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Enhancer redundancy in development and disease

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Abstract

Shadow enhancers are seemingly redundant transcriptional *cis*-regulatory elements that regulate the same gene and drive overlapping expression patterns. Recent studies have shown that shadow enhancers are remarkably abundant and control the majority of developmental gene expression in both invertebrates and vertebrates, including mammals. Shadow enhancers might provide an important mechanism for buffering against mutations in non-coding regulatory regions of genes implicated in human disease. Technological advances in genome editing and live imaging have shed light on how shadow enhancers establish precise gene expression patterns and confer phenotypic robustness. Shadow enhancers can interact in complex ways and may also help drive the formation of transcriptional hubs within the nucleus. Despite their apparent redundancy, the prevalence and evolutionary conservation of shadow enhancers underscore their key role in emerging metazoan gene regulatory networks.

Introduction

Transcriptional enhancers are non-coding DNA elements that are typically 200–2,000 bp in length and drive gene expression patterns in space and time. Enhancers contain numerous binding sites for sequence-specific transcription factors (TFs), which, upon binding to the enhancer, recruit cofactors to activate transcription from a target core promoter. A typical metazoan gene contains multiple cell-type-specific enhancers spread across large genomic distances, which collectively produce a complex gene expression pattern (for general reviews on enhancers see REFS.[1–7]). The classic textbook view is that, within a gene locus, different enhancers drive distinct spatiotemporal aspects of gene expression[1,7]. However, this model is an oversimplification because enhancers regulating the same gene often display overlapping or partially overlapping spatiotemporal activity[8–12]. Examples of such redundant enhancers were often overlooked until 2008 when Mike Levine and colleagues introduced the term “shadow enhancer”[13]. In that study, redundant enhancers were designated either as ‘primary’ (the enhancers closest to the core promoter) or ‘shadow’ (the enhancers located at a greater distance from the core promoter)[13]. This distinction was later revised owing to a lack of functional differences between primary and shadow

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enhancers[14,15]. In this Review, we define ‘shadow’ enhancers as sets of enhancers that regulate a common target gene and drive expression patterns that partially or completely overlap in space and time. This definition has become increasingly accepted in the gene regulation community[14–24]. The degree of overlap required for enhancers to qualify as shadow enhancers depends on what is functionally meaningful in a given biological context. In the early *Drosophila melanogaster* embryo, characterized shadow enhancers typically overlap in more than 50% of their expression domains at a given time point (Table 1). However, in other contexts, such as the nematode nervous system, even an overlap in a single neuron cell can be biologically significant[25].

The existence of shadow enhancers has raised fundamental questions about the purpose and evolutionary origins of this apparent redundancy. In development, multiple mechanisms of regulatory redundancy ensure accurate patterning. Examples include redundant genetic interactions and multiple binding sites for the same TF within an enhancer. Shadow enhancers are increasingly appreciated as another mechanism of redundancy that safeguards against genetic and environmental perturbations. Seminal studies in *D. melanogaster* demonstrated that shadow enhancers improve the precision of gene expression and phenotypic robustness during animal development, especially under conditions of physiological or genetic stress[26–28]. Later work in mammals confirmed that shadow enhancers similarly confer robustness to mammalian development[18,29,30]. Together, these studies suggest that shadow enhancers may be a common mechanism of developmental robustness in animals. Understanding the mechanism of shadow enhancer function will therefore illuminate how multi-enhancer architecture can determine the robustness or fragility of a developmental process to perturbation.

Recent advances in enhancer mapping and novel genetic and imaging tools for enhancer analysis have deepened our understanding of shadow enhancer function and their crucial role in development and human disease. Chromatin and 3D genome profiling by large consortia such as ENCODE, FANTOM, Roadmap Epigenomics and the 4D Nucleome Project have produced genome-wide maps of putative enhancers across many cell types, tissues and time points, both in mice and humans[31–36]. Large-scale transgenic reporter assays have enabled characterization of *in vivo* activity for thousands of *bona fide* enhancers[37–42], revealing an ever growing number of putative shadow enhancers[25,43]. Efficient genome and epigenome editing of enhancers within their native genomic context has enabled analysis of enhancer requirements for organismal function[44–47]. And finally, quantitative live imaging methods have allowed the assessment of shadow enhancer functions in whole embryos[48,49]. These advances have enabled scientists to address key questions in shadow enhancer biology: how common are shadow enhancers in the genomes of different animals? Is there a function of shadow enhancers beyond conferring robustness? How do shadow enhancers work together in the context of the 3D genome? What is the role of shadow enhancers in human disease?

In this Review, we discuss key points that have emerged from these technological advances. We discuss how these advances have provided insight into the prevalence of shadow enhancers in bilaterian genomes and their crucial role in ensuring normal development under conditions of stress. Several lines of evidence suggest that shadow enhancers control the

majority of developmentally regulated genes and are a remarkably widespread feature of bilaterian genomes. Examples in vertebrates show that shadow enhancers are required for normal development under stressful conditions, confirming earlier observations from the *Drosophila* model. We illustrate how the action of multiple shadow enhancers on a single promoter can fine-tune gene expression. We also discuss a potential role of shadow enhancers in organizing 'hubs' of transcriptional activity in the nucleus. We review the evidence that shadow enhancers frequently regulate genes implicated in human disease and buffer against mutations in non-coding regulatory DNA. Lastly, we discuss theories for the origin of shadow enhancers and their unexpectedly high evolutionary constraint. We synthesize mechanistic studies of shadow enhancers in *D. melanogaster* with emerging genetic manipulations of enhancers in mice to provide a cohesive picture of the role of regulatory redundancy in animal systems.

Genomic prevalence of shadow enhancers

Enhancers with redundant activity have been described for more than 30 years, with examples from plants[50,51], flies[8–10,52–56], zebrafish[57], mice[11,12,58,59] and humans[60–63] (Table 1). These individual gene locus studies showed that shadow enhancers are found in a broad set of multicellular organisms, but within a single genome the prevalence of shadow enhancers was unknown. Since these studies were often focused on enhancers that control important developmental regulators, it was also not clear whether shadow enhancers are associated with other classes of genes. Substantial increases in the throughput of enhancer identification and characterization (reviewed in REFS.[1,7,64]) have allowed researchers to determine the prevalence of shadow enhancers genome-wide.

Genome-wide enhancer predictions based on chromatin features, such as chromatin accessibility, histone modifications, and TF binding, have suggested that shadow enhancers might be common in animal genomes. Using a combination of mesodermal TF chromatin immunoprecipitation (ChIP) data and computational models, Cannavo et al. generated an exhaustive catalogue of muscle development enhancers in *D. melanogaster*[16]. They found that nearly two-thirds of examined muscle developmental genes were controlled by shadow enhancers, and the majority of these genes had three or more predicted shadow enhancers[16]. A genome-wide analysis combining ENCODE transcriptomic and epigenomic data from multiple mouse tissues showed ample enhancer redundancy among developmentally regulated genes[29,35]. Whereas housekeeping genes are typically controlled by one enhancer, developmentally regulated genes can have 10 or more shadow enhancers (Table 2).

