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Review

Ever-Changing Landscapes: Transcriptional Enhancers in Development and Evolution

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A class of *cis*-regulatory elements, called enhancers, play a central role in orchestrating spatiotemporally precise gene-expression programs during development. Consequently, divergence in enhancer sequence and activity is thought to be an important mediator of inter- and intra-species phenotypic variation. Here, we give an overview of emerging principles of enhancer function, current models of enhancer architecture, genomic substrates from which enhancers emerge during evolution, and the influence of three-dimensional genome organization on long-range gene regulation. We discuss intricate relationships between distinct elements within complex regulatory landscapes and consider their potential impact on specificity and robustness of transcriptional regulation.

Introduction

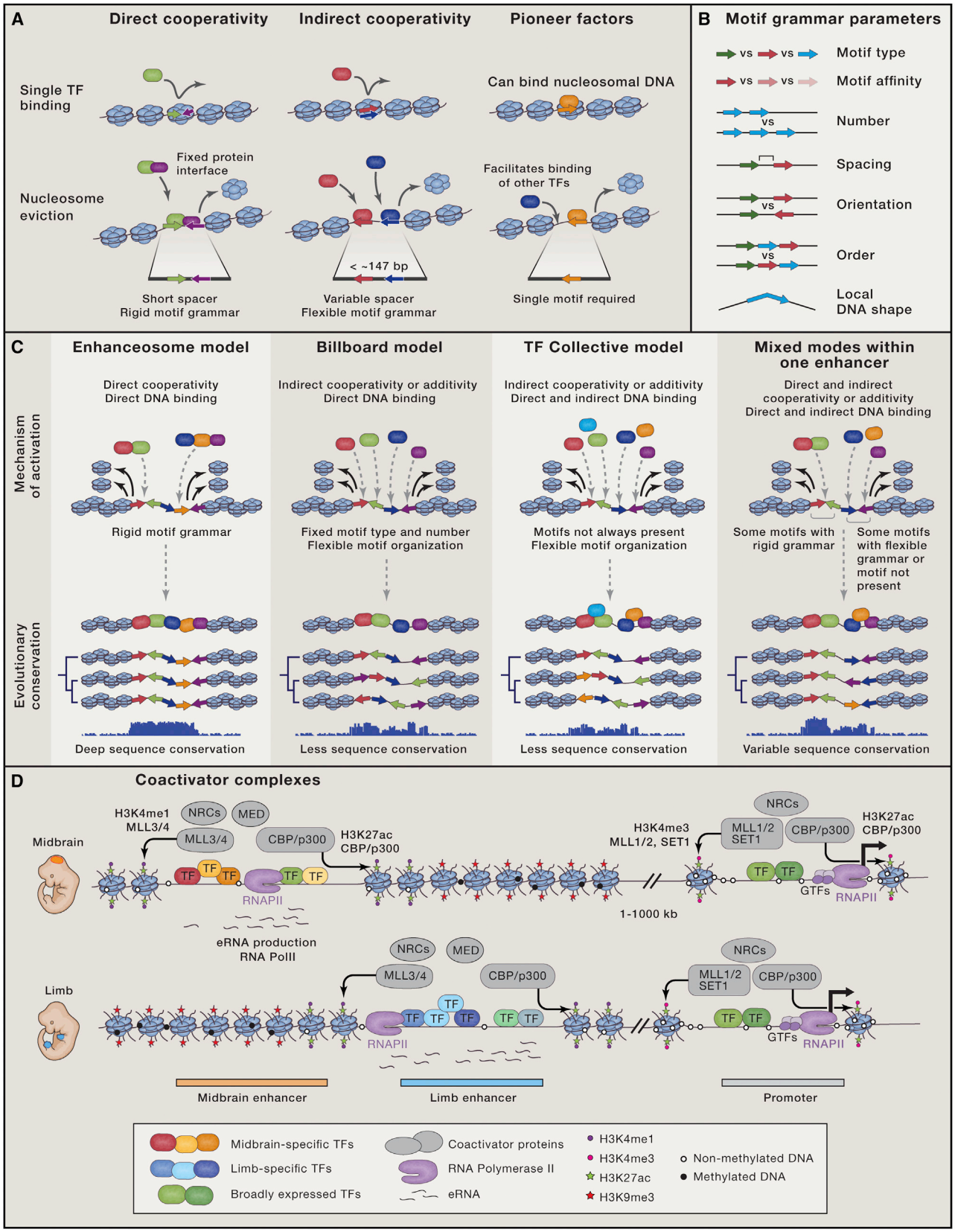
The accurate, precise, and robust regulation of gene expression during development is a cornerstone for complex biological life. Much of this information is encoded by *cis*-regulatory elements called enhancers, canonically defined as short (~100–1,000 bp) noncoding DNA sequences that act to drive transcription independent of their relative distance, location, or orientation to their cognate promoter (for a historical perspective on the discovery of enhancers, see Schaffner, 2015). Although enhancers share many features with other classes of *cis-regulatory* elements, especially promoters (reviewed in Kim and Shiekhattar, 2015), it is their ability to activate transcription over long genomic distances that sets them apart. This feature allows a gene to be regulated by multiple distal enhancers with different spatiotemporal activities, facilitating enormous combinatorial complexity of gene-expression repertoires (and, in turn, a vast array of cellular states) using a relatively limited set of genes. In addition to their central role in developmental gene regulation, enhancers are fertile targets for evolutionary change, as they are both cell-type specific (allowing for modulation of target gene expression in specific tissue context without affecting other pleiotropic gene functions) and commonly exist in groups of redundant elements (facilitating accumulation of genetic variation by buffering the risk of lethality) (Levine, 2010; Wittkopp and Kalay, 2012).

For decades, most of the key insights into enhancer function and divergence have come from *Drosophila* genetics. However, recent years have seen a renewed interest in long-range gene regulation in many species, including mammals, fueled by the increasing availability of genome sequences, great technological progress in the fields of genomics, genome editing, and imaging, as well as the realization that disease- and trait-associated genetic variants frequently map to enhancers. Insights into genome folding have revealed that most metazoan chromosomes are partitioned into self-associating topological domains that restrict enhancer search space for target promoters. Furthermore, comparative epigenomics from both closely related and more distant species have afforded us a better glimpse into *cis*-regulatory evolution and constraint. Here, we discuss these new developments and give an overview of our current understanding of the general principles of enhancer function and evolution.

General Principles of Enhancer Function

Enhancers as Clusters of TF Binding Sites

Functional enhancers are composed of concentrated clusters of transcription factor (TF) recognition motifs. Generally, enhancer activation requires the binding of multiple TFs, often including both lineage-specific factors and sequence-dependent effectors of signal transduction pathways, thereby ensuring integration of intrinsic and extrinsic signaling cues at these elements (reviewed in Buecker and Wysocka, 2012) (Figure 1). One reason why the action of multiple TFs is essential is that regulatory element sequences have high intrinsic affinity for histone octamers (Tillo et al., 2010), creating a strong barrier for access of TFs to the underlying DNA. Cooperative binding of multiple factors in close proximity (i.e., less than one nucleosome length) is thought to play a major role in overcoming the energetic barrier of nucleosome eviction, thus facilitating downstream effector binding and enhancer activation (Spitz and Furlong, 2012).



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Such cooperativity can rely on the direct physical association between TFs before or concurrent with DNA binding, called "direct cooperativity" (Figure 1A, left). Importantly, cooperative binding of TFs to DNA can also occur in the absence of direct protein-protein interactions through a process referred to as "indirect cooperativity" or "collaborative competition," in which a cohort of TFs collectively competes with the same histone octamer for access to underlying DNA, as demonstrated experimentally (Miller and Widom, 2003) and predicted by in silico modeling (Mirny, 2010) (Figure 1A, middle). Thus, as a general rule, increased numbers of TF motifs at an enhancer should be positively correlated with increased nucleosomal eviction, DNA binding, and, consequently, elevated gene-expression output. Numerous studies looking both at endogenous enhancers (Burz et al., 1998) and using synthetic reporters (Erceg et al., 2014; Smith et al., 2013a) support this view but also highlight an often-complex interplay between different regulatory sequences, as discussed in more detail below. In addition, at some developmental enhancers, there is evidence for stepwise licensing by lineage-determining TFs, also known as master regulators, or pioneer factors, which directly bind nucleosomal DNA to prime enhancers for activation (Figure 1A, right). These factors can recruit chromatin remodeling activities, which then facilitate removal and post-translational modification of histones, meaning that subsequent TF and coactivator binding is less strictly dependent on direct competition between TFs and nucleosomes (Zaret and Carroll, 2011).

