Pds5 regulators segregate cohesion and condensation pathways in *Saccharomyces cerevisiae*

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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 reveal that cohesion and condensation are separable pathways and regulated in nonredundant mechanisms. These results are discussed in terms of a new model through which cohesion and condensation are spatially regulated.

cohesin | Elg1 and PCNA | Rad61/WAPL | chromosome condensation | sister chromatid cohesion

DNA tethering activities are tightly regulated and impact numerous cellular processes. For instance, the tethering together of two DNA molecules such as sister chromatids is critical for both high-fidelity chromosome segregation during mitosis and DNA repair. Intramolecular tetherings, on the other hand, generate loops that are critical for chromosome condensation and bring into registration elements required for transcription regulation (1, 2). Cohesins participate in all of these tethering activities. A cohesin complex in part contains Smc1, Smc3, and Mcd1/Scc1 (3). Smc1 and Smc3 dimerize through both hinge-hinge and head binding (4–7). Mcd1/Scc1 caps the SMC head domains and subsequently recruits additional cohesin factors Irr1/Scc3 and Pds5, all of which are essential for cohesion and cell viability (8–11).

Cohesins are of clinical interest due to the fact that mutations lead to precocious sister chromatid separation, chromosome condensation defects, 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) (12). Complicating analyses of cohesin structure-to-function is the likely superimposition of competing posttranslational modifications (SUMOylation, ubiquitination, phosphorylation, and acetylation) through which cohesins may be directed toward one process over another (13). Intriguingly, Ctf7/Eco1 acetylates Smc3 and is required for both cohesion and condensation (14–18). In fact, all cohesion factors tested to date, when mutated, exhibit defects in cohesion and condensation (8, 9, 14, 15, 19), 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 (12, 20). 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 provide a broader understanding of the 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 (19, 21). In contrast, deletion of *ELG1* suppresses *ctf7/eco1* mutant cell conditional growth and cohesion defects (22). 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 (9, 11, 23–31). Our laboratory 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 (32). Here, we exploit these suppressors to isolate, to our knowledge for the first time, Pds5 roles in both cohesion and condensation, findings from which we posit that temporally defined and spatially positioned cues regulate cohesin functions.

Significance

Chromosomes are replicated during S phase to produce sister chromatids that remain tethered together by protein complexes termed cohesins. In preparation for mitosis, sister chromatids condense into discrete, compact chromosomes, a process that also requires cohesins. At anaphase onset, cohesins are inactivated, allowing each chromatid to segregate away from its sister and into newly forming daughter cells. The requirement for cohesin in both cohesion and condensation suggests that these pathways are intimately coupled. Here, we identify cohesin regulators through which cohesin functions for the first time can be isolated from one another. These regulations will be critical for elucidating post-translation modifications through which cohesin roles in chromatin metabolism can be studied in isolation.
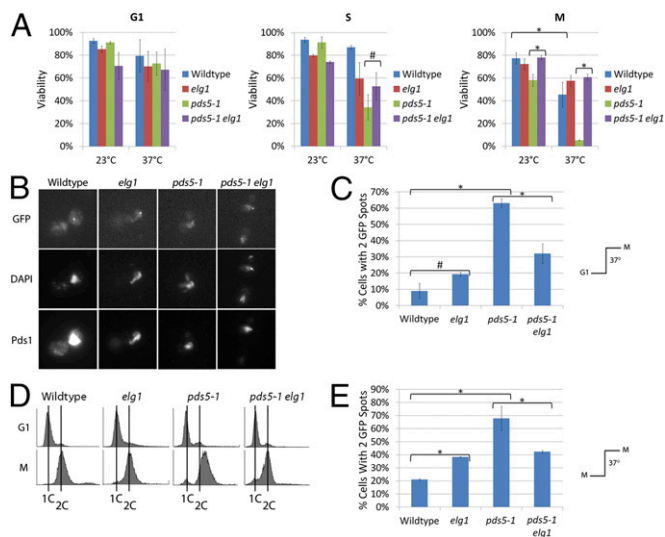
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Fig. 1. *ELG1* deletion promotes Pds5 function. (*A*) Percent viability of yeast strains at 23 °C or 37 °C during G1, S and M phase arrests. Statistically significant differences (*) based on *P* < 0.05 (#, *P* = 0.053). (*B*) Micrographs of sister chromatid foci (GFP) relative to DNA (DAPI) and Pds1. (*C*) Percent of preanaphase cells with precocious sister chromatid separation (#, *P* = 0.057). (*D*) DNA content of cells arrested in G1 at 23 °C, then shifted to 37° and arrested preanaphase. (*E*) Percent of preanaphase cells with precocious sister chromatid separation.

