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Article

Rapid Evolutionary Rewiring of a Structurally Constrained Eye Enhancer

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Summary

Background: Enhancers are genomic *cis*-regulatory sequences that integrate spatiotemporal signals to control gene expression. Enhancer activity depends on the combination of bound transcription factors as well as—in some cases—the arrangement and spacing of binding sites for these factors. Here, we examine evolutionary changes to the sequence and structure of *sparkling*, a Notch/EGFR/Runx-regulated enhancer that activates the *dPax2* gene in cone cells of the developing *Drosophila* eye.

Results: Despite functional and structural constraints on its sequence, *sparkling* has undergone major reorganization in its recent evolutionary history. Our data suggest that the relative strengths of the various regulatory inputs into *sparkling* change rapidly over evolutionary time, such that reduced input from some factors is compensated by increased input from different regulators. These gains and losses are at least partly responsible for the changes in enhancer structure that we observe. Furthermore, stereotypical spatial relationships between certain binding sites ("grammar elements") can be identified in all *sparkling* orthologs—although the sites themselves are often recently derived. We also find that low binding affinity for the Notch-regulated transcription factor Su(H), a conserved property of *sparkling*, is required to prevent ectopic responses to Notch in noncone cells.

Conclusions: Rapid DNA sequence turnover does not imply either the absence of critical *cis*-regulatory information or the absence of structural rules. Our findings demonstrate that even a severely constrained *cis*-regulatory sequence can be significantly rewired over a short evolutionary timescale.

Introduction

Enhancers are genomic *cis*-regulatory sequences that integrate spatiotemporal signals to control the pattern, timing, and levels of gene expression [1, 2]. Enhancers often employ a complex combinatorial logic that allows precisely patterned developmental outputs to be generated from broadly patterned inputs. This regulatory complexity is necessary to restrict gene expression to a specific subset of cells, especially in multicellular organisms, which have many more developmental cell states than transcription factors (TFs). In some enhancers, the organization of TF binding sites is also critical for enhancer function [1–9]. Because *cis*-regulatory elements account for much of the patterning information encoded in the genome, they are also an important evolutionary engine of developmental change [10].

Despite regulatory and structural constraints, genome-scale evolutionary analyses reveal significant sequence turnover within enhancers and TF binding sites [11–15]. *cis*-regulatory sequence evolution can cause changes in gene expression, which can drive morphological differences among populations (e.g., [13, 16–20]). On the other hand, a number of enhancers have retained their function despite sequence divergence and binding-site turnover (e.g., [8, 21–28]). Proposed explanations for the latter phenomenon include binding-site redundancy, compensatory mutations, and organizational flexibility [21, 29–32], but these hypotheses have rarely been tested experimentally. Consequently, both the prevalence and the significance of conserved binding-site "grammars" are debated in the recent literature [2, 5, 6, 8, 21, 23, 29, 33–35].

Here we present a fine-scale evolutionary analysis of the structure and function of the *sparkling* (*spa*) eye enhancer within the genus *Drosophila*. In the developing fly retina, the *spa* enhancer drives expression of *dPax2* in cone cells, where it is required for proper differentiation [36–40]. *spa* is directly bound and regulated by the Notch pathway effector Suppressor of Hairless [Su(H)], the Runx-family protein Lozenge (Lz), and two Ets-family EGFR/MAPK pathway effectors, PointedP2 (PntP2) and Yan/Aop, via motifs resembling Su(H)/Runx/Ets consensus binding sites (Figure 1A) [37]. Regions 1, 4, 5, and 6a of *spa*, which contain no binding sites for the above TFs, also harbor multiple essential regulatory sequences [8, 41]. The linear organization and spacing ("grammar") of these regulatory sites is critically important for both robust transcriptional activation and correct cell-type-specific expression [8].

spa is a good candidate for evolutionary analysis for several reasons. First, its *cis*-regulatory circuitry, though complex, is well characterized; all essential regulatory sequences within a minimal 362 bp version of *spa* have been mapped [8, 37]. Second, unlike some other enhancers whose evolution has been examined, such as the well-studied *even-skipped* stripe 2 enhancer (*eve*S2E), *spa* is regulated by highly conserved cell signaling pathways and TFs [42]. Third, previous in vivo work has revealed strict functional constraints on the structure of *spa*; changing the spacing or arrangement of regulatory sites either kills the enhancer or changes its cell-type specificity [8]. Fourth, as will be discussed below, the sequence of *spa* is unusually rapidly evolving. We therefore investigated the evolutionary dynamics of *spa* to determine the nature and extent of the evolutionary constraints on its sequence and structure.

Results

spa Has Undergone Rapid Sequence Divergence and Structural Rearrangement, While Maintaining Its Function and Cell-Type Specificity

Despite *spa*'s strict regulatory and structural constraints, described above, DNA sequence alignments reveal poor overall conservation within the genus *Drosophila* and poor conservation of most critical regulatory sites (Figure 1B) [8]. Out of 11

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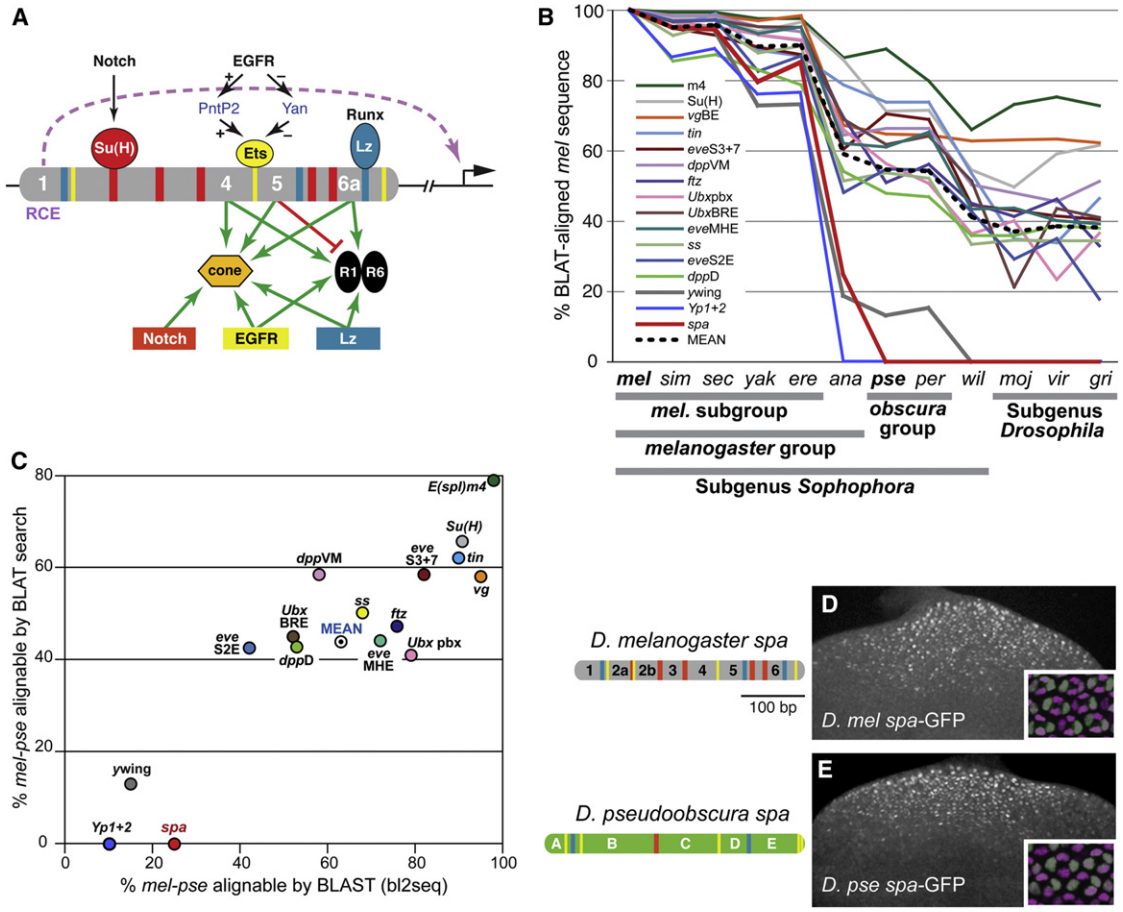

