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Evolution of modular and pleiotropic enhancers

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Abstract

Cis-regulatory elements (CREs), or enhancers, are segments of noncoding DNA that regulate the spatial and temporal expression of nearby genes. Sometimes, genes are expressed in more than one tissue, and this can be driven by two main types of CREs: tissue-specific "modular" CREs, where different CREs drive expression of the gene in the different tissues, or by "pleiotropic" CREs, where the same CRE drives expression in the different tissues. In this perspective, we will discuss some of the ways (i) modular and pleiotropic CREs might originate; (ii) propose that modular CREs might derive from pleiotropic CREs via a process of duplication, degeneration, and complementation (the CRE-DDC model); and (iii) propose that hotspot loci of evolution are associated with the origin of modular CREs belonging to any gene in a regulatory network.

KEYWORDS
evo-devo, enhancers, CREs, evolution, modularity, pleiotropy

1 | INTRODUCTION

Increases in genomic size and complexity in organisms is largely due to duplication of genes or stretches of DNA (Martin, 1999). This happens due to errors in DNA replication or due to multiple insertions of foreign DNA sequences like transposable elements (TE). These duplicated and redundant sequences can then follow a few different fates. Some duplicated sequences may accumulate mutations and become nonfunctional pseudogenes, nonfunctional TEs, or nonfunctional regulatory sequences, or they may evolve a different function (Martin, 1999; Ohno, 1970; Wagner & Lynch, 2010). In the case of genes coding for proteins, duplication followed by diversification of the gene's sequence can give rise to diverse protein families where each family member has a slightly different function (Dulai et al., 1999; Hughes, 2005; Lynch & Conery, 2003; Martin, 1999; Ohno, 1970). Currently, however, it is unclear whether the same mechanism, of duplication followed by diversification, is involved in the origin of modular, tissue-specific *cis*-regulatory elements (CREs) that are found in the vicinity of many genes.

Views on the architecture of gene regulation and on the modularity of CREs have been gradually changing. Early views of gene regulation suggested that genes that were expressed in distinct tissues or traits were largely driven by distinct modular enhancers, each responsible for driving the gene's expression in a single location (Prud'homme et al., 2007). For instance, three independent enhancers of the gene *yellow* were identified early on, each required to drive the gene's expression in the epidermis of the wing, in the body, or in bristles (Geyer & Corces, 1987; Jeong et al., 2006; Martin et al., 1989; Wittkopp et al., 2002). A variety of more recent studies, however, have come to conclude that most CREs are not modular at all (Kalay, et al., 2019; Xin et al., 2020). For instance, more recent work on *yellow*, determined that most regulatory sequences tested with reporter constructs can drive the expression of *yellow* in more than one body structure, that is, the CREs are largely pleiotropic (Kalay, et al., 2019). Furthermore, a variety of studies have also shown that genes have many redundant regulatory sequences, each sequence driving gene expression in the same location or trait (Barrio et al., 1999; Bomblies et al., 1999; Hong et al., 2008; Monteiro & Podlaha, 2009; Thompson, et al., 2018). For instance, different regulatory sequences of *yellow* can drive gene expression in the same tissues, such as the wing epidermis, or the abdomen (Kalay, et al., 2019). Recent work identified multiple pleiotropic enhancers for the gene *optix* in a butterfly which are all essential for the

development of a variety of traits where *optix* is expressed (Lewis et al., 2019). This observation is different from the multiple redundant enhancers observed for the gene *shavenbaby* (*svb*) in *Drosophila* and for genes involved in limb development in mammals, where knocking out single enhancers of those genes did not produce any strong phenotypic effects (Frankel et al., 2011; Osterwalder et al., 2018). How modular, pleiotropic, and redundant or essential CREs originate, however, is still largely a mystery, and a testable model of CRE evolution is lacking in the field of gene regulation.

In this perspective, we will review several hypotheses proposed for the origin of pleiotropic, modular, and redundant CREs, and propose an evolutionary framework to test how these different types of CRE evolved. In addition, we also discuss how the origin of modular CREs might be related to the appearance of hotspot loci in genomes, where most evolutionary change for particular traits is concentrated.

2 | EVOLUTION OF PLEIOTROPIC ENHANCERS VIA GENE-NETWORK CO-OPTION

A simple mechanism that converts a modular CRE into a pleiotropic CRE occurs as a by-product of gene-network co-option. For instance, when a single top regulatory gene, involved in the development of one trait, becomes expressed in a novel developmental context, i.e., at a different developmental stage or in a different tissue, some of its downstream targets might also become expressed in the new context. The expression of the target genes in the new context will be mediated via the same CREs or enhancers that were responsive to the top regulatory gene in the ancestral context. As a result, the CREs of these target genes become pleiotropic, i.e., they become functional in the development of two traits (Glassford et al., 2015; Monteiro & Podlaha, 2009; Murugesan et al., 2022). This is a passive process that does not involve any molecular evolution at any of these CREs and we call it *passive transpleiotropy*. If this process of GRN co-option happens multiple times over the course of evolution, and keeps involving all or part of the same GRN, this can lead to the gradual accumulation of functions to single CREs in hundreds of genes in regulatory networks, that functioned initially as "modular" tissue-specific CREs (Figure 1).

