Enhancer RNAs: Insights Into Their Biological Role

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ABSTRACT: Enhancers play a central role in the transcriptional regulation of metazoans. Almost a decade ago, the discovery of their pervasive transcription into noncoding RNAs, termed enhancer RNAs (eRNAs), opened a whole new field of study. The presence of eRNAs correlates with enhancer activity; however, whether they act as functional molecules remains controversial. Here we review direct experimental evidence supporting a functional role of eRNAs in transcription and provide a general pipeline that could help in the design of experimental approaches to investigate the function of eRNAs. We propose that induction of transcriptional activity at enhancers promotes an increase in its activity by an RNA-mediated titration of regulatory proteins that can impact different processes like chromatin accessibility or chromatin looping. In a few cases, transcripts originating from enhancers have acquired specific molecular functions to regulate gene expression. We speculate that these transcripts are either nonannotated long noncoding RNAs (lncRNAs) or are evolving toward functional lncRNAs. Further work will be needed to comprehend better the biological activity of these transcripts.

KEYWORDS: Enhancer, eRNAs, lncRNAs, chromatin loop, gene expression, chromatin

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General Features and Dynamics of eRNAs

One of the most intriguing questions in biology is how cells achieve transcriptional regulation. In the case of multicellular organisms, development and response to external stimuli are complex processes that require fine regulation of gene expression. At the heart of metazoan transcriptional regulation are found the enhancers,[1] which are DNA elements bound by cofactors and transcription factors (TFs) that are able to increase the transcription levels of their target genes by direct stimulation of their promoters often through chromatin looping.[2] At the chromatin level, enhancers are characterized by prominent accessibility to DNase I and high levels of histone H3K4me1,[3] as well as high levels of histone H3K27ac when active.[4] An interesting discovery in the biology of enhancers came in 2010 when 2 groups independently found that enhancers were transcribed genome-wide into noncoding RNAs termed enhancer RNAs (eRNAs).[5,6] This finding raised the question of the potential role that eRNAs could play in the regulation of gene expression, as several long noncoding RNAs (lncRNAs) have been shown to be functional molecules.[7]

eRNAs have been identified in diverse organisms like *Caenorhabditis elegans, Drosophila melanogaster*, and mouse, and in human cell lines, which suggest that transcription at enhancers is of ancient origin and might have an important role in enhancer activity.[8–11] eRNAs have been described as usually bidirectional, on average separated by 180 base pairs and transcribed at equal levels into nonpoly(A), unspliced molecules.[9] Most eRNAs are transcribed at lower levels than mRNAs and other noncoding RNAs such as lncRNAs and are rapidly degraded by the exosome.[9,12] Interestingly, it has been observed that eRNAs can be subject to methylation.[13,14] Recent single-cell genome-wide data of nascent transcription challenges some of the typical characteristics of eRNAs as it was shown that enhancer transcription is unidirectional, and therefore within a cellular population, some cells transcribe the sense strand and others the antisense strand.[15] In the cases where both strands of eRNAs were detected in the same cell, single-molecule fluorescence in situ hybridization (FISH) experiments showed that colocalization of both RNA molecules was rare. Because typical RNA expression methodologies show an average for total cells, that would explain why eRNAs have been described as bidirectional. Importantly, eRNAs were expressed at similar levels compared with gene promoters in single cells and were detected in just a subset of cells, ie, displaying transcriptional bursting.[15] More single-cell measurements of nascent transcription in different conditions are needed to clarify the real nature of transcripts originating from enhancers.

Active enhancers are the main source of eRNAs, and the transcription of enhancers into eRNAs has been correlated with increased transcription of nearby genes,[5,9,16] suggesting that transcription, the eRNAs themselves, or both, might be important for enhancer activity. In support of this, time course experiments evaluating the response of cells to different stimuli have provided strong evidence of specific timing of transcription of enhancers into eRNAs.[10,16] For example, in mammalian cells, upon stimulation with growth factors and exposure to pathogens, the eRNAs from active enhancers are transcribed first, then mRNAs that code for TFs, and finally non-TFs mRNAs. This suggests that upon stimuli, a concerted transcriptional response is established during which responsive enhancers are transcribed into eRNAs first and then genes coding for TFs which could ultimately lead to genome-wide changes in

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BOX 1. A toolbox to work with eRNAs.

