

Transcriptional control by enhancers and enhancer RNAs

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ABSTRACT

The regulation of gene expression is a fundamental cellular process and its misregulation is a key component of disease. Enhancers are one of the most salient regulatory elements in the genome and help orchestrate proper spatiotemporal gene expression during development, in homeostasis, and in response to signaling. Notably, molecular aberrations at enhancers, such as translocations and single nucleotide polymorphisms, are emerging as an important source of human variation and susceptibility to disease. Herein we discuss emerging paradigms addressing how genes are regulated by enhancers, common features of active enhancers, and how non-coding enhancer RNAs (eRNAs) can direct gene expression programs that underlie cellular phenotypes. We survey the current evidence, which suggests that eRNAs can bind to transcription factors, mediate enhancer-promoter interactions, influence RNA Pol II elongation, and act as decoys for repressive cofactors. Furthermore, we discuss current methodologies for the identification of eRNAs and novel approaches to elucidate their functions.

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Introduction

The transcriptional output of any given cell is largely dictated by the repertoire of active enhancer elements contained within its genome. Enhancers were first described in the 1980's as a sequence of DNA that increases the expression of a linked gene in a manner that is independent of its distance and orientation [1–3]. Interestingly, the first enhancer was a 72-base pair (bp) sequence, cloned out of the simian virus 40 (SV40), that was capable of increasing the expression of the human β -globin gene [1,2]. Since then, enhancers have been found in many metazoan organisms, including humans, and have been shown to recruit specific transcription factors to dictate spatiotemporal gene expression during development, normal physiology, and even in disease [4–9].

The advent of next-generation sequencing technologies has facilitated comprehensive and unbiased identification of enhancers across the genome, and current estimates suggest that there are over 400,000 enhancers scattered throughout the genome [10,11]. Enhancers can be located within genes, in intragenic regions, and even on different chromosomes [12–15].

Interactions between enhancers and their target genes are not exclusive, and frequently promoters are found to interact with multiple enhancers and vice versa [11,16,17]. Of note, enhancers are highly cell-type specific and thus not all potential enhancers are expected to be active at the same time. Instead, within any given cell type, there are tens of thousands of active enhancers orchestrating lineage-specific gene expression [10,11,18–20].

Enhancer malfunction is emerging as a major contributor to human diseases, including cancer, as mounting evidence across many tumor types shows that enhancer networks are rewired by molecular aberrations that collectively lead to the cancer phenotype [21–27]. Translocations, deletions, and mutations within regulatory regions of the genome are frequently observed in cancer patients, which may cause loss of expression of tumor suppressors or overexpression of oncogenes [25,27,28]. For example, in Burkitt lymphoma, genomic translocations relocate the highly active enhancers of the immunoglobulin heavy chain genes in close proximity to the MYC oncogene, leading to deleterious activation of MYC expression [29,30]. In B-cell lymphoma, rearrangements and duplications of benign enhancers

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drive the expression of typical cancer genes, such as *MYC*, *BCL2*, and *NOTCH1* [31]. In addition to enhancer translocation or duplication, cell type-specific enhancers can also be activated, or “hijacked”, by cancer cells for the activation of genes that lead to tumorigenesis or drug resistance [21,23,32]. Thus, delineating the dysregulated enhancer network would certainly extend our knowledge of cancer biology, and provide alternative pathways to treat stubborn cancers lacking driver mutations [33,34]. The increasing appreciation for the role of enhancers in the etiology of disease has placed emphasis on defining the mechanisms of enhancer function, and, although many common features of enhancers have been described over the years, the order-of-events for the activation of enhancers is still unknown.

Common features of active enhancers

Recent studies have focused on the properties of enhancers beyond the binding of sequence-specific transcription factors, which might give clues to their mechanisms of action and aid in their identification. In this regard, cooperative binding of transcription factors, co-factors, chromatin remodeling enzymes, and ultimately RNA Polymerase II complex (RNA Pol II) at enhancers mediate the activation of target gene expression (Figure 1) [35–37]. However, assembly of these proteins and enzymes requires the DNA at enhancers to be open and accessible. Thus, chromatin accessibility is a defining feature of active

enhancers and can be measured using various techniques, such as DNase I Hypersensitivity, Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE), or transposase assisted measurements of chromatin accessibility (ATAC) (Table 1) [3,38–40]. In addition, current models posit that communication between enhancers and their target genes typically occurs via DNA looping, which enables physical interaction between enhancers and target gene promoters (Figure 1) [41–44]. The long-range interactions between enhancers and promoters was initially confirmed using proximity ligation techniques, such as chromosome conformation capture (3C) [45–47], and later coupled to high-throughput sequencing methods such as 4C and HiC-seq [48–51]. Of note, it is thought that chromatin loops are established prior to enhancer activation, suggesting that these networks are pre-determined during the differentiation of the cell.

