MODES OF TRANSCRIPTIONAL REGULATION

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 many 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 crucial players responsible for facilitating enhancer–promoter interactions at some genes. Surprisingly, these interactions may be required not only to facilitate initiation of transcription but also to activate the release of RNA polymerase II (RNAPII) from promoter-proximal pausing.

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.

Differentiation of the various cell types present in multicellular organisms requires the establishment of spatiotemporal 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[2] (FIG. 1). Regulation of transcription is accomplished mainly 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 cases, they can even activate transcription of genes located in a different chromosome[4,5]. A key 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 transcription factor II D (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, decompaction of the chromatin fibre. This task is also carried out by enhancer-bound transcription factors that can recruit histone modifying enzymes or ATP-dependent chromatin remodelling 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 portrays 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], whereas others may play a more active part in the transcription activation process[12]. In addition, it appears that enhancer sequences themselves are not mere binding sites for transcription factors but are also transcribed into non-coding RNAs — which,

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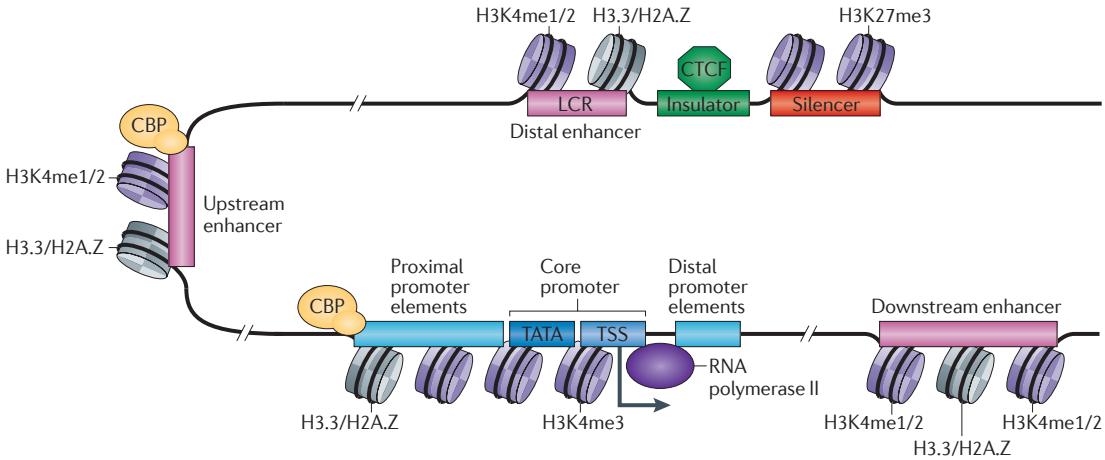


Figure 1 | **Transcriptional regulatory elements in metazoans.** The promoter is typically comprised of proximal, core and downstream elements. Transcription of a gene can be regulated by multiple enhancers that are located distantly and interspersed with silencer and insulator elements, which are bound by regulatory proteins such as CCCTC-binding factor (CTCF). Recent genome-wide data have revealed that many enhancers can be defined by unique chromatin features and the binding of cyclic AMP-responsive element-binding (CREB) protein (CBP). H3K4me1/2, histone H3 mono- or dimethylation 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; TATA, 5′-TATAAAA-3′ core DNA sequences; TSS, transcription start site. Figure is modified, with permission, from REF. 97

together with cohesin, may have 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; the interactions 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 over relatively long distances in an orientation-independent manner with the exquisite precision required to orchestrate the complex process of cell differentiation during development.

Chromatin features of enhancers

The distribution of post-translational histone modifications and the presence of specific histone variants influence gene expression by orchestrating the interaction of transcription factors with the chromatin fibre[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 programmes.

Nucleosome dynamics define 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[20,24,25] (FIG. 2). 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 cells but not in HeLa cells contain unstable double-variant nucleosomes in the $CD4^+$ T cells but not the HeLa cells (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 programmes in prostate cancer cells has revealed a class of enhancers containing androgen receptor and forkhead box protein A1 (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 modelling based on the behaviour of paired H3K4me2

