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Olivier Fosseprez, Olivier Cuvier

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Review

Uncovering the functions and mechanisms of regulatory elements-associated non-coding RNAs

Olivier Fosseprez *, Olivier Cuvier *

Chromatin Dynamics and Cell Proliferation team; Center of Integrative Biology (CBI), Molecular Cellular and Developmental Biology Unit (MCD/UMR5077) Center of Integrative Biology (CBI-CNRS), Université de Toulouse (UPS), F-31000, France

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ABSTRACT

Over the past decade, regulatory non-coding RNAs (ncRNAs) produced by RNA Pol II have been revealed as meaningful players in various essential cellular functions. In particular, thousands of ncRNAs are produced at transcriptional regulatory elements such as enhancers and promoters, where they may exert multiple functions to regulate proper development, cellular programming, transcription or genomic stability. Here, we review the mechanisms involving these regulatory element-associated ncRNAs, and particularly enhancer RNAs (eRNAs) and PROMoter uPstream Transcripts (PROMPTs). We contextualize the mechanisms described to the processing and degradation of these short lived RNAs. We summarize recent findings explaining how ncRNAs operate locally at promoters and enhancers, or further away, either shortly after their production by RNA Pol II, or through post-transcriptional stabilization. Such discoveries lead to a converging model accounting for how ncRNAs influence cellular fate, by acting on transcription and chromatin structure, which may further involve factors participating to 3D nuclear organization.

1. Introduction

The association of RNA polymerase II with DNA at any accessible region of the genome, i.e. depleted of nucleosomes, leads to transcription of a multitude of RNAs that are not translated into proteins, hence named non-coding RNAs (ncRNAs). While ~70-90 % of all ncRNAs in cells are transcribed by RNA polymerase I and III, ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), other small nuclear RNAs (snRNAs), and numerous additional ncRNAs are either directly transcribed by Pol II or indirectly derived from Pol II transcripts. ncRNAs possess a myriad of roles and properties, notably in RNA processing, maturation and regulation [1,2]. The different RNA classes possess numerous and eclectic roles. Notably, extensive literature describes the roles of circular RNAs (circRNAs) (reviewed in [3,4]) and micro RNAs (miRNAs) [5] in transcription and gene expression regulation. Here, the review focuses on subclasses of Pol II-transcribed ncRNAs including enhancer RNAs (eRNAs) and PROMoter uPstream Transcripts (PROMPTs). Upon discovery, these two RNA species were first seen as mere by-products of transcription due to their relatively short half-life, of the order of minutes, yet they have since been the subject of much attention [6]. Several eRNAs have notably been associated with cancers and diseases [7], as well as with the regulation of gene expression and 3D genome organization [8,9]. Intergenic RNAs, sometimes referred to as long non-coding RNAs (lncRNAs, for lengths above 200 bp) may in fact be considered as eRNAs, while PROMPTs and "conventional lncRNAs" represent a minor population [10]. Since lncRNA and eRNAs share a number of properties and roles, these two canonical RNA classes are now thought to define the two extremes of a continuous spectrum [9]. Here, we discuss how the production and processing of these ncRNAs help to describe their roles in the regulation of chromatin processes. Notably, we will focus on the current knowledge regarding how ncRNAs and processing factors may help to regulate transcription, along with the spatial organization of chromatin, and histone Post-Translational Modifications (PTMs).

2. Part I. Birth, life and death of PROMPTs and eRNAs

2.1. Transcription at regulatory regions and its diversity: PROMPTs and eRNAs

eRNAs were first described by de Santa et al. [11] as a widespread bi-directional transcription at active enhancers, a notion that was generalised to all active enhancers and in all cell types, in vertebrates and

* Corresponding authors.
E-mail addresses: olivier.fosseprez@pasteur.fr (O. Fosseprez), olivier.cuvier@inserm.fr (O. Cuvier).

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Abbreviations

ncRNAs	non-coding RNAs
eRNAs	enhancer RNAs
PROMPTs	Promoter Upstream transcripts
RNAPII	RNA Polymerase II

invertebrates. eRNAs were further characterised as mostly short, rarely spliced, 5'capped but non-polyadenylated, and of low-abundance RNAs (Fig. 1A, B) [12–14]. Moreover, a global correlation was demonstrated between the level of eRNA expression and the level of expression of the genes they regulate [12]. Interestingly, a non-negligible fraction of eRNAs tend to be longer, poly-adenylated and more stable, resulting in a bias towards one strand that produces more steady-state transcripts compared to the other strand (Fig. 1B) [15–17]. This bias may reveal that, although eRNAs do not appear to have a conserved sequence, Transcription Start Sites (TSSs) of enhancers may be subject to evolutionary selection to favour accumulation of a specific eRNAs [16,18,19]. In myogenesis, "bi-stable" eRNAs were detected, i.e. where both strands of the enhancer produced more stable eRNAs [20]. Such a heterogeneity in eRNAs may highlight different properties and functions for each subset. For example, enhancers producing longer and more stable eRNAs tend to have higher H3K4me3 deposition, as well as more CCCTC-binding factor (CCTF), transcription factors (TFs) and RNA Pol II binding, which can be speculated to underline different consequences or roles for these enhancers [9,16]. Also, to what extend distinct families of eRNAs can be distinguished according to their transcription levels or to their stability remains to be clarified. In turn, the effects of the various kinds of eRNAs may actually be different depending on their stability, as further discussed below.

