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Review

Actors with Multiple Roles: Pleiotropic Enhancers and the Paradigm of Enhancer Modularity

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The current paradigm in the field of gene regulation postulates that regulatory information for generating gene expression is organized into modules (enhancers), each containing the information for driving gene expression in a single spatiotemporal context. This modular organization is thought to facilitate the evolution of gene expression by minimizing pleiotropic effects. Here we review recent studies that provide evidence of quite the opposite: (i) enhancers can function in multiple developmental contexts, implying that enhancers can be pleiotropic, (ii) transcription factor binding sites within pleiotropic enhancers are reused in different contexts, and (iii) pleiotropy impacts the structure and evolution of enhancers. Altogether, this evidence suggests that enhancer pleiotropy is pervasive in animal genomes, challenging the commonly held view of modularity.

Highlights

Recent genome-wide and locus-specific functional studies uncovered that enhancer pleiotropy is pervasive in gene regulatory regions of animal genomes.

Transcription factor binding sites within pleiotropic enhancers can be reused in different contexts, implying that binding sites can be pleiotropic.

Pleiotropic enhancers are more constrained and have distinctive structural features when compared with tissue-specific enhancers.

The extent of pleiotropy in enhancer function weakens the commonly held view of enhancers as being strictly tissue-specific regulatory elements.

Comprehensive screens of enhancer activity and transcription factor binding site function over multiple contexts and across organisms will shed light on the mechanisms underlying enhancer pleiotropy.

Transcriptional Enhancers and the Paradigm of Modularity

Developmental genes often possess large ***cis*-regulatory regions** (see Glossary) that contain multiple transcriptional **enhancers**. Enhancers are DNA regulatory elements that determine the time, place, and rate of transcription [1,2]. The current paradigm in the field of gene regulation postulates that *cis*-regulatory regions have a modular structure, where each enhancer harbors regulatory information for driving gene expression in a single window of time and space [3–8]. The epitome of this paradigm is the *cis*-regulatory region of the *even-skipped* (*eve*) gene in *Drosophila melanogaster*, where five enhancers generate the seven-striped expression pattern of *eve* in early embryos and each enhancer (module) produces one or two stripes of the whole pattern [9–11].

The modular organization of regulatory information is thought to facilitate the evolution of gene expression by releasing enhancers from the burden of **pleiotropy** [6,7]. However, if enhancers and their **transcription factor binding sites (TFBSs)** were to be active in more than one spatiotemporal context, their sequence would be more constrained, because a mutation affecting expression in one context may affect gene expression in a second context. Although the idea of **modularity** seems to be deeply engrained in the field, recent studies have interrogated the activity of enhancers in multiple developmental contexts, exposing their pleiotropic nature.

Most of the genes that regulate development play multiple roles in different organs and/or at different time points in the ontogeny, a phenomenon that has been named 'gene pleiotropy' [6,12]. The activation of gene expression in different instances can be driven by distinct enhancers or, otherwise, by the same regulatory element (Figure 1A,B, Key Figure). The latter alternative is a deeper level of pleiotropy, which has been termed '**enhancer pleiotropy**' (Box 1) [13]. Enhancer pleiotropy occurs when a small DNA region (a pleiotropic enhancer) encodes the genetic information for driving gene expression in multiple contexts. Necessarily, the chromatin structure of a pleiotropic enhancer has to be similar in the different contexts in which the enhancer is active.

