Defining specificity of transcription factor regulatory activities

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Summary

Mammalian transcription factors (TFs) are often involved in differential cell-type- and context-specific transcriptional responses. Recent large-scale comparative studies of TF recruitment to the genome, and of chromatin structure and gene expression, have allowed a better understanding of the general rules that underlie the differential activities of a given TF. It has emerged that chromatin structure dictates the differential binding of a given TF to cell-type-specific *cis*-regulatory elements. The subsequent regulation of TF activity then ensures the functional activation of only the precise subset of all regulatory sites bound by the TF that are required to mediate appropriate gene expression. Ultimately, the organization of the genome within the nucleus, and crosstalk between different *cis*-regulatory regions involved in gene regulation, also participate in establishing a specific transcriptional program. In this Commentary, we discuss how the integration of these different and probably intimately linked regulatory mechanisms allow for TF cell-type- and context-specific modulation of gene expression.

Key words: Functional genomics, Transcription factors, Cell-type-specific transcription, Chromatin, Coactivators.

Introduction

Although they all contain the same genomic DNA, specialized cell types from multicellular organisms specifically express a limited subset of genes, allowing them to execute the distinct functions that contribute to the vital structure and functions of the tissues of the body. There are many transcription factors (TFs) that can differentially regulate gene expression in multiple cell types. A requirement for our understanding of development and diseases is a comprehensive analysis of the mechanisms that allow a given TF to regulate different genes in different environmental contexts or cell types. Epigenetic modifications (such as DNA methylation and histone post-translational modifications) do not change the DNA coding sequence but are known to play a central role in determining active and inactive chromatin domains (Kouzarides, 2007; Suganuma and Workman, 2008). In this Commentary, we review recent findings indicating that the differential recruitment of a given TF to chromatin, which is potentially guided by epigenetic marks, is a key mechanism for cell-type-specific regulation exerted by mammalian TFs. In addition, we discuss how the functional activities of a given TF can be restricted to only a subset of the bound regulatory elements in specific cellular contexts or cell types, and how this contributes to an appropriate modulation of target gene expression.

Cell-type- and context-specific regulation of gene expression by TFs involves differential recruitment to chromatin

Cell-type-specific recruitment of TFs to chromatin

The post-genomic era of biological research has recently benefited from major technical breakthroughs, which have allowed studies of TF binding to chromatin at the genome-wide scale. Indeed, the combination of chromatin immunoprecipitation (ChIP) and tiling arrays (ChIP-chip) or, more recently, massively parallel sequencing (ChIP-seq) has provided scientists with an unprecedented broad view of chromatin structure and TF recruitment to the genome. Pioneering studies that aimed to identify TF binding sites within the genome revealed that TF recruitment is highly selective. Indeed, only a very limited fraction of all potential recognition motifs for a given TF (a few percent) were found to be occupied in a particular cell type (Carroll et al., 2005; Carroll et al., 2006; Laganiere et al., 2005; Martone et al., 2003). Findings of specific distribution of regulated genes in the vicinity of bound *cis*-regulatory elements, and of differential chromatin structure at bound versus unbound sites, indicated that the data supporting selective TF binding to the genome were not derived from biases or a lack of sensitivity of the experimental approaches used (Eeckhoute et al., 2009; Krum et al., 2008; Lupien et al., 2008). Instead, this selective binding to the genome was shown to be biased towards specific TF target genes.

Several subsequent studies also used these genomic-scale approaches to analyze the correlation between the binding of a given TF to regulatory elements and gene regulation in different cell types. The estrogen receptor (ER), the glucocorticoid receptor (GR), the forkhead family member FOXA1, repressor element 1 silencing transcription factor (REST) and Suz12 (a component of the polycomb repressive complexes, which are indirectly recruited to regulatory elements by TFs) were all found to exhibit significant differences in their recruitment pattern to chromatin in different cell types (Bruce et al., 2009; John et al., 2008; Krum et al., 2008; Lupien et al., 2008; So et al., 2007; Squazzo et al., 2006). Importantly, with the exception of Suz12, these transcription factors bind mainly to enhancers and not to regulatory sites that are in close proximity to target gene transcription start sites (TSS) (Carroll et al., 2006; John et al., 2008; Lin et al., 2007; Lupien et al., 2008; So et al., 2007; Welboren et al., 2009). Accordingly, recent studies have highlighted the key role of enhancers in cell-type-specific transcriptional regulation (Heintzman et al., 2009; Pennacchio et al., 2006; Pennacchio et al., 2007; Xi et al., 2007). This is in line with the finding that transcriptionally competent promoters are mainly similar in different cell types (Heintzman et al., 2009; Komashko et al., 2008; Xu et al., 2007b). Hence, whereas the chromatin