Figure 1. Despite Rapid Sequence Divergence, the Function and Cell-Type Specificity of the *sparkling* Enhancer Is Conserved

(A) Summary of the regulatory logic of the *sparkling* (*spa*) cone cell enhancer of *dPax2*. Colored bars indicate known binding sites for Su(H), PntP2/Yan, and Lozenge (Lz); essential novel regulatory sequences 1, 4, 5, and 6a are numbered. RCE indicates the remote control element in region 1 [8].

(B) Comparison of pairwise ortholog sequence similarity between *D. melanogaster* (*mel*) and 11 other sequenced *Drosophila* species, for 16 developmental enhancers. The following abbreviations are used: *sim*, *D. simulans*; *sec*, *D. sechellia*; *yak*, *D. yakuba*; *ere*, *D. erecta*; *ana*, *D. ananassae*; pse, *D. pseudoobscura*; per, *D. persimilis*; *wil*, *D. willistoni*; *moj*, *D. mojavensis*; *vir*, *D. virilis*; *gri*, *D. grimshawi*. Similarity is measured as the percentage of *mel* enhancer sequence that is aligned to the orthologous region of the comparison genome by BLAT (BLAST-like alignment tool). See Supplemental Experimental Procedures for detailed information on these reference enhancers.

(C) *mel-pse* pairwise sequence similarity for 16 developmental *cis*-regulatory sequences, measured as the percentage of *mel* enhancer sequence that is BLAST-alignable (x axis) or BLAT-alignable (y axis) to the *pse* ortholog.

(D and E) The *D. melanogaster* (*mel*) and *D. pseudoobscura* (*pse*) orthologs of *spa*. Left, enhancer diagrams showing known or predicted Su(H), Ets, and Lz/Runx binding sites (red, yellow, and blue, respectively). Right, eye imaginal discs of transgenic *mel* larvae carrying *mel spa*-GFP (D) or *pse spa*-GFP (E). Insets show 24 hr transgenic pupal eyes stained with antibodies against GFP (green) and the cone cell nuclear marker Cut (magenta).

mapped regulatory binding sites in *spa*—five Su(H) sites, three MGGAW PntP2/Ets sites, and three RACCRCA Lz/Runx sites [37, 43, 44], only two, a closely linked 5′ Lz-Ets pair, are unambiguously preserved throughout the genus (see Figure 5). *spa* sequence appears to be changing unusually rapidly among the 12 sequenced *Drosophila* species; unlike 13 other developmental enhancers used for comparison, no part of *spa* is BLAT (BLAST-like alignment tool)-alignable between the *melanogaster* subgroup and the *obscura* group (Figures 1B and 1C). *spa*'s evolutionary dynamics differ from those of the 13 reference enhancers, including the eve stripe 2 enhancer (*eve*S2E), which is considered to be a rapidly evolving element [24, 45]. When the *yellow* wing enhancer (*y*wing), chosen because of its known functional adaptations within the genus *Drosophila* [46], was added to the analysis, it was comparable to *spa* in its rate of sequence divergence: both enhancers are mostly or entirely unalignable between *D. melanogaster* (*mel*) and *D. pseudoobscura* (*pse*), depending on the alignment method, whereas the original 13 reference enhancers, including *eve*S2E, are highly alignable over this distance (Figure 1C). We also examined the *cis*-regulatory region of the *Yolk protein 1* and *2* genes (*Yp1+2*), which, like *y*wing, is "highly divergent" and thought to be subject to recent adaptive changes [25]. The conservation profile of *Yp1+2* is similar to that of *y*wing and *spa* (Figures 1B and 1C). The poor alignability of *y*wing, *Yp1+2*, and *spa* is likely due to a lack of extended blocks of sequence conservation between *D. mel* and *D. pse* (see Figure S1 available online). *spa*, then, has undergone unusually rapid sequence divergence among *Drosophila* species, resembling that of an enhancer whose expression pattern has undergone significant adaptive change.

Despite *sparkling*'s sequence divergence and its poorly conserved regulatory binding sites, the *D. mel* and *D. pse* orthologs of *spa* drive indistinguishable, cone cell-specific