Both gene and CRE pleiotropy affecting two or more traits might constraint evolution from optimizing the function of the gene, or the expression of the gene, for each of the traits independently. Mutations in those sequences will have effects on multiple traits simultaneously, which might not be ideal. These sequences, thus, might come under strong purifying selection and might not be able to evolve very much at all. Breaking this pleiotropy via genomic duplications, for instance, can allow the evolution and diversification of each protein copy or regulatory sequence copy to be shaped for each trait independently. This process has been extensively investigated for protein family evolution (Hughes, 1994; Konaté

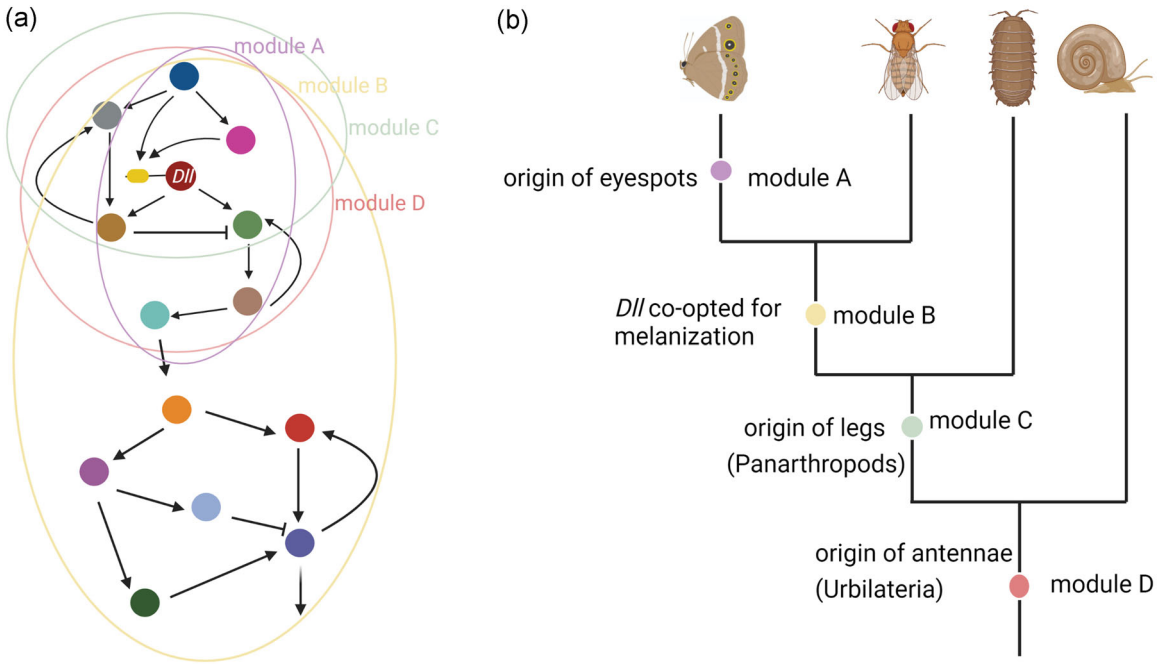

FIGURE 1 Hypothetical scenario for the evolution of pleiotropic enhancers via gene network co-option with *Dll* as an example. (a) Four submodular gene regulatory networks, each containing *Dll* in its core, are co-opted to aid in the development of novel traits (based on the results of Murugesan et al. 2022). Each time *Dll* is co-opted, as part of these larger GRN co-option events, the same original *Cis*-regulatory element (CRE) (yellow ellipse next to the *Dll* gene that responds to the blue and pink inputs) will drive *Dll* in a novel expression domain that will add a novel function to the CRE and make it pleiotropic. (b) Phylogenetic tree highlighting the origin of different traits all expressing the gene *Dll*

et al., 2019; Weiner, et al., 2006), but we currently lack a similar evolutionary framework for CRE evolution.

3 | EVOLUTION OF MODULAR CRES VIA THE CRE-DDC MODEL

One way for pleiotropic CREs to become modular, allowing for specialized regulation of gene expression across different developmental contexts, is via the process of duplication, degeneration, and complementation (DDC) (Monteiro & Gupta, 2016). The CRE-DDC model closely follows the DDC model for gene duplicates that proposes that most genes with modular enhancers should be retained at higher rates in genomes, after the whole locus duplicates, relative to genes without such modularity (Force et al., 1999). The DDC model, however, does not address how multiple modular enhancers for genes evolve in the first place, which is addressed by the CRE-DDC model.

In the CRE-DDC model, ancestral single pleiotropic enhancers regulate the same gene in multiple tissues or traits. After a CRE duplication event, the various CRE copies might subfunctionalize their ancestral pleiotropic enhancer activity. This happens if each copy evolves mutations that allow the enhancer to function in just a subset of the original developmental contexts, and where all copies, together, complement each other and continue to drive gene expression in all the ancestral contexts (Figure 2). This sub-functionalization of CRE duplicates can be driven via purely neutral processes. Once a duplicate CRE can no longer regulate its associated gene in a particular developmental context, natural selection ensures that the other CRE copy remains functional in that context, and vice versa. Alternatively, one of the duplicate CREs might gain a new function, that is, drive gene expression in a novel domain, and become modular in this manner (Figure 2). Once CREs achieve higher levels of modularity, and become active in fewer developmental contexts, further mutations in those CRE, and natural selection, can lead to optimized gene expression for those developmental contexts alone.

There are two other possible outcomes of a pleiotropic CRE duplication that will not achieve either CRE subfunctionalization or CRE modularity. Duplicate enhancers may be retained without subfunctionalization because they might increase a gene's expression or increase robustness in gene expression in the face of environmental challenges Frankel, et al., 2010. Alternatively, if each copy of a pleiotropic CRE accumulates degenerative mutations that lower levels of gene expression across all developmental contexts, then both CREs might become subfunctionalized for total expression levels alone, rather than spatial expression domains. This would be a type of quantitative rather than qualitative subfunctionalization of two CREs that would not lead to CRE modularity nor to CRE redundancy.

