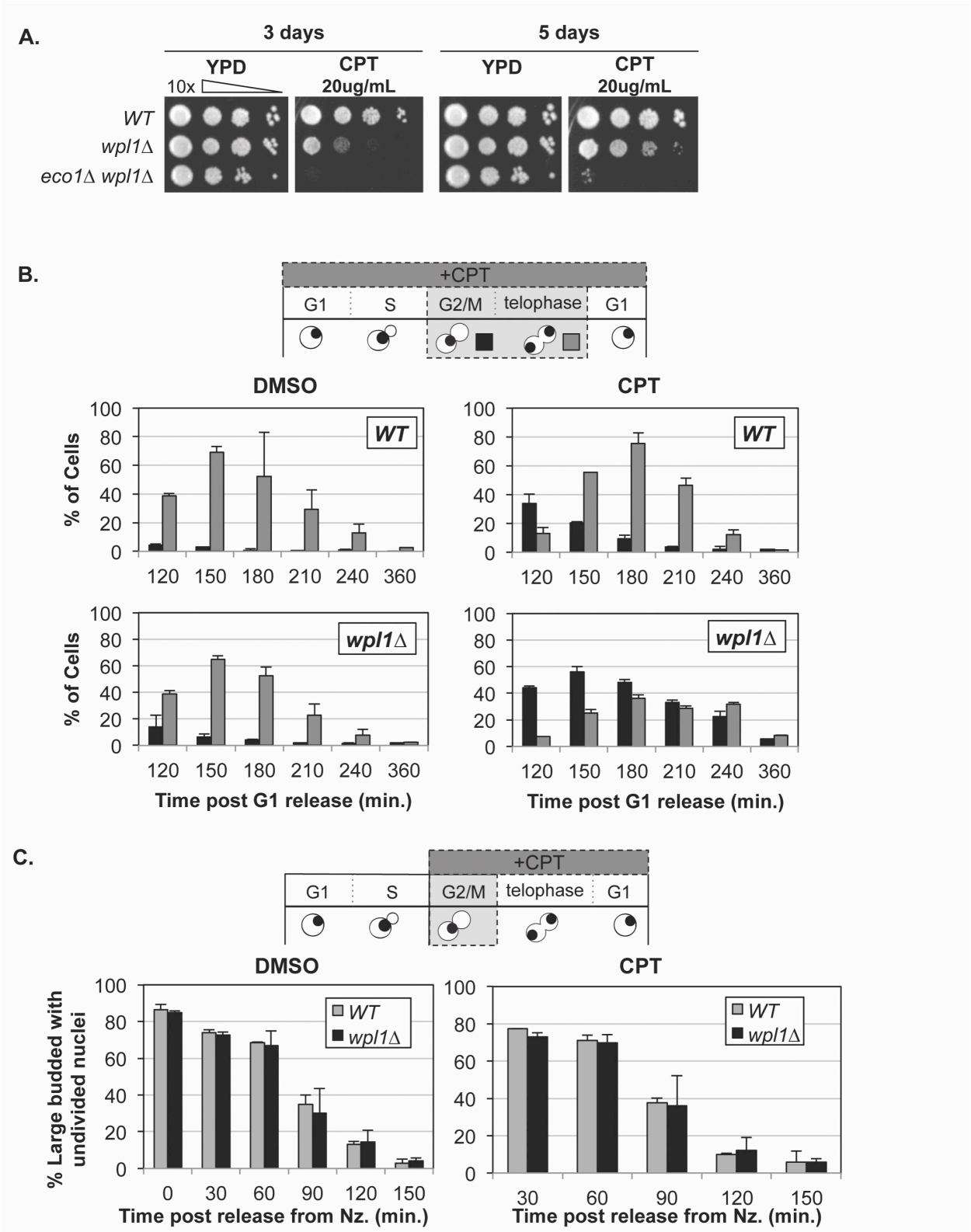

Figure 2.6: Wpl1p is necessary for efficient repair of camptothecin generated DNA damage

(A) *wpl1*Δ cells experience slow growth phenotype when treated with CPT. *WT* (VG3349-1B), *wpl1*Δ (VG3360-3D), and *eco1*Δ *wpl1*Δ (VG3503 #4) cells were serially diluted (each spot represents 10x dilution), and plated on YPD media either with or without 20 µg/ml CPT. Media preparation described in materials and methods. Plates were incubated at 23˚C and assessed at 3 and 5 days post plating. **(B)** Characterization of effect of CPT on *wpl1*Δ cells through a single cell cycle. *WT* (VG3349-1B), and *wpl1*Δ (VG3360-3D) cells were synchronized in G1 by incubation in YPD containing alpha-factor 23˚C for 3 hours and released in to media containing 25mM HEPES pH 7.4 and either DMSO or 20 µg/ml CPT added to the other. Time course is described in Materials and methods. Fixed cells were stained with DAPI for scoring. Cells were scored for bud morphology (no bud, small-medium bud, or large bud). Large budded cells were further classified as containing a single DAPI mass or containing 2 DAPI masses. Graphs are representation of % of cells that are large budded with either a single DAPI mass (black) or two DAPI masses (gray). **(C)** Assessment of S-phase requirement for CPT mediated stalling in *wpl1*Δ cells. *WT* (VG3349-1B), and *wpl1*Δ (VG3360-3D) cells were synchronized in G1 then released into the cell cycle, and re-arrested in mid-M with nocodazole. Cells were then released from mid-M arrest into HEPES buffered media as described in B. Alpha factor was added to cultures followed by splitting of cultures into which either DMSO or 20 µg/ml CPT was added. Time course is described in materials and methods. Fixed cells were stained with DAPI for scoring. Graphs are representation of % of cells that are large budded with a single DAPI mass at each given time-point for WT cells (gray) or wpl1Δ cells (black)

*wpl1*Δ cells were able to divide, indicating that the CPT damage could eventually be repaired. These results are consistent with a role for Wpl1p in the timely repair of CPT induced damage.

CPT-mediated damage is thought to cause DSBs during S-phase. Thus, these results implicate Wpl1p as a factor important for S-phase induced damage. This slow repair of S-phase-induced DSBs leads to subsequent activation of the G2/M checkpoint. To test whether the delay I observed in *wpl1*Δ cells was due to the CPT damage induced during S-phase, I allowed the cells to progress through S-phase in the absence of CPT and arrest in mid-M, with the addition of nocodazole. Following release from this arrest, I treated the cells with CPT and allowed them to undergo anaphase and arrest in the subsequent G1 by the addition of alpha-factor. I analyzed kinetics with which segregation of chromosomes occurred in large-budded cells by collecting cells every 30 minutes. Upon release from nocodazole, wild-type and *wpl1*Δ cells segregated their chromosomes with similar kinetics when treated with either DMSO or CPT (Figure 2.6C & Sup. Figure 2.12B). I conclude that Wpl1p helps to repair S-phase induced DNA damage.

To address whether *WPL1* has general role in mitigating other types of S-phase DNA damage, I analyzed the sensitivity of *wpl1*Δ cells to the alkylating agent, methyl-methanesulfonate (MMS). I first tested the sensitivity of *wpl1*Δ cells by serial dilutions onto media containing 0.01% MMS. Compared to wild-type cells, the growth of *wpl1*Δ cells was significantly impaired (Figure 2.7A). I then tested the role of *WPL1* in mitigating MMS-damage in a single cell cycle by putting wild-type and *wpl1*Δ cells

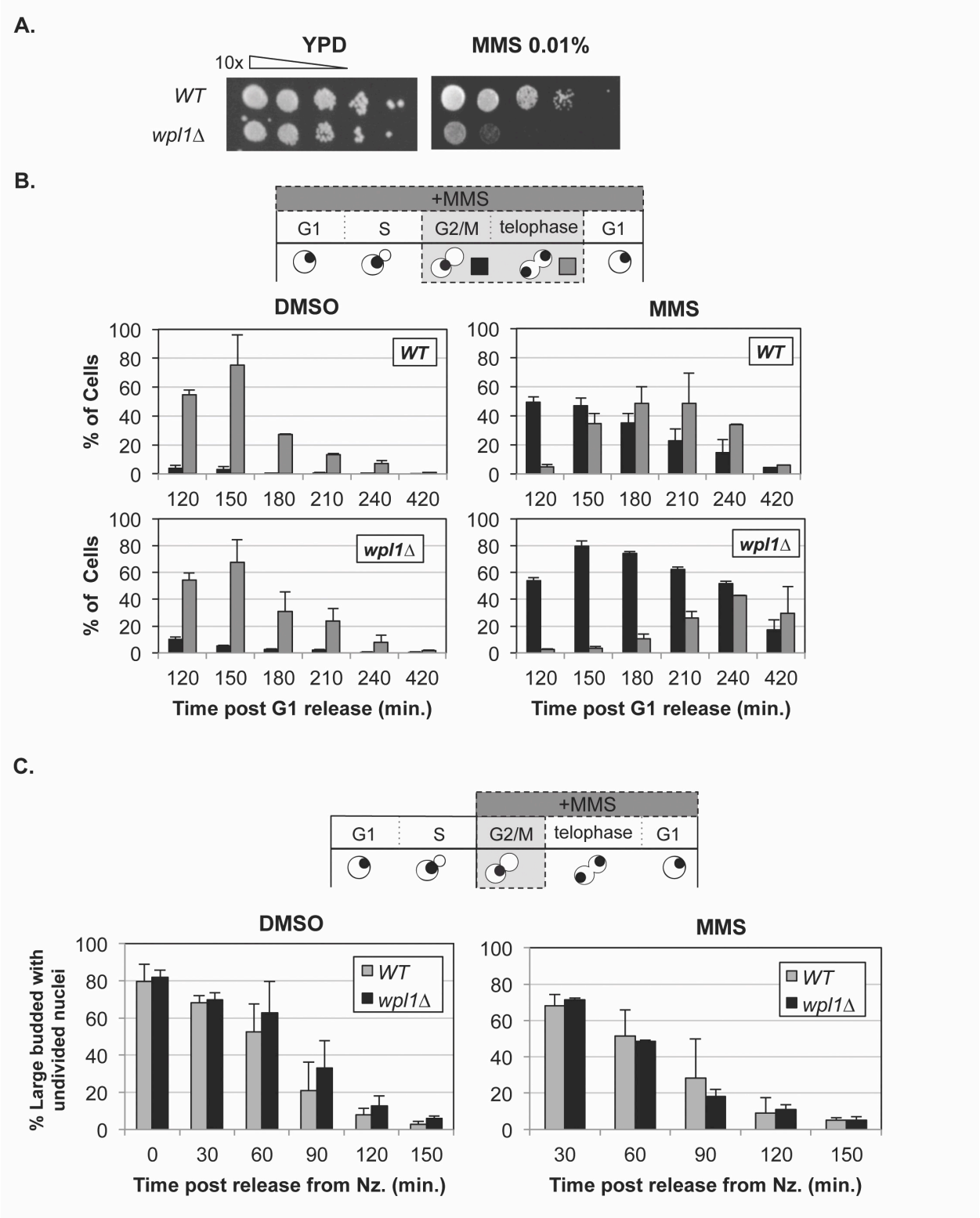

Figure 2.7: Wpl1p is necessary of efficient repair of MMS mediated DNA damage
(A) Assessment of sensitivity of and *wpl1*Δ cells to MMS. *WT* (VG3349-1B) and *wpl1*Δ (VG3360-3D) cells were serially diluted 10x and plated onto YPD media either with or with out MMS to a final concentration of 0.01%. Plates were incubated at 23˚C for 3

days. **(B)** *WT* (VG3349-1B) and *wpl1*Δ (VG3360-3D) cells were synchronized in G1. Upon release into the cell cycle MMS was added to the cultures to final concentration of 0.01%. Cells were collected and processed as described in Figure 2.6B and materials and methods **(C)** Analysis of segregation of DNA in *WT* (VG3349-1B) and *wpl1*Δ (VG3360-3D) cells when treated with MMS in G2/M. Time course and analysis performed as described in Figure 2.6C and materials and methods with addition of MMS to final concentration of 0.01%. Graphs are representation of % of cells that are large budded with a single DAPI mass at each given time-point for *WT* cells (gray) or *wpl1*Δ cells (black)

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through the same G1 to G1 progression as was previously described. Similar to CPT, *wpl1*Δ cells treated with MMS experienced the persistence of a significantly sized 2C peak while wild-type cells returned to a 1C peak, indicating stalling in mid-M (Sup Figure 2.13A). Analysis of nuclear segregation as described for CPT-treated cells, revealed that both wild-type and *wpl1*Δ cells experienced a delay in segregation of chromosomes released from G1 arrest in the presence of MMS with ~50% of cells stalling with a single DNA mass 120-minutes after release into the cell cycle (Figure 2.7B right panels). As with CPT, high levels of arrested *wpl1*Δ cells persisted longer and then took longer to dissipate than wild-type cells. Unlike CPT, after 420 minutes ~20% of MMS-treated *wpl1*Δ cells still remained stalled, suggesting that these cells were unable to recover at all. Finally, like CPT, the stall induced by MMS in *wpl1*Δ cell was not seen when MMS was added to cultures during M-phase rather than during S-phase (Figure 2.7C & Sup. Figure 2.13B). These results show that Wpl1p is important for the efficient repair of multiple types of DNA damage induced during S-phase.

Wpl1p mitigates DNA damage independent of Pds5p

As Wpl1p function in cohesion and condensation was dependent on its interaction with Pds5p, I tested whether these novel roles for Wpl1p in DNA damage were also mediated through its interaction with the Pds5p N-terminus. To test the role of the Pds5p N-terminus in promoting DNA repair, I tested the sensitivity of cells containing the *pds5* N-terminal mutants in either *WPL1* or *wpl1*Δ backgrounds on to media containing either CPT or MMS. These mutants showed significantly greater resistance to CPT or MMS treatment than *wpl1*Δ and looked similar to wild-type. Furthermore, double mutants of *wpl1*Δ with the *pds5* N-terminal mutants were sensitive to both CTP and MMS treatment, to the same degree as the *wpl1*Δ single mutant (Figure 2.8A). These results suggested that Wpl1p function in DNA damage repair was independent of its binding and function with the Pds5p N-terminal domain.

One caveat to this interpretation was that DNA damage might modify Pds5p or Wpl1p to restore the binding and function of Wpl1p with the Pds5p N-terminal domain. I tested this possibility by treating asynchronous cells containing *WPL1-3FLAG* and either a wild-type copy of *PDS5* or the mutation *pds5-P89L* with either DMSO or 20 µg/ml CPT for 3 hours. I then immunoprecipitated Wpl1p-3FLAG and looked for the presence of Pds5p. Upon incubation in media containing CPT, the interaction of Wpl1p with either Pds5p or Pds5p-P89L was not enriched (Figure 2.8B). I take this evidence to show that Wpl1p mediates its DNA damage function independently of its interaction with Pds5p. Finally, given that the *pds*5 N-terminal mutants all exhibit the same cohesion defect as