The epigenetic state of enhancers is also fundamental to their functions and can be used to distinguish between enhancers and gene promoters (Table 1). For example, the nucleosomes surrounding enhancers typically harbor histone H3 lysine 4 monomethylation (H3K4me1) and lack histone H3 lysine 4 trimethylation (H3K4me3). In contrast, active promoters are enriched in H3K4me3 and have reduced H3K4me1 at their transcription start sites [52,53]. The status of H3K4 methylation allows us to differentiate between enhancers and promoters, but this sole criterion is not sufficient to divide all enhancers

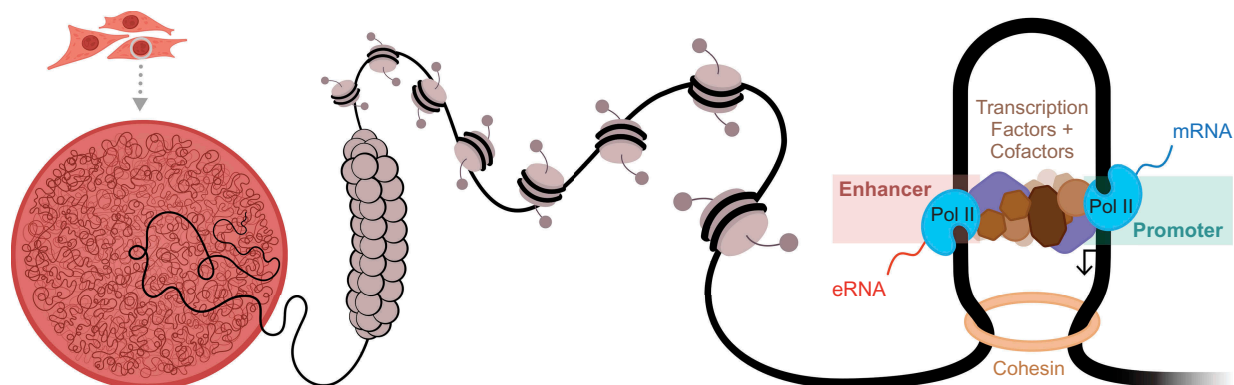


Figure 1. A model for enhancer-promoter interactions within the nucleus of a cell. Enhancers may be located far away in linear distance to their target genes, but are brought into close proximity to these genes via chromatin loops and higher order chromatin structure. Enhancers are bound by sequence-specific transcription factors, which in turn facilitate the cooperative binding of chromatin remodeling enzymes, histone modifying enzymes, other co-factors, and ultimately the RNA polymerase II complex (RNA pol II). Both the target genes and the enhancers are transcribed by RNA pol II. Transcription of the enhancers give rise to non-coding RNA molecules called enhancer RNAs (eRNAs).

Table 1. Common features of an active enhancer.

Feature	Commonly Used Methods for Detection	References
Chromatin accessibility	DNase-seq, ATAC-seq, and FAIRE-seq	[11,38–40]
Histone modification H3K4me1	ChIP-qPCR, ChIP-chip, and ChIP-seq	[10,52,53]
Histone modification H3K27Ac	ChIP-qPCR, ChIP-chip, and ChIP-seq	[10,60,61]
Enhancer-promoter looping	3C, 4C, HiC, and Hi-ChIP	[45–50,163,165–167,176]
Binding of transcription factors, co-factors, and RNA Pol II	ChIP-qPCR, ChIP-chip, and ChIP-seq	[10,18–20,52,55,56,58]
Production of eRNAs	GRO-seq, CAGE, PRO-seq, Total RNA-seq RT-qPCR (using random hexamer primers)	[21,97,152,156–158]

and promoters without ambiguity. Combining H3K4 methylation with other indicators of enhancer activity, for example, transcriptional coactivators p300/CBP or Mediator, can significantly improve the enhancer prediction [18,54–57]. Similarly, binding of RNA Pol II, BRD4, and other TFs, have also been utilized [10,20,52,55,58,59]. Furthermore, studies in embryonic stem cells and several primary cell types uncovered that acetylation of histone H3 lysine 27 (H3K27ac) together with H3K4me1 is typically enriched at active enhancers, whereas H3K4me1 alone designates inactive or “poised” enhancer regions (Table 1) [3,60,61].