To test whether this CRE-DDC mechanism is responsible for the origin of modular CREs it is necessary to first identify orthologous

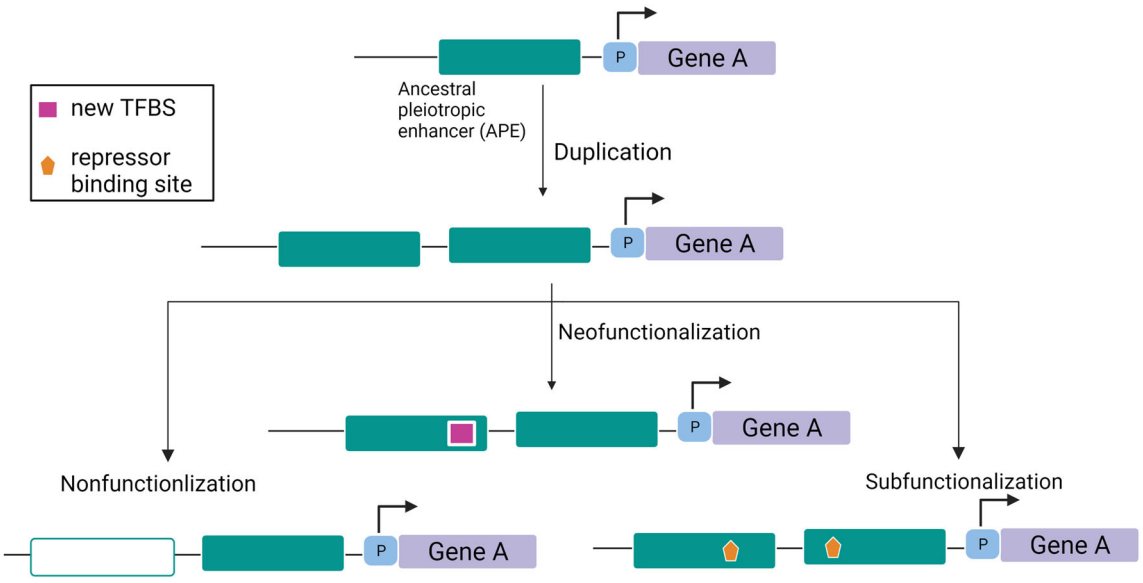

FIGURE 2 Schematic representation of the CRE-DDC Model. In this model ancestral pleiotropic CREs undergo duplication followed by one of three possible fates. Nonfunctionalization—where the duplicated CRE loses its function via the accumulation of degenerative mutations (loss of activating TFBSs or gain of repressive TFBSs). Neofunctionalization—where one of the duplicated CREs gains binding sites for new TFs that drive it in a new developmental context. Subfunctionalization—where each of the duplicated CREs evolves modularity. Each CRE will either each lose some TFBS required for the expression of the gene in a single developmental context, or gain repressive TFBSs (depicted) that allow each CRE to function in only a single developmental context. These two new CREs will now complement the function of the original pleiotropic CRE (modified from Monteiro & Gupta, 2016). CRE-DDC, *Cis*-regulatory element-duplication, degeneration, and complementation; TFBS, transcription factor binding sites

CREs in different lineages on a known phylogeny, before and after a duplication event, and to test the function of those CREs. There should be sequence similarities between duplicate CREs, at least initially, right after the duplication event. However, the accumulation of mutations over time, and developmental systems drift, can quickly render paralogous and homologous CREs (within and between species) unrecognizable (Snetkova et al., 2021; True & Haag, 2001; Wong et al., 2020). To overcome this problem, and to first identify possible CRE duplication events, it might be necessary to examine the function and the expression of candidate orthologous/paralogous CREs rather than their sequences (see Thompson, et al., 2018). Then, from this data collected at the tips of the tree, we need to infer each CRE's expression and function in the ancestors of the species examined. This exercise should indicate whether ancestral CREs are more modular, or more pleiotropic, than derived CREs. The hypothesis under the CRE-DDC model is that ancestral CREs should be more pleiotropic relative to derived CREs, which should be more modular and drive gene expression in a more trait/tissue-specific manner. When mutated, these derived CREs should affect the development of fewer traits relative to the inferred function of ancestral CREs.

Comparative research examining the evolution of *yellow* regulation largely supports the CRE-DDC model. *Yellow* is involved in the melanin biosynthesis pathway in a variety of insect species (Popadić & Tsitlakidou, 2021). GFP reporter assays performed with complete intronic and 5′ intergenic sequences of *yellow*, from six different species of *Drosophila* in the transregulatory environment of *D. melanogaster*, revealed that these regions contained CREs regulating the gene in body, wings, wing veins, and bristles of all six species. The CREs of some species drove GFP expression in more traits/tissues than the CREs of other species. Phylogenetic reconstruction of the ancestral regulatory function of these sequences inferred that the ancestral enhancers of *yellow* were more pleiotropic relative to the more derived enhancers (Kalay & Wittkopp, 2010; Kalay, et al., 2019) (Figure 3).

The gene *shavenbaby* (*svb*) also provides a possible study system for testing the CRE-DDC model. This gene regulates, among other traits, the development of hair-like structures, called trichomes, present along with stripes in the epidermis of first instar larvae of the genus *Drosophila*. Seven orthologous enhancers of *svb* have been identified across several *Drosophila* species (Frankel et al., 2011; Kittelmann et al., 2021). Some enhancers drive *svb* expression in unique parts of the stripes, whereas a few are redundant and drive *svb* expression in the same general stripey areas. *D. sechellia*, a close relative of *D. melanogaster*, lost its dorsal-lateral trichomes due to loss of function of some of its enhancers (Frankel et al., 2011; Kittelmann et al., 2021; McGregor et al., 2007; Noon et al., 2018). This suggests that genetic changes in these derived (duplicate) enhancers of *svb*, presumably more modular than the single hypothetical ancestral pleiotropic enhancer, were targeted by natural selection, which is a prediction of the CRE-DDC model (Figure 4). In other tissues, like pupal epidermis and larval foregut, the seven enhancers drive a similar reporter gene expression pattern (Noon et al., 2018). This suggests that all seven enhancers might have had a common origin, as a single pleiotropic enhancer, as predicted by CRE-DDC model (Figure 4), and that some of the duplicate copies were able to subfunctionalize some of the original functions.

