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Evaluating Enhancer Function and Transcription

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Abstract

Cell-type- and condition-specific profiles of gene expression require coordination between protein-coding gene promoters and *cis*-regulatory sequences called enhancers. Enhancers can stimulate gene activity at great genomic distances from their targets, raising questions about how enhancers communicate with specific gene promoters and what molecular mechanisms underlie enhancer function. Characterization of enhancer loci has identified the molecular features of active enhancers that accompany the binding of transcription factors and local opening of chromatin. These characteristics include coactivator recruitment, histone modifications, and noncoding RNA transcription. However, it remains unclear which of these features functionally contribute to enhancer activity. Here, we discuss what is known about how enhancers regulate their target genes and how enhancers and promoters communicate. Further, we describe recent data demonstrating many similarities between enhancers and the gene promoters they control, and we highlight unanswered questions in the field, such as the potential roles of transcription at enhancers.

Contents

INTRODUCTION

Enhancers are segments of DNA defined by the ability to activate gene expression over large genomic distances, independent of sequence orientation (1). The first such sequence to be identified, more than 35 years ago, is derived from a 72-base-pair (bp) repeat found in the simian virus 40 (SV40) genome. Remarkably, this SV40 element can stimulate expression of the human β-globin gene when placed at arbitrary positions relative to the gene promoter (2). Shortly after this discovery, similar transcriptional enhancers were found in metazoan genomes, where these sequences were implicated in cell-type-specific stimulation of gene expression (3, 4). Sequences with enhancer activity were soon appreciated to harbor a high density of DNA motifs recognized by activating transcription factors (TFs). Thus, a basic model emerged wherein enhancer sequences represent binding sites for a collection of TFs that together increase activity from a target gene promoter. This early model was appealing because it explained how the specific repertoire of TFs present in a given cell type, or active under a particular condition, might cooperate to elicit precise patterns of gene expression. Nonetheless, this model raised a new set of questions concerning how TF binding to an enhancer, located at a great distance from the target gene, functioned to increase levels of transcription.

A good deal of study has defined the features of TF binding at enhancers, illuminating the cooperative nature of TFs in enhancer activation. Current models envision that lineage-specifying factors direct the selection of enhancer sites, where they prime these regions for activation (5). Mechanistically, numerous lineage-specifying TFs are pioneer factors that can bind their consensus motifs on DNA wrapped around nucleosomes, suggesting that these factors are critical to initiating chromatin opening in the locus (6, 7). Importantly, however, the binding of lineage TFs to enhancers is not alone sufficient for enhancer activation. Lineage TFs require cooperation with signal-dependent TFs that bind in response to the cellular environment. In this way, the sites selectively bound by signal-dependent TFs reflect the primed, accessible chromatin landscape that is specific to each cell type. This coordinated occupancy of enhancers by multiple TFs is needed to fully render the enhancer DNA accessible and stimulate gene activity (**Figure 1**). Moreover, TF binding at active enhancers is accompanied by coactivator recruitment. In particular, the Mediator

Promoter: region flanking the transcription start site that harbors core sequence motifs necessary for appropriate assembly of the transcriptional machinery and transcription initiation

Transcription factor (TF): a sequence-specific DNA-binding protein that typically activates transcription through recruitment of coactivators or the transcription machinery

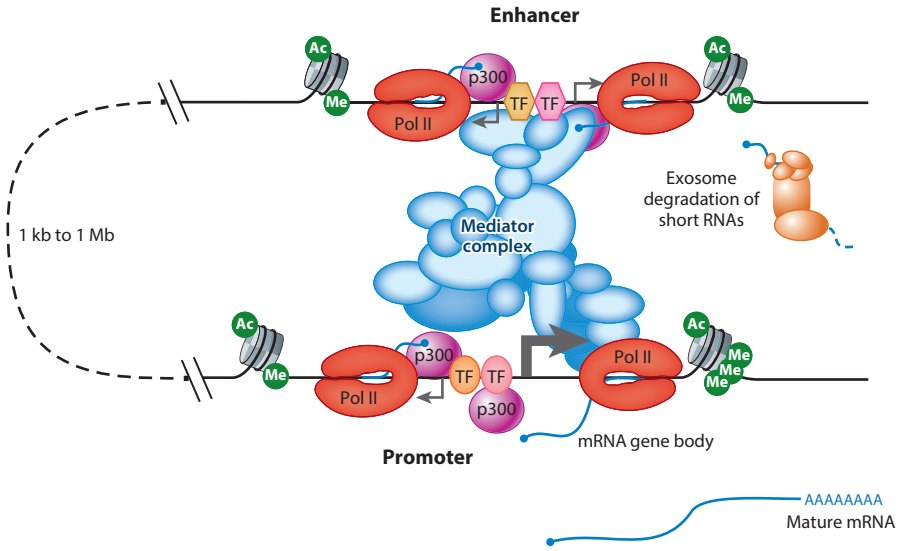

Figure 1

Features of an active enhancer. The molecular features of active enhancers (*top*) and their target promoters (*bottom*) are depicted here with the respective transcription start sites indicated by gray arrows. Enhancers are often transcribed divergently, and there are noncoding RNA promoters located upstream and antisense of mRNA transcription start sites. Both enhancers and promoters are associated with TFs, Pol II (*red*), the Mediator complex (*light blue*), and p300 (*purple*, representative of protein paralogs p300 and CBP). Histone modifications (*green*) are labeled as Ac or Me. RNA (*blue lines*) is extended as Pol II enters productive elongation at mRNA genes or is released during early termination at enhancers and degraded by the exosome (*orange*). Abbreviations: Ac, histone H3 lysine 27 acetylation (H3K27ac); CBP, CREB-binding protein; Me, histone 3 lysine 4 monomethylation/trimethylation (H3K4me1/me3); mRNA, messenger RNA; Pol II, RNA polymerase II; TF, transcription factor.

complex and the lysine acetyltransferases p300 and CREB-binding protein (CBP) associate with active enhancers (8, 9) (**Figure 1**). Accordingly, acetylation on histones flanking the TF-binding site is a general feature of enhancer loci [e.g., histone H3 lysine 27 acetylation (H3K27ac)] (see **Figure 1**). In addition, a vast majority of active enhancers are transcribed (**Figure 1**), suggesting potential roles for this transcription in enhancer function (10, 11).