Motif Organization, Enhancer Grammar, and Models of Enhancer Architecture

Because of this complex interplay and hierarchical logic between DNA-binding regulators at enhancers, the underlying organization of consensus motifs can have dramatic effects on enhancer activity and robustness. The universal principles of this organization—referred to as enhancer "grammar"—are quite complex and still an area of active research. The enhancer lexicon incorporates the type, binding affinity, number, spacing, orientation, order, and local DNA shape of TF motifs, all of which can affect the functional output of any given enhancer and are subject to varying degrees of selective constraint during evolution (Figure 1B). From these principles, two main distinct models for describing enhancer architecture have emerged, with the first "enhanceosome" model requiring rigid motif organization and spacing for function, while the alternative "billboard" model allows greater flexibility of binding, with the presence of a specific set of TFs being more important than the defined order or orientation of their underlying motifs (Figure 1C).

Several examples of enhancers falling at both ends of this architectural spectrum have been described. The archetypal model of an enhanceosome is the mammalian viral-inducible interferon-β (IFNβ) enhancer (Thanos and Maniatis, 1995), which requires the cooperative binding of eight TFs to create a composite surface for DNA binding. While documented examples of enhanceosomes are rare, simulations of TF binding have suggested that this mode of enhancer regulation may be in fact relatively common in mammalian genomes (Guturu et al., 2013). On the other end of the spectrum, so-called "billboard" enhancers preserve function by maintaining transcription factor binding site (TFBS) content but with significant flexibility as to the order or spacing of those sites within the larger enhancer, likely by relying on indirect TF cooperativity for activation and also allowing for independent contribution of multiple sub-elements to gene expression (Arnosti and Kulkarni, 2005). Interestingly, these sorts of enhancers often contain suboptimal binding sites that not only tolerate rapid motif turnover across species but that also may serve an important role in promoting enhancer specificity (Crocker et al., 2015; Farley et al., 2015). For example, the neural plate-specific Otx-a enhancer in Ciona contains GATA and ETS DNA sequence motifs that are imperfect matches to the consensus sequence (Farley et al., 2015). Strikingly, optimization of motif sequence or spacing causes stronger and ectopic enhancer activity patterns, as presumably the enhancer is now responsive to lower levels of the GATA or ETS-domain TFs that recognize these motifs. Thus, combinatorial deployment of multiple suboptimal enhancers may help to promote specificity of regulation without sacrificing signal strength. In addition, often a number of TFs are localized to enhancers without underlying consensus binding sites and are instead recruited through protein-protein interactions, as proposed for the "transcription factor collective" model of enhancer organization (Figure 1C) (Junion et al., 2012).

Both large-scale comparisons of enhancer conservation across species (Taher et al., 2011), as well as synthetic enhancer reporter studies (Smith et al., 2013a), have broadly supported the more flexible organizational models whereby a collection of TF-binding events can drive reporter activity in different orders or orientations. However, reexamination of the prevalence of more rigid motif grammar may be warranted, as recent work has challenged the widely held assumption that direct cooperativity of TFs should be evident on the DNA level by the presence of a composite motif (i.e., complete motifs of both TFs with a defined spacing). In fact, SELEX analysis using consecutive affinity purification to interrogate pairwise TF-binding specificities (CAP-SELEX [Jolma et al., 2015]) has demonstrated that

Figure 1. Nucleosome Eviction, Enhancer Grammar, and Models of Enhancer Architecture

(A) TF mechanisms for overcoming the energetic barrier to nucleosomal eviction and underlying motif requirements.

(B) Parameters of motif grammar at enhancers.

(C) Models of enhancer architecture and their primary mechanism of TF binding, flexibility of motif organization, and selective constraint across evolution.

(D) Coactivator binding and chromatin signatures at active enhancers. Presence of cell-type-specific and broadly expressed TFs, RNA Polymerase II (RNAPII) and associated enhancer RNAs (eRNAs), coactivator complexes such as Mediator (Med), nucleosome remodeling complexes (NRCs), histone acetyltransferases (CBP/p300), and methyltransferases (MLL3/4) at tissue-specific enhancer elements is schematically depicted for a midbrain (top) or limb (bottom) specific enhancer. Select modifications of neighboring nucleosomes associated with active enhancer states are highlighted. An overlapping set of protein complexes and modifications is also present at promoters, with the distinction that enhancers have a high ratio of H3K4me1 to H3K4me3, while the reverse is true at promoters. Both active enhancers and promoters are characterized by DNA hypomethylation, with the methylation status of enhancers being more dynamic and tracking with their cell-type specificity.

the majority of TF-TF interactions have a novel consensus motif, bound only weakly by each TF in isolation. Importantly, these novel long motifs detected by SELEX analysis are enriched in mammalian chromatin immunoprecipitation sequencing (ChIP-seq) TF clusters, suggesting that this sort of cooperative binding is commonplace throughout the genome. Therefore, it was likely the inability to recognize sites of direct cooperativity between TFs, rather than their true scarcity, that contributed to the notion that most mammalian enhancers are devoid of defined TF motif spacing. Indeed, evidence is beginning to emerge that spacing and orientation constraints on pairwise TF binding are more prevalent than initially anticipated (Ng et al., 2014). Thus, in reality, the architecture of most enhancers likely falls into a spectrum between the rigid motif organization of the enhanceosome and flexible organization of the billboard and TF collective, with defined spacing and orientation being required for some motifs, but not others, within a single enhancer (see "Mixed modes within one enhancer," Figure 1C).

Implications of Enhancer Architecture on Regulatory Landscape Evolution

It is interesting to consider what these different models of enhancer architecture could mean during regulatory landscape evolution. At enhanceosomes, any binding site mutation will preclude binding of the entire complex, demanding deep conservation at the DNA-sequence level to maintain function. Such strictly conserved enhancer sequences do indeed exist and have been studied for quite some time (reviewed in Visel et al., 2007). The question remains, however, whether there is anything unique about these deeply conserved enhancers. One hypothesis is that enhanceosomes may have potential roles in mediating switch-like transcriptional activation of genes requiring strict regulatory inputs from multiple signaling pathways (Spitz and Furlong, 2012). Consistent with this theory, deeply conserved enhancers tend to fall as clusters near genes encoding developmentally important transcription factors (see Boffelli et al. [2004] and references within). Because of their developmental importance and strict structural requirements, mutations at conserved regulatory sites are also associated with human disease, as seen for the deeply conserved *Shh* enhancer that drives preaxial polydactyly upon disruption (Lettice et al., 2003).

Despite their association with developmental TFs and human disease, deeply conserved enhancers are an exception rather than the rule. Instead, de novo gains and losses of enhancers appear to be relatively common across evolution (discussed in more detail below), and furthermore, even when an ancestral function is conserved, these enhancers often retain no recognizable sequence similarity or have only very weak conservation signatures. For example, only ~24% of murine human heart enhancers with p300 occupancy showed any detectable conservation with other vertebrates, yet these poorly conserved enhancers still demonstrated heart-specific activity in transgenic mouse embryo reporter assays regardless of their level of sequence conservation (Blow et al., 2010; May et al., 2011). Similarly, poised developmental enhancers identified in human embryonic stem cells drove cell type- and stage-specific expression when introduced to zebrafish embryos, even in the absence of detectable sequence conservation between the two species (Rada-Iglesias et al., 2011). One mechanism that could explain this conservation of ancestral function, but not of sequence, is compensatory TFBS turnover, which is tolerated at enhancers following more flexible organizational principles. A classic example is the even-skipped stripe 2 enhancer (*S2E*) in the *Drosophila* genus, which has undergone dramatic reshuffling of TFBSs across different species, yet directs the same expression pattern in transgenic reporter assays (Ludwig et al., 2000; Arnosti et al., 1996). Chimeric enhancer assays provided strong evidence for stabilizing selection at this site, as swapping the 3′ and 5′ halves of the native *S2E* enhancers from different species did not recapitulate the endogenous activity patterns (Ludwig et al., 2000). A more systematic enhancer discovery strategy, called STARR-seq, confirms that compensatory motif turnover in functionally conserved enhancers is common even between closely related species (Arnold et al., 2014). More generally, TFBS loss or gain without concomitant alteration of enhancer function appears to be a common event at enhancers that employ a more flexible mode of motif grammar over evolutionary timescales.

Impact of Sequences that Affect DNA Shape

In addition to the core TF motif recognition, emerging evidence supports the role of local DNA shape in influencing binding affinity, site accessibility, and binding site choice, known as "shape readout" (Slattery et al., 2014). The DNA sequence at sites flanking core TF-binding sites can thus profoundly influence binding by TFs that are sensitive to DNA shape (Gordân et al., 2013; Levo et al., 2015). Similarly, changes in protein structure that affect shape readout can dramatically affect binding preference. For example, altering amino acids involved in DNA shape recognition by the Scr protein abrogated preferential binding to a narrowed minor groove DNA structure, while introduction of these shape-recognizing residues into another Hox factor, Antp, caused a switch in its binding specificity in vitro and transcriptional targets in vivo to that of Scr (Abe et al., 2015). Thus, at least in some defined cases, local DNA shape readout influences the binding preference of TF family members. Consequently, mutations falling outside core TFBS may affect local DNA shape and contribute to modulation or loss of enhancer activity in both an evolutionary and disease context.

