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Minireview

Long Noncoding RNAs Usher In a New Era in the Biology of Enhancers

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Enhancer-associated long noncoding RNAs act over long distances and across chromosomes to activate transcription at distal promoters. Here, we address the latest advances made toward understanding the role of long noncoding RNA expression and the involvement of these RNAs in enhancer function through association with protein factors and modulation of chromatin structure.

Introduction

Enhancers are intergenic or intragenic regulatory sequences that can activate gene expression independent of their proximity or orientation with respect to their target genes (Bulger and Groudine, 2011). What makes enhancers remarkable is their ability to control temporal- and tissue-specific gene expression during development and their possible misregulation, contributing to a range of human diseases. Enhancer function depends on interplay between DNA sequences, DNA-binding proteins, and chromatin architecture, and in the last 3 years, the functional landscape has gained further complexity with the identification of enhancer-associated noncoding RNAs. Thus, even though enhancers were discovered more than 30 years ago, the molecular basis of their function remains elusive.

Simply identifying enhancers used to be a challenge. In recent years, we have seen a rapid expansion of large-scale prediction and identification of enhancers based on chromatin marks, occupancy by critical enhancer-associated factors, and large-scale transcriptome sequencing (Bulger and Groudine, 2011). Such genome-wide analyses have uncovered general properties of enhancers, resulting in the operational definition of enhancers as distal DNA elements (relative to the transcription start site) occupied by the transcriptional coactivator p300/CBP and RNA polymerase II (RNAPII) that often contain permissive chromatin with nucleosomes bearing histone H3K4 monomethyl (H3K4me1) and H3K27 acetyl (H3K27ac) modifications (Bulger and Groudine, 2011). An estimate of enhancer abundance and nucleotide length suggests that enhancers can make up as much as 10% of human genomic sequences (Bulger and Groudine, 2011).

Active Transcription from Enhancers

There are a large number of long noncoding RNAs that fulfill a range of functions in the nucleus and the cytoplasm (Ulitsky and Bartel, 2013). Nevertheless, the discovery that enhancers express long noncoding RNAs came as something of a surprise, and the observation that these RNAs increase in concentration in a stimulus-dependent manner suggested that they might confer an additional layer of regulation in gene expression (De Santa et al., 2010; Kim et al., 2010).

In several dynamic systems, the transcription of long noncoding RNAs from enhancer regions has been shown to correlate with the expression of neighboring protein-coding genes. In mouse neurons, more than 12,000 activity-regulated enhancers were defined by p300/CBP binding and H3K4Me1, of which 2,000 were shown to bind RNAPII and express bidirectionally long (~2,000 nt) and predominantly nonpolyadenylated noncoding RNAs (Kim et al., 2010). In human macrophages activated with endotoxin, a similar enhancer prediction approach identified several enhancers that express nonspliced, polyadenylated long noncoding RNAs (De Santa et al., 2010). The increase in concentration of the majority of these transcripts following treatment of the cells with the endotoxin preceded the induction of the neighboring protein-coding genes, suggesting a regulatory role for long noncoding RNAs in the upregulation of protein-coding genes in macrophages. A similar pattern of expression was observed in MCF-7 cells treated with estradiol, in which the induction of the majority of long noncoding RNAs also preceded the activation of their target genes (Hah et al., 2013). Additionally, experiments in T lymphocytes have provided evidence for binding of RNAPII along with general transcription factors and transcriptional initiation at several T-lymphocyte-specific enhancers, resulting in the expression of both polyadenylated and nonpolyadenylated long noncoding RNA transcripts (Koch et al., 2011). Interestingly, some of these long noncoding RNA-generating T cell enhancers occupied several kilobase-long transcriptional initiation platforms, reminiscent of recently described super-enhancers (Whyte et al., 2013). Though these studies suggested a functional relationship between long noncoding RNAs generated at enhancer loci and the transcription of their neighboring genes, they did not establish a requirement for long noncoding RNAs in transcriptional activation.

Long Noncoding RNAs Display Enhancer-like Function

Two studies subsequently provided evidence for a causal role in transcriptional activation for enhancer-associated long noncoding RNAs. These reports used knockdown approaches to deplete the levels of several long noncoding RNAs in human cells (Ørom et al., 2010; Wang et al., 2011). Depletion of specific long noncoding RNAs resulted in repression of neighboring