Predictions of enhancer loci based on genomic data sets, pioneered by the Encyclopedia of DNA Elements (ENCODE) Consortium, have identified hundreds of thousands of putative enhancers in the human genome (12), suggesting that enhancers far outnumber gene promoters. Sequences predicted to have enhancer activity are enriched in disease-associated single-nucleotide polymorphisms, underscoring the importance of understanding enhancer biology for human health. Aberrant enhancer activity is also implicated in the misregulation of gene expression patterns (5, 13–15), highlighting the importance of enhancers for the appropriate development and health of metazoan species. Notably, many genes appear to be influenced by multiple discrete enhancer loci (15, 16). This may reflect that distinct enhancers are activated under different conditions, or alternatively, there may be a large degree of redundancy in enhancer function, which has been postulated to convey robustness in gene activity (17, 18).

A majority of metazoan enhancers lie distant from their genic targets, with examples ranging from several kilobases in *Drosophila* to nearly a megabase in mammals (19). Although enhancers can be found in intergenic regions, many are located within gene bodies, with an enrichment of

p300 and CREB-binding protein (CBP): paralogous transcriptional coactivators with acetyltransferase activity; they act on many TFs, as well as histones at enhancers and promoters

Intergenic region: a region of the genome located between genes

enhancers within long intronic sequences. Given these distances, current models for enhancer activity involve the looping of chromatin such that an enhancer and its target promoter (which may be distant on a linear chromosome) are brought into proximity in 3D space (20). Enhancers are often predicted to target the closest active gene (21–25); a study in *Drosophila* estimated that ∼80% of enhancers stimulate their immediately neighboring genes (26). However, several prominent examples from mammalian systems, such as the ZRS enhancer, which controls the sonic hedgehog gene (*Shh*), do not act on the most proximal genes (27). Consistent with this, studies using chromatin conformation capture (3C)-based methods have given a wide range of estimates, from 7% (22) to 46% (28), of enhancer elements that contact the closest gene. Although contact by these assays does not predicate a functional interaction, these studies do highlight the question of how enhancers find their appropriate gene targets.

Chromatin conformation capture (3C)-based methods: NGS techniques that use proximity-based ligation of DNA to infer 3D distances between genomic loci that may be separated in linear DNA

The size of DNA sequences defined as enhancers is highly variable, from <100-bp-long functionally defined elements to 10–100-kilobase stretches of genomic sequence termed stretch enhancers or superenhancers (29, 30). Superenhancers are identified computationally through the identification of neighboring peaks with high-level TF binding, cofactor binding, or histone modifications and are manually or computationally stitched together as coregulated loci. As a category, superenhancers have been defined using a variety of different chromatin and coactivator signatures, and there is generally a lack of data confirming the functionality of the entire locus. As such, it is currently unclear whether they represent a distinct class of large enhancers or consist of a conglomeration of many independent, and perhaps functionally redundant, smaller enhancer elements (31–33).

Importantly, although we have made great progress toward understanding enhancer function over the past few decades, many of the central questions have yet to be fully elucidated. In particular, the molecular mechanisms of enhancer-mediated gene activation are not yet understood, nor are the dynamics and specificity of enhancer–promoter communication. Further, it remains unclear what series of events leads to enhancer activation and what minimal components are necessary for enhancer activity. Here, we discuss each of these aspects of enhancer biology while highlighting our current state of knowledge and key questions for the future.

FUNCTIONAL CHARACTERIZATION AND DISSECTION OF ENHANCERS

There is no single assay to definitively identify enhancer elements and their target genes. For this reason, the field has come to rely on several imperfect assays that together can provide insight into the enhancer potential of a genomic locus (**Figure 2**). Here, we describe the methods used to functionally verify enhancer activity and summarize recent results obtained using these strategies.

Reporter-Based Assays

The most common methods for evaluating the potential of a given sequence to act as an enhancer are reporter assays. The typical construct used in these assays includes a reporter gene (e.g., the genes encoding β-galactosidase, luciferase, or green fluorescent protein) driven by a minimal promoter, which alone is insufficient to drive expression of the reporter. A test sequence is then inserted into the construct, and its influence on reporter gene activity is measured. Reporter assays can be performed with plasmids transfected into cell lines (33, 34) or integrated genomic constructs introduced into developing embryos (14, 26, 35) (see the integrated reporter assay in **Figure 2**). Integrated constructs are a powerful tool when evaluated at key developmental stages since they illuminate the precise spatial and temporal activity of enhancers. However, this

