

Lessons from eRNAs: Understanding transcriptional regulation through the lens of nascent RNAs

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Nascent transcription assays, such as global run-on sequencing (GRO-seq) and precision run-on sequencing (PRO-seq), have uncovered a myriad of unstable RNAs being actively produced from numerous sites genome-wide. These transcripts provide a more complete and immediate picture of the impact of regulatory events. Transcription factors recruit RNA polymerase II, effectively initiating the process of transcription; repressors inhibit polymerase recruitment. Efficiency of recruitment is dictated by sequence elements in and around the RNA polymerase loading zone. A combination of sequence elements and RNA binding proteins subsequently influence the ultimate stability of the resulting transcript. Some of these transcripts are capable of providing feedback on the process, influencing subsequent transcription. By monitoring RNA polymerase activity, nascent assays provide insights into every step of the regulated process of transcription.

Keywords: RNA polymerase II, enhancers, nascent RNA, gene expression, transcription feedback circuit, non-coding RNA function, chromatin structure.

1. Introduction

Transcription varies from cell type to cell type, unlocking the information held within DNA. Understanding transcriptional regulation and the factors that control it is a grand challenge. Despite the many difficulties of deconstructing the biological rules of transcriptional regulation, the importance of this topic cannot be overstated, as most disease associated variation is noncoding and likely regulatory [1, 2]. In fact, a startling 60-76.5% of disease associated single nucleotide polymorphisms (SNPs) are in enhancers, the major regulatory domains of DNA [1–4]. Enhancers are short regulatory regions densely bound by transcription factors [5–7].

Transcription factors (TFs) are the managers of the cellular factory, controlling everything from cellular identity to response to external stimuli [8]. Fundamentally, TFs are proteins that bind to specific DNA sequences and regulate the transcription apparatus. Many high throughput genomics assays have focused on evaluating DNA-protein interactions of TFs, e.g. binding. The workhorse of binding studies is chromatin immunoprecipitation (ChIP), which requires antibodies to the specific TF of interest [9–11]. ChIP experiments are useful in that they can allow researchers to obtain the genomic sequences bound by a particular TF in a specific cellular condition or state. Over the last two decades, ChIP studies have been the backbone of large scale genomics projects such as ENCODE and the Roadmap Epigenomics Project [12, 13]. In general, ChIP studies revealed that most TFs display widespread binding across the genome; though many binding sites may be nonfunctional or spurious [14, 15]. Despite the noise, ChIP data, along with other related assays, has been utilized to characterize the DNA recognition motif preferentially bound by a particular TF [9, 11].

ChIP data informs on protein-DNA interactions but does not provide information on the regulatory function of the TF binding events. To understand the impact of binding on transcription, typically RNA-seq or another steady state RNA detection method is employed. This coupling of expression analysis with ChIP-seq, is preferably carried out in the presence of a TF perturbation, to identify bona fide functional TF binding sites. In some cases, a TF can be rapidly activated by a small molecule, compound, or specific condition. Alternatively a TF can be removed via knockdown or knockout. Unfortunately, it is inherently difficult to distinguish primary from secondary effects of the perturbation on the system if the perturbation itself is not abrupt. The detection of significant RNA changes immediately after perturbation requires either a large deviation in transcript steady state levels (which typically takes time to obtain) or excessive numbers of replicates (which is cost prohibitive [16]). Thus the steady state approach is incapable of reliably detecting small changes at short time points.

In general, the major complication in using expression data is that expression studies are a poor readout on the transcriptional apparatus. RNA-seq, the typical approach to expression studies, is

biased towards the detection of stable transcripts. Hence rapidly degraded RNAs are virtually undetectable. Most critically, steady state expression assays reflect not only transcription but also RNA processing, maturation and stability. So even when a change is detected in RNA-seq, it is unclear whether the change reflects alterations in transcription or transcript stability. Arguably a preferred approach would focus on deciphering the activity and behavior of the key transcriptional machinery, namely cellular RNA polymerases.

In eukaryotic cells, three RNA polymerases are responsible for all nuclear transcription: RNA polymerase I, II, and III. RNA polymerase I and III transcribe primarily ribosomes and structural RNAs, respectively. Critically important for cell growth and replication, these RNA polymerases have relatively well defined promoters [17]. In contrast, RNA polymerase II is the most versatile of the three polymerases, responsible for transcribing all protein coding mRNAs and many ncRNAs. Perhaps a consequence of this versatility, RNA polymerase II interacts with a wide range of transcription factors to regulate the precise locations and levels of its activity. Consequently, most regulators of transcription are known to alter RNA polymerase II activity. Therefore we focus on RNA polymerase II regulation in this review.

Nascent transcription assays provide a more concise readout on the immediate activity of cellular RNA polymerases, e.g. transcription. Generally nascent RNA refers to all transcripts pre-maturation (protocols reviewed in [18]). While many protocols provide insights into transcripts prior to complete maturation, here we specifically refer to nascent protocols that target newborn RNAs; those just coming into existence or those in the process of being made. Some nascent methods (PRO-seq and GRO-seq) label the RNAs with a marked nucleotide via nuclear run-on and then isolate the labeled RNAs via selective precipitation [19, 20]. Other nascent methods select for newborn RNAs via precipitation of molecules that associate with nascent RNA such as polymerase or chromatin (mNET-seq, Chro-seq)[21–24]. In all cases, these methods are complementary to RNA polymerase ChIP but provide higher resolution and strand specific information. In contrast, other protocols are focused on recently synthesized RNA rather than newborn RNA. For example, SLAM-seq and Bru-Seq label live cells with marked nucleotides over extended time frames (hours)[25, 26]. RNAs transcribed within those hours are subjected to processing events such as splicing and 3' end cleavage. Additionally, RNAs with half lives shorter than hours are labeled and subsequently degraded. Here we focus on nascent protocols aimed specifically at newborn RNAs, as these approaches provide the most temporally immediate readout on the impact of regulators on RNA polymerase II.