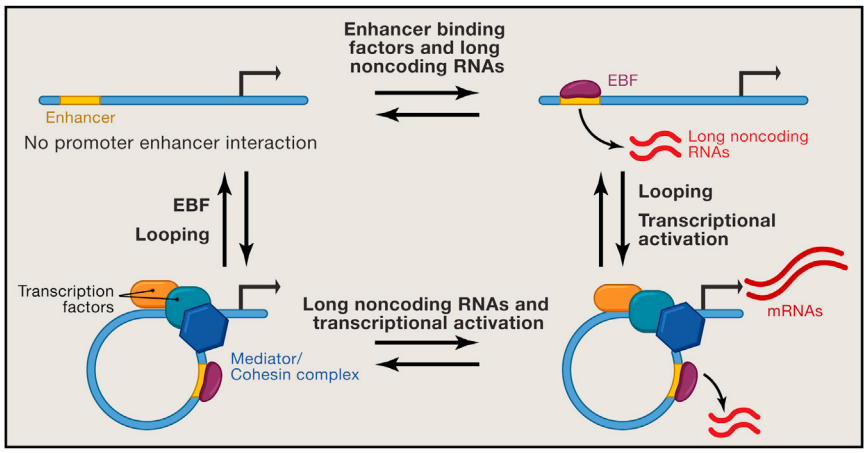

Figure 1. Long Noncoding RNAs in Enhancer Looping and Function

Long noncoding RNAs are proposed to function in the establishment or maintenance of enhancer promoter looping and activation of gene expression. In the absence of looping interactions (upper-left), the enhancer is inactive and transcription is minimal. One model for activation (counterclockwise) features engagement of enhancer-binding factors, transcription factors, and bridging factors such as the Mediator, Cohesin, and WDR5 complexes (lower-left), which leads to gene looping between enhancer and promoter sequences followed by induction of long noncoding RNA expression and transcriptional activation of the target gene (lower-right). A second model proposes that long noncoding RNA transcription precedes target gene activation and looping (clockwise), whereby binding of enhancer-binding factors and transcription of long noncoding RNAs are the first steps in the activation process (upper-right). Subsequently, a loop to the promoter is established through interactions with the transcription factors and bridging factors (lower-right), leading to activation of the neighboring gene.

protein-coding genes in a *cis* regulatory manner. Moreover, transcriptional activation could be recapitulated in classical enhancer assays using heterologous reporters and was shown to be dependent on long noncoding RNA expression (Ørom et al., 2010).

Additional evidence for participation of long noncoding RNAs in transcriptional activation was provided following the analysis of long noncoding RNAs transcribed from p53-binding sites using genome-wide approaches to define p53 chromatin residence (Melo et al., 2013). Depleting RNAs associated with p53-binding sites resulted in decreased expression of the neighboring protein-coding genes targeted by p53. Similarly, a genome-wide study of estrogen receptor-binding sites following estradiol stimulation identified enhancer sites expressing long noncoding RNAs, as revealed by global run-on sequencing (GRO-seq) (Li et al., 2013). Depletion of multiple long noncoding RNAs in this system resulted in diminished transcription of the neighboring genes.

Looking at nuclear hormone repression in mouse macrophages, Lam et al. proposed that the nuclear receptor Rev-Erb represses its target genes by binding predominantly to distal regulatory elements and preventing their ability to transcribe long noncoding RNAs (Lam et al., 2013). Analysis of specific candidate genes *Mmp9* and *Cx3cr1* subject to repression by Rev-Erb revealed the involvement of neighboring long noncoding RNAs. Importantly, most distal Rev-Erb-binding sites expressing long noncoding RNAs and overlapping with domains of H3K4me1 were deemed to be specific in macrophage lineage. This study points to the requirement for both transcription factor binding (Rev-Erb) and the presence of specific nucleotide sequences of the long noncoding RNA in transcriptional regulation. Indeed, the regulation of long noncoding RNA expression by Rev-Erb at distal sites was a more accurate measure of transcriptional repression of target genes than was the occupancy of Rev-Erb at distal regulatory elements alone. Importantly, the authors showed the utility of targeting long noncoding RNAs for locus- and tissue-specific gene regulation in mouse by manipulating the expression of *Mmp9* following depletion of distal *Mmp9* long noncoding RNAs rather than targeting the *Mmp9* gene itself.

Enhancer-associated long noncoding RNAs operate in key physiological processes. For example, experiments analyzing developmental regulation of neurogenin1, a critical transcription factor required for brain development, revealed the presence of distal regulatory elements expressing long noncoding RNAs (Onoguchi et al., 2012). Functional depletion of these long noncoding RNAs, termed *utNgn1*, demonstrated their requirement for transcriptional activation of neurogenin 1 during development (Onoguchi et al., 2012). Moreover, through in vivo experiments in mice, a long noncoding RNA termed *NeST* was shown to have enhancer-like functions by activating the neighboring interferon-γ locus and conferring microbial susceptibility (Gomez et al., 2013). Although most studies have focused on the regulation of protein-coding genes through the action of long noncoding RNAs, there are also examples in which long noncoding RNAs activate a neighboring long noncoding RNA. For instance, the long noncoding RNA *Jpx* regulates transcriptional activation of *XIST*, the long noncoding RNA that is critical for X inactivation in mammalian cells (Tian et al., 2010).