Role of Coactivators in Enhancer Activity

The ability of TFs to activate transcription on chromatin templates is dependent on the recruitment of coactivator proteins (reviewed in Roeder [2005] and Weake and Workman [2010]). Coactivators are defined as factors that are "required for the function of DNA-binding activators, but not for basal transcription per se, and do not show site-specific binding by themselves" (Malik and Roeder, 2010). Coactivators typically act through modifying and remodeling the chromatin context of enhancers and include histone acetyltransferases (such as p300/CBP, SAGA complex, MOF, TIP60, and others), histone methyltransferases (e.g., MLL3/4, CARM1), chromatin remodeling factors (e.g., Brg1, CHD7), and factors that promote crosstalk with the basal transcriptional machinery at promoters (e.g., Mediator complex) (Krasnov et al., 2016; Malik and Roeder, 2010; Taatjes et al., 2004). Coactivator complexes are often broadly and highly expressed and can be recruited to chromatin by a diverse range

of TFs. Consequently, coactivators tend to be associated with most active enhancers in a given cell type, regardless of the examined tissue (see Figure 1D). This property has been widely utilized for annotations of putative enhancers in a myriad of cellular contexts, where occupancy of coactivators (e.g., p300, Mediator, BRG1) or their enzymatic products (e.g., H3K27ac, catalyzed by p300/CBP or H3K4me1, catalyzed by MLL3/4) can be mapped using ChIP-seq (for reviews on enhancer chromatin signatures and epigenomic annotation strategies, see Calo and Wysocka [2013]; Sakabe and Nobrega [2013]; and Whitaker et al. [2015]).

Consistent with the idea that coactivators are the workhorses behind enhancer-mediated transcriptional activation, RNA-guided recruitment of the acetyltransferase domain of p300 fused to dCas9 is sufficient to bypass the requirement for TFs during enhancer activation in an endogenous chromosomal context (Hilton et al., 2015). Conversely, RNA-guided recruitment of the H3K4me1/2 demethylase LSD1 is sufficient to repress enhancer activity and gene transcription (Kearns et al., 2015). A comprehensive study in *Drosophila* used GAL4 DNA-binding domain (DBD) fusions of 338 coactivators and corepressors in enhancer complementation assays to test their capacity to bypass the requirement for key TFs by replacing their motifs with GAL4 recognition sites in 24 distinct enhancer contexts. Interestingly, most (80%) of the examined coactivators and corepressors activated or repressed, respectively, transcription in at least one enhancer context, whereas some coactivators, including p300, Mediator subunits MED15 and MED25, and Lpt1 (a fly homolog of the N-terminal part of MLL3/4), were sufficient to activate transcription in all examined contexts (Stampfel et al., 2015). While not without caveats, taken together, these results suggest that the main role of TFs is to provide a specific genomic address for coactivator complexes, which in turn enable activation of transcription by influencing the activity of RNA polymerase and facilitating establishment of a transcriptionally permissive chromatin environment.

Nonetheless, from an evolutionary perspective, alterations of regulatory sequences themselves are the major driver of change in enhancer activity, as enhancer elements are subject to high rates of turnover (Arnold et al., 2014; Villar et al., 2015) while both coactivator-TF interfaces and TF DNA-binding specificities appear to be well conserved over evolutionary timescales (Minezaki et al., 2006; Nitta et al., 2015). It is therefore important to consider the genomic substrates from which enhancers emerge over evolutionary time and how the availability of such substrates may either act to drive or limit the rates of enhancer genesis.

Genomic Substrates for Evolving New Enhancers

The birth of new enhancers can be mediated by a number of mechanisms, which will be discussed here in turn.

Regulatory Innovation following Genomic Duplication

Genomic duplication is an important event driving evolutionary change, as it generates new gene loci with the potential to evolve divergent functions and drives the formation of new regulatory programs (Taylor and Raes, 2004). Following a duplication event, often one copy of a pair of duplicated genes is simply lost due to lack of selective pressure to maintain both paralogs. However, subfunctionalization of enhancer activity (the loss of activity of a subset of enhancers leading to different expression domains of gene paralogs), distributed gene dosage (Lan and Pritchard, 2015) or rapid divergence in the expression domain of one or both of the gene paralogs by repurposing existing enhancer(s) for novel regulatory functions (Goode et al., 2011) can result in retention of both duplicated genes (Figure 2Ai–iii). For example, teleost fish experienced a relatively recent whole-genome duplication 350 million years ago, with many paralogous duplicated genes retaining an equivalent protein function but showing divergent expression domains that arose through changes in their local *cis*-regulatory landscape (for example, the duplicated pax6a/b in zebrafish [Kleinjan et al., 2008]). Smaller duplications of non-coding DNA can also generate copies of enhancer elements in *cis* to the original enhancer and promoter pair (Figure 2B). While in many cases these duplicated enhancers are lost due to negative selection or genetic drift, in some instances locally duplicated enhancers are retained, for example enhancers at the human apoE/C-I/C-I′/C-IV/C-II gene cluster (Allan et al., 1995). Finally, genomic duplication of previously non-regulatory sequences can also provide new raw material from which primordial regulatory sequences can emerge through neutral drift, as discussed below.

Repurposing of Ancestral Regulatory or Coding Sequences

With or without genomic duplication events, novel regulatory function can arise from functional ancestral DNA sequences through the repurposing of older enhancer elements (Rebeiz et al., 2011). The evolutionary barrier to the formation of a novel enhancer is potentially lower at these sites because they already contain motifs for DNA binding factors, which can act cooperatively with newly emerging sites. Recent genome-wide mapping of DNase hypersensitive sites (DHSs) (Vierstra et al., 2014) revealed that DHSs at orthologous loci between human and mouse were functionally active in distinct tissues, revealing frequent regulatory element repurposing across 90 million years of evolution. Importantly, modulation of existing enhancers can drive regulatory innovation and evolution of morphological change, such as morphogenesis of trichomes in *Drosophila* larvae (Frankel et al., 2011) and limb length in bats (Cretekos et al., 2008).

Interestingly, enhancer function can also arise within protein-coding sequences—for example, in mouse liver, 6% of epigenomically defined enhancers overlap exons (enhancer exons, or eExons), a number of which have been implicated in regulation of nearby liver-specific genes (Birnbaum et al., 2012). A further extensive investigation of this phenomenon in 81 cell types found that ~15% of coding exons have DNase hypersensitivity footprints, which is suggestive that they are dual-use codons with both protein-coding potential and TF-binding capacity, or "duons" (Stergachis et al., 2013). However, whether duons are subject to selective constraint beyond that associated with their protein-coding potential remains controversial (Agoglia and Fraser, 2016; Xing and He, 2015).

Spontaneous Emergence of Enhancer Sequences from Non-regulatory DNA

Attempts to reverse engineer enhancers (Smith et al., 2013b) have revealed that short (i.e, 15-mer) random sequences are