Cap analysis gene expression (CAGE) and global nuclear run-on sequencing (GRO-seq)-based methods as well as other recently developed experimental strategies that target nascent unstable transcripts are the best approaches to start characterizing eRNAs.[9,19–22] The transcriptomic data can then be compared against ChIP-seq data for enhancer chromatin marks, as well as DNase I, and more recently, ATAC-seq data to annotate enhancers and in this way assign eRNAs to those genomic regions.

After identification of transcribed enhancers, the next desirable step is to assign enhancers to target genes. This can be done by assigning an enhancer to the closest gene; however, we find this undesirable and strongly suggest to use genome-wide 3C-based techniques like Hi-C,[23] Capture Hi-C,[24] ChIA-PET,[25] or HiChIP.[26] Alternative, expression has also been used to link enhancers with promoters,[9] and recent computational approaches have been developed to predict enhancer-promoter interactions.[27] Once enhancer-promoter relationships have been established, the next step is to characterize either both the enhancer and its eRNAs or just the function of the eRNAs in this case when the enhancer has already been tested for function. In the former scenario, genome editing tools such as CRISPR-Cas9 can be used to assess the importance of a given transcribed enhancer by direct deletion of the genomic sequence. Evaluation of the expression levels of its target gene by quantitative reverse transcription polymerase chain reaction (RT-qPCR) will inform on the transcriptional effect the enhancer has on that promoter while 3C or 4C-seq can inform on the contribution of that enhancer element on the topology of that locus.

To evaluate the functional contribution of eRNAs to enhancer activity and the regulation of the target promoter, several considerations should be taken. First, validate eRNAs presence by strand-specific RT-qPCR. If possible, perform a Northern blot analysis to identify large RNA molecules derived from the enhancer element. The data from CAGE, GRO-seq, strand-specific RT-qPCR, and Northern blot should provide critical information on the abundance of RNA species, if there is a strand that is particularly more abundant and on the size of the molecules. This information will be fundamental to design knockdown strategies to determine whether the RNA molecule itself is regulating expression. In this case, small interfering RNAs (siRNAs) or locked nucleic acid antisense oligonucleotides (LNAs) can be used to deplete eRNAs and should be preferentially designed against the most abundant RNA specie. However, it is important to notice that RNA interference machinery is found mainly in the cytoplasm, so techniques based on the use of antisense oligonucleotides might be better for loss of function assays to study eRNAs. In this regard, the efficiency of transfection can greatly affect the resulting knockdown efficiency and because of that a careful selection of a cell system remains fundamental.

To evaluate the effect of eRNA knockdown, we advise to measure transcription of target genes as well as possible changes in long-range interactions if it is the case. To further support a function of the mature eRNA, a dead-Cas9–based strategy can be used to bring an eRNA to an enhancer element and evaluate different features like changes in the chromatin organization, histone marks, DNA accessibility, and potency to increase transcription. Finally, to study the potential association of an eRNA with specific regulatory proteins, it is advisable to perform RNA immunoprecipitation against the protein of interest and evaluate enrichment of the eRNA. If there is no a priori knowledge of the involvement of a protein, pull down of the eRNA with nuclear or cytoplasmic extracts, protein purification, and mass-spectrometry of eluted proteins should inform on potential protein partners.

