Introduction

Proper transmission of eukaryotic chromosomes during cell division requires DNA replication and three other DNA-dependent processes: recombination-dependent DNA repair, sister chromatid cohesion, and chromosome condensation. Each of these diverse processes requires protein complexes containing two members of the highly conserved structural maintenance of chromosomes (Smc) family of proteins [1–3]. Smc complexes likely share a common core activity of chromosome crosslinking, either within a chromosome, as in chromosome condensation, or between chromosomes, for sister chromatid cohesion and recombination-dependent DNA repair. How Smc complexes mediate chromosome crosslinking is unknown.

Smc molecules are composed of five structural domains (Figure 1A) [4,5]: a globular N-terminal domain containing a Walker A motif, a globular C-terminal domain with Walker B and Signature motifs, two long α-helical domains, and a globular Hinge domain. Smc monomers fold in half at the Hinge domain, allowing the two α-helices to form a long antiparallel coiled-coil domain [6]. This folding juxtaposes the N- and C-terminal globular domains and the Walker A and B motifs, creating an Smc head domain with ATPase activity. Folded Smc monomers resemble a flexible dumbbell, with the Hinge and head domains separated by ~40 nm of coiled coil [6,7].

Smc complexes are composed of two Smc molecules, a kleisin subunit, and at least one accessory protein [6,8,9]. Smc monomers dimerize primarily through interactions between their Hinge domains [6,10]. The head domains of Smc molecules are also tethered together through the shared binding of a single kleisin subunit and two ATP molecules [6,11,12]. These interactions at both the head and Hinge domains give Smc dimers the potential to form large rings that have been observed in preparations of purified Smc complexes [13].

One of the most studied Smc complexes is cohesin, which mediates sister chromatid cohesion. Cohesin is composed of Smc1, Smc3, Scc3, and the kleisin subunit Mcd1/Scc1 [14–18]. The association of cohesin molecules with chromatin requires the integrity of all cohesin subunits and the ability of the Smc molecules to bind and hydrolyze ATP [19–22]. Multiple cohesins bind proximal to each centromere, forming a large pericentric domain. Cohesins also bind along chromosome arms. In budding yeast, these cohesin-associated regions (CARs) extend over approximately 1 kb of DNA and are spaced at roughly 10-kb intervals [23–25].

Several auxiliary factors contribute to the establishment, maintenance, and eventual dissolution of sister chromatid cohesion. The binding of cohesin to chromosomes at any phase of the cell cycle requires the loading factors Scc2 and Scc4 [26]. However, only cohesin binding in S phase, coupled with the function of Ctf7, results in the establishment of cohesion [16,27]. Pds5 binds cohesin and helps maintain cohesion during S and G2 phases of the cell cycle [28–30].
Finally, separase promotes removal of cohesin at the onset of anaphase by cleavage of Mcd1 [31].

These observations have led to two distinct models to explain how cohesin molecules crosslink sister chromatids. The embrace model posits that cohesin rings encircle chromosomes prior to replication and make no specific contacts with chromatin [6,20]. A topological interaction between cohesin and chromatin is supported by the fact that cohesin can be released from chromatin either by a single cleavage of the DNA or a single proteolytic cleavage of a cohesin subunit [19,20,32]. Passage of the replication fork through cohesin rings leaves both sister chromatids trapped inside, establishing sister chromatid cohesion. In contrast, oligomerization models, on the basis of observations of other Smc complexes, posit that cohesins bind to both sister chromatids. Then, cohesins on one sister chromatid oligomerize with cohesins on the other sister chromatid to generate cohesion [33–36]. To resolve these and other models will require a better understanding of how cohesins bind chromatin and the relationship between this chromatin binding and establishment of cohesion.

Mutagenesis of cohesin subunits provides one approach to gain insight into the chromatin binding of cohesin mutants. Indeed, site-directed mutagenesis of conserved Smc1 residues in the Hinge, Walker A, Walker B, and Signature motifs demonstrated that Smc1 must bind Mcd1 and Smc3, as well as bind and hydrolyze ATP in order for cohesin to bind chromatin [7,21,22,37]. While informative, these mutational analyses of cohesin–chromatin association leave key questions unanswered. Do these mutations define all the domains of cohesin subunits required for chromatin binding? Is chromatin binding of cohesin anywhere on chromatin prior to DNA replication sufficient to generate cohesion? Are additional constraints on cohesin necessary to generate cohesion?

To address these types of questions, one needs a way to identify rare mutant forms of cohesin that modulate rather than eliminate its activity. In the past, knowledge of a protein’s structure (sites for modification or interaction with other subunits) has been used to make dominant negative mutants that alter its activity or incorporation into a fully functional complex. We rationalized that the reciprocal would also be true; surveying an entire polypeptide chain for rare insertions with a dominant negative phenotype should provide a highly efficient means to identify precise regions of a protein important for its activity and/or assembly with other subunits. Furthermore, the study of these allelic should be highly informative for elucidating the molecular mechanism of a protein/complex. With this in mind, we screened a library of random insertion mutations in SMC1 for those that cause a dominant negative phenotype in the budding yeast, *Saccharomyces cerevisiae*. This strategy is henceforth referred to as random insertion dominant negatives (RID). Here, we successfully use RID to identify rare SMC1 alleles that are highly informative in dissecting SMC1’s role in the assembly and function of cohesin. We also use these mutants along with a ctf7 mutant to provide important insights into cohesin and sister chromatid cohesion.

