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# Enhancer function: new insights into the regulation of tissue-specific gene expression

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## Abstract

Enhancer function underlies regulatory processes by which cells establish patterns of gene expression. Recent results suggest that enhancers are specified by particular chromatin marks in pluripotent cells, which may be modified later in development to alter patterns of gene expression and cell differentiation choices. These marks may contribute to the repertoire of epigenetic mechanisms responsible for cellular memory and determine the timing of transcription factor accessibility to the enhancer. Mechanistically, cohesin and non-coding RNAs are emerging as critical players responsible for facilitating enhancer-promoter interactions. Surprisingly, these interactions may be required not only to facilitate initiation of transcription but also to activate the release of RNAPII from promoter-proximal pausing.

### Keywords

epigenetics; histones; chromatin; RNA

Differentiation of the various cell types present in multi-cellular organisms requires the establishment of spatio-temporal patterns of gene expression during development[1]. Transcription of eukaryotic genes is an exceedingly intricate process that requires the precise orchestration of a complex set of interactions among a myriad of proteins and DNA sequences (FIG. 1)[2]. Regulation of transcription is accomplished in large part by enhancers, which are DNA sequences containing multiple binding sites for a variety of transcription factors. Enhancers can activate transcription independent of their location, distance or orientation with respect to the promoters of genes[3]. In some instances, they can even activate transcription of genes located in a different chromosome[4, 5]. A critical issue central to the understanding of enhancer function, is how regulatory elements that show such variability in their relationship to promoters contribute to the precise regulation of transcription.

Our notion of the mechanisms by which enhancers activate transcription has been heavily influenced by the strategies used by bacterial transcription factors, which bind to DNA sequences in close proximity to the promoter and help recruit RNA polymerase[6]. By extension, enhancers are viewed as clusters of DNA sequences capable of binding combinations of transcription factors that then interact with components of the Mediator complex or TFIID to help recruit RNA polymerase II (RNAPII)[2, 7]. In order to accomplish this, enhancer-bound transcription factors loop out the intervening sequences and contact the promoter region, explaining the ability of enhancers to act in a distance-independent fashion[8] (FIG. 1). Activation of eukaryotic genes requires, in addition, de-compaction of the chromatin fiber. This task is also carried out by enhancer-bound transcription factors that

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can recruit histone modifying enzymes or ATP-dependent chromatin remodeling complexes to alter chromatin structure and increase the accessibility of the DNA to other proteins[9].

This view of enhancer function derives from experiments carried out on a variety of genes whose transcription is activated in response to specific cues. Recent advances in molecular and computational biology have allowed the application of genome-wide tools to the analysis of enhancer structure and function[10]. The picture that emerges from these studies is considerably more complex and paints enhancers as sequences that may carry epigenetic information in the form of specific histone modifications. These chromatin marks are first established early in development and are modified as cells differentiate along specific lineages. Interestingly, some of these histone modifications may serve as marks for future gene expression[11] while others may play a more active role in the transcription activation process[12]. In addition, it appears that enhancer sequences themselves are not mere binding sites for transcription factors but they are also transcribed into non-coding RNAs, which, together with cohesin, may play an active role in the control of transcription by stabilizing long-range enhancer-promoter interactions[13]. The role of these interactions is not limited to the regulation of transcription initiation but they may also be required to release RNAPII from promoter-proximal pausing[14]. These findings are beginning to clarify the mechanisms by which enhancers can activate transcription in a distance and orientation-independent manner while doing so with the exquisite precision required to orchestrate the complex process of cell differentiation during development.

## Chromatin features of enhancers

The histone code hypothesis suggests that the distribution of post-translational modifications of histones and the presence of specific histone variants establish specific chromatin environments that influence gene expression by orchestrating the interactions of transcription factors with the chromatin fiber[15]. During the last few years, genome-wide mapping of epigenetically marked nucleosomes has been conducted in an attempt to understand how various histone modifications affect gene expression. These studies have led to new insights into the chromatin landscape of enhancers and its functional significance in the regulation of specific gene expression programs.

### Nucleosome dynamics defines transcriptional enhancers

Assembly, mobilization and disassembly of nucleosomes can influence transcription and other processes that act on eukaryotic DNA[16]. Results from genome-wide mapping studies of nucleosome occupancy in different species indicate that the boundaries of cis-regulatory domains are marked by high rates of histone replacement[17] and transcription start sites are frequently associated with regions of low nucleosome occupancy (normally termed nucleosome-free regions)[10, 18–23]. These findings suggest that nucleosome instability contributes to gene regulation by facilitating the access of transcription factors to promoters and other regulatory elements. The presence of highly unstable nucleosomes containing the histone variants H3.3 and H2A.Z at several well-characterized enhancers suggests that nucleosome dynamics might also be important for their regulatory functions (FIG. 1)[20, 24, 25]. Indeed, this hypothesis is supported by three recent genome-wide studies. Isolation of HeLa cell chromatin with low salt concentration, which maintains the association of unstable histone variants to DNA, has led to the discovery that H3.3/H2A.Z-containing nucleosome core particles are enriched at the nucleosome-free regions of regulatory elements across the genome[26]. Furthermore, regions of the genome that are hypersensitive to DNase I in $CD4^+$ T but not in HeLa cells contain unstable double-variant nucleosomes in the former cells but not the latter (FIG. 2a). This result indicates that the presence of dynamic nucleosomes reflects the activity of the hypersensitive sites, which in turn carry histone modifications correlated with enhancer activity[20, 26] (see below).

