[Cell Cycle 5:21, 2528-2536, 1 November 2006]; ©2006 Landes Bioscience

Report

Intersection Between the Regulators of Sister Chromatid Cohesion Establishment and Maintenance in Budding Yeast Indicates a Multi-Step Mechanism

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Original manuscript submitted: 08/18/06
Revised manuscript submitted: 09/13/06
Manuscript accepted: 09/13/06

Previously published online as a *Cell Cycle* E-publication:
http://www.landesbioscience.com/journals/cc/abstract.php?id=3405

KEY WORDS

CTF7/ECO1, PDS5, sister chromatid cohesion, cohesin complex, acetylase, cohesion, Pds5p, Ctf7p, cohesin complex, mitosis

ABBREVIATIONS

HU	hydroxyurea
Nz	nocodazole
ChIP	chromatin immunoprecipitation

ACKNOWLEDGEMENTS

We thank D. Koshland, and H. G. Yu for critical reading of this manuscript. This work was supported by National Institutes of Health grant GM062178, the Howard Hughes Medical Institute and the National Science Foundation Grant MCB-0212323.

ABSTRACT

Sister chromatid cohesion is established during S phase and maintained until anaphase. The cohesin complex (Mcd1p/Scc1p, Smc1p, Smc3p Irr1p/Scc3p in budding yeast) serves a structural role as it is required at all times when cohesion exists. Pds5p colocalizes temporally and spatially with cohesin on chromosomes but is thought to serve as a regulator of cohesion maintenance during mitosis. In contrast, Ctf7p/Eco1p is required during S phase for establishment but is not required during mitosis. Here we provide genetic and biochemical evidence that the pathways of cohesion establishment and maintenance are intimately linked. Our results show that mutants in *ctf7* and *pds5* are synthetically lethal. Moreover, over-expression of either *CTF7* or *PDS5* exhibits reciprocal suppression of the other mutant's temperature sensitivity. The suppression by *CTF7* is specific for *pds5* mutants as *CTF7* over-expression increases the temperature sensitivity of an *mcd1* mutant but has no effect on *smc1* or *smc3* mutants. Three additional findings provide new insights into the process of cohesion establishment. First, over-expression of *ctf7* alleles deficient in acetylase activity exhibit significantly reduced suppression of the *pds5* mutant but exacerbated toxicity to the *mcd1* mutant. Second, using chromosome spreads and chromatin immuno-precipitation, we find neither cohesin complex nor Pds5p chromosomal localization is altered in *ctf7* mutants. Finally, biochemical analysis reveals that Ctf7p and Pds5p coimmunoprecipitate, which physically links these regulators of cohesion establishment and maintenance. We propose a model whereby Ctf7p and Pds5p cooperate to facilitate efficient establishment by mediating changes in cohesin complex on chromosomes after its deposition.

MATERIALS AND METHODS

Reagents and media. Reagents and media were as described[1] except for YBS191, 192 and 194, which are S288e.[2] All yeast strains used in this study are isogenic A364A strains and are listed in Table 1. Plasmids pVG175 *(2* m *PDS5 URA3)* and *pVG177 (PDS5 CEN TRP1)* were previously described.[3] Plasmids pGF2, pDN14, pDN15 and pDN16 were made by inserting 2.1 kb SacI/SalI fragments bearing *CTF7, ctf7 R222G K223G, ctf7 G225D* and *ctf7 D232G* obtained from pLC3, pAB1034, pAB1035, pAB1036, respectively,[4] between the SacI and SalI sites of plasmid pRS202 *(2* m *URA3)*.

Plasmid shuffle assay. Haploid VG2259-6B contains the sole genomic *PDS5* source disrupted by *URA3* (*pds5::URA3*) but is kept alive by the presence of plasmid pVG177 (*PDS5 CEN TRP1*) and was previously described.[3,5] Haploid strain VG2887-1A was made by converting the *pds5::URA3* allele of haploid VG2259-6B into *pds5*::*ura3::G418* by insertion of G418. The ability of high-copy *CTF7* (pGF2) to suppress the *pds5*::*ura3::G418* disruption was performed as described[3] except that the 2 m test plasmid pGF2 and the positive control pVG175 were maintained by growing cultures in SC-URA media. Cells were plated to YPD, incubated three days at 23°C and replica plated to SC-TRP media to score the presence (TRP$^+$) or absence (TRP-) of the reporter plasmid pVG177.

Chromosome spreads. Chromosome spreads were performed as previously described[1] except that chromosomally bound Mcd1p and Pds5p were detected using affinity purified rabbit 559-1 anti-Mcd1p antibodies (1; 40,000) and rabbit 556-1 anti-Pds5p antibodies (1:40,000), respectively.

Chromatin immunoprecipitation (ChIP) Assay. ChIP assays were performed and analyzed as previously described.[6] Mcd1p and Pds5p were detected using affinity purified rabbit anti-Mcd1p antibodies (559-1) and rabbit anti-Pds5p antibodies (556-1), respectively. 1.3 ul of each antibody was used for each ChIP.

Yeast cell culture conditions. Cells were grown in YPD at 23°C to mid-log phase then treated as follows:

Arrested cells. Cells were arrested in G1, S or mid-M phase cells were arrested using a factor, hydroxyurea (HU) or nocodazole (Nz), respectively, as previously described except the amount of hydroxyurea used to arrest cells was increased to 0.2 M.[1] When necessary, cells were released from G1 phase arrest into YPD containing nocodazole at 37° and grown 2 hrs to arrest in mid-M phase.

Synchronous populations of cycling cells released from S phase at 37°C. Mid-log phase cells were arrested in S phase at 23°C, incubated 30 min at 37°C then released into media containing a factor to allow cell progression through mitosis and arrest in the following G1 phase as described[1,3] except HU was increased to 0.2 M to arrest cells.

Monitoring sister chromatid cohesion at the URA3 locus. A tandem array of Tet operator sequences was integrated at *URA3* (35 kb from *CEN5* on chromosome V) and cohesion monitored by Tet repressor GFP fusion protein binding to the operator, with images collected and recorded as described.[3]

Coimmunoprecipitation of Ctf7p and Pds5p. Ctf7p that was N-terminally tagged with glutathione-S transferase (GST-Ctf7p) or GST alone (negative control) were expressed in bacteria and bound to Glutathione sepharose beads as previously described.[7] Next, yeast extracts from a haploid strain VG2555-9D, which contains Pds5p-6MYC as the sole source of Pds5p, were processed at high salt to generate a clarified supernatant containing soluble proteins as previously described.[7] The clarified supernatant (load) containing Pds5p-6MYC was incubated with beads alone, beads coupled to GST alone or to GST-Ctf7p. The beads were then copiously washed and tightly bound proteins stripped by SDS-denaturation.

