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A novel mechanism for the establishment of sister chromatid cohesion by the ECO1 acetyltransferase

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ABSTRACT Cohesin complex mediates cohesion between sister chromatids, which promotes high-fidelity chromosome segregation. Eco1p acetylates the cohesin subunit Smc3p during S phase to establish cohesion. The current model posits that this Eco1p-mediated acetylation promotes establishment by abrogating the ability of Wpl1p to destabilize cohesin binding to chromosomes. Here we present data from budding yeast that is incompatible with this Wpl1p-centric model. Two independent in vivo assays show that a wpl1Δ fails to suppress cohesion defects of eco1Δ cells. Moreover, a wpl1Δ also fails to suppress cohesion defects engendered by blocking just the essential Eco1p acetylation sites on Smc3p (K112, K113). Thus removing WPL1 inhibition is insufficient for generating cohesion without ECO1 activity. To elucidate how ECO1 promotes cohesion, we conducted a genetic screen and identified a cohesion activator mutation in the SMC3 head domain (D1189H). Smc3-D1189H partially restores cohesion in eco1Δ wpl1Δ or eco1 mutant cells but robustly restores cohesion in cells blocked for Smc3p K112 K113 acetylation. These data support two important conclusions. First, acetylation of the K112 K113 region by Eco1p promotes cohesion establishment by altering Smc3p head function independent of its ability to antagonize Wpl1p. Second, Eco1p targets other than Smc3p K112 K113 are necessary for efficient establishment.

INTRODUCTION Cohesin is an essential, evolutionarily conserved four-subunit complex that tethers sister chromatids from their formation in S phase through metaphase (Guacci et al., 1997; Michaelis et al., 1997; Losada et al., 1998; Sumara et al., 2000; Tomonaga et al., 2000). This tethering (cohesion) enables each sister chromatid pair to achieve a bipolar attachment to the mitotic spindle and thereby promotes high-fidelity chromosome segregation at anaphase. In budding yeast, the cohesin subunits are named MCD1/SCC1, SMC1, SMC3, and SCC3/IRR1 (Figure 1A; Guacci et al., 1997; Michaelis et al., 1997). Cohesin also plays roles in chromosome condensation, transcriptional regulation, and DNA damage repair (Guacci et al., 1997; Rollins et al., 2004; Ström et al., 2004; Unal et al., 2004). These processes are temporally and spatially distinct. For example, cohesion is established during S phase, whereas condensation occurs during mitosis (Oll et al., 2008). Cohesin binds specific sites called cohesin-associated regions (CARs) for cohesion and condensation, whereas it binds any DNA adjacent to double-strand breaks to help facilitate damage repair (Blat and Kleckner, 1999; Megee et al., 1999; Loralaya et al., 2000; Kim et al., 2002; Ström et al., 2004; Unal et al., 2004). Elucidating the mechanisms underlying the temporal and spatial regulation of cohesin is crucial to understanding how cohesin performs its diverse functions and how it tethers chromatins.

A simple model for the regulation of cohesion establishment was built around the cohesin inhibitor, termed WPL1/RAD61 in yeast (Rowland et al., 2009; Sutani et al., 2009; Chan et al., 2012). This WPL1-centric model emerged from three observations in budding yeast. First, the ECO1/CTF7 protein (Eco1p/Ctf7p) acetylates the Smc3p cohesin subunit at lysine residues 112 and 113 (K112, K113),...
and this K113 acetylation is required to allow chromatin-bound cohesin to establish cohesion (Skibbens et al., 1999; Tóth et al., 1999; Rolef Ben-Shahar et al., 2008; Unal et al., 2008; Zhang et al., 2008). Second, deleting WPL1 (wpl1Δ) suppresses the inviability of cells deleted for ECO1 (eco1Δ) or bearing the SMC3 acetylation-null smc3-K113R or smc3-K112R,K113R allele (lysine to arginine; Rolef Ben-Shahar et al., 2008; Rowland et al., 2009; Sutani et al., 2009). Third, the stability of cohesin binding to chromosomes is decreased...
in eco1 mutants relative to wild type but is more stable than wild type in either wpl1Δ or eco1Δ wpl1Δ cells (Chan et al., 2012). Based on these and other studies, it was proposed that a ring-like cohesion molecule topologically entraps both sister chromatids to generate cohesion (Öhn et al., 2008; Chan et al., 2012). In this scenario, Wpl1p inhibits cohesion establishment by destabilizing cohesion binding (Supplemental Figure S1). Acetylation of Smc3p at K112, K113 promotes cohesion by antagonizing Wpl1p, thereby stabilizing cohesion binding/sister entrapment.

Recent data contradict the Wpl1p-centric view of cohesion regulation. Previously we and two other laboratories showed that although viable, eco1Δ wpl1Δ mutants have defects in cohesion as severe as that of eco1 mutants (Rowland et al., 2009; Sutani et al., 2009; Guacci and Koshland, 2012). We extended this result by showing that eco1Δ wpl1Δ cells are defective in the establishment of cohesion (Guacci and Koshland, 2012). These results led us to three conclusions (Guacci and Koshland, 2012). First, since budding yeast can be viable with very little, if any, sister chromatid cohesion, a second mechanism for bipolar attachment of sister chromatids to the spindle must exist. We provided evidence that this alternative mechanism in yeast results from the unusual precocious assembly of the spindle in yeast (Supplemental Figure S5). Acetylation of Smc3p at K112, K113 promotes cohesion by antagonizing Wpl1p, thereby stabilizing cohesion binding/sister entrapment.

Here we provide in vivo evidence that eco1Δ wpl1Δ cells lack sufficient cohesion to promote chromosome segregation. We also conducted a genetic screen to identify mutations that restore cohesion in eco1Δ wpl1Δ cells. One such cohesion activator mutation in the Smc3p head domain changes the highly conserved aspartic acid residue 1189 to histidine (smc3-D1189H) and partially restores cohesion to eco1Δ wpl1Δ cells. The D1189H mutation restores nearly wild-type levels of cohesion to smc3-K112R,K113R. Our results provide strong evidence that Eco1p acetylation of Smc3p modulates its head domain to promote cohesion establishment. This modulation is independent of any antagonism of Wpl1p and occurs at a step distinct from cohesion binding to chromosomes. Our data also suggest that Eco1p has additional targets that promote efficient cohesion establishment other than the Smc3p K112, K113 residues that have been the prime focus of establishment studies.

RESULTS
eco1Δ wpl1Δ cells have little or no functional cohesion
Several groups, including ours, found major cohesion defects in eco1Δ wpl1Δ cells when directly monitoring cohesion at specific chromosome loci, but one group reported significantly less of a defect (Rowland et al., 2009; Sutani et al., 2009; Guacci and Koshland, 2012; Rolef Ben-Shahar et al., 2008). To rule out the possibility that secondary mutations arose during outgrowth of our strains to generate the dramatic cohesion defect, we used a conditional eco1Δ wpl1Δ strain. For this purpose, we constructed a conditional ECO1 allele using the auxin-inducible degron system (AID), which entailed a genetic screen to identify mutations that restore cohesion in eco1Δ wpl1Δ cells (Supplemental Figure S2A). A wpl1Δ strain becomes inviable (Supplemental Figure S2A). A wpl1Δ suppresses the auxin-dependent inviability of the ECO1-AID allele, recapitulating the ability of wpl1Δ to bypass an essential function of ECO1p (Supplemental Figure S2A). Moreover, the ECO1-AID strain also exhibits an auxin-dependent benomyl sensitivity similar to that of eco1Δ wpl1Δ cells, consistent with a cohesion defect.