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Key Figure

Enhancer Pleiotropy and Site Pleiotropy

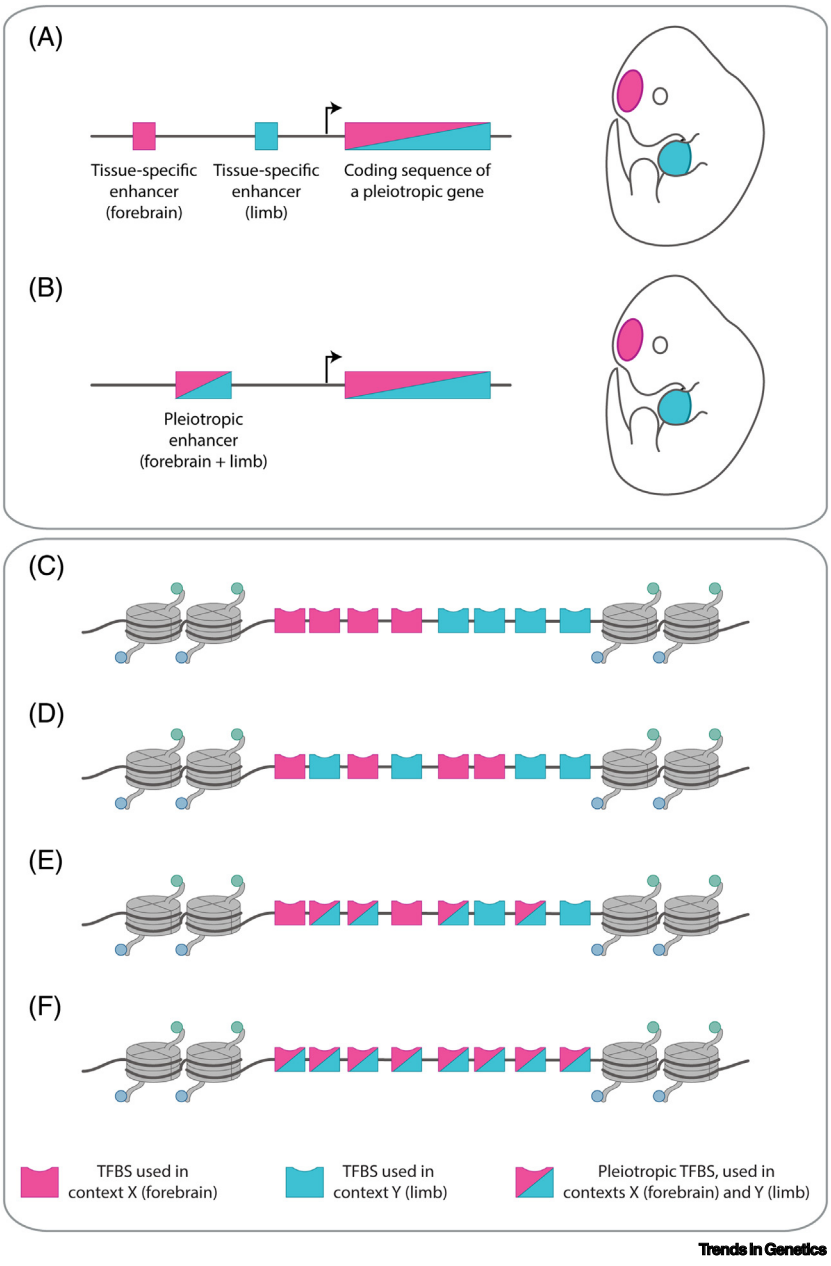

Figure 1. A pleiotropic gene can be expressed in two different organs (forebrain and limb) through two tissue-specific enhancers (A), or through a single pleiotropic enhancer (B). In theory, this pleiotropic enhancer may have different structures: independent sets of transcription factor binding sites (TFBSs) for driving expression in the two organs (C,D), which can be spatially separated (C) or intermingled (D), or TFBS that are used for driving expression in the two organs (E,F). In a scenario where a novel expression pattern evolves within a pre-existing enhancer, only some of the ancestral TFBSs may be co-opted for the new function and, thus, not all the TFBSs will be shared between the two contexts (E). When a complete gene regulatory network (GRN) is co-opted, the enhancers of its genes may use all the same TFBSs in two different contexts (F).

Box 1. The Implications of Enhancer Pleiotropy

A pleiotropic enhancer is a small DNA region that is accessible (devoid of nucleosomes) in multiple contexts and, therefore, serves as a scaffold for driving gene expression in multiple organs and/or at different developmental time points. The existence of enhancer pleiotropy implies that some regulatory information is grouped in the genome. Furthermore, if pleiotropy were to be significant in *cis*-regulatory regions, this would mean that a significant amount of regulatory information lies within a small part of the genome. It is important to emphasize that a pleiotropic enhancer may not use the same TFBSs for driving expression in multiple contexts (i.e., enhancer pleiotropy does not imply site pleiotropy). However, even if there are two different sets of TFBSs (for driving two expression patterns) that are either intermingled within the same DNA segment or are encoded in contiguous but separate DNA segments, the whole region should still be considered a pleiotropic enhancer. This is because the whole region is a platform that acquires a similar chromatin structure (i.e., a similar profile of open chromatin and histone marks) in the multiple contexts in which it is functional [18]. Furthermore, a unique pioneer transcription factor may instruct the 'opening' of the pleiotropic enhancer in different contexts [64].

