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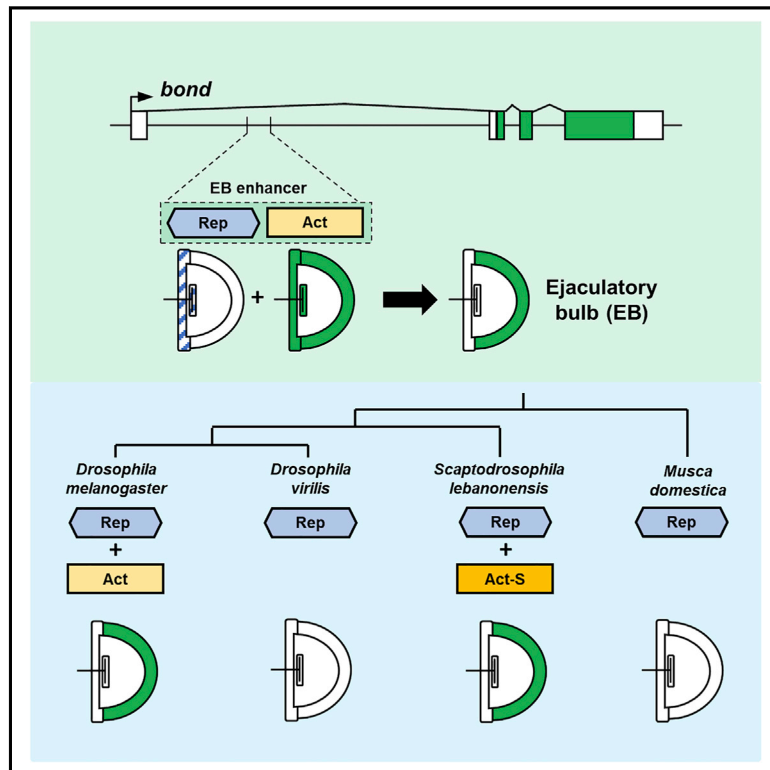
Repression precedes independent evolutionary gains of a highly specific gene expression pattern

Graphical abstract



Highlights

- An enhancer controls a highly specific EB expression of *bond* in *Drosophila*
- This enhancer contains activator and repressor sequences
- A similar enhancer is independently gained in the distantly related *S. lebanonensis*
- Similar repressor sequences are present in other species without *bond* EB expression

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In brief

Pu et al. show that the independent gain of a highly specific expression pattern across distantly related species may be because of the preexistence of repressor sequences that precedes the diversification of these species. This may reflect a general mechanism underlying the evolution of highly specific enhancers.

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Repression precedes independent evolutionary gains of a highly specific gene expression pattern

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SUMMARY

Highly specific expression patterns can be caused by the overlapping activities of activator and repressor sequences in enhancers. However, few studies illuminate how these sequences evolve in the origin of new enhancers. Here, we show that expression of the *bond* gene in the semicircular wall epithelium (*swe*) of the *Drosophila melanogaster* male ejaculatory bulb (EB) is controlled by an enhancer consisting of an activator region that requires *Abdominal-B* driving expression in the entire EB and a repressor region that restricts this expression to the EB *swe*. Although this expression pattern is independently gained in the distantly related *Scaptodrosophila lebanonensis* and does not require *Abdominal-B*, we show that functionally similar repressor sequences are present in *Scaptodrosophila* and also in species that do not express *bond* in the EB. We suggest that during enhancer evolution, repressor sequences can precede the evolution of activator sequences and may lead to similar but independently evolved expression patterns.

INTRODUCTION

Highly specific gene expression patterns are central to the development and evolution of multicellular organisms. These expression patterns are regulated by the action of modular *cis*-regulatory elements called enhancers (Small and Arnosti, 2020), which are controlled at different levels by transcription factors (TFs), such as pioneer factors that open up chromatin and initiate DNA binding by other factors (Zaret and Mango, 2016), transcriptional activators that drive gene expression, and transcriptional repressors that inhibit gene expression (Small and Arnosti, 2020), as well as other transcriptional factors controlled by specific signaling pathways (Barolo and Posakony, 2002). The overlapping and combinatorial actions of these different transcriptional factors within an enhancer control highly specific gene expression in time and space (Spitz and Furlong, 2012). A well-studied example of this logic is the even-skipped (*eve*) stripe 2 enhancer, where the combinatorial actions of two activators and three repressors define a single stripe of *eve* expression in the developing insect embryo (Andrioli et al., 2002; Arnosti et al., 1996; Ludwig et al., 2005; Small and Arnosti, 2020).

In the past few decades, numerous studies have demonstrated that changes in enhancer activities can lead to novel expression patterns and evolutionary innovations in morphology and physiology (Carroll, 2008; Rebeiz and Tsiantis, 2017; Wittkopp and Kalay, 2011). These evolutionary changes in the enhancers include the gain (Jeong et al., 2006) and loss (Jeong et al., 2008; Kvon et al., 2016) of activator-binding sequences, as well as the gain (Preger-Ben Noon et al., 2016) and loss (Sumiyama and Saitou, 2011) of repressor-binding sequences. Although these studies clearly show that evolutionary changes in either activator or repressor sequences can lead to phenotypic changes, fundamental questions remain regarding how these activator and repressor sequences interact during the birth of enhancers that control highly specific expression patterns.

In one of the first studies of *cis*-regulatory changes and evolutionary novelty, the gain of a novel enhancer that underlies a highly specific wing spot in males of *Drosophila biarmipes* is hypothesized to be due to recruitment of at least one activator and one repressor (Arnoult et al., 2013; Gompel et al., 2005; Xin et al., 2020). How do enhancers that rely on the combinatorial effects of both activator and repressor sequences evolve? A reasonable hypothesis would be that activator sequences evolve first, driving a broad expression pattern prior to the appearance of repressor sequences that restricts expression

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of the gene. However, one caveat of this prediction is that for many pleiotropic genes, gains in broad expression patterns may lead to negative fitness effects in the organism, as a result of possible misexpression of these genes in key tissues. Many studies in model organisms, such as *Drosophila*, mice, and zebrafish, have shown that misexpression of key genes in the wrong tissues can lead to negative phenotypic effects on the organism (Furuchi et al., 1996; Morgan et al., 1992; Ungar et al., 1995; Zhang and Odenwald, 1995). In humans, the mis-regulation of genes underlies many disease conditions (Lee and Young, 2013). Therefore, these negative fitness effects may lead to new broad expression patterns being selected against before the gain of repressor sequences. An alternate hypothesis is that repressor sequences precede the evolution of activator sequences, leading to the gain of a specific expression pattern without having evolved broad expression first, thus mitigating the negative fitness effects of broad gene expression patterns. However, the hypothesis also poses a potential quandary: can repressor sequences evolve before activator sequences if no gene expression is driven by repressors alone? How would repressor sequences be maintained during evolution if there is no gene expression output? Determining the evolutionary dynamics between activator and repressor sequences can lead to a better understanding of how enhancers originate and how highly specific expression patterns in nature are generated during evolution.

In this study, we investigated the *cis*-regulatory evolution of the fatty acyl-CoA elongase gene *bond*, a pleiotropic gene involved in *Drosophila* spermatogenesis (Szafer-Glusman et al., 2008), as well as the biosynthesis of the male anti-aphrodisiac, CH503, a male-specific pheromone produced in the ejaculatory bulb (EB) by several *Drosophila* species, which is transferred to the female during mating to prevent remating by other males (Ng et al., 2014; Yew et al., 2009). The specific expression of *bond* in the EB is necessary for the production of CH503. However, species such as *D. erecta* and *D. ananassae* do not produce CH503 but express *bond* in the EB, suggesting that *bond* may also have other unknown roles in the EB of these species (Ng et al., 2015). We investigated the evolution of EB-specific expression of *bond* across a broad phylogeny of 21 *Drosophila* and other related species. We show that there are two independent evolutionary gains of *bond* expression across these species. In *D. melanogaster*, *bond* expression is controlled by an enhancer consisting of an activator region (Act) that drives *bond* expression in the entire EB and a repressor region (Rep) that restricts expression to a specific part of the EB. Interestingly, this Rep region is present even in *D. willistoni* and *D. virilis*, as well as the distantly related housefly, *Musca domestica*, where EB expression of *bond* is not detected. We further show that although *bond* EB expression in *Drosophila* requires the TF *Abdominal-B* (*Abd-B*), *bond* EB expression in *Scaptodrosophila* does not require *Abd-B.* Taken together, our experiments suggest that the evolution of the repressor sequences can precede the evolution of highly specific expression patterns. Repeated evolution of similar specific patterns across lineages may be because of the preexistence of similar repressor sequences, even if the activator sequences may be different.