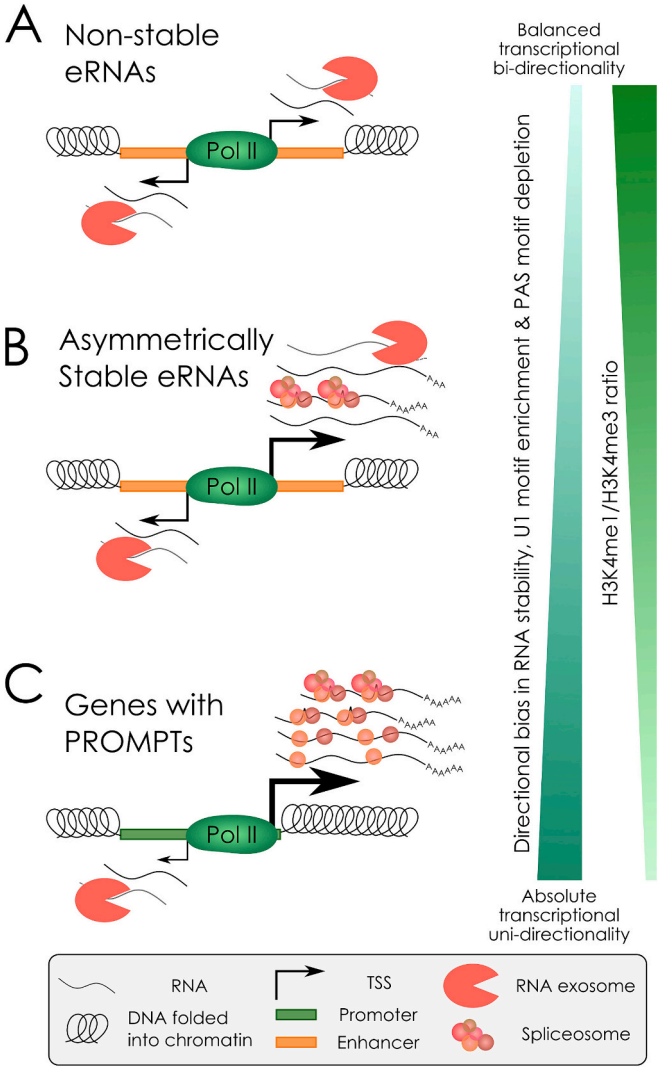

Fig. 1. eRNAs and PROMPTs have specific properties & transcriptional contexts Schemes representing the bi-directional transcription of eRNAs & PROMPTs, their properties and genomic contexts. Non-stable eRNAs (A), like PROMPTs (C), have low steady states levels due to their fast degradation via the exosome, while non-canonical asymmetrically stable eRNAs (B) can have longer half-lives, be spliced and accumulate. The gradients on the right express the gradual increase in directionally-biased transcription, RNA stability, U1 splicing motif enrichment, and PolyAdenylation Signal (PAS) depletion, and the opposite bias in H3K4me1 over H3K4me3 ratio.

PROMPTs, also referred to as Upstream Antisense transcripts (uaRNAs) or Promoter Antisense transcripts (PAS RNAs), were first described as a widely distributed divergent transcription at promoters [21–23], similarly to eRNAs. They are transcribed on the promoters of genes, on the opposite strand and direction from the mRNA (Fig. 1C). The fundamental difference between eRNAs and PROMPTs is still blurry however, as they share the same properties of early termination, low stability and splicing, as well as relatively few polyadenylations [14,24–26]. In fact, this is reminiscent of the old debate of distinguishing promoters and enhancers, as both can initiate transcription. Moreover, promoter can participate to the activation of other, distant genes, in the same manner as enhancers [27–29]. This advocates for a common property of transcription initiating regions to be intrinsically bi-directional which may have then been selected to asymmetrically favour the transcription of one transcript over the other. Such asymmetry may also result from the asymmetric maturation and stability of eRNAs [19,25,30,119], giving rise to "asymmetrically stable eRNAs" (Fig. 1B). Although the stability and length of PROMPTs have been associated with the proximity of additional nearby promoters [25], there is currently no sub-class known to be highly stable, as seen for certain eRNAs. As such, the potential effects of such short-lived PROMPTs onto transcription may be limited, as opposed to eRNAs with higher stability and steady-state.