We next asked whether a severe cohesion defect is indeed generated in the first cell cycle after Eco1p depletion in a wpl1Δ background. After strains were arrested in G1, auxin was added to delete ECO1-AIDp. Cells were released from G1 into media containing auxin and nocodazole to arrest them in mid–M phase under conditions in which ECO1-AIDp is depleted over this entire cell-cycle interval (Supplemental Figure S2B). Yeast cells are termed mid–M phase because sister chromatids have cohesion and are condensed (Guacci et al., 1994). Cohesion was scored at a CEN-distal (LYS4) locus. In this assay, a failure to establish or maintain cohesion leads to the presence of two GFP foci (spots) in cells. Wild-type cells have robust cohesion, as shown by few mid–M phase cells with two GFP spots, whereas ECO1-AID cells have a severe defect, as shown by the high percentage of cells with two GFP spots (Supplemental Figure S2C, left). Of importance, >70% of either ECO1-AID wpl1Δ or eco1Δ wpl1Δ cells have two GFP spots, indicating a cohesion defect as severe as seen in ECO1-AID cells (Supplemental Figure S2C, right).

Few G1 cells from any strain had two GFP spots, demonstrating the absence of preexisting aneuploidy, which indicates that mid–M phase cells with two GFP spots arose due to defective cohesion.
Thus the first cell-cycle depletion of Eco1p in the wpl1Δ background recapitulates studies in which eco1Δ wpl1Δ cells had both major cohesion defects and benomyl sensitivity (Sutani et al., 2009; Guacci and Koshland, 2012). This result makes it highly unlikely that secondary mutations are responsible for these eco1Δ wpl1Δ mutant phenotypes.

Observing major cohesion defects at specific loci in eco1Δ wpl1Δ cells does not rule out the possibility that cohesion exists at other chromosomal regions. If such residual cohesion exists, it should enable bipolar chromosome attachments and thereby promote sister segregation and viability. We assessed this possibility using a nocodazole-arrest release segregation assay (Figure 1B and Materials and Methods). Arrest in mid–M phase using nocodazole abrogates any early S-phase attachments that enable cohesion-independent segregation (Guacci and Koshland, 2012). Subsequent release from nocodazole arrest allows spindles to form, kinetochores to attach to microtubules, and cells to complete mitosis. We labeled the CEN4-proximal (*TRP1*) locus because chromosome IV is the second largest yeast chromosome. As such, it is an excellent substrate to assess whether cohesion exists at other loci along a chromosome and thereby enable bipolar attachment in mitosis to promote subsequent segregation (i.e., segregation-competent cohesion).

To assess segregation-competent cohesion, wild-type and eco1Δwpl1Δ strains were released from G1 into media containing nocodazole to induce arrest in mid–M phase as large-budded cells lacking spindles. Consistent with our previous analyses, cohesion was robust in wild-type cells and severely defective in eco1Δ wpl1Δ cells (Figure 1C). Mid–M phase–arrested cells were allowed to complete mitosis and arrest in G1 by removing nocodazole and adding ω-factor (Figure 1B). We first monitored cell-cycle progression. By 2 h after release from nocodazole arrest, many wild-type cells completed anaphase and exited mitosis, as seen by a decrease in the percentage of large-budded cells and the appearance of cells with 1C DNA content (Figure 1D). In contrast, most eco1Δ wpl1Δ cells remained large budded and had 2C DNA content. By 3 h, virtually all wild-type cells had completed mitosis and arrested in G1, whereas only ~50% of eco1Δ wpl1Δ cells had done so. This mitotic progression delay of eco1Δ wpl1Δ cells is characteristic of checkpoint-mediated delays after global cohesion abrogation via mutants in Eco1p or cohesion subunits (Skibbens et al., 1999; Stead et al., 2003; Noble et al., 2006).

We then scored chromosome IV segregation in large-budded cells 2 h after release from nocodazole arrest (Materials and Methods). More than 80% of large budded wild-type cells had one GFP spot in each daughter nucleus, indicating segregation of sister chromatids (Figure 1E, left). In contrast, only 50% of large-budded eco1Δ wpl1Δ cells showed segregation to the daughter nuclei. We also scored segregation in unbudded cells, which have completed mitosis and rearrested in G1 (Materials and Methods). Approximately 90% of unbudded wild-type cells exhibit proper chromosome IV segregation, whereas only ~50% of unbudded eco1Δ wpl1Δ cells showed segregation (Figure 1E, right). Moreover, sub-1C and >1C peaks are observed in eco1Δ wpl1Δ cells at 4 h after release, consistent with a global defect in segregation (Figure 1D). Random segregation (50%) is expected if there is a complete lack of cohesion, leading us to conclude that eco1Δ wpl1Δ cells have little or no segregation-competent cohesion.

A genetic screen identified smc3-D1189H as a mutation that partially restores cohesion in eco1Δ wpl1Δ cells

Our previous study indicated that cohesion mutants use an alternative mechanism to segregate their sister chromatids despite abrogation of cohesion (Guacci and Koshland, 2012). This cohesion-independent mechanism depends on the persistence of bipolar sister chromatid attachments to kinetochore microtubules generated in early S phase (Figure 2A). Our finding that eco1Δ wpl1Δ cells have little or no segregation-competent cohesion suggests that they also depend on this alternative pathway. This view is consistent with the observation that eco1Δ wpl1Δ cells are inviable when exposed to low levels of the microtubule-depolymerizing drug benomyl (Figure 2A; Sutani et al., 2009; Guacci and Koshland, 2012). These cells are also very sensitive to camptothecin, a topoisomerase I inhibitor that induces DNA damage, possibly because without cohesion, they are less competent to repair DNA damage (Guacci and Koshland, 2012). We reasoned that screening for eco1Δ wpl1Δ cells resistant to either drug should select for suppressor mutations that restore cohesion establishment in the absence of both Eco1p and Wpl1p (see Supplemental Figure S3 and Materials and Methods for screen details).

Only about 1 in 1 million eco1Δ wpl1Δ cells exhibited benomyl or camptothecin resistance.Suppressors were then tested for resistance to the other drug (i.e., benomyl-resistant clones were assayed for camptothecin resistance). Only a small number of clones exhibited dual drug resistance, suggesting that suppressing the cohesion defect of eco1Δ wpl1Δ cells required specific and rare changes in genes. All clones exhibiting dual drug resistance were subjected to whole-genome sequencing. The same amino acid change in Smc3p was found in four independent clones exhibiting dual drug resistance. This mutation is located in the Smc3p head domain and changed aspartic acid residue 1189 to histidine (D1189H; Figure 2B). To assess linkage between the smc3-D1189H mutation and the drug resistance, we reintroduced this allele in place of SMC3 in the parent eco1Δ wpl1Δ (Materials and Methods). We compared one of the screen-derived D1189H suppressors (sup #1) to the rebuilt smc3-D1189H eco1Δ wpl1Δ strain and found that they are phenotypically indistinguishable (Figure 2C). This result confirms that smc3-D1189H is responsible for the drug resistance. The D1189H residue is highly conserved evolutionarily, as the analogous position in all SMC3 family members is either an aspartic acid or a glutamic acid residue (Figure 2D).