Analysis of androgen-mediated transcriptional programs in prostate cancer cells has revealed a class of enhancers containing androgen receptor and FoxA1 binding sites[27]. These enhancers are occupied by a central H2A.Z-containing nucleosome and a pair of flanking nucleosomes with histone H3 dimethylated at lysine 4 (H3K4me2). Androgen stimulation results in the disappearance of the central H2A.Z-containing nucleosome and an increase in the signal of the flanking H3K4me2 nucleosomes (FIG. 2b). This observation is indicative of transcription factors displacing the nucleosome at their binding site. Quantitative modeling based on the behavior of paired H3K4me2 nucleosomes correctly identified known androgen-responsive enhancers as well as predicted novel enhancers from genes that are only activated after prolonged stimulation. A separate study in pre-pro-B cells has shown that expression of the E47 isoform of the E2A transcription factor also alters the genome-wide pattern of monomethylated H3K4 (H3K4me1) from a single peak into a bimodal distribution around E47-binding sites at enhancers (FIG. 2c)[28].

Together, these data suggest that an essential feature of enhancers may be the presence of histone variants that contribute to nucleosome plasticity; replacement of canonical histones for H3.3/H2A.Z must be dependent on the recruitment of specific histone chaperones, perhaps by DNA sequence-specific bound transcription factors. In turn, dynamic nucleosomes at enhancers may be an essential feature to accommodate the binding of other transcription factors, which in turn may recruit other histone modifying enzymes or communicate directly with the transcription apparatus at promoters.

## Histone modifications at enhancers

CBP (CREB-binding protein) and p300 are highly similar proteins that have histone acetyltransferase activity and contain a variety of functional domains involved in interactions with other transcription factors or histone modifications[29]. These two proteins interact with the sequence-specific binding transcription factor CREB (Cyclic-AMP Response Element Binding) and have been previously shown to be involved in several cell signaling pathways by activating the transcription of a variety of genes. Experiments carried out across a 30 Mb region of the human genome have confirmed a correlation between the presence of p300 and enhancer function[10, 30]. Furthermore, i*n vivo* mapping of several thousand p300 binding sites in mouse embryonic forebrain, midbrain, limb and heart accurately identified novel enhancers that could recapitulate tissue-specific gene expression patterns in transgenic mouse assays[31, 32]. These results suggest that cell-type specific occupancy of enhancers by CBP/p300 regulates distinct transcriptional programs in many cell types[12] and, therefore, these proteins may be a general component of a large class of enhancer elements (FIG. 1).

High-resolution maps of multiple histone modifications and transcriptional regulators such as CBP/p300 have provided further insights into the chromatin signatures of different regulatory elements[10]. Active promoters, defined by the presence of RNAPII and TBP-associated factor 1 (TAF1), are marked by nucleosome-free regions with flanking trimethylated histone H3 at lysine 4 (H3K4me3). On the other hand, putative enhancers, predicted by the presence of distant p300 binding sites, are highly enriched in H3K4me1, H3K4me2 and acetylated H3K27 (H3K27ac)[10]. The chromatin state at promoters is largely invariant across different cell types whereas histone modification marks at enhancers are cell-type specific and are also strongly correlated with gene expression patterns[12]. Furthermore, most of the enhancers identified by this approach are functionally active in a cell-type specific manner. These observations held true in several other studies in which H3K4me1 and H3K4me2 marks were found associated with the enhancers of specific genes in multiple cell lines, including E2A-responsive genes in B cells[28], FoxA1-regulated genes in MCF7 and LNCaP cells[27, 33], and a subset of differentiation genes in hematopoietic stem cells/progenitor cells[11]. Since the H3K4me1/2 marks are common to most enhancers, the

cell-type specificity of these regulatory sequences may come from the presence of binding sites for specific factors that may further alter the chromatin structure at the enhancer.

The studies described above used the presence of p300 as an initial definition for enhancer identity. If a broader definition of enhancers is used, results suggest that the chromatin features may not be uniform at all enhancers. When DNase I hypersensitivity[34], excluding insulator and RNAPII binding sites, was used to predict enhancers in $CD4^+$ T cells, expected enhancers were found to be associated with multiple histone modifications, including all three H3K4 methylation states and the presence of histone variant H2A.Z[20, 25]. In addition, subsets of putative enhancers contain distinct histone modifications, including, H3K9me1[20, 25], H3K18ac[25], H3K9ac and H3K14ac[35, 36]. These chromatin features were further validated in well-characterized enhancers of *IL-2RA*, *IL-13*, *CD4* and *IFN-γ* genes in T cells.

There are multiple possible explanations for the apparently contradictory results obtained using the presence of CBP/p300 versus DNase I hypersensitivity as predictors of enhancer identity, specially the presence of H3K4me3 at DNase I hypersensitive regions but not at CBP/p300 sites. It is likely that the presence of CBP/p300 may only identify a subset of DNase I hypersensitive enhancers[10]; this may, in fact, be expected, since CBP/p300 are recruited to chromatin via the sequence-specific DNA binding protein CREB and, therefore, only a subset of enhancers are expected to contain these proteins. The different cell lines and the number of histone modifications examined in various studies may also account for the disparities in histone modification patterns observed at the enhancers. Moreover, enhancers of different genes are found to have distinctive histone modifications in the same cell[25]. Together, these results suggest that, although H3K4me1 appears to be a universal mark of all or most enhancers (FIG. 1), these sequences are probably marked by combinatorial histone modification patterns indicative of their context-dependent functions.

## Chromatin signatures at enhancers are established by multiple factors during transcription activation

Although the enzymes involved in various histone modifications and H2A.Z/H3.3 deposition are well-characterized[37, 38], the timing and mechanisms by which specific enhancers acquire unique chromatin signatures remain poorly understood. Genome-wide mapping of H3K4me3 and H327me3 marks before and after the maternal-zygotic transition in zebrafish has shown that genes acquire histone marks only after transcription has been initiated across the genome[39]. The H3K4me3 mark is deposited at both active and inactive genes in the absence of sequence-specific transcriptional activators or stable association of RNAPII, suggesting that enhancers acquire chromatin marks before distinct transcriptional programs are turned on in embryonic stem cells (ESCs).