Of note, the matrices of both eRNAs and PROMPTs appear to be depleted in U1 motif, while enriched in Poly(A) Sites motifs compared to mRNAs, in agreement with their low splicing and their higher rate of early transcriptional termination, as well as their higher Exosome degradation (Fig. 1) [24,31]. The global epigenetic marking of eRNAs and PROMPTs is also similar, with deposition of H3K4me1 and H3K4me3 next to the Nucleosome Depleted Regions (NDR). However, the ratio between those marks may be different, as PROMPTs templates exhibit low H3K4me1/H3K4me3 ratios, similar to gene promoters, whereas that of eRNAs display higher H3K4me1/H3K4me3 ratios, similar to enhancers. In the case of PROMPTs, it was demonstrated by [32] in Yeast that the deacetylase Rpd3S helps prevent their transcription. On the other hand, the Integrator complex influences RNA Pol II pause release and transcriptional termination [121], and as such, Integrator plays a central role in eRNAs biogenesis by enabling their termination [33]. In the same vein, the ubiquitous Pol II-associated factor SPT6 was shown in Human cells to regulate ncRNA transcription, including both PROMPTs and eRNAs, by ensuring correct H3K36me3 deposition only on coding genes, as well as enabling the recruitment of the Integrator complex to chromatin to favour early termination of ncRNAs [34]. DNA-RNA hybrids (R-loops) were shown to promote the transcription of ncRNAs, both at promoters and enhancers [34–36]. Conversely, the production of eRNAs and PROMPTs may in turn favour the formation of R-loops, as eRNAs and PROMPTs are able to form such structures [8]. Still, most of the possible impacts, functions and even molecular events accounting for differences in the regulations -production or stability- of ncRNAs are yet to be explored.

2.2. PROMPTs & eRNAs targeted degradation by the exosome

When first described, both eRNAs and PROMPTs were suggested to be simple by-products of transcription due to their high turnover and low steady-state level. Both are degraded by the nuclear Exosome machinery, usually shortly after transcription, which explains their very low accumulation levels in conventional RNAseq data. However, the Exosome itself has poor specificity and cannot process RNA secondary structures [37]. To bring specificity, the Nuclear EXosome Targeting complex (NEXT) and the Poly(A) eXosome Targeting complex (PAXT) are the bearers of binding and helicase properties [38–40], even though the exact way by which they target specific RNAs is yet to be determined. The NEXT complex has been demonstrated as being necessary to enable the proper degradation of most PROMPTs and eRNAs, while the PAXT complex tends to target longer, processed and poly-adenylated targets [39–41]. Studies on the secondary structures of ncRNAs are scarce, and the requirement for the NEXT complex to enable their degradation may be due to the formation of secondary structures, such as hairpins. The RNA helicase Mtr4 in particular, present in both PAXT and NEXT, is necessary for the degradation of both PROMPTs and eRNAs, but also of some introns, which may again highlight the formation of secondary structures to be unfolded by this RNA helicase [38–40].

Of interest, it is yet to be understood whether this degradation of PROMPTs and eRNAs can be regulated, or if it is a simple "all you can degrade" mission for the exosome and its targeting complexes. In fact, the studies performed to date were mostly limited to the observation of accumulations of ncRNAs upon degradation of either an Exosome component or a targeting complex factor [37–40]. In the hypothesis of a function for eRNAs and PROMPTs, it would make sense indeed for the degradation of these ncRNAs to be dynamically regulated or even locally prevented, so as to enable their accumulation and enhance their effect in precise contexts. Interestingly, two contradicting studies recently reported that m^6A methylations of eRNAs and PROMPTs appear to either protect them against early termination and degradation [42], or facilitate their degradation via YTHDC1 binding [43], revealing an unexpected layer of regulation that may be context dependent. Supporting this view, the recently published structure of the NEXT complex has