RESULTS

Two independent evolutionary gains of bond EB expression across the *Drosophila* genus and related species

The EB in *D. melanogaster* is made of distinct parts, including a bulbar cavity devoid of cells that holds the ejaculate (Cohen and Wolfner, 2018). Expression of *bond* in the *D. melanogaster* EB is restricted to a specific epithelial region, which we named the semicircular wall epithelium (*swe*) (Figure 1A). To determine the evolutionary history of *bond* expression in the EB, we used *in situ* hybridization of specific *bond* RNA probes to examine a broad phylogeny of 21 different species, including 16 *Drosophila* species, 3 *Scaptodrosophila* species, 1 *Chymomyza* species, and the housefly, *Musca domestica*. EB expression of *bond* is detected in seven *Drosophila* species, *D. melanogaster*, *D. simulans*, *D. erecta*, *D. yakuba*, *D. ananassae*, *D. pseudoobscura*, and *D. subobscura*, as well as three *Scaptodrosophila* species, *S. latifasciaeformis*, *S. lebanonensis*, and *S. rufifrons* (Figure 1B; Figure S1). Because the species expressing *bond* in the EB are divided across two lineages in the phylogenetic tree, we reconstructed the ancestral status of *bond* EB expression using a maximum likelihood model to determine whether EB expression is gained or lost multiple times during the evolutionary history of these species. Our analysis showed there are likely two independent gains of bond EB expression across our phylogeny: one in the lineage before the split of the *melanogaster* group and the *obscura* group in the *Drosophila* genus, and one in the lineage before the diversification of the *Scaptodrosophila* genus (Figure 1B; Figure S1).

cis-regulatory evolution underlies the differential expression of *bond* in different species

To investigate how the expression of *bond* in EB arose, we first sought to locate the enhancer driving this expression pattern in *D. melanogaster*. GFP reporter constructs using non-coding DNA sequences around the *bond* gene (3R: 22547847..22558341, Flybase version: FB2021_01) in *D. melanogaster* showed that the enhancer responsible for driving gene expression in the EB *swe* lies in the first intron of *bond* (Figure 2A; Figure S2). Homologous regions from *D. simulans*, *D. yakuba*, *D. erecta*, *D. ananassae*, and *S. lebanonensis* drove similar GFP expression in the EB *swe*, but homologous regions from *D. willistoni*, *D. virilis*, and *M. domestica* did not (Figure 2B). This observation is consistent with our *in situ* hybridization results for these species (Figure S1), suggesting that differences in *bond* EB expression across these species are due to the evolution of *cis*-regulatory sequences present in the first intron of *bond*.

A 285-bp enhancer, composed of both activator and repressor sequences, recapitulates specific expression of *bond* in the *swe* of the *D. melanogaster* EB

Having confirmed that *cis*-regulatory differences underlie *bond* expression differences in the *swe* of the EB, we sought to identify the evolutionary changes in the *cis*-regulatory sequences that led to the differential expression patterns. Our first step was to determine the minimum enhancer region specific for *bond*

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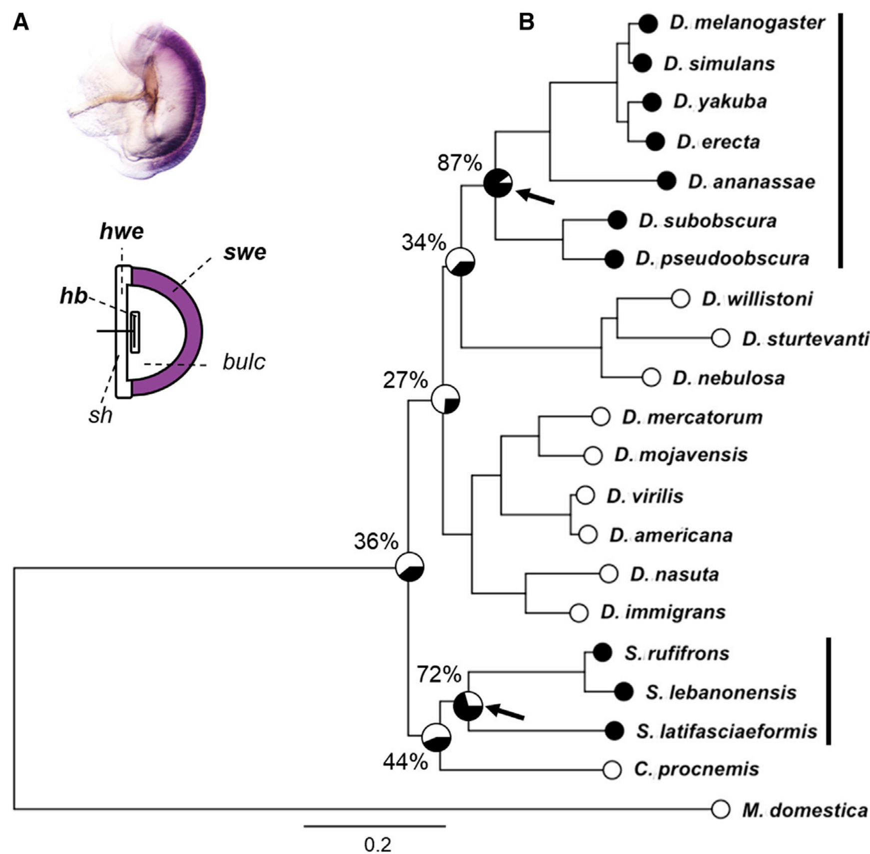

Figure 1. Two independent evolutionary gains of *bond* ejaculatory bulb (EB) expression across 21 species

(A) Schematic of the EB in *D. melanogaster*. The bulbar cavity is devoid of cells and holds the ejaculate. *bond* is specifically expressed in the *swe* of the EB in *Drosophila melanogaster.*

(B) Ancestral trait reconstruction using a maximum likelihood model suggests two independent evolutionary gains of *bond* EB expression across the phylogeny. Arrows indicate likely gains in *bond* EB expression: one in the lineage leading to the *melanogaster* group and the *obscura* group in the *Drosophila* genus, and the other in the lineage leading to the *Scaptodrosophila* genus. Black and white circles for each species indicate the presence or absence of *bond* EB expression, respectively. Percentages on key nodes indicate probability of *bond* EB expression in the ancestor. Phylogeny was constructed using data from Finet et al. (2021). Scale bar indicates the number of nucleotide changes per site.

bc, bulbar cavity; *hb*, handle base; *hwe*, horn wall epithelium; *sh*, sclerite handle; *swe*, semicircular wall epithelium.

expression in the EB *swe*. We performed a systematic dissection of the first intron of *D. melanogaster* to delimit smaller regions for the enhancer by creating smaller overlapping GFP constructs (Figure S2). We first narrowed down a 404-bp region (*bc* fragment) that recapitulates the expression pattern driven by the full intron (Figure 3A; Figure S2). Further dissection of this region allowed us to narrow down an even smaller 285-bp fragment (construct *bc23*) that expresses GFP in the EB *swe* (Figures 3A and 3B). We named this the EB *swe* enhancer. We next set out to identify sequences in this minimal enhancer that potentially underlie *bond* expression differences between species. We initially divided the 285-bp enhancer fragment into overlapping constructs, *bc2* and *bc3*. *bc2* did not drive any GFP expression, but notably, *bc3* drove expression in the entire EB, not just the *swe* of the EB (Figures 3A and 3B). This result suggests that repressor sequences that are present in the *bc2* fragment repress ectopic expression in the horn wall epithelium (*hwe*) and handle base (*hb*) of the EB driven by the *bc3* fragment (Figure 3B). Dissection of *bc3* into two smaller overlapping constructs, *bc3i* and *bc3ii*, shows that although *bc3i* drove expression in the *hb*, *bc3ii* did not drive any GFP expression. This result suggests that *bc3ii* contains activator sequences necessary to drive expression in the whole EB in conjunction with *bc3i* but cannot independently drive expression. Together, our data show that the *bond* EB *swe* enhancer comprises a Rep region and an Act region, which has at least two different transcriptional inputs (Ac1 and Ac2) (Figure 3C).