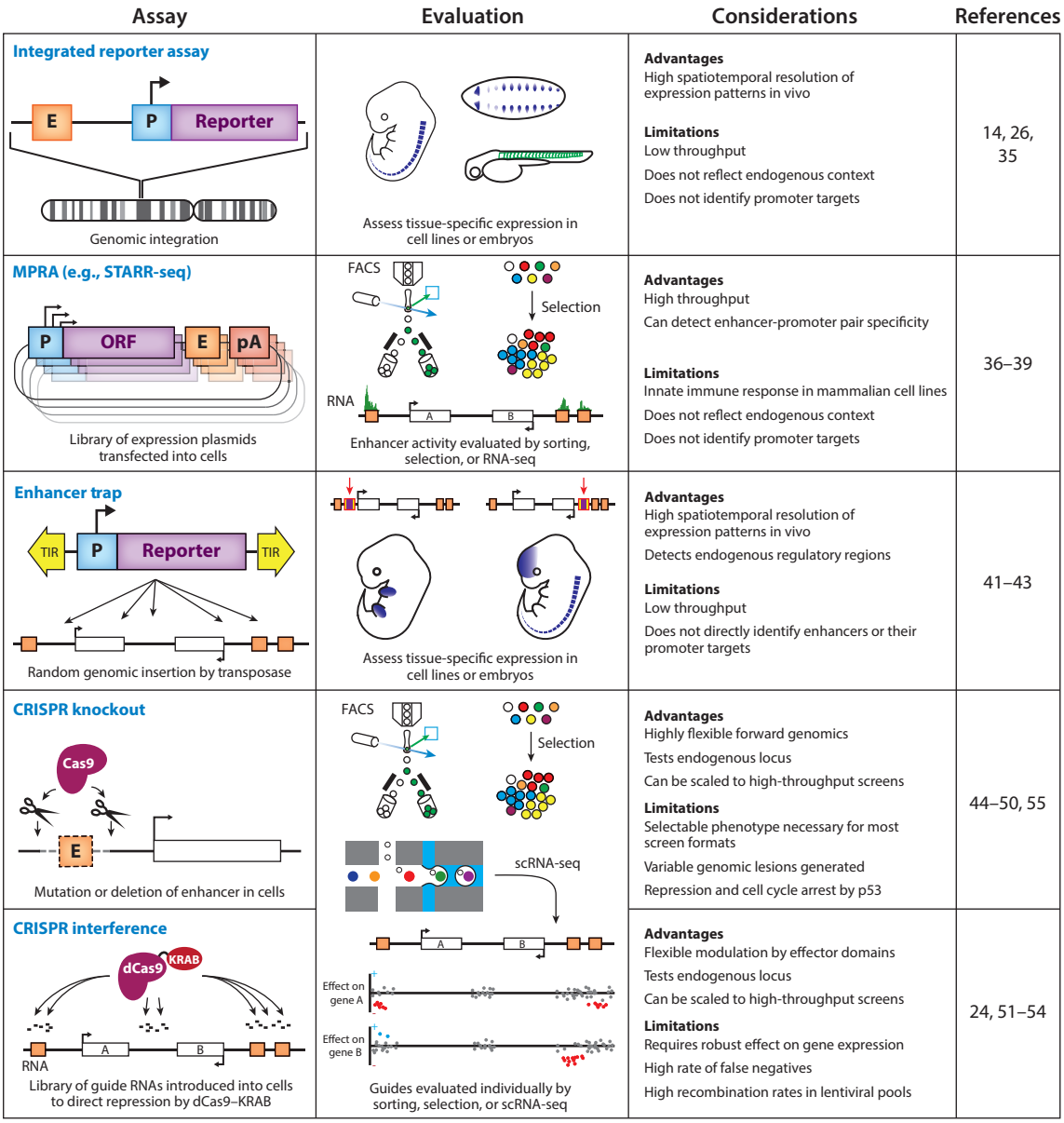

Figure 2

Methods for interrogating enhancer function. Enhancers are evaluated by a variety of experimental approaches, each of which informs on a different aspect of enhancer function. Common methods are depicted, and the means by which the resulting data are evaluated are outlined along with the advantages and limitations of each method. Genes labeled as reporters are those with detectable expression (e.g., the genes encoding β-galactosidase, luciferase, or green fluorescent protein). Selection means that cells displaying a particular phenotype (e.g., cell growth or drug resistance) are enriched. Abbreviations: Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; dCas9, dead Cas9; E, test enhancer sequence; FACS, fluorescence-activated cell sorting; KRAB, Krüppel-associated box; MPRA, massively parallel reporter assay; ORF, open reading frame; P, minimal promoter element; pA, polyadenylation site; RNA-seq, RNA sequencing; scRNA-seq, single-cell RNA-seq; STARR-seq, self-transcribing active regulatory region sequencing; TIR, terminal inverted repeat.

approach often requires a transgenic animal to be made for each test sequence, which is inherently labor intensive and low throughput.

Next-generation sequencing (NGS): techniques that use massively parallel or high-throughput sequencing technology, allowing identification of many DNA or RNA species simultaneously

To increase the scalability of enhancer reporter assays, the field has developed numerous massively parallel reporter assays (MPRAs) (**Figure 2**). These assays expand on traditional plasmid-based systems by introducing a library of test sequences in bulk into amenable cell lines. Using a variety of strategies that make use of next-generation sequencing (NGS) for identification of sequences with enhancer activity, these assays allow for the analysis of tens of thousands of sequences simultaneously (36). Originally implemented to test promoter activity (37), MPRAs have been improved by new construct designs that can pair different gene promoters with a library of enhancer sequences (38). A key result from these assays is that most tested enhancers have preferential activity on particular promoter types (39). This suggests that enhancer target specificity can be revealed in MPRAs, making this topic ripe for further exploration. MPRAs have been particularly effective in *Drosophila*, but they pose some challenges in mammalian systems. The interferon response to transfected plasmid DNA and the high efficiency with which the mammalian transcription machinery initiates from the bacterial origin of replication on most plasmids confound results in many mammalian cell lines (40). Inhibiting the interferon pathway and using the origin of replication site as the promoter element can help mitigate these negative effects, but future work will surely continue to improve these systems.

All reporter-based assays, however, are incapable of concretely linking enhancers to their target genes or accurately reflecting the native chromatin architecture of their endogenous locus. Further, cell-type- or condition-specific enhancers may be active only in a selective cellular context or only with particular promoter partners. Thus, assays that functionally characterize sequences at their endogenous locus are also extremely important for understanding enhancer biology.

Evaluating Enhancers in the Genomic Context

The need for an understanding of the endogenous chromatin environment to assess an enhancer's biological role is illustrated by experiments that use enhancer traps or sensors (**Figure 2**). This technique uses random insertions of a reporter gene construct into the genome by a transposase (41). Inserts within range of an enhancer will be activated in a manner that reflects the spatial and temporal activity of the enhancer, which is evaluated by staining embryos for the reporter gene at key developmental stages. Insertion sites are then mapped to identify developmentally regulated genomic regions. Variations of this technique in mice have shown that tissue-specific enhancers can be found throughout the genome and that this activity is nonuniform within the same insulated neighborhood (42, 43). These findings indicate that enhancer targeting also involves factors such as fine-scale chromatin topology or accessibility. Though enhancer traps do not directly identify enhancers or their genic targets, they emphasize the importance of local context in enhancer function.

Whereas reporter assays can identify regions that are sufficient for enhancer activity and enhancer traps can identify regions potentially influenced by regulatory elements, neither can address the necessity of a region for appropriate gene expression. For this, perturbation of putative enhancers in their genomic context is required. Recently, CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9) directed by guide RNAs (gRNAs) has become a flexible and reliable means of performing such perturbations (44). CRISPR is applied to cell lines or animals by introducing specific gRNAs to delete or disrupt a putative enhancer sequence. Moreover, by using a selectable phenotype—such as cell growth, drug resistance, or expression of a fluorescently tagged protein—paired with NGS detection of guides enriched in a selected population, this method can be scaled to screen genomic loci en masse (45–47). Though

CRISPR deletions are a powerful tool, certain caveats apply: results can be skewed by large genomic lesions, cell cycle arrest, or other cellular defects induced by the DNA damage response (48–50).

