MODES OF TRANSCRIPTIONAL REGULATION

# Transcription factors: from enhancer binding to developmental control

*François Spitz[1] and Eileen E. M. Furlong[2]*

Abstract | Developmental progression is driven by specific spatiotemporal domains of gene expression, which give rise to stereotypically patterned embryos even in the presence of environmental and genetic variation. Views of how transcription factors regulate gene expression are changing owing to recent genome-wide studies of transcription factor binding and RNA expression. Such studies reveal patterns that, at first glance, seem to contrast with the robustness of the developmental processes they encode. Here, we review our current knowledge of transcription factor function from genomic and genetic studies and discuss how different strategies, including extensive cooperative regulation (both direct and indirect), progressive priming of regulatory elements, and the integration of activities from multiple enhancers, confer specificity and robustness to transcriptional regulation during development.

Core promoter
The region of a gene to which RNA polymerase II and the general transcription factors (GTFs) bind to initiate transcription. Core promoters span ~40 base pairs upstream and downstream of the transcription start site.

Silencers
DNA sequences to which repressor factors bind and mediate the silencing of promoters through interactions with the basal transcriptional machinery or enhancers.

[1]*Developmental Biology Unit, European Molecular Biology Laboratory, D-69117 Heidelberg, Germany.*
[2]*Genome Biology Unit, European Molecular Biology Laboratory, D-69117 Heidelberg, Germany.*
*e-mails: spitz@embl.de; furlong@embl.de*
doi:10.1038/nrg3207
Published online
7 August 2012

Cell fates and complex body plans are established through an elaborate succession of signals that define complex and precise patterns of gene expression. Genetic studies have uncovered highly regulated programs of gene expression, which are governed by large interconnected regulatory networks (recently reviewed in REFS 1,2) that drive the stereotypic development of embryos. However, this tightly regulated view of developmental networks is at odds with recent global data revealing many features — including stochastic gene expression, bidirectional promoters, pervasive transcription and widespread binding of transcription factors (TFs) — that suggest transcriptional regulation may be a rather 'leaky' process. This review focuses on the operational properties of TFs at enhancer elements and aims to bridge the current views of transcription that are emerging from genomic studies and our understanding of the highly ordered process of development as shown by genetic studies.

Gene expression is regulated through the integrated action of many *cis*-regulatory elements, including core promoters and promoter-proximal elements (recently reviewed in REF. 3), as well as a various *cis*-regulatory modules that are localized at greater distances from the transcription start sites (TSSs), such as enhancers[4,5], silencers[6–8], insulators[9] and tethering elements[10,11]. Among this constellation of elements, enhancers and their associated TFs have a leading role in the initiation of gene expression, and identifying enhancer locations has been a major focus of many studies. Enhancers are small segments of DNA, typically a few hundred base pairs (bp) in length, that serve as operational platforms to recruit TFs through short, specific DNA sequences (motifs) to regulate transcription. They have the inherent capacity to function in a modular and autonomous manner, with some exceptions (BOX 1). How these pieces of DNA give rise to complex patterns of temporal and spatial activity, and how TFs interpret the genome to 'read' the right sequences at the right time of development, have been the subject of intensive investigation since the discovery of enhancers some 30 years ago[12]. Information on the genome-wide occupancy of individual TFs has provided novel and important insights into their activities. However, as TFs rarely act alone at enhancers, their functions should be considered in a more integrated, combinatorial manner. In the discussion here, we therefore focus on how interactions at different levels (between TFs, and between enhancer elements and other *cis*-regulatory elements) act to convert relatively 'relaxed' individual activities into more specific transcriptional programs that ensure the robust, stereotypic development of embryos.

We first consider models of enhancers, the different modes and rules that govern the binding of TFs to enhancer elements and how they generate different types of transcriptional output. We then discuss how dynamic, sequential changes occur during

Box 1 | **Defining enhancers**

Over many decades, enhancers have been defined predominately on the basis of operational functional assays, such as transgenic reporters in embryos or assays in transiently transfected cells. By their nature, these methods select for compact elements that can function in an autonomous manner. However, it is important to bear in mind that the modularity and boundaries of an enhancer are not always obvious *in vivo* when considering the entirety of the transcriptional control of a given gene. For example, it is possible to define a core minimal enhancer for the *Drosophila melanogaster* even-skipped (*eve*) gene that is sufficient to recapitulate the expression pattern in the stripe 2 domain of the embryo that is seen for the endogenous gene[13]. However, to generate the robust stereotypic output that can withstand variation in natural conditions (for example, temperature fluctuations) requires the presence of additional sequences flanking the core element[156]. The large spread of chromatin modifications — which typically span regions on the order of 2 kb encompassing many enhancer elements[73] — suggests that for many genes, the regulatory landscape may be more continuous and widespread than is generally assumed and that a collection of regulatory elements (modular or continuous) contributes to the overall expression pattern. These large domains may correspond to 'extended' enhancers containing a minimal core flanked by modulatory elements that confer robustness and fine-tuning (both spatial and quantitative) as discussed for *eve*, or may correspond to separable modules with highly overlapping activity (for example, shadow enhancers). Other regulatory elements function as distributed dispersed elements that integrate non-contiguous information over relatively large genomic regions, such as the 1.7 kb element controlling stripe 7 expression of *eve*[157], or the 5 kb region within the *runt* locus that cannot be truncated into different functional sub-elements[158].

developmental progression to regulate enhancer occupancy and output, and end by considering how enhancers act within broader regulatory landscapes that include multiple regulatory elements and complex three-dimensional genomic conformations.

## Modes of TF occupancy and transcriptional output

TFs typically recognize small 6–12 bp-long degenerate DNA sequences. This intrinsic, fairly low sequence specificity suggests that more complex rules, other than the simple affinity of individual TFs for DNA, are involved in controlling both enhancer occupancy and the functional outcome. As discussed in the following sections, TF binding and its impact on gene expression are influenced by many different mechanisms, thus giving rise to a robust, concerted action.

***Combinatorial occupancy and spatiotemporal activities of enhancers.*** TFs typically bind to enhancer regions that contain clusters of different TF binding sites, a property that has been used to define and identify regulatory elements. When the associated TFs are expressed in overlapping spatial domains in an embryo, this combinatorial binding can result in discrete and precise patterns of transcriptional activity[13–17]. For example, the recruitment of broadly expressed activators and spatially restricted repressors gives rise to more refined expression patterns, such as the stripe patterns that are regulated by many gap gene enhancers during the early segmentation of the *Drosophila melanogaster* embryo[18,19]. Similarly, the spatially defined activation of effector TFs in response to signalling cascades can lead to a more restricted activity of enhancers when acting together with additional TFs with overlapping expression domains. For example, in *D. melanogaster*, pMAD (the phosphorylated form of MAD) is the effector of decapentaplegic (DPP) and bone morphogenetic protein (BMP) signalling and provides the competence for cells to adopt particular cell fates. This activity requires the combinatorial binding of pMAD with cell-type-specific TFs on target enhancers, for example, Tinman in the dorsal mesoderm[20,21] and Scalloped in the wing imaginal disc[22], thus restricting a particular cell fate to only those cells that contain both pMAD and the second TF. Similarly, in vertebrates, the SMAD proteins — which are MAD orthologues — require different tissue-specific partners to bind to specific sets of enhancers in different cell types[23,24]. Therefore, cell-type-specific factors, typically key developmental regulators, provide the competence for pMAD or SMADs to bind to specific subsets of enhancer elements, whereas pMAD or SMAD occupancy provide the ability to activate an enhancer at a precise time and place following an extracellular signalling cue. The same TF (for example, pMAD or a SMAD) can thereby induce different cell fates owing to differences in its interaction partners.

Analyses of TF occupancy at different developmental stages or in different conditions demonstrated that TFs can often occupy diverse sets of enhancers depending on the stage or condition considered. For example, examining the binding properties of mesoderm-specific TFs throughout a time course of *D. melanogaster* embryogenesis identified enhancers that were occupied continuously at all stages examined, as well as enhancers that were bound by the same TF exclusively at either early or late stages of development, even though the TF was always present and available[16,25,26]. A similar temporal shift in TF occupancy has been observed in mammals for myoblast determination protein 1 (MYOD1) during myoblast differentiation[27], E2A during B-cell specification[28] and erythroid krüppel-like transcription factor (EKLF; also known as KLF1) during erythrocyte differentiation[29]. These observations highlight that it is not simply the timing of expression of a TF, but rather the timing of its DNA occupancy, that controls the temporal nature of gene regulatory networks (GRNs) driving developmental progression.

How then is temporal binding regulated? In some cases, this context-dependent occupancy may correspond to the relative affinity[30] or number[25] of binding sites for the TF, which would affect occupancy if the concentration of the TF changes over time. However, accumulating evidence from many model systems indicates that the combinatorial interplay of multiple TFs, each with its own partially overlapping temporal windows of expression, is a prominent regulator of context-specific binding: enhancers with context-dependent occupancy have differential motif enrichment for additional TFs[16,27,28,31,32], are co-occupied by other TFs in a time-dependent manner[16,26,31] and require this co-occupancy for additional TF recruitment[23,24,33].

Insulators
Chromatin elements that act as barriers against the influence of positive signals (from enhancers) or negative signals (from silencers and from heterochromatin).

Tethering elements
*Cis*-regulatory elements that contribute to directing a remote enhancer activity towards a specific gene among the surrounding genes. They are usually close to the promoter-proximal region of the target gene.

Bone morphogenetic protein
(BMP). A group of molecules of the transforming growth factor-α (TGFα) family that can induce bone formation and ventralize the vertebrate embryo. Decapentaplegic (DPP) is the fly homologue of the vertebrate BMPs.

***Different modes of binding yield different types of output.*** Combinatorial TF occupancy can lead to diverse types of transcriptional output, depending on how the TFs interact with each other[34]. In some cases, enhancer activation is proportional to the concentration of the individual TF (an additive model), as recently demonstrated for nuclear factor-κB (NF-κB)[35]. In contrast to these graded enhancer outputs that are characteristic of additive TF binding, cooperative binding leads to a nonlinear relationship between TF concentration and the degree of occupancy of their respective sites on specific enhancers. Cooperative binding is often associated with protein–protein interactions between TFs bound to adjacent sites (but not always, as discussed below), and the resulting transcriptional response can produce switch-like effects. Such a binary 'on'/'off' mode of enhancer activity was first observed for the phage-λ cI repressor[36], and it is often seen in developmental contexts in which it acts as a genetic switch for cell-fate decisions or to create sharp boundaries of expression. For example, cooperative homotypic binding of Bicoid only occurs above a very precise concentration, leading to sharp expression patterns of Bicoid target genes along the anteroposterior gradient of Bicoid protein concentration in the early *D. melanogaster* embryo[37]. This mechanism thereby allows the same set of TFs to function at different concentration ranges. For example, low levels of the TFs Dorsal and Twist cooperatively regulate neurogenic genes in ventrolateral regions along the dorsoventral axis of the *D. melanogaster* embryo[38,39], whereas high levels of Dorsal activate enhancers in the ventral region of the embryo[40]. Cooperative binding and switch-like enhancer activation may also have ecological and evolutionary importance, as cooperative binding can buffer variation in the levels of individual TFs, as long as the expression of the relevant TF reaches a threshold level[41]. In this way, embryogenesis may be protected from some variation in the molecular components that drive individual gene expression events.