We now know that nearly all RNA polymerase II loading and initiation sites produce one or more noncoding RNA transcripts, most of which are unstable [27]. These noncoding transcripts are called a variety of names, including long noncoding RNAs (lncRNA) [28], enhancer RNAs (eRNAs) [29], promoter upstream transcripts (PROMPTs) [30], upstream antisense RNAs

(uaRNAs) [31], transcription start site–associated RNAs (TSSa-RNAs) [32] and short-lived non-coding transcripts (SLiTs) [33]. They are classified according to length, stability, and origin relative to protein-coding genes, but the boundaries between the classes are often far from distinct [34–37]. It has been argued that the unstable fraction may simply be a side effect of the transcription process, e.g. noise [38–40]. The term ‘noise’ implies a certain degree of irrelevance; yet, the act of transcription itself can be critical to the regulation of the local genomic context (extensively reviewed in [41]). Furthermore, the mere presence of these transcripts, even when they are apparently non-functional and highly unstable, serve as markers of regulatory activity, and are hence informative about the regulation of RNA polymerase II.

2. Global characteristics of polymerase loading and transcription initiation

Decades of mechanistic studies (reviewed in [42]) have resulted in a well defined cycle of RNA polymerase II activity. Briefly, the first step in the process of transcription is recruitment of RNA polymerase II to the DNA. The pre-initiation complex positions RNA polymerase II at transcription start sites (TSS), adjacent to regulatory regions. This process of loading and initiation of RNA polymerase II was originally thought to occur proximal to promoters and be influenced by distal enhancers. Transcription factors modulate the efficiency of RNA polymerase II recruitment. After loading and formation of the pre-initiation complex (PIC), RNA polymerase II escapes the promoter, initiates RNA synthesis and subsequently pauses (reviewed in [32]). Pause release transitions RNA polymerase II into the elongation phase which gives rise to long pre-processed RNAs. Downstream of encountering a cleavage site, RNA polymerase II terminates transcription. A number of transcript processing steps are co-transcriptional and influence the final product: a mature transcript [43].

Transcription initiation of various RNAs involves the same RNA polymerase II machinery

Nascent transcription methods have widened the view of transcriptional regulation: hugely increasing the number of genomic loci where transcription initiation is known to occur. Moreover, the discovery of extensive genome-wide transcription led to the realization that transcription initiation occurs proximal to regulatory regions more generally. In K562 cells, an astonishing 72% of initiation sites are not promoter associated [44]. Initial inquiries into these nontraditional transcribed regions led to the exciting classification of new RNA subclasses such as PROMPTs and eRNAs [19, 34, 45, 46]. While the function of these transcripts remains hotly debated, it is undeniable that they enrich our understanding of RNA polymerase activity and transcriptional regulation. The emerging picture is of a consistent mechanism underlying RNA polymerase loading and initiation, regardless of the location of initiation or the stability of the resulting transcripts [27].

Basic steps in early transcription such as PIC recruitment and formation, transcription initiation, capping, and promoter proximal pausing appear to be universal at all transcription start sites,

regardless of the stability of the resulting transcript. ChIP studies on various components of the PIC as well as general TFs suggests a common RNA polymerase II machinery underlies bidirectional TSSs, including enhancer transcripts [27, 47, 48]. When the distance between the bidirectional initiation sites is sufficient, distinct Polymerase II, TBP and TFIIB peaks are observed, consistent with PIC formation occurring at two distinct TSS locations for each bidirectional pair [27, 47]. Subsequent to PIC formation and transcription initiation, there is evidence that eRNAs/PROMPTs are capped similarly to mRNAs [34, 49]. More surprisingly, given their relatively short final RNA length, recent studies suggest that in both *Drosophila* and mammalian cells, transcription of PROMPTs and eRNAs is regulated by pausing factors similarly to mRNA transcription [31, 50]. Both mRNAs and eRNAs show the well documented proximal pausing of RNA Polymerase II at the region 20 to 70 base pairs downstream of each TSS [50]. These studies provide further evidence that early stages of transcription of eRNAs and PROMPTs is similar to that of mRNAs and stable noncoding RNAs.

Additionally, Mediator may have a consistent role at both stable and unstable transcripts. Mediator is a large multi-subunit protein complex well established to regulate many vital steps in the process of transcription (reviewed in [51]). The Mediator complex is believed to facilitate PIC assembly, regulate promoter escape of Pol II, and play a role in transcription activation at stimulus responsive genes [51]. Importantly, Mediator may provide a functional bridge to connect DNA bound TFs to the PIC and Pol II (reviewed in [51]). Immunoprecipitation assays have demonstrated that Mediator complex associates with upstream activated sequences (UAS's) regardless of the transcription? level of the associated gene [52]. Mediator has also been localized to and observed to function at long noncoding RNAs and super-enhancers [53–55]. Finally, loss of functional mediator has been demonstrated to decrease the association of RNA polymerase II at essentially all transcribed genes (reviewed in [56]).

Transcription initiation is predominantly bidirectional

The recent use of nascent transcription assays and various 5' cap enrichment assays resulted in the discovery that mammalian transcription initiation is predominantly bidirectional, with two oppositely oriented distinct transcription start sites in close proximity [19, 27, 47, 57–61] (Figure 1). Early recognition of bidirectional transcription at protein coding genes led to the unstable upstream transcript being dubbed either a uaRNA or PROMPT [31, 47, 48, 62]. A large fraction of annotated protein coding genes, roughly 75%, have bidirectional transcription at their promoter [19, 22, 47]. Similarly, genomic regions that show evidence of transcriptional activity but do not produce stable transcripts in either direction, such as enhancer regions, were found to generally contain two opposing TSSs that produce two unstable transcripts termed enhancer RNAs or eRNAs [27, 48, 50, 61, 63]. Importantly, the distinction between enhancers and promoters has blurred with time [64, 65], as they share common underlying sequence patterns, RNA polymerase activity, and chromatin accessibility (Figure 1) [27, 57]. Genome wide, the

distance between observed bidirectional TSS pairs is quite variable, averaging close to 175 base pairs but going up to a few hundred base pairs [47]. Henceforth we refer to the region between the two bidirectional TSSs as the RNA polymerase loading zone.

