

Multiple Layers of Complexity in *cis*-regulatory Regions of Developmental Genes

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Genomes contain the necessary information to ensure that genes are expressed in the right place, at the right time, and with the proper rate. Metazoan developmental genes often possess long stretches of DNA flanking their coding sequences and/or large introns which contain elements that influence gene expression. Most of these regulatory elements are relatively small and can be studied in isolation. For example, transcriptional enhancers, the elements that generate the expression pattern of a gene, have been traditionally studied with reporter constructs in transgenic animals. These studies have provided and will provide invaluable insights into enhancer evolution and function. However, this experimental approach has its limits; often, enhancer elements do not faithfully recapitulate native expression patterns. This fact suggests that additional information in *cis*-regulatory regions modulates the activity of enhancers and other regulatory elements. Indeed, recent studies have revealed novel functional aspects at the level of whole *cis*-regulatory regions. First, the discovery of “shadow enhancers.” Second, the ubiquitous interactions between *cis*-regulatory elements. Third, the notion that some *cis*-regulatory regions may not function in a modular manner. Last, the effect of chromatin conformation on *cis*-regulatory activity. In this article, I describe these recent findings and discuss open questions in the field. *Developmental Dynamics* 241:1857–1866, 2012. © 2012 Wiley Periodicals, Inc.

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INTRODUCTION

The precise control of gene transcription is crucial for the development of an organism. The presence of abnormal mRNA levels during development can modify the adult phenotype and have detrimental fitness effects. The genome contains the necessary information to ensure that genes are expressed in the right place, at the right time and with the proper rate. Developmental genes often possess long stretches of DNA flanking their coding sequences and/or large introns which contain elements that influence

gene expression. Most of these regulatory elements are relatively small and can be studied in isolation (separated from their respective coding sequences). Certainly, the modular nature of most regulatory elements (i.e., their ability to function in isolation) paved the way for their discovery and study. Up to date, four types of elements have been described: transcriptional enhancers (Banerji et al., 1981), transcriptional silencers (Laimins et al., 1986), enhancer-blocking insulators (Udvary et al., 1985), and promoter-tethering elements (Calhoun et al., 2002). Transcriptional enhancers are,

by far, the most studied among the four element types (Levine, 2010). When placed upstream of a minimal promoter and a reporter gene in a transgene, enhancers recapitulate part of the native expression pattern of a gene. Enhancer activity is generated by the binding of transcription factors to short DNA motifs (6–20 base-pairs) present in their sequence. In vivo, chromatin loops mediate the interaction between enhancers and their target core promoters (Akbari et al., 2006). Silencers repress gene expression through interactions with the core promoter and/or enhancer

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elements (Ogbourne and Antalis, 1998). Insulators isolate enhancers from nearby genes, restricting their influence to the target core promoter(s) (Bell et al., 2001). Last but not least, promoter-tethering elements facilitate the interaction between enhancers and target core promoters (Akbari et al., 2008).

The collection of noncoding regulatory elements of a gene constitutes its *cis*-regulatory information. Unlike with coding sequences, there is no simple way to demarcate the physical limits of *cis*-regulatory regions in metazoan genomes; even when flanking and intronic DNA has been explored in detail, it is difficult to rule out the presence of additional distant regulatory elements. For example, the gene *sonic hedgehog* (*shh*), which is involved in vertebrate limb development, is activated by an enhancer located approximately 1 megabase upstream of its coding sequence (Lettice et al., 2003). Despite this challenging issue, studies of reporter expression with large DNA fragments allow us to define discrete functional units which seem to fully recapitulate the expression pattern of a gene. Thus, for some developmental genes, the apparent boundaries of their *cis*-regulatory DNA have been established (Fujioka et al., 2002; Kim and Lauderdale, 2006; Venken et al., 2009).

It has been shown that genes involved in embryonic development, cell differentiation, and pattern specification in *D. melanogaster* and *C. elegans* have significantly more flanking DNA than housekeeping genes (Nelson et al., 2004). However, there is high variance in flanking DNA content among developmental genes (Nelson et al., 2004). This probably means that the regulatory information of a developmental gene can have different degrees of compactness. Indeed, this has been observed within and between genomes. For instance, the regulatory information needed for embryonic expression of *S. purpuratus endo16* appears to be circumscribed to 2,300 base-pairs (Yuh et al., 2001). In contrast, several developmental genes in metazoan genomes have *cis*-regulatory elements scattered over tens or hundreds of kilobases (Maeda and Karch, 2009;

Frankel et al., 2011; Montavon et al., 2011; Visser et al., 2012).

Traditionally, the study of *cis*-regulation was tackled with functional analyses of individual *cis*-regulatory elements (mostly enhancers). Over many years, these analyses have shed light on the logic of *cis*-regulation (Istrail and Davidson, 2005). Furthermore, they have provided invaluable insights into the structural rules (Erives and Levine, 2004; Panne et al., 2007; Swanson et al., 2010) and evolutionary histories (Ludwig and Kreitman, 1995; Crocker et al., 2008) of enhancers. Nevertheless, it is widely recognized in the *cis*-regulation community that, often, small enhancer elements do not faithfully recapitulate native expression patterns (Barolo, 2012). Frequently, reporter constructs drive expression in the wrong cells, a fact known as “ectopic expression” (Summerbell et al., 2000; Chao et al., 2010; Prazak et al., 2010; Frankel et al., 2011; Perry et al., 2011). It is also common to observe reporter expression which does not coincide temporally with that of the native gene, a phenomenon often referred as “heterochronic expression” (Adachi et al., 2003; Lin et al., 2010; Prazak et al., 2010; Frankel et al., 2011; Ludwig et al., 2011). This probably means that additional information is necessary to generate the native expression pattern of a gene. Also, it is known that transgenes are usually subject to position effects; their expression changes depending on the genomic context in which they are located. This suggests that chromatin conformation within and around regulatory elements is vital for their proper function. It is important to clarify that none of these known issues undermine the work with isolated *cis*-regulatory elements (these studies will continue to be useful). Instead, they make us think that additional levels of analysis are necessary to fully comprehend the complexity of *cis*-regulatory regions. In effect, comprehensive studies and methodological breakthroughs have started to expose this complexity. In recent years, numerous investigations have uncovered new aspects of functional complexity at the level of whole *cis*-regulatory regions. First, the discovery that one gene can have multiple

enhancers driving similar expression patterns. Second, the ubiquitous interactions between *cis*-regulatory elements. Third, the notion that some *cis*-regulatory regions may not function in a modular fashion. Last, the effect of epigenetic marks and chromatin conformation on the activity of regulatory elements, and the consequences for gene expression. In this article, I describe these recent findings in detail and evaluate the extent of the discoveries. In addition, I discuss open questions in the field and the state-of-the-art methodological approaches that will facilitate research on *cis*-regulation.

Genes Have Multiple Enhancers With Overlapping or Very Similar Expression Patterns

In 2007, a study of *cis*-regulation with *D. melanogaster* embryos exposed a surprising fact. Whole genome ChIP-chip analyses for three transcription factors involved in dorso-ventral patterning, revealed that the target gene *vnd* is regulated by two enhancers separated by many kilobases, which drive remarkably similar expression patterns in presumptive neurogenic ectoderm (Zeitlinger et al., 2007). This surprising fact was later observed for more genes involved in dorso-ventral patterning. The *Drosophila* genes *sog* and *brinker* also possess two enhancers, separated by many kilobases, with similar embryonic activity (Hong et al., 2008). It was suggested that at least one-third of all *Dorsal* target genes might have this type of regulation (Hong et al., 2008). In the latter study, the authors coined the term “shadow enhancers.” In my opinion, this term is more attractive than descriptive. However, it is difficult to propose an alternative term that is short and descriptive at the same time. Thus, “shadow enhancers” has permeated into the literature and the scientific community.

