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Transcriptional Regulation by (Super)Enhancers: From Discovery to Mechanisms

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Abstract

Accurate control of gene expression in the right cell at the right moment is of fundamental importance to animal development and homeostasis. At the heart of gene regulation lie the enhancers, a class of gene regulatory elements that ensures precise spatiotemporal activation of gene transcription. Mammalian genomes are littered with enhancers, which are frequently organized in cooperative clusters such as locus control regions and superenhancers. Here, we discuss our current knowledge of enhancer biology, including an overview of the discovery of the various enhancer subsets and the mechanistic models used to explain their gene regulatory function.

INTRODUCTION

Molecular biologists have been captivated by how gene expression is regulated ever since Jacob & Monod (71) laid the foundation for our current thinking on transcription control by DNA-binding proteins. This fascination with gene regulation exists for good reasons, since accurate control of how genes are switched on and off in the right cell at the right moment is critical for proper organismal development and homeostasis. In addition, a loss of appropriate regulation of gene expression is a major underlying cause of human disease, emphasizing the importance of studying the basic mechanisms of transcription control (82).

The transformative power of gene regulatory processes is illustrated in **Figure 1**. While neurons, lymphocytes, and macrophages express many genes in a similar manner (referred to as housekeeping genes; genes A and B in **Figure 1**), they each also have a unique transcriptomic signature (genes C–F in **Figure 1**). These cell-type-specific gene expression programs allow for the synthesis of a tailor-made proteome and the phenotypic diversity displayed by these cells. At the same time, aberrant modifications to cell-type- or state-specific transcriptional modules can result in cellular dysfunction and disease (**Figure 1**).

Cell-type-specific gene regulation is established and controlled by transcription factors (TFs), a diverse class of DNA-binding proteins (reviewed in 81, 140). The human genome encodes 1,639 (putative) TFs, approximately half of which are expressed in a tissue-specific manner (81). Many TFs are responsive to microenvironmental signals, and the combined activity of TFs expressed in a particular cell type will dictate a unique gene expression program. The power that TFs command over a cell's phenotype is perfectly illustrated by numerous transdifferentiation and cell reprogramming experiments in which the mere introduction of one or several ectopic TFs induces dramatic phenotypic changes (3, 49).

TFs control gene activity by binding to gene regulatory elements (GREs) scattered across the noncoding genome (**Figure 2**). TFs recognize specific short (6–12-base-pair) DNA motifs, which are enriched in GREs (81). Bound at these elements, TFs in turn recruit chromatin-modifying

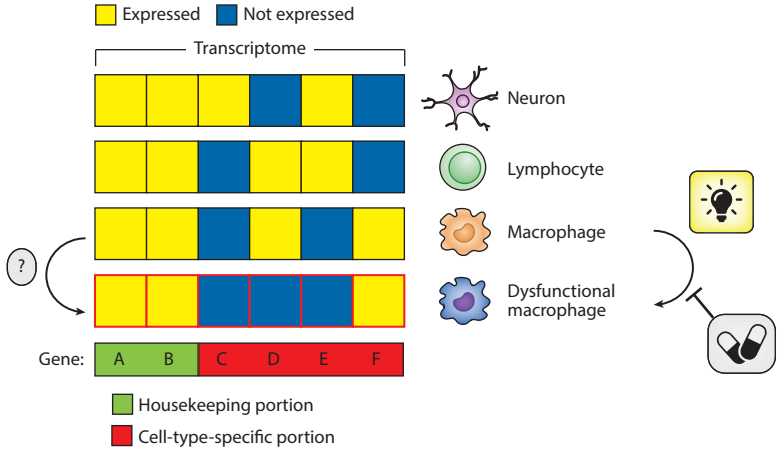

Figure 1

Cell-type-specific gene expression and cellular phenotypic diversity. Neurons, lymphocytes, and macrophages express shared (housekeeping) and unique (cell-type-specific) gene expression programs. Combined, these unique transcriptomes allow for phenotypic diversification. Changes in gene expression can also lead to cellular dysfunction, as exemplified here for macrophages. Insights into how cell-type-specific transcriptomes are controlled or perturbed in disease hold great promise for the development of new therapeutic strategies.

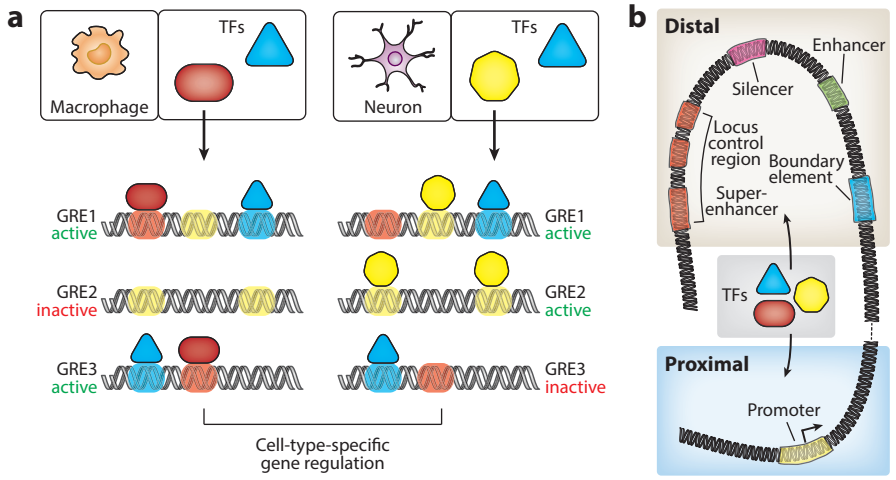

Figure 2

TFs regulating gene expression by binding to gene regulatory elements. (*a*) Macrophages and neurons produce specific combinations of TFs. TFs bind their DNA recognition motifs within GREs in a combinatorial fashion, resulting in a cell-type-specific activity pattern of GREs. As a consequence, each cell type is able to generate a unique gene expression profile. (*b*) GRE subclasses to which TFs are recruited can be divided into two broad categories: proximal (<1 kb from the transcription start site) and distal (>1 kb from the transcription start site). Proximal GREs are located within the promoter region. Abbreviations: GRE, gene regulatory element; TF, transcription factor.