structure at promoters appears mostly conserved in different cell types, the presence of active chromatin marks at enhancers is mainly cell-type-specific and is biased towards enhancers that are in the vicinity of differentially expressed genes. Indeed, Heintzman and co-workers found that genes expressed in cervical carcinoma HeLa cells and leukaemia K562 cells were mostly identical, and that their promoters exhibited a similar pattern of active histone post-translational modifications (methylation of lysine 4 and acetylation of lysine 27 of histone H3) (Heintzman et al., 2009). Conversely, these histone marks were found at distinct enhancers in the two cell types, and the distribution of enhancers that had active chromatin features was biased towards the small fraction of those genes that were differentially expressed (Heintzman et al., 2009). Accordingly, TFs that act mainly through binding to enhancers, such as steroid hormone receptors, were shown to exhibit cell-type-specific cistromes (sets of bound regions in the genome) in correlation with differential gene transcriptional regulation (John et al., 2008; Krum et al., 2008; So et al., 2007). Hence, one important function of chromatin is to determine, on a genome-wide scale, the set of regions bound by a given TF in a given cell type.

Interestingly, a functional link between chromatin structure, TF recruitment and enhancer activity has been suggested to control the transcriptional regulatory activities exerted by FOXA1 and steroid hormone receptors. FOXA1 is thought to probe the chromatin and to selectively promote the binding of other TFs to regulatory regions of chromatin that were initially condensed; FOXA1 is therefore considered to be a 'pioneer factor' that allows for competency of enhancers (Cirillo et al., 2002; Sekiya et al., 2009; Zaret, 1999). Cell-type-specific FOXA1 activities are influenced by the methylation of lysine 4 of histone H3 (H3K4), which directs the binding of FOXA1 to a defined subset of all potential Forkhead motifs (Lupien et al., 2008). Once bound, FOXA1 allows for the recruitment of ER to the same sites, thereby orientating ER activity within the genome (Carroll et al., 2005; Eeckhoute et al., 2006; Laganiere et al., 2005; Lupien et al., 2008). Accordingly, the recruitment of ER to chromatin is markedly different in cell types that express or do not express FOXA1. Indeed, ectopically expressed ER in U2OS osteoblast-like cells and in MDA-MB-231 breast cancer cells binds to a cistrome that differs from the one defined in FOXA1-positive MCF7 mammary breast cancer cells (Fig. 1). In line with these observations, the Forkhead motif is enriched at ER binding sites in MCF7 cells but not at ER binding sites in U2OS or MDA-MB-231 cells (Krum et al., 2008) (our unpublished data). However, H3K4 dimethylation was also linked to ER binding in U2OS cells suggesting that, despite the lack of FOXA1, this epigenetic mark might dictate ER binding in this cellular context (Fig. 1). Chromatin marks that dictate the binding of TFs to chromatin might be deposited through regulatory events that take place before or during cellular differentiation, and might involve other Forkhead factors in the case of FOXA1 (Tagoh et al., 2004; Xu et al., 2007a).

The correlation between H3K4 methylation and TF recruitment to regulatory sites has been extended to other TFs, including signal transducer and activator of transcription 1 (STAT1) and FOXA2, suggesting that a requirement for H3K4 methylation at enhancers might be a general mechanism of regulating TF activity (Heintzman et al., 2009; Robertson et al., 2008). Importantly, epigenetic and dynamic chromatin marks occur in specific combinatorial patterns (reviewed by Cedar and Bergman, 2009; Suganuma and Workman, 2008). For instance, H3K4 methylation is often associated with histone acetylation and histone H2B mono-ubiquitylation (Lee et al., 2007; Wang et al., 2008) but is excluded from regions marked with H3K9, H3R2 and DNA methylation (Guccione et al., 2007; Kirmizis et al., 2007; Li et al., 2008; Okitsu and Hsieh, 2007; Ooi et al., 2007). This is explained, at least in part, by the finding that numerous enzymes that modify DNA or histone tails have chromatin-interaction domains whose binding activities are themselves modulated by pre-existing chromatin marks. This allows for crosstalk between the different DNA and histone modifications (Kouzarides, 2007; Suganuma and Workman, 2008). Hence, control of TF recruitment to chromatin could involve the coordinated action of several chromatin-modifying factors that are required to establish a precise combination of chromatin marks that allow or impede TF binding. Importantly, TFs also have a bidirectional connection with chromatin: TF recruitment is affected by chromatin structure and, in turn, TFs can recruit enzymes that influence the organization of chromatin (Dong et al., 2008; Garcia-Bassets et al., 2007; Knoepfler et al., 2006; Lupien et al., 2009; Metivier et al., 2008; Metivier et al., 2003). For instance, methylation of H3K4 precedes ER recruitment (Garcia-Bassets et al., 2007; Krum et al., 2008; Lupien et al., 2008), whereas its binding to chromatin triggers an ordered mobilization of cofactors such as CBP/p300, CARM1 and DNA methyltransferases that can subsequently induce changes in histone acetylation and in histone and DNA methylation levels (Fig. 1) (Lupien et al., 2009; Metivier et al., 2008; Metivier et al., 2003; Shang et al., 2000). TF-induced changes in chromatin structure are probably required to allow full functional competency of regulatory sites (Garcia-Bassets et al., 2007; Lupien et al., 2009; Metivier et al., 2008; Metivier et al., 2004; Metivier et al., 2003). Further data on the functional relationship between chromatin structure and TF recruitment are central to our understanding of the mechanisms that establish cell-type-specific gene expression patterns.

