REGULATORY ELEMENTS

Enhancers as non-coding RNA transcription units: recent insights and future perspectives

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Abstract | Networks of regulatory enhancers dictate distinct cell identities and cellular responses to diverse signals by instructing precise spatiotemporal patterns of gene expression. However, 35 years after their discovery, enhancer functions and mechanisms remain incompletely understood. Intriguingly, recent evidence suggests that many, if not all, functional enhancers are themselves transcription units, generating non-coding enhancer RNAs. This observation provides a fundamental insight into the inter-regulation between enhancers and promoters, which can both act as transcription units; it also raises crucial questions regarding the potential biological roles of the enhancer transcription process and non-coding enhancer RNAs. Here, we review research progress in this field and discuss several important, unresolved questions regarding the roles and mechanisms of enhancers in gene regulation.

Non-coding
DNA regions or RNA transcripts that do not code for proteins.

Long non-coding RNAs
(lncRNAs). Non-coding RNAs that are longer than 200 nucleotides.

Hypersensitivity
Chromatin regions such as enhancers and other regulatory elements often display extra sensitivity to DNase treatment, reflecting the openness of the regions.

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doi:10.1038/nrg.2016.4
Published online 7 Mar 2016

The unexpected discovery of numerous non-coding DNA and RNA regulatory elements in the genome that far outnumber protein-coding genes[1,2] presents a dramatically altered view of the transcriptional circuits. Intriguingly, regulatory DNA regions are now often found to act as transcription units, as exemplified by the widespread transcription observed at enhancers[2,3]. This finding poses a dual challenge: to elucidate the transcriptional regulation and biogenesis of the non-coding RNAs (ncRNAs) as well as their roles in cognate coding gene control, either dependent or independent of the DNA region. Because of the crucial roles of enhancers in generating cell-type- and state-specific transcriptional programmes[4–6], further understanding of the process of enhancer transcription and its contribution to the overall functionality of enhancers will offer crucial insights into gene regulation, cell identity control, development and disease.

In this Review, we briefly summarize our current understanding of enhancer-mediated gene regulation and then discuss the characteristics and activation process of enhancers as transcription units. We consider whether enhancer RNA (eRNA) production merely reflects the consequences of enhancer activation, or whether the transcription process and eRNAs per se exert functions, with a primary focus on human and mammalian systems. After comparing eRNAs with other transcription units in the genome, we propose a subcategorization of these ncRNAs based on distinguishable properties, their RNA stability and potential functions. Several future directions are suggested that are of importance for a better understanding of enhancer transcription and enhancer biology. We refer readers to several excellent recent reviews that focus on ncRNAs in general and on long non-coding RNAs (lncRNAs)[7–9], as well as on the genome-wide identification of enhancers and their epigenomic properties[10–12].

Evolving concepts of enhancers

Cis-*regulatory DNA elements in gene activation.* In the pre-genomic era, enhancers were initially described as short DNA fragments with several prominent features, including: the ability to positively drive target gene expression; functional independence of genomic distance and orientation relative to the target gene promoter; hypersensitivity to DNase treatment, indicative of a decompacted chromatin state; the presence of specific DNA sequences allowing the binding of transcription factors (TFs); and enriched binding of transcription co-activators and histone acetylation (reviewed in REFS 4,5,13). The first enhancer discovered was a 72 bp-long DNA fragment from the late gene region of simian virus SV40, which increased the expression of a reporter gene promoter by ~200-fold[14,15]. Further work elucidated the existence of cellular enhancers *in vivo*[16,17]. Subsequently, molecular genetic studies have discovered many enhancers that exert important functions in various cell types and developmental systems[4,5,13].

These classic enhancer features have permitted their systematic annotation in the genomic era; for example, chromatin immunoprecipitation (ChIP) of histone

Table 1 | **Tools to detect and study enhancer RNAs**

Method	Description	Advantages	Disadvantages	Refs*
Reverse transcription-PCR (RT-PCR)	A PCR-based method using the capability of reverse transcriptase to convert target RNAs into complementary DNAs, the amount of which can be measured by PCR or real-time PCR	High sensitivity; cost- and time-effective for single-locus experiments	Low throughput; results could be confounded by other transcription/transcripts going through the enhancer region	32,33
RNA fluorescence *in situ* hybridization (RNA-FISH)	A cytogenetic method in which pre-designed fluorescence-labelled oligonucleotides or other long stretches of DNA are used as probes to hybridize target RNAs based on sequence complementarity. The cellular localization of the target RNAs can thus be detected by the fluorescence signal	Single-cell method, suitable for studying inter-molecular spatial relationships	Low throughput and laborious; may be challenging for enhancer RNAs (eRNAs) due to their labile nature	37,108,128
RNA polymerase II chromatin immunoprecipitation coupled with high-throughput sequencing (RNAPII ChIP–seq)	A genome-wide technique to study the chromatin regions associated with RNAPII. It involves immunoprecipitation in the nuclear lysate of cross-linked (for example, formaldehyde) cells using specific antibodies to capture RNAPII, its associated complexes and chromatin DNAs. The resolved DNAs are then subjected to deep sequencing	High throughput, well-established and simple protocol	Only serves as indirect evidence of transcription; the presence of multiple forms of modified RNAPII hinders its direct correlation to transcriptional readout; lacks strand-specificity and of relatively lower resolution; relies on antibody quality	32,33
Global run-on sequencing (GRO-seq)	A genome-wide method to study the nascent transcriptome of a cell population by re-initiating the transcription of RNAPII *in vitro* at its genomic sites (that is, nuclear run-on). To label nascent RNAs during this process with labelled nucleotides allows specific enrichment of nascent RNAs for deep sequencing. A modified version named precision run-on sequencing (PRO-seq) was recently established that provides a nucleotide resolution	High throughput; high-resolution; efficient at detecting dynamic transcriptional activity; highly robust for labile non-coding RNAs of high turnover rates, including eRNAs; maps transcription rates of all three RNA polymerases	Partially *in vitro*; relatively laborious; requires large amounts of material	34,35,42–44, 65,66,73,172, 179,180
5′GRO-seq or GRO-cap	A specialized version of GRO-seq that captures the m7G-capped nascent RNAs generated from the start sites of transcription initiation events; developed independently by Lam *et al.* (5′GRO-seq) and Kruesi *et al.* (GRO-cap)	Similar to GRO-seq, but provides precise start sites of transcription events regardless of the RNA stability; an ideal tool to study transcription initiation of cultured cells	Similar to GRO-seq	43,73,181
BruUV-seq	A technique that takes advantage of UV light-induced DNA lesions to block transcription randomly in the genome prior to BrUTP incorporation and nascent RNA sequencing	Detects nascent RNAs and therefore works well for eRNA identification; the BrUTP incorporation step was performed in intact cells and therefore may preserve the *in vivo* RNAPII position better than GRO-seq; useful for mapping transcription initiation events	Does not provide robust information of transcriptional regulatory steps other than initiation events; UV light treatment induces DNA damage response in cells, which may affect transcriptional outputs	182
RNA-seq (total)	A genome-wide transcriptomic technique to study the RNA composition of a cell or cell population at a given moment. It sometimes involves depletion of ribosomal RNA to enrich signals	High-throughput, well-established and simple to perform	Examines the accumulated RNA end products rather than the dynamic, transient transcriptional activity of a cell; most reads are from highly expressed coding genes	32,33,45,47
RNA-seq (poly(A))	Similar to total RNA-seq, but involves an oligo-dT primer-mediated reverse transcription step, which will enrich RNAs with a poly(A) tail	High-throughput; efficient at detecting RNAs with poly(A) tails; relatively easy to perform; ribosomal RNA signals are excluded	Similar disadvantages as total RNA-seq; not robust at detecting eRNAs as they generally lack poly(A) tails	32,33,42,47, 115
Cap analysis of gene expression (CAGE) followed by deep sequencing	A genome-wide tool to capture the m7G capped RNAs in a transcriptome	Excellent tool to study transcription initiation *in vivo*. The improved protocol uses small amount of materials	Relatively higher background than GRO-cap; less robust in measuring the capping events of labile RNAs than GRO-cap; potential influence by post-transcriptional RNA recapping events	3,38,72

Table 1 (cont.) | **Tools to detect and study enhancer RNAs**

Method	Description	Advantages	Disadvantages	Refs*
Chromatin-bound RNA-seq	An adapted version of RNA-seq in which only chromatin-bound portions of RNA are sampled by biochemical fractionation of the cells, followed by sequencing	No systematic comparison to other methods has been made for studying eRNAs	No systematic comparison to other methods has been made for studying eRNAs	32,42,95
RNA-Seq in isolated 'transcription factories'	An adapted version of RNA-seq in which 'transcription factories' are isolated by biochemical methods, and the RNA components are sequenced	No systematic comparison to other methods has been made for studying eRNAs	No systematic comparison to other methods has been made for studying eRNAs	138
Native elongating transcript sequencing (NET-seq)	A genome-wide approach to map the nascent RNAs associated with transcriptionally engaged RNAPII, based on their co-purification with RNAPII subunit or phosphorylated forms	High throughput; high resolution; provides a complete *in vivo* map of nascent RNAs; measures the 3′ end of RNAs; adjustable to study nascent RNAs associated with specifically phosphorylated forms of RNAPII	Relatively laborious and technically challenging; detailed analysis of eRNAs using this tool has not been published	183,184
RNA capture sequencing (CaptureSeq)	An adapted version of RNA-seq. Instead of sequencing all the RNAs of a cell, only the RNAs of interests are captured by a pre-designed oligonucleotide probes for sequencing	High throughput; cost- and sequencing-depth efficient to detect target RNAs, especially those of low levels or transient expression	Requires pre-designed oligonucleotides and some pre-existing knowledge of the targets, thus may also introduce biases. More efficient at detecting stable RNAs	185
Chromatin isolation by RNA purification (ChIRP-seq)	A high-throughput technique to identify the chromatin associating regions of an RNA of interest, using pre-designed complementary oligonucleotides for the purification of target RNAs and associated chromatin regions. The chromatin association of eRNAs have not been widely studied except in one report	One major tool in the field to study RNA–chromatin association	The relatively low abundance of eRNAs may pose a technical challenge for doing this experiment	44,186

This table lists the currently available tools to detect eRNAs and the advantages and disadvantages of each. Related references, especially those that have interrogated eRNAs, are included. *References are representative.

modifications was coupled with microarray (ChIP–chip) and next-generation sequencing (ChIP–seq) to predict enhancers[18,19]. Commonly used annotation methods for putative enhancers now include: the integration of the DNase hypersensitivity assay with deep sequencing; the detection of a higher ratio of histone H3 lysine 4 monomethylation (H3K4me1) compared with trimethylation (H3K4me3); the presence of histone acetylation (for example, H3 acetylated at lysine 27 (H3K27ac)) and certain histone variants (for example, H2AZ); the binding of co-activator and acetyltransferase (for example, CREB-binding protein and p300 (CBP/p300)); and clustered binding of multiple TFs (reviewed in REFS 10–12,20). The use of epigenomic markers has been transformative for the identification of developmental enhancers with a higher likelihood of driving tissue-specific patterns of gene expression[19,21]. However, annotation by epigenomic features has resulted in an extremely large number of putative enhancers in humans (>400,000 to ~1 million), exceeding that of coding genes by more than ten-fold[10–12]. Importantly, the presence of histone modifications (for example, H3K4me1) per se does not explain the molecular mechanism underlying enhancer activity[19,22]. The arbitrary cut-off to select enhancers based on the H3K4me1/H3K4me3 ratio may omit a portion of functional enhancers[23]. These findings suggest that additional criteria are needed to more precisely annotate functional enhancers in the genome.