proteins and the basal transcription machinery to modulate gene transcription. Chromatin and TFs engage in dynamic crosstalk: While TFs can penetrate nucleosome-dense regions to establish an active chromatin landscape, certain histone or DNA modifications and nucleosome occupancy can also directly prevent TF binding (141). Several classes of GREs have been identified, most notably promoters and enhancers (108). Promoter elements are located around the transcription start sites of genes and include the genomic position on which the RNA polymerase II complex is assembled to initiate transcription (83). While TFs frequently bind gene-proximal promoter sequences, they much more frequently bind to distal enhancer elements. Bound at enhancers, which can be located more than 1 Mb away from their cognate target gene, TFs are able to promote gene transcription from a linear distance (41). Worth mentioning is that while promoters and enhancers are often regarded as separate regulatory entities, the functional distinction between them became substantially more blurry with the identification of enhancers acting as promoters (78) and vice versa (26). Other distal GREs include silencers, which are a still poorly characterized class of transcriptional repressor elements (56), and boundary elements (also referred to as insulators), which are thought to restrict enhancer–promoter communication (108).

In this review, we focus on the enhancer class of GREs. We start by discussing the initial discovery of enhancers and their subsequent diversification into subclasses, putting special emphasis on locus control regions (LCRs) and superenhancers. Subsequently, we review our knowledge of the mechanisms of enhancer action and how the field has now converged on a hub–condensate model for transcriptional regulation. Finally, we identify key questions that remain to be addressed in future research.

THE DISCOVERY OF ENHANCERS

Enhancers are short (~100–300-base-pair) DNA elements that are located upstream or downstream from or within the genes they regulate. The first enhancer activity was reported in 1980

by Capecchi (17), who carried out a series of experiments in which he injected the thymidine kinase (TK) gene into the nuclei or cytoplasm of mouse TK^- cells. He showed that 0.1–0.2% of the nucleus-injected cells survived in a hypoxanthine-aminopterin-thymidine (HAT) medium, which selects for TK activity, but that this frequency increased to approximately 20% of the cells when he included specific SV40 (enhancer) sequences in the same TK gene–containing plasmid. His working hypothesis as to why these viral SV40 sequences enhanced transformation frequency was that they increased the integration of the TK plasmid into the host genome, since SV40 sequences contain palindromic or repetitive sequences that share homology with sequences in the host genome.

One month later, this study was followed by a report where the authors tested the transcription regulation of a sea urchin H2A histone gene by microinjection into *Xenopus* oocytes (50). They showed that deletion of a 300-base-pair fragment located just upstream of the TATA box promoter, which they termed the modulator element, lowered the level of H2A transcription 15–20-fold. To their surprise, inversion of this modulator element—located at a "considerable distance" (50, p. 7102) from the transcription start site—still resulted in stimulation of H2A gene transcription. To explain these results, the authors suggested that the injected DNA was assembled into active chromatin—in contrast to the in vitro transcription systems in use at that time. This difference could explain why modulator activity had not been observed in vitro. They further suggested that the assembly into chromatin may bring GREs together despite being located "far apart" (50, p. 7106) on the linear genome, thereby introducing the idea of looping between the regulatory elements of eukaryotic genes (although they did not use the term looping).

A substantial number of groups following on from the in vitro transcription studies that defined the promoter elements of eukaryotic genes noticed that the expression of genes in vitro and in vivo gave different results. In particular, the expression of gene constructs containing only the gene's promoter sequence was very inefficient. Part of this puzzle was solved shortly after by the correct identification and interpretation of the function of the SV40 viral enhancer (6, 28, 102). Banerji et al. (6) coupled the SV40 enhancer to a β-globin gene and showed that the addition of the enhancer, independent of its orientation relative to the gene, increased β-globin gene expression 200-fold in transient transfections in HeLa cells. De Villiers & Schaffner (28) coupled a similar fragment from the polyoma virus, albeit with a different sequence, to a β-globin gene and showed the same effect. Similar to Gruss et al. (52), Moreau et al. (102) showed that deletion of the 72-base-pair repeat of the SV40 enhancer dramatically lowered the expression of the SV40 T antigen gene, and they went on to also show that coupling this enhancer to an ovalbumin or adenovirus-2 major late gene promoter caused a dramatic increase in gene expression. These landmark papers therefore established that different enhancers exist and that these elements enhance the transcription of a gene in *cis*, independent of its orientation and position relative to the gene. Additionally, Banerji et al. (6) had already speculated about TFs binding to the enhancer element and the occurrence of gene- or tissue-specific enhancers, while Moreau et al. (102) introduced the idea that an enhancer may act as an RNA polymerase entry site from which the polymerase would move to the gene in *cis* [also referred to as tracking; see the section titled (Super)Enhancers and Models for Their Function]. These classic studies firmly established the concept of the enhancer, and their discoveries provided the basis for what has become an entire field of research, which ranges from unraveling the basic mechanisms of transcriptional regulation to understanding diseases caused by nongenic mutations and has yielded biotechnological applications (e.g., gene therapy) (68).