Given this interesting phenomenon, there was an obvious question to answer: why do some genes have enhancers with apparently redundant activities? Is one enhancer sufficient for accurate gene function? The

widespread occurrence of “shadow enhancers” suggested that there was a functional explanation for their presence in genomes. A plausible answer for the above questions came from experimental manipulations of *cis*-regulatory regions. The gene *shavenbaby* (*svb*) encodes a master transcription factor that regulates the formation of trichomes (hair-like structures) in the cuticle of insects (Payre et al., 1999). This gene also contains multiple enhancers which drive expression in the same group of cells (Fig. 1A). The expression patterns of two distant *svb* enhancers named *Z* and *DG2* overlaps with the patterns of enhancers *A* and *E* (Fig. 1A). All these four enhancers are active at the same time in dorso-lateral cells of the embryonic epidermis (Frankel et al., 2010). Then, what happens if the distant enhancers are removed from the native locus through a deletion? If enhancers *Z* and *DG2* are deleted and embryos develop under an optimal growth temperature the larval cuticle has a wild type appearance (Fig. 1B). However, if embryos develop under extreme temperatures, the lack of enhancers *Z* and *DG2* causes a significant loss of cuticular trichomes (Fig. 1B; Frankel et al., 2010). A similar effect is observed with a “stressful genetic background.” *Wingless* is a known regulator of *svb*; if the two alleles of this gene are wild type, the absence of the distant enhancers has no phenotypic effect. In contrast, if one copy of *wingless* is mutated, the lack of *Z* and *DG2* produces a significant loss of trichomes (Frankel et al., 2010). Hence, the activity of the distant enhancers is dispensable under optimal growth conditions but vital under stressful conditions. Thus, we can conclude that enhancers *Z* and *DG2* confer robustness to the phenotype (i.e., canalize the phenotype; Swami, 2010).

A similar set of experiments was also applied to the gene *snail* in *D. melanogaster* (Perry et al., 2010). However, in this case, the experimental manipulations were not made in the native locus but in a BAC (bacterial artificial chromosome) containing the *cis*-regulatory region of the gene. *Snail* is activated by two enhancers (one proximal and one distal to the transcription start site) with similar

activities in the early embryo. Under normal growth conditions, removal of the proximal or the distal enhancer did not affect *snail* function. On the other hand, if the embryo develops at high temperatures or with just one copy of *Dorsal* (a gene that activates *snail*), the lack of one enhancer causes gastrulation defects (Perry et al., 2010). Intriguingly, a recent report with a very similar experimental approach postulated that the distal enhancer of *snail* is essential for gastrulation under normal growth conditions (Dunipace et al., 2011), contending the previous claim about the dispensability of this enhancer (Perry et al., 2010).

In recent years, numerous *cis*-regulatory regions of animal genes have been shown to contain multiple enhancers with overlapping or very similar expression patterns (Jeong et al., 2006; Werner et al., 2007; Hong et al., 2008; McGaughey et al., 2008; Corbo et al., 2010; Frankel et al., 2010; Kalay and Wittkopp, 2010; L'Honore et al., 2010; Naranjo et al., 2010; Perry et al., 2010, 2011; Franchini et al., 2011; Galindo et al., 2011; Ghiasvand et al., 2011; Lee et al., 2011; McBride et al., 2011; Watts et al., 2011; Pauls et al., 2012). Yet, the first observations of overlapping expression from different enhancers date back from the 1990s (Hoch et al., 1990; Kassis, 1990; Tolia and Kafatos, 1990). In addition to these experimentally validated cases, a computational analysis has predicted the occurrence of “shadow enhancers” in many *D. melanogaster* genes that act in the segmentation cascade (Kazemian et al., 2010). The presence of two enhancers with similar activities, however, does not automatically mean that one enhancer is required to canalize the phenotype. For example, a natural deletion of a distal enhancer of *ATOH7* (a gene that has a seemingly redundant proximal enhancer) causes a congenital eye disease in humans without any environmental or genetic perturbation (Ghiasvand et al., 2011). Thus, experimental manipulations or analyses of natural variation are definitely required to establish the dispensability of an enhancer.

The existence of multiple enhancers in *cis*-regulatory regions of developmental genes may be a widespread

buffering mechanism against environmental or genetic variation. This type of *cis*-regulatory architecture could act in concert with other buffering mechanisms, such as the microRNA pathway (Hornstein and Shomron, 2006; Li et al., 2009).

Interactions Between Regulatory Elements

In general, small DNA fragments, isolated from their flanking DNA, are tested for *cis*-regulatory activity using reporter constructs. These reporter constructs are either injected into embryos to generate stable transformants (Venken and Bellen, 2007; Tasic et al., 2011; Frokjaer-Jensen et al., 2012), or electroporated (Funahashi and Nakamura, 2008; Vierra and Irvine, 2012), to observe the transient effect of the construct. In most cases, these small DNA fragments are sufficient to provide coherent functions in vivo. Undoubtedly, modularity is present to some extent in non-coding DNA and this characteristic can be exploited to further dissect the function of regulatory elements. Nonetheless, the fact that *cis*-regulatory elements perform discrete functions which can be tested with isolated DNA fragments does not mean that these elements act alone in their native loci. In fact, several recent reports suggest that interactions between *cis*-regulatory elements are commonplace, and may play relevant roles for the function of *cis*-regulatory regions.

The *HoxD* cluster of vertebrates is active during limb development. The precise spatial and temporal expression of *HoxD* genes in limb buds is vital for appendage formation. This cluster is known to be activated by two proximal enhancer regions named Prox and GCR (Gonzalez et al., 2007). Located toward the centromere, an enigmatic gene desert of 600 kilobases flanks the *HoxD* cluster. Recent work that investigated physical contacts between putative regulatory elements in the gene desert and *HoxD* genes uncovered a surprisingly high number of new regulatory elements and molecular interactions (Montavon et al., 2011). At least five regulatory elements dispersed in the gene desert interacted with the gene *HoxD13*