### Results

**RID Screen Defines Five Regions Necessary for Smc1 Activity and the Establishment of Cohesion**

As the basis of our RID mutagenesis of SMC1, we constructed a minichromosome containing the SMC1-TAP gene under control of the galactose-inducible GAL1 promoter (see Materials and Methods). The level of Smc1-TAP protein expressed from the GAL1 promoter relative to the endogenous SMC1 promoter decreases 0.5-fold under uninducing conditions and increases 100-fold under inducing conditions (unpublished data). The Smc1-TAP protein is functional, as it restores viability and normal growth rate to cells carrying the temperature-sensitive *smc1–2* allele strain deleted for the essential SMC1 gene under repressing, uninducing, and inducing conditions (Figure S1 and unpublished data). The SMC1-TAP gene was mutagenized by a Tn7-based in vitro system that resulted in five amino acid insertions at random positions within the Smc1 protein. For brevity, the initial SMC1-TAP gene is henceforth referred to as the SMC1 or wild-type allele, and the insertion derivatives are named based upon the position of the insertion in the amino acid sequence.

Minichromosomes harboring the mutagenized library of SMC1 were transformed into budding yeast and assayed for their effects on cell viability and cohesion. A total of 13 transformants out of 2,500 candidates showed a marked decrease in cell viability under inducing conditions (Figure S1). The fact that these transformants are viable under uninducing conditions, where significant mutant smc1 expression occurs, indicates that these mutants are not dominant under low expression. To examine whether the mutant proteins are functional, minichromosomes containing inducible wild-type SMC1 or dominant negative smc1 alleles were introduced into cells carrying the temperature-sensitive *smc1–2* allele at the endogenous locus [17]. Under uninducing or inducing conditions, the temperature sensitive growth of the *smc1–2* strain is complemented by expression of wild-type SMC1, but not the insertion alleles (Figure S1B), indicating that the products of the insertion alleles are...
defective for Smc1 function under all conditions. The smc1–2 strains were then used to test the ability of the smc1 insertion alleles to generate cohesion. All smc1 insertion alleles are dramatically impaired for the establishment and maintenance of sister chromatid cohesion (unpublished data) (Figure 1B–1D).

All 13 insertions mapped to the SMC1 ORF, and 11 of them were unique (Figure 2A and 2B). A total of five insertions map to regions of known functional importance: the Walker A motif (35W), Signature motif (1129S), globular Hinge region (657H), and C terminus of Smc1 (1192C and 1215C) [7,21,22]. The remaining six insertions are either in 209L1–1 and 209L1–2 or cluster around Loop1 (189L1, 191L1–1, 191L1–2, and 235L1). Loop1 was previously defined through bioinformatics as one of three small regions within the α-helical domains of Smc molecules that are predicted to disrupt coiled-coil formation [38]. Since the insertions within and around Loop1 exhibit similar phenotypes (see below), these insertions define a new functional region of Smc1, which we call the Loop1 region.

**Figure 1.** Dominant Negative Insertion Mutants Disrupt Sister Chromatid Cohesion

(A) Schematic presents conserved structural and functional features of an Smc protein, folding of an Smc molecule, dimerization with a second Smc protein, and binding of the kleisin subunit (see text).

(B) A LacO/LacI-GFP-based system was used to assess sister chromatid cohesion. The yeast strain YMM-202 contains a tandem array of Lac Operators inserted 9.7 kb from one end of Chromosome IV, LacI-GFP, and the temperature sensitive smc1–2 allele. Under conditions of functional cohesion, sister chromatids are held in close proximity, resulting in a single GFP focus (top). Loss of cohesion allows sister chromatids to precociously separate, resulting in two GFP foci (bottom).

(C) YMM-202 harboring minichromosomes with inducible Smc1 alleles were released from G1 under conditions that inactivate the smc1–2 protein and induce expression of the galactose-regulated allele (see Materials and Methods). Cell cycle progression beyond metaphase was blocked by the addition of n nocodazole. Cohesion in the different strains was assessed by counting the number of metaphase-arrested cells with a single GFP focus.

(D) YMM-202 harboring minichromosomes with inducible Smc1 alleles were treated as described in (C), except cells were removed at the indicated intervals after release from G1 and assessed for the appearance of separated sister chromatids (two GFP foci) following DNA replication (upper graph). Cell cycle progression was determined by cell morphology (lower graph). The appearance of two GFP foci occurs with a similar timing for strains with an empty vector or two representative insertion mutants, indicating that these mutants fail to establish cohesion.

doi:10.1371/journal.pgen.0030012.g001
A Subset of RID Mutants Disrupt Cohesin Complex Assembly

We wanted to determine the efficacy of RID mutagenesis to define regions of Smc1 important for cohesin assembly. For this purpose we analyzed the ability of the 11 RID smc1 mutants (expressed at physiological levels) to coimmunoprecipitate with Mcd1 and Smc3. By comparison with wild-type Smc1, four RID mutants are dramatically reduced in their ability to coimmunoprecipitate with Mcd1, while all can coimmunoprecipitate Smc3 (Figure 3A and 3B). The failure to identify insertions that block Smc3 association is not surprising, since mutants defective in Smc1/Smc3 dimerization do not interact with any other cohesin subunit [21,22,39] and, therefore, are not likely to be dominant negative.

The four Smc1 insertions defective for Mcd1 binding lie within the Walker A motif (35W), the Signature motif (1129S), the HH helix (1192C), or the S15 β strand (1215C) (Figures 2B and 3C). These are four of the five motifs known to be required for Mcd1 binding. The Walker A and Signature motifs, through ATP binding, tether together the Smc1 and Smc3 head domains so that they can both associate with a single Mcd1 [21,22,39]. The helix HH and S15 β strand provide two of the three major contacts between Smc1 and Mcd1 (Figure 3C) [39]. These motifs are widely spaced within the polypeptide chain and constitute a target of only ~2% of the total residues. The efficient identification of these small disperse motifs by RID validates it as an extremely efficient method for generating defined mutations in protein-protein interactions.
means to identify regions of proteins important for complex assembly.