In some cases, it has been shown that the act of transcription rather than the RNA molecule itself can be important and therefore inserting early termination sites as poly(A) signals into the enhancer sequence could be useful to study the role of productive transcription on enhancer activity.

transcription by directly regulating transcription at target genes. A possibility is that early responsive enhancers might produce eRNAs that contribute to a robust transcriptional induction of TF coding genes. It would be interesting to knockdown eRNAs from active enhancers contacting early inducible TF-genes and to characterize if they are actually playing a functional role in transcriptional activation as it might be suggested by their timing of transcription. An intriguing observation in B-lymphocytes during an innate antiviral immune response is that a subset of active enhancers continues to be transcribed despite loss of expression of its target genes.[16] This is in contrast with the majority of cases where enhancer-promoter pairs are concordant in their transcriptional status, ie, enhancer is transcribed first, then its target gene and then loss of enhancer transcription correlates with loss of transcription of its target gene.[16]

Overall, these observations suggest that eRNA transcription at enhancers is a regulated and dynamic process. In many scenarios, stimulus-dependent transcription at enhancers precedes the transcription of associated genes. However, these are mostly correlative observations, and still little is known about the molecular function of eRNAs and how they can impact transcription.

Mechanisms of Action

In the past decade, loss and gain of function assays have been critical to characterize the function of many ncRNAs like the lncRNAs. Most of the described mechanisms involve the interaction of lncRNAs with key regulatory proteins, like Polycomb and Trithorax.[17,18] In many of these cases, the RNA acted as a decoy, a scaffold, a guide, or even as an enhancer.[7] In contrast to lncRNAs, assigning a molecular mechanism of action to eRNAs has been challenging mainly due to their unstable nature as well as their very low abundance (Box 1). However, over the past decade the function of some of these eRNAs, in very specific scenarios, has been characterized. We proceed to describe these examples and discuss their implications for eRNA functions and their impact in the control of transcription.

Stimulation of enzymatic activity

The strongest evidence for a common function of many eRNAs was the finding that eRNAs interact with CREB binding protein (CBP), an acetyltransferase that, alongside with p300, deposits the histone H3K27ac mark in chromatin.[28] Native RNA immunoprecipitation and PAR-CLIP (photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation) experiments demonstrated a CBP-eRNAs interaction in vivo.[28] In vitro assays showed that CBP-eRNAs interactions were independent of sequence, which suggest that a particular secondary structure of these RNAs may influence binding to CBP, as has been suggested for lncRNAs interacting with other regulatory proteins.[29] Of note, CBP-eRNAs interactions are already detected at actively transcribed enhancers where CBP is already bound.[28] This could mean that the interaction between CBP and eRNAs is not required for CBP recruitment. In line