Other studies also support the idea that the epigenetic state of enhancers is intricately orchestrated in a stepwise fashion by multiple protein complexes during development. In particular, many genes in ESCs contain a paused RNAPII and are therefore poised for activation when these cells differentiate along specific lineages. The presence of H3K4me1/2 at the enhancers of many poised differentiation genes in stem cells suggests that deposition of these marks may be the initial event in denoting or specifying an enhancer sequence during the earliest stages of development[11]. For example, the tissue-specific *λ5-VpreB1* enhancer, which is necessary for the expression of the*λ5* and *VpreB1* genes in pro- and pre-B cells, is bound by factors Sox2 and Foxd3 in ESCs[40]. Sox2 contributes to the establishment of the H3K4me2 mark in the enhancers of several B-cell differentiation genes (including *Pax5*, *Blink* and *Dntt*) whereas Foxd3 represses intergenic transcription from the enhancer in ESCs (FIG. 3b). As ESCs differentiate into pro-B cells, recruitment of the lineage-specific transcription factor Sox4 to Sox2 binding sites is required for the enhancer

activity and expression of the*λ5* gene. These data suggest a factor relay model whereby transcription factors bind to enhancer sequences in a stepwise fashion during development; ESC factors establish active epigenetic marks at tissue specific elements before being replaced by cell type-specific factors as cells differentiate[40].

While the timing and the specific factors required for depositing different histone variants at enhancers are not known, several lines of evidence indicate that localization of H3.3 at specific genomic regions is controlled by distinct factors[41–43]. The H3.3 chaperone Hira is required for H3.3 enrichment at active and repressed genes. In yeast, Hartley and Madhani have shown that the chromatin-remodeling complex RSC plays an integral part in the establishment of nucleosome-free regions, which is in turn required for deposition of H2A.Z but not vice versa[44]. Taken together, existing data suggest that specific chromatin signatures at enhancers require the concerted action of multiple proteins complexes, whose activities could in turn be subjected to context dependent regulation.

## Chromatin signatures at enhancers act as epigenetic signals for gene induction

The presence of common −H3K4me1/2 and H3.3/H2A.Z− as well as specific histone variants and covalent histone modifications at enhancers suggest that these epigenetic marks represent historical events in the process of cell differentiation as well as a measure of the transcription activation potential of the enhancer. These epigenetic marks are deposited by distinct histone chaperones or modifying enzymes that need to be recruited to the enhancer by sequence-specific DNA binding proteins or other factors that recognize specific modifications in the chromatin. Current results suggest that combinatorial binding of multiple transcription factors to a single enhancer takes place on a temporal gradient during cell differentiation. Therefore, it is possible that the cell-type specific chromatin features of enhancers that correlate with transcription might provide epigenetic memory for gene expression.

Research aimed at testing this hypothesis suggests that enhancers are associated with specific chromatin signatures in the cell stage before their target genes are expressed. During the differentiation of multipotent human primary hematopoietic stem cells/progenitor cells (HSCs/HPCs) $CD133^+$ cells into erythrocyte $CD36^+$ cells, H3K4me1, H3K9me1 and H3K27me1 marks are associated with the enhancers of differentiation genes prior to their activation, and correlate with their basal expression[11]. Enhancers of the *CD36* and globin genes, which are specifically expressed in erythrocytes, are enriched with these monomethylation marks and H2A.Z in the HSCs/HPCs. This suggests that the critical regulatory elements are epigenetically modified long before gene induction, probably to maintain activation potential required for gene expression[11]. Consistent with this, it has been recently reported that H3K4me2 marks are present at a subset of developmentally poised hematopoietic genes[45]. These chromatin marks at enhancers are then interpreted by transcription factors to effect different gene expression programs.

Consistent with this hypothesis, evidence indicates that pioneer transcription factor FoxA1 switches on specific estrogen and androgen responsive programs in MCF7 and LNCaP cells respectively by its differential binding to selected H3K4me2-marked enhancers.[33] In MCF7 cells, enhancers marked with H3K4me2 have estrogen receptor binding sites whereas in LNCaP cells, H3K4me2-marked enhancers have androgen receptor binding sites (FIG. 3a). Therefore, FoxA1 translates epigenetic signatures at the enhancer into distinct transcriptional programs by binding to unique H3K4me2-marked sites where it can interact synergistically with cell-type specific transcription factors[33].

Taking into account results from a variety of experimental systems, it is possible to postulate a model in which enhancer sequences carry epigenetic information that changes in

complexity and records the differentiation history of cells during development. At each stage, this epigenetic information can be interpreted by cell-type specific transcription factors. The outcome may involve gene expression or alteration in enhancer-encoded epigenetic information that modifies its response capacity and narrows down cell differentiation choices. The initial marks of enhancer identity/function could likely be histone variants H3.3/H2A.Z and H3K4m1/2, elicited by pluripotency factors in embryonic stem cells. Other histone modifications, such as H3K27ac, H3K8ac or H4K16ac may be subsequently added to alter the response of the enhancer and restrict the differentiation potential of the cell.

## The role of long-range interactions in enhancer function

Enhancers were proposed early on to interact with distally located promoters in order to activate transcription. How enhancers find and interact with distant core promoters to trigger transcription and the mechanisms that stabilize these interactions are some of the most enigmatic aspects of the biology of these sequences.