The origin of the multiple enhancers of both *yellow* and *svb* remains unclear. According to the CRE-DDC model, single CREs duplicate to give rise to new CREs. Once duplicate CREs originate in the genome, each copy is free to follow a process of degeneration, neofunctionalization, or subfunctionalization via purely neutral processes. However, since the number of CREs observed around these two genes across *Drosophila* species is conserved, the CREs duplication hypothesis needs to be tested with deeper sampling of additional fly genera or other insects from a different order.

4 | ACTIVE *CIS*-PLEIOTROPY VIA NOVEL TFBS

Another way modular, tissue-specific enhancers, can arise is via de novo evolution from non-regulatory sequences, or via modification of pre-existing enhancers via the loss or gain of new transcription factor binding sites (TFBSs). The latter mechanism is a possibly shorter route to achieving a functional enhancer because a pre-existing enhancer might already have important sequences (i.e., binding sites for pioneer factors) that lead to open chromatin (Fuqua, et al., 2020). If the modified enhancer gains a novel function/expression domain, while retaining its old function/expression domain, this would be an example of enhancer *site modularity* (*sensu* Noon et al., 2018; Sabarís et al., 2019). This term was coined to describe a pleiotropic enhancer that would encode two modular regions, each using distinct sets of TFBSs, either next to each other, or interspersed with each other, in the same general genomic region. A set of TFBSs would drive gene expression in context one, and a different set of TFBSs would drive gene expression in context two. These two sets of TFBS would be independent of each other in regulating the gene (Sabarís et al., 2019). We choose to name this type of pleiotropy, *active cis-pleiotropy*, to focus more on the mechanism that generates the pleiotropy and to distinguish it from the previous *passive trans-pleiotropy*. Active *cis*-pleiotropy and passive *trans*-pleiotropy can both take place in the evolution of an enhancer. A single enhancer might first become pleiotropic passively and then acquire the ability to drive flanking genes in a novel expression domain through the gain (or loss) of TFBSs, making it active in an additional context. Molecular evolution (in *cis*) at the enhancer proper would be required to allow this enhancer to gain the novel expression domain. The occurrence of both mechanisms can also be teased apart on a phylogeny as long as speciation events happened before the CRE evolves the novel expression domains.

The *yellow* spot enhancer in *D. biarmipes* emerging from an ancestral wing blade enhancer is touted as an example of a

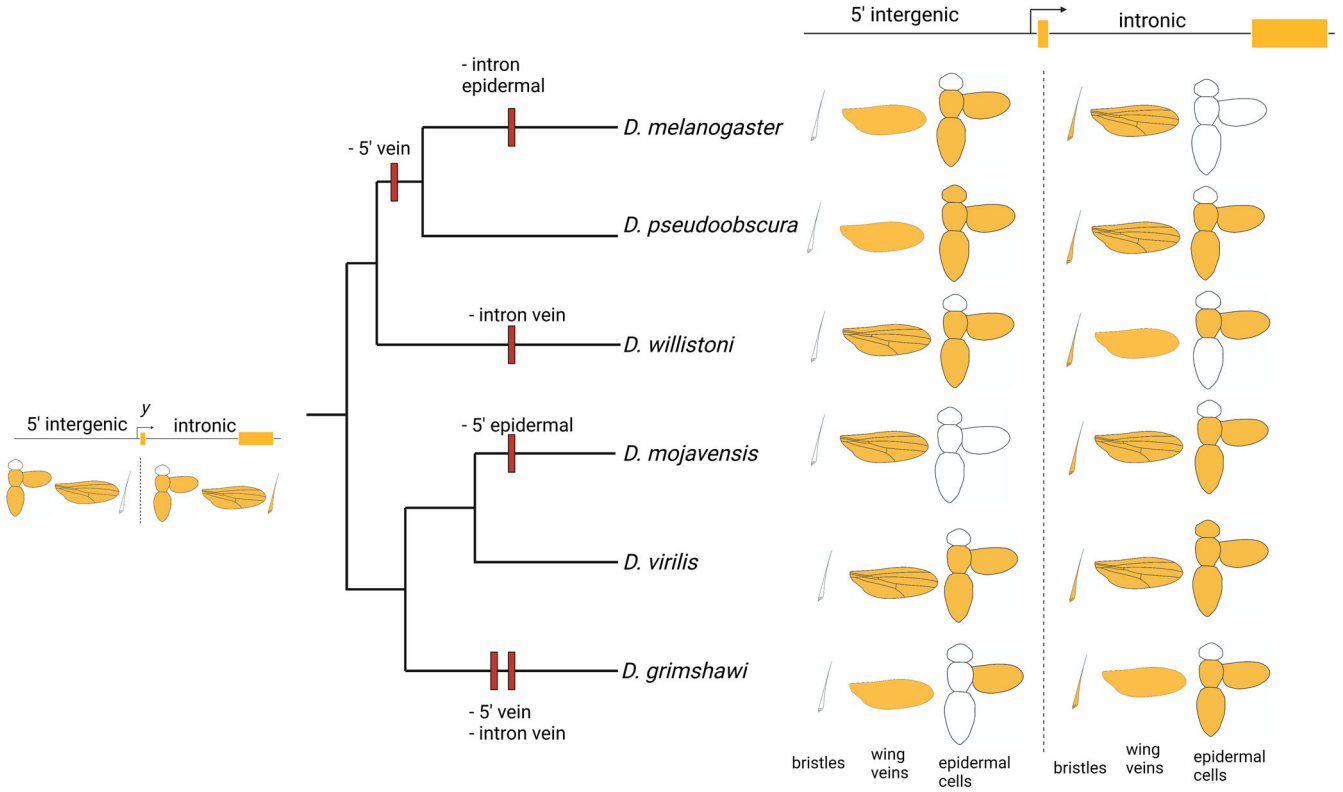

FIGURE 3 Schematic representation of the patterns of *yellow* expression driven by the complete 5′ intergenic and intronic regulatory sequences in six species of *Drosophila*. The ancestral reconstruction of the function of each of these regulatory sequences, suggests the presence of two pleiotropic sequences. One driving expression of *yellow* in epidermal cells in the body and in wing veins (the 5′ intergenic region), and another driving expression in the epidermal cells, wing veins, and bristles (the intronic region). The loss of tissue-specific expression associated with each of these regulatory regions is mapped in the phylogeny by red bars. The regulatory sequences are becoming more modular and tissue specific over the course of evolution. For example, in the case of *D. grimshawi*, the 5′ intergenic enhancer retained expression only in the wing epidermis and lost expression in all other tissues (reproduced from Kalay & Wittkopp, 2010)