Results

The Essential Role of Pds5 in Both S and M Phases Is Supported by Elg1-RFC.

To first assess the extent through which *pds5-1 elg1* double cells exhibit altered conditional growth through the cell cycle, wild-type, *pds5-1* and *elg1* single mutant cells and *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 (nonpermissive for *pds5-1*) for 2 h 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 h before scoring for viability as described (31). As expected, wild-type and *elg1* single mutant cells exhibited robust growth at 23 °C regardless of the cell cycle phase, whereas *pds5-1* mutant cells exhibited only a modest decrease in viability in the M phase. This conditional viability was rescued by *ELG1* deletion (Fig. 1*A*).

Cultures shifted to 37 °C exhibited significant differences in viability depending on the part of the cell cycle under investigation. All strains including the *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 (Fig. 1*A*) and that cohesion pathways, in general, are largely inactive during G1 (8–10, 14, 33–35). In contrast, *pds5-1* mutant cells exhibited significantly decreased viabilities in response to temperature shifts both during S and M phases (9, 31). Importantly, *pds5-1 elg1* double mutant cells instead exhibited viability levels approximating those of *elg1* single mutant cells during both S and M phase (Fig. 1*A*). The bypass suppression obtained through *ELG1* deletion is most notable in mitotic *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.

Elg1-RFC Is a Critical Regulator of Pds5-Dependent Sister Chromatid Cohesion.

pds5-1 mutant cells exhibit severe cohesion and condensation defects (9, 31, 35). It thus became important to test

which, if either, of these Pds5 functions is rescued by *ELG1* deletion. Log phase wild-type, *elg1* and *pds5-1* single mutant cells, and *pds5-1 elg1* double mutant cells harboring cohesion assay cassettes (*TetO* array integrated ~40 kb 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 (nonpermissive for *pds5-1*) rich medium supplemented with nocodazole (herein referred to as a G1 temperature shift). The resulting preanaphase synchronized cultures were harvested and assessed for both DNA content by flow cytometry and premature sister chromatid separation in which one GFP foci indicates tightly tethered sisters and two GFP spots reveals premature sister chromatid separation (Fig. 1 *B–D*). As expected, wild-type cells exhibited minimal (<10%) precocious sister chromatid separation, *elg1* single deletion mutants exhibited only a modest increase in separated sisters (19% cohesion defect), whereas *pds5-1* mutant cells exhibited severe cohesion defects (63%) (Fig. 1*C*). Notably, *pds5-1 elg1* double mutant cells exhibited cohesion defects significantly reduced relative to *pds5-1* mutant cells (compare 32–63%) and instead are comparable to *elg1* single mutant cells (Fig. 1*C*). Thus, *ELG1*-deletion significantly rescues the precocious sister chromatid separation normally observed in *pds5-1* mutant cells.

Could the absence of Elg1 during S-phase rescue cohesion defects that arise upon pds5-1 protein inactivation during mitosis? To address this question, wild-type, *pds5-1*, *elg1*, and *pds5-1 elg1* strains were synchronized in preanaphase at permissive temperature by placing log phase cultures into medium supplemented with nocodazole. The preanaphase arrested cultures were then shifted to nonpermissive temperature for 2 h while maintaining the preanaphase arrest (herein referred to as a mitotic temperature shift) and then assessed for precocious sister chromatid separation (Fig. S1). Under this regimen, wild-type cells exhibited a relatively low level of cohesion defects (<20%) and *elg1* mutant cells exhibited only a modest increase in cohesion defects (39%). In contrast, *pds5-1* mutant cells exhibited severe defects (68%). *pds5-1 elg1* double mutant cells, however, exhibited a significant rescue in the level of cohesion defects (42%) that is comparable to *elg1* single mutant cells (39%) and well below that of *pds5-1* single mutant cells (Fig. 1*E*). This rescue of cohesion observed in *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 (8, 9, 31, 36–39).
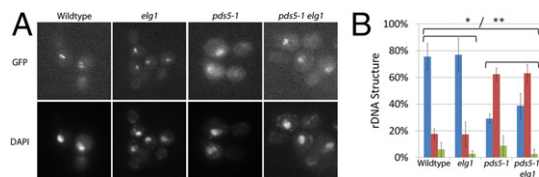