Smc1, Loop1, and Hinge Alleles Assemble into a Cohesin Complex that Binds Chromosomes

In addition to being a powerful tool to dissect cohesin assembly, we anticipated that RID would also efficiently identify informative alleles for cohesin function. The remaining seven RID alleles in the Hinge and Loop1 regions encode mutant Smc1 proteins that assemble with Smc3 and Mcd1 (Figure 3B) as well as Scc3 (unpublished data). Since they assemble with all known cohesin subunits, they were candidates for alleles that blocked cohesin function. All previously published alleles of cohesin subunits block chromatin binding. Therefore, we tested whether cohesins with these RID smc1 mutants were competent for chromatin binding. Minichromosomes were generated that express wild-type Smc1, 657H, or 209L1–2 fused to a 3XHA epitope, again under control of the GAL1 promoter. These alleles will henceforth be referred to as Smc1-HA, 657H-HA, and 209L1–2-HA, respectively. Cultures of smc1–2 cells containing these minichromosomes were released from G1 under conditions that inactivate the smc1–2 protein and induce expression of the galactose-regulated allele. These cells were then arrested prior to anaphase. Nuclei from these cells were spread on slides and processed for immunofluorescence to detect the chromosome association of different Smc1 proteins (Figure 4A). Like wild-type Smc1-HA, the insertion alleles 657H-HA and 209L1–2-HA associate with chromosomes.

We also monitored the chromosome localization of epitope-tagged Mcd1, a cohesin subunit, and Pds5, a cohesin accessory factor whose chromatin binding is mediated by cohesin (Figure 4B) [29,30]. Epitope-tagged Mcd1 and Pds5 localize to chromosomes in cells expressing the wild-type 657H or 209L1–2 allele, but not in cells with only inactivated smc1–2 (empty vector). The fact that the 657H and 209L1–2 alleles mediate Mcd1 and Pds5 localization to chromosomes suggests that these smc1 alleles bind chromosomes as part of the cohesin complex.

While it is clear that upon induction, the Hinge and Loop1 mutant cohesins can associate with chromosomes, their levels of chromosomal staining are reduced compared to induced wild-type cohesin (Figure 4C). Thus, the first question we wanted to ask was whether this reduction was sufficient to explain the cohesion defect of the Hinge and Loop1 mutants. To assess the level of cohesin binding to chromosomes, cells

Figure 3. A Subset of SMC1 RID Alleles Disrupt Cohesin Complex Assembly

Protein extracts were prepared from the strain 1377 A1-4B (MCD1-6HA) or 2258G (SMC3-12MYC) containing minichromosomes with the indicated SMC1 alleles. Cells were grown under repressing conditions to assure physiological levels of the different Smc1 proteins. Smc1 proteins were immunoprecipitated and separated by SDS-PAGE. Immunoblots were probed to detect Smc1-TAP, Mcd1-6HA, and Smc3-12MYC (see Materials and Methods).

(A and B) None of the SMC1 RID alleles disrupt the physical association with SMC3-12MYC. (A) Alleles in the Signature motif, HH Helix, S15 β-strand, and Walker A motif (unpublished data) of Smc1 disrupt the physical association of Mcd1-6HA, whereas (B) alleles in the Hinge and Loop1 regions do not.

(C) A region of the cocrystal of Smc1 (blue) and Mcd1 (brown) is shown (PDB 1W1W). Helix HH and the β-sheets S14 and S15 are indicated. The amino acid positions of 1215 and 1192 are indicated (red).

doi:10.1371/journal.pgen.0030012.g003

Multi-Step Pathway for Cohesion
expressing Smc1, 657H, or 209L1–2 were arrested in mitosis.

Nuclear spreads were prepared, and the levels of Smc1 and 
Pds5 immunostaining on chromosomes were quantified (Materials and Methods). The levels of chromosome binding for induced 657H-HA and its associated Pds5 are nearly identical to the binding of Smc1-HA and Pds5 under uninducing conditions (Figure 4C), a level of binding that is sufficient to generate cohesion and normal cell growth. Therefore, the level of chromosome association for 657H complexes should be sufficient to generate cohesion. The levels of 209L1–2-HA binding and its associated Pds5 are reduced by a maximum of 40%. This reduction may be below a threshold needed to generate cohesion. However, 50% of chromosome-bound cohesins in yeast meiosis and 90% in mammalian mitosis can be removed without eliminating cohesion [40,41]. Therefore, the level of chromosome association for 209L1–2-HA complexes is also likely to be sufficient to mediate at least partial sister chromatid cohesion, yet this seems not to be the case (Figure 1C).

If the quantity of chromatin binding for the mutant

Figure 4. Smc1 RID Mutants 657H and 209L1–2 Bind Chromosomes as Subunits of Cohesion

(A and B) Minichromosomes with the different Smc1 alleles were introduced into YMM10 (smc1–2), YMM11 (smc1–2 MCD1-6HA), or YMM20 (smc1–2 PDS5-6HA). Cultures of these cells were released from a G1 arrest into media that supported either the induced or uninduced expression of the Smc1 alleles, inactivated smc1–2, and caused arrest in mitosis (see Materials and Methods). Nuclei from mitotic arrested cultures were spread on slides and processed for immunofluorescence. The micrographs show representatives images of chromosomes (DAPI) and the chromosome staining of the different Smc1 proteins, Mcd1-6HA, and Pds5-6HA (anti-HA). The allele of SMC1 expressed from the minichromosome is listed on the left.

