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Pause & go: from the discovery of RNA polymerase pausing to its functional implications

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The synthesis of nascent RNA is a discontinuous process in which phases of productive elongation by RNA polymerase are interrupted by frequent pauses. Transcriptional pausing was first observed decades ago, but was long considered to be a special feature of transcription at certain genes. This view was challenged when studies using genome-wide approaches revealed that RNA polymerase II pauses at promoter-proximal regions in large sets of genes in *Drosophila* and mammalian cells. High-resolution genomic methods uncovered that pausing is not restricted to promoters, but occurs globally throughout gene-body regions, implying the existence of key-rate limiting steps in nascent RNA synthesis downstream of transcription initiation. Here, we outline the experimental breakthroughs that led to the discovery of pervasive transcriptional pausing, discuss its emerging roles and regulation, and highlight the importance of pausing in human development and disease.

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Introduction

During transcription, DNA-bound RNA polymerases synthesize nascent RNA molecules along a chromatin DNA template. This process is generally divided into three distinct phases: initiation, elongation and termination (Figure 1) [1,2]. For decades, transcription initiation was thought to be the major regulatory step in the production of RNA [3], but this view was recently challenged by the discovery of widespread RNA polymerase pausing during transcription elongation, suggesting that events downstream of transcription initiation, such as pausing in the promoter-proximal region [4,5], are also key rate-limiting steps [6,7•]. Thus, nascent RNA synthesis is a discontinuous process, during which phases of productive transcription are frequently interrupted by pauses. Although the causes and consequences of transcriptional pausing remain incompletely understood, pausing of RNA polymerase clearly creates opportunities for regulation.

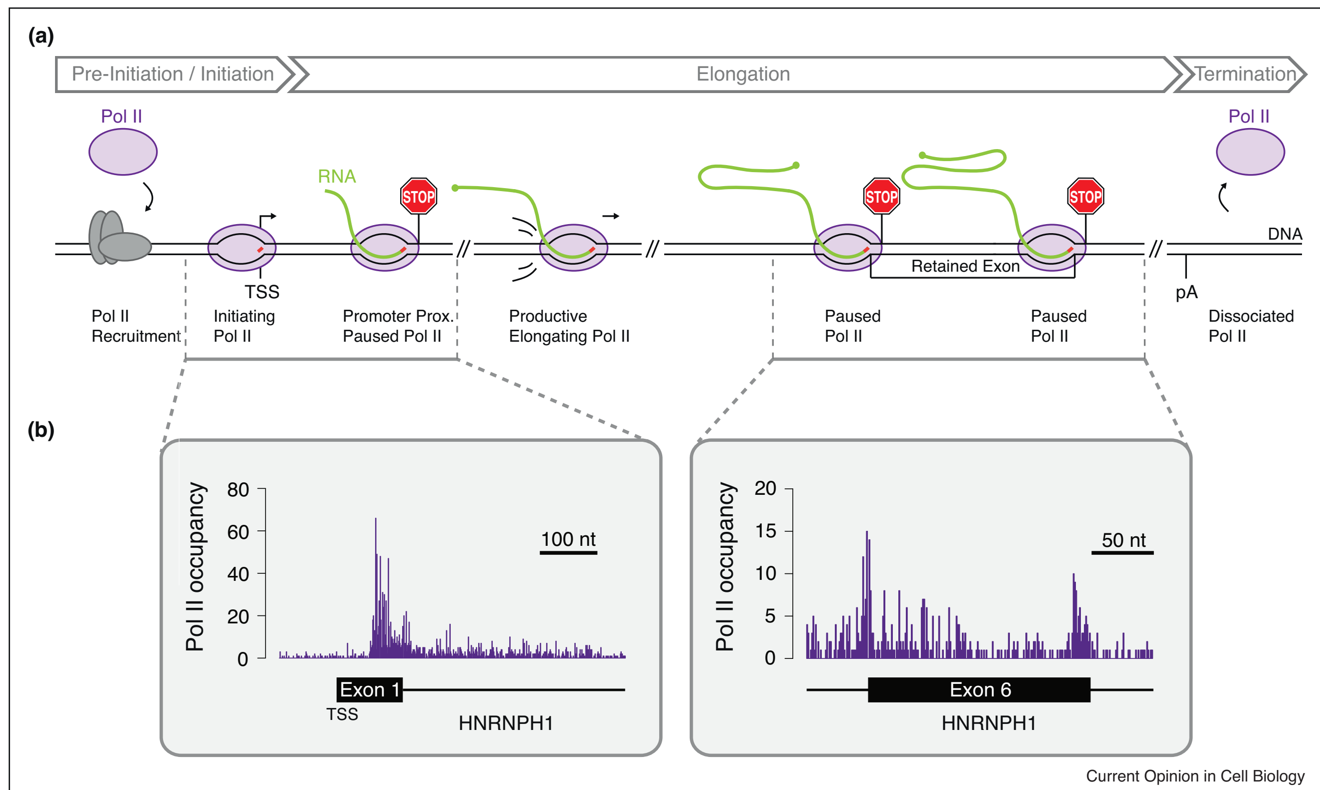
Over the past four decades, analysis of transcriptional pausing has been stimulated by multiple technological advances. To orient the reader, Box 1 provides an overview and comparison of methods used successfully to study transcriptional pausing *in vivo*. All of these experimental approaches reveal the position and relative abundance of RNA polymerase throughout gene bodies. In these data, peaks in RNA polymerase occupancy correspond to pause sites, because locations where RNA polymerase is captured more readily are positions where RNA polymerase spends more time on average (Figure 1). Additionally, these peaks of RNA polymerase are engaged transcription complexes as they are associated with nascent RNA and are capable of resuming transcription. In this review, we describe how technological advances have taken us from the discovery of RNA polymerase pausing to our emerging appreciation of its roles in regulating RNA abundance and identity.