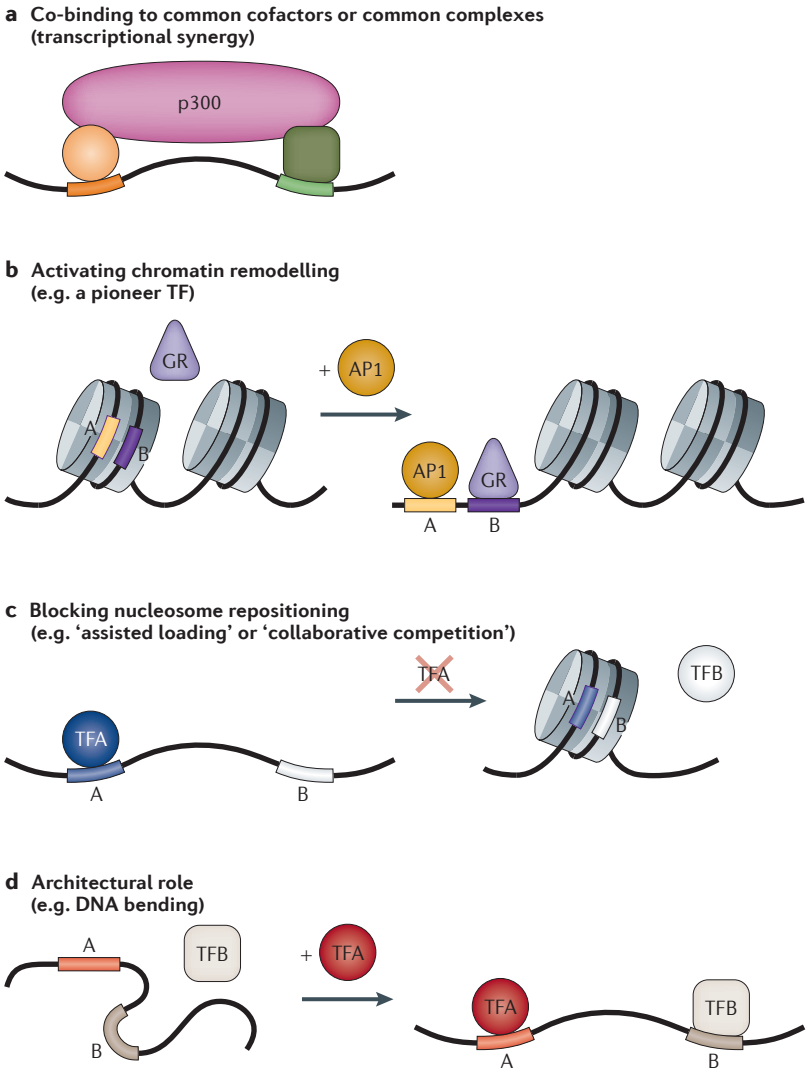



Figure 1 | **Indirect mechanisms of transcription factor cooperativity.** There are many indirect mechanisms by which transcription factors (TFs) can act cooperatively to regulate enhancers. **a** | Two or more TFs co-bound to the same enhancer element may recruit a common cofactor (for example, p300)[43], or different components of a multiprotein complex (for example, the Mediator or SAGA complexes), which may lead to a net increase in the affinity of each TF for their binding site (or may increase the retention time of the TFs at the enhancer). **b** | Some TFs may act cooperatively by activating chromatin remodelling. The binding motifs for the AP1 and glucocorticoid receptor (GR) TFs are located in a nucleosome-bound region of the DNA. Following AP1 binding at site 1, the nucleosome is actively repositioned, which exposes the GR binding site (site 2), allowing GR to bind. AP1 thereby acts as a pioneer TF and assists the occupancy of GR, without a requirement for a direct interaction between the two TFs[101]. **c** | By remaining bound to a given site, a TF (TFA in the figure) can prevent nucleosome repositioning and may therefore serve as a place-holder to facilitate the binding of another factor (TFB in the figure) to a neighbouring site that otherwise could become inaccessible if the place-holder factor was removed[44,45]. This may act as a passive method of enhancer priming. **d** | Some TFs (for example, HMG1) can induce local DNA bending, which can increase the affinity of other TFs for sites in the enhancer[47], or may promote recruitment and complex formation.

## Mechanisms modulating TF occupancy

***Indirect mechanisms of TF cooperativity.*** Cooperative binding to DNA is often thought to result from direct protein–protein interactions between TFs that are bound to adjacent sites on DNA, thus reinforcing each other's occupancy, as discussed above. However, other more indirect modes of cooperativity among TFs exist (FIG. 1). The transcriptional cooperativity between the yeast Gal4 protein and the rat glucocorticoid receptor (GR)[42] was an important demonstration of this alternative, as their cooperativity is unlikely to result from direct protein–protein interactions (as they come from different organisms), but rather from interactions with common conserved components of the transcriptional machinery. Such transcriptional synergy is probably frequent because many TFs interact with common co-activators (such as the p300–CREB-binding protein (CBP) family[43] (FIG. 1a)) or co-repressors (such as Groucho), as well as with members of the Mediator, SAGA and TAFIID complexes.

Cooperativity between two TFs at a given enhancer can also occur without co-binding: co-expression of two TFs that compete for the same binding site does not necessarily result in the quenching of either factor's activity as might be expected, but can lead to an

increased occupancy of each TF[44]. The rapidly alternating occupancy of each TF is proposed to counteract nucleosome repositioning, and therefore lead to a net increase in TF binding. This indirect cooperativity, which the authors termed 'assisted loading'[44] is similar to the 'collaborative competition' model that was previously described in yeast[45] (FIG. 1c). TF binding can also trigger local bending of DNA, which assists the binding of a neighbouring TF to an enhancer[46,47] (FIG. 1d). These mechanisms suggest that a large proportion of the functional cooperativity that is detected between TFs bound to the same enhancer may occur through indirect effects rather than the formation of stable ternary complexes, and these mechanisms may help to convert low-affinity DNA interactions into robust binding events.

***Modulation of TF sequence-specificity by cofactors.*** Cooperative binding between two TFs results in a net increase in the affinity of the two factors for their motifs, while their inherent sequence specificity remains the same. Two recent studies report an alternative mechanism whereby protein–protein interactions with a cofactor can change the DNA sequence specificity of a TF. In yeast, interaction with the cofactor Met28 enables the centromere-binding factor (Cbf1)–Met4 heterodimer to recognize an extended motif, compared with the motif recognized by Cbf1–Met4 alone, even though Met28 does not contain a known DNA-binding domain[48]. Another family of TFs, the homeodomain-containing Hox proteins display very similar binding specificities to each other, both *in vitro*[49] and *in vivo,* at least for the highly bound regions[50]; yet, they have very different phenotypes when mutated *in vivo*. Interactions between extradenticle (EXD) and different *D. melanogaster* Hox proteins were recently shown to induce subtle, but potentially important, changes to the DNA-binding specificities of the Hox proteins *in vitro*, which is referred to as latent specificity[51]. These cofactor-induced differences in sequence specificities may contribute to some of the distinct and specific functions of Hox proteins on different target genes *in vivo*.

***Chromatin accessibility and TF binding.*** TF occupancy is also influenced by nucleosome positioning at an enhancer, where histones and TFs can 'compete' for access to the DNA. TF occupancy patterns *in vivo* are highly correlated with nucleosome-depleted regions (NDRs) in yeast[52], *D. melanogaster*[53] and mammalian systems[54,55], suggesting that nucleosome positioning helps to determine which sites a TF can occupy. Nucleosome displacement is particularly important for the occupancy of low-affinity motifs[56,57], and chromatin remodelling before TF binding is required in some cases: for example, in mammals, loading of GR at a number of sites requires remodelling by the SWI/SNF enzyme BRG1 (REF. 58); and hormone-responsive elements (HREs) require the chromatin-remodelling complexes BAF and PCAF to facilitate receptor binding[59]. Yet, interactions between nucleosomes and TFs are complex and reciprocal: the removal of some TFs (for example, Myb proteins in yeast[60,61]) results in a reduction in the size of NDRs, whereas the binding of other factors (for example, Gal4 in yeast[62,63]) is sufficient to disrupt a well-positioned nucleosome. Thus, TFs can have a role in the modulation of nucleosome positioning.

The interplay between TFs and nucleosomes may also be influenced by post-translational modifications of histone tails within nucleosomes. Specific histone modifications are observed at active or inactive promoters, and in the body of transcribed genes[64–66], and are also found to have dynamic and cell-type-specific patterns at *cis*-regulatory elements[67,68] (see recent reviews[5,69,70]). Histone H3 monomethylation on lysine 4 (H3K4me1) and acetylation on lysine 27 (H3K27ac) are modifications that are strongly associated with *cis*-regulatory elements, and H3K27ac in particular is highly correlated with enhancer activity and the expression of the closest proximal gene[71–73]. Transient placement of H3K79me3 and RNA polymerase II (Pol II) at enhancers is also highly correlated with active developmental enhancers and is predictive of the precise timing of enhancer activity during development[73]. Although chromatin modifications thereby provide a useful readout of enhancer activity, it is currently unclear if the placement of these marks is a consequence or cause of TF occupancy or enhancer activation; their function in enhancer activation, if any, remains unknown. Several studies using similar B-cell model systems showed that binding of TFs (PU.1 in one study[74] and PAX5 in another[75]) is required for the placement or maintenance[76] of H3K4me1, but another study suggested that H3K4me1 is a pre-requisite for TF binding[77]. These somewhat contradictory results probably reflect the complexity, dynamics and diversity of regulatory interactions at active enhancers in any given cell type. In future studies, it will be important to disentangle the role of Pol II and chromatin modifications at enhancers and how they relate to enhancer activation.

## Sequence motifs and enhancer architecture

Cooperative occupancy can in some cases be inferred from the motif architecture of *cis*-regulatory elements, and the presence of specific motif arrangements have indeed provided valuable insights into how an enhancer functions. Two properties of enhancers have been investigated in detail: motif composition and motif positioning. Motif composition is the presence within enhancers of binding motifs for specific TFs that are essential for driving expression in a given cell type. A good example is the regulation of dopaminergic genes by an ETS factor in the *Caenorhabditis elegans* nervous system[78]. In various cases, common signatures of TF occupancy on enhancers are sufficient to predict the temporal and spatial aspects of enhancer activity for large sets of regulatory elements defined by chromatin immunoprecipitation (ChIP)[79]. This study[79] and others[80–82] indicate that there are global predictive regulatory rules, which often represent loose codes of motif composition, that govern enhancer activity.

Motif positioning — often referred to as motif 'grammar' — is the relative order, orientation and spacing of TF motifs within an enhancer (FIG. 2) and is often

Bicoid
A maternally deposited *Drosophila melanogaster* transcription factor that is localized at the anterior end of the embryo and forms a concentration gradient that decreases from the anterior to posterior. This transcription factor regulates transcription at different concentrations along its expression gradient, which is essential for establishing the segmentation of the early blastoderm embryo.

Mediator
The ~30-subunit co-activator complex that is necessary for successful transcription at class II promoters of metazoan genes. Mediator coordinates the signals between enhancers and the general transcription machinery through its interaction with RNA polymerase II and site-specific factors.

SAGA
A multiprotein complex involved in the regulation of transcription that possesses histone acetyltransferase and TATA-box-binding protein (TBP)-binding activities. The budding-yeast complex includes Gcn5, several proteins of the Spt and Ada families, and TBP-associated factors (TAFs); analogous complexes in other species have analogous compositions.

Nucleosome-depleted regions
(NDRs). DNA is usually wrapped around histone octamers, forming nucleosomes that contribute to genome compaction in the nucleus. The distribution of nucleosomes is variable and regulated, and some genomic regions, termed NDRs, show a lower density or absence of nucleosomes.