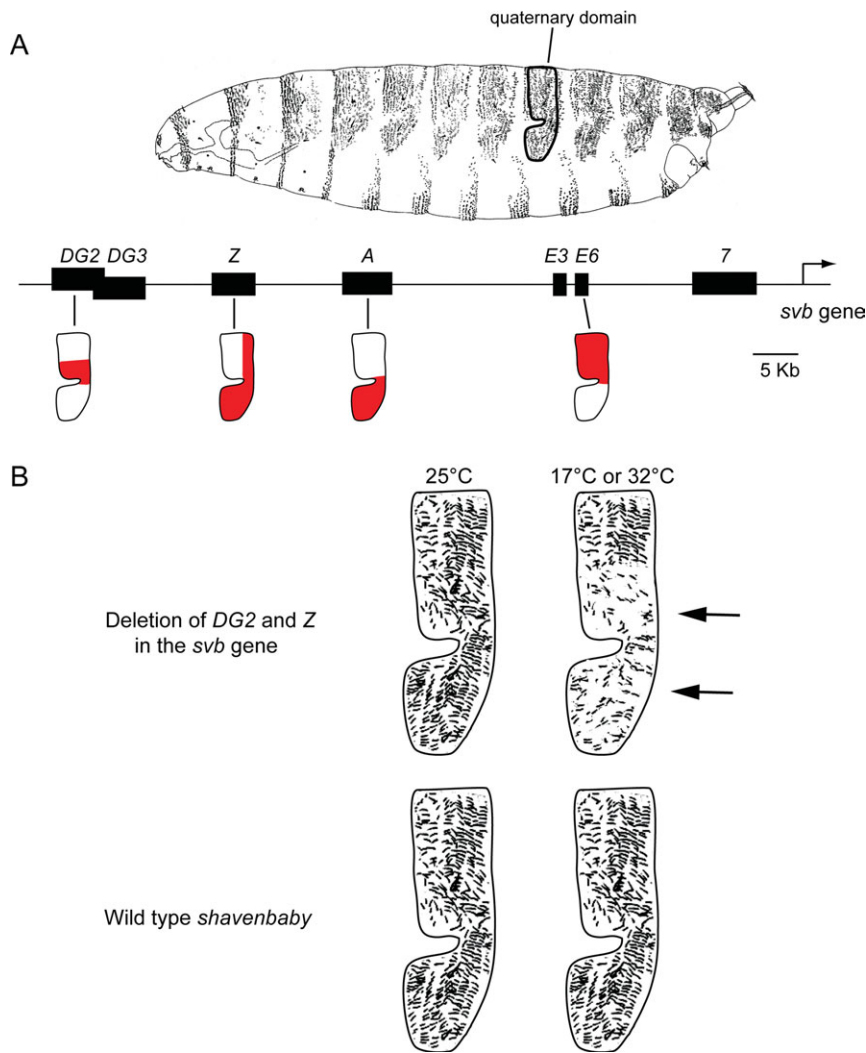


Fig. 1.

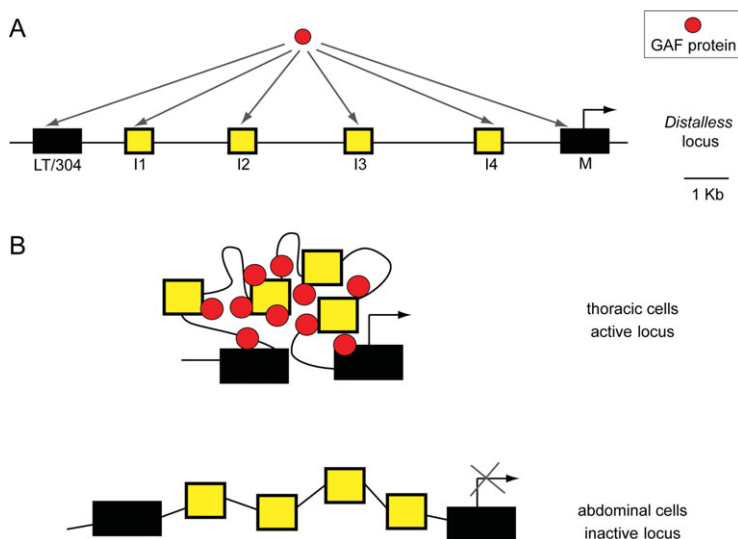


Fig. 2.

during digit development. Moreover, these regulatory elements interacted strongly among themselves during the same developmental period. Four of these noncoding elements, analyzed with *lacZ* reporter constructs in transgenic mice, showed enhancer activity in the developing distal limb. Therefore, in limb development, interactions between many regulatory elements seem to be essential for proper digit patterning. These physical interactions between regulatory elements are not a particular feature of the *HoxD* cluster. In T cells, the TNF gene is activated by two enhancers separated by 12 kilobases. These two enhancers physically interact when T cells become activated, generating a

Fig. 1. The function of *shavenbaby* “shadow enhancers.” **A:** (above) Drawing from the lateral perspective of a *D. melanogaster* first instar larva. The pattern of trichomes (hair-like structures) is depicted in black. The domain producing quaternary trichomes on the fifth abdominal segment is enclosed in a black outline. (below) Diagram of the region upstream of *shavenbaby* (*svb*) transcription start site, showing the positions of the enhancers (black rectangles) for this locus. The overlapping expression driven by enhancers DG2, Z, A, and E6 is shown in red in the diagrams of the quaternary domain (below each enhancer). **B:** Larvae carrying a deletion of enhancers Z and DG2 in the *svb* gene show a wild type number of quaternary trichomes if embryos develop at 25°C, the optimal growth temperature. In contrast, larvae with the deletion display a diminished number of quaternary trichomes in the areas where DG2 and Z drive expression (indicated by black arrows) when embryos are reared at extreme temperatures (17°C or 32°C). Larvae with a wild-type *shavenbaby* develop very similar numbers of quaternary trichomes when grown at different temperatures. Thus, the activity of DG2 and Z confers robustness to the phenotype.

Fig. 2. The architecture of the *Distalless* (*Dll*) locus in *D. melanogaster*. **A:** Linear view of the *Distalless* locus. Black boxes demarcate enhancer regions (LT/304 and M). Yellow boxes indicate regulatory elements without enhancer activity (I1, I2, I3, and I4). The GAF protein, represented here by a red circle, can bind to all six of these regulatory regions. **B:** (Above) In the thoracic cells that will give rise to legs, the chromatin of the *Dll* locus is in a compact state, likely due to GAF activity. In this conformation the gene is active. (Below) In contrast, *Dll* is inactive in the homologous abdominal cells, which do not form legs. In these cells, the chromatin is in an extended conformation. The stoichiometry of GAFs in relation to DNA elements is purely hypothetical. Redrawn with permission from Agelopoulos et al. (2012).

chromatin loop that promotes transcriptional activation (Tsytsykova et al., 2007). In the human β -globin gene, a locus control region (LCR) modulates gene expression. Within this control region there are several elements named MAREs, which contain binding sites for the transcription factors Maf and Bach1. Using atomic force microscopy, investigators showed that distant MAREs physically interact in vitro when Maf and Bach1 are present (Yoshida et al., 1999). These interactions between MAREs might be critical for chromatin structure and gene expression (Yoshida et al., 1999). Although not directly related to the control of gene expression, the case of the *Igh* locus provides another interesting example of interactions between noncoding elements (Guo et al., 2011). This genomic region contains multiple CTCF insulator elements whose interactions are necessary to produce correct V(D)J rearrangements (Guo et al., 2011).

In several *cis*-regulatory regions, active in a wide variety of developing organs, synergistic interactions between *cis*-regulatory elements have been documented. The combined effect of two or more regulatory elements can generate expression in new groups of cells, boost or down-regulate the expression of the target gene, or change the temporal patterns of transcription. A nice example of these phenomena comes from *D. melanogaster* leg development. In this organ, part of the expression pattern of the gene *Distalless* (*Dll*) is controlled by two distant enhancers named LT and M. These two enhancers drive very different expression patterns, and none of them alone recapitulates *Dll* native expression in the leg disc (Estella et al., 2008). However, when these two elements are combined in a *lacZ* reporter construct, the enhancer duo drives expression in all *Dll*-expressing cells (Estella et al., 2008). A similar behavior has been observed in the gene *sloppy-paired-1* during *Drosophila* embryonic development (Prazak et al., 2010). This gene has two enhancers, named DESE and PESE, that drive incomplete or ectopic patterns when analyzed individually with a reporter construct. Again, a composite reporter containing both elements faithfully recapitulates *sloppy-paired-*

1 native expression (Prazak et al., 2010). Finally, a relevant case is provided by the gene *snail*, whose structure was mentioned before to illustrate the function of “shadow enhancers.” As described above, this gene contains a proximal and a distal enhancer with similar activities in the early *Drosophila* embryo. Interestingly, it has been shown that these two enhancers work in a nonadditive manner; the proximal enhancer reduces the activity of the distal enhancer to fine-tune expression levels (Dunipace et al., 2011).