Although various types of RNA polymerase pausing are mentioned, the focus of this review is on the emerging analysis of gene-body pausing by RNA polymerase II (Pol II), the key enzyme that produces all protein-coding RNAs and a large set of non-coding RNAs in eukaryotic cells [8–10]. We refer readers who are especially interested in Pol II promoter-proximal pausing to the following excellent reviews: [4,5,11].

The road to the discovery of pervasive transcriptional pausing

The first evidence of RNA polymerase pausing arose from *in vitro* transcription experiments performed in the early 1970s (Figure 2). In two seminal studies, Maizels et al. and Dahlberg et al. used *in vitro* transcription assays to show that *Escherichia coli* RNA polymerase transcribing the *lac* operon or lambda DNA transiently pauses at discrete sites [12–14]. This *in vitro* pausing was confirmed by multiple laboratories during the 1970s and early 1980s [15–17]. RNA polymerase pausing *in vivo* was first demonstrated in 1981 by the Chambon laboratory, who used a transcriptional run-on assay to show that Pol II accumulates at the 5′-end of the beta-globin gene in hen

Figure 1



RNA polymerase II pausing during the gene transcription cycle.
(a) Different transcriptional states of RNA polymerase II (Pol II) during distinct phases of the gene transcription cycle: pre-initiation/initiation, elongation, and termination. Pol II is shown in purple; nascent RNA is in green with the 3′-end nucleotide labeled in red; double-stranded DNA is indicated by two black lines; TSS: transcription start site; pA: polyadenylation site.
(b) Pol II pausing with single-nucleotide resolution at the promoter-proximal (left panel) and gene-body regions (right panel) as revealed by human NET-seq (Box 1). NET-seq data obtained from HeLa S3 cells are shown for the representative *HNRNPH1* gene [57••]. Exonic and intronic regions are indicated by black boxes and black lines, respectively. TSS: transcription start site.

erythrocytes. This finding provided the first evidence for Pol II promoter-proximal pausing [18].

The advent of chromatin immunoprecipitation (ChIP) and improved transcription run-on protocols (see Box 1) in the mid-1980s enabled detection of Pol II accumulation at the 5′-ends of additional genes in *Drosophila* and mammalian cells. Studies performed in the Lis laboratory revealed that Pol II clusters within a small window at the 5′-end of the *hsp70* heat shock gene in *Drosophila* [19], and that the accumulated polymerases are in a paused state [20]. Pol II accumulation at the 5′-ends of genes was also observed at selected mammalian genes, first in *c-myc* by the Groudine, Bornkamm and Marcu laboratories [21–23], and subsequently in other genes such as *c-myb*, *c-fos*, *c-mos* and ADA [24]. Nuclear run-on experiments at the c-myc gene using short probes revealed that the majority of transcriptionally engaged Pol II was located within the first 169 nucleotides [25,26]. Together, these studies suggested that key rate-limiting steps occur downstream of initiation in a handful of genes.

