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# TFIIIC-based chromatin insulators through eukaryotic evolution

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ABSTRACT

Eukaryotic chromosomes are divided into domains with distinct structural and functional properties, such as differing levels of chromatin compaction and gene transcription. Domains of relatively compact chromatin and minimal transcription are termed heterochromatic, whereas euchromatin is more open and actively transcribed. Insulators separate these domains and maintain their distinct features. Disruption of insulators can cause diseases such as cancer. Many insulators contain tRNA genes (tDNAs), examples of which have been shown to block the spread of activating or silencing activities. This characteristic of specific tDNAs is conserved through evolution, such that human tDNAs can serve as barriers to the spread of silencing in fission yeast. Here we demonstrate that tDNAs from the methylotrophic fungus *Pichia pastoris* can function effectively as insulators in distantly-related budding yeast. Key to the function of tDNAs as insulators is TFIIIC, a transcription factor that is also required for their expression. TFIIIC binds additional loci besides tDNAs, some of which have insulator activity. Although the mechanistic basis of TFIIIC-based insulation has been studied extensively in yeast, it is largely uncharacterized in metazoa. Utilising publicly-available genome-wide ChIP-seq data, we consider the extent to which mechanisms conserved from yeast to man may suffice to allow efficient insulation by TFIIIC in the more challenging chromatin environments of metazoa and suggest features that may have been acquired during evolution to cope with new challenges. We demonstrate the widespread presence at human tDNAs of USF1, a transcription factor with well-established barrier activity in vertebrates. We predict that tDNA-based insulators in higher organisms have evolved through incorporation of modules, such as binding sites for factors like USF1 and CTCF that are absent from yeasts, thereby strengthening function and providing opportunities for regulation between cell types.

## 1. Introduction

RNA polymerase (pol) III synthesises short non-coding RNAs, the most abundant of which are tRNAs (Dieci et al, 2007). With only one exception, transcription of tRNA genes involves TFIIIC interacting with two internal promoter sequences within the transcribed regions of tRNA genes, known as the A and B box (Fig. 1); it recruits TFIIIB, which binds upstream of the transcriptional start site; TFIIIB then assembles pol III at the start site and remains bound to the DNA following initiation to allow for a high rate of transcriptional reinitiation (Schramm and Hernandez, 2002; Ramsay and Vannini, 2018). While tRNA gene promoters recruit pol III to mediate their transcription, additional TFIIIC binding sites exist within genomes, that fail to recruit TFIIIB or pol III (Roberts et al. 2003; Moqtaderi and Struhl, 2004; Noma et al. 2006; Moqtaderi et al. 2010); these are known as extra TFIIIC sites (ETC). In addition to its well-characterised and evolutionarily conserved role in pol III-mediated transcription, TFIIIC is also implicated in genome organisation and insulation (Donze, 2012; Van Bortle and Corces, 2012; Kirkland et al. 2013), roles that have yet to be fully explored.

Insulators are DNA sequences that can block enhancer promoter interactions and/or act as barriers to heterochromatic spread (Van Bortle and Corces, 2012; Kirkland et al. 2013; Phillips-Cremins & Corces,

*Abbreviations:* CTCF, CCCTC-binding factor; ETC, extra TFIIIC site; HAT, histone acetyltransferase; HMR, homothallic mating right; pol, RNA polymerase; Sir, silent information regulator; tDNA, tRNA gene; TFIIIC, transcription factor IIIC; TFIIIB, transcription factor IIIB; TAD, topologically associating domains; USF, upstream stimulatory factor.

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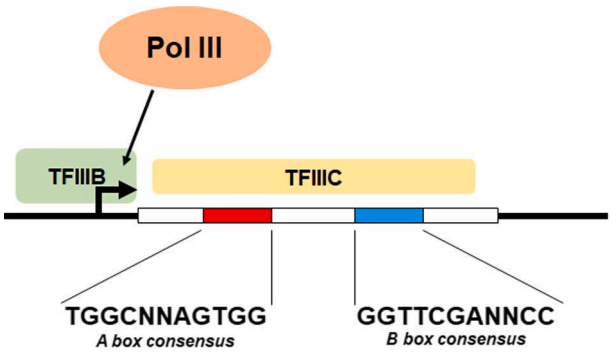



**Fig. 1.** Schematic depicting tRNA gene transcription. TFIIIC binds to the A and B box and recruits TFIIIB, which can then recruit pol III. Consensus sequences described by Galli et al. (1981).

2013). Enhancers are gene-distal sequences that control transcription by forming loops to make physical contact with a target promoter; enhancer blocking insulators prevent this interaction when placed between the enhancer and the promoter. Barrier insulators are important for the segregation of heterochromatin and euchromatin. Heterochromatin can spread into euchromatin both in yeast and mammals by factors deacetylating lysine residues of histones, followed by their subsequent methylation at specific positions associated with transcriptional repression, such as lysine 9 of histone H3 (Oki and Kamakaka 2005; Eissenberg et al. 1990; Bannister et al. 2001; Lachner et al. 2001). Therefore, barrier insulators prevent heterochromatic propagation into euchromatin, and are enriched at the borders of chromatin domains (Dixon et al. 2012; Nora et al. 2012); this prevents detrimental silencing of promoters, thus maintaining necessary gene regulation. A variety of proteins have been implicated in insulator function, among which is TFIIIC (Haldar et al, 2006; Kirkland et al. 2013), a complex of six subunits (Conesa et al. 1993; Dumay-Odelot et al. 2007). Here, we review the role of TFIIIC as an insulator from yeast to humans and identify novel tDNA barriers from an alternative yeast species, *Pichia pastoris*. We highlight the conserved role of TFIIIC in insulation through evolution and propose mechanisms by which it may achieve this function in human cells, where characterisation has lagged behind the more extensive analyses conducted in fungi.

### 1.1. tDNA barriers in Saccharomyces cerevisiae

The first pol III-transcribed gene to be identified as a heterochromatin barrier was the tRNA-Thr gene identified in 2001 by Donze and Kamakaka in the silent HMR (homothallic mating right) locus in *Saccharomyces cerevisiae* (Donze and Kamakaka, 2001). This well-characterised locus can be found to the right of the mating type-determining MAT locus on chromosome 3, where it stores a silenced copy of the MATa allele for mating-type switching in haploid yeast cells (Fig. 2A). Genetic information within the MAT locus determines mating type, so mating-type switching is enabled when the MATa allele from the HMR locus, or conversely the MATα allele from the HML locus, is copied into the MAT locus (Fig. 2B). Transcriptional repression, or silencing, of the MATa allele is mediated by the HMR-E silencer (Hicks et al. 1977). This is achieved by the recruitment of silent information regulator (Sir) proteins SIR2, SIR3 and SIR4. These bind to the tails of nucleosomes and form a multimeric compound which causes the condensation of the chromatin into transcriptionally silent heterochromatin, which has been found to spread towards neighbouring genes (Loo and Rine, 1994; Rine et al, 1979). The observation that silencing can be contained led to the concept of insulator elements and ultimately the discovery that a tRNA-Thr gene can act as a barrier against the spread of heterochromatin..

Early studies into how this canonical tRNA-Thr gene may act as a barrier focused on its transcription initiation machinery, as the gene is actively transcribed. Loss of barrier activity upon introduction of point mutations in the TFIIIC recognition motifs, the B box and, to a lesser extent, the A box, implicated TFIIIC as a key player in this heterochromatin barrier. In support of this, a temperature-sensitive point mutation in a DNA-binding subunit of TFIIIC also compromised barrier activity (Donze and Kamakaka, 2001). An isolated B box that binds TFIIIC but fails to recruit TFIIIB and pol III can suffice to prevent heterochromatin spreading (Simms et al, 2008). These studies provide strong evidence that TFIIIC binding is not only vital, but also sufficient to direct formation of a functional barrier in *S. cerevisiae*.

However, whilst all tRNA genes are bound by TFIIIC, not all tRNA genes have barrier activity (Donze and Kamakaka, 2001; Dhillon et al, 2009). For example, the *SUP53* tRNA-Leu gene is not an effective barrier due to an intron which increases the distance between the A and B boxes from 32 bp to 74 bp (Donze and Kamakaka, 2001). Deleting this intron caused a dramatic increase in barrier activity, which indicates that the distance between these TFIIIC binding sites plays a significant role in barrier activity. TFIIIC has a flexible linker between its domains in order to compensate for the variable distances between the A and B boxes (Schultz et al, 1989, Marzouki et al, 1986, Male et al, 2015), but it is likely that such a large distance in the suppressor tRNA gene weakens TFIIIC function.

It was also demonstrated that the HMR tDNA barrier's flanking sequences contribute to its activity, although insufficient to reconstitute a barrier alone (Donze and Kamakaka, 2001; Dhillon et al, 2009). tDNAs with little or no barrier activity can gain this function when their flanking sequences are replaced with sequences found at the native HMR tRNA-Thr gene (Donze and Kamakaka, 2001). This observation helps explain why many tDNAs show little or no barrier activity, despite being actively transcribed. How the flanking sequences influence this function and the action of TFIIIC has yet to be systematically characterised.

Although not sufficient by itself, histone depletion is necessary for tDNA barrier activity (Oki and Kamakaka, 2005). Amongst the most rapidly turned over nucleosomes in *S. cerevisiae* are those adjacent to tDNAs, where occupancy is substantially depleted (Harismendy et al,

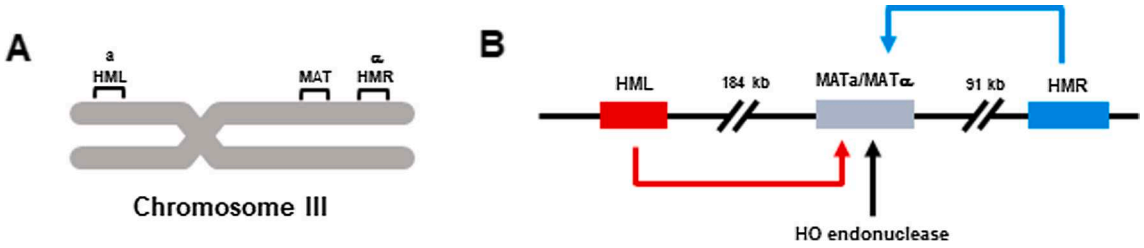



**Fig. 2.** Mating type switching in *S. cerevisiae.* (A) Chromosomal loci of the HML, MAT and HMR loci. (B) Schematic of the Mating-type switching mechanism. Mating-type switching is initiated by a site-specific HO endonuclease, which creates a double-strand break (DSB) in the MAT locus (Strathern et al, 1982). The DSB is repaired by ectopic homologous recombination (gene conversion). One end of the DSB is coated in Rad51, which promotes strand invasion into a short region of homology shared by the MAT locus and the donor sequence. This is followed by copying of the mating-type-specific region, either a or α, and terminated by another short region of homology.

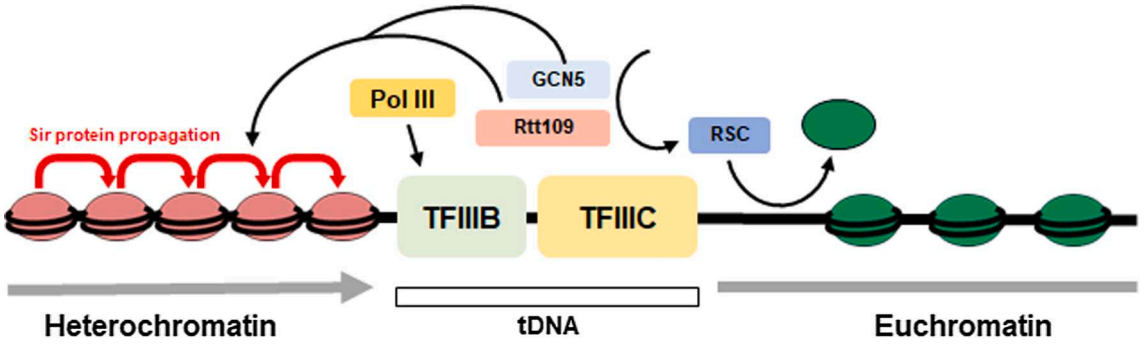



**Fig. 3.** Barrier activity in *S. cerevisiae.* Heterochromatin spread through Sir protein propagation from silencers is disrupted by TFIIIC binding at a tDNA barrier, assisted by TFIIIB, HATs such as GCN5 and Rtt109, the chromatin remodelling complex RSC and cohesin.

2003; Roberts et al, 2003; Moqtaderi and Struhl, 2004; Yuan et al, 2005; Dhillon et al, 2009; Dion et al, 2007; Nagarajavel et al., 2013; Hamdani et al., 2019). Whereas a single nucleosome is excluded at some tDNAs, other tDNAs exclude several. Nucleosome eviction is thought to contribute to barrier activity, as it disfavours propagation of features that facilitate chromatin condensation. This reflects competition by TFIIIC and/or the action of chromatin remodelers, which may contribute towards the formation of the barrier or assist in stabilising the barrier once established by TFIIIC (Saha et al, 2006; Simms et al, 2008). The abundant RSC complex is detected at all tRNA genes in *S. cerevisiae* (Ng et al, 2002) and is important for maintaining nucleosome depletion, along with the Isw1 and Isw2 remodelers (Mahapatra, 2011; Kumar & Bhargava, 2013; Shukla & Bhargava, 2018). The HMR tRNA-Thr gene almost completely loses barrier activity in rsc2Δ mutants, whilst Isw2 also promotes function, albeit weakly (Jambunathan et al, 2005; Dhillon et al, 2009).

Over-expression of Sir proteins reduces insulation (Valenzuela et al, 2009), suggesting that there is competition between TFIIIC binding at the tDNA and binding of Sir proteins. Although TFIIIC binds very strongly to DNA, with an affinity *in vitro* of $< 10^{-10}$ M (Lefebvre et al, 1994), it is likely that its binding to active genes is unstable due to high displacement to make way for Pol III during transcription, as its A and B box recognition motifs are located in the path of the polymerase. Effective tDNA barriers may therefore require additional factors that can remain in position when TFIIIC is displaced during transcription. This property is expected of TFIIIB, which remains bound upstream of the start site when TFIIIC dissociates, sufficing to recruit pol III to its template (Kassavetis et al, 1990). Indeed, a strong reduction in HMR tRNA-Thr barrier activity results from mutations in Brf1 (Donze & Kamakaka, 2001), an essential TFIIIB subunit that contacts TFIIIC. In contrast, a pol III mutation that prevents transcription initiation has minimal effect on this barrier (Donze & Kamakaka, 2001). Thus, full barrier function of the tRNA-Thr gene requires TFIIIB, but not transcription; a role for pol III itself was not excluded, as the mutation prevents initiation but not polymerase recruitment. Weak barriers can be strengthened through multimerisation (Donze and Kamakaka, 2001), which increases the probability of continuous occupancy, as TFIIIC dissociation from one tDNA may be compensated if an adjacent tDNA remains bound.

