Separation of Cohesin-Dependent Pathways in Saccharomyces cerevisiae

Kevin Tong
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Separation of Cohesin-Dependent Pathways in *Saccharomyces cerevisiae*

by

Kevin Tong

A Dissertation
Presented to the Graduate and Research Committee
of Lehigh University
in Candidacy for the Degree of
Doctor of Philosophy

in
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Kevin Tong
Approved and recommended for acceptance as a dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Separation of Cohesin-Dependent Pathways in *Saccharomyces cerevisiae*

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# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>viii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>x</td>
</tr>
<tr>
<td>Abstract</td>
<td>1</td>
</tr>
<tr>
<td>Chapter 1</td>
<td>2</td>
</tr>
<tr>
<td>Figures</td>
<td>12</td>
</tr>
<tr>
<td>References</td>
<td>18</td>
</tr>
<tr>
<td>Chapter 2</td>
<td>28</td>
</tr>
<tr>
<td>Abstract</td>
<td>29</td>
</tr>
<tr>
<td>Introduction</td>
<td>31</td>
</tr>
<tr>
<td>Results</td>
<td>33</td>
</tr>
<tr>
<td>Discussion</td>
<td>45</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>52</td>
</tr>
<tr>
<td>Figures</td>
<td>58</td>
</tr>
<tr>
<td>References</td>
<td>74</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>87</td>
</tr>
<tr>
<td>Abstract</td>
<td>88</td>
</tr>
<tr>
<td>Introduction</td>
<td>89</td>
</tr>
<tr>
<td>Results</td>
<td>91</td>
</tr>
<tr>
<td>Discussion</td>
<td>100</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>105</td>
</tr>
<tr>
<td>Figures</td>
<td>108</td>
</tr>
<tr>
<td>Supplemental Figures</td>
<td>120</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<p>| Figure 1   | 12 |
| Figure 2   | 13 |
| Figure 3   | 14 |
| Figure 4   | 15 |
| Figure 5   | 16 |
| Figure 6   | 17 |
| Figure 1   | 61 |
| Figure 2   | 62 |
| Figure 3   | 63 |
| Figure 4   | 65 |
| Figure 5   | 66 |
| Figure 6   | 68 |
| Figure 7   | 70 |
| Figure 8   | 72 |
| Figure 9   | 73 |
| Figure 1   | 114 |
| Figure 2   | 115 |
| Figure 3   | 116 |
| Figure 4   | 117 |
| Figure 5   | 118 |
| Figure 6   | 119 |</p>
<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>164</td>
</tr>
<tr>
<td>Figure 2</td>
<td>165</td>
</tr>
<tr>
<td>Figure 3</td>
<td>166</td>
</tr>
<tr>
<td>Figure 4</td>
<td>167</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1 58
Table 1 108
Table 1 160
Table 2 161
ABSTRACT

All living organisms consist of cells that undergo a cell cycle to grow and divide so that the resulting daughter cells are exact replicas of the parent cell. To achieve this feat, a parent cell will first replicate its DNA into two identical copies during S phase of the cell cycle, and then segregate those copies into two daughter cells during anaphase of mitosis. Ensuring that daughter cells receive identical sets of chromosomes requires mechanisms that check for accurate DNA replication, compaction of replicated sister chromatids, and proper segregation of chromosomes. The protein complex termed cohesins identify replicated sister chromatids by tethering them together until anaphase. Additionally, cohesins function in proper chromosome condensation and DNA replication. Using the cohesion maintenance factor Pds5, a component of cohesins, this body of work explores the mechanisms of various cohesin-dependent pathways. These studies reveal that 1) sister chromatid cohesion is mediated by a dimeric or oligomeric cohesin complex, 2) cohesin-dependent functions in sister chromatid cohesion and condensation are independently regulated, and 3) Pds5, in combination with Replication Factor C (Elg1-RFC), is required for DNA fork replication progression.
CHAPTER 1

INTRODUCTION
All living organisms consist of cells that undergo a cell cycle to grow and divide. For survival, cells must ensure that the resulting daughter cells are exact replicas of the parent cell. To achieve this feat, a parent cell will first replicate its DNA into two identical copies during S phase of the cell cycle, and then segregate those copies into two daughter cells during anaphase of mitosis. Ensuring that daughter cells receive identical sets of chromosomes requires mechanisms that check for DNA damage, replication errors, and proper segregation of chromosomes. Once all chromosomes are properly oriented to the mitotic spindle and aligned during metaphase, the spindle assembly checkpoint is satisfied, triggering anaphase onset. The products of chromosome replication, the sister chromatids, then separate from each other and move towards opposite ends of the cell. One complex of proteins, called cohesins, tethers together sister chromatid and maintains that pairing (identifying chromatids as sisters) to ensure proper chromosome segregation.

**Sister Chromatid Cohesion Structure**

The cohesin complex acts like “glue” that keeps replicated sister chromatids together from S phase up until anaphase onset. Early studies of cohesin complexes identified 5 components – Mcd1/Scc1, Smc1, Smc3, Scc3/Irr1, and Pds5 (Bialkowska and Kurlandzka, 2002; Guacci et al., 1997; Hartman et al., 2000; Losada et al., 1998; Michaelis et al., 1997; Panizza et al., 2000; Toth et al., 1999), but the structure of cohesins that maintain sister chromatid tethering remains a highly debated topic (Guacci, 2007; Nasmyth, 2005; Skibbens, 2010; Zhang and Pati, 2009). Early studies revealed that Smc1 and Smc3 each contain head and hinge domains, connected by a coiled-coil
domain. Smc1 and Smc3 dimerize, forming a heterodimer stabilized by both hinge-hinge and head-head interactions (Haering et al., 2002). Mcd1 binds to head domains of both Smc1 and Smc3, giving rise to the model that a subset of cohesin subunits form a ring. If cohesins form a ring, the authors speculated that DNA may be trapped within, or that both sisters may become trapped within a single ring to maintain cohesion (Nasmyth and Haering, 2009). Further studies suggested that the ring contains distinct DNA entrance and exit gates from which DNA entry and exit from rings could be regulated (Chan et al., 2012; Gligoris et al., 2014; Huis in ’t Veld et al., 2014).

Despite the prevalence of the one ring model, many studies counter such a simplistic view of cohesin structure. For instance, PDS5 and SCC3 mutations that result in cohesion loss retain chromatin-bound cohesins (Hartman et al., 2000; Kulemzina et al., 2012). These findings provide important challenges to a one ring entrapment model in which cohesion loss must result in cohesin dissociation from one or both sisters. These findings instead support a different model in which each sister chromatid is decorated with cohesins such that cohesin-cohesin interactions result in cohesion (Huang et al., 2005). Even in the absence of a characterized cohesin structure, it is apparent that the process of sister chromatid cohesion is an intricate and highly regulated process.

**Sister Chromatid Cohesion Cycle**

In budding yeast, cohesins are loaded onto DNA during late G1 and early S phase (Guacci et al., 1997; Michaelis et al., 1997). This loading is facilitated by the protein complex Scc2, Scc4 and requires ATP hydrolysis activity (Ciosk et al., 2000). While not sequence specific, cohesins are concentrated in AT rich regions around centromeres and
cohesion attachment regions (CARs) (Blat and Kleckner, 1999; Glynn et al., 2004; Kogut et al., 2009).

While loading of cohesins is essential, cohesins must also become “established” during S phase to stably tether together the sister chromatid pairs. This process of establishment is performed by the protein Ctf7/Eco1. The loss of Ctf7 during S phase results in severe cohesion defects and complete loss in viability. Ctf7 mutant cells, however, show no defects either in cohesin loading or in the successful completion of DNA replication, producing a “cohesin without cohesion” phenotype (Skibbens et al., 1999; Toth et al., 1999). Ctf7 is an evolutionarily conserved acetyltransferase that acetylates lysines on itself, Pds5, and Mcd1 in vitro (Ivanov et al., 2002; Tanaka et al., 2001). It was later discovered that Ctf7 acetylates the lysine residues K112 and K113 (K105 and K106 in human cells) of Smc3 in budding yeast (Rolef Ben-Shahar et al., 2008; Unal et al., 2008; Zhang et al., 2008). Mutation of lysines to mimic acetylation bypasses the need for Ctf7 function, while mutation of lysines to non-acetylatable arginines results in loss in viability and severe cohesion defects (Rolef Ben-Shahar et al., 2008; Rowland et al., 2009b; Unal et al., 2008). These data suggest that Ctf7 establishes cohesion by acetylating conserved lysines on Smc3, which persists until anaphase onset.

Once established, cohesion is maintained throughout G2 phase until anaphase onset in mitosis. This maintenance of cohesion requires Pds5, an essential cohesin subunit that associates with chromatin in a cohesin dependent manner. In budding and fission yeast, loss of Pds5 results in severe cohesion defects, and is critical in both S phase and mitosis (Hartman et al., 2000; Panizza et al., 2000; Stead et al., 2003; Tanaka et al., 2001). PDS5 mutant cells exhibit decreased association of cohesins onto chromatin
However, this may differ in vertebrate model systems as studies in *Xenopus* and human cells show that loss of the human orthologs of Pds5 (Pds5A and Pds5B/APRIN) exhibit higher levels of stably bound cohesin chromosomes and reduced chromosome resolution during early mitosis (Losada et al., 2005; Shintomi and Hirano, 2009). Additionally, vertebrate cells also require Sororin to maintain cohesion, which is not found in yeast (Lafont et al., 2010; Rankin et al., 2005). Outside of the necessity of Pds5 in maintaining proper cohesion during G2/M phase, not much is known about the mechanism of cohesion maintenance. Once chromosomes are aligned at the metaphase plate and properly bi-oriented, Separase cleaves Mcd1, resulting in the dissolution of cohesins allowing for chromosomes to segregate.

Until recently, the factor through which Smc3 is de-acetylated and recycled was unknown. Instead, the main regulatory steps in cohesin function involved transcription of Mcd1 during the G1/S transition, acetylation of Smc3 during S phase and then the degradation of Mcd1 at anaphase onset (Guacci et al., 1997; Michaelis et al., 1997). Recent studies now reveal that a deacetylase Hos1 deacetylates Smc3 (Beckouet et al., 2010; Borges et al., 2010; Xiong et al., 2010). Overexpression of *HOS1* results in increased cohesion defects and growth defects in *eco1* mutants, which is attributed to its Smc3 deacetylation activity (Xiong et al., 2010). This finding now completes the cycle of sister chromatid cohesion, with Smc3 being deacetylated by Hos1 after Mcd1 is cleaved, allowing for Smc3 to be reused in the following cell cycle (Figure 2).

**Cohesin-Dependent Functions**
Sister chromatid cohesion is only one of several pathways that are affected by cohesins. For example, the process of cohesion establishment appears to be linked to DNA replication. The Replication Factor C complex (RFC) subunits Elg1 and Ctf18 are non-essential proteins that are similar to Rfc1, the large subunit of the PCNA clamp loader complex that is required for DNA replication (Bellaoui et al., 2003; Ben-Aroya et al., 2003; Kanellis et al., 2003; Mayer et al., 2001). Intriguingly, deletions of either CTF18 or ELG1 in budding yeast result in cohesion defects (Maradeo and Skibbens, 2009; Mayer et al., 2001; Parnas et al., 2009). While both genetically interact with CTF7, a deletion of CTF18 in combination with a ctf7 mutant allele is synthetically lethal, whereas a deletion of ELG1 rescues ctf7 mutant temperature sensitivity, suggesting opposing regulatory roles played by replication factors (Maradeo and Skibbens, 2009; Maradeo and Skibbens, 2010). The fact that RFCs and PCNA physically interact with Ctf7 suggests that establishment is determined by replication fork progression (Figure 3) (Kenna and Skibbens, 2003; Skibbens et al., 2007b). The converse relationship, that cohesion regulation may affect DNA replication, could also be true. DNA combing techniques provide evidence that establishment affects replication fork progression (Terret et al., 2009) - although the impact thus far appears quite modest. Others have shown a possible link between DNA replication initiation and S phase progression to cohesins and cohesin-associated proteins. In human cells, for instance, a down regulation of cohesins slowed the progression of S phase, and DNA combing techniques also showed a decrease in origin of replication firing (Guillou et al., 2010).

DNA damage response pathways also involve cohesins. In yeast, cells with inactive cohesins have increased sensitivity to DNA double stranded breaks (Sjogren and
Nasmyth, 2001). In the presence of a double strand break, previously established cohesins dissociate from chromatin by the cleaving of Mcd1 by Separase, and new cohesins are loaded by Scc2-Scc4 at the site of the break, facilitating DNA repair (McAleenan et al., 2013; Strom et al., 2007; Strom et al., 2004). In response to DNA damage, cells initiate a new round of cohesion establishment, possibly through Ctf7 re-activation, which establishes new cohesion across the entire genome and not just at the site of damage (Unal et al., 2007). However, in budding yeast, the new round of establishment by Ctf7 acetylates Mcd1 and not Smc3 (Heidinger-Pauli et al., 2009; Strom et al., 2007; Unal et al., 2007).

Cohesins also play an intricate role in chromosome condensation. Early studies of cohesins reveal that cohesin mutants have not only cohesion defects, but also condensation defects (Guacci et al., 1997; Hartman et al., 2000; Lavoie et al., 2002). Cohesin regulators such as Ctf7 and Rad61 also are critical for condensation, but possibly in antagonistic manner. For instance, a deletion of RAD61 bypasses the essential function of Ctf7 (Guacci and Koshland, 2012; Guacci et al., 2015; Rolef Ben-Shahar et al., 2008; Rowland et al., 2009a; Sutani et al., 2009). However, rather than rescuing cohesion defects of ctf7 mutants, RAD61 deletion rescues the condensation defect present in ctf7 mutant cells (Guacci and Koshland, 2012; Guacci et al., 2015). These studies were the first to document a separation of cohesin-dependent functions in cohesion from that of condensation in budding yeast. These studies help explain how mutations in cohesins (such as PDS5) do not result in cohesion defects in higher eukaryotes, yet still produce developmental defects (Zhang et al., 2007).
**Background on Pds5**

Pds5 is an evolutionarily conserved protein that is important for maintaining sister chromatid cohesion in mitosis and meiosis (Hartman et al., 2000; Losada et al., 2005; Panizza et al., 2000; Zhang et al., 2005). Sequence analysis of Pds5 reveals multiple HEAT repeats, similar to the cohesin loading protein Scc2, which suggests protein-protein interactions that center on cohesins (Panizza et al., 2000). Using a combination of RNAi and fluorescence microscopy in human cells, it was determined that Pds5 required chromatin bound cohesins in order to localize to chromatin (Losada et al., 2005). Pds5 (and its homologues) inactivation during S and M phases results in premature sister separation, indicating that it is essential in cohesion maintenance (Hartman et al., 2000; Losada et al., 2005; Panizza et al., 2000; Stead et al., 2003; Tanaka et al., 2001; Zhang et al., 2005). Western blot analysis revealed that Pds5 is SUMOylated and that overexpression of SUMO isopeptidase Smt4 rescues *pds5* mutant viability and cohesion defects. In combination, these findings suggest a role for SUMOylation in regulating Pds5 during maintenance (Stead et al., 2003). Cdc5 is a negative regulator of Smt4 such that overexpression of Cdc5 results in dissociation of Pds5 from chromatin (Baldwin et al., 2009). This posits a model of regulation in which cohesion is maintained until Pds5 is SUMOylated, at which point Pds5 dissociates from cohesin in preparation for anaphase onset. Although it appears that the essential function of Pds5 is tied to cohesion maintenance, temperature sensitive mutants of Pds5 also exhibit decreased viability during S phase (Figure 4), which suggests another function Pds5 outside of cohesion maintenance (Hartman et al., 2000). One such function could be in cohesin loading, as
Pds5-99 mutant cells exhibit reduced cohesin deposition (or decreased stability of deposited cohesins) onto DNA (Panizza et al., 2000).

Pds5 also regulates Ctf7-dependent cohesion establishment pathways. For instance, pds5-1 is synthetically lethal with ctf7-203. Moreover, overexpression of PDS5 rescues temperature sensitivity of ctf7-203 mutant strains (Figure 5) (Noble et al., 2006). Consistent with the genetic interactions, Pds5 and Ctf7 co-immunoprecipitate in vitro (Figure 5) (Noble et al., 2006). This suggests that Pds5 is a pro-establishment factor that interacts with Ctf7. In contrast to the synthetic lethality of pds5-1 ctf7-203, there are also mutations in Pds5 that instead rescue ctf7 mutant strain inviability, suggesting that Pds5 may exhibit an as yet ill-defined “anti-establishment” activity (Rolef Ben-Shahar et al., 2008; Rowland et al., 2009b; Sutani et al., 2009). Co-immunoprecipitation (Co-IP) experiments support this model in that Pds5 can associate with Rad61 - which in turn is a regulator of Ctf7-dependent condensation (Guacci and Koshland, 2012; Guacci et al., 2015; Sutani et al., 2009). The establishment role of Pds5 also affects replication, as depletion of PDS5A rescued the DNA replication fork velocity defect otherwise present in human cells that harbored ESCO1 or ESCO2 (human orthologs of CTF7) mutations (Terret et al., 2009).

In addition to cohesion maintenance and establishment pathways and chromosome condensation, Pds5 participates in additional functions. In Drosophila, a mutation in PDS5 in which the first 5 exons of the gene are removed results in a reduction of cut gene expression, suggesting that Pds5 promotes transcription in higher eukaryotes (Dorsett et al., 2005). A microarray of pds5-1 in budding yeast also support a role of Pds5 in transcription regulation, as inactivation of pds5-1 even for 2 hours showed
significant changes in transcriptional profile compared to wildtype (Ren et al., 2008).
More recently, deletion of ELG1 was found to rescue pds5-1 temperature sensitivity
(Figure 6) (Maradeo et al., 2010; Maradeo and Skibbens, 2010). While little is known
about this suppression, it provides a method to study a cell that can bypass an essential
function of Pds5.

