A *tDNA* establishes cohesion of a neighboring silent chromatin domain

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DNA replication generates sister chromatid pairs that are bound to one another until anaphase onset. The process, termed sister chromatid cohesion, requires the multisubunit cohesin complex that resides at centromeres and sites where genes converge. At the *HMR* mating-type locus of budding yeast, cohesin associates with a heterochromatin-like structure known as silent chromatin. In this report, we show that silent chromatin is necessary but not sufficient for cohesion of the replicating locus. A tRNA gene (*tDNA*) that delimits the silent chromatin domain is also required, as are subunits of the TFIIIB and RSC complexes that bind the gene. Non-*tDNA* boundary elements do not substitute for *tDNA*s in cohesion, suggesting that barrier activity is not responsible for the phenomenon. The results reveal an unexpected role for *tDNA*s and RNA polymerase III-associated proteins in establishment of sister chromatid cohesion.

[*Keywords*: Sister chromatid cohesion; silent chromatin; transcriptional silencing; *tDNA* boundary/barrier element; cohesin; Sir; RNA polymerase III]

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The proliferation and development of all organisms requires high fidelity transmission of intact genomes between dividing cells. Sister chromatid cohesion is one of many processes that evolved to ensure proper chromosome segregation (Nasmyth 2002). DNA replication produces sister chromatids that are held together (cohesed) until mitosis. This ensures that kinetochores of each chromatid pair attach to microtubules from opposing poles of the mitotic spindle. When all kinetochores become properly attached (bioriented), the chromatid pairs separate synchronously with one full set of chromosomes migrating toward each pole.

Sister chromatid cohesion is mediated by a set of evolutionarily conserved proteins that form a protein complex known as cohesin (for reviews, see Nasmyth and Haering 2005; Dorsett 2006). The complex consists of two SMC subunits, Smc1 and Smc3, and two additional subunits, Scc3/Irr1 and Mcd1/Scc1. Cohesin loads onto chromatin late in G1 and becomes activated for cohesion in a replication-coupled process (Lengronne et al. 2006, and references therein). The complex is shaped like a ring with an inner diameter of ~40 nm, large enough for a pair of 10-nm chromatin fibers (Gruber et al. 2003). Bound cohesin embraces DNA in a topological manner (Ivanov and Nasmyth 2005), and one popular model stipulates that both sister chromatids are encircled by a single cohesin ring. Variations on this theme have emerged (Milutinovich and Koshland 2003). Our work at the *HMR* locus, for example, indicates that cohesin binds topologically but not in a way that embraces both chromatids (Chang et al. 2005). At anaphase onset, programmed cleavage of Mcd1/Scc1 by the Esp1 site-specific protease triggers chromosome separation.

Cohesin accumulates at discrete sites on chromosomes (Blat and Kleckner 1999; Laloraya et al. 2000; Glynn et al. 2004; Lengronne et al. 2004). High-density binding occurs in regions surrounding centromeres to facilitate biorientation. The complex also contributes to post-replicative DNA repair by associating with domains that contain double-strand DNA breaks (Strom et al. 2004; Unal et al. 2004). The vast majority of remaining binding sites lie in intergenic regions between pairs of genes oriented toward one another. Active transcription influences the distribution of cohesin in these regions (Glynn et al. 2004; Lengronne et al. 2004). Thus, one theory holds that passage of RNA polymerase pushes cohesin to the ends of genes.

Cohesin also accumulates on large heterochromatic domains that contain few protein-encoding genes. Heterochromatin is a repressive structure that suppresses most transcription, as well as a variety of other DNA transactions (Grewal and Moazed 2003). In *Schizosaccharomyces pombe*, cohesin is maintained at pericentric heterochromatin by interacting with Swi6, a conserved heterochromatin protein (Bernard et al. 2001; Nonaka et al. 2002). In mutants lacking Swi6 or other heterochromatin features, cohesin is lost from pericen-

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tric heterochromatin and chromosomes lag on the elongating anaphase spindle, much like they do in cohesin mutants.

In the budding yeast *Saccharomyces cerevisiae*, cohesin associates with a heterochromatin-like structure, termed silent chromatin, which is found at telomeres and the transcriptionally repressed *HMR* and *HML* mating-type loci. At these locations, *cis*-acting elements termed silencers recruit a complex of silencing factors known as the Sir proteins (for review, see Rusché et al. 2003). Sir2 is an NAD-dependent histone deacetylase. Sir3 and Sir4 bind deacetylated histone tails. Iterative cycles of deacetylation by Sir2 and histone binding by Sir3/4 permit the complex to spread kilobases away from silencers. Binding of cohesin at *HMR* requires Sir3, Sir4, and the deacetylase activity of Sir2 (Chang et al. 2005). In *sir* mutants, cohesion of this locus and probably other silenced domains is lost.

Silent chromatin is restricted from spreading into adjacent domains of active chromatin by barrier elements (Valenzuela and Kamakaka 2006). A tRNA gene (*tDNA*) neighboring *HMR* is a principal component of the right-hand boundary of the silent chromatin domain (Donze and Kamakaka 2001). *tDNA*s within the pericentric repeat elements of *S. pombe* act similarly, serving as barriers to constrain pericentric heterochromatin (Noma et al. 2006; Scott et al. 2006). TFIIIC, an RNA polymerase III (RNA pol III) transcription factor, can form barriers independently of other RNA pol III factors in *S. pombe*. In *S. cerevisiae*, additional proteins of the RNA pol III transcriptional machinery are required (Donze and Kamakaka 2001). Intriguingly, the barrier activity of the *HMR*-proximal *tDNA* is compromised in *smc1* and *smc3* mutants (Donze et al. 1999). Bell and coworkers (Lau et al. 2002) showed that cohesin blocks not only silent chromatin spreading but the de novo establishment of silencing. A unifying interpretation of these findings is that cohesin inhibits heterochromatinization of euchromatic domains.

In this study we investigated the requirements for establishment of silent chromatin cohesion. Using a combination of fluorescence microscopy and site-specific recombination, we analyzed *HMR* alleles that replicate as extrachromosomal DNA circles. Our results identified essential roles for the *HMR*-proximal *tDNA* and components of the RNA pol III machinery in cohesion of silent chromatin.