In theory, regulatory information within pleiotropic enhancers can be encoded in different ways. Two expression patterns can be generated through independent sets of TFBSs that are either spatially separated (Figure 1C) or intermingled (Figure 1D). Alternatively, the same TFBSs (or some of them) can be utilized to generate two expression patterns (Figure 1E,F). The latter phenomenon has been named '**site pleiotropy**' [14].

In the past decade the amount of genomic information has grown exponentially. Despite this fact, we still have a superficial understanding of the function of regulatory information in genomes. In this review we describe and summarize the evidence supporting the idea that pleiotropy is substantial within regulatory regions, and that pleiotropy constrains the evolution of enhancers. In addition, we discuss whether pleiotropic and tissue-specific enhancers might have a distinct structural constitution. Given the extent of pleiotropy, we suggest that the current paradigm of pure modularity needs to be revised, as previously proposed [13].

Enhancer Pleiotropy, a Common Feature of *Cis*-Regulatory Regions

The extent of pleiotropy in enhancer function could only be envisaged in recent years with the proliferation of **genome-wide analyses**. These studies have identified thousands of putative enhancers that are active in different developmental contexts in both mammals and insects [15–22]. Genome-wide **open chromatin** profiling of developing appendages of *D. melanogaster* revealed that the same set of enhancers is active in leg, wing, and haltere primordia [19]. The dynamic changes in chromatin accessibility on each developing primordium are also orchestrated in a similar way, as closing and opening of the regulatory elements on the different structures changes coordinately. However, there are a few differentially accessible elements between the three primordia that correspond to enhancers of master developmental regulators that specify the fate of each individual appendage. These selector genes modulate the activity of shared enhancers, rather than organ-specific enhancers, to create appendage-specific gene expression, thus giving rise to completely different morphological structures [19]. How appendage-specific gene expression is achieved through the same set of enhancers remains largely unknown.

H3K27ac is a **chromatin mark** that is often used to predict the presence of active enhancers [23]. Although this mark is not, by itself, the ultimate proof of the existence of a unique enhancer in a genomic region [24], it is a valid tool for enhancer prediction. A recent study analyzed the genomic location of H3K27ac in order to investigate whether similarities in expression profiles between embryonic limbs and genital tubercle of mice are driven by the same set of putative enhancers [16]. Remarkably, it was shown that a significant proportion of predicted enhancers is shared between the structures: 45.9% of forelimb enhancers overlapped genital tubercle enhancers, and 46.3% of hindlimb enhancers overlapped genital tubercle enhancers. Furthermore, a *de novo* motif search on genital tubercle enhancer elements predicted a significant enrichment of motifs for transcription factors associated with limb development [16]. In addition, a clustering

Glossary

Chromatin marks: post-translational modifications of histones that are correlated with enhancer function (e.g., H3K27ac in active enhancers).
***Cis*-regulatory regions:** noncoding DNA regions that flank coding sequences and carry regulatory information for gene expression.
Enhancer: *cis*-regulatory element that enhances the transcription of a gene through the binding of transcription factors and, in turn, the interaction with the basal transcription machinery.
Enhancer pleiotropy: genetic phenomenon in which a small DNA region encodes the genetic information and acquires the chromatin structure needed to generate gene expression in more than one instance.
eRNA CAGE-seq: technique employed to predict enhancers that detect bidirectional capped RNAs transcribed from enhancers (eRNAs) by using cap analysis of gene expression coupled with high-throughput sequencing (CAGE-seq).
Gene regulatory network (GRN): set of genes that interact with each other by means of transcription factors and enhancers.
Genome-wide analyses: studies that investigate molecular features throughout the whole genome by using mostly high-throughput sequencing techniques (e.g., ChIP-seq for analyzing transcription factor binding and histone modifications, ATAC-seq and DNase-seq for analyzing chromatin accessibility, and CAGE-seq for analyzing RNA expression).
Modularity: this term refers to a possible structure of *cis*-regulatory regions, which can bear multiple modules (enhancers), each containing the information for driving gene expression in a single spatiotemporal context.
Open chromatin: DNA region that is devoid of nucleosomes and, thus, accessible to transcription factors.
Pioneer transcription factors: subset of transcription factors that are capable of binding to their target sequences on nucleosomal DNA and overcoming the repressed chromatin state of an enhancer.
Pleiotropy: biological phenomenon in which a single genetic element is active in multiple contexts.
Site pleiotropy: genetic phenomenon in which a transcription factor binding site is needed for enhancer function in more than one instance.

analysis of H3K27ac signal in predicted enhancers of the hindlimb, forelimb, genital tubercle, eye, and flank, grouped a set of 1585 enhancers as a limb–genital cluster, instead of giving just organ-specific enhancer clusters. This category represents a significant proportion of all predicted enhancers in limb and genitalia. Hence, these results suggest that there is pervasive reuse of enhancers in different developmental contexts [16].

