Being in a loop: how long non-coding RNAs organize genome architecture

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- Long noncoding RNAs (LncRNAs) affect chromatin architecture thus playing an essential role in the regulation of processes such as gene expression.
- Their involvement in chromatin organization occurs both through the act of transcription and the transcript itself.
- The act of transcription of a lncRNA affects local architecture by altering chromatin status or driving the eviction of architectural proteins such as CTCF.
- LncRNA transcripts can hold together chromatin-modifying complexes and guide them to targets throughout the genome. They can also directly and specifically bind to DNA forming R-loops and triple helices.
- Despite fierce scientific interest and recent technical advances, the underlying mechanisms and full extent of lncRNAs' involvement in genome architecture requires further research.

Abstract

Chromatin architecture has a significant impact on gene expression. Evidence in the last two decades support RNA as an important component of chromatin structure [1–3]. Long non-coding RNAs (lncRNAs) are able to control chromatin structure through nucleosome positioning, interaction with chromatin re-modellers and chromosome looping. These functions are carried out in *cis* at the site of lncRNAs transcription or in *trans* at distant loci. While the evidence for a role in lncRNAs in regulating gene expression through chromatin interactions is increasing, there is still very little conclusive evidence for a potential role in looping organisation. Here, we review models for the involvement of lncRNAs in genome architecture and the experimental evidence to support them.

Introduction

Gene expression is dependent on the surrounding chromatin organization within the nuclear environment. Epigenetic processes such as DNA methylation and histone modifications co-ordinately alter the accessibility and chromatin structure, in order to orchestrate tissue- and development-specific gene expression programmes. As far back as 1989, experiments showed that either the digestion or the inhibition of nuclear RNAs could lead to the disruption of interphase chromatin structure[4]. Later analysis of the transcriptome revealed large amounts of long non-coding RNAs (lncRNAs) that are highly enriched in the nucleus [5,6]. LncRNAs are transcribed by RNA polymerase II (RNA polII) into transcripts longer than 200 nucleotides and are devoid of an open reading frame (ORF)[7–9]. Despite initially considered to be the "dark matter of the genome"[10], lncRNAs are currently recognized as key molecules in several biological processes including development and disease[11]. According to their loci of action, lncRNAs can be classified as acting in *cis* near to their point of origin [12] or in *trans* by localizing to distal regions on the same chromosome [13] or

other chromosomes entirely [14].

The ability of lncRNAs to fold into tertiary structures capable of specific interactions with proteins, makes them well suited to regulate chromatin architecture [15,16]. LncRNAs are able to recruit chromatin-modifying complexes that can change gene expression [14,17–20], or act as scaffold molecules to guide or hold complexes at specific chromosomal loci [21]. They can act as decoy or sponge molecules to inhibit or disrupt the binding of transcription factors or RNA binding proteins [22–25]. Furthermore, lncRNAs have been shown to insulate specific portions of the genome by stabilizing CTCFs together with other factors that mediate looping [26]. The functional characterization of most of lncRNAs has been difficult, since their primary sequences are usually not conserved. This is the main reason why several lncRNAs are still uncharacterised. In addition to functions that can be ascribed to the lncRNA transcripts, the act of transcribing any lncRNAs can have consequences on the chromatin template that can affect the expression of adjacent genes[27]. Indeed gene expression is not autonomous and there are many instances within the genome where the expression of a gene affects that of its neighbour. The mechanisms whereby this happens have not been fully explored – and it is important to understand these in an era where gene therapy is fast becoming a reality.