Sir3 and Sir4 bind to the tails of histones H3 and H4 and mutating the lysine residues in the H4 tail to uncharged residues is sufficient to disrupt H4-Sir3 interactions and significantly reduce silencing (Hecht et al, 1995). Acetylation of lysine side-chains removes the positive charge and so histone acetylation counteracts silencing. Hyperacetylation of histones is associated with uncondensed and active chromatin (euchromatin), characterised by an increased general DNase I sensitivity and transcriptional potential of chromosomal domains (Hebbes et al, 1994). Conversely, hypoacetylation is associated with inactive heterochromatin (Lin et al, 1989). Histone acetyltransferases (HATs) have been shown to promote barrier activity by promoting recruitment of RSC and antagonising Sir spreading; accordingly, function of the HMR tDNA-Thr barrier is compromised by mutations in several HATs, including GCN5 and Rtt109 (Donze and Kamakaka, 2001; Oki et al, 2004; Oki and Kamakaka, 2005; Dhillon et al. 2009).

Other cofactors have also been implicated in contributing to TFIIIC-mediated barrier activity in *S. cerevisiae*. An important example is cohesin, a protein complex with the primary role of facilitating cohesion of sister chromatids to ensure proper chromosome segregation, but that also associates with silent chromatin at telomeres and both the HMR and HML loci (Chang et al, 2005). Cohesin is enriched at tDNAs (Glynn et al, 2004) and is implicated in their barrier activity, as mutations in its smc1 and smc3 subunits compromise insulation by the HMR tRNA-Thr gene (Donze et al, 1999). However, the details of its involvement remain unclear. Silencing proteins Sir2, Sir3 and Sir4 are required for cohesin binding at HMR and cohesion at this and other silenced loci is lost in sir mutants (Chang et al, 2005). Cohesin recruitment is also compromised by mutations in RSC (Huang et al, 2004). Thus, cohesin seems to be a downstream contributor in the tDNA heterochromatin barrier mechanism.

### 1.2. tDNA barriers in Schizosaccharomyces pombe

Studies into TFIIIC-associated barriers have also been carried out in the fission yeast *Schizosaccharomyces pombe*, a model species with heterochromatin features that are absent from budding yeast but found in mammalian systems (Allshire and Madhani 2018). Thus, fission yeast silencing involves K9-methylated histone H3 being bound by Swi6, a homologue of human HP1 (Allshire and Madhani 2018). Despite major differences in heterochromatin between *S. cerevisiae* and *S. pombe*, tDNA barriers are found in both species (McFarlane and Whitehall, 2009). Preservation of this phenomenon across these highly diverse species of yeast is suggestive of conserved underlying mechanism(s). In *S. pombe,* tDNA clusters lie at junctions between euchromatin and the centromeres of all three chromosomes, where significant areas of heterochromatin are found (Cam et al, 2005). Indeed, roughly one-third of all the 186 tDNAs are found inside or adjacent to centromeres (Haldar et al, 2006). Heterochromatic domains are formed by RNAi machinery and transcription factors which promote methylation of H3K9 and recruitment of Swi6 (Hall et al, 2002, Jia et al, 2004, Yamada et al, 2005). Deletion of tDNAs that flank the centromeres allows spread of heterochromatin to adjacent regions (Scott et al, 2006). TFIIIC is implicated in this barrier activity, as mutations which disrupt the A box promoter element result in a loss of barrier activity, whereas substitution mutations in the sequence between the A and B boxes had no effect (Scott et al, 2006). Whereas the centromeric tRNA-Ala gene functions as a strong chromatin barrier, a neighbouring tRNA-Glu gene (424 bp away) has very weak barrier activity (Scott et al, 2006). As deletion of both tDNAs was not tolerated, it was hypothesised that the complete barrier involves both the tDNA-Ala and the tDNA-Glu, at least one of which is essential for the centromere. This is reminiscent of mammalian barriers composed of multiple tDNAs (Ebersole et al. 2011; Raab et al. 2012).

### 1.3. Extra TFIIIC (ETC) sites as heterochromatin barriers

Genome-wide mapping of the Pol III machinery in *S. cerevisiae* identified eight untranscribed loci which are bound by TFIIIC, but not TFIIIB or Pol III (Moqtaderi and Struhl, 2004). These were named Extra-TFIIIC (ETC) sites and are found in divergent intergenic regions. They share the B box promoter motif found in tDNAs, but not the A box. Mutational analyses of several tRNA genes have shown that the B box is sufficient for high-affinity binding by TFIIIC. For example, deletion of A boxes from two tDNAs was found to reduce TFIIIC affinity by only 2- to 5-fold, whereas single base B box substitutions resulted in decreases of 43- to 370-fold (Baker et al, 1986). Two of the ETC sites in *S. cerevisiae* were shown to be able to function as heterochromatin barriers; thus, removal of the tDNA-Thr in the HMR barrier results in silencing, but its replacement with an ETC restored protection against Sir-mediated silencing and prevented *ADE2* reporter gene repression, activities abolished by mutation of the B-box (Simms et al, 2008). This provides further strong evidence for TFIIIC as a fundamental component of heterochromatin barriers.

Analysis of the evolutionary conservation of ETC loci across different species of yeast revealed an additional conserved 10 bp sequence extending downstream from the B box (Moqtaderi and Struhl, 2004). The significance of this B box extension at ETC sites has yet to be explored; perhaps it compensates for the absence of TFIIIB, which contributes significantly to the barrier function of tDNAs (Donze & Kamakaka, 2001). It might provide a direct binding site for another factor and/or alter the conformation of TFIIIC to facilitate recruitment of additional machinery.

In *S. pombe*, over 60 loci have been identified as TFIIIC-binding sites without pol III co-occupancy (Iwasaki et al, 2010). Examples are found at the 2 kb identical inverted repeats which flank the silenced mating-type locus in *S. pombe*. A 500 bp fragment of either repeat containing 5 copies of a B box was shown to function as a barrier (Noma et al, 2006). The ETC-like sites in *S. pombe* have been named chromosome-organising clamp (COC) sites, because they are tethered to the nuclear periphery, in proximity to the nucleolus (Noma et al, 2006). Thus, fluorescent *in situ* hybridisation using probes specific to high affinity COC sites, including from centromeres and the inverted repeats at the mat locus, revealed association with the nuclear periphery (Noma et al, 2006). The presence of TFIIIC at these sites was confirmed by immunofluorescence (Noma et al, 2006). These striking observations inspired models of genome organisation involving TFIIIC (Noma et al, 2006). Three-dimensional clustering of TFIIIC is consistent with evidence that tRNA genes in *S. cerevisiae* localise to the boundary of the nucleolus (Thompson et al, 2003; Haeusler et al, 2008). Similarly, six of the eight ETC sites in *S. cerevisiae* are localised at the nuclear periphery, positioning that is lost if the B box is mutated or if TFIIIC is targeted for specific degradation (Hiraga et al. 2012). Tethering of an ectopic chromosomal locus to the nuclear periphery can be induced by insertion of an ETC or anchoring of TFIIIC to the target site (Hiraga et al. 2012). It was speculated that TFIIIC-driven genome organisation would contribute to its barrier function, but an *S. cerevisiae* ETC site was found to retain its ability to prevent heterochromatic spread after release from localisation at the nuclear periphery (Hiraga et al. 2012).

Loci bound by TFIIIC, including tDNAs, also localise to the nuclear periphery in *C. elegans* (Ikegami and Lieb, 2013; Stutzman et al, 2020). About half of all genomic sites bound by TFIIIC in this nematode do not show co-occupancy by TFIIIB and pol III (Stutzman et al, 2020). These sites contain both A and B boxes, in contrast to the ETC sites in yeast and mammals, where B boxes are found without an A box; a large fraction lie within a transcriptionally inactive class of repetitive elements (Stricklin et al, 2005; Stutzman et al, 2020). They are predominantly located adjacently to regions of heterochromatin with high levels of methylated H3K9, often close to protein-coding genes (Stutzman et al, 2020). As such, a barrier role seems a clear possibility, but functional evidence has yet to be obtained.

Understanding of TFIIIC barrier function has been led by studies in yeast and benefited considerably from comparisons between species. *Pichia pastoris* (also known as *Komagataella phaffii*) is a yeast species that is much less characterised than the paradigm models *S. cerevisiae* and *S. pombe* (Fig. 4), but is gaining importance due to its wide range of biotechnological applications (Ahmad et al. 2014; Bernauer et al, 2020). As insights into fundamental molecular mechanisms may be provided by identifying commonalities between *P. pastoris* and the well-studied yeasts, we have tested a series of tDNAs derived from *P. pastoris* for their ability to function as barriers in *S. cerevisiae*. With this approach, we establish that several *P. pastoris* tDNAs display strong activity in the barrier assay. The remainder of this study exploits the wealth of publicly-available data from ChIP-seq experiments to scrutinise epigenetic features of tDNAs in human chromosomes that may be relevant to insulator function, as well as their interactions with regulatory proteins. We focused on data derived from ChIP-seq analyses with K562 cells, an extensively-studied model system. We confirm that in this cell type large numbers of tDNAs attract components of the SWI/SNF remodelling complex, which is homologous to the RSC complex that promotes barrier function at tDNAs in *S. cerevisiae*. We also demonstrate the presence at many human tDNAs of CTCF, which is considered the primary mammalian insulator protein but is absent from yeast. Another well-characterised vertebrate insulator protein is USF1 and we show that this too associates with large numbers of tDNAs in the chromosomes of K562 cells. ChIP-seq data also demonstrate that a substantial fraction of tDNAs attract p300, a vertebrate HAT that may help counteract the spread of facultative heterochromatin in a form that is absent from yeast. Although experimental confirmation has yet to be provided, these bioinformatic analyses lead us to propose that TFIIIC-based mammalian insulators may combine ancient features, that are still found in yeast, with partnerships that have evolved much more recently, such as interactions of TFIIIC with CTCF, USF1 and p300.

## 2. Materials and Methods

### 2.1. Barrier assay in Saccharomyces cerevisiae

Mating assays were performed to test DNA elements for barrier activity in *S. cerevisiae,* as described by Donze et al. (1999). Following transformation of α-expressing strain DDY171 with plasmids containing the HMR-E region with and without a strong barrier between the silencer and a1 gene, pDD371 and pDD442 respectively, a colony of each transformant was selected for mating with a-expressing strain, DDY7. Mating lawns of DDY7 were prepared on YMD -URA -HIS plates. Colonies were spotted directly onto the mating lawn using overnight cultures diluted to an OD600 of 0.2. The mating potential of cells was monitored by the -HIS selection as the his3-11 and his4-519 mutations in DDY7 and DDY171 would be complemented in their diploid progeny, enabling growth on media lacking histidine.

### 2.2. Yeast transformations

Competent yeast strains were prepared from overnight cultures grown in YPD media, which were adjusted to have an OD600 of 1–1.5 before being washed with Li-TE sorbitol (100 mM lithium acetate, 10 mM Tris.HCl pH 7.5, 1.2 M sorbitol, 1 mM EDTA, 200 μM calcium chloride) and stored at −80 °C. Cells were thawed on ice before incubation with plasmid and salmon sperm DNA and 70% PEG-3350 at 30 °C (shaking) for 45 min. Cells were heat-shocked at 42 °C for 20 min before spreading on selective auxotrophic plates.

### 2.3. P. Pastoris genomic DNA isolation

Genomic DNA was extracted from the *P. pastoris* strain X-33 in preparation for the PCR amplification of potential barrier candidates. X-33 cells were harvested from overnight cultures grown in YPD media

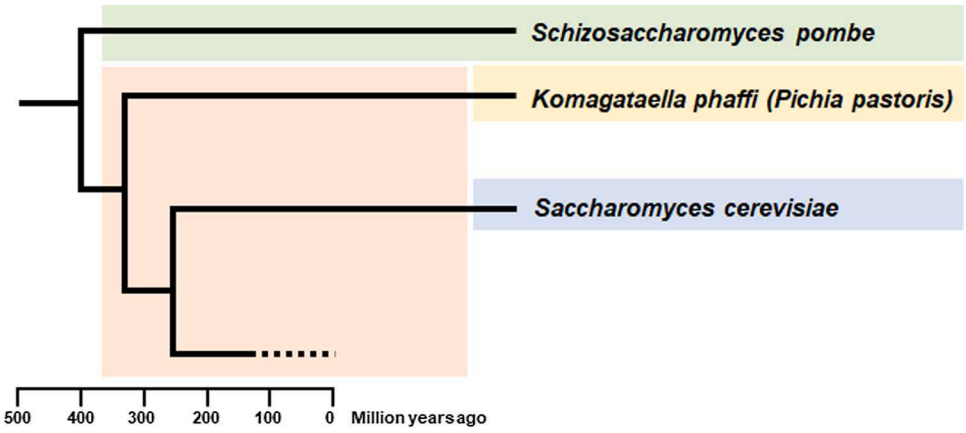



**Fig. 4.** Phylogenetic tree illustrating the evolutionary relationships between *S. pombe, K. phaffi and S. cerevisiae. S. pombe* belongs to the Taphrinomycotina subdivision (green), whereas *S. cerevisiae* and *K. phaffi* are both derived from the Saccharomycotina subdivision (orange), but *S. cerevisiae* belongs to the Saccharomycetaceae family (blue) and *P. pastoris* is a methylotrophic yeast (yellow) (Bernauer et al, 2020). Times of divergence described by Stajich et al. (2009). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and resuspended in 500 μl TE buffer before being lysed through overnight incubation with 3 μl β-mercaptoethanol and 10 μl zymolyase at 37 °C (shaking). Proteins released from the lysed cells were then denatured using two treatments with 500 μl Phenol:Chloroform:Isoamyl alcohol (25:24:1) and vortexed for 5 min after each treatment. DNA was then precipitated through overnight incubation in 50 μl 3 M Sodium acetate and 1 ml 100% ethanol at −80 °C. Precipitated DNA was pelleted and washed 3 times with 70% ethanol and dried at 42 °C. Genomic DNA pellet was then left to suspend overnight in 100 μl dH20.

### 2.4. Plasmid construction

pDD371 was digested by adding 1 μl BamHI-HF (NEB) and 1 μl NotI-HF (NEB) to a mixture containing 1000 ng plasmid DNA, 5 μl 10X Cutsmart buffer (NEB) and dH20 up to a final volume of 30 μl and incubated at 37 °C for 15 min. Digestion products were separated on a 1% agarose gel, excised from the gel using a scalpel and extracted using the GeneJET Gel Extraction Kit (ThermoFisher Scientific), following the manufacturer's instruction with an exception for the elution step, where elution buffer was substituted with an equivalent volume of dH20. Putative barrier sequences were PCR amplified using primers designed using Benchling and obtained from IDT technologies and Merck. Primers contained overlapping regions with the vector, pDD371. Barriers were amplified from *P. pastoris* genomic DNA using the PCRBIO Ultra Polymerase Mix and following the PCRBIO Ultra Mix protocol. Gibson Assembly was used to clone 6*P. pastoris* tRNA-Thr genes and 1 tRNA-Thr and tRNA-Glu gene pair into pDD371. Successful assemblies were obtained when 1.25 μl vector and 1 μl insert were added to 7.5 μl of Gibson master mix. Reaction mixtures were incubated at 50 °C for 1 h and a 0.5 μl aliquot was then transformed into competent DH5α E. coli cells and plated onto selective media containing ampicillin. An equivalent volume of water was used in place of the insert in the negative control.