The Unknown Spatial Distribution of *cis*-Regulatory Information

The physical limits of *cis*-regulatory regions are difficult to demarcate. Currently, it is possible to define regions that contain regulatory elements that affect the expression of a particular gene or group of genes. However, for a developmental gene with a complex expression pattern it would be dubious to state that “the *cis*-regulatory region of gene X starts in nucleotide A and ends in nucleotide B.” Developmental genes bear substantial regulatory information, and, so far, we ignore whether the spatial distribution of regulatory elements follows a particular set of rules. In this regard, recent reports show that some transcriptional enhancers maintain their positions in *cis*-regulatory regions of distant species (Hare et al., 2008; Cande et al., 2009; Frankel, Wang, and Stern, unpublished). These data suggest the existence of constraints in the architecture of *cis*-regulatory regions, although not much more is known about the large scale organization of *cis*-regulatory regions. If we zoom in on smaller regions, we realize that the physical limits of individual *cis*-regulatory elements are difficult to define as well. In particular, transcriptional enhancers can have diverse architectures. These elements can have a compact configuration, where transcription factor binding sites are close together. Alternatively, transcription factor binding sites can be spread over larger regions, forming separate functional units. These two models have been named the “enhanceosome” and the “billboard,” respectively (Arnosti

and Kulkarni, 2005). Certainly, these models represent different loci in a continuum of enhancer configurations. However, not all enhancers may have a somehow modular architecture. In theory, different functional units, all necessary to drive coherent expression, could be separated by many kilobases. This could be the potential architecture of a “femur enhancer” acting on the *D. melanogaster* gene *Ultrabithorax* (Davis et al., 2007). In the latter study, a comprehensive survey of a large genomic region failed to isolate an enhancer of *Ultrabithorax*, suggesting that this enhancer might be structurally complex (Davis et al., 2007). In the case of the “stripes enhancer” of the *Drosophila runt* gene, a large element is necessary to drive a coherent expression pattern (Klingler et al., 1996). Specifically, part of the embryonic expression of *runt* is regulated by a ~5 Kb enhancer, which cannot be subdivided into smaller functional sub-elements (Klingler et al., 1996). It is conceivable that more of these complex enhancers exist in animal genomes. Hence, in some instances, the inability to isolate enhancers could be due to its complex nature. Unfortunately, “negative results” in the search for enhancers are difficult to interpret and will very likely remain unpublished.

A detailed study of the *cis*-regulatory region of the gene *Distalless* (*Dll*) nicely exemplifies the topic discussed in the above paragraph (Fig. 2). As mentioned in previous sections, this gene contains *cis*-regulatory elements, able to work in isolation, that are active in *D. melanogaster* leg discs. These regulatory elements are separated by 12 Kb of DNA without enhancer activity in leg discs (Estella et al., 2008). A recent study revealed that at least four different regions within these 12 Kb harbor functional binding sites for the transcription factor GAF (Fig. 2A; Agelopoulos et al., 2012). GAF (the GAGA factor, encoded by the gene *trl*) is a protein involved in modifying chromatin structure, among other things (Adkins et al., 2006). It was also shown that the whole *Dll cis*-regulatory region appears to be in a compact state in thoracic leg primordia, where the gene is active, whereas the same region is in a loose chromatin state in abdomen cells, where *Dll*

is repressed by *Hox* proteins (Fig. 2B; Agelopoulos et al., 2012). The compact conformation of *Dll* *cis*-regulatory region in thoracic leg primordia suggests that this large region acts as a single functional unit (Agelopoulos et al., 2012). Certainly, the *cis*-regulatory region of *Dll* turned out to be much more complex than previously thought. Initially, its function was explained by the activity of a couple of distant regulatory elements. However, a deeper examination of the locus, performed with a methodology that involves cell-type-specific chromatin immunoprecipitation (cgChIP; Agelopoulos et al., 2012), demonstrated that it is a large functional unit with *cis*-regulatory information dispersed over 14 Kb. The insights on *Dll* regulation also highlight the relevance of chromatin conformation in the control of gene expression. This topic is the theme of the following section.

Epigenetic Marks, Chromatin Conformation, and *cis*-Regulatory Activity

Until a few years ago, most studies of *cis*-regulation were “epigenetics free.” Isolated DNA fragments were analyzed with reporter constructs present in episomes or integrated in random genome locations. In consequence, the chromatin structure of the native locus was not considered as a variable affecting the activity of a particular regulatory element. This bias is probably due to historical and technical reasons; a decade ago, epigenetics was not as omnipresent and easy to tackle experimentally as today. It is now clear that the chromatin state of both regulatory elements and regulatory-element-flanking DNA is important for the regulation of gene expression. Hence, the activity of a regulatory element should ideally be analyzed in the proper genomic context. It could be argued, though, that isolated regulatory elements are subjected to a certain degree of epigenetic regulation. However, this potential regulation could be very different from that occurring at the native location, and, as mentioned before, it does not have the influence of flanking chromatin.

The study of epigenetics has experienced exponential growth in recent years, making the field a rapidly changing ground. The current prominence of the field has motivated the inclusion of epigenetic aspects in some experiments of gene regulation. Moreover, recent technical advances have augmented the resolution of analyses, allowing researchers to obtain deeper insights on epigenetic regulation. In the following paragraphs, I summarize some of the numerous epigenetic processes that impact *cis*-regulatory activity.

The posttranslational modifications of nucleosomal proteins constitute a vital part of gene regulation. Histones are subjected to a large number of posttranslational modifications, being methylation and acetylation the most common epigenetic marks. The action of histone acetylases (HATs), deacetylases (HDACs), methyl-transferases (HMTs), and demethylases (HDMs) generates part of the complex epigenetic landscape, also known as the histone code. Histone marks and chromatin remodeling complexes modify chromatin conformation, regulating the accessibility of transcription factors to *cis*-regulatory regions. Some epigenetic marks have been associated with specific groups of regulatory elements; in humans (Heintzman et al., 2009), zebrafish (Aday et al., 2011), and flies (Negre et al., 2011) the mono-methylation of histone H3 lysine 4 (H3K4me1) is usually found in active enhancer regions, close to areas of open chromatin. A recent study showed that the activity of the protein LSD1 (a demethylase of H3K4 and H3K9) is essential for differentiation of mouse embryonic stem cells (Whyte et al., 2012). With the progression of embryogenesis active enhancers in embryonic stem cells experience a decrease in histone acetylation. In turn, this low level of acetylated histones triggers LSD1-mediated H3K4 demethylation. This modification silences enhancers of genes that need to be shut-down for cell differentiation (Whyte et al., 2012). In addition to the action of histone marks, local chromatin conformation can be influenced by the composition of nucleosomes (Rangasamy et al., 2003; Raisner et al., 2005). It has been reported that histone var-

iants H2A.Z and H3.3 are associated with active enhancers and insulators in the human genome (Jin et al., 2009).

The activity of regulatory elements can be modulated by local chromatin modifications (exerted by nearby nucleosomes) or global chromatin changes, which affect the structure of large regions. In metazoans, GATA and Polycomb proteins interact with specific DNA-motifs in *cis*-regulatory regions and modify the conformation of chromatin. Proteins of the Polycomb group, regulate and interact with epigenetic marks, controlling gene expression programs through cell divisions (Schwartz and Pirrotta, 2008). It has been shown that Polycomb-repressive complexes mediate chromatin compaction in flies and mice (Grau et al., 2011). This large-scale chromatin change represses the expression of many developmental genes (Isono et al., 2005; Boyer et al., 2006; Lee et al., 2006; Lanzuolo et al., 2007; Sing et al., 2009). Another group of proteins, the GATA factors, regulate the conformation of chromatin, influencing gene expression (Vakoc et al., 2005). For example, at the *Kit* locus, the exchange of GATA proteins causes the formation of different chromatin loops that affect transcriptional activity (Jing et al., 2008). In immature erythroid cells, a long chromatin loop mediated by GATA-2 promotes the interaction of an enhancer with the basal promoter. Upon maturation, GATA-1 replaces GATA-2, favoring a repressive loop that down-regulates gene expression (Jing et al., 2008).