Activator sequences for EB expression are present in *D. willistoni*, even though *bond* is not expressed in the EB of this species

To trace the evolutionary origins of this enhancer in *Drosophila*, we examined the activity of the homologous sequences from three other species, *D. ananassae*, *D. willistoni*, and *D. virilis*, based on their *bond* expression in the EB and their phylogenetic relationships (Figure 1B). Our results indicate that the *bc* fragments from these three species are able to recapitulate the EB expression of *bond* (in the case of *D. willistoni* and *D. virilis*, no expression) (Figure 4A). Our *a priori* expectation is that creating smaller fragments of the 285-bp enhancer in *D. melanogaster* and *D. ananassae* would allow us to narrow the region involved in enhancer evolution, and we expected that there would be no GFP expression driven by the smaller fragments in *D. willistoni* and *D. virilis*. Although we did not detect GFP expression in any of the *D. virilis* constructs, to our surprise, the *bc3i* fragment of *D. willistoni* was able to drive GFP expression in the *hb* of the EB, similar to homologous fragments in *D. melanogaster* and *D. ananassae* (Figure 4A). This result suggests that there are activator sequences in *D. willistoni* that can drive partial GFP expression in the EB. Our observation that the *D. willistoni bc3i* fragment can drive GFP expression in the *hb* of the EB, but not the full *D. willistoni bc* fragment, suggests that repressor sequences similar to the Rep region present in *D. melanogaster* may also be present in *D. willistoni* (Figure 4A).

Repressor sequences are present in species that do not express *bond* in the EB

To confirm our observation that repressor sequences may be present in *D. willistoni*, we created GFP reporter constructs

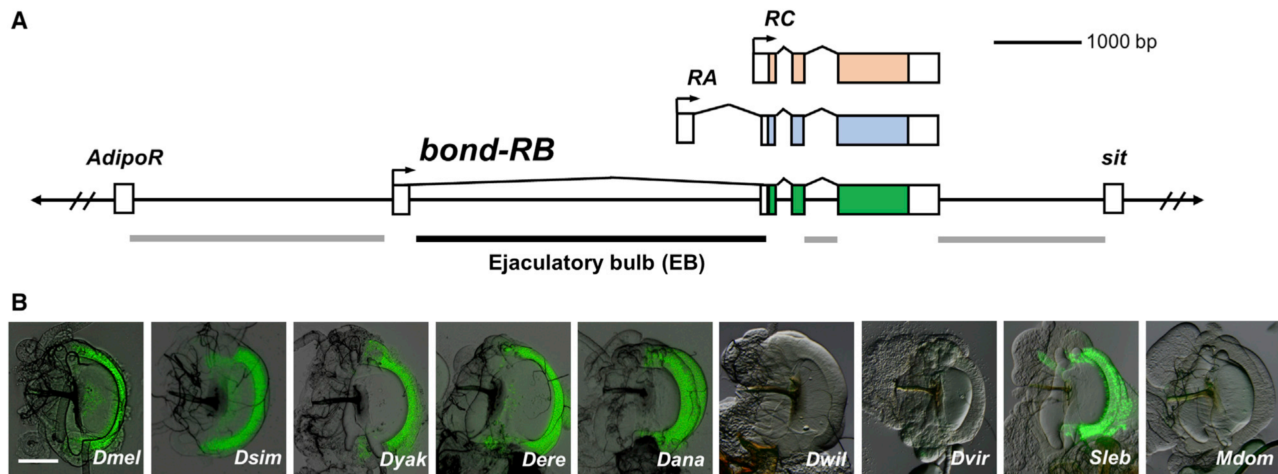

Figure 2. Evolution of *cis*-regulatory sequences in the first intron of *bond* underlies differences in EB expression across species

(A) The non-coding regions around the *D. melanogaster* bond locus were screened for enhancer activity that is able to drive GFP reporter protein expression in the EB. One fragment (black line), the first intron, was able to drive GFP expression in the EB. The other fragments (gray lines) did not drive EB expression.

(B) Intron 1 of *bond* from *D. melanogaster*, *D. simulans*, *D. yakuba*, *D. erecta*, *D. ananassae*, and *S. lebanonensis* drove GFP expression in the EB *swe*. Homologous fragments from *D. willistoni*, *D. virilis*, and *M. domestica* did not. Scale bar, 100 μm.

that fused the region from *D. willistoni* homologous to the Rep region in *D. melanogaster* with the *D. melanogaster bc3* construct that drives expression in the entire EB. If the *D. willistoni* sequence functions as a repressor, it should spatially repress expression of the *D. melanogaster bc3* fragment and restrict expression to the *swe* of the EB in *D. melanogaster*, similar to the repressor sequences in *D. melanogaster* (Figure 4A). Our results confirm this prediction: the *D. willistoni* fragment effectively repressed *bc3*-driven GFP expression in the *hwe* and the *hb* and restricted expression to the *swe*, thus confirming that repressor sequences are present in *D. willistoni* (Figure 4B). Because *D. willistoni* does not express *bond* in the EB and does not have sequences that can drive expression in the EB *swe*, we were intrigued that repressor sequences that can partially repress EB expression are present in *D. willistoni*. This observation motivated us to investigate whether these spatial EB repressor sequences are also present in other species where *bond* is not expressed in the EB. We tested three other *Drosophila* species, *D. mojavensis*, *D. virilis*, and *D. nasuta*, as well as a more distant species, *M. domestica*. We also tested the presence of repressor sequences in *S. lebanonensis*, a species that independently gains *bond* EB expression. Homologous regions from all five of these species can repress expression driven by the *D. melanogaster bc3* construct, restricting GFP expression in the EB *swe* (Figure 4B). Taken together, these observations suggest that repressor sequences are present in these species and, based on the phylogeny, precede the evolution of the complete minimal EB *swe* enhancer.

The *D. melanogaster bond EB* Rep region can repress gene expression of another EB enhancer in a distance-dependent manner and is a short sequence in *Drosophila species*

The presence of similar spatial repression in different species led us to further characterize this repressor. First, we wanted to know if this repressor is modular, i.e., can it repress the expression of other genes expressed in the EB, or does it work only in the context of the *bond* gene? To determine this, we created GFP reporter constructs that fused the *D. melanogaster* Rep region to the 5′ regulatory region of another EB expressed gene, *Cyp312a1* (Figure 4C). A 1-kb construct (*5′Cyp312a1-1kb*) and a 665-bp (*5′Cyp312a1-665bp*) construct of the 5′ regulatory region of *Cyp312a1* drive GFP specifically in the *hwe* and *hb* of the EB (Figure 4C). When we fused the *D. melanogaster* Rep region to these two constructs, there was no change in spatial expression pattern in the *5′Cyp312a1-1kb* construct. However, we did not detect GFP expression in the *5′Cyp312a1-665bp* construct after being fused with the *D. melanogaster* Rep region (Figure 4C). Together, these results suggest that the Rep region is modular, because it is able to repress the EB expression driven by activator sequences of another gene. Moreover, the repressor activity is distance dependent because it can repress EB expression in the shorter 665-bp construct, but not the 1-kb construct. Next, we wanted to determine whether the Rep region is a large region or a short sequence capable of spatial repression in the *hwe* and *hb* of the EB. To narrow down the sequences involved in repression, we made smaller constructs of the *D. melanogaster* 141-bp Rep region and assayed for their ability to repress GFP expression driven by the *bc3* construct (Act region) (Figure 4D). These constructs, coupled with additional site-directed mutagenesis experiments, narrowed the repressor sequence to an 11-bp sequence (5′-AAATTAATTTA-3′) that is able to recapitulate the repressor activity of the *D. melanogaster* Rep region (Figure 4D). A search on Flybase (Thurmond et al., 2019) showed that there are 634 hits to this sequence in the *D. melanogaster* genome, implying the non-uniqueness and pervasiveness of this sequence throughout the genome. An alignment of this region in some of the species tested in this study showed highly similar sequences in some species and dissimilar sequences in other species at the site of