Cohesion around the centromere is essential to enable sister kinetochores to reform bipolar attachments after their transient detachment from spindle microtubules. Therefore we assumed that smc3-D1189H eco1Δ wpl1Δ cells acquired benomyl resistance because cohesion was restored at centromere-proximal regions. To test this idea, we began by comparing the level of cohesion at the centromere-linked *TRP1* locus in wild-type, eco1Δ wpl1Δ, and our reconstructed smc3-D1189H eco1Δ wpl1Δ strain. The smc3-D1189H eco1Δ wpl1Δ cells had restored cohesion relative to eco1Δ wpl1Δ but not fully restored to wild-type levels (Figure 3A). This level of cohesion is sufficient to explain the benomyl resistance, as it is similar to the cohesion level and benomyl resistance seen in a wpl1Δ strain (compare Figures 1C and 3A; Guacci and Koshland, 2012). We then tested whether the cohesion restored by smc3-D1189H as measured via the GFP-spot assay is functional, segregation-competent cohesion using our nocodazole-arrest release segregation assay (Figure 1B). Because smc3-D1189H eco1Δ wpl1Δ and wpl1Δ cells have similar levels of cohesion, we compared their ability to promote segregation. After release from nocodazole arrest, smc3-D1189H eco1Δ wpl1Δ segregated sister chromatids properly in 80% of cells, much better than the random 50% segregation observed in eco1Δ wpl1Δ cells and nearly as well as wpl1Δ or wild-type cells (Figure 3B). Therefore the cohesion restored by smc3-D1189H is segregation competent. Moreover, smc3-D1189H eliminates the sub-1C peak found in eco1Δ wpl1Δ cells, suggesting global restoration of cohesion (Figure 3B). Because smc3-D1189H
restores cohesion to eco1Δ wpl1Δ cells, we term it a cohesion activator mutation.

To further characterize the partial restoration of cohesion in smc3-D1189H eco1Δ wpl1Δ cells, we examined cohesion at TRP1 as cells progressed through the cell cycle as compared with wild-type and eco1Δ wpl1Δ cells. Cells were released from G1 into media containing nocodazole to induce mid–M phase arrest. Cell aliquots were fixed in G1 and at 20-min intervals after G1 release to assess cohesion and DNA content. As expected for wild-type cells, separated sister chromatids were rarely observed, indicative of robust cohesion (Figure 3C). The eco1Δ wpl1Δ cells exhibited separated sister chromatids (two GFP spots) beginning in S phase, consistent with a previously reported establishment defect (Guacci and Koshland, 2012). Fewer smc3-D1189H eco1Δ wpl1Δ cells exhibited two GFP spots than eco1Δ wpl1Δ cells, but the kinetics of cohesion loss was similar, as it also begins during S phase. These results indicate that

FIGURE 2: Genetic screen identifies the smc3-D1189H mutation as a suppressor of eco1Δ wpl1Δ strain sensitivity to benomyl and camptothecin. (A) Rationale for screen. Haploid WT (VG3349-1B) and eco1Δ wpl1Δ (VG3503-4A) were grown to saturation at 23°C, plated at 10-fold serial dilution on YPD alone or containing 12.5 μg/ml BEN, and then incubated at 23°C for 3 d. Left, eco1Δ wpl1Δ cell viability on YPD and schematic showing sister segregation via early S-phase attachments despite failure to establish cohesion. Right, eco1Δ wpl1Δ cell inviability on BEN and schematic showing benomyl-induced loss of S-phase spindle attachments and consequent missegregation and inviability. (B) Cartoon showing localization of the D1189 residue in the SMC3 head domain. (C) Cross drug resistance of the smc3-D1189H suppressor. Haploid WT (3460-2A) and three eco1Δ wpl1Δ background strains, parent SMC3 (VG3502-1A), D1189H suppressor 1 (Sup #1), and rebuilt smc3-D1189H (VG3547-3B), were grown and plated as described in A onto YPD alone or containing 12.5 μg/ml BEN or 10 μg/ml CPT and incubated for 3 d at 23°C. Strains below the red line are eco1Δ wpl1Δ background. (D) Schematic showing evolutionary conservation of budding yeast smc3-D1189H residue (red letter). The black line above the sequence shows the conserved DE residues of the Smc3p Walker B box.
FIGURE 3: smc3-D1189H partially restores sister chromatid cohesion in eco1Δ wpl1Δ cells. (A) Cohesion loss at a CEN-proximal TRP1 locus. Haploid WT (VG3460-2A), eco1Δ wpl1Δ (VG3502-1C), and smc3-D1189H eco1Δ wpl1Δ (VG3547-3B) were arrested in mid–M phase as described in Figure 1C. The percentage of cells with two GFP signals (sister separation) is plotted. The lack of G1 cells with two GFP spots demonstrates absence of preexisting aneuploidy. Data are from four independent experiments; 100–300 cells were scored for each data point in each experiment. (B) Assay for segregation-competent cohesion. Haploid smc3-D1189H eco1Δ wpl1Δ (VG3549-7A) and wpl1Δ (VG3513-1B) cells were treated as depicted in Figure 1B and then assayed for chromosome segregation after release from mid–M arrest. Proper segregation of chromosome IV sister chromatids in large-budded cells 2 h after release (left) and in unbudded cells 3 h after release (middle) and DNA content (right). Random segregation will be 50% and is marked by a dotted red line. Data were generated simultaneously with strains in Figure 1, C–E, in two independent experiments in which 100–300 cells were scored for each data point. (C) Kinetics of cohesion loss at a CEN-proximal TRP1 locus. Strains in A released from G1 and arrested in mid–M phase as described in Figure 1C. The percentage of cells with two GFP.
smc3-D1189H promotes cohesion establishment in the eco1Δ background, but the residual cohesion defect is due to incomplete restoration of establishment rather than a defect in cohesion maintenance. We next examined cohesion at the CEN-distal (LYS4) locus in these strain backgrounds. The smc3-D1189H allele also partially suppresses the cohesion establishment defect of eco1Δ wpl1Δ (Figure 3, D and E). Note that the suppression was less robust than at the CEN-distal locus compared with the CEN-proximal locus.

**smc3-D1189H cohesin responds to regulators as well as wild-type cohesin**

The partial restoration of cohesion by smc3-D1189H in the eco1Δ wpl1Δ background could reflect the inability of smc3-D1189H to fully compensate for cohesion defects caused by the absence of Eco1p and Wpl1p. Alternatively, it could be due to a cohesion defect caused by smc3-D1189H itself. To test whether the smc3-D1189H mutation has inherent defects in cohesion and cohesin function, we generated an otherwise wild-type yeast strain bearing smc3-D1189H as the sole SMC3 allele (Materials and Methods). Wild-type and smc3-D1189H cells were assayed for drug sensitivity and showed similar strong resistance to both benzyl and camptothecin (Figure 4A). We next assayed cohesion at CEN-proximal (TRP1) and CEN-distal (LYS4) loci, as well as cohesin binding to CARs via chromatin immunoprecipitation (ChIP). Wild-type and smc3-D1189H cells were released from G1 and arrested in mid–M phase (Materials and Methods). Few mid–M phase cells had two GFP spots in either wild-type or smc3-D1189H cells, indicating that both strains had robust cohesion (Figure 4B). Analysis of the chromosomal binding of cohesin subunits shows that they colocalize, making the analysis of any one a surrogate marker for cohesin binding (Glynn et al., 2004; Lengronne et al., 2004; Heidinger-Pauli et al., 2010). We used antibodies against Mcd1p (αMcd1p) as a marker to detect cohesin binding to chromosomes. ChiP showed no difference in Mcd1p binding at the CEN-proximal CARC1 or the CEN-distal CARL1 in these two strains (Figure 4C). Thus smc3-D1189H appears fully competent to promote cohesion and to respond normally to cohesion regulation by both Eco1p and Wpl1p. Finally, the Scc2p loader complex is required for cohesion and viability in wild-type cells (Ciok et al., 2000). Smc3-D1189H cells also require Scc2p for both viability and cohesion (Supplemental Figure S4). These results show that smc3-D1189H cohesin functions as well as wild-type (WT) cohesin when normal cohesin regulation is present. Therefore the partial suppression of the cohesion defect of eco1Δ wpl1Δ cells by smc3-D1189H is not due to an inherent cohesin defect associated with smc3-D1189H. Instead, it indicates that smc3-D1189H restores only a subset of the Eco1p and/or Wpl1p activities.