Locus control regions
(LCRs). Genomic elements that elevate expression of linked genes through long-range regulation, in a copy number-dependent and tissue-specific manner. A well-studied example is the LCR upstream of the β-globin gene in erythroid cells.

Enhancers as non-coding RNA transcription units. Enhancer function was linked to their transcriptional activity by several early studies. Interrogation of locus control regions (LCRs) of the β-globin locus led to the discovery that multiple hypersensitivity sites produced transcripts[24,25]. Extragenic transcripts were also found at LCRs in other genomic loci[26,27]. Importantly, these transcripts were expressed in a manner specific to the cell type[24] or stage of differentiation[26,27], correlating with LCR functionality. Extragenic and/or intergenic transcription was also found *in vivo*. Prominent examples include the intergenic RNAs from the infra-abdominal region of *Drosophila melanogaster*[28] and the lncRNA from a mouse enhancer[29]. Subsequently, large-scale transcriptome profiling and RNA polymerase II (RNAPII) ChIP–seq analyses showed that ncRNA species were highly abundant in the human genome[30] and that RNAPII bound a large number of extragenic regions[31], suggesting that enhancers may be commonly transcribed. Finally, two independent studies in 2010 provided unequivocal evidence that putative enhancers with epigenetic marks are pervasively transcribed into largely non-polyadenylated ncRNAs, which were named enhancer-derived ncRNAs or eRNAs[32,33]. Further studies using global run-on sequencing (GRO-seq, TABLE 1) more robustly identified eRNAs regulated by signalling events[34,35]. Transcription of eRNAs has now been pervasively recorded in various cell lineages and in response to different stimuli[3,36–54].

Super enhancers
A group of active enhancers densely clustered in a ~10–30 kb region, highly associated with cell identity genes and disease-associated genomic variations. Also known as stretch enhancers.

Shadow enhancers
A term coined by Michael Levine and colleagues to describe the phenomenon of having another enhancer in the vicinity (or sometimes scattered across larger chromosomal domains) in addition to a primary enhancer to control the expression pattern of an important developmental gene; their biological roles are still under investigation, but, at least in some cases, shadow enhancers act to confer phenotypic robustness under environmental and genetic variability.

Regulatory archipelagos
A term denoting the presence of multiple enhancers in the Hox gene loci during limb development, with each of them playing quantitative or qualitative roles for Hox gene transcription.

Highly occupied target regions
(HOT regions; also known as hotspots). Genomic regions that associate with multiple transcription factors, either simultaneously or sequentially, and that are usually uncovered by chromatin immunoprecipitation followed by sequencing. They are identified in multiple organisms and cell types.

TF collective or MegaTrans enhancer
Highly active enhancers that are bound by multiple transcription factors simultaneously (similar to the definition of HOT regions and super enhancers). However, these two terms have been used to describe a situation in which one (MegaTrans) or several (TF collective) major transcription factors bind target enhancers in *cis* (that is, direct association through a specific DNA motif), which then act to tether other transcription factors in *trans* (that is, bind the major transcription factor through protein–protein interaction).

As defined by the cap analysis of gene expression (CAGE) technique (TABLE 1), the number of eRNAs in humans was reported to be ~40,000–65,000 (REFS 3,38), which constitutes a large portion of the transcription initiation events in the human transcriptome[2].

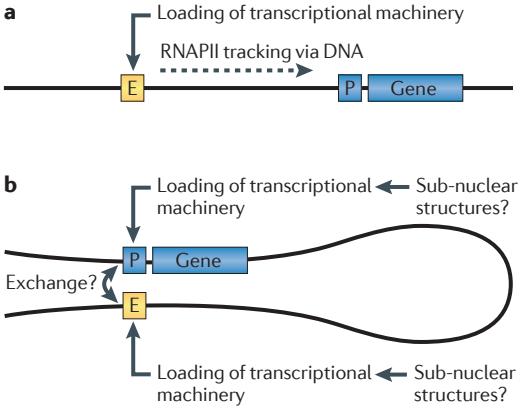
eRNAs as signatures of functional enhancers? Annotation solely by epigenomic marks may not be the optimum way to define active enhancers. A recent examination of the high binding density or affinity of TFs and other chromatin regulators (that is, Mediator) defined a group of clustered enhancers in humans. These so-called super enhancers (also known as stretch enhancers)[55,56] are similar in nature to the existing concepts of shadow enhancers[5,57], regulatory archipelagos[58] or classic LCRs, and they were postulated to be functional enhancers controlling important genes in development and disease[55,56,59]. Others have proposed using the co-binding of multiple TFs on one enhancer site as a mark of a functional enhancer (as in highly occupied target regions (HOT regions)[60,61] and the TF collective or MegaTrans enhancer[62,63]) (reviewed in REFS 20,64). Interestingly, enhancers defined by these criteria also exhibit robust eRNA transcription[63,65]. These and other data support the notion that eRNA induction is a potent, independent indicator of enhancer activity[3,32,33,43,44,53,66–68]. Distinct from non-transcribing enhancers on a genome-wide scale, eRNA-producing enhancers exhibit higher binding of transcriptional co-activators, greater chromatin accessibility and higher enrichment of active histone marks such as H3K27ac[33,66–68], as well as protection from repressive marks, including DNA methylation[69,70]. They have also been highly correlated with the formation of enhancer–promoter loops[71], another indicator of enhancer function (BOX 1). Large-scale reporter assays found that putative enhancers with clear eRNA transcripts are two- to threefold more likely to show significant reporter activity than non-transcribing enhancer-like regions characterized only by their histone marks[3]. However, it is noteworthy that non-transcribing enhancers display a lower probability, rather than complete incapability, of inducing reporter activity[3]. This could reflect levels of eRNAs that are too low for the assays to

Box 1 | Enhancer–promoter looping and higher-order chromosomal confirmation

How do enhancers regulate promoters? Two non-exclusive mechanistic models have been proposed: the tracking model (see the figure, panel **a**), in which RNA polymerase II (RNAPII) and the associated transcriptional machinery track through the intervening DNA in-between enhancers and promoters; and the looping model (see the figure, panel **b**), in which this machinery is loaded at the enhancers and then reaches the promoter due to a physical interaction (that is, through looping)[13,88,154]. In both models, the enhancers are considered to help increase the concentration of transcriptionally active machinery at cognate gene promoters. Over the past two to three decades, increasing evidence has largely backed the looping mechanism. Although the tracking model has been shown to exist in sporadic examples[88,154], it may not be a general rule. The formation of looping may actually allow an 'exchange' of transcriptional machinery from both directions (see the figure, panel **b**), especially given the increasingly realized similarities between enhancers and promoters[149]. This possibility has been implied in a few examples[24,33,108] but remains largely unexplored.

Current studies on the enhancer–promoter interaction are primarily built on the chromosome conformation capture (3C) technique and its modified versions[155–157], including circular chromosome conformation capture (4C), chromosome conformation capture carbon copy (5C), chromatin interaction analysis by paired-end tag sequencing (ChIA-PET), Hi-C, tethered conformation capture (TCC), Capture-C and Capture Hi-C[158,159]. Recent advances have established an understanding that each regulatory element, including enhancers and promoters, is engaged in multiple long-range interactions with many other regions[71,119,136]. All chromatin interactions are created and maintained in a hierarchy of 3D chromatin architectures, including A/B domains, topologically associated domains (TADs, ~1 Mb), and sub-TADs (reviewed in REFS 155,156,160). These findings indicate that any inter-relationships between enhancers and promoters, both as regulated and potentially regulatory transcription units, have to be considered in the context of a highly organized 3D genome.

Technical differences between different versions of 3C-related assays may lead to alternative interpretations of enhancer–promoter interactions and their relationship with enhancer RNAs (eRNAs; see 'Mechanisms of eRNA function in gene activation' section in the main text). For example, although dynamic signal-regulated looping has been reported using 3C followed by conventional PCR or quantitative PCR[44,66,110], in some instances this regulation was only variably detected by 4C[45,161], ChIA-PET[119,162] or Hi-C[163–166]. It has also been noted that results from 3C-related techniques sometimes do not fully agree with findings using microscopy [167]. The discrepancies could be: technical, as a result of biochemical crosslinking or different statistical modelling; or biological, as a result of the dynamic activity of looping formation at different temporal windows, or variations at the single-cell level that may be dampened in populations of cells. A probable interpretation from recent progress is that many pre-existing chromosomal interactions are dynamically strengthened by developmental cues and regulatory signals, reflecting either increased stability or frequency of contacts, and involve important roles of chromatin architectural proteins and/or eRNAs and long non-coding RNAs. A clear understanding of looping requires further advances in experimental techniques and statistical analyses; a confident call of a looping event should be made by both 3C-related and microscopic approaches.



detect, or the lack of a native chromatin environment and enhancer–promoter distance in reporter assays, but it is equally possible that some non-transcribing enhancers are also functional. *In vivo* transgenic reporter assays in mice embryos lend further strong support to the predictive power of eRNA transcription[53]. Based solely on RNA expression, pipelines could independently predict regulatory elements in the genome without using chromatin marks[72,73]. These results not only show the predictive power of eRNA transcription for functional enhancer annotation but also suggest potential functions of enhancer transcription.