[Figure 1 near here]

Recently, single cell sequencing and single molecule imaging techniques have been utilized to refine our understanding of transcription initiation. These methods assay individual cells, in contrast to the population and time averaged data of typical nascent protocols. Within a cell, transcription occurs in regulated bursts of RNA production from individual loci [66–71]. The burst size, or the number of RNA transcripts produced by a single transcriptional burst, is likely influenced by core promoter elements and gene length of a given loci; whereas, the identify of regulating enhancer regions may primarily regulate burst frequency [70–72]. Interestingly, the initial polymerase loading event may bias the direction of subsequent transcriptional events co-occurring within the same transcriptional burst [73]. Exclusive transcription from one TSS within a bidirectional pair may be enforced by the biophysical properties, such as torsional strain and steric hindrance, at the loci induced by the initial transcription event [74]. Thus the bidirectional signal observed from nascent transcription assays reflects averaging across the cellular population rather than simultaneous activity at a single cell level.

Polymerase loading occurs at regions with a general nucleotide bias

Figure 1 shows the genome wide nucleotide composition surrounding all bidirectional sites of RNA polymerase loading and initiation [44]. Notably, some GC bias is present at nearly all sites of RNA polymerase II loading, regardless of the transcription level or stability of the resulting transcript. The GC bias of mRNA promoters is well studied and provides some insights into how nucleotide composition influences transcription [75, 76]. Low GC content regions tend to favor closed chromatin [77, 78]. In contrast, genes that are expressed globally across all tissues, such as housekeeping genes, have higher GC content at their promoters compared to tissue specific genes [78, 79]. Tissue specific genes and enhancers have a somewhat lower GC bias [61, 80, 81]. One reason for the high GC content at TSSs is that transcription initiation coincides with CpG islands, stretches of sequence around 1000 bp that have a higher percentage of C and G bases and a low amount of methylation compared to the global genome [82].

Transcription initiation occurs in nucleosome free regions

DNA accessibility assays [83–85](reviewed in [86]) have consistently demonstrated that regions of polymerase loading and initiation are contained within nucleosome free regions (NFRs) [47, 57, 61, 80, 83–87]. Well placed nucleosomes are found upstream and downstream of the loading zone at both enhancers and promoters, suggesting that the machinery involved in transcription may play a part in maintaining open chromatin at these regions [27, 88, 89]. Most TFs that

recruit RNA polymerase have been shown to bind predominantly (94% in K562 [90]) to open chromatin regions [12]. It remains an important question as to the extent that transcription and transcription factors contribute to the establishment and maintenance of the nucleosome free region.

Transcription levels correlate with histone marks

Factors that influence transcription levels are likely to have a concomitant influence on the local chromatin marks. Transcription levels correlate with a variety of histone marks (Figure 2A). H3K4me1 tends to mark eRNAs, while H3K4me3 tends to mark promoters, providing a functional if imperfect bioinformatic tool for separating the two sets of transcribed genetic regions [80]. However, nascent transcription assays indicate that active histone modifications directly correlate with the transcriptional activity of each bidirectional region, regardless of whether the resulting transcripts are stable or not [27]. For example, H3K4 methylation status correlates with the overall RNA polymerase initiation levels, with increasing transcription leading to higher methylation status. However, studies on a histone demethylase complex (RACK7/KDM5C) suggest the relationship may not be that simple. Loss of RACK7-Histone Demethylase Complex resulted in increased transcription at enhancers and conversion from H3K4me1 to H3K4me3, suggesting RACK7 represses transcription [91]. Interestingly, RACK7 associated genes were unaffected. Therefore, either the RACK7 complex uniquely functions at enhancers or some mechanism drives RACK7 associated promoters to H3K4me3 status despite the presence of the demethylase.

3. Genetically encoded signals regulate transcriptional outcomes.

If RNA polymerase II initiation and loading is mechanistically consistent across the genome, then what regulatory processes influence the frequency of transcription and ultimately the stability of each transcript? A number of sequence features within the RNA polymerase loading zone, including recognition sites for a broad range of transcription factors, influence the efficiency of transcription initiation. The rich profile of transcription provided by nascent assays inherently informs on a broad class of regulatory proteins.

DNA sequences in transcription loading zone influence initiation frequency

Transcription factors are key orchestrators of rapid cellular responses to environmental cues, metabolic demands, and distinct developmental stages. TFs function by binding to DNA and altering the activity of cellular polymerases. Genome profiling of protein-DNA localization, e.g. binding, has led to tremendous insights into how TF binding specificity is achieved (reviewed in [9]). Recruitment of RNA polymerase, and thereby the quantity of transcription initiating from a given TSS, is driven by a wide variety of transcription factors, each with distinct binding profiles across the genome [12]. Transcription factors have distinct genome-wide binding profiles that can vary between cell types or conditions [90, 92, 93]. Some TFs binding predominantly at

promoters whereas others are more enhancer specific (Figure 2B). Though the presence of a TF motif near a gene can be informative, not all TF binding events are functional [5, 8, 14, 15, 94–101]. Therefore, no aspect of TF binding provides information on the subsequent regulatory activity of the TF.

[Figure 2 near here]

The width of the loading zone also influences the overall quantity of transcriptional initiation for each pair of initiation sites [47, 102]. Sites of bidirectional transcription have larger loading zones that are more responsive to induction than unidirectional regions [47]. Larger loading zones may be more responsive simply because there is more genetic real estate for inducible TF binding [47]. Hence the length and identity of sequence elements embedded in the transcription loading zone quantitatively affect transcription initiation at that region [27, 57, 103].

DNA sequences in transcription loading zone influence strand bias of transcription

Decades of transcription research have defined the basic genetic requirements for efficient PIC recruitment at protein coding gene promoters, revealing the importance of core promoter sequences (reviewed in [104]). However, most of these studies have assumed that the majority of TSS regions are unidirectional and result in stable RNAs. The discovery of widespread bidirectional transcription led to questions about the extent that promoter elements influence the balance of transcription from each strand, as well as the quantity of total transcription from a TSS pair. Stable-unstable TSS pairs tend to reveal a large bias of increased transcription initiation from the stable transcript compared to the unstable RNA; whereas, unstable-unstable transcript pairs often reveal a more balanced ratio of initiation from both strands [27]. Genetic analysis of the effects of SNPs on bidirectional TSSs suggests that the sequences within and adjacent to the loading zone tune the ratio of divergent transcription events [102, 105], e.g. the strand bias [78].