Considering that fly appendages share a common ground plan [25,26] and that development of the limb and the genitalia in mammals has similarities [27,28], it is possible to hypothesize that the reuse of regulatory elements in various structures results from the co-option of components of **gene regulatory networks (GRNs)** during organ evolution [13]. Thus, one could think that enhancer reuse is only caused by the sharing of developmental pathways between related organs. Nevertheless, the same enhancers have also been found to be active in seemingly unrelated organs [20,29]. A genome-wide study of H3K27ac enrichment in mouse forebrain, heart, and liver in multiple embryonic and postnatal time points predicted almost 90 000 active enhancers [20]. Subsequently, a calculation of the number of enhancers that were active in more than one organ revealed that most of the putative enhancers in these three organs are pleiotropic: 52% of all enhancers are active in more than one organ, of which 31% are active in two organs, and 21% are active in three organs. Similarly, a study that compared the binding of the cofactor p300 (another signature of active enhancers) in forebrain, midbrain, and limbs of E11.5 mouse embryos, identified 2108 enhancers that function in more than one tissue and 6249 tissue-specific enhancers [29]. Therefore, enhancer reuse seems to be pervasive in mammalian genomes.

Enhancer reuse seems to be common not only under physiological conditions but also during tissue regeneration after damage. The analysis of chromatin accessibility in injured wing imaginal discs of *Drosophila*, uncovered a group of putative enhancers that are activated *de novo* after injury [22]. Remarkably, 58% of the elements that are activated after damage, are also active in other tissues and/or developmental times under normal developmental conditions [22]. These results suggest that the majority of enhancers that are activated in imaginal tissue after damage are pleiotropic.

Genome-wide studies have predicted the existence of thousands of enhancers with pleiotropic roles in animal genomes. However, expression data from reporter constructs in transgenic organisms were needed to confirm this preliminary evidence. An initial confirmation of the reuse of enhancers during development came from a heroic screen of more than 6000 fragments of genomic DNA from *D. melanogaster*, that were tested for their ability to drive Gal4 expression in embryos, imaginal discs, and the adult brain [30–33]. This screen and similar screens that followed [34] have shown that a significant proportion of these genomic fragments drive expression in more than one tissue or developmental stage. However, since the genomic fragments used in these experiments were of 2–3 kb, the resolution was not sufficient to determine whether the expression was driven by a single enhancer or two (or more) enhancers located in tandem within the same genomic fragment [31].

Recently, a number of detailed studies that focused on unique *cis*-regulatory regions have confirmed the prediction from genome-wide studies [14,18,21,35,36]. For example, the dissection of the regulatory landscape of mouse *Hox* loci revealed that at least three enhancers have roles in both limb and genitalia development [18]. Moreover, the physical interaction profile of the locus, as determined by chromatin conformation capture experiments, suggests that additional regulatory elements have pleiotropic roles as well [18]. In addition, another study of *Hox* loci in mice revealed that one enhancer is active in both mammary bud and limb bud, thus confirming the ubiquity of pleiotropic enhancers in *Hox* genes [21].

Transcription factor binding site (TFBS): short DNA motif that is recognized by a transcription factor in a sequence-specific manner.

A recent study demonstrated that enhancers of the *shavenbaby* (*svb*) gene function at multiple instances throughout *D. melanogaster* development. Svb is a transcription factor that participates in many developmental processes that involve actin dynamics, including the development of larval and adult trichomes [14,37–39], leg joints [40], and antennal arista [14]. *svb* is expressed throughout fly development in these tissues and in many other tissues of ectodermal origin, in which the function of *svb* is still unknown [14]. The *cis*-regulatory region of *svb* contains seven enhancers that were originally identified for their embryonic functions [41–43]. A functional characterization of enhancer activity revealed that each one of these enhancers is capable of driving *svb* expression in multiple developmental contexts. Thus, on top of their function in embryonic epidermis, the *svb* enhancers drive expression in the epidermis, digestive system, and brain of the larva, and also in the pupal epidermis at varying levels of redundancy [14].