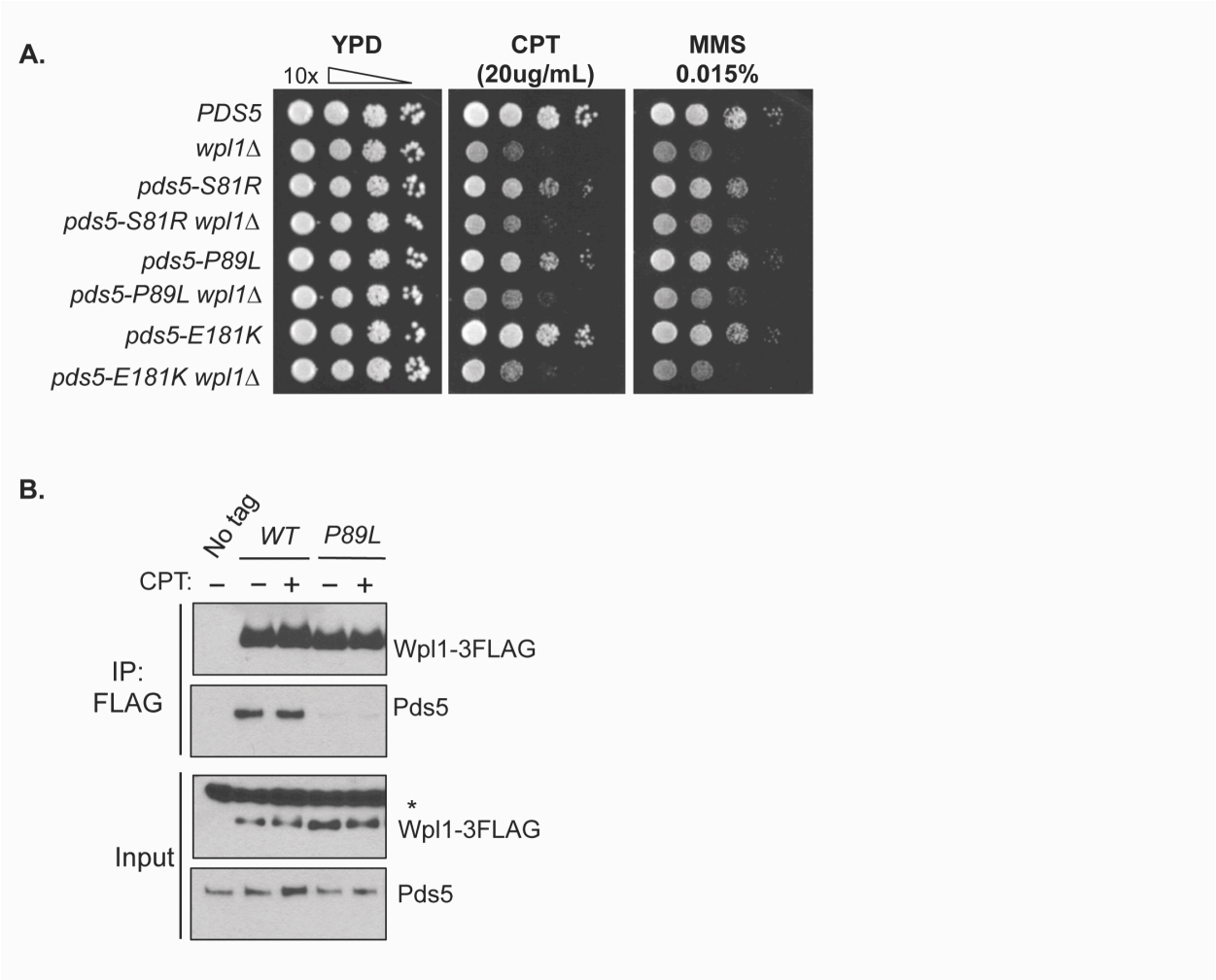


Figure 2.8: Wpl1p function in mitigating DNA damage is independent of Pds5p
(A) Assessment of sensitivity of pds5 N-terminal mutants to CPT and MMS. Cultures of cell in *WPL1* background: *PDS5* (VG3349-1B) pds5-S81R (MSB183-1A), pds5-P89L (MSB184-3A), and pds5- E181K (MSB101-3C), and wpl1Δ background: *wpl1*Δ (VG3360-3B), *pds5-S81R wpl1*Δ (MSB204-1B), *pds5-P89L wpl1*Δ (MSB205-4C), *pds5-E181K wpl1*Δ (MSB223-1A), were serially diluted and plated on YPD media either containing no drug, 20 µg/ml CPT or 0.015% MMS, and incubated at 23˚C for 3 days. **(B)** Assessment of interaction between Wpl1-3FLAG and Pds5 (MSB192-2A) or Pds5-P89L (MSB194-1C) when treated with CPT. Asynchronous cultures were treated either with DMSO or 20 µg/ml CPT for 3 hours before being harvested. No tag control is *WPL1 PDS5* (VG3349-1B) strain that was treated with DMSO. Immunoprecipitation and western blot were performed as described materials and methods.

*wpl1*Δ*,* this DNA damage repair function for Wpl1p appears to be independent of its role in promoting efficient sister chromatid cohesion.

Discussion

Pds5p and Wpl1p are known to form a sub-complex, interacting at least through their N-termini (Kueng et al. 2006; Rowland et al. 2009; Shintomi and Hirano 2009;

Ouyang et al. 2016). Here I showed that Wpl1p and Pds5p function together to both promote cohesion and inhibit condensation. Additionally, I have defined a non-essential regulatory domain of Pds5p that interacts with Wpl1p. As Pds5p is known to be a promoter of condensation, these findings suggest that Wpl1p binding to the N-terminus of Pds5p can toggle Pds5p function between promotion and inhibition of condensation. A parsimonious explanation for both these positive and negative functions is simply that Wpl1p inhibits the ability of Pds5p to promote condensation (Figure 2.9A). The loss of Wpl1p function in the *pds5* N-terminal mutants leads to a restoration of condensation in *eco1*Δ cells. This result indicates that Pds5p that is defective for mediating Wpl1p function can still promote condensation. Conversely, loss of function of the entire Pds5p protein leads to loss of condensation. In addition to the N-terminal domain, Pds5p contains a “hook” domain that is known to interact with Mcd1p residue V137. This interaction is essential for both cohesion and viability (Chan et al. 2013; Eng et al. 2014; Lee et al. 2016; Muir et al. 2016). It is likely that this interaction is also important for condensation. Thus Wpl1p interaction with the Pds5p N-terminus may inhibit condensation by blocking the interaction between Pds5p and Mcd1p. Additionally, this model reinterprets previous conclusions that Pds5p and Wpl1p function together to remove cohesin from DNA *in vitro* (Murayama and Uhlmann 2015). I hypothesize that Pds5p promotes stabilization of cohesin on DNA, and that Wpl1p inhibits this positive function of Pds5p resulting in destabilization and removal of cohesin from DNA.

The *pds5-P89L* allele abrogates the interaction between Wpl1p and Pds5p. This result indicates that the Pds5p N-terminal is required for the interaction of Pds5p with Wpl1p. This finding is distinct from the previous findings for interaction between Pds5B and Wapl in human cells. Mutation of the N-terminus of Pds5B weakened but did not abolish the interaction between the two proteins. Vertebrate Wapl contains FGF repeats, which also interact with Pds5B (Shintomi and Hirano 2009; Ouyang et al. 2016). However, FGF repeats are not conserved in yeast Wpl1p. Thus it is interesting that no other sequence in Wpl1p mediates interactions with Pds5p in yeast.

Pds5p has been shown to bind many positive regulators of cohesin. In *S. pombe* the Eco1p ortholog, Eso1, binds the N-terminus of Pds5p, as does the histone kinase Hrk1. In vertebrates Sororin also bind the N-terminus of Pds5p (Ouyang et al. 2016; Goto et al. 2017; Zhou et al. 2017). Like Wpl1p, all of these factors contain the conserved YSR motif, making it is possible that they all compete for binding to this one domain of Pds5p. Thus a simple model for how Wpl1p could inhibit Pds5p function is by occluding the binding of these other positive regulators. However, I found that Pds5p-S81R and –E181K retain significant amounts of binding to Wpl1p, despite being defective for Wpl1p function. Thus, the interaction between Wpl1p and Pds5p is not sufficient to promote cohesion and inhibit condensation, and Wpl1p must mediate an additional function.

While Wpl1p functions through its interaction with Pds5p to modulate cohesion and condensation, I found that Wpl1p promotes efficient repair of DNA damage independently of Pds5p. This independent function of Wpl1p is, in fact, consistent with my finding that in the absence of interaction between Pds5p and Wpl1p in the Pds5p-P89L mutant, Wpl1p still retains binding to cohesin, albeit at a reduced level. Previous studies have shown that Wpl1p can interact with cohesin independently of Pds5p,

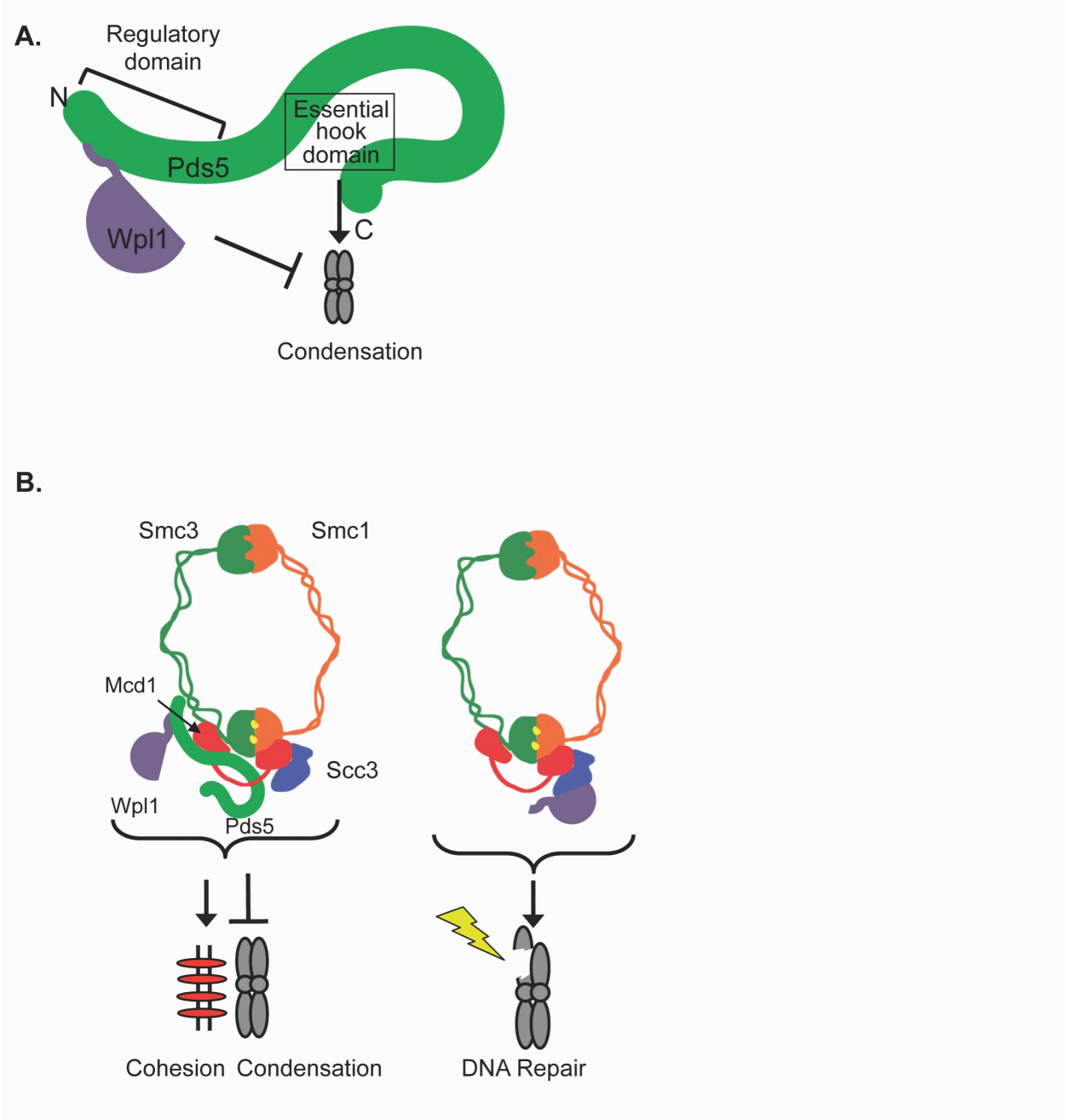


Figure 2.9: Model for mechanism of Pds5p dependent and independent functions of Wpl1p

(A) Schematic for proposed function of Wpl1-mediated condensation of chromosomes. Pds5 (green) essential function is promotion of condensation. Wpl1 interaction with Pds5 N-terminal domain inhibits Pds5 function in condensation. **(B)** Proposed model for Wpl1 functions that are dependent or independent of Pds5 interaction. Left: Wpl1 mediates condensation inhibition and cohesion promotion through interaction with Pds5 N-terminus. Pds5 interaction with cohesin is primarily mediated through interaction of "hook" domain with Mcd1. Right: Wpl1 function in promotion of DNA repair is not dependent on Pds5 interaction. Wpl1 interaction with cohesin may be mediated through interaction with Scc3.

through direct interaction with Scc3p/SA in both vertebrates and yeast (Rowland et al. 2009). Importantly, the interaction between Wpl1p and cohesin must not occur when cohesin is bound to Pds5p because otherwise the Pds5p-P89L allele would co-immunoprecipitate with Wpl1p due to their independent associations with cohesin (Figure 2.9B).

Finally, I found that *wpl1*Δ cells are sensitive to both CPT and MMS damage. This finding indicates that Wpl1p promotes efficient repair of multiple types of DNA damage that occur during S-phase. This result expands our understanding of the role of cohesin in DNA damage repair. DNA damage-induced cohesion has previously been analyzed in the context of double-strand breaks induced after S-phase. However, cells normally encounter DNA damage during S-phase as a result of replication fork stalling and collapse. Thus it is interesting to note that cohesin function is important for mitigation of both double-strand break damage (caused by CPT) and methylation damage (caused by MMS) during S-phase. Cohesin and its regulatory factors have not been implicated in this repair of DNA damage except for the recent finding that Eco1p acetylates PCNA upon damage with MMS (Billon et al. 2016). This function of Eco1p along with this positive function of Wpl1p poses the question as to whether additional distinctions exist between cohesin functions in S-phase DNA damage repair versus canonical S-phase cohesion.

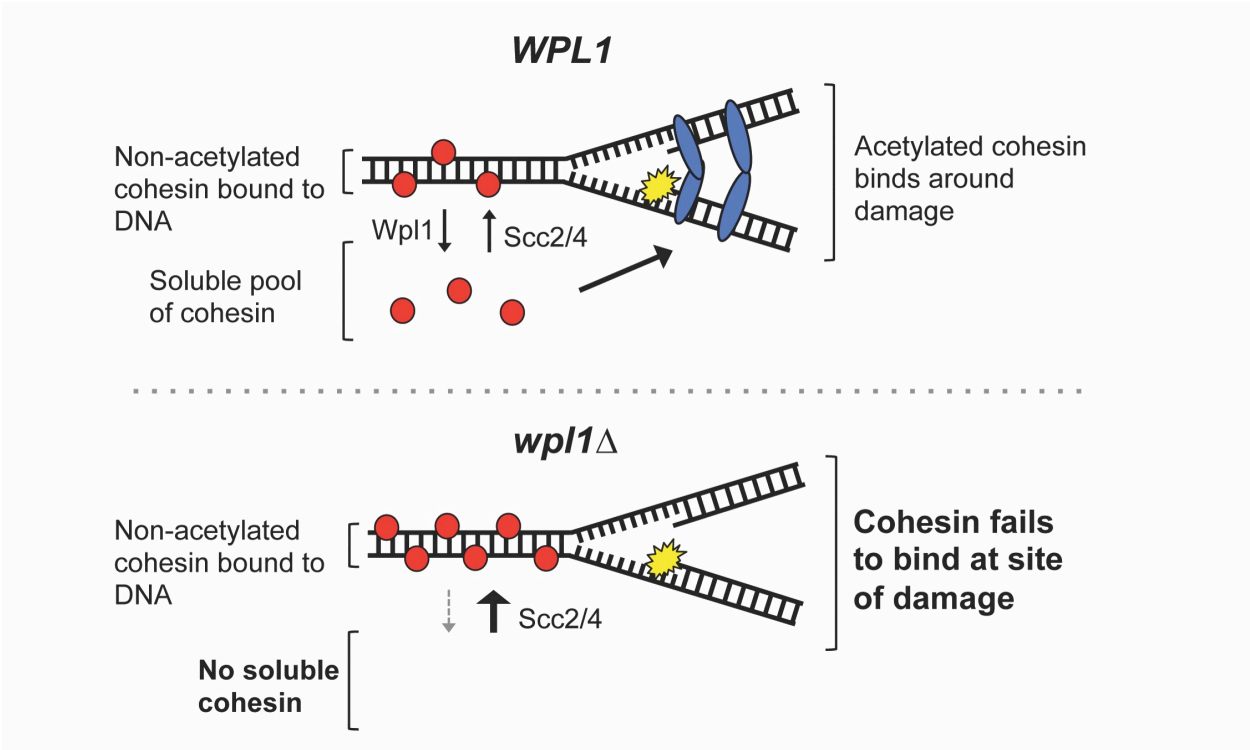


Figure 2.10: Model for Wpl1p-mediated promotion of DNA repair
Wpl1p promotes cohesin dynamism. Top: In the presence of Wpl1p non-acetylated cohesin (red) is loaded and unloaded from DNA by Scc2/4p and Wpl1p, maintaining a soluble pool of cohesin. Upon damage, cohesin loading and cohesion establishment are

promoted around the lesion. Blue indicates acetylated cohesin around break-site that promotes repaid. Bottom: In the absence of Wpl1p, cellular levels of cohesin are decreased. Non-acetylated cohesin (red) is loaded onto DNA but cannot be removed, depleting soluble pool. Cohesin fails to load and cohesion cannot be established around DNA damage.

Additionally, the finding that Wpl1p promotes repair of this damage is in contrast to the inhibitory role that has been ascribed to Wpl1p in post-replicative DNA damage-induced cohesion establishment (Heidinger-Pauli et al. 2009). Both positive and negative roles in DNA damage-induced cohesion establishment could be explained if Wpl1p functions to recycle cohesin and promotes cohesin dynamism. In the presence of Wpl1p, non-acetylated cohesin is constantly loaded and unloaded from DNA by Scc2/4p and Wpl1p respectively, maintaining a soluble pool. Upon replication-dependent DNA damage, cohesin may be loaded around the lesion to stabilize it and promote repair (Figure 2.10 Top). However, in the absence of Wpl1p, there is less cohesin in the cell. Additionally, the soluble pool of cohesin is depleted as cohesin is loaded onto DNA by Scc2/4p, but cannot be removed. Thus, in the event of DNA damage, cohesin cannot be loaded around the lesion, leading to the persistence of damage (Figure 2.10 Bottom). Conversely, failure of Eco1p to inhibit Wpl1p on the newly remodeled cohesin could be detrimental, as Wpl1p could remove cohesin that is important for promoting repair. A similar function in recycling cohesin could explain the positive role of Wpl1p in promoting cohesion as well as cohesin loading onto DNA. Future studies could shed light on how Wpl1p mediates functions independent of Pds5p. Additionally, further analysis of the role of Wpl1p in the DNA damage response could help us gain insight into how Wpl1p regulates cohesin positively and negatively.