Genetic signals dictate RNA processing events that affect differential stability of transcripts

After polymerase initiation, the subsequent transcription and post-transcriptional processing of transcripts is quite diverse. The first major difference between stable processed transcripts and unstable enhancer associated transcripts is the frequency of pause release. In fact, one of the motivating goals of the development of nascent protocols was the study of pause release, a key regulatory step in transcription (reviewed in [106]). Though pausing appears to be regulated by pausing factors at both enhancer and promoter regions, average pause release appears to be faster at enhancer regions than at promoter regions [31, 45, 50]. Studies of pausing related factors NELF/PTefb imply that at some enhancers the eRNA may function in release of pausing factors from partner gene promoters [79].

Ultimately RNA processing signals lead to differential stability [107–109]. Upon pause release at mRNAs, RNA polymerase transitions to elongation and transcripts that encounter a 5' splice site undergo splicing. Splicing conveys many benefits to the transcript including stability and nuclear export (reviewed in [110]). Upon splicing many cofactors associate with both polymerase and the RNA. For instance, a splice site signals snRNPs, SR proteins and nuclear export factors to bind to the RNA (reviewed in [110]). Downstream, cleavage of mRNAs is initiated by the low complexity A rich polyA signal (AAUAAA) or one of its alternatives and a polyA tail is added to the mRNAs further increasing the stability of the transcripts [111]. Meanwhile, the elongating polymerase proceeds past the canonical cleavage motif (reviewed in [112]). Subsequent termination of RNA polymerase II occurs several kb downstream of the cleavage site.

In contrast, unstable transcripts, such as eRNAs and PROMPTs, rarely encounter stabilizing factors. For instance few have splice sites (Figure 2C). Instead, there is evidence that synthesis of these unstable transcripts is often halted relatively close to the TSS. In the case of uaRNAs, transcripts show an enrichment of RNA cleavage signals near the transcription start site [107]. Importantly, when both a splice site and cleavage signal are present, the U1 snRNP protects pre-mRNAs from premature cleavage and polyadenylation [108, 113]. Without splicing, the early occurrence of a cleavage site may target the transcript for premature cleavage and polyadenylation (PCPA), a signal for targeted nuclear degradation of the transcript [114]. Studies demonstrated some enhancer RNAs are polyadenylated while other eRNAs are not, implying there may be multiple mechanisms of termination [46, 48, 115]. Consistent with this idea, there is some evidence that the integrator complex may be essential for cleavage and processing of enhancer RNAs [53]. In any case, the termination of the transcript without splicing is likely sufficient to make the transcript both nuclear and unstable. Therefore, the major known genomic difference that distinguishes unstable RNA classes from stable RNAs is encoded by the initial 5' RNA processing signals (reviewed in [29]).

Mammalian cells have distinct nuclear and cytoplasmic RNA degradation pathways. Therefore differential cellular localization also influences the stability of a transcript (reviewed in [116]). eRNAs and PROMPTs tend to be short and have limited half lives, reportedly in the range of 7.5 to 30 minutes; explaining why early RNA based detection methods initially failed to identify their existence [31, 45]. In fact, the lack of stability complicates measurements of half life. Their rapid degradation involves the nuclear exosome targeting (NEXT) complex [109, 117], as exosome depletion increases the levels of these short unstable transcripts [30, 109, 118].

4. Beyond genetics: using nascent assay analysis to dissect cell type or cellular condition specific deviations in transcriptional regulation

Each cell within an organism contains the same genome and therefore the same encoded regulatory sequences. Yet most multicellular organisms are built from a large variety of different cell types, each with a unique transcriptional program. Therefore, transcriptional regulation is inherently also context dependent -- reliant on the subset of transcription factors and regulators present to not only define cellular state but also cellular responses to perturbations [119]. Nascent transcription provides a unique tool for understanding the context dependent nature of transcriptional regulation.

Transcription factor proteins can themselves be regulated at transcription, translation, post-translationally, or by cellular localization [120–124]. For example, the tumor suppressor transcription factor p53 modulates gene expression to control cell-cycle progression and apoptosis [125, 126] (reviewed in [127]). MDM2 is the principal cellular antagonist of p53 via ubiquitination and subsequent degradation. Consequently, p53 is constitutively transcribed and translated yet its regulatory status is inherently dependent on the tightly regulated p53-MDM2 complex [128]. As each TF is regulated by a unique process, it is difficult to know which set of transcription factors are actually present and actively participating in transcriptional regulation, e.g. active, at any given time within a cell.

Binding of a TF can be readily measured, but it has long been observed that many TF binding sites do not appear to contribute to promoter activity at the nearby target gene [5, 8, 14, 15, 94–101]. The presence of apparently non-functional binding calls into question whether binding is, by itself, sufficient for altering RNA polymerase activity nearby [129, 130]. However, the extent of apparently non-functional binding is difficult to estimate, as the regulatory impact of a binding event is typically accessed by changes in transcription at the associated protein-coding gene. Critically, most TF binding is not at promoters [131]. Therefore, establishing the regulatory impact of binding sites requires distal ChIP peaks to be assigned to the target gene they are thought to regulate. While target gene assignment is defined by the spatial organization of the genome, limits on the availability of high quality 3D data results in wide adoption of the simpler nearest gene approach, an assumption that is often incorrect [132, 133].

Active transcription factor binding sites recruit RNA polymerase II

Intriguingly, nascent transcription studies have demonstrated a tight relationship between the activation of a transcription factor and increased transcription associated with the TF's binding sites [115, 134–137]. Activation of p53 via Nutlin-3a led to a concomitant production of eRNAs from a subset of p53 binding sites [135, 138]. p53-dependent eRNAs are required for efficient transcriptional enhancement of corresponding target genes and induction of p53-dependent cell cycle arrest [138]. Similar results have been observed for activation of other TFs, including estrogen receptor [115], androgen receptor [134, 139], PPAR γ [140] and NF κ B [136] (Figure 3A). In contrast, knocking out the repressive TF Rev-Erb led to recruitment of polymerase and

subsequent eRNA production from the locations that had been bound by the repressive TF [141]. While the mechanistic details may vary, the overall pattern is consistent: activating TFs recruit polymerase proximal to the site of binding whereas repressors inhibit RNA polymerase recruitment (Figure 3B).