The pervasiveness of eRNA production raises several crucial questions. How are enhancers activated as transcription units? What are the specific and common features of eRNAs compared with other transcription units? Do eRNAs serve as important regulators of enhancer activation, or are they merely by-products (FIG. 1)? What is the biological and/or pathological significance of enhancer transcription?

Activation of enhancers

How enhancers are activated during development in response to signalling events has provoked extensive research. One important proposal was that specific histone marks can serve to divide the large numbers of enhancers into distinctive functional states (for example, poised or active)[10–12]. In this regard, interpreting enhancers as transcription units mechanistically links enhancer marks and functions. For example, H3K27ac and the acetyltransferase CBP/p300 were reported to have a causal role in transcription initiation[74,75], and thus their enrichment at enhancers could largely be based on their activity in augmenting enhancer transcription. On the basis of recent findings, we propose a partially hypothetical diagram showing the stepwise events in enhancer activation (FIG. 1). Comparison with classic models of promoter activation indicates that enhancers exhibit many similarities to promoters in terms of the assembly of the transcriptional apparatus[76]. However, there are also important differences. In the following section, we elaborate on these similarities and differences between eRNAs and conventional promoter-produced transcripts, including lncRNAs[8], promoter upstream transcripts (PROMPTs)[77–79] and protein-coding mRNAs. BOX 2 provides an overview of the commonly observed features of these transcription unit categories.

Recruitment of transcription factors to enhancers and promoters. It is well established that sequence-specific TFs conduit regulatory inputs to the chromatin and elicit transcriptional outputs. Although the TFs implicated in enhancer and promoter transcription may act in a similar manner in the early steps of the cascade (FIG. 1), non-overlapping sets of TFs are enriched at enhancers and promoters[1,73]. Many of the lineage-determining and signal-regulated TFs seem to preferentially associate with enhancers[20] (for example, forkhead box protein A1 (FOXA1)[80], oestrogen receptor (ER)[81] and PU.1 (REFS 82,83)), whereas some other TFs are more often bound at promoters (for example, E2F1 (REF. 84)). Analyses have revealed that genomic regions showing co-binding of multiple TFs (that is, HOT regions) are more often enriched at enhancers[60], which also seem to be highly specific to the developmental stage or lineage[85], whereas constitutive HOT regions across developmental stages or lineages preferentially locate at promoters[85]. These results are in accord with the concept that enhancers are responsible for driving lineage-specific gene expression[20]. The difference in TF binding at enhancers and promoters should not all be attributed to distinctive DNA motif frequencies[3,38], but rather is probably modulated by multiple mechanisms, including collaborative and synergistic binding dependent on other TFs[20,60,62,63,86], dynamic chromatin states[80] and even the transcriptional output itself (that is, the ncRNAs)[87]. However, it is currently unclear how distinctive TFs on the two elements contribute to the transcriptional activation of both enhancers and promoters (for example, what is the significance of an enhancer-enriched TF displaying a small percentage of events binding at promoters?). A plausible model is that enhancers and promoters each independently recruit certain TFs, but require collaboration to achieve a full amplitude of transcriptional outputs[88].

Transcriptional features of eRNAs and promoter-produced transcription units. Studies support the concept that similar rules of transcription initiation operate at promoters and enhancers (BOX 2; FIG. 2). Recruitment of general transcription factors (for example, TATA-box-binding protein (TBP)) and the serine 5-phosphorylated form of RNAPII (Ser5p) to enhancers is analogous to their presence at promoters of lncRNAs or mRNAs[89] (but with variable intensities), and Ser5p is perhaps involved in recruiting the RNA capping machinery[90,91]. Results from nuclear run-on followed by 5′cap sequencing (5′GRO-seq or GRO-cap) and CAGE (TABLE 1) show that eRNAs are generally capped[3,43,73]. The DNA sequence, the presence of core promoter elements (for example, TATA boxes) and the nucleosome spacing at the transcription start sites (TSSs) of enhancers and promoters are also similar[73]. In addition, enhancers often, but not invariably, resemble gene promoters in producing bidirectional transcripts[32,33,66,67,72,73] (FIG. 1). Promoter directionality was determined by the relative density of poly(A) cleavage sites (PASs) versus U1 splicing motifs in the downstream regions after TSSs[92,93] — the higher density of U1 motifs in the sense direction allows productive elongation of RNAPII to generate mRNAs. The same mechanism may also apply at enhancers. Computational analyses revealed that PASs exist at a high density in enhancer regions[3,72,73], which are also more likely to locate closer to the enhancer TSSs than U1 motifs[73]. At least one example has been reported in which an intragenic enhancer even functionally substituted for a deleted promoter to drive mRNA expression[94]. These data suggest considerable functional commonality between enhancers and promoters for transcriptional initiation.

Discernible differences between eRNAs, lncRNAs and mRNAs reside in their post-initiation steps, including elongation, termination and RNA processing (BOX 2; FIG. 2). Although the elongation of enhancer

Promoter upstream transcripts
(PROMPTs). Primary transcripts that are generated pervasively from gene promoters but are transcribed in the opposite direction from the sense strand (that is, mRNAs). PROMPTs generally display low stability, lack of splicing and polyadenylation; very similar to enhancer RNAs in many aspects. Also known as upstream antisense RNAs.

General transcription factors
Transcription factors that work together with RNA polymerase II to form the pre-initiation complex at transcription start sites to initiate transcription. They consist of TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH.

Bidirectional transcripts
The two transcripts initially observed to be generated by some coding gene promoters that go to either a sense or an antisense direction, which produces the mRNAs and the promoter upstream transcripts, respectively. A similar phenomenon is now observed to exist for some enhancers.

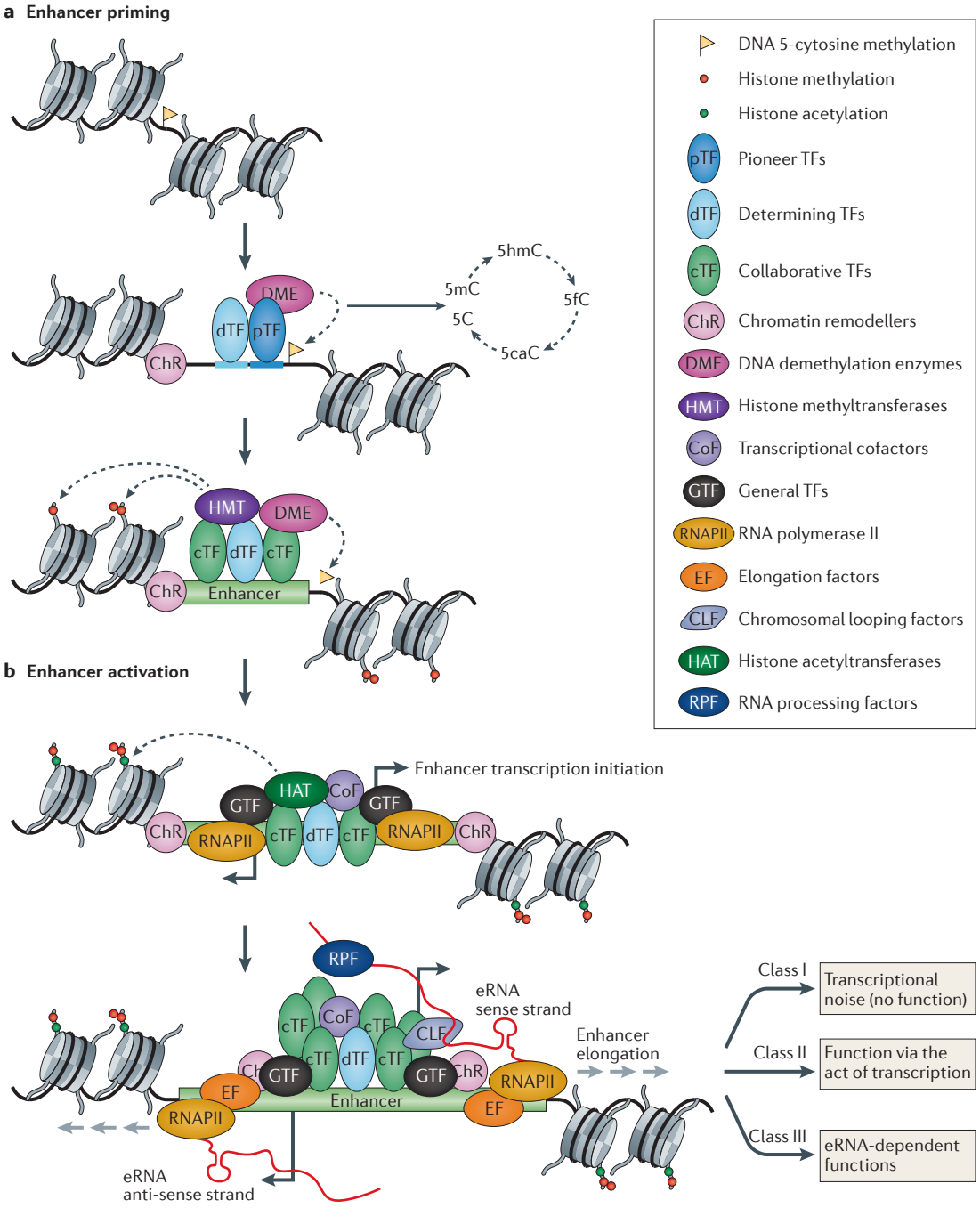
DRB
(5,6-dichloro-1-β-D-ribofuranosylbenzimidazole). An adenosine analogue that acts as an inhibitor of cyclin-dependent kinases needed for efficient RNA polymerase II elongation.