The first review on enhancers appeared in 1983 (73); it primarily discussed viral enhancers and stated that "the presence of enhancer sequences on transposon-like retroviral elements offers a tempting explanation for a wide variety of regulatory events" in eukaryotic cells (p. 313). The authors further speculated that eukaryotic genes may have their own enhancers, referring back to a study by Grosschedl & Birnstiel (50) and the notion of enhancers as an RNA polymerase

entry site proposed by Moreau et al. (102). Importantly, they suggested that "the potential for enhancer sequences to provide clues to an understanding of differential gene activity in both normal and disease states, and their usefulness as experimental tools for examining regulatory systems, will ensure their future importance" (73, p. 314)—a statement that turned out to be quite accurate. Indeed, the first tissue-specific enhancers were reported within the next two months by five different groups, who identified enhancers in the immunoglobulin heavy and kappa light chain loci (5, 46, 97, 116, 120). The discovery of the immunoglobulin enhancers was shortly followed by a whole series of enhancers regulating different types of genes in different tissues (reviewed in 132), and in the next few decades it became clear that there are likely tens of thousands of enhancers in mammalian genomes (see the section titled Enhancer Clustering: A Common Regulatory Scenario). Surprisingly, enhancers were found that are located as far as 1 Mb away from their target gene, residing inside an intron of an unrelated gene (85). Finally, the existence of enhancers was also followed by the identification of silencers, which were thought to represent the opposite of enhancers (12). Silencers also showed tissue-specific activity and appear to be quite abundant (31, 47, 56, 103, 111), although as a class of GREs they remain poorly characterized.

ENHANCERS, CHROMATIN CONTEXT, AND LOCUS CONTROL REGIONS

Like the SV40 enhancer, the first tissue-specific enhancers strongly activated transcription of a linked gene—independent of their orientation and despite the genomic distance separating enhancers from their target genes. However, most of these experiments were carried out by transfection and were therefore outside of the enhancer's native context. In transient transfections, the transferred gene is not incorporated into the genome and is always in an active transcriptional configuration. While stable transfection experiments do involve genomic incorporation of the transgene, the use of a selection marker on the same construct results in maintaining only active configurations. Despite integration in active genomic regions, enhancer-driven expression of model genes in these assays varied and was not as efficient as was observed for the same genes in their in vivo context (18).

Around the same time, several groups developed a novel way of analyzing genes in vivo by injecting purified DNA into the pronuclei of fertilized mouse eggs to establish transgenic mice (13, 48). This technique allowed for the analysis of tissue-specific gene regulation without selection. For example, a hybrid gene consisting of the metallothionein-I gene promoter fused to the coding sequence of a rat growth hormone gene resulted in transgenic mice that expressed growth hormone at different levels (109). Although tissue-specific gene expression could be observed when genes were analyzed in the presence of enhancers identified in cell transfection experiments, transgene expression levels were low and varied substantially among mice carrying the same transgene (19, 57, 138). Part of the discussion regarding why expression was low was the presumed influence of bacterial vector sequences that were part of the injected construct. Nevertheless, similar results were obtained with injected fragments lacking the vector sequences. More importantly, the variable and low expression levels were due to position effects and the inability of an individual enhancer to escape such position effects and promote full transgene expression (see, e.g., 37) (**Figure 3**).

The first report of nearly normal levels of expression was obtained using an α-fetoprotein minigene with 7 kb of flanking DNA sequences (79). The authors concluded that the flanking sequences contained GREs essential for correct tissue- and development-specific expression. However, they did not take into account the transgene copy number and hence did not analyze the level of minigene expression on a per-copy basis. Instead, they focused on the presence or absence of

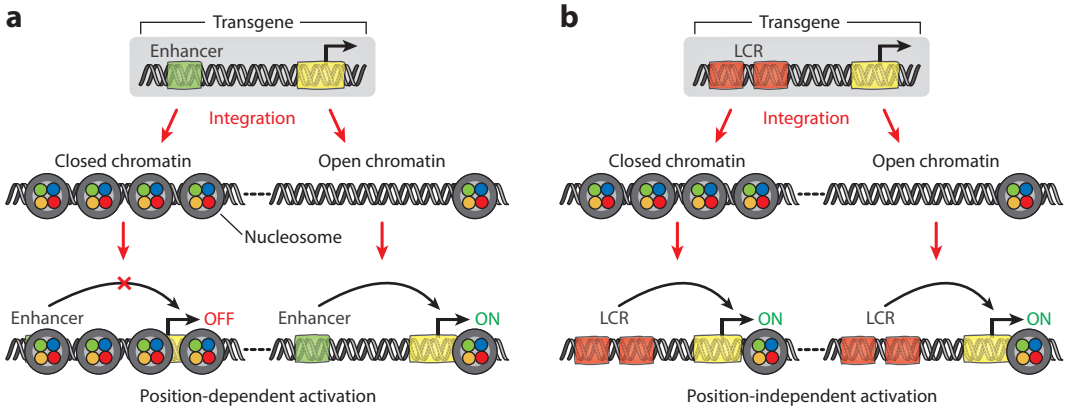

Figure 3

Robust cell-type-specific gene regulation conferred by LCRs. (*a*) When enhancers are randomly integrated into the genome using a transgene, their activity is very sensitive to position effects: If they land in a closed chromatin environment, they are not able to establish robust gene activation. (*b*) By contrast, LCRs can create an open chromatin domain and establish gene activation even when integrated into inaccessible chromatin. This phenomenon of position-independent gene activation is unique to LCRs and is used to operationally define them. Abbreviation: LCR, locus control region.