[Figure 3 near here]

Thus the presence of transcripts immediately proximal to TF binding sites eliminates the need for assigning a binding site to a target gene, as transcription at the binding site itself can be utilized. This allows for a re-evaluation of whether TF binding alone is sufficient for regulatory activity. Careful comparisons of ChIP data to nascent transcription assays indicate that most sites of RNA polymerase initiation overlap multiple different TF binding sites, in line with the fact that regulatory regions are dense with transcription factor binding motifs [104]. For any given TF, it is clear that RNA polymerase initiation associates with only a subset of bound sites -- reinforcing that not all TF binding leads to alterations in RNA polymerase activity [142–145]. This observation led to the intriguing idea that those binding sites with transcription activity may, in fact, be the functional subset. Consistent with this idea, functional enhancers by CapStarr-seq are 5X more likely to have eRNAs associated [44, 146]. Additionally, transcription of a gene is increased when the TF binding site nearby is also transcribed [44]. Therefore binding is a separate, but likely prerequisite, activity of a TF before regulatory activity.

It remains a mystery why some TF binding sites are able to recruit RNA polymerase whereas others do not. Many transcription factors work with cofactors, require a particular chromatin conformation, or prefer a certain DNA methylation status for functionality [9], hence the local context may strongly influence which sites are functional. It has been suggested that TF residency times influence functionality [99], though no comparison has yet been made between residency times and presence of a nascent transcript. Finally, ChIP is not without its own artifacts, including non-specific antibodies and so called phantom peaks [15, 100]. Additionally, it is also worth noting that transcription factors may have regulatory functions that are distinct from RNA polymerase recruitment, for example in altering local chromatin context (Figure 3B) [147, 148]. Chromatin altering functions would not necessarily be reflected in alterations to RNA polymerase, yet may still be crucial to cellular function [149]. Moving ahead, additional studies are necessary to learn what features distinguish functional sites from the nonfunctional.

Profile of transcription initiation is predictive of transcription factor activity

Because active TFs recruit RNA polymerase nearby, the genome wide profile of bidirectional transcription (an indicator of RNA polymerase initiation) can be used to infer when a TF is participating in regulatory activity. Because transcription factor proteins can themselves be

regulated at transcription, translation, post-translationally it is difficult to know which set of transcription factors are actually present and actively participating in transcriptional regulation in a cell.

Transcription factors actively participating in regulation will bind to their cognate motif and contribute to the recruitment (or suppression) of RNA polymerase at multiple loci across the genome. Furthermore, there is a dramatic co-localization of the motif with the RNA polymerase loading zone of the regulated loci [44]. This co-localization suggests that one can utilize nascent transcription to infer when a TF is actively participating in regulation. However, it isn't as simple as looking at an individual locus. The density of motifs and ChIP binding profiles at any one locus precludes assigning responsibility for that transcript to any one factor (or even a small set). But when co-localization is considered genome wide, active TFs show dramatic co-occurrence of the motif with RNA polymerase loading zone, far more than is expected by chance [44, 101]. Hence global TF activity can be inferred from a nascent transcription experiment with high confidence, even when TF responsibility at individual sites is difficult to ascertain.

When comparing across nascent transcription assay samples, it is possible to additionally identify changes in TF activity across conditions [44]. Strikingly, changes can be detected rapidly-- within minutes of a perturbation [150] -- time points early enough to assert that the observed transcription changes are the direct immediate result of the perturbation. Furthermore, analysis of nascent RNA at numerous time points has revealed both short term TF activity pulses and the temporal order in which TFs respond [44]. Thus analysis of nascent transcription data holds tremendous potential to unravel the regulatory network in response to both physiological perturbations and pharmaceuticals.

5. Some transcripts participate in regulation

Many noncoding RNAs, regardless of stability, are important to the transcription regulation process as an RNA. Years of work on lncRNAs indicates that many of these stable transcripts are regulatory [41, 151, 152]. Because of their short length and instability, there was a great deal of initial skepticism on whether enhancer RNAs have a functional role in regulation. However, the mere act of transcription has an important influence on the local chromatin architecture and TF activity profile in a region (reviewed in [41]). Evidence indicates that enhancer transcripts can also be important as RNAs, directly participating in transcriptional regulation through diverse mechanisms such as stabilizing transcription factor binding events or augmenting the activity of epigenetic modifiers (see Figure 4; also reviewed in [29]).

[Figure 4 near here]

Specific protein-eRNA binding interactions can affect transcriptional events

Evidence supports the role of some enhancer RNAs in modulating transcription factor activities at regulatory regions. For example, under immune signaling activation BRD4 binds to enhancer elements primed with acetylated histones to stimulate eRNA transcription. Then bromodomains in BRD4 cooperate to interact with sequence specific eRNAs generated from BRD4 bound enhancers [153]. Additionally, *in vitro* binding assays demonstrate that BRD4 proteins associate more frequently with the acetylated histones H3K27 and H4K16 in the presence of eRNAs from BRD4/p53 positive enhancers. Similarly, the transcription factor Ying Yang 1 (YY1) interacts with eRNAs and, at least in *in vitro* assays, displays preferential affinity for distinct RNA sequences [154]. In this way, sequence specific eRNAs may reinforce the binding/stability of transcription factors at cis regulatory elements and promoters.

In other cases, the importance of the eRNA has been demonstrated, but the mechanism is not well understood. For example, repressive effect that NELF has upon RNA polymerase II elongation can be *transiently* silent under the presence of specific eRNAs [155]. Likewise, key eRNAs upstream of the *Fos* gene appear to be important for the appropriate transcription of *Fos* [156].

Some enhancer transcripts augment epigenetic modifiers

While most studies of the impact of these transcripts have focused on target genes in close proximity to the enhancer, recent evidence demonstrated the ability of eRNAs to act upon distal loci and even across chromosomes. For instance, during the onset of myogenesis, two particular enhancers upstream of the MyoD gene become activated to produce eRNAs. While the eRNA from the most proximal enhancer promotes higher occupancy of RNA polymerase II at the MyoD promoter, the more distal eRNA participates exclusively in the upregulation of downstream myogenic gene effectors [157]. Specifically, the distal enhancer, located on the mouse chromosome 7, generates a stable unidirectional transcript that specifically targets the Myogenin locus on chromosome 1. This regulatory noncoding transcript binds to multiple subunits of the cohesin complex and together they co-localize at the Myogenin gene to regulate its expression. This finding suggests a more elaborate functional role of a regulatory transcript, namely in the recruitment of chromatin organizers to establish transcriptionally active nuclear hot spots.