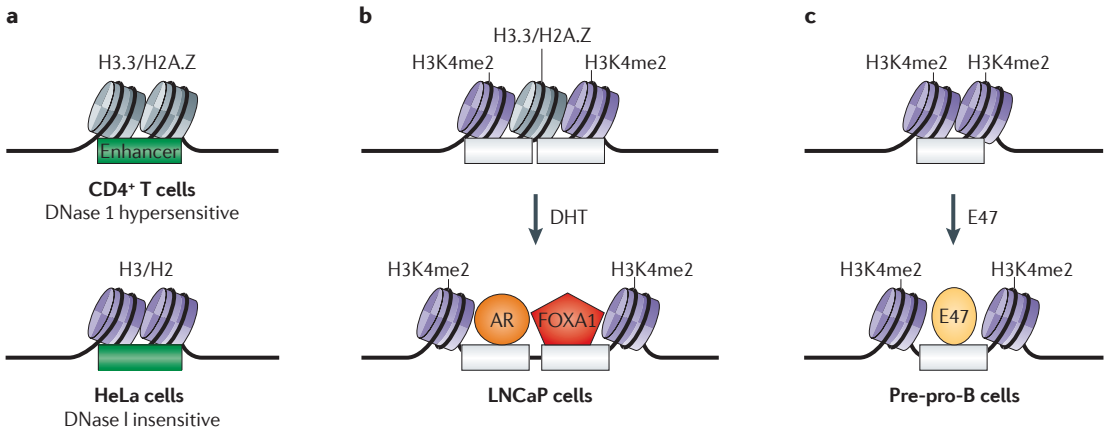


Figure 2 | **Many functional enhancers contain dynamic nucleosomes.** Scenarios are illustrated for sets of enhancers at which nucleosome positioning of composition is distinct — in different cell types or before and after transcription factor binding. **a** | Histone variants H3.3/H2A.Z are found at the enhancer, which is DNase I hypersensitive only in $CD4^+$ T cells but not in HeLa cells. In HeLa cells, the canonical histones H2 and H3 are instead present at the enhancer. **b** | In LNCaP cells (a human prostate cancer cell line), stimulation of androgen-mediated transcription programmes by dihydrotestosterone (DHT) leads to the displacement of the unstable nucleosome at the enhancer by the incoming transcription factors, androgen receptor (AR) and forkhead box protein A1 (FOXA1). **c** | In pre-pro-B cells (precursors of fully differentiated B cells) binding of immunoglobulin E2-box binding protein isoform 47 (E47) to the enhancer is facilitated by outward movement of the H3K4me2-marked nucleosomes at the transcription factor binding site. H3/H2, histone variants H3 or H2; H3.3/H2A.Z, histone variants H3.3 or H2A.Z; H3K4me2, histone H3 dimethylation at lysine 4.

nucleosomes correctly identified known androgen-responsive enhancers and predicted novel enhancers from genes that are only activated after prolonged stimulation. A separate study in progenitor (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[28] (FIG. 2c).

Together, these data suggest that an important feature of many 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. Cyclic AMP-responsive element-binding (CREB) protein (CBP) 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 several sequence-specific binding transcription factors, including CREB, and have previously been shown to be involved in several cell signalling 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, *in 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 and/or p300 regulates distinct transcriptional programmes in many cell types[12] and, therefore, that 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 and 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 histone H3 trimethylated at lysine 4 (H3K4me3). By contrast, putative enhancers, predicted by the presence of distant p300 binding sites, are highly enriched in H3K4me1, H3K4me2 and histone 3 acetylated at lysine 27 (H3K27ac)[10,12]. The chromatin state at promoters is largely invariant across different cell types, whereas histone modifications 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 to be associated with the enhancers of specific genes in multiple cell lines, including E2A-responsive genes in B cells[28], FOXA1-regulated genes in MCF7 breast cancer cells and LNCaP prostate cancer cells[27,33], and a subset of differentiation genes in haematopoietic stem cells and progenitor cells[11]. As the H3K4me1 and H3K4me2 marks are common to many enhancers, the cell type specificity of these regulatory

sequences may come from the presence of binding sites for additional factors that may either further alter the chromatin structure at the enhancer or affect transcription by an alternative mechanism.

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 (REFS 20,25), H3K18ac[25], H3K9ac and H3K14ac[35,36]. These chromatin features were further validated in well-characterized enhancers of the interleukin-2RA (*IL2RA*), *IL13*, *CD4* and interferon-γ (*IFNG*) genes in T cells.

There are several possible explanations for the apparently contradictory results obtained using the presence of CBP or p300 versus DNase I hypersensitivity as predictors of enhancer identity — particularly for the presence of H3K4me3 at DNase I hypersensitive regions but not at CBP or p300 sites. It is likely that the presence of CBP or p300 may only identify a subset of DNase I hypersensitive enhancers[10]; this may, in fact, be expected, since CBP and p300 are recruited to chromatin through different sequence-specific DNA binding proteins, including 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 many enhancers, these sequences are probably marked by combinatorial histone modification patterns indicative of their context-dependent functions.