Context-specific recruitment of TFs to chromatin

As suggested by recent studies, the specific modulation of TF activities by different stimuli also appears to involve differential recruitment of TFs to chromatin (Bhat-Nakshatri et al., 2008; Hartman et al., 2005). For instance, STAT1 and ER bind to different cistromes following cellular activation by interferon-α (IFNα) versus IFNγ (Hartman et al., 2005), or by the serine/threonine protein kinase AKT versus estradiol (E2) (Bhat-Nakshatri et al., 2008). Distinct effects of the stimuli on the interactions of TFs with other proteins could, at least in part, account for TF differential activity in response to the different stimuli. For example, AKT and E2 might have distinct effects on the expression and activity of members of the JUN and FOS protein families, leading to AP1-mediated tethering of ER to different regulatory elements (Bhat-Nakshatri et al., 2008). As mentioned above, differential TF binding to enhancers is mainly responsible for differential gene regulation induced by STAT and ER in response to distinct cellular stimuli (Fig. 2). This is in line with recent findings suggesting that differential gene expression might heavily depend on selective enhancer activities (Heintzman et al., 2009; Lupien et al., 2008). However, promoter-associated mechanisms, including selective binding of factors belonging to the basal transcription machinery, can also be involved in modulating gene transcription (Deato and Tjian, 2007; Liu et al., 2008; Squazzo et al., 2006). Therefore, specific activity of distal regulatory elements together with the ability of a given promoter to receive regulatory signals might ultimately define gene expression (Fig. 2). For instance, most ER target-gene promoters are already bound by RNA polymerase II

(PolII) prior to hormone stimulation (Feng et al., 2008; Kininis et al., 2007; Kininis et al., 2009; Lupien et al., 2009; Welboren et al., 2009). This indicates that modulation of gene expression by E2 probably requires an initial competency of the target-gene promoter afforded by the pre-loading of PolII. ER activation by E2 leads to an increase in PolII binding and/or to post-recruitment activation of PolII (through events that trigger its phosphorylation) at these promoters (Fig. 2) (Feng et al., 2008; Kininis et al., 2009; Welboren et al., 2009). Interestingly, PolII stalling at promoters of genes that need to be rapidly induced has also been observed during *Drosophila melanogaster* and *Caenorhabditis elegans* development (Baugh et al., 2009; Zeitlinger et al., 2007). However, the mechanisms by which distal enhancers control PolII recruitment and activity at target-gene promoters remain to be fully understood (reviewed by Bondarenko et al., 2003; Szutorisz et al., 2005). Therefore, a better understanding of the mechanisms involved in promoter-enhancer communication is required to establish how *cis*-regulatory elements cooperate to ensure specific gene expression and regulation.

Appropriate gene regulation by TFs involves the selective activity of a subset of bound enhancers

As discussed above, the activity of TFs is modulated through controlling their recruitment to chromatin. It has emerged from recent studies that only a precise subset of all bound enhancers needs to be active to appropriately regulate gene expression. Indeed, numerous large-scale studies of TF recruitment to chromatin