The earliest insights into the molecular mechanisms underlying pausing were obtained from *in vitro* transcription experiments that revealed that certain DNA sequences cause RNA polymerase to move backward, a phenomenon known as backtracking [27]. Backtracking results in the misalignment of nascent RNA and the RNA polymerase active site, and can lead to a transcriptional arrest [27–31]. Release of RNA polymerase from a transcriptional arrest is aided by multiple *trans*-acting factors. The first transcription factor to be implicated in the regulation of transcriptional pausing was TFIIS [32,33]. TFIIS induces cleavage of the nascent RNA, restoring a 3′OH of the nascent RNA to the RNA polymerase active site, which is thought to enable the release of arrested Pol II [34–38].

Up to this point, the prevailing view in the field was that gene expression is primarily regulated at two main steps: recruitment of Pol II and transcription initiation. However, this model was challenged by the advent of genome-wide Pol II mapping methods. The first genome-wide

Box 1 Methods for analyzing RNA polymerase pausing *in vivo*.

Chromatin immunoprecipitation (ChIP) of RNA polymerase:

ChIP determines the position of RNA polymerase along genes and genomes *in vivo* [19,39,123]. Chromatin is reversibly crosslinked, typically with formaldehyde [124,125]. After chromatin fragmentation and solubilization, DNA bound by RNA polymerase is purified by immunoprecipitation. After reversal of the crosslinks, the relative amount of captured DNA is assessed by quantitative PCR (ChIP-qPCR), microarray hybridization (ChIP-chip) [39,40], or high-throughput sequencing (ChIP-seq) [123]. Variations on ChIP-seq such as ChIP-exo [50,126] and ChIP-nexus [61••,127•], employ exonuclease digestion to remove DNA that is not directly bound by RNA polymerase, yielding nucleotide resolution and higher sensitivity.

ChIP-based approaches lack DNA strand specificity and suffer from high levels of background signals. Moreover, the spatial resolution of classical ChIP assays is limited, typically >200 bp. Furthermore, because the bound DNA is analyzed rather than the nascent RNA, the transcriptional status of RNA polymerase is not revealed.

Transcription run-on:

Transcription run-on approaches exploit the nascent RNA to reveal the location of transcribing RNA polymerase [20,128]. In nuclear run-on assays, cells are lysed and nuclei are prepared, halting transcription by RNA polymerase. Transcription is then restarted *in vitro* for a short amount of time in the presence of labeled nucleotides. The amount of labeled, nascent RNA at a given region corresponds to the abundance of RNA polymerase, providing a measure of RNA polymerase occupancy at selected genes. Two genome-wide transcription run-on methods have been developed to assess RNA polymerase density: global run-on sequencing (GRO-seq) [51] and precision nuclear run-on and sequencing (PRO-seq) [55,56,129•]. In both techniques, labeled nascent RNA is purified, converted into a DNA sequencing library, and sequenced. PRO-seq is a variant of GRO-seq that reveals the RNA polymerase density at single-nucleotide resolution [55,56].

Transcription-run on assays provide a strand-specific quantitative measure of transcriptionally engaged RNA polymerase. However, these methods rely on the efficient restart of transcription under non-physiological conditions, which depends on the experimental procedure and the transcriptional status of RNA polymerase [130]. Notably, arrested, backtracked RNA polymerase cannot be restarted, and will therefore escape detection [79].

Permanganate footprinting:

This approach detects single-stranded DNA regions as they occur in the transcription bubble of transcriptionally engaged RNA polymerase [131] by exploiting the hyper-reactivity of thymine residues within single-stranded DNA to oxidation by potassium permanganate. After permanganate treatment, the DNA is specifically cleaved at oxidized thymines, and the positions of resulting DNA breaks are determined by ligation-mediated PCR (LM-PCR). This approach was recently adapted for genome-wide analysis [132].

Permanganate footprinting is intended to detect single-stranded DNA within the RNA polymerase transcription bubble. However, single-stranded DNA which arise from other sources, such as DNA replication forks and R-loops, contributes to the experimental background.

