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TFIIIC Bound DNA Elements in Nuclear Organization and Insulation

Jacob G. Kirkland, **Jesse R. Raab**, and **Rohinton T. Kamakaka**
Department of MCD Biology, 249 Sinsheimer Labs, 1156 High Street, University of California, Santa Cruz, CA 95064

Rohinton T. Kamakaka: rohinton@ucsc.edu

Abstract

tRNA genes (tDNAs) have been known to have barrier insulator function in budding yeast, *Saccharomyces cerevisiae,* for over a decade. tDNAs also play a role in genome organization by clustering at sites in the nucleus and both of these functions are dependent on the transcription factor TFIIIC. More recently TFIIIC bound sites devoid of pol III, termed Extra-TFIIIC sites (ETC) have been identified in budding yeast and these sites also function as insulators and affect genome organization. Subsequent studies in *Schizosaccharomyces pombe* showed that TFIIIC bound sites were insulators and also functioned as Chromosome Organization Clamps (COC); tethering the sites to the nuclear periphery. Very recently studies have moved to mammalian systems where pol III genes and their associated factors have been investigated in both mouse and human cells. Short Interspersed Nuclear Elements (SINEs) that bind TFIIIC, function as insulator elements and tDNAs can also function as both enhancer -blocking and barrier insulators in these organisms. It was also recently shown that tDNAs cluster with other tDNAs and with ETCs but not with pol II transcribed genes. Intriguingly, TFIIIC is often found near pol II transcription start sites and it remains unclear what the consequences of TFIIIC based genomic organization are and what influence pol III factors have on pol II transcribed genes and vise versa. In this review we provide a comprehensive overview of the known data on pol III factors in insulation and genome organization and identify the many open questions that require further investigation. \

Keywords

TFIIIC; Insulator; tDNA; Chromatin folding

Introduction

Transcription in the eukaryotic nucleus is mediated by three RNA polymerases [1]. Generally speaking RNA pol I transcribes rRNA genes, while RNA pol II primarily transcribes protein coding genes and most micro RNAs and RNA pol III transcribes 5S rRNA, tRNAs and other small RNAs such as U6, 7SL and vault RNA, as well as short Short interspersed nuclear elements (SINEs).

Correspondence to: Rohinton T. Kamakaka, rohinton@ucsc.edu.

There are three types of promoters that regulate the genes transcribed by RNA pol III [2]. 5s rRNA genes contain class I promoters within the gene and the promoters are bound by the transcription factor TFIIIA, which in turn recruits the transcription factor TFIIIC. tRNA genes (tDNAs) contain a class II promoter, which is also located within the gene and contains two conserved motifs called the A and B boxes. This internal promoter is bound by TFIIIC, which subsequently recruits TFIIIB upstream of the gene. Class III promoters utilize upstream elements instead of promoter elements within the gene and directly recruit TFIIIB via the upstream element. While these promoter-bound factors recruit RNA pol III to mediate transcription, there are additional sites in the genome that bind some of these transcription factors but fail to recruit pol III termed Extra-TFIIIC (ETC) sites.

Recent studies have shown that the RNA pol III transcribed RNAs have roles in numerous processes in the cell besides translation (see reviews by [3, 4]). In addition the RNA polymerase III transcription factors have many roles outside of the transcription of these genes and in this review we focus our discussion on the role of TFIIIC bound sites in insulation and genome organization. Initial studies in yeast and recent studies in mammalian cells have shed light on how this factor mediates these activities. We will highlight the similarities and differences between the species with respect to insulation and genome organization, and also discuss how the location of pol III transcribed genes may impact the expression at pol II loci and vice versa (see [5–7] for general insulator review).

Insulators

The accurate expression of genes during development, or in response to environmental cues, is mediated by a variety of regulatory mechanisms. Enhancers and silencers, acting over large distances, mediate the precise activation and repression of gene promoters while insulators are a class of DNA elements that regulate the activity of enhancers and silencers in a position dependent manner. Barrier insulators are defined by their ability to block the spread of a repressed chromatin domain into an active region thus protecting an active promoter from silencing while enhancer-blocking insulators are defined by their ability to disrupt activation of a promoter by a distal enhancer when placed between the enhancer and the promoter [8].