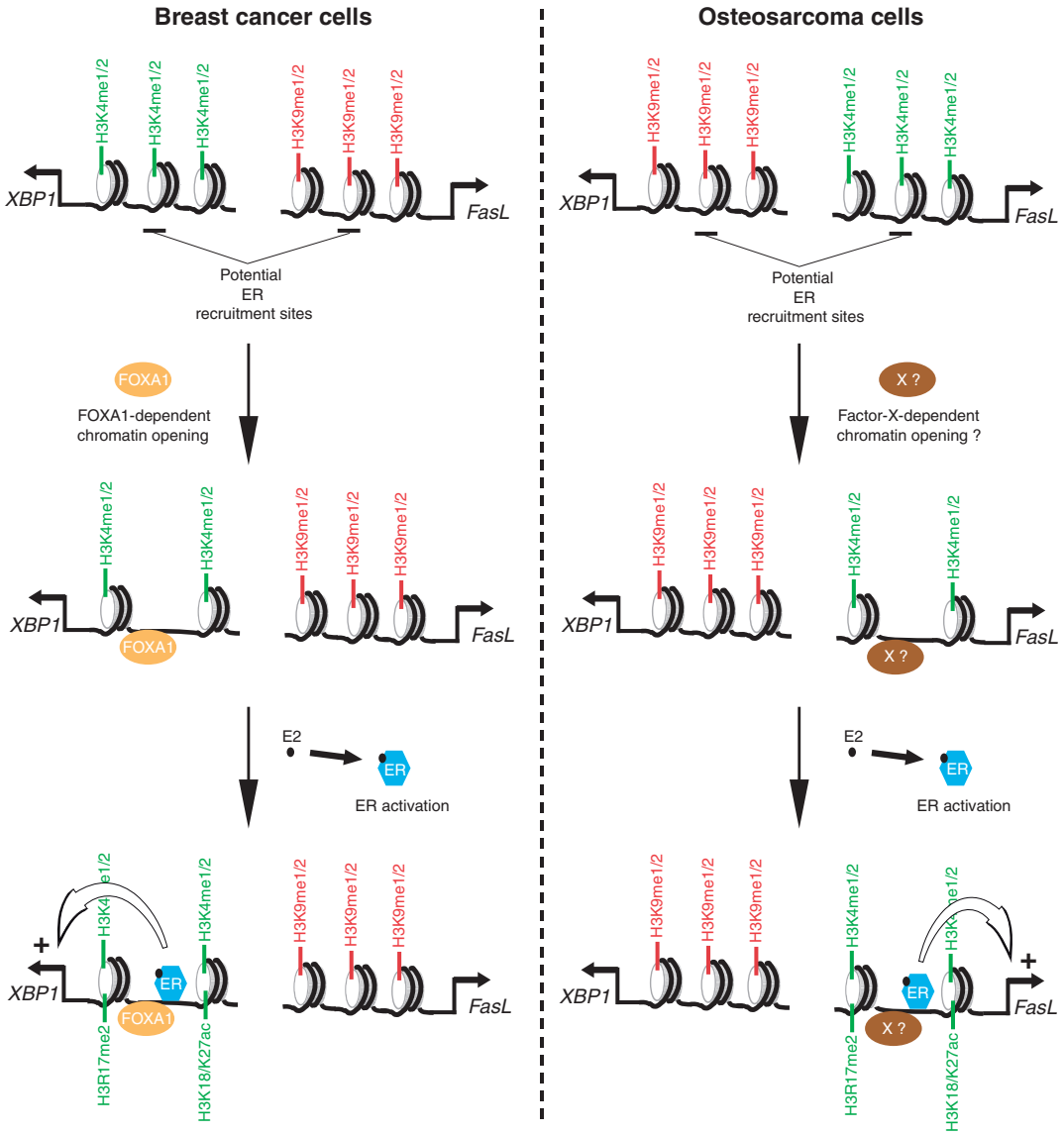

Fig. 1. Cell-type-specific gene regulation by ER involves differential recruitment to chromatin. ER binds to different enhancers and differentially regulates the *XBP1* and *FasL* genes in breast cancer and osteosarcoma cells (Krum et al., 2008). Differential methylation of histone H3 lysines 4 and 9 (higher levels of H3K4me1/2 and H3K9me1/2 are indicated in green and red, respectively) at cell-type-specific enhancers is thought to be directly involved in differential TF recruitment (Krum et al., 2008; Lupien et al., 2008). In breast cancer cells, the pioneer factor FOXA1 is recruited to sites that have high levels of H3K4 methylation and allows for initial chromatin opening (Lupien et al., 2008). Upon E2 stimulation, ER is recruited to the enhancer and induces an increase in dimethylation of histone H3 arginine 17 (H3R17me2) and in acetylation of histone H3 lysines 18 and 27 (H3K18/27ac) (Lupien et al., 2009). In osteosarcoma cells, a different pattern of H3K4 methylation, together with the lack of FOXA1 expression, directs the binding of ER to distinct enhancers. The outcome of this cell-type-specific recruitment of ER to chromatin is a differential regulation of the *XBP1* and *FasL* genes.

