DNA polymerase ϵ , acetylases and remodellers cooperate to form a specialized chromatin structure at a tRNA insulator

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Insulators bind transcription factors and use chromatin remodellers and modifiers to mediate insulation. In this report, we identified proteins required for the efficient formation and maintenance of a specialized chromatin structure at the yeast tRNA insulator. The histone acetylases, SAS-I and NuA4, functioned in insulation, independently of tRNA and did not participate in the formation of the hypersensitive site at the tRNA. In contrast, DNA polymerase ϵ , functioned with the chromatin remodeller, Rsc, and the histone acetylase, Rtt109, to generate a histone-depleted region at the tRNA insulator. Rsc and Rtt109 were required for efficient binding of TFIIB to the tRNA insulator, and the bound transcription factor and Rtt109 in turn were required for the binding of Rsc to tRNA. Robust insulation during growth and cell division involves the formation of a hypersensitive site at the insulator during chromatin maturation together with competition between acetylases and deacetylases.

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Introduction

The packaging of DNA with histones in chromatin and the organization of the chromatin fibre into domains are critical for proper gene regulation during growth, development and differentiation. Two distinct forms of chromatin domains can be visualized in the nucleus—euchromatin and heterochromatin (Huisinga *et al*, 2006; Trojer and Reinberg, 2007). In metazoans, euchromatin domains enriched in acetylated core histones and depleted for linker histones, are present in an accessible configuration, whereas heterochromatin domains contain methylated DNA, histone H3 methylated on

K9 and K27, and repressor proteins that package the chromatin into an inaccessible configuration to transcriptionally silence genes.

Active and inactive chromatin domains are often juxtaposed along the chromosome and this organization requires mechanisms to separate these domains. The junctions of these functionally antagonistic chromatin domains are often marked by elements called insulators (Valenzuela and Kamakaka, 2006). Insulators are integral to proper gene regulation and are conserved in all eukaryotes. The insulator elements restrict the action of enhancer and silencer elements to the specific chromatin domains in which they reside.

Insulators are characterized by a specialized chromatin structure that manifests as a constitutive DNaseI hypersensitive site. Insulators that flank the heat shock locus in *Drosophila* (Udvardy *et al*, 1985), the β -globin locus in chickens (Chung *et al*, 1993) and the silenced *HMR* chromatin domain in yeast (Nasmyth, 1982) are all characterized by the presence of DNaseI hypersensitive sites. Hypersensitive sites signify an alteration in the regular nucleosome repeat of chromatin and are binding sites for sequence-specific transcription factors (West *et al*, 2002; Valenzuela and Kamakaka, 2006). For example, at the heat shock and gypsy insulators in *Drosophila*, transcription factors Su(Hw), Zw5 and BEAF bind to mediate chromatin domain organization, whereas at the β -globin locus in chickens, transcription factors CTCF and USF1 bind to the boundary to mediate insulator function. Although the factors that bind insulators are well characterized, the mechanism by which these factors function to form a DNaseI hypersensitive site at the insulator element and separate distinct chromatin domains is not yet fully understood.

Proteins involved in insulator function in yeast

In yeast, *Saccharomyces cerevisiae*, we have identified a transfer RNA gene as the DNA element that blocks the spread of an inaccessible, transcriptionally silenced heterochromatin domain (Donze *et al*, 1999). The tRNA gene functions as an insulator through the transcription factors TFIIC and TFIIB, but not RNA polymerase III (Donze and Kamakaka, 2001). Furthermore, mutations in histone acetyltransferases, bromo-domain-containing proteins, chromatin remodellers and histone variants also affect insulation (Valenzuela and Kamakaka, 2006). The tRNA-bound transcription factors and histone acetylases function in parallel pathways to mediate robust insulation because loss of either partially affects insulation, whereas loss of both pathways leads to complete loss of insulation (Oki and Kamakaka, 2005).

DNA polymerases and the assembly of chromatin

In metazoans, most insulator-associated DNaseI hypersensitive sites are present throughout different stages of growth and cell division. This constitutive maintenance of DNaseI

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hypersensitive sites at insulators must, therefore, be directly or indirectly linked to replication-coupled chromatin assembly that occurs in every cell cycle, as cells grow and divide. Three DNA polymerases along with accessory proteins, such as PCNA and RFC (Waga and Stillman, 1998) are required for the duplication of the eukaryotic genome. Although DNA polymerase α is required to initiate replication, DNA polymerase ϵ primarily replicates the leading strand with DNA polymerase δ primarily replicating the lagging strand (Stillman, 2008).

All three DNA polymerases are multiprotein enzymes. DNA polymerase ϵ is comprised of four subunits—Pol2, Dpb2, Dpb3 and Dpb4. The N-terminal domain of Pol2, the catalytic subunit, is involved in DNA synthesis and its C-terminal domain interacts with Dpb2, Dpb3 and Dpb4 to form the holoenzyme (Dua *et al*, 2000; Ohya *et al*, 2000; Tsubota *et al*, 2006). Although Dpb3 is solely present in the DNA polymerase ϵ complex, Dpb4 is also found in the Isw2 chromatin-remodelling complex, suggesting links between replication and chromatin remodelling (Iida and Araki, 2004; McConnell *et al*, 2004; Tackett *et al*, 2005).

Chromatin assembly during S-phase is temporally coupled with replication. Nucleosomes are disrupted immediately upstream of the replication fork (within two nucleosomes) (McKnight and Miller, 1977) and re-form 400 bp downstream of the fork (Worcel *et al*, 1978; Annunziato *et al*, 1981; Gasser *et al*, 1996). Histone deposition and chromatin assembly use various protein chaperones and enzyme complexes. Newly synthesized histones are acetylated before their assembly into chromatin during S-phase: lysine residues 5 and 12 in histone H4 are acetylated by the Hat1–Hat2 complex (Parthun *et al*, 1996) and H3 is acetylated at lysine 56 and lysine 9 by the Rtt109–Vps75–Asf1 complex (Fillingham and Greenblatt, 2008).

The chromatin assembly factor CAF1 (Smith and Stillman, 1989) is involved in the replication-coupled histone deposition and binds newly synthesized histones acetylated on specific residues (H3K56 and H4K12) during S-phase (Masumoto *et al*, 2005).

Asf1 is a histone chaperone involved in chromatin assembly (Mousson *et al*, 2007). It is required for both, replication-coupled and replication-independent histone deposition (Green *et al*, 2005; Antczak *et al*, 2006) and is thought to present histones to various histone-modifying enzymes and to proteins involved in histone deposition and eviction from chromatin (Hake and Allis, 2006).

Following deposition of the histones in chromatin, the deposition-specific acetyl marks on histones H3 and H4 are removed (Jackson *et al*, 1976; Annunziato and Seale, 1983). H3 Ac-K9 and Ac-K56 are deacetylated by Rpd3 and Hst3/4, respectively, (Suka *et al*, 2001; Celic *et al*, 2006; Maas *et al*, 2006; Halder and Kamakaka, 2008) whereas H4 Ac-K12 is deacetylated by Rpd3 and Hos2 (Wang *et al*, 2002).