The act of transcribing a lncRNA and the effect on chromatin architecture

Transcription occurs within minutes of activation and can be highly discontinuous, occurring through burst of transcriptional activity, in which many RNAs are transcribed in a short time interspersed with periods of inactivity [28]. While only ~1.5% of the human genome encodes proteins, the 70%–90% of the genome gets transcribed into many diverse non-coding RNAs [29–31]. Despite being heavily transcribed, lncRNAs show low abundance and high tissue specificity [32–34]. Indeed, the low levels of lncRNAs is likely to limit their ability to regulate gene expression in *trans*, which may suggest that the act of transcription is functionally more relevant the RNA molecule itself. Many lncRNAs overlap other genes whose transcription can be affected when the lncRNAs is transcribed [35–37]. Transcription of divergent lncRNA/mRNA gene pairs has been shown to open and maintain active chromatin during embryonic stem cell differentiation [38]. Elongation of lncRNA through a super-enhancer as shown at the *Hand2* locus[39] further indicates that transcription of lncRNA can positively regulate adjacent genes. However, lncRNAs can also negatively regulate nearby genes through a mechanism known as transcriptional interference, where the effect of one transcriptional event suppresses the effects of a second one in *cis* [40]. This has been reported for some imprinted genes where the transcription of a lncRNA from a specific allele (maternal or paternal) drives the inhibition of a set of nearby associated genes at the same allele [41–51]. Transcriptional interference/promoter occlusion has not been conclusively shown in eukaryotes and the physical implications of two polymerases trying to pass on the same DNA template requires a mechanism whereby transcriptionally paused transcripts can be rescued [52,53]. This may also involve transcription-coupled nucleotide excision DNA repair mechanisms [54]. The majority of lncRNAs that have the potential for transcriptional interference are natural antisense RNAs that overlap with, and are transcribed independently from sense RNAs. These lncRNAs can potentially hybridize with sense RNAs to form double strand RNAs (dsRNAs) and silencing interfering RNAs (siRNAs), but their transcription has been associated with the accumulation of repressive chromatin marks such as histone 3 methylation (H3K9me3, H3K27me3, and H3K36me3). What is not known is whether the lncRNAs recruit these chromatin modifying enzymes, or whether these enzymes are part of the transcription elongation complex.

Since transcription alone is enough to change the epigenetic state of chromatin it is easy to envisage that such changes will facilitate interactions between regulatory elements and transcription factor complexes. The probability of loop formation between two anchored points is determined by the flexibility of the chromatin fibre between such points [55]. Transcription of lncRNAs could feasibly change the flexibility of chromatin fibre and the nucleosome composition to modulate interactions between distant loci. An early indication that suggested transcription can reposition nucleosomes and evict CTCF/cohesin complexes was the elegant study by Lefevre et al, who used the inducible chicken lysozyme gene as a model locus to examine

the kinetics of chromatin modifications during transcriptional elongation [56]. More recently a genome-wide study has shown that RNA polII transcription temporally erases chromatin loops in its path, including enhancer-promoter interactions. This is because transcription elongation can occur despite the presence of cohesin, CTCF and transcription factor binding, and other potential epigenetic roadblocks to disrupt CTCF/cohesin mediated chromatin loops [57] (Fig. 1).

A wider role for the low level of pervasive transcription of lncRNAs has been implied in a "cat's cradling" model formulated by Mele and Rinn [58]. In this model several transcribing lncRNAs shape the three-dimensional genome organization by successively opening the chromatin and producing ''grip holds'' to pull and direct looping interactions [58]. What this model does not take into account, is that the transcription of coding genes would also influence genome organisation. Given that transcription can occur at different rates and frequencies across the genome, the three-dimensional organisation is most likely to be dynamically flexible. This may explain the variable intensities observed when visualising Topological Associated Domain (TAD) structures generated by next generation sequencing for chromatin conformation capture.

An experimental based model for transcription-mediated organization of chromatin topology that could explain how enhancers precisely select their cognate promoter regions has been recently been shown for an enhancer RNA at the Bcl11b locus [59]. These authors demonstrated that transcription of a lncRNA, *ThymoD*, promotes demethylation of CTCF sites within the transcribed region which then results in recruitment of CTCF-cohesin complexes, and a change in looping conformation to bring enhancer and promoters together. In this model the enhancer and promoter elements are also sequestered by lncRNAs into a single-loop domain [59]. Further support for this model comes from the discovery of another class of lncRNAs, called trait-relevant long-intergenic ncRNAs (TR-lincRNAs). Strong statistical evidence indicate that TR-lincRNAs frequently arise from enhancer regions and are often located near to the boundaries of TADs suggesting that proximal trait-relevant gene expression is regulated in *cis* through modulating local chromosomal architecture [60]. Recently, topological anchor point RNAs (tapRNAs) have been identified as a new class of conserved lncRNAs that overlap CTCFs binding sites [61]. Further analysis is still required to understand how lncRNA-mediated genome organisation feeds into gene expression networks whether this directly relates to disease.