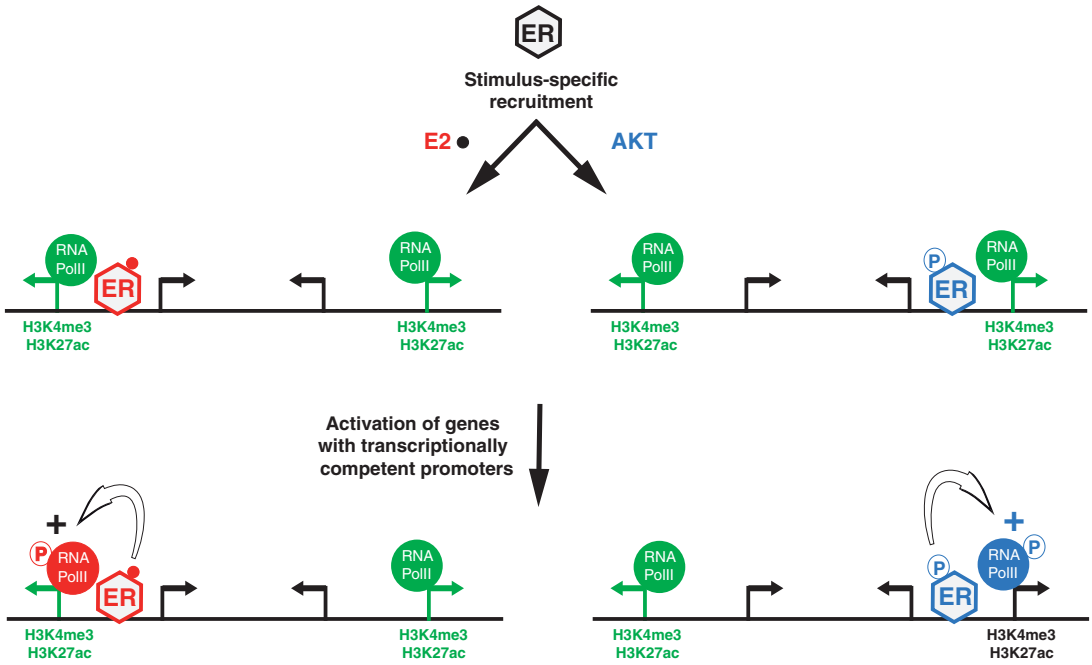

Fig. 2. Differential recruitment of ER to chromatin and the specific transcriptional competence of promoters define the genes that are modulated in response to different ER-activating stimuli. The schematic illustrates how differential gene regulation by ER is achieved following stimulation with E2 or activation of AKT in MCF7 breast cancer cells (Bhat-Nakshatri et al., 2008). Upon activation, ER is recruited to distinct enhancers either through direct DNA binding or through tethering by other TFs such as AP-1. Whether E2- or AKT-stimulated MCF7 cells are characterized by differences in their chromatin structure that could trigger differential ER recruitment remains to be defined. Importantly, genes whose expression is modulated by ER have transcriptionally competent promoters, as indicated by the presence of post-translational modifications of histones typical of active genes (H3K4me3 and H3K27ac), as well as RNA PolII binding, before ER activation. ER-mediated induction of gene expression could involve an increase in RNA PolII recruitment and/or its activation through phosphorylation (indicated by P) of the C-terminal domain. Note that AKT is able to phosphorylate ER, in addition to several ER cofactors and other collaborating TFs.

suggest that only a fraction of all bound regulatory elements is involved in target-gene transcriptional regulation (Bolton et al., 2007; Chen et al., 2008; Jia et al., 2008; Kim et al., 2008; Lupien et al., 2008; Martone et al., 2003; Yang et al., 2006; Zheng et al., 2007). For example, Martone and co-workers found that as much as 38% of nuclear factor κB (NFκB) binding sites that were detected after cellular stimulation with tumor necrosis factor α (TNFα) were not associated with genes whose expression was significantly modulated by this stimulus (Martone et al., 2003). Furthermore, recent investigations of chromatin structure at FOXA1-, androgen receptor (AR)-, ER- and STAT1-bound enhancers revealed that only a subset of bound sites were active and associated with gene regulation (Eeckhoute et al., 2009; Heintzman et al., 2009; Jia et al., 2008; Lupien et al., 2009). For instance, enrichment of acetylated histone H3 was detected only at a fraction of AR-binding sites in prostate cancer cells. Importantly, it was found that genes near acetylated enhancers were more likely to be regulated by dihydrotestosterone (DHT) than genes near AR-binding sites that were deficient for acetylated histone H3 (Jia et al., 2008). Additionally, significant levels of histone H3 methylation at lysines 4 and 17 were observed at a limited fraction of sites to which STAT1 and ER, respectively, were recruited (Heintzman et al., 2009; Lupien et al., 2009). Again, this subset of enhancers that had active chromatin marks was more significantly associated with regulated genes. Finally, analysis of nucleosome density together with acetylated and methylated histone H3 also revealed that only a limited number of all FOXA1-bound enhancers are functionally involved in gene regulation (Eeckhoute et al., 2009).