One of the most robust indicators of an active enhancer is the binding of RNA Pol II and the production of non-coding enhancer RNAs (eRNAs) (Figure 1). The discovery that enhancers are transcribed to produce non-coding RNAs has added another layer of complexity to the mechanisms of gene activation by enhancers and has given rise to an intense area of investigation. Below we detail the current state of enhancer RNAs, what is known about their potential functions, and highlight some emerging paradigms about their role in gene expression (Table 2).

Super enhancers and liquid-liquid phase separation

Advances in enhancer identification using high-throughput sequencing technologies have revealed the presence of large clusters of enhancers that exist in close proximity to each other and seem to work synergistically for the activation of target genes. These clusters of enhancers, named super-enhancers (SEs), are noted for their unusually high occupancy of interacting factors, enzymes, and histone modifications, that work together to drive the expression of prominent lineage specific genes [55,62]. Super-enhancers are larger than typical enhancers, are highly enriched in the classic features of active enhancers (such as H3K27ac, H3K4me1, mediator, p300, BRD4, and RNA Pol II), and are thought to act synergistically for the activation of target genes. Out of 10,000 to 150,000 putative active enhancers in a cell, there are only a few hundred super-enhancers. These SEs drive high levels of transcription and are strongly proposed to be cell fate determinants that dictate cell type-specific gene expression [55,63,64]. Current evidence suggests that super-enhancers contribute to the high expres-

Table 2. Common features of eRNAs.

Common features of eRNAs	Selected References	Comments
Produced at active enhancers	[21,82,104]	
Non-coding transcripts	[78–80]	
Transcribed by RNA Pol II	[8,78,82,88–90].	
Not spliced	[81,96,97]	Spliced eRNA: [14]
Lack of polyadenylation	[78,90,96,100,101]	eRNAs with polyadenylation: [80,96]
Cell-type specific	[21,82,104]	
Bidirectional transcription	[79,81–84]	Unidirectional transcription: [78,80,85–87]
Degraded by RNA Exosome	[78,90,96,100,101]	
Regulated by Integrator	[95]	
Average size 2kb – 5kb	[78–80]	
Bind transcription factors	[58,89,98,117,126–131]	
Release paused RNA Pol II	[90,133–135]	
Contribute to enhancer-promoter looping	[63,89,92,94,99,108,142]	Exceptions: [82,90,143]
Interfere with transcriptional repressors	[90]	

sion of pluripotent genes in embryonic stem cells (mESCs), cell type-specific genes in terminally differentiated cells, and oncogenes in various cancer cells [65,66], and therefore have garnered significant attention.

Emerging evidence suggests that the high levels and very dense assemblies of transcription factors and co-factors at super-enhancers impart the ability of SEs to form biomolecular condensates as a result of liquid-liquid phase separation [67]. Liquid-liquid phase separation refers to the process by which a homologous solution of molecules spontaneously separates into two liquid phases with enriched or depleted molecules [68]. This concept has been widely adopted to explain the dynamics of membrane-less organelles in cells, including centrosome, nucleolus, paraspeckles, P-bodies, and stress granules [68,69]. In contrast to membrane-enclosed sub-cellular compartments, these membrane-less organelles solely consist of a large number of macromolecules, primarily RNAs and proteins, which “condensate” to form a complex based on protein-protein and protein-nucleic acid interactions. Phase separation at SEs are thought to allow for compartmentalization of the transcriptional components needed for robust activation of target genes [67,69–71].

However, this also makes SEs more susceptible to perturbation as compared to most typical enhancers [67]. Recent literature suggests that function of SEs relies on the proper aggregation of the participating transcription factors and coactivators, which depends on the phase-separating properties of their intrinsically disordered regions (IDRs). Chemical compounds with capacity to disrupt liquid-liquid biomolecular condensates, such as 1,6-hexanediol, have been shown to deprive occupation of BRD4, MED1 and RNA Pol II at SEs and attenuate the expression of SE-driven genes [67,70,72,73]. Since SEs are known to regulate the expression of oncogenes, they can be targeted to change the fate of cancer cells by disrupting the biomolecular condensates that mediate their function. The emerging roles of phase separation and its implications on gene expression and human health have been reviewed thoroughly in the following publications [67,68,71,74,75]. Despite the order of magnitude that separates enhancers and super-enhancers, they share many key features that govern their

functions. One of the most interesting of these is the production of eRNAs.