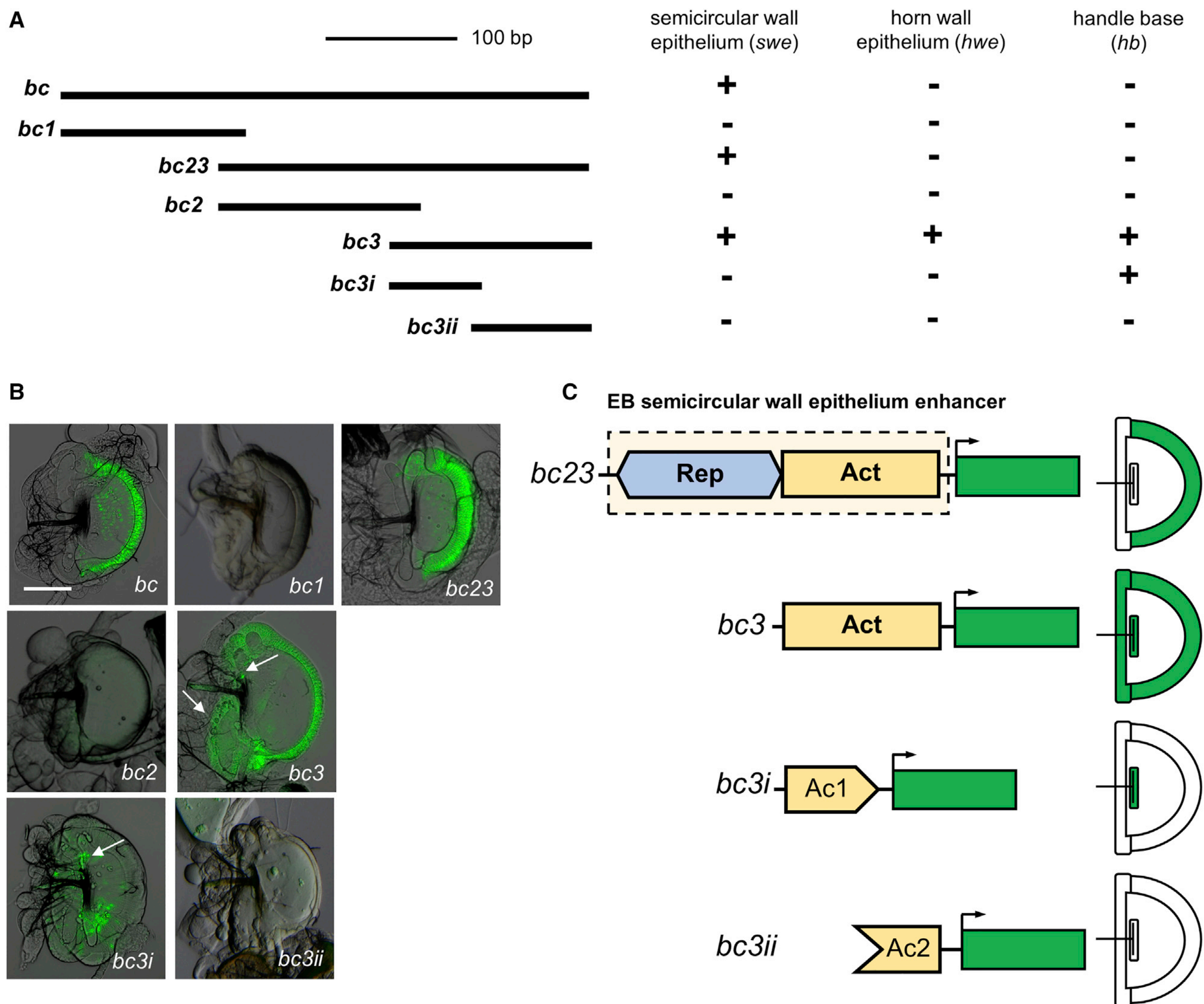

Figure 3. The combination of repressor and activator sequences in the *D. melanogaster bond* EB enhancer drives specific expression in the *swe*

(A) Schematic of the overlapping GFP constructs of the EB enhancer. The *bc23* fragment is the minimum region that can recapitulate the EB expression of *bond* in the *swe*. The *bc3* fragment shows the ectopic GFP expression in horn wall epithelium (*hwe*) and handle base (*hb*). Plus sign (+) indicates the presence of expression; minus sign (–) indicates the absence of detectable expression.

(B) GFP reporter protein expression in the EB corresponding to the different overlapping constructs. Arrows indicate the expression in *hwe* and *hb*. Scale bar, 100 μm.

(C) The *D. melanogaster bond* EB *swe* enhancer contains two modular regions. The activator region (Act) contains activator sequences that drive expression in the whole EB (in *hwe*, *hb*, and *swe*). The repressor region (Rep) represses GFP activity in the *hb* and *hwe* of EB and restricts activity to the *swe* of the EB. The Act region can be divided into two different inputs, Ac1 and Ac2, both of which are needed to drive expression in the whole EB. Ac1 on its own can drive GFP expression in the *hb*, but Ac2 alone does not drive any expression.

this 11-bp sequence (Figure S3). Experiments using the aligned 11-bp sequence from *D. ananassae* (5′-AAATTAAATTA-3′) and *D. willistoni* (5′-CTATTAATTTT-3′) showed that these sequences can repress expression in the *hwe* and *hb* of the EB, whereas the aligned 11-bp sequence from *D. virilis* (5′-ACAAAAAAAAA-3′) could not (Figure S4). Because the full *D. virilis* Rep region can repress expression in the *hwe* and *hb* of the EB (Figure 4B), the results suggest that the 11-bp sequences in distantly related species from *D. melanogaster* may be dissimilar in sequence and location but functionally similar in the ability to spatially repress expression in the EB.

Evolution of putative *Abd-B* activator binding sites is involved in the stepwise evolution of *bond* EB expression in *Drosophila*

We have established that repressor sequences are present in the several species that do not express *bond* in the EB (Figure 4B). One of these species, *D. willistoni*, has sequences homologous