(C) The chromosome association of Smc1-HA and Pds5-6HA was quantitated by determining the average pixel intensity per nuclei following staining for Smc1-HA and Pds5-6HA (see Materials and Methods).

(D) Nuclear spreads were prepared as described above, except half of the cells released from G1 were arrested in mitosis while the other half were arrested in the ensuing G1 (see Materials and Methods). Nuclear spreads were prepared from aliquots of the original G1-arrested cells and those arrested in mitosis and the ensuing G1. The percent of nuclei with chromosomal staining of Smc1-HA, 657-HA, and 209L1–2-HA was determined.

doi:10.1371/journal.pgen.0030012.g004
complexes is sufficient to generate cohesion, then the quality of their chromatin binding must be defective. The mutant complexes may bind chromatin by a nonphysiological mechanism. Previous studies have shown that the binding of cohesin to chromatin requires Mcd1 and ATP. The ATP dependence is mediated through the Walker A, Walker B, and Signature motif. To test whether the mutant complexes also bind in an ATP-dependent manner, we constructed Hinge and Loop1 mutants that carried a second insertion allele in the Signature motif. While the protein products of these double mutants are stable (unpublished data), they failed to associate with the chromatin (Figure 4A), indicating that the mutant complexes bind by ATP-dependent mechanism. One method to address whether the mutant cohesin complexes require Mcd1 to bind chromosomes would be to inactivate the temperature sensitive mcd1–1 in strains harboring the smc1–1 and the smc1 loop or hinge mutants. Unfortunately, mcd1–1 and smc1–1 are synthetically lethal. As an alternative, we followed the chromosome association of the Hinge and Loop1 mutant complexes through a cell cycle. Cohesin binds chromosomes only after G1, when Mcd1 is expressed [14,15], and dissociates from chromosomes upon the onset of anaphase, when Mcd1 is cleaved [19]. Cohesin with 657H-HA or 209L1–2-HA is absent from chromosomes in G1, localizes to chromosomes in metaphase, and is lost by the following G1, a pattern identical to cohesin with Smc1-HA (Figure 4D), strongly suggesting that the mutant complexes, like wild-type complexes, require Mcd1. The fact that, like wild-type, the Hinge and Loop1 mutants bind chromatin in an ATP- and Mcd1-dependent manner suggests that they all bind chromatin by a similar mechanism.

Despite these similarities to wild-type chromatin binding, the mutant complexes could still fail to generate cohesion if their binding is too late in the S phase to establish cohesion or too unstable to maintain cohesion until M. To test if the mutant cohesins bind chromatin in a timely manner, cells were released synchronously from a G1 arrest and analyzed at different intervals for DNA content and chromosome association of wild-type and mutant cohesins (Figure 5A). The timing of chromosome association during S phase for 657H-HA or 209L1–2-HA cohesins is very similar to that seen for uninduced levels of Smc1-HA cohesin, which is sufficient to generate cohesion. Also, the amount of cohesin bound per nuclei during S phase increased with similar kinetics for Smc1-HA, 657H-HA, and 209L1–2-HA cohesins (unpublished data). Hence, cohesin containing 657H or 209L1–2 exhibits a normal timing of chromosome association.

To examine the stability of chromosome binding for cohesin, nuclei were prepared from mitotic cells expressing Smc1-HA, 657H-HA, or 209L1–2-HA and spread in the absence of fixative. Spread nuclei were incubated in a buffer with varying amounts of KCl for 30 min. After incubation, fixative was added, and the association of cohesin with chromosomes was examined by immunofluorescence (Figure 5B). No change in chromosome binding is observed for Smc1-HA in the presence of 150 mM KCl, corroborating previous biochemical analyses that infer stable association between cohesin and chromosomes [32]. Similarly, no significant change in chromosome binding is observed for 657H-HA. Both wild-type and mutant cohesins are extracted completely from spread chromosomes at 250 mM KCl (Figure 5C, unpublished data). Similar results were obtained with the 209L1–2-HA mutant (unpublished data). Therefore, cohesin complexes with wild-type, 657H-HA, or 209L1–2-HA appear to exhibit the same stability of chromosome binding under these conditions. Together, our analyses of the Hinge and
Loop1 mutants reveal a new type of cohesin complex. Like wild-type, these mutant complexes bind stably to chromatin, bind chromatin with proper cell cycle timing, and require ATP and Mcd1. However, unlike wild-type, these mutant complexes fail to generate cohesion. These mutants also suggest that binding, per se, prior to DNA replication is not sufficient to generate cohesion.

Smc1, Loop1, and Hinge Regions Are Required for Cohesin Enrichment at CARs

Since we could not explain the cohesion defect of these mutants by changes in their general chromosome-binding properties, we tested the specificity of their chromatin binding by examining their enrichment at CARs using chromatin immunoprecipitation (ChIP) [23–25]. Cohesin enrichment was analyzed initially at the centromere of Chromosome III and CARC1, a CAR approximately 11 kb from the centromere (Figure 6A and 6B). Under uninduced levels of Smc1 expression, Mcd1-HA is enriched at CARC1 and around CEN3 as reported previously [24,25]. Conversely, Mcd1-HA enrichment at CARC1 and CEN3 is eliminated for cohesin complexes containing 657H or 209L1–2. Similar results were obtained for CARL1 on Chromosome XII (unpublished data). Therefore, cohesin complexes with 657H or 209L1–2 fail to be enriched at CARs.

