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Letter

Knocking Out Enhancers to Enhance Epigenetic Research

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Advances in epigenetics have uncovered a myriad of transcription factor binding sites and chromatin modifications in presumed DNA control elements that regulate gene activation (enhancers) or repression (silencers). Identification of enhancers has relied extensively on the production of animals carrying enhancer reporter transgenes; however, recent studies reporting knockouts of such presumed enhancers have described many cases in which the enhancer is nonessential [1–5]. In one case, knockout of one presumed testis enhancer for *Sox9* had no effect on testis development, whereas knockout of another single testis enhancer reduced expression enough to result in reversal to an ovary fate [4]. In some cases, knockout of two presumed enhancers significantly reduced the expression of a nearby gene and resulted in a physiological defect, thus clearly demonstrating a required function and revealing enhancer redundancy [1–3]. However, two of these studies uncovered examples in which double enhancer knockouts had no effect on gene expression or development; specifically, knockout of two presumed enhancers for *Sox9* in limb bud [2] and two presumed enhancers for *Tbx5* in forelimb bud [5]. It remains to be determined whether the two presumed limb bud enhancers for either *Sox9* or *Tbx5*, or the nonessential testis enhancer for *Sox9*, are redundant with yet more DNA control elements or whether they are nonredundant (i.e., not able to control the nearby gene). Further enhancer knockouts may reveal that some presumed enhancers are indeed vestigial enhancers or pseudoenhancers that are not able to regulate the nearby gene.

Although enhancer reporter transgene technology has been thought to be a good method to identify enhancers that function in specific tissues *in vivo*, the recent knockout studies show that transgene analysis cannot on its own identify enhancers essential for gene regulation. The disconnect is likely to be due to the fact that an enhancer reporter transgene is generated by linking a potential enhancer to a heterologous basal promoter upstream of a marker gene, which is then randomly inserted into the genome of an animal. By doing this, the enhancer is removed from its endogenous location in the genome and placed in a foreign location close to a promoter, instead of being located in a position that is normally far from the promoter of the gene it is proposed to control. These recent studies demonstrate that knockout of the endogenous proposed enhancer in its normal location in the genome is required to validate function.

Genomic studies can identify global changes in transcription factor binding sites and epigenetic marks across the entire genome that are often reported to be responsible for changes in gene expression during development and in the adult. However, now that we know many presumed enhancers are nonessential, it is likely that many transcription factor binding sites and epigenetic marks do not provide true insight into gene regulation as they occur in nonessential DNA control elements. By identifying enhancers that are required to regulate a gene *in vivo*, efforts in epigenetics can be focused on examining the epigenetic changes that occur in these true enhancers. This increase in the signal-to-noise ratio will revolutionize epigenetics.

Recent advances in knockout methodology such as CRISPR/Cas9 make it realistic for journals and funding agencies to require demonstration of functions for presumed enhancers or silencers by *in vivo* genetic loss-of-function studies, thus validating such studies and providing a solid foundation for future studies. Although it is possible to obtain some insight by generating knockouts in cell lines, function *in vivo* is also needed to show how the cell line studies relate to biology *in vivo*. In this way, researchers will be able to make valid conclusions on gene regulation that can be reproduced by others.

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Spotlight

Post-Translational Modification, Phase Separation, and Robust Gene Transcription

Hari R. Singh[1,*] and Yogesh B. Ostwal[2]

A few recent reports reveal fundamental new insights into the intricate regulatory mechanisms that govern RNA polymerase II (Pol II)-mediated gene transcription. Whereas a histidine-rich domain

(HRD) triggers phase separation, promoting transcription elongation, a phosphatase switch promotes transcription termination. A paradigm that might govern the underlying mechanisms leading to robust gene transcription is now starting to emerge.

Not all genes are expressed at the same time and place, and misregulation of gene expression can be detrimental to the organism. The molecular machinery of the general transcription cycle and the mechanisms controlling cell type-specific control of gene expression have been understood in great detail. However, a comprehensive understanding of how intricate regulatory interactions within the molecular machinery regulate faithful transitions in the initiation–elongation–termination steps of the transcription cycle and thereby maintain robustness of Pol II-mediated transcription has been lacking [1].

Pol II-Mediated Gene Transcription

Pol II consists of 12 polypeptides, of which RPB1 is the largest subunit. The Pol II C-terminal domain (CTD) is located within RPB1, and has multiple heptad repeats of the consensus sequence Y-S-P-T-S-P-S, varying in number from 26 in yeast to 52 in humans [2]. The Pol II CTD is known to orchestrate the transcription cycle. First, general transcription factors and the mediator/coactivator complex recruit Pol II containing an unphosphorylated CTD to the promoter to initiate transcription. This is followed by promoter proximal pausing, promoter clearance, and transcription elongation, and finally by termination of the transcription process.