Antibodies. Monoclonal mouse anti-MYC antibody, mouse monoclonal mouse anti-HA antibody 12CA5 and rabbit anti-tubulin antibodies were previously described.[3] Polyclonal rabbit anti-Mcd1p (559-1) antibodies and polyclonal rabbit anti-Pds5p (556-1) were made using bacterially expressed Mcd1p and Pds5p, respectively, which were injected into Elite New Zealand white rabbits (Covance). Antibodies were affinity purified against GST-Mcd1p and GST-Pds5p columns.

Flow cytometry. Analysis of DNA content using Flow cytometry was performed as previously described.[8]

Table 1. ***S. cerevisiae* strains used in this study**

Strain	Genotype
VG2716	*Mata/Matα pds5-1/+ trp1/+ ura3/+ leu2/leu2*
VG2660	*Mata/Matα pds5-2/+ leu2/+ his3/+ ura3/ura3*
VG2718	*Mata/Matα pds5-3/+ trp1/+ ura3/+ leu2/leu2*
VG2720	*Mata/Matα ctf7-203/+ trp1/+ leu2/+ ura3/ura3*
VG2717	*Mata/Matα pds5-1/+ ctf7-203/+ trp1/+ leu2/+ ura3/ura3*
VG2721	*Mata/Matα pds5-2/+ ctf7-203/+ trp1/+ his3/+ ura3/ura3*
VG2719	*Mata/Matα pds5-3/+ ctf7-203/+ trp1/+ leu2/+ ura3/ura3*
VG2887-1A	*Mata pds5::ura3::G418 trp1 leu2 ura3 bar1 w/ plasmid pVG177 (CEN PDS5 TRP1)*
VG986-5B	*Mata pds5-1 trp1 ura3 bar1*
VG988-1C	*Mata pds5-3 trp1 ura3 bar1*
VG2555-9D	*Mata PDS5-6MYC leu2 ura3 his3 bar1*
VG985-7C	*Mata mcd1-1 trp1 ura3 bar1*
VG2125-10A	*Mata smc1-2:LEU2 ura3 leu2 bar1*
VG2460-8A	*Mata smc3-42 leu2 ura3*
VG2551-12A	*Mata scc2-4 trp1 ura3 bar1*
VG2450-7A	*Mata PDS1-3HA-KAN-URA3 TETGFP-LEU2:leu2 trp1 bar1 TetOx224-URA3:ura3-52*
VG2456-5C	*Mata pds5-2 PDS1-3HA-KAN-URA3 TETGFP-LEU2:leu2 trp1 bar1 TetOx224-URA3:ura3-52*
VG2557-5A	*Mata mcd1-1 TETGFP-LEU2:leu2 TetOx224-URA3:ura3-52 trp1 bar1*
VG2898-4C	*Mata pep4D::G418 trp11 leu2 ura3 his3 bar1*
VG3084-2A	*Mata ctf7-203 pep4D::G418 leu2 ura3 his3 bar1*
VG3084-5D	*Mata ctf7-203 leu2 ura3 bar1*
YBS191	*Matα_ctf7D::HIS3 ctf7-201-LEU2:leu2 trp1 ura3 his3 lys2 ade2*
YBS192	*Matα_ctf7D::HIS3 ctf7-202-LEU2:leu2 trp1 ura3 his3 lys2 ade2*
YBS194	*Matα_ctf7D::HIS3 ctf7-203-LEU2:leu2 trp1 ura3 his3 lys2 ade2*

INTRODUCTION

Sister chromatid cohesion ensures that chromosome segregation proceeds with high fidelity. The evolutionarily conserved cohesin complex has properties indicating that it serves a structural role in mediating cohesion.[9] In budding yeast, the cohesin complex (Mcd1p/Scc1p, Smc1p, Smc3p, Irr1p/Scc3p) is required for cohesion from its establishment in S-phase through metaphase.[10,11] Chromatin immunoprecipitation (ChIP) studies have shown that the cohesin complex binds to chromosomes at discrete loci termed CARs for cohesin associated regions.[12-15] Biochemical and EM studies indicate that cohesin complex forms a ring-like structure.[16-19] Based on evidence that cohesion can only be established during S phase, a simple model was proposed whereby cohesin rings are loaded onto chromosomes and then the subsequent passage of the DNA replication machinery through the rings entrap sister chromatids—thereby establishing cohesion.[17,18]

Emerging evidence reveals that cohesion establishment is not such a simple process. Scc2p and Scc4p are important for establishment because they are required for cohesin complex deposition at CARs.[20-24] While establishment requires the deposition of cohesin onto chromosomes prior to DNA replication, it is not sufficient. Ctf7p is the founding member of an evolutionarily conserved class of Establishment Factor Orthologs (EFOs or ESCOs), some of which show cohesion defects when mutated in human cells.[25-27] In budding yeast, *ctf7* mutants exhibit cohesion defects as severe as that of cohesin complex when inactivated prior to S phase, but unlike cohesin complex, once *ctf7* mutants have completed S phase subsequent inactivation of mutant Ctf7p has no effect on cohesion.[28-30] Moreover, the gross cohesin complex localization to chromosomes appears normal in *ctf7* mutants.[29] Ctf7p interaction with Pol30p (PCNA) and Replication Factor C (RFC) subunits provided the first evidence that establishment is intimately linked to DNA replication.[7,28] It is now known that a wide variety of DNA replication/repair factors are required for efficient establishment. These data support a model in which cohesin complex is loaded at CARs and then nascent sister chromatids become paired in a Ctf7p-dependent step during or soon after DNA replication fork passage.[31,32] Thus, establishment involves multiple steps, some before and others either during or soon after fork passage.