Because these mutant complexes do bind to chromatin as assayed by chromosome spreads, they apparently are bound to sites other than CARs. One possibility is that the mutant cohesins bind to chromatin through the Scc2/Scc4 loading factor, but are trapped in a nonproductive preloading complex. To test this, we examined cohesin binding at Scc2/Scc4 chromatin-binding sites [42], but we observed no enrichment of the mutant cohesins at these sites (Figure S2). The failure to observe enrichment of the mutant cohesins in our ChIP experiments does not exclude the possibility that they bind randomly within these regions. The enrichment of wild-type cohesin at CARs is only 10-fold above background, and CARs are spread at approximately every 10 kb, hence the dispersal of this signal to random sites would dilute the signal to background levels. While the position of the ectopic binding remains to be elucidated, the fact that the Hinge and Loop1 mutants cause ectopic binding indicates that Smc1 plays an active role in the specific localization of cohesins to CARs and pericentric regions. Furthermore, since this mislocalization is the only severe cohesin defect we have been able to observe in the Hinge and Loop1 mutants, it suggests that cohesin localization to CARs is critical for cohesion.

CAR Enrichment Is Not Sufficient for Cohesin Function

Our studies show that the Hinge and Loop1 regions of Smc1 are required for the establishment of cohesion and the enrichment of cohesin at CARs and pericentric regions. The accessory protein Ctf7/Eco1 is also required for the establishment of cohesion [16,27]. In a ctf7 mutant, cohesins pellet with chromatin [16]. This technique does not distinguish between ectopic and specific chromatin binding. Indeed, if ctf7, like Hinge and Loop1 mutants, showed ectopic binding, then this would suggest that Ctf7 interacts with the Hinge and Loop1 regions of Smc1 to ensure specific binding to CARs.

To test the function of Ctf7 in cohesin enrichment at CARs, we compared the localization of cohesin on chromatin in...
wild-type and ctf7 temperature-sensitive strains. Wild-type and ctf7 mutant cells expressing Mcd1-HA as the sole source of Mcd1 function were synchronized at the permissive temperature of 23°C in G1. These cells were released to the nonpermissive temperature of 37°C in media containing hydroxyurea (HU) to inactivate mutant ctf7 protein and block DNA replication [27], respectively. Cohesin association to chromatin was monitored by ChIP (Figure 7A and 7B). In wild-type cells arrested in HU, cohesin is enriched at CARs and pericentric regions as described previously [24,25]. In ctf7 strains arrested in HU at the nonpermissive temperature, cohesin is again enriched at CARs and pericentric regions.

Thus Ctf7 is not needed to localize cohesins to CARs or pericentric regions.

The above results indicate that Ctf7 function is distinct from that of the Hinge and the Loop1 regions of Smc1. Previous experiments showed that Ctf7 function is required during S phase; however, this activity was not ordered relative to cohesin loading at CAR sites. To test whether Ctf7 is required coincident with or after cohesin binding to CARs, wild-type and ctf7 mutant cells were allowed to progress from G1 to early S (HU arrest) at the nonpermissive temperature and then shifted to the permissive temperature for the remainder of the cell cycle. The results show that cell viability was 93% ± 4% for wild-type cells and 88.2% ± 5% for ctf7 mutant cells, indicating that Ctf7 function is required after cohesin loads to CARs. This result is strongly supported by cell cycle mapping studies that show inactivation of ctf7 from early S (HU arrest) and prior to the end of DNA replication leads to cell death [16,27,43], indicating that Ctf7 performs its essential function during this window of the cell cycle. Thus, Ctf7 appears to function in the establishment of cohesion after the Smc1-, Loop1-, and Hinge-dependent localization of cohesin to CARs and pericentric regions, and during S phase.

Discussion

Our results validate RID as a very efficient strategy to identify Smc1 alleles that provide important structural and functional information about Smc1 and cohesin. First, of the 11 smc1 RID alleles, ~40% blocked Smc1 association with Mcd1 but not Smc3. Thus, RID efficiently identifies alleles that trap partially assembled cohesin. Furthermore, these Smc1 insertions lie within four of the five small structural elements (each element is approximately ten residues each spread throughout a total of 1,300), which are required for Mcd1 binding. The efficacy and remarkable precision of RID suggests that it can be used effectively on less well-characterized proteins for the de novo identification of candidate regions of that protein that mediate its binding to interacting partners. Second, the remaining seven RID alleles of Smc1 identify a new functional domain (the Loop1 region), and a new function for the Hinge beyond its established function in dimerization. These Hinge and Loop1 alleles also generated cohesin complexes that unlike any previous cohesin mutants retain the ability to bind chromatin. The isolation of these unusual alleles underscores the combined power of using random mutagenesis, which allows one to avoid the inherent biases of directed mutagenesis, and the imposition of the dominant negative phenotype, which allows rapid identification of rare partially functional alleles in a sea of common null mutations caused by misfolding and truncations. Given the success of the RID strategy for Smc1/ cohesin, RID should be a useful tool to dissect the structure and function of many multi-subunit complexes.