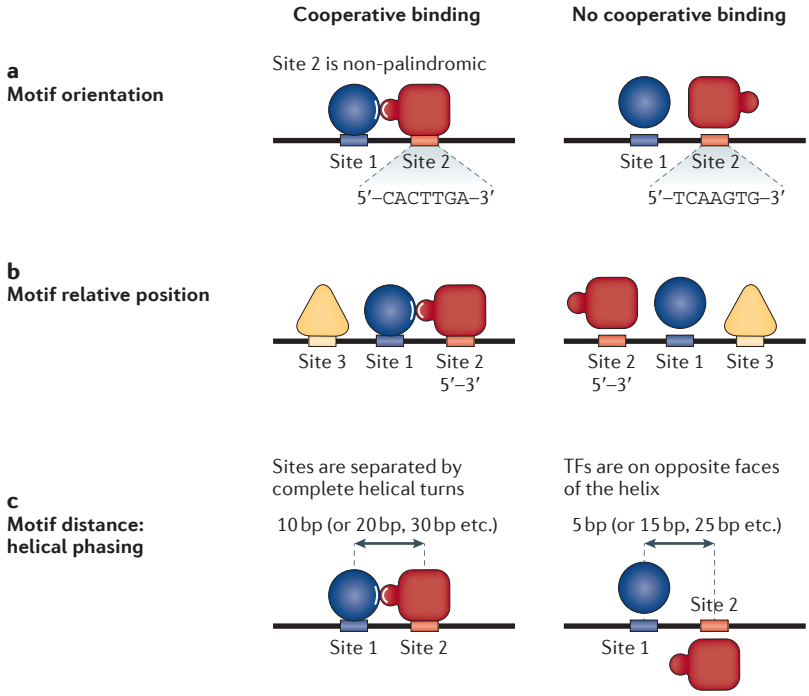


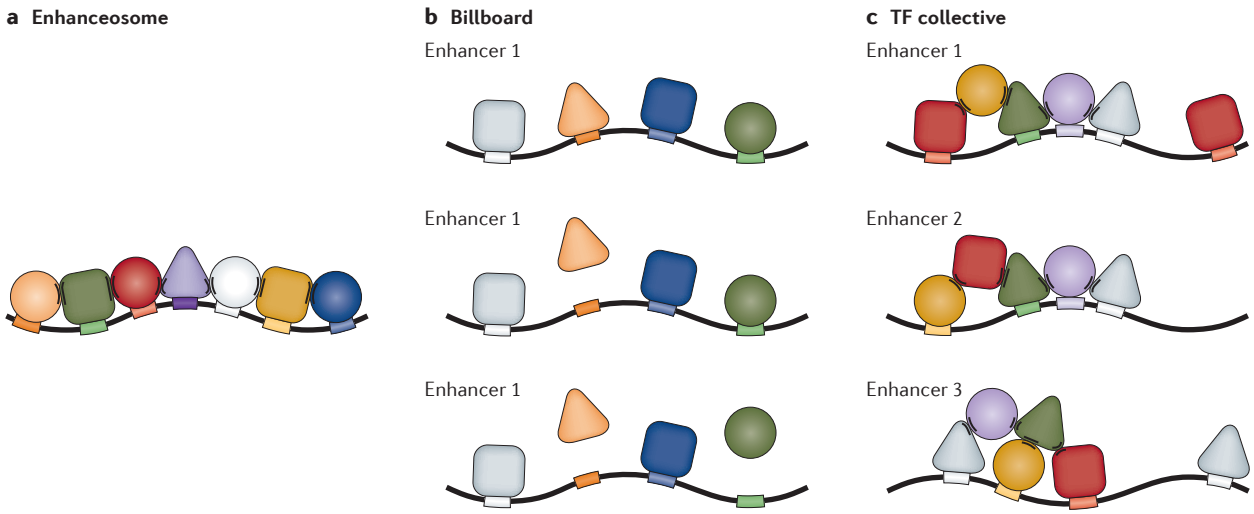

Figure 2 | **Motif positioning (grammar) facilitates direct cooperativity between transcription factors**. The left hand column depicts examples in which two transcription factors (TFs) have a direct protein–protein interaction that promotes their cooperative binding. **a** | One of the TFs (shown in red) binds to a non-palindromic motif (site 2; 5′-CACTTGA-3′). When the relative orientation of site 2 is reversed, that TF cannot be recruited to DNA in a manner that will present the appropriate protein interface to interact with the other TF (shown in blue). **b** | Similarly, when the position of site 2 relative to site 1 is changed, direct protein–protein interactions between the two factors cannot occur. **c** | There are approximately 10 base pairs (bp) per helical turn of DNA. If two TFs cooperatively bind to DNA through a direct protein–protein interaction, they typically need to be positioned on the same face of the DNA. This is facilitated by having a spacing of ~10 bp, 20 bp or 30 bp between the two motifs. When site 1 and site 2 are separated by spacings such as ~5 bp, 15 bp or 25 bp, the TFs will be positioned on opposite faces of the DNA helix, thereby blocking their ability to bind directly to each other (right column). Spacing rules between motifs can also occur at larger distances, which reflects DNA wrapping around nucleosomes. In all three cases, the motif composition has not changed, yet the direct cooperative occupancy of the two TFs cannot occur.

observed as strict sequence constraints within regulatory elements[83,84]. Motif positioning typically ensures that TFs are positioned appropriately to facilitate protein–protein interactions and thereby promote cooperative binding, as well as the recruitment of cofactors and the transcriptional machinery. One of the best-studied examples is the interferon-β enhancer, at which small sequence changes within the 55 bp element alter the binding potential of all eight factors that occupy the enhancer[46,85]. This has led to the 'enhanceosome' model of enhancer activity[85,86] (FIG. 3a), in which the recruited TFs form a highly ordered protein interface that requires a strict and specific positioning of TF-binding motifs relative to each other in the DNA. Cooperative TF recruitment in such an ordered structure leads to a sharp, switch-like activation that may be essential for the rapid responses that are required during viral infection. However, most developmental enhancers do not seem to be occupied in such a highly ordered manner; instead, a subset of TFs may bind to the enhancer cooperatively[83], whereas other TFs bind in an additive or independent manner. This allows a more flexible positioning of motifs and has led to the 'billboard' model of enhancer activity[87], which suggests that enhancers serve as 'information display' elements where TFs can act cooperatively but with few constraints on the relative positioning of their binding sites[87,88] (FIG. 3b).

Accumulating evidence suggests that there may be no generalizable motif positioning rules when examining many enhancers that have a given activity. For example, a single combination of TFs (that is, enhancer input) can generate multiple outputs depending on the relative positions of motifs and on the larger regulatory context[84]. Conversely, highly similar spatiotemporal activity (enhancer output) can be generated from different combinations of TFs (enhancer input)[79,89,90]. At the sequence level, this is indicated by an absence of any consistent motif positioning and by diversity in motif composition among enhancers with similar activities. As an extreme example, a recent study of five TFs that are involved in cardiac development revealed that all five TFs co-bind and regulate a large set of enhancers that harbour motifs for diverse subsets of the occupying TFs[91]. When one TF was removed, all other TFs failed to activate the enhancers *in vitro*, suggesting that this co-recruitment may represent cooperative binding *in vivo*. The previously characterized protein–protein interactions that are known to occur between these TFs probably facilitate this 'all-or-none' recruitment in the absence of any obvious shared motif grammar (FIG. 3c). This mode of regulation — termed a 'TF collective' — suggests that the determinants of the overall output of enhancers extend beyond a linear sequence-based code to include protein–protein-based interactions.

***From occupancy to function?*** In recent years, many regulatory interactions have been inferred from TF occupancy patterns under the assumption that binding implies regulatory function. However, comparisons between TF binding events (in the vicinity of potential target genes) and expression profiling data (identifying genes that are differentially expressed in a loss-of-function mutant for that TF) in yeast[92,93], *D. melanogaster*[25,26,94] and mammalian systems[7], revealed a relatively small overlap between TF occupancy and the expression of neighbouring genes. The overlap was ~50% of TF binding events in yeast and 10–25% in higher eukaryotes. Although there are several features that could lead to an under-estimation of the functionality of TF binding to specific regions (such as a lack of sensitivity of expression profiling, or regulatory redundancy), growing evidence questions the proportion of TF binding events that are indeed functional (BOX 2). Some studies suggest that the vast majority of individual binding events may be non-functional and merely reflect chromatin accessibility[53,54]. Other studies indicate that although some TF binding events do not lead to an immediate response in terms of gene expression, they may have a functional role in chromatin remodelling[27]

Chromatin immunoprecipitation (ChIP). A technique for identifying potential regulatory sequences that are bound by a protein of interest. Covalently crosslinked DNA–chromatin extracts (complexes of DNA and protein) are isolated using antibodies that recognize specific DNA-binding proteins. In ChIP–chip, the ChIP step is followed by microarray analysis, whereas in ChIP–seq, it is followed by high-throughput sequencing.




| | a Enhanceosome | b Billboard | c TF collective |
|---|---|---|---|
| **Protein DNA** | • Highly cooperative DNA binding<br>• DNA sequence acts as scaffold | • Cooperative and additive binding | • Cooperative DNA binding<br>• Both DNA and protein may act as scaffold |
| **Protein interface** | • Fixed (formed from a higher-order TF–DNA complex) | • Variable | • Variable |
| **Motif** | • Fixed motif composition (sites for all factors must be there)<br>• Fixed motif positioning (grammar) | • Fixed motif composition<br>• Flexible motif grammar | • Flexible motif composition (as different TFs directly bind to DNA)<br>• Flexible motif grammar |
| **Output** | • Unitary (requires the integrated activity of all TFs) | • Only requires a subset of factors to be active | • Collective (requires most TFs but not clear if it requires all TFs) |

Figure 3 | **Current models of enhancer activity.** **a** | The enhanceosome model represents a situation in which all transcription factors (TFs) that bind to an enhancer are essential for the cooperative occupancy and activation of the enhancer. The DNA motif composition and its relative positioning (motif grammar) act as a scaffold to cooperatively recruit all TFs, which form a higher-order protein interface to regulate transcription[86]. **b** | The billboard model. For any given enhancer, the positioning of TF binding sites is flexible and subject to loose distance or organizational constraints. Only a subset of sites in the enhancer may be active at a given time[88]. **c** | The TF collective represents a situation in which the same set of TFs bind to many enhancers (five TFs are illustrated). They can occupy each one of these enhancers in a different manner (with a diverse subset of TFs, or all TFs, directly touching DNA). For cases in which the motifs for only a subset of TFs are present, the remaining TFs are still recruited to the enhancer through protein–protein interactions between the participating TFs. The collective binding can therefore occur using diverse motif composition and flexible motif positioning[91].

or nucleosome positioning[56,95], which can influence gene expression at a later developmental stage or have an additional non-transcriptional function (for example, in the case of E2F1 (reviewed in REF. 96)). The functional consequences of TF binding may also depend on temporal dynamics: a recent study in yeast using competitive ChIP demonstrated that measuring TF kinetics (that is, residence time), rather than steady-state occupancy, is a much better measure of functional binding events[97].

## Enhancers and developmental progression

As mentioned above, the composition of TFs bound to a given enhancer can undergo dynamic changes during development[16,25–29], which not only reflects the activity status of the enhancer at a given time point, but may also be relevant for enhancer function at subsequent stages. Developmental enhancers are not simple transcriptional switches: they undergo progressive changes that are triggered by successive waves of TF activity and chromatin remodelling[98,99]. As we discuss below, these successive events, from priming to activation, may have an important functional role in the precision and accuracy of gene expression programs, even though any single event may not have a discernible effect on gene expression.

***Developmental control of accessibility: pioneer factors.*** In the context of embryonic development and cell differentiation, many TFs initiate the assembly of transcriptional complexes at specific enhancers, which in turn trigger nucleosome repositioning so as to increase accessibility for other factors. This 'pioneer' function was first identified in yeast[100] and subsequently at the mouse albumin enhancer during endoderm development (reviewed in REF. 95). Pioneer factors can bind to DNA that is inaccessible to other factors (presumably motifs that are within nucleosomal DNA or where the DNA exits the nucleosome) and can recruit chromatin-remodelling complexes that lead to nucleosome repositioning. For example, the binding of the transcription factor AP1 facilitates the loading of GR to co-bound

Box 2 | **The relationship between transcription factor occupancy and function**

Distinguishing experimentally between functional and non-functional transcription factor (TF) binding events is not a simple task. For example, considering TF occupancy data alone, there are conflicting reports on the usefulness of using the peak height of a chromatin immunoprecipitation (ChIP) signal for a TF to distinguish between functional and non-functional binding events[27,159]. An emerging trend indicates that TF occupancy events that are temporally dynamic or condition-dependent are highly enriched for functional events[26,27,31] and globally predictive of spatiotemporal activity[79]. That is not to say that 'continual' binding over many time-points is non-functional, but rather that condition-dependent binding events are, by their very nature, enriched in regulated binding events, the majority of which are likely to be functional.

To assess the contribution of a TF binding event to gene expression, a functional assay is required. However, the definition of 'functionality' is often dependent on the experimental assay used to assess it. A TF may be able to regulate an enhancer *in vitro* outside of its normal context (for example, in a luciferase reporter assay) but may not be required or sufficient to regulate that enhancer *in vivo*. Similarly, overexpressing a TF *in vivo* outside its normal expression domain will identify genes for which the contribution of the TF is sufficient to activate gene expression, but will miss other genes for which activation requires cooperativity with a tissue-specific cofactor or prior priming of the enhancer. Thus, the set of TF targets identified by overexpression assays can only partially overlap with a set obtained by examining a loss-of-function situation for that TF. Moreover, a particular binding event may not seem to be functional owing to conditional redundancy with other TFs occupying the same enhancer or through redundancy with an alternative (shadow) enhancer for the same gene, as occurs at the *Drosophila melanogaster* eyes absent (*eya*) locus[160]. Redundancy is likely to be much more prevalent in developmental networks than is currently envisaged. In the comparatively simpler yeast system, an extensive genetic analysis of the epistatic relationships between pairs of TFs revealed that site-specific factors function more redundantly in regulating their target genes compared with general components of the basal transcriptional machinery[161].