Interestingly, in some cases the sequence of the transcribed RNA may not matter. A recent study rigorously demonstrated that the histone acetyltransferase (HAT) CBP can bind RNA indiscriminately, e.g. independently of sequence characteristics. Importantly, a diverse set of eRNAs preferentially accumulate at the HAT domain and stimulate its catalytic activity resulting in hyperacetylation of lysines in histones, including H3K27 and H4K5 [158]. Notably, this

eRNA-induced catalytic stimulation decreases at both low and high accumulation levels of eRNAs, suggesting the effect is tuned to respond to only moderate levels of enhancer RNAs.

6. Conclusion and Discussion

By monitoring changes in RNA polymerase II activity, nascent transcription assays paint a more complete picture of regulation than one focused predominantly on protein-coding genes. Importantly, while this review is written from an RNA polymerase II centric viewpoint, some of the processes included may be incorrectly attributed to this polymerase. It remains to be seen to what extent RNA polymerase I and III are influenced by the regulators of RNA polymerase II.

RNA polymerase II steps through multiple stages during the transcription process and nascent transcription protocols have been informative on the regulators participating in every stage of the transcription process. The loading and initiation stage of RNA polymerase II is regulated by a large number of DNA binding transcription factors. Active transcription factor binding sites alter polymerase activity immediately proximal to the binding site. Therefore, evidence of RNA polymerase II loading and initiation, as measured by nascent transcription assays, provides a unique temporal and positional resolution on regulatory activity. Subsequent steps in RNA polymerase II activity: pause release, elongation and termination, depend on not only sequence features but also RNA binding proteins and other regulators. Ultimately this process gives rise to transcripts with a variety of lengths, stability, and post-transcriptional localization.

Transcript properties underly the definitions of the classes of noncoding RNAs (lncRNAs, PROMPTs, uaRNAs, eRNAs, SLITs). Yet transcription studies suggest these features exist on a continuum [58] and therefore there may be substantial overlap between the classes [41]. Intriguingly, some of these properties may even vary according to cell type or perturbation.

What these transcripts do remains a hot topic, but the answer is likely to be diverse (Figure 4), just as proteins have a diverse set of functions. The act of transcription is a critical contributor to the local regulatory context, interacting with and influencing both TFs and histones. Arguably the relationship between nucleosome free regions, histone marks, TF binding and RNA polymerase initiation is one of co-dependence, with each element both interacting and contributing to the local context. Given the vast numbers of transcripts produced in a eukaryotic cell, it is also likely that evolution has found ways to utilize some of the noncoding transcripts as regulators.

7. Figure Captions

Figure 1: A typical RNA polymerase II loading and initiation region

Sequence bias at RNA polymerase loading and initiation sites peaks at the epicenter of bidirectional transcription. Top: The percentage of A (red), T (blue), G (green) and C (yellow) nucleotides for all sites of RNA polymerase II loading and initiation genome wide (adapted from [44]). Bottom: Zoom in on epicenter of RNA polymerase loading and initiation, where typically two bidirectional transcription start sites (black arrows) originate from within a nucleosome free region. Rose barrels are nucleosomes.

Figure 2: The differences between stable mRNAs and other unstable nascent transcripts.

- (A) Cartoon showing the relationship between H3K4 methylation status (me1 and me3) and transcription (adapted from [27]). Stable transcripts include mRNAs and lncRNAs.
- (B) Most TFs preferentially bind non-promoter regions. Using ENCODE k562 ChIP and RefSeq annotations, the heatmap shows the fraction of ChIP sites that overlap promoters. 194 total TFs total but only every 10th row labeled.
- (C) Cartoon depicting early RNA processing decision. Encountering a splice site motif is correlated with message stability whereas encountering a cleavage motif is correlated with instability.

Figure 3: Active transcription factors alter local transcription and chromatin context.

- (A) Activation of a TF leads to recruitment of RNA polymerase II and proximal bidirectional transcription. In four separate papers, activation of specific TFs results in concomitant increases in transcription levels of eRNAs (blue positive strand, red negative strand) associated with that TF's binding events (grey boxes) and motifs (orange dots). Data from: TP53 [135], ANDR [139, 159], ESR1 [115], NF- κ B [136]; motifs from HOCOMOCO [160]. All data mapped to hg19 as described in [44].
- (B) Distinct functions of transcription factors (in orange) include (1) binding to DNA, (2) recruitment of RNA polymerase II (green) and (3) altering the local chromatin landscape (rose). Recruitment of RNA polymerase II may contribute to alterations in chromatin or the TF may alter chromatin distinct from RNA polymerase II recruitment. In the case of a transcriptional repressor, the repressive transcription factor disrupts recruitment of RNA Polymerase II.

Figure 4: Plethora of mechanisms where transcription at enhancers influences regulation.

Top: Instances where the resulting eRNA participates in transcriptional regulation. Enhancer RNAs display functionality through physical interactions with chromatin modulators in a sequence-dependent or independent manner. Most often the presence of eRNAs results in a bias towards gene upregulation. Studies have established direct eRNA effects on transcription regulation by interacting with proteins such as: transcription factors, chromatin writers/readers, 3D chromatin structural proteins, and pausing factors [153–155, 157, 158]. Bottom: Instances where the act of enhancer transcription impacts transcriptional regulation. Indirect ways in

which enhancer transcription itself contributes to transcription regulation include: altering transcription factors, polymerase, or general transcription factor localization, participating in transcriptional interference, or affecting chromatin rearrangements (reviewed in [29, 41]).

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9. Conflicts of Interest

Dr. Dowell is a founder and scientific adviser to Arpeggio Biosciences.