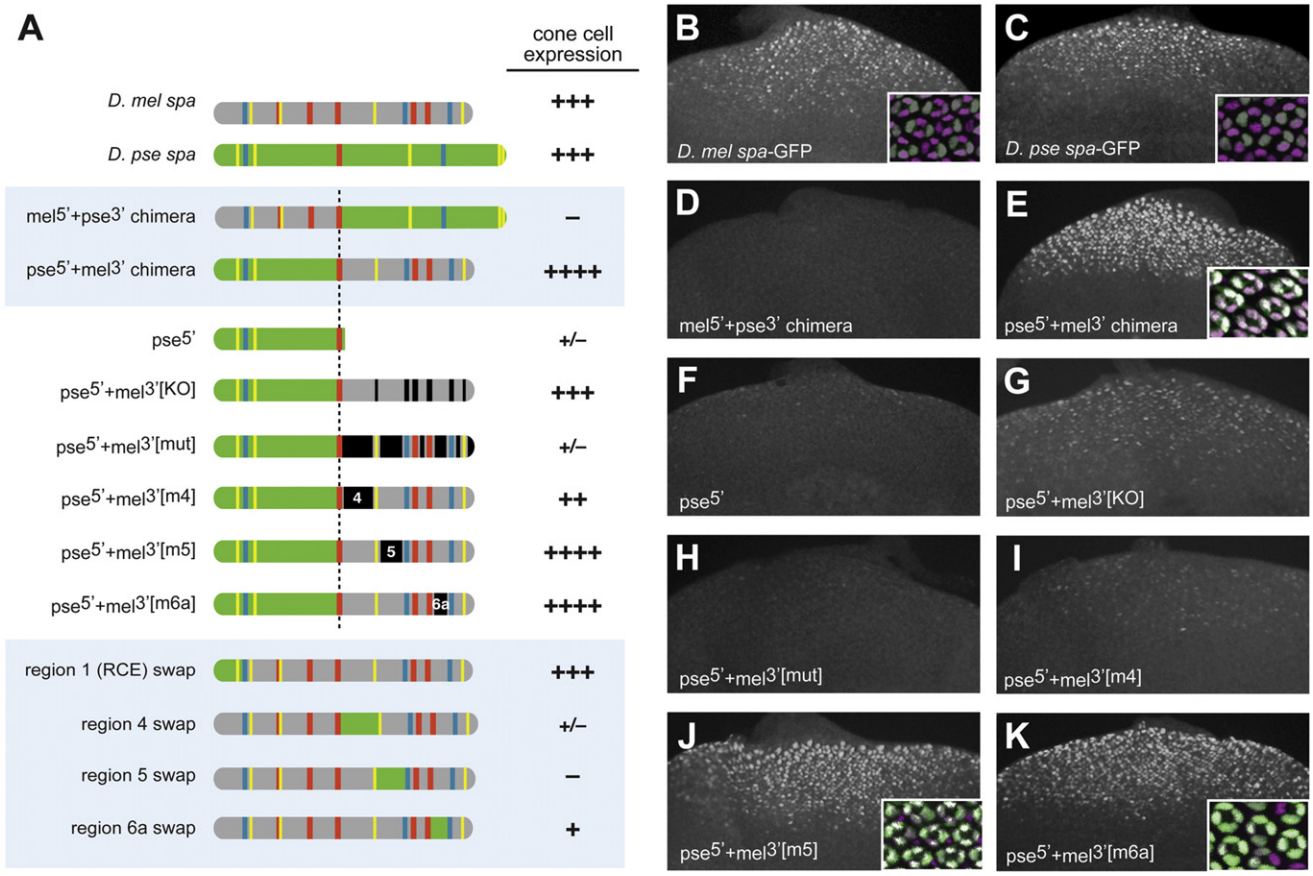

Figure 2. Divergent *cis*-Regulatory Organization of *sparkling* between *D. melanogaster* and *D. pseudoobscura*

(A) Diagrams of *mel-pse* chimeric enhancer constructs, with GFP expression in cone cells of transgenic larvae summarized as follows: +++, wild-type levels of expression in cone cells; ++, moderately reduced; +, severely reduced; +/–, barely detectable or detectable in very few cells; –, no detectable expression; ++++, augmented levels of expression (greater than wild-type).

(B–K) GFP expression in eye discs of transgenic third-instar larvae carrying selected chimeric *spa* reporters depicted in (A). Insets show 24 hr transgenic pupal eyes stained with antibodies against GFP (green) and Cut (magenta).

patterns of gene expression in transgenic *D. melanogaster* (Figures 1D and 1E). Thus, *spa* could be an informative case study in enhancer evolution: a highly constrained *cis*-regulatory element that nevertheless undergoes rapid sequence change and binding-site turnover, while maintaining its function and cell-type specificity. Because *spa* is finely mapped with respect to the in vivo function of its regulatory sequences [8, 37], it provides an opportunity to examine in detail how *cis*-regulatory sequences are rewired over evolutionary time.

To understand how the patterning function of *spa* has been preserved despite extreme sequence divergence, we built chimeric constructs in which halves of the *D. mel* and *D. pse* orthologs of *spa* are spliced together (Figure 2A). When similar manipulations were performed with *eve*S2E in a classic set of experiments, both *mel-pse* chimeras were capable of driving a stripe of gene expression, albeit with shifted boundaries [45]. Our results from *spa* chimeras were very different: the mel$^{5'}$+pse$^{3'}$ construct was inactive in vivo (Figure 2D), whereas pse$^{5'}$+mel$^{3'}$ drives properly patterned cone cell-specific expression, at higher levels than either endogenous enhancer (Figure 2E). Thus, essential regulatory sites in the 3′ half of *D. mel spa* (mel$^{3'}$) could not be replaced by the orthologous *D. pse* sequences, whereas, conversely, mel$^{5'}$ failed to substitute for pse$^{5'}$. This is consistent with a model in which essential activities are recruited to different regions of the orthologous enhancers.

Fine-Scale Chimeric Analysis Tracks the Reorganization of Essential Regulatory Regions

We next undertook a detailed dissection of the hyperactive pse$^{5'}$+mel$^{3'}$ chimera. The 5′ half of *D. pse spa* alone (pse$^{5'}$) was not active (Figure 2F), indicating that not all of the activities in mel$^{3'}$ were duplicated in pse$^{5'}$. In the context of the pse$^{5'}$+mel$^{3'}$ chimera, the Su(H)/Ets/Lz binding sites in mel$^{3'}$ were not required for normal expression levels (construct pse$^{5'}$+mel$^{3'}$[KO], Figure 2G). Note that this construct, which drives expression comparable to wild-type *spa*, contains only one Su(H) site and one Lz/Runx site (Figure 2A). However, altering the mel$^{3'}$ sequences surrounding these 3′ binding sites (regions 4, 5, and 6 of *D. mel spa*) abolished the function of the chimeric enhancer (Figure 2H). These mutations were sequence alterations, not deletions, preserving the native spacing of the remaining enhancer sequences.

Targeted mutations in regions 4, 5, and 6a, in the context of the pse$^{5'}$+mel$^{3'}$ construct, revealed the regulatory contributions of each region to the chimeric enhancer. Mutating only region 4 caused a reduction in expression levels (Figure 2I). By contrast, individually mutating regions 5 and 6a had no

effect on the activity of the chimeric construct in third-instar larval eye discs (Figures 2J and 2K), although the loss of 6a caused a gradual loss of expression in cone cells, as well as ectopic expression in primary pigment cells, during pupal stages (data not shown). Therefore, pse$^{5'}$ contains sequences that can functionally replace region 5 and, to a lesser extent, 4 and 6a.

In finer-scale chimeric swaps, regions 4, 5, and 6a could not be functionally substituted by their *D. pse* orthologous sequences (Figure 2A). However, region 1, the remote control element (RCE) [8]—the best-conserved region of *spa*—was fully substitutable (Figure 2A).

Taken together, these data show that the *cis*-regulatory organization of *spa* differs significantly between *D. mel* and *D. pse*. These structural differences are likely due to individual binding-site turnover, rather than larger-scale DNA rearrangements, as dot-plot comparisons do not suggest sequence rearrangements within *spa* (Figure S2).

Novel Regulatory Motifs with Conserved Functions but Divergent Locations

The above data suggest a model in which rapid losses and gains of regulatory sites have resulted in orthologous enhancers with conserved function but distinct structures. 3′ regions of *D. melanogaster spa* are (wholly or in part) functionally equivalent to sequences in the 5′ half of *D. pseudoobscura spa*. These sequences could recruit the same TFs to different locations in both enhancers; if so, we might be able to identify common novel regulatory sites that have moved since the divergence of *D. mel* and *D. pse*, based on our new understanding of the organization of *D. pse spa*.