Establishing chromatin signatures at enhancers. Although the enzymes involved in various histone modifications and in 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 present at promoters of both active and inactive genes in the absence of sequence-specific transcriptional activators or stable association of RNAPII. Although the distribution of H3K4me1 mark was not examined in these experiments, this observation suggests that enhancers may also acquire specific chromatin marks before distinct transcriptional programmes 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 and/or H3K4me2 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 immunoglobulin λ-like polypeptide 1 (*IGLL1*; also known as *λ5*) –pre-B lymphocyte 1 (*VPREB1)* enhancer, which is necessary for the expression of the *IGLL1* 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 paired box 5 (*PAX5*), B cell linker (*BLNK*) and deoxynucleotidyltransferase, terminal (*DNTT*)), whereas FOXD3 represses intergenic transcription from the enhancer in ESCs (FIG. 3a). As ESCs differentiate into pro-B cells, recruitment of the lineage-specific transcription factor SOX4 to SOX2 binding sites is required for enhancer activity and expression of the *IGLL1* 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].

Although 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-remodelling 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 the importance of reciprocal interactions between chromatin state and transcription factors in gene expression: specific chromatin signatures at enhancers require the concerted action of multiple protein complexes, whose activities can in turn be subjected to context-dependent regulation (see below).

Epigenetic signals for gene induction. The presence of common — H3K4me1 and H3K4me2 marks and H3.3/H2A.Z variants — as well as more specific covalent histone modifications at enhancers suggests 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

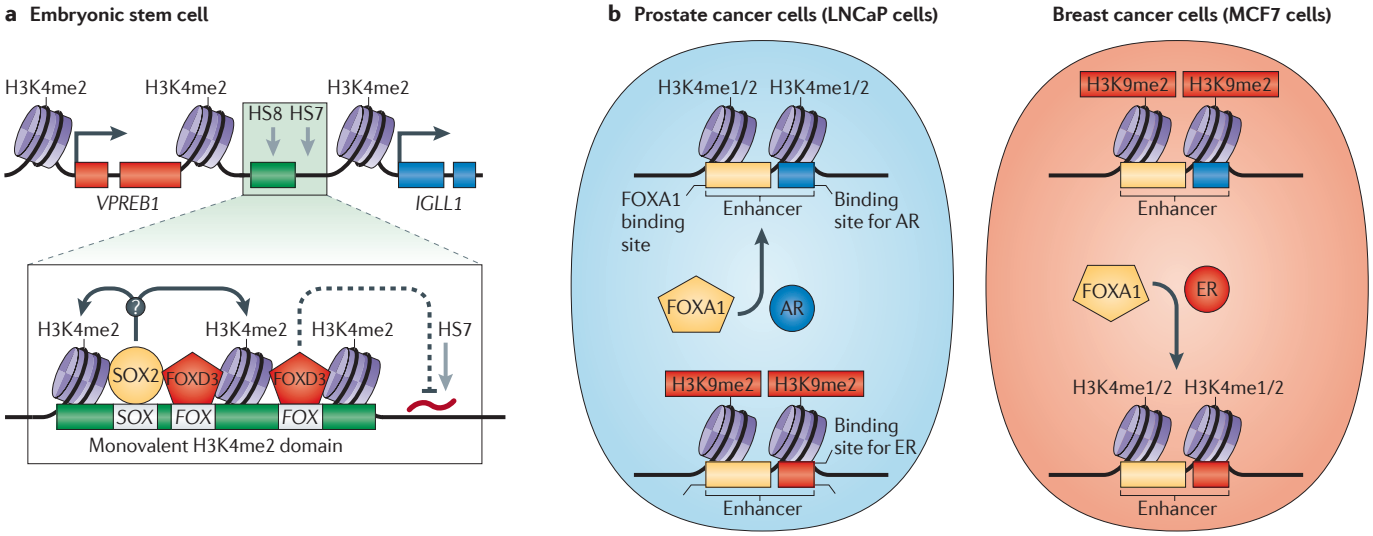


Figure 3 | **Chromatin signatures at enhancers act as epigenetic signals for gene induction. a** | The enhancer of the immunoglobulin λ-like polypeptide 1 (*IGLL1*; also known as *λ5*) and pre-B lymphocyte 1 (*VPREB1*) genes is located between the two, marked by tightly localized monovalent peaks of histone H3 dimethylation at lysine 4 (H3K4me2; shown by a light green box) and two DNase I hypersensitive sites (HS7 and HS8). In embryonic stem cells (ESCs), the transcription factor SOX2 contributes to the establishment of the H3K4me2 mark at the *IGLL1–VPREB1* enhancer, whereas forkhead box protein D3 (FOXD3) represses intergenic transcription. These epigenetic marks at the enhancer are required for tissue-specific expression during differentiation. **b** | In both LNCaP prostate cells and MCF7 breast cancer cells, the cell type-specific distribution of H3K4me2 marks at a set of enhancers allows the differential binding of FOXA1 and the androgen receptor (AR) or oestrogen receptor (ER) transcription factors, which results in the activation of distinct transcriptional programmes. H3K4me1/2, histone H3 mono- or dimethylation at lysine 4; H3K9me2, histone H3 dimethylation at lysine 9.