pleiotropic enhancer that evolved pleiotropy via active *cis* evolution. This enhancer is speculated to have evolved a novel expression domain (in a single wing spot at the apex of the wing) via changes in its sequence (Xin et al., 2020). However, it is equally possible that these changes merely strengthened the expression of *yellow* at that location, not that they were required to drive *yellow*, and melanization, in that novel wing region. It is possible that this enhancer became pleiotropic via the passive mechanism of GNR co-option first, taking place at the base of the melanogaster group. This co-option involved the *yellow* gene as a terminal effector gene of a GRN that contained the essential upstream regulator of *yellow*, the gene *Distal-less* (Arnoult et al., 2013). This alternative scenario would not involve any initial molecular evolution at *yellow in D. biarmipes* to bring about spots (Monteiro & Gupta, 2016). The enhancer would have arisen first as a passive *trans*-pleiotropic enhancer, that later gained additional mutations, in *cis*, to increase the expression of *yellow* in the spot region of the wings of *D. biarmipes*.

The example above highlights the importance of developing an experimental framework that can distinguish the original mechanism that generates pleiotropy at the level of an enhancer, from subsequent mechanisms that may simply modulate levels of gene expression at the novel location. This experimental framework should primarily be able to distinguish pleiotropy arising via the reuse of a pre-exiting CRE in a novel trait with no *cis*-evolution (passive *trans*-pleiotropy), from pleiotropy arising from *cis*-evolution in a pre-existing CRE that allows the CRE to drive the gene in a novel developmental context (active *cis*-pleiotropy) (Figure 5a).

Most of the current experimental approaches try to distinguish passive from active pleiotropy by dissecting the enhancers into small bits of DNA sequences and testing whether the smaller bits are able to drive GFP expression in distinct tissues or traits (Glassford et al., 2015; Rastogi & Liberles, 2005; Trizzino et al., 2016). This experimental approach tries to test whether site modularity, where the TFBS are next to each other, rather than interspersed with each other, is present. Often this is not the case. There are usually some TFBSs that appear to be required for the enhancer to drive gene expression in both traits (Erickson et al., 2015; Jackman & Stock, 2006). A problem with this approach is that dissecting the enhancer into small pieces also affects the activity of the individual sites, as compared with

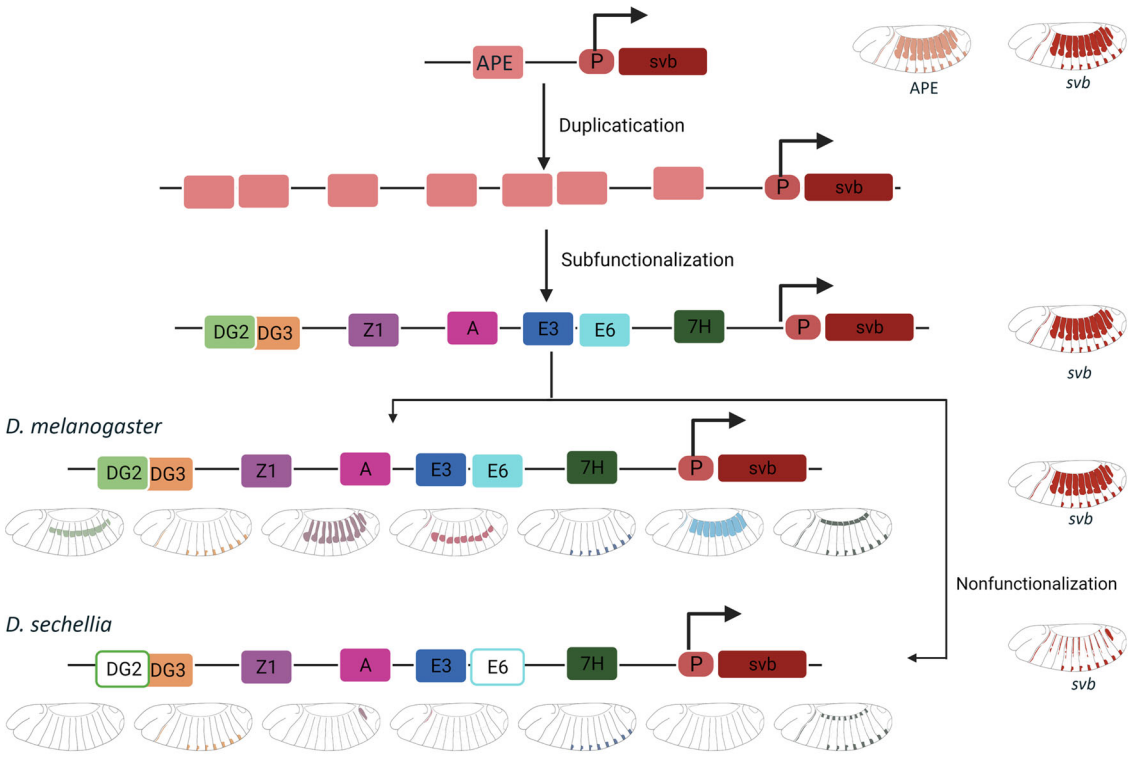

FIGURE 4 CRE-DDC Model for *svb* enhancers. A single ancestral pleiotropic enhancer (APE) regulating *svb* expression in the early embryo (and potentially other tissues) is duplicated in a derived lineage resulting in subfunctionalization of the APE. Following the subfunctionalization, which is maintained in *Drosophila melanogaster*, some of the new "modular" CREs lose their function in the sister lineage *D. sechellia*, resulting in the loss of *svb* expression in particular sections of the embryo. CRE-DDC, Cis-regulatory element-duplication, degeneration, and complementation