Strategies to dissect and uncouple the functions due to the lncRNA transcript and the act of transcription have been developed by using siRNAs [62] or by terminating transcription through the insertion of a polyA cassette [63]. In this regard, while early studies suggested that the presence of the *Airn* lncRNA induces epigenetic silencing by changing the 3D organization allele-specifically to interfere with *Igf2r* promoter [46], it was subsequently demonstrated that merely the act of transcribing *Airn* can evict RNA polII and silence *Igf2r* [48]. Conversely, the same strategy used in experiments to uncouple the *Lockd lncRNA* from its associated *cis* elements revealed that the *Cdkn1b* gene is positively regulated by a *cis* element at the promoter of the adjacent *Lockd* locus, whereas the transcribed *Lockd* lncRNA is dispensable for this function. Therefore, the *Lockd* locus acts as an enhancer for *Cdkn1b* to promote its transcription but not the *Lockd* lncRNA itself [64]. Transcription at enhancer elements in so called enhancer RNA (eRNAs) [65–68] raises the question of whether the transcriptional activity at these loci contributes to the enhancers function as *cis* elements. As with the TR-lincRNAs described above, transcription of an eRNAs can enable enhancers to interact with a specific promoter and allow the engaged RNA polII to recruit chromatin modifiers.

The above evidence points to a role for transcription enabling chromatin conformation at the very least through changing local chromatin structure through the repositioning of nucleosomes, modification of histones, changing DNA methylation states and regulating accessibility of key chromatin architectural proteins such as CTCF and cohesin. Transcriptional elongation through key regulatory elements may mediate the chromatin changes and future analysis should examine the extent to which chromatin modifiers form part of the polymerase elongation complex. Transcription of lncRNAs may serve as the pioneer event to

change chromatin structure to facilitate promoter-enhancer interactions, which can then be stabilised through higher order chromatin looping structures.

There is evidence supporting that some lncRNAs can be functional both through the act of transcription and through the transcript while for others only one or the other has been proven[69–71]. It remains to be understood how these overlap in time or to which extend they are functionally connected.

Effect of lncRNA transcripts

The product of a lncRNA is by definition long, mobile and capable of interaction with proteins and DNA, and can have a far-reaching impact. This can take place either in proximity to the transcription locus to regulate adjacent genes in *trans* [72] or several hundred kb away while still on the same chromosome (intrachromosomal interactions) or on another chromosome entirely (interchromosomal interactions) [21,73–75]. Several lncRNAs interact with chromatin-modifying proteins such as Polycomb and Tritorax protein groups, histone acetyl and DNA methyl transferases[76–78], where they have been reported to function as scaffolds holding the complexes together and/ or recruiters guiding them to genome-wide targets. The capacity of lncRNA transcripts to specifically interact with more than one protein partner further enhances their potential to affect multiple targets in different regions [79,80]. Apart from interacting with chromatin-modifying proteins, lncRNAs can also directly bind to DNA. The latter includes the formation of R-loops or of RNA-DNA triple helices that can act as docking sites during recruitment of chromatin-remodellers [81,82]. *Terra* is an example that showcases the ability of a lncRNA to facilitate several chromatin modifier functions. Thus, besides ensuring telomeric DNA stability by being an integral part of telomere architecture, *Terra* also targets thousands of genomic regions in *trans* and its binding correlates with that of the chromatin remodeller ATRX as well as that of a catalytic subunit of Polycomb group proteins [83]. Telomeric lncRNAs such as *Terra* are encoded by sequences that include telomeric repeats and that allows them to form R-loops at telomeres without the need of an intermediate RNA binding protein [84]. Another example of a lncRNA that forms triple helices illustrating how they can affect looping is the recently discovered lncRNA, *PARTICLE*. *PARTICLE* is located within the *MAT2A* gene and its activation leads to the formation of a triple helix between the lncRNA and the region upstream of the MAT2A gene promoter, where it governs the methylation status of an associated CpG island and thus the expression of MAT2A [81,84,85].