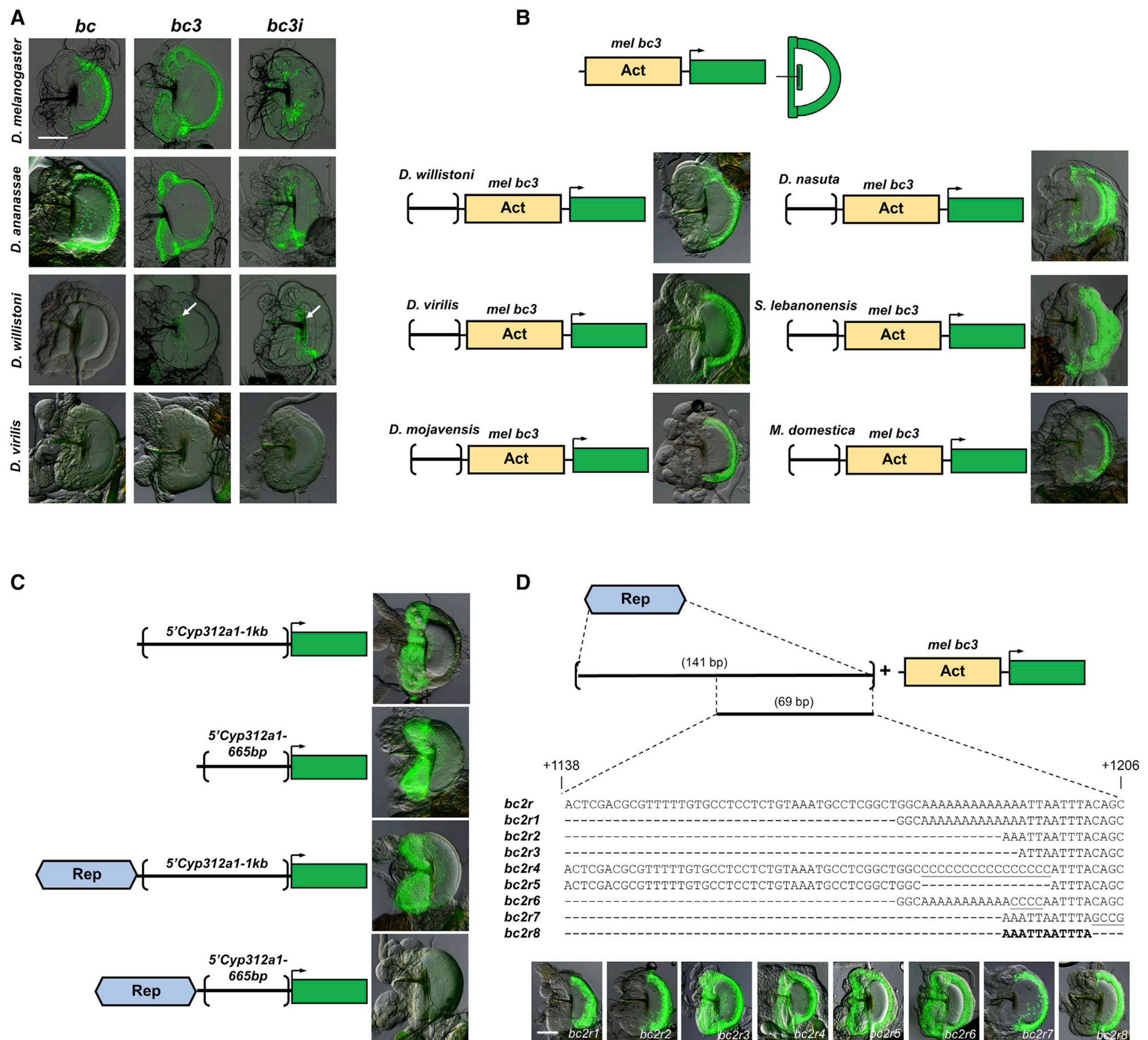

Figure 4. Repressor sequences that can repress *bond* expression in the EB *hwe* and *hb* are present in all species tested, including species that do not express *bond* in the EB

(A) The larger *bc* fragment recapitulates the native expression of *bond* in the EB in *D. melanogaster* and *D. ananassae* and showed no EB GFP expression with *D. willistoni* and *D. virilis* homologous constructs, similar to their native expression. However, the smaller *D. willistoni* bc3 and bc3i constructs could drive GFP expression in the handle base (white arrows), similar to the *bc3i* construct of *D. melanogaster*, suggesting the presence of repressor sequences in the larger *bc* construct of this species. Scale bar, 100 μm.

(B) Homologous sequences to the *D. melanogaster* Rep region from six other species, *D. willistoni*, *D. virilis*, *D. mojavensis*, *D. nasuta*, *S. lebanonensis*, and *M. domestica*, can repress GFP expression in the *hwe* and *hb* driven by the *mel bc3* fragment, suggesting that these regions contain repressor sequences similar to the *D. melanogaster* Rep region.

(C) The *D. melanogaster* Rep region can repress EB GFP expression in the *hwe* and *hb* driven by another activator sequences from another gene, *Cyp312a1*, in a distance-dependent manner.

(D) A series of deletion and mutation constructs are made for the 141-bp Rep region of *D. melanogaster*. The mutated nucleotides are underscored. A 11-bp sequence (5′-AAATTAATTTA-3′) is able to repress GFP expression in the *hwe* and *hb* driven by the *mel bc3* fragment. Scale bar, 100 μm.

to the first transcriptional input (Ac1) in *D. melanogaster* that could drive partial expression in the EB when isolated from the repressor, similar to that of *D. melanogaster* and *D. ananassae* (Figure 4A). This result suggests that the gain of *bond* expression in the EB *swe* could be a stepwise evolutionary process. To investigate how the EB expression of *bond* evolved, we sought to identify potential transcriptional activators in this enhancer that can drive expression in the EB. We performed

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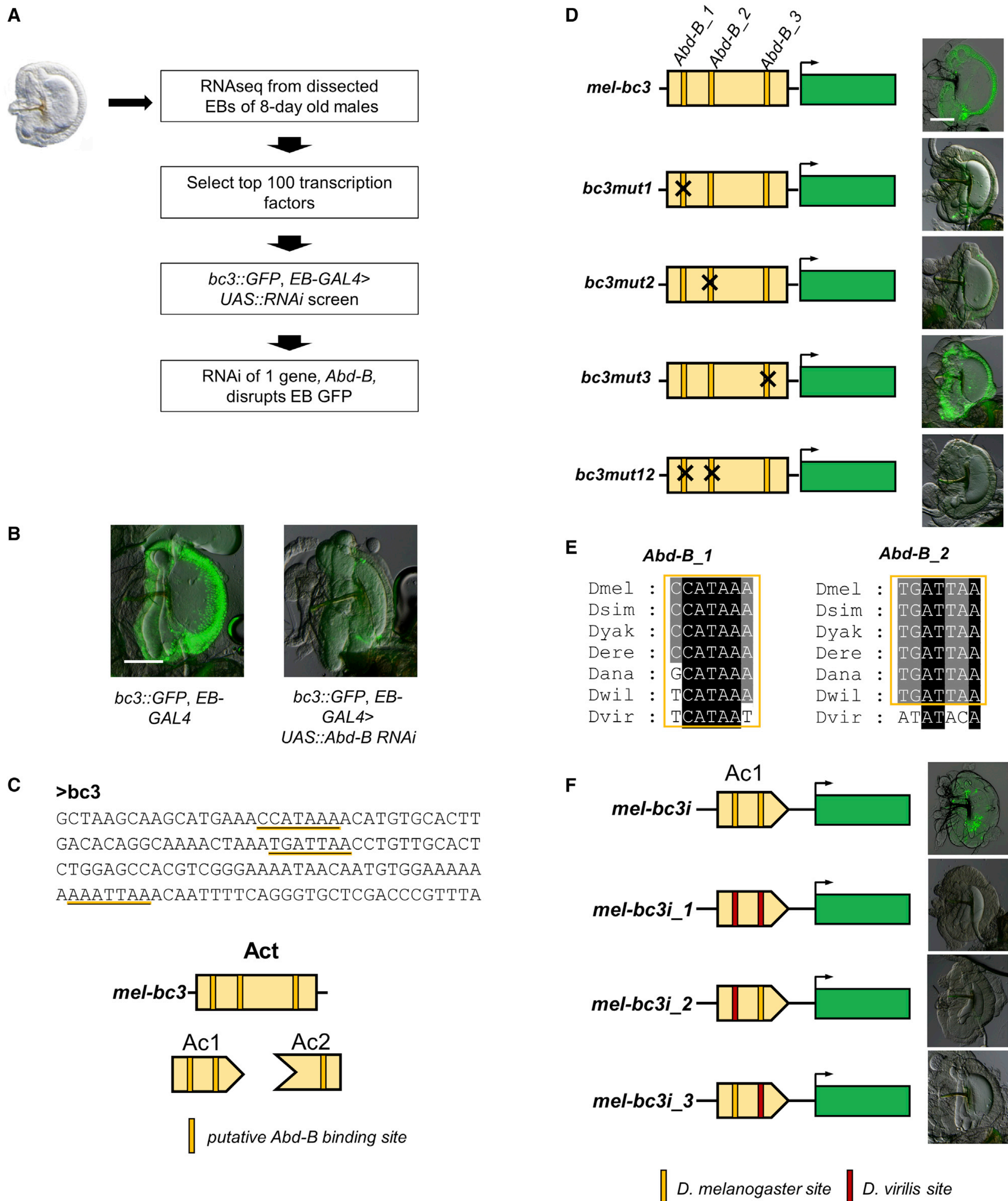

Figure 5. The evolution of two putative Abd-B binding sites is necessary for the expression of bond in the EB

(A) An EB RNAi screen identifies Abd-B as a possible regulator of *bond* in the EB.