DNA methylation also has a big impact on gene expression. The phenomenon of imprinting, where an organism expresses only the maternal or paternal allele, is caused by differential DNA methylation (Feng et al., 2010). Mammalian clusters of imprinted genes contain *cis*-regulatory elements called imprinting control regions (ICRs) which have parental-specific DNA methylation (Barlow, 2011). Besides its role in imprinting, DNA methylation regulates gene expression in lineages of somatic cells during embryogenesis (Oda et al., 2006; Illingworth et al., 2008). Methylation of cytosines in CpG dinucleotides is a common epigenetic mark in

vertebrate *cis*-regulatory regions (Deaton and Bird, 2011). Currently, it is known that CpG methylation changes chromatin architecture, affecting the transcriptional potential of a gene (Blackledge and Klose, 2011).

The mechanisms described in the above paragraphs represent just a part of epigenetic regulation (for a more complete picture see Gibney and Nolan, 2010). Unquestionably, epigenetic processes are complex and dynamic. Furthermore, there seems to be a significant interplay between different epigenetic marks and epigenetic regulators; methylated CpGs, histone modifications, and Polycomb complexes cross-talk in *cis*-regulation (Brinkman et al., 2012; Cedar and Bergman, 2012; Ong and Corces, 2012). Accordingly, it has become clear that developmental genes undergo rapid chromatin changes, orchestrated by several interacting players (Hunkapiller et al., 2012).

Perspectives

I hope I have convinced the reader that *cis*-regulatory regions are more than a collection of modular *cis*-regulatory elements in a string of DNA. Nowadays, it has become evident that the genetic architecture and the epigenetic landscape of *cis*-regions have multiple interrelated pieces. Part of this complex functionality has been depicted in previous sections, but there are many more things that we would like to know. Indeed, we lack the “big picture” of *cis*-regulation; there are many separate bits that need to be put together. Here are some relevant questions that need answers: (i) Is the activity of shadow enhancers additive or interactive? Are shadow enhancers always regulated by the same transcription factors, as in the case of *Dorsal* target genes? (ii) Are there *cis*-regulatory elements with new functions yet to be discovered? Are there genetic elements and/or epigenetic marks that delimit *cis*-regulatory units? (iii) Are there architectural rules in *cis*-regulatory regions? What are the constraints that shape *cis*-regulatory evolution? (iv) What are the molecular mechanisms underlying interactions between regulatory-elements? (v) Can we measure changes in regulatory-

element activity during development? Can we track chromatin conformation dynamics within *cis*-regulatory regions? Can we integrate these data in dynamic models of *cis*-regulation?

Luckily, new techniques can help answer some of these questions. A map of interactions between distant elements can be obtained using 3C (chromatin conformation capture; Miele and Dekker, 2009) and Hi-C (Lieberman-Aiden et al., 2009). Moreover, these techniques can aid in the discovery of new regulatory elements and, thus, provide a better picture of the physical limits of *cis*-regulatory regions.

To avoid biological noise when analyzing chromatin states or interactions, it is very important to examine a homogeneous group of cells (only those cells in the embryo where the gene in question is active). An interesting approach to overcome this difficulty is to FACS-sort embryonic cells expressing the right markers (Spencer et al., 2011; Bonn et al., 2012; Zhou and Pu, 2012). This way, it is possible to obtain accurate temporal maps of chromatin changes.

Another methodological approach is the use of BACs (bacterial artificial chromosomes) containing putative *cis*-regulatory regions. BACs accommodate large DNA regions that can be modified in many ways through BAC recombineering (Warming et al., 2005). Among other modifications, it is easy to add a reporter gene to a BAC. This way, it is possible to monitor the expression driven by large chunks of DNA, and compare this expression with that of the native gene. Furthermore, it is feasible to mutate known regulatory elements or regions of unknown function in the context of a large DNA region.

The techniques mentioned above (combined with traditional methods such as the functional dissection of regulatory regions with reporter assays) are fundamental to get a comprehensive view of *cis*-regulatory activity. Surely, these methodologies coupled to wise experimental designs will generate major discoveries in the field. However, current techniques are not sufficient to obtain a dynamic view of *cis*-regulatory activity in live embryos. Thus, a major breakthrough would be the development of a robust

assay to monitor *cis*-regulatory interactions in real-time.

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REFERENCES

- Adachi Y, Hauck B, Clements J, Kawachi H, Kurusu M, Totani Y, Kang YY, Eggert T, Walldorf U, Furukubo-Tokunaga K, Callaerts P. 2003. Conserved *cis*-regulatory modules mediate complex neural expression patterns of the eyeless gene in the *Drosophila* brain. *Mech Dev* 120:1113–1126.
- Aday AW, Zhu LJ, Lakshmanan A, Wang J, Lawson ND. 2011. Identification of *cis* regulatory features in the embryonic zebrafish genome through large-scale profiling of H3K4me1 and H3K4me3 binding sites. *Dev Biol* 357:450–462.
- Adkins NL, Hagerman TA, Georgel P. 2006. GAGA protein: a multi-faceted transcription factor. *Biochem Cell Biol* 84:559–567.
- Agelopoulos M, McKay DJ, Mann RS. 2012. Developmental regulation of chromatin conformation by Hox proteins in *Drosophila*. *Cell Rep* 1:350–359.
- Akbari OS, Bousum A, Bae E, Drewell RA. 2006. Unraveling *cis*-regulatory mechanisms at the abdominal-A and abdominal-B genes in the *Drosophila* bithorax complex. *Dev Biol* 293:294–304.
- Akbari OS, Bae E, Johnsen H, Villaluz A, Wong D, Drewell RA. 2008. A novel promoter-tethering element regulates enhancer-driven gene expression at the bithorax complex in the *Drosophila* embryo. *Development* 135:123–131.
- Arnosti DN, Kulkarni MM. 2005. Transcriptional enhancers: intelligent enhanceosomes or flexible billboards? *J Cell Biochem* 94:890–898.
- Banerji J, Rusconi S, Schaffner W. 1981. Expression of a beta-globin gene is enhanced by remote SV40 DNA sequences. *Cell* 27:299–308.
- Barlow DP. 2011. Genomic imprinting: a mammalian epigenetic discovery model. *Annu Rev Genet* 45:379–403.
- Barolo S. 2012. Shadow enhancers: frequently asked questions about distributed *cis*-regulatory information and enhancer redundancy. *Bioessays* 34: 135–141.
- Bell AC, West AG, Felsenfeld G. 2001. Insulators and boundaries: versatile regulatory elements in the eukaryotic genome. *Science* 291:447–450.