New evidence reveals that Pds5 mutations lead to various disease states. Recent
studies in the mouse model show that certain mutations in PDS5A and PDS5B/APRIN
cause symptoms consistent with those present in Cornelia de Lange Syndrome (CdLS), a
cohesinopathy that is characterized by mental retardation, heart defects, and abnormal
limb development. Importantly, cells that harbor these PDS5 mutations do not exhibit
cohesion defects, suggesting that development requires a function of Pds5 outside of
sister chromatid cohesion (Zhang et al., 2009; Zhang et al., 2007). The Pds5 paralog
PDS5B/APRIN’s role as a chromatin regulator was implicated in tumor suppression and
stem-cell differentiation (Denes et al., 2010; Maffini et al., 2008). These transcriptional
roles, in addition to cohesion, condensation, and replication pathways, show the immense
versatility of Pds5 in cells. Thus, investigating Pds5 will help expand our knowledge
regarding many processes that Pds5 has in a cell, which may lead to therapeutic
treatments for cohesinopathies and cancers.
FIGURES

Figure 1. Possible model of structural cohesin complex. Figure modified from (Jeppsson et al., 2014b).
**Figure 2.** Proposed model of the cycle of sister chromatid cohesion. Cohesins are loaded during G1/early S phase and established by Ctf7/Eco1 by acetylating Smc3. Mcd1 is cleaved during anaphase onset and Hos1 deacetylates Smc3 prior to loading of cohesins in next cell cycle. Figure modified from (Beckouet et al., 2010).
Figure 3. Model of how the replication fork may participate in cohesion establishment.

Ctf7 interacts with replication factors as replication fork progresses. Upon reaching a cohesin complex, Pds5 binds to Ctf7, localizing it to the complex, allowing for efficient acetylation of Smc3. Modified from (Skibbens et al., 2007a).
**Figure 4.** (A) *pds5* mutant alleles are temperature sensitive and lose viability at 37°C. (B) *pds5* mutant cells show almost no viability when incubated at non-permissive temperatures during M phase arrest, but also show decreased viability when arrested during S phase. Figure modified from (Hartman et al., 2000).
Figure 5. (A-C) Overexpression of PDS5 rescues temperature sensitivity in 3 different ctf7 mutant strains. In vitro Co-IP (rightmost panel) also shows Pds5p physically interacting with Ctf7. Figure modified from (Noble et al., 2006).
Figure 6. *pds5-1 elg1* double mutant strain is viable at non-permissive temperatures. Cells are synthetically sick, suggesting it is not a complete rescue. Figure modified from (Maradeo et al., 2010).
REFERENCES


and Pds5 maintain their stable association with the DNA. *PLoS genetics.* 8:e1002856.


CHAPTER 2

Cohesin without Cohesion: A Novel Role for Pds5 in

*Saccharomyces cerevisiae*
ABSTRACT

High fidelity chromosome segregation during mitosis requires that cells identify the products of DNA replication during S-phase and then maintain that identity until anaphase onset. Sister chromatid identity is achieved through cohesin complexes (Smc1, Smc3, and Mcd1 and Irr1/Scc3), but the structure through which cohesins perform this task remains enigmatic. In the absence of unambiguous data, a popular model is that a subset of cohesin subunits form a huge ring-like structure that embraces both sister chromatids. This ‘one-ring two-sister chromatid’ model makes clear predictions – including that premature cohesion loss in mitotic cells must occur through a substantial reduction in cohesin-DNA associations. We used chromatin immunoprecipitation to directly test for cohesin dissociation from well-established cohesin binding sites in mitotic cells inactivated for Pds5 – a key cohesin regulatory protein. The results reveal little if any chromatin dissociation from cohesins, despite a regimen that produces both massive loss of sister chromatid tethering and cell inviability. We further excluded models that cohesion loss in mitotic cells inactivated for Pds5 arises through either cohesin subunit degradation, loss of Hos1-dependent Smc3 deacetylation or Rad61/Wapl-dependent regulation of cohesion dynamics. In combination, our findings support a model that cohesin complexes associate with each sister and that sister chromatid cohesion likely results from cohesin-cohesin interactions. We further assessed the role that Pds5 plays in cohesion establishment during S-phase. The results show that Pds5 inactivation can result in establishment defects despite normal cohesion loading and Smc3 acetylation, revealing a novel establishment role for Pds5 that is independent of
these processes. The combination of findings provides important new insights that significantly impact current models of both cohesion establishment reactions and maintenance.
INTRODUCTION

Pds5 is a particularly intriguing cohesin-auxiliary protein that highlights the complexity of both establishment reactions and cohesion maintenance. Early findings, in part predicated on pds5-1 and pds5-101 alleles, document that Pds5 both binds cohesins and is required for the maintenance of cohesion during mitosis (Hartman et al., 2000; Panizza et al., 2000; Stead et al., 2003). In contrast, pds5-99 mutant cells maintain cohesion once established, but appear deficient in cohesin loading (or retention) onto DNA (Panizza et al., 2000). A mechanism through which Pds5 may impact Scc2, Scc4-dependent cohesin deposition remains unknown. Pds5 also binds Rad61/Wapl and Irr1/Scc3 (Kulemzina et al., 2012; Rowland et al., 2009b; Sutani et al., 2009), in support of the notion that Pds5 promotes both stable cohesin-DNA association and chromatin condensation. It is thus notable that Pds5 is critical for chromosome condensation, attributes shared by both Eco1 and Mcd1 (Guacci et al., 1997; Hartman et al., 2000; Skibbens et al., 1999b). Pds5 also binds Eco1 in vitro and promotes Eco1-dependent acetylation of Smc3 in vivo, in support of numerous studies that suggest that cohesin deposition and cohesion establishment are temporally coordinated (Chan et al., 2013; Noble et al., 2006; Vaur et al., 2012). Intriguingly, while pds5-1 is lethal in combination with eco1 alleles (Noble et al., 2006), certain other pds5 alleles bypass a requirement for Eco1, even though these pds5 eco1 double mutant cells exhibit significant cohesion defects (Rowland et al., 2009b; Sutani et al., 2009). The extent through which this rescue involves condensation pathways, similar to rad61 eco1 double mutant cells, remains an untested but intriguing possibility (Guacci and Koshland, 2012). Given this surplus of roles, the confusion regarding which activity (cohesin deposition, cohesion anti-
establishment, cohesion maintenance, or chromosome condensation) comprises the essential function of Pds5 is not surprising. Since \textit{PDS5/APRIN} mutations arise in both cancer progression and developmental abnormalities (Denes et al., 2010; Maffini et al., 2008; Zhang et al., 2009; Zhang et al., 2007), resolving these issues remains of significant clinical interest. Here, we characterize a particularly instructive separation-of-function allele of \textit{PDS5} that challenges current paradigms in cohesion maintenance and establishment.
RESULTS

Pds5 is essential for cell viability and cohesion maintenance specifically during mitosis

Despite the essential role that Pds5 plays in budding yeast, its role in cohesion maintenance remains unknown. Using the temperature sensitive allele pds5-1, we first confirmed that Pds5 is essential to retain cell viability and maintain sister chromatid cohesion during an extended metaphase arrest. Wildtype and pds5-1 mutant cells were synchronized in pre-anaphase at a temperature permissive for pds5-1 mutant strains and then shifted to a temperature restrictive for pds5-1, while maintaining the mitotic arrest, to limit in activation to an extended pre-anaphase (Figure 1A). Cells were then plated onto YPD plates at the permissive temperature and viability analyzed by colony growth assays. Wildtype cells exhibit 45% viability after incubation at the non-permissive temperature, consistent with prior studies that this regimen is stressful even to wildtype cells, but that a significant fraction of cells remain viable (Hartman et al., 2000). In contrast, pds5-1 mutant cells are predominantly inviable, exhibiting only 4% colony growth (Figure 1B). We next tested whether pds5-1 mutant cells indeed exhibit cohesion defects upon inactivation specifically during mitosis using a cohesion assay strain in which a TetO array, integrated approximately 40kb from centromere V, is detected through the binding of GFP-tagged TetR protein. This cohesion assay strain also contains epitope-tagged Pds1p (an inhibitor of anaphase onset) so that pre-anaphase cells can be unambiguously identified. Quantification of GFP signals reveal that wildtype pre-anaphase cells show very low levels (~10%) of premature sister chromatid separation. In
contrast, \textit{pds5-1} mutant cells exhibit a significant level (\textasciitilde55\%) of cohesion defects during pre-anaphase (Figure 1C and D), a level identical to that previously reported for this allele (Hartman et al., 2000). In combination, the above results confirm that Pds5 is both essential to retain cell viability and required to maintain sister chromatid cohesion specifically during an extended metaphase arrest (Hartman et al., 2000; Stead et al., 2003).

\textbf{Cohesin enrichment to DNA is retained in cohesion defective \textit{pds5-1} mutant cells during mitosis}

What is the mechanism through which Pds5 inactivation, specifically during mitosis, produces cohesion defects? For the \textit{one-ring two-sister chromatid embrace} model (in which sister chromatids A and A’ are embraced by a cohesin ring), cohesion loss can only occur through one of three possible reactions: either chromatid A exits the ring (A’ is retained), chromatid A’ exits the ring (A is retained), or both A and A’ exit from the ring. If each of the three outcomes occurs with equal probability within a population, then cohesin enrichment onto DNA should drop to approximately 33\% in cohesion deficient cells compared to cells that retain cohesion (Figure 2A). To test this prediction, wildtype and \textit{pds5-1} mutant cells both expressing MYC-tagged Mcd1 were synchronized in pre-anaphase at the permissive temperature, shifted to the restrictive temperature while maintaining the mitotic arrest (Figure 2B), then subjected to chromatin-immunoprecipitation (ChIP) to assess Mcd1 association with chromatin at 13 well-documented Cohesin-Associated Regions (CAR) along chromosome arm and pericentromeric regions of chromosome III (see below). We first analyzed the data \textit{en}
masse} to approximate a genome-wide role for Pds5 on cohesin retention onto DNA. The results show that \textit{pds5-1} mutant cells exhibit 95\% of cohesin binding along chromosome arm CARs compared to wildtype cells (Figure 2C). \textit{pds5-1} mutant cells also exhibit cohesin binding along the pericentromeric domain that was only marginally lower (~75\%) than that observed for wildtype cells (Figure 2D).

We decided to independently assess the global retention of cohesin in \textit{pds5-1} mutant cells using Triton X-100 cell fractionation assays, a documented procedure previously used to demonstrate chromatin-association of cohesin and other factors (Rudra and Skibbens, 2013a; Toth et al., 1999). Log phase wildtype and \textit{pds5-1} mutant cells held at the permissive temperature in medium supplemented with nocodazole to arrest cells pre-anaphase were harvested, lysed and then processed for fractionation analysis. Fractionation of whole cell lysate into soluble and chromatin-associated components was confirmed using Phosphoglycerokinase (PGK) as a cytosolic marker and Histone 2B (H2B) as a chromatin marker, as previously described (Rudra and Skibbens, 2013a). We then assessed fractionation of Mcd1, a core subunit of the cohesin complex, to the chromatin pellet and compared these values to Histone 2B loading control levels. We also assessed Mcd1 fractionation into the soluble pool, using PGK levels as our loading control. Western blot results are shown for each of three independent experiments (Figure 3A). Quantifications of soluble and chromatin-associated Mcd1 are provided as averages from these 3 independent experiments with the level of Mcd1 in \textit{pds5-1} mutant cells normalized to those observed in wildtype cells (Figure 3B). Intriguingly, \textit{pds5-1} mutant cells exhibit Mcd1 levels in whole cell lysates that are significantly lower than the level of Mcd1 in whole cell lysates from wildtype cells (Figure 3B, left panel). Importantly,
however, further analyses of fractionated components reveal that the reduction in Mcd1 levels occurs predominantly in the soluble pool (compare Mcd1 levels in pds5-1 mutant cells in left panel to that in middle right panel of Figure 3B). In contrast, Mcd1 levels in the chromatin fraction are nearly identical to that present in whole cell extracts from pds5-1 mutant cells (compare Mcd1 levels in pds5-1 mutant cells left panel to that in right panel of Figure 3B). To quantify this further, we compared the level of chromatin-bound Mcd1 to that present in the whole cell lysates for both wildtype and pds5-1 mutant cells. The results show that pds5-1 mutant cells are equally competent to wildtype cells in cohesin enrichment to DNA (Figure 3C). In combination, these results reveal that Mcd1 levels are reduced in pre-anaphase pds5-1 mutant cells held at the restrictive temperature, relative to wildtype cells, but that the cohesin retention onto DNA is fully retained in pds5-1 mutant cells. Thus, bulk cohesin-dissociation from DNA is not the basis for the cohesion defects that occur in pds5-1 mutant cells.

We next assessed whether Pds5 inactivation adversely impacts cohesin enrichment within specific loci and well-documented CARs (Figure 4A). We first turned to individual chromosome arm CARs, performing ChIPs on lysates obtained for wildtype and pds5-1 mutant cells maintained at a permissive temperature in medium supplemented with nocodazole to arrest cells in pre-anaphase and then shifting to the restrictive temperature to inactive pds5-1 specifically during the pre-anaphase arrest. The results show that pds5-1 mutant cells overall exhibit levels of cohesin enrichment to DNA at levels nearly identical to those observed in wildtype cells, despite the loss of cohesion in the pds5-1 mutant cells. Careful analyses revealed, however, that cohesin enrichment varies for given loci. Among four individual arm sites comprising two CARs, three
exhibit either equivalent (35) or elevated cohesin enrichment (34 and 36) in pds5-1
mutant cells compared to wildtype cells (Figure 4B). Conversely, only one site (37)
exhibits a reduction (40%) in cohesin enrichment in pds5-1 mutant cells compared to
wildtype cells (Figure 4B). Both the increase and decrease of cohesin enrichment in pds5-
1 mutant cells compared to wildtype cells was intriguing. Thus, we decided to
independently test for cohesin enrichment onto DNA at selected loci using quantitative
PCR (Figure 4C). Results from qPCR reveal that pds5-1 mutant cells contain elevated
levels of cohesin enrichment at site 36 but contain less cohesin enrichment at site 37
compared to wildtype cells that retain cohesion, confirming results obtained through
ChIP.

Does cohesin enrichment remain elevated along the centromere in pds5-1 mutant
cells in which cohesion is abolished? To address this question, we performed similar
analyses on nine individual sites that comprise the pericentromeric domain of
chromosome III. Of the nine sites assayed, six sites (72, 74, 76, 78, 80, 84) retain cohesin
enrichment to DNA in pds5-1 mutant cells at levels nearly identical to that of wildtype
cells (Figure 4D). One site (82) exhibited slightly elevated levels of cohesin-enrichment
in pds5-1 mutant cells, relative to wildtype cells. Only in the remaining two sites (70 and
48) did we find that cohesin enrichment in pds5-1 mutant cells is reduced (25% and 40%
respectively) relative to wildtype cells. Each CAR site was validated using scc2 mutant
cells (see below). The combined results from both chromosome arm and pericentromeric
ChIP studies reveal that the cohesion loss that occurs upon Pds5 inactivation during
mitosis does so despite levels of chromatin-bound cohesins that are similar to wildtype
cells, but that variation in cohesin enrichment occurs within specific loci.
**Cohesin acetylation is retained in cohesion defective pds5 mutant cells during mitosis**

The above findings that cohesin enrichment to DNA is retained in cohesion-deficient pds5-1 mutant cells suggest that ring opening and chromatid release is not the mechanism through which sister chromatids separate. We realized, however, that the above analyses do not exclude the possibility that the chromatin-associated cohesins detected are newly deposited. Eco1/Ctf7 acetylates Smc3 only during S-phase, a modification temporally limited to S-phase by Eco1/Ctf7 phosphorylation (by Cdk1), ubiquitination (by Cdc4/SCF) and degradation upon entry into G2 (Lyons and Morgan, 2011; Rolef Ben-Shahar et al., 2008; Unal et al., 2008; Zhang et al., 2008). If the chromatin-associated cohesins that we detect in mitotic pds5-1 mutant cells are newly (mitotically) deposited, then those cohesins should be devoid of acetylated Smc3. To test which population of cohesins persist in mitotic but cohesion-deficient pds5-1 mutant cells, log phase wildtype and pds5-1 mutant cells expressing HA-tagged Smc3 were synchronized in pre-anaphase, shifted to non-permissive temperature while maintaining the pre-anaphase arrest (Figure 5A), and normalized cell densities lysed and incubated with anti-HA coupled affinity matrix. After washing to remove unbound or weakly associated proteins, Smc3 protein was eluted from the beads and assayed by Western blot. A dilution series confirmed that sample concentrations provide for linear range signal detection (Figure 5B). Smc3 levels in pds5-1 mutant cells were normalized to those observed in wildtype cells and averaged from three different experiments (Figure 5C). Importantly, quantitative analyses from this dilution series reveal that total Smc3
protein levels that are similar (85%) to that of wildtype cells (Figures 5C), suggesting that Smc3 and Mcd1 levels are regulated through different pathways. The same blot was then reprobed (after confirming signal removal) to assess the level of Smc3 acetylation. The results reveal that 85% of Smc3 is acetylated in pds5-1 mutant cells, compared to wildtype (Figure 5D), consistent with the model that the majority of total Smc3 exists in an acetylated state attained during S-phase.

Despite Eco1 degradation upon exit from S-phase, we were concerned that pds5-1 protein inactivation might produce DNA damage during G2 that could in turn induce a new wave of Eco1/Ctf7 establishment activity (Heidinger-Pauli et al., 2009; Lyons et al., 2013; Unal et al., 2007). We therefore decided to test whether pds5-1 inactivation induces DNA damage, which would promote Eco1/Ctf7 re-establishment. We first confirmed that both wildtype and pds5-1 mutant cells are competent to respond to DNA damage after exposure to methyl methanesulfonate (MMS). Importantly, neither mitotic wildtype or pds5-1 mutant cells shifted to the restrictive temperature in the absence of MMS resulted in Rad53 phosphorylation (Figure 5E), negating the model that Eco1 becomes reactivated during G2/M in response to pds5-1 inactivation. In combination, these findings reveal for the first time that the acetylated DNA-enriched cohesins present in pds5-1 mutant cells are the product of Eco1/Ctf7-dependent cohesion establishment reactions that occur during S-phase, not by mitotic loading and subsequent DNA damage-induced response by Eco1.