Results

Silent chromatin is not sufficient for cohesion

In previous work we modified *HMR* to monitor cohesion of the locus selectively (Chang et al. 2005). We integrated an array of lac operators near the *I* silencer and flanked the domain with target sites for the R site-specific recombinase. Inducible recombination uncoupled the construction (termed the simple excision cassette) (Fig. 1A) from the chromosome so that cohesion of *HMR* could be evaluated independently from cohesion of neighboring chromosomal domains. In this case, recombination uncoupled *HMR* from the neighboring *tDNA*. In cells expressing lac-GFP, excised *HMR* circles appeared as bright

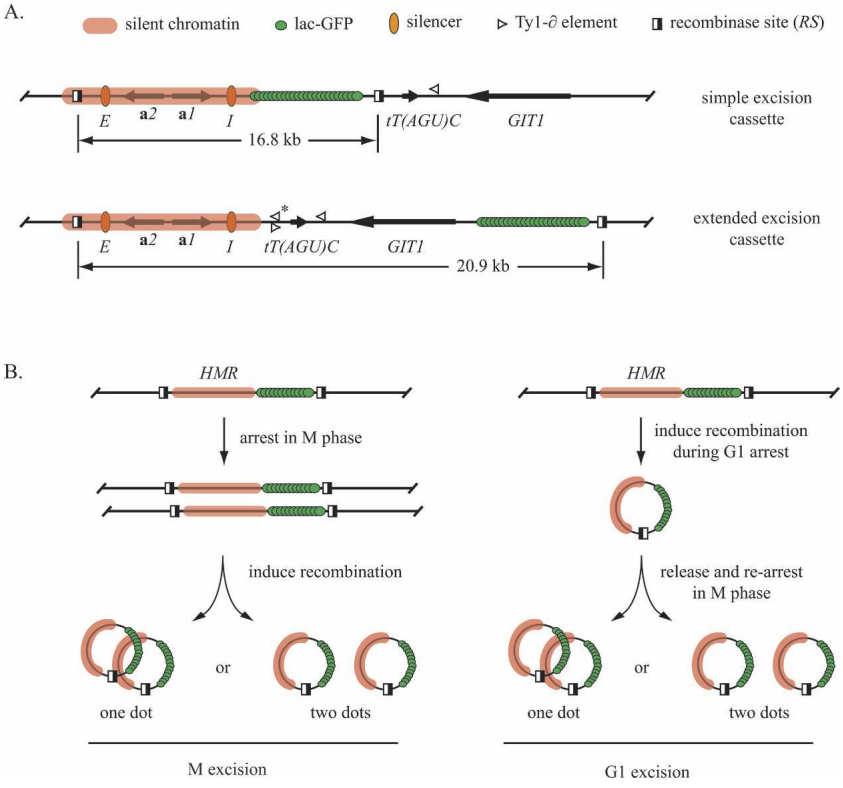

Figure 1. Experimental design for the production of extrachromosomal *HMR* circles. (*A*) Organization of cassettes for excision of the native *HMR* locus, consisting of the **a***1* and **a***2* genes, as well as the *E* and *I* silencers (not drawn to scale). Relevant landmarks are defined graphically at the *top* of the figure. Endpoints of the silent chromatin domain were drawn arbitrarily. The right-hand *RS* site in the simple excision cassette disrupts a pair of overlapping Ty1 solo ∂ elements, entirely deleting one (*) that is present in the W303 but not S288C background. (*B*) Flow charts of the M and G1 excision protocols. Temporary G1 arrest was achieved with α-factor mating pheromone. M-phase arrest was achieved with microtubule destabilizing drugs (see Materials and Methods).

fluorescent dots. Excision during M-phase arrest (referred to as M excision) (Fig. 1B) produced a pair of dots that colocalized in 76% of wild-type cells and only 27% of *sir3*-null cells, in agreement with previous findings (Fig. 2A; Chang et al. 2005). The experiment demonstrates that silencing-dependent cohesion of *HMR* is maintained after unlinking the locus from neighboring chromosomal domains.

In the present study we tested whether replication of the excised locus was sufficient to establish cohesion. To this end, *HMR* was uncoupled from the chromosome in G1 and colocalization was evaluated following a single round of DNA replication. G1 arrest was achieved with α-factor mating pheromone, which produced a uniform population of cells that contained one fluorescent dot per

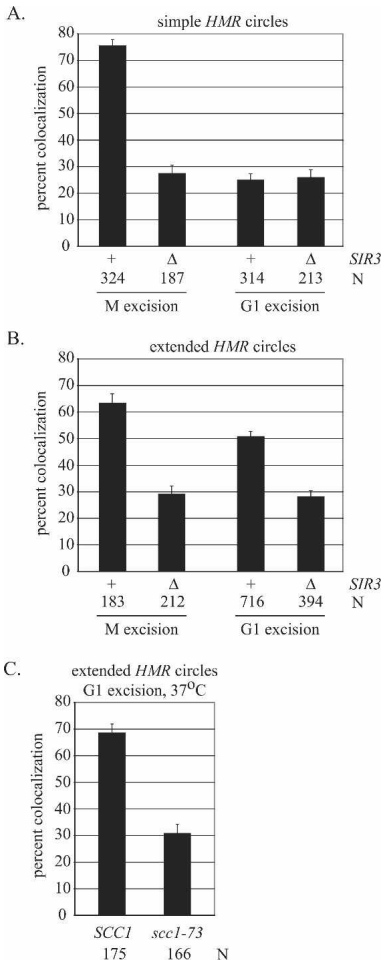

Figure 2. Colocalization of extrachromosomal *HMR* circles produced by the M and G1 excision protocols. (*A*) Simple *HMR* circles were generated in strains RDY143 (*SIR3*) and RDY189 (Δ*sir3*). *N* indicates number of cells examined. (*B*) Extended *HMR* circles were generated in strains RDY152 (*SIR3*) and RDY151 (Δ*sir3*). (*C*) Cohesion of extended *HMR* circles requires *MCD1/SCC1*. Strains RDY152 (*SCC1*) and RDY213 (*scc1-73*) were subjected to the G1 excision protocol. Cells were grown at 24°C until 1 h after removal of α-factor, when the cultures were shifted to 37°C for 2 h. The temperature shift increases cohesion in the wild-type strain (cf. *B* and *C*).

nucleus (data not shown). After galactose-induced recombination, the cultures were released from α-factor arrest and rearrested in the subsequent M phase with the microtubule-inhibiting drugs nocodazole and benomyl. Intact *HMR* silencers, both of which function as chromosomal origins of DNA replication, served to replicate the excised circle during the intervening S phase (Rivier and Rine 1992; Rivier et al. 1999). This is evident in Figure 2A, which shows that most cells emerging from the experimental protocol (hereafter referred to as the G1 excision protocol) contained pairs of dots that did not colocalize. Importantly, *HMR* circles in both wild-type and *sir3*-null strains displayed an equal deficit in colocalization, with only ~25% of cells in each case containing single dots (Fig. 2A). The low values compare with those for M excision without Sir3 (Fig. 2A) or functional cohesin (Chang et al. 2005). Northern blot analysis confirmed that the **a***1* gene at *HMR* remained transcriptionally repressed in the wild-type strain, indicating that absence of cohesion was not due to an unexpected loss of silencing (Supplementary Fig. S1A). The results show that replication of silent chromatin alone is not sufficient to establish cohesion. Simple *HMR* circles lack an important feature required for the process.