Table 2 **Synthetic lethality of pds5 and ctf7 mutants**

Genotype	Tetrads	Viable: Inviable Spores					% Viab	Double Mutants	
		4:0	3:1	2:2	1:3	0:4		Expected	Observed
pds5-1 x WT (VG2716)	9	7	1	0	0	1	86.1	NA	NA
pds5-2 x WT (VG2660)	27	20	4	2	1	0	89.8	NA	NA
pds5-3 x WT (VG2718)	7	6	1	0	0	0	96.4	NA	NA
ctf7-203 x WT (VG2720)	10	9	1	0	0	0	97.5	NA	NA
pds5-1 x *ctf7-203* (VG2717)	17	0	12	2	0	3	58.8	17	0
pds5-2 x *ctf7-203* (VG2721)	18	2	10	6	0	0	69.4	18	0
pds5-3 x *ctf7-203* (VG2719)	13	4	6	3	0	0	76.9	13	0

Another evolutionarily conserved chromosomal protein, Pds5p, is required for cohesion.[1,33-38] Pds5p and cohesin complex physically interact and colocalize at all CARs.[1,34,36,38] Budding yeast *pds5* mutant cells arrested at nonpermissive temperature in metaphase exhibit precocious sister separation at levels similar to cohesin complex mutants.[1] Despite these similarities, Pds5p and cohesin complex are thought to serve distinct functions. In budding and fission yeasts as well as in human cells, Pds5p binding to chromosomes requires cohesin complex function whereas as cohesin complex still localizes to chromosomes when Pds5p function is compromised.[1,34-36,39] Finally, in *pds5* mutants, most sister chromatids do establish cohesion but subsequently exhibit precocious dissociation during mitosis.[3,36] Based on these differences, Pds5p is thought to be a positive regulator of cohesion maintenance rather than a structural component.[1,3,36]

The characterization of Ctf7p and Pds5p as regulators of cohesion establishment and maintenance, respectively, might suggest distinct and unrelated functions. However, in fission yeast, genetic interactions between Eso1p (fission yeast homolog of Ctf7p) and Pds5p were detected suggesting a connection.[36] Here, we provide the first evidence regarding the intersection of regulation and maintenance pathways in budding yeast. We provide genetic and biochemical evidence for an intimate relationship between Ctf7p and Pds5p. Previous in vitro experiments revealed that Ctf7p is an acetylase but a physiological role for this activity remained undocumented.[4,40] Our genetic data provide the first in vivo evidence for a biological role for this acetylase and implicate Pds5p and Mcd1p as likely targets. We further show that Mcd1p and Pds5p localization to chromosomes occur normally in *ctf7* mutants. These data suggest that Pds5p and Ctf7p cooperate in the establishment of cohesion. We propose a model whereby Pds5p recruits Ctf7p to cohesin complex at CAR sites to promote efficient establishment.

RESULTS

Mutants in *PDS5* and *CTF7* exhibit synthetic lethality. In fission yeast, a genetic interaction had been identified between *ESO1* (the homolog of budding yeast Ctf7p) and *PDS5* but it was not extensively characterized.[36] We decided to test for such an interaction between *CTF7* and *PDS5* in budding yeast and if one was detected, to characterize it in detail. First, we assessed the consequences to cells when they contained temperature sensitive alleles of both *CTF7* and *PDS5*. Haploid *ctf7-203* mutant cells were crossed to *pds5-1, pds5-2* and *pds5-3* mutant haploids and the resulting diploids were sporulated, dissected and grown at the permissive temperature (23°C). For controls, the single mutants were crossed to wild-type cells, and as expected, spore viability was high (Table 2). In contrast, the heterozygous (*ctf7-203* x *pds5*) diploids showed greatly reduced spore viability. Moreover, no double mutant spores were obtained (Table 2). Since 48 double mutant spores are expected from the 48 tetrads dissected, our data indicates that *ctf7* and *pds5* alleles exhibit complete synthetic lethality.

***CTF7* overexpression suppresses the temperature sensitivity of pds5 mutants.** We next tested whether over-expression of *CTF7* affects *pds5* mutants. Haploid strains harboring one of two different *pds5* alleles (*pds5-1* and *pds5-3*) were transformed with high-copy (*2* m) plasmids bearing *PDS5*, *CTF7* or no insert. As expected, *pds5* cells bearing empty *2* m plasmid are temperature sensitive whereas those bearing *2* m *PDS5* exhibit robust growth at high temperature (Fig. 1). The *2* m *CTF7* plasmid suppressed the temperature sensitivity of both *pds5* mutant alleles, with stronger suppression seen for *pds5-1* cells.

Since high-copy *CTF7* suppresses *pds5* mutant temperature sensitivity, we asked whether it could also suppress a *PDS5* deletion. *PDS5* is an essential gene in budding yeast so we employed a plasmid shuffle assay (Materials and Methods). Cells deleted for *pds5* were kept viable by the presence of plasmid pVG177, a low copy plasmid bearing *PDS5* (*PDS5 TRP1 CEN*). We assayed whether the presence of a *2* m *CTF7 URA3* plasmid (pGF2) allows loss of pVG177. After 20 generations of nonselective growth, 2263 colonies were assayed but plasmid pVG177 was never lost from cells, indicating that *CTF7* overexpression cannot bypass the essential function of *PDS5*. For a positive control, the presence of a *2* m *PDS5 URA3* plasmid (pVG175) allowed pVG177 loss from more than 90% of colonies analyzed. These results suggest that Ctf7p functions with or modifies some property of the mutant Pds5p to generate suppression.

***PDS5* overexpression suppresses the temperature sensitivity of ctf7 mutants.** We next assayed whether *CTF7* and *PDS5* exhibit reciprocal suppression or whether the suppression described above is unidirectional. Three temperature sensitive haploid *ctf7* mutant strains (*ctf7-201, ctf7-202* or *ctf7-203*) were transformed with *2* m plasmids bearing *PDS5*, *CTF7* or no insert. As expected, *ctf7* mutants bearing *2* m alone are temperature sensitive whereas those bearing *2* m *CTF7* grow robustly at high temperature (Fig. 2). The *2*m *PDS5* plasmid suppressed the temperature sensitivity of all three *ctf7* mutant strains. These data indicate that Ctf7p and Pds5p exhibit a direct relationship. Overexpression of *PDS5* and *CTF7* compensates for reduced *ctf7* and *pds5* activity, whereas simultaneous reduction in the activity of both genes results in lethality. As Ctf7p is not required after S phase, the simplest explanation is that Ctf7p and Pds5p cooperate to modulate efficient establishment of cohesion.

Budding yeast Ctf7p and Pds5p physically interact. The reciprocal suppression exhibited by *CTF7* and *PDS5* suggests a possible physical interaction between Ctf7p and Pds5p. A 2-hybrid interaction between Pds5p and Eso1p in fission yeast also suggested a physical interaction.[36] To more directly determine whether budding

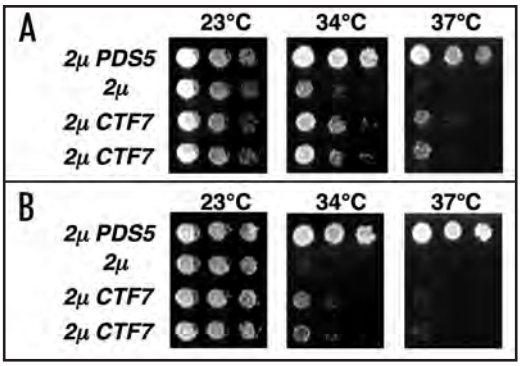

Figure 1. *CTF7* over-expression suppresses the temperature sensitivity of *pds5* mutants. Haploid cells containing high copy (2m) *URA3* plasmids, 2m *PDS5* (pVG175), 2m *CTF7* (pGF2) or 2m alone (pRS202) were grown to saturation in at 23˚C in SC-URA liquid. Cells were diluted to OD_{600} 1.0, plated in 10 fold serial dilutions on SC-URA media and incubate for 72h at 23˚C, 34˚C or 37˚C. (A) *pds5-1* mutant (VG986-5B) cells. (B) *pds5-3* mutant (VG988-1C) cells.