Enhancer RNA discovery and transcriptional properties

The discovery that enhancers are transcribed and produce non-coding RNA transcripts came as a significant breakthrough in the study of enhancer function. This phenomenon was first described at the β -globin locus, where it was shown that the hypersensitive site 2 (HS2) enhancer produced transcripts when active [76,77]. It was not until 2010 that enhancer transcription was demonstrated to be a shared feature of enhancers, as opposed to a mechanism exclusive to the HS2 enhancer. Using RNA Pol II chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq), it was shown that enhancers and super-enhancers produce non-coding RNA molecules, termed enhancer-derived RNAs (eRNAs) [78–80]. The role of eRNAs in enhancer function remains unclear, however, numerous studies have set forth to characterize eRNAs and determine their role, which has highlighted a variety of shared and unique features that are important to enhancers (Table 2).

The simplest definition of an eRNA is a non-coding transcript produced within a validated enhancer region, though the traits that are often shared amongst them are considerably more complex. Current models posit that enhancers are transcribed bidirectionally in a manner resembling gene promoters [79,81–84], with unidirectional transcription occurring at a small subset of these elements [76,78,80,85,86]. Interestingly, a recent single-cell transcriptomic profiling technique has been used to demonstrate that enhancers are primarily transcribed unidirectionally from a single strand on a cell-to-cell basis, challenging the previously accepted belief that both strands are transcribed simultaneously [87]. This study also used single-molecule fluorescence in situ hybridization (smFISH) to show that when both strands are transcribed in the same cell, the eRNAs rarely colocalize. This suggests that enhancers may have additional functionality based on which strand is actively transcribed, though the mechanism by which a strand is selected for transcription has not been defined. Transcription of eRNAs occurs via RNA Pol II and temporally precedes the activation of target gene

promoters [8,78,82,88–90]. These transcripts exist in low abundance and are primarily found in nuclear and chromatin-bound fractions [78,91–93]. Quantification of eRNAs that are thought to exist in higher abundance suggests a range of 0.5–20 copies per cell [94]. eRNA stability and 3'-end processing appears to be regulated by the integrator complex [95] in a poly(A) cleavage site-mediated mechanism that leads to early termination, as compared to promoters [96,97]. Several studies have used northern blots to demonstrate that eRNAs appear as distinct bands, corroborating that they have defined termination sites and are produced at consistent lengths [89,98,99]. eRNAs are predominantly non-polyadenylated and degraded via the exosome, resulting in a high turnover rate with a half-life of approximately 7 minutes [78,90,96,100,101]. Some studies have identified polyadenylated and stable eRNAs, although there is still a debate on whether these transcripts should be classified as long non-coding RNAs instead [22,80,96]. eRNAs are largely unspliced due to a general deficit of U1 splice sites within enhancer regions, resulting in only ~5% of transcripts being spliced (Table 2) [81,96,97].

eRNA production constitutes a large portion of the human transcriptome with previous estimates indicating ~40,000–65,000 events [81,88]. eRNAs are not unique to humans, as they appear in several diverse organisms, suggesting an early evolutionary origin that is critical to enhancer function [81,88,102,103]. Indeed, the production of eRNAs has become widely accepted as one of the most robust indicators of an active enhancer [21,82,104]. Despite this, the lack of a clear mechanism for eRNA requirement has sparked debate regarding their role. Explanations for the role of eRNA production typically fall within three broad schools of thought. The first suggests that eRNAs exist as background noise that occurs as enhancer loci are incidentally transcribed based on close proximity to active gene promoters. The second supposes that transcription of an enhancer region is necessary for its function, likely to establish an open chromatin state, but that eRNAs are merely a byproduct of this and serve no additional purpose. The third model for eRNA production stipulates that eRNAs play an active role post-transcriptionally and are required for proper enhancer function. A growing body of evidence has begun to shed light on the potential functions of

eRNAs, highlighting a complex and fascinating collection of transcripts that do not always fall within convenient classifications (Table 2).

Emerging paradigms for eRNA function

eRNAs are transcriptional noise

A simple explanation for the pervasive production of eRNAs, is that they may arise from leaky transcription events across the genome without significant biological function. It has been suggested that similar to other DNA-binding proteins, RNA Pol II binding to chromatin is not stringent, with only a small percentage (~10%) of RNA Pol II binding leading to productive transcription [105]. Open chromatin structures at active enhancers likely facilitates RNA Pol II binding and may accidentally promote initiation of transcription. Consequently, promiscuous RNA Pol II binding and stochastic transcription events would inevitably produce numerous transcripts as background noise with no apparent biological functions [105]. Most eRNAs are subject to destruction by the exosome complex, therefore, most eRNAs have low abundance and are rapidly degraded, suggesting that they may not have an appreciable biological function in local or long-range transcription events [106–108]. However, this idea has been evolving as improved genomic approaches have revealed that low abundance transcripts are still capable of having a profound effects on biological processes.