Materials and Methods

Yeast strains, media, and reagents

Yeast strains used in this study are A364A background, and their genotypes are listed in Table 2.1. YPD liquid media was prepared containing 1% yeast extract, 2% peptone, 2% dextrose, and 0.01 mg/ml adenine.

Solid Media:

YPD solid media was prepared containing 1% yeast extract, 2% peptone, 2% dextrose, and 2% agar.

Auxin: 1M 3-indoleacetic acid (Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide and added to plates or liquid cultures at a final concentration of 500 μM, with cooling agar used in plates to ~55°C before addition of auxin to each batch.

Camptothecin: Camptothecin (Sigma-Aldrich, St. Louis, MO) was made as a 10 mg/ml stock (in DMSO) and added to final concentration of 20 μg/ ml in YPD media containing 25 mM 4-(2-hydroxyethyl)-1-pipera- zineethanesulfonic acid (HEPES), pH 7.4

Methyl-methane sulfonate: 99% pure MMS (Sigma-Aldrich, St. Louis, MO) was added to a final concentration of 0.01% in YPD media.

Dropout media: 5-FOA was purchased from US Biological Life Sciences (Salem, MA) and used at a final concentration of 1 g/l in URA dropout plates supplemented with 50 mg/L uracil powder (Sigma-Aldrich)

Dilution plating
Cells were grown to saturation in YPD liquid media at 30˚C then plated in 10-fold serial dilutions. Cells containing temperature-sensitive alleles were grown to saturation at 23˚C. Cells were incubated on plates at relevant temperatures or containing drugs as described. For plasmid shuffle assays, cells were grown to saturation in YPD media to allow loss of covering plasmid, then plated in 10-fold serial dilutions on YPD or FOA media.

Cohesin and condensation time course
Cells are inoculated into 5mL YPD starter culture overnight at 23˚C, unless indicated otherwise. Cells are then inoculated from starter cultures into YPD to grow overnight to a final concentration of 0.2 OD. Alpha factor (Sigma-Aldrich) is added to cultures at 10^{-8} M, for 3 hours for cells to arrest in G1. Cells are then washed 3x in YPD containing .2 µg/ml Pronase E, and washed 1x in YPD without Pronase E. Cells are then resuspended into YPD containing 15 µg/ml nocodazole (Sigma-Aldrich) and incubated at 23˚C to allow cell cycle progression until arrest in mid-M (3 hours).

For time courses with auxin treatment, time courses were performed above, with 1M auxin added to final concentration of 500 µM to alpha factor arrested cells for 1 hour. Pronase and YPD washes and nocodazole release were done in the presence of 500 µM auxin.

To assess cohesion, cells were fixed in 4% paraformaldehyde (w/v) 3.4% sucrose (w/v) solution, and then washed and resuspended in 0.1 KPO_4 1.2M sorbitol buffer.

Fluorescence in situ hybridization
Cells were fixed in 3.6% formaldehyde for 2 hours at 23˚C. Cells were then washed 3x with water and resuspended in 1 M sorbitol 20 mM KPO_4 pH4.7. Cells were spheroplasted with beta mercaptoethanol and 0.5% TritonX-100. Cells were then gently spun down and plated on polylysine-coated slides. Cells were then washed with 0.5% SDS. Slides were then submerged in 3:1 methanol/acetic acid and allowed to air dry overnight. Cells were then RNase A treated (100 µg/ml in 2X SSC) at 37% for 1 hr. Slides were washed 4x in fresh 2X SSC and immediately dehydrated through a series of ethanol washes (70%, 80% and 95%, min/wash at -20˚C). Denaturation of chromosomal DNA was done by incubation of slides in 70% formamide in 2X SSC at 70˚C for 2 minutes, followed immediately by ethanol washes as described above (70%, 80%, 90%, and 100%). After slides had dried, cells were treated with proteinase K (10 µg/ml in 20 mM Tris, pH 7.2, 2 mM $CaCl_2$) for 15 minutes at 37˚C. Cells were then stained with ProLong Gold Antifade Moutnant with DAPI (Life Technologies).

CPT and MMS treatment time course

Cells were grown as described above. Cultures were arrested in alpha factor as described above. Cells were washed 3x in YPD containing .2 µg/ml Pronase E, and washed 1x in YPD without Pronase E. Cells are then split and resuspended into YPD containing either DMSO, 20 µg/ml CPT and 25 mM HEPES pH7.4 or 0.01% MMS and incubated at 23˚C to allow cell cycle progression. 90 minutes after release alpha-factor was re-added to cultures at 10^{-8} M to arrest in subsequent G1. Cells were harvested every 30-minutes and fixed in 70% ethanol.

Assessment of chromosome segregation when treated with MMS or CPT after S-phase. Cells were released from initial alpha factor arrest, into media containing 15 µg/ml nocodazole and incubated at 23˚C to allow cell cycle progression until arrest in mid-M (3 hours). Cells were then washed 3x in YPD and split and resuspended into media containing alpha-factor at 10^{-8} M and either DMSO, 20 µg/ml CPT and 25 mM HEPES pH7.4 or 0.01% MMS.

To assess chromosome segregation, fixed cells were washed and resuspended in 1xPBS containing DAPI

Flow cytometry
To assess DNA content, cells were fixed in 70% ethanol. Fixed cells were washed twice in 50 mM sodium citrate (pH 7.2), then treated with RNase A (50 mM sodium citrate [pH 7.2]; 0.25 mg/ml RNase A; 1% Tween-20 [v/v]) overnight at 37˚C. Proteinase K was then added to a final concentration of 0.2 mg/ml and samples were incubated at 50˚C for 2 hr. Samples were sonicated for 30s or until cells were adequately disaggregated. SYBR Green DNA I dye (Life Technologies, Carlsbad, CA) was then added at 1:20,000 dilution and samples were run on a Guava easyCyte flow cytometer (Millipore, Billerica, MA). 20,000 events were captured for each time point. Quantification was performed using FlowJo analysis software.

Microscopy
Images were acquired with an Axioplan2 microscope (100× objective, numerical aperture [NA] 1.40; Zeiss, Thornwood, NY) equipped with a Quantix charge-coupled device camera (Photometrics, Tucson, AZ).

Preparation of cells for immunoprecipitation
Cells were inoculated into 5mL starter cultures and grown overnight at 23˚C. Strains were then inoculated into 60mL cultures and grown to a final OD of 0.8. 20 ODs were then harvested, washed in 1XPBS, spun down, liquid aspirated and cells were flash frozen in LN_2.

For CPT, untreated cells are grown to a final OD_{600} of 0.4. 1 M HEPES pH7.4 is added to cultures to a final concentration of 25 mM. 10 mg/ml CPT stock is added to cells to final concentration of 20 µg/ml and cells are incubated for 3 hours. 20 OD were then harvested and prepared as described above.

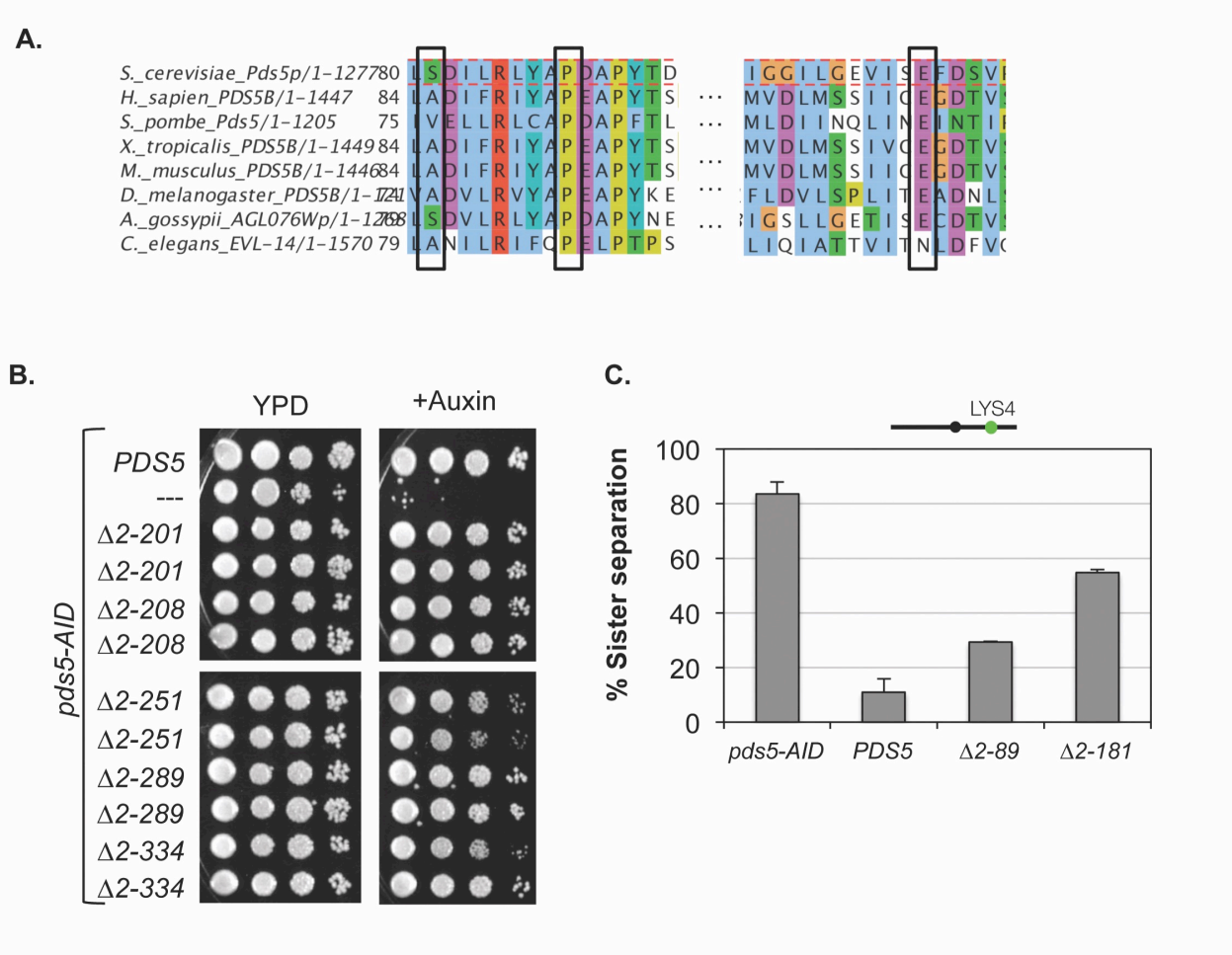
Immunoprecipitation

Cell lysates were prepared by bead beating 30sec on 1 min rest, 4x at 4˚C in GNK100 buffer containing complete mini EDTA free protease inhibitor (Roche), 5mM sodium butyrate, 5 mM beta mercaptoethanol, 1 mM PMSF, and 20 mM b-glycerophosphate. Lysates were cleared of insoluble cell debris. Lysates are incubated with 25 ug monoclonal anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO) and 60 uL Protein A dynabeads for 1 hour at 4˚C. Dynabeads are then washed 4x with GNK100 buffer with additives as described above containing 100 μM MG132. Totals are 1/50 of lysates resuspended in 100 ul Lemmeli buffer and boiled for 5 min. Dynabeads are resuspended in 50 ul Lemmeli buffer and boiled for 5 min

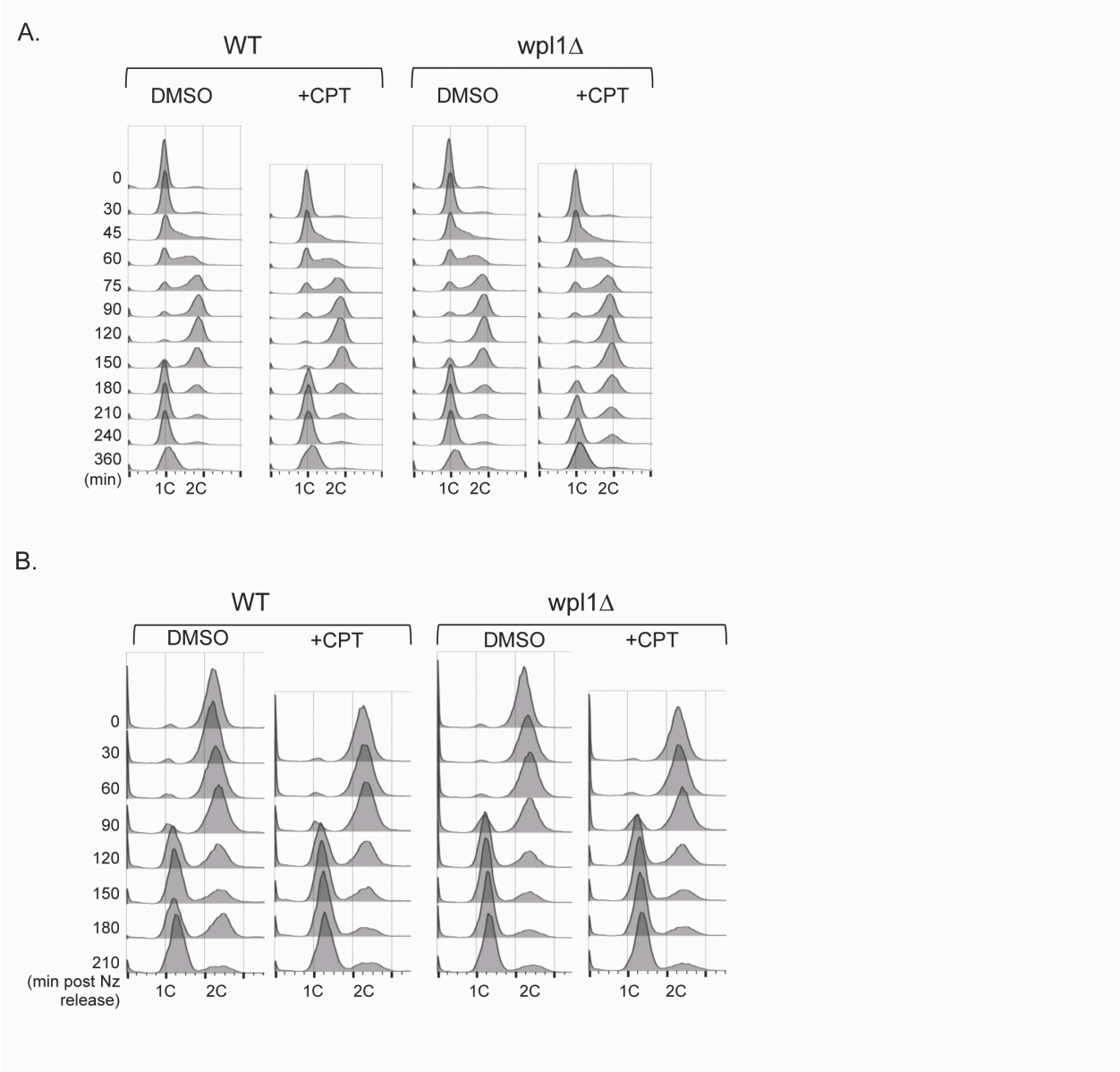
GNK100 buffer
100 mM KCl
20 mM HEPES pH7.5
0.2% NP40
10% glycerol
2.5 mM $MgCl_2$

Western blot
Samples were run on 8% SDS page gels. Pds5 protein was probed with Rabbit anti-Pds5 (556-6 bleed) at 1:20,000 and secondary HRP-conjugated Goat anti-Rabbit (BioRad) at 1:20,000. Inputs were diluted 1:10. Wpl1-FLAG was probed with Mouse anti-FLAG (Sigma-Aldrich, St. Louis, MO) at 1:10,000 and secondary HRP-conjugated Goat anti-Mouse secondary (BioRad) at 1:10,000. IP samples were diluted 1:10.