By multiple EM for motif elicitation (MEME) sequence comparisons among *Drosophila spa* orthologs, we identified five novel motifs (i.e., non-Su(H)/Ets/Lz sites) in *D. mel* regions 4, 5, and 6a, all of which are also found in *D. pse spa*—but not in corresponding positions. We named these motifs α through ε (Figure 3A). Because the proteins binding to these sequences (if any) are unknown, we were unable to define consensus sites based on binding properties. Instead, we chose potential regulatory motifs that fit the following criteria: (1) they reside in essential regulatory regions of *mel spa*, (2) they occur in *spa*-orthologous sequence in most sequenced *Drosophila* species, and (3) they are in noncorresponding positions in the *mel* and *pse* enhancers. Motif degeneracy was adjusted to optimally fit the above criteria.

The α, β, and γ motifs reside in *mel* region 4; in *pse*, α and γ are found near the 3′ end of *spa*, in region E, whereas β motifs are present in both regions B and E (Figure 3A). The motif is present in *mel* region 5 and *pse* region B. The ε motif, AGCCAG, is present in *mel* region 6a and in three copies in *pse* region B, with similar sequences (containing a one-base mismatch) in *pse* regions B, D, and E (Figure 3A). The relocation of any of these novel motifs, if they are functionally significant, could help to explain the evolving *cis*-regulatory structure of *spa*.

Regions 4, 5, and 6a of *mel spa*, which contain motifs α–ε, are all critical for enhancer function in vivo [8]. Finer-scale mutations in *mel spa*, which alter the putative novel regulatory motifs described above, all caused enhancer failure in vivo (Figures 3C and 3G; see also [8]). Targeted mutation of the α motif in *pse spa* weakened its activity, whereas mutation of β, γ, or δ resulted in a severe loss of enhancer function (Figure 3B). These motifs may therefore represent novel, labile regulatory sites present in both *spa* orthologs.

The ε motif was of particular interest to us because it is present in three identical copies in pse$^{5'}$ (plus six more sites with a one-base mismatch), but only one AGCCAG motif is present in *D. mel*, which suggested a possible compensatory mechanism that might account for the reduced numbers of Su(H) and Lz sites in *D. pse spa*, relative to *D. mel*. Mutation of the three ε sites in *pse* region B reduced reporter-gene expression, whereas additionally mutating three sites with a one-base mismatch to the AGCCAG motif resulted in a severe loss of enhancer function (Figures 3B, 3E, and 3F). The latter result suggests that some of the sites containing a mismatch to our ε motif may be functional regulatory sites in vivo. Targeted mutation of the sole ε motif in *D. mel spa* causes a severe loss of reporter-gene expression (Figures 3B and 3G). This loss was fully rescued—to a level of expression greater than that of wild-type *spa*—by altering three "mismatched" ε-like motifs in region 2 to create three "perfect" AGCCAG ε sites (*mel* mut-ε + ε rescue; Figures 3C and 3H). These results are consistent with the possibility that ε is a binding site for an important activator of *spa* in both *D. mel* and *D. pse*—though via different numbers of sites, in different regions of *spa*, in the two species.

Tracking Compensatory Changes to the Regulatory Inputs of *spa*

The identification of novel motifs regulating both *D. melanogaster spa* and *D. pseudoobscura spa* and the importance of Lz, Ets, and Su(H) sites in both orthologs for proper enhancer function ([8, 37]; this study; additional data not shown) suggests that both *spa* orthologs may recruit largely the same set of regulatory factors. However, as the divergent numbers of Su(H), Lz/Runx, Ets, and ε motifs suggest, the relative contributions of individual regulatory factors may have diverged in the two species. *D. mel spa* contains more RACCRCA Lz/Runx motifs than does *D. pse spa* (3 versus 2) and many more Su(H) sites (5 versus 1) but has fewer MGGAW Ets motifs (3 versus 5) and fewer AGCCAG ε motifs (1 versus 3; allowing one mismatch, 4 versus 9).

In order to compare the relative contributions of Lz/Ets/Su(H) to *mel* and *pse spa*, we designed a chimera in which *mel spa*'s Lz/Ets/Su(H) sites were replaced with orthologous sequences from *pse* (Figure 3D), based on a pairwise BLASTZ alignment (Figure S3A). The result of this substitution was the loss of four nonconserved Su(H) sites and one Lz site, along with the gain of two Ets motifs unique to *pse spa*, which were added, at their orthologous positions, to *mel spa* (red arrowheads). The Lz+Ets+Su(H) input into *mel spa* was thus replaced with that of *pse spa.* Despite a reduced number of Lz and Su(H) sites (relative to *mel spa*), this construct drives higher levels of cone cell expression than wild-type *spa* from either *mel* or *pse* (Figure 3I). We also observed occasional ectopic photoreceptor expression in these lines (Table S1).

Note that the *pse*-specific "Ets0" site is immediately adjacent to the conserved Lz1 site (Figures S3A and S3B). Lz (a Runx factor) and PntP2 (an Ets factor) physically interact and synergistically activate gene expression through neighboring DNA binding sites, as do their mammalian Runx and Ets orthologs [47–51]. When the Ets0 site was excluded from the binding-site chimera described above, in vivo gene expression was drastically reduced (Figure 3J), suggesting that this site (a unique innovation of the *obscura* group lineage; see Figure 5 and Figure S3B) helps to compensate for the relative reduction of Lz and Su(H) sites in *D. pse spa*.

We next created a version of *mel spa* that not only contains the Lz/Ets/Su(H) input from *D. pse* but also includes three ε

