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Enhancers

Multi-dimensional signal integrators

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Enhancers play a critical role in regulating tissue-specific gene expression, but their molecular mechanisms of function have not been fully characterized. It is now increasingly clear that enhancers associate with specific protein factors and chromatin modifications and also produce non-coding RNAs known as eRNAs. These predictive signatures have facilitated genomic identification of enhancers and helped characterize tissue-specific gene expression mechanisms. Herein we review recent studies investigating enhancers in mammalian cells, and propose that enhancers function as a central platform integrating lineage-specific transcription factors and epigenetic states with ubiquitous yet signal-dependent transcriptional inputs, culminating in highly specific gene expression programs.

Key words: enhancer, transcription factor, genomics, epigenetics, transcription regulation, cis-regulatory elements, cell type-specificity

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Introduction

The majority of the mammalian genome is composed of non-coding sequences. These sequences contain different types of cis-regulatory elements (i.e., promoters, enhancers, silencers and insulators) that collaboratively control gene transcription.[1] Discovered nearly 30 years ago, enhancers have attracted much attention due to their ability and critical role in regulating tissue-specific expression of a gene from a long distance and in an orientation-independent manner. Conventionally, enhancers are identified using a transient reporter gene system in which an inserted DNA sequence can activate transcription regardless of its orientation or location relative to the promoter. Mechanistically, these elements act by recruiting sequence-specific transcription factors (TFs) and co-activator complexes.[1,2] Until recently, our understanding of enhancer activity and function has been largely based on a limited number of genes, and the specific properties and mechanisms of action remain to be fully characterized. However, high throughput technologies, such as chromatin immunoprecipitation followed by microarray (ChIP-chip) or massive parallel sequencing (ChIP-Sequencing or ChIP-Seq), have led to significant advances in the biology of enhancers.

These recent advances in the study of enhancers are the subject of several excellent reviews.[3,4] Bulger and Groudine[3] summarized various mechanisms through which enhancers may activate target promoters: distal enhancers may activate genes through long-range interaction with promoters, altering nuclear organization, changing chromatin structure, and producing regulatory non-coding eRNA. In another review, Ong and Corces[4] focused on epigenetic mechanisms responsible for enhancer functions such as histone variants and chromatin modifications; these marks may carry cellular memory and provide cues for further enhancer activation. In this point-of-view article, we will focus on the role of enhancers in integrating signaling pathways with lineage specific TFs, ubiquitous TFs and epigenetic states. Based on several recent studies including our own, we propose that besides master lineage-specific TFs, which

establish cell- or tissue-specific enhancers, additional collaborative TFs are often required to create a permissive epigenetic environment for the binding of signal- or ligand-dependent TFs. Such multi-layer regulation mechanisms allow cells to deliver highly cell specific transcriptional responses to ubiquitous cellular signaling pathways.

Properties of Enhancers

It is generally accepted that enhancers function by recruiting sequence-specific transcription factors that recognize short DNA motifs within the enhancers and initiate the assembly of large, multi-protein complexes sometimes referred to as enhanceosomes. The human genome encodes 1,700–1,900 sequence-specific transcription factors,[5] many of which are expressed in a spatial- and temporal-restricted fashion. How do these transcription factors interact with enhancers to mediate tissue-specific gene expression programs? Traditionally, it has been argued that multiple TFs bind to DNA in a combinatorial manner, explaining the diversity of the transcriptional enhancers. In support of this theory, TF binding motifs are often found to exist in clusters along the genome, and certain TFs' binding have been shown to positively or negatively affect the recruitment of another TF at a neighboring location.[6,7] According to this view, the distribution of enhancers in the genome is dictated by the spectrum of TFs expressed in each tissue- or cell-type.

Sequence-specific transcription factors, upon binding to enhancers, recruit several classes of protein complexes, including the mediator complex, histone modifiers and chromatin remodelers to activate transcription of target genes. Mediator is a multi-protein complex that can directly bind both general transcription factors as well as enhancer-bound TFs, thereby facilitating the looping interactions between promoters and distal enhancers.[8,9] Histone modifiers and chromatin remodelers function by changing the chromatin environment around enhancers. The best characterized histone modifiers are histone acetylation transferases (HATs) such as CBP/p300. These enzymes acetylate the N-terminal tails of core histones with differing specificities at enhancers and promoters;[10,11] acetylated histones may in turn act as docking sites to stabilize or further recruit other protein complexes including chromatin remodelers such as the SWI/SNF complex.[12] Chromatin remodelers can reposition or evict nucleosomes along the DNA in an ATP-dependent fashion.[13,14] On enhancers, this activity is responsible for the creation of nucleosome-free regions (NFRs), and for facilitating the binding of sequence-specific TFs.