RSC-mediated remodelling at tRNA genes

The presence of a DNaseI hypersensitive site at most insulators implies not only the binding of a sequence-specific factor to DNA, but also the disruption of the regular nucleosome repeat along the DNA fibre, mediated by chromatin-remodelling factors. The chromatin state of genes is disrupted in every cell cycle as the replication fork passes, but most insulator-associated DNaseI hypersensitive sites are constitu-

tively present through different stages of growth and cell division, suggesting that these sites re-form soon after replication. Indeed, it has been shown that metazoan DNaseI hypersensitive sites are disrupted as the replication fork passes through and re-form soon after histone deposition (Solomon and Varshavsky, 1987).

Most tRNA genes reside in nucleosome-free regions (Yuan *et al*, 2005). Nucleosome depletion is likely to be achieved by chromatin remodellers and two remodellers, Isw2 and Rsc, localize near euchromatic tRNA genes (Damelin *et al*, 2002; Ng *et al*, 2002; Bachman *et al*, 2005; Gelbart *et al*, 2005). Isw2 mediates chromatin remodelling principally by nucleosome sliding, whereas Rsc mediates remodelling through nucleosome eviction (Saha *et al*, 2006). The recruitment of chromatin remodellers to specific DNA sequences is achieved in part by interactions with sequence-specific DNA binding proteins and in part by the interactions of remodellers with specifically modified histones (Saha *et al*, 2006). This principle is likely to be true for the targeting of Rsc to tRNA genes as well. The Rsc complex associates with RNA polymerase III factors and mutations in Rsc4 reduce transcription by RNA polymerase III (Soutourina *et al*, 2006), suggesting that interactions between RNA polymerase III and Rsc might be partially responsible for targeting Rsc to tRNA genes.

The data also suggest a link between histone acetylation and Rsc-mediated remodelling. NuA4 and SAGA, which acetylate histones, stimulate the recruitment of Rsc to nucleosomes *in vitro* (Kasten *et al*, 2004; Carey *et al*, 2006). Studies have also shown that improved remodelling by Rsc is due, in part, to its increased affinity for nucleosomes acetylated on histone H3 and in part due to increased efficiency of histone eviction when the histones in nucleosomes are acetylated (Ferreira *et al*, 2007).

In this study, we show that tRNA insulator is present in a 700-bp region of DNA that is depleted of histones. This specialized chromatin state is maintained by tRNA-bound factors in conjunction with accessory proteins. We show that tRNA-bound transcription factors helped in the recruitment of the chromatin remodeller Rsc to the tRNA to form the histone-depleted region. Furthermore, DNA polymerase ϵ and the histone H3 acetylase, Rtt109, were required for the formation/maintenance of this histone-depleted region and for the recruitment of the transcription factors to the tRNA. We present a model to explain these findings and suggest that these events occur during the S-phase, as newly replicated DNA is packaged into nucleosomes and matures into transcriptionally active and inactive chromatin domains.

Results

To determine the molecular mechanism by which tRNA functions to restrict the spread of silencing, we analysed the chromatin structure of the native boundary, and the function of individual mutants in establishing/maintaining this structure and in transcription factor binding/stability.

A specialized chromatin structure at tRNA insulator

There are multiple copies of individual tRNA genes in the yeast genome and the tRNA gene at the HMR boundary has sequence homologues located elsewhere in the genome. Although histone modifications and transcription factors have been mapped across the entire yeast genome, due to

the repetitive nature of tRNA genes the mapping data for these regions are usually repeat masked and unavailable. We, therefore, decided to quantitatively map the distribution of histones and specific transcription factors at the *HMR* tRNA boundary. Although we could not analyse the highly repetitive TY1 LTR elements located near tRNA insulator, we initially devised probes that would allow us to quantitatively map the distribution of transcription factors and histone modifications around this insulator.

We began by mapping the distribution of the histone H3 across a ~ 1 kb region using ChIP and real-time PCR. For this analysis, all primer pairs were initially tested for: (i) their uniqueness in the genome, (ii) their efficiencies of amplification and (iii) the absence of any potential primer-dimer artifacts. Only primer pairs with equivalent amplification efficiencies for genomic DNA were used. We also used two control probes for all our qChIP analyses—one located 500 bp from telomere 6R and the second probe located 7.5 kb from telomere 6R in an intergenic region. Histone H3 is present at both these loci and we normalized all our values to the 7.5 kb TEL6R probe (Figure 1A). The distribution of H3 measured relative to the telomere probe showed that the *HMR-I* silencer and tRNA were depleted for the core histone and presumably for the entire nucleosome (probes I and III). This is to be expected given that these two elements are bound by transcription factors that exclude nucleosomes. Interestingly, we also observed a depletion of histone H3 on either side of the

tRNA gene, across a 700 bp region of DNA that is immediately flanked by the two TY1 LTR elements (probes II, IV and V). We did not observe complete absence of histone H3 but merely a depletion of this histone across this region. Although histone H3 was depleted in the region flanking the tRNA, we saw less depletion at the 3' end of the *GIT1* gene (probe VI).

Most gene promoters in yeast lack one nucleosome, whereas our data suggested that multiple nucleosomes were missing at this tRNA gene. To confirm our analyses, we decided to redo our qChIP using cross-linked chromatin that had been fragmented to mononucleosomes using micrococcal nuclease. We mapped the histones using more closely spaced primer pairs that spanned the entire region. These data (Figure 1B) show a depletion of histone H3 across an ~ 800 bp region centred on the tRNA gene, confirming our previous results using sonicated DNA (see Supplementary Figure 1 for average size of sonicated and micrococcal nuclease-digested DNA).

At this point, we are unable to determine whether the 700 bp H3-depleted region extends further into the TY1 LTRs or not. Given the average repeat length of nucleosomes in yeast (Thomas and Furber, 1976), these results suggest that approximately four to five nucleosomes were either rapidly turned over or altered in this region; generating a large discontinuity in the chromatin fibre, thereby resulting in the formation of a hypersensitive site. For simplicity, we will refer to this as a histone-depleted region.

Figure 1 Chromatin structure at the *HMR* tRNA. **(A)** Histone H3 at the tRNA. The histogram represents qChIP data, following sonication, for studying the distribution of histone H3 from a wild-type or an *HMR* tRNA Δ strain. Values for ΔC_t were derived from the difference in the real-time amplification of equal amounts of immunoprecipitated and input DNA. Standard error values were calculated using data from at least four immunoprecipitation reactions with at least two independently cross-linked chromatin samples. Data for the histogram were derived by normalizing ΔC_t values for the various amplicons against the ΔC_t value for the 7.5 kb TEL6R amplicon (VIII). **(B)** The histogram represents qChIP data following micrococcal nuclease digestion to study the distribution of histone H3 from a wild-type or an *HMR* tRNA Δ strain. **(C, D)** Sir3 and H4 Ac-K16 distributions are anti-correlative. A histogram representing qChIP data to study the distribution of Sir3 (B) or H4 Ac-K16 (C). Data for the histogram in (C) were normalized as in Figure 1A, whereas the data for (D) were normalized twice: first to amplicon VIII and then to the histone H3 levels at each amplicon. Owing to this, we are unable to provide the standard error values. **(E)** H3 Ac-K56 was found at sites of Sir3 occupancy. A histogram representing qChIP data to study the distribution of H3 Ac-K56 around the tRNA from a wild-type or an *HMR* tRNA Δ strain. Data were normalized as in Figure 1D.