Supplementary Figures and Tables

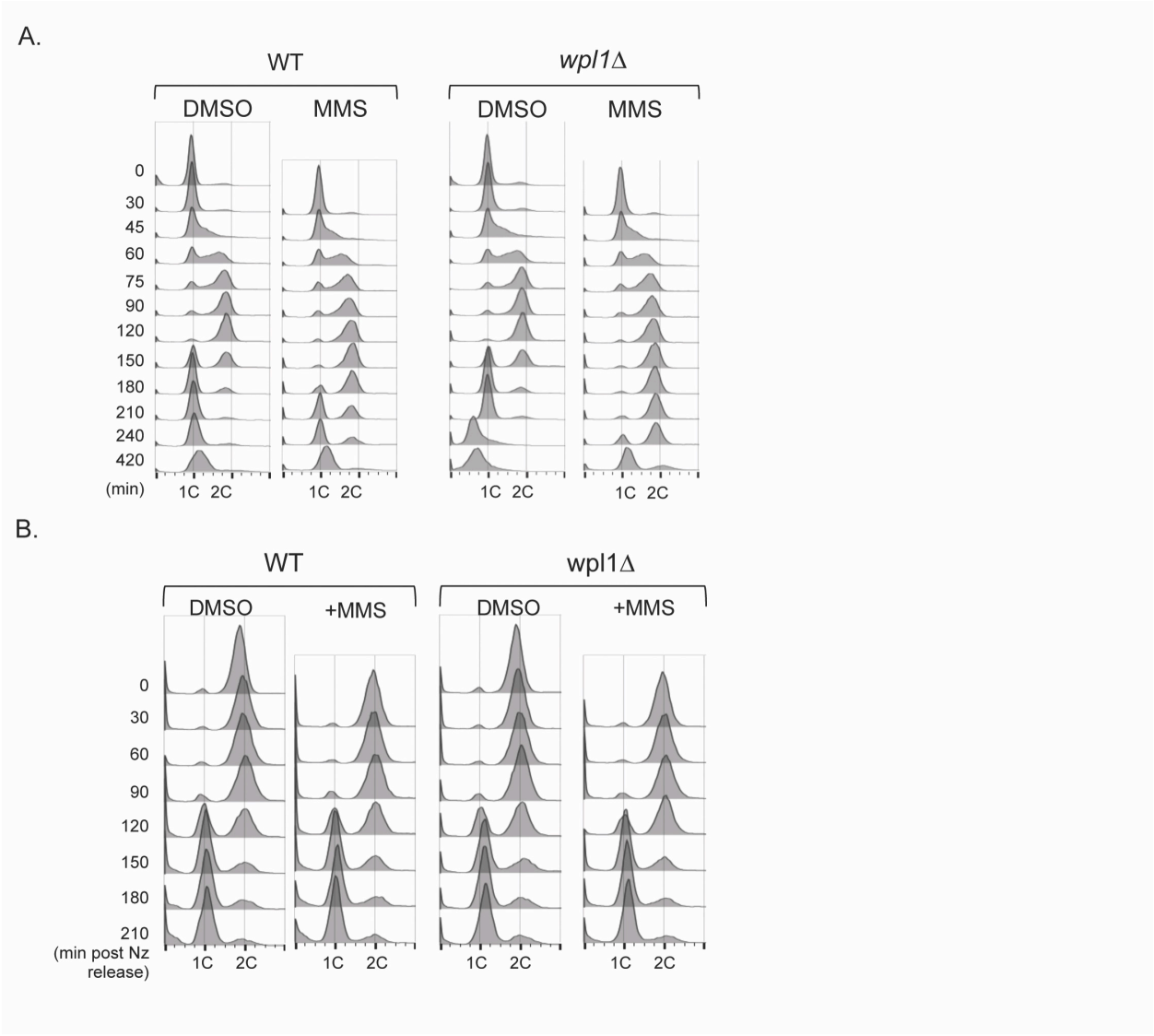


Supplemental Figure 2.11:
(A) Portion of clustal alignment of Pds5 across species. Pds5-S81, -P89 and, -E181 are highlighted in black boxes. **(B)** Assessment of viability of pds5 N-terminal truncation alleles. Full length *PDS5* and truncation alleles *PDS5* Δ*2-201* (MSB224-1A &1B), *PDS5* Δ*2-208* (MSB225-1E &1F), *PDS5* Δ*2-251* (MSB226-1K &1M), *PDS5* Δ*2-289* (MSB227-1P&1Q), *PDS5* Δ*2-334* (MSB228-3E&3F) were integrated at *LEU2* in strain containing second *PDS5-3V5-AID* (TE228) allele. Strains were plated on YPD media either with or without 750uM Auxin and incubated at 23˚C for 3 days. **(C)** Assessment of cohesion of pds5 N-terminal truncations. Cohesion assay adapted from Figure 1D: Pds5 alleles in *PDS5-3V5-AID* (TE228) strain *PDS5* (MSB213-1A&1B), *PDS5* Δ*2-89* (MSB214-2A&2B) and *PDS5* Δ*2-181* (MSB215-3A&3B) were arrested in alpha-factor for 3 hours. 500 uM Auxin was added to alpha-factor arrested cells for 1 hour. Cells were then washed and released into media containing 500 uM auxin and re-arrested in mid-M by addition of nocodazole.



Supplemental Figure 2.12: Wpl1 is necessary for efficient repair of camptothecin generated DNA damage

A) Analysis of replication of DNA in WT and *wpl1*Δ cells in time course described in Figure 2.6B. Cells were processed for flow as described in materials and methods. Alpha-factor cells (0 min) accumulate with a single peak noted as "1C", as replication occurs, cells accumulate in a second peak, noted as "2C". As cells complete the cell cycle (180-360 min), they re-arrest in the subsequent G1 with a "1C" peak. From left to right: *WT* cells treated with DMSO, *WT* cells treated with 20 µg/ml CPT, *wpl1*Δ cells treated with DMSO, and *wpl1*Δ cells treated with 20 µg/ml CPT **(B)** Cells from the time course performed in Figure 2.6C were fixed and processed for analysis of DNA content as described in materials and methods. Cell profiles for time 0 denotes arrest in nocodazole and thus have 2C content. Left to right: *WT* cells treated with DMSO, *WT* cells treated with 20 µg/ml CPT, *wpl1*Δ cells treated with DMSO, and *wpl1*Δ cells treated with 20 µg/ml CPT



Supplemental Figure 2.13: Wpl1 is necessary of efficient repair of S-phase DNA damage formed by MMS

Characterization of effect of MMS on *WT* and *wpl1*Δ cells throughout a single cell cycle. **(A)** Cells were fixed in ethanol, and processed for flow cytometry as described in materials and methods. From left to right: WT cells treated with DMSO, WT cells treated with 0.01% MMS, *wpl1*Δ cells treated with DMSO, and *wpl1*Δ cells treated with 0.01% MMS. **(B)** Assessment of S-phase requirement for MMS mediated stalling in *WT* and *wpl1*Δ cells. Cells from the time course performed in Figure 2.7B were fixed and processed for analysis of DNA content as described in materials and methods. Left to right: *WT* cells (VG3349-1B) treated with DMSO, *WT* cells treated with 0.01% MMS, *wpl1*Δ cells (VG3360-3D) treated with DMSO, and *wpl1*Δ cells treated with 0.01% MMS

Table 2.1: Strain names and genotypes used in this chapter

Description	Strain name	Genotype
pds5-E181K	**MSB101-3C**	*MATa lys4:LacO(DK)-NAT* GAL+*trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-E181K*
pds5-S81R wpl1Δ	**MSB133-3C**	*MATa lys4:LacO(DK)-NAT* GAL+*trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-S81R wpl1Δ::HPH*
pds5-P89L wpl1Δ	**MSB134-1L**	*MATa lys4:LacO(DK)-NAT* GAL+*trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-P89L wpl1Δ::HPH*
pds5-S81R eco1Δ	**MSB138-1K**	*MATa lys4:LacO(DK)-NAT* GAL+*trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-S81R eco1Δ::G418*
pds5-P89L eco1Δ	**MSB139-2J**	*MATa lys4:LacO(DK)-NAT* GAL+*trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-P89L eco1Δ::G418 pBS1030(ECO1 URA3 CEN)*
pds5-E181K eco1Δ	**MSB147-1A**	*MATa lys4:LacO(DK)-NAT* GAL+*trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-E181K eco1Δ::G418 pBS1030(CEN URA ECO1)*
pds5-S81R	**MSB183-1A**	*MATa lys4:LacO(DK)-NAT* GAL+*trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-S81R*
pds5-P89L	**MSB184-3A**	*MATa lys4:LacO(DK)-NAT* GAL+*trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-P89L*
PDS5	**MSB185-1A**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52*
pds5-E181K	**MSB186-2E**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52* pds5-E181K
pds5-E181K eco1-203	**MSB189-2B**	*MATa lys4:LacO(DK)-NAT* GAL+*trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-E181K eco1-207*
pds5-S81R	**MSB190-3E**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-S81R*
pds5-P89L	**MSB191-3A**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-P89L*
PDS5 WPL1-3FLAG	**MSB192-2A**	*MATa lys4:LacO(DK)-NAT* GAL+*trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52*

		wpl1:WPL1-3FLAG-KANMX
pds5-S81R WPL1-3FLAG	**MSB193-1B**	*MATa lys4:LacO(DK)-NAT* GAL+*trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-S81R wpl1:WPL1-3FLAG-KANMX*
pds5-P89L WPL1-3FLAG	**MSB194-1C**	*MATa lys4:LacO(DK)-NAT* GAL+*trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-P89L wpl1:WPL1-3FLAG-KANMX*
pds5-E181K WPL1-3FLAG	**MSB195-2D**	*MATa lys4:LacO(DK)-NAT* GAL+*trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-E181K wpl1:WPL1-3FLAG-KANMX*
pds5-S81R wpl1Δ	**MSB204-1B**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-S81R wpl1Δ::KANMX*
pds5-P89L wpl1Δ	**MSB205-4C**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-P89L wpl1Δ::KANMX*
pds5-E181K wpl1Δ	**MSB206-6A**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-E181K wpl1Δ::KANMX*
pds5-S81R eco1Δ	**MSB210-2A**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-S81R eco1Δ::KANMX pBS1030 (ECO1 URA3 CEN)*
pds5-P89L eco1Δ	**MSB211-2J**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-P89L eco1Δ::KANMX pBS1030 (ECO1 URA3 CEN)*
PDS5 pds5-AID	**MSB213-1A**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 ura3-52:: ADH1-TIR1-URA3 PDS5-3V5-AID2-Kanmx leu2-3,112::pMB8-PDS5-LEU2*
PDS5 pds5-AID	**MSB213-1B**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 ura3-52:: ADH1-TIR1-URA3 PDS5-3V5-AID2-Kanmx leu2-3,112::pMB8-PDS5-LEU2*
pds5-Δ2-89 pds5-AID	**MSB214-2A**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 ura3-52:: ADH1-TIR1-URA3 PDS5-3V5-AID2-Kanmx leu2-3,112::pMB8-*pds5Δ89N-*LEU2*
pds5-Δ2-89 pds5-AID	**MSB214-2B**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 ura3-52:: ADH1-TIR1-URA3 PDS5-3V5-AID2-Kanmx leu2-3,112::pMB8-*pds5Δ89N-*LEU2*

pds5-Δ2-181 pds5-AID	**MSB215-3A**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 ura3-52:: ADH1-TIR1-URA3 PDS5-3V5-AID2-Kanmx leu2-3,112::pMB8-pds5Δ181N-LEU2*
pds5-Δ2-181 pds5-AID	**MSB215-3B**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 ura3-52:: ADH1-TIR1-URA3 PDS5-3V5-AID2-Kanmx leu2-3,112::pMB8-pds5Δ181N-LEU2*
pds5-E181K	**MSB223-1A**	*MATa lys4:LacO(DK)-NAT GAL+trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 pds5-E181K wpl1Δ::HPH*
pds5-Δ2-201 pds5-AID	**MSB224-1A**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 ura3-52:: ADH1-TIR1-URA3 pds5::PDS5-3V5-AID2-Kanmx leu2-3,112::pMB8- pds5Δ201N-LEU2*
pds5-Δ2-201 pds5-AID	**MSB224-1B**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 ura3-52:: ADH1-TIR1-URA3 pds5::PDS5-3V5-AID2-Kanmx leu2-3,112::pMB8- pds5Δ201N-LEU2*
pds5-Δ2-208 pds5-AID	**MSB225-1E**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 ura3-52:: ADH1-TIR1-URA3 pds5::PDS5-3V5-AID2-Kanmx leu2-3,112::pMB8- pds5Δ208N-LEU2*
pds5-Δ2-208 pds5-AID	**MSB225-1F**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 ura3-52:: ADH1-TIR1-URA3 pds5::PDS5-3V5-AID2-Kanmx leu2-3,112::pMB8- pds5Δ208N-LEU2*
pds5-Δ2-251 pds5-AID	**MSB226-1K**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 ura3-52:: ADH1-TIR1-URA3 pds5::PDS5-3V5-AID2-Kanmx leu2-3,112::pMB8- pds5Δ251-LEU2*
pds5-Δ2-251 pds5-AID	**MSB226-1M**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 ura3-52:: ADH1-TIR1-URA3 pds5::PDS5-3V5-AID2-Kanmx leu2-3,112::pMB8- pds5Δ251-LEU2*
pds5-Δ2-289 pds5-AID	**MSB227-1P**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 ura3-52:: ADH1-TIR1-URA3 pds5::PDS5-3V5-AID2-Kanmx leu2-3,112::pMB8- pds5Δ289-LEU2*
pds5-Δ2-289 pds5-AID	**MSB227-1Q**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 ura3-52:: ADH1-TIR1-URA3 pds5::PDS5-3V5-AID2-Kanmx leu2-3,112::pMB8- pds5Δ289-LEU2*
pds5-Δ2-334 pds5-AID	**MSB228-3E**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 ura3-52:: ADH1-TIR1-*

		URA3 pds5::PDS5-3V5-AID2-Kanmx leu2-3,112::pMB8- pds5∆334-LEU2
pds5-∆2-334 pds5-AID	**MSB228-3F**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 ura3-52:: ADH1-TIR1-URA3 pds5::PDS5-3V5-AID2-Kanmx leu2-3,112::pMB8- pds5∆334-LEU2*
pds5-AID	**TE228**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 ura3-52:: ADH1-TIR1-URA3 pds5::PDS5-3V5-AID2-Kanmx*
eco1-203	**VG3223-12B**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 ctf7-203*
PDS5/WT	**VG3349-1B**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52*
wpl1∆	**VG3360-3D**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 rad61∆::HPH*
eco1∆	**VG3499-1B**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 eco1∆::KANMX pBS1030 (ECO1,CEN,URA3)*
eco1∆ wpl1∆	**VG3502 #A**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 wpl1∆::HPH eco1∆::G418 pBS1030(ECO1,URA3,CEN)*
eco1∆ wpl1∆	**VG3503 #4**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 wpl1∆::HPH eco1∆::G418 pBS1030(ECO1,URA3,CEN)*
wpl1∆	**VG3513-1B**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 leu2-3,112 ura3-52 wpl1∆::HPH*
eco1-AID	**VG3633-2D**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 leu2-3,112 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 eco1::ECO1-3V5-AID2-KANMX TIR1-CaTRP1*

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CHAPTER 3: ECO1P PROMOTES COHESION AND CONDENSATION BY MODULATING COHESIN STRUCTURE

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Introduction

The acetyltransferase, Eco1p, plays a critical role in the regulation of the cohesin complex. Eco1p positively regulates the establishment of cohesion as well as the promotion of condensation (Skibbens et al. 1999; Tóth et al. 1999). Eco1p acetylates Smc3p K112 and K113 in budding yeast, two conserved lysine residues in the head domain (Rolef Ben-Shahar et al. 2008; Unal et al. 2008; Zhang et al. 2008). Acetylation of these two residues is essential to promote viability, cohesion and condensation (Guacci and Koshland 2012). K112 and K113 acetylation happens after cohesin binds DNA, suggesting this modification affects cohesin function after it is loaded onto DNA. How acetylation of Smc3p regulates cohesin function is an important unanswered question in the field.

Initially the acetylation of Smc3p was thought to promote cohesin's functions by making cohesin refractory to the antagonistic functions of Wpl1p. Support for this conclusion came from the finding that deletion of *WPL1* (*wpl1*Δ) restored viability to cells containing either *eco1*Δ or an *eco1-ts* allele. Additionally, *wpl1*Δ also restored viability to cells blocked specifically for acetylation of K112 and K113 (*smc3-K112R K113R*, referred to as *smc3-RR*), suggesting that Eco1p antagonized Wpl1p specifically through the acetylation of these two residues (Rolef Ben-Shahar et al. 2008). It was surprising when it was found that *eco1*Δ *wpl1*Δ cells had normal condensation but remained severely compromised for cohesion. This result suggests that Eco1p acetylation must promote a step in cohesion in addition to inhibition of *WPL1* function (Guacci and Koshland 2012).

To better understand the function of Smc3p acetylation in regulating cohesin, I collaborated with Dr. Vincent Guacci to study suppressors that restored cohesion in the absence of the regulators *ECO1* and *WPL1*. His previous results suggested that *eco1*Δ *wpl1*Δ cells were viable due to a cohesin-independent mechanism of bi-polar spindle attachment that exists in budding yeast. This alternative pathway required persistent attachment of the kinetochore to spindle microtubules from S-phase through mitosis (an unusual feature of budding yeast). These cells were therefore sensitive to transient disruption of kinetochore-microtubule interactions by benomyl. Additionally, the absence of cohesion in this strain made them very sensitive to DNA damage induced by camptothecin (CPT) (Guacci and Koshland 2012). He took advantage of these deficits to identify suppressors in *eco1*Δ *wpl1*Δ cells that restored resistance to these drugs, presumably because they reactivated normal cohesion (Figure 3.1)(Guacci et al. 2015).

One of these suppressors was an allele of *SMC3*, located in the head domain that changed aspartic acid residue 1189 to histidine (D1189H). Initial characterization of *smc3-D1189H* showed that it 1) could partially restore cohesion and drug resistance to

*eco1*Δ *wpl1*Δ cells, 2) could support viability in an otherwise wild-type background, and 3) had a minor cohesion defect on its own (Guacci et al. 2015).