Several mechanisms might account for the enhancer-specific activity of TFs. A difference in the ability of different TFs to recruit cofactors might be a key determinant to the selective activity of some TF-recruiting enhancers (Fig. 3) (Lupien et al., 2009; Rogatsky et al., 2002). Such cofactors are typically enzymes that tightly control the activity of *cis*-regulatory elements through post-translational modification of histones and chromatin-bound regulatory factors (Feng et al., 2006; Garcia-Bassets et al., 2007; Metivier et al., 2003; Subramanian et al., 2008; Wang et al., 2008). Importantly, the recruitment of cofactors by TFs can be influenced by various parameters, which allows for context-specific interactions. For instance, TF activity can be influenced by the primary sequence of the DNA it binds to, as the sequence can directly affect TF structure and its interactions with cofactors (Hall et al., 2002; Leung et al., 2004; Loven et al., 2001; Meijsing et al., 2009; Wood et al., 1998). Alternatively, TF-mediated recruitment of required cofactors might depend on a specific stimulus. For example, the interaction of ER with some of its cofactors is differentially modulated by various ligands (Ozers et al., 2005; Weatherman et al., 2002), which, in turn, could underlie ligand-specific modulation of gene expression (Belandia et al., 2002; Shang and Brown, 2002; Sheng et al., 2008; Welboren et al., 2009). Moreover, the ability of a given TF to recruit other specific cofactors might be regulated by the presence of other TFs or cofactor enzymes at a given enhancer, which might alter its activity through direct interaction or post-translational modifications that induce changes in conformation (Panne et al., 2004; Zwart et al., 2007).

Regulatory regions are composite elements that recruit several TFs, which often intimately cooperate functionally, and the

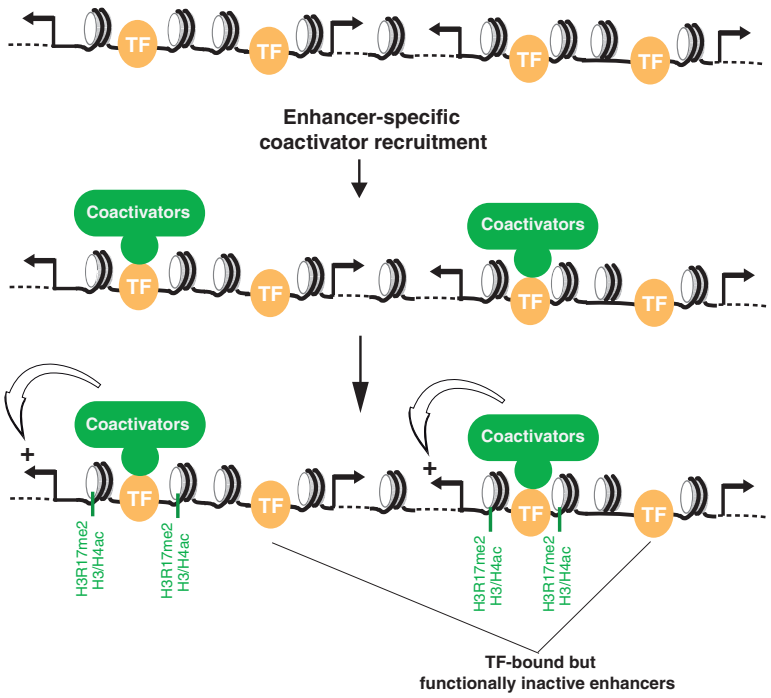

Fig. 3. The selective activity of a subset of TF-bound enhancers defines specific gene regulation. The schematic shows four TF-bound enhancers, of which only two have the potential to actively regulate transcription. Enhancer-specific recruitment of cofactors (such as CARM1) is illustrated here by selective coactivator engagement to two of the TF-bound sites. This selective coactivator mobilization is correlated with enrichment for active histone post-translational modifications at functionally active enhancers, including H3R17me2 and histone H3 and H4 acetylation (H3/H4ac). This mode of regulation defines TF-bound but inactive enhancers as indicated.

appropriate combination of TFs at a given enhancer is central to its activity (Chen et al., 2008; Kim et al., 2008; Lupien et al., 2008; Wang et al., 2007). For example, some FOXA1-bound enhancers that are conserved between different cell types exhibit cell-type-specific activities owing to the recruitment of nuclear receptors that are expressed in a cell-type-specific manner (Eeckhoute et al., 2009). The cooperation of FOXA1 with nuclear receptors is thought to involve its ability to relax the chromatin structure, allowing for recruitment of nuclear receptors and modulation of gene expression (Carroll et al., 2005; Eeckhoute et al., 2006; Laganiere et al., 2005; Lupien et al., 2008). Such cooperation of different TFs might be central to the function of most, if not all, enhancers.

In summary, the control of TF functions that occurs subsequent to their recruitment to gene regulatory regions plays a crucial role in defining precise gene expression programs. Therefore, sites that are bound by TFs but are inactive might represent regions that were active at some stage of cellular differentiation or that have the potential to become active under particular stimulus conditions.