Fig. 2. *ELG1* deletion fails to rescue *pds5-1* condensation defects. (*A*) Micrographs reveal changes in rDNA condensation as detected by Net1-GFP (GFP) and DNA counterstained with DAPI. (*B*) Percent of cells that contain condensed (Lines) or uncondensed (Puffs) rDNA chromatin detected using Net1-GFP (*/**, statistical differences between wild-type and *pds5-1* mutant cells and also between wild-type and *pds5-1 elg1* double mutant cells).
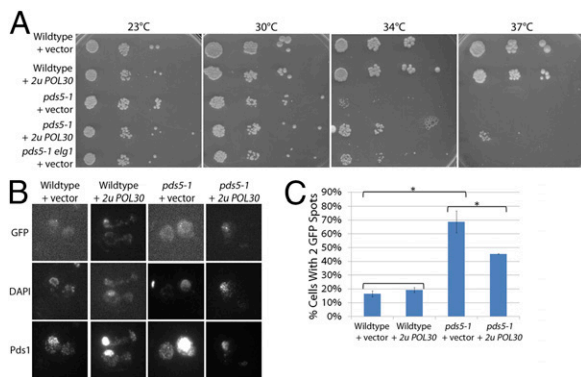
Fig. 3. PCNA promotes Pds5 function in cohesion. (*A*) Serial dilutions of cells harboring either vector or vector directing overexpression of PCNA (*POL30*). (*B*) Micrographs of sister chromatids (GFP), DNA (DAPI), and Pds1. (*C*) Percent of cells that exhibit precocious separated sister chromatids quantified as described in Fig. 1*C*.

We first validated this system in our own laboratory by recapitulating the efficacy of Net1-GFP to detect changes in rDNA condensation in response to Mcd1 inactivation (Fig. 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 wild-type cells but form highly amorphous puffs in mitotic *pds5-1* mutant cells (9, 31, 36). We performed a G1 temperature shift on log phase wild-type, *elg1* and *pds51* single mutant cells, and *pds5-1 elg1* double mutant cells, all harboring Net1-GFP. The resulting synchronized preanaphase cells were then scored for DNA content by flow cytometry (Fig. S3) and condensation defects (rDNA puffs instead of loop or lines) by microscopy (Fig. 2 *A* and *B*). As expected, the majority of wild-type 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%) (Fig. 2*B*). 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 (40–43). Elevated levels of PCNA rescue *ctf7/eco1* mutant cell conditional growth (14). Intriguingly, *ELG1* mutation results in higher levels of chromatin bound PCNA (44, 45), raising the possibility that PCNA overexpression may similarly rescue *pds5-1* mutant cell phenotypes. To test this hypothesis, wild-type 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 before assessing growth. Wild-type cells grew robustly at all temperatures regardless of PCNA overexpression. As expected, *pds5-1* mutants harboring only vector exhibited robust growth at 23 °C and 30 °C but were predominantly inviable at elevated temperatures (Fig. 3*A*). 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 with *pds5-1 elg1* double mutant cells (Fig. 3*A*). In combination, these findings reveal, to our knowledge 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 wild-type and *pds5-1* cells harboring either vector alone or vector plus *POL30* and all harboring the cohesion assay cassettes described above. The resulting preanaphase cultures were then analyzed for DNA content (Fig. S4) and premature sister chromatid separation as described above (Fig. 3 *B* and *C*). As expected, the majority of wild-type 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 (Fig. 3*C*). 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 overexpression 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 wild-type and *pds5-1* mutant cells that express Net1-GFP and harbor vector or vector plus *POL30*. The resulting preanaphase cells were then assessed for DNA content (Fig. S5) and rDNA structure as described above (Fig. 4 *A* and *B*). Wild-type 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 (Fig. 4*B*). Notably, PCNA overexpression failed to suppress *pds5-1* mutant cell defects in rDNA structure and instead exhibited an identical level (59%) of puffs (Fig. 4*B*). The PCNA-dependent rescue in both viability and cohesion, but not condensation, confirms a common mechanism through which *ELG1* deletion and PCNA overexpression 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 the viability and condensation defects exhibited by *ctf7/eco1* mutant cells are rescued
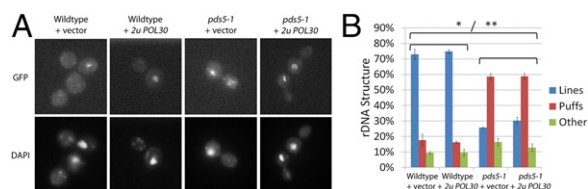