Cohesion of HMR requires a neighboring chromatin domain

We hypothesized that establishment of cohesion at *HMR* requires a *cis*-acting element in the neighboring chromosomal DNA. Such an element would be linked to *HMR* during normal chromosomal replication (the M excision protocol) but unlinked when the locus replicates extrachromosomally (the G1 excision protocol). To test this hypothesis, we expanded the excision cassette to include additional neighboring chromosomal sequences. Specifically, the telomere-proximal recombinase site was moved downstream to an intergenic region ~4 kb away. The new excision cassette (termed the extended excision cassette) (Fig. 1B) produced a larger ring that contained *HMR*, the *GIT1* gene, a set of Ty1 retrotransposon long terminal repeats (solo ∂ elements), and the threonine tRNA gene that creates the right boundary of the silent chromatin domain [designated *tT(AGU)C*]. Following M excision, the pair of extended *HMR* circles colocalized in 63% of cells, a value that roughly parallels the result obtained for simple *HMR* circles (Fig. 2B). Deletion of *SIR3* reduced colocalization of the extended circles to approximate background values (29%). These results indicate that pairing of the extended circles, like simple circles, relies on a Sir-dependent mechanism.

The extended *HMR* circles colocalized in 51% of cells following G1 excision. This represents a significant increase relative to simple circles produced by the same procedure ($P > 0.001$) (Fig. 2, cf. A and B). Moreover, in a *sir3*-null mutant, extended circles colocalized in only 28% cell, indicating that cohesion relied on silent chromatin. We repeated the G1 excision experiment with a conditional mutation in *MCD1/SCC1* to test whether cohesin was responsible for the colocalization phenom-

enon. Cultures bearing newly formed circles were shifted from permissive to nonpermissive temperature following release from α-factor arrest. Figure 2C shows that the temperature shift eliminated colocalization in the cohesin mutant but not the wild-type strain. Taken together, these findings demonstrate that the extended circles contain elements that are both necessary and sufficient to establish cohesin-mediated cohesion of silent chromatin at *HMR*.

Cohesion of HMR requires the adjacent tRNA gene

We made deletions in sequences unique to the extended excision cassette to identify the element(s) necessary for establishment of cohesion at *HMR*. *tT(AGU)C* was examined first because of the role of this gene as a silent chromatin barrier. A 100-base-pair (bp) fragment spanning the gene was replaced with a *loxP* site. The alteration reduced *HMR* pairing to background levels in the G1 excision protocol (24% colocalization) (Fig. 3A). Loss of the *tDNA* also disrupted pairing of the circles produced by M excision (31% colocalization) (Fig. 3A). The deletion had no effect on the unrecombined chromosomal arm, indicating that global cohesion was unperturbed (Fig. 3B). We conclude that *tT(AGU)C* promotes cohesion of the neighboring silent chromatin domain.

The role of *tT(AGU)C* in cohesion was also examined in a strain carrying the simple excision cassette. In this construct, integration of the lac operator array displaces the *tT(AGU)C* from *HMR* by ~14 kb. A *TRP1* marker gene linked to the array expresses equally well in both wild-type and *sir3* strains, indicating that silencing does not spread across the integrated DNA to the new distal *tDNA* position (Supplementary Fig. S2). Following the M excision protocol, *HMR* colocalizes in only 32% of the cells if the distal *tDNA* is deleted (cf. 76% in the wild type). This indicates that *tT(AGU)C* exerts its influence on cohesion even though it does not abut the silent chromatin domain and is not present on the excised DNA circle. Presumably the gene acts in *cis* to establish cohesion before *HMR* is uncoupled from the chromatin fiber by recombination.

The experiments above show that *tT(AGU)C* is necessary for cohesion of silent chromatin but is not necessarily sufficient. *tT(AGU)C* and the neighboring *GIT1* gene are transcribed toward one another, like other convergent gene pairs where cohesin has been found (Glynn et al. 2004; Lengronne et al. 2004). It therefore seemed possible that convergent transcription of the *tDNA* and *GIT1* was important for cohesion of *HMR*. To test this notion, we replaced the *GIT1* ORF and 600 bp of upstream sequence with the *URA3* gene from *Kluyveromyces lactis (klURA3)*, orienting transcription of the new gene away from *tT(AGU)C* (see Fig. 6C, below). The results in Figure 3D show that the modified locus maintains cohesion following M excision (69% colocalization). We conclude that the role of *tT(AGU)C* in cohesion at *HMR* does not involve convergent transcription of the gene with *GIT1*. While undocumented transcription units cannot be ruled out, we note that large-scale transcriptome analysis did not identify nearby cDNAs that were oriented convergently with *tT(AGU)C* (Miura et al. 2006).

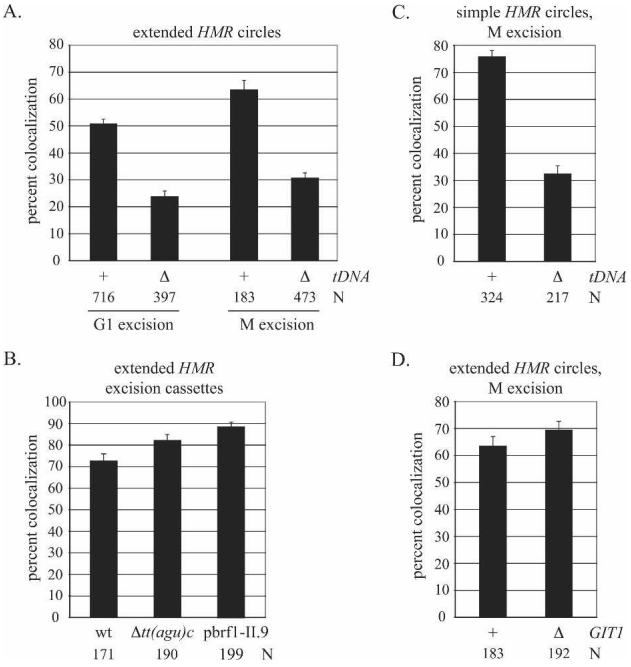

Figure 3. *tT(AGU)C* establishes cohesion of *HMR*. (*A*) Colocalization of extended *HMR* circles produced by the G1 and M excision protocols using strains RDY152 (wt) and RDY180 [Δ*tt(agu)c*::*loxP*]. (*B*) Colocalization of the unexcised chromatids in strains RDY152 (wt), RDY180 [Δ*tt(agu)c*::*loxP*], and RDY209 (Δ*brf1* with plasmid pbrf1-II.9, grown at 24°C). Cultures were supplemented with dextrose rather than galactose in the M excision protocol. (*C*) Colocalization of simple *HMR* circles requires *tT(AGU)C*. Circles were produced by the M excision protocol in strains RDY143 (wt) and RDY279 [Δ*tt(agu)c*::*loxP*]. (*D*) Colocalization of extended *HMR* circles does not require *GIT1*. Circles were produced by the M excision protocol using strains RDY152 (wt) and RDY226 (Δ*git1*::*klURA3*).