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. In human ESCs, enhancer elements with H3K4me1 and H3K27ac marks are located proximally to actively expressed genes. By contrast, enhancers associated with H3K4me1 and H3K27me3 are linked to inactive genes. These genes are turned on in differentiation processes that take place later during embryonic development, at which time H3K27me3 is replaced by H3K27ac. These results indicate that active and poised enhancers can by distinguished by the presence of H3K27ac or H3K27me3, respectively[45,46]. A second example of epigenetic chromatin alterations associated with developmental processes has been described during the differentiation of multipotent $CD133^+$ human primary haematopoietic stem cells (HSCs) 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 transcription[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. This suggests that the crucial 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 the enhancers of a subset of developmentally poised haematopoietic genes[47]. These chromatin marks at enhancers can then be interpreted by transcription factors to effect different gene expression programmes.

Also consistent with this hypothesis, it has been found that pioneer transcription factor FOXA1 switches on specific oestrogen- and androgen-responsive programmes in MCF7 and LNCaP cells, respectively, by its differential binding to selected H3K4me2-marked enhancers[33]. In MCF7 cells, enhancers marked with H3K4me2 have oestrogen receptor binding sites, whereas in LNCaP cells, H3K4me2-marked enhancers have androgen receptor binding sites (FIG. 3b). Therefore, FOXA1 translates epigenetic signatures at the enhancer into distinct transcriptional programmes 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 at least some enhancer sequences may 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

Basal transcription
Low levels of transcription that can occur in the absence of an activator.

Pioneer transcription factor
The first transcription factor to access a regulatory region of tissue-specific genes. Its association with chromatin initiates decompaction of nucleosomes and the cascade of events that culminates in transcriptional activation.

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 and/or function are likely to be histone variants H3.3/H2A.Z and H3K4m1 or H3K4m2, elicited by pluripotency factors in embryonic stem cells. Other histone modifications, such as H3K27ac, H3K8ac or H4K16ac may subsequently be added to alter the response of a subset of enhancers and restrict the differentiation potential of the cell.

The role of long-range interactions

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 interactions occur 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[48–54]. This hypothesis has been tested experimentally by fluorescence *in situ* hybridization (FISH) and, more recently, by chromosome conformation capture (3C)[55]. 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 programmes at a large number of gene loci[56–58]. Recent studies on the mechanisms underlying the cross-talk 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 fruitful model systems for the study of long-range interactions in gene regulation[51,59,60]. The mouse β-globin locus contains a cluster of β-chain variants of haemoglobin that are developmentally regulated by multiple elements spanning a region of 100 kb. These regulatory elements include the locus control region (LCR), which acts as an enhancer and is located approximately 25 kb upstream of the εy-globin gene (*HBE1*), and a group of DNase I hypersensitive sites located approximately 20 kb downstream of the locus (3′HS1) and upstream of the LCR (–60 HS). Expression of the β-globin gene (*HBB*) requires the presence of specific transcription factors (erythroid transcription factor (GATA1) and Krüppel-like factor 1 (KLF1)) that mediate the clustering of these elements with the β-globin gene promoters through long-range interactions to form what has been described as an 'active chromatin hub'[61,62].

The formation of a chromatin hub by looping of distant sequences has also been detected in the T helper type 2 (T_H2) cytokine locus. Long-range interactions among multiple enhancer and promoter elements within the locus increases significantly upon T_H2 cell differentiation, suggesting that the three-dimensional organization of poised co-regulated genes is important for rapid activation upon induction[63,64]. The ability of enhancers to interact with promoters is not limited to genes located in *cis* on the same chromosome as the enhancer; the olfactory H enhancer has been shown to interact with multiple olfactory receptor genes both 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 interchromosomal 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[65]. 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.