In human cells, ChIP-based profiling of TFs, cofactors, chromatin regulators and enhancer-associated histone modifications revealed that hundreds of key cell identity genes are regulated by large clusters of putative transcriptional enhancers (super-enhancers and stretch enhancers), which could be clusters of shadow enhancers[65–70]. Many mammalian enhancers including human enhancers are actively transcribed, and the presence of enhancer-derived RNAs (eRNAs) was suggested to be predictive of enhancer activity[31,71,72]. Profiling of eRNAs using cap analysis of gene expression (CAGE) across hundreds of human cell lines and tissues revealed that ~80% of 2,206 examined genes were associated with two or more co-transcribed enhancers[31], suggesting that enhancer redundancy is common in the human

genome. Computational approaches have also found widespread evidence for shadow enhancers in the human genome, particularly in association with developmental and disease-causing genes[73].

Most chromatin and TF profiling methods are based on indirect measures of enhancer activity, which is why they have to be followed by functional testing. Large-scale transgenic enhancer-reporter screens have verified that *bona fide* redundant enhancers are common in the *D. melanogaster* and *Caenorhabditis elegans* genomes. An analysis of nearly 8,000 enhancer fragments during *D. melanogaster* embryogenesis revealed that many developmentally regulated genes are controlled by two or more enhancers with overlapping activities (Table 1)[43]. Single-neuron imaging data from hundreds of enhancer-reporter constructs in *C. elegans* demonstrated that shadow enhancers control nearly all 23 studied pan-neuronal genes[25]. Even within a single cell type, massively parallel reporter assays (MPRAs) have shown that hundreds of genes in *D. melanogaster* cell lines are potentially controlled by two or more redundant enhancers[74]. Taken together, these studies indicate that enhancers driving overlapping expression patterns are common in organisms from worms to insects to mammals and are preferentially, albeit not exclusively, associated with the control of developmental genes.

Shadow enhancers confer robustness

Several early studies in *D. melanogaster* demonstrated that shadow enhancers are required to drive normal development under conditions of stress, but they may be dispensable in 'ideal' conditions. For example, the TF Snail is required for normal gastrulation, and its expression in mesoderm is controlled by two shadow enhancers. Deletion of either of *snail*'s shadow enhancers caused no apparent gastrulation defect[27]. However, individual shadow enhancer deletion led to abnormal gastrulation under elevated temperatures or in a sensitized genetic background where the dosage of an upstream regulator, Dorsal, was reduced[27]. Similarly, a deletion of three of six epidermal shadow enhancers of *shavenbaby* had no phenotype under normal conditions but caused a decrease of trichome numbers under temperature or genetic stress conditions[26].

Advances in genome editing have enabled the efficient introduction of multiple mutations in mice[75–77], enabling experiments to test whether shadow enhancers similarly provide developmental robustness in vertebrates. Whereas single shadow enhancer deletions in mice typically show either mild or no observable phenotypes, double enhancer deletions show severe phenotypes, often comparable to complete gene loss-of-function in relevant tissues[18,29,30,68,78]. Together, these observations indicate that both enhancers regulate the gene, and at least one shadow enhancer is required for normal development in ideal conditions (FIG. 1). Despite driving similar expression patterns, the individual shadow enhancers are not strictly redundant. In a sensitized genetic background with a reduced dosage of the target gene, single enhancer deletions show abnormal phenotypes, indicating that shadow enhancers can confer robustness to genetic perturbations (FIG. 1). This pattern has been demonstrated for *Pax6*, a gene required for early eye lens morphogenesis[18], *Shh* in developing teeth[30] and several limb development loci[29].

Taken together, both fruit fly and mouse studies emphasize that, while ostensibly redundant in the expression patterns they drive, the necessity of shadow enhancers is revealed when enhancer-deficient organisms are placed in stressful conditions. How shadow enhancers provide this robustness remains an area of open investigation and more than one mechanism may be at play. One potential scenario is that each enhancer alone can drive sufficient levels of gene expression for normal development, similar to the haplosufficiency of many developmental genes. By having multiple enhancers the probability that at least one is active increases, improving the chance for normal development[14,24]. A second potential mechanism was suggested by the observation that a pair of *D. melanogaster* shadow enhancers controlling the gene *Krüppel* are regulated by different combinations of TFs[17]. By responding to different sets of TFs, but converging on a single output, shadow enhancers could provide a mechanism to buffer against not only mutations in their sequences but, more importantly, perturbations in one of their upstream TFs (FIG. 2). Experimental measurements show that *Krüppel's* independently controlled shadow enhancers drive lower expression noise than single or duplicated enhancer configurations, suggesting that simple enhancer duplications may not be sufficient to provide phenotypic robustness[79]. Independent regulation of shadow enhancers may be a widespread mechanism to confer robustness, as many mesodermal shadow enhancers are bound by different combinations of upstream TFs[16].

Modes of shadow enhancer interactions

The interactions between shadow enhancers can fine-tune the expression pattern of their target gene. Within an individual cell, shadow enhancers can interact in one of four ways: additively, super-additively (driving more expression than the sum of the individual enhancer activities), sub-additively (driving less expression than the sum of the individual enhancer activities), or repressively (FIG. 3). The classic view of enhancers is implicitly additive, as each enhancer functions independently to build up a gene's total expression pattern[80]. Several studies in the fruit fly embryo used live mRNA tracking of reporter constructs to measure shadow enhancer interaction. Shadow enhancers can act additively, with a pair of shadow enhancers driving expression roughly equal to the sum of the individual enhancers' expression output. For example, such additive behaviour is seen for the shadow enhancers controlling the genes *knirps* and *hunchback*[81]. However, this behaviour can change depending on the cell type or time point because of the varying levels and identities of TFs bound to each enhancer. For example, the *knirps* shadow enhancers act additively at some time points, and super-additively at others, indicating the presence of synergistic interactions between shadow enhancers[81]. The shadow enhancers controlling the mouse *Pomc* gene also show super-additivity at some time points and additivity at others[82]. In the case of the *D. melanogaster hunchback* gene, the behaviour depends on the concentration of its primary activating TF, Bicoid (Bcd). In cells where Bcd is low, the two enhancers combine additively, but in cells where Bcd is high, the enhancers combine sub-additively[81]. Such sub-additive behaviour could indicate the presence of competition between shadow enhancers for promoter occupancy. Sub-additivity has also been observed in the case of strong enhancers in the *Krüppel* locus[83].

In addition to the above interactions, one shadow enhancer can partially or completely repress the other, decreasing or shutting off expression entirely. In *D. melanogaster* embryonic cells at the boundary of the *knirps* and *Krüppel* expression domains, shadow enhancers can repress each other's activity, yielding sharper expression patterns than either enhancer alone[84]. Examples from the *sog, snail,* and *defective proventriculus* loci show some shadow enhancer deletions can lead to higher expression levels, suggesting that one shadow enhancer represses the other[81,85–87]. The mechanisms that explain this repression are still unclear and could include quenching, interference of enhancer–promoter looping, or the spread of repressive chromatin marks.