Differential gene regulation is linked to differential three-dimensional organization of the genome

An additional level of control of TF-bound enhancers is provided by the three-dimensional organization of the genome in the nucleus. The genome of higher eukaryotes appears precisely organized at both the level of the individual chromosome and the level of the full complement of chromosomes. For instance, each chromosome occupies a specific region of the nucleus called ‘territory’ [reviewed in (Bartova and Kozubek, 2006; Cremer and Cremer, 2001; Meaburn and Misteli, 2007)]. This non-random organization probably explains the finding that there are functional subdomains within the nucleus (Sexton et al., 2007; Takizawa et al., 2008). In particular, several reports have found that actively transcribed genes gather within transcription ‘factories’ where PolII is highly concentrated (Carter et al., 2008; Mitchell and Fraser, 2008; Osborne et al., 2004; Sutherland and Bickmore, 2009; Xu and Cook, 2008). In addition, induction of immediate early genes including *Myc* is correlated with their relocation to such transcription factories (Osborne et al., 2007). Hence, a direct functional connection between gene nuclear localization and activity has been suggested (Osborne et al., 2004; Osborne et al., 2007). Importantly, cell-type-specific differences in gene locations have been observed, thereby suggesting the spatial environment of genes in the nucleus might be involved in defining differential gene expression (Kosak et al., 2007; Rajapakse et al., 2009; Schneider and Grosschedl, 2007). For instance, the differentiation of hematopoietic progenitors into either erythroid cells or neutrophils is associated with differential spatial relocation of chromosome domains in the two cell types, which relates to differentially expressed genes (Kosak et al., 2007).

Accumulating data indicate that the spatial localization of an enhancer, with respect to the gene it regulates and the functional subdomains of the nucleus, is integral to controlling its activity. Although the mechanisms that govern such three-dimensional organization are still poorly understood, evidence suggests an important role for chromatin loops in shaping interphase chromosomes (Jhunjhunwala et al., 2008; O’Sullivan et al., 2004; Perkins et al., 2008; Wallace and Felsenfeld, 2007). Chromatin loops might connect distal enhancers to their target-gene promoters (Dekker, 2008; Kadauke and Blobel, 2009). However, insulation (the sheltering of genes from the transcriptional effects of neighboring regulatory elements, including enhancers) might also rely on the formation of chromatin loops. Indeed, insulators (which are the *cis*-regulatory elements that are responsible for transcriptional insulation) might use loops to delineate chromatin domains between which regulatory control by enhancers is impeded (Chan and Song, 2008; Cuddapah et al., 2009; Wallace and Felsenfeld, 2007). CCCTC-binding factor (CTCF) is a zinc-finger TF that is central to the function of insulators (Wallace and Felsenfeld, 2007; Zlatanova and Caiafa, 2009). Interestingly, the identification of CTCF-bound sites suggested that many insulators are conserved between different cell types, but that a significant fraction of insulators (20-50%) could be cell-type-specific (Bushey et al., 2009; Cuddapah et al., 2009; Kim et al., 2007; Phillips and

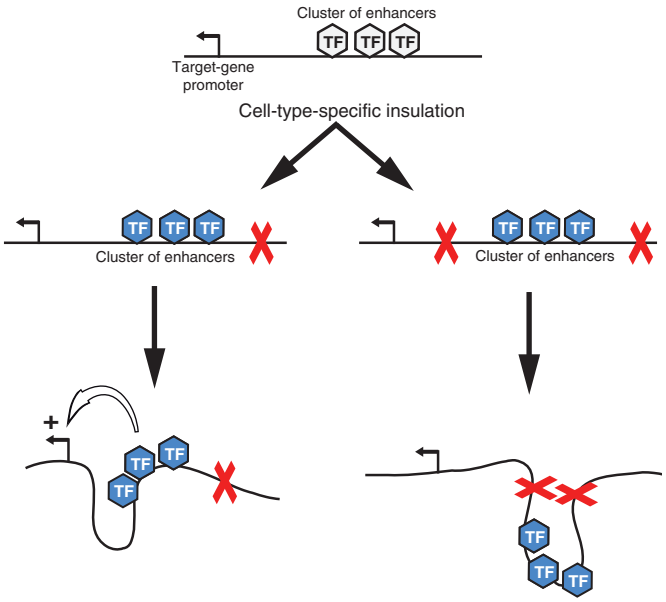

Fig. 4. Cell-type-specific three-dimensional organization of chromatin could control enhancer activity. The schematic illustrates how cell-type-specific insulators such as CTCF-bound elements (red crosses) might direct differential regulation of transcription of a given gene in the presence of conserved enhancers. Enhancers are often found in clusters, as shown. A chromatin loop that allows physical contact between enhancers and a target-gene promoter is shown on the lower left, and an illustration of how CTCF-mediated chromatin looping is thought to restrict enhancers from a promoter is shown on the lower right.