Figure 1 Continued.

tRNA is necessary for the formation of the large nucleosome-free region at the insulator

The presence of a large histone-depleted region was interesting. At RNA polymerase II-transcribed genes, there is a single nucleosome that is absent/alters at the 5' end (Yuan *et al*, 2005). Likewise, it is possible that tRNA was responsible for the eviction/alteration of a single nucleosome, whereas the flanking sequences bound by other transcription factors were responsible for the removal of additional nucleosomes. We, therefore, determined whether removal of the tRNA-bound factors was sufficient for the formation/maintenance of this large histone-depleted region on either side of the

gene. The promoter for all tRNA genes resides within the gene and mutations of this internal promoter result in a loss of tRNA transcription (Geiduschek and Kassavetis, 2001). We analysed the distribution of histone H3, using qChIP, in a strain deleted for the internal promoter for the tRNA gene. An analysis of histone H3 showed a dramatic increase in H3 occupancy around the tRNA gene and extending to the 3' end of the downstream TY1 LTR, but not at the *HMR-I* silencer (Figure 1A). We confirmed this analysis by qChIP of chromatin that had been fragmented using micrococcal nuclease instead of sonication. These data (Figure 1B) corroborated the previous observation that tRNA gene was responsible for

maintaining the large 800 bp region of histone depletion. Thus in the absence of the tRNA-bound transcription factors, histone occupancy increases across the entire region. The observation that this increase was not restricted to the tRNA gene but was present across the entire 700 bp region suggests that the tRNA gene-bound transcription factors prevent stable histone binding and presumably nucleosome occupancy over a large region of the DNA.

Histone modification profiles at the tRNA insulator

Silenced chromatin is restricted from spreading, in part, by the action of histone acetylases. These enzymes function to restrict the spread of heterochromatin, most probably, by modifying core histones in direct competition to the spreading Sir proteins (Kimura *et al*, 2002; Suka *et al*, 2002; Oki and Kamakaka, 2005). We mapped the modification state of the histones around the tRNA insulator relative to H3 occupancy. The distribution of the Sir proteins and histone H4 acetylated on K16 showed a clear anti-correlation between these two proteins at the tRNA boundary (Figure 1C and D, probes III and IV). There is large enrichment of Sir3 at the *HMR-I* silencer, and at the region between the silencer and tRNA gene with a sharp decline outside of tRNA gene. In contrast, there are low levels of H4 Ac-K16 at regions occupied by Sir3 and a sharp increase outside the silenced domain.

The acetylation of histone H3 at K56 has been suggested to affect chromatin compaction and gene regulation and it has been shown that K56 acetylation affects silencing (Xu *et al*, 2007). Next, we analysed the distribution of histone H3 modified on K56 relative to H3 (Figure 1E). Although acetylated K56 was reduced at *HMR-I*, there was significant acetylation of this histone immediately outside the silencers showing that H3 Ac-K56 was present in regions occupied by Sir3 (see probes II and III). This region of co-occupancy of Sir3 and H3 Ac-K56 was phenotypically repressed based on the expression of the *MAT α 1* reporter gene (Supplementary Figure 2). These data suggest that either the Sir proteins can interact with chromatin-containing H3 Ac-K56, or there are two populations of cells: one active and associated with H3 Ac-K56, and a second silent population reflecting Sir protein occupancy.

The deletion of the tRNA insulator results in the spread of Sir proteins into neighbouring heterochromatin (Figure 1C). We measured the histone modifications in strains lacking a functional tRNA, and found that the loss of tRNA results in a significant loss of histone acetylation at H4 K16 and H3 K56 beyond the tRNA insulator in neighbouring euchromatin (Figure 1D and E).

Synthetic genetic array screen for mutants that affect the tRNA insulator

To identify proteins that function through the tRNA gene to block silencing, we used the synthetic genetic array (SGA) screen methodology (Tong *et al*, 2001) to identify deletions in all non-essential *S. cerevisiae* genes that impaired the tRNA insulator (Figure 2). We built a yeast strain with a modified *HMR* locus (*HMR::URA3*), in which the *URA3* coding sequence was under the control of *MAT α 1* promoter. A tRNA insulator was inserted between the *HMR-E* silencer and *URA3* gene. A NatMX gene located centromere proximal to the *HMR* locus marked the modified locus. This strain also carried a

HIS3 gene transcribed from an *MFA1* promoter thus allowing us to select for *MAT α* haploid cells by growth on plates lacking histidine. This *MAT α* strain was crossed with *MAT α* KanMX deletion collection and *MAT α* haploid spores that were KanMX–NatMX positive were generated by growth on plates containing G418, nourseothricin and lacking histidine. The haploid colonies generated by this selection were then screened for the expression of the *URA3* reporter gene at *HMR* by growth on YMD plates containing 5-FOA.

In strains, in which the insulator was active, silencing emanating from the silencer would be blocked thus keeping the *URA3* reporter on and the OMP decarboxylase activity of the *URA3* gene would render these strains inviable on plates containing 5-FOA. However, if tRNA barrier were inactivated due to the loss of a gene important for its function, then silencing would spread to the *URA3* reporter allowing the strain to grow on media containing 5-FOA.

Using this assay, we screened over 4400 gene deletions and scored each deletion over a range of 0–3, with 0 denoting no loss of activity and 3 denoting a complete loss of barrier activity. Using these criteria, we identified 377 mutants that had significantly or completely lost tRNA-mediated boundary function. As we identified greater than 5% of all non-essential genes that directly or indirectly affected expression of the *URA3* reporter, we decided to carry out the entire SGA screen using a different reporter to eliminate mutants that specifically affected expression of the reporter gene.

In the second screen, we used *MAT α 2* gene as a reporter in place of *MAT α 1* gene (Figure 2). This gene is normally repressed at the *HMR* locus but repression is lost when tRNA barrier is inserted between the silencer and reporter genes. However, inactivation of the barrier in a deletion mutant will cause the gene to be repressed allowing barrier activity to be assayed by monitoring *MAT α 2* expression.

We generated a strain in which the *MAT α* locus was marked by inserting *URA3* adjacent to *MAT α* (*MAT α -URA3*). We also modified the *HMR* locus so that it contained *MAT α 2* with the tRNA boundary between the *HMR-E* silencer and reporter gene. The modified locus at *HMR* was also marked with NatMX. This strain was crossed with approximately 4400 non-essential *MAT α* deletion (KanMX) strains, and following sporulation, we selected *MAT α* haploids carrying the gene deletion and the *HMR*-insulator–*MAT α 2* reporter by selecting for NatMX, KanMX and FOA resistant (*MAT α*) strains. These strains were assayed for the loss of barrier activity using a mating assay with a *MAT α* tester. If the gene knockout did not affect barrier activity, then the strain would remain a non-mater and conversely, if barrier activity was lost in the mutant, then the silent domain would spread to repress the *MAT α 2* gene and the strain would mate.

We scored each deletion over a range of 0–3 with 0 denoting no loss of activity and 3 denoting a complete loss of barrier activity. We identified 135 mutants in this screen that significantly affected the tRNA boundary function, resulting in the silencing of the *MAT α 2* reporter gene.