Barrier activity of TFIIIC bound elements in *S. cerevisiae*

Insulation mediated by the RNA Pol III complex was first shown in *Saccharomyces cerevisiae* at the silent mating type locus *HMR* [9]. At *HMR*, silencing is initiated by proteins bound at the E-silencer, which in turn recruit the Sir 2/3/4 proteins [10]. Sir protein binding to histones in nucleosomes leads to the formation of heterochromatin that spreads along the chromatin fiber. A search for the DNA element that restricted the spread of heterochromatin at *HMR* led to the discovery that a $tRNA^{Thr}$ gene located downstream of the *HMR* locus was the insulator. This tDNA can function as an insulator in an orientation independent manner [9]. Further analysis demonstrated that its activity depends upon binding of TFIIIC since point mutations in the promoter A and B-box as well as temperature sensitive mutants in TFIIIC partially compromise barrier activity [11]. Additionally, an array of 9 B-boxes (that only bind TFIIIC) is sufficient for barrier function [12]. These data suggest that TFIIIC is necessary and may be sufficient for minimal barrier activity.

Interestingly, not all tRNA genes have the ability to block the spread of silenced chromatin [11–13]. While it is not fully understood why some tDNAs have barrier activity and others do not, it appears that sequences flanking the tRNA genes play a role in insulation. tDNAs, which are weak barriers or lack barrier activity, gain the ability to function as an insulator when their flanking sequences are replaced with sequences found at the native *HMR* tDNA insulator. However, the flanking sequences of the barrier tDNA alone are not sufficient for

barrier activity on their own [11, 14]. Flanking sequences are known to affect the stability of binding of the transcription factors to the tRNA gene promoter [15–17]. Therefore, it is likely that differences in the on/off rates of TFIIIC/IIIB at tDNA promoters determine the ability of a specific gene to function as an insulator. This is supported by a series of experiments where a weak tRNA insulator is transformed into a robust insulator by multimerizing the gene [11]. Interestingly, insulation by the multimerized tDNA insulator is reduced when the Sir proteins are over-expressed [12], suggesting that insulation is a competition between binding of TFIIIC to the tDNA promoter and Sir protein binding to chromatin in the vicinity of the insulator.

Analysis of the chromatin structure around the tDNA insulator shows that the gene is nucleosome depleted [14, 18, 19] and most likely resides in a region of high histone turnover [20]. Mutant analysis suggests that histone depletion is necessary for tDNA mediated insulation though it may not be sufficient for robust insulation [18]. The flanking regions are also depleted of histones in comparison to the genomic average, but to a lesser extent than the tDNA [14]. The histones present in nucleosomes flanking the tDNA gene are highly acetylated and modestly methylated and nucleosomes containing both modifications are refractory to Sir binding.

In *S. cerevisiae*, mapping studies using ChIP-chip show that TFIIIC, TFIIIB and RNA pol III subunits are bound to all known pol III transcribed genes including all tDNAs [21–23]. Surprisingly, the TFIIIC subunit Tfc4 is found at additional sites in the genome that are devoid of RNA Pol III [21, 22] and these sites are termed ETC (Extra-TFIIIC) sites. Similar to tDNAs, ETC sites can also function as barrier insulators dependent upon TFIIIC [12], but these data indicate that transcription is not necessary for robust insulation since ETC loci lack pol III and temperature sensitive mutants in pol III subunits also have no effect on tDNA mediated insulation [11].

Insulation mediated by the tDNA or by a minimal synthetic insulator or ETC sites requires additional co-factors [11, 12, 14, 18, 24]. Mutations in the SAS-I complex that acetylates histone H4K16, the NuA4 complex that acetylates other sites on histone H4, the SAGA complex that acetylates histone H3 tails and the Rtt109/Vps75 or Rtt109/Asf1 complexes that acetylates histone H3K56 all lead to a significant reduction in insulation even in the presence of the endogenous tDNA insulator. Furthermore, mutations in the chromatin remodelers RSC and Isw2 also affect insulation. Molecular analyses suggest a pathway for tDNA mediated insulation whereby acetylation of histones during replication enables the transcription factors and the RSC complex to be recruited to the insulator thus setting up a disrupted chromatin state that is necessary for stable TFIIIC binding and is simultaneously antithetical to the spread of Sir protein repressors. It should be pointed out that this molecular pathway may not be specific to the *HMR* tDNA but is likely utilized by all tDNAs in the nucleus since nucleosome depletion and RSC binding is observed at all tRNA genes in *S. cerevisiae.*

Consistent with the model that these chromatin modifying and remodeling factors play a role in insulation was the demonstration that direct recruitment of specific histone acetylases and remodelers to a synthetic insulator is sufficient to partially block the spread of silenced chromatin [25]. These data collectively suggest that the key to barrier insulation is stable binding of the TFIIIC transcription factor coupled with nucleosome eviction and local acetylation of nucleosomes in the vicinity of the insulator.