TF binding can also have functions other than directly regulating the expression of a target gene, but such contributions can be difficult to evaluate experimentally. For example, a pioneer function of a TF may not be observed in a transgenic enhancer-reporter assay (or in *in vitro* systems) as the enhancer is not acting within its normal chromatin and regulatory context. An example of the scale of such pioneer functions for a TF residing at the top of a gene regulatory network (GRN) comes from the mammalian master regulator myoblast determination protein 1 (MYOD1). Integrating MYOD1 binding with expression profiling data from mouse C2C12 cells revealed that only ~4% of genes with a MYOD1 binding event in their vicinity had significant changes in their expression after removal of MYOD1 (REF. 27). Examining the other 96% of MYOD1-bound genes revealed that although their expression levels were not altered, MYOD1 occupancy was associated with a significant increase in histone H4 lysine acetylation at these gene loci[27]. What this response to binding means in terms of gene expression remains unclear, but it may render genes in a primed state for subsequent transcriptional activation that is dependent on the occupancy of additional factors.

regions by recruiting BRG1, which keeps chromatin in an open state[101] (FIG. 1b). Pioneer TFs thereby provide the sequence specificity to endow general chromatin-remodelling complexes with enhancer-specific roles.

The binding of a pioneer TF by itself is usually not sufficient to form an activating complex at an enhancer[76] (that is, occupancy does not imply an immediate function) but they nevertheless play an essential part by initiating a series of changes that allow the enhancer to subsequently recruit its full complement of TFs, thus triggering activation at a later stage in development (FIG. 4). Given this priming function, many pioneer TFs — such as MYOD1 (REF. 102), PAX5 (REF. 75), PU.1 (REF. 76), forkhead box protein A1 (FOXA1; also known as HNF3α)[103], and CCAAT/enhancer-binding protein-β (C/EBPβ)[104] — act at the top of GRNs. At some enhancers, they have a transient early role and are replaced by other TFs as the enhancer becomes fully active[105–107], whereas in other situations they confer multi-lineage priming of enhancers that are used differentially in subsequently distinct lineages[77].

Recent studies in *D. melanogaster* identified a new transcriptional regulator, Zelda (also known as Vielfaltig), that is required for the activation of many enhancers in the early embryo at the maternal-to-zygotic transition (MZT)[108,109]. Zelda has two unusual properties. First, Zelda occupies ~64% of its TAGteam motifs in the genome[110]. This is in contrast to other factors, which generally occupy a small fraction of their potential target sites throughout the genome (for example, ~2.5% of MYOD1 E-box sites in the mouse genome are occupied by MYOD1[27]). Second, Zelda binding is tightly linked to changes in gene expression[109,110]. It remains to be determined whether Zelda acts as a global pioneer factor for the activation of early zygotic transcription, which is suggested by its prior binding to enhancer elements before the MZT[110] and its requirement for the occupancy of additional TFs[110,111]. The temporal delay in activation that is observed for some genes in *zelda* mutants[109] indicates that in some cases Zelda potentiates, rather than being strictly required for, gene expression. This suggests that Zelda may function to lower the threshold for gene activation in these cases. Given its important and global role, it is surprising to note that Zelda is not conserved in vertebrates. This suggests that either a sequence-diverged functional orthologue performs this role or that this major transcriptional event in development, the MZT, is supported by an alternative mechanism in vertebrates.

In addition to nucleosome repositioning, protection from DNA methylation within enhancers may also play a part in the enhancer occupancy of TFs during development. The methylation of cytosines in DNA generally negatively influences TF binding (recently reviewed in REF. 112).

Maternal-to-zygotic transition
(MZT). A period of embryonic development that coincides with the transcriptional activation of the zygotic (embryonic) genome. Most of the RNA and proteins that are present in the embryo before the MZT are provided by the mother, through maternal loading into the oocyte.

TAGteam motifs
A collection of related heptamer motifs (consensus sequence CAGGTAG) that are enriched in the vicinity of genes that are transcribed during the early blastoderm stages of *Drosophila melanogaster* embryogenesis; these motifs are recognized by the transcription factor Zelda.

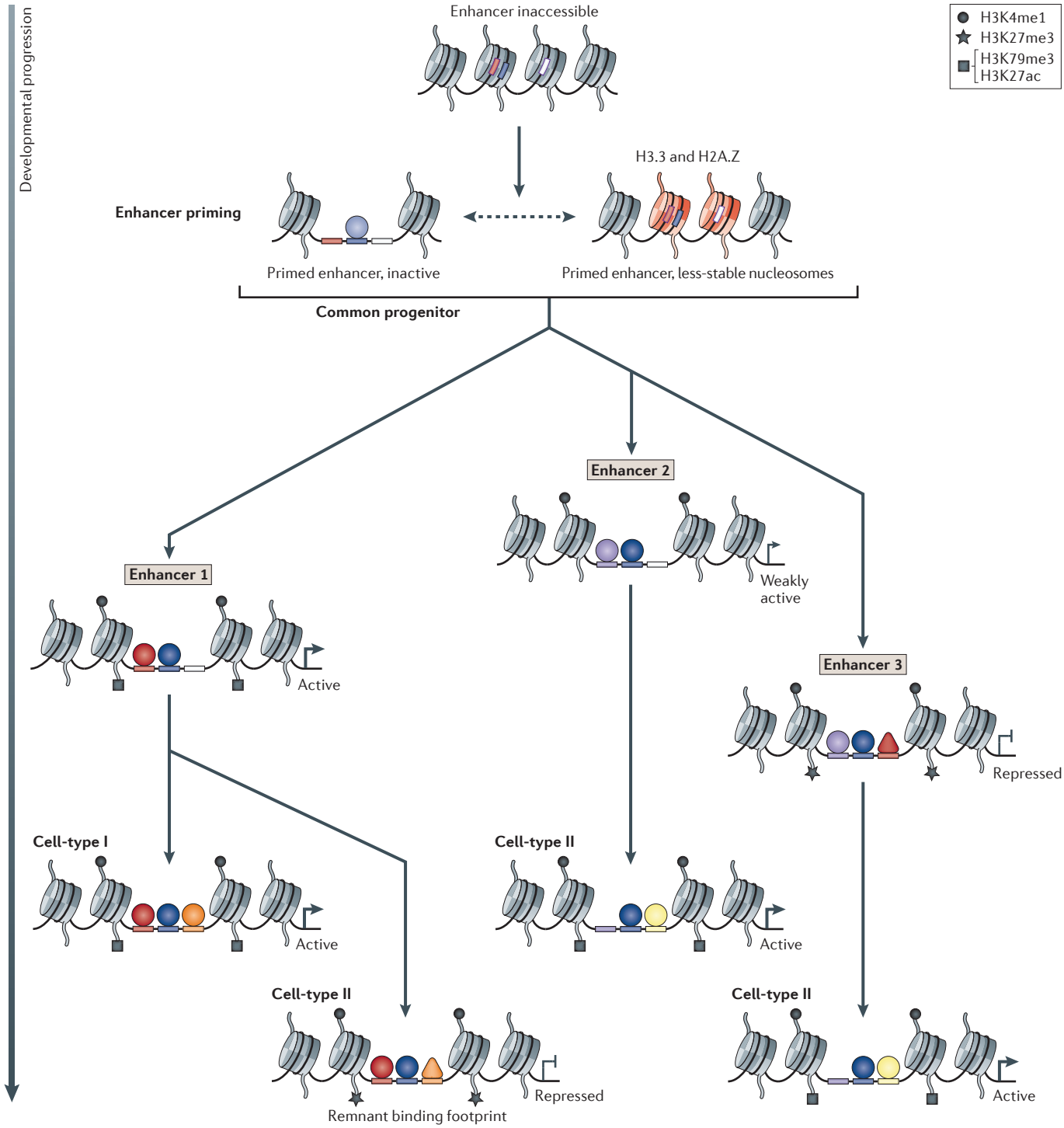



Figure 4 | **Dynamic changes at enhancers during developmental progression.** At early stages of development, some enhancer elements are covered by a condensed nucleosomal array that prevents access to transcription factor (TF) binding sites. Expression of a pioneer factor (the light blue circle) at a particular stage in development can initiate chromatin remodelling, reposition nucleosomes (perhaps in a dynamic manner, with competition between TFs and nucleosomes), and prime the enhancer for future activation. At later stages, the pioneer factor can be maintained or replaced by a different factor (dark blue circles). Depending on the context (for example, the presence of binding sites for other TFs), an enhancer may become active and thus associated with the corresponding epigenetic signature (H3K4me1, H3K79me3, H3K27ac and RNA polymerase II binding[71–73]), or may stay repressed[73,120] (or poised[72]) as manifested by the presence of repressive histone marks (such as H3K27me3), even though activating TFs might be bound. As development continues, the binding of additional TFs or changes in cofactor availability may change the state of enhancer activity. For example, enhancer 1 may become bound by additional factors (orange circle) in cell-type I to maintain enhancer activity, whereas in cell-type II, binding of a repressor (orange triangle) could suppress enhancer activity. This repression may occur even though the binding of the earlier activating TF or TFs is maintained, thus forming a remnant footprint of the developmental history of the cell[91]. Expression of new factors (yellow circles) may lead to the activation of enhancers that were poised or repressed at an earlier stage, as shown for enhancers 2 and 3.

A recent study of mammalian embryonic stem cells revealed that several enhancers seem to be protected from DNA methylation[113], suggesting that specific mechanisms may prevent enhancer silencing to facilitate their subsequent activation later in development. For example, motifs bound by FOXA1 have generally low DNA methylation levels, and their methylation levels are further reduced after binding of FOXA1 (REF. 114). This could represent a general feature of mammalian enhancers, as a recent study reported that many enhancers have low levels of DNA methylation that are cell-type specific and are influenced by TF binding[115].

***Enhancer priming and developmental progression.*** Developmental progression and cell determination is regulated by large, highly interconnected GRNs. Recent studies suggest that the sequential expression of structurally related TFs that bind to similar target sequences and participate in a given developmental process (for example, SOX factors in neurogenesis[116], or MYF factors in myogenesis[117]), play a central part in the progressive deployment of GRNs. For example, as noted above, early binding of FOXD3 to the albumin enhancer prevents DNA methylation of a crucial FOXA1 binding site that is required at later stages of development[106]. Similarly, in embryonic stem cells, SOX2 is bound to multiple regions: some are active enhancers that are required to maintain embryonic stem cell pluripotency and others are inactive but will become active after the cells are specified to become neurons[118] or B cells[105]. Importantly, following differentiation, SOX2 is replaced at these sites in a tissue-specific manner by SOX3 and SOX11 in neurons and by SOX4 in B cells. It is unclear if this 'priming' role of SOX2 is actively regulated or is a passive mechanism whereby it acts as a 'place-holder' to avoid nucleosome repositioning or DNA methylation over generic SOX binding sites, but it suggests that such a system could be used to prepare crucial enhancers that control regulatory activity at the next level of the GRN or the next stage of development.

These findings imply that TF binding alone is not always indicative of activity in the traditional sense (BOX 2), but it might 'label' regions that will be used subsequently, perhaps by different TFs (FIG. 4). Priming specific enhancers may therefore facilitate developmental transitions by reducing the rate-limiting step of chromatin remodelling. Indeed, many enhancers are decorated with specific histone tail modifications in progenitor cells (for example, H3K4me1 or H3K4me2 (REFS 71,72,103,119)), even though these elements are not associated with strong regulatory activity at that stage. It has been suggested that the presence of H3K4me1, in the absence of H3K27ac, indicates enhancers that are in a 'poised' state ready for rapid upregulation of their activity[72]. However, as the majority of active enhancers do not seem to be poised at the previous developmental stage[72], this type of epigenetic priming is not a pre-requisite for enhancer activation, and may only be required for a subset of elements, as is observed when human embryonic stem cells are differentiated into neuroectodermal spheres[72]. The presence of H3K4me1 in the absence of H3K27ac can also correspond to enhancers that are actively repressed — which are often also marked by H3K27me3 (REFS 72,73) — to avoid inappropriate target gene expression, as was recently observed at developmental enhancers in *D. melanogaster*[73] and in mammalian cells[120].

The importance of active gene repression is further underscored by data examining TF occupancy. In some cases, enhancer priming can lead to inappropriate TFs being bound at later stages of development; such TF binding events may reflect a molecular 'relic' of the developmental history of the cells. For example, TFs that are expressed in the *D. melanogaster* cardiogenic mesoderm occupy enhancers that are active in the neighbouring visceral mesoderm in addition to cardiac enhancers[91]. As both cell types are derived from the splanchnic mesoderm, this dormant TF occupancy signature may represent a footprint of the developmental history of these cells, reflecting earlier priming of these enhancers throughout the splanchnic mesoderm. Such promiscuous binding of TFs at enhancers that were made accessible owing to events at earlier stages of development indicates a requirement for repression to prevent inappropriate activation of primed enhancers (as depicted in FIG. 4 for enhancer 1 in cell-type II).