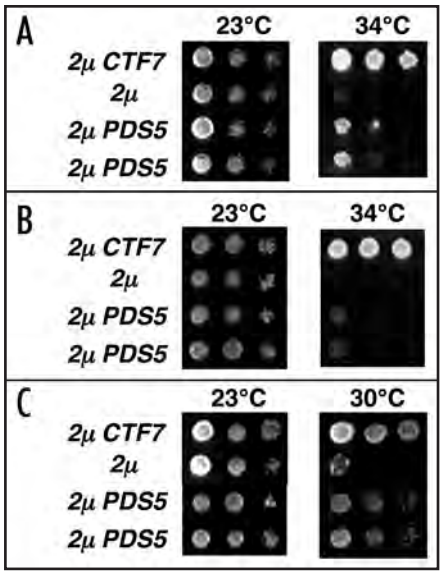

Figure 2. *PDS5* over-expression suppresses the temperature sensitivity of *ctf7* mutants. Haploid cells containing high copy (2m) *URA3* plasmids 2m *CTF7* (pGF2), 2m *PDS5* (pVG175) or 2m alone (pRS202) were grown and plated as described in (Fig. 1). (A) *ctf7-201* mutant (YBS191) cells. (B) *ctf7-202* mutant (YBS192) cells. (C) *ctf7-203* mutant (YBS194) cells.

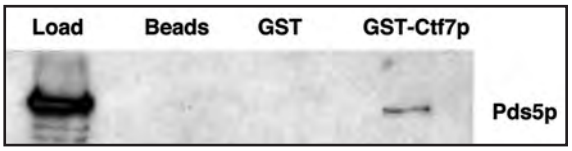

Figure 3. Pds5p and Ctf7p coimmunoprecipitate. Pull-down assay demonstrating that Pds5p binds to GST-Ctf7p beads but not to GST beads or Sepharose beads alone.

yeast Ctf7p and Pds5p physically interact, we preformed a GST pull-down experiment. GST-Ctf7p or GST alone were expressed in bacteria and bound to Glutathione sepharose beads (Materials and Methods). Yeast extracts from haploid strain VG2555-9D, which contains Pds5p-6MYC as the sole source of Pds5p, were processed at high salt to generate a clarified supernatant containing soluble proteins as previously described.[7] This supernatant (load) containing Pds5p-6MYC was incubated with beads alone, beads coupled to GST-Ctf7p or to GST alone. The beads were copiously washed and then tightly bound proteins eluted by SDS-denaturation. Western blot analyses revealed that Pds5p-6MYC bound specifically to GST-Ctf7p but not to GST or beads alone (Fig. 3). The result that budding yeast Ctf7p and Pds5p coimmunoprecipitate provides the first direct evidence that these regulators of cohesion establishment and maintenance physically associate.

***CTF7* overexpression is toxic to a subset of cohesin complex mutants.** To better understand the mechanism of Ctf7p function, we examined whether *CTF7* over-expression is a general suppressor of mutants defective in sister chromatid cohesion or specific for *pds5* mutants. For this purpose we transformed a *2* m *CTF7* plasmid or *2* m alone into haploid strains mutant for either cohesin complex subunits (*mcd1*, *smc1* and *smc3*) or a protein required for cohesin complex localization onto chromosomes (*scc2*). For positive controls, haploid *pds5* and *ctf7* mutants were also transformed with *2* m *CTF7* and *2* m alone. As expected, *2* m *CTF7* suppressed both *ctf7* and *pds5* mutants (Fig. 4). Surprisingly, instead of suppression, the *2* m *CTF7* plasmid was toxic to the *mcd1* mutant as indicated by increased temperature sensitivity at both 30˚C and 32˚C compared to that of *2* m alone. The *2*m *CTF7* had no obvious effect on the temperature sensitivity of *smc1*, *smc3* or *scc2* mutants. These results indicate that *CTF7* mediated suppression is specific for *pds5* mutants. The fact that toxicity is specific to the *mcd1* mutant but not to the *smc*1 or *smc3* mutants suggest that Ctf7p, possibly through its interaction with Pds5p, can modulate cohesin complex function with the Mcd1p being the likely target. Mutants in *scc2* are defective for cohesin complex deposition to chromosomes but not cohesin complex formation.[20,21,23] Failure to detect any effect on the *scc2* mutant is consistent with the observation that Ctf7p does not play a role in cohesin complex deposition but rather acts afterwards.

Mutations in the *ctf7* acetylase domain alter its effect on *pds5* and mcd1 mutants. Our genetic data that *CTF7* affects only *pds5* and *mcd1* mutants suggest they are key targets for Ctf7p action. Previous in vitro experiments revealed that Ctf7p exhibited acetyltransferase activity with Pds5p, Mcd1p, Scc3p and Ctf7p serving as substrates.[40] Therefore, it may be that Ctf7p through its acetyltransferase activity on Pds5p and Mcd1p facilitates efficient establishment. However, subsequent in vivo testing of three different acetyltransferase deficient *ctf7* mutants (*ctf7ac*) revealed no adverse effect on cell viability or chromosome transmission casting doubts as to the in vivo relevance of this acetylase.[4] Our findings that elevated *CTF7* rescues conditional growth in *pds5* mutant cells but exacerbates the conditional growth in *mcd1* mutant cells provides two sensitive assays to test the role of Ctf7p acetyltransferase activity in vivo. We envisioned two possible outcomes. If elevated levels of *ctf7ac* alleles do not alter either the suppression of *pds5* mutants or the toxicity to the *mcd1* mutant, then this domain is not relevant to Ctf7p function. Alternatively, if elevated *ctf7ac* levels fail to rescue *pds5* mutant cells and are not toxic to *mcd1* mutant cells, then the acetyltransferase function is likely an important part of the mechanism regulating cohesion establishment.