In *S. cerevisiae*, the rDNA locus has characteristics of heterochromatin and binds the Sir2 repressor protein, which aids in the repression of genes. Adjacent to this locus is a tRNA gene that also has barrier activity and restricts the spread of Sir2 from the rDNA [26]. This

barrier activity is also dependent upon the SAS-I and SAGA complexes suggesting that similar factors are utilized by most tDNAs to restrict repressive chromatin domains.

Barrier activity of TFIIIC bound elements in *S. pombe*

Silencing in the fission yeast *Schizosaccharomyces pombe* relies on the RNAi machinery and "silencers" for the recruitment and binding of the Swi6 (HP1 homolog) repressor protein to hypoacetylated H3K9me3 chromatin (see reviews [27, 28]). Similar to *S. cerevisiae, S. pombe* has heterochromatic domains at the cryptic mating type loci (*MAT2P* and *MAT3M*) as well as at telomeres and additionally, at centromeres.

Studies in *S. pombe* to map active and repressed chromatin domains by ChIP using antibodies against specific histone modifications identified chromatin domains and their inferred boundaries. One such transition at the silenced *MAT3M* locus coincides with inverted repeat regions (IR-R) [29, 30]. Further studies revealed that the IR-R sites are bound by TFIIIC and deletion of these binding sites leads to the spread of heterochromatin into neighboring euchromatin [30]. Intriguingly although transcripts originate from the IR-R repeats, no RNA Pol III was found at these repeats raising the possibility that RNA pol II is involved in the generation of these transcripts but the role of TFIIIC in this process is unclear.

In addition to the TFIIIC mediated insulation at the *MAT3M* locus, TFIIIC bound tDNAs function as barriers at other silenced loci as well. Fission yeast centromeres are composed of a central domain (cnt) flanked by *dg* and *dh* repeat regions that are packaged into heterochromatin. Clusters of tDNAs flank these heterochromatin domains at the centromeres. Deletions of the tDNAs lead to a spread of centromeric heterochromatin into the adjacent domains [31]. Mutations specifically disrupting the tRNA gene promoter (A-box) also led to loss of barrier activity suggesting this phenotype is mediated by TFIIIC. However, it is unclear if *S. pombe* chromatin remodeling and histone modifying enzymes are involved in restricting the silent chromatin. Recently the chromatin-remodeling factor Fission yeast Fun 30 (Fft3) was identified as a chromatin remodeling protein that localizes to tDNAs and the centromeric domain. In the absence of Fft3, the normally heterochromatic and H3K9me2 modified domain becomes acetylated on H3K9 [32]. These data suggest that Fft3 is localized to a tDNA barrier region where it is involved in limiting the spread of active chromatin marks. It does not appear to do this by evicting nucleosomes from the tRNA gene as H3 occupancy levels at the *S. pombe* tDNA-containing insulator remain depleted in *fft3Δ* strains nor is it known whether the tDNAs themselves are directly recruiting Fft3.

Similar to the ETC loci in *S. cerevisiae*, a significant number of sites in *S. pombe* are bound by TFIIIC but not by RNA polymerase III and are termed COC sites (Chromosome Organizing Clamps) [30]. While the IR-R COC sites function as barrier insulators, most of the other COC sites are found between divergently transcribed genes and ~90% of COC sites were within a few hundred base pairs of promoters of pol II transcribed genes, but it is unclear whether and how COC sites regulate these genes.

Collectively, the data from *S. pombe* and *S. cerevisiae* demonstrate that despite the different mechanisms of heterochromatin formation in these two yeasts, tDNAs and TFIIIC bound loci function as barriers in both species. This raises the possibility that TFIIIC mediated barrier activity is conserved across many species and may be an intrinsic property of tDNAs.