Pds5 role in cohesion maintenance occurs independent of Rad61/WAPL
Rad61/WAPL binds Pds5 and is implicated in regulating cohesin dynamics (Gause et al., 2010; Rowland et al., 2009b; Sutani et al., 2009). While cohesin binding to DNA is not globally decreased upon Pds5 inactivation during mitosis (Figures 2-4), we decided to test whether deletion of Rad61/WAPL might rescue pds5-1 mutant cell inviability. Log phase wildtype, pds5-1 and rad61 single mutants, and pds5-1 rad61 double mutant cells were synchronized in pre-anaphase and then shifted to the non-permissive temperature while retaining the mitotic arrest (Figure 6A). Normalized cell numbers from the resulting cultures were then plated onto rich medium and assessed for cell viability as previously described. Both wildtype and rad61 mutant cells exhibited fairly robust levels of cell viability (approximately 60%). In contrast, pds5-1 mutant cells exhibited a dramatically reduced level of viability (8%), confirming prior results (Figures 6B and 1). Importantly, pds5-1 rad61 double mutant cells exhibited a nearly identical low level of cell viability (9%) as pds5-1 single mutant cells (Figure 6B). Thus, loss of cell viability upon Pds5 inactivation during mitosis is not due to a Rad61-dependent increase in cohesin dynamics.

Rad61 is known to bypass the lethality of eco1 mutant cells, not by rescuing the cohesion defect but rather by rescuing the condensation defect that occurs upon Eco1 inactivation (Guacci and Koshland, 2012; Rowland et al., 2009b; Sutani et al., 2009). Since deletion of RAD61 from pds5-1 mutant cells failed to rescue cell inviability, we hypothesized that pds5-1 mutant cells are not deficient in maintaining chromosome condensation during an extended pre-anaphase arrest, even though prior evidence reveals that Pds5 inactivation starting from G1 does produce condensation defects (Hartman et al., 2000). Net1-GFP is well-established as a tool suitable for detecting changes in rDNA
chromatin architecture (Lopez-Serra et al., 2013; Machin et al., 2005). Wildtype and *pds5-1* mutant cells expressing Net1-GFP were arrested in mitosis and shifted to the restrictive temperature while maintaining the mitotic arrest. We then quantified Net1-GFP as forming either linear/loop structures (in which the rDNA loci are clearly distinguishable as well-defined axial elements which often form a tight loop) or puff-like structures in which no clear axial resolution is discernible (Guacci et al., 1997; Hartman et al., 2000; Lopez-Serra et al., 2013). The results show that mitotic wildtype and *pds5-1* mutant cells both contained similar levels of condensed "linear" rDNA structures (58% to 50% respectively) that exceeded the level of uncondensed "puffed" structures (30% to 39% respectively) (Figure 6C,D).

To confirm previous reports that *pds5-1* mutant cells exhibit condensation defects when shifted to the restrictive temperature prior to S-phase, we repeated our analysis but now arresting wildtype and *pds5-1* mutant cells in late G1 at a permissive temperature in medium supplemented with alpha-factor and then releasing those cultures into fresh medium held at the restrictive temperature. The results of the Net1-GFP analysis show that *pds5-1* has a significant condensation defect (65% puffed structures) when compared to wildtype (31%) (Figure 6E, F). In combination, these results confirm the condensation defect shown previously when Pds5 is inactivated during cohesion establishment (Hartman et al., 2000) and reveal for the first time that, once established, Pds5 plays only a marginal role in maintaining chromosome condensation. Herein, we refer to this as a condensation establishment reaction that depends on Pds5 and that occurs concomitantly with cohesion establishment.
Pds5 role in cohesin loading during S-phase is separate from its essential role in cohesion establishment

Does Pds5 function during S-phase, when cohesion is first established, differ from its role during mitosis when cohesion is maintained? Numerous studies document a role for Pds5 during cohesion establishment (Kulemzina et al., 2012; Noble et al., 2006; Rowland et al., 2009b; Stead et al., 2003; Sutani et al., 2009; Tanaka et al., 2001; Vaur et al., 2012) and at least one study suggests that Pds5 is critical for cohesin enrichment to DNA during S-phase (Panizza et al., 2000). To address this latter possibility, log phase wildtype, eco1-1, scc2-4, and pds5-1 mutant cells, all expressing Mcd1-3HA as the sole source of Mcd1, were synchronized in G1 and then released to the non-permissive temperature in fresh media supplemented with nocodazole to arrest cells pre-anaphase (Figure 7A). The resulting mitotic cells were then harvested and ChIP performed to assess the level of Mcd1 enrichment onto DNA at CAR arm sites. Quantification of ChIPs averaged from 3 independent experiments document that wildtype cells retain high levels of Mcd1 enrichment to DNA (Figure 7B). As expected, scc2-4 mutant cells instead exhibit a massive reduction in Mcd1 enrichment to chromatin (about 20% compared to wildtype cells) whereas eco1-1 mutant cells retain high levels of chromatin-bound cohesins (Figure 7B), despite a regimen that produces significant cohesion defects (Milutinovich et al., 2007; Skibbens et al., 1999b; Toth et al., 1999). This latter ‘cohesin without cohesion’ phenotype typifies establishment mutations (Skibbens et al., 2007a). Importantly, pds5-1 mutant cells retained Mcd1 enrichment onto DNA (about 80% compared to wildtype and about 90% compared to eco1-1 mutant cells), recapitulating the establishment phenotype (Figure 7B).
To further validate both the *scc2-4* mutant cell control strain and the pericentromeric CAR sites employed throughout this study, we performed ChIP using the primer pairs previously analyzed (Figure 4). As before, cells synchronized in G1 at the permissive temperature were released into fresh medium supplemented with nocodazole to arrest cells pre-anaphase prior to performing ChIP analyses. The results show that cohesin enrichment to DNA was substantially reduced along the entire pericentromeric DNA region in *scc2-4* mutant cells (Figure 7C), consistent with the loss of cohesin enrichment along the chromosome arm (Figures 7B). Western blot analyses confirmed that Mcd1 was present in all strains, obviating the model that cohesion loss occurs predominantly through premature Mcd1 proteolysis (Figure 7D).

We further tested the possibility that cohesin dissociated early during the cell cycle (S or G2 phases) and that the cohesin detected by ChIP was redeposited late in the cell cycle during pre-anaphase. Wildtype and *eco1, scc2, and pds5* mutant cells synchronized in G1 at the permissive temperature were released at the restrictive temperature into fresh medium supplemented with nocodazole. In this case, however, culture samples were harvested at 40 minute time increments to map cell cycle progression (Figure 7E). ChIP analyses reveals that, except for *scc2* mutant cells, all other strains retain cohesin enrichment to DNA throughout the time course of the experiment (Figure 7F). These results exclude the possibility that cohesin was lost early in the cell cycle and reloaded during the mitotic arrest. In combination, these studies document that the essential role for Pds5 during cohesion establishment is independent of cohesin enrichment onto chromatin.
**Pds5 is not required for Smc3 acetylation during cohesion establishment**

Our finding that cohesin acetylation is retained upon Pds5 inactivation during mitosis does not exclude the possibility that Pds5 plays a key role in cohesin acetylation during S-phase. To test this possibility, log phase wildtype and *pds5-1* strains expressing HA-tagged Smc3 were synchronized in G1, then released at the non-permissive temperature into fresh medium containing nocodazole (Figure 8A). As before, we performed a dilution series to confirm that sample concentrations fell within the linear range of Smc3 and acetylated Smc3 signal detection (Figure 8B). The results show that cells that progress through S-phase in the absence of Pds5 contain 90% of total Smc3 protein levels compared to wildtype cells (Figure 8C). Moreover, Pds5-deficient cells contain over 90% of acetylated Smc3 compared to wildtype cells (Figure 8D). Thus, the essential role of Pds5 during S-phase occurs independent of Smc3 acetylation.
DISCUSSION

Prior studies reveal that Pds5 exerts many functions throughout the cell cycle: promoting both cohesin deposition and cohesion establishment during S-phase, inhibiting cohesin deacetylation upon mitotic exit, and regulating cohesin dynamics (Chan et al., 2013; Hartman et al., 2000; Kulemzina et al., 2012; Losada et al., 2005; Noble et al., 2006; Panizza et al., 2000; Shintomi and Hirano, 2009; Stead et al., 2003; Sutani et al., 2009; Vaur et al., 2012; Wang et al., 2002). One of the major revelations of the current study is that the essential role of Pds5 in maintaining cohesion during mitosis is not necessarily dependent on any of these activities – even if various pds5 alleles exhibit such defects. Notwithstanding, Pds5 inactivation during mitosis clearly results in cell inviability and premature separation of sister chromatids, despite the retention of cohesins to both chromosome arm and centromere CAR sites. We note recent supporting evidence that cohesion loss during mitosis can occur despite cohesin retention on sister chromatids, although that study focused primarily on establishment reactions (Kulemzina et al., 2012). Our results further document that pds5-1 mutant cells retain Smc3 acetylation – negating the possibility that this population of cohesin is newly deposited. The inability to detect DNA damage in pds5 mutant cells reported here and previously, and that Eco1 acetylates Mcd1 (not Smc3) in response to DNA damage, further support the assertion that the acetylated Smc3 detected in the current study is retained from Eco1-dependent S-phase activity (Heidinger-Pauli et al., 2009; Kulemzina et al., 2012). Finally, we found no evidence of Smc3 deacetylation being sufficient to account for the loss of cohesion or that cohesion loss occurs through an increase in Rad61-dependent cohesion dynamics. In
combination, these findings negate prior models that the essential role of Pds5 is to either prevent Hos1-dependent deacetylation of Smc3 or preclude Rad61 destabilization of cohesins (Chan et al., 2013; Rowland et al., 2009b; Sutani et al., 2009). Importantly, we also provide novel evidence that Pds5 plays a greatly diminished role in maintaining chromosome condensation during mitosis once it is established during S-phase. While our results do not preclude roles for Pds5 in cohesin enrichment onto DNA, cohesin acetylation/deacetylation, altering cohesin dynamics or chromatin architecture - activities all attributed to Pds5 based on analyses of separation-of-function alleles (Chan et al., 2013; Panizza et al., 2000; Rowland et al., 2009b; Sutani et al., 2009), our results are clear in revealing that these reported roles are not the essential mechanism through which Pds5 maintains cohesion during mitosis.

Pds5 inactivation during mitosis results in cell death and loss of sister chromatid cohesion, even while both cohesin enrichment and cohesin acetylation are retained. What then, is the role of Pds5 in maintaining cohesion during mitosis and what can we infer about the mechanism through which sister chromatids remain tethered together during mitosis? We initiated the current study to test the presiding model that both sisters reside within a single cohesin ring (one-ring two-sister chromatids embrace model). Based on this model, cohesin loss upon Pds5 inactivation must be mediated through cohesin ring opening and dissociation from one or both sisters - either through increased cohesin dynamics (Rad61), possibly in association with loss of Smc3 acetylation (Hos1), or cohesin degradation. The second revelation of the current study is that each of the predictions fail to be borne out by the data. We thus favor instead a preceding model that each sister is individually decorated with cohesins (Skibbens, 2000; Skibbens et al.,
1999b). Do cohesin rings entrap each sister chromatid? While cohesin rings remain a popular model, we note evidence of Mcd1 dimerization, analogous to Mre11 dimers in MRN complexes that contain the SMC-like Rad50 protein, consistent with a model that each sister chromatid may be held between SMC heads and an Mcd1 capping complex (Mockel et al., 2012; Rudra and Skibbens, 2013b; Schiller et al., 2012; Zhang et al., 2013). The intimate positioning of DNA between Smc1,3 ATPase heads and an Mcd1 capping structure, as opposed to passively retained within a cohesin ring lumen distal from these active sites, provides a satisfying model for not only the regulation of cohesion, but also for condensation and DNA repair properties of SMC-type complexes (Figure 9). Regardless of the cohesin structure through which cohesins remain associated to DNA, a one cohesin per sister model allows for cohesion loss through cohesin-cohesin dissociation - even while both sisters retain cohesin binding and Smc3 acetylation (Figure 9). We further hypothesize that chromatin looping in cis, which brings enhancer/promoter elements into close apposition for transcription, is similarly stabilized by cohesin-cohesin assemblies (Rudra and Skibbens, 2013b). Note that this conserved one ring per sister model is supported by numerous findings that cohesion loss can occur despite full cohesin enrichment and acetylation (Chan et al., 2013; Hartman et al., 2000; Kulemzina et al., 2012; Losada et al., 2005; Milutinovich et al., 2007; Sharma et al., 2013; Skibbens et al., 1999b; Toth et al., 1999). In light of our current study, results that removing the deacetylase Hos1 fails to significantly recover cohesion defects in pds5 mutant cells are well accommodated (Chan et al., 2013).

In many respects, the long-lived popularity of a one-ring two-sister chromatid embrace model is surprising. Early studies of both Eco1/Ctf7 and Pds5 provided ample
proof-of-principal that cohesin deposition and subsequent DNA replication, mainstays of the one-ring two-sister chromatid embrace model, were inadequate to engender sister chromatid cohesion (Hartman et al., 2000; Milutinovich et al., 2007; Skibbens et al., 1999b). Recent analyses of Chl1 DNA helicase, coupled with a prior study that mapped Scc2 function to S-phase, confirm that cohesin loading during G1 (a mainstay of the one ring two sister embrace model) is insufficient for subsequent establishment reactions that occur during S-phase. Instead, both cohesion deposition and Eco1-dependent cohesin modification occur in the wake of the DNA replication fork (Ciosk et al., 2000; Rudra and Skibbens, 2013a). Gartenberg and colleagues demonstrated that cohesion between sister chromatids can be mediated by different complexes (for instance, Sir2 complex association with cohesins), in which each resides on a sister chromatid and linked together (Chang et al., 2005). The finding that histone modifications are central to cohesion maintenance, and that cohesin is retained in H2A.Z mutant cells that exhibit cohesion defects, provides compelling evidence for a model in which cohesin deposition and modification occur in concert with chromatin-assembly reactions (Rudra and Skibbens, 2013b; Sharma et al., 2013). The apparent bias in favoring a one-ring two-sister chromatid embrace model is perpetuated by the erroneous notion that there is a difference in capabilities (‘strong’ versus ‘weak’) among cohesin structures (Nasmyth and Haering, 2009). By definition, every model must include as a founding principal that the protein associations required for cohesion are sufficient to withstand mitotic forces – regardless of architecture.

What is the consequence of a one ring per sister chromatid model beyond cohesion maintenance? We are particularly intrigued by the findings that, while cohesins
are maintained at most CARs upon Pds5 inactivation, some regions show a modest decrease in cohesins while other regions show a modest increase in cohesin enrichment compared to wildtype. From this, we propose that cohesins tethered together to maintain cohesion are relatively restricted from migrating along DNA. Upon cohesion inactivation, our data suggests that each cohesin complex is able to diffuse along DNA – some cohesin towards CAR sites (resulting in increased enrichment) and some away from CAR sites (resulting in decreased enrichment). This implies that Pds5 not only maintains the tethering together of sister chromatids, but also ensures cohesin enrichment at specific locations on DNA, possibly to ensure transcriptional identity between sisters. Currently, it remains unknown whether the cohesin-sliding phenomenon posited here requires transcription (Lengronne et al., 2004) or occurs independent of the presumptive transcription-driven migration of cohesin along DNA. We note that a transcriptional mechanism of migration does not appear to be a conserved feature – even in yeast (Kogut et al., 2009; Lengronne et al., 2004; Lengronne et al., 2006; Misulovin et al., 2008; Parelho et al., 2008). Thus, the emerging model of cohesin-cohesin interactions also impacts the current view of a single cohesin ring stabilizing DNA looping in cis during transcription (Dorsett, 2011; Gartenberg, 2009). Notably, mutations within Pds5 are implicated in both cancer progression and birth defects (Denes et al., 2010; Maffini et al., 2008; Zhang et al., 2009; Zhang et al., 2007) – the latter of which appears attributable to transcription dysregulation (Denes et al., 2010; Maffini et al., 2008). Thus, insights into novel mechanisms through which Pds5 inactivation might enable each cohesin complex to exert different transcriptional effects – even in the absence of complex sister-sister separation, may prove to be of clinical interest.
Recently, an article published by D’Ambrosio and Lavoie (D'Ambrosio and Lavoie, 2014) reported both that Mcd1-6HIS-3FLAG is reduced in whole cell extracts obtained from \textit{pds5-1} mutant cells. Our results reveal that it is the soluble pool of cohesin that is predominantly targeted from degradation – the chromatin-bound cohesins appear relatively refractile to Pds5 inactivation during mitosis. D’Ambrosio and Lavoie also reported that binding to chromatin was reduced in \textit{pds5-1} mutant cells, relative to wildtype (D'Ambrosio and Lavoie, 2014). The decrease reported for the single site in the D’Ambrosio and Lavoie study, however, does not necessarily conflict with our results in that we exploit different epitope tags and quantify cohesin binding at 13 sites different from the site reported in the D’Ambrosio study. As noted above, one mechanism consistent with this loci-specific variability is diffusional mobility upon cohesin-cohesin de-anchoring through loss of cohesion. A more interesting explanation, however, is that the role of cohesins in a particular function (cohesion, condensation, repair, silencing or transcription) within discrete chromatin contexts and along the chromosome length are uniquely sensitive to Pds5 alterations. In the broader context, these and other studies bring to light an amazing range, revealed within individual \textit{pds5} alleles, through which Pds5 functions in cohesin loading, cohesion establishment, cohesion maintenance and chromosome condensation (Chan et al., 2013; Hartman et al., 2000; Panizza et al., 2000; Rowland et al., 2009a; Sutani et al., 2009). It is not, however, the phenotypic range of \textit{pds5} mutant cells that impacts models of cohesion maintenance, but rather the identification of allelic (\textit{pds5-1}) inactivation that results in both cell inviability and loss of cohesion but in the relative absence of either cohesin loss or Smc3 deacetylation. A growing body of evidence supports this model of cohesin without cohesion and allel-
specific roles of Pds5 in cohesin retention (Hartman et al., 2000; Kulemzina et al., 2012; Losada et al., 2005; Milutinovich et al., 2007; Skibbens et al., 1999b; Toth et al., 1999; Zhang et al., 2005). The simplest model emanating from these findings is that cohesin complexes associate with each sister chromatid as they emerge from behind the DNA replication fork and that cohesion is maintained through cohesin-cohesin interactions.
MATERIALS AND METHODS

Genetic manipulations for epitope-tagging or gene deletion

Deletion of *RAD61* was performed and independently confirmed as previously described (Maradeo and Skibbens, 2010). C-terminal tags were engineered as previously described (Longtine et al., 1998) within endogenous encoding genes *MCD1* and *SMC3* (Mcd1-3HA and Smc3-3HA). Primers used for *MCD1* are (forward primer) 5'-AGAAGCATTCGGAAATATTAAATAGACGCCAACCTGCACATTTTGAAAGGTTTATCAATGCTCGGATCCCCGGGTTAATTAA-3' and (reverse primer) 5'-AAGAAGATTGTTTTGGGCTGGGAAAACTTTCTAGACGTTGCTTTATTACCCAGGGTTGTGTGTAAGTTAGAATTCGAGCTCGTTTAAAC-3'. Primers used for *SMC3* are (forward) 5'-GGTTATTGAGGTCAATAGAGAAGAAGCAATCGGATTCATTAGAGGTTAGCATTAACTTCAAGGCA-3' and (reverse) 5'-TTTAGGTAAGAAGAAGCCAAGTTGGATTTGCATCATTAATAAAAGATATTGTGTGGATTTACCCAGGGTTGTGTGTAAGTTAGAATTCGAGCTCGTTTAAAC-3'. Integrations were confirmed by PCR using primers 5'-CTGGCGAATTACTTCAAGGCA-3' (*MCD1*) and 5'-GCGGCTCGAGATTCTTGTTCAATCGTTGTACACAC-3' (*SMC3*) in combination with 5'-AACTGCATGGAGATGAGTGGT-3' (*TRP1*). Epitope-tagged protein production was confirmed by Western blot.