To test the relevance of the Ctf7p acetylase, haploid *pds5-1* mutant cells were transformed with *2* m plasmids bearing wild type *CTF7*, three *ctf7ac* domain mutants or no insert. All three *ctf7ac* mutants exhibited significantly reduced suppression of the *pds5* mutant

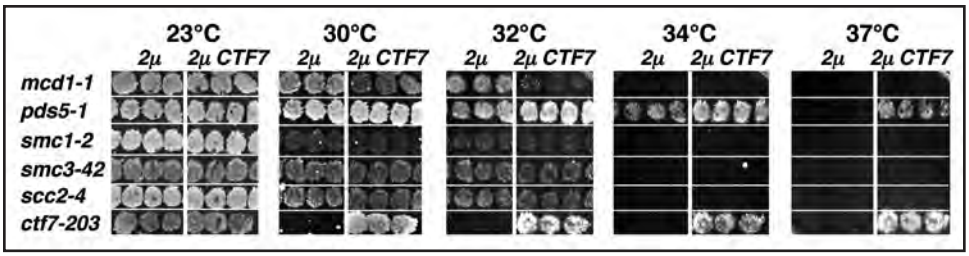

Figure 4. Effect of *CTF7* over-expression on mutants defective for cohesion. Haploid *mcd1-1* (VG985-7C), *pds5-1* (VG986-5B), *smc1-2* (VG2125-10A), *smc3-42* (VG2460-8A), *scc2-4* (VG2551-12A) and *ctf7-203* (VG3084-5D) cells containing high copy (2m) *URA3* plasmids, either 2m *CTF7* (pGF2) or 2m alone (pRS202) were patched to SC-URA plates and grown for 2 days at 23°C. Cells were replica plated to SC-URA media, incubated for 24h at 23°C, 30°C, 32°C, 34°C or 37°C then replica plated to another SC-URA plate and incubated for 48h at 23°C, 30°C, 32°C, 34°C or 37°C.

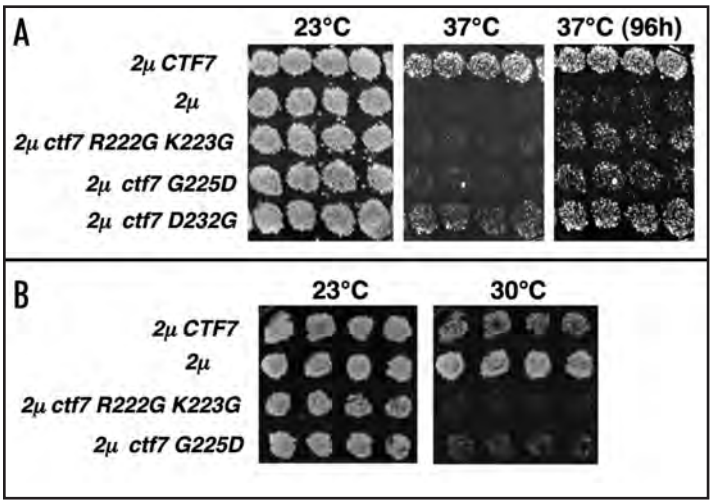

Figure 5. Effect of overexpressing *ctf7* acetylase domain mutant alleles. Haploid cells containing high copy (2 m) *URA3* plasmids, 2 m *CTF7* (pGF2), 2 m alone (pRS202) or three different acetylase domain mutant alleles, 2 m *ctf7 R222G K223G* (pDN14), 2m *ctf7 G225D* (pDN15) and 2 m *ctf7 D232G* (pDN16) were patched to SC-URA plates and grown as described in Figure 4. (A) *pds5-1* (VG986-5B) mutant cells. (B) *mcd1-1* (VG985-7C).

temperature sensitivity as compared to wild type *CTF7* (Fig. 5A). It is important to note that the *ctf7ac* mutants still exhibit some level of suppression (compare to *2* m alone). Previous in vitro data revealed that residual acetylase activity remained in all *ctf7ac* mutants we analyzed here.[4] Thus, strength of suppression directly correlates with in vitro Ctf7p acetylase activity, providing evidence that the Ctf7p acetylase activity is biologically relevant. Next, we asked whether the *CTF7* mediated toxicity to *mcd1* mutant cells correlates with acetylase activity. Haploid *mcd1-1* mutant cells were transformed with *2* m plasmids bearing wild type *CTF7*, or two *ctf7ac* domain mutants. As before, the *2* m *CTF7* is toxic as compared to *2* m alone (Fig. 5B). Surprisingly, overexpression of either *ctf7ac* mutant is even more toxic to *mcd1-1* cells than wild type *CTF7*. These data suggest that the *mcd1* mutant is exquisitely sensitive to the levels and or the timing of Ctf7p acetylase activity. The increased toxicity of the *ctf7ac* alleles to *mcd1-1* mutant cells is consistent with the idea that acetylase activity is important for establishment.

pds5 mutants may have a weak establishment defect. Given the connection between Pds5p and the essential establishment factor Ctf7p, one might expect that *pds5* mutants would also exhibit some defect in establishment. Our previous experiments using cells released from early S phase showed that most sisters did establish cohesion but approximately 20% of *pds5* cells showed precocious separation after DNA replication but prior to anaphase.[3] To better assess this putative establishment defect, we compared sister separation in *pds5* mutant cells to that of *mcd1* mutant cells and wild-type cells. Haploid wild-type, *pds5-1* mutant and *mcd1-1* mutant strains were arrest in early S phase using hydroxyurea (HU) then shifted to nonpermissive temperature (37°C) to inactivate the mutant proteins. Cells were released from arrest at 37°C in YPD media containing a factor to allow cells to complete DNA replication and mitosis at nonpermissive temperature then arrest in G_1 phase (Materials and Methods). We monitored cohesion at the *URA3* locus using a Tet repressor-GFP fusion protein (Materials and Methods).

The number of GFP signals was scored and cells containing 2 GFP signals indicate that sister chromatids have separated (Fig. 6). In the wild-type strain, cells with separated sister were seen 60 minutes after release from HU arrest, which corresponds to anaphase onset as indicated by Pds1p degradation (Fig. 6A and B). In contrast, in the *mcd1-1* strain, significant sister separation is observed at 30 minutes while DNA is undergoing replication and is greater than 50% by 45 minutes, which is post-replication but prior to anaphase in wild type cells. In *pds5* mutant cells, there is a slight increase in sister separation at 30 minutes, when DNA replication is occurring. This reaches nearly 20% by 45 minutes, the time replication is completed but prior to anaphase initiation in wild type cells. As we previously reported,[3] the *pds5* mutant cells show large increases in precocious sister separation during mitosis but remain delayed in mitosis as indicated by Pds1p stabilization and accumulation of large budded cells (Fig. 6B and C). The *mcd1-1* mutant cells also delay in mitosis, presumably due to precocious sister dissociation (data not shown). Thus, sister separation is delayed in *pds5* cells as compared to *mcd1-1* cells, consistent with cohesion being established on most, but not all sister chromatids in *pds5* cells. With the exception of mutants in *ctf7*, mutants in establishment exhibit approximately a 20% defect in cohesion following S phase. As this level of defective cohesion is observed in *pds5* mutants after replication but prior to anaphase, our data suggests that there is indeed a weak establishment defect in *pds5* cells.