Native elongating transcript sequencing (NET-seq):

In NET-seq, the 3′-ends of nascent RNAs are detected by to high-throughput sequencing, thereby revealing both the genomic location and abundance of RNA polymerase [133,134•,135•]. Nascent RNA is co-purified with the extremely stable RNA–DNA–RNA polymerase ternary complex, either by immunoprecipitation or cell fractionation. Because transcriptional restart *in vitro* is not required, all RNA polymerases can be monitored, regardless of their pausing status. The 3′-ends of nascent RNAs are converted into a DNA sequencing library, and the identity and abundance of the library fragments are determined by deep sequencing. Mapping the sequencing reads to the reference genome reveals the strand-specific genomic position of the RNA polymerase active site with single-nucleotide precision.

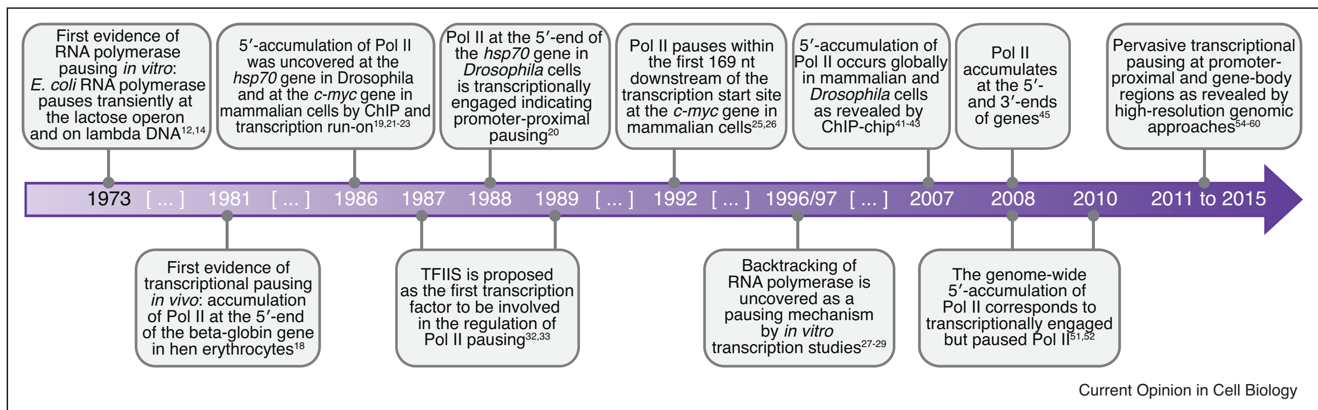
In addition to the 3′-ends of nascent RNAs, NET-seq libraries capture RNA processing intermediates from splicing and 3′-end cleavage, as well as fully processed mature RNAs. These background sequences can be computationally identified and removed, but processing the data in this manner decreases the fraction of informative reads.

approach that has been used to study transcriptional pausing was ChIP coupled with microarray hybridization (ChIP-chip), initially developed in the mid-2000s [39,40] (Box 1). This experimental tool revealed that Pol II clusters in the promoter-proximal regions of a large set of active genes in various model organisms [41–44]. ChIP-chip, and subsequently ChIP coupled with deep sequencing (ChIP-seq), revealed that Pol II accumulation is not restricted to 5′-ends of genes, but also occurs globally at 3′-ends [45–48,49••]. With a high-resolution ChIP study, ChIP-exo (Box 1), Pol II was observed to accumulate on average 50 bp downstream of the TSS at the majority of genes in human K562 cells [50].

Although these genomic Pol II ChIP studies suggested that peaks in Pol II density are widespread, the transcriptional status of Pol II, that is whether it is engaged in transcription, was uncertain. Critically, it remained unclear whether the clusters of Pol II corresponded to paused Pol II. This limitation was initially overcome by combining genomic ChIP with potassium permanganate footprinting assays at selected genes (Box 1) [41,42] and later by the development of methods for mapping the locations of transcriptionally engaged Pol II throughout the genome, for example, global run-on sequencing [51] (GRO-seq, Box 1), as well as sequencing of short and capped RNAs (scRNA-seq) [52,53•].

Recently, techniques for mapping Pol II at nucleotide resolution throughout the genome have enabled pausing to be analyzed across gene bodies, outside of promoter-proximal regions. Native elongating transcript sequencing

Figure 2



Milestones on the road to the discovery of pervasive transcriptional pausing.