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Figure 1:

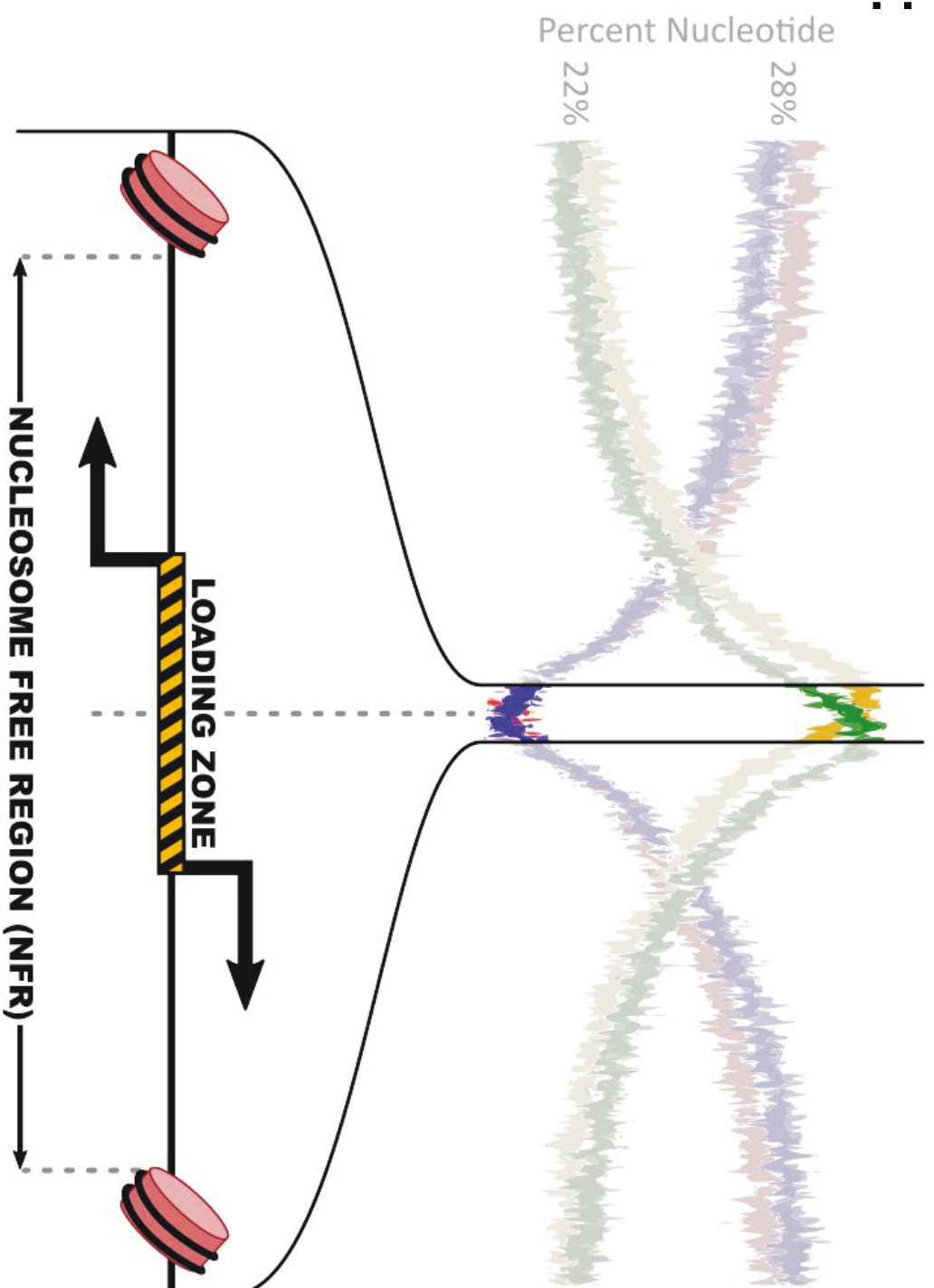


Figure 2:

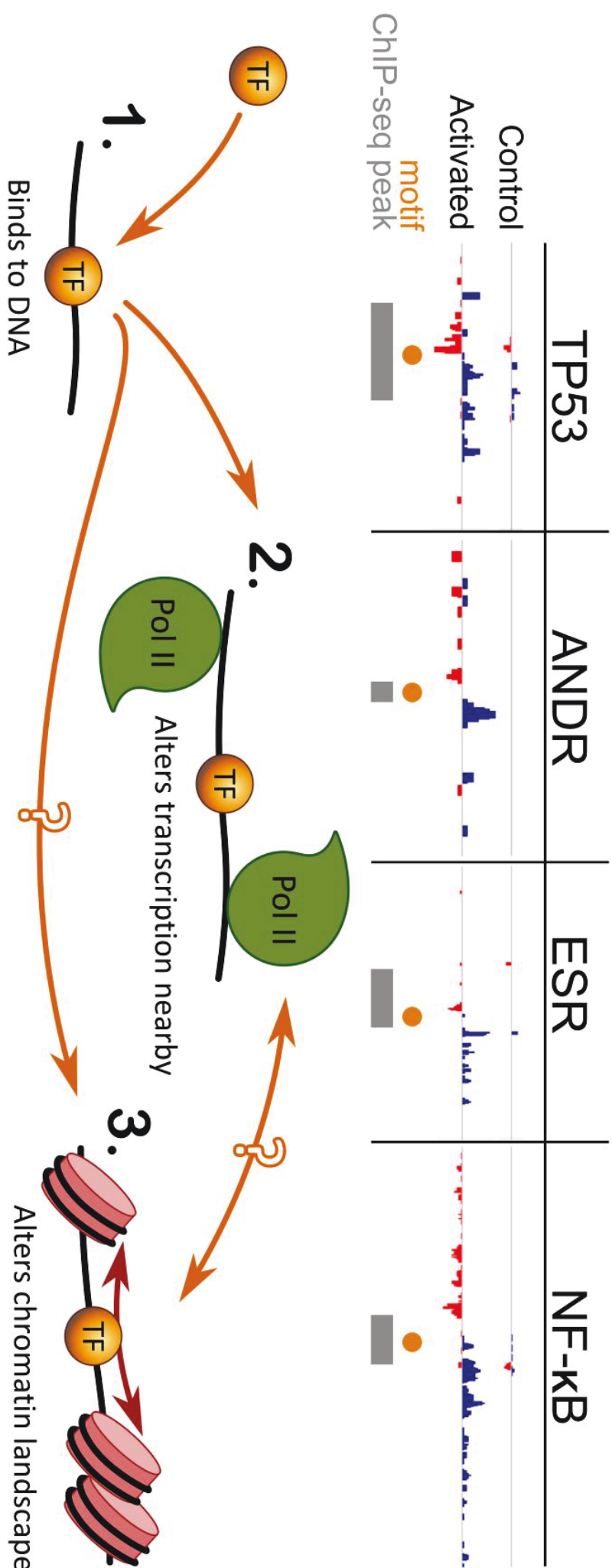
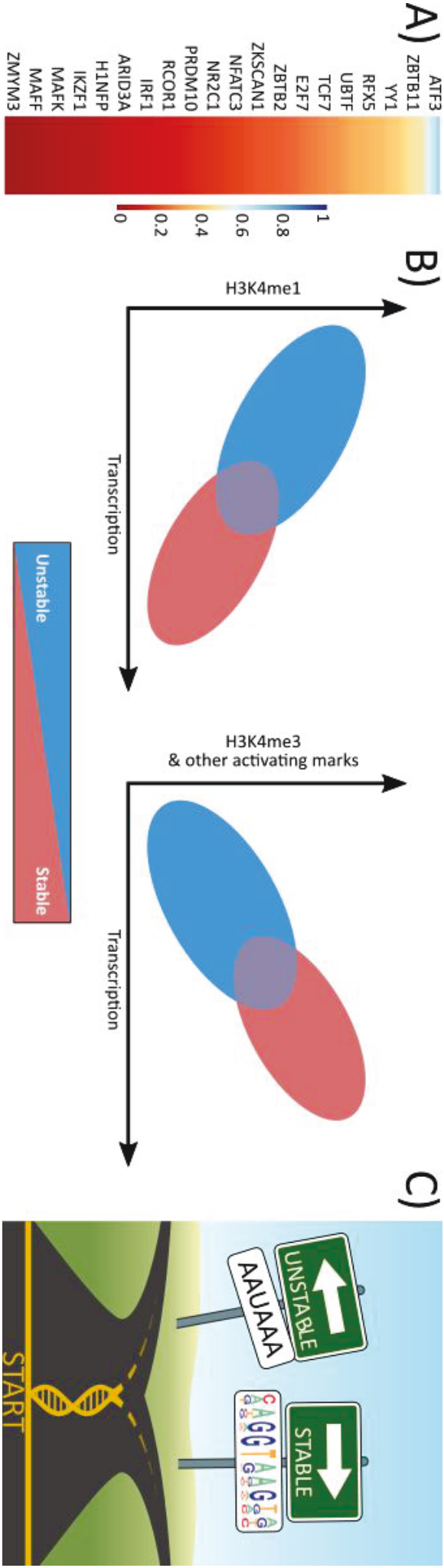