- Blackledge NP, Klose R. 2011. CpG island chromatin: a platform for gene regulation. *Epigenetics* 6:147–152.
- Bonn S, Zinzen RP, Girardot C, Gustafson EH, Perez-Gonzalez A, Delhomme N, Ghavi-Helm Y, Wilczynski B, Riddell A, Furlong EE. 2012. Tissue-specific analysis of chromatin state identifies temporal signatures of enhancer activity during embryonic development. *Nat Genet* 44:148–156.
- Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, Levine SS, Wernig M, Tajonar A, Ray MK, Bell GW, Otte AP, Vidal M, Gifford DK, Young RA, Jaenisch R. 2006. Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* 441:349–353.
- Brinkman AB, Gu H, Bartels SJ, Zhang Y, Matarese F, Simmer F, Marks H, Bock C, Gnirke A, Meissner A, Stunnenberg HG. 2012. Sequential ChIP-bisulfite sequencing enables direct genome-scale investigation of chromatin and DNA methylation cross-talk. *Genome Res* 22:1128–1138.
- Calhoun VC, Stathopoulos A, Levine M. 2002. Promoter-proximal tethering elements regulate enhancer-promoter specificity in the *Drosophila* Antennapedia complex. *Proc Natl Acad Sci U S A* 99:9243–9247.
- Cande J, Goltsev Y, Levine MS. 2009. Conservation of enhancer location in divergent insects. *Proc Natl Acad Sci U S A* 106:14414–14419.
- Cedar H, Bergman Y. 2012. Programming of DNA methylation patterns. *Annu Rev Biochem* 81:97–117.
- Chao CH, Wang HD, Yuh CH. 2010. Complexity of cis-regulatory organization of *six3a* during forebrain and eye development in zebrafish. *BMC Dev Biol* 10:35.
- Corbo JC, Lawrence KA, Karlstetter M, Myers CA, Abdelaziz M, Dirkes W, Weigelt K, Seifert M, Benes V, Fritsche LG, Weber BH, Langmann T. 2010. CRX ChIP-seq reveals the cis-regulatory architecture of mouse photoreceptors. *Genome Res* 20:1512–1525.
- Crocker J, Tamori Y, Erives A. 2008. Evolution acts on enhancer organization to fine-tune gradient threshold readouts. *PLoS Biol* 6:e263.
- Davis GK, Srinivasan DG, Wittkopp PJ, Stern DL. 2007. The function and regulation of *Ultrabithorax* in the legs of *Drosophila melanogaster*. *Dev Biol* 308:621–631.
- Deaton AM, Bird A. 2011. CpG islands and the regulation of transcription. *Genes Dev* 25:1010–1022.
- Dunipace L, Ozdemir A, Stathopoulos A. 2010. Complex interactions between cis-regulatory modules in native conformation are critical for *Drosophila* snail expression. *Development* 138:4075–4084.
- Erives A, Levine M. 2004. Coordinate enhancers share common organizational features in the *Drosophila* genome. *Proc Natl Acad Sci U S A* 101:3851–3856.
- Estella C, McKay DJ, Mann RS. 2008. Molecular integration of wingless, decapentaplegic, and autoregulatory inputs into *Distalless* during *Drosophila* leg development. *Dev Cell* 14:86–96.
- Feng S, Jacobsen SE, Reik W. 2010. Epigenetic reprogramming in plant and animal development. *Science* 330:622–627.
- Franchini LF, Lopez-Leal R, Nasif S, Beati P, Gelman DM, Low MJ, de Souza FJ, Rubinstein M. 2011. Convergent evolution of two mammalian neuronal enhancers by sequential exaptation of unrelated retroposons. *Proc Natl Acad Sci U S A* 108:15270–15275.
- Frankel N, Davis GK, Vargas D, Wang S, Payre F, Stern DL. 2010. Phenotypic robustness conferred by apparently redundant transcriptional enhancers. *Nature* 466:490–493.
- Frankel N, Erezylmaz DF, McGregor AP, Wang S, Payre F, Stern DL. 2011. Morphological evolution caused by many subtle-effect substitutions in regulatory DNA. *Nature* 474:598–603.
- Frokjaer-Jensen C, Davis MW, Ailion M, Jorgensen EM. 2012. Improved *Mos1*-mediated transgenesis in *C. elegans*. *Nat Methods* 9:117–118.
- Fujioka M, Yusibova GL, Patel NH, Brown SJ, Jaynes JB. 2002. The repressor activity of *Even-skipped* is highly conserved, and is sufficient to activate engrailed and to regulate both the spacing and stability of parasegment boundaries. *Development* 129:4411–4421.
- Funahashi J, Nakamura H. 2008. Electroporation in avian embryos. *Methods Mol Biol* 461:377–382.
- Galindo MI, Fernandez-Garza D, Phillips R, Couso JP. 2011. Control of *Distal-less* expression in the *Drosophila* appendages by functional 3' enhancers. *Dev Biol* 353:396–410.
- Ghiesvand NM, Rudolph DD, Mashayekhi M, Brzezinski JA IV, Goldman D, Glaser T. 2011. Deletion of a remote enhancer near *ATOH7* disrupts retinal neurogenesis, causing NCRNA disease. *Nat Neurosci* 14:578–586.
- Gibney ER, Nolan CM. 2010. Epigenetics and gene expression. *Heredity (Edinb)* 105:4–13.
- Gonzalez F, Duboule D, Spitz F. 2007. Transgenic analysis of *Hoxd* gene regulation during digit development. *Dev Biol* 306:847–859.
- Grau DJ, Chapman BA, Garlick JD, Borowsky M, Francis NJ, Kingston RE. 2011. Compaction of chromatin by diverse Polycomb group proteins requires localized regions of high charge. *Genes Dev* 25:2210–2221.
- Guo C, Yoon HS, Franklin A, Jain S, Ebert A, Cheng HL, Hansen E, Despo O, Bossen C, Vettermann C, Bates JG, Richards N, Myers D, Patel H, Gallagher M, Schlissel MS, Murre C, Buslinger M, Giallourakis CC, Alt FW. 2011. CTCF-binding elements mediate control of V(D)J recombination. *Nature* 477:424–430.
- Hare EE, Peterson BK, Iyer VN, Meier R, Eisen MB. 2008. Sepsid even-skipped enhancers are functionally conserved in *Drosophila* despite lack of sequence conservation. *PLoS Genet* 4:e1000106.
- Heintzman ND, Hon GC, Hawkins RD, Kheradpour P, Stark A, Harp LF, Ye Z, Lee LK, Stuart RK, Ching CW, Ching KA, Antosiewicz-Bourget JE, Liu H, Zhang X, Green RD, Lobanov VV, Stewart R, Thomson JA, Crawford GE, Kellis M, Ren B. 2009. Histone modifications at human enhancers reflect global cell-type-specific gene expression. *Nature* 459:108–112.
- Hoch M, Schroder C, Seifert E, Jackle H. 1990. cis-acting control elements for Kruppel expression in the *Drosophila* embryo. *EMBO J* 9:2587–2595.
- Hong JW, Hendrix DA, Levine MS. 2008. Shadow enhancers as a source of evolutionary novelty. *Science* 321:1314.
- Hornstein E, Shomron N. 2006. Canalization of development by microRNAs. *Nat Genet* 38(Suppl):S20–S24.
- Hunkapiller J, Shen Y, Diaz A, Cagney G, McCleary D, Ramalho-Santos M, Krogan N, Ren B, Song JS, Reiter JF. 2012. Polycomb-like 3 promotes polycomb repressive complex 2 binding to CpG islands and embryonic stem cell self-renewal. *PLoS Genet* 8:e1002576.
- Illingworth R, Kerr A, Desousa D, Jorgensen H, Ellis P, Stalker J, Jackson D, Clee C, Plumb R, Rogers J, Humphray S, Cox T, Langford C, Bird A. 2008. A novel CpG island set identifies tissue-specific methylation at developmental gene loci. *PLoS Biol* 6:e22.
- Isono K, Fujimura Y, Shinga J, Yamaki M, J OW, Takihara Y, Murahashi Y, Takada Y, Mizutani-Koseki Y, Koseki H. 2005. Mammalian polyhomeotic homologues *Phc2* and *Phc1* act in synergy to mediate polycomb repression of *Hox* genes. *Mol Cell Biol* 25:6694–6706.
- Istrail S, Davidson EH. 2005. Logic functions of the genomic cis-regulatory code. *Proc Natl Acad Sci U S A* 102:4954–4959.
- Jeong Y, El-Jaick K, Roessler E, Muenke M, Epstein DJ. 2006. A functional screen for sonic hedgehog regulatory elements across a 1 Mb interval identifies long-range ventral forebrain enhancers. *Development* 133:761–772.
- Jin C, Zang C, Wei G, Cui K, Peng W, Zhao K, Felsenfeld G. 2009. H3.3/H2A.Z double variant-containing nucleosomes mark 'nucleosome-free regions' of active promoters and other regulatory regions. *Nat Genet* 41:941–945.
- Jing H, Vakoc CR, Ying L, Mandat S, Wang H, Zheng X, Blobel GA. 2008. Exchange of GATA factors mediates transitions in looped chromatin organization at a developmentally regulated gene locus. *Mol Cell* 29:232–242.
- Kalay G, Wittkopp PJ. 2010. Nomadic enhancers: tissue-specific cis-regulatory elements of yellow have divergent genomic positions among *Drosophila* species. *PLoS Genet* 6:e1001222.
- Kassis JA. 1990. Spatial and temporal control elements of the *Drosophila* engrailed gene. *Genes Dev* 4:433–443.