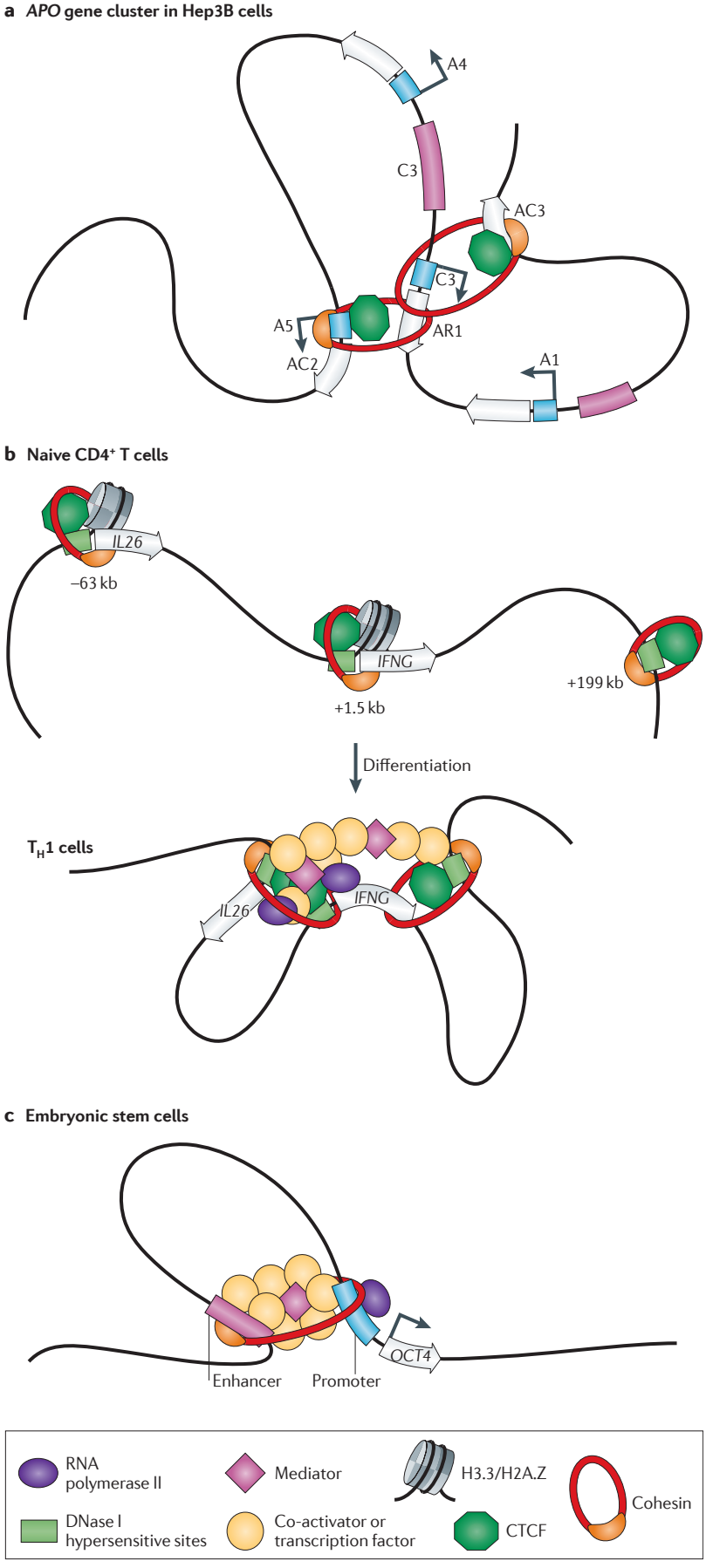
Cohesin stabilizes long-range interactions. Although the role of transcription factors in mediating association between specific regulatory elements in the genome is well-characterized, the nature of the protein complexes required for stabilizing these interactions has remained elusive. Recent studies suggest that cohesin complexes might be, at least in part, responsible for this task. Cohesins are known to mediate sister chromatid

Figure 4 | **Cohesins stabilize enhancer–promoter interactions by different strategies.** Chromatin loops that are mediated by interactions between CCCTC-binding factor (CTCF) and cohesin bring enhancer and promoters into close proximity at different gene loci. **a** | In the human apolipoprotein (*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 and A5 promoters reside in one loop, whereas the A1 promoter is present in a different loop. **b** | The interferon-γ (*IFNG*) locus is reorganized by CTCF and/or cohesin-mediated interactions during T helper type 1 (T_H1) cell differentiation. **c** | Association of enhancers and promoters at several genes in embryonic stem cells (ESCs) — for example, octamer-binding protein 4 (*OCT4*; also known as POU domain, class 5, transcription factor 1) is mediated by physical interactions between Mediator and cohesin complexes. H3.3/H2A.Z, histone variants H3.3 or H2A.Z; *IL26*, interleukin-26; RNAPII, RNA polymerase II. Part **a** is modified, with permission, from REF. 79 © (2009) Macmillan Publishers Ltd. All rights reserved.