### Long-range interaction between regulatory elements occurs at many gene loci

It has been proposed that the effects of enhancers on transcription require the formation of chromatin loops through direct physical association of distant elements within the nucleus[46–52]. This hypothesis has been tested experimentally by fluorescence *in situ* hybridization (FISH), and, more recently, by Chromosome Conformation Capture (3C)[53]. By detecting the frequency of physical association between genomic elements within the nucleus, results from 3C-based analyses suggest that interactions between distal enhancers and promoters are necessary for the regulation of specific transcriptional programs at a large number of gene loci[54–56]. Recent studies on the mechanisms underlying the crosstalk between enhancers and promoters are beginning to shed light on how chromatin loops are formed by long-range interactions as well as the strategies enhancers employ to communicate with their cognate promoters during gene activation.

The α-globin and β-globin loci have been a fruitful model system for the study of long-range interactions in gene regulation[49, 57, 58]. The mouse β-globin locus contains a cluster of beta-chain variants of hemoglobin that are developmentally regulated by multiple elements spanning a region of 100 kb. These regulatory elements include the LCR, which acts as an enhancer and is located approximately 25 kb upstream of the εy-globin gene, and a group of DNase I hypersensitive sites located ~20 kb downstream of the locus (3′HS1) and upstream of the LCR (-60 HS). Expression of the β-globin gene requires the presence of specific transcription factors (GATA-1 and EKLF) that mediate the clustering of these elements with the β-globin gene promoters via long-range interactions to form an “active chromatin hub”[59, 60].

The formation of a chromatin hub by looping of distant sequences has also been detected in the T helper (Th) type 2 cytokine locus. Long-range interactions among multiple enhancer and promoter elements within the locus increases significantly upon Th2 cell differentiation, suggesting that the three-dimensional organization of poised co-regulated genes is important for rapid activation upon induction[61, 62]. The ability of enhancers to interact with promoters is not limited to genes located in cis on the same chromosome; the olfactory H enhancer has been shown to interact with multiple olfactory receptor genes on the same and different chromosomes in epithelial tissues where these genes are specifically expressed[5].

Consistent with the idea that enhancers are able to interact with other regulatory sequences over long distances, highly expressed co-regulated genes associate together at specific nuclear locations called transcription factories. Analyses of globin gene expression in

erythroid cells has revealed extensive intra- and inter-chromosomal transcription interactomes where active globin genes associate with other transcribed genes. The Klf1 transcription factor, which binds both enhancer and promoter elements, is necessary for mediating preferential co-associations of Klf1-regulated genes at a limited number of specialized transcription factories[63]. These data indicate that long-range interaction between enhancers and promoters is important for proper gene regulation. An interesting possibility is that, as a consequence of these interactions necessary for transcription, enhancers contribute to the establishment of a cell-type specific three-dimensional nuclear architecture.

### Cohesin stabilizes long-range interactions between some enhancers and promoters

Although the role of transcription factors in mediating association between specific regulatory elements in the genome is well-characterized, the nature of the protein complexes required for stabilizing these interactions has remain elusive. Recent studies suggest that cohesin complexes might be at least in part responsible for this task. Cohesins are known to mediate sister chromatid cohesion necessary for proper chromosome segregation and DNA repair[64–66]. Earlier studies in various model organisms have also highlighted their involvement in gene regulation[67–72]. However, the mechanism underlying their transcription regulatory function only became evident recently when cohesins were shown to interact with CCCTC-binding factor (CTCF)[73–76]. CTCF is a highly conserved zinc finger protein with diverse gene regulatory functions. CTCF has been proposed to organize global chromatin architecture by mediating intra- and inter-chromosomal contacts[50].

The role of cohesins in transcription appears to go beyond mediating interactions between distant CTCF sites. Several recent studies suggest that cohesin may facilitate enhancer-promoter interaction via two distinct strategies. In human Hep3B cells, the *APO A1/C3/A4/A5* gene region is demarcated by three CTCF and cohesin (RAD21) binding sites, in which two of the sites overlap. High resolution 3C analysis of the locus reveals the formation of two transcribed chromatin loops, such that the C3 enhancer and APOC3/A4/A5 promoters reside in one loop, and the APOA1 promoter in a different loop (FIG. 4a). Depletion of either CTCF or RAD21 disrupts the chromatin loop structure, causing significant changes in APO expression and the reduced localization of the transcription factor hepatocyte nuclear factor (HNF)-4a and the transcriptionally active form of RNAPII specifically at the APOC3 promoter[77]. Similar cohesin-mediated chromatin loops were also detected at the developmentally regulated *IFN-γ* locus (FIG. 4b)[78], imprinted *IGF2-H19* locus[79] and the β-globin locus[80]. These results suggest that CTCF/cohesin-mediated chromatin looping facilitates enhancer-promoter interaction by bringing them into close proximity.

Studies of transcription in mouse embryonic stem cells suggest that cohesin stabilizes looping necessary for the direct interaction between enhancers and promoters in the absence of CTCF. ChIP-seq analyses show that mediator, cohesin and the cohesin loading factor Nipbl co-localize at thousands of sites within the ESC genome[81]. Nipbl is generally found at the mediator/cohesin-bound enhancer and promoter regions, but rarely at CTCF/cohesin co-occupied sites. Knockdown of cohesin, Nipbl or mediator led to changes in the expression profiles of the genes whose regulatory elements were occupied by cohesin and mediator. Furthermore, chromatin looping between enhancers and promoters of active genes is dependent on the cell-type specific occupancy of mediator and cohesin. These data suggest that mediator and cohesin physically and functionally connect the enhancers and core promoters of active genes in murine ESCs (FIG. 4c)[81].