(NET-seq, Box 1) and precision nuclear run-on and sequencing (PRO-seq, Box 1) revealed that RNA polymerase pausing occurs throughout gene-body regions in various model organisms, ranging from bacteria to human cells [54–56,57••,58••,59,60]. These high-resolution techniques reveal that in yeast and bacteria, RNA polymerase pauses approximately every 20–100 basepairs [54,59]. These studies determined that the relative magnitude of promoter-proximal pausing varies between genes, and in many cases Pol II density in the region around the TSS is more than 10-fold higher than the rest of the gene body [55,57••]. Nevertheless, genome-wide Pol II density averages show that prominent pauses occur at the boundaries of retained exons and near polyadenylation sites [55,57••,58••,61••].

Together, these studies provide strong evidence for pervasive RNA polymerase pausing and imply that it represents a general feature of gene transcription (Figure 2). Efforts currently underway in our group and others aim to better understand the causes and functional roles of transcriptional pausing.

Causes of RNA polymerase pausing

Diverse mechanisms modulate the frequency and lifetime of RNA polymerase pauses. Many *in vitro* biochemical and biophysical studies have revealed the detailed molecular mechanisms that are responsible for Pol II pausing and are reviewed elsewhere [62,63]. Whether *in vivo* transcriptional pausing occurs through these same biophysical mechanisms is not clear, but it is likely that many pauses exploit them.

RNA polymerase has an intrinsic capacity for pausing and alterations in any component of the RNA–DNA–RNA polymerase ternary complex can trigger this natural propensity to pause. Specific amino acid residues in Pol II, particularly in the trigger loop domain, are critical for transcription elongation and pausing. Mutating these residues leads to an increase or decrease in the transcription elongation rate and tendency to pause [64–66]. Certain DNA sequences, on their own, cause RNA polymerase pausing [12,16,67]. For example, regions around promoters and termination sites often have high G/C content followed by A/T-rich sequences [52,68••]. This sequence feature is thought to induce RNA polymerase backtracking and pausing by abruptly reducing the melting temperature and stability of the DNA–RNA hybrid that forms within the transcription elongation complex [69,70]. Other sequence elements associated with transcriptional pausing include ‘A’ nucleotides, which are present at sites of pausing in *Saccharomyces cerevisiae* [54], and a 12-bp consensus sequence detected at *E. coli* pause sites both *in vivo* [59,60,71•] and *in vitro* [59,67]. Nascent RNA can also induce pausing by forming either secondary structures or R-loops that interfere with the transcription elongation complex. For example, RNA hairpins that form within or upstream of the RNA exit channel cause pausing [30,72•]. R-loops are also implicated in Pol II pausing during transcription termination [73,74]. Thus, each of the core components of transcription – Pol II, DNA, and RNA – are intrinsically capable of inducing pausing.

Extrinsic factors, such as nucleosomes and regulatory factors, are also capable of inducing RNA polymerase pausing. For most of these, the biophysical mechanisms by which Pol II pausing occurs are not known, however we propose that extrinsic factors likely act by perturbing some aspect of the RNA–DNA–RNA polymerase ternary complex. In other words, the biophysical mechanisms for pausing outlined above are likely utilized when extrinsic factors manipulate Pol II elongation.

Nucleosomes, which compact and organize chromatin, present a significant barrier to transcription. *In vitro* transcription through nucleosomes is punctuated by

increased pausing compared to naked DNA [75], which can be alleviated by elongation factors such as TFIIS [76,77] and histone chaperones such as FACT [78]. Nucleosomes also hamper transcription *in vivo* [54,55,79], but to a lesser degree due to the presence of chromatin remodelers [80,81] and transcription elongation factors [79]. Consistent with the idea that nucleosomes represent a transcriptional barrier, depletion of histones from human cells increases the speed of transcription elongation [82••].

Beyond the chromatin itself, dozens of regulatory factors interact with the transcription elongation complex as it moves along a gene [46,61••,83–87], and many influence pausing and transcription elongation rates. Promoter-proximal pausing is regulated in part by negative elongation factors such as NELF and DSIF, which interact with Pol II to induce pausing by a mechanism that has yet to be revealed (for review, see Ref. [88]). DNA binding factors, such as CTCF, YY1, and Reb1, are thought to induce Pol II pausing by creating 'roadblocks' on the DNA template that obstruct transcription elongation [57••,89,90••]. Many features of these pausing factors remain to be elucidated, but their existence demonstrates that transcriptional pausing is highly regulated and likely a core component of diverse gene regulatory mechanisms.