Another study demonstrated that the appearance of a morphological novelty in the male genitalia of *D. melanogaster* was caused by the co-option of an entire GRN, which was ancestrally involved in the formation of respiratory structures of the larva. The study showed that at least seven enhancers of this network that are used in the formation of larval respiratory structures, are redeployed during the development of male genitalia [35]. In the same vein, it was shown that an enhancer with an ancestral function in the ventral ganglion and retina of *Drosophila* species, was co-opted for optic lobe expression in the lineage of *Drosophila santomea* [36].

Even the paradigmatic example of a gene that is supposedly regulated by tissue-specific enhancers provides evidence of enhancer pleiotropy. Besides its activity in the early embryo of *D. melanogaster,* the gene *eve* is also expressed at later stages of embryogenesis in the developing nervous system and the posterior end of the embryo. Remarkably, a pleiotropic enhancer of 800 base pairs generates expression in both ganglion mother cells and neurons and the anal plate ring [9].

Altogether, the previously mentioned studies suggest that enhancer pleiotropy is a common feature of *cis*-regulatory regions. These findings raise new questions, such as how pleiotropic enhancers encode their multiple expression patterns, and what are the consequences of pleiotropy for the evolution of noncoding DNA?

The Structure of Pleiotropic Enhancers

In theory, pleiotropic enhancers can encode their multiple expression patterns in diverse manners (Figure 1). TFBSs may be reused to drive expression in various contexts (i.e., there is site pleiotropy) or, alternatively, a different set of TFBSs (within the same enhancer sequence) might drive each expression pattern (i.e., there is no site pleiotropy). So far, most of the functional evidence supports the first mechanism, but this might reflect ascertainment bias, since only a handful of pleiotropic enhancers have been functionally dissected to identify TFBSs in more than one developmental context.

It has been shown that the *E6* enhancer of *svb* contains multiple binding sites for the transcriptional activators Arrowhead and Pannier [44]. Mutating the Arrowhead and Pannier sites, either separately or in combination, dramatically reduced enhancer function in both embryonic and pupal epidermis [14]. Therefore, in this enhancer, the same TFBSs are reused during development, which means that these TFBSs are pleiotropic. However, another enhancer of *svb,* named *Z*, appears to have a contrasting architecture: embryonic and pupal expression patterns are encoded by contiguous but separate DNA regions, suggesting that the TFBSs driving the two expression patterns are independent [14].

Site pleiotropy was also detected in a pleiotropic enhancer of the *Bmp6* gene in sticklebacks. This 190 base pair enhancer is required for driving *Bmp6* expression in both developing fins and tooth

epithelia, and mutation of a conserved Smad6 binding site within this enhancer completely eliminated its function in both contexts [45]. Interestingly, enhancers with shared functions in fin/limb and teeth have also been described for the zebrafish *Dlx2* [46] and mouse *Bmp4* [47] genes. Thus, it is conceivable that the sharing of enhancers between teeth and limb is due to co-option of an entire GRN, as is known to be the case for limb and genitalia. Indeed, most of the pleiotropic enhancers that have been functionally characterized to date are part of co-opted GRNs. As expected, these enhancers play their new roles by using ancestral TFBSs [35,36,48]. In two of the enhancers that were co-opted for the development of the posterior lobe of *D. melanogaster* male genitalia [35] the same TFBSs activate expression in two contexts. For example, in an enhancer of the *Pox neuro* gene, disruption of highly conserved STAT and Abd-B sites reduced enhancer function in both the male genitalia and larval respiratory system [35]. Similarly, it was recently shown that binding sites for the transcriptional activator Grainy head in a wound response element upstream of the *Dopa decarboxylase* (*Ddc*) gene are redeployed for driving *Ddc* expression in the pigmented abdominal tergites of *D. melanogaster* males [48].

Although being reused, pleiotropic sites may not necessarily play the same role in different instances. Pleiotropic TFBSs may be bound by different transcription factors or mediate a different transcriptional output in distinct developmental contexts. Indeed, a recent study showed that a single nucleotide substitution in an enhancer of the *scute* gene of *Drosophila santomea* alters a binding site for the Hox protein Abd-B in the developing genitalia (leading to bristle loss), while the same mutation affects the binding of an unknown transcription factor, which is not Abd-B, in the developing leg (contributing to bristle gain) [49]. Another study demonstrated that a highly conserved region in a thoracic enhancer of the *Distalless* (*Dll*) gene, named the *Dll* conserved regulatory element (DCRE), has a contrasting function in thoracic leg primordia and abdomen of *D. melanogaster* embryos. While DCRE represses *Dll* expression in the abdomen, it enhances expression in thoracic leg primordia. It has been shown that the DCRE element contains three neighboring Hox/cofactor binding sites [50,51]. In the abdomen, the Hox proteins Ubx and Abd-A bind to these sites and repress *Dll* expression. In contrast, in thoracic leg primordia the Antp protein binds these Hox TFBSs and enhances the expression of *Dll* [51]. Likewise, in enhancers of the *forkhead* and *rhomboid* genes, two distinct transcription factors were found to bind to the same Hox TFBS in different regions of the *Drosophila* embryo [52,53]. Thus, the use of one binding site by distinct Hox factors along the anteroposterior axis may be common in animal development.