Barrier activity of Pol III transcribed elements in human cells

Genome-wide bioinformatic analysis based on published ENCODE data sets for histone modifications and RNA polymerase III has found tDNAs near the transition between active and repressed chromatin in human cells. Since the positioning of tDNA relative to the junctions of chromatin domains appears evolutionarily conserved and is similar to that of the well-studied vertebrate insulator protein CTCF [33, 34], tDNAs were tested for their ability to function as insulators in human cells [35]. These studies showed that tDNAs have the ability to protect a transgene from silencing, demonstrating their ability to function as a barrier insulator. This effect is partially dependent on the B-box promoter since a significant reduction in expression of the reporter is seen when constructs containing a three base pair mutation in the B-box were assayed. In other experiments tDNAs inserted between a tethered polycomb repressor protein and a promoter can also protect the promoter from repression [35, 36] demonstrating that they can act as barriers to different forms of repressed chromatin.

It was recently reported that tDNAs can also function as barrier insulators in murine cells [37], and this activity is dependent on the A box promoter element. As in *S. cerevisiae*, flanking sequences play a role in insulation and the presence of AT rich flanking sequences increases barrier activity compared to GC rich sequences. It will be interesting to know if tDNAs function as barriers at their native loci in human and murine cells.

tRNA genes in mammals are often found in clusters along the DNA fiber, separated by 300–600bp of intervening DNA [35, 38]. While a single tDNA does not possess robust barrier activity, arrays of tDNAs containing either two or four genes in tandem are a robust barrier. Clustering of tDNAs along the DNA fiber is common in many higher eukaryotic species making it tempting to speculate that whether a tDNA is part of a cluster or not determines which tDNAs can function as barrier insulators in higher eukaryotes [35]. As is the case for *S. pombe,* it remains unclear if mammalian tDNAs function as barriers by the same mechanisms as tDNAs in *S. cerevisiae,* namely through high turnover of histones creating a nucleosome depleted region at the tDNA. In support of this model, active tDNAs in vertebrates are located in nucleosome-depleted regions and flanking nucleosomes contain histone modifications associated with active promoters. In addition histone acetylases are recruited to mammalian tRNA genes suggesting that the mechanisms of insulator function in vertebrates may have similarities to those in *S. cerevisiae* [38–41].

While numerous ETC sites have also been identified in mammalian cells [38, 40], it is currently unknown if these sites function as barrier insulators., Unlike tDNAs, ETC loci do not cluster along the DNA (JR unpublished results) and synthetic multimerized B-box sequences are not sufficient for barrier activity in murine cells [37] suggesting that these sites may have different roles but the role of these loci is unclear at this time.

Mechanism of Barrier Activity

Mechanisms of silencing vary from yeast to mammals [42]. However, common to all these systems is that silencing proteins appear to spread from nucleation sites and that silenced and active regions are located in distinct regions of the nucleus despite being juxtaposed on the linear DNA fiber. How do barriers function to block the spread of the repressive signal and how do pol III barriers function to restrict the various forms of heterochromatin in all of these organisms?

Mechanistically, our current barrier insulator models are primarily based on data from *S. cerevisiae* and chicken cells [8]. The first component of these models relies on the fact that all known barrier insulators are nucleosome depleted. A gap in the chromatin fiber created

by a nucleosome-depleted region is an impediment to the binding and spread of repressor proteins that rely on histone tails to spread and silence genes. In *S. cerevisiae* barrier activity requires chromatin remodelers and histone acetylases. The absence of these factors results in loss of barrier activity and, importantly, loss of the nucleosome depleted region. It remains unknown if human and *S. pombe* barriers require binding of chromatin-remodeling components for barrier activity, although active tDNAs in both species are nucleosome depleted.

A second conserved feature of barriers is the high level of histone acetylation in nucleosomes flanking the barrier. These modifications are seen at the chicken HS4 insulator as well as tRNA insulators in *S. cerevisiae*, *S. pombe* and humans. In chickens, the recruitment of histone modifying enzymes to the barrier is critical for blocking the spread of repressed chromatin [43] and the same is true at synthetic insulators in *S. cerevisiae* [25]. Therefore, barrier activity could result from histone acetylation and nucleosome eviction at the barrier. This would allow stable binding of transcription factors to the barrier and simultaneously preclude the spreading of the repressor proteins, which require deacetylated histones to spread.

It will be necessary in the future to test the role of tDNAs in barrier function at their native loci in mammals as well as other model organisms. Further work in other organisms besides yeast will also be required to clarify the role of accessory factors in TFIIIC mediated barrier insulation.