Our study of RID smc1 alleles has provided important new insights into cohesin binding to chromatin. First, our results show that in vivo the Hinge and Loop1 regions are needed for binding to CARs, two domains not implicated in chromatin binding by either the embrace or snap model. The joint requirement for these two domains is even more surprising given their apparent physical separation (~40 nm) based upon electron micrographs of cohesin [13]. Interestingly, in vitro analyses of bacterial Smc complexes have implicated the
The second interesting feature of cohesins containing the RID smc1 mutants is that they appear to bind chromatin ectopically. This conclusion is based upon the observations that cohesins exhibit general chromatin binding as assayed by chromatin spreads but are no longer enriched at CARs. It is possible that the cohesin binding is compromised at CARs in some way that they bind there but subsequently dissociate. Consistent with this, the level of binding of overexpressed RID mutant proteins is reduced compared to overexpressed wild-type protein. However, under our assay conditions using chromatin spreads, the Hinge, Loop1, and wild-type cohesins appear to be bound with similar stability. Furthermore, the level of chromatin binding of cohesin, when the Hinge mutant is overexpressed, is still comparable to the level of binding for uninduced wild-type, which is sufficient for cohesion function. Alternatively one could argue that the ectopic binding observed by chromatin spreads reflects some artifact of this assay. This is extremely unlikely, as chromatin binding observed by spreads for the RID mutants share many of the chromatin-binding features of wild-type cohesin, including Mcd1 dependence, ATP dependence, and proper cell cycle loading in S and unloading in M. Together these results suggest that cohesins are capable of general chromatin binding, mediated by Mcd1 binding and ATP functions, but are then targeted to CARs through an additional function(s) mediated by the Hinge and Loop1 regions.

In one scenario the Loop1 and Hinge regions may target cohesins to CARs by binding targeting factors that recognize specific chromatin features; the RID mutants perturb the binding of the targeting factors. There are precedents for this idea, since the Hinge and Loop1 regions mediate interactions with non-SMC factors in other Smc-related proteins [45–47]. In addition, histone modifications have already been demonstrated to be critical to target cohesion binding to heterochromatin and double strand breaks [48–50]. In this model, cohesion binding to chromatin resembles RNA polymerase; both are targeted to specific sites through specificity factors that recognize changes in chromatin, and in their absence load with lower efficiency at abundant cryptic sites.

As an alternative, cohesin itself may be capable of recognizing specific features of chromatin. Indeed the MutS mismatch repair complex, which shares similarities to Smc complexes, forms a ring that topologically traps generic DNA, while residues within the ring specifically recognize mismatch DNA [51–53]. In this scenario, the SMC1, Hinge, and Loop1 mutant subunits allow a topological interaction of cohesin with chromatin, but perturb its ability to make intimate chromatin/DNA interactions. Interestingly, in vitro studies have implicated residues on the interior of the bacterial Smc ring for DNA binding and DNA-dependent stimulation of the ATPase [44].

The chromatin-binding properties of cohesin in ctf7 and RID mutants also provide important insights into the mechanism of sister chromatid cohesion. First, the binding of cohesin to chromatin in early S at CARs (ctf7 mutants) or ectopic sites (Hinge and Loop1 mutants) is not sufficient to generate cohesion. Second, cohesin binding to CARs and centromeres appears necessary for cohesion. Third, an auxiliary factor, Ctf7 enables cohesins already bound at CARs to generate functional cohesion. These observations all contradict a one-step mechanism for cohesion like the simple embrace model, which requires only chromatin binding of cohesin anywhere on the chromosomes followed by DNA replication.

Rather, our results support tethering of sister chromatids by two chromatin-binding events, which require specific binding of cohesins at CARs followed by a Ctf7-dependent step. Given these new constraints, we propose the following working model for the establishment of cohesion: cohesins bind to CAR sites as they emerge from the replication fork and are subsequently activated by Ctf7 to initiate the capture of the homologous CAR on the sister chromatid. This capture could occur by activating a second chromatin binding event by a single complex (for example, a second embrace) or by activating oligomerization of cohesins bound to each CAR (the oligomerization models) [33–36]. In either version of this two capture model, the specific binding to CARs and Ctf7 steps would be critical. Because of local proximity during replication, a cohesin molecule bound to a CAR on one sister chromatid will find the sister CAR and/or cohesin bound to that site before finding other sites/cohesins in the genome. We speculate further that the activation by Ctf7 is programmed to be local/transient. As a result, cohesin bound to a random site on a chromatid will be unlikely to remain active long enough to find a random CAR or another randomly bound cohesin. Thus, the targeting of cohesin by CAR binding and its local activation by Ctf7 would provide spatial and temporal regulation of cohesion to activate the second capture and ensure that cohesin generates a crosslink only between only sister chromatids and not random chromatin. Finally, the specificity of tethering by SMC complexes in other DNA processes may be achieved through specific chromatin binding coupled with Ctf7-like activators.

Materials and Methods

Media and strains. Yeast strains were grown in YEP, SC-URA, or SC-URA-TRP media [54] supplemented with 2% dextrose (D), 2% raffinose 2% galactose (RG), 3% raffinose 3% lactic acid (LA), or 3% glycerol 2% lactic acid 2% galactose (LAG), as indicated. Glucose, raffinose, and galactose were purchased from Sigma-Aldrich (http://www.sigmaaldrich.com), glycerol from EMD Biosciences (http://www.emdbiosciences.com), and lactic acid (40% v/v stock, [pH 5.7]) from Fisher Scientific (http://www.fishersci.com). A PCR-based strategy was used to generate a complete genomic replacement of SMC1 with the Schizosaccharomyces pombe His5+ gene [55], creating the yeast strain Ymm1. Ymm1 is dependent on the plasmid pMM26 for viability. The genotypes of strains used in this work can be found in Table S1. Yeast transformation and genetic methods were as described previously [56].

Plasmids. The plasmids described were generated through use of the Echo Cloning System (Invitrogen, http://www.invitrogen.com), which results in the expression of genes fused to COOH-terminal V5 epitope under control of the GAL1 promoter. In all cases, the V5 epitope was replaced by a TAP tag using a PCR-based tagging strategy [57,58]. The plasmid pMM14 contains the SMC1 ORF. Restriction digestion by SmaI and Pmel, followed by religation, destroyed a unique Pmel site and generated AMH4.