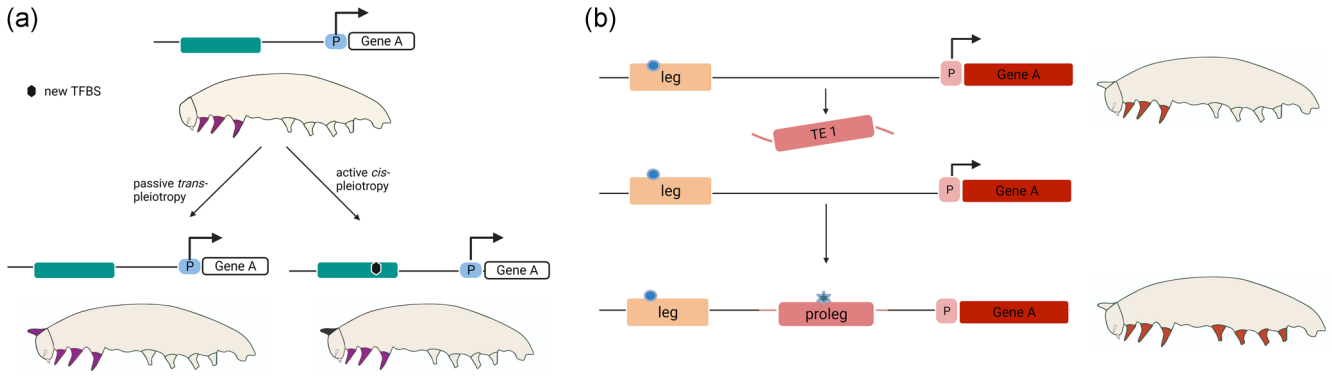

FIGURE 5 Types of enhancer pleiotropy and TE-based origin of enhancer. (a) In an ancestral state, *Gene A* is regulated by a single enhancer in thoracic legs. In this hypothetical example, head horns derive from the co-option of the leg GRN to the horn region. The enhancer of *Gene A* is reused in the horn tissues via a passive process and becomes pleiotropic (passive *trans*-pleiotropy). Alternatively, the enhancer evolves new TFBSs allowing it to be expressed in horn tissue. The enhancer becomes pleiotropic via an active process of *cis*-evolution (active *cis*-pleiotropy). (b) *Gene A* is expressed in legs, but after a TE insertion, it gains a novel enhancer enabling *Gene A* to become expressed in prolegs. TE, transposable elements

testing a full enhancer, even in their own native environment (Gompel et al., 2005; Kalay et al., 2019). Another larger problem, however, is that most of the enhancer constructs (enhancers attached to GFP) are tested in species that do not carry the novel trait (e.g., wing spots) that might have made the enhancer pleiotropic in the first place (via GNR co-option) (Gompel, et al., 2005; Prud'homme et al., 2006; Xin et al., 2020). This means that the transregulatory environment necessary for an ancestral enhancer to become passively active in the location of a novel trait may not be available.

An alternative and perhaps more productive way to address the mechanism that led to the origin of pleiotropic enhancers is based on the *yellow* enhancer work in *Drosophila* and the origin of novel wing spots (Arnoult et al., 2013). It involves a comparative transgenic study where orthologous enhancers from basal lineages, that don't have wing spots, are expressed in species with spots, where the trans-environment essential for a potential passive enhancer to become active in the novel trait is present. If the enhancer from a basal lineage without the novel trait can drive the expression of GFP in the novel region, in the transregulatory environment of the species with the trait, this means no *cis*-regulatory evolution is required at the enhancer for it to drive gene expression in the novel trait. This would indicate that the enhancer in the focal species with the novel trait became pleiotropic though a passive process of GRN co-option. If the orthologous enhancer cannot drive GFP in the novel region, this means that *cis*-regulatory evolution in the enhancer of the focal species was required for it to acquire the novel expression domain (active *cis*-pleiotropy). Arnoult et al. (2013) produced a version of this experiment where they showed that *cis*-regulatory evolution in a *yellow* CRE was necessary for *yellow* to respond to Distal-less in *D. biarmipes*, a lineage that evolved spot patterns at the tips of its wings. However, it is still unclear whether the spot expression, per se, required specific *cis*-regulatory changes in the CRE beyond those that were required to drive *yellow* both across the whole wing blade and in the spot region. Another way of detecting passive co-option events being responsible for the origin of pleiotropic CREs is through the analysis of TFBS usage. If a pleiotropic enhancer depends on exactly the same TFBSs for its function in two contexts, then, co-option is the likely cause of pleiotropy (Sabarís et al., 2019).