### 2.5. Bioinformatic analysis

#### 2.5.1. Analysis of chromatin modifications and pol III transcriptional machinery around tRNA genes in UCSC

Pre-loaded ChIP-Seq datasets from the encyclopaedia of DNA elements (ENCODE; accession numbers listed in Table 1) for histone modifications, Pol III transcription complex components, and CpG methylation determined by Methyl 450 K Bead Arrays were viewed using UCSC genome browser (available at https://genome.ucsc.edu) and aligned to the GRcH37 (Hg19) genome (release date 2009) (Kent et al. 2002; Rosenbloom et al. 2013; Lander et al. 2001; ENCODE Project Consortium 2012; Barrett et al. 2013; Edgar et al. 2002). This genome was chosen because (1) there is a large amount of publicly available data published on the UCSC genome browser for this genome assembly, and (2) previously tested mammalian tRNA gene barriers and ETC site coordinates align to this genome.

**Table 1**
Accession numbers for datasets acquired from ENCODE (available at www.encodeproject.org/).

| Encode Dataset | Laboratory | Dataset | Files |
|---|---|---|---|
| K562 H3K27me3 ChIP-Seq | Bernstein - Broad Institute | ENCSR000AKQ | ENCSR445KAE |
| K562 H3K9me3 ChIP-Seq | Bernstein - Broad Institute | ENCSR000APE | ENCSR445KAE |
| K562 H3K27ac ChIP-Seq | Bernstein - Broad Institute | ENCSR000AKP | ENCSR445KAE |
| K562 H3K9ac ChIP-Seq | Bernstein - Broad Institute | ENCSR000AKV | ENCSR445KAE |
| K562 H3K36me3 ChIP-Seq | Bernstein - Broad Institute | ENCSR000AKR | ENCSR445KAE |
| K562 H3K4me3 ChIP-Seq | Bernstein - Broad Institute | ENCSR000AKU | ENCSR445KAE |
| K562 GTF3C2 ChIP-Seq | Snyder- Stanford | ENCSR000DOD | ENCSR661PUN |
| K562 BRF1 ChIP-Seq | Snyder- Stanford | ENCSR000DOJ | ENCSR661PUN |
| K562 POLR3G ChIP-Seq | Snyder- Stanford | ENCSR000EHQ | ENCSR661PUN |
| K562 Methyl Array | Myers - Hudson Alpha | ENCSR000ACM | ENCSR147DVS |
| Brf1 ChIP-Seq on human K562 | Struhl, HMS | ENCAN065YBG | ENCFF000YGK<br>ENCFF000YGM<br>ENCFF000YGL |
| GTF3C2 ChIP-Seq human K562 | Struhl, HMS | ENCAN269GJS | ENCFF000ZBT<br>ENCFF000ZBS<br>ENCFF000ZBU |
| POLR3A ChIP-Seq human K562 | Struhl, HMS | ENCAN152NED | ENCFF000YYM<br>ENCFF000YYN |
| CTCF ChIP-Seq Human K562 | Bernstein - Broad Institute | ENCAN121OSK | ENCFF000BWB<br>ENCFF000BWC |
| EP300 ChIP-Seq Human K562 | Snyder - Stanford | ENCAN509LOQ | ENCFF366ROL<br>ENCFF703ULA |
| USF1 | Myers - HAIB | ENCSR000BKT | ENCFF318WJA<br>ENCFF528NQV |
| USF2 | Snyder - Stanford | ENCSR000EHG | ENCFF351ZZA<br>ENCFF842LBP |
| SMARCC2 | Snyder - Stanford | ENCSR519WMW | ENCFF662JMK<br>ENCFF269TSX |
| SMARCE1 | Snyder - Stanford | ENCSR157TCS | ENCFF115WLQ<br>ENCFF868URB |

#### 2.5.2. Downloading datasets

Previously published ENCODE (available at https://www.encodeproject.org/) ChIP-Seq datasets were used to analyse the binding signal intensity of CTCF, USF1, p300 and Pol III machinery at tRNA genes (ENCODE Project Consortium 2012; Davis et al. 2018). Filtered alignment ChIP-Seq datasets from K562 cells were downloaded in BAM format (accession numbers are listed in Table 2). R-ChIP D210V K562

**Table 2**
Accession numbers for datasets acquired from Gene Expression Omnibus (available at www.ncbi.nlm.nih.gov/geo/).

| GEO Dataset | Laboratory | GEO Sample Accession | GEO Series Accession |
|---|---|---|---|
| K562 R-ChIP D210N | Chen - University of California | GSM2551008 GSM2551007 | GSE97072 |

Sequence Read Archive (SRA) files were extracted in FastQ format using the Galaxy web platform (available at usegalaxy.org) (Afgan et al. 2018; Chen et al. 2017). Bowtie2 was then used to map reads against the Hg19 reference genome and convert the file to a BAM format. Hg19 aligned tRNA genes and sno-miRNA genes were downloaded from UCSC using the Table Browser function and converted to a.txt file (Karolchik et al. 2004).

#### 2.5.3. Data visualisation

ChIP-Seq and R-ChIP datasets, and tRNA genes and sno-miRNA regionsets were loaded into EAseq (available at https://easeq.net) (Lerdrup et al. 2016). Isogenic replicates were merged using the 'pool' function provided by EAseq. All heatmaps were generated using the 'heatmap' function provided by EAseq. Hg19 tRNA genes or sno-miRNA genes were aligned at their centre point, and the signal intensity of R-loops, CTCF, USF1 and p300 signals from both strands were plotted 5,000–10,000 bp either side. Signal intensity was segmented into 200 bins and sorted according to increasing intensity, calculated using the 'Quantify' function provided in EAseq.

The 'FillTrack' function on EAseq was used to visualise signal intensity of R-loops, CTCF or p300 around tRNA genes. Specific tRNA genes of interest were gated using the 'gate' function and enrichment of R-loop, CTCF or p300 10,000–20,000 bp either side were plotted for both strands. Signal intensity was segmented into 400 bins and smoothed for 1 bins.

## 3. Results

### 3.1. Pichia pastoris tDNAs demonstrate strong barrier activity in S. Cerevisiae

Chromatin organisation and epigenetic regulation in *P. pastoris* has not been described extensively and little is known about how it segregates its transcriptionally active and inactive subsets of genes. As a step towards this, we tested if *P. pastoris* tRNA-Thr genes are capable of acting as heterochromatin barriers in *S. cerevisiae,* using the assay that first identified a tDNA barrier (Donze and Kamakaka, 2001). This uses expression of the *S. cerevisiae* a1 mating-type gene, responsible for the 'a' mating type, to assess the strength of putative barriers. *P. pastoris* tRNA-Thr genes with 250 bp flanking sequences were amplified and cloned in between the HMR-E silencer and *a1* gene. *S. cerevisiae* strains of the opposite mating type (α-maters) were then transformed with plasmids containing a potential barrier in between the HMR-E silencer and *a1* gene (Fig. 5A). If no barrier or a weak barrier is present, the a1 gene is silenced by the HMR-E silencer and the cell retains its α mating type; it can mate with the a-mating strain on the mating lawn and its progeny grow on the appropriate selective plates. However, strong barriers prevent silencing of the a1 gene, so that the strain expresses both mating types, giving it a non-mating phenotype that produces no progeny. Barrier activity can therefore be measured through the strain's mating phenotype, which is revealed by the number of daughter colonies growing on selective plates. Lack of growth on YMD -URA -HIS plates indicates that mating did not take place for the strains containing the *P. pastoris* tRNA genes, suggesting that they were effective at preventing a1 silencing and caused a non-mating phenotype. Whereas only one of the 16 tRNA-Thr genes in *S. cerevisiae* demonstrated strong barrier activity in this assay (Donze and Kamakaka, 2001), each of the seven *P. pastoris* tRNA-Thr genes we tested (six individually and one as part of a tRNA gene pair) was found to protect effectively against heterochromatic silencing in *S. cerevisiae* (Fig. 5B). Indeed, some of the *P. pastoris* tRNA-Thr genes outperformed the paradigm *S. cerevisiae* tRNA-Thr barrier when tested in parallel in budding yeast.

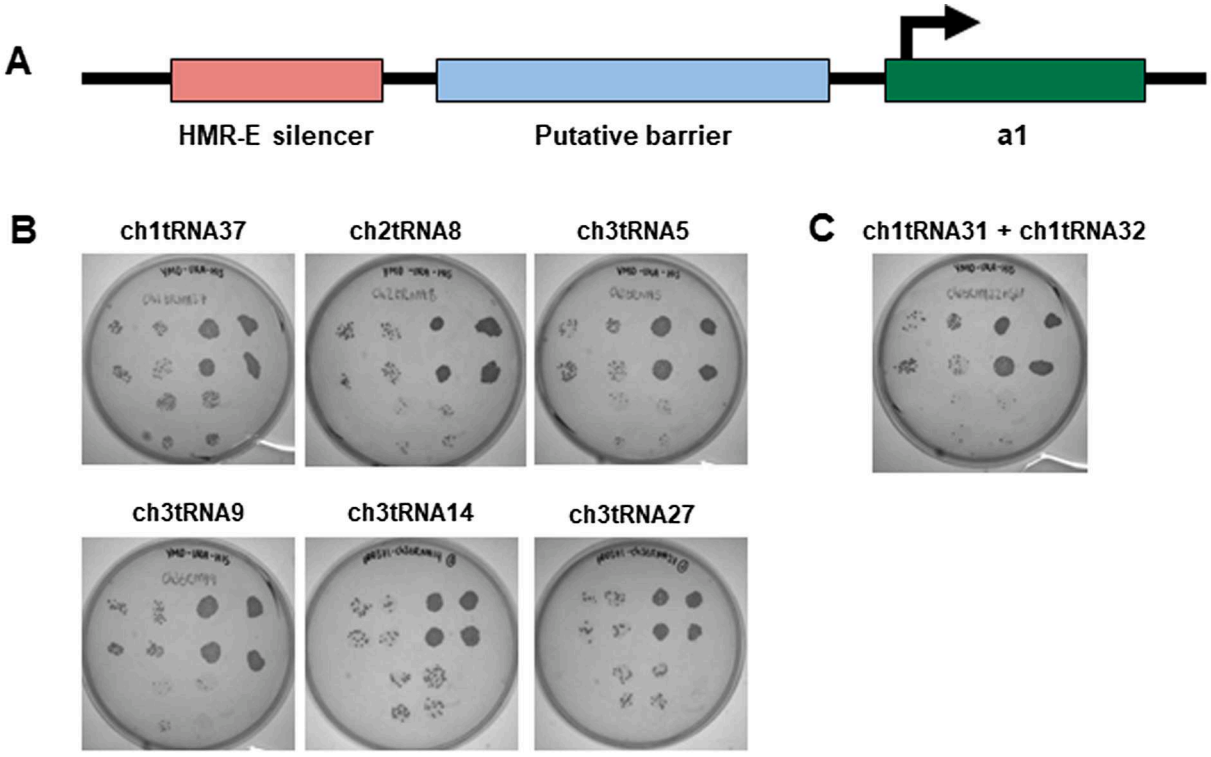
The sequences of the seven *P. pastoris* tRNA-Thr genes tested are highly similar and they each have A and B boxes that conform to a consensus defined in yeast. Despite this, they exhibit varying levels of strength against the spread of heterochromatin from the HMR-E silencer. This suggests that differences in barrier activity may reflect features besides affinity for TFIIIC. Nucleosome occupancy predictions suggest that ch2tRNA8 and the ch1tRNA31 (tRNA-Glu)-ch1tRNA32 (tRNA-Thr) gene pair are flanked with nucleosome-free regions due to AT-rich sequences and poly (dA:dT) tracts (Fig. S1), which render the DNA strand less flexible and therefore resistant to nucleosome binding (Jansen and Verstrepen, 2011); this may strengthen barrier activity in these cases, but is probably not a dominant feature, as comparable nucleosomal depletion is not predicted for some of the other *P. pastoris* tRNA-Thr genes (Fig. S2).

### 3.2. Mammalian TFIIIC-based barriers

The first experimental evidence that tRNA genes can work as barriers in mammalian systems came from a transgenic reporter assay conducted in murine erythroid leukemia cells (Ebersole et al, 2011). A cluster of four mouse tRNA genes, derived from chromosome 1, was able to protect an eGFP reporter gene against H3K9me3-mediated silencing from a bacterial/yeast artificial chromosome sequence (Ebersole et al, 2011). ChIP-PCR confirmed recruitment of TFIIIC, TFIIIB and pol III in this context (Ebersole et al, 2011). A tDNA pair from the cluster functioned as efficiently in this assay as all four tDNAs together, but neither showed barrier activity when tested individually (Ebersole et al, 2011). Deletions and substitutions in the sequences between the tDNAs of this cluster did not compromise barrier function in this context, but it was abolished by deletion of A boxes from the four tDNAs (Ebersole et al, 2011). Although an A box is required for tRNA gene transcription, the B box is expected to suffice for binding TFIIIC; it is therefore noteworthy that the barrier function was lost without the A box, as this suggests that this mouse tDNA barrier requires TFIIIB, pol III and/or transcription in this context, as shown for the HMR tRNA-Thr gene in *S. cerevisiae* (Donze and Kamakaka, 2001). Consistent with this, four or six copies of a B box were also inactive as barriers in this assay (Ebersole et al, 2011). Although the study did not confirm the predicted recruitment of TFIIIC to orphan B boxes in the absence of A boxes, these observations suggest that TFIIIB and pol III may be necessary for TFIIIC to exert barrier activity in mammalian cells.

ChIP-seq data, accessible through ENCODE, allows analysis of interactions with the human equivalent of this mouse tDNA cluster, in its natural context on chromosome 1. This shows robust binding *in situ* of TFIIIC and pol III to the four tDNAs in the cluster, whereas the Brf1 subunit of TFIIIB gives a less consistent signal (Fig. 6A). Chromatin features support the possibility that this tDNA cluster functions as a barrier in its natural chromosomal context; thus, strong peaks of histone acetylation, characteristic of euchromatin, overlap the tDNAs, whereas heterochromatic marks decline abruptly at the edge of the cluster (Fig. 6B).