Enhancer-blocking activity of elements associated with the RNA polymerase III machinery

Recent high-resolution mapping of RNA pol III components in murine and human cells indicate that unlike yeast, in mammalian cells only half of the tRNA genes are bound by the pol III machinery. Furthermore, the majority of TFIIIC bound loci do not contain pol III in these cells. In murine cells more than 80% of TFIIIC containing sites are identified as ETCs [44]. In human cells most of these ETC sites localize adjacent to RNA pol II transcription start sites (TSS) [38, 40, 41]. The link between RNA pol III components and RNA pol II genes is not limited to ETCs as very few unoccupied tDNAs are found close to active RNA pol II transcribed genes, while 20% of all occupied tDNAs are found near the promoters of active pol II transcribed genes. Additionally, the pol II factor TFIIS is found near RNA pol III genes in murine ES cells as well as in *S. cerevisiae* [44, 45].

RNA pol II transcription factors are found in close association with tRNA genes. CBP and ETS1, which are typically found together at enhancers, are also found at approximately half of the pol III bound sites [38]. A separate study found that at least one of the RNA pol II transcription activators c-Myc, c-Fos, or c-Jun are found at >75% of pol III bound regions, with all three being found at 60% of pol III targets [46]. Prior work *in vitro* and *in vivo* has shown that c-Myc directly stimulates RNA pol III transcription via an interaction with TFIIIB [47–49]. Together, these links between RNA pol II and RNA pol III components and regulatory factors blurs the distinction between pol II and pol III specific factors in gene regulation.

Enhancer-blocking insulators are defined by their ability to disrupt enhancer-mediated activation of a gene when placed between the enhancer and promoter [5, 7]. Some of the TFIIIC bound sites are located between enhancers and promoters of genes and there is a discordance between the expression levels of adjacent genes separated by tDNAs suggesting that tDNAs have the potential to function as enhancer blocking insulators thus influencing expression of RNA pol II transcribed genes.

Most vertebrate enhancer blocking insulators that have been characterized bind the protein CTCF [50]. However, recent work from several labs has shown that RNA Pol III transcribed elements possess the ability to function as an enhancer-blocking insulator. Two studies in mice found that short interspersed nuclear elements (SINEs) are able to function as enhancer-blocking insulators [51, 52]. SINEs are retrotransposable elements that are highly repetitive elements transcribed by both RNA pol II and pol III (reviewed in [4, 53, 54]). SINE B1 elements are derived from 7SL RNA while SINE B2 elements are derived from pre-tRNA genes. SINE B2 elements contain the canonical pol III internal B-box promoter but also contain an external Pol-II promoter that is bound and activated in a developmentally controlled manner.

A SINE element in the murine growth hormone locus (GH) was found to possess insulator function [51]. Early in murine development a single domain of H3K9me3 encompasses the entire GH locus. Later in development this domain is truncated and the site of transition coincides with a SINE B2 element and deletion analysis suggests that the SINE B2 element does function as a barrier in these cells. The SINE B2 element has robust enhancer blocking activity in reporter assays. Interestingly, both RNA pol II and RNA pol III transcripts originate from this locus in an antisense configuration. Testing the requirement for the different promoter elements in insulation suggests that while the full SINE B2 possesses the most robust activity, only the RNA pol II promoter is absolutely essential [51]. The precise role of TFIIIC and these transcripts in GH regulation remain to be elucidated.

Recently a second SINE element was found to function as an insulator in murine cells [52]. In this case a SINE B1 element was thoroughly examined for enhancer-blocking activity. Again, this element generates transcripts dependent on RNA pol II and RNA Pol III, however in contrast to SINE B2, they are found on the same strand. Stimulation of the pol II transcript occurs after binding of the transcription factors SLUG and AHR while over-expression of these factors leads to increased enhancer-blocking activity. Because these transcription factors bind to regions near the pol III promoter (A and B box) the authors looked at potential competition between RNA pol II and RNA pol III transcription factors for binding. Their result suggests instead that the pol III promoter may be required for basal enhancer blocking activity, which is augmented by replacement of pol III factors by the transcription factors SLUG and AHR. It remains unclear in both SINE studies whether transcription, either by pol II or pol III, is required for insulation or if simply binding of factors associated with transcription is sufficient.

Recent work has shown that human tRNA genes can also function as enhancer-blocking insulator in a TFIIIC dependent manner [35]. While clusters of tDNAs are capable of enhancer-blocking activity single tDNAs lack this ability. Since a significant percent of tDNAs in the human genome cluster along the DNA fiber it is possible that clustered tDNAs function as enhancer blocking insulators *in vivo* though direct testing at their native loci is necessary for unambiguous conclusions.