On the tissue or organismal level, shadow enhancers can interact in nuanced ways to fine-tune both the levels and patterns of gene expression. The way that multiple shadow enhancers interact can vary from cell to cell, depending on the *trans*-regulatory environment. Multiple potential mechanisms may explain the variety of behaviours observed. For example, sub-additive behaviours between two strong shadow enhancers might occur because their target promoter has reached its maximum expression rate or because the enhancers are competing with each other for promoter access[3,81,83]. Super-additive behaviours might arise if there is synergy between the TFs bound at each shadow enhancer[3,88]. Additional experiments that manipulate number of shadow enhancers in a locus or their TF binding content, combined with experiments that probe the molecular details of shadow enhancer function (described below) may further illuminate the mechanisms at play.

Shadow enhancers and nuclear organization

The experiments described in the previous section measured the gene expression driven by shadow enhancers across the entire organism. How do shadow enhancers operate on a molecular level? Enhancers can regulate their target core promoters over long distances, sometimes up to several megabases (Table 1), a process mediated by TFs, co-activators and RNA polymerase II. Many studies observe the establishment of enhancer–promoter interactions coordinately with gene transcription. Various mechanisms and models of enhancer–promoter communication have been proposed, including tracking, linking, looping, and combinations thereof (for general reviews on enhancer–promoter interactions see REFS.[2,4,89,90]). The prevalence of shadow enhancers raises an intriguing question about how multiple enhancers interact with a single core promoter. Do shadow enhancers loop to the target promoter in a coordinated fashion, or is it a dynamic process with multiple enhancers competing for the same promoter (FIG. 4a)? Distinguishing between these possibilities may help illuminate how multiple shadow enhancers combine their activities to specify patterns and levels of gene expression.

Experiments based on chromosome conformation capture provide indirect support for simultaneous promoter activation, as individual shadow enhancers often form contacts between each other and the target gene in the same cell[68,91–94]. These capture experiments were performed in populations of fixed cells and do not reflect the dynamics of enhancer–promoter interactions and transcription from the target promoter. Live imaging of transcription in *D. melanogaster* embryos suggests that a single enhancer can simultaneously

activate two different promoters, even those located on different chromosomes, leading to synchronized transcription bursts[95,96]. Together, these studies suggest that enhancer–promoter loops can include more than two DNA elements. Therefore, it seems plausible that several shadow enhancers could simultaneously coordinate the expression of a single target promoter (FIG. 4a). A direct demonstration of such coordinated expression is challenging as it requires simultaneous labelling of several shadow enhancers and transcription from a target promoter. With the development of new live imaging tools, it may soon be possible to visualize how shadow enhancers activate target promoters in live nuclei[97–100].

The concept of dynamic ‘transcriptional hubs’ (or the related concepts of ‘nuclear microenvironments’ or less dynamic ‘transcriptional condensates’) challenges the simple enhancer–promoter looping model and provides a plausible model for promoter regulation by multiple shadow enhancers[90,101–106]. These large hubs (>300 nm) are formed by TFs, components of the core transcriptional machinery[102,103] and RNA polymerase II[105,107] and may explain why some enhancers activate promoters even in the absence of close enhancer–promoter proximity[108,109]. The hub model suggests that shadow enhancers and their target promoter can simultaneously participate in the same microenvironment, forming a multi-enhancer hub. The observation of transcriptional coactivator condensates on super-enhancer-associated genes provides support for this model[65,102,103] Recent work on the *D. melanogaster shavenbaby* locus showed that deleting one of the shadow enhancers results in decreased local density of the key activating TFs, suggesting that shadow enhancers are critical for maintaining high concentrations of TFs within the transcriptional hub (FIG. 4b)[110]. Through the formation of multi-enhancer transcriptional hubs with high concentrations of TFs, transcriptional coactivators and RNA polymerase II, shadow enhancers may improve phenotypic resilience to stress by buffering against environmental and genetic perturbations.

Shadow enhancers and human disease

Many human genetic disorders are caused by mutations in developmental genes. A strong association of shadow enhancers with developmental genes suggests that enhancer redundancy provides an important safeguard against deactivating non-coding mutations in *cis*-regulatory regions of disease-causing genes (FIG. 5). Indeed, evidence from human genetics studies and experiments in mice suggests that disease-associated genes contain shadow enhancers that likely buffer against the effect of loss-of-function (LoF) non-coding mutations.

A recent study used chromatin profiling data across 127 human tissues from the Roadmap Epigenomic Consortium to calculate an ‘enhancer-domain score’ for each human gene[33,73]. Enhancer-domain scores indicate the amount of redundant regulatory DNA for each gene, based on the total number of predicted enhancers and the redundancy of TF motifs within them. High enhancer-domain scores are predictive of gene pathogenicity, suggesting that the number of shadow enhancers is closely related to the gene’s importance in human disease[73]. This analysis is consistent with previous observations in fruit flies and mice, where important developmental genes tend to have larger regulatory domains[111] and contain more enhancers per tissue[29].

The strong association between shadow enhancers and developmental and disease-associated genes explains why many targeted deletions of enhancers of these genes cause fairly mild phenotypes or no observable phenotypes in mice[12,112–116]. Moreover, deletions of ultraconserved enhancers, which retain almost perfect sequence conservation across vertebrates and are located next to important developmental genes, have also led to viable mice with subtle phenotypes[78,117,118]. With the availability of highly efficient CRISPR–Cas9 genome editing, the number of enhancer knockout mice that lack observable phenotypes has grown[18,29,68,78,119]. These studies further suggest that a significant fraction of LoF mutations in human shadow enhancers will cause relatively subtle phenotypes in patients.

Shadow enhancer buffering predicts that LoF genetic variants in human shadow enhancers would have less severe effects on gene expression and phenotypes than variants in non-redundant enhancers. Indeed, genes with redundant enhancer domains are depleted for common and rare non-coding variants associated with gene expression changes[73]. This pattern indicates that genes with shadow enhancers are more resilient against naturally occurring non-coding mutations in the human population[73].

It remains to be seen whether shadow enhancers also buffer against gain-of-function mutations in enhancers that cause misexpression of disease-associated genes. Studies in *D. melanogaster* showed that one shadow enhancer can repress another shadow enhancer in a dominant fashion[84], suggesting that enhancer mutations causing gene misexpression could, in principle, be buffered by repression from another shadow enhancer. By contrast, both rare and common gain-of-function enhancer variants are associated with congenital malformations[120], heart disease[121], intellectual disabilities[122] and cancer[123–125], potentially through misexpression or upregulation of important developmental genes. In these examples, it is not always clear whether an additional shadow enhancer was also present. Systematic mutagenesis of human enhancers using MPRAs followed by *in vivo* validation in mice will help identify how frequently such gain-of-function mutations affect enhancers[40,126–129].

Evolution of shadow enhancers

Evolutionary origin of shadow enhancers.