eRNAs are a byproduct of necessary transcription at enhancers

Instead of the eRNA transcript itself, the act of transcription could promote chromatin remodeling around the enhancer to establish and maintain an open chromatin state required for proper activation of target genes. For example, it has been shown that initiation and elongation of RNA Pol II at its binding sites leads to the remodeling of local chromatin and deposition of histone modifications necessary for active transcription [109,110]. In addition, there are several histone modifying enzymes that “piggy back” onto RNA Pol II, such as p300, which can acetylate histones co-

transcriptionally [111]. Thus, perturbations of transcription at the enhancers often result in reduced target gene activation [17,112,113]. Although transcription of the enhancer region appears critical for enhancer function, it is difficult to dissociate transcription and the resulting RNA products since both are impaired upon interference. However, recent advances have made it possible to knockdown eRNAs transcripts and study their functions without affecting the enhancers from which they are produced. For example, at TLR4 signaling induced enhancer sites, RNA Pol II transcription induced changes in histone methylation, and this methylation was solely dependent on RNA Pol II transcription instead of the eRNA transcripts [83]. Beyond the chromatin remodeling capacity of RNA Pol II transcription, a recent study demonstrates that transcription induced-supercoiling drives chromatin loop extrusion, therefore, playing a role in stimulating enhancer-promoter contacts during enhancer transcription [114].

eRNA transcripts are required for enhancer function

The association between eRNA production and enhancer activity has become so widely accepted that production of eRNAs alone has been used as a method to identify novel enhancers [21,104]. Several studies have demonstrated that eRNA production temporally precedes the upregulation of target gene mRNAs [8,78,82,88–90] and occurs with similar kinetics, as shown by single-cell profiling [93]. Given that eRNAs are predominantly located within the nucleus and chromatin-bound fractions, this suggests that enhancer transcription and/or eRNAs are critical for enhancer-mediated target gene upregulation [78,91–93]. Dissecting the requirement for the eRNA transcript apart from the act of transcription has proven challenging for investigations into enhancer function. This has been primarily overcome through the use of short hairpin RNAs (shRNAs), small interfering RNAs (siRNAs), and locked nucleic acids (LNAs) that knockdown eRNAs without affecting transcription at the enhancer; providing some of the most compelling evidence for eRNA function [89,90,92,94,98,99,115–123]. These studies showed that knockdown of the eRNA was associated with a reduction in target gene

expression, strongly suggesting a functional role for the eRNA. Conversely, tethering of eRNA transcripts to inactive enhancers has been shown to elicit features of target gene activation [92,98,124,125]. Taken together, these data suggest that the eRNA transcripts are required for proper enhancer function, although their mechanisms of action are less clear.

Several studies have utilized a variety of methods at individual enhancer loci to determine how eRNA transcripts might be involved in enhancer mechanisms (Figure 2). Enhancers are characterized by their ability to bind transcription factors (TFs) needed for efficient upregulation of their target genes [91]. It has therefore been suggested that eRNAs may be involved in binding these transcription factors to stabilize or trap them in place [126]. Several studies have identified interactions that occur between eRNAs and key transcription factors known to bind at a broad array of enhancers, such as mediator, CBP, BRD4, cohesin, P300, YY1, and p53 (Figure 2(b)) [58,89,98,117,126–131]. In-vitro assays have further demonstrated that eRNAs capable of interacting with CBP do so in an eRNA sequence independent manner, suggesting that CBP may recognize specific RNA secondary structures that are inherent to many eRNAs [130]. The association between eRNA transcripts and TF binding is so strong that production of eRNAs has even been used as a method to predict TF activity [132]. These observations demonstrate how eRNA functionality can be tied into a fundamental characteristic of enhancer activity, transcription factor binding and stabilization (Table 2).

eRNAs have also been implicated in the release of paused RNA Pol II from target gene promoters to allow for productive elongation. Studies into this potential role have demonstrated two distinct ways that eRNAs control RNA Pol II elongation. Similar to TF trapping, eRNAs have been shown to bind and activate the positive transcription elongation factor B (P-TEFb) complex (Figure 2(c)), which uses phosphorylation to promote the release and elongation of RNA Pol II [133–135]. Specifically, the PSA eRNA has been shown to interact with the kinase CYCLIN T1, a subunit of the P-TEFb complex, and to be necessary for serine-2 phosphorylation and RNA Pol II release [134]. eRNAs have also been shown to act as decoys that bind repressive cofactors, enabling productive transcription of their target genes (Figure 2(d)). This mechanism was best