## Enhancer function can be mediated by non-coding RNAs

Recent studies indicate that non-coding RNAs (ncRNA) may also have important regulatory roles in activating transcription. In mouse cortical neurons, genome-wide analyses have

revealed that stimuli-dependent binding of CBP occurs at approximately 12,000 enhancers that are pre-marked by H3K4me1 modification. Interestingly, CBP recruits RNAPII at a subset of these enhancers to transcribe a novel class of non-coding RNA[82]. The transcription of enhancer RNA (eRNA) positively correlates with the level of mRNA synthesis at nearby genes and requires the presence of an intact promoter. This suggests that the long-range association between enhancers and promoters of actively transcribed genes might be necessary for the synthesis of eRNAs (FIG. 5a). Although the significance of eRNAs has not been addressed experimentally, it is possible that transcription of eRNAs establishes a chromatin landscape at the enhancer that in turn facilitates gene activation. Alternatively, eRNAs may play an integral role in forming a chromatin hub by interacting with other factors, analogous to the role of RNA in the formation of chromatin insulator complexes[83, 84].

A second class of ncRNAs that exhibit enhancer-like function has been found in a recent survey of the human genome with GENCODE annotation[13]. These long ncRNAs are necessary for robust expression of their neighboring protein-coding genes in multiple cell lines as well as transcription activation from the thymidine kinase promoter in luciferase reporter assay. The activating role of these ncRNAs on heterologous promoters can be explained if the ncRNAs serve as a scaffold for the assembly of transcription factors or other chromatin remodeling enzymes at the promoter (FIG. 5b). This mechanism would be similar to that of the human HOTAIR RNA, where ncRNA-mediated assembly of different histone modifying complexes is necessary for their DNA binding and silencing of *HOXD* gene expression[85]. However, more experiments are needed to test this hypothesis.

## Enhancers communicate information to the core promoter through multiple mechanisms

Distinct chromatin signatures at enhancers are hypothesized to act as epigenetic marks that poise specific genes for rapid induction[11, 45]. Studies aimed at testing this hypothesis indicate that different signaling pathways may employ separate molecular strategies to disseminate the epigenetic information embedded at the enhancer to the core promoters during transcriptional activation. Classically, it has been thought that enhancers activate transcription by facilitating the recruitment and assembly of the transcription complex[2]. Nevertheless, recent results suggest that enhancers may also affect downstream processes such as the release of RNAPII from promoter-proximal pausing. Phosphorylation of the serine 10 of histone H3 (H3S10ph) is required for transcriptional activation of cytokine-induced genes[86], *Drosophila* heat shock genes[87] and 20% of MYC target genes[88]. The serum-inducible FOSL1 gene has been a useful model system for dissecting the molecular events at the enhancer leading to transcriptional activation[14]. Serum stimulation induces PIM1-mediated phosphorylation of preacetylated H3S10 at the FOSL1 enhancer. The adaptor protein 14-3-3 binds to H3S10ph nucleosomes and recruits the histone acetyltransferase MOF, which in turn triggers the acetylationof histone H4 at lysine 16 (H4K16ac). The resulting H3K9acS10ph/H4K16ac nucleosomes at the enhancer, and possibly the promoter[89], act as a platform for the binding of the bromodomain protein BRD4. BRD4-mediated recruitment of positive transcription elongation factor b (P-TEFb) is then necessary for the release of the promoter-proximal paused RNAPII (FIG. 6). Therefore, the FOSL1 enhancer appears to facilitate the recruitment of histone modifying complexes that trigger RNAPII elongation and productive transcription from its cognate promoter. This role of enhancers in the release of RNAPII from promoter-proximal pausing is different from the generally accepted role in transcription initiation. It will be important to investigate whether other enhancers use a similar strategy, since transcription regulation through promoter-proximal pausing of RNAPII is broadly used by eukaryotic genes[90].

## Conclusions and Perspectives

Advances in sequencing technologies and molecular techniques (ChIP-seq and 3C-based assays) have helped illuminate two critical aspects of enhancer function: their complex but largely invariant chromatin structure and the mechanisms underlying their long-distance influence on promoters. The definition of a specific chromatin signature at enhancers -the presence of H3K4me1/2 modifications, H3.3/H2A.Z variants, p300 occupancy and hypersensitivity to DNase I - has allowed the prediction of putative enhancers across the genome. In addition, the finding that cohesin, and perhaps ncRNAs, can mediate long-distance interactions between enhancers and promoters to facilitate initiation and elongation of transcription has substantially contributed to understanding how enhancers can play a role that is both flexible and specific in the regulation of transcription.

The emerging picture suggests that enhancers impart epigenetic memory and dictate context-dependent signaling outcomes through their unique chromatin features. Histone modification patterns at the enhancers may determine cell-fate choices by fine-tuning the transcriptional output via differential recruitment of other histone modifying complexes[9, 91]. In the future it will be important to understand the regulatory mechanisms that maintain or edit chromatin modification patterns at enhancer sequences during cell differentiation. It is clear that many histone modifying enzymatic complexes contain protein domains for both synthesizing and recognizing specific chromatin marks[92, 93]. Such inter-domain crosstalk may provide a means for editing specific chromatin modifications at enhancers during development[45]. It is also possible that, in addition to regulating transcription activation, expression of eRNAs/ncRNAs from enhancers might be required for maintaining their histone modification patterns through successive cell divisions. Integration of genomics, proteomics and reverse genetics approaches on various developmental systems will ultimately provide more information on how changes in chromatin features at specific enhancers can be regulated and how they impact signaling outcomes as cells differentiate along diverse lineages.

Chromatin features and cohesin-mediated chromatin loops are inseparable facets of enhancer function. However, as recruitment of protein complexes to enhancers is necessary for long-range interactions between regulatory elements, it is reasonable to conclude that unique chromatin features at enhancers precede chromatin loop formation. Interestingly, cohesin complexes are involved in stabilizing many enhancer-promoter interactions and long-range associations between distant CTCF-insulator sites. This suggests that enhancer and insulators are both actively involved in high-order nuclear organization. It will be of great interest to identify regulatory mechanisms that segregate the differential functions of cohesin in maintaining these diverse chromatin loops. These studies will be instrumental for the understanding of the processes by which cell-type specific patterns of gene expression can be established and maintained.