Genome-Wide Mapping of Enhancers

The unique properties of enhancers have permitted high-throughput identification of these elements in the genome. Enhancers can be experimentally identified by genome-wide location analysis (GWLA) of the above-mentioned enhancer-associated proteins using ChIP-chip or ChIP-Seq. GWLA of some HATs, such as CBP/p300, or a combination of multiple TFs, have identified numerous enhancers with high accuracy.[15,16] However, one problem with such approaches is that these proteins only function at a subset of enhancers in certain cell types.

Two alternative methods have been developed that exploit other features of enhancers for their identification. The first involves genomic mapping of NFRs, as enhancer (and promoter) elements are often devoid of nucleosomes due to TF binding and hence sensitive to nuclease digestion. Genomic methods include mapping of DNase I hypersensitivity sites[17] and FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements).[18] The second method, on the other hand, maps enhancers through enhancer-associated histone modifications, such as histone acetylation. Recent genomic data revealed a universal chromatin signature for unbiased enhancer identification. This method searches for locations with high H3K4 mono-methylation (H3K4me1) but low H3K4 tri-methylation (H3K4me3) levels. The H3K4 methylation signature is proven to be highly predictive of enhancer locations, and has been applied to identify enhancers in different tissue- or cell-types of several species.[19,20]

Enhancers Drive Cell-Specific Transcription Programs

One important finding from genome-scale analysis of enhancers is that these elements are associated with histone modifications and co-activator proteins in a highly cell type-specific manner.[19,21] This is in contrast to promoters (marked by H3K4me3) and insulator elements (bound by CCCTC-binding factor or CTCF), which are largely invariant across different cell types. Furthermore, cell-specific enhancer distribution correlates well with cell-specific gene expression,[19] suggesting that enhancers are the major contributors to cell specificity. These observations are supported by another large-scale enhancer mapping study using the co-activator p300.[16]

How do enhancers contribute to cell-specific gene expression programs? In multicellular organisms, virtually every cell contains the same set of genetic material but has very different properties and functions. For example, when facing the same environmental perturbation, different types of cells display specific responses and activate distinct transcription programs, even if the same signaling pathways and target TFs are activated. One prevailing explanation for such cell specificity involves the combinatorial regulation by other transcription factors at target promoters.[2] Recent genome-wide data, including our own, have highlighted the critical role of enhancers in driving cell-specific transcription programs in different cell types.[19,22-24] On one hand, pre-existing enhancers with open chromatin have a dominant role guiding the binding of signal dependent TFs; on the other, binding of different TFs synergistically prepare enhancers for target gene activation. We will discuss the new genomic evidence from both aspects.

Enhancers Govern TF Binding

Pre-existing enhancers with open chromatin have long been hypothesized to regulate TF binding in the genome. Supporting this idea, a few genome-wide studies observed preferential binding of signal or hormone dependent TFs and nuclear receptors, including STAT1,[25]