**smc3-D1189H does not suppress the cohesion defect of a wpl1Δ**

The residual cohesion defect in smc3-D1189H eco1Δ wpl1Δ (35% sister chromatid separation at TRP1) is very similar to that of a wpl1Δ mutant alone (compare Figures 1C and 3A; Guacci and Koshland, 2012). This similarity could reflect that smc3-D1189H robustly suppresses the eco1Δ without affecting the cohesion defect caused by a wpl1Δ. Alternatively, it could reflect complete suppression of wpl1Δ, but only partial suppression of the eco1Δ. We first addressed whether smc3-D1189H suppresses a wpl1Δ. One signature of a wpl1Δ is a reduction in cohesin bound at CARs as compared with WT cells (Rowland et al., 2009; Sutani et al., 2009). In contrast, an eco1 mutant does not reduce cohesin binding at CARs (Noble et al., 2006). Therefore, if smc3-D1189H in eco1Δ wpl1Δ suppressed the loss of Wpl1p, we would expect it to increase cohesin binding to WT levels. We examined whether smc3-D1189H affects cohesin binding to chromosomes in the eco1Δ wpl1Δ background. Cells were released from G1 and then rearrested in mid–M phase (Materials and Methods). Mid–M phase cells were processed to assess cohesin binding to chromosomes using both chromosome spreads and ChiP.

We first used chromosome spreads to qualitatively assess cohesin binding to chromosomes. We detected robust Mcd1p staining on chromosomal DNA in wild-type, eco1Δ wpl1Δ, and smc3-D1189H eco1Δ wpl1Δ cells, indicative of broad cohesin binding (Figure 5A). We then used ChiP to perform a quantitative analysis of cohesin binding at two CARs—one in the pericentric region of chromosome III (CARC1) and one at a CEN-distal locus (CARL1). Mcd1p binding at either CAR site was reduced by twofold to threefold in the eco1Δ wpl1Δ cells compared with wild type (Figure 5B). This degree of reduction was previously reported for wpl1Δ and eco1Δ wpl1Δ cells (Sutani et al., 2009). The smc3-D1189H eco1Δ wpl1Δ cells exhibit the same reduced cohesin binding as the parent eco1Δ wpl1Δ cells (Figure 5B). Thus smc3-D1189H did not suppress the cohesin-loading defect characteristic of a wpl1Δ. Moreover, this result indicates that the improved sister chromatid cohesion engendered by smc3-D1189H in eco1Δ wpl1Δ cells occurs via a mechanism distinct from increasing cohesin localization to CARs.

To assess specifically whether smc3-D1189H affects the wpl1Δ cohesion defect, we analyzed its effect when Eco1p is present. For this purpose, we constructed an smc3-D1189H wpl1Δ strain and compared its phenotype to that of a wpl1Δ strain. The smc3-D1189H is unable to suppress the slight camptothecin sensitivity of a wpl1Δ (Figure 4A). In addition, the smc3-D1189H wpl1Δ strain exhibited a very similar cohesion defect as the wpl1Δ strain (Figure 5C). These results suggest that smc3-D1189H partially restores cohesion in eco1Δ wpl1Δ cells by recapitulating Eco1p activity rather than compensating for the loss of Wpl1p.

**smc3-D1189H suppresses the requirement for Eco1p-mediated Scc3p K112 K113 acetylation in cohesion generation**

Eco1p acetylates sites on cohesin subunits and cohesin regulators (Ivanov et al., 2002; Rofel Ben-Shahar et al., 2008; Unal et al., 2008; Zhang et al., 2008). Scc3p is acetylated at both K112 and K113, but
Mcd1p binding was assessed by quantitative PCR. Data are presented as percentage of total DNA assayed using the same primer pairs at each site. Left, chromosome III pericentric CARC1. Seven primer pairs used to assay Mcd1p binding at loci spanning an ∼2.6-kb region including CARC1 of chromosome III. Right, chromosome XII cen-distal CARL1. Seven primer pairs used to assay Mcd1p binding at loci spanning an ∼4.5-kb region including CARL1 of chromosome XII. WT (gray line, gray squares) and smc3-D1189H (black line, open circles).

K113 had been shown to be critical for cohesion (Unal et al., 2008; Zhang et al., 2008). The function and identity of other sites remain largely untested. Therefore it was possible that smc3-D1189H suppressed the eco1Δ wpl1Δ cohesion defect by providing the function of all the Eco1p substrates or of only a subset of them.

To distinguish between these possibilities, we first tested whether smc3-D1189H suppressed inviability and major cohesion defects associated with the failure to acetylate Smc3p K112 and K113 (Unal et al., 2008; Zhang et al., 2008). To assess bypass of the smc3-K112R, K113R acetyl-null (smc3-RR) inviability, we used an SMC3 shuffle strain in which the endogenous SMC3 is deleted but cells are kept viable by the presence of plasmid pEU42 (SMC3 URA3 CEN). We integrated a second “test SMC3 allele”—wild-type SMC3, smc3-D1189H, the K112R, K113R acetyl-null (smc3-RR), or a chimeric smc3-RR-D1189H allele—into the shuffle strain at the LEU2 locus. These test alleles were assayed for ability to support viability as the sole SMC3 source by growth on media containing 5-fluoroorotic acid (FOA; Materials and Methods). FOA selectively kills URA3 cells, thereby selecting for cells that had lost plasmid pEU42. As expected, all strains grew well on URA− medium because of the presence of WT SMC3 on pEU42 (Figure 6A). On FOA medium, cells with WT SMC3 and smc3-D1189H test alleles grew well, whereas the smc3-RR allele could not support viability. Cells bearing the chimeric smc3-RR-D1189H allele grew as well as on FOA medium as WT and smc3-D1189H cells, demonstrating that the smc3-D1189H mutation suppresses the inviability of the RR mutation (Figure 6A). We then compared the drug sensitivity of cells bearing only the WT SMC3, smc3-D1189H, or chimeric smc3-RR-D1189H test alleles as the sole SMC3. All strains showed equal resistance to both benomyl and camptothecin, suggesting that chimeric allele efficiently generated cohesion (Figure 6B).
FIGURE 5: Smc3-D1189H fails to suppress the characteristic wpl1Δ defects of reduced cohesin binding and partial cohesion loss. (A–C) Haploid wild-type (WT; VG3349-1B), eco1Δ wpl1Δ (VG3503-4A), and smc3-D1189H eco1Δ wpl1Δ (VG3549-7A) cells were arrested in mid–M phase as described in Figure 1C. (A) Chromosome spreads of mid–M phase cells. WT (top), eco1Δ wpl1Δ (middle), and smc3-D1189H eco1Δ wpl1Δ (bottom). Cells were processed to detect chromosomal DNA (4′,6-diamidino-2-phenylindole) and cohesin using αMcd1p antibodies. (B) ChIP of Mcd1p in mid–M phase cells. Cells were fixed and processed for ChIP using αMcd1p antibodies. WT (gray line, gray diamonds), eco1Δ wpl1Δ (red line, red circles), and smc3-D1189H eco1Δ wpl1Δ (black line, gray triangles). Mcd1p binding was assessed as described in Figure 4C. Top, chromosome III pericentric CARC1. Bottom, chromosome XII CEN-distal CARL. (C) Effect of smc3-D1189H on cohesion loss in a wpl1Δ background. Haploid WT, wpl1Δ, and smc3-D1189H wpl1Δ cells were arrested in mid–M phase as described in Figure 1C. Left, cohesion loss at CEN-proximal TRP1 locus assayed in haploid WT (VG3460-2A), wpl1Δ (VG3626-4C), and smc3-D1189H wpl1Δ (VG3605-5D) strains. Right, cohesion loss at CEN-distal LYS4 locus assessed in haploid WT (VG3349-1B), wpl1Δ (VG3626-2E), and smc3-D1189H wpl1Δ (VG3627-3C) strains. Bottom, DNA content. The percentage of cells with two GFP signals (sister separation) is plotted. Data were derived from two independent experiments; 100–300 cells were scored for each data point in each experiment.
FIGURE 6: smc3-D1189H robustly suppresses the cohesion defect of the smc3-K112R, K113R (RR) mutation. (A) Plasmid shuffle to assess viability of the chimeric smc3-RR-D1189H allele. Haploid shuffle strain VG3464-16C bearing plasmid pEU42 (SMC3 CEN URA3) and a second SMC3 “test allele,” SMC3, smc3-D1189H, smc3-RR, or chimeric smc3RR-D1189H, was grown and plated as described in Figure 2A onto URA–dropout or FOA-containing media. Plates were