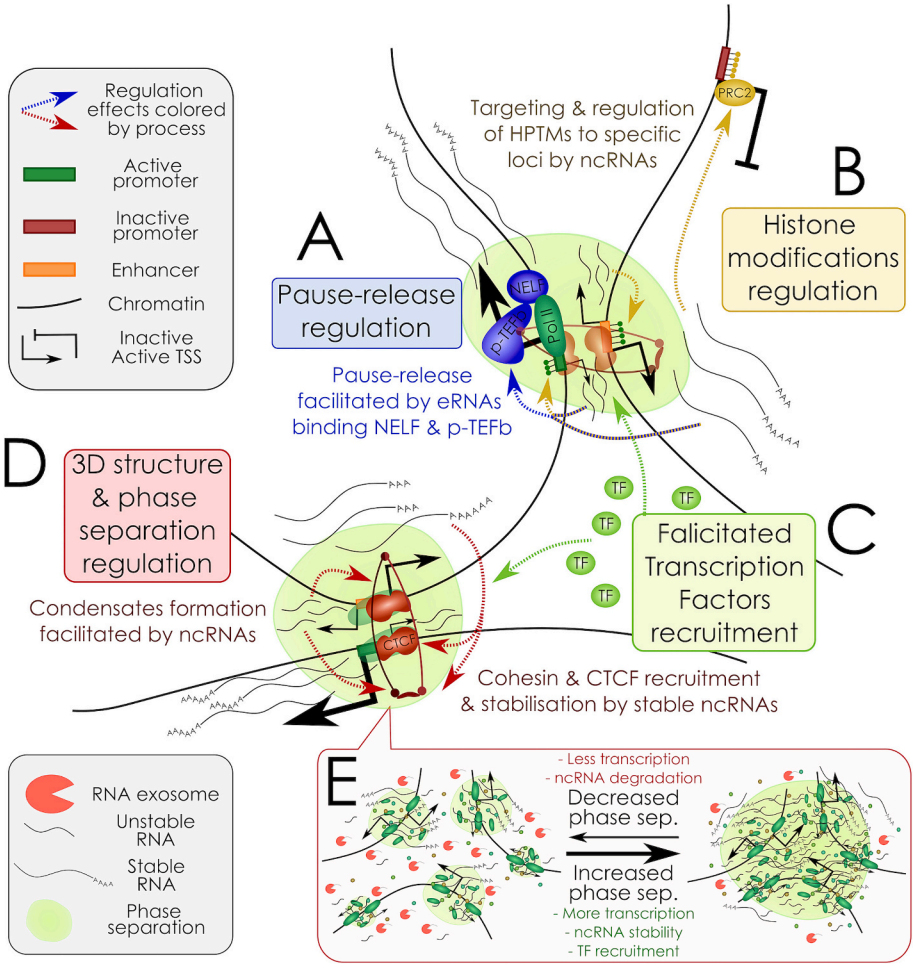

Fig. 2. Non-coding RNAs regulate transcription, chromatin & genome 3D organization. Schematic summarizing some of the roles identified for eRNAs & PROMPTs to date. The upper and lower parts represent distinct types of factors that can associate with 3D clusters of genes for the same genomic context. (A) Color-coded in blue, exemplifies the regulation of Pause-release by eRNAs & PROMPTs, notably through inhibition of the pausing factor NELF, and the activation of the p-TEFb kinase. (B) Color-coded in yellow, showcase of the contexts in which stable and unstable ncRNAs may facilitate the regulation of histone PTMs deposition, such as for H3K27me3 at silenced genes, or H3K4me3 and histone acetylation at promoters & enhancers. (C) Color-coded in green, Transcription Factors recruitment to promoters & enhancers is facilitated & targeted by binding to various eRNAs. (D) Color-coded in red, formation of chromatin loops & phase separated condensates are regulated by stable and unstable PROMPTs & eRNAs to favour gene regulation and transcription. (E) Model of the formation and dynamics of phase-separated transcriptional condensates. The main actors of phase separation in this context are the abundance of RNAs including ncRNAs, and of RNA binding proteins (RBPs) such as transcription factors or CTCF and cohesin. As such, an increase in transcription or in RNA stability, including of ncRNAs, will facilitate condensates formation, whereas a decrease of RNA production or an increase of RNA degradation will challenge formation of condensates. Importantly, this model is not linear, as an excessive concentration of negatively charged RNAs, will also cause the condensates to crumble.

revealed regulatory configurations preventing processing of the bound RNA, especially via the ZCCHC8 subunit [38]. Furthermore, the NEXT complex interacts with various factors outside of the Exosome, notably via ZC3H18 [44], which leaves ample space to elucidate the molecular mechanisms by which different classes of ncRNAs are recognized and targeted for degradation by the Exosome. The PAXT complex also interacts with the PolyA polymerase gamma (PAPγ) [41], which in turn regulates the polyadenylation of ncRNAs, involving the ability of PAXT to favour the recruitment of PAPγ. Such interactions may in turn regulate both polyadenylation and degradation of mRNAs and PROMPTs, though the specificity of the targeting is unclear.

3. Part 2. A life worth living: PROMPTs' and eRNAs' roles in functional mechanisms

The aforementioned assumption that eRNAs and PROMPTs are useless transcriptional by-products, based on their rapid degradation, has been overturned by the discovery of regulatory roles, particularly for certain eRNAs. Notably, studies have suggested roles for these non-coding RNAs in Pol II Pause release, Histone PTMs deposition, TFs recruitment and 3D genome organization (Fig. 2). However, the question of the role of the RNAs per se, or of their transcription, was raised. As such, transcription of the enhancer may function by maintaining the enhancer accessible, and in this instance, only transcription may be really necessary, while eRNAs can be targeted for degradation. Alternatively, eRNAs or PROMPTs might act locally, before their degradation. Finally, more stable ncRNAs have been suggested to regulate the pausing of RNA Pol II or even the organization of chromatin in 3D, possibly through their ability to stabilize the binding of RNA-binding proteins. Thus, stable ncRNAs could possibly act in *trans,* i.e. further away from their loci of transcription. As many ncRNAs are predicted to have secondary structures, it is also plausible that their structure, rather than their sequences, is necessary to mediate their roles [6]. For a more detailed review of the existing links between eRNAs structures and their functions, see Harrison & Bose [122]. In this section, we discuss the various mechanism in which ncRNAs have been attributed functions, while considering the features cited above, i.e. the stability of ncRNAs, their spatial and temporal localization, and their sequence or structure.