RNA exosome

A multi-protein complex capable of degrading various types of RNA molecules in the cytoplasm, nucleus and the nucleolus; it bears both exo- and endo-ribonucleolytic functions in eukaryotes.

transcription involves some common regulators (for example, bromodomain-containing protein 4 (BRD4)[95]) as lncRNAs and mRNAs (BOX 2; FIG. 2), it is distinguished by the low recruitment of serine 2-phosphorylated RNAPII (Ser2p, a RNAPII form involved in elongation) and minimal levels, if any, of the H3K36me3 modification (a histone mark enriched in gene bodies of lncRNAs and mRNAs)[22,89]. The low H3K36me3 level may actually be a consequence of low Ser2 phosphorylation of RNAPII[96] and a lack of splicing[97] (BOX 2; FIG. 2). DRB-mediated inhibition of cyclin-dependent kinase 9 activity repressed the elongation of many eRNAs and most mRNAs[32,87], but exhibited a lack of effect on several specific eRNAs tested in macrophages[32]. These results suggest that alternative mechanisms other than those used for mRNAs may underlie eRNA elongation, at least for specific enhancer subsets.

Distinct from mRNAs, eRNAs generally display low stability and abundance (BOX 2), consistent with their presence largely in the nuclear and chromatin-bound fractions[2,32,47]. Specific eRNAs considered to be of higher abundance were quantitated at ~0.5–20 copies per cell[44]. Analogous to events at PROMPTs, the stability of eRNAs is probably regulated by PAS-mediated early termination[72,73], and their decay is conducted by the RNA exosome[3,49,72,79] (FIG. 2). The lack of U1 splice sites in



RNAPII carboxy-terminal domain
(RNAPII CTD). An evolutionary conserved tandem repeat of heptapeptides $Y_1S_2P_3T_4S_5P_6S_7$ that is present in the C terminus of RPB1, the largest subunit of RNA polymerase II (RNAPII).

Small nuclear RNAs
A class of small RNAs in the nucleus of eukaryotic cells that have been found to largely take part in regulating splicing (for example, U1 and U2 RNAs) and occasionally in transcriptional control of RNA polymerase II (for example, 7SK RNA).

Enhancer–promoter inter-regulation
A hypothesis in which active enhancers affect promoter expression, and some promoters also control enhancer transcription.

enhancer regions[72,73] also explains the finding that eRNAs are rarely spliced (splicing is observed in ~5% of eRNAs)[3], which is in sharp contrast to the fact that ~80% of mRNAs show splicing[3] (BOX 2). Falling in-between, lncRNAs display a splicing rate of ~30% in human embryonic stem cells[51] (BOX 2) and exhibit an intriguing bias for containing two exons[98]. Recruitment of the U1 splicing machinery has been reported to depend on, and modulate, the H3K4me3 modification at promoters[99,100]. The observed differences in transcriptional activities on enhancers versus promoters can be linked to a long-known, but unexplained, difference in their H3K4me3-to-H3K4me1 ratio[19]. Enhancers with more stable eRNAs exhibited stronger H3K4me3 marks[73], whereas H3K4me3-marked enhancers have been suggested to be more active[23]. These correlations call into question whether transcriptional activities are the cause or the consequence of the histone methylation states at enhancers versus promoters[101].

Transcriptional termination at enhancers is just beginning to be understood. Several experimentally validated eRNAs migrate as distinctive bands in northern blot analyses[40,45,50], suggesting uniform initiation and termination. However, the generality of this feature requires further experimental evidence. An important regulator of eRNA termination is Integrator[42], a complex that interacts with the RNAPII carboxy-terminal domain (RNAPII CTD) and was originally found to be important in the termination of small nuclear RNAs[102]. Depletion of Integrator subunits reduced levels of chromatin-bound processed eRNAs while unexpectedly increasing the transcription activity at enhancers and the amount of polyadenylated eRNAs, suggesting that eRNA termination was compromised[42] (FIG. 2). Intriguingly, small nuclear RNA genes differ from eRNAs by containing distinctive 3′ box elements at their terminus[102]. This finding raises an important question as to how Integrator carries out termination differently for these two types of ncRNAs. Proper eRNA termination also requires the function of WD repeat-containing protein 82 (WDR82), an adaptor protein that is involved in targeting the SET1 H3K4 methyltransferase to chromatin. This was shown by increased eRNA abundance and length due to defective termination after depletion of WDR82 (REF. 103). An intriguing feature of active enhancers is that the tyrosine 1-phosphorylated form of RNAPII (Tyr1p) is particularly enriched at active enhancers and PROMPT regions, but not at the sense strand of gene promoters[104,105] (BOX 2; FIG. 2). This feature is potentially associated with eRNA termination because such a role has been ascribed to Tyr1p in yeast[91,106]. Chemical modifications of RNA molecules are increasingly recognized to regulate RNA processing and function[8]. A recent study found that the NOP2/Sun RNA methyltransferase (NSUN7) deposits 5-cytosine methylation to some eRNAs and thereby affects their stability[107] (FIG. 2). This finding opens a door to the exciting area of RNA epigenetics in the control of eRNA transcription and enhancer function.

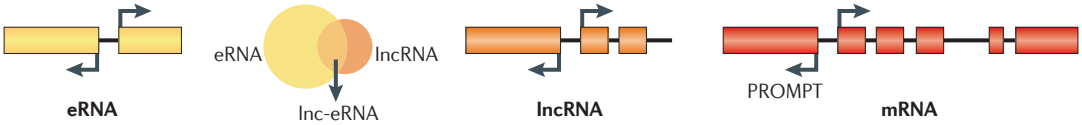
Enhancer transcription and enhancer–promoter looping. Another key distinction between enhancers and promoters is associated with their inter-regulation mediated by the formation of enhancer–promoter looping (BOX 1). Despite being a widely accepted model, loop formation is largely enigmatic in terms of the underlying mechanisms. The physical proximity between looped pairs of enhancers and promoters and the fact that both elements serve as transcription units raise some key questions about this process. Do enhancers and promoters exchange certain transcriptional machinery dependent on looping? For looped enhancer–promoter pairs, do enhancers generally instruct promoters for their activation, or can promoters also instruct enhancers (BOX 1)? Genetic studies often focus on deleting enhancers to examine the impact on promoters[5]; fewer investigations have been conducted with a focus on promoter deletion, without clear conclusions being drawn[24,33,94,108]. Clues to understand the order of enhancer–promoter inter-regulation have been provided by studies of transcriptional kinetics from both single locus[32,40,66,109,110] and large-scale profiling[38], which revealed that certain eRNAs were the first transcription units to respond to stimuli, temporally preceding the activation of promoters. This evidence supports an argument that the 'earliest responder' group of enhancers is loaded with transcriptional machinery first, subsequently participating in the activation of the promoters (FIG. 3). This also raises the possibility that enhancers and promoters exhibit a certain hierarchy in the loading of transcriptional machinery, but the underlying mechanism for such a temporal hierarchy is unresolved.

Notwithstanding the hierarchy, from where do enhancers and promoters acquire their transcriptional machinery? A role of sub-nuclear structures has been suggested. Early work on the β-globin locus showed that LCRs assist the β-globin locus in associating with transcriptionally engaged RNAPII foci[111]. In a recent

Figure 1 | **Activation of an enhancer.** A generic diagram showing stepwise enhancer activation as a transcription unit in the presence of developmental or other cues. **a** | The assembly of the transcriptional apparatus at an enhancer is first initiated by the binding of pioneer transcription factors (pTFs)[170], which bind the DNA in nucleosomes to generate open chromatin[170], allowing the recruitment of lineage-determining transcription factors (dTFs) to dictate the enhancer site for activation in a specific cell lineage[20]. Collaborative transcription factors (cTFs)[20,62,63,86] and important cofactors (CoFs) are further recruited, such as histone methyltransferases, which 'write' mono- and dimethylation on the H3 tail at lysine 4 (H3K4me1 and H3K4me2, respectively)[82]. Each red circle represents a methyl group. **b** | These previous steps prepare the enhancer for further recruitment of other CoFs, such as histone acetyltransferases (HATs; for example, CREB-binding protein and p300 (CBP/p300)) that deposit histone acetylation marks (green circles), as well as general transcription factors (GTFs) and RNA polymerase II (RNAPII) holoenzymes to initiate bidirectional transcription. The acetylated histone tail recruits additional CoFs, such as bromodomain-containing protein 4 (BRD4), which probably works together with positive transcription elongation factor-b complex (pTEFb) (not shown) to promote transcriptional elongation of enhancer RNAs (eRNAs)[82,87,95]. The recruitment of chromatin looping factors (CLFs) facilitates enhancer–promoter interactions. However, the order of their recruitment and roles in enhancer transcription is not fully understood[171,172]. DNA methylation was dynamically regulated during enhancer activation. Hydroxylated 5-methylcytosine (5hmC) or the oxidized 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) were found to be enriched at enhancers during enhancer priming in stem cells[173,174], with potential roles in modulating p300 binding[174]; the underlying mechanisms and the order of demethylation events in this cascade remain areas of active investigation[70,173–175]. In addition, the local chromatin structure, including nucleosome spacing, is under regulation by chromatin remodellers (ChRs) in various stages of this cascade, which may also affect eRNA transcription[176]. This diagram represents a generic model and may be subject to changes for different enhancer subsets. The functional importance of enhancer transcription could involve three possible, non-exclusive models as shown.

Box 2 | Features of enhancer RNAs and other transcription units

Based on their length, enhancer RNAs (eRNAs) would logically be considered as a subcategory of long non-coding RNAs (lncRNAs). However, most Cap analysis of gene expression (CAGE)-defined eRNAs are not recorded in lncRNA databases such as GENCODE[3,38,98]. The reasons for this are twofold: the discrepancy in definition criteria — eRNAs are generally defined by their transcription from regions with enhancer-like chromatin features (for example, histone H3 lysine 4 monomethylation (H3K4me1))[3,38,98], whereas lncRNAs are primarily defined arbitrarily on the basis of RNA length (that is, >200 nucleotides)[8,98]; and many eRNAs are too unstable and/or of too low an abundance to be effectively captured by methods commonly used in constructing lncRNA databases[98]. There is therefore an inevitable grey area in which independently defined eRNAs and lncRNAs exhibit overlap. A search of lncRNAs from the ENCODE database identified 2,695 lncRNAs that overlapped with tissue-specific enhancers, and their expression correlates with the predicted enhancer activities[168]. This group of RNAs may thus be termed as both eRNAs and lncRNAs. A revised definition system to categorize eRNAs in relation to lncRNAs will be useful in this field. We propose that, before their functional roles can be established, eRNAs could be divided into at least two subcategories based on current genomics data, namely: lnc-eRNAs, to define transcripts with initiation sites overlapping enhancer regions with appropriate histone marks (for example, H3K4me1 and H3K27 acetylation) and presence in current lncRNA databases, including GENCODE[98]; and eRNAs, to denote the remaining group of transcripts from enhancer-like regions not currently recorded in lncRNA databases. This arbitrary classification is useful given the current ambiguity of terminologies before functional characterization can be achieved. We further propose an empirical categorization of eRNAs based on functional roles (FIGS 1,3 and main text). The figure below presents a generic structure of eRNAs compared with lncRNAs and mRNAs. The Venn diagram shows the overlap between eRNAs and lncRNAs annotated by the current definition.