Enhancers and Chromosome Conformation

Although the experiments with reporter genes described above indicated that these long noncoding RNAs can work over short distances, many enhancers are distant from the promoters that they regulate, and there must exist augmenting mechanisms such as chromatin looping and perhaps formation of noncoding RNA-RNA or noncoding RNA-DNA interactions that allow for distal long noncoding RNAs to achieve target specificity in vivo. Indeed, one of the main hypotheses of how enhancers mediate their function is predicated on the idea that enhancers and their target promoters associate through a three-dimensional genomic architecture, bringing about a "chromatin loop" (Bulger and Groudine, 2011). This regulation has been shown to involve the interaction of the transcriptional coactivator Mediator and the chromosome segregation machinery the Cohesin complexes (Kagey et al., 2010; Phillips-Cremins et al., 2013). In addition, sequence-specific DNA-binding factors and their designated transcriptional coactivators play a prominent role in enhancer function (Figure 1).

Following the observations that long noncoding RNAs are required for enhancer activity, recent work has supported the involvement of long noncoding RNAs in the establishment or maintenance of chromosome conformation (Lai et al., 2013; Li et al., 2013). The studies by Lai et el. provided evidence that the Mediator complex and enhancer-like long noncoding RNAs are involved in the establishment or maintenance of chromatin looping between the long noncoding RNA loci and their regulated promoters. Knockdown of either long noncoding RNAs or Mediator subunits abrogated the chromosomal interactions, demonstrating an involvement of both the Mediator and the long noncoding RNA in chromatin looping and enhancer-promoter interactions. It was further shown that such long noncoding RNAs could associate with the Mediator complex and could stimulate its kinase activity toward phosphorylation of histone 3 serine 10 (H3S10), an important chromatin mark in transcriptional activation (Lai et al., 2013).

A subsequent study assessing the role of long noncoding RNAs induced by treatment of MCF-7 human breast cancer cells with 17B-oestradiol (E2) used a different approach and confirmed the importance of long noncoding RNAs in chromosomal conformation (Li et al., 2013). Using a strategy named three-dimensional DNA selection and ligation (3D-DSL), the authors identify DNA looping events that are induced between enhancers and their target promoters. Importantly, depletion of a number of E2-induced long noncoding RNAs diminished the looping events concomitant with decreased transcriptional activation (Li et al., 2013). Importantly, the authors identified the cohesion complex as a binding partner of E2-induced long noncoding RNAs and proposed a role for Cohesin in enhancer-derived chromosome looping. In a related study (Hah et al., 2013), long noncoding RNA production at estrogen-receptor-binding sites following estradiol treatment of MCF-7 cells was strongly correlated with not only the genomic features associated with enhancers (H3K4me1, H3K27ac, p300, RNAPII, and open chromatin architecture), but also with the DNA looping between enhancers and their target promoters. However, inhibition of long noncoding RNA production using the transcriptional elongation inhibitor flavopiridol did not affect the chromatin looping between enhancers and their target promoters. Though it is difficult to have a clear interpretation of these results, as flavopiridol also inhibits the expression of protein-coding genes, it may suggest that the prior synthesis of long noncoding RNAs may be sufficient to maintain chromatin looping. These results may, therefore, delineate a role for long noncoding RNAs in the maintenance of the DNA looping rather than its establishment.

It is also likely that not all genomic loci require long noncoding RNAs for DNA looping between enhancers and promoters. Indeed, although a role for the long noncoding RNA *HOTTIP* in chromatin looping across the human HOXA locus was suggested, depletion of *HOTTIP* did not affect the overall chromatin architecture, as measured by high-throughput chromosome conformation capture (Wang et al., 2011). A similar scenario was reported in studies analyzing p53-mediated transcriptional activation (Melo et al., 2013), wherein chromatin looping between p53-binding sites and their targets was deemed to be p53 independent, whereas long noncoding RNA expression at p53-binding sites was p53 dependent. The authors proposed that chromatin looping is established prior to p53-mediated activation and therefore prior to synthesis of long noncoding RNAs at p53 sites. Given these distinct results, further experiments are required to understand the precise contribution of long noncoding RNAs to chromatin architecture at specific genomic loci.