- Kazemian M, Blatti C, Richards A, McCutchan M, Wakabayashi-Ito N, Hammonds AS, Celniker SE, Kumar S, Wolfe SA, Brodsky MH, Sinha S. 2010. Quantitative analysis of the *Drosophila* segmentation regulatory network using pattern generating potentials. *PLoS Biol* 8.
- Kim J, Lauderdale JD. 2006. Analysis of Pax6 expression using a BAC transgene reveals the presence of a paired-less isoform of Pax6 in the eye and olfactory bulb. *Dev Biol* 292:486–505.
- Klingler M, Soong J, Butler B, Gergen JP. 1996. Disperse versus compact elements for the regulation of runt stripes in *Drosophila*. *Dev Biol* 177:73–84.
- L'Honore A, Ouimette JF, Lavertu-Jolin M, Drouin J. 2010. Pitx2 defines alternate pathways acting through MyoD during limb and somitic myogenesis. *Development* 137:3847–3856.
- Laimins L, Holmgren-Konig M, Khoury G. 1986. Transcriptional "silencer" element in rat repetitive sequences associated with the rat insulin 1 gene locus. *Proc Natl Acad Sci U S A* 83:3151–3155.
- Lanzuolo C, Roure V, Dekker J, Bantignies F, Orlando V. 2007. Polycomb response elements mediate the formation of chromosome higher-order structures in the bithorax complex. *Nat Cell Biol* 9:1167–1174.
- Lee AP, Brenner S, Venkatesh B. 2011. Mouse transgenesis identifies conserved functional enhancers and cis-regulatory motif in the vertebrate LIM homeobox gene *Lhx2* locus. *PLoS One* 6:e20088.
- Lee TI, Jenner RG, Boyer LA, Guenther MG, Levine SS, Kumar RM, Chevalier B, Johnstone SE, Cole MF, Isono K, Koseki H, Fuchikami T, Abe K, Murray HL, Zucker JP, Yuan B, Bell GW, Herbolsheimer E, Hannett NM, Sun K, Odom DT, Otte AP, Volkert TL, Bartel DP, Melton DA, Gifford DK, Jaenisch R, Young RA. 2006. Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell* 125:301–313.
- Lettice LA, Heaney SJ, Purdie LA, Li L, de Beer P, Oostra BA, Goode D, Elgar G, Hill RE, de Graaff E. 2003. A long-range *Shh* enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. *Hum Mol Genet* 12:1725–1735.
- Levine M. 2010. Transcriptional enhancers in animal development and evolution. *Curr Biol* 20:R754–R763.
- Li X, Cassidy JJ, Reinke CA, Fischboeck S, Carthew RW. 2009. A microRNA imparts robustness against environmental fluctuation during development. *Cell* 137:273–282.
- Lieberman-Aiden E, van Berkum NL, Williams L, Imakaev M, Ragoczy T, Telling A, Amit I, Lajoie BR, Sabo PJ, Dorschner MO, Sandstrom R, Bernstein B, Bender MA, Groudine M, Gnirke A, Stamatoyannopoulos J, Mirny LA, Lander ES, Dekker J. 2009. Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science* 326:289–293.
- Lin Q, Lin L, Zhou J. 2010. Chromatin insulator and the promoter targeting sequence modulate the timing of long-range enhancer-promoter interactions in the *Drosophila* embryo. *Dev Biol* 339:329–337.
- Ludwig MZ, Kreitman M. 1995. Evolutionary dynamics of the enhancer region of even-skipped in *Drosophila*. *Mol Biol Evol* 12:1002–1011.
- Ludwig MZ, Manu, Kittler R, White KP, Kreitman M. 2011. Consequences of eukaryotic enhancer architecture for gene expression dynamics, development, and fitness. *PLoS Genet* 7:e1002364.
- Maeda RK, Karch F. 2009. The bithorax complex of *Drosophila* an exceptional Hox cluster. *Curr Top Dev Biol* 88:1–33.
- McBride DJ, Buckle A, van Heyningen V, Kleinjan DA. 2011. DNaseI hypersensitivity and ultraconservation reveal novel, interdependent long-range enhancers at the complex Pax6 cis-regulatory region. *PLoS One* 6:e28616.
- McGaughey DM, Vinton RM, Huynh J, Al-Saif A, Beer MA, McCallion AS. 2008. Metrics of sequence constraint overlook regulatory sequences in an exhaustive analysis at *phox2b*. *Genome Res* 18:252–260.
- Miele A, Dekker J. 2009. Mapping cis- and trans- chromatin interaction networks using chromosome conformation capture (3C). *Methods Mol Biol* 464:105–121.
- Montavon T, Soshnikova N, Mascrez B, Joye E, Thevenet L, Splinter E, de Laat W, Spitz F, Duboule D. 2011. A regulatory archipelago controls Hox genes transcription in digits. *Cell* 147:1132–1145.
- Naranjo S, Voesenek K, de la Calle-Mustienes E, Robert-Moreno A, Kokotas H, Grigoriadou M, Economides J, Van Camp G, Hilgert N, Moreno F, Alsina B, Petersen MB, Kremer H, Gomez-Skarmeta JL. 2010. Multiple enhancers located in a 1-Mb region upstream of *POU3F4* promote expression during inner ear development and may be required for hearing. *Hum Genet* 128:411–419.
- Negre N, Brown CD, Ma L, Bristow CA, Miller SW, Wagner U, Kheradpour P, Eaton ML, Loriaux P, Sealfon R, Li Z, Ishii H, Spokony RF, Chen J, Hwang L, Cheng C, Auburn RP, Davis MB, Domnanus M, Shah PK, Morrison CA, Zieba J, Suchy S, Senderowicz L, Victorsen A, Bild NA, Grundstad AJ, Hanley D, MacAlpine DM, Mannervik M, Venken K, Bellen H, White R, Gerstein M, Russell S, Grossman RL, Ren B, Posakony JW, Kellis M, White KP. 2011. A cis-regulatory map of the *Drosophila* genome. *Nature* 471:527–531.
- Nelson CE, Hersh BM, Carroll SB. 2004. The regulatory content of intergenic DNA shapes genome architecture. *Genome Biol* 5:R25.
- Oda M, Yamagiwa A, Yamamoto S, Nakayama T, Tsumura A, Sasaki H, Nakao K, Li E, Okano M. 2006. DNA methylation regulates long-range gene silencing of an X-linked homeobox gene cluster in a lineage-specific manner. *Genes Dev* 20:3382–3394.
- Ogbourne S, Antalis TM. 1998. Transcriptional control and the role of silencers in transcriptional regulation in eukaryotes. *Biochem J* 331(Pt 1):1–14.
- Ong CT, Corces VG. 2012. Enhancers: emerging roles in cell fate specification. *EMBO Rep* 13:423–430.
- Panne D, Maniatis T, Harrison SC. 2007. An atomic model of the interferon-beta enhanceosome. *Cell* 129:1111–1123.
- Pauls S, Smith SF, Elgar G. 2012. Lens development depends on a pair of highly conserved Sox21 regulatory elements. *Dev Biol* 365:310–318.
- Payre F, Vincent A, Carreno S. 1999. *ovo/svb* integrates Wingless and DER pathways to control epidermis differentiation. *Nature* 400:271–275.
- Perry MW, Boettiger AN, Bothma JP, Levine M. 2010. Shadow enhancers foster robustness of *Drosophila* gastrulation. *Curr Biol* 20:1562–1567.
- Perry MW, Boettiger AN, Levine M. 2011. Multiple enhancers ensure precision of gap gene-expression patterns in the *Drosophila* embryo. *Proc Natl Acad Sci U S A* 108:13570–13575.
- Prazak L, Fujioka M, Gergen JP. 2010. Non-additive interactions involving two distinct elements mediate sloppy-paired regulation by pair-rule transcription factors. *Dev Biol* 344:1048–1059.
- Raisner RM, Hartley PD, Meneghini MD, Bao MZ, Liu CL, Schreiber SL, Rando OJ, Madhani HD. 2005. Histone variant H2A.Z marks the 5' ends of both active and inactive genes in euchromatin. *Cell* 123:233–248.
- Rangasamy D, Berven L, Ridgway P, Tremethick DJ. 2003. Pericentric heterochromatin becomes enriched with H2A.Z during early mammalian development. *EMBO J* 22:1599–1607.
- Schwartz YB, Pirrotta V. 2008. Polycomb complexes and epigenetic states. *Curr Opin Cell Biol* 20:266–273.
- Sing A, Pannell D, Karaiskakis A, Sturgeon K, Djabali M, Ellis J, Lipshitz HD, Cordes SP. 2009. A vertebrate Polycomb response element governs segmentation of the posterior hindbrain. *Cell* 138:885–897.
- Spencer WC, Zeller G, Watson JD, Henz SR, Watkins KL, McWhirter RD, Petersen S, Sreedharan VT, Widmer C, Jo J, Reinke V, Petrella L, Stromer S, Von Stetina SE, Katz M, Shaham S, Ratsch G, Miller DM, 3rd. 2011. A spatial and temporal map of *C. elegans* gene expression. *Genome Res* 21:325–341.
- Summerbell D, Ashby PR, Coutelle O, Cox D, Yee S, Rigby PW. 2000. The expression of Myf5 in the developing mouse embryo is controlled by discrete and dispersed enhancers specific for particular populations of skeletal muscle precursors. *Development* 127:3745–3757.