As an overview, the table below summarizes prominent features of eRNAs as an overall group (the subcategory of lnc-eRNAs is expected to share most features with lncRNAs) compared with other promoter-produced transcription units. We list promoter upstream transcripts (PROMPTs) as a separate category here due to the many similarities between eRNAs and PROMPTs[72,73,78,79]. Of course, almost all of the generalized features listed here are invariantly accompanied by anecdotal cases of exceptions, reflecting the complexity and current ambiguity in classifying ncRNAs. In addition, we did not discuss in this Review the small RNAs reported to be generated from active enhancers[1,3,169], as their identity and abundance awaits more experimental confirmation.

Features	eRNA	PROMPT	lncRNA	mRNA
DNase HS	Yes	Yes	Yes	Yes
H3K4me1	High	High	Medium	Low
H3K4me3	Low	Low to medium	Medium	High
H3K36me3	No	No/low	Yes	Yes/high
H3K27ac	High	High	High	High
RNA polymerase II (RNAPII)	Yes	Yes	Yes	Yes
RNAPII Tyr1p	High	High	Unclear	Low
RNAPII Ser2p	No	Yes/low	Yes	Yes/high
RNAPII Ser5p	Yes	Yes	Yes	Yes
RNAPII Ser7p	Yes	Yes	Unclear	Yes
CpG island	Low	High	Medium	High
Splicing	Rare	Rare	Common (2-exon bias)	Yes
Polyadenylation	Some	Some	Mostly	Mostly
Stability	Low	Low	Low to medium	High
Number*	~40,000–65,000	Several thousands to ~10,000	Several to tens of thousands	~23,000
Conservation	Low	Unclear	Medium to high	High
Small RNAs	Yes	Yes	Unclear	Yes
Tissue specificity	Extremely high	Unclear	High	Low
Preferential subcellular enrichment	Nuclear and chromatin-bound	Nuclear and chromatin-bound	Nuclear and chromatin-bound and cytoplasmic	Mostly cytoplasmic
Exosome targets	Yes	Yes	Partially yes	Mostly not

The features listed here are mainly based on data generated in human and mammalian cells. *The numbers listed denote those of genomic regions producing transcripts, rather than the numbers of distinctive RNA transcripts, which could be more complex as a result of post-transcriptional processing. The number for coding mRNAs could be much larger than 23,000 if isoforms or only the transcriptional initiation events are counted[38]. Also, it is the number of eRNA transcription units, but perhaps not the number of eRNA molecules, that accounts for a major constituent of the human transcriptome[2,3].

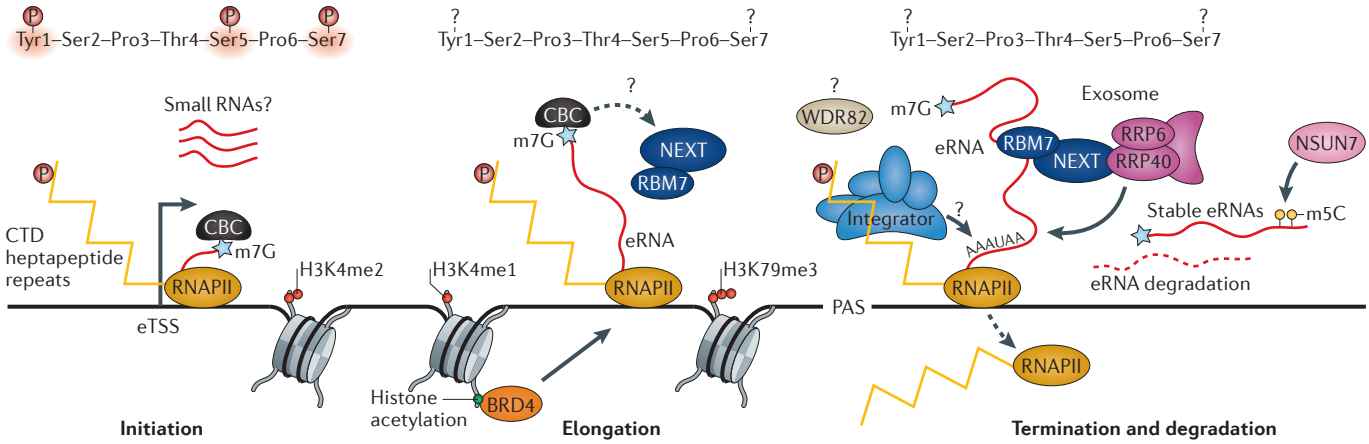


Figure 2 | **Enhancer transcription process and enhancer RNA processing.** A schematic diagram showing the known process and regulators of enhancer transcription, with some postulations. The recruitment of cap-binding complex (CBC) is postulated to bind enhancer RNAs (eRNAs) through a 5′ end 7-methylguanosine (m7G) cap (blue star). The transcription elongation of enhancers is controlled by at least partially overlapping machinery as coding genes, including the positive transcription elongation factor-b complex (pTEFb; not shown here) and bromodomain-containing protein 4 (BRD4)[82,87,95], which is recruited by acetylated histone tails on enhancers (green circles)[95]. The Mediator complex is known to associate with enhancers[59] and has been reported to directly bind eRNAs[40,115] (not shown in this diagram). Transcription termination of eRNAs was carried out by the Integrator complex[42], potentially after the poly(A) cleavage site (PAS, AAUAAA) in the nascent eRNA[72,73]. The adaptor protein WD repeat-containing protein 82 (WDR82) has also been attributed a role in the termination of eRNAs[103]. The nuclear RNA exosome complex is responsible for the degradation of eRNAs, with two of its components shown in the diagram, which are exosome component 10 (EXOSC10; also known as RRP6) and EXOSC3 (also known as RRP40). The targeting of the exosome to RNAs in human cells is, at least partially, mediated by a trimeric nuclear exosome targeting (NEXT) complex, comprising Ski2-like RNA helicase 2 (SKIV2L2), zinc finger CCHC domain-containing protein 8 (ZCCHC8) and RNA-binding protein 7 (RBM7)[79]. Among these, RBM7 directly binds eRNAs[79]. Recruitment of NEXT may also be facilitated by the CBC[79,90] (dashed line and arrow in the centre). The red-coloured small 'p' in a circle represents phosphorylation of the RNA polymerase II (RNAPII) C-terminal domain (CTD) (yellow zigzag tail). The 5-methylcytosine (m5C) chemical modification was found to be present at some eRNAs, and was deposited by the NOP2/Sun RNA methyltransferase 7 (NSUN7)[107]. Question marks denote unclear modifications or processes. In addition, small RNA transcripts have been reported to exist in enhancer regions[1,3,169], but their identity, abundance and functions require further interrogation. eTSS, enhancer transcription start site.

study, attachment to a sub-nuclear structure enriched in matrix-3, a protein related to the nuclear matrix, was found to be necessary for the optimum transcription of both eRNA and target genes in pituitary cells[52]. These results could be interpreted to suggest a model in which transcriptional machinery is 'transferred' from certain sub-nuclear structures to enhancers and/or promoters (BOX 1); it is equally possible that enhancers and promoters relocate in the nucleus to such structures, or generate such structures *in situ*, and the sequential order of their relocation may determine their hierarchy during inter-regulation. Despite some efforts[52,112], it remains technically challenging to specifically up- or downregulate a looping event, or detach or attach a genomic region to sub-nuclear structures. New tools and further investigation are required to delineate the inter-relationship between enhancer and promoter transcription, the roles of enhancer–promoter looping in transcriptional control, and the link of these events to sub-nuclear structures.

Transcriptional noise
A term used to denote non-productive and perhaps random transcriptional activity of an RNA polymerase on a DNA template, which is proposed to take place due to open chromatin regions.

The function of enhancers as transcription units

Whether enhancer transcription has a functional role is a central question in our understanding of enhancers and gene regulation (FIG. 1). An early hypothesis, which may still hold true in some instances, is that pervasive ncRNA transcription probably represents transcriptional noise[113] (FIGS 1,3A). However, both the robustness of eRNA transcription and their regulated expression pattern argue for some potential functional roles. Some reports have ascribed functions to at least some eRNAs[37,40,41,43–45,47,48,50,82,87,110,114–117]. This raises another key question: if enhancer transcription is indeed functional, what specifically is responsible for such a function? Is it the actual eRNA transcripts, the act of transcription, or both (FIGS 1,3)? We first discuss this topic with the null hypothesis that enhancer transcription represents non-functional incidental events, and then consider the published evidence supporting functional roles for some subsets of studied eRNAs (FIG. 3).

eRNA: transcriptional noise? It is postulated that RNAPII constantly scans the genome, and the specificity of a RNAPII initiation at an optimum site could be ~10^4-fold higher than at an average, random site[113]. The relatively low transcriptional levels[2,3], poor evolutionary conservation[118] and high chromatin accessibility[10–12] (BOX 2) of active enhancers has encouraged the proposal that most eRNAs result from a non-productive or random 'scanning' of the RNAPII machinery, merely acting to load the transcriptional machinery for promoters to access (FIG. 3Aa). Alternatively, enhancer transcription may simply be a consequence of proximity to the high

De novo enhancers
A group of enhancers that were not marked by any epigenomic marks during cell lineage determination, but rather are generated acutely in a mature cell type after treatment with acute stimulation; they are also known as latent enhancers.

concentration of transcriptional machinery at active promoters, especially because promoters have been suggested to nucleate chromosomal interactions involving multiple enhancers[119] (FIG. 3Ab).