Constraints on the Evolution of Pleiotropic Enhancers

The function of most proteins is inherently pleiotropic, since their activity is needed in diverse contexts. Thus, the multiple roles of proteins impose a constraint on the evolution of their coding sequences [7,54]. Likewise, the existence of pleiotropy in noncoding DNA may constrain the evolution of enhancers and promoters as well. In fact, it has been shown that promoters of ubiquitously expressed genes in mammalian genomes are highly conserved [55], and that promoters used at multiple stages of development in *Heliconius erato* are more conserved than their stage-specific counterparts [17].

There is also substantial evidence that supports the notion that pleiotropic enhancers are more constrained than stage-specific enhancers. A recent study used **eRNA CAGE-seq** to generate an atlas of active enhancers across human tissues and primary cell lines [56]. A hierarchical clustering of the samples uncovered a set of ubiquitous enhancers that are actively transcribed, and thus are presumably active in all tissues and cell types. These are, by definition, pleiotropic enhancers. This study also uncovered sets of enhancers that are active in more than one tissue or cell type (also pleiotropic, but to a lesser extent) and enhancers that are active in just one context. Remarkably, ubiquitous enhancers are twice as conserved as context-specific enhancers

[56]. Likewise, another study used a probabilistic model to analyze the molecular evolution of enhancers and its relation to pleiotropy and showed that pleiotropy is positively correlated with the amount of purifying selection in a large dataset of human enhancers [57]. These findings support the hypothesis that pleiotropy constrains the evolution of enhancer sequence.

A clear example of the relationship between pleiotropy and sequence conservation is that of amniote limb enhancers [16]. The analysis of a validated set of mouse limb enhancers across distant amniotes unveiled a surprising fact: the sequence of these enhancers is conserved in several species of snakes [16]. Paradoxically, under the prevailing paradigm of modularity, it is expected that elements driving gene expression in limbs would decay in limbless species, due to the relaxation of selective constraints. The fact that snake enhancer sequences can be easily aligned with those of other amniote species, suggests that snake enhancers are still constrained, likely due to their utilization during genitalia development [16].

Enhancer pleiotropy is also connected to functional conservation. A recent investigation found a correlation between conserved enhancer activity across mammalian species and pleiotropy (as measured by activity in multiple contexts and number of target genes). Enhancers that are active in many species are predominantly pleiotropic, whereas enhancers that are species-specific tend to be more context-specific [15]. In the same way, conservation of transcription factor binding in mouse and human orthologous enhancers predicts the degree of pleiotropy of regulatory elements [58]. When human and mouse cell lines and tissues are compared, it can be observed that enhancers having the same ChIP-seq peaks in both species (meaning that binding sites for the same transcription factors are conserved) are mostly elements that are presumably active across multiple contexts [58]. Also, it has been shown that pleiotropic enhancers are less likely to be deleted during evolution. Thousands of conserved noncoding DNA elements (CNEs), which are likely to be enhancers, were lost through deletions in different mammalian lineages [29]. Interestingly, lost CNEs shared features mostly with tissue-specific enhancers. Specifically, both tissue-specific enhancers and lost CNEs were shorter, had a lower level of sequence constraint, and were evolutionarily younger than non-lost CNEs and pleiotropic enhancers [29]. These results suggest that deletions of pleiotropic enhancers are under stronger purifying selection than deletions of tissue-specific enhancers. In the same vein, the analysis of a large dataset of predicted enhancers in the human genome has uncovered that pleiotropic enhancers have less sequences derived from transposable elements than tissue-specific enhancers, and that pleiotropic enhancers are depleted of evolutionarily young transposable elements [59].

Are Pleiotropic Enhancers Intrinsically Different from Tissue-Specific Enhancers?