Cohesin binding at HMR

We used chromatin immunoprecipitation (ChIP) of TAP-tagged Mcd1/Scc1 to evaluate the role of *tT(AGU)C* in binding cohesin at *HMR*. Excised circles were first formed during G1 arrest in strains carrying extended excision cassettes. Cross-links were then generated with formaldehyde during the subsequent mitotic arrest. Association of Mcd1/Scc1 with the *HMR* **a***2* gene (Fig. 4A) was compared with a *SIR*-independent binding site on chromosome V (549.7). Figure 4B shows that the protein bound *HMR* **a***2* and that deleting *SIR3* reduced binding (nearly threefold) to a level comparable with other well-characterized cohesin-free sites (534 on chromosome V and *ACT1*) (Chang et al. 2005; data not shown). Deleting the *tDNA* also reduced binding, albeit to an intermediate level. Deleting the *tDNA* from the *sir3*-null strain did not further diminish Mcd1/Scc1 binding. The results

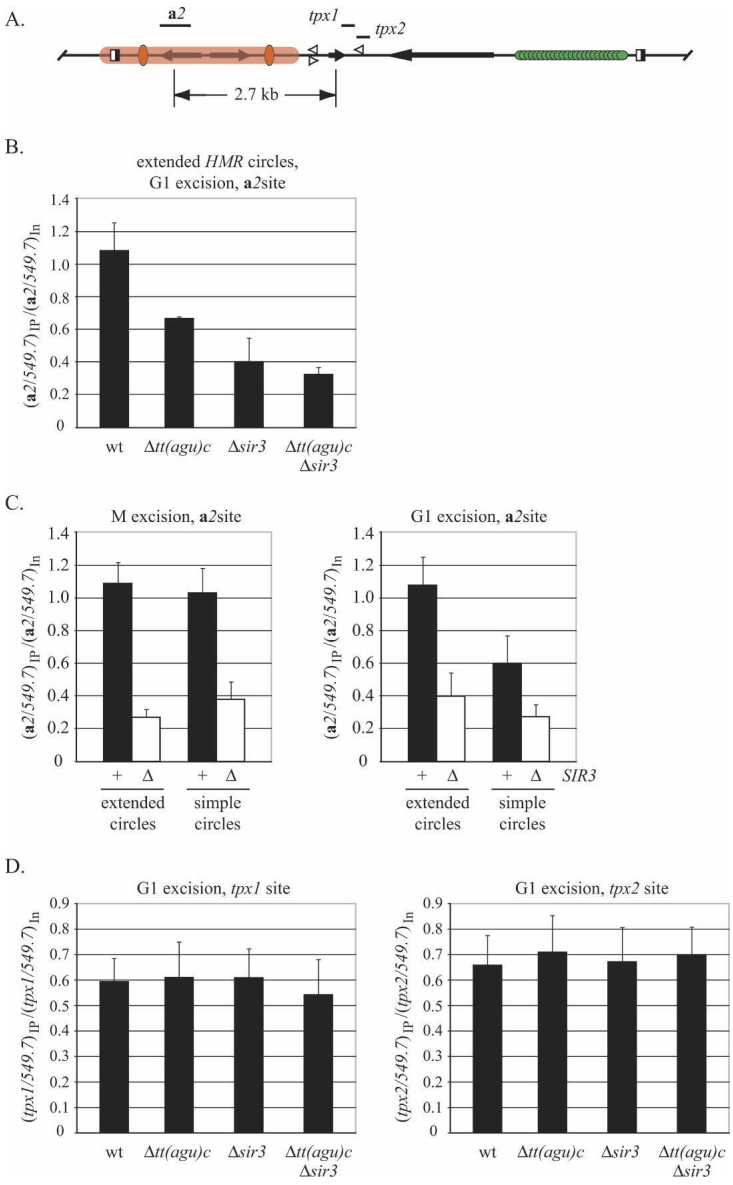

Figure 4. ChIP of Mcd1/Scc1-TAP. (*A*) Relative positions of the PCR-amplified sites. Primers are listed in Supplementary Table S2. (*B*) Mcd1/Scc1 binding at the **a***2* site of extended circles formed by G1 excision. Cross-links were generated during the subsequent M-phase arrest. The ratio of immunoprecipitated material (the specific site relative to the 549.7 control) was normalized to the same ratio of input material. The mean and standard deviation of three or more independent trials are presented (see Supplementary Fig. S3 for sample gels). Strains RDY177 (*MCD1/SCC1-TAP*), RDY179 (Δ*sir3 MCD1/SCC1-TAP*), RDY181 [Δ*tt(agu)c*::*loxP MCD1/SCC1-TAP*], and RDY272 [Δ*tt(agu)c*::*loxP* Δ*sir3 MCD1/SCC1-TAP*] were used. (*C*) Mcd1/Scc1 binding to **a***2* of simple and extended excision cassettes. Circles were formed by either the G1 or M excision protocols. Strains RDY178 (*MCD1/SCC1-TAP*) and RDY190 (Δ*sir3 MCD1/SCC1-TAP*) were used to produce simple *HMR* circles. (*D*) Mcd1/Scc1-TAP binding to *tT(AGU)C*-proximal sites *tpx1* and *tpx2*. Strains are listed in *B*.

show that *tT(AGU)C* facilitates cohesin binding at *HMR*. In the absence of the *tDNA*, residual Mcd1/Scc1 either is bound in a nonproductive manner or is not present in sufficient quantity to establish cohesion.

We examined the binding of cohesin to simple *HMR* circles as well. When we uncoupled *HMR* from *tT(AGU)C* in M phase after establishment of cohesion, the level of Mcd1/Scc1 bound to *HMR* **a***2* compared with the level on extended circles (Fig. 4C). On the contrary, when we uncoupled *HMR* from *tT(AGU)C* in G1 (and cross-linked in the subsequent M phase), the simple circles associated with a reduced level of Mcd1/Scc1 (Fig. 4C). Notably, the amount of Mcd1/Scc1 on these circles compared with the amount on extended circles lacking the *tDNA* (Fig. 4, cf. B and C). Deleting *SIR3* reduced Mcd1/Scc1 binding further in all cases. Collectively, the simple circle findings agree fully with the extended circle findings. *tT(AGU)C* must be present in *cis* during passage from G1 to M phase for cohesin to bind efficiently at *HMR*.