We collated all the mutants in which the tRNA boundary had been adversely affected from the two independent SGA screens and identified ones that affected insulation in both screens. Approximately one-third of the genes identified (45) were unknown ORFs, whereas another one-third (41) were mutants for cell wall and cell membrane components or affected cellular transport and were most probably scored

Figure 2 A schematic representation of the two SGA screens and the modified *HMR* locus.

positive because of the reporter assays used. We have not analysed these any further. Approximately 10% (12) of the mutants were involved in chromatin function and another 10% (14) were involved in protein modification and genome integrity, whereas the remaining nine were involved in other diverse functions.

Various chromatin modifying and remodelling factors affect *tRNA* insulator

We next confirmed the effects of the identified mutants on boundary function using a wild-type *HMR* locus as well as three specific mutants for the insulator at *HMR*. We generated four isogenic *MAT α* strains that differed only at the *HMR* boundary. In all four strains, the native promoter of the *MATa1* gene at *HMR* was deleted (*HMRa Δ p*) and a functionally active *MATa1* gene under its own promoter was inserted in the intergenic region between the TY1 LTR and the *GIT1* gene, such that *MATa1* promoter was proximal to *HMR-I* silencer. In the wild-type strain, the *tRNA* boundary and the flanking TY1 LTR elements were intact and blocked the spread of silencing. In the barrier-deleted strain, the *tRNA*

as well as the two TY1 LTR elements were deleted and replaced with an equivalent length of DNA from a pRS vector. In the *tRNA*-deleted strain, a region containing the *tRNA* gene was deleted whereas in the LTR-deleted strain the two flanking TY1 LTR elements were replaced with equivalent lengths of pRS vector DNA leaving the *tRNA* gene and 100 bp of flanking DNA intact (Figure 3A).

The four *MAT α* strains were monitored for expression of the *MATa1* reporter gene using mating assays. *MAT α* strains with an active copy of the *MATa1* gene at *HMR* are phenotypically diploid and will not mate with a tester *MATa* strain to form diploids and are therefore unable to grow on diploid selective media. When the *MATa1* reporter is repressed, the strains mate with the tester strain, form diploids and grow on selective plates. Several groups have used *URA3* or *ADE2* to monitor insulation using colour assays or by growth on plates containing 5-FOA (Bi, 2002; Jambunathan *et al*, 2005; Tompa and Madhani, 2007). These reporters are excellent for monitoring loss of silencing (van Leeuwen and Gottschling, 2002; Valenzuela *et al*, 2006) but are not very sensitive for monitoring the spread

Figure 3 Chromatin modifiers and remodellers affected the *HMR* tRNA boundary. (A) A phenotypic assay for the *HMR* tRNA boundary. The *HMR* tRNA boundary mating assay shown with wild-type cells carrying either (I) the wild type boundary, (II) a full deletion of the boundary, (III) a 70-bp tRNA deletion of the boundary or (IV) the 70 bp tRNA with 100 bp flanks. Tenfold serial dilutions of overnight cultures with a starting A_{600} of 1.0 were spotted on a fully supplemented minimal medium (growth control) or minimal medium with the mating tester lawn (mating). (B) The *HMR* tRNA boundary mating assay shown with isogenic wild-type, *eaf3Δ* or *sas2Δ* cells. (C) Differential effects of remodeller mutants on the *HMR* tRNA boundary. The *HMR* tRNA boundary mating assay shown with isogenic wild-type, *rsc2Δ*, *isw2Δ* or *swr1Δ* cells. (D) Mutants for DNA polymerase ϵ and, to a significantly lesser degree, *Isw2* compromised the *HMR* tRNA boundary. The *HMR* tRNA boundary mating assay shown with isogenic wild-type, *dpb3Δ*, *dpb4Δ* or *dls1Δ* cells. (E) Mutants in all three subunits of the Rtt109 acetylase complex weakened the *HMR* tRNA boundary. The *HMR* tRNA boundary mating assay shown with isogenic wild-type, *asf1Δ*, *rtt109Δ* or *vps75Δ* cells. (F) Mutants in H3 Ac-K56 Histone deacetylases compromised the *HMR* tRNA boundary. The *HMR* tRNA boundary mating assay shown with isogenic wild-type or *hst3Δ-hst4Δ* cells.

Figure 3 Continued.

of the repressed state following loss of insulator function because these assays depend on stable silencing of the reporter gene for many generations. The use of *MATa1* as a reporter provides an extremely sensitive and 'acute' assay that can detect repression of the *MATa1* reporter gene over a 10^5 -fold range and repression can be easily quantified using serial dilutions.

In a wild-type strain, the *MATa1* gene residing outside the *HMR* boundary is fully active and this strain is unable to mate and generate diploid colonies on selective YMD plates. In the barrier- and tRNA-deleted strains, there is no apparent insulation and the *MATa1* gene is almost fully repressed whereas deletion of the TY1 LTRs leads to a minor reduction in insulation function (Figure 3A).

These four reporter strains were systematically crossed to various deletion mutants of interest and the mutants were then assayed for their effects on boundary function. Mutants for SAS-I, NuA4 and SAGA histone acetylases affect tRNA boundary function (Oki and Kamakaka, 2005) and were also identified in our SGA screen. We re-tested some of these mutants in the constructs described above and consistent with previous data, these acetylases were required for insulation (Figure 3B). The loss of Sas2 or Eaf3 led to a significant loss of insulation as manifested by repression of the reporter gene.

We identified an *isw2Δ* mutant as adversely affecting insulation and therefore analysed the effect of mutations in various chromatin remodellers on boundary activity. We analysed mutants for Isw2, Rsc2 and Swr1 (Figure 3C). Interestingly, *isw2Δ* and *rsc2Δ* mutants affected boundary activity to different degrees: *isw2Δ* had a weak effect on the tRNA boundary, whereas *rsc2Δ* mutants almost completely lost tRNA boundary function. However, Swr1 (Figure 3C) and Htz1 mutants (data not shown) had no effect on tRNA boundary activity as measured by this mating assay.

Another gene identified in the SGA screens was *dpb4Δ*. Dpb4 is found in two distinct protein complexes in yeast cells: one is the Isw2 complex and the second is the DNA polymerase ϵ protein complex. Dpb3 and Dls1, however, are unique to the DNA polymerase ϵ complex and the Isw2 complex, respectively. We, therefore, analysed the effects of mutations in Dpb3, Dpb4 and Dls1 to determine which of these complexes contributed to the boundary function (Figure 3D). Our mating assays showed that loss of Dpb3 or Dpb4 profoundly diminished the tRNA boundary, whereas the contribution of Dls1 to the boundary was relatively small. This result suggests that a fully functional DNA polymerase ϵ was required for a robust tRNA boundary. Rrm3 is a DNA helicase required for DNA replication. Consistent with the observation that *dpb3* mutants affected insulation, we also

found a significant effect on insulator function in an *rrm3* mutant but not mutants in other helicases, such as *Sgs1*, *Srs2* and *Slx4* (data not shown). *Esc2* also does not affect tRNA-mediated insulation.

Finally, we isolated *vps75 Δ* as a mutant that adversely affected the tRNA boundary. *Vps75* is part of the Rtt109 acetyltransferase complex that is involved in acetylating free histones during replication- and repair-coupled chromatin assembly. We analysed mutations in Rtt109, *Vps75* and *Asf1*, the three subunits of the Rtt109 acetyltransferase complex (Figure 3E). In all three mutants, insulation was severely compromised. For further experiments, we focused on the *rtt109 Δ* mutant for two reasons: *vps75 Δ* cells are extremely sick and are very prone to acquiring growth suppressors (data not shown), and *Asf1* is present in multiple protein complexes with diverse functions whereas Rtt109 is the catalytic subunit of this complex.