Fig. 4. Pds5 role in condensation appears independent of PCNA. (*A*) Micrographs of cells harboring either vector alone or vector directing PCNA overexpression and assessed for rDNA chromatin as described in Fig. 2*A*. (*B*) Percent of cells exhibiting condensed (Lines) or uncondensed (Puffs) rDNA structures as described in Fig. 2*B*.

by *RAD61*/*WAPL* deletion (17, 19, 21, 29, 30). Does *RAD61*/ *WAPL* deletion rescue *pds5-1* mutant cell condensation defects? We performed a G1 temperature shift on log phase wild-type, *pds5-1* and *rad61* single mutant cells, and *pds5-1 rad61* double mutant cells all modified to express Net1-GFP. The resulting preanaphase cultures were assessed for DNA content (Fig. S6*A*) and rDNA condensation (Fig. 5 *A* and *B*). The majority of wild-type and *rad61* cells contained tight loop/line rDNA chromatin structures (76% and 79%, respectively), whereas *pds5-1* mutant cells exhibited severe condensation defects (29% loop/line rDNA chromatin structures) (Fig. 5*B*). Notably, *pds5-1 rad61* double mutant cells exhibited a significant reduction in condensation defects compared with *pds5-1* mutant cells such that over 60% of *pds5-1 rad61* cells contained distinct loop/line rDNA chromatin structures (Fig. 5*B*). 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, wild-type and *rad61* mutant cells exhibited robust growth at all temperatures, whereas *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 (Fig. 5*D*) (31). 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. Because *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 wild-type, *pds5-1*, *rad61*, and *pds5-1 rad61* cells. The resulting preanaphase cells were assessed by flow cytometry (Fig. S6*B*) and for cohesion defects. Wild-type cells exhibited minimal cohesion defects (<20%), whereas *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 (Fig. 5*C*). These results are notable for several

Fig. 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 (31). (*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.

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 (17, 19, 21, 29, 30). 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 (12, 46). 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 irrevocably, entangled (8, 9, 14). 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 (17, 19, 21, 29, 30), 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. During final revision of the current manuscript, Orgil and colleagues identified *IRR1*/ *SCC3* separation of function alleles that provide further insight into cohesion roles (47). These tools will be critical in assessing how regulatory factors direct cohesin modifications and structures toward cohesion, condensation, and transcription (13).

Prior observations that deletion of *ELG1* or *RAD61* rescue cohesion mutant cell growth defects might suggest that Elg1 and Rad61 each directly antagonize some aspect of cohesion, activities termed "antiestablishment" (16, 17, 29, 30, 32, 48). Instead, our results provide a clear template regarding the mechanism of bypass suppression. *ELG1* deletion results in increased PCNA retention onto chromatin (44, 45) such that simply overexpressing PCNA fully supplants the requirement for *ELG1* deletion to rescue both *ctf7*/*eco1* and *pds5-1* mutant cell conditional growth (14, 22, 32, 48, 49). Thus, Elg1 does not directly antagonize cohesion reactions but instead its deletion results in the elevated retention of a positive regulator of cohesion: PCNA (14, 50). We posit a
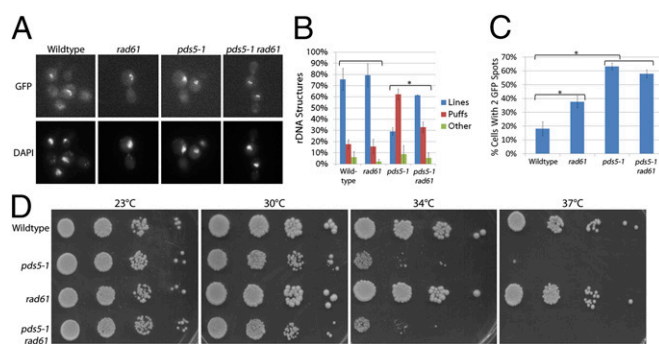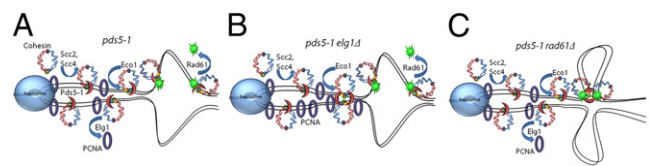