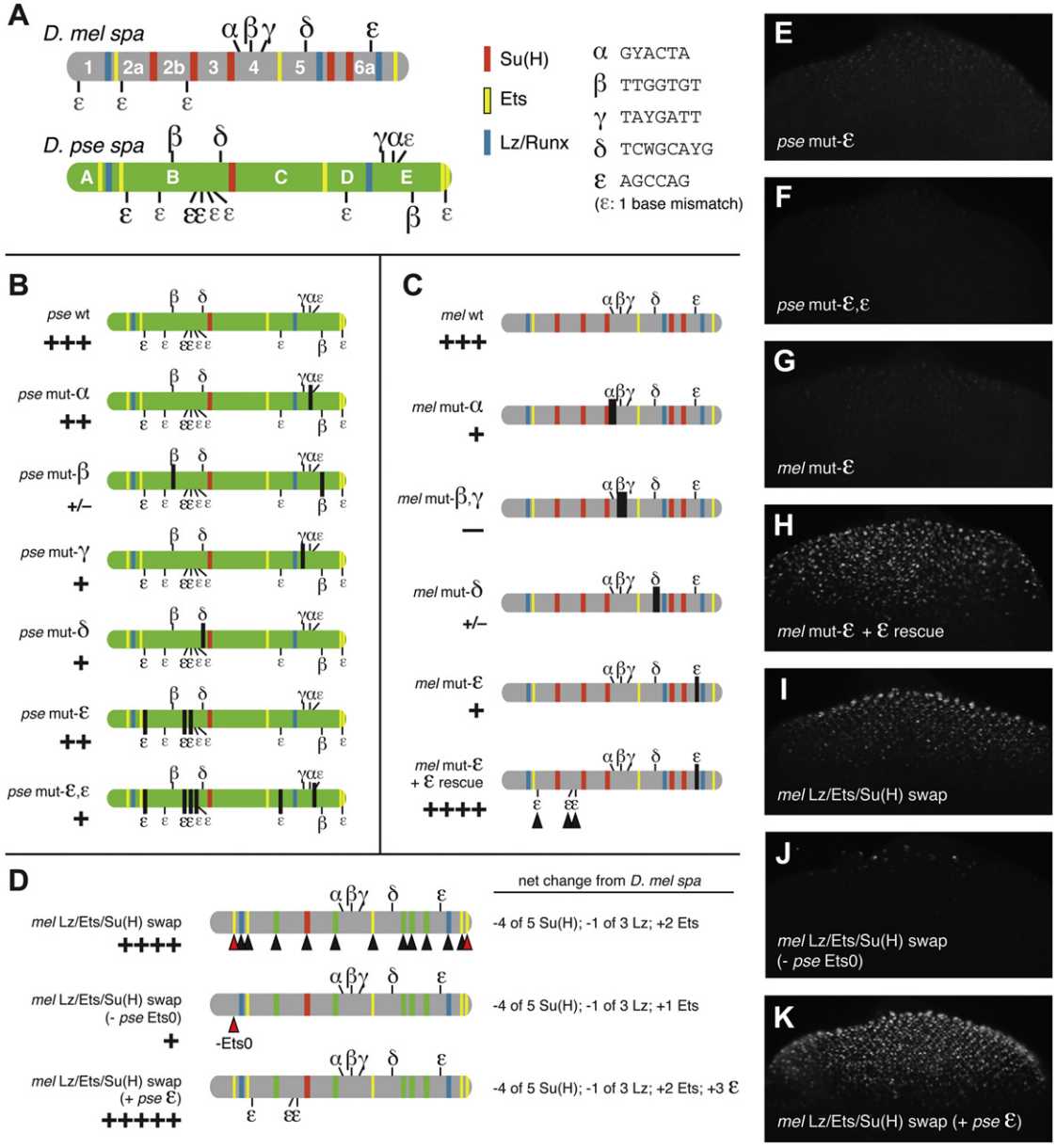

Figure 3. Cross-Species Sequence Comparisons Identify Novel *cis*-Regulatory Motifs at Rapidly Changing Positions

(A) Distribution of Su(H), Ets, and Lz/Runx binding sites, along with putative novel regulatory motifs α, β, γ, δ, and ε, in *D. mel* and *D. pse* orthologs of *spa*. Sequence motifs are listed to the right.

(B and C) In vivo mutational analysis of novel regulatory motifs in *D. pse spa* (B) and *D. mel spa* (C).

(D) Experiments in which the Lz/Ets/Su(H) sites in *mel* are replaced with their orthologous *pse* sequences. Black arrowheads show *D. mel* Lz, Ets, and Su(H) sites (blue, yellow, and red bands) that have been replaced by orthologous *D. pse* sequences; green indicates that the orthologous *D. pse* sequence is not a predicted site. Red arrowheads show *D. pse*-specific Ets and ε sites added to *D. mel spa*, including the 5′ site Ets0.

(E–K) GFP expression in eye discs of transgenic third-instar larvae carrying mutated or chimeric *spa* reporters depicted in (B)–(D).

motifs (AGCCAG) taken from the 5′ half of *pse spa*. The resulting Frankensteinian sequence drives augmented cone cell expression and also exhibits some ectopic activity in photoreceptors and primary pigment cells (Figures 3D and 3K; Table S1; additional data not shown). Important intersite spatial relationships may have been disrupted in this chimeric context, resulting in ectopic enhancer activity (see below and [8] for further discussion of site arrangement). The above data suggest that the 5′ Ets and ε motifs that are unique to the *obscura* group are functionally significant compensatory adaptations.

Evidence for Selective Pressure Maintaining Low Binding Affinity for Su(H)

The predicted Su(H) binding sites in all 12 *spa* orthologs are almost exclusively nonconsensus, low-affinity sites (see Figure 5B). All five of the confirmed Su(H) sites in *D. mel spa*, as well as the lone predicted site in *D. pse spa*, deviate from the well-established high-affinity consensus YGTGRGAAM by one to four bases (Figure 4A), and four of these six sites also deviate from the lower-affinity binding consensus RTGRGAR [21, 37, 43]. These suboptimal Su(H) sites are essential in

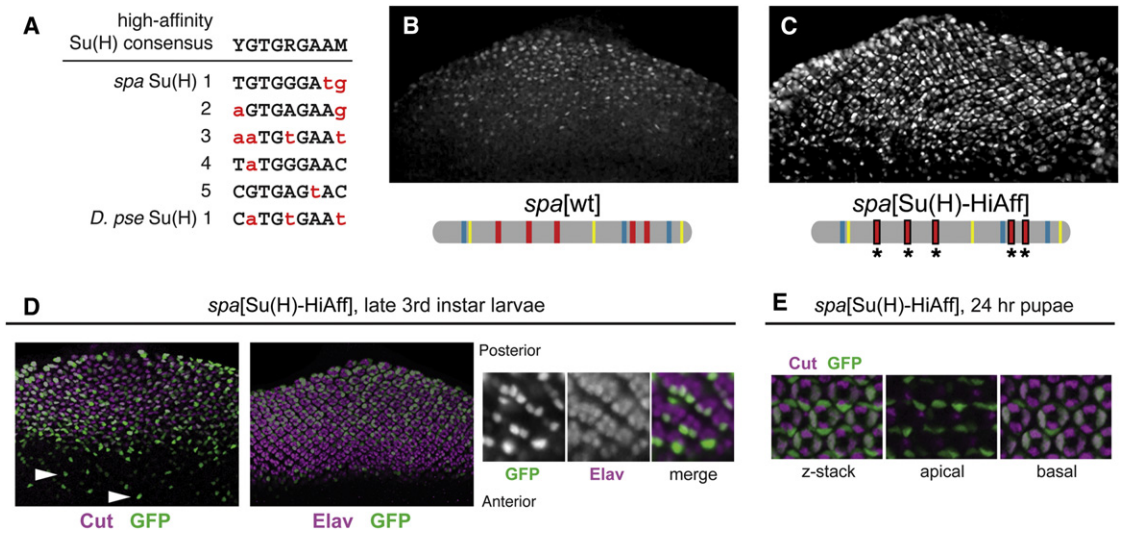

Figure 4. Low Binding Affinity for Su(H) Is Essential for Proper Cell-Type Specificity

(A) The five previously identified Su(H) binding sites in *D. mel spa* [37], and the single identifiable putative Su(H) site in *D. pse spa*, deviate from the high-affinity consensus binding motif (nonmatching bases are in red lowercase).