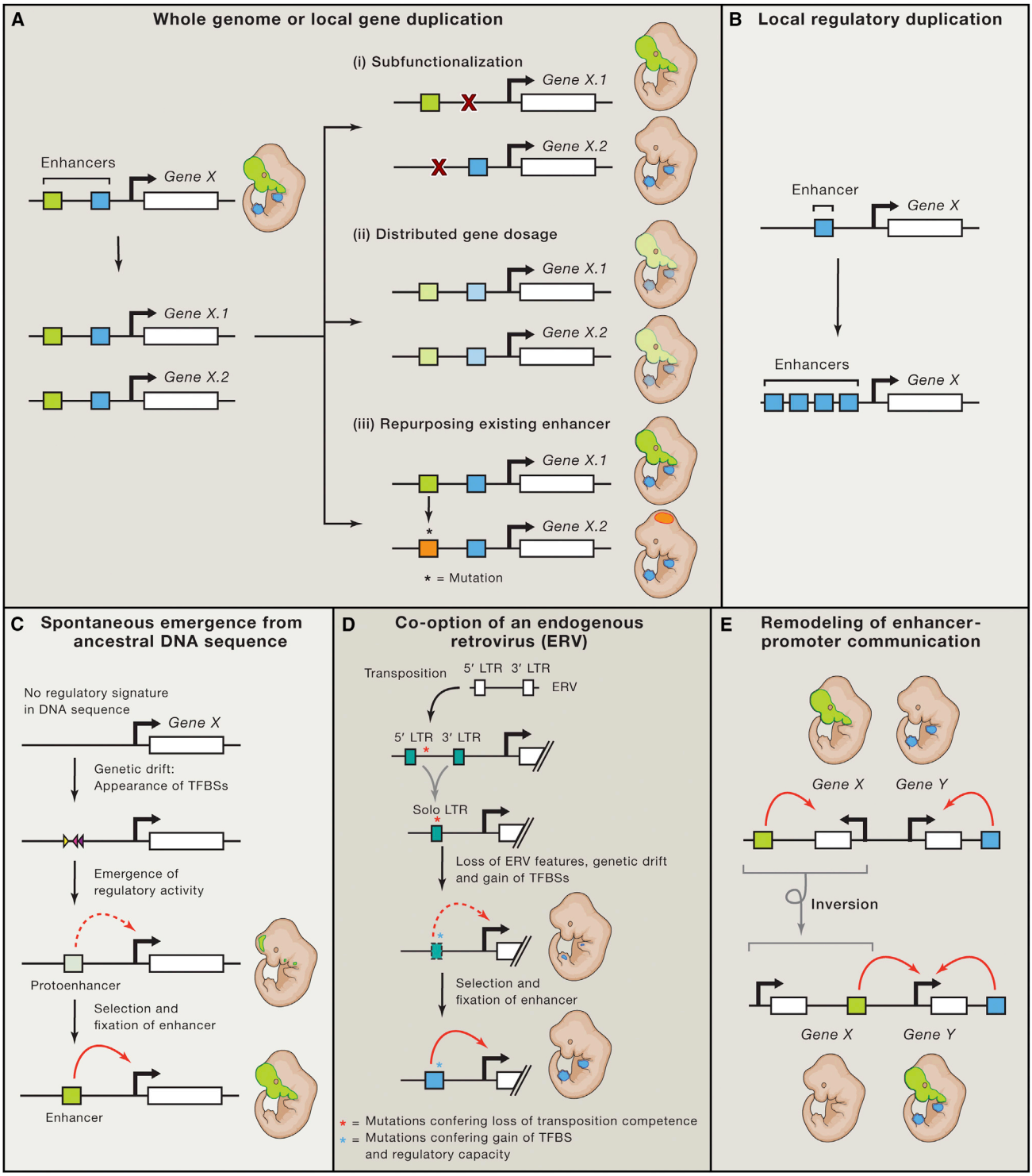

Figure 2. The Birth and Death of Enhancers during Evolution

(A) Following whole-genome or local duplication events, enhancers and associated genes can become duplicated (left) and subsequently undergo (i) loss of enhancer function and subfunctionalization of associated gene activity, (ii) reduction in enhancer activity and gene dosage sharing between alleles, or (iii) enhancer repurposing with novel tissue or developmental stage expression for the duplicated gene.

(B) Local duplication events can lead to increased enhancer copy-number.

(C) Novel enhancer activity can emerge from ancestral DNA through genetic drift and spontaneous appearance of TFBSs and protoenhancer activity.

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often sufficient to drive tissue-specific expression, suggesting that enhancer activity can emerge spontaneously from neutral DNA sequences purely by random genetic drift. Modeling theoretical rates of enhancer genesis in *Drosophila* concluded that it should take 0.5–10 million years to evolve novel anterior-posterior (A-P) patterning enhancer elements from neutral DNA sequence (Duque and Sinha, 2015). These predictions match well with observations from across the *Drosophila* genus whereby hundreds of species-specific enhancers have arisen over similar timescales (Arnold et al., 2014) and provide an initial framework for understanding emergence of new enhancers from the genetic void. Recent studies emphasize that indeed most species-specific enhancers evolve de novo from previously non-regulatory non-coding DNA (Figure 2C). For example, using comparative epigenomic analysis of enhancers from livers of 20 diverse mammalian species, Villar et al. (2015) demonstrated that the majority (52%–77%) of species-specific enhancers arose through exaptation of ancestral DNA sequences (i.e., present in the genomes of the ancestral species for at least 100 million years), with the remainder being derived primarily from transposable elements (Villar et al., 2015).

Co-option of Transposable Elements

A large proportion of eukaryotic genomes, including almost half the human genome, are composed of repetitive transposable elements (TEs) (Lander et al., 2001). TEs are a rich source of regulatory innovation, as they are disseminated throughout the genome via TE expansion, carrying with them *cis*-regulatory sequences that exploit the host *trans* environment for their transcription (Feschotte, 2008) and have the potential to acquire mutations that allow for their tissue-specific exaptation for host gene regulation (Bourque et al., 2008). Indeed, evidence is beginning to emerge that TEs play a central role in rewiring gene regulatory networks and can facilitate rapid evolution of ecologically relevant traits, such as sweet perception in primates (Ting et al., 1992) or the diversification of tissues at the boundary between mother and fetus in eutherian mammals (Lynch et al., 2011).

Indeed, according to some estimations, the majority of primate-specific regulatory sequences are derived from TEs (Jacques et al., 2013). For example, Alu elements, which comprise ~10% of the human genome, contain suboptimal TFBSs and can acquire enhancer-like features with few mutations (Su et al., 2014), while ERV1 elements are enriched at sites of *cis*-regulatory divergence in primate neural crest cells (Prescott et al., 2015), suggesting that many enhancers that changed their activity since the separation of humans and chimpanzees did so through exaptation of pre-existing TEs. Importantly, direct evidence for LTR-mediated gene regulation has been demonstrated for human innate immunity genes (Chuong et al., 2016). This is consistent with the emerging idea that motif-rich LTRs of endogenous retroviruses are particularly fertile substrates for evolving new enhancers, with many LTRs acquiring inducible or cell type-specific enhancer function during evolution (Figure 2D). As endogenous retroviruses are remnants of ancient retroviral infections, one can speculate that the ancestral evolutionary history of viral infections and endogenization can and will continue to influence the future *cis*-regulatory evolution of a species.

DNA Methylation and Biased Gene Conversion in Regulatory Evolution

The frequency with which TFBSs emerge by chance from ancestral DNA sequences and TEs is greatly influenced by local mutational rate. Certain processes can act to increase or to suppress the basal mutagenesis rate and have been implicated in rapid evolution of sequences and emergence of novel regulatory function. For example, the majority of vertebrate genomes are methylated at cytosine residues in the context of CpG dinucleotides (Jones, 2012). Mutation of methylated cytosines to thymine due to spontaneous deamination is subject to error-prone repair (Duncan and Miller, 1980). Thus, C to T mutation is the most common mutation among all base substitutions (Sved and Bird, 1990), favoring de novo genesis of TFBSs that contain TpG dinucleotides. For example, there is evidence that spontaneous deamination has helped to generate thousands of p53 binding sites genome-wide (Zemojtel et al., 2009).Therefore, in addition to its role in genome silencing, DNA methylation may play a role in regulatory evolution. A second mechanism causing rapid change of local nucleotide composition is known as GC-biased gene conversion (gBGC), in which mismatch repair processes following meiotic recombination favor G or C alleles over A or T alleles, thus leading to increase in GC content at these regions (Galtier and Duret, 2007). gBGC might have played an important role in recent human evolution, as around 20% of sequences which are conserved across vertebrates but have undergone rapid change in the human lineage (e.g., human accelerated regions [HARs]) can be explained by gBGC alone (Kostka et al., 2012).

Remodeling of the Chromosomal Context

Novel enhancer function can arise not only through change of the enhancer sequence itself, but also through the rearrangement of the neighboring chromosomal context (Figure 2E). For example, in the beetle *Tribolium castaneum*, the expression of the *ladybird* gene is absent from the dorsal mesoderm as compared to the honeybee *Apis mellifera* and fruit fly *Drosophila melanogaster* and is replaced by expression of *C15*, a neighboring gene. This switch in expression from *ladybird* to *C15* appears to have arisen from a genomic inversion redirecting a conserved ladybird 3′ enhancer to regulate *C15* (Cande et al., 2009). Thus, despite minimal alteration to the enhancer itself, change in its genomic location can rapidly introduce a new gene or enhancer into a pre-existing gene-regulatory network in one step. Recently, much progress has been made toward understanding how three-dimensional genome organization influences enhancer function, poising the field to uncover the frequency with which structural genomic variation can drive evolutionary rewiring of enhancer targets.

(D) Enhancer elements can be exapted from transposable elements. For example, following endogenization and unequal homologous recombination, the long-terminal repeats (LTRs) of endogenous retroviral (ERV) elements can gain tissue-specific regulatory activity through accumulation of mutations and emergence of TFBSs.

(E) Enhancer activity can be transferred to a new gene target, for example, through a genomic inversion event.