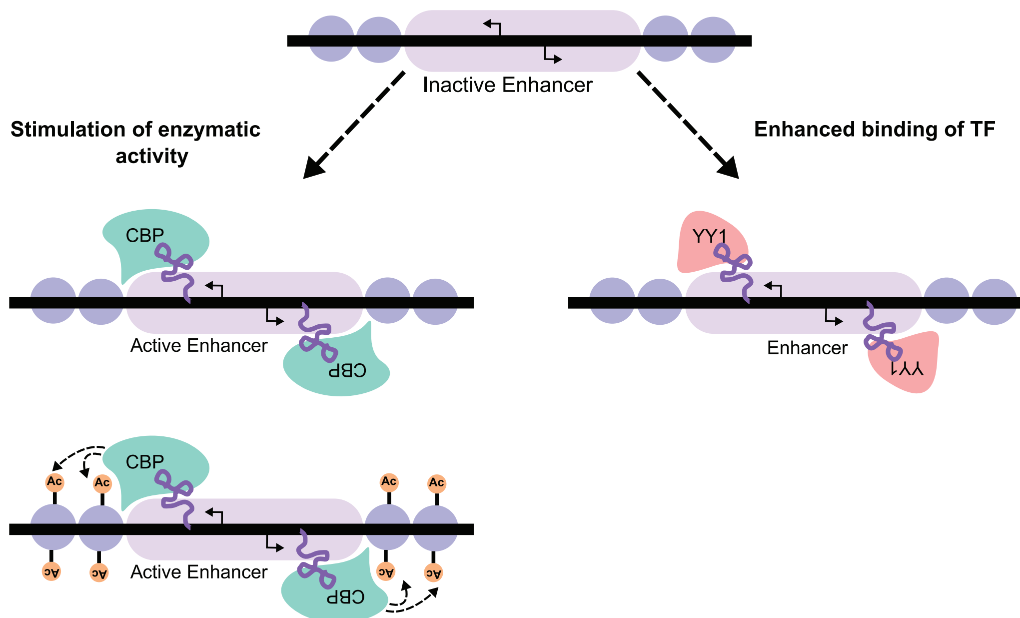

Figure 1. Enhancer RNAs (eRNAs) can influence catalytic activity of chromatin modifier proteins or act as traps for transcription factors. *Left*, eRNAs can directly interact with CBP, a histone acetyltransferase, and stimulate its enzymatic activity which results in a eRNA concentration dependent increase of H3K27ac as well acetylation in other amino acid residues of histones. *Right*, eRNAs at enhancers can trap transcription factors, like YY1, which results in an increase of signal for YY1 as evaluated by ChIP. This could mean that association with eRNAs increases residency time of TFs. CBP indicates CREB binding protein; TF, transcription factors.

with this, CBP-eRNA's interaction stimulates the histone acetyltransferase activity of CBP by increasing its affinity for a histone substrate which results in higher deposition of H3K27ac as well as H4K5ac histone marks[28] (Figure 1, *Left*). Knockdown of different eRNAs using antisense oligonucleotides resulted in a decrease in the histone H3K27ac level at the corresponding enhancer of origin and at the adjacent gene promoter with a concomitant reduction in the mRNA levels for target genes.[28] These data suggest that transcription of enhancers into eRNAs could enhance CBP acetyltransferase activity, which in turn might promote the acquisition of a chromatin environment permissive for enhancer function. In line with this, it has been shown that depletion of eRNAs during myogenesis results in a decrease in DNase I accessibility,[30] although the chromatin remodeling complex responsible for this effect remains elusive.

TFs trapping

lncRNAs can interact with different proteins involved in chromatin modification or organization, such as PRC2, MLL1, and CTCF.[17,18,31] Because enhancers are platforms for the binding of a variety of TFs, and Zinc Finger Domains of different TFs can interact with RNA[31–33]; therefore, eRNAs could interact with TFs, possibly influencing their residency time at enhancer elements. In line with this, YY1, a ubiquitously expressed TF in mammals, can interact in vivo with RNA when bound both at promoters and enhancers.[33] Inhibiting transcription or the exosome resulted in a decrease of YY1 recruitment at enhancers. This suggests that it is the nascent transcription of the eRNAs that is important for YY1 binding at enhancers. In line with this, tethering a specific eRNA in the vicinity of six enhancers bound by YY1 resulted in an increase in the binding of YY1 to those sites[33] (Figure 1, *Right*). Although the increase of YY1 binding to enhancers after eRNA tethering was modest, it supports a model where eRNAs might help to increase binding or residency time of YY1 at enhancers.

eRNAs can also interact with bromodomain-containing protein 4 (BRD4),[34] which is a member of the bromodomain and extraterminal domain (BET) family of proteins. It can bind to acetylated histones and stimulates elongation of protein-coding and noncoding RNAs. The interaction between eRNAs and BRD4 increases BRD4 binding to acetylated histones in vitro and to its target enhancers in vivo.[34] Knockdown of BRD4 interacting eRNAs results in loss of BRD4 and RNA polymerase II (RNA Pol II) binding at enhancer as well as loss of transcription of the target gene.[34]

Because other TFs have been shown to specifically interact with mRNAs and lncRNAs, like CTCF,[31] it remains to be determined if they can also interact with eRNAs as well. In addition, it is currently unclear what could be the effect of increasing the residency time of a TF at enhancer elements. An attractive possibility is that eRNAs could increase the binding of TFs to low affinity sites at enhancers; however, this remains to be elucidated.