As described above, many eRNAs can modulate enhancer activity by coordinating chromatin looping between enhancers and promoters [86,87]. Interestingly, down-regulation of eRNAs reduces the looping and the expression of nearby target genes hinting at their direct involvement in the looping and the importance of their presence [87,88]. They may therefore directly contribute to looping by interacting with proteins bound to target promoters. This was shown for example in the case of ncRNA-a7, which physically interacts with the Mediator complex that allows the enhancer–promoter looping interaction and causes the activation of target genes [89]. Recently, Fanucchi *et al* identified the lncRNA *UMLILO* and showed that it also interacts with a super-enhancer in order to prime immune genes for activation [90]. Furthermore, a study on the myogenesis-involved lncRNA *Charme,* led to a model in which *Charme* associates with chromatin and mediates the formation of a loop at its site of expression [91]. Other enhancer-like lncRNAs, such as *HOTTIP* [92] and *CCAT1-L* [93], can interact with CTCF to orchestrate the chromatin loop and regulate gene expression in *cis* [94].

The phenomenon of X chromosome inactivation (XCI) efficiently demonstrates several ways in which lncRNA function can orchestrate genome architecture (Fig. 2). *Xist*, the most extensively studied of all lncRNAs, directs XCI by coating the whole chromosome ensuring that the genes that need to be active or suppressed are repositioned accordingly [35,95]. *Xist* covers and repositions the extra X chromosome by binding to many locations across the chromosome non-specifically but with preference to gene-dense locations while avoiding the genes that need to escape XCI. Additionally, it interacts with RNA-binding proteins such as SHARP and ATRX and recruits Polycomb group complexes [96–99]. There are other lncRNAs that are involved in

the accurate orchestration of XCI, including *Tsix* which ascertains that the active X chromosome escapes silencing by determining methylation status of *Xist* CpG islands [100,101]. *Jpx* is a another lncRNA encoded proximal to the *Xist* locus and it is thought to activate *Xist* by interacting with CTCF and removing it from the Xist promoter [102,103]. This interaction requires the existence of an RNA binding region (RBR) within CTCF. In a recent study, the RBR was identified and shown to interact with *Wrap53* in order to regulate expression of p53 [104]. Given the known role of CTCF in higher order chromatin structure, further exploration of direct interactions between CTCF and lncRNAs is required to determine whether CTCF-RNA interactions are globally essential for chromatin conformation. This will require the identification of additional RNAs that can bind to CTCF.

Beyond a potential function in enabling chromatin looping conformation to facilitate gene regulation. lncRNAs have also been reported to play a role in the formation of subnuclear structures such as the nucleolus (pRNA) and paraspeckles (*NEAT1* and *MALAT1*) [105–109].

Future considerations

LncRNAs clearly exert varied functions and in recent years this has become an intense focus in the field of chromatin architecture. However, there is still little insight into the mechanisms whereby they function. Indeed, despite advances in computational, biochemical and genome wide technologies, we still lack the capability to accurately predict and resolve the secondary structure of lncRNAs, how they interact with proteins and DNA. Most lncRNAs are not evolutionary conserved and present in as little ten copies per cell. To envisage how such lncRNAs can have multiple genome-wide targets, technologies are required whereby we can identify and manipulate the effects of transient interactions. The rapid advances in CRISPR-based methods provide a glimpse into the future exploration of this field. One example is CRISPR-interference (CRISPR-i), a variation of the technique that can be used to block transcription by causing heterochromatinization of a specific target sequence [110,111]. Used in conjunction with siRNA or antisense nucleotides (ASOs) which target the produced transcript, can help to distinguish between the effects of the transcript and those caused by the act of transcription. Similarly, other newly developed CRISPR-based methods can be used to either specifically knock-out lncRNA promoters or knock-in polyA stop sites thus complementing existing methodologies and further characterizing the function of lncRNAs[112]. Other variations such as CRISPR-genome organiser (CRISPR-GO) which can be used to reposition a sequence in the genome [113] are particularly promising for understanding how lncRNAs are involved in the determination of chromatin structure. Techniques for the study of genome architecture [114] and dissecting the function of lncRNAs [115] have been extensively reviewed elsewhere. It is now time to build upon these technologies to develop streamlined methodologies whereby the mechanistic intersection of lncRNAs and genome architecture can be studied.