Here, I analyzed the relationship between *smc3-D1189H* and acetylation of the K112 and K113 residues. My data suggest that Eco1p acetylation of K112 and K113 can promote at least two separate pathways: 1) the inhibition of Wpl1p to promote condensation, and 2) a Wpl1p-independent function to promote cohesion. Furthermore my experiments suggest that Eco1p acetylates other targets in addition to K112 K113 to mediate the antagonism of Wpl1p.

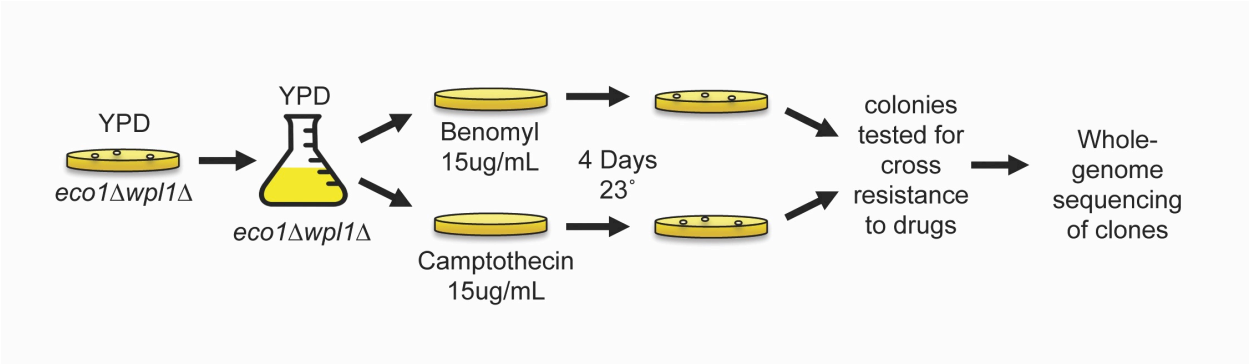

Figure 3.1: Screen for cohesion activator mutants
Schematic of the suppression screen of *eco1*Δ *wpl1*Δ cells. Screen performed as described in Materials and Methods

Results

*smc3-D1189H restores some cohesion but not viability to eco1*Δ

The *smc3-D1189H* suppressor was identified in the *eco1*Δ *wpl1*Δ background. To understand how *smc3-D1189H* was promoting cohesion in this mutant background, I wanted to test the ability of *smc3-D1189H* to bypass either Eco1p function or Wpl1p function. To that end, I generated two strains that contained either the *smc3*Δ or *smc3-D1189H* mutations. These deletions were covered by plasmid containing *SMC3 URA3* to maintain viability. Similarly, I also generated a strain containing *eco1*Δ *smc3-D1189H* covered by an *ECO1 URA3* plasmid. As expected, upon counter selection on media containing 5-FOA to remove the covering plasmid, the *smc3-D1189H* was viable but the *smc3*Δ strain was not. Importantly, the *eco1*Δ *smc3-D1189H* strain was not viable (Figure 3.2A) unlike *eco1*Δ *wpl1*Δ strain. This difference suggested that *smc3-D1189H* could not suppress the *WPL1* function normally antagonized by Eco1p. This phenotype has also been noted by other independent studies (Beckouët et al. 2016)

The inviability of *eco1*Δ *smc3-D1189H* suggested a failure to restore condensation. To test whether it also failed to suppress cohesion, Dr. Guacci utilized the conditional auxin-inducible system by building the *smc3-D1189H* allele into a strain containing an *eco1-AID* allele as the sole copy of *ECO1*. He measured cohesion loss at both the CEN-distal and –proximal loci, *LYS4 and TRP1*, by depleting Eco1p by adding auxin to alpha-factor arrested cells, followed by release into media containing auxin and nocodazole to arrest them in mid-M.

As expected, strains containing wild-type *ECO1* had robust cohesion when arrested in mid-M while *eco1-AID* had a dramatic cohesion loss at both CEN-distal and proximal loci (50%-70%). The *smc3-D1189H eco1-AID* cells had a decreased cohesion defect from *eco1-AID* alone, where cohesion loss occurred in ~50% of cells at both CEN-distal and proximal loci. This cohesion restoration was similar to what was seen in *smc3-D1189H eco1*Δ *wpl1*Δ cells compared to *eco1*Δ *wpl1*Δ alone (Figure 3.2B) (Guacci et al. 2015). These results suggest that the *smc3-D1189H* allele can partially restore cohesion to cells lacking *ECO1* even in the presence of *WPL1*.

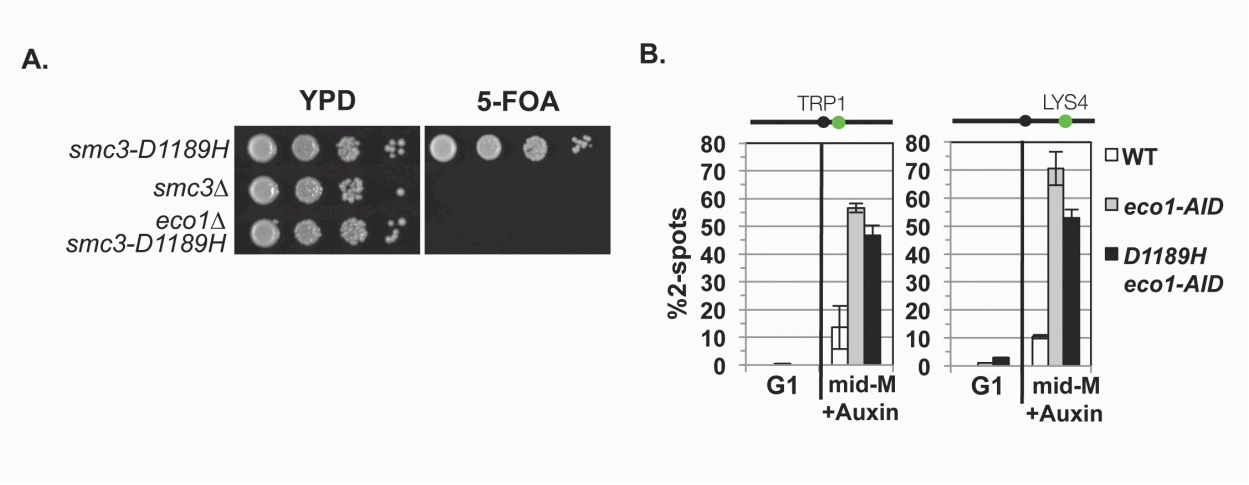

Figure 3.2: characterization of cohesion activator, smc3-D1189H
(A) *smc3-D1189H* cannot restore viability to *eco1*Δ by plasmid shuffle assay. *smc3-D1189H* (MSB46-1A), and *smc3*Δ (VG3464-16C) contain covering plasmid pEU42 (*SMC3 CEN URA3*), and *smc3-D1189H eco1*Δ (MSB91-1A) contains covering plasmid pBS1030 (*ECO1 CEN URA3*). Strains were grown to saturation overnight, and serially diluted 10X, then spotted onto YPD or 5-FOA media, and incubated at 23˚ for 3 days. **(B)** Cohesion loss after auxin-mediated *ECO1* depletion from G1 cells through mid–M phase arrest. Left, cohesion loss at *CEN*-proximal *TRP1* locus assayed in haploid strains WT (VG3460-2A), *ECO1-AID2* (VG3659-1A) and *smc3-D1189H ECO1-AID2* (VG3663-2E) strains. Right, cohesion loss at *CEN*-distal *LYS4* locus assessed in haploid WT (VG3620-4C), *ECO1-AID2* (VG3646-1A) and *smc3-D1189H ECO1-AID2* (VG3650-1E) strains as explained in *materials and methods*. The percentage of cells with two GFP signals (sister separation) is plotted.

smc3-D1189H bypasses the need for acetylation of K112 K113 for viability and cohesion

Although, Smc3p-K112 and K113 are known to be critical targets of Eco1p, Eco1p also acetylates other targets. However, the biological significance of those events is unknown (Ivanov et al. 2002; Unal et al. 2008; Heidinger-Pauli et al. 2009; Chao et al. 2017). The relatively weak suppression of *eco1*Δ by *smc3-D1189H* had a number of explanations. For one, it was possible that D1189H might only bypass the need to acetylate K112 and K113 but not Eco1p's other targets. Alternatively, it was

possible that D1189H bypassed acetylation of other Eco1p targets, but not K112 K113 or that D1189H was a weak suppressor of Eco1p function.

I first tested if *smc3-D1189H* could bypass the requirement of K112 K113 acetylation for viability. I created a chimeric allele of *smc3* in which *D1189H* was mutated in *cis* with the *K112R K113R* mutations (*smc3-RR-D1189H*). I integrated *smc3-RR* or *smc3-RR-D1189H* into a strain containing a deletion of the endogenous *SMC3* gene and a *SMC3 URA3* plasmid. I then plated these cells onto media containing 5-FOA to counter-select for the cells that had lost the *SMC3*-bearing plasmid. As expected, cells containing *smc3-RR* alleles were inviable. Additionally, as expected, *smc3-D1189H* (containing wild-type K112 K113) was viable. Importantly, the *smc3-RR-D1189H* chimeric protein was also viable (Figure 3.3A). Thus *D1189H* could suppress the inviability due to the loss of acetylation at K112 K113, but could not suppress loss of Eco1p function (compare to Figure 3.2A). These results suggest that a number of Eco1p-acetylation targets including K112 and K113 are required for viability (and likely condensation). The D1189H mutation can bypass the need for acetylation at K112 K113 but not these other targets.

To assess the ability of D1189H to restore cohesion to *smc3-RR*, I used the same strains that were used to assess viability and measured cohesion at both *LYS4* and *TRP1*. In mid-M the *smc3-RR-D1189H* cells exhibited robust cohesion, with a defect only slightly elevated relative to wild-type and *smc3-D1189H* (Figure 3.3B). To directly compare the cohesion defect seen in the *smc3-RR-D1189H* to that of *smc3-RR*, I built *smc3-RR* into an *smc3-AID* background. *smc3-AID* was depleted with auxin while arrested in G1 and synchronously released into the cell cycle in the presence of auxin. Cells were arrested in mid-M with nocodazole. Under these conditions, *smc3-RR* had a severe cohesion defect where 70-80% of cells experienced cohesion loss compared to cells with an *SMC3* allele, which only exhibited minor cohesion loss (Figure 3.3C). Thus D1189H can restore cohesion to *smc3-RR*. Notably the level of cohesion in *smc3-RR-D1189H* was much greater than that seen in the *eco1-AID smc3-D1189H* strain in the presence of auxin. This result indicated that D1189H could suppress the cohesion defect of sm*c3-RR* significantly better than it could suppress that of *eco1*Δ. Together, these cohesion results corroborate the previous interpretation that there are multiple Eco1p-acetylation targets required for cohesion. The D1189H allele can bypass the need for acetylation at K112 K113 to promote cohesion but not these other targets.

smc3-D1189H activates cohesion through a Wpl1p-independent mechanism

It has previously been reported that *wpl1*Δ suppresses lethality of the *smc3-RR* (Rolef Ben-Shahar et al. 2008). After observing that the D1189H allele restored viability to *smc3-RR*, we wondered if it was blocking the anti-cohesion function of *WPL1*. Should that be the case, I would expect the *smc3-RR-D1189H* to phenocopy *smc3-RR wpl1*Δ in other metrics.

I began testing these metrics by assessing the viability and drug sensitivity of *smc3-RR* and *smc3-D1189H* in the *wpl1*Δ background. I knocked out *WPL1* in both the *smc3-RR* and *smc3-D1189H* strains covered with a *SMC3 URA3* plasmid. I then

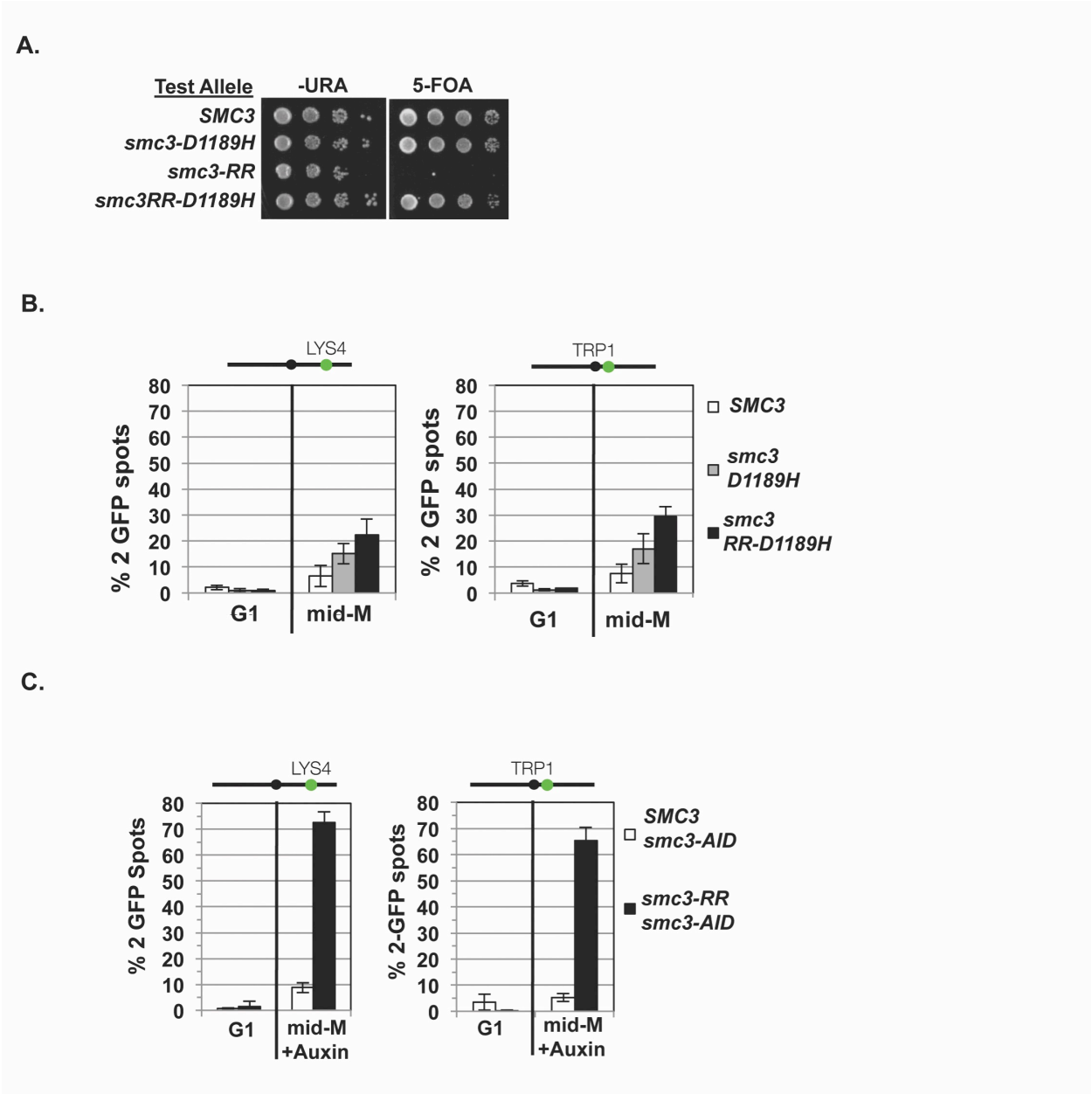

Figure 3.3: *smc3-D1189H* bypasses acetylation of Smc3-K112 K113.
(A) *smc3-D1189H* restores viability to *smc3-K112R K113R* mutant. Plasmid shuffle assay. Haploid shuffle strain VG3464-16C bearing plasmid pEU42 (*SMC3 CEN URA3*) and a second *SMC3* "test allele," *SMC3* (MSB45-1A), *smc3-D1189H* (MSB46-1A), *smc3-RR*, or chimeric *smc3RR- D1189H* (MSB47-1A), was grown, serially diluted 10X and plated as onto URA–dropout or 5-FOA-containing media. Plates were incubated at 23˚ for 3 days. **(B)** Cohesion loss of *smc3-RR-D1189H*. Haploid *SMC3, smc3-D1189H* and chimeric *smc3-RR-D1189H* were arrested in mid–M phase as described in materials and methods. Left, cohesion loss at *CEN*-distal *LYS4* locus assessed in haploid strains from A. Right, cohesion loss at *CEN*-proximal *TRP1* locus assessed in haploid wild-type (*SMC3*; MSB65-1A), *smc3-D1189H* (MSB66-1A), or chimeric *smc3-RR-D1189H* (MSB67-1A) strain. The percentage of cells with two GFP spots (sister

separation) is plotted. **(C)** Cohesion loss in *smc3-RR*. Haploids bearing *SMC3-AID* and a second *SMC3* allele, either WT or *smc3-RR*, were depleted for *SMC3-AID* from G1 through mid–M phase arrest. Left, cohesion loss at *CEN*-distal *LYS4* locus assessed in haploid *SMC3 SMC3-AID* (MSB81-1A) and *smc3-RR SMC3-AID* (MSB79-1A) strains. Right, cohesion loss at *CEN*-proximal *TRP1* locus assessed in haploid *SMC3 SMC3-AID* (MSB84-1A) and *smc3-RR SMC3-AID* (MSB83-1A) strains. The percentage of cells with two GFP spots (sister separation) is plotted.

assessed the viability of each strain by plating on 5-FOA to counter-select for the plasmid. As expected when *SMC3* is present, *wpl1*Δ can support viability. Similarly, *smc3-D1189H wpl1*Δ cells were viable, which was unsurprising as both mutants were viable on their own. Additionally, *wpl1*Δ could restore viability to *smc3-RR* (Figure 3.3A, compare to Figure 3.4A). When tested for drug sensitivity, *smc3-RR wpl1*Δ was highly sensitive to both benomyl and CPT, similar to what has been reported for *eco1*Δ *wpl1*Δ cells (Guacci and Koshland 2012; Guacci et al. 2015). Conversely, both *wpl1*Δ and *smc3-D1189H wpl1*Δ were not sensitive (Figure 3.4B). Additionally, *smc3-RR-D1189H* was resistant to both benomyl and CPT to similar levels as wild-type cells and *smc3-D1189H* cells, which was drastically different from the sensitivity of *smc3-RR wpl1*Δ (Figure 3.4C). The difference in drug sensitivity between *smc3-RR wpl1*Δ and *smc3-RR-D1189H* indicates that D1189H suppresses lethality of *smc3-RR* through a different mechanism than *wpl1*Δ.