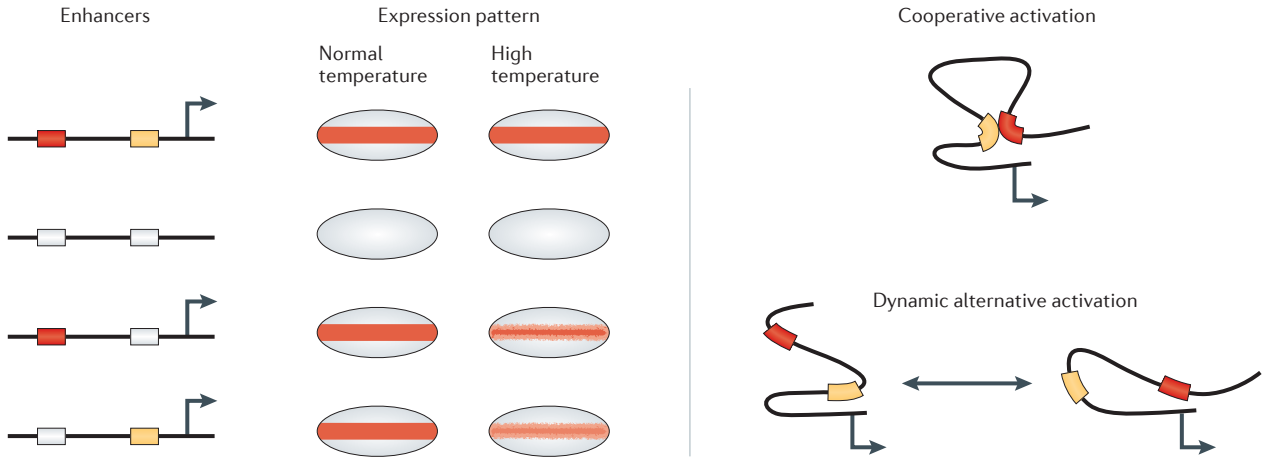
## Regulatory landscapes

Similar to the activity of an individual enhancer, which is regulated by the combined activities of many TFs that can function over different timescales, the expression of a gene typically requires the interplay between multiple enhancer elements, different 'available' promoter elements and a three-dimensional arrangement that is only beginning to be explored. As discussed below, the spatiotemporal expression of a gene often requires a specific genomic architecture, which can involve hierarchical interactions between regulatory elements, to ensure both precise and robust expression.

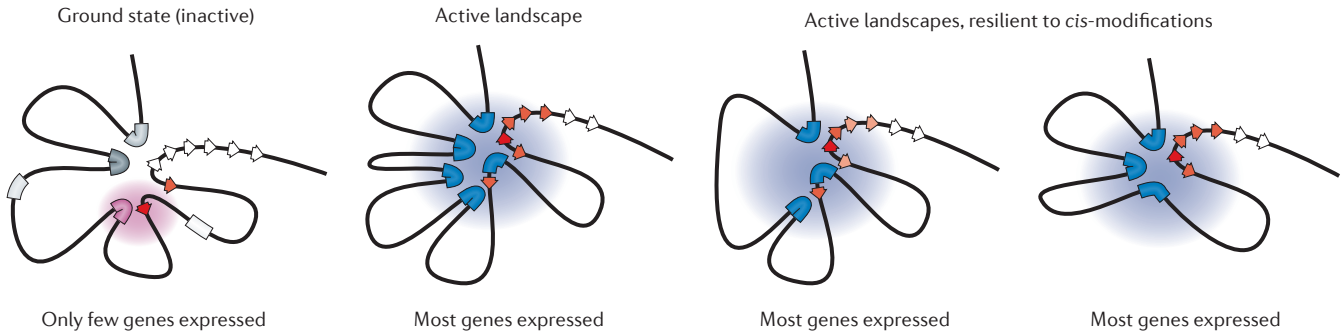
***Integrating the activity from multiple enhancers for gene expression.*** Most developmental genes are regulated by multiple enhancer elements, each controlling a specific spatiotemporal aspect of the expression of the gene. Recent studies showed that this multiplicity of enhancers may also include 'secondary' enhancers that display overlapping, or even identical, spatial activities to primary enhancers that are closer to the gene promoters[121,122] (FIG. 5a). This distinction between 'primary' and 'secondary' elements is generally based purely on the physical distance to the gene promoter or on the order in which the enhancers were discovered: it does not imply any functional hierarchy or relative functional importance per se. Often, the activities of the two elements seem to be almost equivalent, leading to the hypothesis that the secondary enhancer is a 'shadow enhancer' that may provide the phenotypic robustness that is essential for a highly deterministic process such as embryonic development[123]. For example, this secondary enhancer could act by shielding gene expression from environmental perturbations or fluctuations that could otherwise affect the primary enhancer[124,125] (FIG. 5a).

Splanchnic mesoderm
The part of the mesoderm that associates with the endoderm and that will develop later into gut muscles and heart. In *Drosophila melanogaster* the splanchnic mesoderm is located dorsally, whereas in vertebrates it is ventral.

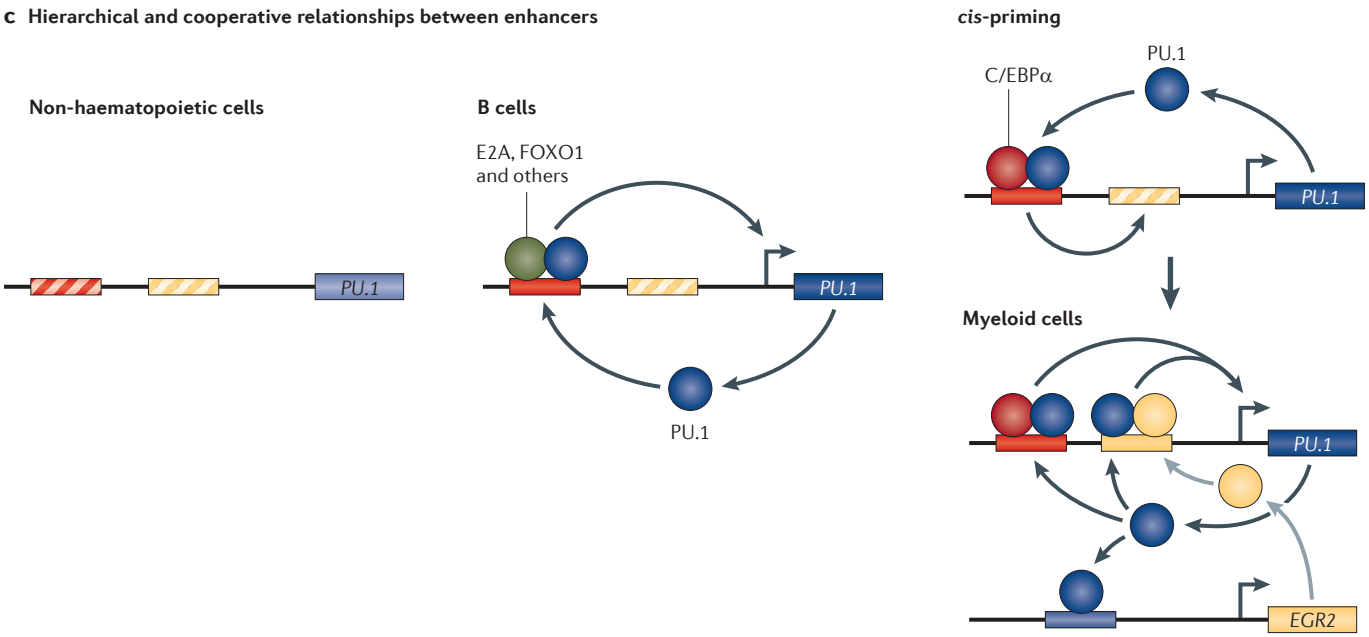
**a** **Shadow enhancers : redundancy and cooperativity**




**b** **Robustness and regulatory archipelagos**




**c** **Hierarchical and cooperative relationships between enhancers**



◀ Figure 5 | **Robustness through enhancer–enhancer interactions and redundancy.** **a** | Shadow enhancers: between redundancy and cooperativity. The presence of two enhancers with overlapping activity provides robustness to gene expression (left panel), especially across a range of environmental conditions (for example, high temperature), thus establishing precise boundaries of gene expression. However, these boundaries may become blurred and result in variegated expression when the activity of one of the enhancers is compromised[124,125]. It is currently unclear if this robustness is associated with the formation of a stable, three-dimensional, multi-enhancer complex that contacts the promoter, or through dynamic alternative activation by each element separately (depicted in the right panel), which would reduce periods of transcriptional inactivity in a manner that is analogous to 'assisted loading' of transcription factors (TFs). **b** | Robustness of the regulatory archipelago. The regulatory archipelago at the mouse *HoxD* locus is an example of *cis*-cooperativity between multiple enhancer elements. These elements are spread across >800 kb and form a complex structure that is characterized by multiple cross-interactions (that are either simultaneous or dynamic)[132]. Some interactions are already present in a ground state (in 'inactive' cells) even though some enhancers could act locally on individual genes. In distal limbs, many enhancers become active and engage in new or stronger interactions, thereby activating the expression of several genes in their vicinity. In the limb, the absence of some elements (through deletions or other chromosomal rearrangements) may not be detrimental as long as other enhancer elements can compensate. Thus, the archipelago seems to be resilient to changes and provides robustness to *HoxD* expression through a dense network of *cis*-three-dimensional interactions between limb regulatory elements, each of which displays slightly different autonomous enhancer activities. **c** | *cis*-priming and hierarchical cooperation. The mouse *PU.1* (also known as *Sfpi1*) gene is regulated by at least two enhancers that have different roles in different haematopoietic cell types[136]. In B cells, binding of TFs — including E2A, forkhead box O1 (FOXO1) and PU.1 — to the upstream element (red box; hatched = inactive, filled = active) is sufficient to activate *PU.1* gene expression. In myeloid cells, CCAAT/enhancer-binding protein-α (C/EBPα), instead of an E2A–FOXO1 complex, binds to this element, which triggers chromatin changes over a neighbouring regulatory element (yellow box, switching from inactive to active). This allows the binding of PU.1 and other factors (for example, early growth response protein 2 (EGR2)) to this neighbouring element, the activity of which is essential to maintain expression of PU.1. Thus, in myeloid cells, the autoregulatory loop that controls PU.1 expression is formed by *cis*-priming of the proximal enhancer by TFs that are bound elsewhere. These two elements and their interaction play a central part in the distinct PU.1-dependent gene regulatory networks (GRNs) in myeloid and B cells.

Yet, how the separate inputs provided by distinct elements are integrated remains poorly understood. At a mechanistic level, this may be achieved in different ways. In some cases, the overall output (the spatial expression patterns of a gene) is different from the sum of the individual activities of each enhancer element. This may correspond to cross-repression, whereby repressors that are bound to one enhancer can also restrict the activity of another enhancer that is regulating the same gene[126,127]. Alternatively, enhancer integration may lead to synergistic action, as occurs at, for example, the *D. melanogaster brk*[128] and *slp1* (REF. 129) genes. In mice, many enhancers that drive diverse patterns of activity in the developing limb bud are spread across a large 800 kb region upstream of the *HoxD* gene cluster[130–132]. Chromatin conformation capture experiments revealed pervasive physical proximity between these different modules, suggesting that what appears as a dispersed 'regulatory archipelago' may come together to act on *HoxD* gene promoters as a single regulatory unit[132] (FIG. 5b), leading to an expression pattern that is wider than the sum of the activities of the isolated modules.

This type of enhancer synergy can also provide robustness to the expression of a gene, as shown for the *HoxD* locus[132]. Multiple enhancers with overlapping activities could stabilize target gene expression by increasing the frequency of transcriptional bursts[133]. Accordingly, in mouse embryos, *Shh* transcription is robust in the floor plate (where it is controlled by at least two distinct and autonomous enhancers), whereas its transcription is much more sporadic in the limb (where a single element is used)[134,135].

The action of distinct modules can also be associated with hierarchal relationships. For example, PU.1 expression in myeloid cells and B cells relies on a common enhancer (known as the upstream regulatory element (URE)), but in myeloid cells, an additional nearby element (known as the –12 kb enhancer) is required for full expression[136]. Remarkably, C/EBPα binding to the URE is required to modify the chromatin over the –12 kb enhancer, so as to allow binding of its associated TFs, including PU.1 itself (FIG. 5c). In this system, the URE not only regulates gene expression in its own right, but also primes a distinct adjacent regulatory element depending on the cellular context and available TFs. Such complex hierarchal *cis*-interactions are probably common and could play a central part in controlling the progressive activation of a given gene during development.