(B and C) Levels of eye disc GFP expression driven by wild-type *spa* (B) and *spa*[Su(H)-HiAff] (C), in which the Su(H) sites were altered to the high-affinity sequence CGTGGGAA.

(D) *spa*[Su(H)-HiAff]-GFP is ectopically expressed in a subset of photoreceptors. Left, GFP expression (green) precedes cone cell specification, as marked by Cut expression (magenta). Middle, GFP expression overlaps temporally and spatially with Elav, a photoreceptor marker (magenta). Right, GFP labels a varying subset of early-specified photoreceptors.

(E) Unlike *spa*[wt], *spa*[Su(H)-HiAff]-GFP (green) is strongly active in Cut-negative, apically located, Notch-responsive primary pigment cells in the 24 hr pupal eye.

both *spa* orthologs for normal expression in cone cells, which respond to Notch ([37, 42]; additional data not shown). To determine whether *spa*'s low affinity for Su(H) might be a significant functional adaptation, we made targeted nucleotide substitutions to increase the affinity of the five Su(H) sites in *D. mel spa*, based on previous binding data [37, 43]. The resulting enhancer, *spa*[Su(H)-HiAff], drives increased levels of GFP expression in cone cells (Figures 4B and 4C). Perhaps more significantly, *spa*[Su(H)-HiAff] is ectopically active in multiple noncone cell types of the eye. Expression was observed in a variable subset of larval photoreceptors (Figure 4D); Notch plays complex, sequential roles in photoreceptor specification [42, 52]. We also observed robust reporter-gene expression in the primary pigment cells of 24 hr pupal eyes (Figure 4E). Primary pigment cells depend on high levels of Notch signaling ([42, 53]; see Supplemental Discussion). We take these findings to be strong evidence supporting the idea that the Notch/Su(H) input into *spa* must be balanced at a relatively weak level: some input is required for activation in Notch-responsive cone cells, but strong input enables the ectopic activation of *dPax2* elsewhere in the eye.

Evolutionary Dynamics of the Binding-Site Grammar of *sparkling*

Given that the arrangement of *cis*-regulatory sites within *spa* plays a critical role in enhancer function and proper patterning [8], we were surprised to see little obvious conservation of binding-site structure (Figure 5; Figure S3). Very few TF binding sites are indisputably conserved across the genus, or even between *mel* and *pse*. How can an enhancer obey structural rules without highly conserved binding sites?

Even if regulatory sites are rapidly turned over, they may still be preferentially found in structurally optimal positions, relative to other sites. For example, although Lz and PntP2 (Runx and Ets) sites may be rapidly lost and gained, the fact that they physically interact could bias the location of newly acquired sites [30]. This could result in the preservation of characteristic arrangements of sites, even if the sites themselves are recently derived. In principle, such configurations could move around the enhancer as individual sites are gained and lost [29, 33].

In order to identify any such "grammar elements," as we will refer to them here, we inspected the sequence of *spa* orthologs, including 5′ and 3′ flanking DNA, in the 12 sequenced *Drosophila* species. Because no preferred arrangements or spacings are known for any of the regulators of *spa* (excepting the Su(H) "paired site" [43], which does not appear in *spa*), all possible configurations were considered. The results of this analysis are depicted in Figure 5.

Spacing between the well-conserved core of the RCE and the Lz1 site is maintained at 27–31 bp across the genus (grammar element *b*; Figure 5A; Figure S3B). We identified an additional Lz/Runx motif, Lz0, 5′ of the RCE (just outside of the minimal 362 bp *spa* element); Lz0-RCE spacing is exactly 11 bp in all species (grammar element *a*), suggesting that Lz0 may be a significant regulatory site. However, it is not very surprising that, within the only well-conserved region of *spa*, binding-site spacing is also conserved [24, 29, 54]; grammar elements in highly divergent sequences, described below, are potentially more informative.

Of the potential grammar elements that we were able to identify, a large proportion consisted of Lz and Ets sites (labeled *c–i* in Figure 5A). Only one such element, *i*, occurs in both *Drosophila* subgenera, and no single Lz-Ets element is identifiable in all species. Most of these elements are fairly recently derived: for example, *c* and *h* are unique to