In the above study (Ebersole et al, 2011), the murine tDNAs were tested for their ability to prevent the spread of constitutive heterochromatin characterised by H3K9me3. As this type of heterochromatin predominates in fission yeast (*S. pombe)*, one can postulate that TFIIIC and its associated proteins have retained through evolution activities that arose in unicellular organisms and resist the spread of H3K9me3. This model is supported by the finding that several human tDNAs with barrier activity in K562 erythroleukaemia cells can also prevent heterochromatin spread when tested in fission yeast (Raab et al, 2012). These tDNAs exist in clusters on human chromosome 17 and mark a



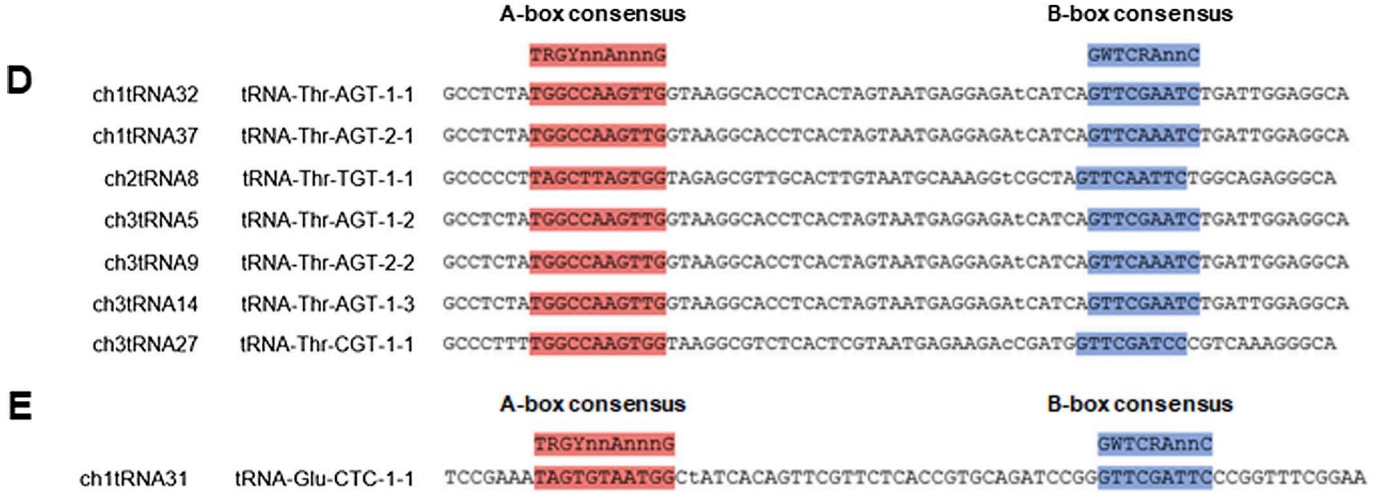




**Fig. 5.** (A) Map of the mating-type region in barrier assay. Potential barriers can be tested for their ability to prevent silencing of the a1 gene by being placed between the HMR-E silencer region and the a1 gene. Barrier activity is assessed through expression of the a1 gene, which determines the mating phenotype. (B) Mating assay plates demonstrating that *P. pastoris* tRNA genes display strong barrier activity in *S. cerevisiae*. Lack of colony formation on YMD-URA-HIS plates indicates that the tRNA-Thr genes effectively prevented silencing of the a1 gene from the *S. cerevisiae* HMR-E silencer. Each plate shows tests in quadruplicate for cells with the *S. cerevisiae* HMR tRNA-Thr gene (top left), no barrier (top right), or a *P. pastoris* tRNA-Thr gene (bottom) inserted between a1 and the HMR-E silencer. Identity of the *P. pastoris* tRNA-Thr genes is indicated above each plate. (C) As in B, except using a fragment of *P. pastoris* DNA carrying closely-spaced tRNA-Thr and tRNA-Glu genes. (D) tRNA-Thr alignments with yeast A- and B-box consensus motifs (E) tRNA-Glu alignments with yeast A- and B-box consensus motifs. Consensus sequences TRGYnnAnnnG (A-box) and GWTCRAnnC (B-box) were identified by Marck et al (2006) through global distance analysis of all tDNA sequences in 10 different yeast species; *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Saccharomyces castellii, Candida glabrata, Kluyveromyces waltii, Kluyveromyces lactis, Eremothecium gossypii, Debaryomyces hansenii, Candida albicans* and *Yarrowia lipolytica* (Marck et al, 2006).

region of transition between silent and active protein-coding genes (Raab et al, 2012). Approaching from the silenced region, the first tDNAs encountered are a tDNA-Lys-tDNA-Gln pair that form half of a cluster of four alongside the ALOXE3 gene. When flanking an eGFP reporter, this tDNA pair was found to protect against silencing after random integration into the genome of K562 cells, a barrier function that was compromised by deletion of the B-boxes (Raab et al, 2012). The chicken HS4 insulator is probably the best-characterised example of a vertebrate barrier, but it was less effective than the ALOXE3 tDNA-Lys-tDNA-Gln pair at maintaining eGFP expression, when tested in parallel in this assay (Raab et al, 2012).

It is likely that random integration of reporters into mammalian chromosomes will result in some exposure to facultative heterochromatin characterised by H3K27me3. This is a form of chromatin

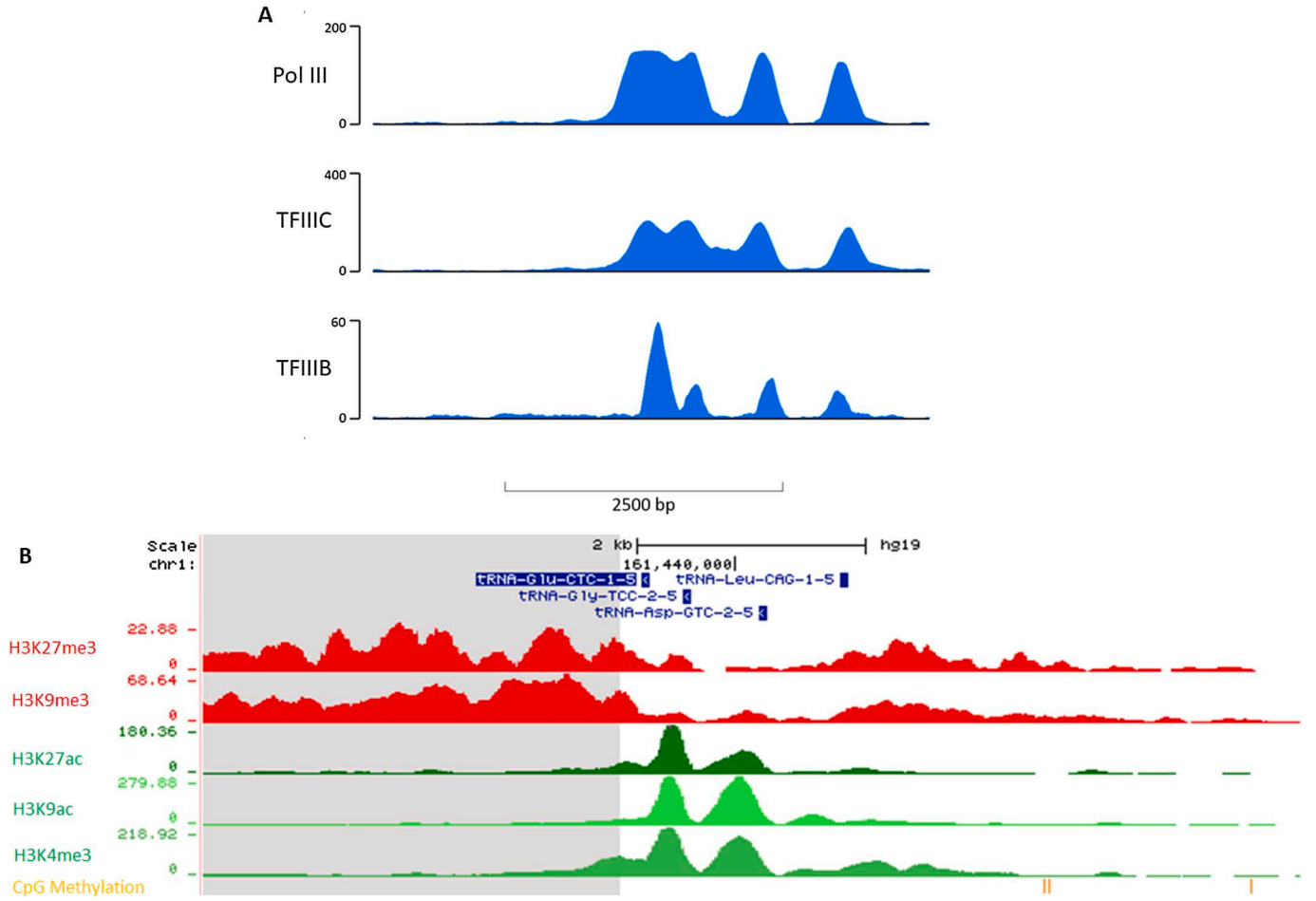



**Fig. 6.** A tRNA gene barrier resides at the border of a heterochromatic domain on chromosome 1 and is marked by histone acetylation, H3K4me3 and binding of TFIIIC, TFIIIB and pol III. (A) ChIP-Seq data quantifying the binding of TFIIIC subunit GTF3C2, TFIIIB subunit Brf1 and pol III subunit POLR3A at tRNA-Glu-CTC-1–5, tRNA-Gly-TCC-2–5, tRNA-Asp-GTC-2–5, tRNA-Leu-CAG-1–5. Due to the repetitive nature of tRNA genes on chr1, no ChIP-Seq data were available to view in UCSC genome browser for TFIIIB, TFIIIC and Pol III. Therefore, publicly available ENCODE ChIP-Seq datasets were downloaded and analysed to view quantitative Pol III transcriptional machinery enrichment at this cluster (see Methods and Materials). (B) Screenshots of UCSC genome browser (*http://genome.ucsc.edu*) in K562 cells in hg19 assembly displaying histone modifications and CpG methylation surrounding a cluster of four tRNA genes located on chromosome 1 (tRNA-Glu-CTC-1–5, tRNA-Gly-TCC-2–5, tRNA-Asp-GTC-2–5, tRNA-Leu-CAG-1–5). Blue vertical lines represent tRNA gene positions. CpG methylation status: blue line = unmethylated CpG; orange = methylated; purple = partially methylated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

deposited by polycomb complexes, which is not encountered in model yeasts; it presents a challenge to TFIIIC-dependent barriers that was probably absent during early evolution. The ability of tDNAs to resist this challenge was tested directly using a luciferase reporter downstream of nine Gal4-binding sites, integrated into HEK293 cell chromatin at a specific site by recombination-mediated cassette exchange; luciferase was expressed by cells transfected with a vector encoding the DNA-binding domain of Gal4, but silenced if this domain was fused to the polycomb protein CBX4 (Raab et al, 2012). When inserted between the Gal4 sites and the promoter driving luciferase transcription, the ALOXE3 tDNA-Lys-tDNA-Gln pair provided robust protection against polycomb-driven repression, which was abolished by B box deletion (Raab et al, 2012). Once again, the barrier activity of these tDNAs outperformed that of the chicken HS4 insulator (Raab et al, 2012). These data establish that certain tDNAs have the capacity to resist the spread of both H3K9me3-dependent constitutive heterochromatin and H3K27me3-dependent facultative heterochromatin in mammalian cells.

Evidence that human tDNAs function this way *in situ* and not just in reporter assays can be gathered by studying chromatin marks detected by ChIP-seq studies and accessible through ENCODE (Fig. 7). For example, a region enriched for H3K27me3 stops alongside the ALOXE3 tDNA-Lys-tDNA-Gln genes in K562 cells, consistent with a barrier to the spread of facultative heterochromatin; the tDNAs themselves are occupied by TFIIIC, TFIIIB and pol III and coincide with peaks of histone acetylation and H3K4me3, euchromatic marks that correlate strongly with pol III transcription (Barski et al. 2010). The tDNA cluster on chromosome 1, referred to above, shows similar euchromatic features in K562 cells and coincides with the edge of a heterochromatin domain that is highly enriched for both H3K9me3 and H3K27me3 (Fig. 6B). Troughs in various histone marks coinciding with the tDNAs are very apparent in Fig. 7; these are likely to reflect nucleosomal depletion where the pol III machinery binds, as documented collectively for human tDNAs and many histone modifications (Barski et al. 2010).

Further bioinformatic analysis of ENCODE data reveals other examples of putative tRNA gene barriers, which are localised at interfaces between heterochromatic marks (H3K9me3 and/or H3K27me3) and euchromatic marks (H3K4me3, H3K9ac and H3K27ac); for example, the tRNA-Ile-TAT-2–3 gene on human chromosome 6 (Fig. 8) and the tRNA-Arg-TCG-1–1 gene on human chromosome 15 (Fig. 9). ChIP-Seq shows that these putative tDNA barriers are enriched for TFIIIC, TFIIIB and pol III, strongly suggestive of active transcription. Both these cases involve an isolated tDNA, in contrast to the mouse chromosome 1 cluster and the human ALOXE3 cluster of tDNAs that have been confirmed experimentally as functional barriers; indeed, single tDNAs from the

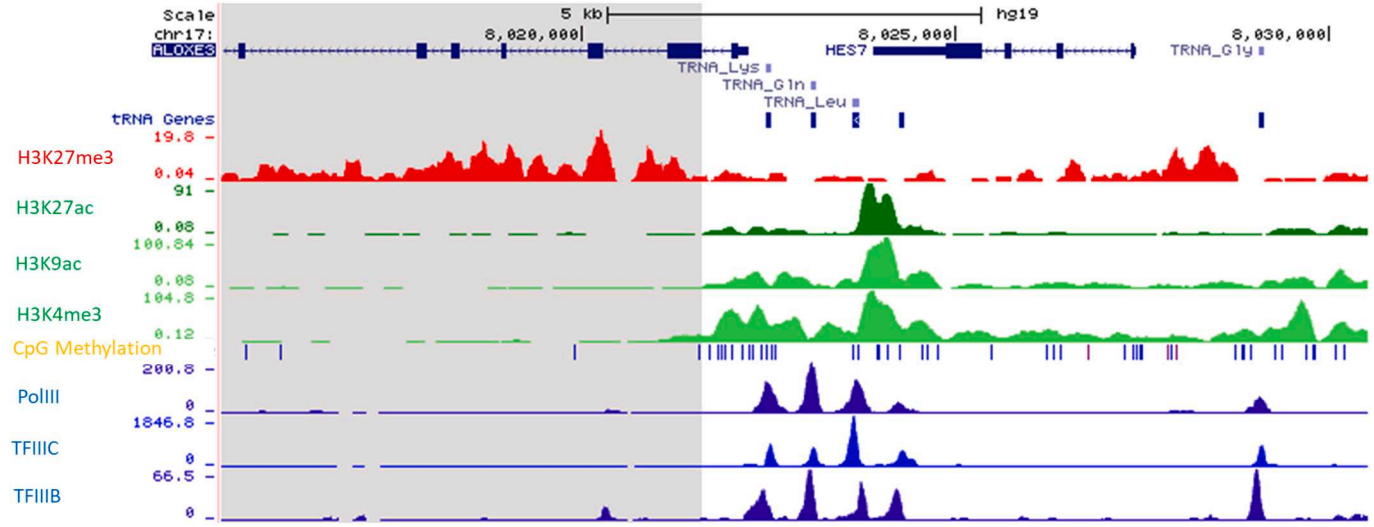



**Fig. 7.** The ALOXE3 tRNA gene cluster partitions chromatin marks at its native loci. Screenshots of UCSC genome browser (*http://genome.ucsc.edu*) showing ENCODE ChIP-Seq peaks for active (green) and repressive (red) chromatin marks, CpG methylation, and Pol III transcription complex components (blue) at the ALOXE3 tRNA gene barrier cluster (tRNA-Lys-TTT-3–5, tRNA-Gln-CTG-1–5, tRNA-Leu-TAG-1–1, tRNA-Arg-TCT-2–1) in K562 cells located on chromosome 17. CpG methylation status: blue line = unmethylated CpG; orange = methylated; purple = partially methylated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

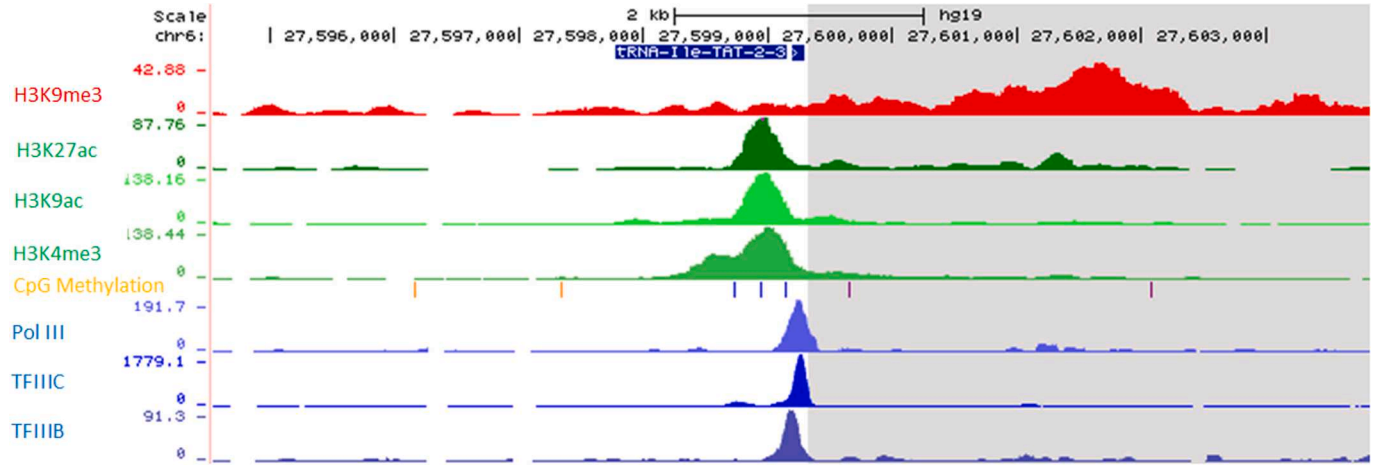



**Fig. 8.** Identification of putative tRNA gene barrier resistant to constitutive heterochromatin spread in K562 cells. Screenshots of UCSC genome browser (*http://genome.ucsc.edu*) showing ENCODE ChIP-Seq data for the distribution of H3K9me3, H3K27ac, H3K9ac, H3K4me3, CpG methylation, Pol III, TFIIIC and TFIIIB at the tRNA-Ile-TAT-2–3 gene on chromosome 6. CpG methylation status: blue line = unmethylated CpG; orange = methylated; purple = partially methylated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

characterised clusters were inactive when tested in isolation (Ebersole et al, 2011; Raab et al, 2012). It will be interesting to see whether the individual human tRNA-Ile-TAT-2–3 and tRNA-Arg-TCG-1–1 genes act as barriers when tested in reporter assays. In the latter case, function may be supported by close proximity to an active pol II promoter that is driving transcription, as demonstrated by trimethylation of H3K36 throughout the POLG gene (Fig. 9).