Despite their importance, the evolutionary origins of the majority of shadow enhancers are unclear. Like non-redundant enhancers (reviewed in REF.[3]), shadow enhancers may arise by one of several mechanisms: *de novo* from existing non-coding DNA, duplication of existing enhancers, or co-option of transposable elements or unrelated enhancers. Another potential mechanism is splitting an enhancer with redundancy across its length into two parts through the insertion of non-functional DNA (FIG. 6). The redundancy of shadow enhancers suggests that they may emerge as a result of duplication events, an idea proposed for some *Drosophila* shadow enhancers[13]. However, there are only few documented examples of such origins[63,130], and many shadow enhancers seem to have little sequence similarity[79,131]. Some shadow enhancers can arise from transposon co-option events. For example, MER41 endogenous retroviruses (ERVs) have been co-opted to redundantly regulate genes involved in the interferon response[132]. Mammalian-apparent long terminal repeat (LTR) and short interspersed element (SINE) retrotransposons were independently co-opted to redundantly

regulate the brain expression of the *Pomc* gene, which is important for the control of food intake[133] (Table 1). A recent study used enhancer predictions based on eRNA profiling across hundreds of human and mouse cell lines[31] to estimate that 31% of all redundant enhancer pairs in human and 17% of those in mouse have evolved by transposon co-option. Interestingly, for most transposon-derived redundant enhancer pairs, both enhancers have evolved through independent transposons co-option events, suggesting that duplication may not be a dominant route of shadow enhancer acquisition[131]. Most shadow enhancers have only partially overlapping activity patterns (Table 1), suggesting that one of the main mechanisms of shadow enhancer birth could be through co-option of enhancers with initially non-overlapping activities. Selection may favour the recruitment of shadow enhancers to genes whose robust expression is required for a newly emerging key developmental process (for example, pectoral fins in jawed fish[19]).

Evolutionary conservation of shadow enhancers.

Given the redundancy of shadow enhancers, it was initially hypothesized that they would be subject to relaxed evolutionary constraint, allowing them to evolve novel regulatory functions[13]. If true, this hypothesis would predict a greater rate of mutations in shadow enhancers than in non-redundant enhancers. In a large group of *D. melanogaster* mesoderm-specific enhancers, shadow enhancers have higher sequence conservation than non-redundant enhancers, and there is no evidence of relaxed constraint on shadow enhancers[16]. Among ultraconserved enhancers, many have activity that is redundant with another ultraconserved enhancer in the locus[29,78,117,118]. This observation is again in contrast to the prediction that shadow enhancers are subject to weak evolutionary constraint. Growing evidence also suggests that evolution acts on groups of shadow enhancers as regulatory units, instead of on each enhancer individually. Similar to the stabilizing selection that maintains a single enhancer's function[134], mutations that cause a reduction in the activity of one shadow enhancer could be compensated by other mutations that increase the activity of another shadow enhancer and vice versa. Indeed, stabilizing selection has been shown to act on shadow enhancers to maintain conserved expression levels across different species[17,135,136].

A full understanding of the evolutionary patterns of shadow enhancers remains to emerge, but the data collected so far suggest that shadow enhancers may not be an evolutionarily special and distinct class of enhancers *per se*. The conservation of shadow enhancers and the growing evidence that individual shadow enhancers can have distinct functions suggest that shadow enhancers can be fine-tuned for multiple purposes[79,84,86].

Perspectives

Work over the last 10 years has shown that enhancer redundancy is a common feature of animal genomes, with shadow enhancers potentially controlling the majority of developmental genes. The primary purpose of this redundancy seems to be providing a mechanism to drive robust developmental patterning, irrespective of genetic and environmental stress. Shadow enhancers can also interact in complex ways to drive finely-tuned expression patterns, similar to the intricate interactions between TF binding sites

within an enhancer. Shadow enhancers may also drive the formation of transcriptional condensates or hubs via increased TF recruitment, which may increase the fidelity of transcription. Evidence from human genetics studies indicates that shadow enhancers are key to regulating many disease-associated genes. The importance of shadow enhancers is also underscored by their surprising evolutionary conservation.

There remain a number of open questions in the shadow enhancer field. One of the most persistent questions about shadow enhancer prevalence is whether multiple enhancers are intrinsically capable of regulation that is unachievable by a single enhancer. Many of the ways that shadow enhancers interact, that is, synergistically or repressively, are reminiscent of interactions observed between TF binding sites within a single enhancer. So why have more than one enhancer? It may be possible that there is a limit on the stretch of DNA that can serve as an enhancer, so multiple enhancers allow for more room to encode complex biological functions. Or perhaps the formation of stable transcriptional hubs requires multiple clusters of TF binding sites spread throughout a locus to recruit the necessary transcriptional machinery. Alternatively, the flexibility of 3D genome organization may allow regulatory information to be either encoded in a single enhancer or multiple shadow enhancers located within the same topologically associating domain (TAD). If true, this suggests that shadow enhancers exist in the genome because there is no selective pressure to consolidate them into a single enhancer. A comprehensive answer to these questions will require several types of experiments. Measuring the activity of large numbers of individual enhancers and shadow enhancer sets may identify the behaviours that are possible with multiple enhancers, but not a single enhancer. Experiments that visualize the dynamic 3D conformation of loci with multiple enhancers would improve our ability to predict how multiple enhancers interact to control a single target gene.

Despite the prevalence of shadow enhancers in animal genomes, their evolutionary origins are largely a mystery. Once present in a genome, shadow enhancers are typically more conserved than other enhancers[16]. Some shadow enhancers are even among the most conserved sequences in the genome, that is, ultraconserved enhancers[38,118]. Since many shadow enhancers seem to be dispensable for organismal function and display superficial redundancy, their high degree of evolutionary conservation is puzzling. Most shadow enhancer knockout studies have been performed in lab conditions, which do not recapitulate native environments. Therefore, it may be hard to observe the potentially small reductions in fitness that can result in strong purifying selection. Future studies of shadow enhancer mutants in more natural environments may generate a fuller picture of the contributions of enhancer redundancy to organismal fitness.

Finally, our ability to predict the effect of enhancer sequence variation on human phenotypes is still limited. Most trait- and disease-causing variants discovered in genome-wide association studies (GWAS) fall outside coding sequences and are hypothesized to affect enhancer sequences[137,138]. Similarly, whole-genome sequencing (WGS) of patients has identified a growing number of rare non-coding variants that affect developmental genes and are linked to disease[139–142]. In contrast to findings from GWAS and WGS studies, disease-associated genes with large redundant regulatory domains show a relative depletion of functional non-coding variants[73]. How can we synthesize the fact that shadow enhancers can

buffer sequence variation with the prevalence of disease-associated enhancer mutations? It is possible that disease-causing non-coding variants primarily affect genes lacking shadow enhancers or cause a gain of enhancer activity, which may not be buffered by the presence of shadow enhancers. Alternatively, variants in shadow enhancers may have a fairly small effect on target gene expression, which can be amplified by the presence of other mutations or the environment, leading to disease. The rapid increase in WGS of individuals[143–145] combined with large-scale functional assays of human enhancer variant activity[127,146] will shed more light on the role shadow enhancers play in human disease.

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Glossary

Shadow enhancers
Sets of enhancers that drive overlapping expression patterns and regulate the same gene.

Expression domains
The specific tissues or cell types where an enhancer drives expression of its target gene.

Phenotypic robustness
The ability of a system to reliably produce a wild-type phenotype in the presence of environmental (e.g., temperature) or genetic (e.g., decreased expression levels of an upstream TF) stress.

Super-enhancers (or the closely-related stretch enhancers)
Clusters of enhancers that are strongly bound by TFs, co-activators, or modified histones (as measured by ChIP-seq) and that control key cell identity genes.