More Than One Kind of Activating RNA

The repertoire of long noncoding RNAs displaying enhancer-like activity is wider than originally thought. The initial identification of long noncoding RNAs with activating function focused on a class of long noncoding RNAs with gene loci that do not overlap with protein-coding genes. These long noncoding RNAs are expressed from independent transcription units and are predominantly spliced and polyadenylated (Gomez et al., 2013; Onoguchi et al., 2012; Ørom et al., 2010; Wang et al., 2011). Based on their genomic localization, these long noncoding RNAs were named intergenic or intervening long noncoding RNAs (lincRNAs). LincRNAs with activating function were operationally called *ncRNA-activating* (*ncRNA-a*). Many of these *ncRNA-a* are developmentally regulated and are responsive to differentiating stimuli (Onoguchi et al., 2012; Ørom et al., 2010; Wang et al., 2011).

On the other hand, long noncoding RNAs expressed from distal regulatory elements, though responsive to stimulus-dependent activation, comprise a class of bi- and unidirectional RNAs that contain a mixture of nonpolyadenylated and polyadenylated species (De Santa et al., 2010; Kim et al., 2010). These stimulus-induced long noncoding RNAs are termed enhancer RNAs (eRNAs) and constitute a heterogeneous class of long noncoding RNAs that is still not well annotated. Although the precise overlap between *ncRNA-a* and eRNAs is not clear, growing evidence suggests that, despite perceived differences in the biogenesis of these long noncoding RNA classes, they exert their functional effects on neighboring protein-coding genes through a common molecular underpinning by promoting chromatin looping to allow enhancer-promoter interactions (Lai et al., 2013; Li et al., 2013).

Do Long Noncoding RNAs Exert Their Function through a *cis*- or *trans*-Mediated Mechanism?

An important mechanistic question underlying long noncoding RNA function is whether they achieve transcriptional activation through a *cis*- or *trans*-mediated mechanism. A number of characteristics of activating long noncoding RNAs favor their function through a *cis*-mediated manner (that is, acting in proximity to the region from which they are transcribed). These characteristics include their low levels of expression, their predominant effect on neighboring genes, and their nuclear localization, as they are often closely associated with chromatin. However, there are examples in which long noncoding RNAs may activate their targets in *trans*. *Jpx*, an activating long noncoding RNA, was proposed to mediate its effects on the *XIST* RNA in *trans* (Tian et al., 2010) by evicting CTCF from the *XIST* promoter (Sun et al., 2013). One of the strongest examples of an activating long noncoding RNA functioning in *trans* is *NeST*, a long noncoding RNA that activates the IFN-γ locus contributing to microbial susceptibility (Gomez et al., 2013). The functional consequences of loss of *NeST* in mice could be rescued through overexpression

of the long noncoding RNA in transgenic animals, underscoring the importance of the long noncoding RNA in this immune context.

Long noncoding RNAs with activating function may also recruit transcriptional activators or critical chromatin regulatory complexes to enhancers or their corresponding targets. Such physical recruitment of regulatory complexes could be achieved either in *cis* via locally produced activating RNAs or in *trans*, where activating RNAs direct the recruitment of a specific complexes to a target loci in vivo. Taken together, the previous designation of enhancers as distal DNA regulatory elements allowed only for *cis*-mediated regulation of their target genes through transcription factor binding and chromatin looping. However, the emergence of long noncoding RNAs as dynamic molecular mediators of transcriptional activation provides for novel mechanisms of gene activation by which the long noncoding RNAs, through recruitment of coactivator complexes and regulatory factors, could mediate transcriptional activation in *trans* (Figure 1).

Outlook

The discovery of long noncoding RNAs with activating function and their intimate association with distal regulatory elements (enhancers) has profound implications for our understanding of gene regulation during differentiation, development, and disease progression. We have spent the last 20 years analyzing gene expression programs in healthy tissues and in disease states by profiling expression changes in protein-coding genes. The realization that tissue- and temporal-specific regulation of gene expression is governed by long noncoding RNAs expressed at enhancers provides us with an opportunity to analyze additional key regulatory molecules that may provide a greater insight into locus-specific gene regulation during disease development and progression. Moreover, although recent experiments have shed some light on the mechanisms by which this class of long noncoding RNAs functions, we are still in the early stages of fully understanding the long noncoding RNA code(s) required for activating function. It will be highly informative to understand the long noncoding RNA sequence determinants or structural requirements (secondary or tertiary) that govern enhancer function. It is also of prime importance to determine the allele specificity of the RNAs' activating function. Though multiple studies have pointed to *cis*-mediated function of some activating RNAs, whether activation is restricted to a single allele or can extend to both alleles has not been addressed and is a critical question that may reveal a broad and critical function for activating long noncoding RNAs in dosage compensation.

Given that RNA is an evolutionary predecessor of proteins, long noncoding RNA-mediated regulation of transcription may represent an ancient mechanism by which gene expression was regulated long before the appearance of proteins. As we continue to accumulate insights into these regulatory RNAs, it only becomes more apparent that there are new and unanticipated layers of regulation for tissue- and temporal-specific gene expression waiting to be discovered.

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