vector sequences to explain their results, a concern that had been noted by others (19, 57, 138). The results obtained by Townes et al. (138) and Chada et al. (19), which included a previously described enhancer just 3′ of the β-globin gene, as well as results from our own laboratory (76) were very reminiscent of findings obtained from a β-thalassemia patient who displayed an intact β-globin gene but lacked the upstream sequences (74). Hence, a β-globin gene construct with both upstream and downstream sequences, containing a cluster of DNase hypersensitive sites (139), was introduced into transgenic mice and analyzed for expression per copy of the transgene (51). These experiments showed that including the set of enhancers normally located more than 60 kb from the β-globin gene resulted in full tissue-specific expression of the gene. Importantly, β-globin gene expression in these mice was dependent on the copy number but independent of the site of integration in the genome (**Figure 3**), with the notable exception of the inactivated X chromosome (144). Deletion of the upstream sequences resulted in very low β-globin expression. The responsible enhancer cluster became known as the LCR. On the basis of a series of experiments that showed that the individual enhancers of the LCR are functionally distinct (15) but could not provide full expression, it was proposed that the enhancers would work together as one regulatory unit (34, 35, 39)—similar to what was later proposed for superenhancers (see the section titled Superenhancers). Interestingly, the regulatory function of the LCR can be captured in engineered DNA fragments as small as 1 kb (stitching together several of the LCR enhancers) (126), which has enabled the design of efficient gene therapy vectors to treat hemoglobinopathies (137). The discovery of the β-globin LCR was rapidly followed by the description of several other LCR sequences (reviewed in 87).

ENHANCER CLUSTERING: A COMMON REGULATORY SCENARIO

An impressive collection of experimental evidence has firmly positioned enhancers as the central drivers of cell-type-specific gene expression, with the majority of enhancers showing a tightly restricted developmental window of activity (104). The identification and characterization of LCRs provided the first evidence for cooperation among several enhancers to control gene expression. The exact nature of enhancer cooperation—i.e., whether the individual GREs have

additive, redundant, or synergistic effects on transcription—remains controversial (90). For the globin loci, studies in which individual or combinations of LCR enhancers were deleted in vivo point toward additive and partially redundant functions (10, 59). Notably, not all enhancers within an LCR are functionally equivalent, as certain individual elements exert a much stronger effect on expression output than others (e.g., the β-globin HS2 enhancer) (10, 59). This was nicely illustrated by a recent study of the *BCL6* LCR in germinal center B cells (25), in which systematic silencing of LCR components using CRISPR interference separated essential from nonessential constituent enhancer elements. Still, with only a handful of LCRs characterized, it remained unclear how pervasive gene regulation by cooperative enhancer clusters really was: Are only selected genes controlled by multiple GREs, or is this a much more common regulatory theme?

Since the introduction of high-throughput sequencing-based chromatin profiling technologies, in particular chromatin immunoprecipitation and sequencing (ChIP-seq), it has become increasingly feasible to scan an entire genome for regions that show a biochemical signature indicative of regulatory activity. Key components of this signature are local nucleosome depletion, the synthesis of short enhancer RNAs, and the appearance of specific histone modifications (reviewed in 16, 18). Whereas promoters are generally enriched for histone 3 lysine 4 di- and trimethylation (H3K4me2/3), enhancers are marked by H3K4me1/2. A fully activated status of both promoters and enhancers is associated with high levels of histone acetylation (e.g., H3K9ac or H3K27ac), while many active enhancers also show enhancer RNA production. On the basis of such signatures, the human genome is estimated to harbor a staggering 668,000 candidate enhancer-like sequences, encompassing approximately 5.6% of the entire genome (36). It is important to note here that the presence of chromatin features indicative of transcriptional control activity does not necessarily mean that a genomic region has a bona fide enhancer function in living cells. It is currently not entirely clear what percentage of biochemically predicted enhancer sequences are able to actually regulate gene expression in functional assays, with reports ranging from up to 70% (36) to as low as ~10% (7, 42) depending on the specific validation strategy used. Additionally, current validation methods struggle to take into account relevant biological phenomena, such as in vivo enhancer redundancy or microenvironmental dynamics (38, 115). Nevertheless, with these drawbacks in mind, the use of biochemical enhancer (or promoter) signatures provides an excellent starting point for more in-depth studies of gene regulatory processes.

The observation that animal genomes are home to many more (putative) enhancers than genes immediately implies that controlling the expression of a single gene often involves multiple enhancers (90). Not long after the introduction of high-throughput methods to identify putative enhancer regions, several groups observed frequent clustering of open chromatin regions and TF binding sites in animal genomes (43, 44, 75, 80, 93, 101). These clusters carry diverse names [e.g., highly occupied target (HOT) regions, transcriptional initiation platforms (TIPs), and clusters of open regulatory elements (COREs)] and could often be linked to tissue-specific gene expression patterns (43, 75, 80). This clustering of cell-type-specific enhancers around genes implies a substantial degree of cooperation among individual GREs, reminiscent of LCRs. In line with studies of the globin LCRs, others have reported that the coexistence of multiple enhancers within a locus provides transcriptional accuracy and robustness (38, 40, 100, 106). An important conclusion drawn from these studies is that most individual enhancers can be inactivated without severe phenotypic consequences, since other functionally redundant enhancers partially compensate for the loss. This regulatory buffering mechanism provides obvious advantages for complex biological processes such as animal development, dampening the potential severe consequences of genetic insults or hazardous environmental changes (106). Whereas enhancer redundancy appears to be widespread, notable examples of single enhancer deletions causing severe phenotypes have been reported—e.g., loss of the long-range *Shh* enhancer, leading to limb malformations (84, 127).