Enhancer RNAs (eRNAs)
Short, non-coding RNAs that are transcribed from the DNA of enhancer sequences and whose transcription correlates with enhancer activity.

Genes expression noise
Variability in gene expression across either time or space, owing to the stochastic nature of the molecular interactions underlying gene expression.

Quenching
A form of repression whereby the binding of repressive TFs within an enhancer sequence blocks the binding of activating TFs.

Topologically Associating Domains (TADs)
Large genomic domains (~1 megabase) that display more frequent physical contacts between sequences within the same domain than between sequences from different domains.

Evolutionary constraint

Factors that serve to limit the divergence of a particular phenotype; conserved DNA sequences are interpreted as evidence of evolutionary constraint.

Haplosufficiency
A property of an allele whereby a single copy of that allele in a diploid organism is sufficient to drive a wild-type phenotype.

Transcriptional bursting
Periods of rapid transcription interspersed with periods of transcriptional silence.

Ultraconserved enhancers
Enhancers overlapping “ultraconserved” sequences, which are stretches of DNA that share perfect sequence conservation between human, mouse, and rat.

Transposon co-option
The process by which a transposon changes its function (e.g., becomes a new gene or enhancer) through the introduction of sequence mutations.

Transcriptional hubs
Three-dimensional nuclear compartments (>300 nm) formed around actively transcribed genes with a high local concentration of TFs, co-activators, RNA polymerase II and other components of the core transcriptional machinery.

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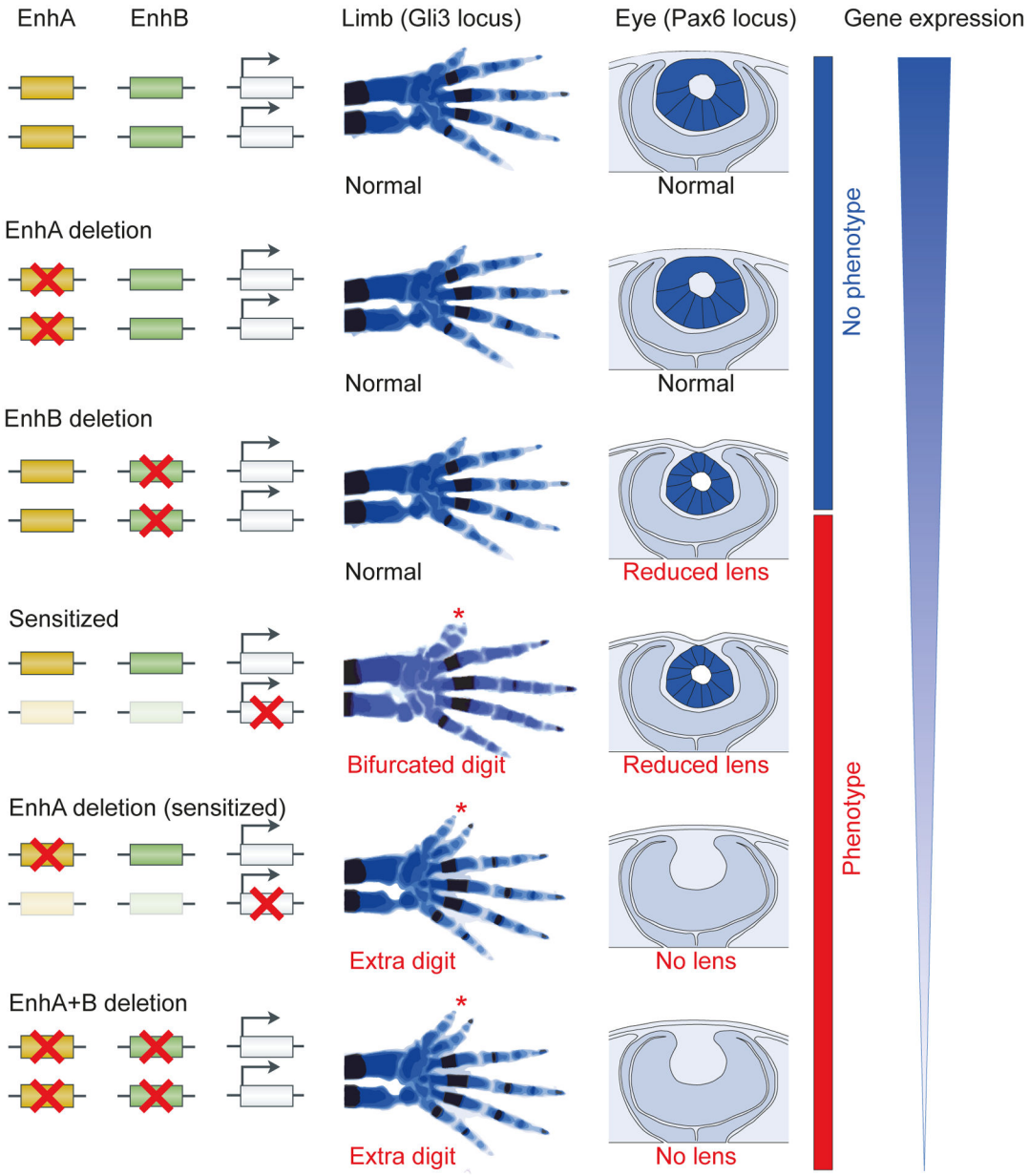

Fig. 1 |. Shadow enhancers confer phenotypic robustness in mammals.

In mice, many individual shadow enhancer deletions yield no observable phenotypes. However, either the deletion of individual shadow enhancers in a sensitized background or the deletion of pairs of shadow enhancers leads to observable phenotypes. Schematics of perturbations (left) and resulting phenotypes in mice (right) are shown for two gene loci: *Gli3*[29] and *Pax6*[18,160]. GLI3 is critical for proper limb development, and its knockout causes the formation of extra digits (among other phenotypes)[161]. Skeletal phenotypes in the absence of individual *Gli3* shadow enhancers, pairs of shadow enhancers, or an individual shadow enhancer in a sensitized background are shown (centre). Red asterisks indicate the presence of extra digits. *Pax6*-deficient mice have arrested eye development and no lens formation[162,163]. A schematic diagram of an eye section showing a developing lens in the

absence of individual PAX6 shadow enhancers, pairs of shadow enhancers, or an individual shadow enhancer in a sensitized background is shown (center-right). A schematic of gene dosage in the mutants is shown on the right. Figure adapted with permission from REF [29].