Topological Constraints on Enhancer Function and Relationships between Enhancers within Complex Regulatory Landscapes

Topological-Associated Domains as Structural Elements of Chromatin Organization

A defining feature of enhancers is their ability to activate transcription at a distance. Indeed, in mammalian genomes, such distances can be over a megabase long, as exemplified by the Shh limb enhancer (Lettice et al., 2003), raising the question as to what constrains enhancer search space such that high-regulatory precision is preserved. A key insight into this question came from studies of chromosome folding using techniques such as Hi-C and 5C, which are chromosome conformation capture (3C)-based assays and rely on the principle that digestion and re-ligation of chromatin can capture the three-dimensional proximity of DNA sequences in the nucleus (extensively reviewed elsewhere, see de Wit and de Laat [2012]). These studies have revealed that the chromosomes of many eukaryotic genomes are segmented into self-interacting domains reported as physical domains in *Drosophila* (Hou et al., 2012; Sexton et al., 2012) and topological-associated domains (TADs) in mammalian genomes (Dixon et al., 2012; Nora et al., 2012) (Figures 3A and 3B). High-resolution mapping has estimated TAD median length at 185 kb in mammals, although importantly a subset of domains are over a megabase in size (Rao et al., 2014). Importantly, compartmentalization of the genome in this manner partitions the chromosome into ''regulatory neighborhoods'' by limiting the activity of *cis*-regulatory elements to genes that fall within the same TAD and by preventing spreading of heterochromatic marks (Dixon et al., 2012; Nora et al., 2012) (Figures 3B and 3C, also discussed in more detail below).

Strikingly, TAD boundaries appear to be largely invariant between cell types and are conserved across even distantly related species, suggesting that TADs represent a fixed structural unit of chromatin organization within which tissue-specific regulatory interactions can occur and evolve (Dixon et al., 2012; Vietri Rudan et al., 2015; Harmston et al., 2016). Such an organizational role for TADs is supported by the demonstration of a one-to-one correspondence between TADs and bands on polytene chromosomes from *Drosophila* larval salivary glands, with interbands corresponding to highly transcribed TAD boundary regions (Eagen et al., 2015) (Figure 3D). Endoreplicated polytene chromosomes have long served as a cytological model for understanding the relationship between chromatin structure and function, with highly stereotyped banding patterns observed across cells within a given cell type and only minor variations seen between tissues (Bridges, 1935; Mavragani-Tsipidou et al., 1990). This recent observation further suggests that TADs and their boundaries represent fundamental features of chromatin organization. Notably, the invariance of TADs across cell states is not due to a passive maintenance from one cell division to the next, as TAD structures are lost during mitosis to accommodate an alternative conformational state of metaphase chromosomes (Naumova et al., 2013) (Figure 3E). This implies that with each cell division, TADs must reproducibly refold, but the mechanisms that drive their reformation and maintenance are still under investigation.

Conservation of Topological Domain Structures across the Eukaryotic Tree of Life

Given the importance of TAD boundaries in demarcating the limits of gene-regulatory domains, changes to domain architecture would represent a means for saltatory (or non-gradual) change in gene regulation over evolutionary time, as shifting of domain boundaries would expose multiple genes to a novel regulatory environment. However, this sort of restructuring of TADs appears to be rare during evolution, as TADs tend to overlap with highly syntenic genomic blocks of conserved enhancers and non-coding elements (Harmston et al., 2016). Interestingly, structural rearrangements between mammalian genomes appear to instead occur between the boundaries of adjacent domains (Farré et al., 2015; Vietri Rudan et al., 2015) (Figure 4Ai–ii).

In the context of high conservation of TAD boundaries in vertebrates and their ostensible role in organizing regulatory landscapes from *Drosophila* to man, it is surprising that some multicellular organisms appear to lack conventional TAD organization. For example, in *C. elegans*, TAD-like structures seem to predominantly occur on the X chromosomes of XX hermaphrodites and are driven by the dosage compensation complex (DCC), whereas autosomes are largely devoid of long-range chromosomal interactions (Crane et al., 2015). This could be perhaps rationalized by the fact that *C. elegans* has a compact genome, with most of the *cis*-regulatory information usually contained within 10 kb from the TSS, thus alleviating the need for long-range gene-regulatory domains. In plants, which appear to have long-range cell-type-specific enhancers (Zhu et al., 2015), TAD structures are also not detectable by Hi-C, albeit both boundary-like elements and self-interacting heterochromatic regions having been reported (Feng et al., 2014; Grob et al., 2014). Interestingly, both *C. elegans* and *A. thaliana* do not encode a CTCF homolog (Heger et al., 2012), suggesting that these species exploit alternative mechanisms of genome organization and functional segmentation (the role of CTCF in mammalian TAD formation is discussed below). Interestingly, the inactive mammalian X chromosome also mostly lacks TADs and is instead divided into two megadomains (Giorgetti et al., 2016). Therefore in addition to being absent in a number of eukaryotic species, TAD-like structures are not a required feature of mammalian interphase chromosome folding.

It is unclear when and how TAD-like structures first appeared during evolution. However, recent studies from both bacteria and yeast suggest that self-interacting domains may be an ancient feature of chromosome organization. Hi-C experiments in *Caulobacter* cells found evidence of independent spatial domains called ''chromosomally interacting domains'' (CIDs), ranging in length from 30 to 420 kb, which are proposed to correspond to supercoiled DNA loops, or plectonemes, arranged in a bottle-brush-like fiber and flanked by highly transcribed genes at CID boundaries (Le et al., 2013). In the fission yeast *S. pombe*, the cohesin complex has been shown to be important for the formation of globule structures at the 50–100 kb scale (Mizuguchi et al., 2014). Further explorations in *S. cerevisiae* also found CIDs which are much shorter self-interacting regions than those detected in other organisms on the order of around 2–10 kb in size and bounded once again by

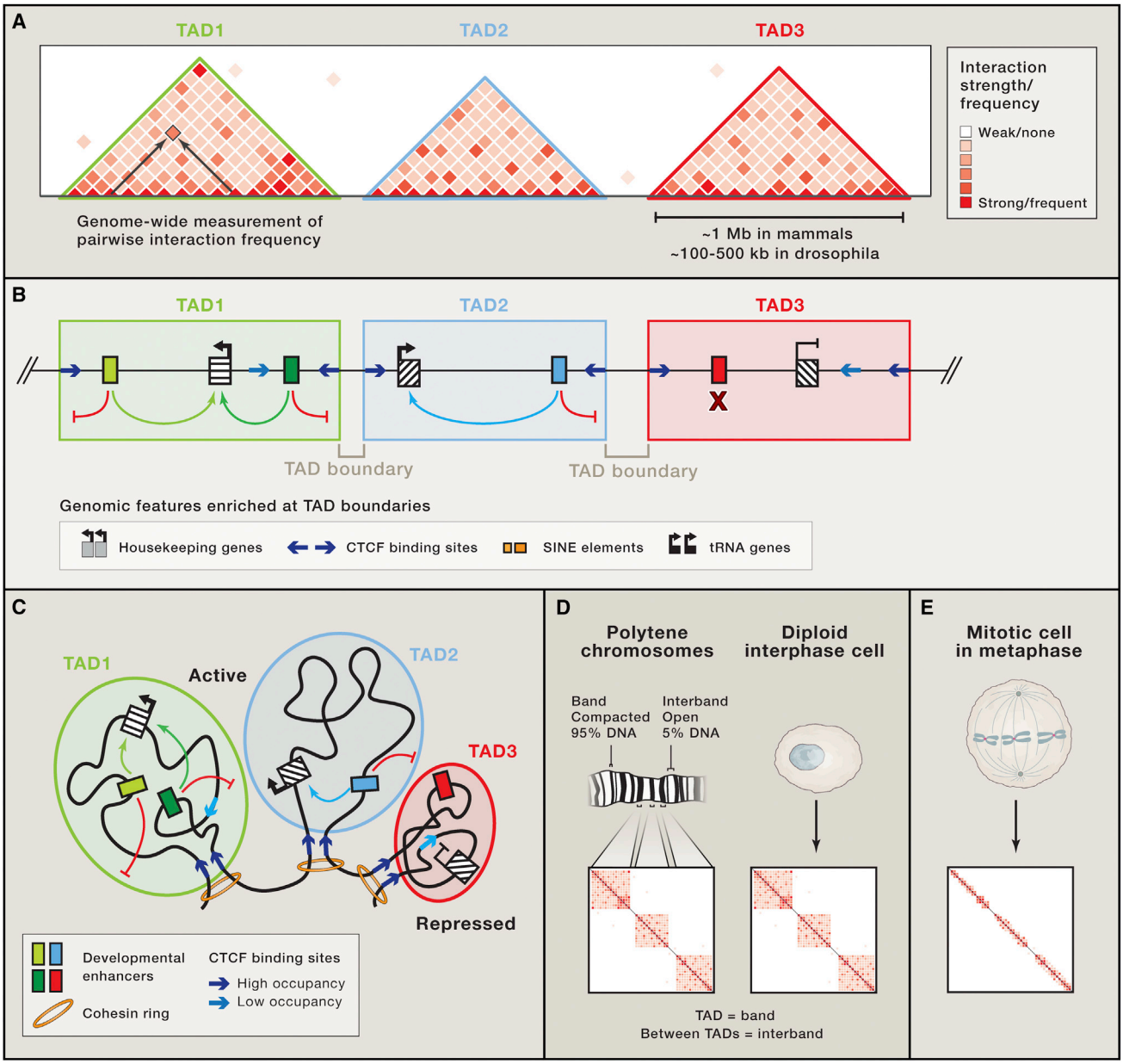

Figure 3. Organization of Chromatin into Topologically Associated Domains

(A) Hi-C or 5C heatmaps visualize three-dimensional interactions or compartmentalization of chromosomes into TADs, visible as triangular blocks of increased interaction frequencies.