C.

E.

F.
To test the suppression of the smc3-RR cohesion defect by smc3-D1189H, we compared cohesion at CEN-proximal and CEN-distal loci in cells expressing only chimeric smc3-RR-D1189H allele with cells expressing only the smc3-RR allele. Making this comparison was complicated by the fact that the smc3-RR allele cannot support viability (Unal et al., 2008; Zhang et al., 2008). Consequently, the smc3-RR allele was assayed in a strain bearing an smc3 temperature-sensitive allele at nonpermissive temperature and shown to have a major cohesion defect (Unal et al., 2008; Zhang et al., 2008). However, we wanted to assess the smc3-RR allele at low temperature (23°C) used for our previous assays. Therefore we constructed a parent strain bearing a conditional SMC3-AID allele, which enables the SMC3-AIDp to be rapidly degraded at 23°C upon auxin addition, rendering cells inviable and severely defective for cohesion (Supplemental Figure S5, A–C). We integrated a second SMC3 allele, either wild-type SMC3 or smc3-RR, into the SMC3-AID-bearing strain. As expected, the SMC3 allele suppressed the SMC3-AID inviability on auxin, whereas the smc3-RR failed to do so (Supplemental Figure S5C). We next assessed cohesion at CEN-proximal and CEN-distal loci in cells released from G1 and arrested in mid–M phase under conditions in which SMC3-AIDp was depleted over this entire cell-cycle window (Supplemental Figures S2B and S5D). The SMC3 allele enabled robust cohesion, whereas the smc3-RR cells were severely compromised for cohesion, as ∼70% of cells had two GFP spots (Figure 6C), a value similar to that for strains bearing only SMC3-AID as the sole SMC3 (Supplemental Figure S5B). We then examined cohesion at mid–M phase in strains in which the sole SMC3 allele is wild-type SMC3, smc3-D1189H, or the chimeric smc3-RR-D1189H allele (Supplemental Figure S5E). Only 20–30% of the cells in the chimeric smc3-RR-D1189H strain exhibited two GFP spots, a dramatic improvement over the cohesion defect of the smc3-RR strain and similar to that seen in either WT SMC3 or smc3-D1189H cells (Figure 6D). These results indicate that the smc3-D1189H mutation strongly suppresses the cohesion defect generated by blocking K112, K113 acetylation.

Evidence that Eco1-dependent acetylation of sites other than Smc3-K112, K113 are required for efficient cohesion establishment

We next addressed whether smc3-D1189H could suppress loss of all Eco1p acetylation targets in cells as well as it suppressed the smc3-K112, K113 acetyl null. Because eco1Δ cells are not viable, we used strains bearing a conditional ECO1-AID allele and either wild-type SMC3 or smc3-D1189H as the sole SMC3 alleles in cells. As expected, wild-type haploids grew on yeast extract/peptone/dextrose (YPD) medium either alone or containing auxin (Figure 6E). Auxin-containing medium prevented growth of ECO1-AID cells, but a low-level of viability was detected when the smc3-D1189H allele was present, indicating at best a weak suppression of the essential ECO1 function (Figure 6E).

We then examined cohesion at CEN-proximal and CEN-distal loci in these three strains after auxin-mediated ECO1-AIDp depletion from G1 through arrest in mid–M phase (Supplemental Figures S2B and S5F). As expected, the wild-type cells had robust cohesion (10% two GFP spots), whereas the ECO1-AID strain had a severe cohesion defect (∼60–70% two GFP spots; Figure 6F). The smc3-D1189H ECO1-AID strain also showed a strong cohesion defect, with only modest improvement in cohesion at CEN-proximal and CEN-distal loci, respectively. Thus the chimeric smc3-RR-D1189H allele in a WT background generated cohesion more efficiently than the smc3-D1189H allele did in an ECO1-AID background: 15 and 30% better at TRP1 and at LYS4, respectively (compare Figure 6, D and F). Therefore, whereas the smc3-RR and ECO1-AID alone have a similar ∼70% cohesion defect, smc3-D1189H suppresses the smc3-RR cohesion defect much better than that of an Eco1-AIDp depletion. These results suggest that the D1189H mutation effectively provides the K112, K113 acetylation function but that other Eco1p acetylation targets are necessary for efficient cohesion generation.

smc3-D1189H activates cohesion by a Wpl1p-independent mechanism

Previous work showed that a wpl1Δ suppresses the smc3-RR allele lethality (Rolef Ben-Shahar et al., 2008). This result raised the prospect that in the chimeric smc3-RR-D1189H allele, the D1189H mutation merely blocked the anticohesion function of Wpl1p. If true, then a wpl1Δ smc3-RR strain should be phenotypically identical to the chimeric smc3-RR-D1189H strain. Therefore we compared wild-type and smc3-RR wpl1Δ strains for viability, drug sensitivity, and cohesion generation. We first used a wpl1Δ SMC3 shuffle strain to assess smc3-RR wpl1Δ viability and drug sensitivity compared with SMC3 wpl1Δ. As previously reported, the wpl1Δ suppresses
containing media. Plates were incubated 2 d at 30°C. (B) Assessment of ∆wpl1
∆smc3-D1189H (allele—WT (smc3-RR wpl1), ∆smc3-D1189H wpl1, ∆smc3-RR wpl1Δ, or ∆smc3-R
were scored for each data point in each experiment.

DNA content (bottom). Data are derived from two independent experiments; 100–300 cells
arrested in mid–M phase as described in Figure 1B. Cells were scored for cohesion loss (top) and
4 d for BEN and CPT plates. (C) Cohesion loss at
alone or containing BEN (10 μg/ml) or CPT (10 μg/ml) and incubated at 23°C for 3 d for YPD and
3 d for YPD and CPT plates. (C) Cohesion loss at CEN-proximal locus LYS4. Haploid wild-type
(WT; VG3627-3C), wpl1 (SMC3 wpl1Δ; MB48-1A), and smc3-RR wpl1Δ (MB50-1A) strains arrested in mid–M phase as described in Figure 1B. Cells were scored for cohesion loss (top) and DNA content (bottom). Data are derived from two independent experiments; 100–300 cells
were scored for each data point in each experiment.