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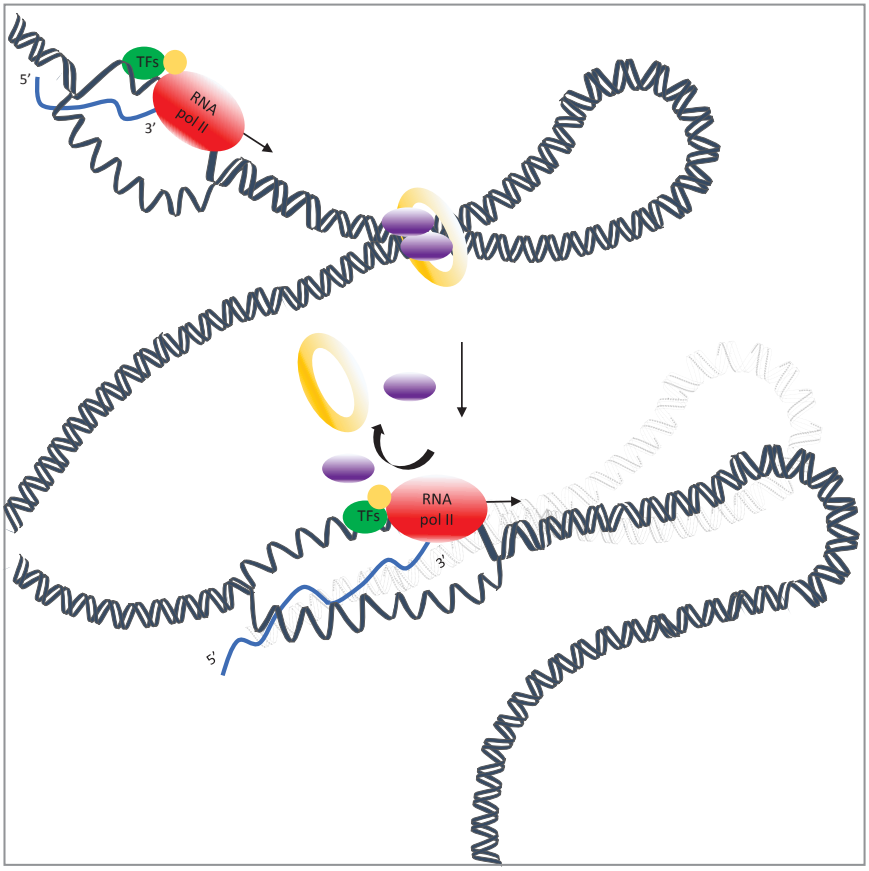
Figure Legends:

Figure 1: A model for how transcription of a lncRNA can interfere with the genome architecture. The transcriptional machinery of a lncRNA (transcript shown in blue) can temporally erase chromatin loops by evicting CTCF/cohesin (violet/yellow) complexes that are in its path.

Figure 2: A model for how a lncRNA transcript can alter three-dimensional genome architecture. A lncRNA (blue) can bring together distant regions of the chromosome into closer proximity to its locus of transcription (yellow). These structural changes caused by the lncRNA may propagate by pulling new regions toward the same genomic locus to finally coat the locus or even the whole chromosome. (An example would be *Xist*).


TFs
RNA pol II
5'
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TFs
RNA pol II
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