SUPERENHANCERS

In 2013, the Young laboratory (145) reported the identification of yet another form of enhancer clustering: the superenhancer. They reasoned that large, multienhancer regions occupied by exceptional amounts of TFs and components of the general transcription machinery would typically have a higher capacity to boost transcription as compared with small, single enhancers. This concept had already been shown for LCRs (as described above), but Young and colleagues (145) devised a clever method to systematically identify LCR-like enhancer clusters. Using ChIP-seq data for lineage-specific TFs, the Mediator complex, and histone modifications such as H3K27ac, the authors first concatenated enhancers in close proximity (<12.5 kb) to each other. They then ranked these stitched enhancers and the remaining individual enhancers by their ChIP-seq signal, resulting in an occupancy curve. Enhancer regions with the highest occupancy levels for gene regulatory proteins (~3% of all enhancer regions, based on the slope of the curve) are considered superenhancers, separating them from typical enhancers (61, 145). Whereas the latter showed a median size of approximately 700 base pairs in mouse embryonic stem cells, superenhancers were more than 10-fold larger. Later that year, Parker et al. (113) described stretch enhancers, which represent a somewhat similar class of large enhancer regions defined by a size of >3 kb. Although no systematic comparisons between superenhancers and other types of enhancer clusters have been made [with the exception of superenhancers versus stretch enhancers (142)], the general consensus is that considerable overlap exists among them. This overlap was illustrated by recent findings that various LCRs emerge as top-ranked superenhancers (59, 135, 145).

The superenhancer concept quickly gained traction in the scientific community. Despite their rather arbitrary definition (e.g., the use of 12.5 kb as a stitching cutoff), superenhancers show extraordinary enrichment for being located close to key cell identity genes (61, 145), including critical oncogenes in cancer cells (91). Notably, genes encoding lineage-determining TFs that bind superenhancers are often themselves associated with superenhancers, suggesting the existence of interconnected TF–superenhancer autoregulatory loops that stabilize cell-type-specific gene expression programs (145). Hence, the superenhancer definition enables researchers to define the core transcriptional network of their cell type of interest through a relatively simple experiment (i.e., H3K27ac ChIP-seq).

THE SUPERENHANCER: GREATER THAN THE SUM OF ITS PARTS?

At the same time, the superenhancer concept continues to stir debate. The controversy mainly revolves around a single question: Is a superenhancer greater than the sum of its parts, or is a superenhancer simply a collection of conventional enhancers (33, 118)? Several studies have addressed this question by genetically removing individual or multiple enhancers from the larger superenhancer domain (4, 10, 24, 59, 62, 66, 72, 119, 133). Their findings paint a complex and heterogeneous picture. Various superenhancers show additive and redundant relationships between their constituents, often accompanied by hierarchies among the individual enhancers (i.e., one enhancer element exerting a more powerful effect than the others). Importantly, in some—but not all—superenhancers, deletion of one enhancer constituent resulted in reduced signs of activity at other enhancer elements, including reduced histone acetylation, TF binding, enhancer RNA production, and promoter–enhancer or enhancer–enhancer interactions (65, 66, 72, 119, 133). These last findings suggest that for a subset of superenhancers, functional crosstalk occurs between individual enhancer components. Interestingly, a superenhancer driving expression of the *Myc* proto-oncogene appeared to be active in multiple hematopoietic cell types, exploiting a cell-type-specific combinatorial activity of individual enhancer modules to control *Myc* expression in different cells (4). Together, these studies reveal complex relationships among the different parts

of a superenhancer, which differ substantially among individual superenhancers and depend on biological context. Nevertheless, it currently seems reasonable to conclude that superenhancers can indeed be greater than the sum of their enhancer parts, although this is not a general feature of superenhancers (as exemplified by the globin LCRs). Important to note is that these conclusions are based on the assumption that the most relevant cell type was used to evaluate the strength of each enhancer, and constituent enhancer activity might be (partially) cell-type specific. Synergy between GREs inside the same *cis*-regulatory domain has been convincingly reported outside the specific context of a superenhancer (reviewed in 90). Examples include the mouse *Fgf8* locus, in which multiple GREs act synergistically as a coherent unit to control accurate *Fgf8* expression during development (95). Enhancer synergy was also observed at the *Drosophila hunchback* locus in developing embryos (115).

PUTATIVE UNIQUE PROPERTIES OF SUPERENHANCERS

Other observations point toward superenhancers possessing features that distinguish them from conventional enhancers. Superenhancer domains harbor large quantities of chromatin-associated proteins, including coactivators (e.g., BRD4) and kinases (e.g., CDK7) that play critical roles in the process of transcription (122, 128). One particularly interesting observation that emerged from early studies of superenhancers is their sensitivity to chemical inhibition of these general components of the transcriptional machinery (20, 91). Upon treatment of cancer cell lines with a BRD4 inhibitor in vitro, superenhancer regions showed a disproportionate loss of BRD4 binding and linked oncogene transcription as compared with typical enhancers (91). In addition, superenhancers were preferential targets of the effector TFs of several signaling pathways (e.g., the TCF3 TF activated by Wnt signaling), and genes associated with superenhancers were more sensitive to manipulation of these signals than genes linked to typical enhancers (62). The increased sensitivity of superenhancers to chemical perturbations or environmental signals has been confirmed in various biological contexts (53, 55). While this vulnerability of superenhancers provides promising new putative therapeutic options in the context of cancer and inflammatory disease (114, 131), it appears to be somewhat at odds with the concept that most genes are regulated by multiple functionally redundant enhancers to increase regulatory robustness (106) (also see above). Given that superenhancer sensitivity to chemical inhibitors is quite heterogeneous (20, 91), one explanation for these contradictory roles of enhancer clustering could be that only a subset of superenhancers (as identified using the current algorithms) are exceptionally vulnerable to perturbations. In this scenario, the remaining nonsensitive superenhancers function in more conventional ways by providing a powerful additive transcriptional boost to highly expressed genes or by increasing transcriptional robustness to key cell identity genes. Refined algorithms (e.g., different combinations of TFs or histone modifications) for superenhancer detection might improve the process of separating vulnerable from more robust superenhancers.