In addition to the conservation of sequence and function, other features may distinguish pleiotropic enhancers. Considering that pleiotropic enhancers are active in many contexts, the concomitant increase in their regulatory information may define a distinct architecture. Indeed, it has been shown that enhancers that drive various expression patterns, and therefore have complex regulatory tasks, are significantly larger than tissue-specific enhancers, in both mammals and *D. melanogaster* [15,29,60]. This characteristic may reflect the presence of more TFBSs. In fact, mammalian enhancers with a conserved function across species, which are mostly pleiotropic, have greater density and diversity of TFBSs than species-specific enhancers [15]. Finally, it has been found that pleiotropic enhancers interact with more promoters than tissue-specific enhancers [15]. This means that there are many pleiotropic enhancers that modulate the expression of multiple genes [18,61]. Thus, there are two nonexclusive versions of enhancer pleiotropy: a single enhancer that drives multiple expression patterns of the same gene, and a single enhancer that drives multiple expression patterns of different genes.

The architectural complexity of pleiotropic enhancers may further facilitate the acquisition of new functions in additional contexts, thus increasing the degree of pleiotropy. If there is a highly diverse set of TFBSs in a pleiotropic enhancer, the element could be prone to co-option, either by changes in *cis* or in *trans* [62]. The gain of extra TFBSs in *cis* would result in the binding of novel transcription factors that can interact with pre-existing transcription factors [63], generating a novel expression pattern. Besides, the gain of transcription factor expression (a *trans* change) would result in the activation of an enhancer in a novel context. Certainly, more studies that