(B) TAD boundaries restrict the influence of regulatory elements to genes within a given TAD and limit the spread of chromatin modifications. The boundary regions between TADs have been observed to be associated with CTCF binding sites, housekeeping genes, SINE elements, and tRNA genes.

(C) A model of chromosome folding corresponding to (B), with convergent CTCF sites and cohesin depicted at loop anchors.

(D) Correspondence between TADs and cytological bands on polytene chromosomes and TAD boundaries with decondensed interbands.

(E) During mitosis, TAD structure is lost.

highly transcribed genes (Hsieh et al., 2015). Interestingly, these self-associating topological domains in *S. cerevisiae* encompass ~1–5 genes, approximately the same order of gene number as mammalian TADs, suggesting that the size of genes and intergenic spacing may influence the size and formation of topological domains. Further interrogation of the topological landscapes at high resolution across the eukaryotic tree of life will be necessary to understand the evolution and functional role of topological domain architecture.

Formation of TAD Boundaries and CTCF-Mediated Loops

Since TAD boundaries appear to restrict both enhancer function (Dowen et al., 2014; Flavahan et al., 2016; Guo et al., 2015; Lupiáñez et al., 2015) and spreading of chromatin marks

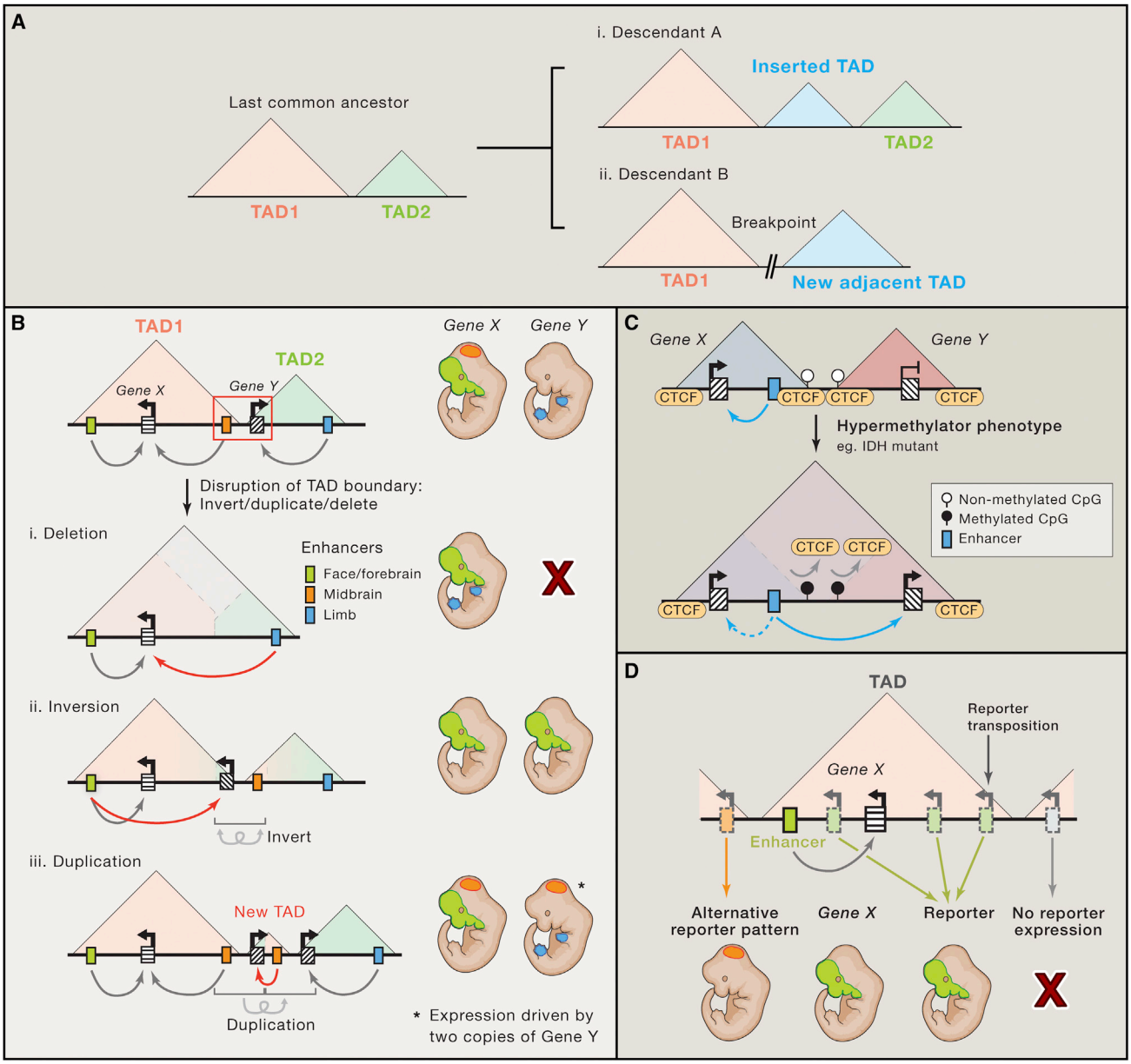

Figure 4. Topologically Associated Domains Define Discrete Units of Gene Regulation

(A) TADs appear to be highly conserved across mammals, therefore structural changes between species tend to occur at TAD boundaries, for example (i) insertion of a new TAD or (ii) chromosomal breaks.

(B) Disruption of a TAD boundary (red box) can impact gene expression. (i) Boundary deletion fuses two adjacent TADs facilitating de novo regulation of genes from one TAD by the enhancers in another. (ii) Boundary inversion can translocate genes or enhancers into an adjacent TAD, where they are then incorporated into the regulatory environment of the new TAD. (iii) TAD boundary duplication can create a new TAD and potentially expose any duplicated genes to a novel regulatory environment.

(C) Aberrant DNA methylation can abrogate CTCF binding, causing TAD boundary defects and misregulation of gene expression.

(D) Reporter constructs transposed into different regions of a TAD containing a developmental enhancer, but not into genomic regions outside the TAD, can recapitulate the gene-expression pattern of the endogenous gene (*Gene X*) controlled by this enhancer.

(Narendra et al., 2015), they fulfill a canonical definition of insulator elements. Indeed, TAD boundaries are enriched for insulator-binding proteins, such as CP190, CTCF, and BEAF-32 in *Drosophila* (Van Bortle et al., 2014; Sexton et al., 2012; Phillips-Cremins et al., 2013) and are enriched for CTCF and cohesin in mammals (Dowen et al., 2014; Sofueva et al., 2013). In fact, high-resolution Hi-C maps in mammalian cell lines revealed that 86% of roughly 10,000 long-range contact peaks, interpreted as anchors for chromatin loops, are associated with CTCF (Rao et al., 2014). Moreover, CTCF sites that are engaged

in these contacts are characterized by a unique motif orientation, with convergent (inward-facing) CTCF motifs found in > 90% of loop anchors (Figure 3B) (Rao et al., 2014; Vietri Rudan et al., 2015). Subsequent work has led to an as of yet experimentally untested "loop extrusion model" of TAD formation whereby a chromosomal loop is randomly initiated then continuously fed through an extrusion complex containing a cohesin ring until convergent CTCF binding sites are encountered, at which point the loop structure is stabilized, likely via CTCF dimerization (Fudenberg et al., 2015; Sanborn et al., 2015). Although this hypothetical model is attractive, at present it remains unclear to what extent convergent CTCF-cohesin sites are the major driver of genome segmentation into TAD domains. Interestingly, neighboring topological domains do not completely collapse or merge upon deletion of domain boundary regions nor when cohesin is depleted in non-cycling cells (Seitan et al., 2013; Sofueva et al., 2013; Zuin et al., 2014). This can be rationalized by inherent interactions within a TAD mediating TAD structure organization by preventing interactions with a neighboring TAD and thus contributing to the presence of a boundary between them (Giorgetti et al., 2014; Ulianov et al., 2016). Taken together, current results suggest that multiple parallel mechanisms may contribute to separating the genome into highly reproducible TAD structures.