Emerging roles of transcriptional pausing

RNA polymerase pausing occurs throughout transcription elongation. Even though the precise biophysical mechanisms of *in vivo* Pol II pausing have yet to be established for each step in the transcription cycle, a number of functions for pausing have been proposed [63]. The main consequence of Pol II pausing are the windows of opportunity for regulation and coordination with other processes at different stages during transcription elongation.

During early transcription elongation, promoter-proximal pausing represents a key rate-limiting step in RNA synthesis that can impact mRNA abundance [91–93]. Promoter-proximal pausing is also thought to control gene expression by maintaining an open chromatin state near promoters [94–96]. Additionally, RNA polymerase pausing is implicated in diverse gene regulatory mechanisms, including coupling between transcription and translation in bacteria [59,97], suppressing genome instability [98••], and promoting RNA folding [99].

Transcriptional pausing also occurs downstream of the promoter-proximal region. A great deal of work over the past decade has suggested that pausing plays a role in splicing. Splicing often occurs co-transcriptionally, while the nascent RNA is attached to chromatin [100], and in close proximity to transcribing Pol II [101•]. The proximity between the splicing and transcription machineries suggests that transcription elongation could impact splicing through kinetic coupling since the pausing of Pol II creates a 'window of opportunity' for splicing to occur (proposed by Refs. [102–104] and recently reviewed in Refs. [105,106]). Several lines of evidence support this kinetic coupling model. First, mutations in Pol II that alter the transcription elongation rate cause widespread changes in alternative splicing [107–110], suggesting that proper transcription rate is important for alternative splicing regulation. Second, Pol II density is elevated around splice sites [55,57••,58••,61••,111]. Third, Pol II pausing induced by the transcription factor, CTCF, can cause retention of weak exons, as demonstrated in the *CD45* gene [89].

At the 3′-ends of protein-coding genes, Pol II must be released from DNA in order to be recycled and to prevent read-through transcription into downstream genes. Pol II density increases after polyadenylation sites [45–48,49••,51] and at putative termination sites [68••], suggesting that transcription termination requires pausing or slowing [112]. Consistent with this model, Pol II mutants with altered transcription elongation rates display different sites of transcription termination [113,114••]. The proposed causes of RNA polymerase pausing during termination in eukaryotes include Pol II backtracking [68••,115,116••], R-loop formation [73,74], and Reb1-mediated transcription blockage [90••].

Analysis of the functional roles of transcriptional pausing is still in its infancy. Future work will likely uncover a broad spectrum of mechanisms that manipulate Pol II elongation, particularly in relation to disease development. Promoter-proximal pausing and aberrant pause release have been implicated in various human pathologies [117•] such as in c-Myc driven cancers [25,118] and cardiac hypertrophy [119], and also plays a role in inflammatory responses [120], as well as in development [11,121]. Therefore, we expect other classes of transcriptional pausing, for example the elevated polymerase density around splice sites and polyadenylation signals, to also play critical roles in human pathologies.

Future directions

Following the advent of high-resolution tools for analyzing RNA polymerase pausing throughout the genome, the next steps are to elucidate the regulation and function of these pauses in normal conditions and in disease. To achieve this, additional tools will be necessary. First, we need better methods for inducing transcriptional pausing. RNA polymerase mutants with altered transcription elongation rates have contributed greatly to our understanding of the connections between transcription elongation rates and other processes. However, little mechanistic insight has been gleaned from these systems because the impact of these mutations on transcriptional pausing remains poorly characterized. Altering DNA sequence to induce transcriptional pausing or to impact the binding of a roadblock protein is a necessary complementary approach

to uncovering the functional roles of RNA polymerase pausing. Second, we need to resolve the temporal order of RNA polymerase pausing in relation to the arrival of regulatory factors and RNA processing machinery, in order to determine whether pausing kinetics truly influence the window of opportunity for co-transcriptional events. Finally, imaging studies have shown that *in vivo* elongation rates vary significantly among individual Pol II enzymes [122]. Thus, it will be important to understand whether transcriptional pausing is a purely stochastic event or differentially regulated for individual Pol II enzymes.

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