(B) RNAi knockdown of bond in the EB led to a big decrease in GFP expression driven by the *D. melanogaster bc3* construct. Scale bar, 100 μm.

(C) JASPAR analysis identified three putative *Abd-B* binding sites in the *D. melanogaster* bc3 construct.

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high-throughput transcriptomic sequencing of EBs from 8-day-old male *D. melanogaster* and identified putative TFs that are expressed in the EB (Table S1). Next, we carried out a reverse genetic screen by using an EB-specific GAL4 driver (EB-GAL4) to drive *UAS::RNAi* constructs of the most highly expressed TFs (Table S2) in order to ascertain which TF(s) can affect the *mel bc3* construct-driven GFP expression (Figure 5A). Out of the 100 *UAS::RNAi* constructs tested, one line, driving RNAi knockdown of the homeobox gene, *Abd-B*, was able to disrupt *bc3*-driven GFP expression in the EB, suggesting that *Abd-B* may function as a transcriptional activator for the expression of *bond* in the EB (Figure 5B). The RNAi of *Abd-B* in the EB also led to morphological changes in the EB, suggesting that *Abd-B* may also be required for EB development. Bioinformatics analyses using JASPAR (Fornes et al., 2020) predicted three putative *Abd-B* binding sites (Figure 5C).

To determine whether the putative *Abd-B* binding sites function *in vivo*, we systematically mutated each site in the *bc3* GFP construct (Figure 5D). Our experiments show that site-directed mutagenesis of the first two putative *Abd-B* binding sites, *Abd-B_1* and *Abd-B_2*, reduced GFP expression drastically when mutated individually and abolished GFP expression completely when mutated in combination. In contrast, mutation of the third putative *Abd-B* binding site, *Abd-B_3*, did not affect GFP expression (Figure 5D). Our results suggest that *Abd-B_1* and *Abd-B_2* sites are both necessary to drive *bond* expression in the EB of *D. melanogaster*. Because *Abd-B_1* and *Abd-B_2* are both in the Ac1 region (bc3i construct), we expect that these two sites will be conserved in all *melanogaster* group species tested and *D. willistoni*, but not *D. virilis.* To trace the evolution of *Abd-B_1* and *Abd-B_2* in these species, we performed an alignment of the *bc23* region of these species (Figure S3). We used JASPAR to predict potential *Abd-B* binding sites in all these species and found that all *Drosophila* species have putative *Abd-B* binding sequences at *Abd-B_1*, and species in the *Sophophora* subgenus have putative *Abd-B* binding sequences at *Abd-B_2*, but not in *D. virilis* (Figure 5E). These predictions are consistent with the activity of homologous *bc3i* fragments (Ac1 region) from the four species, in which *D. melanogaster, D. ananassae*, and *D. willistoni* sequences drove GFP reporter expression in the *hb* of the EB, but *D. virilis* did not (Figure 4A). To determine whether the predicted difference at the *Abd-B* binding sites could underlie differences in expression driven by the *D. melanogaster* and *D. virilis bc3i* constructs, we swapped in the corresponding *D. virilis* sequences at the *D. melanogaster Abd-B_1* and *Abd-B_2* sites individually in the *D. melanogaster bc3i* construct. Our results show that the *D. virilis* sequence at both sites either individually or together led to the loss of GFP expression in the *hb* of the EB (Figure 5F). Taken together, the findings suggest that evolution in both *Abd-B_1* and *Abd-B_2* sites between these species could contribute toward the evolution of *bond* expression in the EB.

The *S. lebanonensis* enhancer does not require *Abd-B* to drive gene expression in the EB *swe*

Because the expression of *bond* in the EB *swe* is independently gained in two lineages, we wanted to determine whether the parallel evolution of this trait has a similar or different genetic basis. We had shown that the *bond* EB *swe* enhancer lies in intron 1 of *bond* in both lineages (Figure 2B). To investigate whether *bond* EB *swe* expression pattern in *S. lebanonensis* requires *Abd-B*, we use a *S. lebanonensis bond* intron 1 GFP construct that can drive EB *swe* GFP expression in transgenic *D. melanogaster*. The 3,112-bp *S. lebanonensis* B1 construct (*Sle_B1*) contains 64 putative *Abd-B* sites as predicted by JASPAR. However, when we drove RNAi of *Abd-B* in the EB of transgenic *D. melanogaster* carrying this *S. lebanonensis* B1 construct, we did not see obvious changes in GFP expression compared with the control (Figure 6). In contrast, RNAi of *Abd-B* drastically reduced GFP expression in a *D. melanogaster bond* EB *swe* enhancer-driven construct. In both cases, RNAi caused morphological changes to the EB, suggesting that EB RNAi of *Abd-B* is successful. Our results showed that *bond* EB expression in *S. lebanonensis* is likely to be *Abd-B* independent (Figure 6).

DISCUSSION

The generation of highly specific gene expression patterns is often due to the combinatorial actions of activators, repressors, and other transcriptional inputs that form a modular enhancer (Small and Arnosti, 2020; Spitz and Furlong, 2012). However, the evolutionary history that leads to the origin of these enhancers is not always clear. In this study, we have identified and characterized an enhancer that drives precise expression of the elongase gene *bond* in the male EB *swe* of *D. melanogaster* and several other species in the *Drosophila* genus. EB expression of *bond* has been shown to be necessary for the production of the male anti-aphrodisiac pheromone, CH503 (Ng et al., 2015). Our experiments show that the EB *swe* enhancer is made up of at least one Rep region and one Act region, which can be subdivided in two transcriptional inputs (Ac1 and Ac2). Together, the combined action of these transcriptional inputs restricts *bond* expression to the *swe* of the EB in *D. melanogaster* (Figure 3C). We further show that the Rep region containing sequences capable of repressing gene expression in parts of the EB is present in all *Drosophila* species tested in our study, as well as in distantly related species, *Scaptodrosophila lebanonensis* and *Musca domestica*, even though many of these species do not express *bond* in the EB *swe* (Figure 4B). These findings suggest that the spatial repressor precedes the evolution of the Act regions (Figure 7).

(D) Site-directed mutagenesis of these putative *Abd-B* binding sites individually shows that the *Abd-B_1* and the *Abd-B_2* sites are necessary for GFP expression driven by the bc3 construct, but not the *Abd-B_3* site. Scale bar, 100 μm.

(E) Evolutionary analysis of the *Abd-B_1* and the *Abd-B_2* sites shows that all species have putative *Abd-B* binding sequences at *Abd-B_1*, and most species have putative *Abd-B* binding sequences at *Abd-B_2* except *D. virilis*.

(F) Swapping in the *D. virilis* sequence at both sites either individually or in combination led to the loss of GFP expression in the *hb* of the EB driven by the *D. melanogaster* bc3i construct.