Lastly, we examined binding of Mcd1/Scc1 at sites near *tT(AGU)C* (designated *tpx1* and *tpx2* in Fig. 4A). Cohesin was previously shown to bind robustly to this region, which lies immediately downstream but adjacent to the *tDNA* (Laloraya et al. 2000). Cross-links were generated in M-phase-arrested cells that had undergone the G1 excision protocol. Only strains with extended excision cassettes were examined. Figure 4D shows that Mcd1/Scc1 bound the *tDNA*-proximal sites equally well in both wild type and a *sir3* mutant, in agreement with earlier results (Kobayashi et al. 2004). More importantly, deletion of *tT(AGU)C* did not significantly reduce Mcd1/Scc1 binding in either strain. Like the residual cohesin at **a***2* described above, cohesin bound at this site does not support cohesion (Figs. 2, 3). The results indicate that cohesin binds to a *tDNA*-proximal region in a

manner that requires neither the *tDNA* nor silent chromatin. Association with this site, like the residual cohesin at **a***2*, is not sufficient for cohesion.

Cohesion of HMR requires the RNA pol III transcription machinery

Select mutants were used to evaluate the role of the RNA pol III machinery in cohesion of *HMR*. RNA pol III transcription requires a hierarchical structure that utilizes two intragenic promoter elements at *tDNA*s, *boxA* and *boxB* (Schramm and Hernandez 2002). Transcription factor TFIIIC binds independently of other RNA pol III factors and recruits TFIIIB, which then recruits RNA pol III. A single point mutation in *boxB* (a *c56/g* transversion) prevents TFIIIC binding, and consequently blocks *tDNA* transcription and boundary function (Newman et al. 1983; Baker et al. 1986; Donze and Kamakaka 2001). When this single base-pair change was made in the extended excision cassette, colocalization of circles produced by the M excision protocol dropped to 26% (Fig. 5A). **a***1* transcripts from *HMR* could not be detected in this strain (Supplementary Fig. S1B). Therefore, the reduction in cohesion does not arise from an unexplained loss of silencing. These findings indicate that cohesion of *HMR* requires TFIIIC binding or some subsequent RNA pol III-related event.

TFIIIB is composed of three polypeptides TBP, Bdp1, and Brf1. Select mutations in Brf1 block association of TBP, preventing assembly of TFIIIB on DNA (Andrau et al. 1999). We crossed one of these mutations (*brf1-II.9*) into our strain bearing the extended excision cassette. Previous work had shown that the conditional allele disrupted barrier function at *HMR*, even at the permissive temperature of 24°C (Donze and Kamakaka 2001). Figure 5B shows that colocalization of *HMR* circles formed by the M excision protocol dropped from 66.5% in the wild type to 37% in the mutant (Fig. 5B). This defect cannot be attributed to loss of silencing, which was found to be intact (Supplementary Fig. S1B). Colocalization was fully restored by reintroducing a plasmid-borne copy of the *BRF1* gene, indicating that the cohesion defect was indeed due to mutation of the TFIIIB subunit. Furthermore, the defect appears to be localized near *HMR* since cohesion of the unrecombined chromosomal arm was not reduced (Fig. 4B). Taken together, these results indicate that recruitment of TFIIIB or a subsequent step in the RNA pol III transcription pathway is required for cohesion of *HMR*.

Heterologous tDNAs support cohesion of HMR

We entertained the possibility that *tT(AGU)C*-mediated cohesion of *HMR* was related to the boundary the gene creates. Therefore, we tested whether other *tDNA*s with barrier activity could substitute for *tT(AGU)C*. For this purpose, we utilized *tT(UGU)G1* and twin copies of *tT(AGU)N2*, both of which block the spread of silencing when placed near *HMR*, and a single copy of *tT(AGU)N2*,

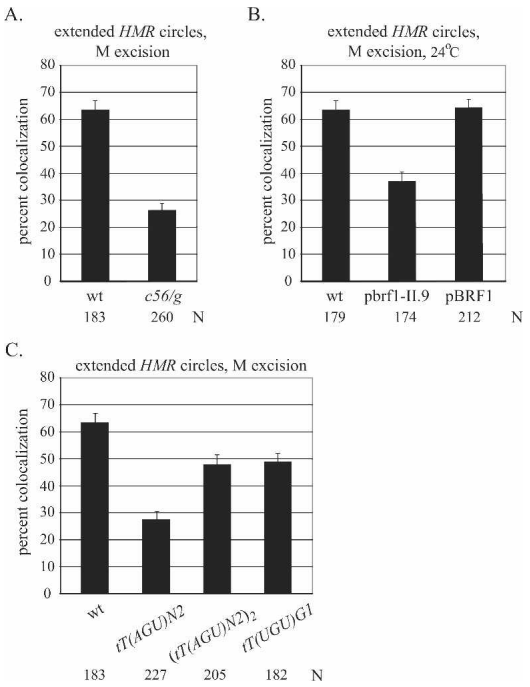

Figure 5. Cohesion of *HMR* requires RNA pol III machinery on *tT(AGU)C*. (*A*) Colocalization of extended *HMR* circles produced by the M excision protocol in strains RDY152 (wt) and RDY204 (*c56/g*). (*B*) Colocalization of extended *HMR* circles produced by the M excision protocol in strains RDY152 (wt), RDY209 (Δ*brf1* with plasmid pbrf1-II.9), and RDY227 (Δ*brf1* with plasmid pBRF1). Cultures were grown in nonselective rich media at 24°C. The difference between the values for the wild-type and mutant *brf1* alleles (plasmids pBRF1 and pbrf1-II.9, respectively) is significant ($P > 0.001$). (*C*) Comparison of extended *HMR* circles with replacement *tDNA*s following M excision using strains RDY152 (wt), RDY231 [Δ*tdna*::*tT(AGU)N2*], RDY241 [Δ*tdna*::*tT(AGU)N2*]$_2$], and RDY205 [Δ*tdna*::*tT(UGU)G1*]. The difference between the values for the single and double *tT(AGU)N2* replacement copies is significant ($P > 0.001$).

which does not (Donze and Kamakaka 2001). Figure 5C shows that only the *tDNA* replacements with boundary-forming capacity supported colocalization of excised circles. In these cases, the Sir2 inhibitor splitomicin (Bedalov et al. 2001) reduced colocalization to background levels, indicating that the heterologous *tDNA*s also act through a silencing-dependent mechanism (Supplementary Fig. S4). The data show that cohesion at *HMR* can be established by *tDNA*s that create silent chromatin boundaries. These results, however, cannot distinguish whether cohesion relies on barrier activity per se or some upstream event, like transcription of the gene required to generate barrier activity.