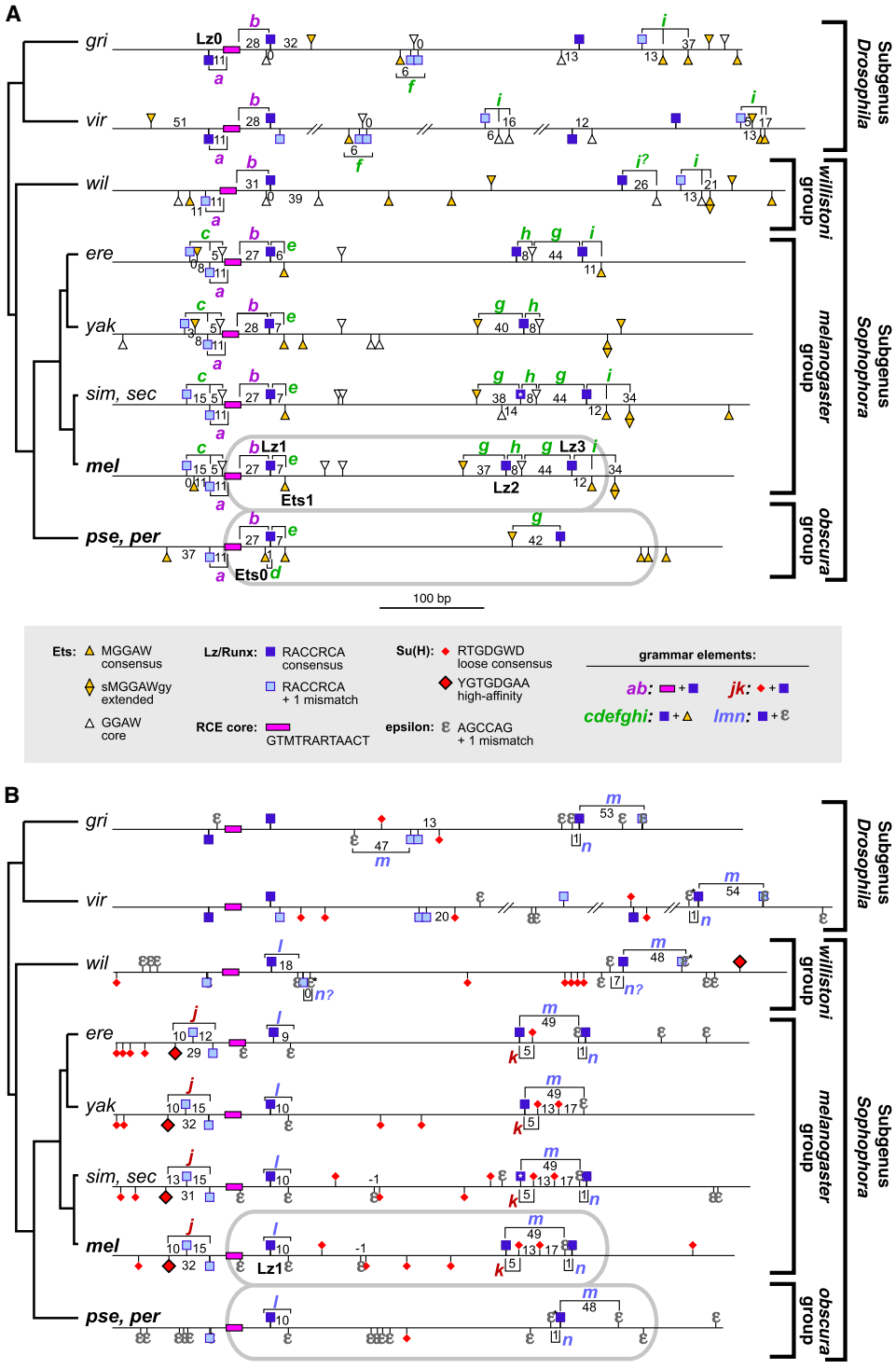

Figure 5. Evolutionary Dynamics of the Binding-Site Grammar of *sparkling*

Diagram of selected *cis*-regulatory motifs within *spa* orthologs of several *Drosophila* species.

(A) Grammar elements involving Lz, Ets, and/or RCE motifs.

(B) Grammar elements involving Lz, Su(H), and/or ε motifs. Brackets, lettered *a* through *n*, show spatial relationships among binding sites ("grammar elements") that are identifiable in multiple species; numbers indicate the base-pair spacing between two motifs. Gray ovals show the sequences included in the minimal *mel* and *pse* enhancer reporter constructs. Here, ε denotes a 5/6 or 6/6 match to the AGCCAG motif; selected sequences with a weaker match are designated ε*. The Lz site with a white asterisk in *sim/sec* indicates that the site has a mismatch in *sim* only.

the *melanogaster* group, whereas *d* is a novel feature of the *obscura* group, and *f* is found only in the subgenus *Drosophila* (Figure 5A; additional data not shown). Nevertheless, all *spa* orthologs contain at least one recognizable Lz-Ets grammar element that is shared with other orthologs.

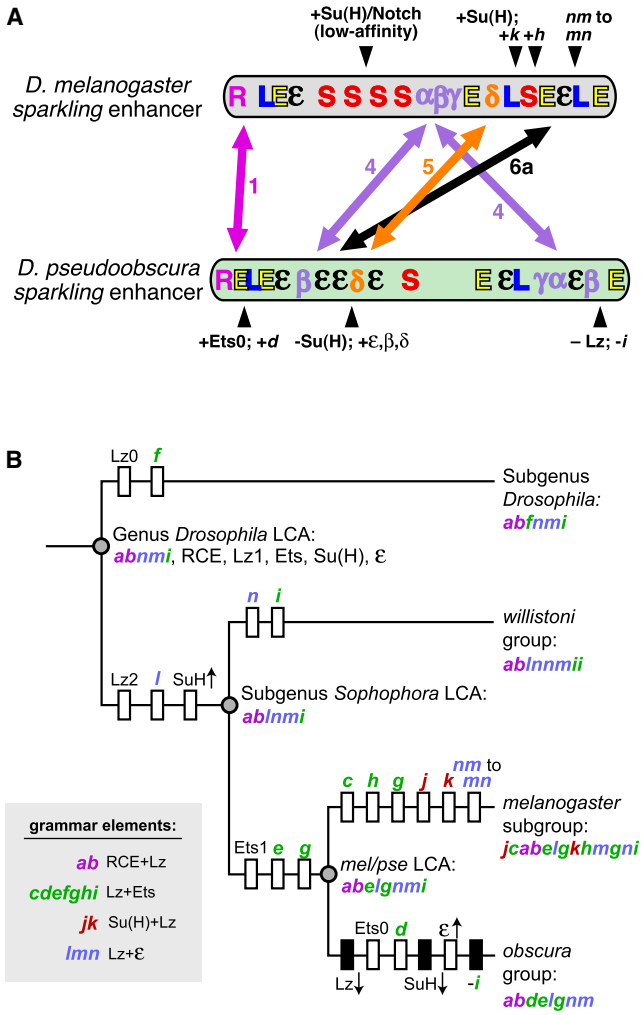

Figure 6. Inferred Evolutionary History of the Vocabulary and Grammar of the *sparkling* Enhancer

(A) Summary of conserved, divergent, and relocated *cis*-regulatory features of *sparkling* in *D. melanogaster* (top) and *D. pseudoobscura* (bottom). Symbols represent binding sites for known regulators [L, Lz/Runx; E, Ets; S, Su(H)], the remote control element (R), and novel regulatory motifs α, β, γ, δ, and ε. Double-headed arrows show changes in position of the regulatory motifs comprising essential enhancer regions 1, 4, 5, and 6a (arrows). Selected lineage-specific innovations are indicated with black arrowheads. Letters in italics refer to grammar elements described in Figure 5.

(B) A maximum-parsimony tree describing the *cis*-regulatory features of the *sparkling* enhancer in the inferred last common ancestors (LCAs) of four *Drosophila* subtaxa, based on sequence analysis and functional assays. Acquired novel features—binding sites or grammar elements—are shown as white boxes crossing a particular lineage, whereas lost ancestral features are shown as black boxes. Arrows pointing up or down, next to the name of a transcription factor, indicate increased or decreased input of that regulator. Grammar elements are listed in 5′–3′ order.

Three potential Lz-ε grammar elements older than the *melanogaster* subgroup were identified: *l* is restricted to the subgenus *Sophophora*, whereas *m* and *n* could be identified in both subgenera (Figure 5B; Figure S3C). Elements *m* and *n* appear to have switched their relative positions in *mel* and *pse*. Because of poor sequence conservation in this region, it is difficult to trace the most likely scenario of site gain and loss explaining the rearrangement of *m* and *n*. However, based on outgroup species comparisons (Figure 5B), the *nm* arrangement found in *pse* seems to be the ancestral state.

We could only identify two grammar elements involving Su(H) sites in the vicinity of *sparkling* (*j* and *k*), both of which are exclusive to the closely related species of the *melanogaster* subgroup (Figure 5B). Further, we could detect no conservation of individual Su(H) sites beyond the *melanogaster* subgroup. In this respect, *dPax2* differs from other Notch targets such as *numb*, *Su(H)*, and genes of the *Enhancer of split* complex, whose enhancers contain many conserved Su(H) binding sites [55–57]. Interestingly, the highly conserved Su(H) sites in those enhancers are generally of much higher predicted affinity than the poorly conserved, but functionally significant, Su(H) sites in *spa*.

Discussion

Because of *spa*'s rapid structural evolution and binding-site turnover, multispecies sequence alignments do not reveal many conserved features. Only the extreme 5′ end of *spa* is unequivocally alignable across 12 *Drosophila* genomes [8] (Figure 5A; Figure S3B). Given *spa*'s complex regulatory circuitry and structure, its unusually rapid sequence divergence between *D. mel* and *D. pse* was surprising, especially because both orthologs of *spa* have identical cell-type specificities. Here we demonstrate that even an enhancer that is subject to structural constraints can be evolutionarily flexible; therefore, an apparent lack of conserved *cis*-regulatory structure does not imply an absence of organizational rules within an enhancer.