Below we discuss a specific example on how *trans* and *cis*-pleiotropy can be distinguished in the origin of a pleiotropic CRE. The *Distal-less* (*Dll*) enhancer *Dll319* of *Bicyclus anynana* butterflies when disrupted, using CRISPR-cas9, deleted multiple traits including legs, antennae, wings, and eyespots, a novel trait in this lineage of butterflies (Murugesan et al., 2022). This is clearly a pleiotropic enhancer, but was pleiotropy achieved passively or actively? Did this enhancer evolve a novel expression domain (and function) in the eyespots via changes in its sequence or did it become pleiotropic via a passive mechanism, where *Dll* was co-opted to the eyespots as part of a larger GRN co-option event? This enhancer can be validated for the type of pleiotropy using the above-mentioned approach. First, we could try and identify an orthologous sequence to *Dll319* in a species like the Monarch butterfly, *Danaus plexippus*, which does not have the novel trait—eyespots (Figure 6). Then, we could insert this sequence from *D. plexippus* upstream of a reporter gene, such as GFP, and insert the construct into the genome of *B. anynana*, which has the transregulatory environment for eyespots to develop. If the enhancer of *D. plexippus* can drive the expression of GFP in *B. anynana* eyespots, this supports the hypothesis that *Dll* in *B. anynana* is reusing an ancestral CRE for its regulation in a novel location (passive *trans*-pleiotropy). If, on the other hand, the Monarch CRE drives GFP expression in the legs, antennae, wing margin, but not in the eyespot centres, this supports the hypothesis that evolution in the *Dll319* sequence in *B. anynana* was required to drive *Dll* in the novel trait - eyespots (active *cis*-pleiotropy) (Figure 6).

5 | TE MEDIATED ORIGINS OF MODULAR AND PLEIOTROPIC ENHANCERS

Novel genomic insertions of transposable elements (TEs) next to genes is another mechanism that can generate modular, tissue-specific enhancers and also pleiotropic enhancers. TEs are known to play an important role in evolution, and in organismal diversity (Bourque et al., 2018; Chen et al., 2020; Martin, 1999). TE are very versatile at inserting or removing their own DNA sequences from genomes, and these sequences can have regulatory activity (Lynch et al., 2011; Rebeiz & Tsiantis, 2017; Sundaram & Wysocka, 2020). TEs can, thus, function as novel CREs that drive a diversity of genes (next to them) in the same novel trait/tissue or in multiple tissues, depending on the TFBS present within the TE (Chuong et al., 2016; Lynch et al., 2011; Sundaram & Wysocka, 2020; Trizzino et al., 2016). For example: if a TE element with regulatory activity is inserted next to a gene it might drive the gene in a novel spatial-temporal location or tissue (Figure 5b).

6 | EVOLUTION OF ENHANCER MODULARITY AND HOTSPOT LOCI

Many studies have shown that genomic mutations that led to modifications or losses of particular traits (e.g., pigmentation, trichomes, and pelvic fins) are often concentrated at specific loci, called hotspot loci of repeated evolution (Martin & Orgogozo, 2013). These mutations could be both in coding and in noncoding regulatory regions but the mutations that are repeatedly found in natural populations are those that typically have few pleiotropic effects. For example, *Pitx1* functions in the development of jaws, pelvic fins, and the pituitary gland. Disruptions of the *Pitx1* coding sequence have been lethal to laboratory animals due to pituitary gland abnormalities (Domyan et al., 2016; Shapiro et al., 2004; Szeto et al., 1999), whereas deletions of more modular regulatory regions, shown to affect *Pitx1* expression in pelvic fins alone, have been repeatedly observed in natural populations of stickleback fishes (Chan et al., 2010, Shapiro et al., 2004). These observations led researchers to propose that *Pitx1* was a hotspot locus for pelvic fin evolution. A different gene, *bric-a-brac* (*bab*), has also been proposed as a hotspot locus for the evolution of male-specific traits. The gene plays a role in pattern formation along the proximal-distal axis of legs and antennae, and in specifying male-specific traits in lepidopterans and flies (Ficarrotta et al., 2021, Unbehend et al., 2021, Williams et al., 2008). This last function of the gene appears to be connected to changes in the first intron of the *bab* gene, which may contain a CRE regulating a smaller sub-set of this gene's functions. Evolution of male-specific abdominal pigmentation in *D. melanogaster* (Williams et al., 2008), of male specific UV-iridescence patterns in *Colias eurytheme* (Ficarrotta