Synchronization of Log Phase Cells and Flow Cytometry
Synchronization of yeast cultures and assessment of DNA contents by flow cytometry were performed as previously described (Maradeo and Skibbens, 2010). All strains and genotypes are listed in Table 1.

**Viability Assay**

Cultures were grown in high nutrient YPD media to an OD$_{600}$ of 0.2, synchronized in G1 (alpha factor) or pre-anaphase (nocodazole) at permissive temperature (23°C) for 3 hours, shifted to non-permissive temperature (37°C) for 1-2 hours in the presence of fresh media supplemented with either alpha factor or nocodazole and then placed on high nutrient YPD media for 16 hour at 23°C. Viability was scored by the ability to form microcolonies (colonies with over 30 cells).

**Cohesion Assay**

Cohesion assays were performed as previously described with the following modifications (Maradeo and Skibbens, 2009). Cells were normalized to 0.1-0.2 OD$_{600}$ and incubated in rich medium supplemented with nocodazole for 2.5 hours at 23°C to synchronize in pre-anaphase. Cells were then shifted to 37°C for 1 hour in the presence of fresh media supplemented with nocodazole to maintain the mitotic arrest. Cell aliquots were harvested at indicated time points, incubated in paraformaldehyde fixation solution. Large budded cells in which both DNA (DAPI) and Pds1 detection (A-14 anti-MYC (Santa Cruz Biotechnology) followed by goat anti-rabbit Alexa 568 (Molecular Probes, Inc., Eugene, OR) were analyzed. Cells images captured using a Nikon Eclipse E800 microscope equipped with a cooled CD camera (Coolsnapfx, Photometrics) and IPLab.
software (Scanolytics). Cells were scored for separation of TetR-GFP signal in the presence of Pds1, indicating premature sister chromatid separation. Cohesion analyses were repeated three times and a total of at least 300 cells counted.

**Chromatin Immunoprecipitation**

ChIP was performed as previously described (Glynn et al., 2004; Rudra and Skibbens, 2013a) with the following modifications. Log phase growth yeast (minimum of 0.6 OD$_{600}$) grown in high nutrient YPD broth were synchronized in either G1(alpha factor) or pre-anaphase (nocodazole) for 3 hours, shifted to the non-permissive temperature of 37°C for 2 hours and then fixed in 1% formaldehyde for 2 hours. Mcd1 enrichment was obtained by incubating extracts with EZ-view Red Anti-C-Myc affinity matrix (Sigma) or EZ-View Red Anti-HA affinity matrix (Sigma) overnight at 4°C. Beads were collected by centrifugation, washed with TSE-150 (0.1% SDS; 1% Triton X-100; 2mM EDTA; 150mM NaCl; 20mM Tris-Cl pH 8.1) and LiCl/Detergent Wash (0.25M LiCl; 1% IPEGAL; 1% DOC; 1mM EDTA; 10mM Tris-Cl pH 8.1) and the remaining bead-bound proteins harvested using 1%SDS; 0.1M NaHCO3. DNA-protein crosslinks were reversed in 5M NaCl. DNA precipitation from the resulting lysate was performed by overnight incubation at -20°C in ethanol. Precipitates were extracted in series using 25:24:1 phenol:chloroform:isoamylalcohol and pure chloroform prior to reprecipitation of DNA overnight at -20°C in ethanol. DNA was resuspended in water and analyzed by PCR using CAR site primers previously described (Glynn et al., 2004; Unal et al., 2007). PCR products were resolved using 1% agarose gels, and histograms of pixel densities quantified in Photoshop. Mcd1 enrichment was calculated as ratio of
pulldown (minus background obtained from GST control) over total chromatin minus background.

For quantitative-PCR, DNA collected from ChIP was measured for Ct values using Rotor-gene (Corbett) and E-values were calculated for each individual primer set (between 1.8-2.0). Immunoprecipitation efficiency was determined by E-value^((C_{Total} - C_{ChIP})-(C_{Total} - C_{GST\text{[negative]}}))

**Chromatin Binding Assay**

Chromatin binding assay was performed as previously described with modifications (Rudra and Skibbens, 2013a). Briefly, cells were cultured to an OD_{600} of 0.4, arrested in pre-anaphase (nocodazole), pelleted and washed with 1.2M Sorbitol. Cells were resuspended in CB1 buffer (50mM Sodium citrate, 1.2M Sorbitol, 40mM EDTA, pH 7.4). Cells were spheroblasted, and resuspended in 1.2M Sorbitol and frozen in liquid nitrogen. Cells were thawed on ice and supplemented with Lysis buffer (500mM Lithium Acetate, 20mM MgSO_{4}, 200mM HEPES, pH 7.9), protease inhibitor cocktail (Sigma), and TritonX-100. Lysate was centrifuged at 12,000xg for 15 minutes and supernatant containing soluble fraction and pellet containing chromatin bound fraction were collected and supplemented with 4X Laemelli (Amresco). Whole cell extracts, supernatant, and pellet were resolved by SDS-PAGE and analyzed using c-Myc(9E10) (Santa Cruz), H2B (Santa Cruz), and PGK (Invitrogen).

**Acetylation Assay**
C-terminally tagged Smc3 strains were grown to 0.1-0.3 OD_{600}, arrested in pre-anaphase (nocodazole), pelleted by centrifugation, resuspended in IPH150 (150mM NaCl, 50mM TRIS pH 8, 5mM EDTA, 0.5% IGEPAL-CA 630 (Sigma), 1mM DTT, 10mM Sodium Butyrate, Roche protease inhibitor cocktail, and immediately frozen in liquid nitrogen. Cells were mechanically lysed (Bead-beater, BioSpec) and extracts incubated with EZ-View Red Anti-HA affinity matrix (Sigma). Beads were washed with IPH50 buffer (50mM NaCl, 50mM TRIS pH 8, 5mM EDTA, 0.5% IGEPAL-CA 630 (Sigma), 1mM DTT, 10mM Sodium Butyrate, Roche protease inhibitor cocktail), and bead-bound proteins harvested using 4X Laemmli loading buffer (Amresco). Acetylation status was determined by Western blot using 1:5000 dilution of anti-Acetylated Lysine (Calbiochem) and band densities quantified using Photoshop.

**Condensation Assay**

*NET1* was genetically modified as previously described (Longtine et al., 1998) to include DNA sequence that encodes GFP using the following primers: 5’-
TTTAGGTAAGAAGAAGAAGCCAAGTGGTGATTTGCATCATATATAAAAGAT
TTCAAGAAAAACGGATCCCCCGGGGTTAATTAA-3’ and 5’-
TGCTTGATTATTTTTTTTACTAGCTTTTCTGTGACGTTGTTATTCTACTGAGACTT
TCTGGTATCAGAATTTCGAGCTCGTTAAAC-3’. Integrations were confirmed by PCR using the following primers: 5’-CGGATTCCAGTTCAGATTCTA-3’ and 5’-
AACTGCATGGAGATGAGTGGT-3’. Net1-GFP strains were grown to 0.1-0.2 OD_{600}, then incubated for 2.5 hours at 23°C in rich YPD medium supplemented with nocodazole or alpha-factor to arrest cells in pre-anaphase or G1 respectively. Cells were shifted to
37°C for 1 hour in fresh media supplemented with nocodazole to maintain the mitotic arrest. Following 4% paraformaldehyde fixation (10 min at 30°C), cells were assayed using an E800 light microscope (Nikon) equipped with a cooled CD camera (Coolsnapfx, Photometrics) and imaging software (IPLab, Scanalytics, Inc).

**DNA damage and Rad53 phosphorylation Assay**

Wildtype and *pds5-1* mutant strains were grown to 0.1-0.3 OD$_{600}$, arrested in pre-anaphase (nocodazole), pelleted by centrifugation, resuspended in water, and immediately frozen in liquid nitrogen. Cells were mechanically lysed (Bead-beater, BioSpec) in the presence of Trichloroacetic acid (TCA). The precipitated extracts were then solubilized in 4X Laemelli loading buffer (Amresco) and resolved by SDS-PAGE prior to transfer to PVDF membrane. Western blot analysis to assess the level of Rad53 modification was performed using Goat-anti-Rad53 (Santa Cruz, yC-19), Donkey-anti-Goat HRP secondary and signal detection performed following ECL Prime (GE) manufacturer instructions.
FIGURES

Table 1. Yeast Strain Table

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$\text{can1-100 pds5-1}$  
$\text{SMC3:3HA::TRP1}$ | This study |
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$\text{can1-100 rad61Δ::URA3}$ | This study |
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$\text{leu2-3,112 trp1-1 ura3-1}$  
$\text{can1-100 pds5-1}$  
$\text{rad61Δ::URA3}$ | This study |
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$\text{can1-100 NET1:GFP:TRP1}$ | This study |
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$\text{can1-100 pds5-1}$  
$\text{NET1:GFP:TRP1}$ | This study |

*all strains are in W303*
**Figure 1.** Pds5 is essential for cohesion maintenance. (A) Flow cytometry analyses revealing DNA content of wildtype and *pds5-1* mutant cells prior to and following 3 hour incubation in nocodazole (cultures were shifted to the restrictive temperature during the final hour of incubation in medium supplemented with nocodazole). (B) Percent viability of wildtype and *pds5-1* mutant cells in the presence or absence of the final shift to the restrictive temperature during mitotic arrest. (C) Percent cohesion defects of wildtype and *pds5-1* mutant cells after incubation at non-permissive temperature as described in (A) above. (D) Micrographs of wildtype and *pds5-1* mutant cells showing separated sisters (GFP-TetR), DNA (DAPI) and retention of Pds1 indicative of a pre-anaphase state.
Figure 2. Inactivation of Pds5 during mitosis results in cohesion loss in the absence of cohesin dissociation from DNA. (A) Schematic highlights possible mechanisms through which cohesion loss may occur in the one-ring around two sister chromatids embrace model. See text for details. (B) DNA content of wildtype and pds5-1 mutant cells treated as described in Figure 1A. (C and D) Average IP efficiency along arm and pericentromeric CAR sites obtained from wildtype (normalized to 1) and pds5-1 mutant cells.
Figure 3. *pds5-1* mutant cells exhibit reduced Mcd1 levels but retain high levels of Mcd1 cohesin enrichment to DNA. (A) Triton X-100 fractionation assays of wildtype and *pds5-1* mutant cells expressing Mcd1-MYC. Western blots performed on the resulting whole cell extracts (WCE), soluble fractions (S) and chromatin-bound pelleted fractions (P). Histone 2B (H2B) and Phosphoglycerate kinase (PGK) serve as controls for soluble and chromatin-bound proteins, respectively. Results shown for three independent fractionation studies. (B) Quantifications of Mcd1 in whole cell extracts, supernatants, and chromatin pellet fractions. Mcd1 enrichment to DNA is based on the ratio of Mcd1 to Histone 2B levels obtained from 3 independent experiments while the soluble pool of Mcd1 is based on the ratio of Mcd1 to PGK levels from 3 independent experiments. Wildtype Mcd1 is normalized to 1. Note that while total Mcd1 levels in whole cell extracts are decreased in *pds5-1* mutant cells relative to wildtype, analyses of cell
fractionation studies reveal that Mcd1 levels was mostly absent from $pds5-1$ supernatants but significant levels are retained in the chromatin pellet fraction, relative to the total Mcd1 level. (C) Ratio of chromatin bound Mcd1 in pellet to total levels in whole cell extracts (normalizing to H2B) reveal that equivalent proportions of Mcd1 remains chromatin bound in both wildtype and $pds5-1$ mutant cells.
Figure 4. Sister chromatid cohesion loss occurs despite retention of cohesin enrichment along chromosome arm and pericentromeric CAR sites. (A) Position of primers used in ChIP along individual arm (comprising two CAR sites) and pericentromeric CAR sites for chromosome III. (B) Average IP efficiency along chromosome arm CARs obtained from four oligo pairs (34, 35, 36 and 37) in wildtype and pds5-1 mutant cells. (C) Quantitative PCR performed on CAR sites 36 and 37 confirm cohesin enrichment levels observed using ChIP in both wildtype and pds5-1 mutant cells. (D) Average IP efficiency along pericentromeric CARs obtained from oligo pairs (70, 72, 74, 76, 48, 78, 80, 82 and 84) in wildtype and pds5-1 mutant cells. All primer design and designations from (Glynn et al., 2004; Unal et al., 2007).
Figure 5. (A) Sister chromatid cohesion loss occurs despite retention of Smc3 acetylation and in the absence of DNA damage. (A) DNA content of wildtype and pds5-1 mutant cells treated as described in Figure 1A. (B) Dilution series of Smc3 immunoprecipitated from wildtype and pds5-1 mutant cells revealing total Smc3 protein (HA) and acetylation (Acetyl-Lys) levels. (C-D) Quantification of total Smc3 protein and Smc3 acetylation levels in wildtype (normalized to 1) and pds5-1 mutant cells. (E) Wildtype and pds5-1
mutant cells are competent to phosphorylate Rad53 in response to DNA damage (MMS),
but do not phosphorylate Rad53 in the absence of MMS.
Figure 6. Pds5 is not required to maintain condensation during an extended pre-anaphase arrest. (A) DNA content of wildtype cells and rad61 and pds5-1 single mutant cells and pds5-1 rad61 double mutant cells as described in Figure 1A. (B) Percent viability of wildtype cells and rad61 and pds5-1 single mutant cells and pds5-1 rad61 double mutant cells following the regimen described in Figure 1A. (C) Percent of wildtype and pds5-1 mutant cells showing condensed (Lines) and uncondensed rDNA (Puffs) rDNA structures following regimen described in Figure 1A. (D) Micrograph of wildtype and pds5-1 mutant cells highlights rDNA structure through Net1-GFP detection (GFP) and DNA (DAPI). Pds5 inactivation specifically during S-phase impacts chromosome condensation. Cells were synchronized in G1 (alpha factor arrest) at
permissive temperature and then shifted to the non-permissive temperature and
synchronized in G2/M (nocodazole arrest).(E) Percent of wildtype and pds5-1 mutant
cells that exhibit either condensed (Lines) or decondensed (Puffs) rDNA structures. (B)
Micrographs of wildtype and pds5-1 mutant cells reveal changes in rDNA architecture.
Figure 7. Pds5 is not essential for cohesin enrichment onto DNA during cohesion establishment. (A) DNA content of wildtype cells and eco1-1, scc2-4 and pds5-1 mutant cells synchronized G1 (alpha factor arrest) at permissive temperature and then shifted to the non-permissive temperature in fresh media supplemented with nocodazole (NZ arrest). (B) Overall IP efficiency of Mcd1 on chromosome arm sites for each of the four strains and treated as described above. Wildtype was normalized to 1. (C) Validation of both scc2 mutant strains and each of the nine pericentromeric primer sites in which Scc2
inactivation results in substantially reduced Mcd1 enrichment to DNA. (D) Western blot analyses revealing that Mcd1 is present in whole cell extracts obtained from wildtype cells and eco1-1, scc2-4 and pds5-1 mutant cells. (E and F) Kinetic ChIP analyses of wildtype, eco1-1, scc2-4 and pds5-1 mutant cell aliquots harvested at 40 minute increments starting from the G1 release and processed for ChIP.
Figure 8. Establishment of sister chromatid cohesion during S-phase is abrogated by loss of Pds5 despite normal levels of Smc3 and Smc3 acetylation. (A) DNA content of wildtype and pds5-1 mutant cells synchronized G1 (alpha factor arrest) at permissive temperature and then shifted to the non-permissive temperature in fresh media supplemented with nocodazole (NZ arrest). (B) Dilution series of Smc3 immunoprecipitated from wildtype and pds5-1 mutant cells revealing similar levels of both total Smc3 protein (HA) and acetylated (Acetyl-Lys) Smc3. (C-D) Quantification of total Smc3 protein (left) and Smc3 acetylation levels (right) in wildtype (normalized to 1) and pds5-1 mutant cells.
Figure 9. Proposed model of cohesin architecture and Pds5 function throughout the cell cycle. (A) Scc2, Scc4-dependent cohesin loading during S-phase onto nascent sister chromatids is coordinated with Eco1-dependent Smc3 acetylation, leading to stable cohesin-cohesin interactions. Many cohesin structures are possible; shown is one model that reflects recent advances in SMC-like crystal structure studies through which chromatin is captured between SMC head domains and an Mcd1 cap complex (Rudra and Skibbens, 2013b). Note the role of Pds5 and Eco1-dependent Smc3 acetylation in regulating hinge-hinge interactions and additional roles for Pds5 in establishing condensation and transcription regulation (not shown). (B) Summary of results that, upon Pds5 inactivation during mitosis, sister chromatid cohesion is lost despite retention of cohesin to DNA and Smc3 acetylation.
REFERENCES


Ben-Aroya, S., A. Koren, B. Liefshitz, R. Steinlauf, and M. Kupiec. 2003. ELG1, a yeast gene required for genome stability, forms a complex related to replication factor


CHAPTER 3

Pds5 Regulators Segregate Cohesion and Condensation

Pathways in *Saccharomyces cerevisiae*
ABSTRACT

Cohesins are required both for the tethering together of sister chromatids (termed cohesion) and subsequent condensation into discrete structures – processes fundamental for faithful chromosome segregation into daughter cells. Differentiating between cohesin roles in cohesion and condensation would provide an important advance in studying chromatin metabolism. Pds5 is a cohesin-associated factor that is essential for both cohesion maintenance and condensation. Recent studies revealed that ELG1 deletion suppresses the temperature sensitivity of pds5 mutant cells. However, the mechanisms through which Elg1 may regulate cohesion and condensation remain unknown. Here, we report that ELG1 deletion from pds5-1 mutant cells results in a significant rescue of cohesion, but not condensation, defects. Based on evidence that Elg1 unloads the DNA replication clamp PCNA from DNA, we tested whether PCNA overexpression would similarly rescue pds5-1 mutant cell cohesion defects. The results indeed reveal that elevated levels of PCNA rescue pds5-1 temperature sensitivity and cohesion defects, but do not rescue pds5-1 mutant cell condensation defects. In contrast, RAD61 deletion rescues the condensation defect, but importantly, neither the temperature sensitivity nor cohesion defects exhibited by pds5-1 mutant cells. In combination, these findings provide novel evidence that cohesion and condensation are separable pathways and regulated in non-redundant mechanisms. These results are discussed in terms of a new model through which cohesion and condensation are spatially regulated.
INTRODUCTION

Cohesins are of clinical interest due to the fact that mutations lead to chromosome mis-segregation, premature chromosome decondensation, decreased DNA repair efficiencies, impaired rDNA production and transcription deregulation - the latter of which is now considered the basis of severe developmental maladies that include Robert Syndrome (RBS) and Cornelia de Lange Syndrome (CdLS) (Skibbens et al., 2013). Complicating analyses of cohesin structure is the likely superimposition of competing post-translational modifications (SUMOylation, ubiquitination, phosphorylation and acetylation) through which cohesins may be directed toward one process over another (Rolef Ben-Shahar et al., 2008; Unal et al., 2008). Additionally, many cohesion factors when mutated exhibit defects in cohesion and condensation (Guacci and Koshland, 2012; Guacci et al., 1997; Hartman et al., 2000; Skibbens et al., 1999b; Toth et al., 1999), raising the question of whether these cohesion-related processes are so intimately entwined as to be potentially inseparable.