FIGURE 7: A wpl1Δ suppresses the inviability of smc3-RR-bearing cells but not the cohesion
defect. (A) Plasmid shuffle to assess viability of smc3-RR wpl1Δ cells. Haploid wpl1Δ SMC3 shuffle strain (VG3578-1A) bearing pEU42 (SMC3 CEN URA3) and a second integrated test
SMC3 allele—WT (SMC3 wpl1Δ), smc3-D1189H (smc3-D1189H wpl1Δ), or smc3-RR (smc3-RR
wpl1Δ)—were grown and plated as described in Figure 2A onto URA-dropout or FOA-
containing media. Plates were incubated 2 d at 30°C. (B) Assessment of smc3-RR wpl1Δ strain
drug sensitivity. Haploid SMC3 wpl1Δ (MB48-1A), smc3-D1189H wpl1Δ (VG3627-3C), and
smc3-RR wpl1Δ (MB50-1A) strains were grown and plated as described in Figure 2A onto YPD
alone or containing BEN (10 μg/ml) or CPT (10 μg/ml) and incubated at 23°C for 3 d for YPD and
4 d for BEN and CPT plates. (C) Cohesion loss at CEN-proximal locus LYS4. Haploid wild-type
(WT; VG3627-3C), wpl1 (SMC3 wpl1Δ; MB48-1A), and smc3-RR wpl1Δ (MB50-1A) strains arrested in mid–M phase as described in Figure 1B. Cells were scored for cohesion loss (top) and DNA content (bottom). Data are derived from two independent experiments; 100–300 cells
were scored for each data point in each experiment.

DISCUSSION
The prevailing Wpl1p-centric model posits that the Eco1p acetyltransferase promotes cohesion establishment simply by antagonizing Wpl1p-mediated inhibition (Rowland et al., 2009; Sutani et al., 2009; Chan et al., 2012). Contrary to this model, we previously showed that eco1Δ wpl1Δ cells have little or no cohesion, based on in vivo monitoring of specific chromosomal GFP-tagged loci (Guacci and Koshland, 2012). We suggested that Eco1p promotes cohesion establishment by a Wpl1p-independent mechanism. Here we provide additional in vivo metrics to support the absence of cohesion in eco1Δ wpl1Δ cells. First, we used a nocodazole arrest-release assay, which uses proper chromosome segregation as a sensitive readout for the presence of residual cohesion on a chromosome. We find that eco1Δ wpl1Δ cells, like cohesin subunit mutants, lack segregation competent cohesion (this study; Guacci and Koshland, 2012).

The lack of cohesion in eco1Δ wpl1Δ is also supported by published cytological observations from other laboratories. Before anaphase, yeast cells have a short mitotic spindle overlaid by a short bilobed barrel of centromeres and a single DNA mass (Pearson et al., 2004; Yeh et al., 2008). However, cohesion-defective yeast precociously elongate their spindle to generate two widely spaced centromere clusters and two separated DNA masses (Yeh et al., 2008). Both an eco1 mutant and an eco1Δ wpl1Δ double mutant exhibit the same phenotype of widely spaced centromere clusters and two separated DNA masses (Skibbens et al., 1999; Chan et al., 2012), indicative of an equivalent global failure in spindle-restraining cohesion.

We also identified smc3-D1189H as a mutation that significantly restores cohesion
in *eco1Δ wpl1Δ* cells as measured by both the GFP-spot assay and presence of segregation-competent cohesion. Taken together, these independent in vivo measures of cohesion strongly corroborate our previous conclusion that wpl1Δ poorly, if at all, suppresses the cohesion defect of *eco1Δ*. Moreover, because both *eco1Δ wpl1Δ* and *wpl1Δ smc3-RR* cells exhibit major defects in cohesion and are very sensitive to both benomyl and campothecin, we propose that these dual drug sensitivities represent signatures of cohesion-defective cells. Thus *Eco1p* promotes cohesion establishment by a *Wpl1p*-independent mechanism that was missed by previous studies.

The characterization of the *smc3-D1189H* cohesion activator mutation provides important clues about this unrecognized *Wpl1p*-independent mechanism for cohesion generation. *Smc3-D1189H* robustly suppresses the cohesion defect of an acetylation-null *smc3-K112R, K113R*, whereas a *wpl1Δ* fails to suppress the *K112R, K113R* cohesion defect. This stark difference between *smc3-D1189H* and *wpl1Δ* further validates that *Eco1p* acetylation of Smc3p K112, K113 promotes cohesion by *Wpl1p*-independent mechanism (Figure 8A, arrow 1). The K112, K113, and D1189 and residues are in the Smc3p head domain, suggesting a common mechanism for modulating cohesion. No crystal structure of the Smc3p head is available, and so we used the structure of the related Smc1p head bound to the Mcd1p C-terminus as a surrogate for Smc3p head binding to the Mcd1p N-terminus (Haering et al., 2004). Although D1189 does not appear to be in close proximity to K112, K113 residues, it is in a region that could alter interactions with Mcd1p and or affect Smc3p ATPase function (Figure 8B). The latter is intriguing, as we previously proposed a connection between K112, K113 acetylation and Smc3p ATPase function based on genetic interactions between mutations in K112, K113 and mutations of Walker B residues required for hydrolysis residues (Heidinger-Pauli et al., 2010).

The *smc3-D1189H* allele suppresses the cohesion defect of *smc3-K112R, K113R* much better than the defect when *Eco1p* function is lost. This difference suggests that *Eco1p* acetylates other targets besides Smc3p K113 to promote cohesion (Figure 8A, arrow 1). The view is supported by the observations that Smc3 K113 and K112, K113 acetyl mimics (glutamine or asparagine residues) only partially suppress the cohesion defect of reduced *Eco1p* activity (Unal et al., 2008). *Eco1p* acetylates itself, Mcd1p (Scc1p), Scc3p, and the cohesion regulator, Pds5p (Ivanov et al., 2002). Of these, only the Mcd1p substrate has been examined, and its acetylation has no role during S-phase cohesion but instead promotes DNA damage repair cohesion (Heidinger-Pauli et al., 2009). Further studies of other *Eco1p* targets are needed to elucidate how cohesion is regulated.

We find that although a *wpl1Δ* does restore viability to *smc3-RR* or *eco1Δ* cells, it fails to restore cohesion. Similarly, the *smc3-K112R, K113Q* acetyl-mimic allele restores viability without restoring cohesion (Guacci and Koshland, 2012). We previously showed that the *smc3-K112R, K113Q* allele, like a *wpl1Δ* in *eco1Δ* background, restores condensation (Guacci and Koshland, 2012). This led to the idea that Smc3p acetylation overcomes *Wpl1p* inhibition of an essential cohesion function, likely condensation (Figure 8A, T-bar 2; Guacci and Koshland, 2012). *smc3-D1189H* restores viability to *smc3-RR* cells without restoring cohesion, which fits with the idea that *D1189H* mimics acetylation to promote condensation. The limited ability of *smc3-D1189H* to suppress the inviability of *ECO1-AID* depletion suggests that other *Eco1p* targets besides *smc3-K112, K113* possibly *Wpl1p* itself, also contribute to condensation (Figure 8A, T-bar 2).

The fact that cohesion is defective in both *eco1Δ wpl1Δ* and *smc3-K112R, K113R* cells provides insights into the mechanism of cohesion (this study; Guacci and Koshland, 2012). Cohesin binding to chromosomes is as stable in *smc3-K112R, K113R* cells as in wild-type cells but is more stable in *eco1Δ wpl1Δ* cells (Rowland et al., 2009; Chan et al., 2012). Therefore the failure to acetylate Smc3p does not compromise cohesin binding to chromatin but likely mediates a subsequent step necessary for cohesion. Consistent with this conclusion, *smc3-D1189H* does not alter cohesin binding levels on chromosomes in *eco1Δ wpl1Δ* cells despite partially restoring cohesion. We suggest that Smc3p acetylation/*smc3-D1189H* promote...
cohesion establishment by converting the stably bound cohesin to a tethering form (Figure 8A, arrow 1). Recent studies support the idea that a second step distinct from stable cohesin binding to DNA is required for cohesion maintenance (Eng et al., 2014; Tong and Skibbens, 2014).