Chromatin loop formation

Enhancer-promoter interactions through chromatin looping are important for tight control of gene expression either during development or cell differentiation, or in response to specific stimuli.[35,23] Defects in these interactions can have profound

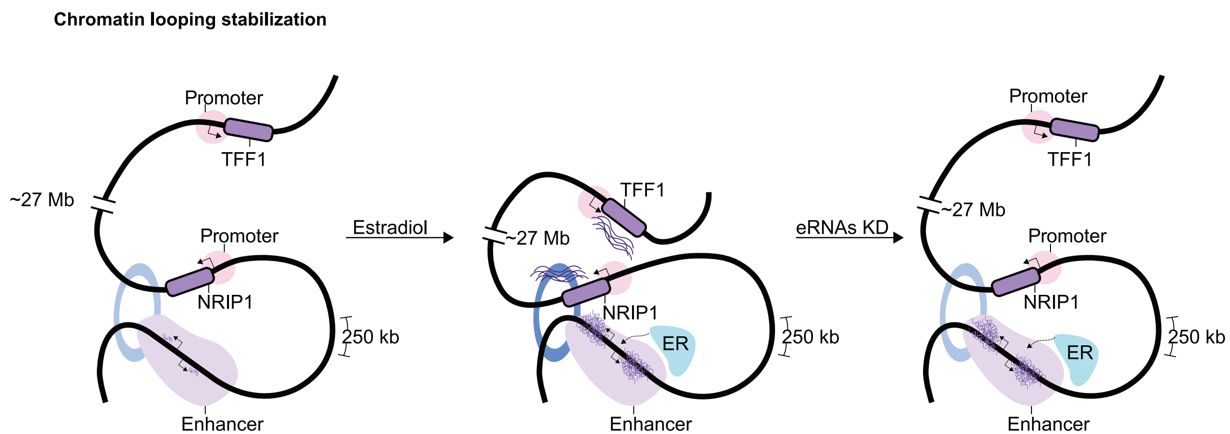

Figure 2. eRNAs can stabilize chromatin long-range interactions between transcribing enhancers and target promoters. The *NRIP1* promoter is in close proximity with an enhancer located ~250 kb upstream. Upon stimulation with estradiol, the transcription of both the enhancer and *NRIP1* is induced. This transcriptional effect is accompanied by increased deposition of the cohesin subunits Rad21 and SMC3 at the enhancer, as well as an increased frequency of interaction between the enhancer and the *NRIP1* promoter gene. This local reorganization is also accompanied by the gain of a novel interaction with *TFF1*, located 27 Mb away from *NRIP1* gene that is also transcriptionally induced during estradiol treatment. Depletion of *NRIP1*-associated eRNAs results in loss of *NRIP1* transcription, decreased deposition of cohesin subunits at the enhancer, and importantly, a decreased frequency of long-range interactions between the enhancer and the *NRIP1* promoter gene as evaluated by 3C and the loss of the 27 Mb interaction as evaluated by fluorescence in situ hybridization. Of note, eRNAs can directly interact with cohesin subunits. eRNA indicates enhancer RNAs; KD, knockdown; ER, Estrogen Receptor.

implications in development and disease generally due to changes in gene expression.[36–38] Different architectural proteins have shown to be important for promoting chromatin looping between regulatory elements, among them CTCF, YY1, and cohesin, as well as proteins involved in the general machinery of transcription like Mediator, RNA Pol II, Integrator, and cell-type-specific TFs.[23,39–44] These proteins can bind, for example, at enhancers and promoters and induce the establishment or the stability of long-range interactions. Because enhancers can be transcribed into eRNAs, it has been tempting to speculate that they might influence looping interactions between enhancers and promoters, which could ultimately result in regulated changes in gene expression. In line with this, several studies have reported a contribution of eRNAs to chromatin looping between selected activated enhancers and promoter elements.[12,16,40,45,46] In most of these studies, knockdown of eRNAs from selected enhancers either by small interfering RNAs (siRNAs) or locked nucleic acid antisense oligonucleotides (LNAs) results in quantitative and qualitative changes in looping contacts between transcribed enhancers and promoters which correlates with decreased transcription of their target genes.