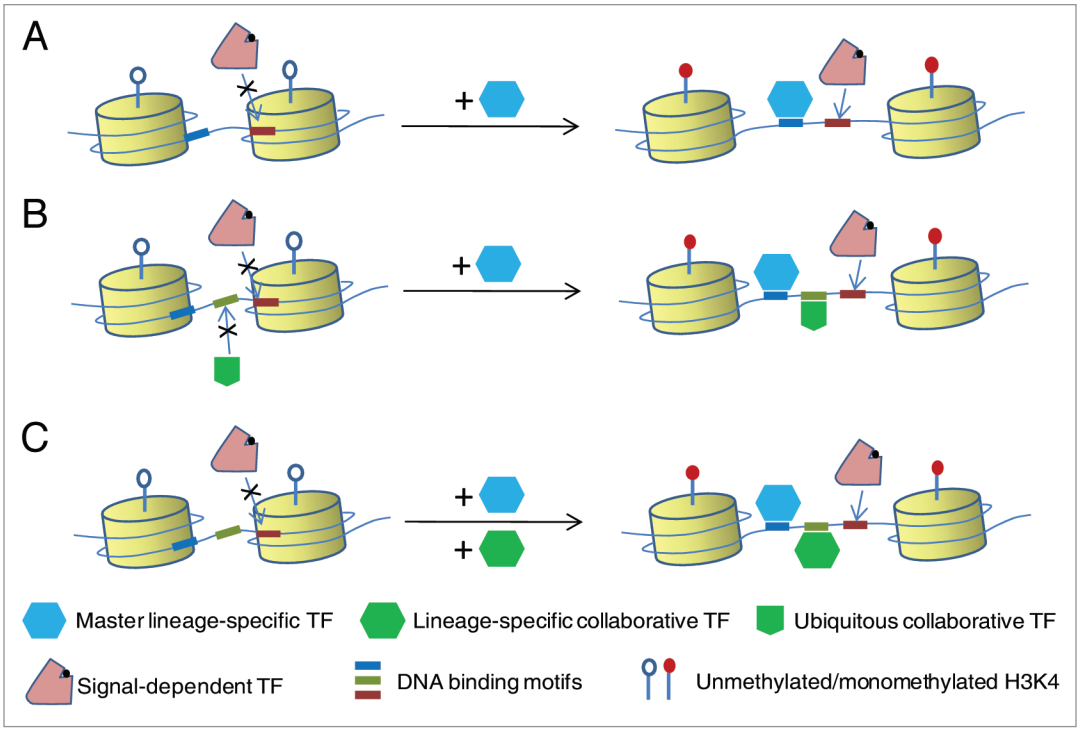

Figure 1. Mechanisms for cell-specific binding of signal-dependent TFs. (A) Simple selection model. Signal-dependent TFs cannot access their motifs until lineage-specific TFs open the chromatin and generate H3K4me1-marked enhancers. (B) Selection triggering model. In this model, cells already express the collaborative TF necessary for signal-dependent TF binding. Expression of the master lineage-specific TF triggers binding of the collaborative TF and allows binding of signal dependent TFs. (C) Hierarchical selection model. A master lineage-specific TF defines a selective set of enhancers, while binding of signal-dependent TFs also requires a second collaborative factor. In this case, the collaborative TF is also specifically expressed and provides another layer of selection.

LXRβ[26] and PPARγ[27], to locations that are marked by active chromatin modifications before cell stimulation. We recently systematically examined the binding sites of the TF NFκB (p65) after tumor necrosis factor-α (TNFα) treatment in HeLa cells and the human monocytic cell line THP-1.[22] Only ~30% of p65 binding sites in each cell type are common in both cell lines, and the differential p65 binding is highly correlated with cell-specific gene induction. Strikingly, we found that p65 preferentially binds to pre-existing enhancers: more than 80% of all p65 binding sites are already occupied by p300 or H3K4 methylation marks before cell stimulation. Importantly, cell-specific p65 binding sites can be strongly predicted by differential enhancer distribution.[22] These findings suggest a dominant role of pre-existing enhancers in determining the cell-specific recruitment of TFs.

This raises the question of how these enhancers regulate the DNA-TF interaction. One simple explanation is that enhancers define accessible genome locations for TFs to engage in vivo (**Fig. 1A**). Some genome-wide studies have linked epigenetically marked enhancers with open chromatin structures, as revealed by DNase I hypersensitivity,[28] FAIRE enrichment[18] or dynamic nucleosome positioning.[29] The SWI/SNF complex is also found at promoter distal regions, which are also likely to be enhancers.[30] Consistent with this model, in a recent genome-wide study that mapped DNase I hypersensitive sites, Stamatoyannopoulos and colleagues found that the majority of glucocorticoid receptor (GR) binding sites have pre-existing open chromatin before stimulation.[23] All these results support a simple selection model that signal dependent TF binding is pre-determined by differential baseline chromatin accessibility patterns (**Fig. 1A**).

TFs Change the Epigenetic Status of Enhancers

Recent genomic studies also revealed that a variety of events may occur on enhancers when new TF-DNA interactions are introduced and demonstrated that, using a few genomic assays, it is possible to determine the status or activity of all enhancers. For example, DNaseI-Seq revealed that signal-dependent GR binding invariably increased chromatin accessibility.[23] In another study, when macrophages were stimulated with lipopolysaccharide (LPS), only a subset of H3K4me1-marked enhancers near induced genes showed increased p300 binding, while levels of H3K4me1 on these locations stayed constant,[31] suggesting that H3K4me1 is a stable marker for enhancers both before and after transient stimulation. Consistent with this study, two recent papers showed that during differentiation, many H3K4me1-marked enhancers in embryonic stem cells gain the H3K27 acetylation mark (presumably by recruiting HATs such as p300) while losing repressive H3K27me3 mark, suggesting a switch from "poised" to active status.[32,33] These results suggest that H3K4me1 marks enhancers before they are activated. Although how this

modification is catalyzed or incorporated into enhancers is still unknown, it has been shown that lineage-specific TFs can facilitate the formation of H3K4me1 on enhancers.[26,31,34] In macrophages, loss of the ETS family transcription factor PU.1 leads to lower H3K4me1 levels on a subset of macrophage-specific enhancers; overexpressing this factor in fibroblasts or macrophage progenitor cells can create H3K4me1-marked enhancers.[26,31] In addition, re-introduction of the B-cell specific transcription factor E2A in E2A-deficient B cells can change the abundance and pattern of the H3K4me1 mark on target enhancer regions.[34] Collectively, these results confirmed the role of cell-specific TFs in establishing the enhanceosome during cell differentiation.