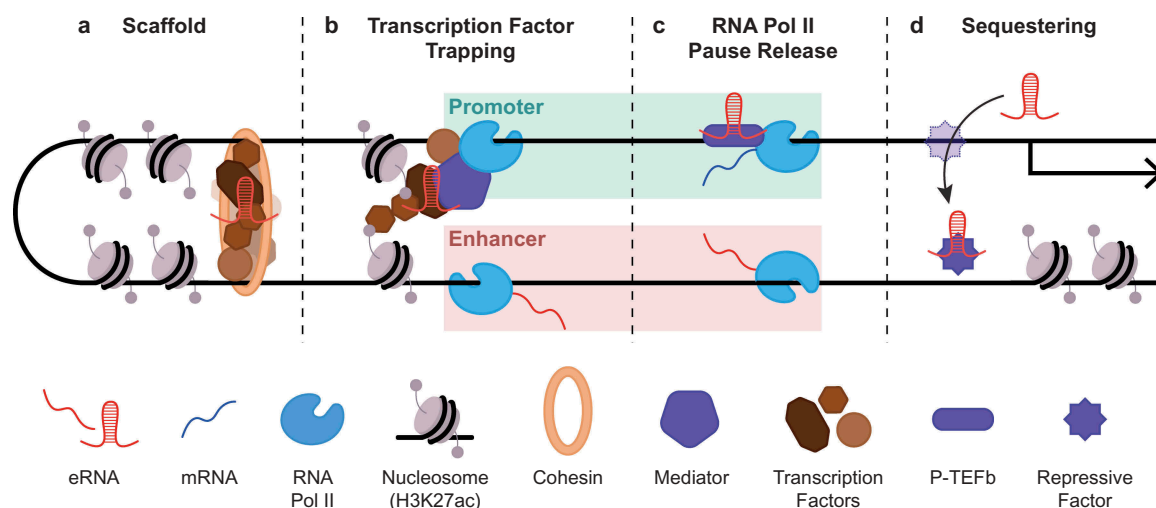


Figure 2. Emerging paradigms for eRNA function. eRNAs have been implicated as critical components of active enhancers in several ways. (a). eRNAs can interact with chromatin looping factors to act as scaffolding for proper enhancer-promoter loop formation. (b). Binding to various transcription factors enables eRNAs to participate in TF trapping, RNA Pol II loading, and histone modification. (c). eRNAs can facilitate RNA Pol II pause release via activation of the P-TEFb complex. (d). eRNAs can also act as decoys to sequester cofactors that repress transcription at target genes.

demonstrated through the observation that neuronal eRNAs were required for binding and sequestering away the negative elongation factor (NELF), allowing for the release of paused RNA Pol II from cognate target promoters (Table 2) [90].

Perhaps the most notable feature of enhancers is the formation of chromatin loops that allow for direct interaction with target gene promoters. While the specific mechanisms that create these loops are not well understood, it is known that several architectural proteins are crucial for their creation, such as CTCF, YY1, cohesin, mediator, and integrator [95,136–141]. As discussed earlier, several investigations have provided considerable evidence to show that eRNAs are capable of interacting with many of these elements, suggesting that they may be required to establish and/or stabilize chromatin loops (Figure 2(a)). To further emphasize their importance, several studies have demonstrated that knockdown of eRNAs disrupt the loop structures and result in decreased target gene transcription [63,89,92,94,99,108,142]. eRNAs also exhibit the ability to bind signal regulated transcription factors to facilitate enhancer-promoter interactions, as shown with the estrogen receptor in breast cancer cells [94]. However, this should not necessarily be considered as a universal mechanism for eRNAs considering that other studies have identified enhancers that do not rely on the eRNA transcript for loop formation/stabilization [82,90,143]. It is likely that at

these particular loci, the eRNAs serve a disparate purpose unassociated with the formation of chromatin loops (Table 2).

Though enhancers are commonly considered to be *cis* acting elements, some do have the ability to operate in *trans* and there is evidence to suggest that eRNAs also have *trans* activity in rare cases. It has recently been demonstrated that an eRNA produced at the MyoD locus work in *trans* to colocalize with Myogenin nascent transcripts and associate with the cohesin complex to enable recruitment to the Myogenin locus [14]. Despite the rare occurrence, this study demonstrates that eRNAs are capable of performing diverse roles and are not limited to acting in *cis*. Interestingly, this particular eRNA is spliced, unidirectional, and polyadenylated. This allows for speculation that the transcript is more stable than its non-polyadenylated counterparts and these features may aid in defining a subgroup of eRNAs that have *trans* functionality. The accumulated evidence for eRNA active participation in the enhancer mechanism is very compelling and continues to grow as the field moves forward. In fact, eRNAs have very recently been implicated in the formation of liquid-liquid phase separation [67,144]. Taken together, these studies suggest that eRNA activity should always be considered at novel enhancers unless the loss of eRNA

transcript can be deemed non-essential through experimental testing. While there are some shared mechanisms of eRNA behavior, there does not appear to be a defining feature and each eRNA should be assessed on an individual basis, perhaps even within a specific enhancer locus.