The nature of this second step remains to be determined. A new study on the related Smc condensin complex provided evidence for a two-step mode of DNA binding (Piazza et al., 2014). One can envision cohesin having two sites of different DNA binding that are differentially regulated—one used for cohesin binding and the second for sister tethering. Alternatively, models from studies of the S. pombe Rad50 or bacterial Smc complexes suggest that Smc complexes oligomerize, potentially by changes in the coiled-coil domain conformation (Hopfner et al., 2002; Woo et al., 2009; Bürmann et al., 2013). Finally, a recent study of bacterial Smc complexes revealed a two-step mode of DNA binding (Piazza et al., 2014).

The idea that a second step distinct from stable cohesin binding to DNA is required for cohesion maintenance (Eng et al., 2014) is supported by the finding that the kleisin (Mcd1p) subunit binds the coiled coils as well as the Smc heads (Bürmann et al., 2013). This structure provides a potential mechanism by which Smc3p acetylation and/or ATPase could alter kleisin (Mcd1p) binding to the coiled-coil domain. Whether this alters Mcd1p-Smc head binding, Smc3p head conformation, or longer-range cohesin structure remains an open question. Testing these ideas will require in vitro biochemical assays for which the D1189H mutation provides a powerful new reagent.

Do other organisms require additional Eco1p acetylation sites and the Wpl1p-independent function of Eco1p for cohesion establishment? One functional study in Schizosaccharomyces pombe suggested that additional sites besides the Smc3p K112, K113 equivalent lysines (Psm3-K105R, K106R) are needed for centromere-proximal cohesion (Feytout et al., 2011). In contrast, the existence of a Wpl1p-independent function for Eco1p might appear less likely, as the cohesion defect associated with reduced Eco1p activity in S. pombe and vertebrate cells appears to be significantly suppressed by reduction in Wpl1 activity (Gandhi et al., 2006; Feytout et al., 2011; Vaur et al., 2012). However, closer scrutiny suggests that this conclusion may be premature. In vertebrates, WAPL (Wpl1p orthologue) removes ~95% of chromosomally bound cohesin from prophase to metaphase (Losada et al., 1998; Sumara et al., 2000; Gandhi et al., 2006; Kueng et al., 2006). The remaining 5% of cohesin is sufficient to tether sisters, although cohesion is less robust and sisters become more separated and resolved. Small interfering RNA (siRNA) depletion of the Eco1 orthologue ESCO2 generates metaphase chromosomes with significantly defective cohesion, whereas codepletion of WAPL and ESCO2 was reported to reduce this cohesion defect (Gandhi et al., 2006). However, these experiments compared distinct chromosomal states—prophase (siRNA both WAPL and ESCO2) versus metaphase (siRNA of just ESCO2)—that have greatly different levels of cohesin bound. The extra bound cohesin on the prophase-like chromosomes could obscure a significant cohesin defect. Therefore, the existence of a WAPL-independent function of Eco1p remains an open question in vertebrates.

In S. pombe, unlike budding yeast and vertebrates, acetylation of Smc3p at K112, K113 equivalents (Psm3-K105R, K106R) is not essential (Feytout et al., 2011). Similarly, the cohesin regulator Pds5p is not essential in S. pombe but is essential in budding yeast and metazoans (Hartman et al., 2000; Tanaka et al., 2001; Dorsett, 2005; Vaur et al., 2012). This difference remains despite the fact that physical interactions between Pds5p and Eco1p have been demonstrated in both yeasts (Tanaka et al., 2001; Noble et al., 2006). Perhaps cohesion regulation in S. pombe has been simplified during evolution so as to contain natural variants that function analogous to budding yeast smc3-D1189H, bypassing the need for Eco1p-mediated activation of cohesion establishment. Indeed, our initial characterization of other cohesion activators in eco1Δ wpl1Δ cells suggests multiple targets for potential natural variants.

Although additional studies will be necessary to tease out these potential differences of cohesin regulation between species, the underlying mechanism of its cohesion activity is undoubtedly conserved. Further studies of the D1189H mutation and other eco1Δ wpl1Δ cohesion activators will provide powerful new reagents to elucidate cohesin regulation and function.

MATERIALS AND METHODS

Yeast strains and media

Yeast strains used in this study are A364A background, and their genotypes are listed in Supplemental Table S1. SC minimal and YPD media were prepared as described (Guacci et al., 1997). Benomyl (a gift from Dupont, Wilmington, DE) and camptothecin (Sigma-Aldrich, St. Louis, MO) plates used to assess drug sensitivity were prepared as previously described (Guacci and Koshland, 2012). Preparation of media containing auxin (Sigma-Aldrich) for depletion of AID tagged proteins was as previously described (Eng et al., 2014).

Dilution plating assays

Cells were grown to saturation in YPD medium at 23°C (or 30°C when listed) and then plated in 10-fold serial dilutions. Cells were incubated on plates at relevant temperatures or containing drugs as described. For plasmid shuffle assays, cells were grown to saturation in YPD medium to allow loss of plasmid pEU42 (SMC3 CEN URA3) and then plated in 10-fold serial dilutions.

G1 arrest and release into mid–M phase arrest

G1 arrest. Asynchronous cultures of cells were grown to mid log phase at 23°C in YPD medium, and then αFactor (Sigma-Aldrich) was added to 10⁻⁶ M. Cells were incubated for 3 h to induce arrest in G1 phase. This incubation time was increased to 3.5 h for all strains in any experiment in which an eco1Δ wpl1Δ background strain was used. For depletion of AID tagged proteins, auxin was added (500 μM final) and cells incubated an additional 1 h in αFactor-containing medium.

Release from G1 into mid–M phase arrest. G1 arrested cells were washed three times in YPD containing 0.1 mg/ml Pronase E (Sigma-Aldrich) and once in YPD and then resuspended in YPD containing nocodazole (Sigma-Aldrich) at 15 μg/ml final. Cells were incubated at 3°C for 3 h to arrest in mid–M phase. For depletion of AID-tagged proteins, auxin was added (500 μM final) in all wash media and in resuspension media containing nocodazole to ensure depletion at all times.

Nocodazole arrest–release assay for chromosome IV segregation

Cells were arrested in G1 phase and then released and rearrested in mid–M phase using nocodazole as described. Cells were washed three times with YPD and then resuspended in YPD containing αFactor (10⁻⁸ M) and incubated 4 h at 23°C.

Scoring segregation in large-budded (telophase) cells. Large-budded cells after nocodazole arrest–release were scored for proper chromosome IV sister segregation. Only cells with two GFP signals were scored. Cells were scored as exhibiting segregation when there was one GFP signal in each daughter bud. The percentage of chromosome IV sister chromatids scored as having segregated was calculated as (large-budded cells with segregated sisters/total number of large-budded cells) × 100.
Scoring segregation in unbudded (G1 phase) cells. After cell division, segregation generates two cells with one GFP spot each, whereas missegregation generates one cell with two GFP spots and one cell with no GFP spots. Therefore the percentage of unbudded cells showing segregation was calculated as (unbudded cells with one GFP spot/total number of unbudded cells) × 100.