In some cases, it has been shown that eRNAs can directly recruit architectural proteins like cohesin to their enhancer elements and this could directly contribute to changes in long-range interactions.[46] For example, in breast cancer cells the promoter of the *NRIP1* locus interacts in cis through chromatin looping with and enhancer located ~250 kb away. Upon stimulation with estradiol, the estrogen receptor binds to the enhancer. This results in strong transcription into eRNAs, increased binding of cohesin subunits, and higher frequency of long-range interaction with the *NRIP1* promoter which correlates with upregulation of the *NRIP1 mRNA* levels.[46] Importantly, reduction of eRNAs by the use of LNAs leads to the loss of looping interactions and transcription at *NRIP1* locus. These changes are also accompanied by decreased recruitment of cohesin subunits to the enhancer element. For this locus, the changes in long-range interactions are not just restricted to its enhancer element because depletion of eRNAs also results in loss of a ~27 Mb long-range interaction with the *TFF1* locus, which itself has an inducible enhancer transcribed upon estrogen stimulation (Figure 2).

Other proteins like Mediator, an important regulator of RNA Pol II activity, have been documented to play a role in promoting looping interactions between enhancers and promoters.[39,45,47] In regard to eRNAs, some reports have suggested that eRNAs interact with Mediator to promote long-range interactions between transcriptionally active enhancers and their target promoters.[45,47] However, a careful examination of current annotations for those locus suggest that those eRNAs are in fact lncRNAs that act in cis to regulate neighboring genes through direct interaction with Mediator and cell-type -specific TFs. Therefore, the role of Mediator in eRNA function requires further clarification.

The proper biogenesis of eRNAs is also important for stimulus-dependent enhancer activity and the establishment of long-range interaction between such enhancers and their target promoters. In this regard, it has been documented that Integrator, a multisubunit complex associated with RNA Pol II, is recruited to enhancers in a stimulus-dependent manner and is necessary for release of eRNA transcripts for elongating