The function of the eRNA transcription process. Two facts prompt consideration of the potential impact of the transcribing RNAPII: it is a DNA motor that has dramatic architectural effects on local chromatin[120], and its CTD serves as a 'landing pad' that can bind more than 100 proteins with various functions to 'travel' together[91]. In an early endeavour, Gribnau *et al.*[121] suggested that the act of intergenic transcription in the β-globin locus is important for specific chromatin acetylation and remodelling because RNAPII can 'piggyback' histone acetyltransferase during transcription (FIG. 3Ba). In support of a role of the transcription process, the insertion of transcriptional terminators disrupted the eRNA transcription from two different LCRs and concomitantly reduced the adjacent mRNA levels[122,123]. However, these experiments did not completely exclude a potential role of the eRNAs per se because the forced termination also truncated eRNA transcripts. In a recent study on a specific cohort of *de novo* enhancers[82] (also called 'latent enhancers' (REF. 83)), inhibition of enhancer transcriptional elongation reduced the deposition of H3K4me1 and H3K4me2 owing to compromised 'travelling' of the responsible histone methyltransferase associated with RNAPII[82]. Importantly, this effect seemed to be independent of eRNAs[82]. In addition

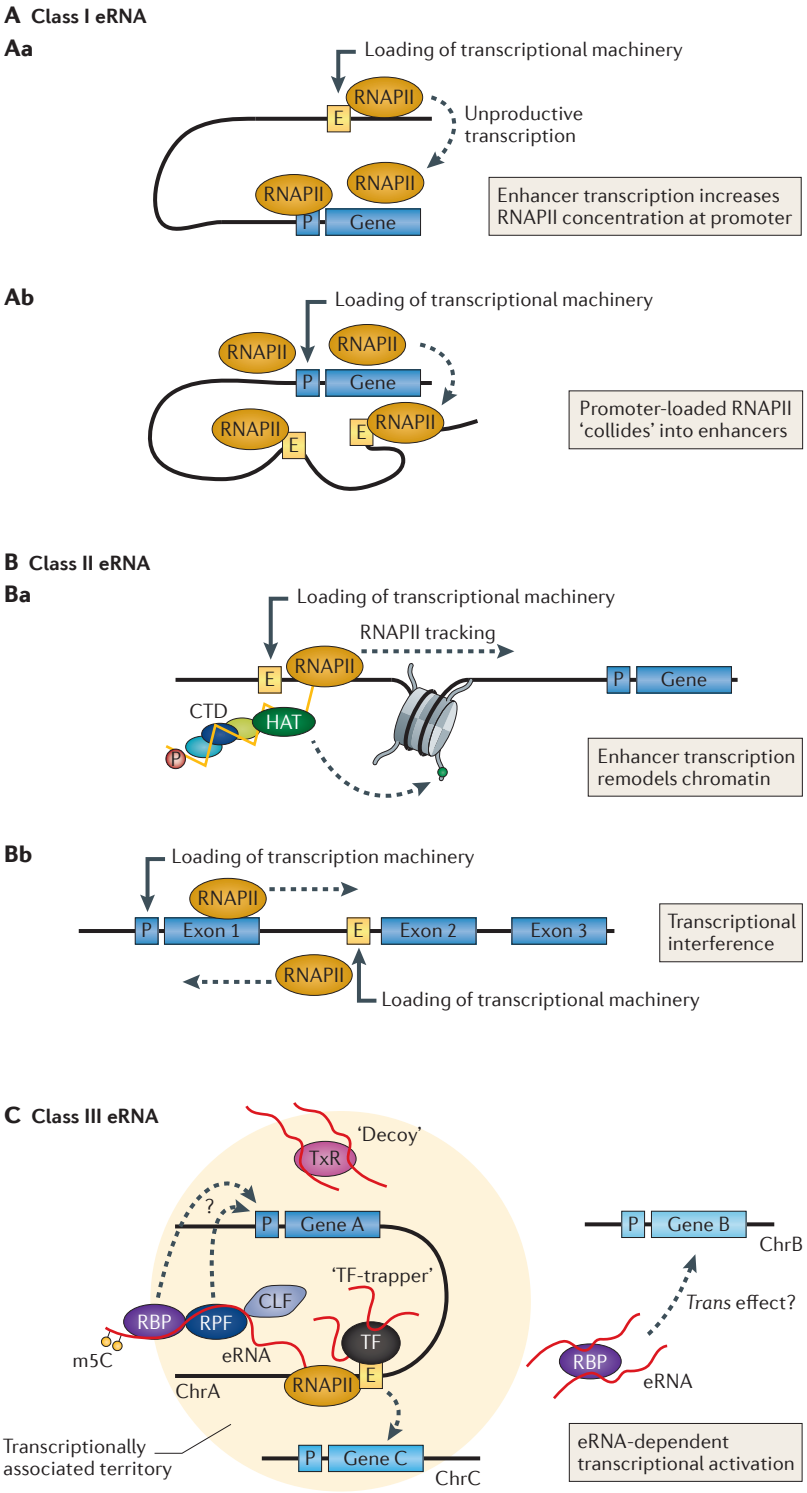


Figure 3 | **Functional roles of enhancer transcription in gene regulation.** Three non-exclusive models may underlie the functions of enhancer transcription: the transcription process and enhancer RNAs (eRNAs) are non-functional and are merely transcriptional noise (part **A**); the act of enhancer transcription mediates function (part **B**); and genes on the same chromatin fibre (*cis*), or potentially on other chromosomes (*trans*), are regulated by an eRNA (part **C**). Although perhaps many transcriptional regulators can be potentially 'transferred' between enhancers and promoters, RNA polymerase II (RNAPII) is used here as a representative for simplification. **Aa** | Enhancer transcription merely increases the local concentration of transcriptional machinery to augment promoter activities. **Ab** | Promoter-loaded machinery passively or randomly collides with enhancers due to their physical proximity. **Ba** | The transcription of some enhancers by RNAPII could remodel the intervening chromatin between enhancers and promoters and activate target genes over a long range[122,123]; transcribing RNAPII could carry histone modifiers such as histone acetyltransferases (HATs) or histone methyltransferases (not shown) to modify the enhancer region and the intervening DNA. **Bb** | For other enhancers, especially those in introns[124], their transcription may interfere with the overlapping gene transcription. **C** | Functional mechanisms of eRNAs per se include: eRNAs interact with chromosomal looping factors (CLFs) to positively influence enhancer–promoter looping and gene transcription; eRNAs bind transcription factors (TFs) to help 'trap' them at enhancers; and eRNAs act as a 'decoys' or 'repellents' to inhibit transcriptional repressors (TxRs). *Trans* roles could be achieved by eRNA translocation to distant sites (right side of panel) or proximity-based regulation in which eRNAs and target gene(s) reside in certain transcriptionally associated territory (light yellow area). A common, emerging theme regarding eRNA function is that the 5′ end of the nascent eRNA interacts with protein partners (RBPs), with the 3′ end still attached to its transcribing loci. In turn, such an interaction can modulate the functions of RBPs or RNA processing factors (RPFs) allosterically, as exemplified by the cyclin D1 (*CCND1*) promoter long non-coding RNA and *HOTTIP* RNA[127,177].

to regulating chromatin remodelling, enhancer transcription may have other roles, as shown by the report of two intronic transcribing enhancers modulating the isoform decision of the overlapping sense coding genes by 'transcriptional interference' (REF. 124) (FIG. 3Bb). Together, these data suggest that the act of enhancer transcription has important functional roles, sometimes independent of the eRNA transcripts.

Roles of eRNAs. In the previous examples where enhancer transcription affected the expression of the target gene[122,123], eRNAs were forced to terminate early, leaving much shorter eRNA transcripts. Potential roles of these transcripts can therefore not be excluded. Several recent lines of evidence support a functional role of at least a subset of eRNA transcripts. Two different measures, including short hairpin RNAs and small interfering RNAs[37,40,44,45,47,48,50,110,114–117,124] as well as locked nucleic acids[41,43,44,124] were used in a set of experiments that effectively knocked down eRNA transcripts in the nucleus. Specific eRNA knockdown was accompanied by downregulation of the cognate coding genes, suggesting the functional importance of eRNAs. In addition, RNA-tethering experiments in reporter assays[43–45,125] demonstrated a quantitative effect of eRNA transcripts in conferring gene activation, independent of the act of transcription.

Given the extremely large number of eRNAs in the human genome[2,3,38], these results together suggest that all three models underlying eRNA functions may exist non-exclusively (FIG. 3). Although both the transcription process and some eRNAs by themselves have functional roles, considerable additional evidence is required to fully elucidate the biological roles, if any, of the large majority of eRNAs in the genome. Nevertheless, most evidence supports the view that the transcription of eRNAs, regardless of their additional functions, seems to reliably serve as an indicator of enhancer activity.

Mechanisms of eRNA function in gene activation. A number of studies have illustrated several functional mechanisms underlying the actions of eRNAs per se. One mechanism is that eRNA regulates the chromatin accessibility of target promoters and the subsequent RNAPII binding[47,116]. These studies implied a functional relationship between eRNAs and chromatin remodelling complexes at promoters; however, they do not answer how these eRNAs act over long distances to reach promoters. Several other studies asked whether eRNAs could be involved in the formation or stabilization of enhancer–promoter loops (BOX 1; FIG. 3C) and, indeed, found impaired looping in interrogated loci on eRNA knockdown[40,44,50,115]. In support of this role, reduced eRNA levels were accompanied by concomitant defects of looping on Integrator knockdown[42]. In this regard, specific eRNAs were found to interact with either cohesin[44] or the Mediator complex[40,115] to facilitate the formation or stability of enhancer–promoter loops. A similar mechanism has been reported for several lncRNAs with activating roles, such as *HOTTIP*, *CCAT1-L* and *LUNAR1*, each by interacting with distinct protein partners[126–128]. However, there are examples that argue against eRNAs being necessary for enhancer–promoter looping. The inhibition of RNAPII elongation using flavopiridol reduced eRNA and coding gene expression from two interrogated loci in breast cancer cells, but did not seem to affect the interrogated loops[66]. Similarly, no significant change in looping was observed on knockdown of two functional eRNAs in depolarized neurons[110]. In interpreting the basis of these differing results, in addition to technical differences (BOX 1), it must be taken into consideration that the cause–consequence relationship between enhancer transcription and loop formation is perhaps not invariant — enhancer activation and eRNA transcription might be the cause of looping for some loci, but consequential or unrelated for others. This is consistent with a recent report that enhancer–promoter loops pre-exist for some, but not all, enhancers with stimulus-induced eRNAs[38].