- Swami M. 2010. Transcription: shadow enhancers confer robustness. *Nat Rev Genet* 11:454.
- Swanson CI, Evans NC, Barolo S. 2010. Structural rules and complex regulatory circuitry constrain expression of a Notch- and EGFR-regulated eye enhancer. *Dev Cell* 18:359–370.
- Tasic B, Hippenmeyer S, Wang C, Gamboa M, Zong H, Chen-Tsai Y, Luo L. 2011. Site-specific integrase-mediated transgenesis in mice via pronuclear injection. *Proc Natl Acad Sci U S A* 108:7902–7907.
- Tolias PP, Kafatos FC. 1990. Functional dissection of an early *Drosophila* chorion gene promoter: expression throughout the follicular epithelium is under spatially composite regulation. *EMBO J* 9:1457–1464.
- Tsytzykova AV, Rajsbaum R, Falvo JV, Ligeiro F, Neely SR, Goldfeld AE. 2007. Activation-dependent intrachromosomal interactions formed by the TNF gene promoter and two distal enhancers. *Proc Natl Acad Sci U S A* 104:16850–16855.
- Udvardy A, Maine E, Schedl P. 1985. The 87A7 chromomere. Identification of novel chromatin structures flanking the heat shock locus that may define the boundaries of higher order domains. *J Mol Biol* 185:341–358.
- Vakoc CR, Letting DL, Gheldof N, Sawado T, Bender MA, Groudine M, Weiss MJ, Dekker J, Blobel GA. 2005. Proximity among distant regulatory elements at the beta-globin locus requires GATA-1 and FOG-1. *Mol Cell* 17:453–462.
- Venken KJ, Bellen HJ. 2007. Transgenesis upgrades for *Drosophila melanogaster*. *Development* 134:3571–3584.
- Venken KJ, Carlson JW, Schulze KL, Pan H, He Y, Spokony R, Wan KH, Koriabine M, de Jong PJ, White KP, Bellen HJ, Hoskins RA. 2009. Versatile P[acman] BAC libraries for transgenesis studies in *Drosophila melanogaster*. *Nat Methods* 6:431–434.
- Vierra DA, Irvine SQ. 2012. Optimized conditions for transgenesis of the ascidian *Ciona* using square wave electroporation. *Dev Genes Evol* 222:55–61.
- Visser M, Kayser M, Palstra RJ. 2012. HERC2 rs12913832 modulates human pigmentation by attenuating chromatin-loop formation between a long-range enhancer and the OCA2 promoter. *Genome Res* 22:446–455.
- Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG. 2005. Simple and highly efficient BAC recombineering using galK selection. *Nucleic Acids Res* 33:e36.
- Watts JA, Zhang C, Klein-Szanto AJ, Kormish JD, Fu J, Zhang MQ, Zaret KS. 2011. 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.
- Werner T, Hammer A, Wahlbuhl M, Bosl MR, Wegner M. 2007. Multiple conserved regulatory elements with overlapping functions determine Sox10 expression in mouse embryogenesis. *Nucleic Acids Res* 35:6526–6538.
- Whyte WA, Bilodeau S, Orlando DA, Hoke HA, Frampton GM, Foster CT, Cowley SM, Young RA. 2012. Enhancer decommissioning by LSD1 during embryonic stem cell differentiation. *Nature* 482:221–225.
- Yoshida C, Tokumasu F, Hohmura KI, Bungert J, Hayashi N, Nagasawa T, Engel JD, Yamamoto M, Takeyasu K, Igarashi K. 1999. Long range interaction of cis-DNA elements mediated by architectural transcription factor Bach1. *Genes Cells* 4:643–655.
- Yuh CH, Bolouri H, Davidson EH. 2001. Cis-regulatory logic in the endo16 gene: switching from a specification to a differentiation mode of control. *Development* 128:617–629.
- Zeitlinger J, Zinzen RP, Stark A, Kellis M, Zhang H, Young RA, Levine M. 2007. 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.
- Zhou B, Pu WT. 2012. Isolation and characterization of embryonic and adult epicardium and epicardium-derived cells. *Methods Mol Biol* 843:155–168.