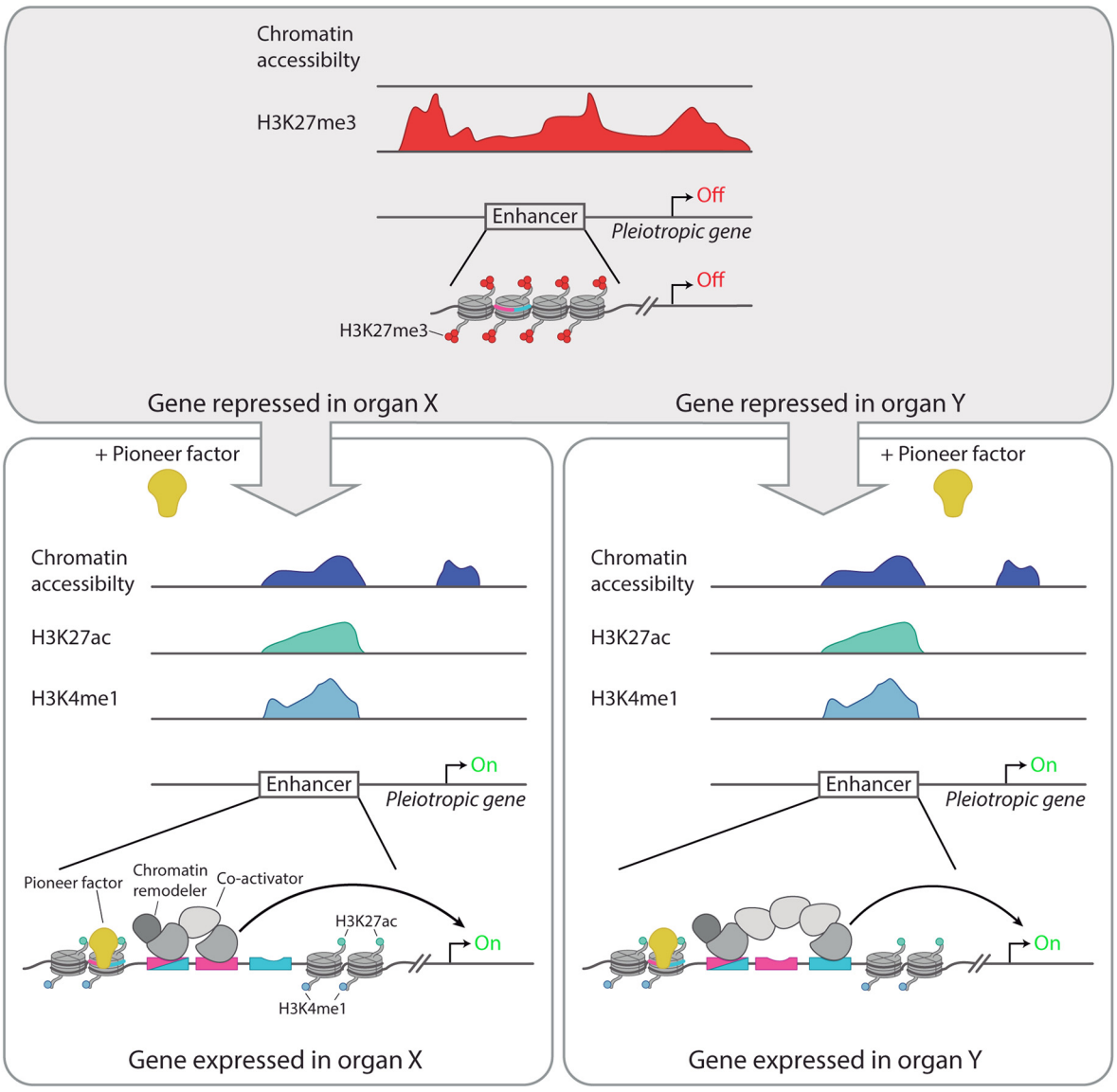

Figure 2. Mechanisms of Enhancer Pleiotropy. The enhancer of a gene is in an inactive state in two different organs, as evidenced by the repressive chromatin marks that it harbors in both contexts (top). Consequently, the gene is repressed. A possible way to overcome this repressed state in the two organs is through the binding of a pioneer transcription factor, which leads to chromatin opening and acquisition of activating chromatin marks by the enhancer (bottom). Through the binding of transcription factors that recognize pleiotropic transcription factor binding sites (TFBSs) (cyan–magenta sites) and organ-specific TFBSs (cyan or magenta sites), the enhancer activates gene expression in organ X and organ Y.

analyze the relationship between pleiotropy and enhancer complexity are needed to shed light on the evolution of *cis*-regulatory DNA.

Concluding Remarks and Future Perspectives

Altogether the work discussed in this review suggests that enhancer pleiotropy is pervasive in animal genomes, challenging the commonly held view that each enhancer is a genetic module regulating gene expression in a single window of time and space. Furthermore, the extent of pleiotropy indicates that a substantial quantity of regulatory information is not randomly located in the genome, but crowded in small DNA regions.

A possible explanation for the existence of pleiotropic enhancers is that some DNA regions are propitious places for the buildup of regulatory information. These DNA regions might have a chromatin structure that permits the binding of transcription factors and, in turn, the interaction with the basal transcriptional machinery in multiple contexts (Figure 2). Indeed, the same **pioneer transcription factors** might mediate the opening of an enhancer in multiple contexts, as has been recently shown for Grainy head [64] (Figure 2). It is also conceivable that the distance of an enhancer to the basal promoter influences the strength of the interaction between these two elements and fine-tunes the transcriptional output. Thus, a specific distance to the basal promoter might give the appropriate transcriptional output for a gene in different contexts, favoring the accumulation of regulatory information in pleiotropic enhancers. Another possible explanation for the appearance of pleiotropic enhancers is the co-option of part or a whole GRN [35,65]. In this scenario, tissue-specific enhancers become pleiotropic upon the redeployment of genes (of a GRN) in a new context. These novel pleiotropic enhancers may then duplicate and subfunctionalize, becoming tissue-specific enhancers again [65].

So far, functional studies have demonstrated that many developmental genes have pleiotropic enhancers. In addition, there are data confirming the existence of site pleiotropy, where TFBSs are reused in different contexts. Nevertheless, the fact that, in general, pleiotropic enhancers are longer, more complex, and have higher density and diversity of predicted TFBSs than tissue-specific enhancers, suggests that at least some pleiotropic enhancers encode their many functions by using different sets of TFBSs. In this context, there are unresolved issues regarding the function of pleiotropic enhancers in the genome (see Outstanding Questions). Untangling the architecture of regulatory regions and pleiotropic enhancers will shed light on these unresolved questions. This will require more functional dissections of pleiotropic enhancers in multiple species, which implies obtaining information on the distribution of epigenetic marks, open chromatin, TFBS usage, and enhancer–promoter contacts in different organs and developmental stages across organisms.

Outstanding Questions

What are the target genes of pleiotropic enhancers? Is there a specific group of genes that are enriched in pleiotropic enhancers?

Do pleiotropic enhancers function differently from tissue-specific enhancers? What structural features distinguish pleiotropic enhancers from tissue-specific enhancers?

What are the mechanisms underlying enhancer pleiotropy? Do pleiotropic enhancers function in a similar fashion in different contexts?

How do pleiotropic enhancers encode their multiple expression patterns? How common is site pleiotropy?

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