Pds5p and Mcd1p localization to chromosomes are normal in ctf7 mutants. Given the essential role that Ctf7p serves in establishment, one might expect that cohesin complex and or Pds5p localization would be perturbed in *ctf7* mutants. It was previously shown using chromosomes spreads that cohesin complex is still broadly bound to the bulk chromosomal mass. As we have identified an intimate link between Pds5p and Ctf7p, it could be that a defect in Pds5p binding is the cause of the establishment defect in *ctf7* mutants. To test this possibility we compared the chromosomal localization of Pds5p in wild type and *ctf7-203* haploid cells. Cells were arrested in G1 phase (a factor) at 23°C, then released from G1 phase into YPD containing nocodazole (Nz) at 37°C to arrest cells in mid-M phase at nonpermissive temperature (Materials and Methods). Cells were fixed and processed for chromosomes spreads and for chromatin immunoprecipitation (ChIP).

We assayed the chromosomal localization of cohesin complex by monitoring Mcd1p and also monitored Pds5p. The chromosome spreads revealed no difference between wild type and *ctf7* cells as Mcd1p and Pds5p are detected over the entire DNA mass and exhibited similar intensity of staining (Figs. 7A & 7B). To more finely characterize cohesin complex and Pds5p localization, we used ChIP to examine their binding at the centromere of chromosome III (*CEN3*) and a *CEN3* proximal locus. Mcd1p and Pds5p binding was similar in wild type and *ctf7* mutants arrested in mid-M phase at 37°C (Fig. 7C). We then examined the binding at a CAR site on the arm of chromosome III (CARC1) as well as the adjacent DNA sequences. Once again, the binding patterns of Mcd1p and Pds5p are similar in wild type and *ctf7* mutant cells, both in overall level of binding as well as distribution along the chromosome (Fig. 7D). Thus, even though *ctf7* mutants exhibit defects in cohesion comparable to that of cohesin complex mutants, the cohesin complex and Pds5p appear to be bound normally to chromosomes.

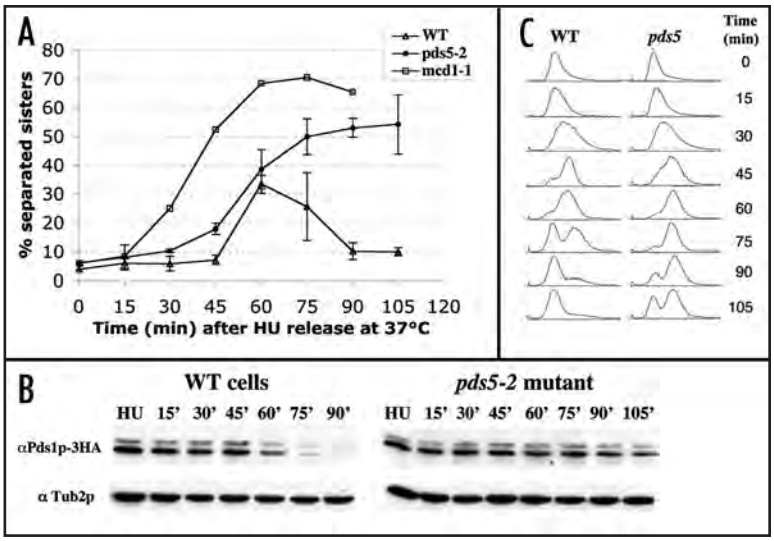

Figure 6. Comparison of precocious sister chromatid dissociation in wild type, *pds5* and *mcd1* cells. Haploid wild-type (VG2450-7A), *pds5-1* (VG2456-5C) and *mcd1-1* (VG557-5A) mutant cells were synchronously released from S phase arrest at 37° into YPD liquid (Materials and Methods). (A) Percentage of *pds5* cells with separated sister chromatids. Cohesion near *URA3* was monitored and the number of cells with 1 or 2 GFP signals determined. Cells with separated sisters (2 GFP signals) were plotted as a percentage of total cells. Open triangle (wild type), closed circles (*pds5-2*) and open squares (*mcd1-1*). Data was obtained from three independent experiments for wild type cells and *pds5-1* mutant cells and a single experiment for *mcd1-1* mutant cells. 100-300 cells at each time point in each experiment scored to generate data. (B) Western Blot of Pds1p levels. Wild type (VG2450-7A) and *pds5-1* (VG2456-5C) cells bearing PDS1-3HA were grown as described above. Western Blots of total proteins using anti-HA (Pds1-3HA) or anti-b tubulin (Tub2) antibodies. One of two independent experiments is shown. (C) DNA content of cells by FACS.

DISCUSSION

Cohesin deposition occurs during telophase in vertebrate organisms occurs during late G_1 or at the G_1/S phase transition in budding yeast.[1,11,38,41,42] Sister chromatid cohesion is formed during or soon after replication and requires functional cohesin complex. The Scc2p/Scc4p complex is required for cohesin complex localization to chromosomes but cohesion establishment also requires the activity of several proteins during S phase.[32] Ctf7p is the most critical of these establishment factors as its inactivation during S phase generates precocious sister dissociation at levels comparable to cohesin complex mutants.[28,29,43] However, a molecular mechanism describing Ctf7p function remained elusive. Our studies uncover an intimate relationship between Ctf7p and Pds5p, thus revealing an intersection between the regulators of establishment and maintenance. These data provide new insights into the molecular mechanism of cohesion establishment and the functions of Ctf7p and Pds5p.