To avoid issues associated with DNA damage, the use of dead Cas9 (dCas9), which lacks endonuclease activity, has gained popularity. The dCas9 protein can be fused to elements such as the Krüppel-associated box (KRAB) domain to block enhancer activity at a specific locus by depositing repressive histone 3 lysine 9 trimethylation (H3K9me3) (51, 52). This method, known as CRISPR interference (CRISPRi), has been used for phenotypic screens in cell lines by selecting for gRNAs that influence cell growth rate, confer drug resistance, or induce fluorescent reporter gene expression (24, 44, 53). Both CRISPR knockout and CRISPRi can also be paired with single-cell RNA sequencing (scRNA-seq) to further increase throughput (54, 55), offering a promising approach to functionally test the hundreds of thousands of potential mammalian enhancers identified by ENCODE (15, 16).

Single-cell RNA sequencing (scRNA-seq): method that captures and amplifies RNAs from single cells, allowing for NGS-mediated definition of expression profiles for individual cells within a population

DNase I–hypersensitive site sequencing (DNase-seq): DNA sequencing technique using sensitivity of DNA regions to cleavage by DNase to identify accessible chromatin regions of the genome

Assay for transposase-accessible chromatin using sequencing (ATAC-seq): DNA sequencing technique using accessibility of DNA to Tn5 transposase to identify chromatin regions that lack nucleosomes

Mixed-lineage leukemia (MLL) complexes: a conserved family of multisubunit complexes containing histone methyltransferases that modify H3K4 at promoters and enhancers

MOLECULAR FEATURES OF AN ACTIVE ENHANCER

Chromatin Signatures of Enhancers

In the absence of facile, high-throughput functional assays for mammalian enhancer activity, the field has turned toward approaches to predict enhancer loci on the basis of characteristic genomic features (**Figure 1**). The binding of chromatinized DNA by pioneer factors (6, 7) facilitates the binding of other TFs through the recruitment of chromatin-remodeling machines, stimulation of DNA breathing off the nucleosome surface, or cooperative DNA binding (5, 6). The resulting region of nucleosome-depleted DNA allows for detection of the region by DNase I–hypersensitive site sequencing (DNase-seq) or assay for transposase-accessible chromatin using sequencing (ATAC-seq) (56, 57). TF binding is also accompanied by the recruitment of the transcriptional coactivators p300 and CBP (14, 58) and the Mediator complex (59).

Active enhancers are enriched for several chromatin modifications, such as H3K27ac, mediated by p300 and CBP, and H3K4 methylation (58, 60). H3K4 can be differentially methylated by a family of mixed-lineage leukemia (MLL) complexes, with monomethylation (H3K4me1) more common at enhancers and trimethylation (H3K4me3) enriched at promoters (58, 60–62). As a result, the ratio of H3K4me1 to H3K4me3 has been used as a means to distinguish enhancers from promoter loci. However, this method of enhancer identification is problematic since H3K4 methylation typically correlates with transcription activity, such that highly active enhancers display H3K4me3 and lowly active promoters exhibit H3K4me1 (63–66). Furthermore, several studies have demonstrated that enhancers may retain H3K4me1 after inactivation (67, 68), rendering it a poor marker of the current enhancer landscape in mature cell types.

Although chromatin marks can be useful for identifying putative enhancer regions, these identification methods tend to overestimate the number of true enhancers. Several computational approaches have attempted to gather published data sets from multiple cell types and fit them to statistical models to predict active enhancers (69) and their promoter targets (70, 71). These approaches have proven that no one genomic feature is highly predictive of an active enhancer, stressing the need for supporting data from multiple assay types—such as chromatin immunoprecipitation followed by sequencing (ChIP-seq) of H3K27ac plus nascent RNA sequencing (RNA-seq) to identify enhancer transcription—to improve predictions (70). For example, an estimated 40–50% of candidate enhancers predicted by multiple chromatin signatures do not stimulate reporter gene expression in the anticipated tissue or condition (72). More strikingly, whereas CRISPR methods paired with scRNA-seq have confirmed that H3K27ac, p300, and RNA polymerase II (Pol II) occupancy faithfully mark active enhancer sites, a comprehensive study detects enhancer activity at

<10% of sequences predicted to be enhancers (55). It is not yet clear whether this low positive rate is driven primarily by the poor predictive potential of chromatin marks, by a high occurrence of redundant enhancers that can compensate for lost activity (17, 18), or by weaknesses in current technical approaches (73, 74). However, in human embryonic stem cells, just 12% of H3K27ac peaks function as enhancers in plasmid-based MPRAs (33), indicating that only a fraction of predicted enhancers possess validated enhancer activity. Therefore, despite the widespread utility of enhancer prediction tools like ChromHMM (69), their output still requires careful manual curation and rigorous functional validation.

Nonchromatin Features of Enhancer Regions

A subset of enhancers are also bound by CCCTC-binding factor (CTCF) and cohesin, which have been proposed to aid in communication between an enhancer and its target promoter by stabilizing looping interactions. However, CTCF infrequently contributes to enhancer–promoter interactions (75), and ablation of cohesin-mediated looping has only minor effects on the maintenance of gene expression (76–78). Thus, the CTCF and cohesin proteins appear to play a more structural role in genome organization and have less of a direct effect in facilitating transient, small-scale promoter–enhancer interactions. Recently, the broadly expressed zinc finger TF YY1 has been suggested to mediate enhancer loops since it is enriched at enhancer–promoter contacts (79, 80). Interestingly, rapid loss of YY1 achieved by using a degron approach yielded decreased contact frequencies for selected enhancer–promoter pairs (80), but more work is needed to determine whether these YY1-mediated contacts are supportive of enhancer function.