***Cis-regulatory architecture of the genome.*** For many genes that are regulated by remote enhancers[130,137–140], large chromatin loops are generally thought to bring enhancers and target genes into close physical proximity, leading to simple pairwise interactions. Indeed, chromatin conformation capture technologies have provided many examples of this phenomenon[141]. Promoter-specific elements[142], tethering elements[10,11] and insulators[9] are thought to provide logic, specificity and boundaries to such interactions. Yet, many key developmental enhancers act not only on their biologically relevant target gene, but also on unrelated neighbouring genes[130,143–145]. In fact, most locations of the genome seem to be at least 'scanned' by tissue-specific enhancers[146]. These 'bystander' effects suggest that the systems governing promoter–enhancer interactions may not be as specific as is typically depicted. It is conceivable that such bystander interactions could fine-tune gene expression levels, either by titrating enhancer activity or by contributing to the stabilization of complex regulatory structures.

It is important to consider the integration of enhancer modules and enhancer–promoter communication in the context of the three-dimensional organization of the genome. This is emphasized by various developmental phenotypes that are associated with structural changes in genome organization that affect the loci of developmental genes[147–149], suggesting that the relative arrangement of regulatory elements is essential to their collective output, particularly when remote enhancers are involved. In keeping with this, enhancer locations with respect to genes and other *cis*-regulatory elements are often under evolutionary constraint[150–152]. Thus, similar to the output of an enhancer, which can be influenced by the relative positioning of TF binding sites[84], the transcriptional output may be shaped by the overall architecture of a locus, including the relative position of genes, enhancers and possible interspersed structural elements.

## Concluding remarks

Enhancers and other regulatory elements constitute key intermediate nodes in GRNs (reviewed in REFS 1,2): to fully understand the flow of information through these networks, it is essential to decipher the complex relationships between TFs and *cis*-regulatory elements and their contribution to gene expression. The combinatorial action of multiple TFs thereby constitutes a key feature of GRN interconnectivity. The widespread binding profiles of individual TFs, and the questionable extent of their functionality, emphasizes the importance of integrative processes at multiple levels, from combinatorial TF binding, sequential and ordered occupancy of enhancers to interdependencies of regulatory modules and their genomic and chromatin context. Collectively, such features ensure stereotypic and specific gene expression patterns. A central foundation of developmental gene regulation is that enhancers are not always activated as simple on/off switches, but undergo progressive and regulated changes that are essential for their function and for the timing of gene expression. The different strategies that lead to regulatory priming, particularly for the *cis*-regulatory elements that control key TFs in a GRN, may have important roles in the gradual deployment of GRNs and could act as a mechanism to safeguard against the ectopic or premature activation of a gene caused by the inappropriate binding of individual 'master' TFs.

The past five years have seen excellent progress in identifying the location of *cis*-regulatory elements (using ChIP and other methods). An essential future goal is to understand how these elements function in terms of their regulation of temporal and spatial expression and how they interact with other *cis*-regulatory elements. Crosstalk among regulatory elements is emerging as an important feature in the control of gene regulation, so gaining insight into the mechanisms that govern the intricate interactions between enhancers and their target genes will be an important step forwards. Rapid progress is being made in elucidating three-dimensional chromosomal organization[153–155]; integrating this information and discerning its functional implications will be a key challenge, the results of which will yield new insights into the general principles of transcriptional regulation and how it leads to robust developmental progression.


1. Davidson, E. H. Emerging properties of animal gene regulatory networks. *Nature* **468**, 911–920 (2010).
2. Peter, I. S. & Davidson, E. H. Evolution of gene regulatory networks controlling body plan development. *Cell* **144**, 970–985 (2011).
3. Lenhard, B., Sandelin, A. & Carninci, P. Metazoan promoters: emerging characteristics and insights into transcriptional regulation. *Nature Rev. Genet.* **13**, 233–245 (2012).
4. Levine, M. Transcriptional enhancers in animal development and evolution. *Curr. Biol.* **20**, R754–R763 (2010).
5. Bulger, M. & Groudine, M. Functional and mechanistic diversity of distal transcription enhancers. *Cell* **144**, 327–339 (2011).
6. Petrykowska, H. M., Vockley, C. M. & Elnitski, L. Detection and characterization of silencers and enhancer-blockers in the greater CFTR locus. *Genome Res.* **18**, 1238–1246 (2008).
7. Vokes, S. A., Ji, H., Wong, W. H. & McMahon, A. P. A genome-scale analysis of the *cis*-regulatory circuitry underlying sonic hedgehog-mediated patterning of the mammalian limb. *Genes Dev.* **22**, 2651–**2663** (2008).
8. Ayer, S. & Benyajati, C. Conserved enhancer and silencer elements responsible for differential Adh transcription in *Drosophila* cell lines. *Mol. Cell. Biol.* **10**, 3512–3523 (1990).
9. Gaszner, M. & Felsenfeld, G. Insulators: exploiting transcriptional and epigenetic mechanisms. *Nature Rev. Genet.* **7**, 703–713 (2006).
10. Ohtsuki, S., Levine, M. & Cai, H. N. Different core promoters possess distinct regulatory activities in the *Drosophila* embryo. *Genes Dev.* **12**, 547–556 (1998).
11. Calhoun, V. C., Stathopoulos, A. & Levine, M. Promoter-proximal tethering elements regulate enhancer-promoter specificity in the *Drosophila Antennapedia* complex. *Proc. Natl Acad. Sci. USA* **99**, 9243–9247 (2002).
12. Banerji, J., Rusconi, S. & Schaffner, W. Expression of a β-globin gene is enhanced by remote SV40 DNA sequences. *Cell* **27**, 299–308 (1981).
13. Small, S., Blair, A. & Levine, M. Regulation of even-skipped stripe 2 in the *Drosophila* embryo. *EMBO J.* **11**, 4047–4057 (1992).
14. Halfon, M. S. *et al.* Ras pathway specificity is determined by the integration of multiple signal-activated and tissue-restricted transcription factors. *Cell* **103**, 63–74 (2000).
15. Yuh, C. H., Ransick, A., Martinez, P., Britten, R. J. & Davidson, E. H. Complexity and organization of DNA-protein interactions in the 5′-regulatory region of an endoderm-specific marker gene in the sea urchin embryo. *Mech. Dev.* **47**, 165–186 (1994).
16. Sandmann, T. *et al.* A core transcriptional network for early mesoderm development in *Drosophila melanogaster*. *Genes Dev.* **21**, 436–449 (2007).
17. Lettice, L. A. *et al.* Opposing functions of the ETS factor family define Shh spatial expression in limb buds and underlie polydactyly. *Dev. Cell* **22**, 459–467 (2012).
18. Stanojevic, D., Small, S. & Levine, M. Regulation of a segmentation stripe by overlapping activators and repressors in the *Drosophila* embryo. *Science* **254**, 1385–1387 (1991).
19. Ip, Y. T., Levine, M. & Small, S. J. The bicoid and dorsal morphogens use a similar strategy to make stripes in the *Drosophila* embryo. *J. Cell Sci. Suppl.* **16**, 33–38 (1992).
20. Xu, X., Yin, Z., Hudson, J. B., Ferguson, E. L. & Frasch, M. Smad proteins act in combination with synergistic and antagonistic regulators to target Dpp responses to the *Drosophila* mesoderm. *Genes Dev.* **12**, 2354–2370 (1998).
21. Lee, H. H. & Frasch, M. Nuclear integration of positive Dpp signals, antagonistic Wg inputs and mesodermal competence factors during *Drosophila* visceral mesoderm induction. *Development* **132**, 1429–1442 (2005).
22. Guss, K. A., Nelson, C. E., Hudson, A., Kraus, M. E. & Carroll, S. B. Control of a genetic regulatory network by a selector gene. *Science* **292**, 1164–1167 (2001).
23. Mullen, A. C. *et al.* Master transcription factors determine cell-type-specific responses to TGF-β signaling. *Cell* **147**, 565–576 (2011).
24. Trompouki, E. *et al.* Lineage regulators direct BMP and Wnt pathways to cell-specific programs during differentiation and regeneration. *Cell* **147**, 577–589 (2011).
25. Sandmann, T. *et al.* A temporal map of transcription factor activity: mef2 directly regulates target genes at all stages of muscle development. *Dev. Cell* **10**, 797–807 (2006).
26. Jakobsen, J. S. *et al.* Temporal ChIP-on-chip reveals Biniou as a universal regulator of the visceral muscle transcriptional network. *Genes Dev.* **21**, 2448–2460 (2007).
27. Cao, Y. *et al.* Genome-wide MyoD binding in skeletal muscle cells: a potential for broad cellular reprogramming. *Dev. Cell* **18**, 662–674 (2010).
28. Lin, Y. C. *et al.* A global network of transcription factors, involving E2A, EBF1 and Foxo1, that orchestrates B cell fate. *Nature Immunol.* **11**, 635–643 (2010).
29. Pilon, A. M. *et al.* Genome-wide ChIP-Seq reveals a dramatic shift in the binding of the transcription factor erythroid Kruppel-like factor during erythrocyte differentiation. *Blood* **118**, e139–e148 (2011).
30. Gaudet, J. & Mango, S. E. Regulation of organogenesis by the *Caenorhabditis elegans* FoxA protein PHA-4. *Science* **295**, 821–825 (2002).
31. Wilczynski, B. & Furlong, E. E. Dynamic CRM occupancy reflects a temporal map of developmental progression. *Mol. Syst. Biol.* **6**, 383 (2010).
32. Yanez-Cuna, J. O., Dinh, H. Q., Kvon, E. Z., Shlyueva, D. & Stark, A. Uncovering *cis*-regulatory sequence requirements for context specific transcription factor binding. *Genome Res.* 25 Apr 2012 (doi:10.1101/gr.132811.111).
33. Zeitlinger, J. *et al.* Program-specific distribution of a transcription factor dependent on partner transcription factor and MAPK signaling. *Cell* **113**, 395–404 (2003).
34. Biggar, S. R. & Crabtree, G. R. Cell signaling can direct either binary or graded transcriptional responses. *EMBO J.* **20**, 3167–3176 (2001).
35. Giorgetti, L. *et al.* Noncooperative interactions between transcription factors and clustered DNA binding sites enable graded transcriptional responses to environmental inputs. *Mol. Cell* **37**, 418–428 (2010).
36. Johnson, A. D., Meyer, B. J. & Ptashne, M. Interactions between DNA-bound repressors govern regulation by the lambda phage repressor. *Proc. Natl Acad. Sci. USA* **76**, 5061–5065 (1979).
37. Lebrecht, D. *et al.* Bicoid cooperative DNA binding is critical for embryonic patterning in *Drosophila*. *Proc. Natl Acad. Sci. USA* **102**, 13176–13181 (2005).
38. Zinzen, R. P., Senger, K., Levine, M. & Papatsenko, D. Computational models for neurogenic gene expression in the *Drosophila* embryo. *Curr. Biol.* **16**, 1358–1365 (2006).
39. Szymanski, P. & Levine, M. Multiple modes of dorsal-bHLH transcriptional synergy in the *Drosophila* embryo. *EMBO J.* **14**, 2229–2238 (1995).
40. Chopra, V. S. & Levine, M. Combinatorial patterning mechanisms in the *Drosophila* embryo. *Brief. Funct. Genomic. Proteomic.* **8**, 243–249 (2009).
41. Masel, J. & Siegal, M. L. Robustness: mechanisms and consequences. *Trends Genet.* **25**, 395–403 (2009).
42. Lin, Y. S., Carey, M., Ptashne, M. & Green, M. R. How different eukaryotic transcriptional activators can cooperate promiscuously. *Nature* **345**, 359–361 (1990).
43. Merika, M., Williams, A. J., Chen, G., Collins, T. & Thanos, D. Recruitment of CBP/p300 by the IFNβ enhanceosome is required for synergistic activation of transcription. *Mol. Cell* **1**, 277–287 (1998).
44. Voss, T. C. *et al.* Dynamic exchange at regulatory elements during chromatin remodeling underlies assisted loading mechanism. *Cell* **146**, 544–554 (2011).
45. Miller, J. A. & Widom, J. Collaborative competition mechanism for gene activation *in vivo*. *Mol. Cell. Biol.* **23**, 1623–1632 (2003).