3.1. ncRNAs direct influence on Pol II transcription

Various studies have demonstrated that ncRNAs can regulate gene expression possibly at distinct stages of transcription. In particular, certain eRNAs were shown to participate to the regulation of their enhancers' target genes. Most of these studies were based on approaches such as depletions, or more sporadically accumulations of target eRNAs, to demonstrate a deregulation of the gene of interest (i.e. not necessarily local) or of the genes in the vicinity of the deregulated enhancer [45–53]. In 2013, Melo et al. [54] performed a Gal4-MS2 targeting of specific exogenously transcribed eRNAs to a promoter to demonstrate their capacity to enhance gene expression, though the precise mechanism by which these eRNAs could impact gene regulation remains unclear. Since then, a CRISPR-dCas9-based method was developed to target ncRNAs [55–58], revealing variable influence in the regulation of enhancers activity. Another study also observed that RNA-exosome-regulated ncRNAs, i.e. mostly PROMPTs and eRNAs, were influential in the regulation of super-enhancers activity [35]. Finally, hnRNPL, a protein involved in RNA alternative splicing and transcriptional regulation has been shown by several reports to bind eRNAs to regulate gene expression levels. It may do so at several steps of the transcription process, as one of these reports observed repressing effect of the ncRNA-hnRNPL interaction, while the two others found up-regulating effects [20,59,60].

Interestingly, the structures of ncRNAs may participate in their regulatory functions, particularly to provide specificity to proteins binding. Although poorly investigated in the context of eRNAs and PROMPTs compared to other functional ncRNAs (such as *7SK*, tRNAs and ribosomal RNAs), this hypothesis agrees with the observed lack of sequence conservation among most eRNAs and PROMPTs, and especially for the less stable ones. Yet only few eRNAs/PROMPTs structures have been experimentally characterised [55,58,61,62], although many have been predicted to be spatially folded [6]. In a few cases however, the structures of eRNAs and PROMPTs have been directly associated with their role [58,62]. In the case of ^{DDR}eRNA in myogenesis for example, it is striking to see different domains, and their respective unpredicted but experimentally confirmed structures, regulate separate sets of genes, through different mechanisms [62].

Aside of these rather general association of gene regulation with modifications of ncRNA levels, certain eRNAs were observed to directly impact Pol II pausing (Fig. 2A). They do so by acting as decoys and/or destabiliser for the Negative Elongation Factor (NELF), hence facilitating pause release into productive elongation [61,63,64]. This action was demonstrated in vitro to be mediated through direct interactions between eRNAs and NELF-A/E subunits, yet with no effects of structures nor sequences, needing only a certain length of RNA (>200 bases) and unpaired guanosines to be effective [61]. This argues for a role of the RNA themselves, regardless of motif recognition [61], in tune with the previous demonstration that NELF-E binds a large range of RNA with no sequence or structure preference [65]. In the same vein, the pause-release kinase p-TEFb was shown to be bound and activated by an eRNA, thus promoting RNA Pol II phosphorylation and productive elongation. In this case, the structure-motif of the eRNA was shown to be influential, as it substitutes the well characterised p-TEFb inhibitor RNA *7SK* [66]. It remains to be determined whether these mechanisms can be generalised to all eRNAs, as suggested for NELF [61]. Nonetheless, it is clear that at least eRNAs and maybe other ncRNAs have regulatory roles on Pol II pause release and gene expression, both dependent and independent on their structures-motif.

The accumulation of ncRNAs has also been reported to participate in the formation of transcriptional hubs, which may in turn favour or regulate transcription at several stages. Notably, transcription initiation has been proposed to be substantially impacted by the concentration of ncRNAs in the transcriptional condensates [67,68]. In effect, transcriptional condensates are formed mainly via the Intrinsically Disordered Regions (IDR) of TFs, via the global process of phase separation (Fig. 2A, E) [69–73]. Interestingly, these IDRs are canonical RNA-binding domains in many proteins involved in transcriptional condensate formation [74]. In 2021, Henninger et al. proposed a model, where the synthesis of relatively small and quickly degraded eRNAs and PROMPTs would, at low concentrations, facilitate the formation and efficiency of the initial transcriptional condensates (Fig. 2A, E). Upon induction of the transcriptional burst however, the massive accumulation of mRNAs would then concentrate too many negative charges, making the phase crumble on itself, and thus terminating the period of hyper-efficient transcription. This model was actually supported by other studies [68,75–77], and it provides a brilliantly simple mechanistic framework to explain transcriptional bursts (see [78] for a more extensive discussion of Transcriptional Bursting). Still, a great number of additional transcription factors may be involved, and additional parameters from alternative transcriptional models also suggested how separate phases may correspond to initiation and productive elongation (see [79]). Also, the former model was challenged by quantitative live-imaging and super-resolution that argue for enhancer transcription to antagonize molecular crowding of transcription factors, suppressing transcriptional bursting of the linked genes, while certain developmental enhancers may be structurally optimized to support both coding and non-coding transcriptional activation [80]. They argue that enhancer function would be tuneable through non-coding transcription and hub formation to perform gene regulation. Importantly, this study uses synthetic constructions where the effect of non-coding transcription is to displace TFs bound at the enhancer, perhaps explaining the contradicting effect observed, which may reflect the configurations of a few natural – and

mostly developmental – enhancers. Also, distinguishing the effects of non-coding transcription versus accumulation of ncRNAs may help understanding such paradox.