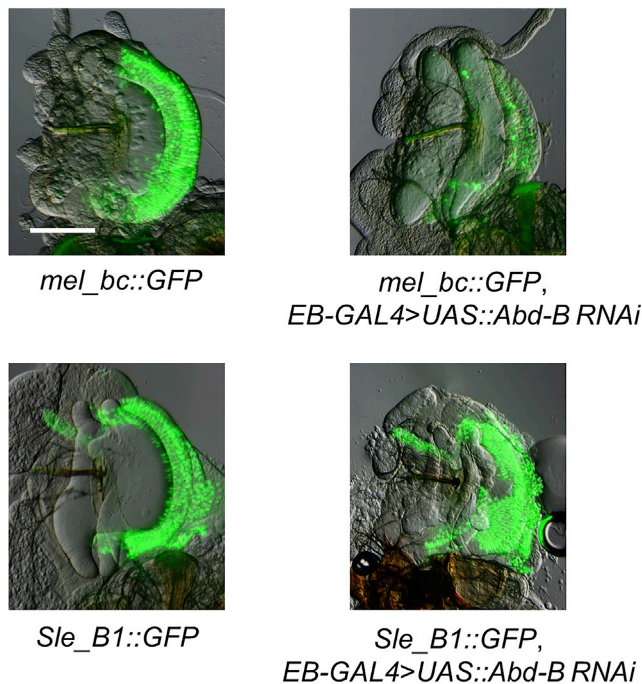

Figure 6. *bond* EB expression in *S. lebanonensis* is independent of *Abd-B*

EB-specific RNAi of Abd-B greatly reduces expression of EB *swe* GFP driven by the *D. melanogaster bc* construct but has no observable effects on EB *swe* GFP driven by the *S. lebanonensis* B1 construct. Scale bar, 100 μm.

During the divergence of the *Sophophora* and *Drosophila* subgenus in the genus *Drosophila*, the Ac1 activator regulatory input, containing putative binding sites for the TF *Abd-B*, evolved in the *Sophophora* subgenus. However, as seen in *D. willistoni*, the presence of Ac1 was not sufficient for *bond* expression in the EB due to the overlapping action of the repressor sequences. The Ac2 transcriptional input that evolved in the ancestor leading to the *melanogaster* and *obscura* groups allowed for expression in the entire EB with Ac1 but was not able to drive expression on its own (Figure 4A). Together, the combinatorial action of the Rep region and the full Act (Ac1 + Ac2) region form a modular enhancer driving specific expression in the *swe* of the EB in some *Drosophila* species (Figures 3C and 7). Based on our ancestral trait reconstruction analyses (Figure 1B), we show that there is likely to be an independent gain of *bond* EB expression in *Scaptodrosophila* species. We also showed that *bond* EB expression in *S. lebanonensis* is *Abd-B* independent, providing further evidence that it is likely to be an independent evolutionary gain.

The evolution of modular enhancers

The evolution of new enhancers activity can occur from at least four different evolutionary mechanisms: transposition, promoter switching, co-option, and *de novo* generation (Rebeiz and Tsiantis, 2017). Although the previous studies have provided evidence for the first three mechanisms (Chung et al., 2007; Guo et al., 2016; Koshikawa et al., 2015), *de novo* evolution of enhancers has been difficult to study because of their rarity (Rebeiz and Tsiantis, 2017). Identifying a *de novo* origin of an enhancer is challenging partly because these enhancers are usually composed of multiple binding sites or sequences that bind different transcriptional activators or repressors. It is therefore more parsimonious to evolve a new enhancer by co-opting existing regulatory sequences (Rebeiz et al., 2011). However, we suggest that the *de novo* generation of an enhancer is possible. Previous genome-wide studies in *Drosophila* and human suggest that a large fraction of these genomes is bound by TFs (ENCODE Project Consortium, 2012; Roy et al., 2010). Although there is a vigorous scientific debate on whether these TF-bound sequences are functional (Graur et al., 2013; Kellis et al., 2014), the fact that TF binding sites are short, degenerate, and dispersed widely and randomly across the genome suggests that a small number of mutations generating new TF binding sites around these existing TF-bound sequences could form a new enhancer based on the combinatorial effects of these sequences. Our study shows that the gain of the Ac2 transcriptional input led to the evolution of an EB *swe* enhancer in *Drosophila* species. However, Ac2 is unable to drive expression on its own, relying on the combinatorial activity with the Ac1 transcriptional input and the Rep region to drive expression specifically in the EB *swe*. Because we did not detect any other enhancer activities within 1 kb of this region, we suggest that this could be a case of *de novo* generation of a novel enhancer, although we cannot rule out that co-option could also be a possibility (see later discussion on the origin of the Rep region).

The presence of repressor sequences before the evolution of highly specific expression patterns

Numerous studies have demonstrated the significance of repressor binding sites or sequences in modular enhancers to constrain and shape gene expression (Gompel et al., 2005; Preger-Ben Noon et al., 2016; Small et al., 1992; Struffi et al., 2011). However, to our knowledge, there are no studies that investigate the order of whether activator sequences or repressor sequences evolved first in the origin of enhancers driving highly specific expression patterns. The presence of functionally similar repressor sequences (Rep) in multiple *Drosophila* species and *Musca domestica* that do not express *bond* in the EB suggest that these sequences may be present at least 150 million years ago, which is the estimated divergence time between *Musca* and *Drosophila* (Thomas et al., 2020). What could be the putative function(s) of these sequences before being part of the *bond* EB *swe* enhancer? We propose three different hypotheses.

The first hypothesis is that this repressor sequence could be part of another enhancer in the *bond* intron and is co-opted into the *bond* EB *swe* enhancer. However, our experiments in *D. melanogaster* showed that there are no other apparent enhancer activities within 1 kb of the Rep region aside from Ac1 and Ac2 (Figure S2). Our experiments show that the Rep region represses gene expression in a distance-dependent manner (Figure 4C) and is not able to repress gene expression that is more than 1 kb away. This observation could suggest that the repressor associated with the Rep region functions as distance-dependent ''short-range repressors'' like knirps and Krüppel, which can repress gene expression only around 100 bp away (Small and Arnosti, 2020), rather than ''long-range repressors,'' such as Hairy, which mediates repression of *cis*-

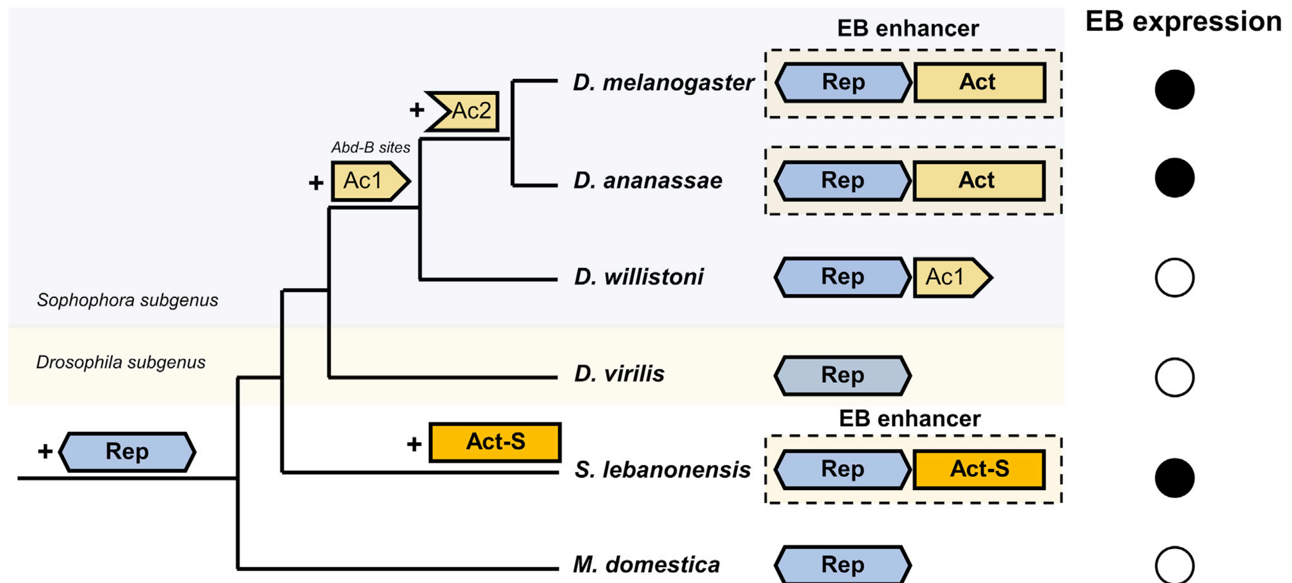

Figure 7. Repression precedes independent evolutionary gains of a highly specific gene expression pattern
The EB *swe* enhancer present in several *Drosophila* species is made up of an Act region, which contains two distinct transcriptional inputs (Ac1 and Ac2) that drive expression in the whole EB (*hb*, *hwe*, and *swe*), and a Rep that represses expression in the *hb* and *hwe*, resulting in the expression of *bond* in the EB *swe*. The first transcriptional input (Ac1) evolved in the *Sophophora* subgenus, possibly because of the gain of *Abd-B* binding sites. The Ac1 region is present in *D. willistoni*, but there is no expression of *bond* in the EB of this species due to the presence of the Rep region. The second transcriptional input (Ac2) evolved before the split of the *melanogaster* group and the *obscura* group and can drive expression in the whole EB in conjunction with Ac1, but because of the presence of Rep, *bond* expression is restricted to the *swe* in these species. An independent gain of EB gene expression occurs in *Scaptodrosophila* species that do not require *Abd-B* as a transcriptional input. The Rep region is present in all species, including the housefly *M. domestica*, and precedes the evolution of the activator sequences.