The plasmid pMM26 was derived from pMM14. GAL1 was replaced with a 412-basepair fragment immediately 5′ of the SMC1 ORF using AgeI and XhoI restriction sites, creating pMM24. Loss of the URA3 gene by BsgI digestion, followed by T4 blunting and religation, generated pMM26. Expression of SMC1-TAP from pMM24 and
pMM25 is capable of rescuing growth of a temperature sensitive smc1–2 strain (1360–7C) and a strain deleted for endogenous SMC1. The plasmid pMM25–3HA expresses SMC1 fused to a COOH-terminal TAP-3HA tag under control of the GAL1 promoter. Through a PCR-based strategy, a BamHI site within the TAP coding sequence of pMM14 was introduced by a silent mutation and replaced by a BamHI site inserted immediately 5’ of the TAP stop codon [57, TRP1 gene]. Plasmid DNA was linearized by restriction digestion, followed by religation, resulting in pMM14–PIBS. The XbaI/Smal fragment of pMM14–PIBS was subcloned into pRS305, resulting in pMM27. A 3XHA coding sequence was inserted in frame at the BamHI site of pMM27, resulting in pMM27–3HA. Replacing the XbaI/Smal fragment of pMM27–3HA resulted in pMM25–3HA. The plasmid pMM25–3HA is able to rescue viability of an SMC1 deleted strain under either uninduced or induced expression conditions. The plasmids 2091L–2–3HA and 657–HA were generated by replacing the XbaI/Smal fragment of pMM25–3HA with those from 2091L–2 and 657–HA.

Construction of a smc1 deletion library. Insertion mutagenesis of AMH4 was performed using the GPS-Linker Scanning system (New England Biolabs, http://www.neb.com). In vitro mutagenized AMH4 was transformed into Escherichia coli. Approximately 3,100 colonies were scraped from plates, and plasmid DNA was isolated using a Plasmid Maxi kit (Qiagen, http://www.qiagen.com). The majority of plasmids received only a single insertion (unpublished data). Plasmid DNA in the primary library was linearized by PmeI digestion, gel purified, and transformed into E. coli. Plasmid DNA from this secondary library was isolated from approximately 4,000 colonies from plates using the Plasmid Maxi kit (Qiagen, http://www.qiagen.com). The majority of plasmids received only a single insertion (unpublished data). Plasmid DNA from the primary library was linearized by PmeI digestion, gel purified, and transformed into E. coli. Plasmid DNA from this secondary library was isolated from approximately 4,000 colonies from plates using the Plasmid Maxi kit (Qiagen, http://www.qiagen.com). The majority of plasmids received only a single insertion (unpublished data).

Screen for dominant negative and functional smc1 alleles. The secondary library was transformed into YMM1/pMM26, with transformants selected for on SC-URA-TRP-D plates at 23 °C. Individual transformants were patched and patched to new SC-URA-TRP-D plates and grown at 23 °C. Patches were replica plated to an identical plate and a SC-URA-TRP-RG plate and grown for 3–5 d at 23 °C. From patches that were impaired for growth on SC-URA-TRP-RG plates, plasmids were isolated from the identical patch growing on the SC-URA-TRP-D plate and retested. For plasmids that retested, the insertion mutations were mapped by restriction endonuclease digestion and sequenced. To isolate functional alleles, the secondary library was transformed into an smc1–2 strain, 1360–7C. Transformants on SC-URA-TRP-D plates at 23 °C were patched to new SC-URA-D plates, and grown at 23 °C. Patches were replica plated to an SC-URA-RG plate and grown at 37 °C. Plasmids were isolated from patches that grew at 37 °C and retested. For plasmids that retested, the insertion mutations were mapped by restriction endonuclease digestion and sequenced.

Cell synchronization. Exponentially dividing cell cultures were initially grown in SC-URA-LA at 25 °C. a-Tfactor (1.5 × 10^9 M [Sigma]) was added to cultures in mid-log phase (approximately 0.5 × 10^9 cells/ml). To simultaneously inactivate smc1–2 and release them from G1, cells were washed twice in 37 °C YEP-LA containing 0.1 mg/ml Pronase (Sigma). Cells were resuspended in either 37 °C YEPLA-YEP-LA and incubated for 10 min. Each slide was then placed in a single coplin jar containing ~50 ml 0.25% Triton X-100 in PHEM buffer with varying amounts of KCl for 30 min, while gently shaking. Following this incubation, nuclei were fixed for 4 % PFA with 0.25% Triton X-100 in PHEM buffer, as before. Immunofluorescence was performed as above.

Supporting Information

Figure S1. Colony Growth of Cells Expressing Dominant Negative smc1 Alleles

Cultures of 1377A1–4B (SMC1, odd numbers) and 1360–7C (smc1–2, even numbers) cells containing minichromosomes expressing the indicated SMC1 allele were grown in SC-URA-LA media at 23 °C. Cultures were diluted to an identical cell density, and 5-fold serial dilutions were grown on either SC-URA-LA (uninduced) or SC-URA-LAG (induced) plates at (A) 25 °C or (B) 37 °C for 3–5 d. Cultures of dominant negative smc1 alleles. Protein extracts were prepared from the strain 1,377 A1–4B (SMC1 MCD1-HA) containing minichromosomes expressing the indicated SMC1 allele. Cultures were grown as in Figure 3B and 3C.

Found at doi:10.1371/journal.pgen.0030012.sg001 (490 KB PDF).

Figure S2. Analysis of Cohesin Binding to Scc2/Scc4 Chromatin-Binding Sites

Cultures of YMM11 (smc1–2 MCD1-HA) cells containing minichromosomes with different SMC1 alleles were grown and arrested in metaphase (see Figure 4C legend). Cells were processed for ChIP (see Materials and Methods). Mcd1-HA binding to specific chromosomal loci was analyzed by PCR. Two Scc2/Scc4 binding sites on Chromosome VI (SGD 75200–76000 and SGD 220000–226000) were tested for Mcd1-HA binding [42]. A representative dataset is shown for the SGD 75200–76000 region.