One unexpected phenomenon that emerged from genome-wide deep sequencing data is the finding that a significant fraction of enhancers can produce RNA, called enhancer RNA (eRNA).[35,36] eRNAs are low-abundance, short, non-coding, bidirectional and non-polyadenylated RNAs, and their transcription might require the presence of a target promoter. Although production of eRNA has been shown to correlate with enhancer activity,[37] it is still not clear how eRNA may regulate enhancer functions in general.

Enhancers are Integrators for Crosstalk of Multiple TFs

In addition to lineage specific TFs, other non-specifically expressed TFs may also contribute to a cell-specific transcription program (**Fig. 1B**). This is probably best exemplified by so called "pioneer factors" such as Fox family TFs. These factors are so named because their DNA binding domains are structurally similar to linker histones and can "explore" DNA motifs wrapped around nucleosomes without activating target genes. One pioneer factor, FoxA1, is expressed in both MCF7 (breast cancer cell line) and LNCaP cells (prostate cancer cell line), but binds to different enhancer locations correlating with differential marking by H3K4 methylation.[24] FoxA1 binding appears to create open chromatin at enhancers and is required for binding of ligand-dependent nuclear receptors, such as androgen or estrogen receptor (AR or ER) relevant to these cells. Knock-down of FoxA1 in LNCaP cells leads to dramatic reprogramming of the prostate-specific AR binding pattern.[37] Interestingly, reduction of H3K4 methylation by overexpression of histone demethylase LSD1 impairs the binding of FoxA1, suggesting that H3K4 methylation is an early mark on enhancers and acts upstream of pioneer factors.[24] It is likely that FoxA1 gains its binding specificity through other lineage-specific factors, and determines which enhancers will be used by AR or ER activation.

Our recent genome-wide results also revealed the synergistic effect of two THP-1-specific TFs (PU.1 and C/EBPα) in regulating NFκB-dependent transcription program. For several NFκB targeted cytokine genes examined, we observed TNFα response only when both PU.1 and C/EBPα were overexpressed in HeLa cells.[22] Intriguingly, although both TFs are specifically expressed in THP-1 cells, they seem to regulate NFκB binding through different mechanisms: (1) Only PU.1 motifs, but not C/EBPα motifs, are enriched in THP-1-specific NFκB binding sites; (2) Only a portion of PU.1 binding sites are associated with active chromatin, and these sites are likely to be bound by NFκB upon cell stimulation; (3) C/EBPα binding sites are enriched in those active PU.1 sites.

Although further experiments, such as knocking-down or overexpressing relevant proteins, are needed to dissect the crosstalk between PU.1 and C/EBPα on each NFκB target enhancer, our genome-wide results already suggested that monocyte-specific enhancers seem to be selected by two THP-1-specific TFs acting at different levels: PU.1 binding initiates the formation of potential monocyte-specific enhancers, while C/EBPα may further select and activate a subset of PU.1 associated locations. We speculate that this multi-level hierarchical selection mechanism (**Fig. 1C**) might be important for allowing cells to precisely control the production of cytokines, which is essential for proper functioning of the immune system. Interestingly, a previous report also showed that a combination of PU.1 and C/EBPα can convert mouse fibroblast cells into macrophage-like cells,[38] suggesting that the enhancer profile is key for maintaining cell identity.

Summary

Recent advances in genomic technology and epigenetics have provided unprecedented opportunities to advance the understanding of enhancers. In this article, we have discussed the interactions between TFs and enhancers from two angles: (1) how TFs create enhancers or affect their status and; (2) how enhancers in turn govern TF binding in a cell type specific manner. We propose that regulation of gene expression by enhancers is governed by two types of TFs. Some factors are master regulators (such as PU.1 in THP-1 monocytes), which act to initiate enhancer formation at specific genomic locations. Other TFs, which we call collaborative factors, may be required to generate fully active enhancers. Collaborative TFs can be either lineage-specific TFs (such as C/EBPα, **Fig. 1C**) or non-specifically expressed TFs (such as FoxA1, **Fig. 1B**). Importantly, binding of collaborative TFs is likely also dependent on master lineage-specific TFs, as exemplified by the selective binding of FoxA1 protein in different cell types. We speculate that such multi-level TF regulatory networks are a common mechanism controlling the complex transcription program in different cells. Dissecting such TF regulatory networks requires integrative systematic analysis involving not only genetic but also genomics studies.

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