Cells from RBS patients typically exhibit heterochromatic repulsion (regionalized condensation defects) absent in cells from CdLS patients. The presence of aneuploidy and failed mitosis also appears to differentiate, at the cellular level, otherwise highly similar developmental abnormalities (Mehta et al., 2013; Skibbens et al., 2013). Therefore, the identification of pathways through which cohesion and condensation are experimentally separated would provide important tools useful in dissecting each pathway in isolation and providing a broader understanding of this multifaceted cohesin complex. A limited number of genes (RAD61/WAPL and ELG1), when deleted, suppress
$ctf7/eco1$ mutant cell growth deficiencies. $RAD61$ deletion rescues the conditional growth and condensation defect, but not cohesion defect, of $ctf7/eco1$ mutant cells (Guacci and Koshland, 2012; Guacci et al., 2014). In contrast, deletion of $ELG1$ suppresses $ctf7/eco1$ mutant cell conditional growth and cohesion defects (Maradeo and Skibbens, 2009). The mechanisms, however, through which either rescues $ctf7/eco1$ mutant cell conditional growth remains elusive. $pds5$ alleles have proved tremendously informative given their differential impact on cohesin deposition, cohesion establishment and maintenance and also transcription, placing Pds5 at a convergence of cohesin-related developmental defects and cancers (Denes et al., 2010; Hartman et al., 2000; Maffini et al., 2008; Zhang et al., 2009; Zhang et al., 2007). Our lab previously reported that $ELG1$ deletion suppresses $pds5-1$ mutant cell conditional growth, providing an important platform from which to initiate an effort to dissect and isolate various roles for Pds5 in cohesin pathways. Here, based on previous findings of ELG1 deletion bypassing $pds5-1$ conditional cell growth (Maradeo et al., 2010), we exploit these suppressors to isolate for the first time Pds5 roles in both cohesion and condensation – findings from which we derive a new model regarding cohesin functions.
RESULTS

The essential role of Pds5 in both S and M phases is supported by Elg1-RFC.

To first assess the extent through which \textit{pds5-1 elg1} double cells exhibit altered conditional growth through the cell cycle, wildtype, \textit{pds5-1} and \textit{elg1} single mutant cells and \textit{pds5-1 elg1} double mutant cells were grown to log phase at 23°C and then synchronized in either G1 (alpha factor), S (hydroxyurea), or M phase (nocodazole) portions of the cell cycle. The resulting cultures were divided in two and one half shifted to 37°C (non-permissive for \textit{pds5-1}) for 2 hours while maintaining the respective cell cycle arrests and then plated onto rich medium plates and incubated at the permissive temperature of 23°C for 18 hours prior to scoring for viability as previously described (Tong and Skibbens, 2014). As expected, wildtype and \textit{elg1} single mutant cells exhibited robust growth at 23°C regardless of the cell cycle phase while \textit{pds5-1} mutant cells exhibited only a modest decrease in viability in the M phase. This conditional viability was rescued by \textit{ELG1} deletion (Figure 1A).

Cultures shifted to 37°C exhibited significant differences in viability depending on the part of the cell cycle under investigation. All strains including the \textit{pds5-1} single mutant strain exhibited high levels of viability following a shift to 37°C during G1, suggesting that Pds5 plays only a minimal role during this portion of the cell cycle (Figure 1A) and that cohesion pathways, in general, are largely inactive during G1 (Ciosk et al., 2000; Guacci et al., 1997; Hartman et al., 2000; Michaelis et al., 1997; Milutinovich et al., 2007; Skibbens et al., 1999b; Stead et al., 2003). In contrast, \textit{pds5-1} mutant cells exhibited significantly decreased viabilities in response to temperature shifts.
both during S and M phases (Hartman et al., 2000; Tong and Skibbens, 2014).

Importantly, \textit{pds5-1 elg1} double mutant cells instead exhibited viability levels approximating those of \textit{elg1} single mutant cells during both S and M phase (Figure 1A). The bypass suppression obtained through \textit{ELG1} deletion is most notable in mitotic \textit{pds5-1} mutant cells. These results suggest that Elg1 impacts Pds5 function in S phase (cohesin loading or cohesion establishment), which appears to affect Pds5 function during maintenance.

\textbf{Elg1-RFC is a critical regulator of Pds5-dependent sister chromatid cohesion}

\textit{pds5-1} mutant cells exhibit severe cohesion and condensation defects (Hartman et al., 2000; Stead et al., 2003; Tong and Skibbens, 2014). It thus became important to test which, if either, of these Pds5 functions is rescued by \textit{ELG1} deletion. Log phase wildtype, \textit{elg1} and \textit{pds5-1} single mutant cells, and \textit{pds5-1 elg1} double mutant cells harboring cohesion assay cassettes (\textit{TetO} array integrated approximately 40kb from centromere V detected through binding of TetR-GFP) were synchronized in G1 at the permissive temperature of 23°C, washed and released into 37°C (non-permissive for \textit{pds5-1}) rich medium supplemented with nocodazole (herein referred to as a G1 temperature shift). The resulting pre-anaphase synchronized cultures were harvested and assessed for both DNA content by flow cytometry and premature sister chromatid separation in which 1 GFP foci indicates tightly tethered sisters while 2 GFP spots reveals premature sister chromatid separation (Figure 1B-D). As expected, wildtype cells exhibited minimal (<10%) precocious sister chromatid separation, \textit{elg1} single deletion mutants exhibited only a modest increase in separated sisters (19% cohesion defect).
while \textit{pds5-1} mutant cells exhibited severe cohesion defects (63\%) (Figure 1C). Notably, \textit{pds5-1 elg1} double mutant cells exhibited cohesion defects significantly reduced relative to \textit{pds5-1} mutant cells (compare 32\% to 63\%) and instead are comparable to \textit{elg1} single mutant cells (Figure 1C). Thus, \textit{ELG1}-deletion significantly rescues the precocious sister chromatid separation normally observed in \textit{pds5-1} mutant cells.

Could the absence of Elg1 during S-phase rescue cohesion defects that arise upon \textit{pds5-1} inactivation during mitosis? To address this question, wildtype, \textit{pds5-1}, \textit{elg1}, and \textit{pds5-1 elg1} strains were synchronized in pre-anaphase at permissive temperature by placing log phase cultures into medium supplemented with nocodazole. The pre-anaphase arrested cultures were then shifted to non-permissive temperature for 2 hours while maintaining the pre-anaphase arrest (herein referred to as a mitotic temperature shift) and then assessed for precocious sister chromatid separation (Supplemental Figure S1). Under this regimen, wildtype cells exhibited a relatively low level of cohesion defects (<20\%) and \textit{elg1} mutant cells exhibited only a modest increase in cohesion defects (39\%). In contrast, \textit{pds5-1} mutant cells exhibited severe defects (68\%). \textit{pds5-1 elg1} double mutant cells, however, exhibited a significant rescue in the level of cohesion defects (42\%) that is comparable to \textit{elg1} single mutant cells (39\%) and well below that of \textit{pds5-1} single mutant cells (Figure 1E). This rescue of cohesion observed in \textit{pds5-1 elg1} double mutant cells suggests that the cohesion-promoting effect produced by the absence of Elg1-RFC during S phase persists through the cell cycle and into mitosis. In combination, these results reveal that Elg1 is a key negative regulator of Pds5 function in cohesion and that this rescue correlates with increased cell viability.
Pds5 functions in cohesion and condensation are separable through Elg1-RFC

Does ELG1 deletion similarly promote proper chromosome condensation in pds5-1 mutant cells? Net1-GFP provides for quantification of cohesin-dependent changes in rDNA chromatin architecture and condensation, a well-established indicator of condensation defects in cohesin mutants (Cuylen et al., 2011; D'Ambrosio et al., 2008; Guacci et al., 1997; Hartman et al., 2000; Lopez-Serra et al., 2013; Machin et al., 2005; Tong and Skibbens, 2014). We first validated this system in our own lab by recapitulating the efficacy of Net1-GFP to detect changes in rDNA condensation in response to Mcd1 inactivation (Supplemental Figure S2). Next, we focused on mitotic inactivation of pds5-1 mutant protein given that rDNA condenses into well-defined loop or line-like structures in mitotic wildtype cells but form highly amorphous puffs in mitotic pds5-1 mutant cells (Hartman et al., 2000; Lopez-Serra et al., 2013; Tong and Skibbens, 2014). We performed a G1 temperature shift on log phase wildtype, elg1 and pds5-1 single mutant cells, and pds5-1 elg1 double mutant cells, all harboring Net1-GFP. The resulting synchronized pre-anaphase cells were then scored for DNA content by flow cytometry (Supplemental S3) and condensation defects (rDNA puffs instead of loop or lines) by microscopy (Figure 2A, B). As expected, the majority of wildtype cells exhibited high levels (76%) of distinct rDNA loop or line structures, indicative of condensed chromosomes. Interestingly, elg1 mutant cells exhibited nearly an identical level (77%) of rDNA loops/lines, revealing that Elg1 exerts separable effects on cohesion versus condensation reactions. On the other hand, pds5-1 mutant cells exhibited significant condensation defects (only 29% of cells with loops or lines) with the majority of cells (62%) instead containing highly decondensed puff-like structures. Surprisingly, pds5-1
elg1 double mutant cells exhibited nearly identical levels of decondensed puff-like structures (63%) (Figure 2B). Thus, ELG1 deletion does not rescue the condensation defects in pds5-1 mutant cells, indicating that the loss of pds5-1 cell viability correlates only with elevated levels of cohesion defects, not condensation defects.

PCNA overexpression rescues pds5-1 viability

Elg1 comprises an alternative Replicaton Factor C (RFC) complex that regulates PCNA (Proliferating Cell Nuclear Antigen encoded by POL30) association with DNA (Bellaoui et al., 2003; Ben-Aroya et al., 2003; Kanellis et al., 2003; Parnas et al., 2010). Elevated levels of PCNA rescue ctf7/eco1 mutant cell conditional growth (Skibbens et al., 1999b). Intriguingly, ELG1 mutation results in higher levels of chromatin bound PCNA (Kubota et al., 2013; Shiomi and Nishitani, 2013), raising the possibility that PCNA over-expression may similarly rescue pds5-1 mutant cell phenotypes. To test this hypothesis, wildtype and pds5-1 mutant cells were transformed with vector alone or vector that directed elevated expression of PCNA (POL30). We included pds5-1 elg1 double mutant cells for comparison. Log phase cultures were diluted in series, plated onto rich medium and then incubated at 23°C, 30°C, 34°C, and 37°C prior to assessing growth. Wildtype cells grew robustly at all temperatures regardless of PCNA over-expression. As expected, pds5-1 mutants harboring only vector exhibited robust growth at 23°C and 30°C but were predominantly inviable at elevated temperatures (Figure 3A). Importantly, elevated PCNA expression suppressed pds5-1 conditional growth, providing for robust growth at 34°C and even limited rescue at 37°C. Notably, pds5-1 mutant cells expressing elevated levels of PCNA exhibited both improved growth kinetics and
viability compared to pds5-1 elg1 double mutant cells (Figure 3A). In combination, these findings reveal for the first time that PCNA is a critical regulator of Pds5 and suggest that the growth and viability benefits obtained through ELG1 deletion occur through PCNA.

We hypothesized that the suppression of pds5-1 mutant cell temperature sensitivity by elevated PCNA levels may be due to the rescue of sister chromatin cohesion. To directly test this model, we performed a G1 temperature shift on wildtype and pds5-1 cells harboring either vector alone or vector plus POL30 and all harboring the cohesion assay cassettes described above. The resulting pre-anaphase cultures were then analyzed for DNA content (Supplemental Figure S4) and premature sister chromatid separation as described above (Figure 3B, C). As expected, the majority of wildtype cells contained tightly tethered sister chromatids regardless of the status of PCNA overexpression (16% and 19%, respectively). pds5-1 mutant cells, in contrast, exhibited a significant loss of cohesion (69%) that was significantly rescued (45%) by elevated PCNA levels (Figure 3C). In combination, these results reveal that PCNA promotes Pds5-dependent sister chromatid cohesion and rescues pds5-1 mutant cell conditional growth.

**Pds5 functions in cohesion and condensation are separable through PCNA**

We noted that the rescue of pds5-1 conditional growth by elevated levels of PCNA appears superior to that obtained through ELG1 deletion. We speculated therefore that PCNA over-expression might bypass the conditional chromosome condensation defect, in addition to the cohesion defect, exhibited by pds5-1 mutant cells. To test this possibility, we performed a G1 temperature shift on wildtype and pds5-1 mutant cells that
express Net1-GFP and harbor either vector or vector plus POL30. The resulting pre-anaphase cells were then assessed for DNA content (Supplemental Figure S5) and rDNA structure as described above (Figure 4A,B). Wildtype cells predominantly contained distinct loop/line rDNA structures regardless of PCNA expression (73% and 75%, respectively). In contrast, pds5-1 mutant cells exhibited significantly decreased incidence of rDNA loop/lines (26%), instead exhibiting predominantly (59%) puff-like decondensed rDNA chromatin structures (Figure 4B). Notably, PCNA over-expression failed to suppress pds5-1 mutant cell defects in rDNA structure and instead exhibited an identical level (59%) of puffs (Figure 4B). The PCNA-dependent rescue in both viability and cohesion, but not condensation, confirms a common mechanism through which ELG1 deletion and PCNA over-expression rescue pds5-1 sister chromatid cohesion defects and isolates Pds5 function in cohesion from that of condensation.

**RAD61/WAPL deletion rescues the condensation defects, but not conditional cell inviability, of pds5-1 mutant cells**

Is there a pathway through which pds5-1 mutant cell condensation defects can be rescued? Prior analysis revealed that viability and the condensation, defect exhibited by ctf7/eco1 mutant cells is rescued by RAD61/WAPL deletion (Guacci and Koshland, 2012; Guacci et al., 2014; Rolef Ben-Shahar et al., 2008; Rowland et al., 2009a; Sutani et al., 2009). Does RAD61/WAPL deletion rescue pds5-1 mutant cell condensation defects? We performed a G1 temperature shift on log phase wildtype, pds5-1 and rad61 single mutant cells and pds5-1 rad61 double mutant cells all modified to express Net1-GFP. The resulting pre-anaphase cultures were assessed for DNA content (Supplemental Figure
S6A) and rDNA condensation (Figure 5A,B). The majority of wildtype and rad61 cells contained tight loop/line rDNA chromatin structures (76% and 79%, respectively) while pds5-1 mutant cells exhibited severe condensation defects (29% loop/line rDNA chromatin structures) (Figure 5B). Notably, pds5-1 rad61 double mutant cells exhibited a significant reduction in condensation defects compared to pds5-1 mutant cells such that over 60% of pds5-1 rad61 cells contained distinct loop/line rDNA chromatin structures (Figure 5B). These results reveal that RAD61 deletion suppresses pds5 condensation defects and suggest that Pds5 and Ctf7/Eco1 promote condensation through a common mechanism regulated by Rad61.

Is the RAD61-deletion dependent rescue of condensation defects sufficient to rescue the conditional growth otherwise present in pds5-1 mutant cells, similar to that observed in ctf7/eco1 mutant cells? We spotted serial dilutions of each of the four strains onto rich medium and incubated replicant plates at 23°C, 30°C, 34°C, and 37°C. As expected, wildtype and rad61 mutant cells exhibited robust growth at all temperatures while pds5-1 mutant cells were inviable at temperatures tested above 30°C. As opposed to rescuing pds5-1 conditional growth, however, deletion of RAD61 either had no impact or further exacerbated the temperature sensitive growth of pds5 mutant cells (Figure 5D) (Tong and Skibbens, 2014). Thus, Eco1/Ctf7 and Pds5 roles are separable based on their differential responses in cell viability to RAD61 deletion.