Instances can also be found of tDNAs in heterochromatic regions despite being occupied by the pol III machinery. For example, Fig. 10 shows three tRNA genes with strong peaks of TFIIIC, TFIIIB and pol III, but nevertheless embedded within a > 15 kb region enriched for heterochromatin marks H3K27me3 and H3K9me3. Two tentative conclusions can be inferred from such cases: (1) assembly of pol III transcription complexes does not guarantee barrier function; and (2) such complexes can exist within heterochromatin. A strong caveat to these inferences is that the apparent coincidence of these marks might in fact result from a mixed population of cells and/or alleles, with some chromosomes carrying active tDNAs and others carrying tDNAs silenced by heterochromatin. This issue could be resolved by single molecule assays or sequential ChIP (ChIP-reChiP).

Mammalian genomes contain large numbers of short-interspersed nuclear elements (SINEs) that evolved from tRNA or 7SL genes and spread by retrotransposition. Many of these retain functional A and B box promoters that can recruit TFIIIC and direct transcription by pol III. A subset of these SINEs also possess insulator activity. This was first demonstrated for one of the ~ 120,000 B2 SINEs that are scattered throughout mouse genomes, which was implicated in protecting the growth hormone gene against silencing (Lunyak et al, 2007). Other examples include specific members of the murine B1 family (Roman et al., 2011) and the human MIR and Alu families of SINEs (Wang et al, 2015). It is noteworthy that in the best-characterised cases of B1 and B2 barriers, inducible pol II promoters located within the SINEs were found to be important for resisting heterochromatic spread (Lunyak et al., 2007; Roman et al., 2011).

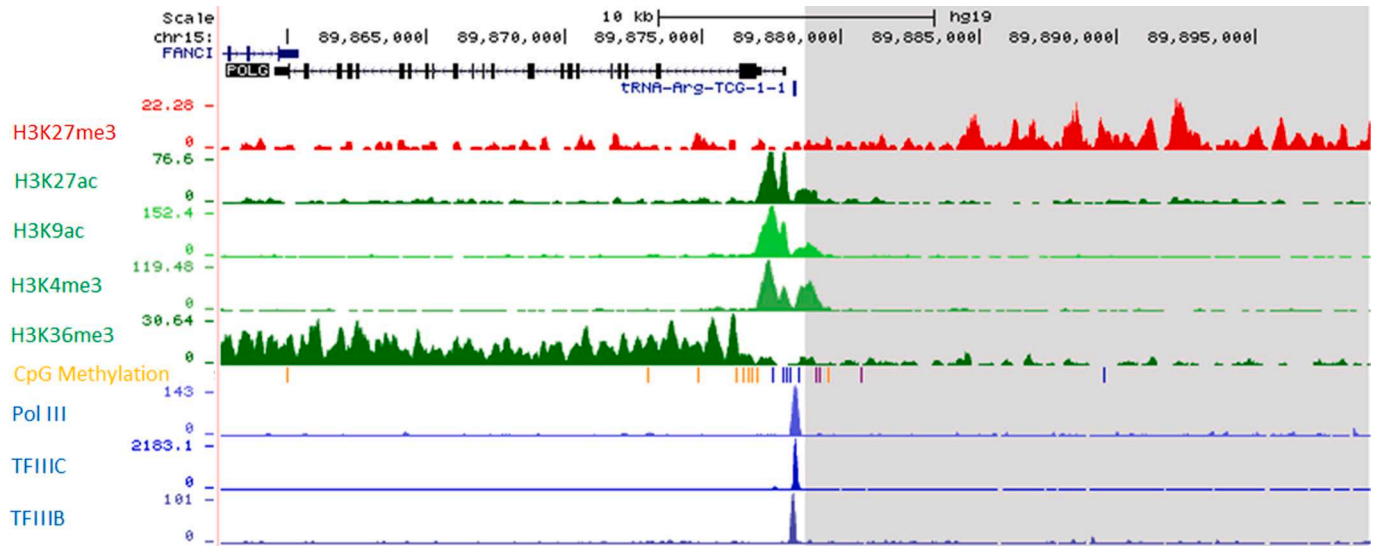



**Fig. 9.** Identification of putative tRNA gene barrier resistant to facultative heterochromatin spread in K562 cells. Screenshots of UCSC genome browser (*http://genome.ucsc.edu*) showing ENCODE ChIP-Seq data for the distribution of H3K27me3, H3K27ac, H3K9ac, H3K4me3, H3K36me3, CpG methylation, Pol III, TFIIIC and TFIIIB at the chromosome 15 domain. tRNA-Arg-TCG-1–1 position is indicated. CpG methylation status: blue line = unmethylated CpG; orange = methylated; purple = partially methylated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

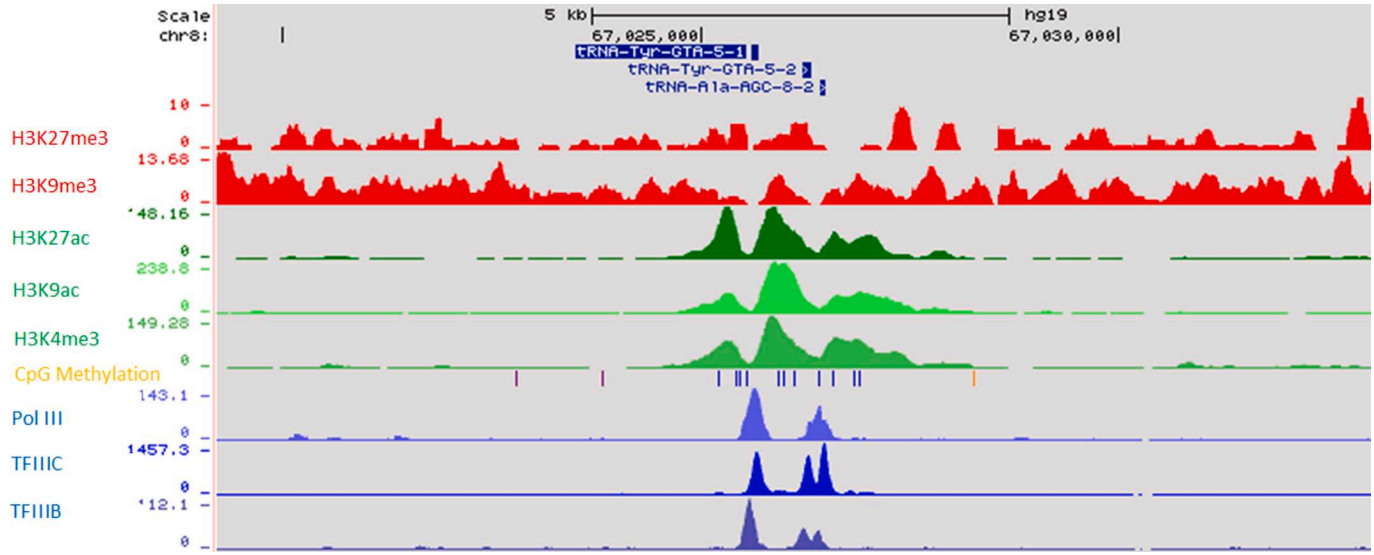



**Fig. 10.** Apparently active tDNA cluster on chromosome 8 appears not to disrupt heterochromatin. Screenshots of UCSC genome browser (*http://genome.ucsc.edu*) showing ENCODE ChIP-Seq data for the distribution of H3K27me3, H3K9me3, H3K27ac, H3K9ac, H3K4me3, CpG methylation, Pol III, TFIIIC and TFIIIB at the tRNA-Tyr-GTA-5–1, tRNA-Tyr-GTA-5–2, tRNA-Ala-AGC-8–2 genes on chromosome 8. CpG methylation status: blue line = unmethylated CpG; orange = methylated; purple = partially methylated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### *3.3. Enhancer-blocking by tDNAs and SINEs*

In addition to acting as barriers to the spread of chromatin states, vertebrate insulators frequently display an ability to disrupt communication between remote enhancers and promoters of protein-coding genes. This behaviour is referred to as enhancer-blocking and is shown by many of the vertebrate pol III-transcribed genes with barrier activity. For example, when transfected into K562 cells the ALOXE3 cluster of four tDNAs can block reporter activation by a beta-globin enhancer with similar efficiency to the duplicated HS4 insulator (Raab et al, 2012). The tDNA-Lys-tDNA-Gln pair from the ALOXE3 quartet is sufficient to function in this enhancer-blocking assay, but its performance is doubled by duplication (Raab et al, 2012). This activity is independent of tDNA orientation, but compromised severely if the B boxes are deleted to prevent recruitment of TFIIIC and hence TFIIIB and pol III (Raab et al, 2012). When other clusters of two to four human tDNAs were tested in this assay, some showed significant enhancer-blocking activity, whereas others were inactive (Raab et al, 2012); the molecular basis of this variation has yet to be dissected.

The enhancer-blocking activity of the HS4 insulator results from its recruitment of the ubiquitous CCCTC-binding factor CTCF (Bell et al, 1999), which has been described as “the primary insulator in mammals” (Phillips-Cremins & Corces, 2013). A heatmap of ChIP-seq data from ENCODE reveals substantial enrichment in K562 cells of CTCF binding close to many tRNA genes (Fig. 11a), but only a small minority of snoRNA and miRNA genes (Fig. 11b). CTCF also localises to tDNAs and ETC sites in a variety of other mammalian cell types (Moqtaderi et al, 2010; Oler et al, 2010; Carrière et al, 2012; Yuen et al, 2017; Van Bortle

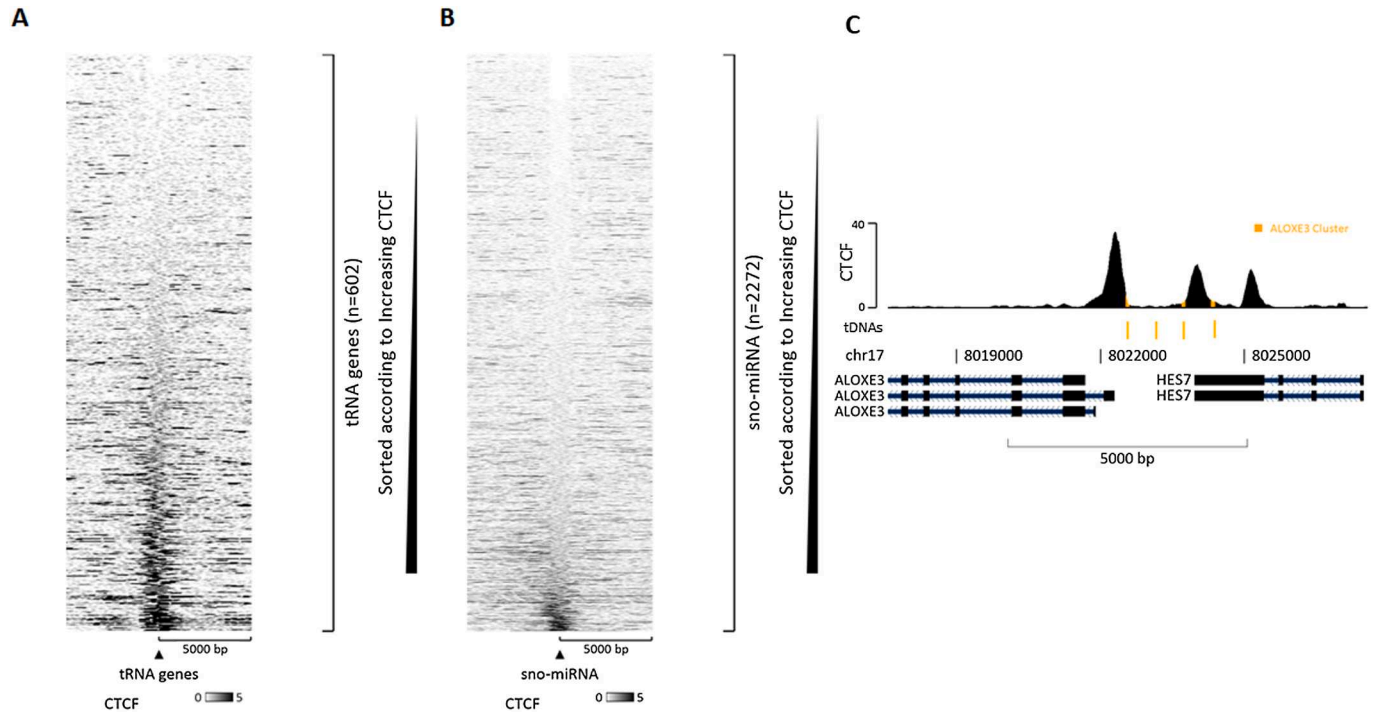



**Fig. 11.** CTCF is frequently enriched in the vicinity of tRNA genes, but not snoRNA and miRNA genes. (A) Heat map depicting CTCF binding 5 kb either side of Hg19 tRNA genes in K562 cells. (B) Heat map depicting CTCF binding 5 kb either side of snoRNA and miRNA genes. (C) CTCF signal intensity in K562 cells at the ALOXE3 tRNA gene barrier cluster (tRNA-Lys-TTT-3–5, tRNA-Gln-CTG-1–5, tRNA-Leu-TAG-1–1, tRNA-Arg-TCT-2–1). Orange lines depict tRNA gene positions. ChIP-Seq datasets downloaded from ENCODE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al. 2017). The discovery that CTCF can co-immunoprecipitate with TFIIIC (Ferrari et al, 2020) suggests that these factors might interact at tDNAs, but the interaction is sensitive to context, being evident in serum-starved breast cancer cells and disappearing after serum stimulation (Ferrari et al, 2020). Co-immunoprecipitation of CTCF with TFIIIC was not observed from mESC or HEK293 cells (Yuen et al, 2017), but this may have reflected their culture with serum. Peaks of CTCF binding are observed within and adjacent to the ALOXE3 tDNA cluster in K562 cells (Fig. 11c), but these binding sites were eliminated from the tDNA-Lys-tDNA-Gln fragment that retained enhancer-blocking activity (Raab et al, 2012). CTCF recruitment may therefore be dispensable for enhancer-blocking by tDNAs, although it is likely to contribute in some contexts.