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## Glossary

**Cluster analysis** A mathematical algorithm that organizes a set of observations into subsets (called clusters) according to their similarity. It is a common

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| | statistical technique used for data mining and establishing specific patterns |
| **Basal transcription** | Low levels of transcription that can occur in the absence of an activator |
| **Pioneer transcription factors** | The first transcription factors to access the regulatory regions of tissue-specific genes. Their association with chromatin initiates de-compaction of nucleosomes and the cascade of events that culminates in transcriptional activation |
| **Mediator** | The ~30-subunit co-activator complex that is necessary for successful transcription at class II promoters of metazoans genes. Mediator coordinates the signals between enhancers and the general transcription machinery through its interaction with RNA polymerase II and site-specific factors |
| **GENCODE** | The GENCODE annotation aims to identify and map all gene features within the ENCODE (ENCyclopedia Of DNA Elements) regions by experimental validation. The results will include protein-coding genes with alternatively transcribed variants, non-coding RNAs and pseudogenes |

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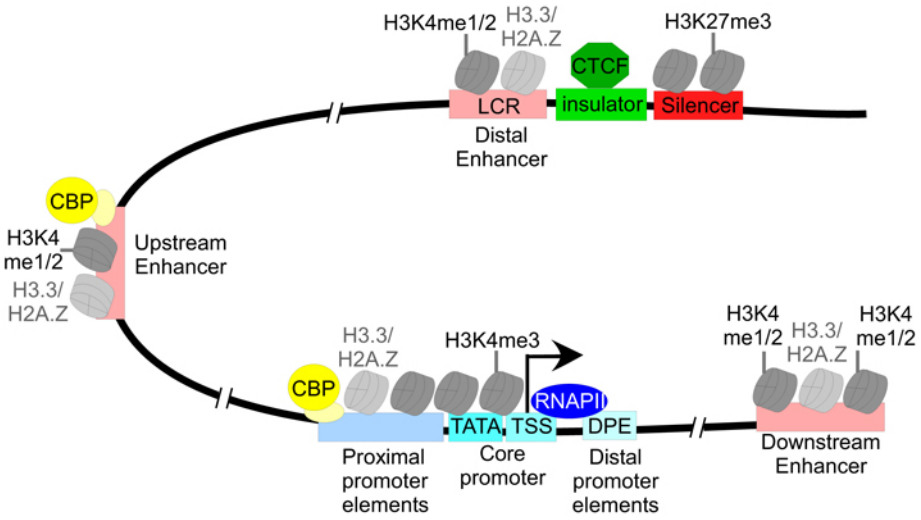


**Figure 1. Regulatory elements of transcription in metazoans**

The promoter is typically comprised of proximal, core and downstream elements. Transcription of a gene can be regulated by multiple enhancers that are located distantly, interspersed with silencer and insulator elements. Recent genome-wide data have revealed that many enhancers can be defined by unique chromatin features and the binding of CBP.

| | |
|---|---|
| CBP: | CREB (Cyclic-AMP Response Element Binding)-binding protein. |
| CTCF: | CCCTC-binding factor. |
| DPE: | Distal promoter elements. |
| H3K4me1/2: | histone H3 mono/di-methylation at lysine 4. |
| H3K4me3: | histone H3 trimethylation at lysine 4. |
| H3K27me3: | histone H3 trimethylation at lysine 27. |
| H3.3/H2A.Z: | histone variants H3.3 and H2A.Z. |
| LCR: | Locus control region. |
| RNAPII: | RNA polymerase II. |
| TATA: | 5′-TATAAAA-3′ core DNA sequences. |
| TSS: | Transcription start site. |

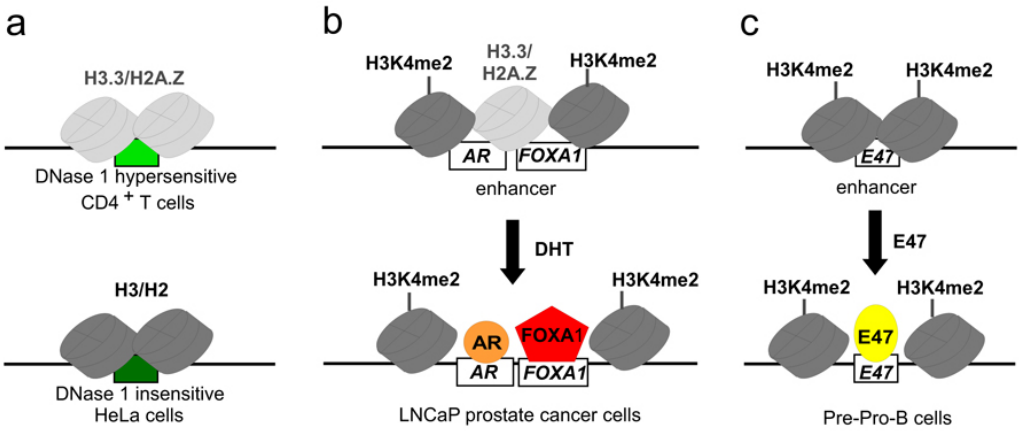


**Figure 2. Many functional enhancers contain dynamic nucleosomes**

**a.** Histone variants H3.3/H2A.Z are found at the enhancer, which is DNase I hypersensitive only in $CD4^+$ T cells but not in HeLa cells. **b.** In LNCaP cells, stimulation of androgen-mediated transcription programs by dihydrotestosterone (DHT) leads to the displacement of the unstable nucleosome at the enhancer by incoming transcription factors. **c.** Binding of E47 protein to the enhancer is facilitated by outward movement of the H3K4me2-marked nucleosomes at the transcription factor binding site.