Another feature of superenhancers that has emerged from recent studies using chromosome conformation capture technologies is extensive interactions between individual enhancer constituents in the three-dimensional nuclear space. Ing-Simmons et al. (69) reported that interactions within superenhancers were substantially stronger than interactions between conventional enhancers or promoters. The formation of elaborate chromosomal intradomain contacts was also observed within the globin LCRs, including simultaneous interactions between multiple individual enhancer elements (2, 107). Superenhancer regions also engage in strong interactions with each other or with promoter regions over large chromosomal distances (8, 105). Interestingly, the mechanisms that mediate long-range superenhancer colocalization appear to depend on cellular context. Removal of the cohesin complex strengthened superenhancer colocalization in

cancer cells (123), whereas cohesin depletion weakened superenhancer interactions in embryonic stem cells (124). Huang et al. (65) reported a subclassification of hierarchical superenhancers based on their high three-dimensional connectivity with other genomic regions. The enhancer elements mediating these interactions within hierarchical superenhancers, termed hub enhancers, control the local superenhancer chromatin landscape and showed the highest enrichment for disease-associated genetic variation (65). Aligning with such a concept of superenhancers carrying a functionally dominant hub enhancer module is the observation by Mansour et al. (94) that an 8-kb superenhancer can be formed de novo by a somatic mutation, creating a single TF (MYB) binding site upstream of the *TAL1* oncogene in a subset of acute T cell lymphoblastic leukemias. It will be of interest for future studies to investigate whether hierarchical superenhancers and their hub enhancer modules are also most sensitive to chemical inhibitors or signaling perturbations.

Together, these findings reveal putative unique properties of superenhancers and contribute to the idea that superenhancers—or at least a subset of those detected using the current methodology—represent a distinct class of GREs.

(SUPER)ENHANCERS AND MODELS FOR THEIR FUNCTION

Ever since the discovery of enhancers in the 1980s, scientists have pondered how enhancers get the job done. How do they achieve precise spatiotemporal control of their target genes from a distance? Enhancers are thought to affect virtually all aspects of promoter activation, including opening of the chromatin, transcription initiation, and transcription elongation (reviewed in 60, 117). But how do enhancers reach a specific promoter from large genomic distances, independent of its orientation and its relative position (i.e., upstream or downstream of the gene, or inside an intron)? Tackling this question has led to several different models for enhancer function (41) (**Figure 4**). One possibility could be tracking. Here, a TF complex recruits RNA polymerase II to the enhancer, after which the polymerase would transcribe (or track) in the direction of the target gene to arrive at its promoter. This process could pull along the enhancer to establish direct enhancer–promoter contacts or involve a continuous transcription (tracking) process independent of close proximity between the enhancer and promoter (the latter of which is shown in **Figure 4**). Advocating against this mechanism is the original observation that enhancers are active independent of their orientation and relative positioning (77). Hence, a strict tracking mechanism is difficult to reconcile with intragenic enhancers and enhancers that must skip one or more genes to reach their target gene. Enhancer tracking might still operate over some distance, perhaps through chromatin loop extrusion driven by cohesin complexes sliding along the DNA (41).

The linking model was inspired by the *Drosophila* Chip protein, which was proposed to oligomerize between enhancer and promoter (14, 32) (**Figure 4**). However, its mammalian equivalent (Ldb1) can homodimerize to form chromatin loops between a promoter and enhancer, resulting in gene activation (29). In addition, as with the tracking model, it is difficult to envision a widespread role for linking in the crowded and complex regulatory landscape of mammalian genomes.

Another influential model for enhancer function is the relocation model. This model proposes that both the enhancer and the promoter are relocated to a nuclear compartment, where they would interact with each other (**Figure 4**). In this compartment, referred to as a transcription factory (70, 143), high local concentrations of RNA polymerase II would facilitate efficient transcription. Hundreds of such transcription factories have been observed per cell, with a given factory estimated to contain a handful of genes (112). Although the precise stability of transcription factories in living cells remains debated (25, 45), the RNA polymerase II factory model is compatible