Several SINEs have also shown enhancer-blocking function, both in cultured cells and in animals (Lunyak et al., 2007; Roman et al., 2011; Wang et al., 2015). The first example was provided by the B2 SINE located upstream of the murine growth hormone gene, which requires both its A and B boxes to block enhancer activity fully (Lunyak et al, 2007). A DNA motif recognised by CTCF is conserved within subclasses of the B2 family and propagation of these SINEs through retrotransposition has spread $>$ 10,000 CTCF binding sites throughout the mouse genome (Bourque et al, 2008; Schmidt et al. 2012).

In contrast to enhancer-blocking, a study in human breast cancer cells detected long-range stimulatory interactions between TFIIIC-bound Alu SINEs and distal promoters of pol II-transcribed genes (Ferrari et al, 2020). For example, an Alu close to the FEM1A gene contacts the UHRF1 gene $\sim$ 150 kb downstream following serum-deprivation; furthermore, deletion of this Alu using CRISPR-Cas9 compromised expression of its remote target (Ferrari et al, 2020). This long-distance interaction was shown by RNAi to require TFIIIC (Ferrari et al, 2020). Histone acetyltransferase activity associated with TFIIIC was implicated in this positive regulation (Ferrari et al, 2020). A notable feature of this Alu-mediated control is its sensitivity to context, as contact with the downstream UHRF1 gene was replaced by an interaction with genes located $\sim$ 200 kb upstream following serum stimulation of the cells (Ferrari et al, 2020).

### 3.4. *Topologically associating domains*

HiC technology reveals that genomes are partitioned into megabase-sized topologically associating domains (TADs) that are conserved between cell types and species (Dixon et al, 2012). A defining feature is that loci interact more frequently with local sites within the same TAD than with loci outside; enhancers share TADs with their target genes and tend not to regulate genes in neighbouring TADs (Dixon et al. 2012; Beagan and Phillips-Cremins 2020). The spread of heterochromatin is constrained by TADs and their boundaries correlate with regions that display barrier activity and are enriched for insulator proteins (Dixon et al. 2012; Beagan and Phillips-Cremins 2020). tRNA genes, SINEs and ETC sites are also enriched at TAD boundaries in mouse and human cells (Dixon et al, 2012; Yuen et al, 2017) and similar observations have been made in Drosophila (Van Bortle et al, 2014). These correlations may at least partly reflect the association of TAD boundaries with high densities of actively-transcribed housekeeping genes.

In budding and fission yeast, the architectural protein condensin interacts with tDNAs and ETC/COC sites and mediates their three-dimensional organisation within the nucleus (Thompson et al. 2003; Noma et al. 2006; Haeusler et al. 2008; Hiraga et al. 2012). Drosophila TFIIIC localises to TAD boundaries enriched for condensin, cohesin and other architectural proteins and the relative occupancy of these proteins scales with boundary strength (Van Bortle et al, 2014). A similar relationship exists between mammalian TADs and TFIIIC, CTCF, cohesin and condensin (Van Bortle et al, 2014). In human and mouse cells, condensin II colocalises with tDNAs and ETC sites with strong TFIIIC occupancy (Yuen et al, 2017). Indeed, 60% of all TAD boundaries contain ETC associated with condensin II in mouse ES cells (Yuen et al, 2017). ETC clusters correlate with stronger boundaries, whereas ETC with lower TFIIIC occupancy have less condensin II associated and are also less enriched at TAD boundaries (Yuen et al, 2017). Human TFIIIC and

condensin II can be co-immunoprecipitated from extracts of HEK293T cells (Yuen et al, 2017), as is the case for TFIIIC and condensin in budding yeast extracts (Haeusler et al. 2008). RNAi-mediated depletion of TFIIIC abolished recruitment of condensin II to ETC in mouse ES cells, whereas the converse was not observed, demonstrating that TFIIIC recruits condensin II to these loci, rather than *vice versa* (Yuen et al, 2017). As at active tRNA genes (Barski et al. 2010), H3K4me3 is enriched at ETC sites bound by condensin II (Yuen et al, 2017). Histone H3 peptides marked with H3K4me3 can bind condensin II *in vitro* (Yuen et al, 2017). ETC occupancy by both condensin II and TFIIIC was severely reduced when H3K4me3 levels were reduced in HEK293T cells by RNAi-mediated depletion of the WDR5 subunit of the H3K4 methylase complex COMPASS (Yuen et al, 2017). Thus, stable association of condensin II with ETC sites in human cells requires both TFIIIC and H3K4me3 (Yuen et al, 2017). In contrast to these data implicating TFIIIC in TAD formation, specific removal of the largest pol III subunit (POLR3A/RPC1) from mouse ES cells using an auxin-inducible degron had minimal effect on the TAD boundary insulation score (Jiang et al. 2020). A similar approach with TFIIIC could be informative.

### 3.5. Mechanisms underlying TFIIIC-based insulators in mammalian cells

Although the mechanistic basis of TFIIIC-based barrier function has been studied extensively in *S. cerevisiae* and *S. pombe*, far less is known in metazoa. Nucleosome depletion of tDNAs in yeast is thought to be important in preventing the propagation of heterochromatin (Yuan et al, 2005; Dhillon et al, 2009). Active human tRNA genes are also highly depleted of nucleosomes (Barski et al. 2010), which is why they frequently coincide with troughs of histone modification, flanked by peaks where histone occupancy resumes (clear examples can be seen in Figs. 9 and 10). For example, a tRNA-Leu on human chromosome 14 has a nucleosome-depleted region in K562 cells that extends from ~ 100 bp upstream to > 200 bp downstream of the transcription start site, thereby encompassing the entire gene (Helbo et al. 2017). Just as yeast TFIIIC can compete effectively with nucleosomes (Burnol et al. 1993), human TFIIIC remains at tDNAs in highly compacted metaphase chromosomes where many transcription factors are displaced by chromatin condensation (Fairley et al, 2003; Fairley et al, 2012). Although pol III dissociates when human metaphase chromosomes condense, the Brf1 subunit of TFIIIB remains at genes with TFIIIC (Fairley et al, 2003; Fairley et al, 2012); this might be important to maintain insulation, as Brf1 mutations cause a strong reduction in barrier activity of budding yeast HMR tDNA-Thr (Donze & Kamakaka, 2001).

The HMR tRNA-Thr gene loses barrier activity in rsc2Δ mutants of the abundant RSC remodelling complex (Jambunathan et al, 2005; Dhillon et al, 2009), which occupies all tRNA genes in *S. cerevisiae*, assisting in the displacement of nucleosomes (Ng et al, 2002). Human SWI/SNF remodelling complexes are homologous to RSC and were detected at 65% of pol III-occupied loci in HeLa cells, including many that are remote from pol II-transcribed regions (Euskirchen et al, 2011). Heat maps of SWI/SNF subunits SMARCC2 (BAF170) and SMARCE1 (BAF57) show that the complex also localises to many tRNA genes in K562 cells (Fig. 12A and C), but only a very few snoRNA and miRNA genes (Fig. 12B and D). A role in nucleosome displacement at tDNAs seems likely for SWI/SNF and this might contribute to barrier activity. Experiments are required to test the extent to which remodelling complexes contribute to tDNA barrier function in mammalian cells. It also remains to be determined how SWI/SNF complexes are recruited to tDNAs. TFIIIB, TFIIIC and pol III were not amongst the 158 proteins found by mass spectrometry to co-immunoprecipitate with SWI/SNF from HeLa cells (Euskirchen et al, 2011). However, stable association was detected with RelA (Euskirchen et al, 2011), which itself interacts with TFIIIB (Graczyk et al, 2015). With the caveat that RelA/TFIIIB association was not tested in HeLa or K562 cells, it is possible that SWI/SNF is recruited to human tDNAs via TFIIIB and RelA.

Barrier function of the *S. cerevisiae* HMR tRNA-Thr gene is compromised by mutations in the architectural protein cohesin (Donze et al, 1999). A conserved role at tDNA barriers is probable, as cohesin co-localises with TFIIIC on chromosomes of budding and fission yeasts, flies, mice and humans (Dubey & Gartenberg, 2007; Van Bortle et al, 2014; Kim et al, 2016; Büchel et al, 2017; Yuen et al, 2017). Human cohesin was found to co-immunoprecipitate with TFIIIC from neuroblastoma cells, but not from HEK293 cells, despite strong ChIP evidence for recruitment to tDNAs and ETC sites in both cell types (Büchel et al, 2017; Yuen et al, 2017). This discrepancy might simply reflect differences in assay stringency or may be caused by the presence of distinct post-translational modifications or binding partners in the two contexts. For example, recruitment of cohesin to ETC sites by TFIIIC is assisted in neuroblastoma cells by N-MYC (Büchel et al, 2017), which is absent from HEK293 cells. Cohesin is required for insulation by CTCF (Phillips-

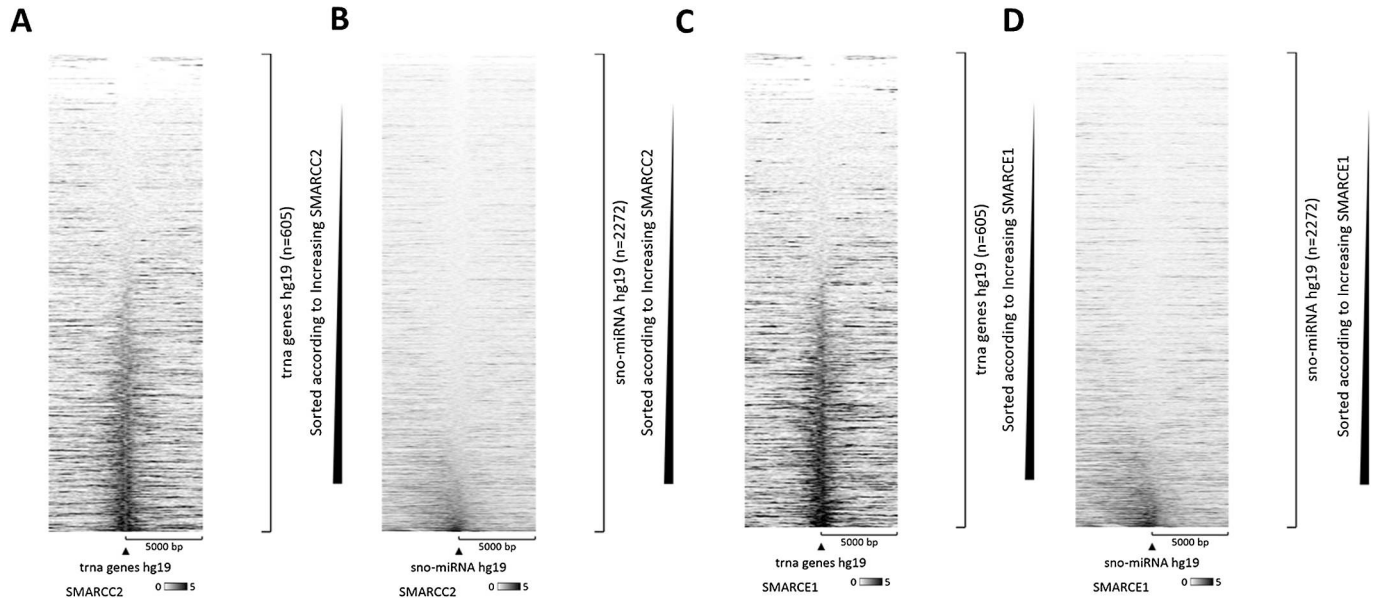



**Fig. 12.** SWI/SNF subunits are selectively enriched at tRNA genes in K562 cells. Heatmaps showing signal intensity of SMARCC2 (A and B) and SMARCE1 (C and D) reads across 10 kb centred around tRNA genes (A and C) and sno-miRNA genes (B and D). ChIP-Seq datasets were downloaded from ENCODE.

Cremins & Corces, 2013), and ChIP-seq shows CTCF at many, but not all, tDNA and ETC loci occupied by both cohesin and TFIIIC in mouse and human cells (Moqtaderi et al. 2010; Oler et al, 2010; Carrière et al, 2012; Büchel et al, 2017; Yuen et al, 2017). CTCF may assist insulation by tDNAs and ETC, but seems not to be essential (Raab et al, 2012), which is consistent with the fact that cohesin and TFIIIC cooperate at barriers in yeasts that have no CTCF. Some studies have found that CTCF contributes specifically to the enhancer-blocking function of insulators and not to barrier activity (Bell et al, 1999; Nora et al, 2017), whereas other studies found evidence of barrier function (Cuddapah et al, 2009; Lu et al, 2016); this discrepancy may reflect context, such as different cell types (Cuddapah et al, 2009; Lu et al, 2016). As mentioned above, stable association of CTCF with TFIIIC was found to be sensitive to culture conditions (Ferrari et al, 2020). When bound in proximity to tDNAs, it seems likely that CTCF will contribute to insulation, at least under some circumstances. However, barrier activity is displayed by some tDNAs in the apparent absence of CTCF.

Several HATs contribute to the barrier activity of tDNAs in *S. cerevisiae*, including Rtt109 and GCN5 (Kirkland et al. 2013). Although Rtt109 is only found in fungi, homologues of GCN5 are present in mammals. Indeed, human GCN5 was shown to be recruited to tDNAs in HeLa cells and stimulate their expression (Kenneth et al, 2007; Sadeghifar et al, 2015). In response to GCN5, acetylation of histone H3 increases at tDNAs, including at H3K9 (Kenneth et al, 2007 Sadeghifar et al, 2015). As acetylation and methylation of lysine side chains are mutually exclusive, this is expected to oppose formation of the repressive H3K9me3 mark that is characteristic of constitutive heterochromatin. Thus, GCN5 may be important for barrier function at tDNAs in mammals, as in budding yeast. Its recruitment to mammalian tDNAs is promoted by MYC (Kenneth et al, 2007; Sadeghifar et al, 2015), which was detected by ChIP-seq at 74% of pol III-occupied genes in K562 cells (Raha et al, 2010). The presence of MYC at these sites can be explained by stable interactions with both TFIIIB (Gomez-Roman et al 2003; Ernens et al, 2006; Steiger et al, 2008; Sadeghifar et al, 2015) and TFIIIC (Koch et al, 2007; Büchel et al, 2017), associations that have been detected in several cell types and species. MYC depletion by RNAi reduces H3K9 acetylation at tDNAs substantially (Sadeghifar et al, 2015). This dependence on MYC is noteworthy, because MYC expression decreases markedly when cells stop growing, which might be predicted to reduce GCN5 occupancy and H3K9 acetylation. Although it has not been tested, MYC-dependence of GCN5 recruitment could potentially compromise the efficacy of tDNA barriers following growth arrest.