Genetic screen of eco1Δ wpl1Δ cells for cohesion activator suppressors

Our results indicate that eco1Δ wpl1Δ cells have little or no cohesion and are sensitive to benomyl (BEN) and camptothecin (CPT; Guacci and Koshland, 2012). BEN destabilizes microtubules and so induces detachment of early S-phase kinetochore–microtubule attachments essential for cohesin-independent segregation (Figure 2A). CPT inhibits topoisomerase I, which induces single-strand nicks that can become double-strand DNA breaks (DSBs) during replication in S phase. Because cohesin/cohesion play a role in DSB repair (Ström et al., 2004; Unal et al., 2004), CPT lethality is likely due to a combination of reduced repair of DSBs and DNA-damage checkpoint–induced cell-cycle delays, increasing the likelihood of loss kinetochore–microtubule attachments formed in S phase.

We wanted to identify mutations that restore cohesion to eco1Δ wpl1Δ cells. We reasoned that suppressors that restore cohesion in eco1Δ wpl1Δ cells will be resistant to both drugs, and so we conducted a genetic screen on this basis. To set the parameter for the screen, we assayed haploid eco1Δ wpl1Δ cells for the BEN and CPT concentrations that induce complete lethality when 10^10 cells are plated (Supplemental Figure S3A). For the screen, haploid eco1Δ wpl1Δ cells were dilution streaked on YPD plates and grown at 23°C to enable formation of colonies from single cells (Supplemental Figure S3B). A small amount of one single colony was inoculated into YPD liquid medium and grown to saturation at 23°C. Aliquots containing ~2 × 10^6 cells were plated onto YPD containing either BEN or CPT at 12.5–15 μg/ml and then incubated 4–5 d at 23°C. Twenty-four different single colonies were subjected to this regimen to generate a pool of suppressors with independent origins. A few drug-resistant colonies (suppressors) arose on each plate. Suppressor clones were retested for resistance to the same drug and for cross-resistance—that is, BEN-resistant clones tested for CPT resistance and vice versa. Suppressors that exhibited cross drug-resistance were subjected to whole-genome sequencing using TruSeq DNA Sample v2 Kit (Illumina, San Diego, CA).

Monitoring cohesion using LacO-GFP assay

Cohesion was monitored using the LacO-Lac system, in which cells contained a GFP-LacI fusion and tandem LacO repeats integrated at one chromosomal locus, which recruits the GFP-LacI (Straight et al., 1996). CEN-distal cohesion was monitored by integrating LacO repeats at LYS4, located 470 kb from CEN4. CEN-proximal cohesion is monitored by integrating LacO at TRP1, located 10 kb from CEN4. Cells were fixed and processed to allow the number of GFP signals in each cell to be scored and the percentage of cells with two GFP spots determined as previously described (Guacci and Koshland, 2012). Bulk chromosomal DNA for imaging was visualized as previously described (Guacci and Koshland, 2012).

Plasmid constructs

Site-directed mutagenesis using the QuikChange Site Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) was used to generate the smc3-D1189H allele on URA3 or LEU2 integration plasmid pVG441 D1189H (smc3-D1189H URA3) or pVG419 D1189H (smc3-D1189H LEU2), respectively. The smc3-D1189H mutation was confirmed by sequencing the entire open reading frame (ORF), as well as the promoter region, to ensure that it was the only change.

Strain construction

SMC3 shuffle strain construction. Haploids containing plasmid pEU42 (SMC3 URA3 CEN) had their endogenous SMC3 gene deleted and replaced by the HPH cassette (encodes resistance to hygromycin B [Roche Biologicals, Indianapolis, IN]) using standard PCR-mediated, homology-based recombination.

Assessment of SMC3 test alleles by integration at the LEU2 locus. A second SMC3 “test allele” was cloned onto an integrating vector (pVG419; SMC3 LEU2) and linearized within LEU2 by BstEII digestion. Linearized plasmid pVG419 bearing WT or smc3 mutant alleles was transformed into shuffle strains to integrate them at the LEU2 locus, and LEU+ transformants were selected. These “test alleles” were assayed for their ability to support viability as the sole SMC3 source as follows. LEU+ clones were grown to saturation in YPD medium at 23°C to allow loss of plasmid pEU42 and then plated in 10-fold serial dilutions on medium containing FOA (US Biologicals, Salem, MA). FOA selectively kills URA3 cells, thereby selecting for loss of pEU42, which allows assessment of test allele ability to support viability as the sole SMC3 in cells. As a control, cells were also plated on either YPD or URA–medium. This shuffle strategy was used to create WT, wpl1Δ, and eco1Δ wpl1Δ cells with smc3-D1189H alleles integrated at the LEU2 locus as the sole SMC3.

Insertion of SMC3 alleles at the endogenous locus. Two different strategies were used. One used SMC3 shuffle strains described earlier for one step-gene replacement. A linear DNA fragment containing the desired SMC3 ORF allele, SMC3 promoter, and 3′ untranslated region were transformed into shuffle strains, plated on YPD, and grown overnight. Plates were replica plated to FOA, and FOA-resistant clones were selected and tested for sensitivity to hygromycin B, which occurs when smc3Δ::HPH is replaced by the transformed linear SMC3 allele. Transplantation alleles were confirmed by PCR screening and PCR sequencing.

The second strategy to replace the SMC3 allele with smc3-D1189H allele in haploid eco1Δ wpl1Δ cells or to replace SMC3 with SMC3-AID in WT cells bearing TIR1 was as follows. Plasmid pVG441 D1189H (smc3-D1189H URA3) or pVG465 (SMC3-AID URA3) was linearized within the SMC3 ORF by PshAI digestion. Linearized pVG441 or pVG465 was transformed into haploid eco1Δ wpl1Δ strains (VG3502-1A and VG3503-4C) or a wild-type TIR1 strain (VG3620-4C, respectively. URA+ colonies contain the SMC3 URA3 plasmid integrated at the SMC3 locus to create tandem SMC3 genes. URA+ transformants were replica plated onto YPD and then dilution streaked on FOA to excise the URA3 marker and thereby select for loss of one SMC3 allele. PCR-mediated sequencing was used to identify clones containing only the smc3-D1189H or SMC3-AID allele.

Strains containing AID-tagged proteins

Details of the auxin-mediated destruction of AID-tagged proteins in yeast was previously described (Eng et al., 2014). Briefly, the TIR1 E3-ubiquitining ligase placed under control of the GPD promoter and marked by Candida albicans TRP1 replaced the TRP1 gene on chromosome IV. ECO1 and SCC2 were C-terminally tagged with 3V5-AID2 sequences by standard PCR techniques and transformed into yeast strains bearing TIR1 to generate ECO1-AID or SCC2-AID allele, respectively. For SMC3-AID, a BglII site was inserted after Smc3p amino acid residue N607 and then the 3V5-AID1 cassette
inserted on a BamHI/BgIII fragment via standard cloning techniques. We replaced SMC3 with SMC3-AID at the endogenous locus as described. PCR screening and auxin-mediated sensitivity were used to identify clones containing AID-tagged genes.

**Chromosome spreads**

Chromosome spreads were performed as previously described, except primary that antibody was diluted in 5% bovine serum albumin, 0.2% milk, 1x phosphate-buffered saline, and 0.2% Triton X-100 (Hartman et al., 2000). Mcd1p was detected using rabbit anti-Mcd1p antibodies (Rb#555; αMcd1p) at a 1:10,000 dilution.

**Chromatin immunoprecipitation**

ChIP was performed as previously described (Wahba et al., 2013; Eng et al., 2014).

**Microscopy**

Images were acquired with a Zeiss Axioplan2 microscope (100x objective, numerical aperture, 1.40) equipped with a Quantix charge-coupled device camera (Photometrics).

**Flow cytometry analysis**

Flow cytometry analysis was performed as previously described (Eng et al., 2014).

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