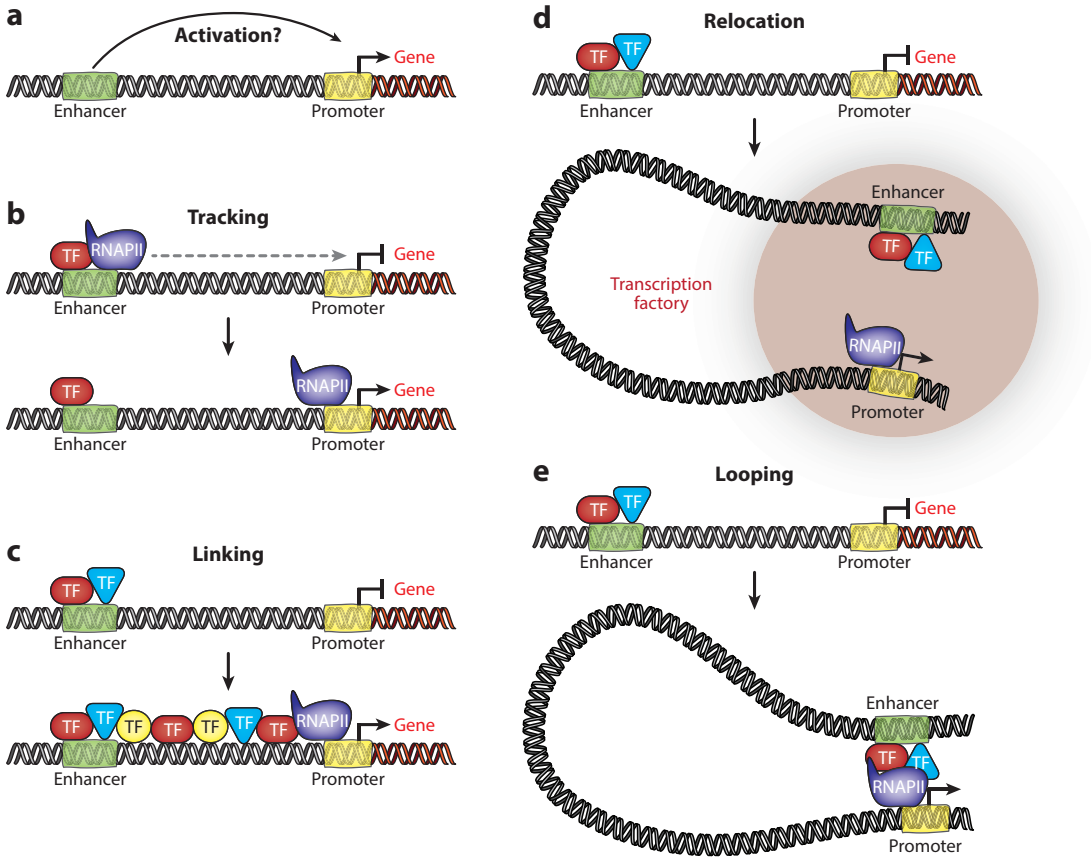

Figure 4

Models of enhancer–promoter communication. (*a*) A central question in the field is how enhancers can activate promoters across large genomic distances. (*b*) Enhancer tracking involves transcription from the enhancer in the direction of the promoter (either with or without pulling along the enhancer). (*c*) For linking, TFs oligomerize between the enhancer and the promoter to bridge the distance. (*d*) Relocation of both the enhancer and the promoter to a transcription factory allows for promoter activation. (*e*) In the looping model, the enhancer and promoter come into close physical proximity through the formation of a chromatin loop. Abbreviations: RNAPII, RNA polymerase II; TF, transcription factor.

with dynamic gene regulation by enhancers (89, 130). Recruitment of β-globin genes to transcription factories depends on the LCR (121). Interestingly, Schoenfelder et al. (130) described specialized factories containing a cell-type-specific TF and its canonical target genes. What drives the formation of transcription factories remains unclear, although the clustering of enhancers and their associated regulatory proteins in the three-dimensional nuclear space through weak multivalent interactions was recently proposed to create such transcriptional hubs (89) (further discussed in the next section).

The looping model proposes that enhancers and promoters meet in three-dimensional nuclear space through chance with high probability, because they act as two entities tethered on a string (**Figure 4**). This notion fits with the observation that genes compete for a set of enhancers, in which the more proximal gene would simply have a higher chance of meeting the enhancer(s) (30, 58). Unlike the tracking and linking models, the looping model can readily explain how an enhancer can skip more proximal genes to find its distal target promoter and aligns well with the

orientation-independent nature of enhancers. The looping and relocation models share features, as clustering of multiple enhancers (e.g., an LCR or superenhancer) via looping was proposed to create active chromatin hubs that concentrate the basal transcription machinery for efficient expression (27, 136). In line with the cell-type-specific activity of enhancers, enhancer–promoter loop formation is a dynamic and developmentally regulated process (110). Mechanistically, loops may also be formed by the process of loop extrusion, in which the cohesin complex actively extrudes a chromatin loop until it encounters the extrusion barrier protein CTCF (reviewed in 108). Since CTCF is constitutively expressed in all cells and binds in a largely tissue-invariant manner (21), it has been proposed that many cell-type-specific promoter–enhancer loops are mediated by TFs (29, 41, 134), possibly through an interplay with cohesin (88, 134). Loop extrusion in this scenario would need to be compatible with an LCR or superenhancer that can interact with multiple genes simultaneously on the same allele (2) and with the observation that the most enhancer-proximal of two identical genes has a competitive advantage for activation (30).

ENHANCER CLUSTERS, PHASE SEPARATION, AND THE HUB–CONDENSATE MODEL

Eukaryotic cells frequently organize biochemical reactions in membrane-less compartments, such as nucleoli and Cajal bodies (67). These compartments possess liquid-like properties and are formed through a process called phase separation, which is mediated by weak multivalent interactions among molecules (67). With this phenomenon in mind, Hnisz et al. (63) presented a model that proposed that the high density of TFs, RNA polymerases, cofactors, and enhancer RNAs would facilitate dynamic local phase separation through weak multivalent interactions among superenhancer-associated molecules—a process that would be much more difficult to achieve at conventional enhancers. Local phase separation, or condensate formation, would allow the concentration of the transcriptional apparatus to ensure robust transcription. At the same time, the model provides a theoretical framework for superenhancer vulnerability to perturbations (63). Although more evidence is required to definitively prove that phase separation is actually relevant for transcriptional regulation in vivo (98), the concept has attracted a lot of interest, as it provides a mechanism for rapidly creating a high-concentration and dynamic environment for efficient transcription (64). In favor of the model, exciting recent work has shown that in live cells, RNA polymerase II and key coactivators such as Mediator form dynamic superenhancer-associated clusters with properties of phase-separated condensates (22, 125).