Mammalian TFIIIC has also been shown to interact with p300, a HAT that is not present in yeast (Mertens et al, 2008). Binding of endogenous p300 to a tRNA-Gln gene in HeLa cells was demonstrated by ChIP-PCR (Mertens and Roeder, 2008). Whether this interaction is widespread has not been reported, so we plotted a heat map of ENCODE ChIP-seq data to assess how frequently p300 associates with tDNAs across the whole genome of K562 cells; this demonstrates substantial enrichment of p300 at many tRNA genes (Fig. 13A). There is specificity to this interaction, as it is only detected at a tiny minority of snoRNA and miRNA genes (Fig. 13B). Peaks of p300 binding can be found at each of the four tDNAs at the ALOXE3 barrier cluster (Fig. 13C).

The presence of p300 may counteract the spread of H3K27me3 and facultative heterochromatin, as H3K27 is acetylated by p300, thereby precluding its trimethylation. The SWI/SNF complex might also help some tDNAs to counteract the spread of H3K27me3, as it has been shown to displace EZH2, the histone methylase responsible for depositing H3K27me3 (Kia et al, 2008; Kadoch et al. 2017). Such action might additionally reduce the spread of DNA methylation, since EZH2 binds directly to DNA methyltransferases and recruits them to target loci (Viré et al, 2006). Thus, SWI/SNF can be predicted to bolster the barrier function of tDNAs not only through nucleosome displacement but also as an opponent of methylation on DNA and H3K27. However, the impact of SWI/SNF and other remodelling complexes on insulation in mammalian cells have yet to be tested in reporter assays.

The paradigm vertebrate insulator HS4 utilises CTCF for enhancer-blocking, as already discussed, but its barrier activity is mediated by the USF1 and USF2 transcription factors that recruit histone acetylases and methyltransferases to prevent encroachment of heterochromatin

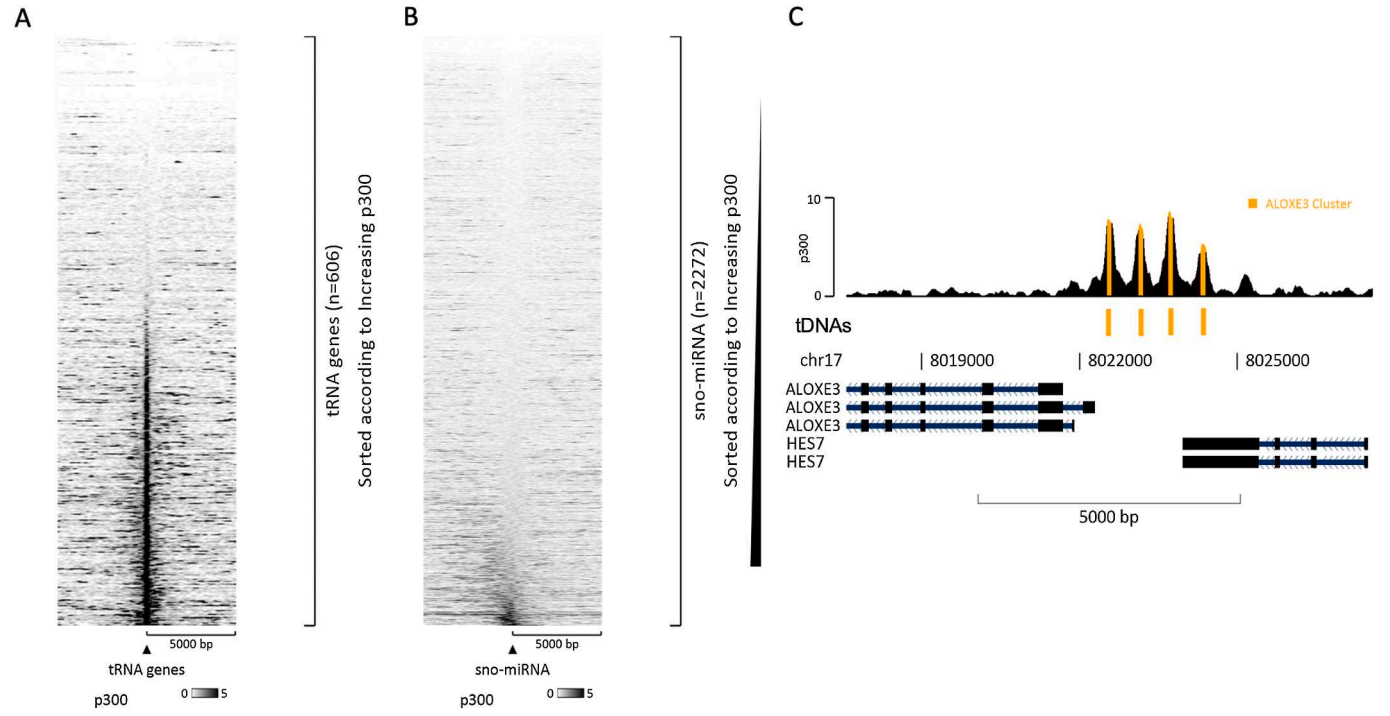



**Fig. 13.** p300 is enriched at tRNA genes, but not snoRNA and miRNA genes. (A) Heat map depicting p300 binding 5 kb either side of Hg19 tRNA genes in K562 cells. (B) Heat map depicting p300 binding 5 kb either side of snoRNA and miRNA genes. (C) p300 signal intensity in K562 cells at the ALOXE3 tRNA gene barrier cluster (tRNA-Lys-TTT-3–5, tRNA-Gln-CTG-1–5, tRNA-Leu-TAG-1–1, tRNA-Arg-TCT-2–1). Orange lines depict tRNA gene positions. ChIP-Seq datasets were downloaded from ENCODE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(West et al, 2004). A proteomic screen identified the essential GTF3C1 subunit of TFIIIC amongst the proteins that interact with USF1 when overexpressed in HeLa cells (Huang et al, 2007). We therefore investigated if USF1 is commonly associated with tRNA genes *in vivo*. Heatmaps of ChIP-seq data from ENCODE reveal substantial enrichment of USF1 close to many tRNA genes (Fig. 14A), in contrast to other noncoding RNA genes (Fig. 14B). A similar pattern is seen for USF2, although enrichment at tDNAs is less strong than for USF1 (Fig. S3A and S3B). It seems likely that the presence of this metazoan insulator protein could strengthen the barrier function of at least some tDNAs, but the data do not show enrichment of USF1 or USF2 at the ALOXE3 tDNA cluster in K562 cells (Fig. S3C), so it is probably not essential. As TFIIIC is readily detected at the ALOXE3 tDNA genes (Fig. 7), the apparent absence of USF1 in this case suggests that its interaction with TFIIIC may be regulated *in vivo*. Perhaps this provides a mechanism for controlling barrier activity.

DNA methylation is an important mechanism of epigenetic control that is found in vertebrates, whereas little or no DNA methylation is found in yeast (Proffitt et al, 1984). It can suppress gene expression by reducing the affinity of transcription factors for DNA and/or by recruiting repressor proteins that recognise methylated DNA specifically (Tate and Bird, 1993; Choy et al, 2010). Methylation occurs predominantly on CpG dinucleotides and is associated with condensed nuclease-resistant heterochromatin (Razin and Cedar, 1977; Clark et al, 1995). The level of DNA methylation is low at most actively transcribed tRNA genes, but is much higher at most SINEs (Meissner et al. 2008; Varshney et al. 2015). However, binding of TFIIIC, TFIIIB and pol III to Alu, B1 and B2 SINEs *in vivo* is not impeded by DNA methylation, even within the A and B boxes (Varshney et al. 2015). Expression of SINEs is unchanged in cells with DNA methyltransferases knocked out and SINE transcription is unaffected by short-term treatment with 5-azacytidine that is sufficient to release methylCpG-binding proteins and restore expression of mRNAs that are silenced by DNA methylation (Varshney et al. 2015).

A large proportion of active tDNAs reside in CpG islands that overlap promoters of housekeeping genes (Oler et al, 2010). Indeed, expression of tRNA genes is very strongly correlated with local CpG density (Thornlow et al. 2020). This is well-illustrated in Figs. 7-10, where the frequency of CpG dinucleotides is highest in the vicinity of these active tDNAs, relative to more distal regions. Furthermore, in all these cases the CpGs overlapping the tDNAs are unmethylated, a feature of CpG islands. In general, this absence of methylation extends well beyond the tDNAs and in the case of the ALOXE3 locus a broad region that is enriched in unmethylated CpG reaches from upstream of the tDNA cluster to encompass the pol II-transcribed HES7 gene and an isolated tDNA further downstream (Fig. 7). In contrast, the tRNA-Arg-TCG-1–1 gene on human chromosome 15 is surrounded by methylated CpG, although the CpGs overlapping the tDNA itself are unmethylated (Fig. 9). Such highly localised effects may reflect the presence of R-loops, RNA-DNA hybrids that form when nascent transcripts displace one strand of a DNA duplex, as these structures suppress the *de novo* methylation of CpG by DNMT3b (Ginno et al, 2012). R-loop formation is consistently detected at active tRNA genes, reflecting their high rates of transcription (El Hage et al, 2014). This can be seen clearly in a heatmap plotting R-loops relative to tDNAs in K562 cells (Fig. 15A). The strong presence of R-loops at most tRNA genes contrasts with their absence from the majority of snoRNAs and miRNAs, two other classes of short non-coding RNA genes that are less strongly transcribed (Fig. 15B). More detailed analyses (Fig. 15C-15G) confirm individual R-loop peaks at each of the tDNAs bound by pol III in Figs. 6-10. However, R-loops are not detected at ETC sites occupied by TFIIIC in the absence of pol III

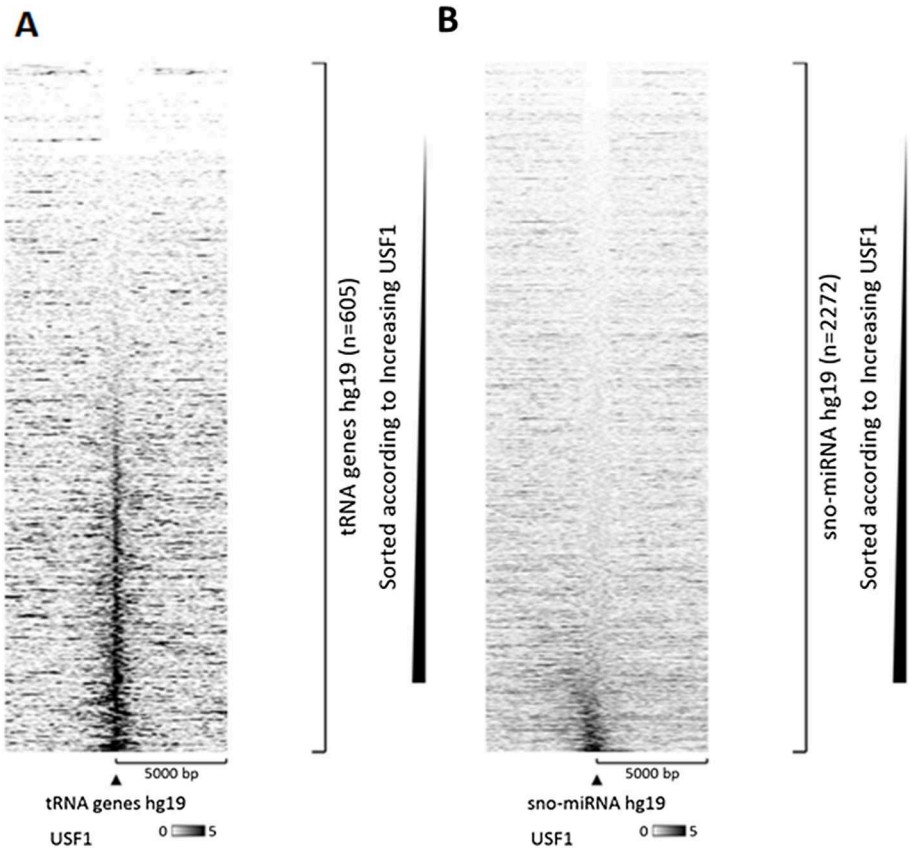



**Fig. 14.** USF1 is selectively enriched at tRNA genes in K562 cells. Heatmaps showing signal intensity of USF1 reads across 10 kb centred around tRNA genes (A) and sno-miRNA genes (B). A similar approach for USF2 provided less convincing evidence for enrichment at tDNAs in this cell type. ChIP-Seq datasets were downloaded from ENCODE.