| | |
|---|---|
| AR: | Androgen receptor. |
| CD4: | Cluster of differentiation 4. |
| DHT: | Dihydrotestosterone. |
| E47: | Immunoglobulin E2-Box Binding Protein isoform 47. |
| FoxA1: | Foxhead box A1. |
| H3/H2: | histone H3 and H2. |

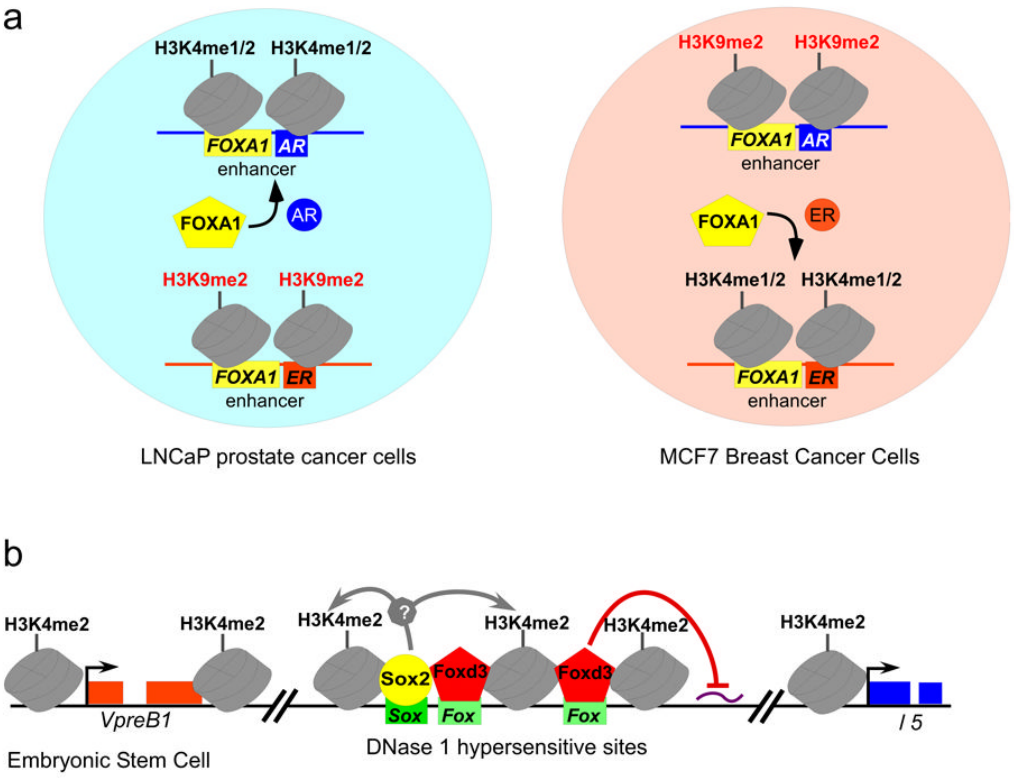


**Figure 3. Chromatin signatures at enhancers act as epigenetic signals for gene induction**

**a.** Cell-type specific distribution of H3K4me2 marks at the enhancers allows the differential binding of FoxA1 and specific transcription factors to the chromatin sites, which results in the activation of distinct transcriptional programs. AR: androgen receptor, ER: estrogen receptor. **b.** In ESCs, Sox2 contributes to the establishment of the H3K4me2 mark at the *λ5-VpreB1* enhancer whereas Foxd3 represses intergenic transcription. These epigenetic marks at the enhancer are required for tissue-specific expression during differentiation.

| | |
|---|---|
| AR: | Androgen receptor. |
| ER: | Estrogen receptor. |
| FoxA1: | Foxhead box A1. |
| Foxd3: | Foxhead box D3. |
| H3K9me2: | histone H3 dimethylation at lysine 9. |
| Sox2: | SRY (sex determining region Y)-box 2. |

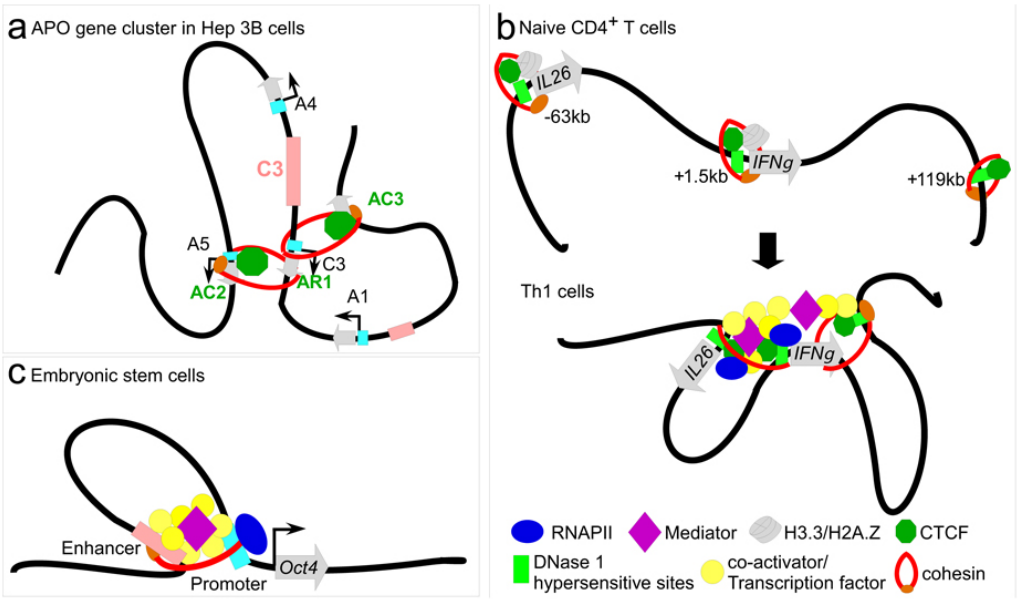


**Figure 4. Cohesins stabilize enhancer-promoter interactions by different strategies**

**a** and **b.** CTCF/Cohesin-mediated chromatin loops bring enhancer and promoters into close proximity at different gene loci. **a.** In the human APO gene cluster, long-range interactions between the AC2, AR1 and AC3 insulator sites result in the formation of two chromatin loops. The C3 enhancer and C3/A4/A5 promoters reside in one loop, whereas the A1 promoter is present in a different loop. **b.** The *IFN-γ* locus is reorganized by CTCF/cohesin-mediated interactions during Th1 differentiation. **c.** Association of enhancers and promoters at several genes in ESCs is mediated by physical interactions between mediator and cohesin complexes.