CTCF and cohesin have a well-defined role in enhancer blocking in vertebrates. CTCF and cohesin subunits are found in the vicinity of the most highly occupied tRNA genes and ETC sites [38, 40, 44]. As is the case for the link between RNA pol II and pol III machinery, it remains unclear whether the high levels of TFIIIC and pol III occupancy found at ETCs and tDNAs is a cause or consequence of CTCF binding. At the chicken HS4 insulator, CTCF cooperates with USF1 and VEZF1 to form an extremely robust insulator capable of both enhancer blocking and barrier activity [55–58], although these activities are separable. It is possible that a large percentage of tDNAs and ETC sites could function as insulators when found in conjunction with accessory proteins such as CTCF, c-Fos, c-Myc, or c-Jun.

Assays and results for enhancer blocking are well described in vertebrates and *Drosophila* but budding yeast lack classic distal enhancers. However, in yeast the Upstream Activating Sequence (UAS) can be viewed as "enhancer-like." Interestingly, tRNA gene placement between the UAS of the GAL1–GAL10 genes results in the reduction of transcription of these gene showing that tRNA genes can function as UAS-blocking elements in budding yeast [13]. Both a tRNA gene and an *ETC* site also function in this assay to inhibit the UAS. Furthermore the *TRT2* tRNA gene also functions as a UAS-blocking element at its native locus blocking activation of the *CBT* gene by the UAS binding transcription activator Mcm1 [59]. A similar effect is found at *ETC6*, which is located between the divergently transcribed *TFC6* and *ESC2* genes [13, 60] though the molecular factors and mechanisms involved in this process are not fully known. While the majority of COC (ETC) sites in *S. pombe* are located between divergently transcribed genes and may function in a similar manner [30], "UAS-blocking" activity has not yet been experimentally described in *S. pombe*.

These studies in human, mice and yeast cells highlight potential cross regulation between RNA pol II and RNA Pol pol III transcribed genes. The data suggest cross talk between the two sets of transcription machineries that has not yet been fully appreciated or explored. It will be important to determine if intact pol III promoter elements are required for proper regulated expression from pol II loci or vice versa. It remains to be seen in which direction the information flows. Do the pol III bound loci influence expression from the neighboring pol II loci, or do the binding of the pol II transcription factors allow for pol III to become active or is there a co-dependency for interaction?

Mechanism of enhancer blocking

Enhancers are believed to activate promoters via specific contacts between factors bound to the enhancer and promoter. Enhancer/promoter interactions may be stabilized by sequestration to sites in the nucleus of high local concentrations of transcriptional machinery termed transcription factories. Two non-mutually exclusive models exist for enhancer-blocking insulators [8]. In the first model, insulators aggregate within the nucleus via interactions with each other, the result of which partitions the chromatin fiber into chromatin loop domains such that enhancers in one loop domain cannot interact with promoters in another loop [7]. A second model is based on the observation that some enhancer blocking insulators can physically or functionally interact with promoters or enhancers and it has been suggested that these interactions block the propagation of the signal from the enhancer to the promoter [61].

None of the evidence for how enhancer-blocking insulators function relies on data involving TFIIIC bound sites, which raises the question of how TFIIIC bound regions fit into current models for enhancer-blocking activity. The demonstration that tDNAs cluster with other tDNAs and localize to specific sites in the nucleus [35, 62–66] suggests that tDNA interactions with each other, or with nuclear sub-structures could function as a mechanism for insulation. It is believed that the nucleus contains many transcription factories each specific for certain transcription factors or polymerases. One possible model postulates that tDNA clustering at RNA pol III transcription factories [67] could move neighboring RNA pol II transcribed genes away from pol II factories thus turning off their activity. Alternatively, the tDNAs could be functioning simply by separating enhancers and promoters into separate chromatin loops even if they do not drastically alter spatial nuclear positions. This local separation could be sufficient to disrupt enhancer mediated gene activation. Missing from current studies on pol III insulators is what effect nuclear position has on the gene regulatory abilities of TFIIIC bound sites. By identifying the cofactors involved in both enhancer-blocking activity and three-dimensional architecture of the pol III

bound genome it should be possible to determine if positioning and gene regulation are linked.