Chromosome conformation capture
(3C). A technique used to study long-distance interactions between genomic regions, which in turn can be used to study the three-dimensional architecture of chromosomes within a cell nucleus.

Locus control region
(LCR). *Cis*-acting element that organizes a gene cluster into an active chromatin domain and enhances transcription in a tissue-specific manner.

Transcription factory
A nuclear subcompartment that is rich in RNA polymerases and transcription factors where dispersed genes gather to become active.



cohesion, which is necessary for proper chromosome segregation and DNA repair[66–68]. Earlier studies in various model organisms have also highlighted their involvement in gene regulation[69–74]. However, the mechanism underlying their transcription regulatory function only became evident recently when cohesins were shown to interact with CCCTC-binding factor (CTCF)[75–78]. CTCF is a highly conserved zinc finger protein with diverse gene regulatory functions and has been proposed to organize global chromatin architecture by mediating intra- and interchromosomal contacts[52].

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 through two distinct strategies. In human Hep3B cells, the region containing the apolipoprotein genes *APOA1*, *APOC3*, *APOA4* and *APOA5* 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 the *APOC3*, *APOA4* and *APOA5* 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 substantial changes in APO expression and the reduced localization of the transcription factor hepatocyte nuclear factor 4A (HNF4A) and the transcriptionally active form of RNAPII specifically at the *APOC3* promoter[79]. Similar cohesin-mediated chromatin loops were also detected at the developmentally regulated *IFNG* locus (FIG. 4b)[80], the imprinted *IGF2–H19* locus[81] and the β-globin locus[82]. These results suggest that CTCF- or cohesin-mediated chromatin looping facilitates interactions between enhancers and promoters by bringing them into close proximity.

Studies of transcription in mouse embryonic stem cells suggest that cohesin stabilizes the looping necessary for the direct interaction between enhancers and promoters in the absence of CTCF. Chromatin immunoprecipitation followed by sequencing (ChIP–seq) analyses show that Mediator, cohesin and the cohesin loading factor nipped B-like protein (NIPBL) co-localize at thousands of sites within the ESC genome[83]. NIPBL is generally found at the Mediator- and cohesin-bound enhancer and promoter regions, but rarely at CTCF and 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[83] (FIG. 4c).