The enthusiasm with which the field welcomed the phase-separated condensate model is perhaps not surprising. The concept of concentrating key biochemical processes for gene regulation in condensate-like structures was previous proposed for genes regulated by enhancer clusters that form active chromatin hubs through looping [e.g., the β-globin locus (2, 27) and the *Myb* locus (136)] or through relocation of genes and enhancers to transcription factories (112). Integrating the latest insights from (superenhancer-driven) phase separation with the more conventional mechanisms for enhancer–promoter communication described above results in a unified hub–condensate model of transcriptional activation (41). In this model, enhancer clusters such as LCRs and superenhancers function as nucleation sites for the formation of chromatin hubs or condensates (**Figure 5**). Other transcription units or genes can be added to these hubs in a stochastic manner, which can explain why different combinations of genes can be present in a particular hub in otherwise identical cells (120). Hub and condensate formation is achieved by a multitude of interactions among not only multivalent proteins (such as RNA polymerase II) and chromatin-associated proteins, but also TFs. Several studies have suggested that weak sequence-specific protein–protein interactions among intrinsically disordered regions in regulatory factors

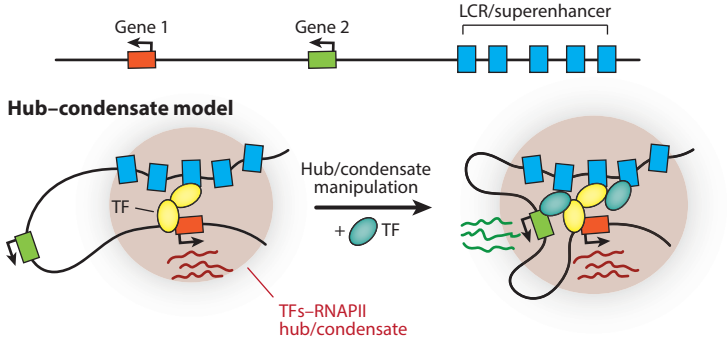

Figure 5

The hub–condensate model for enhancer function. The formation of transcriptional hubs or condensates is initiated from enhancer clusters (e.g., LCRs or superenhancers) through numerous weak interactions among TFs, coactivators, and RNAPII complexes. Hubs or condensates create a high-concentration environment that enables robust transcription (RNA is depicted as *wave-shaped lines*) of recruited target genes. Hubs or condensates can be dynamically altered by regulatory proteins such as TFs. Abbreviations: LCR, locus control region; RNAPII, RNA polymerase II; TF, transcription factor.

are critical for condensate formation (11, 23, 92). However, very recent findings indicate that recruitment of Sox2 and Brd4 into nuclear clusters depends on their DNA- or chromatin-binding modules, respectively, and not on their intrinsically disordered regions (86). Cell-type-specific TF involvement, exemplified by Klf1-mediated recruitment of genes to specialized transcription factories (130), allows for specificity in the formation and dynamic modification of local hubs or condensates as cells respond to microenvironmental signals (**Figure 5**). Mechanistically, phase separation is a likely candidate for driving this process, although alternative mechanisms have been proposed (96, 99).

Akin to the looping model, the hub–condensate model indicates the close physical proximity of enhancers and promoters, as they share a hub or condensate (**Figure 5**). These contacts could be (partially) preformed or established de novo (41), which might involve recruitment of the cohesin loop extrusion complex by cell-type-specific TFs (88). The model also accounts for instances where gene activation appears to not be accompanied by (increased) promoter–enhancer proximity (1, 9). In this scenario, one could envision that hubs or condensates formed at distal enhancers might grow large enough to encompass and activate target gene(s) without a requirement for frequent promoter–enhancer interactions.

CONCLUSION AND FUTURE PERSPECTIVES

Our view of how enhancer–promoter communication takes place has changed drastically since the discovery of enhancers in 1981. Although much remains to be discovered, it has become very clear that animal enhancer biology is extremely complex and likely difficult to generalize using a single model. Integrated or combined mechanisms show strong potential to paint a more comprehensive picture. For example, tissue-invariant loops could be established by a tracking mechanism such as CTCF–cohesin loop extrusion. Within such an insulated domain that is maintained in various cell types, functional loops may be formed by a largely stochastic looping process where distance plays a role and genes can compete for enhancers. Alternatively or as part of this process, loop formation could take place by relocation to active chromatin hubs or condensates that emerge from interactions between multivalent factors, such as TFs, bound at powerful enhancer clusters.

Many questions remain unanswered. Apart from validating the hub–condensate model in vivo, exactly how the specificity of enhancer–promoter interactions is achieved remains a major conundrum (129). The observation that enhancers frequently skip closely located genes when searching for their target genes (42) illustrates the ability of enhancers to pick their target genes with impressive precision. Additionally, what is the temporal relationship between three-dimensional enhancer–promoter proximity and promoter activation in single cells? What is the exact role of TFs in establishing promoter–enhancer communication? That is, do TF complexes directly induce precise cell-type-specific interactions [like Ldb1 (113)], or is their main role to drive hub–condensate formation and facilitate stochastic enhancer–promoter pairing? Are (large) differences in TF binding affinity dictating the strength of enhancer–promoter interactions? Superenhancers remain a heterogeneous group of GREs that lack a solid biological definition. Do subsets of superenhancers exist with distinct functions? How are the individual enhancer constituents coordinated within their broader regulatory circuit, and what are the roles of redundancy and synergy? Are cooperating enhancers within a superenhancer orientation dependent? Do superenhancers display unique biophysical properties compared with typical enhancers, e.g., regarding their nuclear mobility in transcriptionally active states (54)? Finally, it will be interesting to better understand enhancer function in the context of the cell cycle. How is enhancer–promoter communication so rapidly resumed after cells exit from mitosis (146)? With the support of rapidly improving single-cell and imaging technologies, we anticipate that answering these questions will uncover new principles of enhancer biology and gene regulation.

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