The inability of RAD61 deletion to rescue pds5-1 mutant cell temperature sensitivity suggests that the essential function of Pds5 remains in deficit. Since pds5-1 rad61 double mutant cells exhibit nearly normal levels of condensation, we speculated that pds5-1 rad61 double mutants are deficient in cohesion. To test this possibility, we
performed a G1 temperature shift on log phase wildtype, *pds5-1*, *rad61*, and *pds5-1 rad61* cells. The resulting pre-anaphase cells were assessed by flow cytometry (Supplemental Figure 6B) and for cohesion defects. Wildtype cells exhibited minimal cohesion defects (<20%) while *rad61* mutant cells exhibited a modest increase in precocious sister separation (37%). In contrast, *pds5-1* mutant cells exhibited significant cohesion defects (63%). Importantly, *pds5-1 rad61* double mutant cells exhibited a high level of cohesion defects (58%) similar to that of *pds5-1* single mutant cells (Figure 5C). These results are notable for several reasons. First, the rescue in condensation evident in *pds5-1 rad61* double mutant cells is uncoupled from increased viability, opposite to the situation that arises in *ctf7/eco1 rad61* mutant cells (Guacci and Koshland, 2012; Guacci et al., 2014; Rolef Ben-Shahar et al., 2008; Rowland et al., 2009a; Sutani et al., 2009). Second, our findings identify a second and distinct facet of chromatin regulation in which cohesion and condensation can be experimentally isolated.
DISCUSSION

Cohesion and condensation are separable through analysis of Pds5

The expanding roles of cohesins include cohesion, condensation, DNA repair, replication, ribosome maturation, and transcription regulation. Cohesin mutation not only results in aneuploidy, but transcription deregulation, a deficit now firmly implicated in severe birth defects (Dorsett, 2007; Skibbens et al., 2013). Thus, ascertaining the extent through which these activities are separable, and then differentiating between competing forms of cohesin regulation, becomes of increasing clinical interest. One of the major findings of the current study is the development of a genetically tractable system through which each of the essential functions of Pds5 in cohesion and condensation are isolated. Early findings that revealed that cohesin (Mcd1 and Pds5) and cohesin regulatory (Ctf7/Eco1) factors are uniformly required for both cohesion and condensation suggested that these processes might be intimately, if not irreversibly, entangled (Guacci et al., 1997; Hartman et al., 2000; Skibbens et al., 1999b). Our findings that either deletion of ELG1 or overexpression of PCNA rescues the cohesion defect, but not the condensation defect, in pds5-1 mutant cells provide critical tools through which one facet of separation-of-function analyses can proceed. Complementing those findings is that deletion of RAD61 rescues the condensation defect, but not the cohesion defect, in pds5-1 mutant cells. These results augment the RAD61 deletion-dependent rescue of ctf7/eco1 mutant cells noted previously (Guacci and Koshland, 2012; Guacci et al., 2014; ROLEF Ben-Shahar et al., 2008; Rowland et al., 2009a; Sutani et al., 2009), bringing cohesin separation-of-function analyses full circle. The current study thus reveals that the
pathways of condensation and cohesion can be separated and that the critical role of Pds5 is biased toward maintaining sister chromatid cohesion. These tools will be critical in assessing how regulatory factors direct cohesin modifications and structures toward cohesion, condensation and transcription (Rudra and Skibbens, 2013b).

Prior observations that deletion of \textit{ELG1} or \textit{RAD61} rescue cohesion mutant cell growth defects might suggest that Elg1 and Rad61 each directly antagonize some aspect of cohesion – activities termed “anti-establishment” (Maradeo et al., 2010; Maradeo and Skibbens, 2010; Rolef Ben-Shahar et al., 2008; Rowland et al., 2009a; Sutani et al., 2009; Unal et al., 2008). Instead, our results provide a novel yet clear template regarding the mechanism of bypass suppression. \textit{ELG1} deletion results in increased PCNA retention onto chromatin (Kubota et al., 2013; Shiomi and Nishitani, 2013) such that simply over-expressing PCNA fully supplants the requirement for \textit{ELG1} deletion to rescue both \textit{ctf7/eco1} and \textit{pds5-1} mutant cell conditional growth (Maradeo et al., 2010; Maradeo and Skibbens, 2009; Maradeo and Skibbens, 2010; Parnas et al., 2009; Skibbens et al., 1999b). Thus, Elg1 does not directly antagonize cohesion reactions but instead its deletion results in the elevated retention of a positive regulator of cohesion - PCNA (Moldovan et al., 2006; Skibbens et al., 1999b). We posit a similar situation may exist for Rad61: that it is not the deletion of \textit{RAD61} per se that provides bypass suppression of \textit{ctf7/eco1} and \textit{pds5-1} mutant cells, but that \textit{RAD61} deletion results in the recruitment/retention of a factor that positively impacts condensation. This model represents a major shift in paradigm from the current view that non-essential Rad61 directly precludes stable cohesin binding to DNA by revealing some conjectured
destabilizing activity of Pds5 and Scc3 (Marston, 2014) – factors which are essential to maintain cohesion.

**Cohesion and condensation occur independently of each other behind the DNA replication fork**

Prior characterization of cohesins led to a model that cohesin deposition precedes condensin deposition onto DNA, suggesting that cohesion and condensation establishment occur in a temporally and spatially defined manner (Arumugam et al., 2003; Ciosk et al., 2000; D'Ambrosio et al., 2008; Eng et al., 2014; Rudra and Skibbens, 2013b). This study reveals that cohesion and condensation each can be established and maintained in the absence of the other, extending prior evidence that *RAD61* deletion rescues condensation, but not cohesion (Guacci and Koshland, 2012; Guacci et al., 2014). These findings inform new models through which cohesin-dependent processes proceed.

How do PCNA or Rad61-dependent auxiliary factors differentially direct Pds5 roles in cohesion and condensation? In logarithmically growing cells, PCNA functions almost exclusively behind DNA polymerase to both promote replication processivity and serve as a landing pad for numerous DNA modulating factors (nucleosome deposition complexes, chromatin remodeling complexes). Elevated PCNA levels may augment or bias Pds5 function toward cohesion, suggesting a post-DNA polymerase replication-coupled mechanism. In parallel, we hypothesize that a Rad61-dependent factor may augment Pds5 function toward condensation. Presently, there is a paucity of evidence that physically links Rad61 to the DNA replication fork. Based on this, we speculate that a Rad61-dependent regulatory factor promotes chromosome condensation at sites that trail
the DNA replication fork, a context that does not significantly impact the role of Pds5 in cohesion. It is tempting to further speculate that this activity may be influenced by Okazaki lagging strand maturation given that Scc2 binding to DNA (required for both cohesin and condensin deposition) is regulated by Chl1 DNA helicase that appears to function in the context of Okazaki maturation (Bharti et al., 2014; Rudra and Skibbens, 2012; Rudra and Skibbens, 2013a).

A new model for cohesion and condensation establishment reactions

Until recently, the structural basis through which cohesins establish and tether sister chromatids together was highly debated. One notion was that huge cohesin rings are deposited during G1 and that passage of the DNA replication fork through cohesin rings entraps within both sister chromatids (Lengronne et al., 2006). However, it is now clear that cohesin deposition is essential only during S phase and that cohesins deposited prior to S phase are unstable regardless of acetylation state (Gause et al., 2010; Gerlich et al., 2006; Kueng et al., 2006; Rudra and Skibbens, 2013a; Song et al., 2012). In addition, there is direct evidence that each sister chromatid is individually decorated by cohesins and that Mcd1 can bridge different Smc1,3 heterodimers (Gruber et al., 2003; Haering et al., 2004; Kulemzina et al., 2012; Tong and Skibbens, 2014; Zhang et al., 2013), providing support of an early model that cohesion is mediated through cohesin-cohesin interactions (Skibbens, 2000). Intriguingly, Pds5 (and Nse5 for Smc5,6 complexes) bind both the head and hinge domains (Jeppsson et al., 2014a; Mc Intyre et al., 2007) – in support of findings that cohesins fold over to promote head-hinge interactions (Anderson et al., 2002; Sakai et al., 2003). Our findings regarding PCNA-dependent rescue further
support a higher-order cohesin assembly model in that mutant pds5-1 protein becomes resistant to temperature shift inactivation during mitosis. We posit that PCNA promotes assembly of cohesin oligomers that may stabilize pds5-1 protein against thermal fluctuations (Figure 6). It is exciting to consider a context-based mechanism through which cohesin assemblies and modifications required to promote cohesion and condensation are regulated (Rudra and Skibbens, 2013b)(Figure 6).
MATERIALS AND METHODS

Yeast strains, Synchronization of Log Phase Cells and Flow Cytometry

Yeast strain genotypes used in the current study are listed in Table 1. Synchronization of yeast cultures and assessment of DNA content by flow cytometry were performed as previously described (Tong and Skibbens, 2014).

Viability Assay

Cultures were grown to log phase in high nutrient YPD medium to an OD_{600} of approximately 0.2, synchronized in G1 (alpha factor), S (hydroxyurea), or pre-anaphase (nocodazole) at permissive temperature (23°C) for 3 hours, shifted to non-permissive temperature (37°C) for 2 hours in fresh media again supplemented with either alpha factor, hydroxyurea, or nocodazole to maintain respective G1, S, or pre-anaphase arrests and then placed on high nutrient YPD medium plates for 16 hours at 23°C. Viability was scored by the ability to form microcolonies (colonies with over 30 cells) as previously described (Tong and Skibbens, 2014).

Cohesion Assay

Cohesion assays were performed as previously described with the following modifications (Tong and Skibbens, 2014). Cells in log phase growth were normalized to 0.1-0.2 OD_{600} and incubated in rich medium supplemented with alpha factor or nocodazole for 2.5 hours at 23°C to synchronize in G1 or pre-anaphase respectively. Resulting cultures were harvested, washed through medium exchange and centrifugation
and cells suspended in fresh media supplemented with nocodazole and maintained at 37°C for 3 hours. Cell aliquots of the resulting pre-anaphase arrested cells were harvested at indicated time points and structure preserved by the addition of paraformaldehyde to a final concentration of 3.7%. Large budded cells that exhibited co-incident DNA (DAPI) and Pds1 staining (A-14 anti-MYC (Santa Cruz Biotechnology) followed by goat anti-rabbit Alexa 568 (Molecular Probes, Inc., Eugene, OR) were analyzed for disposition of 1 versus 2 GFP signals. Cell images were captured using a Nikon Eclipse E800 microscope equipped with a cooled CD camera (Coolsnapfx, Photometrics) and IPLab software (Scanalytics). Cohesion analyses were repeated three times and a total of at least 300 cells counted.

**Condensation Assay**

*NET1* was genetically modified as previously described (Tong and Skibbens, 2014). Condensation assays were done as previously described (Lopez-Serra et al., 2013; Tong and Skibbens, 2014). Briefly, log phase Net1-GFP strains were grown to 0.1-0.2 OD$_{600}$ and then incubated for 2.5 hours at 23°C in rich YPD medium supplemented with alpha-factor to arrest cells in G1. The resulting cells were harvested, washed in fresh medium before resuspension in fresh media supplemented with nocodazole and incubated at 37°C for 2-3 hours. The resulting pre-anaphase cultures were persevered by paraformaldehyde fixation (3.7% final concentration) for 10 min at 30°C, prior to analyses. Cells were assayed using an E800 light microscope (Nikon) equipped with a cooled CD camera (Coolsnapfx, Photometrics) and imaging software (IPLab, Scanalytics, Inc).
**Statistical Analyses**

Statistical analyses were performed for all viability, cohesion and condensation assays. Student’s T-Tests were used to assess the statistical significance differences between cell viabilities. ANOVA was used to assess the statistically significant differences in all cohesion and condensation assays. Statistical significant differences (*) are based on P < 0.05. Whereas (*) indicates statistical significance, (#) indicate P values close to significance. Comparisons resulting in P values farther above 0.05 are indicated by a lack of asterisk. Statistical analyses typically obtained from average values based on a minimum of 300 cells from three independent experiments.
**FIGURES**

**Table 1.** Yeast Strain Table

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*all strains are in W303 background*
**Figure 1.** *ELG1* deletion promotes Pds5 function (A) Percent viability of yeast strains at 23°C or 37°C during G1, S and M phase arrests. Statistically significant differences (*) based on P < 0.05 (#, P = 0.053). (B) Micrographs of sister chromatid foci (GFP) relative to DNA (DAPI) and Pds1. (C) Percent of pre-anaphase cells with precocious sister chromatid separation (#, P = 0.057). (D) DNA content of cells arrested in G1 at 23°C, then shifted to 37°C and arrested pre-anaphase. (E) Percent of pre-anaphase cells with precocious sister chromatid separation.
**Figure 2.** *ELG1* deletion fails to rescue *pds5-1* condensation defects. (A) Micrographs reveal changes in rDNA condensation as detected by Net1-GFP (GFP) and DNA counterstained with DAPI. (B) Percent of cells that contain condensed (Lines) or uncondensed (Puffs) rDNA chromatin detected using Net1-GFP (*/**, statistical differences between wildtype and *pds5-1* mutant cells and also between wildtype and *pds5-1 elg1* double mutant cells).
**Figure 3.** PCNA promotes Pds5 function in cohesion. (A) Serial dilutions of cells harboring either vector or vector directing overexpression of PCNA (*POL30*). (B) Micrographs of sister chromatids (GFP), DNA (DAPI) and Pds1. (C) Percent of cells that exhibit precocious separated sister chromatids quantified as described in Figure 1C.
Figure 4. Pds5 role in condensation appears independent of PCNA. (A) Micrographs of cells harboring either vector alone or vector directing PCNA overexpression and assessed for rDNA chromatin as described in Figure 2A. (B) Percent of cells exhibiting condensed (Lines) or uncondensed (Puffs) rDNA structures as described in Figure 2B.
Figure 5. *RAD61* deletion suppresses the condensation defect of *pds5-1* mutant cells. (A) Micrographs of rDNA chromatin structure and DNA as described in Figure 2A. (B) Percent of cells exhibiting condensed (Lines) or uncondensed (Puffs) rDNA structures as described in Figure 2B. (C) Serial dilutions of cells performed as described in Figure 3A. (D) Percent of pre-anaphase cells with precocious sister chromatid separation as described in Figure 1.
Figure 6: Model of Pds5-dependent cohesion and condensation. (A) *pds5-1* mutant cells exhibit both cohesion and condensation defects with cohesins retaining their acetylation state (Tong and Skibbens, 2014). (B) Elevated PCNA retention onto DNA (*ELG1* deletion) rescues the cohesion establishment (but not condensation) defect otherwise present in *pds5-1* mutant cells. (C) Elevated retention of an as yet unidentified factor (green star) in *RAD61* deletion strains rescues the condensation (but not cohesion) defect otherwise present in *pds5-1* mutant cells.
SUPPLEMENTAL FIGURES

**Figure S1.** ELG1 deletion rescues cohesion defects in *pds5-1* mutant cells during mitosis.

(A) DNA content of wild-type, *elg1* and *pds5-1* single mutant cells, and *pds5-1 elg1* double mutant cells. Synchronization obtained following exposure to nocodazole for 2 h at 23 °C (M 23 °C) and subsequent exposure to nocodazole for 2 h at 37 °C (M 37 °C).

(B) Representative micrographs of wild-type, *elg1* and *pds5-1* single mutant and *pds5-1 elg1* double mutant reveal the disposition of sister chromatid foci (GFP) relative to DNA (DAPI) and persistence of the anaphase inhibitor Pds1.
Figure S2. Net1-GFP detection of rDNA condensation defects in cohesin mutants. (A) DNA content obtained by Flow cytometry as described in Fig. 1D. (B) Representative micrographs of wild-type and mcd1-1 mutant cells. rDNA chromatin structure detected as described in Fig. 2A. (C) Percent of cells that contain condensed (Lines) or uncondensed (Puffs) rDNA chromatin detected using Net1-GFP. Results represent average values obtained from 200 cells obtained over two independent experiments.
Figure S3. DNA content obtained using flow cytometry shows cell synchronization in G1 following exposure to alpha factor for 3 h at 23 °C and subsequent cell synchronization in pre-anaphase (M) following exposure to nocodazole for 3 h at 37 °C.
Figure S4. DNA content assayed as described in Fig. S3.
Figure S5. DNA content assayed as described in Fig. S3.
Figure S6. DNA content assayed as described in Figure S3. See text.
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cohesin loader determines the distribution of cohesin on budding yeast


CHAPTER 4

Combinatorial Effects of $PDS5$ and $ELG1$ Mutants in Replication Progression
ABSTRACT

Cohesins play critical roles in sister chromatid cohesion, chromosome condensation, gene regulation, and DNA damage repair. There is a paucity of data, however, that suggests that cohesins significantly impact DNA replication fork progression to the extent that S phase is substantially delayed. Pds5 is a critical component of cohesins, and is essential for all cohesin-dependent functions. We previously reported deletion of replication factor ELG1 or overexpression of PCNA rescues mutant pds5-1 viability and cohesion defects. However, a role for Pds5 in DNA replication was not tested. Here, we report that pds5-1 elg1 double mutant cells exhibit a substantial delay near to the G1/S phase transition and during S phase progression. This progression defect appears independent of DNA damage. We further report synthetic lethal interactions of pds5-1 with CTF4, a DNA polymerase alpha/primase binding protein, further linking Pds5 to DNA replication. Despite the cell cycle delay, we report that early and late origins of replication are still able to fire, suggesting that S phase progression is slowed due to fork stalling or instability. These combinatorial Pds5 and Elg1 effects suggest a new model regarding an unanticipated role in DNA replication fork progression.
INTRODUCTION

DNA replication is a critical portion of the cell cycle. The cell’s genome must be accurately and efficiently replicated to ensure the survival of the resulting daughter cells.

Replication also provides a mechanism through which DNA is scanned for damage and repaired (Edenberg et al., 2014). Upon encountering DNA damage, DNA polymerase stalls and checkpoint activation both inhibits subsequent firing of replication origins and promotes assembly of fork stability factors that ensure an efficient restart once the DNA damage is repaired (Yekezare et al., 2013). High fidelity repair requires proximity of an identical DNA template. Template proximity is facilitated by cohesins, which tether together the products of DNA replication (sister chromatids) and are further recruited to sites of DNA damage to ensure efficient repair (Dorsett and Strom, 2012).

Cohesins complexes decorate an extensive portion of the genome. For instance, cohesins tether together replicated sister chromatids - binding DNA at roughly 12kb intervals in yeast from the beginning of S phase until anaphase onset of mitosis (Laloraya et al., 2000). The cohesins that promote sister chromatid cohesion must resist robust spindle forces that otherwise would precociously separate the sisters. However, very few of these chromatin-associated cohesins are actually required for cohesion, implicating that the majority of cohesins are used for other functions (Heidinger-Pauli et al., 2010). For instance, cohesins impact gene expression by associating with a host of transcription factors- revealing that cohesins are recruited to all forms of DNA from heterochromatic and silenced DNA to euchromatic and actively transcribed DNA (Dorsett and Merkenschlager, 2013; Merkenschlager and Odom, 2013). Additionally, cohesins are required for proper chromosome condensation - possibly by stabilizing DNA loops akin
to those through which cohesins regulate transcription (Guacci and Koshland, 2012; Guacci et al., 1997; Hartman et al., 2000; Tong and Skibbens, 2015). In light of the fact that cohesins bind genome-wide to produce DNA-bound complexes that can be both robust and durable, the relative paucity of data that suggest that cohesins significantly impact DNA replication fork progression is surprising. A single report identified a minor impact on fork progression by reduced cohesin modification due to mutation of the SMC3 acetyltransferase ESCO2 (human ortholog of Ctf7/Eco1). Unfortunately, characterization of the impact was limited to DNA combing: no effect on cell cycle progression or S phase progression was provided nor was the impact on fork progression localized to cohesin binding sites (Terret et al., 2009). In a second paper, S phase progression again was found to be slowed by mutation in ECO1 (Lengronne et al., 2006). However, no evidence was provided that directly linked mutation of structural cohesins to fork stalling or significant S phase progression delay.