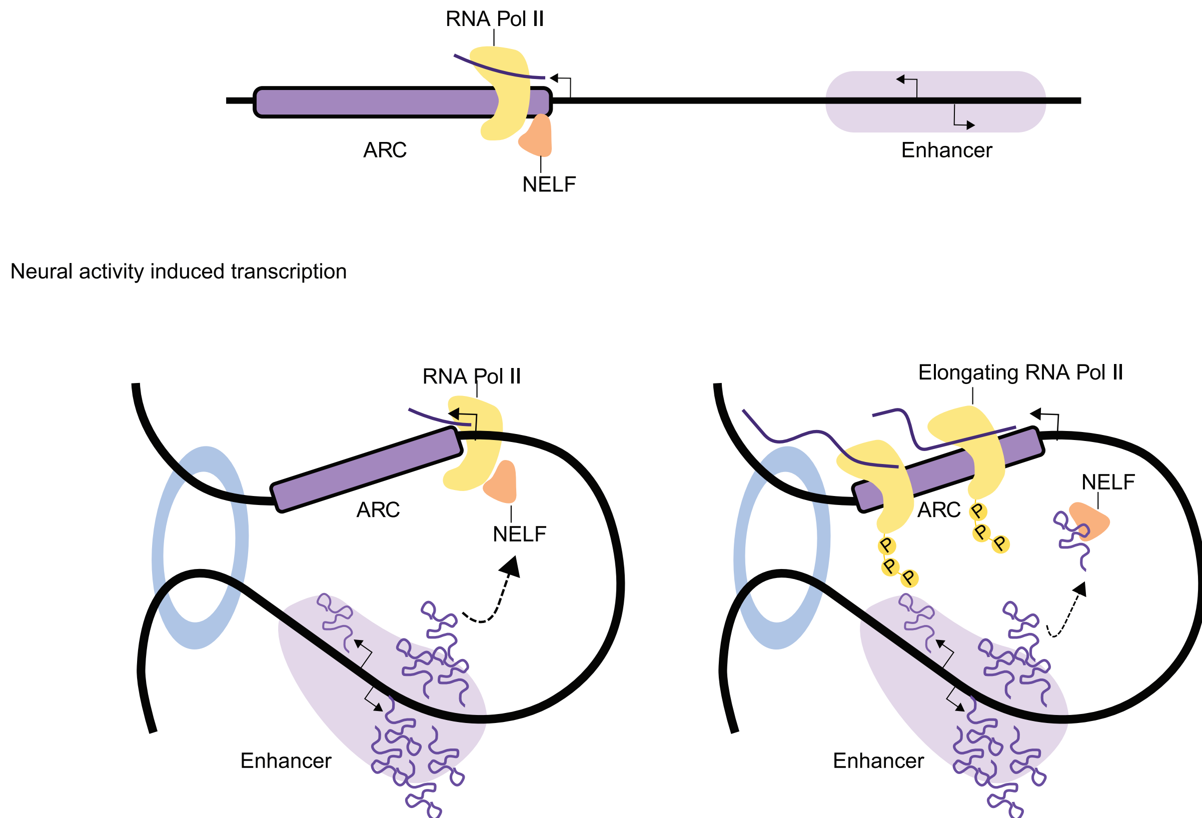

Figure 3. Enhancer RNAs (eRNAs) can promote exit of RNA Pol II pausing at promoters by mimicking nascent transcripts and interacting with NELF. The *ARC* locus is in physical proximity with an enhancer element throughout chromatin looping. Upon neuron stimulation, the transcription of both the enhancer and *ARC* gene is induced. Interestingly, transcription of one of the enhancer strands is more abundant. This eRNA can directly interact with NELF acting as a decoy for that protein. This results in loss of NELF at *ARC* promoter and the release of the paused RNA Pol II which engages in productive elongation. NELF indicates negative elongation factor; RNA Pol II, RNA polymerase II.

RNA Pol II.[40] Loss of Integrator results in accumulation of eRNAs primary transcripts. This effect compromises enhancer function, resulting in loss of chromatin looping between selected responsive enhancers and their target promoters with a corresponding loss of transcriptional induction. These data suggest that mature eRNAs, but not the transcription of enhancers, are required for long-range interactions between enhancers and promoters.

In this regard, it may be tempting to speculate that at stimulus-dependent enhancers, the induction of transcription by binding of specific TFs results in the generation of mature eRNAs by the Integrator complex. Then, these eRNAs in conjunction with boundary proteins like CTCF or YY1, could promote the stalling of loop-extrusion factors, like cohesin, through direct RNA-protein interactions.[48] Therefore, depletion of eRNAs could result in the loss of an RNA-dependent boundary that results in a decrease of specific long-range interactions between pairs of enhancer-promoter sequences. Interestingly, an RNA-dependent function of CTCF for chromatin looping has been recently reported.[49–51]

RNA Pol II productive elongation

eRNAs can regulate RNA Pol II elongation by interacting with proteins that either promote or inhibit elongation.[52,53] For example, eRNAs can promote exit of RNA Pol II pausing at activity-dependent neuronal promoters throughout direct interaction with the negative elongation factor (NELF), which promotes RNA Pol II pausing.[52] Knockdown of eRNAs associated with the enhancers of the neuronal genes *ARC* and *GADD45B* did not affect long-range interactions between the enhancers and promoters but resulted in an increase of the NELF at their promoters, which was accompanied by a decrease of the corresponding mRNA[52] (Figure 3). Because these eRNAs can interact with NELF via an RNA-binding domain, it was suggested that eRNAs could trap NELF, mimicking nascent transcription at target promoters. Therefore, eRNAs could promote RNA Pol II productive elongation by acting as a decoy for NELF, which in turn might be facilitated by the close physical proximity between the enhancers and promoters by means of chromatin looping.