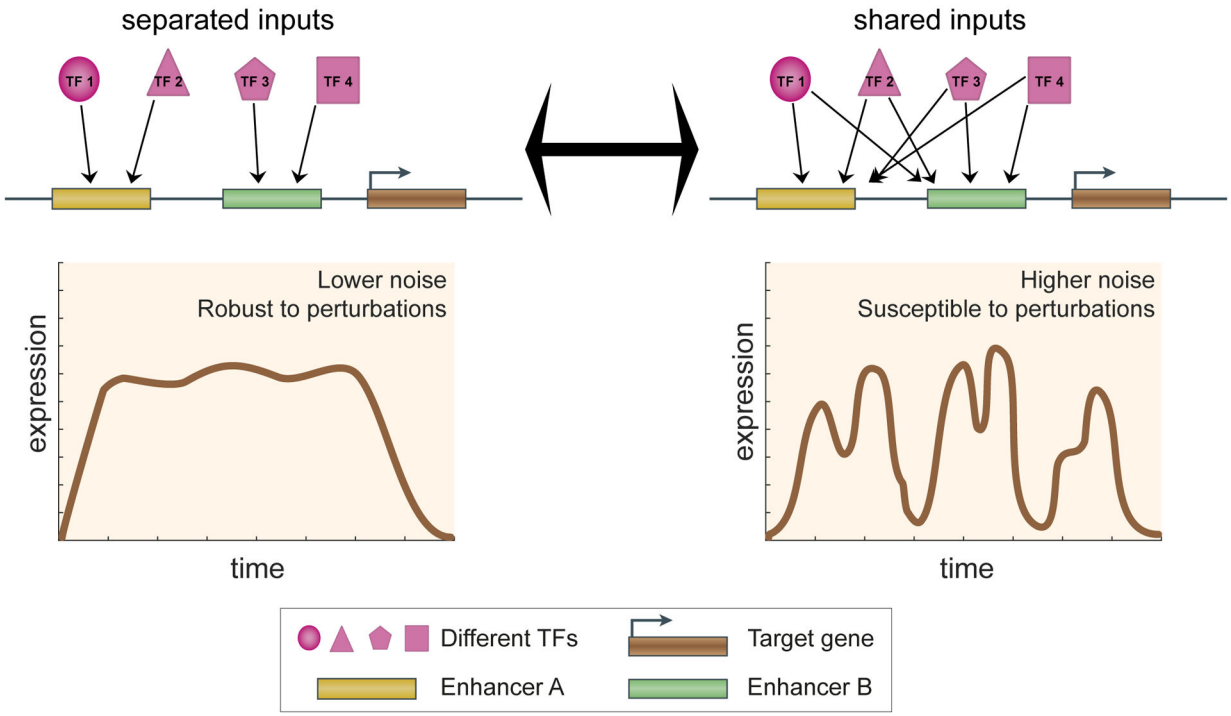

Fig. 2 |. Independent TF inputs to shadow enhancers lead to more robust transcriptional output. Shared and separated TF inputs to the individual shadow enhancers can have different effects on gene expression noise. In case of separated inputs, shadow enhancers regulating the same target gene do not share any of the same TF regulators (top left), while in case of shared inputs, shadow enhancers are regulated by the same set of TFs (top right). Below these two different models, we show the corresponding target gene expression dynamics in single cells as a function of time. Lower expression noise is seen with shadow enhancers with separated TF inputs than with shadow enhancers using shared TF inputs. See REF [79] for more details.

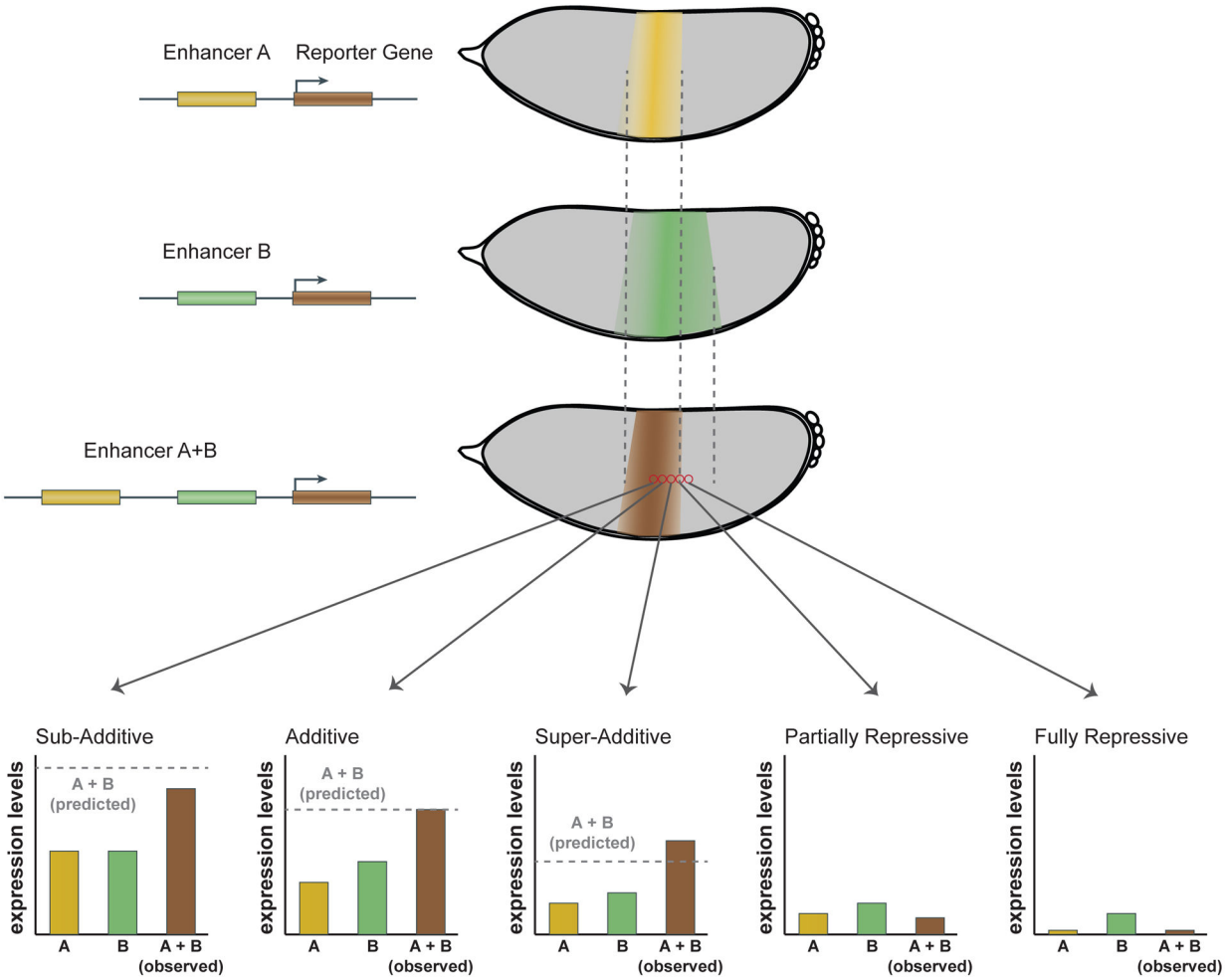

Fig. 3 |. Shadow enhancers can combine in complex and varied ways.

In an individual cell (red circles), enhancers can interact additively, sub-additively, super-additively, partially repressively, or fully repressively, as shown in the bar graphs. Cartoon embryos depicting individual (yellow and green) and combined (brown) enhancer activities, measured in transgenic *D. melanogaster*, are shown. Notably, the mechanism of interaction can vary from cell to cell, highlighting the importance of performing experiments in whole embryos. For example, two shadow enhancers can show each of these behaviours in different parts of their expression stripe, allowing them to combine to produce a sharper and stronger stripe than that produced by either individual enhancer.