Conclusions and future perspectives

The study of enhancers has gained significant interest recently as research into human diseases has shifted from the protein coding regions of the genome to the vast and primarily unexplored non-coding regions. As more mutations and genomic alterations of the non-coding genome become associated with disease, the investigation of novel enhancers and aberrantly regulated enhancers will continue to increase. These investigations will likely focus on a few key areas: (1) the identification of enhancers that drive disease, (2) the network of genes that they regulate, and (3) the mechanisms by which they function. The current body of evidence for enhancer function clearly demonstrates the necessity for enhancer transcription and their cognate eRNAs, while also depicting that enhancers lack a common unifying mechanism. Therefore, it is important to view the current models as general outlines for how eRNAs can behave and to continue searching for novel mechanisms. Furthermore, the pervasiveness of eRNA production raises several crucial questions about the nature of these RNAs and biological or pathological significance of enhancer transcription. We hypothesize that eRNAs act locally at the enhancer from which they originate, functioning as a scaffold for assembling components of an active enhancer complex. We also anticipate that eRNAs may be a critical participant in liquid-liquid phase separation of chromatin as emerging literature provides compelling evidence for this phenomenon [144,145]. In addition, it is now evident that eRNA-producing enhancers are highly cell type specific, represent the most highly active enhancers (as compared to non-eRNA-producing enhancers), and consequently drive the critical gene expression pathways of any given cell type.

The enhancers discussed in this review serve as a reminder that each locus has unique features, and most vary in how they utilize their produced

transcripts. Therefore, the role of enhancer transcription and also the eRNA transcript itself should be assessed when studying a particular enhancer or set of enhancers. Some reports have identified active enhancer loci that fail to produce eRNAs [91,92,146]. However, there is still a debate regarding whether these enhancers do not produce eRNAs or simply produce them at an undetectably low level [103]. In consideration of these points, it is important to reiterate that eRNAs do not always fall within well-defined classifications. It is possible and likely that some currently assigned eRNAs are actually long non-coding RNAs (lncRNAs), and the reverse. It is also critical to remember that the current definitions for these two groups of transcripts are not exclusive and that several transcripts can be fairly labeled as both an eRNA and lncRNA. For example, *linc-p21* and *lockd* are annotated lncRNAs that have been shown to exhibit cis-regulatory effects on target genes, independent of the RNA transcript, suggesting that the regulatory effects of these particular lncRNAs are due to DNA enhancer elements found within the locus [147,148]. These findings have prompted the idea that lncRNAs may evolve from eRNAs, whereby spurious splicing and polyadenylation of ancestral eRNAs, due to the accidental presence of splicing and 3' processing sequences in the vicinity of the respective enhancers, would lead to the stabilization of the eRNAs and the acquisition of novel biochemical properties of these RNA transcripts [149]. It is also equally likely that enhancers may simply exist within lncRNA genes, similar to how they are found within mRNA genes, and thus co-exist as “lncRNAs with enhancers within them”; negating the need to relabel these particular lncRNA as eRNAs. Thus, the boundaries that define enhancer/eRNA function remain broad and allow for several potential avenues that must be explored. Below we provide some context for methods commonly used to study enhancers and their eRNAs that will hopefully serve as a primer for those launching inaugural studies in this field.

Common methods used for detecting eRNAs

Several genomic techniques have been used to measure RNA production, however the majority of eRNA transcripts were originally missed because these

genome-wide transcriptomic methods relied on polyadenylation priming. While valuable poly(A)-based datasets have been analyzed to draw worthwhile conclusions about enhancers and their transcripts, this limits the eRNAs studied to a relatively small subset of all potential eRNAs [22]. Future experiments should utilize techniques that focus on detecting nascent transcripts, such as global run-on sequencing (GRO-Seq) [21,82,94,95,97,118,150–155], precision run-on nuclear sequencing (PRO-Seq) [156], and cap analysis gene expression (CAGE) [157,158], to ensure that all eRNAs are surveyed. Total RNA-sequencing using random hexamer primers for reverse transcription, coupled to very high depth of sequencing, can also be used to measure eRNA transcripts. These techniques are particularly useful for the identification of previously unannotated eRNAs in a high-throughput manner, which has proven to be a successful approach to discover novel active enhancers [21].