As the pair of sirtuin histone deacetylases Hst3–Hst4 deacetylate residues acetylated by Rtt109, whereas Sin3–Rpd3 complex deacetylates H4 K16, we also analysed tRNA insulator function in an *hst3–hst4* double mutant and found that loss of this histone deacetylase also weakened insulation (Figure 3F), as did a mutation in *Sin3* (data not shown). We have not determined whether these effects are attributable to the direct loss of boundary function or are indirect effects of Sir protein levels or localization in the nucleus. It is quite plausible that a cycle of acetylation and deacetylation mediated by Rtt109 and Hst3–Hst4 complex is necessary for nucleosome remodelling and boundary function.

Effect of replication proteins, chromatin modifiers and remodellers on nucleosome depletion

Once we had shown that the tRNA formed a specialized chromatin structure depleted in four nucleosomes, we next turned our attention to the various mutants that were characterized in our boundary assays. We were interested in understanding how these proteins might contribute to insulation. One mechanism could be that these factors are required to form or stabilize the specialized chromatin structure at the tRNA. We carried out qChIP to study the distribution of histone H3 around the tRNA in various mutant strains. Our data (Figure 4) clearly show that the loss of *Isw2*, *Sas2* or *Eaf3* had almost no effect on the histone levels at the tRNA boundary. H3 was depleted at the tRNA in all these strains to the same extent as in the wild-type strain (Figure 4A–C, probes II, III and IV). In contrast, loss of *Dpb3*, *Rsc2* or Rtt109 resulted in varying degrees of increased H3 occupancy. The loss of *Rsc2* or Rtt109 resulted in a fourfold increase of H3 levels at the tRNA but not in the flanking regions (Figure 4D and E compare probes II, III and IV). Similarly, the loss of *Dpb3* resulted in a modest but reproducible twofold increase in H3 levels at the tRNA (Figure 4F). These results suggest that the three protein complexes independently or together function to destabilize nucleosomes thus creating the specialized chromatin structure at the tRNA.

***Dpb3*, *Rsc2* and *Rtt109* function in the same genetic pathway to evict nucleosomes**

To determine whether these factors function in the same or in separate pathways to realize a robust tRNA barrier, we first analysed the phenotypes of various double mutants in our *MATa1* boundary mating assays. We analysed *dpb3 Δ –rtt109 Δ*

and *dpb3 Δ –rsc2 Δ* for their effects on the tRNA boundary. The phenotypic analysis indicated that the double mutants showed approximately the same degree of loss of boundary function as shown by *rsc2 Δ* or *rtt109 Δ* single mutant, suggesting that they function in the same genetic pathway (Figure 5A). We were unable to carry out a double mutant analysis using an *rsc2 Δ –rtt109 Δ* strain due to the presence of very severe growth defect in these mutants.

We next analysed the effects of the double mutant on histone depletion at the tRNA. Although *dpb3 Δ* had a small effect on H3 levels at the tRNA and *rtt109 Δ* had a larger effect, H3 levels in the double mutant (*dpb3 Δ –rtt109 Δ*) were same as those for *rtt109* single mutant (Figure 5B). Similarly, H3 levels in the *dpb3 Δ –rsc2 Δ* strain were the same as those observed for *rsc2 Δ* alone (Figure 5C). These results collectively argue that the three complexes Rsc, Rtt109 and DNA polymerase ϵ probably function in the same genetic pathway to promote the tRNA boundary.

The boundary is not formed during replication pausing

Identifying a role for a replication protein in chromatin insulation was intriguing and raised the question of how *Dpb3* might function. Transcription factors bound to tRNA genes cause replicating polymerases to pause (Deshpande and Newlon, 1996) and recovery from a replication pause site requires a fully functional DNA polymerase ϵ as well as Rtt109 (Han *et al*, 2007; Celic *et al*, 2008; Roberts *et al*, 2008). We reasoned that the lack of *Dpb3* or Rtt109 might result in alterations in replication pausing at the tRNA causing mis-assembly of nucleosomes, thereby weakening the boundary. To test this model, we investigated replication pausing at the tRNA using two-dimensional gels (Brewer and Fangman, 1991). We probed the gels with a restriction fragment containing the tRNA gene. This region lacks an origin of replication and we simply monitored the Y-arc. Replication pausing should result in the presence of a focal spot on the Y-arc. Our data show that the tRNA boundary at *HMR* was not very robust at blocking the movement of the replication fork (Figure 6; wt panel), as we did not observe any significant spot on the Y-arc. Furthermore, mutations in the tRNA, *Dpb3*, Rtt109 or *Rsc2* had no discernible effect on replication pausing. The evidence from this set of experiments argues against a model that recovery from a paused replication fork marks the genesis of a boundary though it is still possible that slower fork progression across this region without appreciable pausing could be involved in establishing the boundary.

Stable recruitment of *Rsc2* to the insulator requires the tRNA and *Rtt109*

To connect the dots between the functions these complexes have in generating a specialized chromatin structure at the tRNA, we mapped Rtt109, *Dpb3* and *Rsc2* across the tRNA. Our qChIP experiments using a TAP-tagged Rtt109 strain were not successful in that we were unable to observe any significant enrichment of Rtt109-immunoprecipitated DNA over input DNA at any of the loci we tested. This is consistent with recent studies on Rtt109 (Selth *et al*, 2009). This is not too surprising because there is no evidence to date suggesting Rtt109 being directly associated with chromatin. We observed a slight enrichment of *Dpb3* at the tRNA (data not shown) but

currently we are not sure whether this effect is significant or not.

We next carried out a qChIP with TAP-tagged Rsc2 at the boundary (Figure 7A) and plotted the data relative to *HMR-I*, in which we observed a modest enrichment of Rsc2. We observed enrichment of the remodeller specifically in the region centred on the tRNA insulator (see probes II, III and

IV). This binding was reduced in a strain in which the tRNA gene was deleted, suggesting that the tRNA transcription factors were important for the recruitment/stabilization of Rsc2 to the tRNA boundary. This is consistent with previous reports showing that Rsc directly interacts with the RNA polymerase III transcription machinery *in vitro* (Soutourina *et al*, 2006).

Figure 4 Only some chromatin modifiers and remodellers influenced the histone-depleted *HMR* tRNA region. (A–C) Histone H3 occupancy at the *HMR* tRNA boundary was unaltered in *sas2Δ*, *isw2Δ* and *eaf3Δ* mutants. Histograms representing qChIP data to study the distribution of histone H3 from a (A) *sas2Δ*, (B) *isw2Δ* or (C) an *eaf3Δ* strain. The H3 distribution in a wild-type strain from Figure 1A is plotted alongside for ease of comparison. (D–F): Histone H3 occupancy at the *HMR* tRNA increased in *rtt109Δ*, *rsc2Δ* and *dpb3Δ* mutants. Histograms representing qChIP data to study the distribution of histone H3 from (D) an *rtt109Δ*, (E) an *rsc2Δ* or (F) a *dpb3Δ* strain. The H3 distribution in a wild-type strain from Figure 1A is plotted alongside for ease of comparison.