Interestingly, the m^6A modification of eRNAs has been advanced as potentiating the formation of transcriptional condensates through its phase-separating reader YTHDC1, in turn activating gene expression [81]. It remains to be investigated whether this effect is directly exerted by the m^6A modification, for which the eRNAs would be mere carriers, or if it is really the eRNAs that are effective, in which case the m^6A modification may play its part in regulating the stability of the eRNAs [42,43]. A global analysis by Quinodoz et al. [82] based on the development of the RD-SPRITE technique, enabling the simultaneous observation of RNA and DNA spatial repartition genome-wide, allowed to propose a general model whereby ncRNAs globally act as seeds and recruiters of various protein factors to form specific and localised genome 3D structures. Such ncRNA-driven clustering in 3D may then regulate and shape gene expression, DNA contacts, but also heterochromatin assembly. The great asset of this model, as well as that of the rather complementary transcriptional condensate model exposed above, is that they finally provide a general rule and framework to understand how ncRNAs can influence gene transcription.

3.2. ncRNAs influence on chromatin regulation through histone PTMs

Alternative mechanisms by which ncRNAs may regulate genes have been proposed, notably through various indirect changes in chromatin, either at the level of nucleosome occupancy or histone Post-Translational Modifications (PTMs), or at the level of transcription factor recruitment (Fig. 2B,C). This notably includes the extensively studied Xist ncRNA, which mediates X-chromosome Inactivation in Mammals by triggering gene silencing and structural reorganization through the recruitment of a vast array of chromatin modifiers (reviewed by [83]), which will not be further reviewed here. Of note, what had been demonstrated for the X chromosome inactivation, deserve consideration to test whether it also stands in the less-studied roles and multitude of other ncRNAs.

In the specific case of enhancers, it was demonstrated that transcription of the enhancer, independently of eRNA, is what precedes and facilitates H3K4 methylation at enhancers, independently of the eRNA stability [84]. Interestingly, this argues that eRNA transcription is not a simple consequence of enhancer function, but rather is necessary to enable the activity of certain enhancers. Accordingly, the transcription of a distal enhancer, and particularly one of the eRNAs produced, was shown to influence the modifications of several histone PTMs at both enhancers and promoters (Fig. 2B) [85]. In this instance, a simple knockdown of only one strand of the bi-directional eRNA is sufficient to cause a loss of H3K27ac at the enhancer and replacement by H3K27me3, coinciding with H3K4me3 loss at the promoter. Of note, the authors used an experimental strategy acting solely post-transcriptionally, hence arguing against a direct role of transcription, but rather by the ncRNAs themselves.

ncRNAs further antagonize H3K27me3 deposition by PRC2, even though PRC2-RNA interactions are necessary for proper targeting of the silencing complex to chromatin. In this instance, JARID2, a specific subunit of PRC2, may timely inhibit RNA-binding when necessary, in order to enable PRC2 catalytic activity [86–89]. Although most of these studies do not distinguish different RNA species, at least three studies demonstrate that specific ncRNAs, localised away from their target genes, also mediate their regulatory role through the PRC2-RNA interaction mechanism [90–92]. This suggests a model in which many, if not all ncRNAs would participate in regulating H3K27me3 deposition to setup and maintain transcriptional programs (Fig. 2B). In another illustration of histone PTMs being regulated by ncRNAs, PROMPTs have also recently been shown to stabilize the binding to promoters of the demethylases of H3K9me3, KDM4B and KDM4C, thereby regulating transcription of numerous genes [58]. Similar to histone methylation, acetylation has also been shown to be regulated by eRNAs. The Histone AcetylTranferase (HAT) complex CBP/p300 binds locally-transcribed eRNAs directly, targeting and stimulating histone acetylation at enhancer, and hence transcription of target genes (Fig. 2B) [55,93,94]. This mechanism is further supported by the eRNA-binding property of BRD4, a Bromodomain-containing protein that binds to histone acetylation to enhance transcription. This direct eRNA binding again increases BRD4 binding to acetylated histones and thus its recruitment to enhancers to function as a transcriptional cofactor (Fig. 2C) [95]. Taken together, a number of studies have therefore highlighted key aspects of the regulation by which eRNAs or their transcription empower transcriptional regulation, notably by snowballing the deposition of several histone PTMs favourable to transcription, but also by prohibiting deposition of repressive marks, thus making chromatin more permissive.

3.3. eRNAs and PROMPTs impact chromatin by recruiting TFs and forming R-loops

ncRNAs may also control transcription through the recruitment of various transcription factors and cofactors. Several Bromodomains-containing factors, analogous to BRD4 as cited above, have been shown to bind eRNAs, even though a complete description of the genes they may regulate through ncRNA binding is still lacking [95]. The ncRNA ARIEL has also been shown to enhance the activation of an oncogenic transcriptional program by a specific TF named TAL1, through the recruitment of the Mediator complex [96]. Similarly, other oncogenic TF, such as c-JUN [64] and NF-κB [97,98], were documented to depend on eRNAs for their recruitment to regulate gene expression. Finally, Sigova et al. demonstrated in 2015 that the ubiquitously expressed YY1 is also affected by eRNAs, as they facilitate its recruitment and retention on chromatin at enhancers (Fig. 2C). Surprisingly however, general accumulation of eRNAs and PROMPTs through exosome depletion caused a global decrease of YY1 at enhancers. The authors interpret this result as a titration of YY1 by the excessive accumulation of eRNAs, keeping too many YY1 molecules away from chromatin and with no possibility to bind anything but the eRNAs. Clearly, YY1 has been involved in 3D clustering of distant sites, and the interplay between YY1 and eRNAs may also be complicated by the saturating levels of eRNAs.

