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 TFIIB and RSC complexes that bind the gene. Non-tDNA boundary elements do not substitute for tDNAs in cohesion, suggesting that barrier activity is not responsible for the phenomenon. The results reveal an unexpected role for tDNAs 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-
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]. tDNAs 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](https://example.com/figure1.png)
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 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 α1 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 d elements], and the threonine tRNA gene that creates the right boundary of the silent chromatin domain [designated t(TAGU)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-
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 unexcised 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 \( Kluveromyces\) \( lactis\) (\( klURA3\)), 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].

Cohesin binding at HMR

We used chromatin immunoprecipitation (ChIP) of TAP-tagged Mcd1/Scc1 to evaluate the role of \( tt(AGU)C\) in binding cohesion 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 a2 gene [Fig. 4A] was compared with a SIR-independent binding site on chromosome V [549.7]. Figure 4B shows that the protein bound HMR a2 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 3. \( tt(AGU)C\) establishes cohesion of HMR. [A] Colocalization of extended HMR circles produced by the G1 and M excision protocols using strains R DY152 [wt] and R DY180 [att(agu)c::loxP]. [B] Colocalization of the unexcised chromatin cassettes in strains R DY152 [wt], R DY180 [att(agu)c::loxP], and R DY209 [Δbrf1] 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 [att(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 [Δgit1::klURA3].

\( tDNAs\) and silent chromatin cohesion
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 a2 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-TAP binding to tT(AGU)C-proximal sites tpx1 and tpx2. Strains are listed in B.
manner that requires neither the tDNA nor silent chromatin. Association with this site, like the residual cohesin at a2, 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 tDNAs, 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]. a1 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 tDNAs with barrier activity could substitute for tT(AGU)C. For this purpose, we utilized tT(UGU)C1 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, 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 tDNAs also act through a silencing-dependent mechanism [Supplementary Fig. S4]. The data show that cohesion at HMR can be established by tDNAs 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 tDNAs. 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 ΔtDNA 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.

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 Δtt(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 tDNAs 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 aI 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 tDNAs 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 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 [Δtt(agu)c::loxP], and RDY206 [Δtt(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 [Δtt(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 nocardazole. (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 [Δtt(agu)c::loxP], RDY251 [Δtt(agu)c::CHA1p] and RDY263 [Δtt(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.
ences between wild type, yta7 and (Δyta7) were used. The difference between values for the tDNA was present in tT(AGU)C silent chromatin cohesion, we generated extrachromatin domain. The replication fork. To investigate establishment of S phase and is thought to involve events at or near the origin of replication. 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 a2, either by sliding along the chromatin fiber or by looping out the intervening DNA and transferring directly to HMR.

**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).

tDNAs and silent chromatin cohesion

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 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 in 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

**Figure 7.** Influence of trans-acting factors on cohesion of HMR. Strains RDY176 (Δrsc2), RDY208 (Δyta7), and R DY225 (Δisw2) 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.

**Discussion**

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

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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 in 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

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In this regard, we note that active tDNAs cause secondary events that, in turn, promote cohesion. A third possibility is that transcription, via either the elongating polymerase or nascent RNA chain. A third possibility is that transcription activates a nonfunctional pool of cohesin

**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 tp1 and tp2 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 a2 gene (Fig. 4), the a1 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 tDNAs impede the movement of replication forks (Deshpande and Newlon 1996; Iveysa 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 tDNAs to establish cohesion at HMR suggests that these and other tDNAs might function in cohesion at their endogenous locations, which distribute across every chromosome. We envision that other tDNAs are paired with secondary sites, like silent chromatin in the case of tT(AGU)C, and that these secondary sites capture activated cohesin. Individual tDNAs may serve dedicated functions. In S. pombe, for example, tDNAs 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 tDNAs 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 R5 target site upstream of HMR-E (at the SnBI site). A second R5 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. sec1-73 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 RY5 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 pDD60], the c56/g mutation [plasmid pDD450], or the tT(UGU)G1, tT(AGU)N2 or tT(AGU)N2], tDNAs (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, nutMX, 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 pbriI-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 to YPRA [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%), nocardazole (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 nocardazole. 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, unpub.). 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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