Found at doi:10.1371/journal.pgen.0030012.sg002 (45 KB PDF).

Table S1. Strain Table

Found at doi:10.1371/journal.pgen.0030012.s001 (29 KB DOC).

Acknowledgments

We thank Vivian Zheng and M. Andrew Hoyt for constructive comments on the manuscript; Hong-Guo Yu, Catherine Huang, Vincent Guacci, Eileen Hogan, Cathy Mistrot, Ellen Cammon, and Patricia Cammon for technical support; and Terence Murphy, Judith Yanowitz, and members of our laboratories for advice and helpful discussions.

Author contributions. MM, EU, RVS, and DK conceived and designed the experiments. MM, EU, CW, and RVS performed the experiments. MM, EU, CW, and DK analyzed the data. RVS contributed reagents/materials/analysis tools. MM and DK wrote the paper.

Funding. This investigation was supported by the Howard Hughes Medical Institute and the US National Science Foundation (MCB-0212323).

Competing interests. The authors have declared that no competing interests exist.

References

1. Strunnikov AV, Jesberger R (1999) Structural maintenance of chromosomes (SMC) proteins: Conserved molecular properties for multiple biological functions. Eur J Biochem 263: 6–13.

2. Fujioka Y, Kimata Y, Nomaguchi K, Watanabe K, Kohno K (2002) Identification of a novel non-structural maintenance of chromosomes (SMC) component of the SMC5-SMC6 complex involved in DNA repair. J Biol Chem 277: 21585–21591.

3. Hirano T (2005) SMC proteins and chromosome mechanics: From bacteria to humans. Philos Trans R Soc Lond B Biol Sci 360: 507–514.

4. Jesberger R (2002) The many functions of SMC proteins in chromosome dynamics. Nat Rev Mol Cell Biol 3: 767–778.
Cohesion's binding to chromosomes depends on a separate complex consisting of Scc2 and Scc4 proteins. Mol Cell 5: 243–254.

Baker EA, Celniker SE, Rokos J, Liang W, Dickinson H, et al. (2004) The Drosophila genome as an expression manual. Science 306: 1763–1787.

Kann CH, Wohlschlegel J, Riedel D, Imai M, Kwon JS, et al. (2005) The yeast genome as a physical map. Science 310: 765–770.

Southwick MM, Jones SM, Lister R, Kantor HL, Green MR, et al. (2006) The mouse genome as a physical map. Nature 439: 966–970.

Celniker SE, Levine MD, Lois C, Balasubramanian S, Chia JY, et al. (2007) The worm genome as a physical map. Nature 445: 173–180.

Huang CE, Milutinovic M, Koshland D (2005) Rings, bracelet or snap: Fashionable alternatives for SMC complexes. Philos Trans R Soc Lond B Biol Sci 360: 537–542.

Chiu A, Revenkova E, Jescherer B, Peters JM (2004) Characterization of vertebrate cohesin complexes and their regulation in prophase. J Cell Biol 161: 749–762.

Yu HG, Koshland D (2005) Chromosome morphogenesis: Condensin-dependent cohesin removal during meiosis. Cell 122: 397–407.

Lengronne A, McIntyre J, Katou Y, Kanoh Y, Hopfner KP, et al. (2006) Establishment of sister chromatid cohesion at the S. cerevisiae replication fork. Molecular Cell 23: 787–799.

Hirano M, Hirano T (2006) Opening closed arms: Long-distance activation of the yeast cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish cohesion between sister chromatids during DNA replication. Genes Dev 20: 320–333.

Acharya S, Foster PL, Brooks P, Fishel R (2003) The coordinated functions of SMC ATPase by hinge-DNA interactions. Mol Cell 21: 175–186.

Unal E, Arbel-Eden A, Sattler U, Shroff R, Lichten M, et al. (2004) DNA damage response pathway uses histone modification to assemble a double-strand break-specific cohesin domain. Mol Cell 16: 591–1002.

Nonaka N, Kitajima T, Yokobayashi S, Xiao G, Yamamoto M, et al. (2002) Recruitment of cohesin to heterochromatic regions by Swi6/HP1 in fission yeast. Nat Cell Biol 4: 89–93.

Acharya S, Foster PL, Brooks P, Fishel R (2003) The coordinated functions of the E. coli MutM and MutL proteins in mismatch repair. Mol Cell 12: 233–246.

Junop MS, Obmolova G, Rausch K, Hsieh P, Yang W (2000) Composite active site of an ABC ATPase: MutS uses ATP to verify mismatch recognition and authorize DNA repair. Mol Cell 1: 1–12.

Obmolova G, Ban C, Hsieh P, Yang W (2000) Crystal structures of mismatch repair protein MutS and its complex with a substrate DNA. Nature 407: 705–710.

Guthrie C, Fink G (1982) The yeast genome. New York: Cold Spring Harbor Laboratory Press. 198 p.

Schneider BL, Seufert W, Steiner B, Yang QH, Fisher AL, et al. (1995) Use of polymerase chain reaction epitope tagging for protein tagging in Saccharomyces cerevisiae. Yeast 11: 157–167.

Robertson KE, Pringle JR (1989) Monolayer growth of yeast. Yeast 5: 437–450.

Megee PC, Mistrot C, Guacci V, Koshland D (1999) The centromeric sister chromatid cohesion site directs Mcd1p binding to adjacent sequences. Mol Cell 4: 445–450.