Non-coding RNAs and enhancer function

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

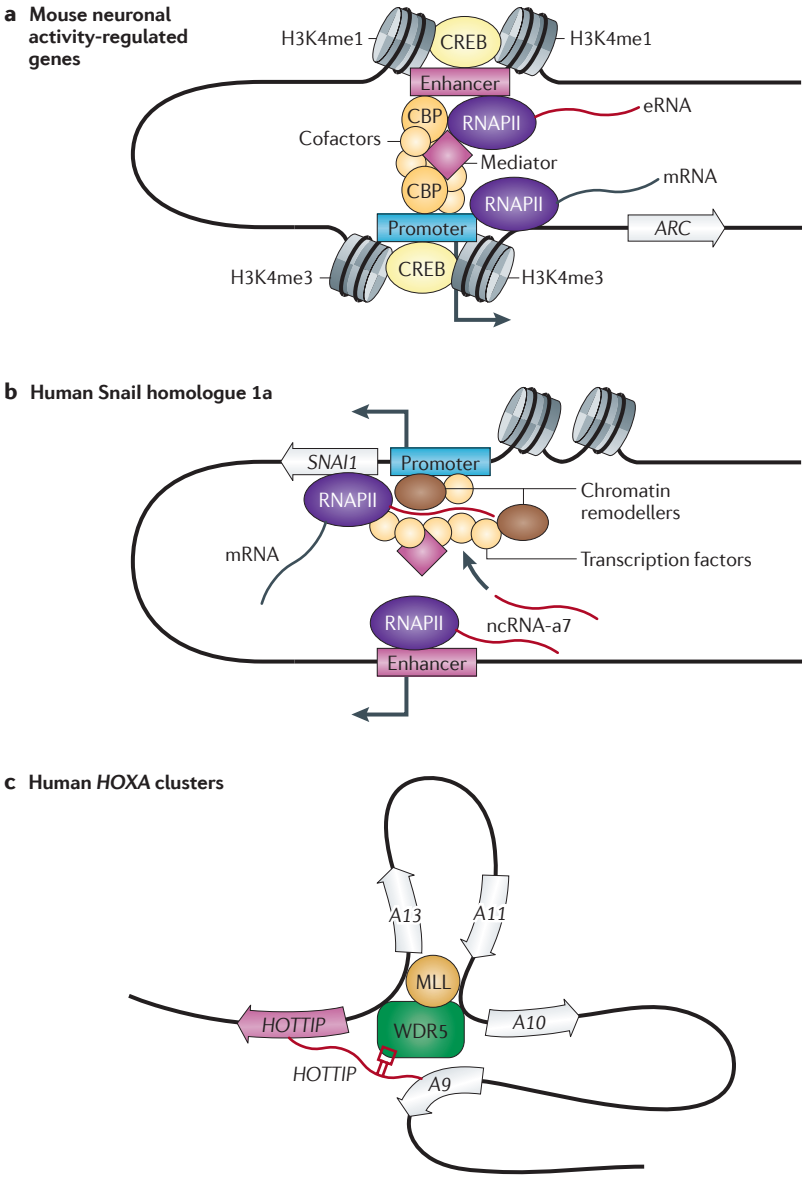


Figure 5 | **Non-coding RNAs mediate enhancer function. a** | In mouse cortical neurons, neuronal activity-regulated enhancers are bound by cyclic AMP-responsive element-binding (CREB) and the general transcriptional co-activator CREB protein (CBP) in an activity-dependent manner. The transcription of enhancer RNA (eRNA) positively correlates with the level of mRNA synthesis at nearby genes, such as activity-regulated cytoskeleton-associated (*ARC*). Transcription of eRNA 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 (shown by a purple diamond) and the specific cofactors (shown by yellow circles) that are involved remain to be determined. **b** | A hypothetical model, based on findings using A549 human alveolar epithelial cells, in which the long non-coding RNA (ncRNA-a7) serves as a scaffold for the assembly of transcription factors (shown by the yellow circles) or other chromatin remodelling enzymes (shown by brown ovals) at the Snail homologue 1a (*SNAI1*) promoter to facilitate gene activation. **c** | In human foreskin fibroblasts, chromosome looping brings the *HOTTIP* (*HOXA* transcript at the distal tip) long intergenic ncRNA in close proximity to its target genes (homebox A9 (*HOXA9*), *HOXA10*, *HOXA11*, *HOXA12* and *HOXA13*). HOTTIP binds WD repeat-containing protein 5 (WDR5), which in turn recruits the mixed-lineage leukaemia (MLL) histone methylase complex. Targeting of this complex to the 5′ *HOXA* locus by HOTTIP drives histone H3 trimethylation at lysine 4 (H3K4me3) and gene transcription. CBP, CREB-binding protein; CREB, cyclic-AMP response element binding protein; H3K4me1, histone H3 monomethylation at lysine 4; H3K4me3, histone H3 trimethylation at lysine 4; RNAPII, RNA polymerase II.

analyses have revealed that stimulus-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[84]. The transcription of this 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 part in forming a chromatin hub by interacting with other factors — analogous to the role of RNA in the formation of chromatin insulator complexes[85,86].

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 intergenic ncRNAs (lincRNAs) are necessary for robust expression of their neighbouring protein-coding genes in multiple cell lines as well as transcription activation from the thymidine kinase promoter in luciferase reporter assays. 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 remodelling enzymes at the promoter (FIG. 5b). This mechanism would be similar to that of the human HOX antisense intergenic RNA (HOTAIR RNA): this ncRNA mediates the assembly of different histone-modifying complexes and is necessary for their DNA binding and the resulting silencing of homeobox D cluster (*HOXD*) gene expression[87]. Indeed, recent data indicate that transcription of the *HOXA* transcript at the distal tip (HOTTIP) lincRNA from the 5′ end of the *HOXA* locus is required for the activation of multiple 5′ *HOXA* genes[88] (FIG. 5c). HOTTIP binds the adaptor protein WD repeat-containing protein (WDR5), which in turn recruits the mixed-lineage leukaemia (MLL) histone methyltransferase complex. HOTTIP is brought into close contact with its target genes through chromosome looping, targeting bound WDR5 and MLL1 complexes to these genes, and resulting in trimethylation of H3K4 and transcription activation. Interestingly, the activity of HOTTIP is *cis*-restricted and distance-dependent. These results suggest that lincRNAs can recruit specific chromatin-modifying factors to different genomic locations by organizing higher-order chromosome structures through DNA looping.

Enhancer–promoter communication

Distinct chromatin signatures at enhancers are proposed to act as epigenetic marks that poise specific genes for rapid induction[11,47]. Studies aimed at testing this hypothesis indicate that different signalling pathways may employ distinct molecular strategies to disseminate the epigenetic information embedded at the enhancer to the core promoters during transcriptional activation.