| | |
|---|---|
| APO: | Apolipoprotein. |
| CTCF: | CCCTC-binding factor. |
| IL26: | Interleukins 26. |
| IFNγ: | Interferon-gamma. |
| Oct4: | octamer-binding transcription factor 4. |
| RNAPII: | RNA polymerase II. |

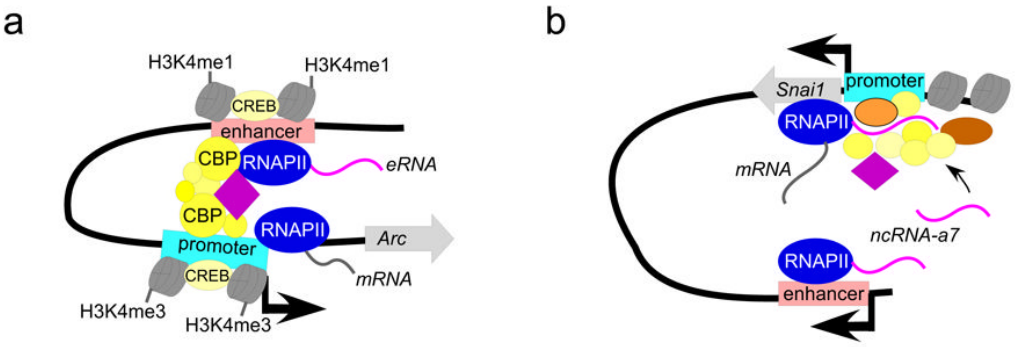


**Figure 5. Non-coding RNAs mediate enhancer function**

**a.** The transcription of eRNA positively correlates with the level of mRNA synthesis at nearby genes and requires the presence of an intact promoter at the gene, suggesting the presence of long-range associations between the enhancer and promoter. The type of mediator (purple diamond) and cofactors (yellow circle) involved remains to be determined. **b** A hypothetical model in which long ncRNA-a7 serves as a scaffold for the assembly of transcription factors (yellow circle) or other chromatin remodeling enzymes (brown oval) at the *Snai1* promoter to facilitate gene activation.

| | |
|---|---|
| Arc: | Activity-regulated cytoskeleton-associated protein |
| CREB: | Cyclic-AMP Response Element Binding Protein. |
| CBP: | CREB-binding protein. |
| eRNA: | enhancer RNA. |
| mRNA: | messenger RNA. |
| ncRNA: | non-coding RNA. |
| Snai1: | Snail homolog 1a. |

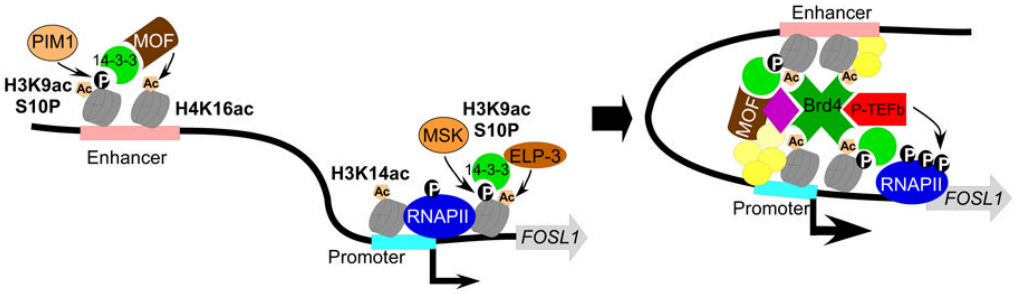


**Figure 6. Histone crosstalk between enhancers and promoters regulates RNAPII elongation**

Serum stimulation induces PIM1-mediated phosphorylation of preacetylated H3K9S10 at the *FOSL1* enhancer. Binding of 14-3-3 to H3S10ph nucleosomes recruits MOF, which in turn acetylates H4K16 (H4K16ac). In *Drosophila*, Elp3 and JIL-1 respectively acetylate and phosphorylate H3K9S10ph at the promoter. The modified nucleosomes act as a platform for the sequential recruitment of BRD4 and P-TEFb, which then facilitates the release of the promoter-proximal paused RNAPII. The type of mediator (purple diamond) and co-factors (yellow circle) involved remains to be determined.

| | |
|---|---|
| Brd4: | Bromodomain containing 4 |
| ELP-3: | Elongator complex protein 3. |
| FOSL1: | FOS-like antigen 1. |
| H3K9acS10P: | histone H3 acetylation at lysine 9 and phosphorylation at serine 10. |
| H4K16ac: | histone H4 acetylation at lysine 16. |
| MOF: | Male absent on the first, histone H4 lysine 16-specific acetyltransferase. |
| MSK: | Mitogen and Stress-Activated Protein Kinase. |
| PIM1: | Proviral integration site 1, a proto-oncogene serine/threonine-protein kinase. |
| P-TEFb: | positive transcription elongation factor b (P-TEFb). |
| RNAPII: | RNA polymerase II. |