Pds5 is a core component of cohesin that is essential for all cohesin-dependent pathways including cohesion, condensation, DNA repair, and transcription regulation (Gause et al., 2010; Hartman et al., 2000; Panizza et al., 2000; Ren et al., 2008; Tong and Skibbens, 2014; Tong and Skibbens, 2015; Zhang et al., 2007). Numerous lines of evidence suggest that some of these activities may be coupled to or depend on the DNA replication fork. For instance, Pds5 interacts with Ctf7 during S phase to regulate cohesion establishment (Chan et al., 2013; Noble et al., 2006; Rowland et al., 2009; Sutani et al., 2009). Pds5 is also sensitive to the dosage of DNA replication factors such as RFC Elg1 and PCNA (Maradeo et al., 2010; Tong and Skibbens, 2015). During further characterization of PDS5 mutant cells, we discovered that Pds5 plays a role in
DNA replication that is coordinated with PCNA. Here, we characterize a unique cell cycle delay phenotype thus far observed only in $pds5-1\, elg1$ double mutant cells. This S phase progression delay is independent of a DNA damage response. Given that chromatin remodeling, critical to confer cell identity, is tightly coordinated with DNA replication (Ma et al., 2015; Singh, 2014), our findings raise the possibility that decoupling of replication from transcription may contribute to cohesinopathic maladies such as Roberts Syndrome and Cornelia de Lange Syndrome.
RESULTS

*pds5-1 elg1* cells have a cell cycle delay

Temperature sensitivity of mutant *pds5-1* cells can be bypassed by either deletion of *ELG1* or elevated levels of PCNA (Maradeo et al., 2010; Tong and Skibbens, 2015). However, the mechanism through which Elg1/PCNA impacts Pds5 function remains entirely unknown. Importantly, the rescue of *pds5-1* by the *elg1*/*PCNA* pathway is unique in that other cohesin mutations combined with *elg1*/*PCNA* exhibit exacerbated phenotypes. For example, *mcd1-1 elg1* mutants exhibit significant growth defects even at permissive temperature (Maradeo and Skibbens, 2009). In pursuing further characterization of Pds5 function during S phase, log phase wildtype, *pds5-1*, *elg1*, and *pds5-1 elg1* strains were synchronized in G1 (alpha factor arrest) at the permissive temperature of 23°C. Cells were then released from G1 arrest and moved to fresh YPD media supplemented with nocodazole to synchronize in pre-anaphase at non-permissive temperature of 37°C. Samples were collected every 30 minutes for flow cytometry (Figure 1A). Wildtype cells progressed as expected through the cell cycle, with cells progressing into S phase within 60 minutes and fully synchronizing in M phase in approximately 90 minutes. *pds5-1* and *elg1* cells similarly synchronized in M phase in approximately 90 minutes. This is in contrast to previous reports that *elg1* mutant cells have a slight (20 minute) cell cycle delay (Bellaoui et al., 2003). *pds5-1 elg1* cells, however, failed to exhibit any noticeable change in DNA content via flow cytometry until an hour from alpha factor release. Moreover, full synchronization in M phase required 3
hours. Thus, *pds5-1 elg1* double mutant cells exhibit a significant delay in cell cycle progression compared to wildtype and single mutant strains.

We were concerned that the observed cell cycle delay in *pds5 elg1* double mutant cells might be due to hyper-sensitivity to the arrest strategy (alpha-factor). To test this possibility, we analyzed cell cycle progression using time-lapse video sequences of unsynchronized and naturally dividing cells. Log phase wildtype and *pds5-1 elg1* cells were diluted and placed on high nutrient YPD agar maintained at 37°C and individual cells tracked and imaged using Biostation IM (Nikon). Cells were recorded and video playback analyzed to measure the length of time required to progress from initial bud formation (S phase entry) to cytokinesis (mitotic exit). Wildtype cells required only 52 minutes, ranging between 48-60 minutes to progress from bud emergence to cytokinesis. In contrast, *pds5-1 elg1* cells required 82 minutes, ranging between 78-90 minutes, to complete the same bud emergence-to-cytokinesis sequence (Figure 1B). Thus, *pds5 elg1* double mutant cells exhibit a significant cell cycle delay that occurs independent of cell synchronization or alpha factor sensitivity.

The cell cycle delay of *pds5-1 elg1* mutant cells is independent of Rad53 DNA damage response

The cell cycle delay exhibited solely in the *pds5-1 elg1* double mutant cells, and not in the *pds5-1* or *elg1* single mutants, implies a combinatorial effect. Despite evidence that *elg1* mutant cells exhibit relatively normal replication kinetics in our background, we considered the possibility that the combined inactivation of both Pds5 and Elg1 produces DNA damage which impedes the replication fork (Segurado and Tercero, 2009). To test
this possibility, log phase wildtype and *pds5-1 elg1* double mutant cells were
synchronized in G1 and released into 37°C fresh YPD supplemented with nocodazole.
Samples were taken every 20 minutes after release from G1 arrest and DNA content
assessed by flow cytometry. In parallel, cell samples were harvested, lysed, and the
resulting protein samples assessed for Rad53 phosphorylation which is an indicator of
DNA damage response activation (Allen et al., 1994; Sun et al., 1996). Wildtype cells
entered S phase as soon as 20 minutes after release from G1 synchronization, and fully
arrested with a 2C DNA content by 80 minutes. In contrast, *pds5-1 elg1* cells did not
progress into S phase until almost 100 minutes after release from G1, finally arresting in
M phase, 180 minutes after release from alpha factor. We then compared Rad53
phosphorylation at timepoints matched based on DNA content assessed using flow
cytometry (Figure 3A). As expected, wildtype cells did not exhibit elevated levels of
Rad53 phosphorylation throughout the course of the experiment. Similarly, *pds5-1 elg1*
double mutant cells exhibited Rad53 phosphorylation levels identical to those of wildtype
cells as similar stages of cycle progression (Figure 3B). The absence of Rad53
phosphorylation, however, does not exclude the possibility that DNA damage is present
and that *pds5-1 elg1* double mutant cells are deficient in appropriate responses to DNA
damage. To test this possibility, we compared the response of wildtype and *pds5-1 elg1*
double mutant cells after exposure to methyl methanesulfonate (MMS). The results show
that, similar to wildtype cells, *pds5-1 elg1* double mutant cells fully retain the ability to
respond to MMS and phosphorylate Rad53, even at non-permissive temperature (Figure
3C). In combination, these results reveal that *pds5-1 elg1* double mutant cells exhibit a
significant cell cycle delay that occurs independent of overt DNA damage.
Cell cycle delay does not rescue viability of \textit{pds5-1} mutants

Deletion of \textit{ELG1} rescues viability of \textit{pds5-1} in part by suppressing the cohesion defect otherwise present (Tong and Skibbens, 2015). How is this cohesion defect suppressed? Prior studies revealed that Pds5 functions during DNA replication to promote cohesion establishment (Chan et al., 2013; Noble et al., 2006). We postulated that a prolonged S phase might therefore provide additional time for cohesion establishment to occur. To emulate a slow S phase progression, serial dilutions of log phase wildtype and \textit{pds5-1} cells were plated on rich medium that contain a range of hydroxyurea concentrations (0.1M, 0.05M, and 0.01M concentrations). The plated cells were then incubated at 23°C and 37°C for 3 days. As expected, wildtype cells grew robustly at both temperatures and at all hydroxyurea concentrations except for 0.1M concentration - which is known to block DNA replication (Jong et al., 1995). \textit{pds5-1} cells grew at rich medium maintained at 23° but were inviable at 37°C. Importantly, the addition of hydroxyurea failed to suppress \textit{pds5-1} growth defects at 37°C. Thus, simply slowing S phase progression does not appear to be the mechanism through which \textit{ELG1} deletion rescues \textit{pds5-1} temperature sensitivity.

We decided to pursue a complementary approach to test whether an extended S phase was beneficial to \textit{pds5-1} mutant cells. Ctf4 recruits replication initiation factors, MCM complexes and DNA polymerase alpha to chromatin (Zhu et al., 2007). \textit{ctf4} mutants at elevated temperatures exhibit delayed entry into S phase and also progress through S phase slowly, compared to wildtype (Wang et al., 2010), mimicking the delay seen in \textit{pds5-1 elg1} cells. We decided to test whether we could recapitulate the cell cycle delay of \textit{pds5-1 elg1} double mutant cells in \textit{pds5-1 ctf4} double mutant cells. \textit{pds5-1} and
ctf4 mutant strains were crossed, diploids selected and sporulated. The resulting tetrads were then dissected. Of the 27 tetrads dissected, wildtype (13), pds5-1 (14), and ctf4 (20) strains were recovered at expected frequencies. However, we were unable to recover pds5-1 ctf4 double mutant cells (Table 1). These results reveal a novel synthetic lethality between mutations in a core cohesin subunit and a DNA polymerase alpha-binding protein.

**Replication fork progression is impeded in pds5-1 elg1 cells**

The severity of the S phase progression defect in pds5-1 elg1 mutant cells is especially intriguing. The basis of this defect could arise through one of several mechanisms. For instance, pds5-1 elg1 double mutant cells could either fail to initiate replication or instead deregulate origin firing. Conversely, origin firing might be normal and pds5-1 elg1 double mutant cells are deficient in fork stability. To differentiate between these possibilities, log phase wildtype, pds5-1, elg1 single mutant cells, and pds5-1 elg1 double mutant cells (competent for thymidine analog 5-bromo-2′-deoxyuridine (BrdU) uptake) were synchronized in G1 at 23°C and then released into 37°C fresh YPD supplemented with nocodazole and BrdU. Samples were taken every 15 minutes and analyzed for DNA content by flow cytometry (Figure 4A). In parallel, cells were analyzed for BrdU incorporation using chromatin-immunoprecipitation (Viggiani and Aparicio, 2006; Viggiani et al., 2010). We tested for origin firing at both early (ARS607) and late (ARS609) sites (Lai et al., 2012). Wildtype cells exhibit a gradual rise in BrdU incorporation at the early origin of replication, peaking at 75 minutes. Similar results are also seen in pds5-1 and elg1 cells, with BrdU enrichment at ARS607 peaking
at 75 minutes. Interestingly, *pds5-1 elg1* cells exhibit identical BrdU incorporation kinetics at the early origin of replication (Figure 2B). Thus, there is no observable effect on early origin of replication firing in *pds5-1 elg1* mutants.

At a late origin of replication (ARS609), wildtype cells exhibit BrdU integration later in S phase progression; rising at 30 minutes after G1 release, and increasing as the cells progress through S phase. *pds5-1* cells exhibit a gradual increase in BrdU IP efficiency at ARS609 similar to wildtype. Surprisingly, while *elg1* cells exhibit BrdU incorporation at 30 minutes, BrdU IP efficiency only increases at 90 minutes, even though no cell cycle delay was observed. Similarly, *pds5-1 elg1* cells are able to incorporate BrdU at ARS609 30 minutes after release from G1 arrest. However, *pds5-1 elg1* cells show no increase in late origin BrdU integration, even after 90 minutes (Figure 4B,C). Thus, *pds5-1 elg1* cells are fully competent to initiate replication from early and late origins of replication. The lack of BrdU incorporation over time, however, indicates that *pds5-1 elg1* mutant cells are unable to continue replication – consistent with a defect in fork stability.
DISCUSSION

*PDS5 genetic interactions support a role for cohesins in DNA replication*

Pds5 is a cohesin subunit required for the maintenance of cohesion and condensation (Hartman et al., 2000; Panizza et al., 2000; Stead et al., 2003; Tong and Skibbens, 2014; Tong and Skibbens, 2015). One of the revelations of the current study is the extent to which *PDS5* genetically interacts with DNA replication factor genes. For instance, *pds5-1* mutant cell growth and cohesion defects are both rescued by either *ELG1* deletion or *PCNA* overexpression (Maradeo et al., 2010; Tong and Skibbens, 2015). These positive genetic interactions so far are unique to other core cohesin genes (Maradeo et al., 2010; Maradeo and Skibbens, 2009). We further report interactions between *PDS5* and *CTF4*, although here the genetic interaction is negative to produce synthetic lethality. *ELG1* and *CTF4* are also synthetically lethal (Ben-Aroya et al., 2003). Intriguingly, *CTF4* (*AND-1* in vertebrates) promotes replication initiation through the tethering recruitment of MCM helicases to POL alpha primase complex (Wang et al., 2010; Zhu et al., 2007). In combination, these findings are consistent with a new and novel role for Pds5 in DNA replication.

*Mutant PDS5 in combination with loss of ELGI results in fork stalling*

Despite the prevalence of binding throughout the genome, and tenacity of binding in specific instances, there is little data reported previously that structural cohesins significantly impact DNA replication fork progression. A second major revelation of the current study is that S phase progression in *pds5-1 elg1* double mutant cells is
significantly delayed and that this delay thus far appears unique to other cohesin mutations. S phase progression defects could arise either through the inability to initiate replication or maintain fork stability during DNA replication. Our findings reveal that both early and late origins fire in \emph{pds5-1 elg1} double mutant cells, albeit at reduced levels, which suggests that the S phase progression defect is most likely due to fork stalling. There are 4 mechanisms through which DNA replication forks stall - DNA polymerase mutations, DNA damage checkpoint activation, reduction of nucleotide pools, or a replisome impediment (Edenberg et al., 2014). Here, we exclude the first two of these scenarios given that \emph{pds5-1 elg1} double mutant cells do not harbor polymerase mutations nor do we detect overt activation of the Rad53 DNA damage response pathway. While we cannot formally exclude a model that \emph{pds5-1 elg1} cells are deficient in nucleotide metabolism, there is no evidence to support such a model. In contrast, the role of Pds5 in the assembly of high-order chromatin-bound complexes (i.e. cohesins) is well documented (Hartman et al., 2000; Panizza et al., 2000). Thus, we favor a model that \emph{pds5-1 elg1} double mutant cells form defective cohesin complexes that impede fork progression.

\textbf{Models of cohesins impeding fork progression}

How might cohesins form a barrier that is unique to \emph{pds5-1 elg1} double mutant cells? We previously reported that \emph{pds5-1} cells still retain the majority of chromatin-bound cohesins. However, these cohesins are non-functional in cohesion and condensation, suggesting that these cohesins are not properly regulated (Tong and Skibbens, 2014; Tong and Skibbens, 2015). We speculate that these non-functional
cohesins may be hyper-stable and thus form a structural barrier through which the DNA replication fork is unable to proceed - resulting in replication stress. Normally, cells deficient in Pds5 are able to circumvent this stress - but how? PCNA has a large role in replicative stress, and is modified by ubiquination or SUMOylation to promote DNA damage bypass, translesion synthesis, or template switching (Edenberg et al., 2014; Fox et al., 2011). Interestingly, Elg1 aids in removal of SUMOylated PCNA and deubiquination of PCNA (Fox et al., 2011; Parnas et al., 2010). These PCNA modifications must be properly regulated, to ensure fork stability (Mailand et al., 2013).

Based on our findings that S phase progression is significantly slowed only when *pds5-1* is coupled to *elg1* (PCNA overexpression), we posit that elevated levels of chromatin-bound PCNA dilute out those modifications that would otherwise promote translesion synthesis and fork progression.

An alternative model to cohesin-barriers is that the observed, S phase progression defects could occur due to the inability to resolve processes behind the replication fork. There is some evidence that DNA replication fork progression is impacted by defects in Eco1/Ctf7 (Terret et al., 2009). Pds5 interacts with Ctf7 and appears to play a critical role during cohesion establishment (Noble et al., 2006; Rowland et al., 2009; Sutani et al., 2009). In this scenario, Pds5 defects may impact cohesion, resulting in replication stress behind the fork causing fork stalling. In support of this model is evidence that cohesion establishment occurs behind the replication fork. Ctf7 associates with helicase Chl1 and flap-endonuclease Fen1, both of which promote lagging strand maturation (Rudra and Skibbens, 2012). Additionally, Elg1 interacts with Fen1, indicating a role of Elg1 in Okazaki fragment maturation (Kanellis et al., 2003). PCNA is also ubiquinated when
Okazaki fragments are unligated, resulting in replication fork stalling (Nguyen et al., 2013). It will be important in future studies to test the extent through which this combination of inefficient cohesion establishment and defective Okazaki fragment maturation results in replicative stress and fork instability. In summary, the models posited here provide new insights into potential targets for cohesinopathies, several of which appear to be associated with DNA replication mechanisms (Pehlivan et al., 2012).
MATERIALS AND METHODS

Yeast strains, Synchronization of Log Phase Cells and Flow Cytometry

Yeast strains used in the current study are listed in Table 2. Synchronization of yeast cultures and assessment of DNA content by flow cytometry were performed as previously described (Tong and Skibbens, 2014).

Live Cell Imaging

Cells in log phase growth were normalized to 0.2 OD₆₀₀ and plated on glass bottom microwell dishes, with YPD agar plug on top of plated cells (MatTek). Cells maintained at 37°C in a humidified chamber were imaged using Biostation IM (Nikon). Phase contrast images were obtained using an 80x objective and collected at 2 minute intervals for 5 hours. S phase entry was scored by identifying the video frame in which bud emergence was first detectable and cytokinesis was scored by identifying the video frame image in which lateral independent movements between daughter and parent cell were apparent.

DNA Damage and Rad53 Phosphorylation Assay

Wildtype and pds5-1 elg1 mutant strains were grown to log phase, normalized to 0.2-0.3 OD₆₀₀, and then incubated for 2.5 hours at 23°C in rich YPD medium supplemented with alpha-factor to arrest cells in G1. The resulting cells were harvested, washed in fresh medium before resuspension in fresh media supplemented with nocodazole and incubated at 23°C or 37°C for 3 hours. Cells were collected at 20 minute
intervals and assessed for DNA content by flow cytometry. Cells were also collected at 20 minute intervals for protein analysis as previously described (Tong and Skibbens, 2014). Western blot analysis to assess the level of Rad53 modification was performed using Goat-anti-Rad53 (Santa Cruz, yC-19), Donkey-anti-Goat HRP secondary and ECL Plus (GE).