TRANSCRIPTION AT ENHANCERS

Discovery of and Relationship Between Enhancer Transcription and Activity

Early studies of the β-globin locus control region and other enhancer loci showed that transcription from the DNase I–hypersensitive sites was concurrent with enhancer activity (81–84). Subsequent Pol II ChIP-seq revealed considerable Pol II occupancy of intergenic loci, suggestive of transcription at *cis*-regulatory elements (85). It was not until more recently, however, that transcription was appreciated as a general feature of active enhancers (10, 11, 86, 87). Enhancer RNAs (eRNAs) are short (typically <200-nucleotide-long) RNAs that are transcribed from the area of open chromatin that results from TF occupancy of enhancer regions (10, 65, 88, 89). eRNAs often initiate at the edges of the nucleosome-depleted regions, with many enhancers being transcribed divergently (**Figure 1**). Most eRNAs are not spliced or polyadenylated (10, 88), making them targets for rapid degradation by the exosome (90).

The instability of eRNAs makes them difficult to detect reliably in steady-state RNA-seq. Therefore, methods for nascent RNA-seq such as global run-on sequencing (GRO-seq) (91), precision run-on sequencing (PRO-seq) (92), and Start-seq (93, 94) are used for eRNA detection (64, 65, 95–97). These methods isolate RNAs during the act of transcription, while they are still engaged with Pol II and are protected from degradation. Evaluation of nascent RNAs has shown that eRNA transcription is activated within minutes of cell signaling cues (86, 87) and that the level of nascent RNA transcription at a locus correlates well with the activity of the enhancer as defined in reporter assays (65). These assays also provide a sensitive approach for identifying enhancers. A recent study in *Drosophila* S2 cells, where the full repertoire of active enhancers has been defined using MPRAs, found that ∼94% of enhancers undergo transcription (65) that is detectable with Start-seq (94). Nonetheless, not all intergenic transcription represents enhancers. The above study found that only ∼50% of unannotated transcription start sites (TSSs) corresponded to

Global run-on sequencing (GRO-seq): cell nuclei are isolated, and transcription is resumed in the presence of labeled nucleotides; labeled RNAs are then enriched and sequenced to determine regions of active transcription genome-wide

Precision run-on sequencing (PRO-seq): modification of GRO-seq wherein transcription is resumed with biotinylated nucleotides; Pol II adds one nucleotide before stopping, which enables nucleotide-level resolution of actively engaged Pol II

Start-seq: chromatin-associated or nuclear RNA is isolated and enriched in 20–80-nucleotide-long TSS-associated RNAs; RNAs with 5′ caps are isolated and sequenced to define TSSs at single-nucleotide resolution

Transcription start site (TSS): the position at which transcription initiates, corresponding to the 5′ end of the RNA product

enhancers (65), in line with other work (89). Despite this caveat, using a nascent transcription assay to identify putative enhancers yields fewer false negatives than using the repertoire of ChIP-seq data sets needed for ChromHMM or similar computational predictions (and a comparable number of false positives) (65, 69, 70, 96, 98). Indeed, putative enhancers with clear eRNA transcription were found to be two to three times more likely to show activity in reporter assays than sequences identified by histone marks alone (90). These promising results, coupled with the high sensitivity and resolution provided by Start-seq and PRO-seq, are making nascent RNA approaches increasingly popular methods for enhancer identification (96, 98).

General transcription factor (GTF): part of the basal transcriptional machinery that binds to promoters and is necessary for efficient, accurate transcription initiation

Evaluating Potential Functions for Transcription at Enhancers

Although the presence of eRNAs has proven to be a strong indicator of enhancer activity, it has been challenging to define clear roles for eRNAs in enhancer function. Due to the poor sequence conservation, short length, and rapid degradation of most eRNAs, models postulating specific protein binding partners or activities in *trans* are unlikely to be broadly applicable (99, 100). A central question in the field is thus, what function(s), if any, might be served by enhancer transcription? On one hand, due to the generally accessible nature of DNA at active enhancers and the promiscuity of Pol II initiation, it has been speculated that transcription could merely be an accidental by-product of TF binding that is tolerated by the cell (101). On the other hand, the presence of core-promoter-like motifs and the recruitment of the general transcription factors (GTFs) and Mediator at enhancers suggest coordinated activation of transcription at enhancers that is similar to that observed at promoters (65, 88, 102). Enhancer transcription is also regulated at the level of pause release, which is not consistent with spurious or uncontrolled transcriptional activity (65, 103). Thus, despite an absence of evidence for sequence-specific functions of eRNAs, the existing data suggest that the act of transcription itself could facilitate enhancer activation (100, 104–107).

One straightforward prediction is that transcription at enhancers will influence local chromatin structure. Transcription elongation across a region disrupts nucleosome occupancy (108), and Pol II pausing just downstream of TSSs protects the region from nucleosome assembly (65, 109), helping to maintain accessible chromatin (**Figure 3*a***). Moreover, a large number of protein factors interact with and are stabilized by Pol II and the general transcription machinery, including TFs and Mediator (110). The C-terminal domain of the largest Pol II subunit consists of a highly conserved YSPTSPS repeat motif that serves as a landing pad for a multitude of TFs and epigenetic regulators. Therefore, the association of factors with enhancer regions, stimulated by interactions with the Pol II C-terminal domain, could contribute to a local environment supportive of transcriptional activation (**Figure 3*b***).

In particular, histone-modifying enzymes including acetyltransferases and methyltransferases interact with Pol II and deposit active chromatin marks during transcription elongation (5) (**Figure 3*c***). The proteins p300 and CBP, which are the primary acetyltransferases responsible for H3K27 acetylation at metazoan enhancers, associate with actively transcribing Pol II (14, 62). Mammalian complexes responsible for H3K4me1, the MLL complexes MLL3 and MLL4, have also been found to specifically localize at transcribed enhancers and deposit H3K4 methylation along the transcription unit (111, 112). However, in this context, whereas the MLL3 and MLL4 complexes are essential for enhancer maintenance and function, the H3K4me1 mark is largely dispensable (113, 114). Expression of enzymatically dead MLL3 and MLL4, or the *Drosophila* counterpart Trr, abrogates H3K4me1 without effects on eRNA production or gene expression (113, 114). Consequently, the critical role for MLL3 and MLL4 in gene regulation is not the methyltransferase activity.