Because of the similar drug sensitivity of *smc3-RR wpl1*Δ and *eco1*Δ *wpl1*Δ, I wondered whether *smc3-RR wpl1*Δ would also phenocopy the cohesion defect of *eco1*Δ *wpl1*Δ. I therefore assessed the cohesion defects of *smc3-RR wpl1*Δ cells in mid-M at *LYS4. smc3-RR wpl1*Δ cells had a severe cohesion defect, of ~65% similar to what has previously been reported for *eco1*Δ *wpl1*Δ cells (Figure 3.4D). Additionally, *smc3-D1189H wpl1*Δ cells had a cohesion defect of ~30%, similar to that of *wpl1*Δ alone. Thus *smc3-D1189H* does not ameliorate the cohesion defect of *wpl1*Δ further indicating that *smc3-D1189H* cannot counteract *WPL1* function. Together these results indicate that *wpl1*Δ suppresses the inviability of the *smc3-RR* allele but not the cohesion defect. Thus acetylation of K112 and K113 is necessary for a step in promoting cohesion in addition to antagonizing Wpl1p. Furthermore, the acetylation requirement for both these steps are bypassed by *smc3-D1189H*.

Additional Smc3p acetylation sites do not promote cohesion establishment

The implication that Eco1p has additional targets that contribute to the activation of cohesion prompted me to test potential other targets. As Eco1p has been shown to acetylate all of the cohesin subunits and regulators *in vitro*, there are many substrates that could be tested for their contribution to cohesion (Ivanov et al. 2002; Chao et al. 2017). I chose to begin this analysis with six additional acetylation sites that were identified on Smc3p: K309 K316, K699 K700 and, K931 K940 (Figure 3.5A)(Unal et al. 2008).

To test the importance of these residues in promoting cohesion I mutated all six of these lysines to arginine, *smc3-6R*. The *smc3-6R* allele was integrated into a strain containing *smc3-AID* and tested for its ability to sustain viability when plated on media

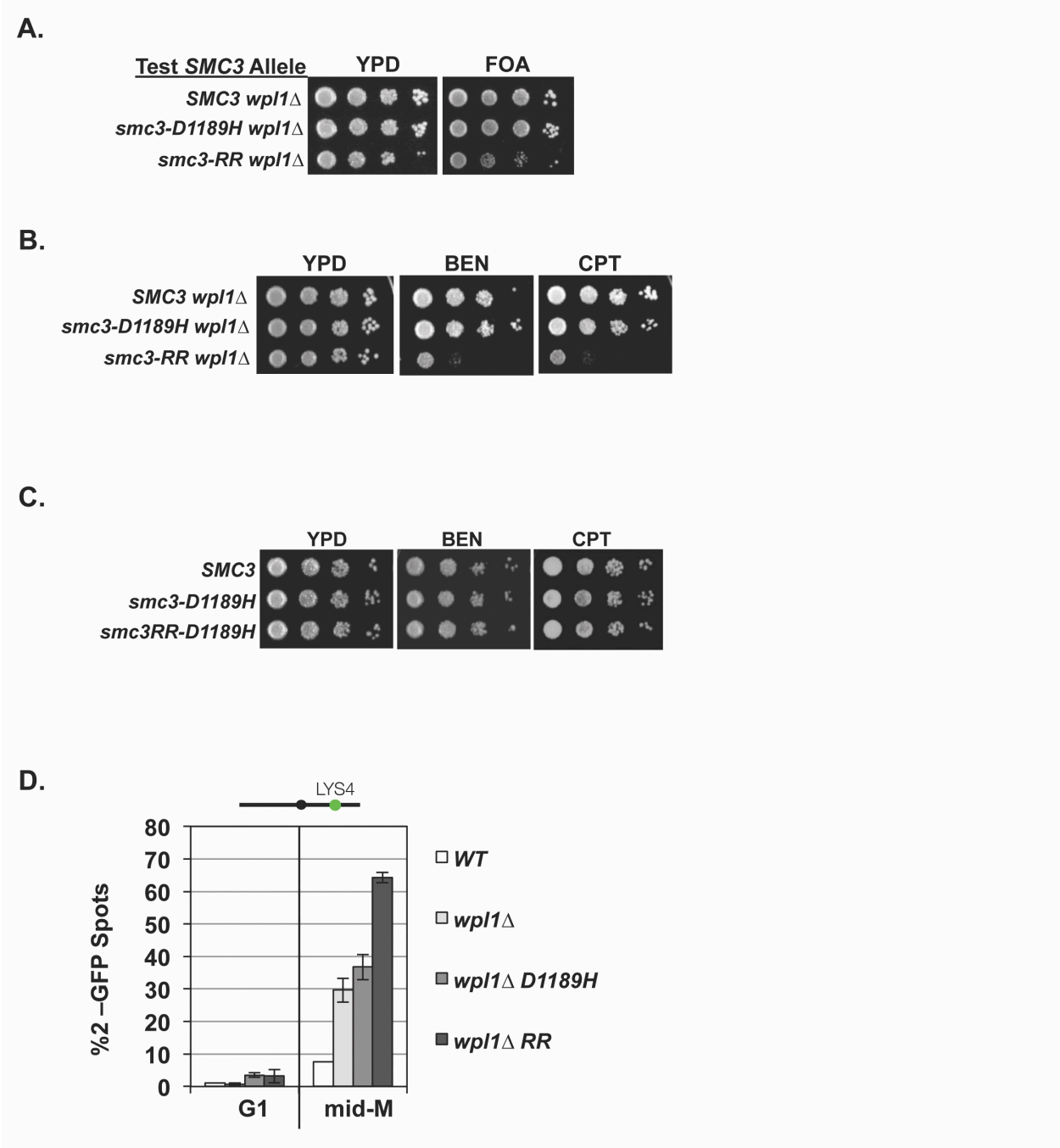

Figure 3.4: *smc3-D1189H* activates cohesion through a Wpl1p-independent mechanism

(A) Plasmid shuffle to assess viability of *smc3-RR wpl1*Δ cells. Haploid *wpl1*Δ *SMC3* shuffle strain (VG3578-1A) bearing pEU42 (*SMC3 CEN URA3*) and a second integrated test *SMC3* allele—WT (*SMC3 wpl1*Δ; MSB48-1A), *smc3-D1189H* (*smc3-D1189H wpl1*Δ; MSB49-1A), or *smc3-RR* (*smc3-RR wpl1*Δ; MSB50-1A)—were grown and plated as described in Figure 3.2A onto YPD or 5-FOA- containing media. Plates were incubated 2 days at 30°C. **(B)** Assessment of *smc3-RR wpl1*Δ strain drug sensitivity. Haploid *SMC3 wpl1*Δ (MSB48-1A), *smc3-D1189H wpl1*Δ (MSB49-1A), and *smc3-RR wpl1*Δ (MSB50-1A) strains were serially diluted 10X and plated onto YPD media alone

or containing BEN (10 µg/ml) or CPT (10 µg/ml) and incubated at 23°C for 3 days for YPD and 4 days for BEN and CPT plates. **(C)** Assessment of drug sensitivity. Haploid *SMC3* (MSB45-1A), *smc3-D1189H* (MSB46-1A), or chimeric *smc3-RR-D1189H* (MSB47-1A) strains serially diluted 10x and plated onto YPD, BEN, and CPT as described in B and incubated at 30°C for 2 days. **(D)** Cohesion loss at *CEN*-distal locus *LYS4*. Haploid wild-type (WT; VG3627-3C), *wpl1*Δ (*SMC3 wpl1*Δ; MSB48-1A), *smc3-D1189H wpl1*Δ (MSB49-1A), *smc3-RR wpl1*Δ (MSB50-1A), and *smc3-RR-D1189H wpl1*Δ (MSB51-1A) strains arrested in mid–M phase as described in materials and methods. Cells were scored for cohesion loss (sister separation) Data are derived from two independent experiments; 100–300 cells were scored for each data point in each experiment.

containing auxin. Cells containing *SMC3* grew robustly on auxin compared to the strain lacking a second, copy of *SMC3*. Importantly, the *smc3-6R* grew as well as *SMC3* on media containing auxin. When tested for sensitivity to benomyl, the *smc3-6R* did not have any noticeable sensitivity compared to *SMC3*. These results indicate that the *smc3-6R* does not compromise the ability to sustain viability in this background (Figure 3.5B).

I also assessed the *smc3-6R* mutant for its ability to mediate cohesion. I depleted *smc3-AID* in G1 by the addition of auxin to cultures containing *SMC3*, *smc3-6R*, or lacking a second *SMC3* allele. Cells were then synchronously released in the presence of auxin and re-arrested in mid-M arrest with nocodazole. As expected, the strain bearing only the *smc3-AID* allele had a severe cohesion defect of~90% compared to cells containing *SMC3* which had robust cohesion. Similar to *SMC3*, the *smc3-6R* strain was not compromised for cohesion, and only had ~10% of cells that exhibited cohesion loss (Figure 3.5C). Thus, I conclude that acetylation of the six non-K112 K113 lysines identified by Unal et al. (2008) do not contribute to establishment of cohesion or promotion of viability.

Discussion

I showed that the allele, *smc3-D1189H*, specifically promotes cohesion and viability in the absence of acetylation of K112 K113. As restoration of viability has been correlated with the ability to promote chromosome condensation, we can infer that the D1189H also promotes condensation in the absence of acetylation at K112 K113. These findings indicate that both cohesion and condensation are promoted through a common mechanism. In contrast, *wpl1*Δ restores viability but not cohesion to *smc3-RR*, indicating that D1189H promotes these functions through a Wpl1p-independent manner (Figure 3.6 #2). This is contrary to the long standing model that acetylation of K112 K113 promoted cohesion by inhibiting Wpl1p function. Instead, the location of D1189 in the Smc3p head suggests that it is due to alteration of cohesin function itself. A recent *in silico* analysis of D1189H suggested that this amino acid change may hinder the formation of a salt bridge between residues in Smc1p and Smc3p leading to destabilization of this interface (Huber et al. 2016). Thus, the promotion of cohesion and condensation may be due, in part, to destabilization of the Smc1p-Smc3p interface.

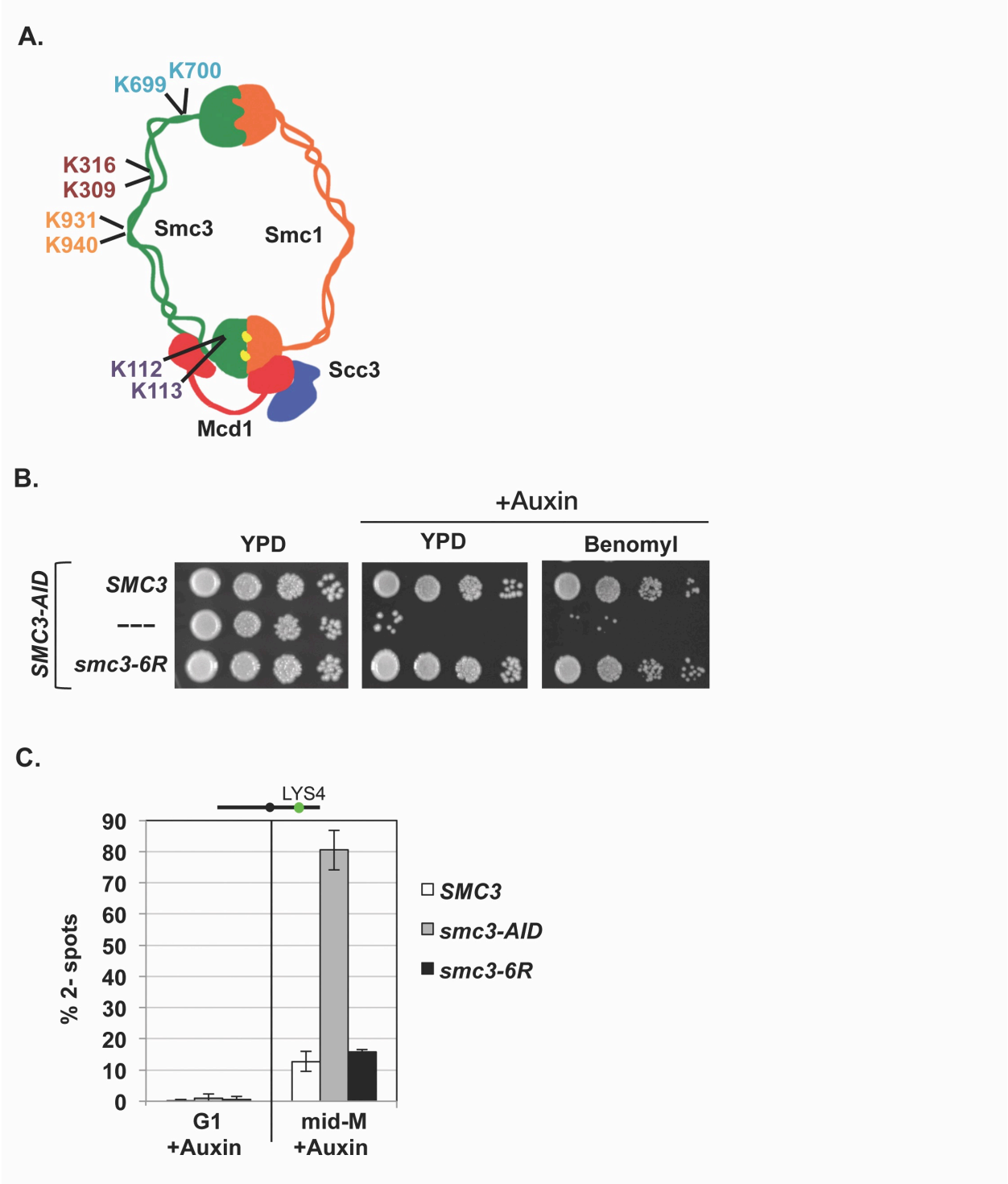

Figure 3.5: Acetylation of additional Smc3p lysines do not contribute to cohesion establishment

(A) Location of acetylated lysines in Smc3 identified in (Unal et al. 2008). Schematic of cohesin complex: Smc1 (orange), Smc3 (green), Mcd1 (red), Scc3 (blue). Clusters of acetylated lysines are indicated by color with black lines indicating approximate location: K112 K113 (purple), K309 K316 (maroon), K699 K700 (light blue), K931 K940 (light

orange). **(B)** Assessment of viability and drug sensitivity. *SMC3* alleles WT (MSB81-1A) or smc3-6R (MSB92-3A) were integrated at *LEU2* in strain containing a second *smc3-3V5-AID* allele (VG3651-3D). Strains were plated on YPD media either with or without 750 µM Auxin, and YPD media containing 750 µM Auxin and 10 mg/mL Benomyl and incubated at 23˚C for 3 days. **(C)** Cohesion loss at *CEN*-distal locus *LYS4*. *smc3-AID* strains containing either *SMC3 (MSB81-1A)*, *smc3-6R (MSB92-3A)* or no allele (VG3651-3D) from part B were tested for cohesion loss in mid–M phase at *CEN*-distal locus *LYS4,* as described in materials and methods.

Mapping of K112, K113, and D1189 on the crystal structure of the Smc3p head domain shows that all three residues lay in close proximity of the ATP binding pocket, hinting that the D1189H mutation might affect ATPase function (Figure 3.7). Consistent with this hypothesis, other cohesion activators that were also picked up in our screen are located in highly conserved motifs of the Smc3p ATP binding pocket. One of these mutants, Smc1p-D1164E, lowers ATP hydrolysis, indicating that this attenuation promotes cohesion *in vivo* and stable cohesin binding *in vitro* (Çamdere et al. 2015). In fact, after the *in vivo* analysis of *smc3-D1189H* presented here, Dr. Gamze Camdere found that purified *S. pombe* cohesin containing the equivalent mutation also had decreased ATPase activity compared to wild-type (not shown). Previous study of mutants in either the Walker A or Walker B motifs of the cohesin ATPases resulted in the inability of cohesin to associate with DNA, precluding analysis of the function of the ATPase in any step past cohesin binding to chromosomes (Arumugam et al. 2003; Heidinger-Pauli et al. 2010). Thus the identification of these cohesion-activating mutants is the first implication of cohesin ATPase function in a step beyond cohesin loading onto chromosomes. Together, these results suggest that K112 K113 acetylation promotes cohesion and condensation by down-regulating the ATPase in a step past cohesin loading. In order to mediate both of these functions, modulation of the ATPase likely promotes tethering of two genomic loci. It could achieve tethering either by promoting capture of the second locus by opening the cohesin ring, or by promoting oligomerization with a second cohesin complex. The failure of the *smc3-RR* allele to complement other *smc3* mutant alleles supports the model that acetylation might promote direct interactions between cohesin subunits (Eng et al. 2015).