Knowledge of the gene(s) regulated by a particular enhancer is necessary to draw meaningful conclusions about the role of the enhancer, but this can prove to be challenging. It was previously thought that enhancers act upon their nearest neighboring gene, however we now know that enhancers are capable of upregulating genes located farther than 1 Mbp away and can skip over closer genes [36,37,159,160]. This is further complicated by the discovery that individual enhancers can interact with and influence several different promoters [16]. Fortunately, there are several approaches to identify target gene(s) and investigate the mechanisms of enhancer function.

Chromatin loops allow physical interaction between enhancers and their target gene promoters, thus the ability to visualize the 3D structure of the genome is extremely valuable [8,42,44,89,161,162]. There are several chromatin conformation techniques that enable the mapping of enhancer-promoter interactions, such as chromatin conformation capture (3C) [46,47,163], Hi-C [164,165], and recently Hi-ChIP [166,167]. These techniques can be used to identify new enhancer-promoter interactions or describe the loss of contact following disruption of enhancer activity. As our general understanding of higher order chromatin structure and phase separation grows, these techniques will likely increase in value as they can provide a unique insight into a fundamental aspect of enhancer activity.

As with chromatin loops, we know that enhancers and eRNAs interact with key enzymes and TFs that have significant impacts on target gene upregulation. Therefore, techniques to discover the identity of proteins interacting with specific enhancers or eRNA transcripts are extremely useful. Some methods used to accomplish this are RNA immunoprecipitation (RIP) [168,169], RNA antisense purification coupled with mass spectrometry (RAP-MS) [120,170], variations of ChIP-seq [78,79], and several other RNA-protein interaction techniques, as discussed in a recent review [171]. Additionally, there are several valuable databases, such as FANTOM [172,173] and ENCODE [10,59], that contain annotated enhancers using datasets generated by several of these approaches.

Methods for eRNA perturbation

Beyond observing the enhancer-promoter physical interactions that occur, another common approach is to perturb the activity of the enhancer and assess the effects on target gene(s) expression. This can be accomplished through several avenues that typically involve either altering the DNA sequence of the enhancer, the transcriptional status of the enhancer, or the eRNA transcript produced. A classical approach is to delete the enhancer region entirely [174], or insert a transcriptional terminator sequence within the enhancer [85,112], to cause a loss of enhancer function. Both of these methods involve altering the sequence of the enhancer; however, insertion of a terminator sequence is considered to be less invasive since it inhibits transcription while having a smaller impact on the enhancer sequence. Another recently popular technique for disrupting enhancers is the use of clustered regularly interspaced palindromic repeat interference (CRISPRi) that features a catalytically dead Cas9 (dCas9) fused to a Krüppel associated box (KRAB) domain to epigenetically inhibit transcription at a targeted enhancer and thereby prevent its ability to upregulate target gene(s) [17,175–180]. This technique allows researchers to manipulate the function of the enhancer element without altering the DNA sequence. Interestingly, the reverse can also be performed using CRISPR activation (CRISPRa), a method that can specifically activate or *turn on* enhancers to study their effects [128,177,181–185]. In a step to further refine these approaches, several

researchers have employed siRNAs [142] or antisense mediated decay [89,128] to knockdown the eRNA transcripts directly without altering the transcriptional status of the enhancer. These methods are considered to be the most targeted approaches as they only disrupt mechanisms associated with the post-transcriptional function of eRNA transcripts. The converse approach to this would be CRISPR-Display, which involves tethering an eRNA transcript to dCas9 to stimulate eRNA-based mechanisms in the absence of transcription [125].

Production of eRNAs can also be inhibited by treatment with small molecule inhibitors of RNA Pol II elongation such as flavopiridol [82], or inhibitors of transcription initiation such as Triptolide [186]. It is important to note that these small molecule inhibitors are not specific to just eRNAs and will disrupt global transcription. The rapid degradation of eRNA transcripts has thwarted the use of common RNA sequencing approaches that measure steady-state RNA levels [187,188]. To combat this issue, investigators have used exosome inhibition to stabilize eRNA transcripts and allow for further study into their molecular function [81]. Taken together, the techniques discussed here demonstrate the variety of approaches that can be taken to identify and study enhancers and their eRNAs, from classical genetic engineering to cutting edge epigenetic perturbations. The combination of several methodologies has been the greatest asset to these investigations and should be regarded as the best avenue for future studies into enhancer function. This field will continue to be shaped by the constantly evolving technology that allows researchers to answer novel questions and address past queries with greater precision.

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