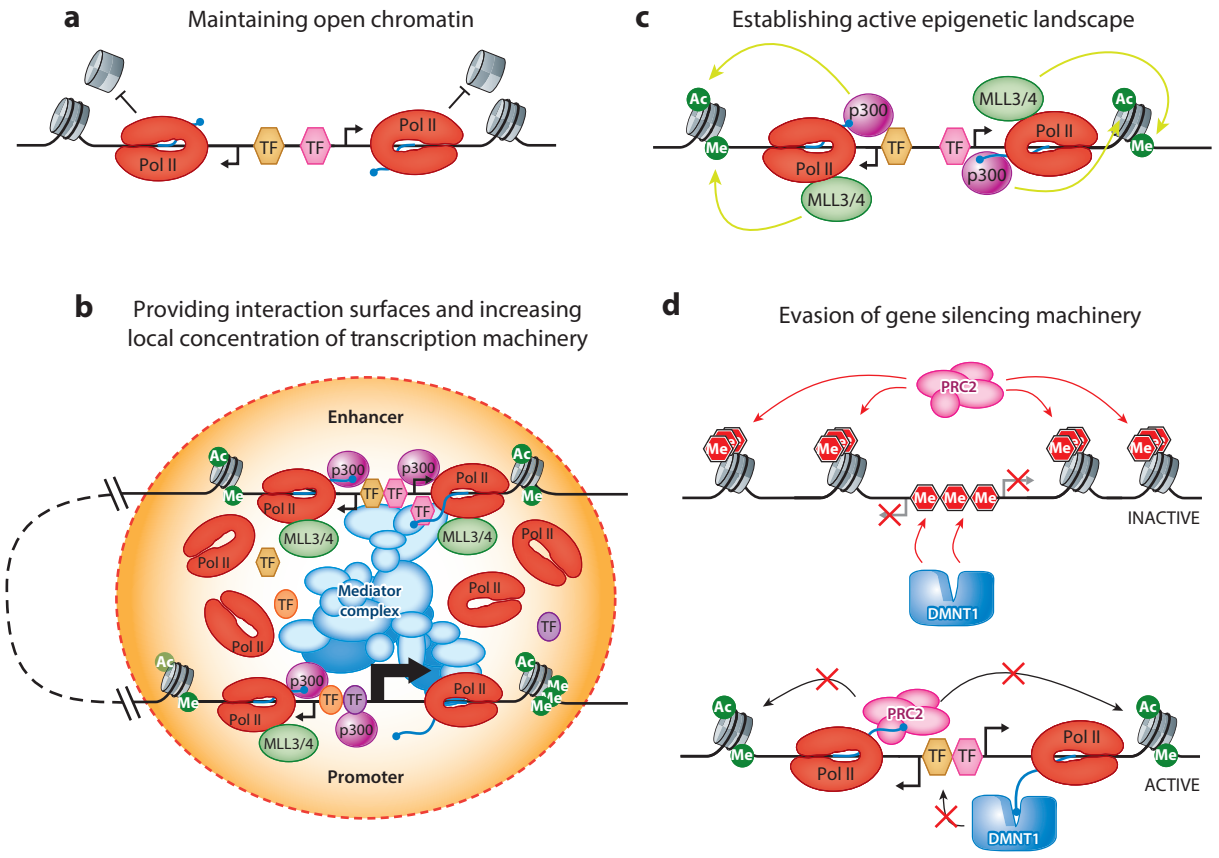

Figure 3

Models for the function of transcription at enhancers. (*a*) Transcription elongation by Pol II (*red*) could evict nucleosomes locally, and Pol II pausing prevents reassembly, serving to maintain accessible chromatin. (*b*) The presence of the transcription machinery and RNA at enhancers provides numerous surfaces for stabilizing interactions with TFs and coactivators. This could serve to create a high local concentration of factors (*orange oval*) that is favorable to transcriptional activity at the target promoters. (*c*) Complexes with acetyltransferase (e.g., p300 and CBP; *purple*) or methyltransferase (e.g., MLL3 and MLL4; *light green*) activity associate with the elongating Pol II. The act of transcription could serve to spread activating marks (Ac of histone H3 lysine 27 and Me of histone H3 lysine 4; *dark green*) across the enhancer region. (*d*) RNA binding inhibits repressive machinery. Without transcription (*top*), PRC2 (*pink*) deposits repressive Me on histone H3 lysine 27 (*red*) and DMNT1 methylates DNA (*red*). When enhancers are transcribed (*bottom*), nascent RNA could inhibit both PRC2 and DMNT1 activity, preventing the accumulation of negative regulatory marks. Abbreviations: Ac, acetylation; CBP, CREB-binding protein; DMNT1, DNA methyltransferase 1; Me, methylation; MLL, mixed-lineage leukemia; Pol II, RNA polymerase II; PRC2, polycomb repressive complex 2; TF, transcription factor.

Epigenetic regulators also interact in a sequence-independent manner with nascent RNA in mammalian and invertebrate systems, with RNA binding serving to alter protein localization or catalytic activity. These data offer tantalizing evidence that the act of transcription could directly influence the assembly and activity of transcription cofactors at enhancers. For example, binding of RNA by CBP stimulates its acetyltransferase activity in vitro, and there is evidence that RNA at enhancers and promoters could regulate activity in vivo (115). Likewise, RNA binding by factors that deposit repressive histone and DNA modifications—polycomb repressive complex 2 (PRC2) and DNA methyltransferase 1 (DNMT1), respectively—inhibits their catalytic activity (**Figure 3*d***). As a result, the generation of RNA at enhancers could protect them against

repression (116–118). In fact, PRC2 target sequences integrated into RNA-rich environments between active enhancers and promoters are protected from repressive H3K27 methylation (119). This protection was lost under conditions where the enhancer and promoter were no longer transcriptionally active. Similarly, PRC2 binding to the genome increases globally upon chemical inhibition of Pol II (120), consistent with a model of active transcription preventing PRC2's repressive function. Finally, most DNA-binding TFs also have affinity for RNA, such that RNA production from an enhancer might provide additional, low-affinity binding sites that help retain TF occupancy in the region (**Figure 3*b***). A particularly appealing example of this comes from YY1, which has separable RNA- and DNA-binding surfaces (79). Intriguingly, RNA binding by YY1 improves DNA binding, suggesting coordination between these interfaces (79).

SIMILARITIES BETWEEN GENE PROMOTERS AND ENHANCERS

Classically, a promoter is defined as the core regulatory sequences that are necessary for the assembly of the transcription machinery and initiation of RNA synthesis (121). The core promoter, also known as the minimal promoter, encompasses ∼50 bp on either side of the TSS. This region contains numerous sequence motifs such as the TATA box, Initiator, and downstream promoter element that facilitate recruitment of the GTFs and Pol II (121, 122). These motifs are fairly well defined in *Drosophila*, where many of them were first described, but they are more degenerate in mammals (121, 122).