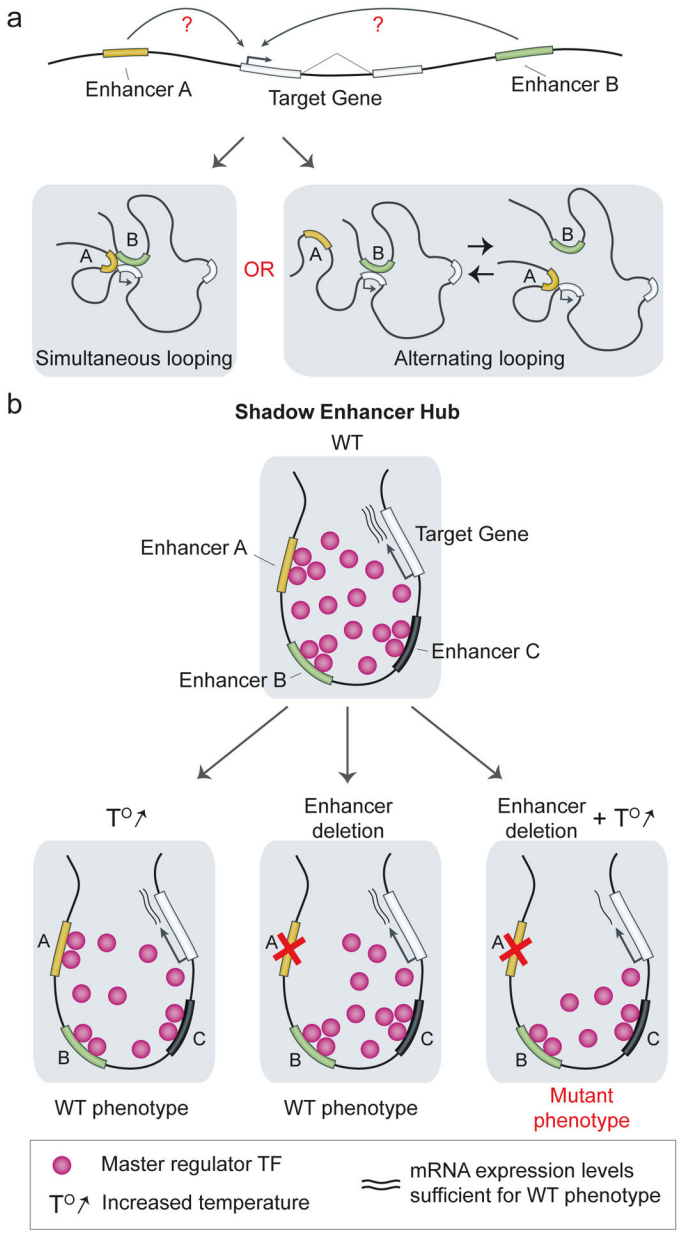

Fig. 4 |. Interactions of shadow enhancers with target promoters.

a | There are two possible models of enhancer–promoter looping. In the simultaneous looping model, enhancer A and B coordinately loop to the target core promoter to initiate transcription. In the alternating looping model, enhancer A competes with enhancer B, so, at a given time point, only one of the enhancers contacts the promoter. **b** | Multiple shadow enhancers may aid in the formation of transcriptional hubs by recruiting a high local amount of a master regulator TF (pink). Such transcription hubs can buffer against environmental stress and genetic perturbations[110].

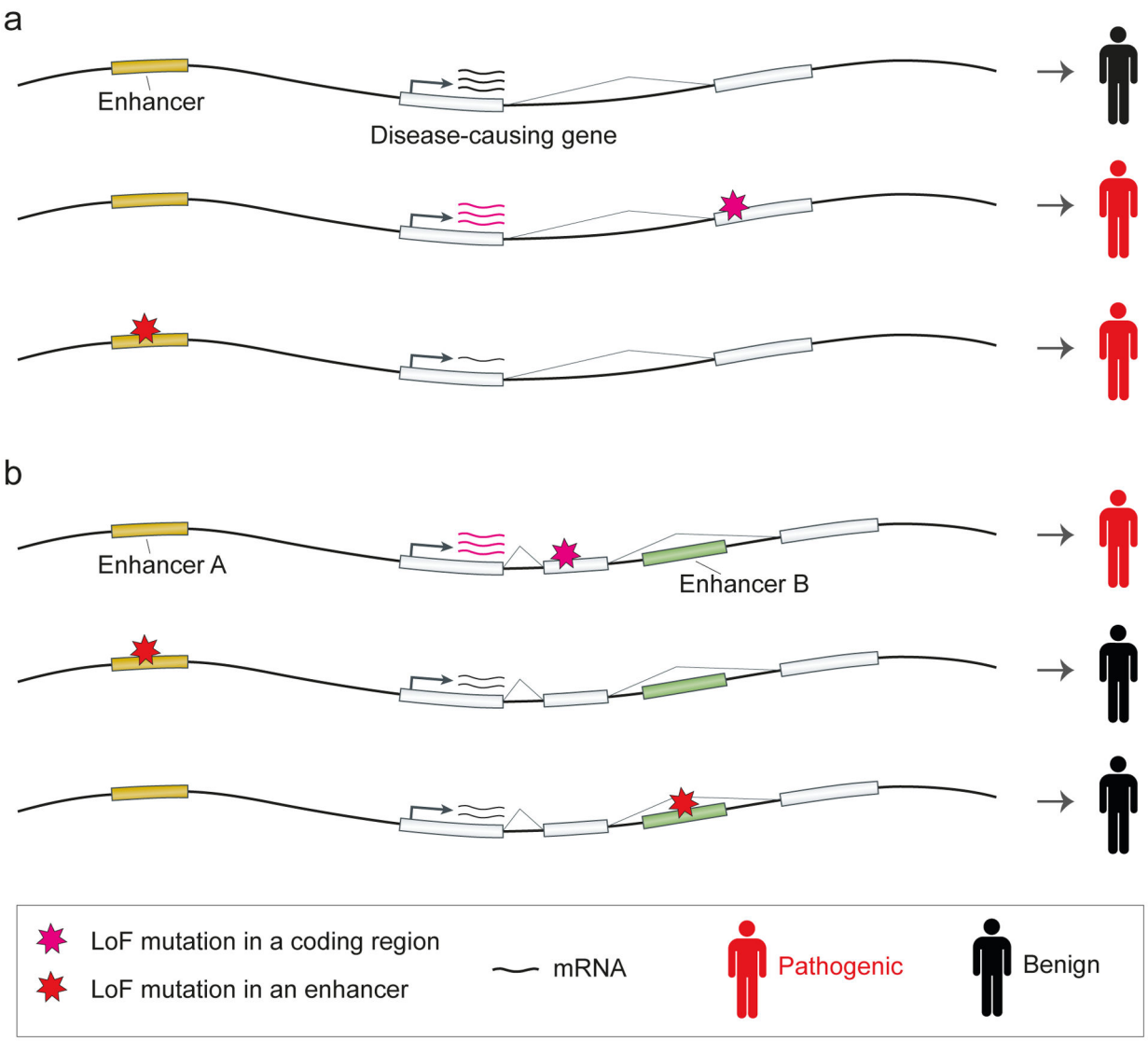

Fig. 5 |. Shadow enhancers buffer against non-coding mutations in disease-causing genes.
a | Many genetic disorders are caused by loss-of-function (LoF) mutations in coding regions of disease-causing genes. If the gene is controlled by a single enhancer, a LoF mutation in the enhancer will mimic the loss of gene function in the tissue and the time point of enhancer activity. **b |** If a disease-causing gene is controlled by shadow enhancers, non-coding mutations that deactivate one of the shadow enhancers will be buffered by another shadow enhancer.