Figure 3:



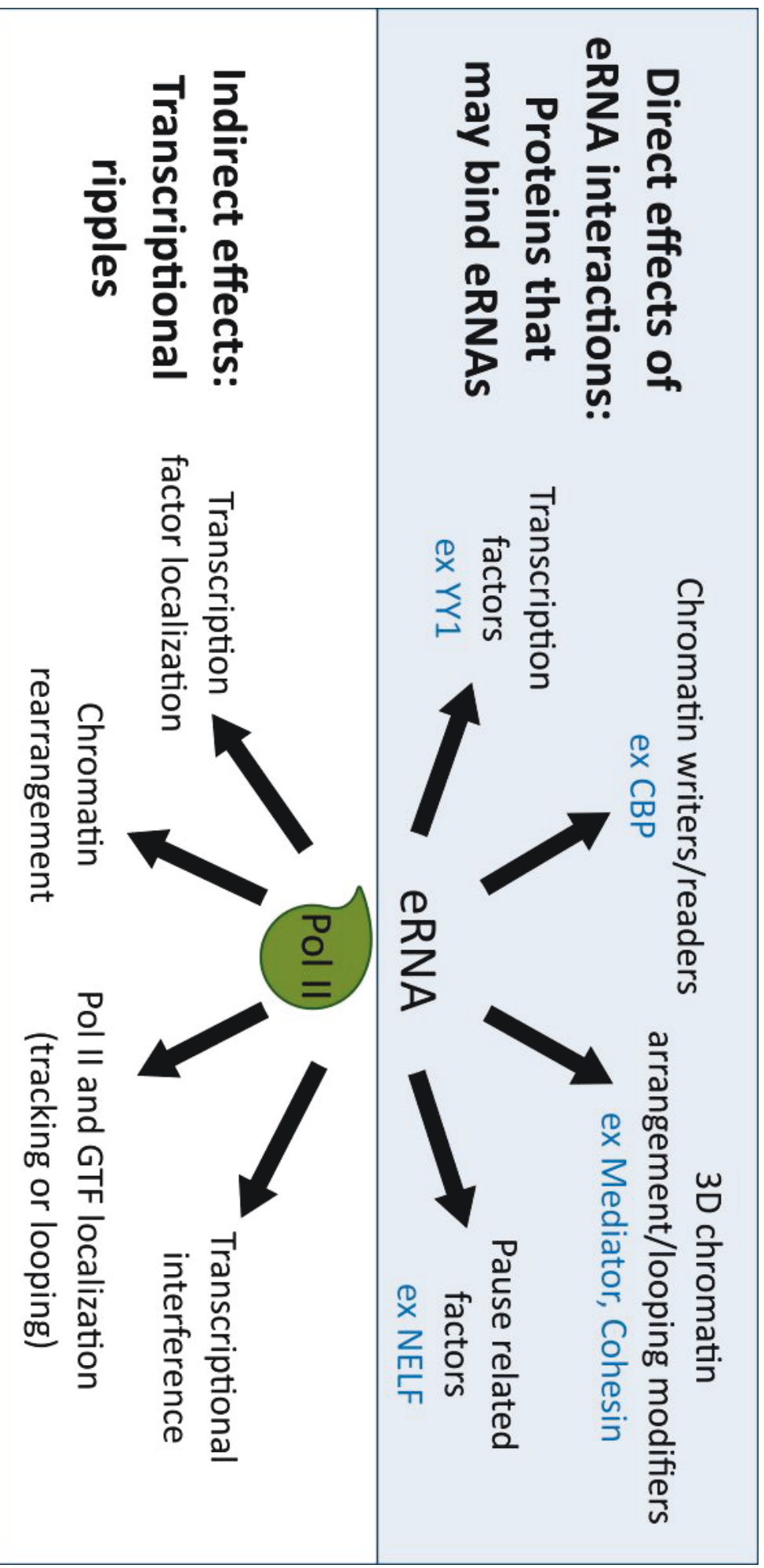


Figure 4