The localization of pol III components in the *S. cerevisiae* nucleus

The nuclear positioning of a locus can be important for its regulation as evidenced by studies on RNA pol II transcribed loci [68, 69]. How are components of the pol III machinery positioned within the nuclear volume and what are the effects of this on insulation? Fluorescent *in situ* hybridization studies (FISH) in budding yeast show that many but not all tRNA genes cluster on the outer periphery of the nucleolus and co-localize with the 5S ribosomal DNA and U14 small nucleolar RNA genes [62]. This observation was confirmed by genome-wide chromosome confirmation capture (Hi-C) in *S. cerevisiae,* which show that tRNA genes are found in two distinct clusters [63]. Of the 274 yeast tRNA genes, approximately 75 cluster in 3D space with the nucleolar rDNA genes on ChrXII while 125 tRNA genes are found to cluster with the centromeres in 3D space and the remaining either do not cluster or interact infrequently with other tRNA genes.

Cohesin and condensin proteins are involved in the higher order organization of chromatin. Genome-wide mapping studies show that the condensins Smc2/4 preferentially localize to tDNAs [70, 71], and it is likely that they are recruited to these loci via interactions with tDNA bound factors. However, whether the pol III machinery directly recruits the cohesin/condensing subunits is not clear. The 3D clustering of tDNAs at the nucleolus is also dependent on condensins [70] but the loss of condensin mediated tDNA clustering within the nucleus does not appear to lead to a change in tDNA transcription suggesting that clustering is separable from tDNA transcription. Interestingly, the tRNA gene insulator adjacent to the silenced *HMR* locus does not localize with the nucleolus or centromere but associates with the nuclear pore [64, 72] though pore localization is also not necessary for insulation. Whether this tDNA recruits condensins is not known but this tDNA insulator does recruit cohesins [66], and mutations in the cohesins affect insulation [9], though the exact mechanism by which cohesins contribute to insulator activity is also unknown.

Similar to the *HMR* tDNA insulator, the ETC loci in *S. cerevisiae* also localize to the nuclear periphery in a B-box and TFIIIC dependent manner [60]. Additionally ETC tethering to the nuclear periphery is dependent on Mps3, an inner nuclear membrane protein. Like the *HMR* tDNA, loss of ETC localization to the periphery does not affect insulator function. These results bring up numerous questions of how TFIIIC functions in genome organization and how this affects TFIIIC bound loci function in insulation.

S. pombe tDNAs and pol III machinery are involved in nuclear organization

In *S. cerevisiae* tRNA genes are not present in clusters along the DNA fiber. In contrast, approximately 30% of the tDNAs in *S. pombe* are found in arrays flanking the centromeres. The pol III machinery in *S. pombe* has also been shown to cluster in three-dimensional space. 5–10 foci containing TFIIIC can be visualized by immunofluorescence [30, 65, 73], while only a single focus is seen for pol III. This focus is coincident with the centromeres and tDNAs.

Mapping TFIIIC in *S. pombe* identified ~ 60 COC (ETC) sites [30, 65, 73] which appear to coalesce into 5–10 TFIIIC foci in the nucleus. This long-range association between the sites is dependent upon the TFIIIC binding sequence. In *S. pombe*, condensins play a role in the 3D clustering of TFIIIC bound sites in the nucleus. Further analysis suggests a competition between condensin and TFIIIC for binding at the tDNA and this competition is responsible for changes in nuclear position of the locus [65]. Taken together, these results in *S. pombe* suggest TFIIIC and condensin play opposing roles in localizing pol III genes within the

nucleus and this localization is important for proper transcription of these regions. When condensin is bound to these sites, pol III binding and transcription of the tDNA is reduced, and the region is localized to a distinct region of the nucleus adjacent to the centromeres. When TFIIIC is bound, condensin binding is reduced, transcription from the tDNA is increased, and the region is found clustered with the nucleolus. This situation is in contrast to *S. cerevisae* where condensin binding at tDNAs has not been shown to play a role in transcription, though it is required for localization of the tDNAs to the nucleolus. It remains to be seen if localization of tDNA genes to specific sub-nuclear structures plays a role in regulating neighboring RNA pol II transcribed genes and whether localization also plays a role in insulation in *S. pombe*.