During initiation, the CTD is phosphorylated at Ser5 (Ser5-P) by the TFIIH-associated kinase CDK7. During elongation, Ser5 phosphorylation is gradually removed while Ser2 is phosphorylated (Ser2-P) by the P-TEFb (positive transcription elongation factor B) complex, which consists of the kinase enzyme CDK9 and another subunit, cyclin T1 (CCNT1). This leads to a typical CTD phosphorylation pattern wherein Ser5-P levels peak around the transcription start-site (TSS) while Ser2-P levels peak at the 3′ ends of genes. P-TEFb can also phosphorylate elongation factors, for example, Spt5. Interestingly, Spt5 also possess a CTD (Spt5-CTD) that consists of nonapeptide repeats of the consensus sequence T-P-A-W-N-S-G-S-K, another target for CDK9 activity [2].

However, many facets of Pol II-catalyzed transcription regulation remain unresolved. For example: (i) how does P-TEFb bring about efficient elongation and, in particular, what is the role of CCNT1 subunit? and (ii) what signal triggers the elongation-to-termination transition? From many recent studies it appears that post-translational modification (PTM) switches, phase separation, and cooperative interaction hubs are in part responsible.

A PTM Switch

Two recent reports by Parua *et al.* [3] and Kecman *et al.* [4] demonstrate the presence of a phosphatase switch that antagonizes CDK9 signaling and thereby mediates transition from elongation to termination during the transcription cycle (Figure 1).

Parua *et al*. [3] describe a CDK9–PP1 switch in *Schizosaccharomyces pombe*, wherein CDK9 and the PP1 isoform Dis2 both determine the phosphorylation status of the Spt5 CTD. CDK9 phosphorylates Dis2 and negatively regulates its activity, while it also phosphorylates the Spt5 CTD. Dis2 can dephosphorylate the Spt5 sites targeted by CDK9, thereby antagonizing CDK9 signaling as well as its elongation enhancement function. High CDK9 activity during elongation is inactivated upon its phosphorylation. Dis2 becomes active when the transcription complex traverses the cleavage and polyadenylation signal (CPS), resulting in dephosphorylation of the Spt5 CTD and the accumulation of Pol II CTD Ser2-P.

Kecman *et al*. [4] further show that Dis2 not only dephosphorylates Spt5 but also Thr4-P of Pol II CTD that has been shown to be essential for mRNA 3′-end processing in vertebrates [5]. Termination defects and cell death in Dis2 catalytically inactive and Thr4Glu (phosphomimetic) mutants, respectively, support the PTM-based elongation-to-termination switch. The authors also show that Seb1, a factor essential for 3′-end processing and termination, can bind to Thr4-P of Pol II CTD and can compete with Spt5 for binding to Pol II *in vitro*, suggesting a possible release mechanism for Spt-5 and Pol II from the DNA (Figure 1). Further research on how the phosphatase activity of Dis2 is regulated and its possible involvement in RNA Pol II recycling would prove useful in understanding the complete transcription cycle.

Phase Separation and Pol II Transcription

Another recent report by Lu *et al.* [6] shows that a HRD in CCNT1 can trigger phase separation to locally increase the concentration of the elongation factors, thereby increasing the efficiency of Pol II CTD phosphorylation by P-TEFb (Figure 1).

CCNT1 regulates the activity of the CDK9 kinase. The authors tested which region of CCNT1 is responsible for the regulation of CDK9 activity-dependent transcriptional activation. They discovered that mutations in the HRD of CCNT1 led to Pol II transcription defects *in vitro* and *in*