The proximal enhancer is the region just upstream of the core promoter that contains binding sites for specific TFs that are needed for gene regulation. The TF-binding sequences in this region are often highly conserved across evolution, strongly matched to the optimal consensus motifs, and tightly spaced or even overlapping (121, 123, 124). This latter feature leads to a highly concentrated binding platform for TFs, within ∼200 bp upstream of the TSS (125), that stimulates local recruitment of the transcription machinery. Thus, the region near the TSS contains two distinct segments with distinct properties: a core promoter that specifies where transcription will initiate and a proximal enhancer region that stimulates and regulates transcription activity at this locus.

Enhancers were long appreciated to harbor a number of TF binding sites, but these were assumed to function only toward recruiting the transcription machinery at the distal promoter. However, the fact that enhancers are generally transcribed by Pol II has changed the prevailing views on this subject (10, 11), blurring the distinction between enhancers and promoters. Indeed, transcription at enhancers arises from core-promoter-like motifs (64, 65, 126) and involves recruitment of the full repertoire of general transcription factors (65, 102). However, the core promoter elements and TF motifs at enhancers are more degenerate from consensus sequences than those at mRNA promoters (124, 127, 128), and enhancer regions are much more rapidly evolving than gene promoters (129, 130). Notably, it has been suggested that the suboptimal nature of TF motifs at enhancers is important for the specificity of activity, since this feature obligates cooperation between TFs for chromatin opening and stable binding (124, 127, 128).

As might be anticipated given the similarities of the sequences at promoters and enhancers, these regions share many molecular features, including accessible chromatin flanked by active histone modifications; the recruitment of transcriptional coactivators like Mediator, p300, and CBP; and active transcription (126). These similarities have led to suggestions that we revise our strict definitions of promoters and enhancers as discrete entities (126). Such suggestions have gained recent popularity, spurred by the discovery that many gene promoters can, in fact, act as enhancers for other genes (38, 47, 97, 106, 131, 132). This basic principle was reported many years ago with some of the first reports of enhancer activity originating from promoter loci (1). Thus,

since enhancer and promoter activity frequently overlap, it may be difficult to disentangle the sequence requirements for each.

The breadth of this enhancer-like promoter (E-promoter) phenomenon has been revealed by several genomic techniques. Many promoters display enhancer activity in reporter assays (14, 38, 40, 97, 132), affect other genes in *cis* when disrupted with CRISPR (45, 47, 106, 132), and participate in 3D looping interactions with other promoters (132, 133). Moreover, many tested ∼200-bp sequences could exhibit both promoter and enhancer activities in reporter assays (131). Enhancers are generally more weakly transcribed than promoters, in agreement with their weaker core promoter motifs, but the strength of transcriptional activity is often correlated with enhancer activity within each class (65, 97, 131). When comparing enhancers with E-promoters, however, the two perform similarly in enhancer reporter assays (131, 134). Taken together, these results suggest that sequences that assemble a collection of TFs—like enhancers and proximal promoter regions—have an inherent tendency to drive nearby transcription initiation. Strong core promoter motifs in the vicinity might enable high-level transcription, whereas more degenerate motifs would yield the less efficient transcription initiation characteristic of enhancers. There is therefore great interest in defining the relationship between the enhancer and promoter potential of individual sequences and determining whether these activities are truly separable.

ENHANCER–PROMOTER COMMUNICATION

Enhancers are thought to communicate with promoters through looping interactions between chromatin domains, which bring the enhancer and its target promoter within close proximity in 3D space. However, the mechanism, duration, and timing of such contacts with respect to gene activity are still debated (72, 135). This controversy is complicated by inconsistencies in the definitions of chromatin structural features based on 3C-based methods and fueled by the scarcity of single-cell imaging data that document sustained interactions between promoters and enhancers.

The largest scale of defined chromatin organization is that of compartments. Compartments are higher-order structures, on the scale of megabases, of generally active euchromatin in the A compartment and inactive heterochromatin in the B compartment (136). This large-scale organization is dynamic during cell differentiation (137), with chromatin regions transitioning from A to B compartments and vice versa. However, chromatin domains at the megabase scale, which include hundreds of genes, are not proposed to directly influence smaller-scale interactions at individual enhancers and promoters. At the next level of organization are topologically associated domains (TADs), which are cohesin- and CTCF-associated substructures whose boundaries divide and segment regions of the genome (72, 135). Unlike compartment structure, however, TAD structure is largely invariant across cell types (76, 137), rendering these domains insufficient to explain the highly dynamic nature of enhancer activity in different cell states. Moreover, within a single TAD, enhancers and promoters can vary widely in activity level, arguing against domain-wide regulation of gene expression. As an example, CTCF-binding sites facilitate long-range enhancer–promoter interactions within the well-characterized *Shh* locus. However, inserted regulatory sensors across the region revealed that the ZRS enhancer had nonuniform activity within the TAD (138), indicating that enhancer–promoter specificity depends on more than proximity within a shared TAD.

Importantly, CTCF-binding sites at TAD boundaries can serve as insulators that restrict enhancer interactions to target genes within the same domain (72, 135, 139). It is clear, however, that not all CTCF-binding sites exhibit this insulator activity; recent studies have reported mixed results on whether nearby gene expression is changed upon removal of CTCF-binding sites between enhancers and nontarget genes (140, 141). These studies highlight that there is still much to be learned about CTCF-mediated boundary function and demonstrate that there is more to insulator

function than CTCF occupancy. Perhaps the most striking recent results are those wherein rapid removal of CTCF, the cohesin subunit RAD21, or the cohesin loader NIPBL completely ablates TAD structures but has only minor effects on steady-state gene expression and enhancer activity (76–78). Whereas it remains possible that CTCF and cohesin play a role in the establishment of newly activated enhancers under dynamic conditions (142), the field is coming to appreciate that cohesin and CTCF-mediated TADs are generally not sufficient to explain enhancer specificity, nor are they necessary for the maintenance of enhancer activity.