Fig. 5. *RAD61* deletion suppresses the condensation defect of *pds5-1* mutant cells. (*A*) Micrographs of rDNA chromatin structure and DNA as described in Fig. 2*A*. (*B*) Percent of cells exhibiting condensed (Lines) or uncondensed (Puffs) rDNA structures as described in Fig. 2*B*. (*C*) Serial dilutions of cells performed as described in Fig. 3*A*. (*D*) Percent of preanaphase cells with precocious sister chromatid separation as described in Fig. 1.

similar situation may exist for Rad61: That it is not the deletion of *RAD61* per se that provides bypass suppression of *ctf7/eco1* and *pds5-1* mutant cells, but that *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 nonessential Rad61 directly precludes stable cohesin binding to DNA by revealing some conjectured destabilizing activity of Pds5 and Scc3 (51), factors that are essential to maintain cohesion.

Cohesion and Condensation Occur Independently of Each Other Behind the DNA Replication Fork. In the current study, we document that cohesion and condensation each can be established and maintained in the absence of the other. These findings can now 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 (52–54).

An Integrated Model of 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 the DNA replication fork passes through cohesin rings to entrap within both sister chromatids (55). However, it is now clear that cohesin deposition is essential only during S phase and that cohesins deposited before S phase are unstable regardless of acetylation state (27, 52, 56–58). In addition, there is direct evidence that each sister chromatid is individually decorated by cohesins and that Mcd1 can bridge different Smc1,3 heterodimers (4, 6, 31, 59, 60), providing support of an early model that cohesion is mediated through cohesin-cohesin interactions (61). Intriguingly, Pds5 (and Nse5 for Smc5,6 complexes) bind both the head and hinge domains (62, 63), in support of findings that cohesins fold over to promote head-hinge interactions (64, 65)—further challenging a simplistic ring model. 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 (Fig. 6). It is exciting to consider a context-based mechanism through which cohesin assemblies

and modifications required to promote cohesion and condensation are regulated (13) (Fig. 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 S1. Synchronization of yeast cultures and assessment of DNA content by flow cytometry were performed as described (31).

Viability Assay. Cultures were grown to log phase in high nutrient YPD medium to an OD_{600} of ~0.2, synchronized in G1 (alpha factor), S (hydroxyurea), or preanaphase (nocodazole) at permissive temperature (23 °C) for 3 h, shifted to nonpermissive temperature (37°) for 2 h in fresh media again supplemented with either alpha factor, hydroxyurea, or nocodazole to maintain respective G1, S, or preanaphase arrests and then placed on high-nutrient YPD medium plates for 16 h at 23 °C. Viability was scored by the ability to form microcolonies (colonies with over 30 cells) as described (31).

Cohesion Assay. Cohesion assays were performed as described with the following modifications (31). 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 h at 23 °C to synchronize in G1 or preanaphase, 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 h. Cell aliquots of the resulting preanaphase 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 coincident DNA (DAPI) and Pds1 staining [A-14 anti-MYC (Santa Cruz Biotechnology) followed by goat anti-rabbit Alexa 568 (Molecular Probes)] were analyzed for disposition of one versus two GFP signals. Cell images were captured using a Nikon Eclipse E800 microscope equipped with a cooled CD camera (Coolsnapfx, Photometrics) and IPLab software (Scanolytics). Cohesion analyses were repeated three times and a total of at least 300 cells counted.

Condensation Assay. *NET1* was genetically modified as described (31). Condensation assays were done as described (31, 36, 66). Briefly, log phase Net1-GFP strains were grown to 0.1–0.2 OD_{600} and then incubated for 2.5 h 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 h. The resulting preanaphase cultures were persevered by paraformaldehyde fixation (3.7% final concentration) for 10 min at 30 °C before analyses. Cells were assayed using an E800 light microscope (Nikon) equipped with a cooled CD camera (Coolsnapfx, Photometrics) and imaging software (IPLab, Scanalytics).

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.

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