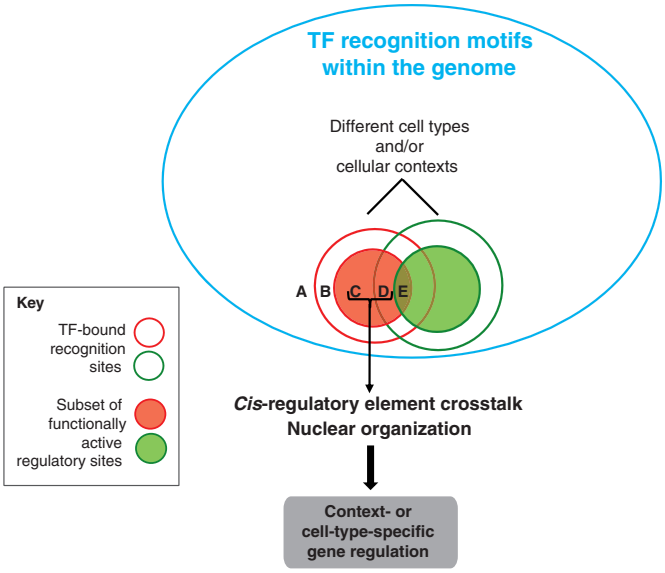

Fig. 5. The selective activity of a limited fraction of all potential regulatory sites defines the function of a given TF in a context- and/or cell-type-specific manner. The schematic illustrates mechanisms defining context- or cell-type-specific activities of a given TF. A, unbound recognition motifs; B, bound but inactive regulatory sites; C, regulatory sites specifically bound and active in a given cellular context or cell type; D, regulatory sites bound in two different cellular contexts or cell types that nevertheless exhibit context- or cell-type-specific activities; E, regulatory sites that are bound and active in two different cellular contexts or cell types.

Corces, 2009). The differential recruitment of CTCF in different cell types might therefore contribute to differential gene expression patterns by helping to establish cell-type-specific chromatin domains that control enhancer activities (Fig. 4). For instance, a cell-type-specific CTCF binding site in erythroid cells is required to induce the spatial organization of the β-globin locus found in these cells (Phillips and Corces, 2009; Splinter et al., 2006). On a more general basis, interactions mediated by several remote *cis*-regulatory elements within the β-globin locus are involved in the induction of globin gene expression during the differentiation of erythroid cells (Palstra et al., 2003; Splinter et al., 2006). Interestingly, recent high-throughput approaches that applied chromosome conformation capture (3C) techniques (Dekker et al., 2002) and are referred to as 4C or 5C techniques (Simonis et al., 2007) have allowed investigators to start large-scale mapping of the physical interactions that take place between distal chromatin regions (Sexton et al., 2009; Simonis and de Laat, 2008). These novel approaches should help in better understanding how modulation of physical contacts between remote *cis*-regulatory sites is involved in cell-type-specific gene expression.

Notably, clusters of enhancers seem to be involved in gene regulation (Bolton et al., 2007; Carroll et al., 2005; Krum et al., 2008; Welboren et al., 2009). There is an uneven organization of protein-coding genes found in the genomes of higher eukaryotes, which contain gene-rich and gene-poor regions. Moreover, it has been found that many co-regulated genes are clustered (Mishiro et al., 2009; Saccone et al., 1997; Saccone et al., 1999; Tang et al., 2008; Tschopp et al., 2009). Whether the different enhancers found in a cluster are required for gene regulation because they have diverse roles**,** or redundant but quantitatively additive roles**,** still remains to be established (Visel et al., 2009). However, it is becoming clear that the overall genomic context in which enhancers are found is an important additional level of control of gene expression.

Conclusion and perspectives

The general mechanisms that determine the specific functions of TFs have emerged from recent genomic-scale investigations of TF recruitment and chromatin structure at regulatory sites. Evidence indicates that a multi-layer regulatory system allows for selective recruitment to and activity of *cis*-regulatory sites in different cellular contexts (Fig. 5). Indeed, the chromatin structure (including epigenetic modifications) and the presence of collaborating factors (such as transcriptional cofactors) act in combination to modulate the activities of a given TF. Furthermore, nuclear organization and crossregulation between different *cis*-regulatory elements (enhancers, insulators and promoters) probably also play crucial roles in determining a specific transcriptome. Further understanding of the functional connections and the relative contributions to transcriptional regulation of these successive layers is a major challenge for the future. In particular, the relative influence of chromatin structure versus the primary DNA sequence is still a matter of debate (Anway et al., 2005; Wilson et al., 2008). Finally, a better understanding of the mechanisms involved in communication between different *cis*-regulatory sites is needed to appreciate how gene regulation is achieved in vivo.

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