As noted above, TADs are thought to be established early in development and remain fixed thereafter (137), though several studies have argued some degree of TAD plasticity (23, 25, 143, 144). These contacts tend to be shorter in length and lower in frequency than the strong TAD boundaries described in most studies, leading some to refer to such interactions as sub-TADs (135). Indeed, a recently developed technique with subkilobase resolution termed Micro-C has revealed short-range chromatin contacts that can occur in a CTCF-dependent or CTCF-independent manner and appear more cell type specific than traditional TADs (145, 146). Thus, the development of higher-resolution assays may provide new information on enhancer–promoter interactions. It seems possible, however, that enhancer–promoter interactions are far more transient and stochastic than 3C-based methods could efficiently detect. In this view, the population averaging of these strategies makes them ill-suited for observing rare and highly dynamic structures. Accordingly, both microscopy-based and sequencing-based single cell Hi-C studies have failed to support that TADs form consistent, stable structures detectable in individual cells (147–150).

Several recent live-cell imaging studies have worked to address the dynamics of enhancer–promoter communication. This work has revealed that active gene and enhancer regions, containing decondensed chromatin, are often more mobile and dynamic than inactive regions. For example, recently developed dCas9-based live-cell imaging strategies that monitor an enhancer as it is activated during cellular differentiation found that the observed rate of enhancer diffusion is increased during activation, as is the spatial territory it explores (151). These studies argue against a view that stable, persistent contacts are formed between enhancers and promoters. Instead, the data suggest a mixing model whereby, upon chromatin opening, the enhancer and promoter regions increase their diffusion rates, allowing for more frequent, transient contacts between these elements (151).

In support of highly dynamic enhancer–promoter interactions, live-cell imaging of labeled Pol II has revealed that clustering of the polymerase near the promoter is extremely transient, on the order of seconds (152). This is consistent with a model of transcriptional bursting wherein the transcription machinery rapidly accumulates at the locus and Pol II is fired into the gene in sporadic surges. That bursts of transcription result from transient enhancer–promoter interactions rather than long-lived contact is supported by studies measuring transcription burst frequency and enhancer–promoter interactions in *Drosophila* (153). These studies found that enhancer strength and transcriptional output are connected to the bursting frequency and not the duration of enhancer–promoter interaction (153). This is consistent with computational modeling of scRNA-seq data in mouse cells, which predicts that promoters control transcriptional burst size while enhancers largely affect burst frequency (154). Intriguingly, Fukaya and colleagues (153) also showed that the same enhancer can target two genes concurrently, further challenging the notion of stable enhancer looping to one target promoter at a time. Another live-cell imaging study of reporter gene expression in the even-skipped (*eve*) locus in *Drosophila* demonstrated that transient enhancer–promoter proximity, on the order of minutes, is correlated with gene activity (155). Interestingly, the lifetime of enhancer–promoter colocalization increases when transcription is activated, supporting a model wherein the high local concentration of TFs at promoters and enhancers that accompanies bursts of transcription facilitates transient enhancer–promoter communication

(156). At this point, only a handful of enhancer–promoter interactions have been closely evaluated with live-cell imaging methods, and none at endogenous loci. Thus, the general mechanism and dynamics of enhancer–promoter communication remain to be fully elucidated. Further development of these technologies will be key for understanding the functional enhancer–promoter interactions that govern gene regulation.

CONCLUSIONS AND FUTURE PERSPECTIVES

The field has made great progress toward detecting and identifying putative enhancers; however, we still lack the ability to accurately predict which regions will have enhancer activity in a specific cell type, condition, or tissue. Although using a combination of chromatin signatures, transcription activity, and chromatin interaction maps improves our predictive power, these combinatorial approaches suffer from a high rate of false positives (69–71). We suggest that some of the complications associated with these approaches stem from the fact that such predictions rely on chromatin characteristics that are not unique to enhancer elements. In particular, careful analysis of chromatin and transcriptional signatures across regulatory elements has revealed that many features of active enhancers are shared with weak promoters (64, 65, 96, 126).

Without a comprehensive molecular definition, functional assessment of putative enhancer sequences is vital to ensure their validity and designate their boundaries. Reporter assays can define sequences that are sufficient to drive tissue-specific transcriptional regulation. Perturbation of a locus (e.g., by using CRISPR), on the other hand, can identify sequences that are necessary for gene expression in the endogenous context and pinpoint genic targets of enhancer activity. Future advances are anticipated to increase the throughput of such assays, which is needed to evaluate the hundreds of thousands of predicted enhancer elements identified in mammalian genomes. In this regard, massively parallel approaches such as MPRAs and CRISPRi screens have shown great promise, paving the path toward large-scale validation of predictions based on genomic features.

The mechanisms by which enhancers communicate with promoters and the ways in which promoter target specificity is defined have also remained elusive. Current data paint a picture of active enhancer and promoter elements diffusing and exploring their local chromatin environment, allowing for short-lived contacts between these loci (153, 156, 157). Proximity between a promoter and an enhancer, both bound by a repertoire of TFs and coactivators, could lead to sharing or exchange of protein and RNA species between the regions (**Figure 3*b***). Intriguingly, this high local concentration of factors with compatible activation domains has been suggested to enable the formation of higher-order assemblies, perhaps triggering a transient phase separation (156, 158) that stimulates gene activity. Testing of these models and others will be an active area of research for many years to come.

The rapid pace of technology development in live-cell imaging, genome engineering, protein perturbation, and organoid growth is enabling detailed mechanistic investigations into enhancer activities. This work holds the promise of providing novel insights into how enhancers function in their endogenous context, advancing us toward the goal of enhancer manipulation in disease states or defective developmental processes. In particular, such approaches are enabling the field to probe how enhancers are selected and activated in differentiating or signal-responsive systems. To deduce the required molecular components of enhancer assembly, we can now turn to reductionist biochemistry-inspired approaches in vivo. The development of specific small-molecule inhibitors (159, 160) or protein degradation (e.g., degron) systems (161–163) allows for the rapid inactivation or removal of specific proteins and activities. These approaches are powerful in that they allow for the evaluation of enhancer function just minutes to hours after perturbation to avoid potential secondary effects. In this way, the immediate effect of losing a critical enhancer component can

be accurately evaluated. By pairing this approach with recently developed high-resolution live-cell microscopy to observe enhancer activation in real time, detailed mechanistic dissection of enhancer components may soon become possible.

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