Complementary to these mechanisms, the R-loops formed by eRNAs and PROMPTs can enhance transcription by serving as promoters through the opening of the DNA helix [36]. Importantly, R-loops are DNA-RNA hybrids which have been extensively studied in recent years, and were shown to be highly regulated, and to have ambivalent roles both in causing genomic instability, as well as regulating important gene regulation and chromatin accessibility (for a more extensive review, see [99]). Of note, the exact mechanisms afoot to regulate ncRNA-mediated formation of R-loops are still under investigation. Aside from this direct regulation of transcription initiation, the ncRNA named KHPS1 may control, via the formation of R-loops, various chromatin regulators such as CTCF, p300 and an eRNA, to target the regulation of a gene, SPHK1 [100]. This rather complex mechanism highlights the possibility for ncRNAs to form DNA-RNA hybrids as a mean of targeting to specific regions, as will be discussed in depth in the next section regarding spatial genome regulation by ncRNAs. Cooperatively, it was suggested that m^6A modifications of a subset of eRNAs and PROMPTs forming R-loop might participate in their targeting to degradation via YTHDC1 [43]. This modification would in turn inhibit TFs recruitment and histone PTM deposition, allowing for the abrogation of the otherwise self-sustaining mechanism of transcription activation.

To conclude, substantial evidence demonstrates that ncRNAs enable the setup and enforcement of transcriptional programs by regulating chromatin. In particular, the deposition of histone PTMs and the recruitment of TFs facilitated by eRNAs is influential to regulate various genes and processes. However, it is still unclear in most cases whether these observations can be generalised, or if ncRNAs act in a context

specific manner to regulate limited numbers of genes. As such, it will be of great interest to develop an exhaustive view of the transcription factors and histone modifiers that necessitate or are regulated by the repertoire of ncRNAs.

3.4. ncRNAs influence on 3D genome organization

The 3D structure of the genome has substantial impacts on various cellular processes, and in particular gene expression. The regulation of enhancer-promoters contacts is notably influential. The current model is that a loop-extrusion mechanism mediated by the Cohesin ring enables most contacts to happen, while certain contacts are enriched due to the formation of domain borders (mainly by CTCF in Mammals), more rarely due to random collisions. Domain borders stop or slow loops extrusion to limit spurious enhancer-promoter contacts between two contiguous Topologically associating Domains (TADs). Upon certain interactions, DNA looping between a regulatory element and its "promoter target" are favoured through the stabilization of Cohesin and/or other factors. For a better understanding of the intricacies of genome 3D architecture and the various factors involved, see e.g. [101].

ncRNAs have been involved in regulating this spatial architecture of the genome by binding and recruiting various architectural factors (Fig. 2D), also involving transcriptional condensates (Fig. 2E). Cohesin was first shown to bind several eRNAs in 2013 by Li et al., with implications in gene regulation by promoting Cohesin binding at enhancers. This finding was later supported by studies on the previously mentioned ^{DDR}eRNA, which interacts with the Cohesin complex and is required for its proper loading and activity to regulate the *Myogenin* gene [62,102]. Another biochemical investigation also demonstrated that the SA1 and SA2 subunits of Cohesin directly bind not only single stranded RNA (ssRNA), but also double stranded RNA (dsRNA) and RNA:DNA hybrids, opening the door to many potential effects of ncRNA transcripts on Cohesin recruitment and its mediation of 3D interactions [103]. YY1 was actually shown to enable the formation of certain *E*-P interactions by anchoring Cohesin in an eRNA-binding dependent manner, because it is regulated by eRNA interactions, as detailed in the previous section [57,104]. Similarly, Mediator binding to ncRNAs was demonstrated to promote chromatin looping [49]. Given the ongoing debate regarding Mediator's role in chromatin looping [105,106], we may speculate that the RNA-Mediator interaction effect on chromatin looping also involves Cohesin. In the same vein, the observed modification in chromatin looping stabilized by eRNAs in the regulation of Nanog and Dppa3 by super-enhancers could possibly involve Cohesin [107].