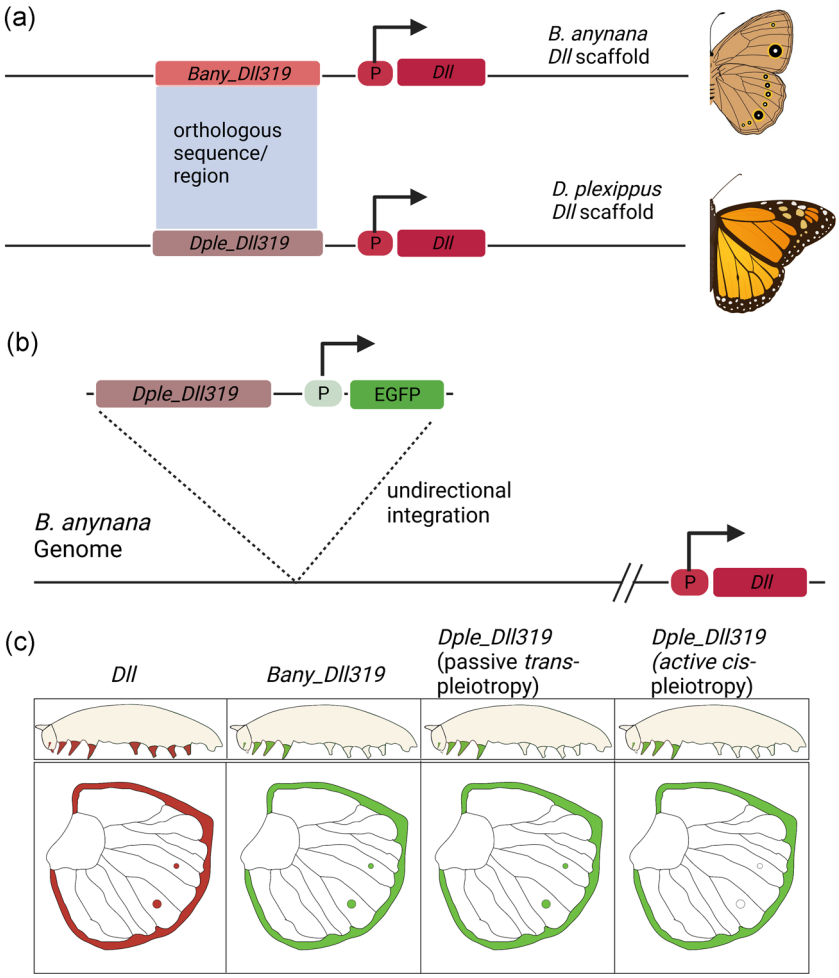

FIGURE 6 Distinguishing the mode of origin of pleiotropic enhancers using reporter constructs. (a) The *Bany_Dll319* is a pleiotropic enhancer that, when disrupted, leads to disruptions of antennae, legs, and eyespots (Murugesan et al., 2022). To identify whether this enhancer became pleiotropic via an active or passive mechanism, we can investigate the function of the orthologous sequence of *Danaus plexippus*, which does not have eyespots, but has legs and antennae. (b) This orthologous region, *Dple_Dll319*, can be placed upstream of GFP, and inserted into the genome of *Bicyclus anynana*. (c) The expression pattern of the *Dll* gene in larval legs, prolegs, antennae, mouthparts, wing margin, and eyespots in *B. anynana*. The predicted expression pattern of GFP based on the CRE knockout phenotypes of *Bany_Dll319* (Murugesan et al., 2022). If the pattern of GFP driven by *Dana_Dll319*, in the trans regulatory environment of *Bicyclus* is found in eyespots as well as other tissues, similar to *Bany_Dll319*, then the *Dll319 Bicyclus* CRE became pleiotropic via a passive process. If the pattern of GFP is observed in legs, antennae, and wing margin but not in eyespots, then the *Dll319 Bicyclus* CRE became pleiotropic via an active process. CRE, *cis*-regulatory elements; GFP, green fluorescent protein

et al., 2021), and of asymmetric male preference towards different female pheromones in the European corn moth, *Ostrinia nubilalis*, all involve changes in this intron (Ficarrotta et al., 2021, Unbehend et al., 2021, Williams et al., 2008).

Stern and Orgogozo (2008) proposed that *svb*, a gene involved in larval trichome development, was a hotspot locus because of its special position in a GRN. This position was in the middle of an "hour-glass" shaped GRN, with *svb* behaving as a master regulator of trichome development. We argue, instead, that hotspot loci can be found anywhere in a GRN. The only pre-condition for a hotspot locus to appear is that it has to have evolved modular enhancers (or modular protein-coding sequences), either de novo or via the neutral CRE-DDC process. We propose that the evolution of modular CREs, or site-modularity within a CRE, is the only pre-condition for the emergence of hotspot loci in regulatory regions of genes. The presence of modular CREs, or site modularity within a pleiotropic CRE, will allow evolution to tinker with these CREs, including removing the expression and function of the associated gene in a trait or tissue-specific manner, without jeopardizing the expression and function of the gene in other traits. For example, the gene *yellow* is considered a hotspot locus of evolution, yet this gene does not have the same position as *sbv* in a GRN. This gene is clearly an effector gene, at the end of a pigmentation GRN, rather than in the middle of an hourglass-shaped GRN. Species of *Drosophila* that lose spots of pigmentation on their wings (Gompel, et al., 2005; Prud'homme et al., 2007), or pigmentation in other areas of their body (Geyer & Corces, 1987; Kalay & Wittkopp, 2010; Kalay, et al., 2019; Wittkopp et al., 2002), have all sustained mutations in the regulatory regions of *yellow*, that contain multiple CREs, with varying levels of modularity.

7 | CONCLUDING REMARKS

In this perspective we highlighted the multiple ways modular enhancers can originate in genomes and proposed an empirical evolutionary comparative approach for how to distinguish among these different modes of enhancer evolution. According to the CRE-DDC model, most enhancers start off as pleiotropic enhancers, via a passive process of GNR co-option, then they duplicate to create redundant enhancers, and finally they degenerate, via purely neutral processes, to create modular CREs. We also make a new proposal—that the origin of enhancer modularity is intimately linked with the origin of hotspot loci. These ideas and models need testing. ATAC-seq that allows the identification of candidate enhancer sequences in a much more targeted manner can be used to identify orthologous enhancers across species (Tissières et al., 2020). Comparative work on enhancer function across a phylogeny should continue to test whether modular CREs evolve primarily from ancestral pleiotropic CREs (Box 1).

BOX 1 – Definitions of key terms used in this manuscript

Cis-regulatory elements (CREs): CREs are noncoding DNA elements present in and around genes that can regulate them, that is, activate or repress gene transcription via the binding of transcription factors.

Co-option: A mechanism whereby a gene is reused to perform a novel function or a CRE is reused to drive a novel gene expression pattern during development.

Enhancers: We use this term interchangeably with CREs.

Modular CRE: A CRE that regulates expression of a nearby gene in a context-specific manner, i.e., in a particular developmental stage or in a particular tissue or cell type.

Pleiotropic CRE: A CRE that regulates expression of a nearby gene in multiple contexts, i.e., across more than one developmental stage or more than one tissue or cell type.

Active *cis*-pleiotropy: A mechanism whereby a CRE becomes pleiotropic via the evolution of novel transcription factor binding sites (TFBS) inside the CRE, which allow a nearby gene to become expressed in a novel context.

Passive *trans*-pleiotropy: A mechanism whereby a CRE becomes pleiotropic via GRN co-option. No evolution at the CRE takes place, but the presence of similar *trans* factors in the novel context allow the CRE to also function in that context.

Hotspot locus: Genomic region that often carries independent mutations in different lineages, causing similar phenotypic variations. Hotspot loci are often associated with the loss of specific traits in different species.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Suriya N. Murugesan and Antónia Monteiro conceived the work and wrote the manuscript.

DATA AVAILABILITY STATEMENT

The article uses no data.

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