Figure 4 Continued.

In vitro data also suggest a link between histone acetylation and remodelling. NuA4 and SAGA, which acetylate histones, stimulate the recruitment of RSC to nucleosomes *in vitro* (Carey *et al*, 2006). Studies have also shown that remodelling by Rsc is increased in nucleosomes with acetylated histone H3 but not H4 (Kasten *et al*, 2004; Ferreira *et al*, 2007). We, therefore, also measured the binding of TAP-tagged Rsc2 at the boundary in a strain deleted for Rtt109. We found that Rsc recruitment was slightly reduced at probes flanking the tRNA but not at the tRNA itself (Figure 7B, see probes II, III and IV).

***Rsc2* and *Rtt109* are required for the stable recruitment of tRNA transcription factors to the insulator**

If the tRNA-bound factors and K56-acetylated histones are important for the recruitment of the Rsc complex to the boundary, we inquired whether the loss of either Rsc2 or Rtt109 affected the binding of the RNA polymerase III transcription factors (Figure 7C). Bdp1 is a part of the TFIIB complex, which binds upstream of the tRNA gene and is exclusively required for RNA polymerase III transcribed genes. We generated a tagged Bdp1 allele in a wild-type background as well as in strains lacking Rsc2 or Rtt109. qChIP was used to measure the binding of Bdp1 across the tRNA boundary. In the wild-type strain, we observed binding of Bdp1 upstream (Figure 7C, probes II and III) but not downstream of the tRNA gene as would be expected for this protein. In strains mutated for Rsc2 or Rtt109, we observed a small but reproducible decrease in the levels of Bdp1 at the tRNA. This result suggests that the histone modifier and chromatin remodeller are required for the optimal recruitment/binding of the RNA polymerase III machinery to the tRNA insulator and are consistent with recently published data describing a function for Rsc in the

recruitment of the RNA polymerase III machinery (Parnell *et al*, 2008).

Discussion

Insulators are DNA elements that partition the epigenome into transcriptionally active and silenced domains, and the best-characterized insulators seem to function constitutively during growth and cell division (Valenzuela and Kamakaka, 2006). Insulators in yeast and chickens use various sequence-specific transcription factors and histone-modifying complexes to mediate insulation although the exact mechanism by which these elements function is not well understood. The metazoan chicken β -globin HS4 insulator is characterized by constitutive DNaseI hypersensitive sites that bind the transcription factors CTCF and USF. CTCF functions in enhancer blocking, whereas USF1 mediates insulation by recruiting specific histone acetylases—PCAF, CBP, and p300—which acetylate histones in the vicinity of the insulator and block the spread of neighbouring heterochromatin into the active β -globin domain in erythroid cells (Bell *et al*, 1999; West *et al*, 2004).

In yeast, the *HMR*-silenced domain is restricted from spreading by a tRNA gene through its transcription factor TFIIC. Molecular genetic analyses indicate that similar to the chicken globin insulator, the histone acetylases, SAGA, NuA4 and SAS-I function to restrict the spread of silenced chromatin (Donze and Kamakaka, 2001) but unlike the chicken insulator, the yeast histone acetylases are not preferentially localized to the tRNA boundary (Oki and Kamakaka, 2005). Current data indicate that the silenced chromatin at *HMR* is blocked from spreading by two independent mechanisms: by the presence of RNA polymerase III transcription complexes bound to the tRNA promoter and by competition from

Figure 5 Genetic and molecular pathways to the *HMR* tRNA boundary. (A) Double mutants for *dpb3* Δ and *rsc2* Δ or *dpb3* Δ and *rtt109* Δ weakened the *HMR* tRNA boundary to the same extent as an *rsc2* or *rtt109* single mutant, respectively. The *HMR* tRNA boundary mating assay shown with isogenic wild-type, *dpb3* Δ , *rsc2* Δ , *rsc2* Δ *dpb3* Δ , *rtt109* Δ or *rtt109* Δ -*dpb3* Δ cells carrying either (I) the wild-type boundary or (II) the 70 bp tRNA with 100 bp flanks. (B, C) Histone H3 occupancy at the *HMR* tRNA boundary in *dpb3* Δ -*rtt109* Δ or *dpb3* Δ -*rsc2* Δ double mutants was unchanged from that of an *rtt109* Δ or *rsc2* Δ single mutant, respectively. Histograms representing qChIP data to study the distribution of histone H3 from a (C) *dpb3* Δ -*rtt109* Δ or (D) *dpb3* Δ -*rsc2* Δ strain. The H3 distribution in the single mutants from Figure 4D–F is plotted alongside for ease of comparison.

histone acetylases SAS-I, SAGA and NuA4 that counteract the Sir2 histone deacetylase activity present in silenced chromatin. Changes in the relative local concentration of repressors or acetylases affect this equilibrium, thereby affecting the extent to which silenced chromatin spreads. Increased expression of Sir proteins or increased recruitment and/or retention at silenced loci will enhance the spread of silenced chromatin at that locus. In contrast to the competition mode

of insulation by the acetylases, the exact mechanism by which the tRNA-bound transcription factors function in insulation is not yet understood.

A specialized chromatin structure marks the tRNA insulator

RNA polymerase III factors act as insulators in numerous organisms (Haldar and Kamakaka, 2006) and therefore

Figure 6 Replication fork pausing at the *HMR* tRNA and insulation were not linked.

understanding how the tRNA helps establish a heterochromatin boundary is important. One key finding of this study is that the tRNA adjacent to *HMR* mediated the disruption/destabilization of a large region of chromatin encompassing at least 700 bp and maybe more. A recent report has found that tRNA genes located in euchromatin affect nucleosome density in their immediate vicinity (Parnell *et al*, 2008) and our results were consistent with these observations. The size of the histone-depleted region at the tRNA insulator suggests that four to five nucleosomes were disrupted, altered or rendered unstable in some manner. We do not believe that this region is completely devoid of nucleosomes, as we observed a reduction but not a complete absence of histone H3. All tRNA genes have internal promoters that bind the transcription factor TFIIC. We have shown that deletion of the TFIIC binding site within the tRNA gene led to the stabilization/re-formation of nucleosomes across this region, suggesting that TFIIC had a function in organizing this large altered chromatin structure.

Recent ChIP-Seq data (Mavrich *et al*, 2008) indicate positioned nucleosomes downstream of the tRNA insulator. However, the number of reads in this region is smaller relative to other regions on this chromosome, suggesting that these nucleosomes may be unstable or absent in some cells in the population. Several studies have shown that nucleosomes near tRNA genes are in a constant flux and the histones in these nucleosomes rapidly turnover (Dion *et al*, 2007; Rufiange *et al*, 2007; Kaplan *et al*, 2008). It is, therefore, possible that the histone depletion that we observe is a manifestation of rapid histone turnover of positioned nucleosomes and future experiments should help clarify this.

Rsc and insulation

Chromatin remodellers are required for nucleosome sliding, histone eviction and the exchange of histone variants. We tested chromatin remodellers that affect all three processes.