46. Panne, D., Maniatis, T. & Harrison, S. C. An atomic model of the interferon-β enhanceosome. *Cell* **129**, 1111–1123 (2007).
47. Falvo, J. V., Thanos, D. & Maniatis, T. Reversal of intrinsic DNA bends in the IFNβ gene enhancer by transcription factors and the architectural protein HMG I(Y). *Cell* **83**, 1101–1111 (1995).
48. Siggers, T., Duyzend, M. H., Reddy, J., Khan, S. & Bulyk, M. L. Non-DNA-binding cofactors enhance DNA-binding specificity of a transcriptional regulatory complex. *Mol. Syst. Biol.* **7**, 555 (2011).
**This is an interesting study demonstrating that the binding of a cofactor that does not contain a DNA-binding domain can alter the sequence specificity of a transcription factor complex.**
49. Noyes, M. B. *et al.* Analysis of homeodomain specificities allows the family-wide prediction of preferred recognition sites. *Cell* **133**, 1277–1289 (2008).
50. Carr, A. & Biggin, M. D. A comparison of *in vivo* and *in vitro* DNA-binding specificities suggests a new model for homeoprotein DNA binding in *Drosophila* embryos. *EMBO J.* **18**, 1598–1608 (1999).
51. Slattery, M. *et al.* Cofactor binding evokes latent differences in DNA binding specificity between hox proteins. *Cell* **147**, 1270–1282 (2011).
52. Liu, X., Lee, C. K., Granek, J. A., Clarke, N. D. & Lieb, J. D. Whole-genome comparison of Leu3 binding *in vitro* and *in vivo* reveals the importance of nucleosome occupancy in target site selection. *Genome Res.* **16**, 1517–1528 (2006).
53. Li, X.-y. *et al.* The role of chromatin accessibility in directing the widespread, overlapping patterns of *Drosophila* transcription factor binding. *Genome Biol.* **12**, R34 (2011).
**The authors found that TF occupancy correlates better with DNA accessibility (estimated by DNaseI hypersensitivity) than with predicted sequence affinity, suggesting that this simple property could explain the clustering of TF binding.**
54. John, S. *et al.* Chromatin accessibility pre-determines glucocorticoid receptor binding patterns. *Nature Genet.* **43**, 264–268 (2011).
55. Degner, J. F. *et al.* DNase I sensitivity QTLs are a major determinant of human expression variation. *Nature* **482**, 390–394 (2012).
56. Buck, M. J. & Lieb, J. D. A chromatin-mediated mechanism for specification of conditional transcription factor targets. *Nature Genet.* **38**, 1446–1451 (2006).
57. Carr, A. & Biggin, M. D. Accessibility of transcriptionally inactive genes is specifically reduced at homeoprotein-DNA binding sites in *Drosophila*. *Nucleic Acids Res.* **28**, 2839–2846 (2000).
58. John, S. *et al.* Interaction of the glucocorticoid receptor with the chromatin landscape. *Mol. Cell* **29**, 611–624 (2008).
59. Vicent, G. P. *et al.* Two chromatin remodeling activities cooperate during activation of hormone responsive promoters. *PLoS Genet.* **5**, e1000567 (2009).
60. Hartley, P. D. & Madhani, H. D. Mechanisms that specify promoter nucleosome location and identity. *Cell* **137**, 445–458 (2009).
61. Yarragudi, A., Miyake, T., Li, R. & Morse, R. H. Comparison of ABF1 and RAP1 in chromatin opening and transactivator potentiation in the budding yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **24**, 9152–9164 (2004).
62. Workman, J. L. & Kingston, R. E. Nucleosome core displacement *in vitro* via a metastable transcription factor-nucleosome complex. *Science* **258**, 1780–1784 (1992).
63. Morse, R. H. Nucleosome disruption by transcription factor binding in yeast. *Science* **262**, 1563–1566 (1993).
64. Barski, A. *et al.* High-resolution profiling of histone methylations in the human genome. *Cell* **129**, 823–837 (2007).
65. Kolasinska-Zwierz, P. *et al.* Differential chromatin marking of introns and expressed exons by H3K36me3. *Nature Genet.* **41**, 376–381 (2009).
66. Mikkelsen, T. S. *et al.* Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature* **448**, 553–560 (2007).
67. Heintzman, N. D. *et al.* Histone modifications at human enhancers reflect global cell-type-specific gene expression. *Nature* **459**, 108–112 (2009).
68. Ernst, J. *et al.* Mapping and analysis of chromatin state dynamics in nine human cell types. *Nature* **473**, 43–49 (2011).
69. Heintzman, N. D. & Ren, B. Finding distal regulatory elements in the human genome. *Curr. Opin. Genet. Dev.* **19**, 541–549 (2009).
70. Ong, C. T. & Corces, V. G. Enhancer function: new insights into the regulation of tissue-specific gene expression. *Nature Rev. Genet.* **12**, 283–293 (2011).
71. Creyghton, M. P. *et al.* Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proc. Natl Acad. Sci. USA* **107**, 21931–21936 (2010).
72. Rada-Iglesias, A. *et al.* A unique chromatin signature uncovers early developmental enhancers in humans. *Nature* **470**, 279–283 (2011).
**References 71 and 72 identified a strong correlation between the presence of H3K27ac on *cis*-regulatory elements and the activity of the neighbouring gene, providing the first indication that this mark, rather than H3K4me1, is a good indicator of active regulatory elements.**
73. Bonn, S. *et al.* Tissue-specific analysis of chromatin state identifies temporal signatures of enhancer activity during embryonic development. *Nature Genet.* **44**,148–156 (2012).
74. Heinz, S. *et al.* Simple combinations of lineage-determining transcription factors prime *cis*-regulatory elements required for macrophage and B cell identities. *Mol. Cell* **38**, 576–589 (2010).
75. McManus, S. *et al.* The transcription factor Pax5 regulates its target genes by recruiting chromatin-modifying proteins in committed B cells. *EMBO J.* **30**, 2388–2404 (2011).
76. Ghisletti, S. *et al.* Identification and characterization of enhancers controlling the inflammatory gene expression program in macrophages. *Immunity* **32**, 317–328 (2010).
77. Mercer, E. M. *et al.* Multilineage priming of enhancer repertoires precedes commitment to the B and myeloid cell lineages in hematopoietic progenitors. *Immunity* **35**, 413–425 (2011).
78. Flames, N. & Hobert, O. Gene regulatory logic of dopamine neuron differentiation. *Nature* **458**, 885–889 (2009).
79. Zinzen, R. P., Girardot, C., Gagneur, J., Braun, M. & Furlong, E. E. Combinatorial binding predicts spatio-temporal *cis*-regulatory activity. *Nature* **462**, 65–70 (2009).
80. Brody, T. *et al.* Use of a *Drosophila* genome-wide conserved sequence database to identify functionally related *cis*-regulatory enhancers. *Dev. Dyn.* **241**, 169–189 (2012).
81. Kaplan, T. *et al.* Quantitative models of the mechanisms that control genome-wide patterns of transcription factor binding during early *Drosophila* development. *PLoS Genet.* **7**, e1001290 (2011).
82. Narlikar, L. *et al.* Genome-wide discovery of human heart enhancers. *Genome Res.* **20**, 381–392 (2010).
83. Senger, K. *et al.* Immunity regulatory DNAs share common organizational features in *Drosophila*. *Mol. Cell* **13**, 19–32 (2004).
84. Swanson, C. I., Evans, N. C. & Barolo, S. Structural rules and complex regulatory circuitry constrain expression of a Notch- and EGFR-regulated eye enhancer. *Dev. Cell* **18**, 359–370 (2010).
**By carrying out an extensive mutagenesis analysis, this elegant study found that the sequence of a *D. melanogaster* enhancer is highly constrained, with the presence and relative positioning of almost all sequence motifs being essential for some aspect of the enhancer's activity. Interestingly, in other *Drosophila* species, the homologous enhancer adopted different 'solutions' to give the same activity.**
85. Thanos, D. & Maniatis, T. Virus induction of human IFNβ gene expression requires the assembly of an enhanceosome. *Cell* **83**, 1091–1100 (1995).
86. Merika, M. & Thanos, D. Enhanceosomes. *Curr. Opin. Genet. Dev.* **11**, 205–208 (2001).
87. Kulkarni, M. M. & Arnosti, D. N. Information display by transcriptional enhancers. *Development* **130**, 6569–6575 (2003).
88. Arnosti, D. N. & Kulkarni, M. M. Transcriptional enhancers: intelligent enhanceosomes or flexible billboards? *J. Cell. Biochem.* **94**, 890–898 (2005).
89. Brown, C. D., Johnson, D. S. & Sidow, A. Functional architecture and evolution of transcriptional elements that drive gene coexpression. *Science* **317**, 1557–1560 (2007).
90. Liberman, L. M. & Stathopoulos, A. Design flexibility in *cis*-regulatory control of gene expression: synthetic and comparative evidence. *Dev. Biol.* **327**, 578–589 (2009).
91. Junion, G. *et al.* A transcription factor collective defines cardiac cell fate and reflects lineage history. *Cell* **148**, 473–486 (2012).
92. Gao, F., Foat, B. C. & Bussemaker, H. J. Defining transcriptional networks through integrative modeling of mRNA expression and transcription factor binding data. *BMC Bioinformatics* **5**, 31 (2004).
93. Ucar, D., Beyer, A., Parthasarathy, S. & Workman, C. T. Predicting functionality of protein-DNA interactions by integrating diverse evidence. *Bioinformatics* **25**, i137–i144 (2009).
94. Krejci, A., Bernard, F., Housden, B. E., Collins, S. & Bray, S. J. Direct response to Notch activation: signaling crosstalk and incoherent logic. *Sci. Signal.* **2**, ra1 (2009).
95. Zaret, K. S. & Carroll, J. S. Pioneer transcription factors: establishing competence for gene expression. *Genes Dev.* **25**, 2227–2241 (2011).
96. Biswas, A. K. & Johnson, D. G. Transcriptional and nontranscriptional functions of E2F1 in response to DNA damage. *Cancer Res.* **72**, 13–17 (2012).
97. Lickwar, C. R., Mueller, F., Hanlon, S. E., McNally, J. G. & Lieb, J. D. Genome-wide protein-DNA binding dynamics suggest a molecular clutch for transcription factor function. *Nature* **484**, 251–255 (2012).
**The authors tagged a transcription factor with two different tags and carried out a competitive ChIP assay to measure how long it took for the newly transcribed tagged TF to outcompete its alternatively tagged counterpart for chromatin binding. The resulting information provides a nice way to measure TF residence time on each site throughout the genome, which seems to be a better indicator of functional binding events than static occupancy data.**
98. Tagoh, H. *et al.* Epigenetic silencing of the *c-fms* locus during B-lymphopoiesis occurs in discrete steps and is reversible. *EMBO J.* **23**, 4275–4285 (2004).
99. Krysinska, H. *et al.* A two-step, PU.1-dependent mechanism for developmentally regulated chromatin remodeling and transcription of the *c-fms* gene. *Mol. Cell. Biol.* **27**, 878–887 (2007).
100. Almer, A., Rudolph, H., Hinnen, A. & Horz, W. Removal of positioned nucleosomes from the yeast PHO5 promoter upon PHO5 induction releases additional upstream activating DNA elements. *EMBO J.* **5**, 2689–2696 (1986).
101. Biddie, S. C. *et al.* Transcription factor AP1 potentiates chromatin accessibility and glucocorticoid receptor binding. *Mol. Cell* **43**, 145–155 (2011).
102. de la Serna, I. L. *et al.* MyoD targets chromatin remodeling complexes to the myogenin locus prior to forming a stable DNA-bound complex. *Mol. Cell. Biol.* **25**, 3997–4009 (2005).
103. Lupien, M. *et al.* FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription. *Cell* **132**, 958–970 (2008).
**This study shows that FOXA1 occupancy correlates with the prior presence of H3K4me2 at specific enhancer elements. This histone modification directs FOXA1 to induce further chromatin remodelling and TF occupancy specifically at those enhancers. This suggests that even what is perceived as a pioneer factor requires a prior chromatin context.**
104. Siersbæk, R. *et al.* Extensive chromatin remodelling and establishment of transcription factor 'hotspots' during early adipogenesis. *EMBO J.* **30**, 1459–1472 (2011).
105. Liber, D. *et al.* Epigenetic priming of a pre-B cell-specific enhancer through binding of Sox2 and Foxd3 at the ESC stage. *Cell Stem Cell* **7**, 114–126 (2010).
106. Xu, J. *et al.* Transcriptional competence and the active marking of tissue-specific enhancers by defined transcription factors in embryonic and induced pluripotent stem cells. *Genes Dev.* **23**, 2824–2838 (2009).
107. Hoogenkamp, M. *et al.* Early chromatin unfolding by RUNX1: a molecular explanation for differential requirements during specification versus maintenance of the hematopoietic gene expression program. *Blood* **114**, 299–309 (2009).
108. Liang, H. L. *et al.* The zinc-finger protein Zelda is a key activator of the early zygotic genome in *Drosophila*. *Nature* **456**, 400–403 (2008).
109. Nien, C. Y. *et al.* Temporal coordination of gene networks by Zelda in the early *Drosophila* embryo. *PLoS Genet.* **7**, e1002339 (2011).