Barrier function alone is not sufficient for cohesion of HMR

To further examine a possible relationship between silent chromatin barriers and cohesion, we analyzed barriers formed by sequences other than *tDNA*s. For this

purpose, we replaced *tT(AGU)C* of the extended excision cassette with the serine-inducible *CHA1* promoter (*CHA1p*) that normally resides on the right side of *HML*. We also replaced the *tDNA* with a series of binding sites for the bacterial lexA protein (six copies of the ColE1 operator, each containing two overlapping binding sites) (Ansari and Gartenberg 1997). Previous work revealed that inducing the *CHA1* promoter or binding of lexA at high density blocks the spread of silencing (Donze and Kamakaka 2001; Bi et al. 2004). In this study we induced *CHA1p* with 4 mM serine. Figure 6A shows that circles containing the promoter colocalized in only 16% of the nuclei examined. Colocalization of wild-type and Δ*tDNA* circles, on the other hand, was not affected by the inducer (cf. Figs. 6A and 4A). *HMR* circles bearing lexA sites (*lexOPs*) were examined in strains that carried either a lexA expression plasmid or empty vector. In neither case was colocalization observed (Fig. 6B). Colocalization of wild-type circles was unaffected by the added plasmids. Collectively, these experiments show that neither *CHA1p* nor bound lexA can substitute for the role of a *tDNA* in silent chromatin cohesion.

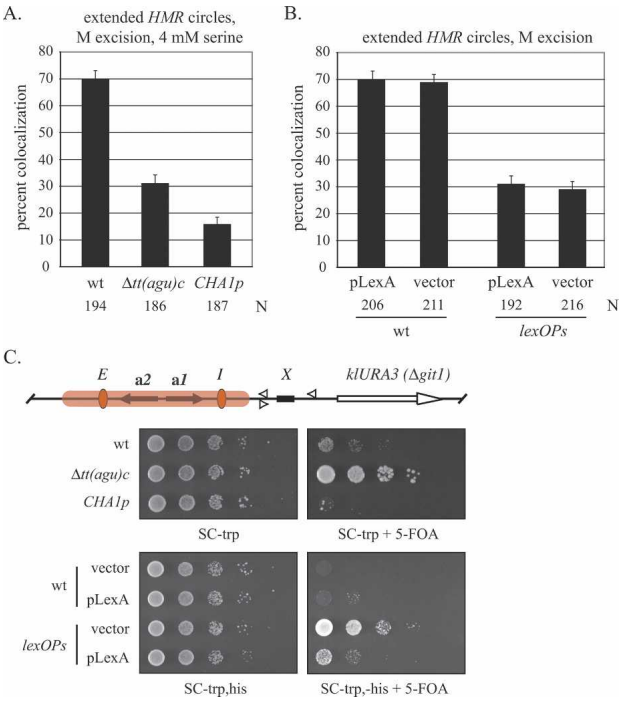

Figure 6. Non-*tDNA* barrier elements do not support *HMR* cohesion. (*A*) Colocalization of *HMR* circles containing a *CHA1p* barrier following M excision. Strains RDY152 (wt), RDY180 [Δ*tt(agu)c*::*loxP*], and RDY206 [Δ*tt(agu)c*::*CHA1p*] were examined in rich media supplemented with 4 mM serine. (*B*) Colocalization of *HMR* circles bearing lexA operators following M excision. Strains RDY152 (wt) and RDY242 [Δ*tt(agu)c*::*6lexOPs*] were transformed with a plasmid expressing lexA (pLexA) or empty vector (pRS413). Plasmids were maintained by overnight growth in SC-trp,his + raffinose prior to replacing media with rich media containing raffinose and nocodazole. (*C*) Organization of the *HMR* region in RDY226 with *GIT1* replaced by the *K. lactis URA3* (*klURA3*). X marks the *HMR*-proximal *tDNA* that was substituted with heterologous sequences. (*D*) Barrier function of RDY226 (wt) and *tDNA* replacement strains RDY249 [Δ*tt(agu)c*::*loxP*], RDY251 [Δ*tt(agu)c*::*CHA1p*] and RDY263 [Δ*tt(agu)c*::*6lexOPs*]. Tenfold serial dilutions of each strain were spotted in rows on selective media containing or lacking 0.1% 5-FOA. All strains are prototrophic for tryptophan and grow on SC-trp media containing 4 mM serine. Plasmids pLexA and empty vector were maintained by SC-his selection.

To be certain that *CHA1p* and lexA create silencing barriers in our constructs, we integrated the *klURA3* reporter gene ~1.5 kb downstream from *HMR* at the *GIT1* locus (Fig. 6C). Repression of the gene permits growth on 5-FOA, a drug that the *klURA3* gene product converts to a toxic metabolite. Robust growth of the Δ*tt(agu)c*::*loxP* mutant relative to the wild-type strain demonstrates that this assay can measure variations in barrier activity over a 100-fold range (Fig. 6C). Similar results were found when comparing the *c56/g* mutant with wild type (data not shown). Growth of the *CHA1*-modified strain was completely blocked on media containing 5-FOA and serine, indicating that the heterologous promoter equals or exceeds the potency of the native *tDNA* barrier. Growth of strains expressing lexA was also hindered on 5-FOA, but only when *lexOPs* sites replaced the *tDNA* (Fig. 6C). Thus, lexA binding also creates a boundary at *HMR*. We conclude that a boundary between silenced and active chromatin domains is not sufficient for cohesion of *HMR*.

Cohesion of HMR requires Rsc2 but not Yta7 or Isw2

Rsc2 and Yta7 are bromodomain proteins that contribute to the natural silent chromatin barriers at *HMR* (Jambunathan et al. 2005; Tackett et al. 2005). Rsc2, as part of the RSC chromatin remodeling complex, binds *tDNA*s but does not appear to regulate their expression (Ng et al. 2002; Soutourina et al. 2006). Yta7 associates with silent chromatin boundaries as part of a Dpb4–chromatin remodeling complex (Tackett et al. 2005). The available evidence indicates that Yta7 and the *tT(AGU)C* function in distinct pathways at *HMR*. Deleting both elements causes a greater boundary defect than deleting either one alone (Jambunathan et al. 2005). In agreement with earlier work (Chang et al. 2005), we found that a *RSC2* deletion impairs cohesion of extended *HMR* circles without causing derepression of the **a***1* gene (Fig. 7; Supplementary Fig. S1). Deletion of *YTA7*, on the other hand, had no impact (64% colocalization), and deletion of *YTA7* from a *tT(AGU)C*-null strain did not exacerbate the colocalization defect. This result indicates that barrier function can be compromised without untoward effects on cohesion. The data lend weight to the idea that the *tDNA* promotes cohesion to *HMR* by means other than serving as a silent chromatin boundary.

Isw2 is the ATPase subunit of the yeast ISWI chromatin remodeling complexes that also bind *tDNA*s and modulate silent chromatin barrier activity at *HMR* (Gelbart et al. 2005; Oki and Kamakaka 2005; Tackett et al. 2005). The remodeler alters target site selection of Ty1 retrotransposons that integrate preferentially near

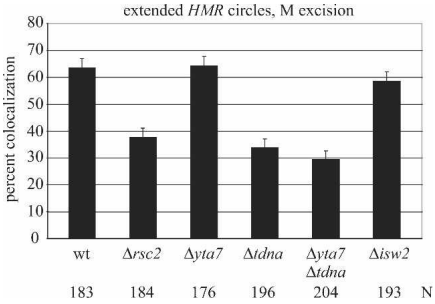

Figure 7. Influence of *trans*-acting factors on cohesion of *HMR*. Strains RDY176 (Δ*rsc2*), RDY208 (Δ*yta7*), and RDY225 (Δ*isw2*) were used. The difference between values for the *rsc2* and *yta7* strains is significant ($P > 0.001$), whereas the differences between wild type, *yta7*, and *isw2* is not.