eRNAs might exert function in the process of RNAPII pause release at cognate promoters through interacting with the NELF-E protein, a known RNA-binding subunit of the NELF complex that represses gene transcriptional elongation[110]. Putatively, this interaction could 'lure' the NELF complex away from the target promoters to allow productive elongation and gene activation[110]. In addition, a recent paper proposed a simple mechanism underlying eRNA function that the authors named 'transcription factor trapping' (REF. 87) (FIG. 3C). In this report, the TF YY1 was found to widely interact with both the DNA and ncRNAs of active regulatory enhancers. Using a CRISPR–Cas9-mediated RNA-tethering assay, it was found that each interrogated eRNA could specifically, albeit modestly, augment YY1 binding to its respective enhancer DNA, indicating a role of the transcripts per se in stabilizing YY1 binding[87]. This study suggested a potentially generalizable role for a large group of eRNAs (and other ncRNAs from regulatory elements) in facilitating the binding of TFs. However, the broad interaction between eRNAs and YY1 is reminiscent of the recently reported 'promiscuous' RNA binding of Polycomb repressive complex 2 (PRC2) and CCCTC-binding factor (CTCF)[129–132], apparently calling into question the molecular basis mediating the specificity and affinity of such interactions. Together, these mechanistic studies demonstrated that functional eRNAs are involved in perhaps all stages of gene activation, from controlling promoter chromatin accessibility, RNAPII loading, loop formation and pause release to modulating TF–DNA binding. Future studies should aim for a more open-ended search for the protein partners[133–135] of functional eRNAs to provide further and more complete biochemical insights into their mechanisms of action.

Cis *versus* trans *roles of eRNAs.* In search of potential *trans* targets of an eRNA, one study from our laboratory carried out chromatin isolation followed by RNA purification (ChIRP-seq) (TABLE 1) of an oestrogen-induced eRNA (*FOXC1-eRNA*) but failed to reveal confident *trans* targets[44]. By contrast, at least two other eRNAs, once depleted, affected the expression of many genes[40,47], some of which resided on other chromosomes. These two eRNAs are *KLK3-eRNA*, generated next to the kallikrein

NELF complex
(Negative elongation factor complex). A four-subunit complex consisting of NELF-A, NELF-B, NELF-E and either NELF-C or NELF-D. As denoted by the name, it negatively affects transcription by RNA polymerase II.

related peptidase 3 (*KLK3*) gene in human prostate cancer cells, and *DRR-eRNA*, generated next to the myogenic differentiation 1 (*MyoD1*) gene in mouse muscle cells. These results raise the possibility that some eRNAs may have *trans* targets. In accord with this possibility, the overexpression of *DRR-eRNA* upregulated some of the potential *trans* target genes, many of which are on another chromosome[44]. However, it cannot be ruled out that these two eRNAs affected some TFs, cofactors or signalling molecules that are important in a broad signalling or differentiation programme, thus indirectly influencing the expression of many genes. Therefore, additional, direct evidence of eRNA–gene loci association (for example, ChIRP-seq, or RNA and DNA fluorescence *in situ* hybridization (FISH); TABLE 1) and functional assays (for example, RNA tethering to target loci[87,125]) are required to further support such potential *trans* roles. Both *KLK3-eRNA* and *DRR-eRNA* are polyadenylated, whereas *FOXC1-eRNA* is not[40,44,47]. This leads to the hypothesis that polyadenylation and/or other post-transcriptional processing may confer a relatively higher stability to eRNAs to allow their *trans* action and thus more stable eRNAs, including many 'lnc-eRNAs' may have wide roles in gene regulation (BOX 2). Knockdown of an enhancer-like lncRNA (*ncRNA-a*), a typical example of a lnc-eRNA, affected hundreds of genes, which is compatible with a *trans* role for this transcript[117].

It should be stressed that none of the reported eRNA functions has been proved to be enhancer-independent — that is, eRNAs detached from the enhancer DNA and relocated into other chromatin regions. This is likely to be because the abundance and stability of most eRNAs is seemingly too low to sustain such a function. At a more speculative level, it is likely that eRNAs and the transcribing enhancers influence a target (or targets) on the basis of spatial proximity in the three-dimensional genome (FIG. 3C), such as in certain transcriptionally associated sub-nuclear territories. This is supported by several types of evidence showing: the confined locale of RNA-FISH signals of some eRNAs and lncRNAs[37,108,128]; that single enhancers can interact with several promoters[136]; that enhancers form visually discernible clusters that sometimes colocalize with RNAPII foci[137]; that eRNAs were enriched in biochemically isolated transcription factories[138], one type of such transcriptionally associated territory; and that ncRNAs can serve as organizing modules of distinctive sub-nuclear structures[135,139]. Further studies identifying any bona fide *trans*-acting eRNAs and their sub-nuclear location relative to their target genes will be instrumental in testing this model.

Functional categorization of eRNAs. These functional roles of eRNAs provide an opportunity to categorize them into more meaningful subgroups. We propose a possible functional categorization of eRNAs into three classes: class I eRNAs, for which neither their transcription nor transcripts have so far been shown to exert a discernible function (FIG. 3A), although the enhancers that produce such eRNAs could be needed for target gene expression; class II eRNAs, for which the act of their transcription serves as the important contributing factor to their function (FIG. 3B); and class III eRNAs, which fulfil RNA-dependent functions — this class is probably enriched in relatively abundant or stable eRNAs, especially lnc-eRNAs, and may act through binding protein partners (with a spectrum of specificity or affinity) to control gene expression or other nuclear activities (FIG. 3C). These classes can be further divided on the basis of functional models, such as 'decoy', 'looper' or 'TF-trapper'. This categorization will require extensive functional characterization of various eRNAs or eRNA groups.

Enhancer transcription in biology and disease

In addition to their roles in gene regulation, enhancer transcription and eRNAs have begun to be associated with biological functions — for example, two specific eRNAs are involved in cell cycle arrest and cell growth in cancer cells[40,45], whereas several erythrocyte-specific eRNAs regulate red blood cell maturation[37]. Enhancer transcription may also be involved in other nuclear activities, such as the regulation of genome stability and the generation of genomic variations at enhancers.

Enhancer transcription, R-loops and genomic instability. Transcription often leads to the formation of RNA–DNA hybrid structures referred to as R-loops, which severely compromise genome stability if left unresolved and can lead to single- or double-stranded DNA breaks or replication stress[140]. In mouse stem cells and B cells, the depletion of RNA exosome subunits increased levels of eRNAs and concomitantly upregulated R-loop formation at several interrogated enhancers[49]. This study further revealed that the resolution of these R-loops involved not only exosome subunits, but also heterochromatin protein 1γ (HP1γ) and histone H3K9 dimethylation[49] (FIG. 4a). Several factors of the DNA damage response (DDR) pathway, such as DNA topoisomerase I, MRE11 and DNA-dependent protein kinase (DNA-PK), have been found to be enriched at active enhancers and are needed to optimize the eRNA transcription induced by ligands[63,141] (FIG. 4a). Consistent with the known roles of DDR factors in R-loop regulation[140], these findings imply an intricate link between the transcriptional activities, DNA structure and genome instability at enhancers. An example illuminating this link comes from the study of activation-induced cytidine deaminase (AID). As a critical DNA mutator in activated mammalian B cells, AID is responsible for generating antibody diversity through somatic hypermutation and class switch recombination, but its mis-targeting often causes B cell malignancy[46,142]. Two reports have shown that enhancer transcription, especially that of intronic super enhancers, is responsible for AID mis-targeting to induce the subsequent genomic instability in malignancy[46,142] (FIG. 4a). In this sense, abnormal enhancer transcription and associated genomic instability could be widely involved in tumorigenesis (FIG. 4a). The increasing appreciation of R-loops[140] and the advent of genome-wide tools[143] have paved the way for systematic investigations of the location and strength of R-loops at transcribing enhancers and their physiological and pathological roles.

Myogenic differentiation 1 (*MyoD1*). A gene encoding a key transcription factor that promotes muscle-specific gene transcription programmes that are required for myogenic determination.

Genomic variations
The varied DNA sequences in alleles of certain genes carried by individuals within and among populations, which may or may not result in phenotypic variations.

R-loops
RNA/DNA hybrid structures in the genome, in which nascent RNA binds the transcribing DNA strand through sequence complementarity, leaving the non-bound single-strand DNA displaced and prone to damage. Once thought to be transcriptional by-products, R-loops have now been found to be involved in the regulation of gene expression, epigenetic modifications, DNA replication and genome stability.

Somatic hypermutation
A biological process mainly conducted by activation-induced cytidine deaminase in activated B cells, in which the immunoglobulin genes are highly mutated to generate a library of diversified antibodies.

Class switch recombination
A biological mechanism enabling B cells to switch their production of immunoglobulin from one type to another (for example, IgM to IgG), which involves an activation-induced cytidine deaminase-mediated specific DNA double-strand break and recombination.

Single nucleotide polymorphisms
(SNPs). Genomic variations that involve a single nucleotide.

Genomic variations of enhancers in disease and evolution. Enhancer transcription and the associated chance of DNA mutation coincide with the recent findings that enhancer-like regions contain a high density of genomic variants, many of which are linked to human diseases[3,55,56,144] (FIG. 4a). These variants can potentially lead to the loss or gain of enhancer activity (for example, altered TF binding sites) and result in aberrant gene expression. A subset of putative enhancers enriched in disease-associated single nucleotide polymorphisms (SNPs) is often transcriptionally active in pathologically relevant cell types[3,145]. For autoimmune diseases, Farh *et al.*[152] found that ~60% of putatively causal variants map to enhancers related to immune cells, many of which are eRNA-producing on immune stimulation. The same study also showed that <20% of putative causative SNPs interfere with recognizable TF binding sites, suggesting additional mechanisms[152]. This begs the question of whether the alteration of eRNA transcription or function has an active role in SNP-associated enhancer malfunction.