110. Harrison, M. M., Li, X.-y., Kaplan, T., Botchan, M. R. & Eisen, M. B. Zelda binding in the early *Drosophila melanogaster* embryo marks regions subsequently activated at the maternal-to-zygotic transition. *PLoS Genet.* **7**, e1002266 (2011).
**References 109 and 110 indicate that Zelda is a major global regulator of the MZT in *D. melanogaster*. Zelda binds to thousands of regulatory elements prior to the MZT and prior to the occupancy of other known factors and is required for the zygotic gene activation of a large number of genes.**
111. Struffi, P. *et al.* Combinatorial activation and concentration-dependent repression of the *Drosophila* even skipped stripe 3 + 7 enhancer. *Development* **138**, 4291–4299 (2011).
112. Bell, O., Tiwari, V. K., Thoma, N. H. & Schubeler, D. Determinants and dynamics of genome accessibility. *Nature Rev. Genet.* **12**, 554–564 (2011).
113. Xu, J. *et al.* Pioneer factor interactions and unmethylated CpG dinucleotides mark silent tissue-specific enhancers in embryonic stem cells. *Proc. Natl Acad. Sci. USA* **104**, 12377–12382 (2007).
114. Serandour, A. A. *et al.* Epigenetic switch involved in activation of pioneer factor FOXA1-dependent enhancers. *Genome Res.* **21**, 555–565 (2011).
115. Stadler, M. B. *et al.* DNA-binding factors shape the mouse methylome at distal regulatory regions. *Nature* **480**, 490–495 (2011).
116. Pevny, L. & Placzek, M. SOX genes and neural progenitor identity. *Curr. Opin. Neurobiol.* **15**, 7–13 (2005).
117. Olson, E. N. & Klein, W. H. bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. *Genes Dev.* **8**, 1–8 (1994).
118. Bergsland, M. *et al.* Sequentially acting Sox transcription factors in neural lineage development. *Genes Dev.* **25**, 2453–2464 (2011).
119. Cui, K. *et al.* Chromatin signatures in multipotent human hematopoietic stem cells indicate the fate of bivalent genes during differentiation. *Cell Stem Cell* **4**, 80–93 (2009).
120. Watts, J. A. *et al.* Study of FoxA pioneer factor at silent genes reveals Rfx-repressed enhancer at Cdx2 and a potential indicator of esophageal adenocarcinoma development. *PLoS Genet.* **7**, e1002277 (2011).
121. Zeitlinger, J. *et al.* Whole-genome ChIP-chip analysis of Dorsal, Twist, and Snail suggests integration of diverse patterning processes in the *Drosophila* embryo. *Genes Dev.* **21**, 385–390 (2007).
122. Hong, J. W., Hendrix, D. A. & Levine, M. S. Shadow enhancers as a source of evolutionary novelty. *Science* **321**, 1314 (2008).
123. Barolo, S. Shadow enhancers: frequently asked questions about distributed *cis*-regulatory information and enhancer redundancy. *Bioessays* **34**, 135–141 (2012).
124. Frankel, N. *et al.* Phenotypic robustness conferred by apparently redundant transcriptional enhancers. *Nature* **466**, 490–493 (2010).
125. Perry, M. W., Boettiger, A. N., Bothma, J. P. & Levine, M. Shadow enhancers foster robustness of *Drosophila* gastrulation. *Curr. Biol.* **20**, 1562–1567 (2010).
**Developmental genes often have multiple enhancers that seem to have overlapping activities and functions under normal developmental conditions. References 124 and 125 showed that the removal of one (or both) of the enhancers leads to developmental defects when the environmental conditions are more extreme, indicating that 'shadow' enhancers provide robustness to gene expression in the midst of fluctuating environmental temperatures.**
126. Barolo, S. & Levine, M. hairy mediates dominant repression in the *Drosophila* embryo. *EMBO J.* **16**, 2883–2891 (1997).
127. Perry, M. W., Boettiger, A. N. & Levine, M. Multiple enhancers ensure precision of gap gene-expression patterns in the *Drosophila* embryo. *Proc. Natl Acad. Sci. USA* **108**, 13570–13575 (2011).
128. Yao, L.-C. *et al.* Multiple modular promoter elements drive graded brinker expression in response to the Dpp morphogen gradient. *Development* **135**, 2183–2192 (2008).
129. Prazak, L., Fujioka, M. & Gergen, J. P. Non-additive interactions involving two distinct elements mediate sloppy-paired regulation by pair-rule transcription factors. *Dev. Biol.* **344**, 1048–1059 (2010).
130. Spitz, F., Gonzalez, F. & Duboule, D. A global control region defines a chromosomal regulatory landscape containing the *HoxD* cluster. *Cell* **113**, 405–417 (2003).
131. Gonzalez, F., Duboule, D. & Spitz, F. Transgenic analysis of *Hoxd* gene regulation during digit development. *Dev Biol.* **306**, 847–859 (2007).
132. Montavon, T. *et al.* A regulatory archipelago controls Hox genes transcription in digits. *Cell* **147**, 1132–1145 (2011).
**Genetic and transgenic analyses showed that mouse *HoxD* genes are controlled by many remote regulatory elements that are separated by large distances. Chromatin conformation capture experiments revealed that these elements come together in cells where they contribute to *HoxD* gene expression, providing a three-dimensional view of the complex folding of regulatory landscapes during embryonic development.**
133. Suter, D. M. *et al.* Mammalian genes are transcribed with widely different bursting kinetics. *Science* **332**, 472–474 (2011).
134. Amano, T. *et al.* Chromosomal dynamics at the *Shh* locus: limb bud-specific differential regulation of competence and active transcription. *Dev. Cell*, **16**, 47–57 (2008).
135. Jeong, Y., El-Jaick, K., Roessler, E., Muenke, M. & Epstein, D. J. A functional screen for sonic hedgehog regulatory elements across a 1 Mb interval identifies long-range ventral forebrain enhancers. *Development* **133**, 761–772 (2006).
136. Leddin, M. *et al.* Two distinct auto-regulatory loops operate at the PU.1 locus in B cells and myeloid cells. *Blood* **117**, 2827–2838 (2011).
137. Jack, J. & DeLotto, Y. Structure and regulation of a complex locus: the *cut* gene of *Drosophila*. *Genetics* **139**, 1689–1700 (1995).
138. Wunderle, V. M., Critcher, R., Hastie, N., Goodfellow, P. N. & Schedl, A. Deletion of long-range regulatory elements upstream of *SOX9* causes campomelic dysplasia. *Proc. Natl Acad. Sci. USA* **95**, 10649–10654 (1998).
139. Sagai, T., Hosoya, M., Mizushina, Y., Tamura, M. & Shiroishi, T. Elimination of a long-range *cis*-regulatory module causes complete loss of limb-specific Shh expression and truncation of the mouse limb. *Development* **132**, 797–803 (2005).
140. Visel, A., Rubin, E. M. & Pennacchio, L. A. Genomic views of distant-acting enhancers. *Nature* **461**, 199–205 (2009).
141. Splinter, E. & de Laat, W. The complex transcription regulatory landscape of our genome: control in three dimensions. *EMBO J.* **30**, 4345–4355 (2011).
142. Butler, J. E. & Kadonaga, J. T. Enhancer-promoter specificity mediated by DPE or TATA core promoter motifs. *Genes Dev.* **15**, 2515–2519 (2001).
143. Zuniga, A. *et al.* Mouse limb deformity mutations disrupt a global control region within the large regulatory landscape required for Gremlin expression. *Genes Dev.* **18**, 1553–1564 (2004).
144. Cajiao, I., Zhang, A., Yoo, E. J., Cooke, N. E. & Liebhaber, S. A. Bystander gene activation by a locus control region. *EMBO J.* **23**, 3854–3863 (2004).
145. Lower, K. M. *et al.* Adventitious changes in long-range gene expression caused by polymorphic structural variation and promoter competition. *Proc. Natl Acad. Sci. USA* **106**, 21771–21776 (2009).
146. Ruf, S. *et al.* Large-scale analysis of the regulatory architecture of the mouse genome with a transposon-associated sensor. *Nature Genet.* **43**, 379–386 (2011).
147. Klopocki, E. *et al.* A microduplication of the long range SHH limb regulator (ZRS) is associated with triphalangeal thumb-polysyndactyly syndrome. *J. Med. Genet.* **45**, 370–375 (2008).
148. Kurth, I. *et al.* Duplications of noncoding elements 5′ of SOX9 are associated with brachydactyly-anonychia. *Nature Genet.* **41**, 862–863 (2009).
149. Dathe, K. *et al.* Duplications involving a conserved regulatory element downstream of BMP2 are associated with brachydactyly type A2. *Am. J. Hum. Genet.* **84**, 483–492 (2009).
150. Cande, J., Goltsev, Y. & Levine, M. S. Conservation of enhancer location in divergent insects. *Proc. Natl Acad. Sci. USA* **106**, 14414–14419 (2009).
151. Mongin, E., Dewar, K. & Blanchette, M. Long-range regulation is a major driving force in maintaining genome integrity. *BMC Evol. Biol.* **9**, 203 (2009).
152. Engstrom, P. G., Ho Sui, S. J., Drivenes, O., Becker, T. S. & Lenhard, B. Genomic regulatory blocks underlie extensive microsynteny conservation in insects. *Genome Res.* **17**, 1898–1908 (2007).
153. Dixon, J. R. *et al.* Topological domains in mammalian genomes identified by analysis of chromatin interactions. *Nature* **485**, 376–380 (2012).
154. Lieberman-Aiden, E. *et al.* Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science* **326**, 289–293 (2009).
155. Nora, E. P. *et al.* Spatial partitioning of the regulatory landscape of the X-inactivation centre. *Nature* **485**, 381–385 (2012).
156. Ludwig, M. Z., Manu, Kittler, R., White, K. P. & Kreitman, M. Consequences of eukaryotic enhancer architecture for gene expression dynamics, development, and fitness. *PLoS Genet.* **7**, e1002364 (2011).
157. Janssens, H. *et al.* Quantitative and predictive model of transcriptional control of the *Drosophila melanogaster* even skipped gene. *Nature Genet.* **38**, 1159–1165 (2006).
158. Klingler, M., Soong, J., Butler, B. & Gergen, J. P. Disperse versus compact elements for the regulation of runt stripes in *Drosophila*. *Dev. Biol.* **177**, 73–84 (1996).
159. MacArthur, S. *et al.* Developmental roles of 21 *Drosophila* transcription factors are determined by quantitative differences in binding to an overlapping set of thousands of genomic regions. *Genome Biol.* **10**, R80 (2009).
160. Liu, Y. H. *et al.* A systematic analysis of Tinman function reveals Eya and JAK-STAT signaling as essential regulators of muscle development. *Dev. Cell* **16**, 280–291 (2009).
161. Zheng, J. *et al.* Epistatic relationships reveal the functional organization of yeast transcription factors. *Mol. Syst. Biol.* **6**, 420 (2010).



### Acknowledgements
We are very grateful to J. Erceg, D. Harnett and P. Khoueiry for useful comments, with particular thanks to R. Zinzen and D. Garfield for some rephrasing. We apologize to all colleagues whose work was omitted due to space limitations; we unfortunately could not comment on all the interesting papers in the field. F.S. is supported by grants from the Human Frontier Science Program (HFSP), a European Commission grant (EC-FP7, grant Health 223210/CISSTEM). E.E.M.F. is supported by grants from the HFSP, the European Research Area for Systems Biology (ERASysBio) for the ModHeart project and the German Research Foundation (DFG; grant FU 750/1-1).


### Competing interests statement
The authors declare no competing financial interests.

### FURTHER INFORMATION
**François Spitz's homepage:** http://www.embl.de/research/units/dev_biology/spitz/index.html
**Eileen E. M. Furlong's homepage:** http://furlonglab.embl.de
**ENCODE and modENCODE:** http://www.genome.gov/10005107
**REDfly enhancer database:** http://redfly.ccr.buffalo.edu
**VISTA Enhancer browser:** http://enhancer.lbl.gov

**ALL LINKS ARE ACTIVE IN THE ONLINE PDF**