Although Isw2 is present at tRNA genes (Bachman *et al*, 2005; Gelbart *et al*, 2005), the *isw2Δ* mutant had no significant effect on H3 distribution around the tRNA gene and it is not clear whether its role in insulation is direct or indirect. Swr1 is a histone remodeller involved in exchanging histone H2A with Htz1. Htz1 has been shown to restrict silenced chromatin (Meneghini *et al*, 2003) and localizes adjacent to histone-depleted regions (Raisner *et al*, 2005), but we did not observe any role for Htz1 or Swr1, in insulation at *HMR*. The reason for this discrepancy is currently unclear.

The Rsc complex had a significant, direct function in restricting the spread of silencing. The loss of Rsc2 phenotypically impaired the insulator and increased histone localization at the tRNA, which is consistent with recent observations showing that histone eviction around euchromatic tRNA genes is reduced in Rsc mutants (Parnell *et al*, 2008). We also showed that the tRNA gene was required to recruit/stabilize the Rsc remodelling complex to the insulator. This correlates well with the genome-wide distribution data showing that a large number of Rsc targets are tRNA genes (Damelin *et al*, 2002; Ng *et al*, 2002). Furthermore, studies have shown that Rsc interacts with subunits of the RNA polymerase III machinery (Soutourina *et al*, 2006), suggesting that the recruitment/stabilization of Rsc to the insulator could be through its physical interactions with this machinery.

We also found that a mutation in Rtt109 modestly reduced the recruitment of Rsc around the insulator. Rsc has been shown to interact with acetylated histones and evicts acetylated histones more efficiently than unacetylated ones (Kasten *et al*, 2004; Carey *et al*, 2006; Ferreira *et al*, 2007; VanDemark *et al*, 2007). Therefore, the presence of H3 Ac-K56 in the vicinity of the tRNA gene together with the RNA polymerase III machinery could help recruit/stabilize Rsc to the insulator. Vps75, which is a member of the Rtt109 complex also preferentially localizes to tRNA genes and may (Selth *et al*, 2009) and could aid in the recruitment of Rsc.

Given these observations, we were surprised to find that a mutation in Rsc2 led to a small reduction in the RNA polymerase III machinery at the tRNA insulator. This would suggest that Rsc-mediated histone eviction/remodelling of nucleosomes was required for the stable binding of the RNA polymerase III machinery. Although an intriguing result, it is consistent with recent data showing that tRNA transcription is attenuated in Rsc mutants (Soutourina *et al*, 2006; Parnell *et al*, 2008). The study by Parnell *et al*, also showed that Rsc is required to maintain a nucleosome-free region at tRNA genes and in its absence, tRNA genes are susceptible to be packaged into nucleosomes during replication-coupled chromatin assembly.

The conundrum of the co-dependency between Rsc and the polymerase III machinery can be resolved if one posits that the two function together aiding each other to allow: (i) stable binding to the tRNA and (ii) disrupting nucleosomes around the tRNA which further augments the binding thus self-reinforcing each other. This co-dependency is reminiscent of the situation at the *ARG1* promoter where Gcn4 helps recruit various remodellers and modifiers, and the recruitment of these factors is highly co-dependent (Govind *et al*, 2005, 2007).

Figure 7 tRNA transcription factors at *HMR* co-operated with a chromatin remodeller and a modifier to form a boundary. **(A)** The *HMR* tRNA was important for Rsc2 localization. Histograms representing qChIP data to study the distribution of Rsc2-TAP centred on the tRNA from a wild-type or an *HMR* tRNA Δ strain. **(B)** *Rtt109* was necessary for Rsc2 localization. Histograms representing qChIP data to study the distribution of Rsc2-TAP centred on the tRNA from a wild-type or an *rtt109* Δ strain. **(C)** Stable recruitment of the tRNA transcription factors required *Rsc2* and *Rtt109*. Histograms representing qChIP data to study the distribution of Bdp1-TAP centred on the tRNA from a wild-type, an *rsc2* Δ or an *rtt109* Δ strain.

Although Rsc markedly affected insulation, nucleosome and Bdp1 occupancy at the tRNA were only slightly reduced in an Rsc mutant. Owing to this, we are unable to unequivocally conclude that Rsc-mediated insulation is due, solely, to nucleosome eviction. It is entirely possible that part of the effect of Rsc on insulation is because of alterations in the distribution and spreading of Sir proteins in the nucleus.

DNA polymerase ϵ and *rtt109* affect insulation

What is the role of DNA polymerase ϵ in insulator function? It is possible that in the absence of Dpb3, histone deposition in chromatin is adversely affected. Replication accessory proteins such as PCNA mark newly synthesized DNA during S-phase (Shibahara and Stillman, 1999) and are used by chromatin assembly factors, such as CAF-1 and Asf1 to target

the newly replicated DNA, and assemble nucleosomes. We have observed a small enrichment of Dpb3 at the tRNA in asynchronously growing cells, and Dpb4 and Dpb3 localize to the tRNA insulator during S-phase (Tackett *et al*, 2005). It is, therefore, possible that these proteins mark the *HMR* tRNA gene after passage of the replication fork and these marks are subsequently recognized by other co-factors. Interestingly, mutant analysis of Dpb3 indicated that the histone fold domain of Dpb3, which binds replicated double-stranded DNA (Asturias *et al*, 2006), had an important function in insulation (ND unpublished results). The double-mutant analyses between Dpb3 and Rtt109 or Rsc2 show that phenotypically, *dpb3 Δ rtt109 Δ* and *dpb3 Δ -rsc2 Δ* mutants were as impaired in insulation as either single mutant, suggesting that they function in the same pathway. This would be consistent with the model that Dpb3 directly or indirectly helps mark the tRNA for chromatin remodelling and modifying enzymes that function in chromatin assembly and maturation.

We have shown that all three subunits of the Rtt109 complex affected the tRNA insulator, suggesting that K56 acetylation directly or indirectly affected the insulator. One possibility is that K56-acetylated histone H3 is a poor substrate for the Sir proteins to bind, and regions adjacent to silenced chromatin that are K56 acetylated would block the spread of silencing. To investigate this possibility, we mapped the distributions of Sir3, histone H3 acetylated on K56 and histone H4 acetylated on K16. Deacetylation of histone H4 at K16 by Sir2 is a pre-requisite for the spread of silenced chromatin, whereas acetylation of histone H4 on K16 by the SAS-I complex has been shown to be important for insulation. Consistent with these observations, we found a clear anti-correlation between Sir3 and H4K16 acetylation. Furthermore, deletion of Sas2 (Oki and Kamakaka, 2005) or the tRNA results in an increased spread of Sir proteins and a concomitant retraction of acetylated K16 in regions of the genome in which Sir3 spreads. In contrast to these observations, we found significant levels of H3 acetylated at K56 in regions in which Sir3 was also present. We found elevated levels of both Sir3 and acetylated K56 in the region between the *HMR-I* silencer and tRNA insulator. This region was transcriptionally repressed in a Sir-dependent manner arguing that the presence of acetylated K56 was not antithetical to Sir3 binding and repression. One possibility is that the two marks (Sir3 and acetylated K56) are present in two distinct cell populations. Alternatively, the Sir proteins can bind histone H3 acetylated on K56 and repress genes. We favour the latter scenario because the distribution patterns for K56 did not mirror those for K16, which would be expected if the first scenario were the case.