In contrast to the full restoration of cohesion and condensation in *smc3-RR*, D1189H fails to restore viability (and thereby condensation) and only modestly restores cohesion to cells lacking Eco1p function. This discrepancy indicates that Eco1p promotes cohesion and condensation by at least 2 pathways: through K112 K113 acetylation, and through acetylation of targets other than K112 K113 (Figure 3.6 #1). What are these additional targets of Eco1p? I tested the contribution of 6 additional Smc3p acetylation sites and found that they were dispensable for both cohesion and viability. Until recently, these were the only residues identified as targets of Eco1p acetylation, besides Mcd1p residues K84 and K210, are important for DNA damage-induced cohesion, but have no significant function in S-phase cohesion establishment (Heidinger-Pauli et al. 2009). Thus the task of determining the relevant targets for cohesion and condensation still remains. A recent study identified targets of Eco1p through *in vitro* acetylation and mass-spectrometry. This experiment identified peptides on all of the cohesin sub-units (Smc1p, Smc3p, Mcd1p, Scc3p), and its known

regulators (Scc2p, Scc4p, Pds5p, Wpl1p) that were acetylated, (Chao et al. 2017). This list of acetylated peptides provides a jumping off point for future studies to search for the biologically significant substrates.

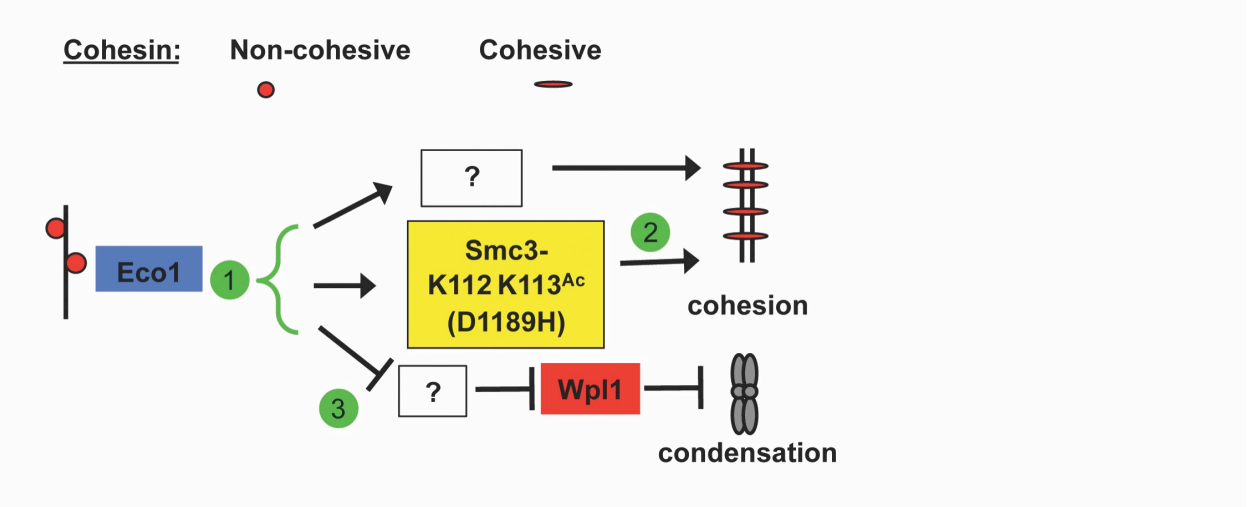

Figure 3.6: Additional targets of Eco1p promote cohesion activation and condensation
Proposed model for promotion Eco1 function in mediating cohesion and condensation. **(1)** Eco1 acetylates unknown targets to promote at least three pathways in promoting cohesion and condensation **(2)** Acetylation of K112 K113 promotes cohesion in Wpl1p-independent pathway. Smc3-D1189H compensates for function of K112 K113 acetylation. **(3)** Eco1p acetylates unknown targets to inhibit Wpl1p function in inhibition of condensation.

One question that arises from the implication of additional Eco1p targets for both cohesion and condensation is if both cohesion and condensation are promoted through acetylation of the same targets or distinct targets. As previously described, inhibition of Wpl1p is important for promoting condensation (Guacci and Koshland 2012). Thus it is possible that Wpl1p is inhibited by acetylation of other targets (such as Pds5p), or Eco1p directly acetylates Wpl1p (Figure 3.6 #3). Further studies will be needed to understand differences between the targets and mechanisms of acetylation in promoting cohesion and inhibition of Wpl1p.

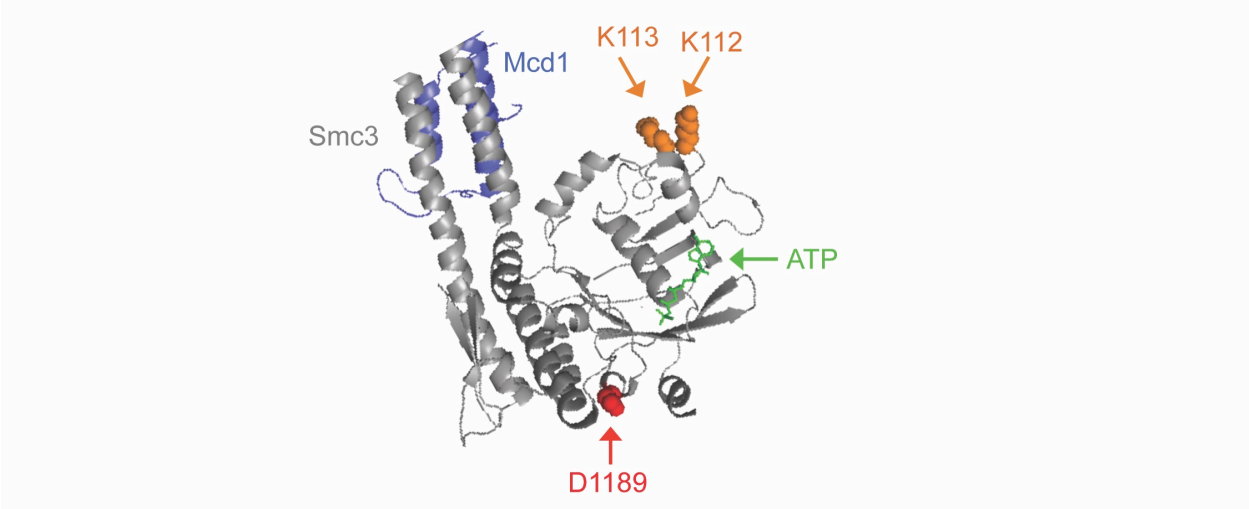

Figure 3.7: Mapping of acetylation sites and cohesion activator on Smc3p head domain
Smc3-D1189 located near ATP binding pocket. Crystal structure of *S. cerevisiae* Smc3p head domain and coiled-coil DOI: 10.2210/pdb4ux3/pdb from (Gligoris et al. 2014). Grey: Smc3, Blue: Mcd1, Yellow: ATP, Orange: acetylation sites K112 K113, Red: D1189 location of cohesion activating mutant.

Materials and Methods

Yeast strains, media, and reagents

Yeast strains used in this study are A364A background, and their genotypes are listed in Table 3.1. YPD liquid media was prepared containing 1% yeast extract, 2% peptone, 2% dextrose, 0.01 mg/ml adenine.

Solid Media:
YPD solid media was prepared containing 1% yeast extract, 2% peptone, 2% dextrose, 2% agar.

Additives:

Auxin: 1M 3-indoleacetic acid (Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide and added to plates or liquid cultures at a final concentration of 500 µM, with cooling agar used in plates to ~55˚C before addition of auxin to each batch.

Benomyl: Benomyl (a gift from DuPont, Wilmington, DE) made as a 10 mg/ml stock (in DMSO) was added to a final concentration of 10 µg/ml in media cooled to 55°C.

Camptothecin: Camptothecin (Sigma-Aldrich, St. Louis, MO) was made as a 10 mg/ml stock (in DMSO) and added to final concentration of 20 µg/ ml in YPD media containing 25 mM 4-(2-hydroxyethyl)-1-pipera- zineethanesulfonic acid (HEPES), pH 7.4

Dropout media: 5-FOA was purchased from US Biological Life Sciences (Salem, MA) and used at a final concentration of 1 g/l in URA dropout plates supplemented with 50 mg/L uracil powder (Sigma-Aldrich)

Dilution plating
Cells were prepared and plated as described in chapter 2

Cohesin time course
Time course and cell preparation for microscopy was performed as described in chapter 2

Flow cytometry
Cells were prepared and analyzed as described in chapter 2

Microscopy
Images and cells were scored as described in chapter 2

Cohesion activator suppressor screen
Screen originally published in (Guacci et al. 2015). Haploid *eco1*Δ *wpl1*Δ cells were dilution streaked onto YPD and incubated 3 days at 23°C to allow colony formation from single cells. A small amount of a single colony was inoculated into 5ml YPD and grown overnight at 23°C to saturation. ~10^7 of saturated cells were plated onto BEN (12.5 mg/ml or 15 mg/ml) or CPT (12.5 mg/ml or 15 mg/ml) then grown for 4 days at 23°C to select drug resistant suppressor mutants. A different single colony from YPD plates was used for each selection trial to generate independent suppressors. Colonies were then tested for cross resistance to drugs (i.e. colonies from BEN plate tested for CPT resistance and vice-versa). Colonies exhibiting cross-resistance were then sent for whole genome sequencing.

Supplementary Table

Table 3.1: Strain names and genotypes used in this chapter

Description	Strain name	Genotype
SMC3	**MSB45-1A**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-TRP1 smc3Δ::HPH leu2-3,112:pVG419-SMC3-LEU2*
smc3-D1189H	**MSB46-1A**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-TRP1 smc3Δ::HPH leu2-3,112:pVG419-smc3-D1189H-LEU2*
smc3-D1189H	**MSB46-1A**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-TRP1 smc3Δ::HPH leu2-3,112:pVG419-smc3-D1189H-LEU2*
smc3-RR-D1189H	**MSB47-1A**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-TRP1 smc3Δ::HPH leu2-3,112:pVG419-smc3 K112R K113R D1189H-LEU2*

SMC3 wpl1Δ	**MSB48-1A**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-TRP1 smc3Δ::HPH leu2-3,112:pVG419-SMC3-LEU2 wpl1Δ::KANMX*
smc3-D1189H wpl1Δ	**MSB49-1A**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-TRP1 smc3Δ::HPH leu2-3,112:pVG419-smc3-D1189H-LEU2 wpl1Δ::KANMX*
smc3-RR wpl1Δ	**MSB50-1A**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-TRP1 smc3Δ::HPH leu2-3,112:pVG419-smc3 K112R K113R-LEU2 wpl1Δ::KANMX*
smc3-RR-D1189H wpl1Δ	**MSB51-1A**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-TRP1 smc3Δ::HPH leu2-3,112:pVG419-smc3 K112R K113R D1189H-LEU2 wpl1Δ::KANMX*
SMC3	**MSB65-1A**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-TRP1 smc3Δ::HPH leu2-3,112:pVG419-SMC3-LEU2*
smc3-D1189H	**MSB66-1A**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-TRP1 smc3Δ::HPH leu2-3,112:pVG419-smc3-D1189H-LEU2*
smc3-RR-D1189H	**MSB67-1A**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-TRP1 smc3Δ::HPH leu2-3,112:pVG419-smc3 K112R K113R D1189H-LEU2*
smc3-AID smc3-RR	**MSB79-1A**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 smc3::SMC3-N607-3V5-AID TIR1-CaTRP1 leu2-3,112:smc3 K112R K113R-LEU2*
smc3-AID SMC3	**MSB81-1A**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 smc3::SMC3-N607-3V5-AID TIR1-CaTRP1 leu2-3,112:SMC3-LEU2*
smc3-AID smc3-RR	**MSB83-1A**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 smc3::SMC3-N607-3V5-AID TIR1-CaTRP1 leu2-3,112:smc3 K112R K113R-LEU2*
smc3-AID SMC3	**MSB84-2A**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 smc3::SMC3-N607-3V5-AID TIR1-CaTRP1 leu2-3,112:SMC3-LEU2*
smc3-D1189H eco1Δ	**MSB91-1A**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-TRP1 smc3Δ::HPH leu2-3,112:smc3-D1189H-LEU2 eco1Δ::KANMX pBS1030 (ECO1 URA3 CEN)*
smc3-AID smc3-6R	**MSB92-3A**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 bar1 leu2-3,112 his3-11,15:pHIS3-GFPLacI-HIS3 smc3::SMC3-N607-3V5-AID TIR1-CaTRP1 ura3-52:smc3-K309R K316R K699R K700R K931R K940R-URA3*

WT	**VG3460-2A**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-HIS3*
smc3Δ	**VG3464-16C**	*MATa lys4:LacO(DK)-NAT GAL+ leu2-3,112 trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-TRP1 smc3Δ::HPH pEU42 (SMC3 CEN URA3)*
smc3Δ wpl1Δ	**VG3578-1A**	*MATa lys4:LacO(DK)-NAT GAL+ leu2-3,112 trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-TRP1 smc3Δ::HPH pEU42 (SMC3 CEN URA3)* wpl1Δ::KANMX
WT	**VG3620-4C**	*MATa lys4:LacO(DK)-NAT GAL+ leu2-3,112 trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 TIR1-CaTRP1*
smc3-D1189H wpl1Δ	**VG3627-3C**	*MATa 10Kb-CEN4:LacO(DK)-NAT trp1-1 ura3-52 bar1 GAL+ pHIS3-GFPLacI-TRP1:his3-11,15 smc3Δ::HPH leu2-3,112:pVG419-smc3-D1189H-LEU2 wpl1Δ::KANMX smc3Δ::HPH*
eco1-AID	**VG3646-1A**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-HIS3* leu2-3,112*:SMC1-LEU2 smc1Δ::HPH eco1::ECO1-3V5-AID2-KANMX TIR1-CaTRP1*
eco1-AID smc3-D1189H	**VG3650-1E**	*MATa lys4:LacO(DK)-NAT GAL+ trp1-1 leu2-3,112 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-TRP1 eco1::ECO1-3V5-AID2-KANMX TIR1-CaHIS3 smc3Δ::HPH leu2-3,112:smc3-D1189H-LEU2*
smc3-AID	**VG3651-3D**	*MATa lys4:LacO(DK)-NAT GAL+ leu2-3,112 trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-HIS3 smc3::SMC3-N607-3V5-AID TIR1-CaTRP1*
eco1-AID	**VG3659-1A**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-HIS3* leu2-3,112*:SMC1-LEU2 smc1Δ::HPH eco1::ECO1-3V5-AID2-KANMX TIR1-CaTRP1*
eco1-AID smc3-D1189H	**VG3663-2E**	*MATa 10Kb-CEN4:LacO(DK)-NAT GAL+ trp1-1 ura3-52 bar1 his3-11,15:pHIS3-GFPLacI-HIS3* smc3Δ::HPH *eco1::ECO1-3V5-AID2-KANMX TIR1-CaTRP1 leu2-3,112:smc3-D1189H-LEU2*

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CHAPTER 4: CONCLUDING REMARKS

Cohesin mediates diverse functions

The discovery of the cohesin complex marked a turning point in our understanding of the structure and function of chromosomes both during interphase as well as during cell division. Most of our understanding of cohesin function comes from studying its role in sister chromatid cohesion. However, cohesin is also involved in other functions that mediate complex chromosomal structure including chromosome condensation, DNA damage repair, and transcription regulation. It is likely that cohesin mediates these diverse functions through a common mechanism in tethering two genomic loci as many cohesin accessory proteins regulate cohesin in multiple contexts. Much of the data presented in this dissertation aims to understand the mechanism through which these regulatory proteins alter cohesin function and how they discern between cohesin functions.

Much of the research presented here has focused on the regulator Wpl1p, whose function has long been enigmatic and complex. The studies presented here focused on how both the positive and negative functions of Wpl1p mediated, either in a complex with Pds5p, or independent of Pds5p. I also presented analysis of how cohesion establishment is achieved by both inhibition of Wpl1p, and independent of Wpl1p. I then showed that this Wpl1p-independent step is mediated by modulation of the ATPase. In reality, all of these functions and regulators are intimately linked. Understanding the relationships among all the regulators and cohesin will be more insightful than studying each one individually.