*tDNA*s (Bachman et al. 2005). Here we find that deletion of *ISW2* does not impair colocalization of extended *HMR* circles (Fig. 7). The result indicates that action of Isw2 at *tT(AGU)C* is not required for cohesion of the silent chromatin domain.

Discussion

tT(AGU)C establishes cohesion of the neighboring silent chromatin domain

Establishment of sister chromatid cohesion occurs during S phase and is thought to involve events at or near the replication fork. To investigate establishment of silent chromatin cohesion, we generated extrachromosomal *HMR* circles that replicated autonomously inside living cells. Despite remaining transcriptionally repressed, replicated circles failed to colocalize with one another unless the *HMR*-proximal tRNA gene *tT(AGU)C* was present in *cis*. Mutations in Brf1 and Rsc2, both subunits of complexes that associate with RNA pol III and bind the gene, attenuated the *tDNA* effect. Mutation of a critical residue in the *tDNA* promoter yielded similar consequences. Taken together, this work identifies *tT(AGU)C* and the associated RNA pol III machinery as a cohesion establishment complex at *HMR*.

tDNA-independent silent chromatin barriers do not establish cohesion

tT(AGU)C is distinguished by its ability to block silent chromatin from encroaching on the adjoining active chromosomal domain (Oki and Kamakaka 2005). The gene creates a discontinuity in arrayed nucleosomes that acts as a chain terminator to the propagation of chromatin-bound Sir proteins. The gene also abuts with one of the first documented cohesin-associated regions (CARC4) (Laloraya et al. 2000). Thus, we considered the possibility that the boundary between silent and nonsilent chromatin was responsible for cohesion at *HMR*. The failure of *tDNA*-independent boundaries to generate *HMR* cohesion, however, showed that barrier activity alone was not sufficient (Fig. 6). That two genes with documented roles in barrier activity at *HMR*, *YTA7*, and *ISW2* had no measurable effect on cohesion of the locus reinforced these results (Fig. 7). Moreover, we found that *tT(AGU)C* mediated cohesion even when the *tDNA* was displaced from silent chromatin by an intervening lac operator array and active reporter gene (Fig. 3B). Collectively the evidence points to a role for *tT(AGU)C* in cohesion that is independent of the boundary it creates.

tT(AGU)C promotes binding of cohesin to the neighboring silent chromatin domain

Binding of cohesin throughout the *HMR* domain has been examined in a rigorous manner (at sites **a***1*, 3′ untranslated region of **a***1*, *HMR-I* in Chang et al. 2005, and sites *tpx1*, *tpx2*, and **a***2* above). At the **a***2* gene near the center of the silenced region, Mcd1/Scc1 binding diminished upon removal of the *tDNA* and fell to background levels in the absence of Sir3. At the *tT(AGU)C*-proximal sites *tpx1* and *tpx2* ~2.7 kb away from **a***2*, Mcd1/Scc1 binding was independent of both the *tDNA* and Sir3. Importantly, binding at these sites in strains lacking the *tDNA* (and the residual binding at **a***2*) did not produce cohesion (Fig. 3). The results indicate that replication of cohesin-bound chromatin does not necessarily lead to cohesion. Maps of chromosomal cohesin-binding sites must be interpreted carefully since cohesin binding cannot be equated with cohesin function. Similar conclusions were reached when cohesin was found at locations where cohesin-independent mechanisms account for sister chromatid pairing (Lam et al. 2006; Shimada and Gasser 2007).

Our findings lead us to propose the existence of two pools of bound cohesin within the *HMR* domain: an active pool that associates with silent chromatin and participates in cohesion, and an inert pool that binds near the *tDNA* (and to a limited extent on silent chromatin). We view *tT(AGU)C* as an initiator element that promotes cohesion in one of two ways. In the first scenario, the gene functions by activating the inert pool. Alternatively, the gene loads and activates a second pool of cohesin de novo. In either case, activated cohesin could then migrate to silenced positions like **a***2*, either by sliding along the chromatin fiber or by looping out the intervening DNA and transferring directly (Fig. 8).

A role for RNA pol III transcription complex in silent chromatin cohesion

How might the RNA pol III machinery participate? The simplest scenario is that Brf1 (or another pol III factor dependent on Brf1 for binding) directly recruits factors dedicated to cohesion. Precedent for such a scaffolding model comes from the targeting of yeast retrotransposons near pol III genes (Devine and Boeke 1996; Bachman et al. 2005). In the case of Ty3 viral-like particles of yeast, Brf1 and TBP alone are sufficient for targeting in vitro, presumably by interacting directly with the integrase (Yieh et al. 2002).

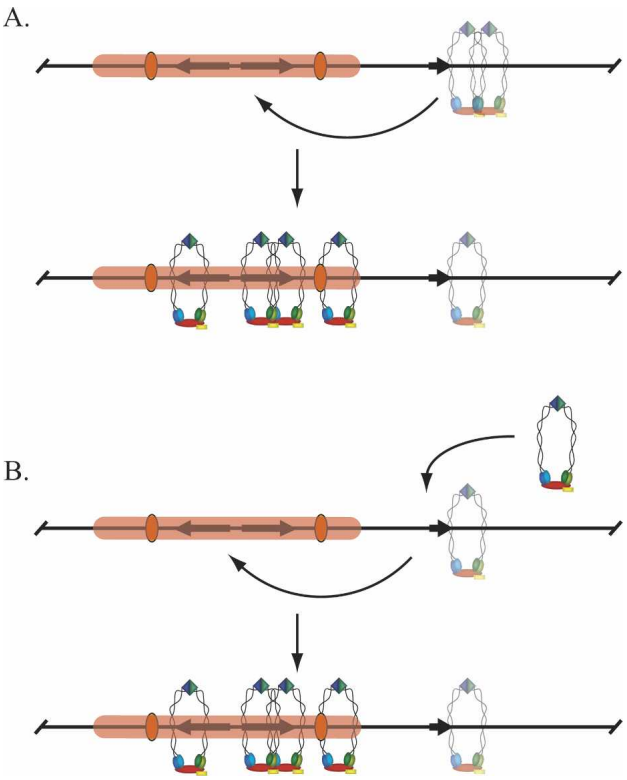

Figure 8. Models for the role of *tT(AGU)C* in cohesion of *HMR*. (*A*) *tT(AGU)C* activates a nonfunctional pool of cohesin on the adjacent tpx1 and tpx2 sites (represented as a translucent complex) that then migrates to the neighboring silenced chromosomal domain. (*B*) *tT(AGU)C* loads an active pool of cohesin, which then migrates to the adjacent silenced chromosomal domain. Silencing-dependent cohesin has thus far been detected on the **a***2* gene (Fig. 4), the **a***1* gene, and the *HMR-I* silencer (Chang et al. 2005).