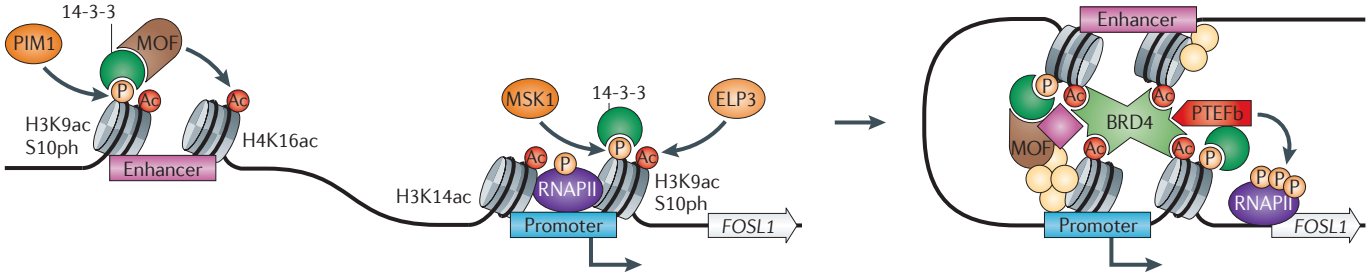


Figure 6 | **Crosstalk between histones at enhancers and promoters regulates RNAPII elongation.** Serum stimulation induces proviral integration site 1 (PIM1)-mediated phosphorylation (P) of pre-acetylated (Ac) H3K9S10 (serine 10 of histone H3 lysine 9) at the FOS-like antigen 1 (*FOSL1*) enhancer. PIM1 is a proto-oncogene-encoded serine/threonine protein kinase. Binding of 14-3-3 to nucleosomes that carry histone H3 acetylation at lysine 9 and phosphorylation at serine 10 (H3K9acS10ph) recruits MOF, a histone H4 lysine 16-specific acetyltransferase, which in turn acetylates H4K16 (H4K16ac). In *Drosophila melanogaster*, elongator complex protein 3 (ELP3) and JIL1 (a serine/threonine kinase that is related to nuclear mitogen- and stress-activated protein kinase 1 (MSK1 in humans)) respectively acetylate and phosphorylate H3K9S10 at the promoter. The modified nucleosomes act as a platform for the sequential recruitment of bromodomain-containing 4 (BRD4) and positive transcription elongation factor b (PTEFb). PTEFb then facilitates the release of the promoter-proximal paused RNA polymerase II (RNAPII). The type of Mediator (shown by a purple diamond) and cofactors (shown by yellow circles) involved remains to be determined.

Traditionally, it has been thought that enhancers activate transcription by facilitating the recruitment and assembly of the transcription complex[2]. However, 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[89], *Drosophila melanogaster* heat shock genes[90] and 20% of MYC target genes[91]. The serum-inducible FOS-like antigen 1 (*FOSL1*) gene has been a useful model system for dissecting the molecular events at the enhancer that lead to transcriptional activation[14]. Serum stimulation induces proviral integration site 1 (PIM1)-mediated phosphorylation of pre-acetylated 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 acetylation of histone H4 at lysine 16 (H4K16ac). The resulting H3K9acS10ph and H4K16ac nucleosomes at the enhancer, and possibly the promoter[92], act as a platform for the binding of the bromodomain-containing protein (BRD4). BRD4-mediated recruitment of positive transcription elongation factor b (PTEFb) 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 occurs at many eukaryotic genes[93].

Conclusions and perspectives

Advances in sequencing technologies and molecular techniques (ChIP–seq and 3C-based assays) have helped to illuminate two key 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 and H3K4me2 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 part that is both flexible and specific in the regulation of transcription.

The emerging picture suggests that enhancers impart epigenetic memory and dictate context-dependent signalling outcomes through their unique chromatin features. Histone modification patterns at the enhancers may determine cell fate choices by fine-tuning the transcriptional output through differential recruitment of other histone modifying complexes[9,94]. 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[95,96]. Such interdomain crosstalk may provide a means for editing specific chromatin modifications at enhancers during development[47]. It is also possible that, in addition to regulating transcription activation, expression of eRNAs and other ncRNAs from enhancers might be required for maintaining the histone modification patterns at enhancers through successive cell divisions. Integration of genomics, proteomics and reverse genetics approaches in various developmental systems will ultimately provide more information on how changes in chromatin features at specific enhancers can be regulated and how they impact signalling outcomes as cells differentiate along diverse lineages.

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.

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 enhancers and insulators are both actively involved in high-order nuclear organization. It will be of great interest to identify the regulatory mechanisms that segregate the differential functions of cohesin in maintaining these diverse chromatin loops. These studies will be instrumental for understanding the processes by which cell type-specific patterns of gene expression can be established and maintained.

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Competing interests statement
The authors declare no competing financial interests.

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