One problem in understanding the molecular basis of Ctf7p function in establishment was the inability to connect it with cohesin complex. Ctf7p is a nuclear protein but has not been shown to localize at CAR sites. Moreover, previous in vivo or in vitro analyses failed to detect any coIP between Ctf7p and cohesin complex subunits Mcd1p and Scc3p.[7,29] Our observation that Ctf7p and Pds5p physically associate provides some insight. Pds5p and cohesin complex are intimately associated as they bind to each other and colocalize at all CAR sites.[1,36,37,44] We propose that Pds5p helps recruit Ctf7p to CAR sites, and thus brings Ctf7p into close proximity to cohesin complex to promote efficient establishment. Additional evidence supporting the biological relevance of the Pds5p-Ctf7p association comes from fission yeast, where a 2-hybrid interaction between Pds5p and Eso1p (fission yeast homolog of Ctf7p) is detected but this interaction is lost when a mutant *eso1* allele defective in establishment is assayed.[36]

It is necessary to understand the molecular basis of sister chromatid cohesin in order to elucidate how Ctf7p functions to mediate establishment. A popular model for cohesion posits that once the cohesin ring is loaded onto chromosomes, replication through the rigid ring ensures that cohesion is established and maintained until the Mcd1p subunit is cleaved at anaphase.[17] However, we find that in *ctf7* mutants, both cohesin complex and Pds5p are bound at CARs and broadly distributed along chromosomes at levels similar to wild type cells. An independent result using ChIP of Mcd1p and Pds5p also shows their binding to CARs is not perturbed in *ctf7* mutants (Ünal E, Koshland D, personal communication). This result does not appear consistent with the replication through a rigid ring model. Thus, to promote establishment, Ctf7p mediates events that occur after both cohesin complex and Pds5p deposition.

We propose three models explaining how Ctf7p and Pds5p cooperate to mediate efficient establishment. First, we consider a model where cohesion is mediated by a single cohesin ring that is wrapped around both sisters (Fig. 8A). Prior to DNA replication, cohesin complex and Pds5p are at CARs. Since it is not known how Pds5p interacts with cohesin complex, a generic cohesin ring is depicted. We place cohesin complex closer to the DNA than Pds5p and term this the preestablishment complex (pre-EC) (Fig. 8A, left side). This arrangement is consistent with data that Pds5p localization to chromosomes is dependent on cohesin complex whereas cohesin complex still localizes to chromosomes when Pds5p function is compromised.[1,34-36,39] In the pre-EC form, Pds5p is positioned on cohesin complex to prevent ring opening. To promote establishment, Ctf7p interacts with Pds5p to either change its conformation or orientation on cohesin complex, which generates a state permissive for establishment. Ctf7p then acts on cohesin complex, presumably Mcd1p or possibly Scc3p, to facilitate ring opening and allow the newly replicated sister to enter the ring. Finally, the ring is reclosed and locked by the return of Pds5p to form a protected site of cohesion. A second

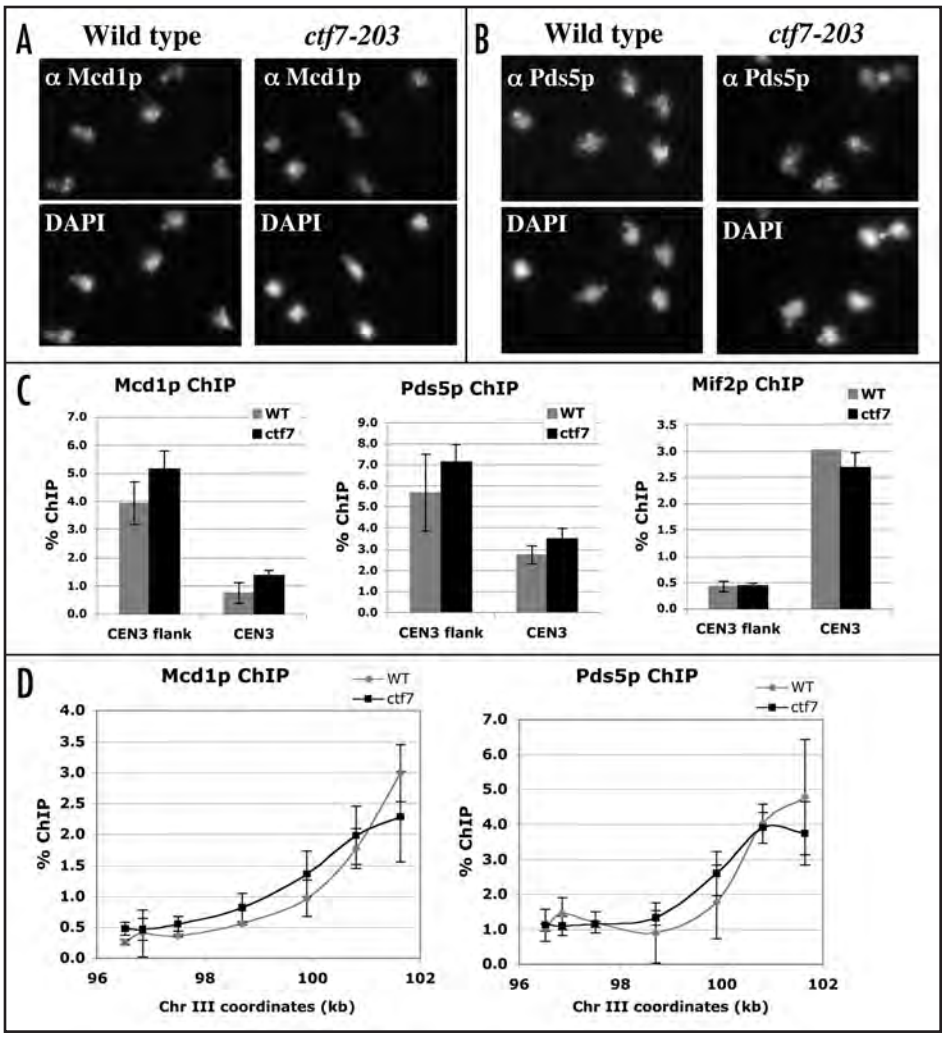

Figure 7. Mcd1p and Pds5p localization to chromosomes in wild type and *ctf7-203* cells. Haploid wild-type (VG2898-4C) and *ctf7-203* (VG3084-2A) cells arrested in G_1 phase at 23°C were released from G_1 phase then arrested at 37°C in mid-M phase (Materials and Methods). Aliquots of mid-M phase arrested cells were processed for chromosome spreads and for ChIP. (A and B) Chromosome spreads. (A) Mcd1p assayed using rabbit anti-Mcd1p antibodies (Mcd1p) and chromosomal DNA (DAPI). (B) Pds5p assayed using rabbit anti-Pds5p antibodies (Pds5p) and chromosomal DNA (DAPI). (C and D) ChIP of Mcd1p and Pds5p. Mcd1p and Pds5p were assayed using rabbit anti-Mcd1p and rabbit anti-Pds5p antibodies, respectively. (C) ChIP at *CEN3* and a *CEN3* proximal locus. (D) ChIP at *CARC1* and adjacent loci. (E) DNA content of cells by FACS.