Cohesin might also be linked to RNA pol III transcription, via either the elongating polymerase or nascent RNA chain. A third possibility is that transcription causes secondary events that, in turn, promote cohesion. In this regard, we note that active *tDNA*s impede the movement of replication forks (Deshpande and Newlon 1996; Ivessa et al. 2003) and that two replisome-associated proteins responsible for the stalling, Tof1 and Csm3, are required for efficient sister chromatid cohesion (Mayer et al. 2004; Calzada et al. 2005; Tourriere et al. 2005).

A universal role for tDNAs in cohesion?

The ability of heterologous *tDNA*s to establish cohesion at *HMR* suggests that these and other *tDNA*s might function in cohesion at their endogenous locations, which distribute across every chromosome. We envision that other *tDNA*s are paired with secondary sites, like silent chromatin in the case of *tT(AGU)C*, and that these secondary sites capture activated cohesin. Individual *tDNA*s may serve dedicated functions. In *S. pombe*, for example, *tDNA*s at the boundaries of pericentric heterochromatin may establish cohesion for chromosome segregation, in addition to providing barrier activity (Noma et al. 2006). However, it is not likely that the RNA pol III acts alone in cohesin loading/activation. Cohesion of the unrecombined chromosomal arm, for example, persists in the *brf1* mutant (Fig. 3). Moreover, large stretches of the genome, including the 80-kb domain surrounding *HML*, are devoid of all RNA pol III components (Harismendy et al. 2003), and minichromosomes lacking pol III transcription units establish cohesion efficiently (Chang et al. 2005).

Recent reports have highlighted roles for the RNA pol III pathway in spatial organization of genomes. In *S. cerevisiae*, tRNA gene families cluster near the nucleolus (Thompson et al. 2003). In *S. pombe*, TFIIIC concentrates in foci at the nucleolus and nuclear periphery where TFIIIC-bound sequences reside (Noma et al. 2006). It is tempting to speculate that the RNA pol III promoters embedded within the highly repetitive and dispersed *Alu* elements in mammalian chromosomes do the same (Deininger and Batzer 2002). Cohesin mediated by *tDNA*s may thus represent an additional layer of genome-wide chromosome organization.

Materials and methods

Strain and plasmid construction

Strains used in this study are listed in Supplementary Table S1. Those that contain the extended excision cassette were derived in several steps from the progenitor strain MRG2277, which has integrated copies of the lac-GFP (S65T) expression vector pGVH60 and the recombinase R-inducible expression vector pRINT, as well as a single *RS* target site upstream of *HMR*-E (at the SnaBI site). A second *RS* site and *lacOP* array were added with a single plasmid (pAFS52-RS-GIT1u), which integrates 1071 bp upstream of the *GIT1* start codon. A cross between MRG2227 and strain CRC25 produced segregants RDY151 and RDY152. *scc1-73* was introduced by crossing RDY152 with CRC83. PCR-mediated gene replacement (PMGR) was used to substitute *tT(AGU)C* with a *loxP–URA3–loxP* cassette from strain RRY5 to create RDY173. The module was then replaced using PMGR with templates that contain a single *loxP* site (strain RDY174), six ColE1 operators (plasmid pAA6), the *CHA1* promoter (plasmid pDD560), the *c56/g* mutation (plasmid pDD450), or the *tT(UGU)G1*, *tT(AGU)N2* or $[tT(AGU)N2]_2$ *tDNA*s (plasmids pDD454, pDD451, and pDD589, respectively) (Ansari and Gartenberg 1997; Donze and Kamakaka 2001). The heterologous *tDNA* replacement fragments carried with them ~250 bp of flanking DNA from their native chromosomal position. TAP tagging of *MCD1/SCC1* was achieved by PMGR using strains from Chang et al. (2005) as templates. Various null mutants were obtained by PMGR using *kanMX*, *natMX*, or *hphMX*. The marker on the pRS plasmid bearing *brf1-II.9* was swapped from *LEU2* to *URA3* by PMGR in strain DDY412. The new plasmid, as well as the brf1 genomic deletion, was crossed into RDY152 to generate RDY209. The pbrf1-II.9 plasmid in RDY209 was replaced with a plasmid bearing a wild-type copy of the gene (Conesa et al. 2005) to generate RDY227. PMGR was used to replace the *GIT1* ORF and 624 bp upstream with the *loxP–klURA3–loxP* cassette of pUG72 (Güldener et al. 1996). Transcription of the integrated *klURA3* gene was oriented in the

opposite direction of *GIT1*. The *TRP1* marker of the high-copy lexA expression vector pBTM116 was swapped to *HIS3* using PMGR to generate pLexA. All strain modifications were confirmed by PCR and/or functional tests. Sequences of engineered loci are available on request.

Cell growth and microscopy

The M excision protocol was performed as described in Chang et al. (2005). For G1 excision, freshly streaked cells were grown to mid-log density in SC-trp media + 2% dextrose before diluting 1/200 into YPA (rich media) + raffinose for overnight growth. α-Factor was added (Cf = 20 nM) when cultures reached an OD of 0.2. Galactose (Cf = 2%) was added to induce excision 2.5 h later, when nearly all cells had adopted the "shmoo" morphology. Two hours after the addition of galactose, cells were collected by centrifugation, washed, and resuspended in rich media containing galactose (2%), nocodazole (10 µg/mL), and pronase E (100 µg/mL). Benomyl (Cf = 10 µg/mL) was added 1.5 h after resuspension, and cells were harvested 1.5 h later by centrifugation. Exceptions to this protocol are described in the figure legends. Paraformaldehyde fixation, mounting of cells on slides, fluorescence microscopy, and error analysis were as described in Chang et al. (2005).

ChIP

Cross-linking after M-phase excision utilized the standard cell growth protocol described above. The G1 excision protocol was modified by collecting cells 1.5 h after release from α-factor into media containing nocodazole. Immunoprecipitation procedures were as described in Chang et al. (2005) with the noted exceptions. PCR reactions were run in multiplex using oligo sets listed in Supplementary Table S2. Specificity of the ChIP reactions was confirmed with an additional set of primers that detected little immunoprecipitation of the cohesin-free *ACT1* promoter (Lengronne et al. 2004; R.N. Dubey, unpubl.). Gels were stained with EtBr and destained in water before digital photography and quantization (Alpha Innotech, Inc). Individual bands were found to be nonsaturating and within the linear range.

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