We propose a model for the structural divergence of *spa* between the *melanogaster* and *obscura* groups (Figure 6A), based on our sequence analyses and experimental data. Although the RCE and its flanking Lz1-Ets1 pair are relatively stable, many other essential regulatory sites have been relocated. Within regions 4, 5, and 6a, we have identified putative novel regulatory motifs, essential for full-strength activation of both *spa* orthologs, whose movements are consistent with our experimental data on *spa*'s evolutionary restructuring (Figure 6A).

Important changes to the Lz/Ets/Su(H) inputs have also occurred: *D. pse* has fewer Su(H) and Lz sites, relative to the *melanogaster* group—which can be compensated by newly acquired, functionally significant 5′ Ets and ε sites. Meanwhile, the *melanogaster* group has gained a new Lz site and also has a relative abundance of Su(H) sites, which may compensate for relatively few ε and Ets sites (Figures 6A and 6B).

By tracking the reorganization of Su(H), Lz, Ets, and ε motifs across multiple species, we can propose a speculative phylogeny of the *spa* enhancer within the genus *Drosophila* and predict the *cis*-regulatory content of the last common ancestors (LCAs) of several species groups, by reconstructing the gain and loss of sites, and the changing strengths of *trans*-regulatory inputs, in specific lineages (Figure 6B). The main conclusions to be drawn from this evolutionary view of *spa*, informed by our functional experiments, are: (1) significant enhancer rewiring has occurred since the divergence of the *mel* and *pse* lineages; (2) this rewiring involves the loss and gain of individual regulatory motifs, as well as compensatory changes in the overall strength of several *trans*-regulatory inputs through changes in binding-site number, position, and possibly affinity; (3) despite very rapid site turnover, characteristic configurations of sites ("grammar elements") can be identified; (4) these grammar elements can be relocated within the

enhancer, suggesting that a specific arrangement of sites can be more ancient than the individual sites that compose it. These last two points, taken together, may explain how *spa* can continue to obey structural rules while being significantly reconfigured.

A large proportion of the grammar elements that we have identified involve Lz/Runx and Ets motifs. Unlike the case of linked sites for Dorsal, Twist, and other factors in insect neurogenic enhancers [3, 21, 58], there is no single, clearly preferred arrangement of Lz and Ets sites within *spa*: we identified seven distinct types of Lz/Ets grammar element that are at least as ancient as the LCA of the *melanogaster* group (Figure 5). Perhaps Runx and Ets factors, which are known to directly interact and to cooperatively activate transcription in flies and vertebrates [47–51], can synergize productively in several different spatial configurations. This is consistent with mapped Runx and Ets sites in vertebrate genomes, which are frequently associated with one another in target enhancers, but not with a single rigid arrangement or spacing [59–61].

We have also discovered a nonstructural constraint on the sequence of *spa*: a requirement for nonconsensus, low-affinity Su(H) sites for proper cone-specific patterning. Because ectopic *dPax2* expression in photoreceptor precursors causes faulty cell fate specification and differentiation, resulting in defective eye morphology [40], it is reasonable to suppose that the expression pattern of *spa*[Su(H)-HiAff] would have negative fitness consequences for the fly. Taken together with previous work, the data presented here suggest that *spa* requires input from Notch/Su(H) but also requires that input to be attenuated at the *cis*-regulatory level, in order to generate the proper levels and cell-type specificity of *dPax2* expression in a tissue with widespread Notch signaling.

Like Notch/Su(H), EGFR/Ets signaling and Lz are also used to specify multiple cell types in the retina, which presents a challenge for combinatorial gene regulation: enhancers must be able to make fine qualitative distinctions in regulatory inputs and often must translate this information into relatively sharp on/off decisions [2, 6, 40–43, 53, 62, 63]. These pressures could result in a *cis*-regulatory logic for genes like *dPax2* in which many weak inputs are independently tuned—and spatially arranged—to maximize activation in the proper cell type, while minimizing ectopic activation. Our previous studies of *spa* present a picture of an enhancer operating just above a functional threshold, such that the loss of a single regulatory site, or a loss of proper grammar, can result in transcriptional failure in cone cells. One of our main conclusions from this study is that, over a relatively short evolutionary timescale, a *cis*-regulatory module can find multiple solutions to this complex computational problem.

The presence of weak, nonconsensus binding sites for signal-regulated TFs is a common, but little remarked upon, feature of developmental enhancers [64]. Low-affinity TF binding sites have well-documented functions in shaping a stripe of gene expression across a morphogen gradient and in determining temporal responses to developmental regulators [58, 65–68]. Here, we provide direct evidence supporting a role for weak signal response elements in preventing ectopic transcriptional responses to highly pleiotropic signaling pathways such as Notch.

There is one striking question not addressed by this study: why is this enhancer evolving at an unusually high rate, given that its expression pattern is stable? We can think of two plausible explanations for which supporting data exist. First, *dPax2* is on chromosome 4, the "dot" chromosome of *Drosophila*, which has a severely reduced recombination rate, resulting in inefficient selection and relaxed sequence constraint [69]. No other *cis*-regulatory module on the fourth chromosome has been subjected to an extensive evolutionary analysis, nor are any as well-mapped as *sparkling*, but enhancers of the fourth-chromosome genes *eyeless* and *toy* contain fairly large blocks of sequence conservation, compared to *spa* [70]. An alternative explanation for the rapid turnover observed within *spa* involves the presence of nonconsensus, predicted low-affinity sites for Su(H) and, in some cases, Lz and PntP2 (Figure 4; Figure 5). For a typical TF, there are many more possible low-affinity binding sites than high-affinity sites: for example, the highest-affinity Su(H) consensus YGTGDGAAM encompasses only 12 variants (TGTGGGAAA, etc.), whereas the lower-affinity consensus of the same length nRTGDGWDn, which accommodates all of the known Su(H) sites within *spa*, contains 576 possible sequences. Accordingly, it is much more likely that an enhancer will acquire a low-affinity binding site via a single mutational event than a high-affinity site. Thus, an enhancer that does not require high-affinity binding sites for given *trans*-regulators may rapidly sample a variety of configurations of weak sites and may thereby undergo considerable sequence turnover without losing the input from that regulator. In other words, an enhancer such as *spa*, which must maintain a weak regulatory linkage with Notch/Su(H), may be less constrained than a high-affinity target with respect to the sequence, number, and position of its Su(H) binding sites. Whatever the reason for the rapid sequence divergence of *spa*, it provides an opportunity to examine in detail the evolutionary mechanisms by which a complex *cis*-regulatory module can be significantly reorganized, while still conforming to specific constraints of combinatorial logic and grammar.

Supplemental Information

Supplemental Information includes three figures, Supplemental Discussion, Supplemental Experimental Procedures, and one table and can be found with this article online at doi:10.1016/j.cub.2011.05.056.

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