The other major regulator of chromatin 3D organization in vertebrates, CTCF, has also been shown in Human and Mouse to bind RNAs. First reported in a case study with the SRA ncRNA, the RNA-binding property of CTCF was then proposed as necessary for its insulating activity at a few loci [108]. The RNA-binding regions of CTCF were later identified, and demonstrated to bind a large variety of transcripts, including ncRNAs, with surprisingly no particular sequence or motif preference [109–111]. Even more intriguing, although CTCF is well known to be required for virtually all TAD borders and loops in vertebrates, the deletion of its RNA Binding Regions (RBR) impacted only about half of all loops and TADs mediated by CTCF [109]. This result is mirrored by the specific disruption of CTCF binding to DNA only in certain loci that depend on the deleted RBR [109,111]. Two classes of loops could even be distinguished, between RBR dependent loops that are abolished upon RBR deletion, and RBR independent loops that are not affected. A new report from Harris et al. [112] has shown using ultra-deep sequencing that the RBR of CTCF may actually distinguish different types of loops. Hence, the more diffusive loops would depend on CTCF's RBR, while the punctuate loops remain unaffected, perhaps due to different mechanisms behind their formation. The model proposed for the influence of RNA on CTCF is that RNAs would facilitate the oligomerization and clustering of CTCF, hence maintaining loops, perhaps somewhat independently from Cohesin [109,111]. Yet a proper demonstration of this speculative model remains to be generated, as it is still obscure how exactly RNA binding by CTCF would cause or favour loop formation in some cases and not others. The lack of specific sequence motif is just as puzzling, as the current understanding would mean that any RNA can be bound by CTCF, making it conceptually hard to appreciate context specificities. The potential of RNA structures mentioned above may bring a first layer of specificity but remains to be demonstrated as impacting RNA-CTCF interactions. RNA-based condensates may form depending on RNA binding proteins (RBP) that set up multivalent interactions with easily tuneable concentrations of RNAs, including ncRNAs. As such, production or stabilization of RNAs may lead to highly concentrated condensates of RNAs and factors that do not respond to equilibrium dynamics with the outside environment, thereby defining the bona fide conditions for phase separation (Fig. 2D, E) [69–73,113]. Yet another layer of comprehension may be derived from two studies of HOTTIP, a ncRNA involved in the setup of the leukemic transcription profile [114,115], as well as two studies of Jpx, a ncRNA involved in Xist expression for X-chromosome inactivation [116,117]. These studies show that these two ncRNAs are targeted widely in the genome, via the formation of sequence specific R-loops in the case of HOTTIP, to regulate a large transcriptional program. To mediate this regulation, HOTTIP seemingly helps to recruit CTCF and Cohesin to form TAD boundaries where it forms R-loops, in a sort of targeted enhancement CTCF boundary formation [115]. These findings, if generalised to other ncRNAs, could mean that CTCF does not need to bind only free RNAs, but rather chromatin-tethered RNAs that would retain or empower CTCF at certain loci, hence the specificity. Oppositely, Jpx seem to act as a competitor for CTCF binding, displacing it from low-affinity sites, thus favouring the formation of other loops and possibly TAD frontiers, although a complete description of the effects of Jpx on TADs and Cohesin recruitment remains to be produced. Regarding ncRNAs, their formation of R-loops and their binding to chromatin proteins may thus be shown influential to regulate CTCF architectural purposes in precise contexts.

In conclusion, although comprehensive models are still lacking to explain how architectural proteins are impacted by ncRNAs, it is clear that such a layer of regulation does exist in Mammals. It is however surprising that all of the studies to date are limited to Human and Mouse models. In fact, a very limited number of studies analysed the effects of ncRNAs on architectural proteins present in Drosophila and Yeast, two widely used models for the study of 3D genome organization. Yet an original study performed in Drosophila by Lei and Corces in 2006 highlighted an RNA-dependent interaction between the insulator protein CP190 and an RNA helicase, Rm62, though the potential role of RNA remains to be shown. A second study tackle how mRNAs may participate in the formation of insulator complexes in Drosophila, reporting the interaction of CP190 and Su(Hw) with RNAs [118,120]. To summarize, the potential effects of ncRNAs on 3D genome organization in these organisms are still vastly unexplored, and left to be speculated from observations made in Mammals, which is an error-prone task in regards of the poor homology of architectural factors between Mammals and Invertebrates.

4. Concluding remarks

Altogether, the various genome-wide and loci-specific data produced to date argue for both promiscuous, local effect of ncRNAs and their transcription, as well as more long-range effects of specific and more stable ncRNAs. Even though most studies do not examine the stability versus transcription of the ncRNAs, complicating interpretations as to whether they exert their effect locally or distantly. Importantly, the myriad of mechanisms through which ncRNAs impact chromatin, 3D genome organization and transcription support a general and prevalent role for these sometimes-rare ncRNAs, albeit not in all contexts nor by all ncRNAs. It may be expected that the diversity of mechanisms in which eRNAs act, for example, is comparable to the variety in enhancers and

transcription factors bound to them. Clearly, a comprehensive model of how ncRNAs impact genome regulation is yet to be achieved, even though interesting models have been proposed. The development of new methods to study ncRNAs in their native contexts while decoupling their effects from that of their transcription will be a major challenge and prospect to understand the multitude of ncRNAs functions. Eventually, a better understanding of these mechanisms may permit to harness the regulatory potential of ncRNAs in a variety of therapeutic contexts.

Author statement

The authors declare that all procedures related to this manuscript were performed in compliance with relevant laws and institutional guidelines.

CRediT authorship contribution statement

Olivier Fosseprez: Writing – review & editing. **Olivier Cuvier:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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