Consequences of TAD Organization on Enhancer Function and Gene Regulation

Regardless of the specific mechanisms, genome segmentation into TADs has profound implications on how *cis*-regulatory landscapes are organized and evolve by limiting the genomic search space within which enhancers can act. As such, perturbations of TAD boundary elements, either through structural variation (Figure 4B) or aberrant DNA methylation resulting in loss of CTCF binding (Figure 4C), can lead to misregulation of genes by exposing them to the regulatory influence of enhancers in the adjacent TAD, in some cases resulting in congenital malformations or cancer (Nora et al., 2012; Gómez-Marín et al., 2015; Guo et al., 2015; Flavahan et al., 2016; Lupiáñez et al., 2015).

Importantly, genes lying within the same topological domain are often co-regulated (Nora et al., 2012; Gómez-Marín et al., 2015), suggesting that enhancers may sample multiple, if not all promoters confined within a TAD. This idea is supported by mouse reporter assays, in which transposed LacZ reporter sensors maintain a similar expression readout across a range of distances from a known enhancer until they cross a TAD boundary (Symmons et al., 2014) (Figure 4D). However, given that in many cases genes in the same TAD in fact exhibit distinct expression patterns, this apparent capacity of enhancers to activate transcription across their designated topological domain raises an issue of enhancer-promoter specificity. More quantitative analysis of genomic space sampling by enhancers is warranted, as some genomic positions within a topological domain may be favored targets due to preferential intra-domain chromatin folds. Moreover, additional mechanisms, such as promoter-specific repression, complex synergistic, or competitive relationships between simultaneously active enhancers within the same TAD or biochemical compatibility between enhancers and promoters, may all contribute to achieving greater regulatory specificity of enhancer-promoter interactions within a topological domain.

What is also becoming clear is that, while there are certainly well documented examples of enhancer-promoter loops, typical enhancer-promoter contacts are likely less stable and/or less frequent than structural loops mediated by CTCF that are readily detectable by Hi-C methods. For example, most enhancers and promoters active in a given cell type are not detected as peak points (a.k.a. loop anchors) on high-resolution Hi-C maps (Rao et al., 2014). Regrettably, the authors chose to de-emphasize this point by focusing on a subset of promoters and enhancers that coincide with stable loop anchor sites. However, the elements in this subset are distinct from most enhancers and promoters, as they are bound by CTCF and therefore may only represent the minority of enhancer-promoter pairs, which serve dual roles as both structural and regulatory elements.

Intriguingly, recent live-imaging studies in *Drosophila* uncovered the capacity of a single enhancer to drive synchronized transcriptional bursting from two equidistant promoters (Fukaya et al., 2016), raising the question of whether or how chromatin topology regulates this behavior. Another recent study supports a highly dynamic view of enhancer-promoter contacts in mammalian cells and shows that enforcing promoter-enhancer looping can increase transcriptional burst frequency, but doesn't affect burst size (Bartman et al., 2016). One should also consider that alternative mechanisms of communication may be at play, such as the previously proposed tracking model (Zhu et al., 2007), lateral propagation of torsional strain, indirect association in transcriptional hotspots or "factories" (Kolovos et al., 2012), enhancer-mediated trapping of activator complexes in a "reaction vessel," or other yet-to-be-conceived mechanisms. With carefully measured parameters of transcriptional bursting in relation to changes in nuclear positions of relevant regulatory elements and intervening sequences in living cells, it may soon become possible to gain insights into the role of chromatin topology in long-range gene regulation by enhancers.

Regulatory Relationships among Simultaneously Active Enhancers

With our greater appreciation of the complexity of genomic organization, it is becoming apparent that *cis*-regulatory elements do not function in isolation. Many (if not most) developmental genes are regulated by multiple enhancers with both overlapping and distinct spatiotemporal activities. Indeed, key lineage genes in a given cell type tend to be associated with dense cluster(s) of highly active enhancers, often referred to as super-enhancers (Hnisz et al., 2013; Whyte et al., 2013). These observations beg a couple of questions: what are the regulatory relationships among simultaneously active enhancers? What are the consequences of multiple enhancers regulating one gene?

The classic mode of enhancer behavior is described as autonomous, modular, and additive—a feature which has been suggested to confer evolvability to enhancers over other functional parts of the genome (Carroll, 2008) (Figure 5A). Many demonstrations of the modular and additive behavior of enhancers exist, both for elements controlling the same locus across different tissues and for simultaneously active individual elements within a super-enhancer (for example, Hay et al., 2016; Maeda and Karch, 2011; Visel et al., 2009). This relative modularity is thought to confer highly tissue-specific phenotypes of enhancer deletions near pleiotropically expressed genes.

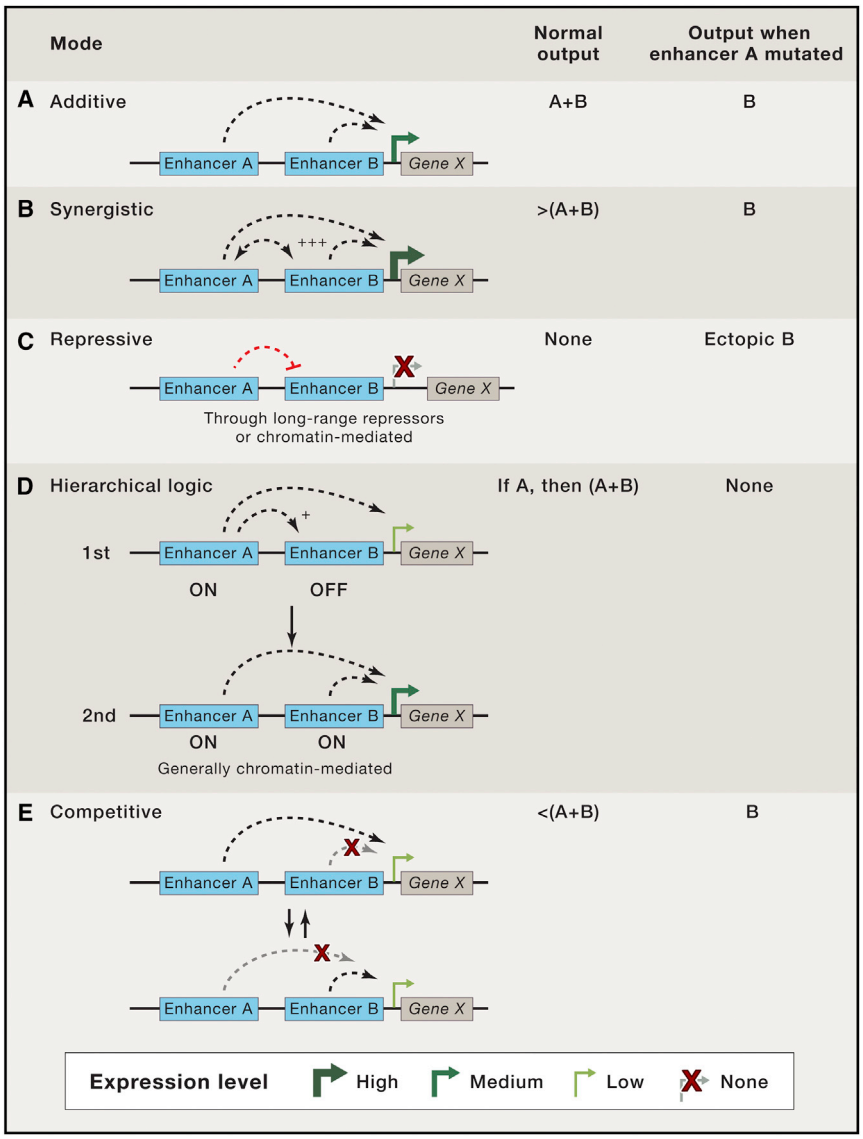

Figure 5. Regulatory Crosstalk between Enhancers Operating within the Same *Cis-Regulatory* Landscape

(A–E) Potential modes of enhancer relationships and their effect on transcriptional output under wild-type or mutant conditions.

In other cases, however, the overall expression pattern of a given gene is different from the sum of the individual activities of each enhancer element. While the pervasiveness of such non-additive interactions between enhancers acting within regulatory landscapes is still unclear, some studies suggest that such interactions may be in fact extremely common. For example, comparison of individual enhancer activity with endogenous gene-expression patterns estimate that the activity of up to ~37% of enhancers may be modulated by their extended endogenous loci in *Drosophila* (Kvon et al., 2014).

The first appreciation of this regulatory complexity came from detailed analysis of the *endo16* regulatory region in sea urchins. Using a quantitative reporter and in situ hybridizations, these studies discovered that some elements within the broader regulatory region were required for the activity of other elements, or could linearly amplify the output of another element by a factor of ~4 (Yuh and Davidson, 1996; Yuh et al., 1998). Since this early proof of principle, multiple studies in other species, including mammals, have documented similar multiplicative or greater-than-multiplicative effects of enhancers acting together to boost expression (for example, Maekawa et al., 1989; Stine et al., 2011) (Figure 5B).