Three structural changes in cohesin have been identified that correlate with the establishment of cohesion: Acetylation of K112 K113, down-regulation of the ATPase, and stabilization of the Smc3p/Mcd1p interface (Rolef Ben-Shahar et al. 2008; Unal et al. 2008; Çamdere et al. 2015; Beckouët et al. 2016). It is likely that all three of these functions are linked, though not many experiments have directly analyzed their relationships. The next steps to understanding cohesin function are to analyze how these structural changes are linked mechanistically and to analyze the molecular basis for how Wpl1p, Eco1p, and Pds5p mediate these changes. In my analysis, I have proposed models for functions of Pds5p, Wpl1p, and Eco1p, which pose questions that may provide a direction for future experimentation:

- How does Wpl1p positively and negatively regulate cohesin?
- How can Pds5p and Wpl1p functions be reconciled?
- How does Eco1p regulate cohesin beyond acetylation of K112 K113?

How does Wpl1p positively and negatively regulate cohesin?

The best-characterized function of Wpl1p is its removal of cohesin from chromosome arms during prophase of mitosis. However, in yeast, where no prophase removal of cohesin has been noted, Wpl1p is conserved and appears to play inhibitory roles. Additionally, loss of Wpl1p function is not severely detrimental to yeast cells. Thus it is unclear why Wpl1p-antagonization of cohesin is important in yeast. However, as Wpl1p also regulates cohesin positively, it is possible that Wpl1p has a single function that is a double-edged sword, acting positively or negatively depending on its context in

the cell cycle. How can we assign Wpl1p a single function that could act positively and negatively?

I described in chapter two a model in which Wpl1p promotes cohesin recycling. In this model, the antagonistic functions of Eco1p and Wpl1p create two pools of cohesin within the nucleus: a stable pool that is "locked" on DNA, and a dynamic pool that is free to bind and dissociate from DNA (Figure 4.1). Eco1p promotes the stable pool on DNA by acetylating cohesin. Wpl1p promotes the dynamic pool by removing cohesin from DNA. If cohesin can only mediate one function at a time (i.e. cohesion, or condensation, but not both) an advantage to maintaining a dynamic pool is that cohesin can be re-loaded onto DNA to mediate multiple functions.

Evidence supports that this soluble pool is needed to mediate non-cohesion functions of cohesin. First, it has been estimated that only about 10% of cohesin is acetylated during a cell cycle (Zhang et al. 2008). Second, fluorescence recovery after photo-bleaching (FRAP) experiments show that cohesin is dynamic in the absence of Eco1p function. When Eco1p is present, some cohesin becomes stabilized, though, a significant portion remains dynamic, which is consistent with the small portion of cohesin that is acetylated (Rowland et al. 2009). Finally, there is a hierarchy to cohesin functions. Experiments in which cohesin levels were lowered within the cell revealed that cohesion persists, while the ability to promote condensation and DNA repair were lost. Additionally, when cohesin levels were extremely limited (~13% of wild-type levels), cohesin was preferentially loaded at the centromere, but not on chromosome arms, and cells still remained viable (Heidinger-Pauli et al. 2010). The hierarchy revealed in these experiments suggests that each cohesin molecule can only mediate a single function at a time, and thus a prioritization of functions exists.

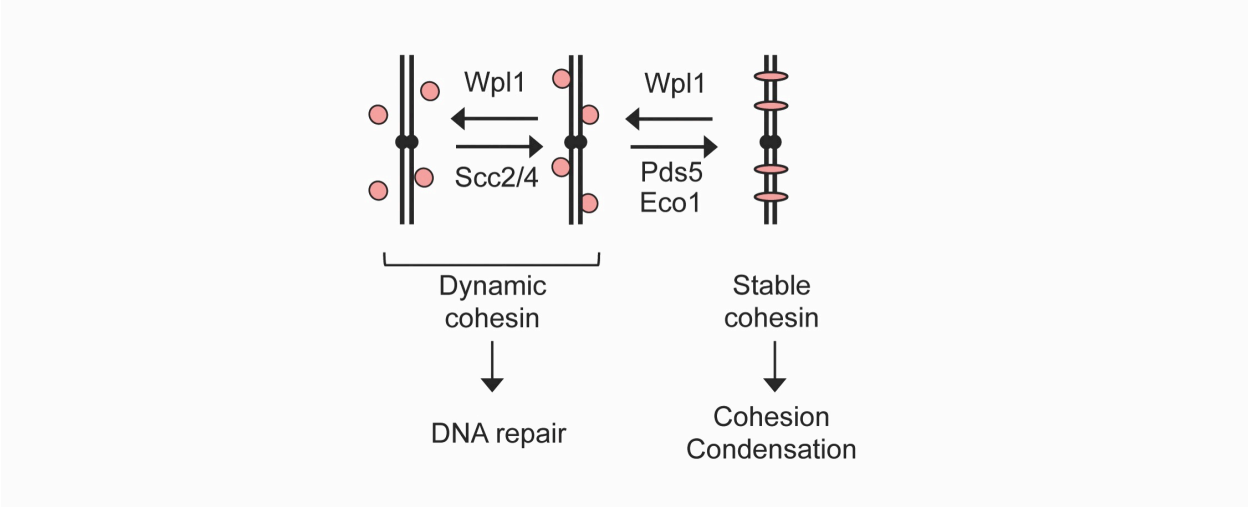

Figure 4.1: Model for cohesin recycling
Left: Cohesin exists in two states, dynamic (pink balls) which can bind DNA, facilitated by Scc2/4, and unbind DNA, facilitated by Wpl1. Right: Cohesin is stabilized on DNA (pink ovals) facilitated by Pds5 and Eco1, and opposed by Wpl1.

The model in which Wpl1p recycles cohesin suggests that when Wpl1p function is lost, cohesin would still be loaded throughout the cell cycle, but would be hyper-stabilized, a phenomenon shown to be true (Chan et al. 2012). Consistent with this finding, *in vitro* and *in vivo* experiments showed that cohesin is stable on chromosomes in the absence of Eco1p and Wpl1p, even though cohesion is not established (Çamdere et al. 2015; Murayama and Uhlmann 2015). This model also suggests that without Wpl1p function, cohesin would not be able to be removed and reloaded in the proper place, for example around a DNA break. This inability to promote a dynamic form of cohesin could explain why Wpl1p appears to play a positive role in DNA repair.

Wpl1p promotion of cohesin turnover could be bad, however, if not balanced by Eco1p function promoting cohesin stabilization. In this scenario, Wpl1p removes cohesin from DNA before it can mediate tethering. Thus Eco1p inhibition of Wpl1p is important to prevent removal of cohesin that is needed for cohesion and condensation during a significant portion of the cell cycle. My experiments in chapter 3 revealed that Eco1p promotes cohesion establishment by altering the properties of cohesin itself in addition to inhibiting Wpl1p. Thus Eco1p needs to prevent Wpl1p from premature removal of cohesin from DNA, in addition to promoting a structural change that mediates tethering.

How can Pds5p and Wpl1p functions be reconciled?

In chapter two, I proposed that Wpl1p binds to the N-terminal regulatory domain of Pds5p to inhibit Pds5p function. My model, however, does not explain the molecular mechanism of how Pds5p may promote cohesion and condensation. If Wpl1p inhibits Pds5p function to destabilize cohesin on DNA, then Pds5p may promote stabilization. Thus Pds5p may promote any of the three structural changes to cohesin that have been shown to achieve stable binding to DNA: stabilization of the Smc3p/Mcd1p interface, acetylation of cohesin, or modulation of the ATPase.

Pds5p modulates stabilization of the Smc3p/Mcd1p interface

It has been suggested that the interface between the N-terminus of Mcd1p and the coiled-coil of Smc3p is a key interface for the mediating the stability of cohesin on DNA. It is thought that destabilization of this interface allows for cohesin to dissociate from DNA (Chan et al. 2012). Experiments which use levels of crosslinking between Smc3p and Mcd1p as a metric for stability of the interface, show that Wpl1p function destabilizes this interaction. Additionally, the *pds5* N-terminal mutants analyzed in chapter 2, like *wpl1*Δ, have been shown to stabilize this interaction (Beckouët et al. 2016). These findings suggest that there may exist two forms of cohesin: a “closed” form of cohesin that is stably bound to DNA when Smc3p and Mcd1p interact, and an “open” form of cohesin that is unstable when Mcd1p and Smc3p are not interacting (Figure 4.2). I hypothesize that Pds5p promotes the stabilization of the Smc3p/Mcd1p interface by physically interacting in that region (Figure 4.2). Additionally, I hypothesize that Wpl1p binding to the N-terminus of Pds5p prevents it from stabilizing the Smc3p/Mcd1p interface. Crosslinking experiments support this model, showing that the N-terminus of Pds5p interacts with cohesin in this region (Huis in 't Veld et al. 2014). These interactions were corroborated by high resolution electron microscopy of the cohesin complex bound to Pds5p (Hons et al. 2016). This model predicts that loss of

Pds5p or an N-terminal truncation (*pds5 Δ2-181*) would lead to destabilization of the Smc3p/Mcd1p interface. Additionally, this destabilization would be independent of Wpl1p function, and *wpl1Δ* would not re-stabilize the interface in the absence of Pds5p as it does when the Pds5p is present.

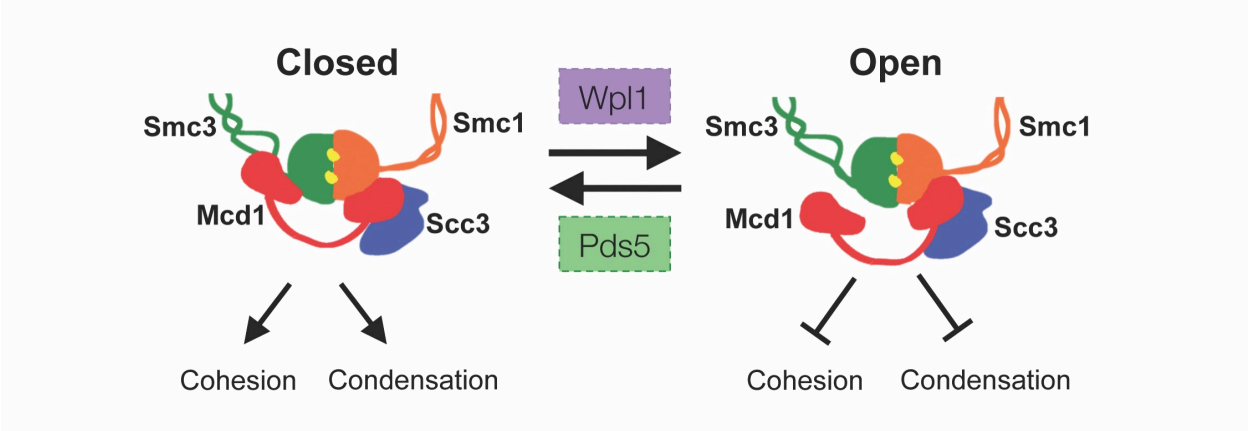

Figure 4.2: Model for Pds5p and Wpl1p function is cohesin stabilization and destabilization
Left: Stabilization of Smc3/Mcd1 interface ("closed" form) promotes cohesion and condensation. Proposed Pds5p function is to stabilize Smc3/Mcd1 interaction. Right: Destabilization of Smc3/Mcd1 interface ("open" form) inhibits cohesion and condensation. Wpl1p functions to destabilize Smc3/Mcd1 interaction.

Pds5p promotes acetylation of cohesin

Cohesin acetylation is important for both counteracting Wpl1p function as well as modulating ATPase function. The model I proposed for Pds5p-mediated promotion of tethering would predict that Pds5p is involved in promoting acetylation. In fact, it has been previously shown that Pds5p is involved in both promotion and protection of acetylation (Chan et al. 2013). Additionally, genetic and physical interactions between Pds5p and Eco1p support this model (Noble et al. 2006). A study recently showed that Eso1, the *S. pombe* ortholog of Eco1p, contains a YSR motif that mediates its physical interaction with the N-terminus of Pds5p (Goto et al. 2017). Thus it is possible that Eco1p is recruited to cohesin by Pds5p to promote acetylation of cohesin. In addition, it has been shown that Pds5p also protects Smc3p from deacetylation by Hos1 (Chan et al. 2013). How Pds5p physically protects acetylation is unknown but a simple explanation could be that Pds5p blocks either the acetylation sites or blocks binding of Hos1p.

Pds5p modulates ATPase function

There is no current evidence that Pds5p directly affects ATPase function. However ATPase function is intimately connected to both stabilization of the Smc3p/Mcd1p interface and acetylation of K112 K113. For instance, the cohesion activator mutant, Smc1p-D1164E, bypasses Eco1p function, down-regulates the ATPase and stabilizes the Smc3p/Mcd1p interface (Çamdere et al. 2015; Beckouët et

al. 2016). Thus it is possible that either modulation of the ATPase affects Pds5p interaction with cohesin, or Pds5p interaction with cohesin leads to modulation of the ATPase.

Mechanism for Wpl1p removal of cohesin from DNA

How Wpl1p mediates removal of cohesin from DNA is unknown, though there must be another step beyond binding Pds5p. Wpl1p removal of cohesin could occur either by inducing a conformation change in Pds5p so that it can no longer stabilize cohesin, or by acting on cohesin directly. Comparison of the Pds5p *apo* structure versus Wpl1p-bound have not been published, though slight conformation change has been noted upon Mcd1p binding to Pds5p, suggesting that Wpl1p binding could similarly affect Pds5p structure. There is support, though, that Wpl1p may effect cohesin directly by binding the head domain of cohesin near K112 K113, suggesting that acetylation physically blocks Wpl1p from interaction with Smc3p (Chatterjee et al. 2013). Additionally it has been suggested that through interaction with the head, Wpl1p may serve to alter the stability between Smc1p and Smc3p, which could directly destabilize cohesin, or promote the ATPase cycle, to disengage it from DNA (Elbatsh et al. 2016).

How does Eco1p regulate cohesin aside from acetylation of K112 K113?

An important conclusion from my analysis of the cohesion activator, *smc3-D1189H*, was that Eco1p acetylates other targets aside from Smc3p-K112 K113 in order to promote cohesion and condensation. This model poses the question as to whether cohesion and condensation are promoted through the same targets or distinct targets (Chapter 3 Figure 3.6). Recently, targets of Eco1p were identified on each of the cohesin subunits and regulators through *in vitro* acetylation and mass spectrometry, leaving a long list of potential candidates for promoters of cohesion and condensation (Chao et al. 2017).

I ruled out acetylation of six previously identified Eco1p targets in the Smc3p coiled-coil, showing that mutation of them did not lead to and detectable defects in viability or cohesion. Though the coiled-coil regions of cohesin are not generally thought to play an active role in cohesin function, a recent study showed that post-translational modifications (PTMs) in these regions may be important functionally important. This study found that mutating all of the lysines in certain regions of the coiled coils and in certain combinations in Smc1p and Smc3p could lead to inviability or defects in cohesion (Kulemzina et al. 2016). Though this study does not point to specific residues important for cohesion, it does highlight functional importance of the coiled-coils of the Smc proteins. Additionally, this study suggests that PTM of a single residue in the coiled-coil may not be critical for signaling cohesin function, but rather, these modifications change the general properties of the coiled-coils to help facilitate tethering.

In addition to promotion of cohesion, Eco1p must inhibit Wpl1p function. However, the mechanism by which Wpl1p inhibition occurs is unknown. A simple hypothesis is that Eco1p acetylation prevents interaction between Pds5p and Wpl1p, as Pds5p is needed to maintain cohesion. However, I found that the interaction between Pds5p and Wpl1p is constant throughout the cell cycle (data not shown). This finding is consistent with my conclusion that Wpl1p binding to Pds5p is not sufficient for Wpl1p

function and suggests that Eco1p may directly acetylate Wpl1p to inactivate it. In support of Eco1p acetylation of Wpl1p, during the course of my work, I detected a second Wpl1p species in lysates containing Wpl1p-3FLAG through immunoblotting. Though I have not characterized this species, further analysis its appearance in the cell cycle, and its dependency on Eco1p function may give insight into the mechanism of Wpl1p function.

Perspective

Though the architecture of the genome as been studied for decades, there still remain many open questions about how architecture is regulated by cohesin and other SMC complexes in order to mediate different functions of the genome. The advances in technology recently have allowed for global analysis of genome structure. Additionally, advances in the ability to purify cohesin will allow us to directly analyze cohesin structure and function. These analyses will hopefully connect structure and function to form a simple picture for how cohesin orders the genome.

In my opinion, Wpl1p is the key to understanding how each cohesin molecule can alter its function through tethering different genomic loci. The current data in the field complicate the functions of Wpl1p and Pds5p to give them active roles in both loading and unloading of cohesin from DNA. However, at their core, their functions must be simple: Pds5p to stabilize cohesin on DNA, and Wpl1p to destabilize cohesin through inactivation of Pds5p. It will be interesting to probe further into how Pds5p and Wpl1p together mechanistically affect stabilization of the Smc3p/Mcd1p interface, acetylation and modulation of the ATPase. Additionally, in studying other targets of Eco1p, we may also identify additional structural changes that occur in cohesin to achieve tethering. Finally, the big question as to whether cohesin mediates tethering by entrapment of both DNA molecules within a single ring, or through oligomerization of cohesin molecules, is still undetermined. The models that I have proposed do not preclude one model or the other. Understanding how the structure of cohesin changes upon tethering will help greatly to answer this question. The task now, is to use the models I proposed and others as jumping off points for future studies. Further understanding of the connection between cohesin structure and function will also be able to highlight similarities and differences between other SMC complexes in how they define chromosome structure and function.

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