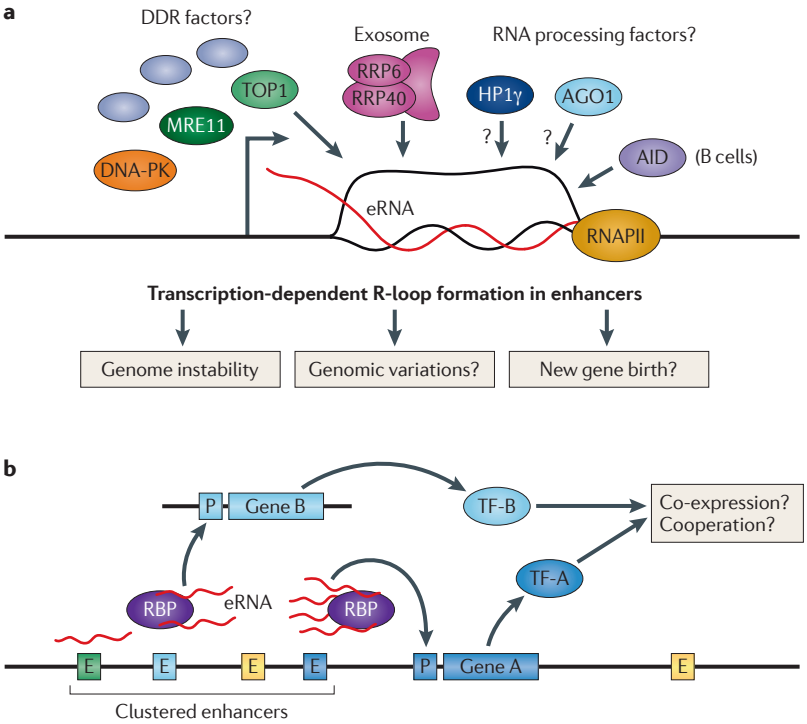


Figure 4 | **Biological significance of enhancer transcription and enhancer RNAs.** **a** | Transcriptional activity at enhancers may result in nucleic acid structures including R-loops[49,140], although the prevalence of R-loops at enhancers still requires further genome-wide elucidation. Various factors, as shown here, are presumably involved in the process of R-loop resolution or enhancer transcription[49,63,141]. Despite their binding to enhancers, most DNA damage response (DDR) factors are still mechanistically enigmatic in enhancer transcription or function. The additional factors in the figure include Argonaute 1 (AGO1), which has been shown to bind active enhancers depending on enhancer transcription[178], and activation-induced cytidine deaminase (AID), the mis-targeting of which in the B cell genome has recently been associated with enhancer transcription[46,142]. Question marks denote unclear functional roles or mechanisms. The transcription process of enhancers may be linked to several important biological, pathological or evolutionary functions, as shown in the diagram. Exosome component 10 (EXOSC10; also known as RRP6) and EXOSC3 (also known as RRP40) denote the two components of the RNA exosome complex. **b** | Roles of enhancer RNAs (eRNAs) related to the phenomenon of multiple or clustered enhancers. Clustered enhancers and the resultant eRNAs may cooperate to confer the co-regulation of distantly located important developmental genes, as illustrated in the regulatory region of myogenic differentiation 1 (*MyoD1*)[47], which meets the criteria for being a super enhancer[47]. One of the clustered eRNAs regulates the expression of the neighbouring key lineage-determining transcription factor A (TF-A; for example, MyoD1), while one or more other eRNAs controls the expression of a collaborative transcription factor (TF-B; for example, myogenin (MyoG)) or other cofactors (not shown) that reside distantly. This would confer co-expression and cooperative action of TF-A and TF-B in development and is consistent with the 'functional hierarchy' observed in some cases of multiple enhancers, such as primary and shadow enhancers[57]. That the deletion of a 'shadow enhancer' exhibited no obvious phenotype unless under stress may be because the shadow enhancer or eRNA regulates a 'cooperative' factor active only in response to stress. DNA-PK, DNA-dependent protein kinase; HP1γ, heterochromatin protein 1γ; RBPs, RNA-binding proteins; TOP1, DNA topoisomerase I.

eRNAs and clustered enhancers in development. In the development of metazoans, important genes may have multiple enhancers, which sometimes form a cluster, as exemplified by shadow enhancers[5,57], the regulatory archipelago[58] and LCRs. This phenomenon has emerged as a common theme for controlling gene transcription associated with cell identity or disease, as shown by the discovery of super and stretch enhancers[55,56,59]. It has been hypothesized that multiple enhancers may confer the robustness, diversity, flexibility or precision of important genes[5,20,57]; however, the relationship between individual constituents in a cluster and the underlying molecular logic remain unclear. In some instances there is only a minimal or quantitative contribution from individual constituents (that is, redundancy)[58,150], whereas in other instances one constituent but not others in the cluster proved to be functionally crucial (that is, functional hierarchy)[8,150]. Computational analyses of the human transcriptome suggest that there are various models of contribution from multiple transcribing enhancers to cognate gene expression[3].

The facts that clustered enhancers are among the highest transcribed and that each constituent is an active transcription unit[65,150] provide some new mechanistic

perspectives of clustered enhancers. In a hypothetical scenario in which transcribing enhancers transfer certain transcriptional machinery to promoters (BOX 1; FIG. 3Aa), every constituent could independently and quantitatively transfer a portion of the associated machinery to the target promoter. Therefore the loss of one or more constituents can, in some instances, only result in minimal or quantitative defects, emphasizing the phenomenon of enhancer redundancy. This model can be tested by examining eRNA levels and enhancer–promoter loops of each constituent when other constituents are deleted. In a second scenario, functional hierarchy among individual constituents may stem from the differential roles of individual eRNAs. An illuminating example was the regulatory region of *MyoD1*, which falls under the definition of a super enhancer[47]. Two major eRNAs (that is, *CE-eRNA* and *DRR-eRNA*) were identified in this enhancer cluster, but only *CE-eRNA* seemed to control *MyoD1* expression[47] (FIG. 4b), with *DRR-eRNA* serving, surprisingly, an important role for another set of genes, including myogenin (*MyoG*) (FIG. 4b). Intriguingly, both MyoD1 and MyoG are key TFs for myogenesis and act cooperatively[47]. These results suggest that some eRNAs in a cluster (for example, *CE-eRNA*) are dominant for expression of the *cis* target gene, whereas others (for example, *DRR-eRNA*) are designed to confer additional, functionally cooperative roles (FIG. 4b). Large-scale CAGE analyses of human eRNAs have identified multiple examples in which an enhancer cluster associates with multiple genes that themselves are collaborative partners or subunits of protein complexes[3]. We speculate that this 'functional cooperation' model might be common during animal development to coordinate the expression and functions of important lineage-determining (and/or disease-related) TFs (FIG. 4b). Given the large number of constituents in a typical clustered enhancer, redundancy and hierarchy could coexist in various combinations to ensure the versatile control of both the quantitative expression and functional cooperation of their target gene(s).

Conclusions and future perspectives

Enhancers are at the heart of deciphering contemporary regulatory biology[4,6]. The discovery that many or most functional enhancers are eRNA-producing transcription units has profound implications in understanding gene regulation, development and disease. To solve the remaining enigmas surrounding enhancers will require many additional studies, including further evaluation of the proposal that eRNA transcription stands as an independent criterion to predict an active enhancer, and to ascertain the roles of non-transcribing enhancers. Blocking the decay of eRNAs by the RNA surveillance pathway can be exploited to detect less stable eRNAs, facilitating the characterization of unannotated, putatively non-transcribed enhancers[79]. Measuring eRNA levels could be used to identify enhancer signatures on a much larger and wider scale, including those in human samples[151] of both physiological and pathological conditions[3,152], potentially offering diagnostic and therapeutic targets for human disease based on the exceptional specificity of eRNAs towards cell type and state.

The co-transcriptional regulation and RNA processing of eRNAs is only beginning to be understood. It remains to be tested whether the various regulators and pathways that govern mRNA processing[91] also have a role in eRNA processing, and whether additional players are involved. For example, cracking the CTD code of enhancers, especially the roles of Tyr1p[104], will uncover fundamental differences between eRNAs and other transcription units. Importantly, how RNA processing factors are involved in enhancer–promoter interactions will be a major topic to pursue, as suggested by such roles of the RNA exosome and Integrator complexes[42,49]. In addition, RNA processing factors are known to control nucleic acid structures and genome stability[140]; future investigations to study the interplay among these factors, the DDR machinery and enhancer transcription will be crucial in elucidating the basis of disease-associated genomic variations and genome instability at enhancers.

There remains a continued uncertainty about the clear and direct roles for eRNAs at a global level. Compared with the large number of mammalian enhancer transcription units (~40,000–65,000)[3,38], the anecdotal examples reported for eRNA functions represent merely the tip of the iceberg. Extensive functional studies of individual eRNAs or eRNA groups will be important in future work and will help to functionally categorize all of the eRNAs, as proposed in this Review (FIG. 3). Proteomic studies identifying specific interacting proteins of functional eRNAs[133–135] are the key to biochemical insights into eRNA functions. Exploratory interrogation needs to be conducted to unravel the association of functional eRNAs with specific chromatin regions and other functional RNAs[153], as well as to understand their possible functional motifs, secondary structures and chemical modifications[8,107]. Progress in these directions will then allow the mechanistic dissection of eRNA regulation and functions by using genome-editing tools to mutate such structures or motifs.

An important future goal will be a better understanding of enhancer–promoter looping, including its formation process, specificity and dynamic behaviour, preferably in real-time in live cells and at the single-cell level. This will corroborate any identified roles of eRNAs in modulating looping and further delineate the cause–consequence relationship between looping and transcription of eRNAs and genes. Given the increasingly recognized commonalities between enhancers and promoters[149], it will be important to delineate their inter-regulation and potential hierarchy in loading transcriptional machinery (BOX 1; FIG. 3). Therefore the systematic deletion of promoters and enhancers to investigate their relationship in the three-dimensional genome will be illuminating. Although we have witnessed an explosion of studies revealing a subset of enhancers as functional transcription units in the past five years, we can look forward in the next five years to unravelling the many remaining enigmas of enhancer transcription and function that will renew our fundamental understanding of gene regulation, development and disease.

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Acknowledgements
The authors thank M. Chuang for her helpful comments on the manuscript. This work was supported by the US Department of Defense breast cancer research programme postdoctoral fellowships (BC110381 to W.L. and BC103858 to D.N.) and grants from the US National Institutes of Health to M.G.R. M.G.R. is an investigator with the Howard Hughes Medical Institute. The authors apologize for not being able to cite many important other publications owing to the imposed limit.

Competing interests statement
The authors declare no competing interests.