Although histone H3 K56 acetylation may not preclude Sir protein binding, mutations in Rtt109 did affect insulation. Multiple scenarios can be postulated to explain our results and they may not be mutually exclusive. One possibility is that in cells lacking Rtt109 (or Rsc2), there is a redistribution of Sir proteins in the nucleus, which might then result in an increased spread of silencing at *HMR*. This model probably explains the Sas2 mutant phenotype and could also explain the Rtt109 phenotype.

The promoters of RNA polymerase II- and polymerase III-transcribed genes are sites of acetylation (Kurdistani *et al*, 2004; Zhang *et al*, 2005) and high histone turnover (Dion *et al*, 2007; Rufiange *et al*, 2007), and Asf1 and K56

acetylation help create a histone-depleted region at promoters (Adkins *et al*, 2004; Kaplan *et al*, 2008). A high turnover outside *HMR-I* could prevent stable Sir association with nucleosomes. In this scenario, Rtt109 along with Hst3 and Hst4 would mark the tRNA gene by creating a region of high histone turnover. However, whether Rtt109 is targeted to tRNA genes through other proteins is not clear as we did not observe any localization of this protein to the tRNA. Although we could not ChIP Rtt109, Vps75 does localize to tRNA genes and could target K56 acetylation to the insulator (Selth *et al*, 2009).

An alternative possibility is that the region is nucleosome free as a consequence of tRNA transcription-mediated nucleosome eviction mediated by Asf1/Rtt109. Although the 70 bp tRNA gene is constitutively bound by transcription factors and is transcribed (Donze and Kamakaka, 2001), it is unlikely that tRNA transcription alone accounts for the large 700 bp histone-depleted region, especially because mutants that affect tRNA transcription do not affect insulation (Donze and Kamakaka, 2001).

Another model is that K56-acetylated histones, deposited during replication, help recruit/stabilize the RNA polymerase III transcription factor binding to the tRNA gene, which then establishes the boundary between heterochromatin and euchromatin. Consistent with this, we showed a reduction in the recruitment of RNA polymerase III transcription factor Bdp1 to the tRNA and a concomitant increase in the spread of silencing in the absence of K56 acetylation of histone H3. This model is consistent with the observation that both, DNA polymerase ϵ and Rtt109 are important for insulation and although insulation occurs on chromatin, Rtt109 is unable to acetylate chromatin-bound histones and only acetylates histones free in solution. The DNA polymerase ϵ complex could coordinate chromatin assembly through direct or indirect interactions with histone chaperones and remodellers. In the absence of Dpb3 or histone H3 acetylated on K56, deposition may be inefficient, or the deposited nucleosomes may not be optimally positioned or may not be dynamic enough to rapidly turnover. DNaseI hypersensitive sites are disrupted as the replication fork passes through and re-form soon after histone deposition (Solomon and Varshavsky, 1987). In the absence of Rtt109, histones deposited on newly replicated DNA would already be unacetylated. This may prevent or slow down the process of eviction/turnover of nucleosomes by Rsc post-replication. Rsc has a preference for binding and evicting acetylated nucleosomes (which would be lacking in a *rtt109 Δ* cell), and in an Rsc and Rtt109 mutant the histones at the tRNA are stabilized. Furthermore, the absence of acetylated nucleosomes (in a *rtt109 Δ* cell) may reduce the ability of RNA polymerase III transcription factors to bind their cognate sites at the tRNA promoter. *In vitro* RNA polymerase III transcription factors have a preference for binding acetylated histones, and consistent with this observation we have found that in an Rtt109 mutant binding of Bdp1 is reduced. All these scenarios need not be mutually exclusive and, for example, mutations in Rsc or Rtt109 could reduce complex formation at the tRNA and simultaneously alter Sir protein distribution in the nucleus, the consequence of which would be to overwhelm the tRNA gene, resulting in a spread of silencing beyond the insulator. It will be interesting to determine whether similar mechanisms function during the activation of tRNA genes located at other sites in the

genome, and additional studies should help dissect the detailed molecular pathway involved in tRNA-mediated insulation and transcription.

Materials and methods

Strain details and oligomer details are provided in Supplementary Table 1 and 2.

Boundary mating assays were carried out as described previously by Valenzuela *et al* (2006).

SGA screens

SGA screens were carried out as described previously by Tong *et al* (2001).

ChIP

Chromatin for immunoprecipitation reactions was prepared as described previously by Meluh and Broach (1999). Following cross-linking of chromatin with formaldehyde and neutralization, the cells were lysed using a glass beater and the chromatin was sequentially sheared into ~300-bp fragments using a Bioruptor (Diagenode, Belgium) and a cup-horn (Branson, USA) sonicator. Immunoprecipitation reactions were carried out in duplicate using antibodies against histone H3 (Abcam, UK), Ac-K16 H4 and Ac-K56-H3 (Millipore, USA) or polyclonal anti-Sir3 antibodies. Immune complexes were collected using Protein G/A beads (Calbiochem, EMD Biosciences). Tap-tagged protein–chromatin complexes were directly precipitated using Protein A-Sepharose (Pharmacia, Sweden). Immunoprecipitated and input DNA were purified using Chelex 100 (BioRad) using the Fast ChIP method (Nelson *et al*, 2006), and quantified using the Picogreen dsDNA quantitation kit (Invitrogen, USA) and a fluorescent spectrophotometer. Equal amounts of input and immunoprecipitated DNA (between 50 and 200 pg) were then used for qPCR using SybrGreen.

Only primer pairs with equivalent relative efficiencies of amplification were used and their locations/sequences are provided in the Supplementary Table 1 and 2. Relative enrichment of DNA loci was calculated as a ratio of $2^{\Delta C_t}$ (between immunoprecipitated and input DNA) of the experimental locus to $2^{\Delta C_t}$ (between

immunoprecipitated and input DNA) of a reference locus (either Tel6R/7.5 kb probe or the *HMR-I* probe).

Micrococcal nuclease-digested chromatin was prepared as described. Following cross-linking of chromatin with formaldehyde and neutralization, the cells were washed and then lysed with glass beads in 250 μ l of supplemented H-NT buffer (50 mM HEPES–KOH pH (7.5), 50 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 0.1% Triton X-100 with 0.5 mM spermidine and 1 mM β -mercaptoethanol and protease inhibitors). Micrococcal nuclease digestions were carried out in 600 μ l aliquots of cell lysate in 1.5-ml microfuge tubes. The concentration of MNase required to yield ~85% of mononucleosomes was determined empirically for each strain. The buffer of the digested chromatin was changed to FA-140 by the addition of 50 mM HEPES–KOH (pH 7.5), 140 mM NaCl, 0.1% sodium deoxycholate and 1% Triton X-100. All components were added drop-wise with vortexing. The chromatin was left on ice for ~30 min to equilibrate, transferred to 4 $\times$ 1.5 ml microfuge tubes and spun at 15 000 g for 15 min at 4°C, and the supernatant was used for immunoprecipitation reactions.

Analysis of replication pausing

Two-dimensional (2-D) gel electrophoresis was carried out as described on the website <http://fangman-brewer.genetics.washington.edu>. Probes were radioactively labelled using a MegaPrime DNA labelling kit (Amersham Pharmacia) and detected on a phosphor-imager (Molecular Dynamics, Storm 860 or BioRad FX scanner). A *Bam*H1–*Xho*I DNA fragment encompassing the *HMR* tRNA was used as the probe.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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