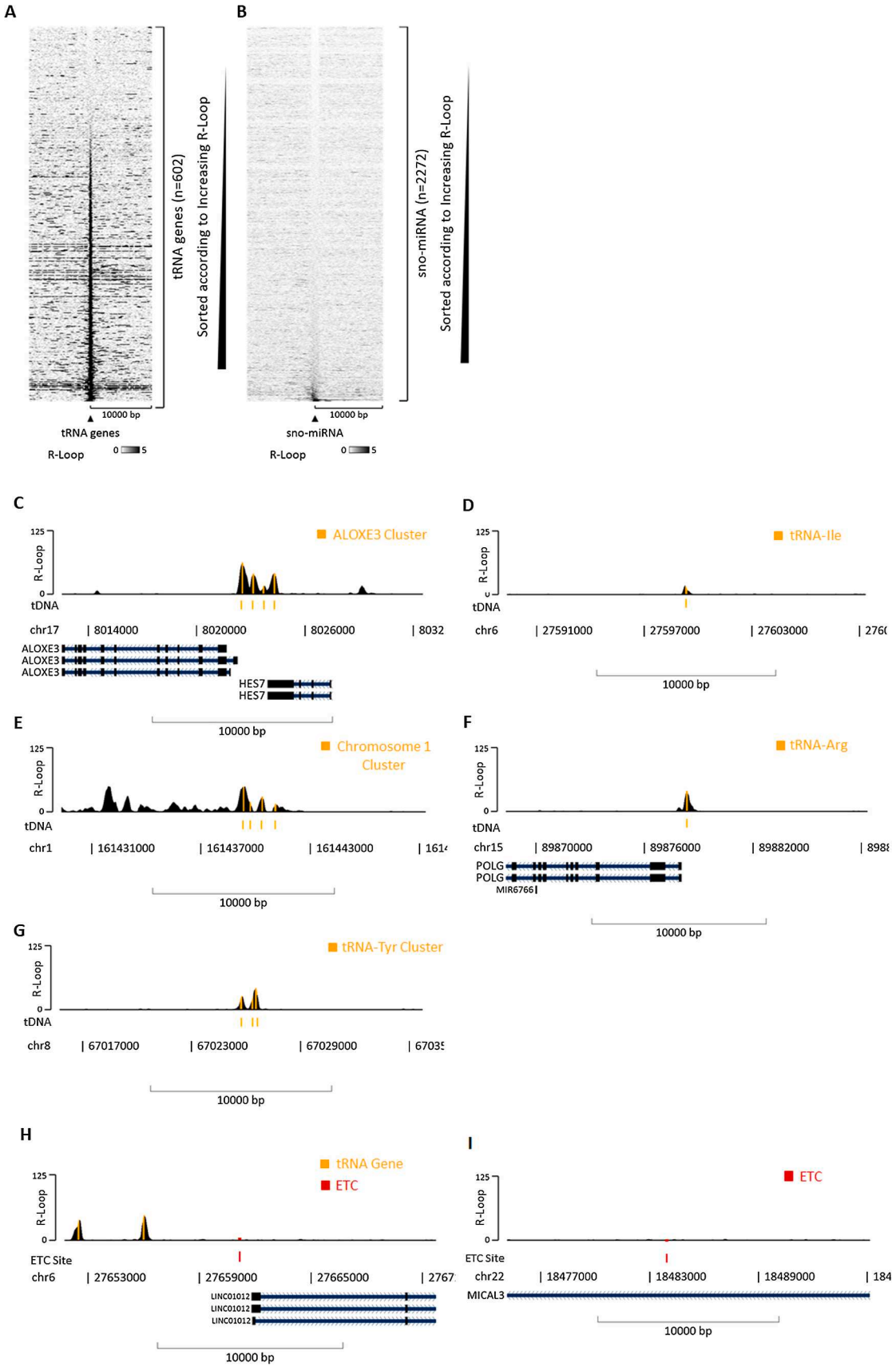



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**Fig. 15.** R-Loops are enriched around tRNA genes. Heatmap showing R-ChIP signal intensity reads covering 20,000 bp centered around (A) tRNA genes and (B) snomiRNA genes. R loop signal intensity on both strands and 20,000 bp surrounding the (C) ALOXE3 tRNA gene cluster (tRNA-Lys-TTT-3–5, tRNA-Gln-CTG-1–5, tRNA-Leu-TAG-1–1, tRNA-Arg-TCT-2–1), (D) the tRNA- Ile-TAT-2–3 located on chromosome 6, (E) the chromosome 1 tRNA gene cluster (tRNA-Glu-CTC-1–5, tRNA-Gly-TCC-2–5, tRNA-Asp-GTC-2–5, tRNA-Leu-CAG-1–5), (F) the tRNA-Arg-TCG-1–1 located on chromosome 15, (G) the tRNA-Tyr cluster located on chromosome 8 (tRNA-Tyr-GTA-5–1, tRNA-Tyr-GTA-5–2, tRNA-Ala-AGC-8–2). R loop signal intensity on both strands at ETC sites and 20,000 bp surrounding the ETC sites located at (H) Chr6:27661001–27662472 and (I) Chr22:18483080–18486926. ETC site coordinates were identified by Moqtaderi et al. (2010). Orange lines depict tRNA gene positions, red lines depict approximate ETC positions. ChIP-Seq datasets were downloaded from NCBI (Chen et al. 2017). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 15H and 15I), as expected due to the absence of transcription. By protecting CpG sites from the action of DNMT3b, R-loops may provide a mechanism that assists tDNAs to counteract propagation of heterochromatin through DNA methylation. This possibility has yet to be tested experimentally.

## 4. Discussion

The tRNA genes are the most highly conserved barriers in eukaryotic evolution. This provides opportunities to gain mechanistic insights by comparisons between diverse organisms where the properties of chromatin differ. For example, heterochromatin is very different in *S. cerevisiae* from that in *S. pombe*, the two species where tRNA and ETC barriers have been studied most extensively to date. To assist in bridging that gap in both evolution and understanding, we plan to characterise barrier function in *P. pastoris*, a methylotrophic yeast that is used extensively in biotechnology (Ahmad et al, 2014; Bernauer et al, 2020). As a first step in this endeavour, we have tested six individual tRNA-Thr genes and one tRNA gene pair from *P. pastoris*, that were selected solely on the basis of their shared isotype with the first discovered tDNA barrier, the HMR tRNA-Thr gene (Donze and Kamakaka, 2001). It was a surprise to find that all six function efficiently in *S. cerevisiae* to protect a reporter against the spread of heterochromatin from the HMR-E silencer, as did a tRNA-Thr-tRNA-Glu pair (Fig. 5). This contrasts strikingly with the finding that only one of the 16 $tRNA^{Thr}$ genes in *S. cerevisiae* displayed strong barrier activity in the same assay (Donze and Kamakaka, 2001). Indeed, the *P. pastoris* tRNA-Thr genes outperformed the *S. cerevisiae* HMR tRNA-Thr gene when tested in parallel in budding yeast, despite the latter's home advantage. This unexpected observation offers a hint that the chromatin environment of *P. pastoris*, which is largely uncharacterised to date, might have driven tDNAs to evolve more robust barrier activities than those of most tDNAs in *S. cerevisiae*. Much more work will be required to test the accuracy of this speculation.

The vertebrate chromatin environment resembles that of *S. pombe* more closely than that of *S. cerevisiae*. Indeed, the tDNA-Lys-tDNA-Gln pair from the ALOXE3 cluster on human chromosome 17 can protect a reporter gene against silencing in *S. pombe* with efficiency comparable to that of the HMR tRNA-Thr gene from *S. cerevisiae* (Raab et al, 2012). Thus, the human tDNA pair has features that allow it to perform as a barrier to heterochromatic spread in fission yeast. Minimally, it may be that the ability to recruit TFIIIC robustly is sufficient in this context, as ETC/COC sites have barrier activity in yeast (Noma et al, 2006). The B box responsible for TFIIIC recruitment to tRNA genes is under stringent evolutionary constraint for at least two reasons: it is required for expression of the gene but, in addition, it is transcribed into a critical part of the tRNA product. A conserved B box that can recruit TFIIIC and its associated proteins and retain them with sufficient stability may suffice to prevent silencing of the reporter in the fission yeast barrier assay.

Chromatin is more complex in metazoa than in fungi and presents additional challenges to barriers. These include DNA methylation, as well as facultative heterochromatin that forms at histones marked with H3K27me3 by polycomb complexes. Has TFIIIC evolved novel activities to counteract these threats? Perhaps not, as RSC is important for tDNA barriers in yeast and the homologous SWI/SNF complexes in mammalian cells can effectively oppose the polycomb protein EZH2, that trimethylates H3K27 and also recruits DNA methyltransferases (Kia et al, 2008; Kadoch et al. 2017). Retaining the ability to recruit SWI/SNF family remodelers may therefore have been sufficient for tDNAs to meet new chromatin challenges, if those remodelers themselves have adapted to deal with the evolving environment. The high levels of R-loops that form at tRNA genes due to their rapid transcription may also protect against DNA methylation. This hypothesis might explain why ETC sites were found to be insufficient to act as barriers to reporter silencing in mouse cells (Ebersole et al, 2011), whereas they display this activity in yeasts, where DNA methylation is absent; R-loops are absent from ETC sites because they are not transcribed (Fig. 15), which may undermine their barrier potential specifically in higher organisms where DNA becomes methylated.

Mammalian insulators are generally modular and combine distinct mechanisms to achieve their functions effectively; a good example is the use of both CTCF and USF by the HS4 insulator (Phillips-Cremins & Corces, 2013). Although not yet confirmed experimentally, we can expect that several factors not found in yeasts are exploited in metazoa to augment the security of tDNA barriers. For example, *Drosophila* TFIIIC was found by co-immunoprecipitation to associate with CP190 and Mod (mdg4), two insulator proteins in fruit flies (Van Bortle and Corces, 2012). Human TFIIIC interacts in HeLa cell extracts with overexpressed USF1 (Huang et al, 2007), the factor responsible for barrier activity of the HS4 insulator (West et al, 2004). Heat maps show specific localisation of endogenous USF1 at many tDNAs in K562 cells (Fig. 14). Perhaps TFIIIC promotes USF1 recruitment to some tDNAs, both through physical interaction and also by pioneering to generate a region of nucleosome depletion; USF1 might then be expected to bolster barrier function at these sites by recruiting additional histone methyltransferases and acetylases. Similarly, some tDNA insulators may exploit proximal binding of CTCF (Fig. 11; Moqtaderi et al, 2010; Oler et al, 2010; Carrière et al, 2012; Yuen et al, 2017; Van Bortle et al. 2017). Nevertheless, it is important to remember that chromatin borders only coincide with 4–6% of sites bound by CTCF genome-wide, so the presence of this "primary insulator" protein is by no means sufficient to create a functional barrier (Phillips-Cremins and Corces, 2013). Distinct modules may be combined with specific tDNAs to create composite insulators with mechanisms of action that might vary between different loci (Fig. 16).

The chromatin environment of mammalian genes can change between cell types, which may require barriers to adapt to varying challenges. Some barriers may only be used in specific cell types, whereas others may be needed constitutively. TFIIIC is an essential transcription factor that can be assumed to be functional in all nucleated cells and ~63% of tRNA genes are active throughout mouse development in tissues as diverse as brain and liver (Kutter et al. 2011, Schmitt et al. 2014). In contrast, ~9% of murine tDNAs are only expressed at specific stages of brain and liver development (Schmitt et al. 2014); it is not yet known how such regulation is achieved, as the essential pol III machinery must remain active. Transcriptional repression of tDNAs tends not to be accompanied by dissociation of TFIIIC from DNA, as recruitment of TFIIIB and/or pol III are most commonly the steps that are targeted by regulators (Sutcliffe et al. 2000; Crighton et al. 2003; Kenneth et al. 2007; Cabart et al., 2008; Vannini et al. 2010; Fairley et al. 2012; Orioli et al. 2016). This is well-illustrated by the silencing of tDNAs in mitotic HeLa cells, where TFIIIC remains in position whilst pol III and part of TFIIIB are displaced (Fairley et al, 2003; Fairley et al, 2012); in this way, the tDNAs that were active prior to division are bookmarked for

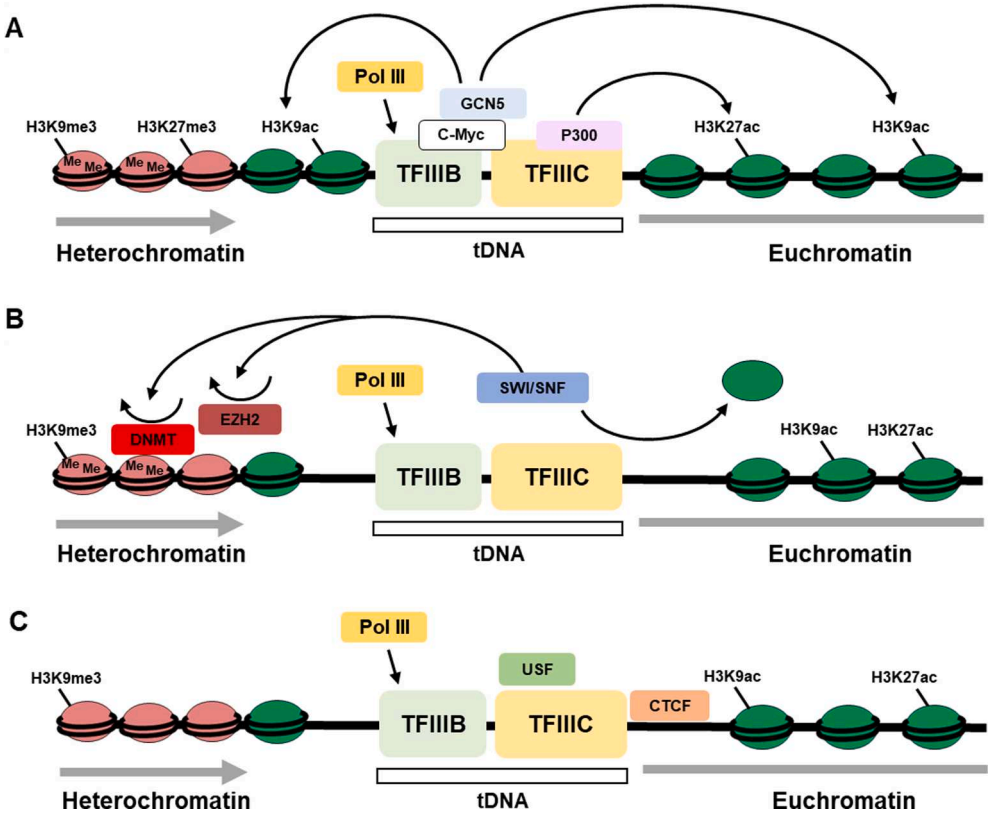


**Fig. 16.** Schematic depicting putative mechanisms by which mammalian TFIIIC may work as a barrier. (A) Pol III transcription complex recruits acetyltransferases GCN5 and P300; these deposit euchromatic marks H3K27ac and H3K9ac, preventing the spread of heterochromatic marks H3K27me3 and H3K9me3. (B) The chromatin remodelling complex SWI/SNF is recruited to tRNA genes and ejects nucleosomes; this creates a gap in the nucleosome occupancy, inhibiting the propagation of heterochromatin. SWI/SNF has also been shown to counteract EZH2, the histone methyltransferase responsible for the facultative heterochromatin mark H3K27me3; as EZH2 can recruit DNA methyltransferases, its ejection by SWI/SNF may help oppose the spread of DNA methylation. In addition, the presence of R-loops at highly transcribed tDNAs may prevent local methylation of DNA. (C) Insulation at some tDNAs may also be buttressed in some contexts by interaction of TFIIIC with factors such as USF and CTCF.

preferential reactivation in daughter cells. Thus, retention of TFIIIC may allow barriers to endure even when transcription is inhibited. On the other hand, regulation of its interactions may impact insulation; a potential example of this is suggested by the sensitivity to culture conditions of TFIIIC binding to CTCF (Ferrari et al, 2020). Mechanistic understanding of tDNA barrier activity in metazoa will be required before we can predict with confidence how it might vary between cell types.

A surprising fact is that TFIIIC is poorly conserved at the sequence level, despite its functions in transcription and barriers that are retained through evolution. For example, the Sfc6p subunit of TFIIIC in *S. pombe* is only 25% identical to TFC6p, its homologue in *S. cerevisiae*, and 26% identical to its human homologue GTF3C2 (TFIIIC110); similarly weak conservation is found for other TFIIIC subunits (Huang et al, 2000). In contrast, the Brf subunit of *S. pombe* TFIIIB is 37% and 47% identical to its budding yeast and human homologues, respectively, and the largest pol III subunit shows 57% and 51% identity between these species (Huang et al, 2000).

Our attempts to understand barriers based on TFIIIC have drawn on a variety of assay types and experimental systems, as well as model organisms that span the evolutionary spectrum. Such an approach can be extremely powerful in uncovering commonalities, which are likely to underlie the key functional requirements, but risks overlooking context-specific details that are almost certainly important for each individual barrier, reflecting its immediate surroundings in terms of sequence, chromatin context and the proximity and nature of other genes. Conclusions drawn for a strong barrier may be less appropriate for a weaker one. Although the first discovery of a TFIIIC-based barrier was in the last century (Donze et al. 1999) and multiple studies have followed, we remain a very long way from the goal of defining predictive rules that will allow the design of customised insulators that are tailored to the needs of synthetic biology.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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*Author contributions*

RES and NC wrote drafts of this manuscript and generated all data, except Fig. 13: NC was responsible for Figs. 1-5 and RES for Figs. 6-12, 14 and 15. SPB generated Fig. 13. DD provided reagents, protocols and barrier expertise. NJB and RJW were responsible for supervision. The study was conceived and guided by RJW, who also helped write the manuscript. All authors contributed to discussion of the project and manuscript.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2022.146533.

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## Further reading

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