model has one cohesin ring wrapped around each sister immediately following replication to form two pre-EC rings (Fig. 8B). Ctf7p action on Pds5p and Mcd1p would enable transient ring opening as described above but the end result is two interlocked cohesin rings. The third model is one where cohesion is mediated by an association of two cohesin complexes (Fig. 8C). Immediately after fork passage two cohesin complexes are on chromosomes in the pre-EC configuration. Ctf7p acts on Pds5p to displace it, which exposes an interaction domain on both cohesin complex complexes. Ctf7p activity on Mcd1p then alters the cohesin complex conformation to favor association. Atomic force microscopy reveals that SMC complexes can exist in different conformations, where the hinge region can be away from the globular heads and another in a compact form where it folds back upon the globular heads.[45] Perhaps Ctf7p activity can change cohesin into the conformation that favors complex associations, or alternatively, make the complex more dynamic in alternating between both conformations. Finally, the end of Ctf7p activity enables the cohesin association to stabilize and Pds5p to be repositioned such that it protects the junction, thereby forming a protected site of cohesion. This model enables a distinction between the mechanism responsible for cohesin complex loading onto chromosomes and that mediating cohesion. In models 2 and 3 (Fig. 8B and C), there is no need for a cohesin ring to actually wrap around each sister chromatid as cohesin association with a sister is also consistent with these models.

How might Ctf7p mediate the changes to cohesin complex and Pds5p described in our models above? We favor the idea that acetylation is the mechanism of action. Ctf7p acetylates itself, Pds5p, and the cohesin subunits- Mcd1p and Scc3p in vitro. Moreover, we find that the acetylase activity of Ctf7p was required for robust suppression of *pds5* mutants and its decreased acetylase activity exacerbated the toxicity to the *mcd1* mutant. Together, these data implicate Ctf7p acetylase activity on cohesin complex and Pds5p as being important for establishment. However, we cannot rule out that the

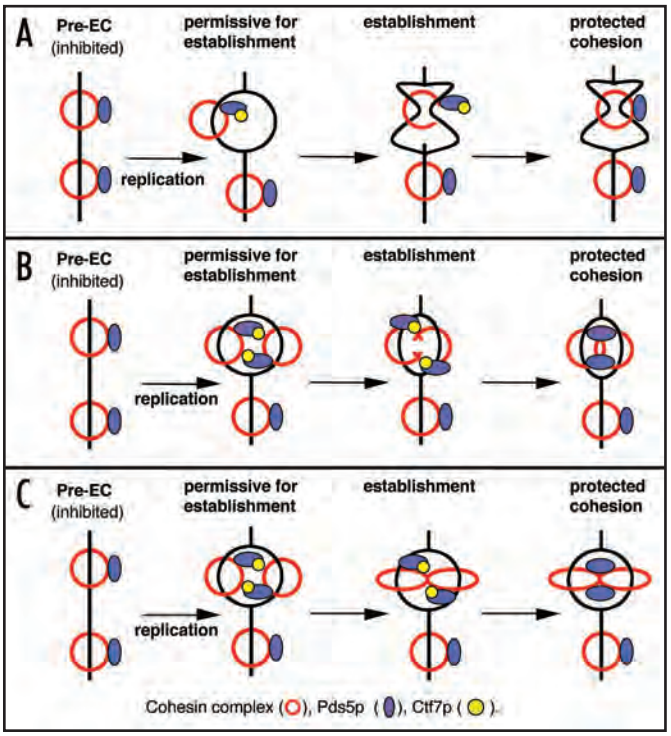

Figure 8. Model illustrating how Pds5p and Ctf7p cooperate to regulate cohesion establishment. Cohesin complex (red circles), Pds5p (blue ovals), Ctf7p (yellow circles).

auto-acetylation of Ctf7p regulates another property of Ctf7p important for establishment. For example, Ctf7p contains a zinc finger domain, which when mutated induces chromosome instability, so Ctf7p acetylation could regulate the zinc finger domain.[4] Two lines of evidence argue against a physiological role for Mcd1p acetylation. First, mutations in the Ctf7p acetylase domain that greatly reduce in vitro acetylation activity have no effect on yeast cells.[4] Second, mutation of the lysine residue of Mcd1p that is acetylated in vitro by Ctf7p had no effect on budding yeast cell growth.[40] It may be that only a small amount of acetylation is required for establishment function or that another acetylase can substitute for Ctf7p. Furthermore, the in vitro Ctf7p assay may not mimic the putative in vivo Mcd1p acetylation. Alternatively, a functional redundancy may exist so that either Scc3p or Mcd1p acetylation could modulate cohesin complex. Finally, perhaps Ctf7p acetylation of Pds5p is sufficient to enable changes in cohesin complex that facilitate establishment.

Whether or not acetylation is the sole or most important activity of Ctf7p, Pds5p recruitment of Ctf7p to CARs can also be viewed as antagonistic to the initial Pds5p protective position. This would explain the opposite effects of Ctf7p over-expression on *pds5* and *mcd1* mutants. It is also consistent with genetic data from fission yeast where Pds5p and Ctf7p appear to function antagonistically.[36] If Pds5p were the sole mechanism for recruiting Ctf7p to CARs sites, one would expect *pds5* mutants to have an establishment defect as severe as *ctf7* mutants. However, the *pds5* defect is observed on only 20% of sisters, less severe than *ctf7* mutants. The mutant *pds5* protein is still present in cells but at a lower level than wild type (Guacci V, unpublished results), making it possible that this mutant protein can still facilitate some Ctf7p recruitment to CARs. Alternatively, Ctf7p also associates with DNA replication factors, such as PCNA and the alternative RFC complex.[7,28] Mutants in PCNA and alternative RFC subunits exhibit only 10% to 20% precocious sister separation.[7,28,46,47] Therefore, there may be multiple mechanisms to bring Ctf7p near cohesin complex at the right time, but all are required for maximal efficiency. Ctf7p interaction with Pds5p may only occur during replication fork passage or immediately afterwards. The transient nature of the interaction would explain the failure to detect Ctf7p localization to CARs or in vivo acetylation of Pds5p, Mcd1p or Scc3p. Outside of S-phase, Ctf7p is dispensable for cohesion suggesting it no longer antagonizes Pds5p. This would enable Pds5p to remain on the cohesin complex as a molecular shield to promote cohesion maintenance until anaphase onset.[3]

We previously proposed that sumoylation of Pds5p modulates its function to promote cohesion dissolution.[3] Our data is consistent with acetylation of Pds5p and Mcd1p playing a role in establishment. A recent study suggested that cohesin sub-units Mcd1p and Smc1p are sumoylated.[48] Sumoylation and acetylation both occur at lysine residues. This raises the idea that different post-translational modifications of Pds5p and cohesin complex generated at distinct cell cycle stages could modulate their functions in both cohesion establishment and maintenance.

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