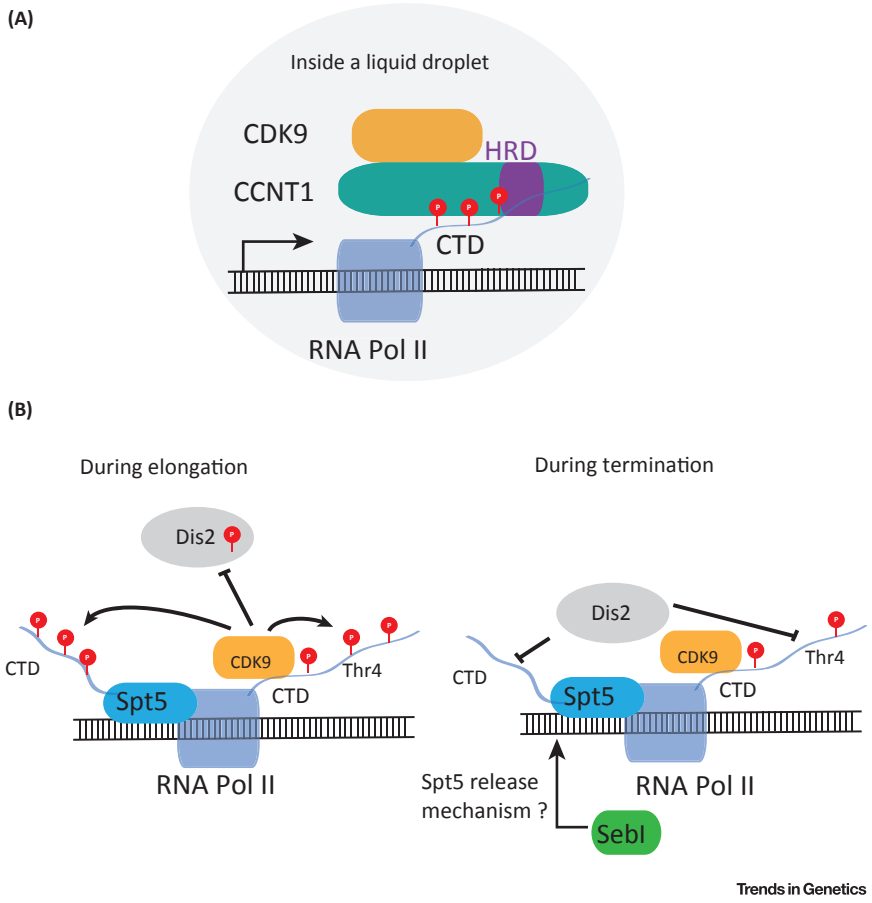

Figure 1. Two Facets of the P-TEFb Complex of CDK9 and CCNT1. (A) A phase-separated liquid droplet increases the chances of functional interactions among the components. In particular, the evidence suggests that, upon phase separation, better transcription elongation can be achieved owing to more efficient phosphorylation (P) of the RNA polymerase II (Pol II) C-terminal domain (CTD) by CDK9. (B) A network of regulatory interactions among Dis2, Spt5, and the P-TEFb kinase subunit CDK9 creates a switch that regulates the elongation-to-termination transition. During elongation, CDK9 inactivates Dis2 while keeping the Spt5 CTD phosphorylated. At the 3′ end, owing to a drop in CDK9 activity, Dis2 is activated and therefore can dephosphorylate Spt5, facilitating 3′-end pausing and transcription termination. In another study, Dis2 was shown to dephosphorylate Thr4 on the RNA Pol II CTD during termination, while Seb1 can bind to phosphorylated Thr4 (Thr4-P) of Pol II CTD, thus competing with Spt5 for binding to Pol II *in vitro*. Abbreviations: HRD, histidine-rich domain; P-TEFb, positive transcription elongation factor B.

vivo. HRDs in proteins are low-complexity intrinsically disordered regions (IDRs). IDRs have long been correlated with phase separation, a process that compartmentalizes biomolecules inside cells via dynamic cooperative multivalent interactions, locally concentrating biochemical reactions [7]. *In vitro* phase-separation experiments showed that the HRD of CCTN1 is indeed involved in phase separation, wherein a GFP-tagged wild-type CCNT1 formed micrometer-sized spherical droplets, but CCTN1 lacking the HRD did not. *In vivo* imaging experiments further corroborated the findings wherein CCNT1 clusters, called speckles, could be visualized in the nucleus. When treated with a compound that disrupts phase separation, 1,6-hexanediol, the clusters were dispersed. The authors propose that phase separation leads to local accumulation of Pol II together with P-TEFb, leading to more efficient hyperphosphorylation of the Pol II CTD (Figure 1). Interestingly, phosphorylated Pol II CTD can either drive phase separation or prevent phase separation, depending on which proteins are in its vicinity, indicating the possibility of a switch-like reversible transition of the underlying processes [8,9].

The discussed reports unify many facets of the transcription cycle in which CDK9/Dis2-mediated phosphorylation/dephosphorylation of CTDs achieves a switch-like regulation over the elongation-to-termination transition as well as efficient and robust transcription elongation. The logic for utilizing PTM action on CTDs is clear – what better way to achieve quick and reversible assembly of supramolecular complexes other than modifications of

scaffold-like repetitive sequences? A common central theme [3,4,6] appears to be the modulation of the same complex, P-TEFb, to achieve efficient Pol II elongation and termination, two distinct steps in Pol II-mediated transcription, albeit as of now in two different model systems.

As it stands today, these reports [3,4,6] provide a paradigm for the mechanisms that might govern the Pol II transcriptional cycle, wherein PTM switches (which can provide quick control towards maintaining transcriptional integrity) combined with phase separation achieve robust and dynamic transcriptional control. Further, genetic and biochemical studies will allow dissection of the roles of phase separation and PTM switches in the context of P-TEFb and the transcription cycle. How these mechanisms achieve robust transcriptional control is a key question.

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