**BrdU Incorporation and ChIP**

Wildtype, *pds5-1, elg1, and pds5-1 elg1* cells were transformed with p403-BrdU-Inc vector (Viggiani and Aparicio, 2006). Resulting wildtype, *pds5-1, elg1, and pds5-1 elg1* strains were grown to log phase, normalized to 0.2-0.4 OD$_{600}$, and then incubated for 2.5 hours at 23°C in rich YPD medium supplemented with alpha-factor to arrest cells in G1. The resulting cells were harvested, washed in fresh medium before resuspension in fresh media supplemented with nocodazole and incubated at 23°C or 37°C for 90 minutes. Cells were harvested at 15 minute intervals and BrdU-labeled DNA immunoprecipitated and analyzed by PCR as previously described with the following modifications (Viggiani et al., 2010). Briefly, cells were mechanically lysed (Bead-beater, BioSpec) in the presence of Lysis Buffer (100mM Tris pH 8.0, 50mM EDTA, 1% SDS). Supernatant was collected and DNA was sheared by sonication. DNA was extracted from lysates using 25:24:1 phenol:chloroform:isoamylalcohol prior to precipitation in ethanol. DNA was purified using QIAquick PCR Purification Kit (QIAGEN) and eluted in TE (pH 7.6). BrdU incorporated DNA enrichment was obtained by incubating DNA extracts with Agarose conjugated BrdU (Santa Cruz, II B5) overnight at 4°C. Beads were collected by centrifugation, washed with IP Buffer (0.0625%
TritonX-100, PBS) and TE (pH 7.6). Bead-bound DNA was harvested using Elution Buffer (TE pH 7.6, 1% SDS) and purified using NucleoSpin PCR Clean-up Kit (Macherey-Nagel). DNA was analyzed by PCR using primers to ARS607 and ARS609 (Lai et al., 2012). PCR products were resolved using 1.1% agarose gels, and histograms of pixel densities quantified in Photoshop. IP efficiency was calculated as ratio of pulldown (minus background obtained from no BrdU control) over total chromatin minus background.
FIGURES

Table 1. *pds5-1 ctf4* is synthetically lethal.

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<td><em>ctf4::HIS</em></td>
<td>20</td>
<td>27</td>
</tr>
<tr>
<td><em>pds5-1 ctf4::HIS</em></td>
<td>3*</td>
<td>27</td>
</tr>
<tr>
<td>Dead</td>
<td>58</td>
<td>0</td>
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(*) Cells were unable to grow on new YPD plates after colony purification
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<td>Tong and Skibbens, 2014</td>
</tr>
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<td>LEU2:tetR-GFP&lt;br&gt;TRP1:PDS1-MYC13 pds5-1 &lt;br&gt;elg1::KAN</td>
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*All strains are in W303 background*
Figure 1. *pds5-1 elg1* mutant cells exhibit a cell cycle delay. (A) Flow cytometry analyses of DNA content in wildtype, *pds5-1* and *elg1* single mutant cells and *pds5-1 elg1* double mutant cells. Log phase cells were synchronized in G1 (alpha factor) at permissive temperature of 23°C for 2.5 hours, then released into 37°C fresh YPD supplemented with nocodazole to arrest cells pre-anaphase. Samples were collected every 30 minutes. (B) Live cell imaging of wildtype and *pds5-1 elg1* cells on Biostation IM (Nikon). Video frames were analyzed to determine the time interval between initial budding and cytokinesis.
Figure 2. Cell cycle delay in pds5-1 elg1 double mutant cells occurs independent of the Rad53-dependent DNA damage response. (A) Flow cytometry analyses of DNA content of wildtype and pds5-1 elg1 mutant cells. Log phase cells were synchronized in G1 (alpha factor) at permissive temperature of 23°C for 2.5 hours, then released into 37°C fresh YPD supplemented with nocodazole to arrest cells pre-anaphase. Cell samples were collected every 20 minutes. Samples shown were selected to represent similar levels of cell cycle progression. (B) Rad53 phosphorylation state monitored by Western upon release of cells from G1 arrest. Samples shown were selected to represent similar levels of cell cycle progression based on 2A. (C) Wildtype and pds5-1 elg1 cells are competent to phosphorylate Rad53 in response to DNA damage (MMS) at both 23°C and 37°C, but do not phosphorylate Rad53 in the absence of MMS.
**Figure 3.** Viability is not rescued by fork stalling. (A) Serial dilutions of wildtype and *pds5-1* cells plated on YPD rich medium plates supplemented increasing levels of hydroxyurea (HU). Plates were incubated at 23°C or 37° and grown for 3 days.
**Figure 4.** *pds5-1 elg1* double mutant cells exhibit reduced levels of late origin of replication firing (A) Flow cytometry analyses of wildtype, *pds5-1*, *elg1*, and *pds5-1 elg1* mutant cells. Log phase cells were synchronized in G1 (0) at 23°C, then released into 37°C YPD rich medium supplemented with nocodazole. Samples were collected every 15 minutes and assessed for DNA content. (B) BrdU chromatin-immunoprecipitation of early (ARS607) and late (ARS609) origins of replication. (C) Representative gel of BrdU ChIP.
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CHAPTER 5

Concluding Remarks and Future Directions
Cohesins are critical for multiple cellular pathways. The primary function of cohesins is the tethering of sister chromatids together to maintain proper pairing after replication and through mitosis. Mutations in cohesins result in segregation defects and aneuploidy, a hallmark of cancer cells. However, cohesins also affect chromosome condensation, with many cohesin mutations in yeast having condensation defects (Guacci and Koshland, 2012; Guacci et al., 1997; Hartman et al., 2000; Tong and Skibbens, 2014). Additionally, cohesins have been implicated in gene regulation and transcription (Dorsett, 2007; Dorsett, 2011). Finally, cohesins and its regulators alike have genetic and physical interactions with several replication factors, implying a link between cohesins and replication (Maradeo et al., 2010; Maradeo and Skibbens, 2009; Maradeo and Skibbens, 2010; Moldovan et al., 2006; Rudra and Skibbens, 2012; Tong and Skibbens, 2015). With the assortment of functions cohesins have a role in - it is imperative to understand how cohesins and their associated factors affect each pathway.

**Cohesin structure is still unknown**

The structural basis through which cohesins tether sister chromatids together remains highly debated. Early studies suggest that coiled-coil domains residing between bound heads and hinges of Smc proteins remain flexible and kink out to form a lumen (Gruber et al., 2003; Haering et al., 2002; Haering et al., 2004). This gave rise to the notion of cohesins as huge rings that entrap DNA, even two DNA molecules, within a single lumen (Nasmyth and Haering, 2009). However, this "one-ring entrapment" model
makes very bold predictions on how cohesins must function. Fortunately, these predictions can be readily and systematically tested.

The "one-ring" model requires that cohesion loss results from one or both sisters dissociate from cohesin. However, this does not appear to always be the case. Pds5 is an essential cohesin protein that is critical for cohesion maintenance, and numerous studies document that inactivation of Pds5 during mitosis results in severe cohesion defects (Hartman et al., 2000; Tong and Skibbens, 2014). However, cohesins still remain chromatin-bound when Pds5 is inactivated during mitosis (Kulemzina et al., 2012; Tong and Skibbens, 2014). We further excluded the possibility that this population of chromatin-bound cohesin was newly deposited in that it remained acetylated - a modification that occurs during S phase (Tong and Skibbens, 2014). Thus, the one ring model is not sufficient to explain the mechanism through which sister chromatid cohesion is maintained.

Besides the "one-ring" model, several other models have been posited consisting of multiple rings or higher order cohesin structures (Huang et al., 2005). The "two-ring" models suggest that instead of a single ring entrapping both chromatids, each chromatid is decorated with cohesins, which are then linked via cohesin-cohesin interactions. For instance, the "handcuff" model suggests that cohesin rings are interlocked (Zhang and Pati, 2009). Another possible formation would be cohesin-cohesin interactions without interlocking rings. The human homolog of Mcd1 (Rad21) interacts with each other, providing evidence that two cohesin complexes are linked together (Zhang et al., 2013). In yeast, cross-linking studies were used to interactions between Mcd1, Smc3, and Smc1, and it would be of great interest to expand on this method to test if Mcd1 can cross-link
with another Mcd1 subunit (Gligoris et al., 2014; Haering et al., 2008; Huis in ’t Veld et al., 2014). If cohesin subunits do interact in a more varied way than currently postulated, it would provide a much greater insight into the various functions cohesins.

**Identifying and mapping cohesin-dependent pathways**

Every cohesin subunit and regulator mutation tested to date produces both cohesion and condensation defects (Guacci and Koshland, 2012; Guacci et al., 1997; Hartman et al., 2000). Despite the fact that cohesin mutations result in both cohesion and condensation defects, these two processes now appear separable and each pathway is independently regulated (Guacci and Koshland, 2012; Orgil et al., 2015; Tong and Skibbens, 2015). Our studies reveal that overexpression of PCNA (or by proxy, deletion of *ELG1*) rescues cell viability and cohesion of *PDS5* mutants, whereas deletion of *RAD61* results in a rescue of condensation, but not viability (Tong and Skibbens, 2015). With distinct regulations that bias cohesin function towards cohesion or condensation, we posit a model that the context, and possibly the components of a cohesin complex, is critical to drive cohesins to perform various and different functions. For example, *RAD61* deletion promotes condensation pathways, and rescues condensation defects of cohesin mutants (Guacci and Koshland, 2012; Tong and Skibbens, 2015). Rad61 forms a complex with Pds5, which is displaced after acetylation of Smc3, suggesting a change in cohesion components after Smc3 is modified (Rowland et al., 2009a; Sutani et al., 2009). Further, a recent study shows that Scc3 has distinct domains required for cohesion and condensation, which are separable (Orgil et al., 2015). These findings reveal that altering the factors that assemble into a cohesin complex can alter which pathway cohesins
function in. Exploring the stoichiometry of cohesin components and correlating these changes in cohesin composition to cohesin function would be critical in further elucidating the true structure(s) of cohesin.

In addition to different combinations of cohesin subunits, post-translational modifications also appear to significantly alter cohesin function. Many cohesin subunits are modified by various molecular groups (Rudra and Skibbens, 2013b). For instance, Pds5 is modified by SUMOylation, which promotes cohesion maintenance (Stead et al., 2003). Mcd1 is phosphorylated and acetylated to re-establish cohesion in the presence of DNA damage (Heidinger-Pauli et al., 2008; Heidinger-Pauli et al., 2009; Unal et al., 2007). Finally, Smc3 is acetylated at evolutionarily conserved lysines, which promotes cohesion (Milutinovich et al., 2007; Rolef Ben-Shahar et al., 2008; Rowland et al., 2009a; Unal et al., 2008). A systematic screen of mutations at modifiable sites and correlating these modifications to function would provide a great deal of information of which modifications are required in determination of cohesin "fate".

If cohesins exhibit functional differences in both subunit assembly and post-translational modifications, how do cohesin roles change in response to DNA replication? The finding that deletion of ELG1 from pds5-1 cells results in a rescue of cohesion raises the possibility that PCNA may directly promote cohesion outside of S phase. Overexpression of PCNA only in M phase in pds5-1, or degradation of PCNA during M phase in a pds5-1 elg1 strain, would test a novel function of PCNA that is currently undocumented. Alternatively, the role of PCNA in establishment could be more intricate than previously believed. Overexpression of PCNA in ctf7 also results in a restoration of viability (Skibbens et al., 1999b). The nature of these rescues, even after a decade,
remains surprisingly unknown. Given the similar rescue of viability in both *ctf7 elg1* and *pds5-1 elg1* double mutant cells (Maradeo et al., 2010; Maradeo and Skibbens, 2009; Maradeo and Skibbens, 2010; Tong and Skibbens, 2015), it is worth speculating how elevated PCNA retention onto DNA stabilizes newly deposited cohesins and promotes cohesion establishment even in cells diminished in Ctf7 or Pds5 function. Excess PCNA could act as a steric barrier, preventing cohesins from sliding from specified CAR sites and thus promote registration that is important for cohesin-cohesin tethering reactions. Alternatively, PCNA could directly interact with cohesins such that PCNA on one sister (the lagging strand) interacts with cohesins on the opposing sister to transiently maintain cohesion until bipolar attachment occurs - thus ensuring high fidelity chromosome transmission (Guacci and Koshland, 2012). The exploration of PCNA and its function in cohesion pathways will provide important new insights into the linked processes of replication and cohesion.

**Possible role of cohesin components independent of cohesins**

We revealed that *pds5-1 elg1* has a cell cycle delay in G1-to-S phase progression (Tong and Skibbens, unpublished). While our findings are consistent with a cohesin "barrier" model that impedes fork progression, it may also be possible that the S phase delay is cohesin independent. To address these possibilities, an important first step would be to prevent cohesin loading (induced degradation of Scc2 or, more specifically, Mcd1) in *pds5-1 elg1* to test whether the S phase delay is facilitated by cohesins. A finding that the delay persists even in the absence of chromatin bound cohesins would provide novel evidence of a cohesin subunit regulating a process outside of the cohesin complex.
There are several possible candidates for Pds5 function apart from cohesins. For instance, cohesins, condensins, and the DNA repair complex all contain SMC heterodimers (Smc1,3 and Smc2,4 and Smc5,6 respectively) (Jeppsson et al., 2014b). Pds5 might aberrantly associate with other SMC complexes and impede progression of the DNA replication fork. Induced degradation of Smc2 or Smc5 would similarly test for roles of other SMC complexes in producing a cell cycle delay. Alternatively, we may find that the cell cycle delay is independent of any SMC complex. Here, we would pursue a model that Pds5 interacts directly with DNA polymerase or other replication initiation factors, a model predicated on \textit{pds5-1} synthetic lethality with \textit{ctf4} (Tong and Skibbens, unpublished). Pds5 also binds Top2 isomerase, which appears independent of cohesion and thus supports a model that Pds5 may directly impact DNA metabolism factors outside of cohesin pathways (Aguilar et al., 2005).

\textbf{Pds5 can be used to characterize cohesin-dependent functions}

While our studies focused on the mutant \textit{pds5-1}, different \textit{pds5} mutants result in cohesion defects but exhibit phenotypes that differentiate between Pds5 functions. For example, \textit{pds5-1}, \textit{pds5-99}, and \textit{pds5-101} all exhibit cohesion defects, yet each exhibit distinct phenotypes (Hartman et al., 2000; Panizza et al., 2000; Stead et al., 2003). For example, \textit{pds5-1} and \textit{pds5-101} mutants still retain chromatin bound Mcd1, though the soluble pool of Mcd1 is depleted in \textit{pds5-1} mutants (Chan et al., 2013; D'Ambrosio and Lavoie, 2014; Hartman et al., 2000; Tong and Skibbens, 2014). \textit{pds5-99} mutants, however, do exhibit defects in retention of Mcd1 to chromatin (Panizza et al., 2000). Additionally, \textit{pds5-101} and \textit{pds5-99} mutants show a reduction in Smc3 acetylation,
indicating a defect in facilitating Ctf7 establishment function, whereas \textit{pds5-1} does not (Chan et al., 2013; Tong and Skibbens, 2014). In contrast, \textit{pds5-r10} has a smaller cohesion defect than compared to others, and rescues \textit{ctf7} mutant temperature sensitivity, opposite of the synthetic lethality seen in \textit{pds5-1 ctf7-203} (Noble et al., 2006; Sutani et al., 2009). Further characterizing other \textit{pds5} mutants is likely to reveal important details of the processes of cohesin loading, establishment, and maintenance in a genetic system in which each process can be studied in isolation, similar to our studies of \textit{pds5-1} roles of cohesion maintenance from condensation (Tong and Skibbens, 2015).

\textbf{Implications of future studies}

Understanding the roles of cohesins is crucial in understanding many underlying causes of several diseases. Cohesinopathies such as Roberts Syndrome, SC-Phocamelia, and Cornelia deLange show a wide range of symptoms, ranging from mental disorders to severe cranial facial and malformations. The segregation defects of cohesin mutants can also lead to aneuploidy, a hall mark of many cancers. While cohesinopathies are primarily due to mutations in cohesins, not all mutations result in segregation defects (Bose and Gerton, 2010; Liu and Krantz, 2008; Mehta et al., 2013; Skibbens et al., 2013). Therefore, studying the roles of cohesins outside of sister chromatid cohesion is even more crucial to understand the molecular basis of many of these developmental diseases.

\textit{Pds5} remains a powerful tool from which to understand various functions of cohesins, especially outside of cohesion. A great advantage is being able to study the process of cohesion maintenance without altering the loading or retention of cohesins onto DNA (Tong and Skibbens, 2014). Furthermore, we have shown separable pathways
of cohesion and cohesin-dependent condensation, revealing the ability to bypass the individual functions in yeast (Tong and Skibbens, 2015). The ability to isolate and study cohesin-associated pathways in budding yeast represents a critical advancement, as we begin to better mimic cohesin regulation in higher eukaryotes and humans. For example, Pds5 homolog knockdowns in mice exhibit developmental defects similar to CdLS, yet do not show cohesion defects (Zhang et al., 2009; Zhang et al., 2007). Additionally, Pds5 and its human orthologs reveal changes in transcriptional regulation, implicating those changes to cancer cell progression (Denes et al., 2010; Maffini et al., 2008; Ren et al., 2008). Elucidating the various functions of cohesins and their regulators in a simpler model organism will make recognizing, understanding, and treating developmental diseases much easier, and will advance the field that much quicker moving forward.
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191


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Publications


Posters

3. Tong, Kevin and Robert V. Skibbens (2012) " Cell Cycle Dependent Separation of Function in the Cohesion Pathway."
5. Tong, Kevin and Robert V. Skibbens (2014/2015) “Sister chromatid cohesion and chromosome condensation are separable pathways”

Seminar Presentations

1. Tong, Kevin (2011) “Two Diseases, a Protein, and a Ph.D Student: Studying Cancer and Developmental Disorders in Yeast”
2. Tong, Kevin (2013) "Sister Chromatids: If You Like ‘Em, You Should’ve Put Some Rings On ‘Em"