eRNAs can also promote elongation by interaction with the positive transcription elongation factor b (p-TEFb) complex

which promotes RNA Pol II elongation by phosphorylation of different targets including NELF and Pol II-Ser2.[53–55] An eRNA transcribed from an enhancer that increases the expression of the target gene *PSA* can directly interact with the kinase CYCLIN T1 which is part of the p-TEFb complex.[53] Loss of this interaction correlates with a diminishment of Pol II-Ser2 phosphorylation and *PSA* expression. Interestingly, the interaction between CYCLIN T1 was achieved via an HIV-1 TAR RNA-like motif found in the eRNA. The motif present in the eRNA is also similar to the one found in the 7SK small nuclear RNA which can inhibit p-TEFb function. Therefore, this eRNA could favor elongation by RNA Pol II by competing with 7SK for the interaction with the p-TEFb complex thought CYCLIN T1. This is an example of a functional embedded structure in an eRNA and opens up the possibility that a subset of eRNAs act through specific sequence motifs. However, as we know that enhancers poorly conserved, this case might rather be an exception.

Concluding Remarks

The discovery of the pervasive transcription of the genome into ncRNAs has opened a whole new field of study. Enhancer transcription is of interest as these regulatory elements are at the heart of transcriptional regulation in processes as diverse as development, cell differentiation, and response to stimuli. One of the most important questions in the field is if eRNAs have a function. Even though it has been suggested that most eRNAs might be transcriptional noise,[56] for over almost a decade, different mechanisms of action have been characterized by which eRNAs could influence gene expression. Overall, eRNAs seem to exert their function by interacting with different regulatory proteins, similar to what has been discovered for lncRNAs. eRNAs can affect the chromatin environment of their enhancer, either by promoting chromatin accessibility, stimulating the histone acetyltransferase activity of CBP or enhancing the binding of TFs.

eRNAs can also stabilize chromatin looping contacts between enhancers and promoters by the recruitment of cohesin and can affect the transcriptional machinery already poised at promoters. In those cases, eRNAs can act as decoys for proteins like NELF, promoting elongation or influence the catalytic activity of other proteins associated with RNA Pol II.

Although progress has been made over the past years to uncover the potential molecular function of eRNAs, it should be pointed out that most of the examples covered here are restricted to a few loci. In many cases, although transcription of both strands of the enhancer is detected, one strand is prominently transcribed into an ncRNA that has a molecular function important for enhancer activity or gene transcription. This rise the concern of whether the molecular functions already reported for this subset of eRNAs could be a general feature of eRNAs or in fact they represent specific cases were eRNAs have acquired functions similar to lncRNAs or have been misannotated as eRNAs.[57] In this regard, a clear working definition that helps to distinguish eRNAs from lncRNAs might be of great help.

Based on current evidence, we propose that transcription of enhancers into eRNAs could be an RNA-based mechanism to trap proteins relevant for enhancer activity. In support of this, an increasing number of proteins with key regulatory functions like CTCF, YY1, and CBP have been shown to interact with RNA, and this association can have important consequences for their activity. In this regard, transcription at enhancers could be a means to increase the residency time of both DNA-binding proteins and cofactors.

For example, upon cell stimulation, specific TFs could bind to already CBP-bound enhancers and potentiate transcription. Those eRNAs could further stimulate the histone acetyltransferase activity of CBP which in turn could trigger BRD4 binding. Because eRNAs can also interact directly with BRD4 and increase the binding of this protein to the enhancer element, and BRD4 can promote elongation by recruitment of p-TEFb, Mediator, and RNA Pol II, these could lead to increased transcription of the enhancer into eRNAs.[34] All these can result in a net increase of RNA species at the enhancer that could lead to the establishment of a feed-forward loop where increasing eRNA molecules keep on promoting elongation at enhancer as well as acetylation of histones resulting in a chromatin structure that could boost enhancer activity over target promoters. Therefore, based on current evidence, we envision a general mechanism by which eRNAs favor the maintenance or reinforcement of a chromatin environment optimal for enhancer function. In some cases, these eRNAs could have evolved to acquire a specific molecular function and might be in a path toward becoming lncRNAs. Further work is needed, particularly on developing high-throughput genome-wide tools, to assess the functional role of eRNAs and clarify their relevance on transcriptional regulation.

Author Contributions

JC-FL, RGA-M and FR-T wrote and revised the manuscript.

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