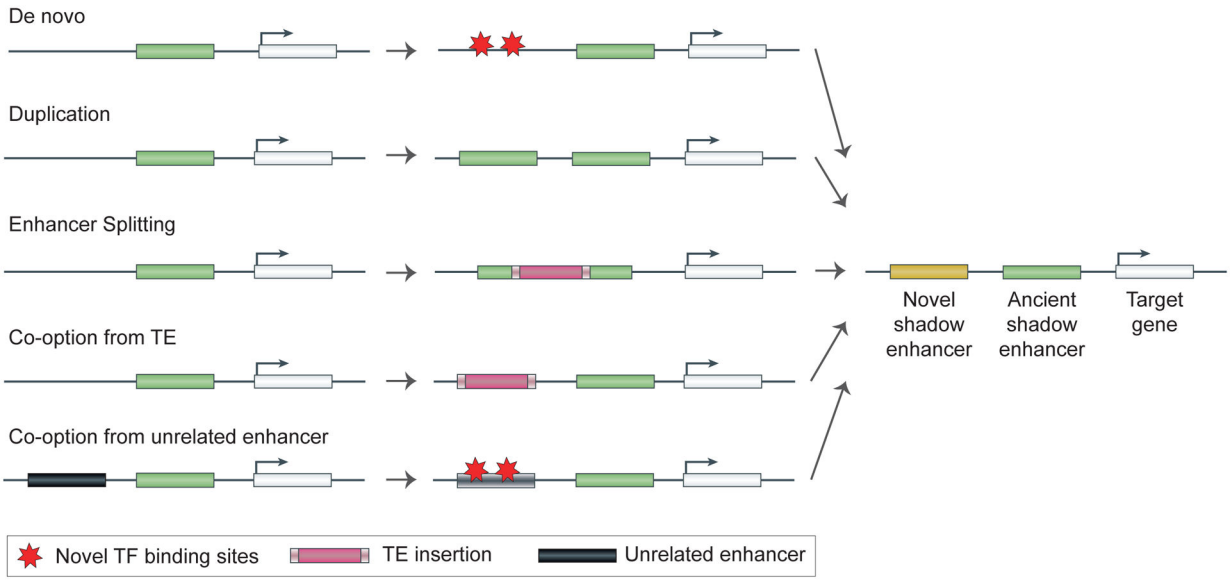

Fig. 6 |. Many evolutionary routes potentially lead to shadow enhancer birth.
Proposed mechanisms include mutations in non-coding regions that generate a novel enhancer, duplication of an existing enhancer, splitting of a large enhancer into two by TE insertion, and co-option of either a transposable element (TE) or an unrelated enhancer to become a shadow enhancer.

Table 1 |

Examples of shadow enhancers in different organisms

Tissue or cell type	Gene(s) with reported shadow enhancers	Gene class	Maximum distance between shadow enhancers	Refs
Plant				
Anthers, pollen	*LAT*	Signalling	~ 3 kb	50
Leaf cells	*rbcS-8B*	Signalling	~ 1 kb	51
Worm				
Nervous system	*cog-1, ric-4, ric-19, snb-1, unc-10, unc-11, unc-31, unc-64, unc-108*	TF, pan-neuronal genes	~ 10 kb	25,147
Fruit fly				
Neurogenic ectoderm	*vnd, brk, sog, dan, SoxN*	TF, signalling	~ 40 kb	10,13,43
Dorsal ectoderm	*tup*	TF	~ 20 kb	43
A–P blastoderm	*slp1, wg, hb, Kr, kni, gt, oc/otd, ems, hkb, fkh, Abd-B, prd*	TF, signalling	~ 30 kb	28,148,149
Mesoderm	*sna, miR-1, ade5, Traf1, rols, CG42788, CadN*	Various	~ 10 kb	10,16,27
Salivary glands	*sens*	TF	~ 2 kb	43
Epidermis	*Ser, svb, y*	Various	~ 40 kb	26,53,150
Wing imaginal disc	*brk*	Signalling	~10 kb	56
Nervous system	*Ddc*	Signalling	~ 1 kb	151
Eye	*Dve, dac*	TF, signalling	~ 15 kb	55,87
Zebrafish				
Brain	*krox20*	TF	~ 100 kb	152
Fin	*Shh*	Signalling	~ 2 kb	19
Neural tube	*Shh*	Signalling	~ 2 kb	57
Mouse				
Brain, neural tube	*Otx2, Pomc, Shh, Arx, Ngn1*	TF, signalling	~ 800 kb	11,12,58,78,153,154
Neural crest	*Pax3*	TF	~ 30 kb	155
Eye	*Pax6, Cryaa*	TF, structural	~ 150 kb	18,156
Blood	*α-globin and β-globin genes, igk, igH*	Haemoglobin subunits, immune response	~ 25 kb	68,91,114,130
Limb	*Gli3, Sox9, Shox2, Ihh, HoxD, HoxA, Tbx4*	TF, signalling	~ 1.2 Mb	29,59,104,157,158
Tooth	*Shh*	Signalling	~ 100 kb	30
Gut	*Cdx2*	TF	~ 7 kb	23
Human				
Liver	*APOE*	Metabolism	~ 10 kb	63
Blood	*β-globin genes*	Haemoglobin subunits	~ 15 kb	60,61
Eye	*ATOH7*	TF	~ 20 kb	22,159
Kidney	*REN*	Signalling	~ 6 kb	62

Only non-adjacent enhancers were included in the table. TF, transcription factor.

Table 2 |

Examples of genes controlled by more than two shadow enhancers

Tissue or cell type	Enhancer identification method	Method of assigning enhancers to genes	Genes	Number of shadow enhancers per gene	Refs.
Fruit fly					
S2 cells (macrophage-like)	MPRA	Genomic proximity	Various (203 genes)	5 or more	74
Embryonic mesoderm	Mesoderm TFs ChIP	Genomic proximity and correlation with gene expression	Various (150 genes)	3 or more	16
Mouse					
Embryonic limb	H3K27ac ChIP	Genomic proximity within a TAD and correlation with gene expression	Limb TFs (41 genes)	Median of 8	29
Embryonic heart	H3K27ac ChIP	Genomic proximity within a TAD and correlation with gene expression	Heart TFs (27 genes)	Median of 10	29
Embryonic forebrain	H3K27ac ChIP	Genomic proximity within a TAD and correlation with gene expression	Forebrain TFs (21 genes)	Median of 4	29

ChIP, chromatin immunoprecipitation; H3K27ac, histone 3 lysine 27 acetylation; MPRA, massively parallel reporter assay, TAD, topologically associating domains.