Distribution of pol III components in vertebrates

The 3D clustering of the pol III machinery and of pol III transcribed genes has been well demonstrated in yeast. The picture of global organization of the pol III transcriptome in vertebrates is much less clear but also indicates clustering of these genes. Early studies using confocal and electron microscopy and incorporation of Br-UTP into nascent RNA pol III transcripts show approximately 2000 discrete pol III “transcription factories” [67]. Biochemical analysis of these same cells suggested approximately 10,000 pol III transcripts in each cell, thus suggesting that each discrete pol III factory contains on average 5 transcripts. Recent work in human and mouse mapped the RNA polymerase III machinery in several cell types and showed that only 50% of tDNAs appeared to be active, suggesting that individual 3D foci may only contain one to two genes [38, 40, 41, 44, 46].

Proteomic analysis of purified nucleoli identified the presence of RNA pol III machinery in this sub-nuclear compartment of human cells [74] and recent genomic analysis of nucleolar associated DNA fragments clearly showed association of tDNAs with this organelle [75]. These data are consistent with observations in yeast showing clustering of these genes with nucleoli.

Recent work using 4C also indicate that tDNAs are in close physical proximity with each other in 3D space. Using chromosome conformation capture followed by high-throughput sequencing it was shown that human tRNA genes located on chromosome 17 cluster in 3D space with other nearby tDNAs though whether these interactions were stable or transient is not clear [35]. This raises the possibility that intra-chromosomal interactions among tDNAs over hundreds of kilobases may be important for chromosome folding and packaging. The identification of factors controlling tDNA-tDNA interactions in 3D space will address the question of whether localization of tDNAs within the mammalian nucleus plays a role in the regulation of gene expression *in vivo*. Furthermore, it is unknown how transcription states contribute to tDNA interactions. Do active tDNAs only interact with other active tDNAs or do they also interact with inactive tDNAs?

Conclusions

Recent studies have shown that the pol III machinery has a more complex role in gene regulation than previously appreciated. tDNAs in yeasts have long been known to function as barrier insulators, but recent results have expanded that activity to mammals as well (Table 1). Vertebrate TFIIIC bound elements also have the additional ability to function as enhancer-blocking insulators though the precise role of these elements at their native loci is awaited. Furthermore little is known about how tDNAs and ETC (Table 2) mediate their activity in metazoans, and what precise role tDNAs play in the regulation of RNA pol II transcribed genes. Ties between pol III transcription machinery bound regions and pol II genes suggest that the pol III machinery may regulate some genes directly, rather than

through insulator activity. The observation that TFIIIC bound loci across species recruit histone modifying enzymes and remodeling proteins and cluster in 3D space in the nucleus, suggest that these properties are likely to be critical factors in their ability to act as barriers, enhancer-blocking insulators and possibly direct regulators of RNA pol II transcribed genes in all species as well. Further studies are necessary to determine if these activities are separable or rely on a common set of factors and whether metazoan tDNAs function through mechanisms analogous to those in yeast.

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Highlights

- TFIIIC is a key transcription factor in tRNA gene transcription
- Some TFIIIC bound sites, function as gene insulators across species.
- Chromatin remodeling/modifying cofactors contribute to TFIIIC mediated insulation
- TFIIIC also coordinates higher order chromatin folding in the nucleus

Table 1

tDNAs as Insulators and Chromosome Organizers

Organism	2D tDNA Cluster	3D tDNA organization	Barrier Insulation	Enhancer Blocking	Proposed Mechanism
Budding yeast	No	Nucleolus, centromeres, nuclear pores	Yes, at native *HMR*	Yes, in UAS blockers	Nucleosome depletion Acetylated histones Chromosome remodelers Cohesin recruitment
Fission Yeast	Yes, Centromeres	Centromere	Yes, at native centromeres/Mat3	Unknown	Nucleosome depletion Acetylated histones Chromosome organization
Mouse	Yes, across genome	Unknown	Yes, in transgene assay and BAC reporter assay	Yes, SINES in enhancer blocking assay	Nucleosome depletion Acetylated histones Pol II transcription
Human	Yes, across genome	Intra- chromosomal cluster, Nucleolus	Yes, in transgenes assays and repressor blocking assays	Yes, tDNAs in enhancer blocking assay	Nucleosome depletion Acetylated histones CTCF and cohesin

Table 2

ETC Loci as Insulators and Chromosome Organizers

Organism	# of ETC sites	Genomic context	Genome Organization	Insulator Activity
Budding yeast	~9	Divergent genes	Peripheral tethering	Barrier activity UAS blocking activity
Fission yeast	~60	Divergent genes	Adjacent to centromeres	Barrier activity
Mammals	~1800	Pol-II TSS Not clustered along chromosome	Unknown	No barrier activity