Importantly, enhancers acting together can drive not only quantitative but also qualitative changes in spatiotemporal activity patterns in vivo. A number of reports using transgenes in *Drosophila* and mice have demonstrated that the spatial expression patterns of multiple enhancers tested together can be distinct from the sum of each enhancer tested individually and that this interplay is required to recapitulate the endogenous expression pattern of their target genes (examples include Dib et al., 2011; Dunipace et al., 2011; Prazak et al., 2010). Interestingly, one recurrent observation from such studies is that often the effect of multiple enhancers acting together is not to generate novel activity domains but to prevent ectopic expression outside of its proper context. Several careful examples of this in *Drosophila* have been described, one being the restriction of gap-gene-expression patterns to proper contexts by specific enhancers near *hunchback* and *knirps* (Perry et al., 2011). Similarly, synergistic enhancers restrict aberrant activity domains at the murine *Fgf8* locus (Marinić et al., 2013). One suggested mechanism for this behavior is through the recruitment of long-range repressors, which suppress the activity of the neighboring enhancer in ectopic regions (Dunipace et al., 2011; Perry et al., 2011) (Figure 5C). Thus, it appears that fine-tuning of gene activation patterns in vivo is in part mediated by repressive influences between neighboring regulatory elements.

In some cases, *cis*-regulatory landscapes appear to follow a more complex hierarchical logic. A classic example of this is seen at the *Drosophila* bithorax complex (BX-C), responsible for the restricted expression of Ubx, abd-A, or Abd-B along the fly's A-P axis. A shared ~300 kb regulatory region for the BX-C is divisible into nine parasegment-specific chromosomal domains, with each domain shown to control the activation of one of the three BX-C homeotic genes in a pattern appropriate for that segment (reviewed in Maeda and Karch, 2011).

Interestingly, while each domain contains several regulatory elements, generally only one or two of these enhancers is limited in activity along the A-P axis to their correct parasegment, while the rest drive activity in tissues along the entire axis when tested alone. These elements with limited activity were termed "initiator" elements, as they were proposed to read the appropriate parasegmental address and determine if the surrounding regulatory domain is to be altogether active or silenced (Iampietro et al., 2010; Mihaly et al., 2006). Importantly, when initiator elements are switched between domains, it results in a homeotic transformation (Iampietro et al., 2010), demonstrating that the initiator is sufficient to coordinate the various enhancers within the domain. While such a well-characterized domain-restricted system hasn't been discovered in mammals, some examples of hierarchical logic at enhancers have been described, such as a conditional relationship between two enhancers near the PU.1 locus in mouse myeloid cells where an upstream regulatory element (URE) directly initiates the activity of a nearby enhancer (the "-12 kb enhancer"), possibly through a chromatin-mediated mechanism (Leddin et al., 2011) (Figure 5D).

Enhancer Redundancy and Competition

Another mode of non-additive behavior in *cis*-regulatory regions is enhancer redundancy, with functionally redundant enhancers referred to as "shadow enhancers" (reviewed in Barolo [2012]), as coined by Mike Levine and colleagues to describe "remote secondary enhancers mapping far from the target gene and mediating activities overlapping the primary enhancer" (Hong et al., 2008). Importantly, under this definition, shadow enhancers are redundant only in that they have overlapping activity patterns and are not necessarily functionally identical. For example, despite having overlapping activity, shadow enhancers may have different temporal dynamics or may serve to fine-tune temporal or spatial gene-expression boundaries (see below). Interestingly, shadow enhancers may also confer robustness against environmental or genetic variability (also known as canalization), since mutation of a shadow enhancer can remain cryptic under normal conditions but be revealed under stress conditions (Frankel et al., 2010).

A potential mechanism for enhancer redundancy would be a competition model, where two enhancers compete for association with a shared promoter, buffering individual enhancer activity to facilitate a constant transcriptional output (Figure 5E). In this model, strongly activated enhancers would function sub-additively, while weaker enhancers would be expected to function in a more additive manner as a consequence of lower rates of promoter competition. Live monitoring of transcription at the *hunchback* and *snai* loci in *Drosophila* suggests that enhancer pairs operate in exactly this sub-additive manner when they are strongly activated due to competition for the target promoter (Figure 5E) (Bothma et al., 2015). This model could explain phenotypic robustness (or canalization) in a population, as loss-of-function polymorphisms in one enhancer would be compensated for by increased frequency of promoter contacts of the remaining enhancer, resulting in maintenance of normal expression levels of the target gene. In addition, this enhancer competition model could also create sharp boundaries of gene expression during development through the recruitment of long-range repressors. For example, interrogation of the primary and shadow enhancers at the *knirps* and *Kruppel* loci suggests that they act additively at the center of an expression domain but can become dominantly repressive in the posterior regions, facilitating establishment of a sharp expression boundary (El-Sherif and Levine, 2016). Therefore, enhancer competition can buffer against fluctuations in gene expression levels in the "on" state, yet impart sharp on/off boundaries at the edges of expression domains when combined with tissue-specific recruitment of repressors.

The ability of enhancers to cooperate or compete adds an additional layer of complexity to understanding how regulatory landscapes evolve. Redundant "shadow" enhancers, for example, may facilitate accumulation of neutral mutations, which may then be unmasked during times of environmental stress, conferring "evolvability" to the species by increasing the phenotypic diversity to allow more rapid selection to a changing fitness landscape. In contrast, cooperative enhancers may experience weak negative selection to co-evolve across evolution, with potential positional constraints to remain within the same topological domain or to retain enough space to minimize short-range cross-repressive interactions (Dunipace et al., 2011). Consequently, changes in synergistic relationships may be a source of evolutionary innovation that cannot be easily captured by conventional sequence comparisons and will require more complex functional analyses to detect systematically, such as genetic perturbation screens to manipulate function of enhancers in their native chromosomal context.

Concluding Remarks and Future Perspective

As ever, progress in our mechanistic understanding of gene regulation has thrown into relief the remaining gaps in our knowledge and opened up many new questions regarding enhancer function. First, despite identification of a huge number of putative enhancer sequences, our understanding of the importance of orientation, spacing, and copy-number of TFBSs for enhancer function remains rudimentary. Efforts to engineer synthetic enhancers have made strides toward unpicking the enhancer lexicon; however, thus far, they had limited combinatorial power, were often performed in an episomal context, and were designed without much appreciation for the distinct sequence preferences of TFs in cooperative versus individual binding contexts. Given that (1) the sampling size for potential enhancer sequences is so large, (2) multiple distinct sequences may confer equivalent activities, and (3) the local regulatory context may alter enhancer output, uncovering a universally predictive set of rules for enhancer grammar, activity, and specificity in different tissues will be a major challenge for future research.

Chromosomal conformation studies (3C and derivatives) have recently provided key insights into long-range regulation by showing that most metazoan genomes are partitioned into TADs, which delimit boundaries of self-interacting chromatin and thus organize regulatory landscapes. However, 3C-type approaches also have significant limitations, as they involve chromatin crosslinking, are typically performed on the population level, and there are associated challenges in normalizing the data to reflect realistic background models. As a result, not only may the extent to which enhancers and promoters form "loops" have been overestimated, but also the kinetic

information underlying enhancer activation at a single-cell level has been lost. Orthogonal approaches, including high-resolution imaging of fixed cells, has already provided some insight into the dynamics of enhancer-promoter interactions (Giorgetti et al., 2014; Fabre et al., 2015; Williamson et al., 2016). We anticipate that future live-cell imaging approaches will further help to address how the information from enhancers is dynamically transduced to promoters to allow for precise activation of transcription.

It recently became clear that the majority of genetic variants associated with human complex disease or normal range trait variation maps to the non-coding parts of the genome (reviewed in Tak and Farnham [2015]). A substantial fraction of such variation is thought to modulate *cis*-regulatory element function, and a number of examples of disease-associated non-coding mutations that ablate or change enhancer function or impact the folding of topological domains have been identified (reviewed in Scacheri and Scacheri [2015]). Indeed, genetic variation within the human population coupled with quantitative epigenomic approaches can be leveraged to link sequence changes to chromatin-state divergence both locally and distally within interacting chromosomal regions, providing an avenue for the interpretation of GWAS studies and future investigation of mechanisms underlying disease traits (Grubert et al., 2015; Waszak et al., 2015). Similarly, evidence has accumulated that enhancer sequence changes mediate morphological divergence between species, and "cellular anthropology" approaches utilizing pluripotent stem cells from great apes have enabled investigation of recent hominid *cis*-regulatory landscape evolution in developmentally and evolutionary relevant cell types (Prescott et al., 2015; Gallego Romero et al., 2015). Further understanding of the contribution of enhancer sequence variation to human disease susceptibility, normal range variation, and evolutionary innovation will likely soon come from human genetics and follow-up functional studies in cells and model organisms. A key challenge will be to understand how combinations of such regulatory variants make us uniquely human and uniquely individual.

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