regulatory sequences more than 1 kb away (Li and Arnosti, 2011). A recent paper showed that other *cis*-regulatory sequences, such as silencers, may have dual roles as transcriptional enhancers when the cellular context is different (Gisselbrecht et al., 2020). The Rep region identified in our study, which functions as a Rep region in the *bond* EB *swe* enhancer, may have other unidentified and undetermined roles in other cell types. We do not exclude the possibility that the Rep region of the *bond* EB *swe* enhancer was co-opted from another regulatory function that it may have, but determining other functions is beyond the scope of this paper.

A second hypothesis is the Rep region is evolutionarily conserved because of this region overlapping with the exon of an antisense non-coding RNA, *CR44062*, which resides on the opposite DNA strand to *bond* (Figure S5). Although the function of *CR44062* is unknown, one possible scenario is that the Rep region is conserved because of potential functional constraint on the evolution of *CR44062*; i.e., evolutionary changes in the sequence of *CR44062* may have negative fitness effects.

A third hypothesis is that binding sites for the transcriptional activators or repressors are usually very short (6–10 bp long) (Payne and Wagner, 2014; Stewart et al., 2012) and due to this short length are pervasive and randomly distributed throughout the genome. We narrowed down the putative repressor binding site to an 11-bp sequence in *D. melanogaster* (Figure 4D). Bioinformatics analyses showed that this exact 11-bp sequence has 634 complete matches in the *D. melanogaster* genome. In addition, homologous 11-bp sequences from *D. ananassae* and *D. willistoni* with inexact matches to the *D. melanogaster* 11-bp sequence also showed the ability to transgenically repress GFP expression in the *hwe* and *hb* of the *D. melanogaster* EB. Because the sequences of these binding sites are usually degenerate in nature, this may suggest that these sequences would be commonly distributed through the whole genome. Therefore, the likelihood of these short sequences randomly distributed across the genome without any apparent function is high. These sequences may not produce any phenotypes until activator sequences that produce an overlapping expression pattern with the repressor sequences evolve.

Regardless of whether each of the three hypotheses is correct, the phenomenon that repressor sequences precede the gain of activator sequences in enhancer evolution could be a common mechanism during the evolution of highly specific gene expression patterns.

The independent evolution of highly specific gene expression patterns

In this study, we showed a possible example of independent evolutionary gains of a highly specific expression pattern of *bond* in the EB *swe* of species in two distant lineages. Although the repressor sequences in these enhancers that drive this specific expression are likely to be ancestral, we found that the transcriptional activators are likely to be different; i.e., *bond* EB expression in *Drosophila* species requires the transcriptional factor *Abd-B*, but *bond* EB expression in *Scaptodrosophila* does not. Due to our limited RNAi screen, we did not identify all the TFs involved in driving expression in the *Drosophila*

enhancer, so we could not determine whether some transcriptional inputs are similar between these two enhancers. However, we propose that preexisting spatial repressor sequences may preconfigure similar highly specific gene expression patterns in different lineages, even though other transcriptional inputs that led to the appearance of these patterns are different. This may reflect a general evolutionary mechanism for enhancer origins.

Limitations of the study

Our study is limited by the species we can obtain from stock centers, as well as the available genomes for the species. A more extensive sampling of species may shed more light into the origins of the repressor sequences and the independent gains of different activator sequences. In addition, our RNAi screen used only one RNAi resource (DRSC/Harvard TRiP) and focused on only the top 100 potential TFs that could affect the expression of *bond* in the EB. A more extensive screen, using both the DRSC/Harvard TRiP RNAi collection and the Vienna *Drosophila* Resource Center (VDRC) and focusing on more TFs identified in our EB transcriptome, may allow us to identify more transcriptional regulators that regulate the expression of *bond* in the EB. This can provide a more comprehensive picture of the evolutionary events described in this manuscript.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.109896.

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AUTHOR CONTRIBUTIONS

J.P. and H. Chung designed research; J.P., Z.W., H. Cong, J.S.R.C., J.J., C.F., J.Y.Y., and H. Chung performed research; J.P., Z.W., C.F., J.Y.Y., and H. Chung analyzed data; and J.P. and H. Chung wrote the paper with input from other authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

Andrioli, L.P.M., Vasisht, V., Theodosopoulou, E., Oberstein, A., and Small, S. (2002). Anterior repression of a Drosophila stripe enhancer requires three position-specific mechanisms. Development *129*, 4931–4940.

Arnosti, D.N., Barolo, S., Levine, M., and Small, S. (1996). The eve stripe 2 enhancer employs multiple modes of transcriptional synergy. Development *122*, 205–214.

Arnoult, L., Su, K.F., Manoel, D., Minervino, C., Magriña, J., Gompel, N., and Prud'homme, B. (2013). Emergence and diversification of fly pigmentation through evolution of a gene regulatory module. Science *339*, 1423–1426.

Barolo, S., and Posakony, J.W. (2002). Three habits of highly effective signaling pathways: principles of transcriptional control by developmental cell signaling. Genes Dev. *16*, 1167–1181.

Capella-Gutiérrez, S., Silla-Martínez, J.M., and Gabaldón, T. (2009). trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics *25*, 1972–1973.

Carroll, S.B. (2008). Evo-devo and an expanding evolutionary synthesis: a genetic theory of morphological evolution. Cell *134*, 25–36.

Chung, H., Bogwitz, M.R., McCart, C., Andrianopoulos, A., Ffrench-Constant, R.H., Batterham, P., and Daborn, P.J. (2007). Cis-regulatory elements in the *Accord* retrotransposon result in tissue-specific expression of the *Drosophila melanogaster* insecticide resistance gene *Cyp6g1*. Genetics *175*, 1071–1077.

Chung, H., Sztal, T., Pasricha, S., Sridhar, M., Batterham, P., and Daborn, P.J. (2009). Characterization of Drosophila melanogaster cytochrome P450 genes. Proc. Natl. Acad. Sci. USA *106*, 5731–5736.

Cohen, A.B., and Wolfner, M.F. (2018). Dynamic changes in ejaculatory bulb size during Drosophila melanogaster aging and mating. J. Insect Physiol. *107*, 152–156.

Edgar, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. *32*, 1792–1797.

ENCODE Project Consortium (2012). An integrated encyclopedia of DNA elements in the human genome. Nature *489*, 57–74.

Finet, C., Kassner, V.A., Carvalho, A.B., Chung, H., Day, J.P., Day, S., Delaney, E.K., De Ré, F.C., Dufour, H.D., Dupim, E., et al. (2021). DrosoPhyla: Resources for Drosophilid Phylogeny and Systematics. Genome Biol. Evol. *13*, evab179.

Fornes, O., Castro-Mondragon, J.A., Khan, A., van der Lee, R., Zhang, X., Richmond, P.A., Modi, B.P., Correard, S., Gheorghe, M., Baranašić, D., et al. (2020). JASPAR 2020: update of the open-access database of transcription factor binding profiles. Nucleic Acids Res. *48*, D87–D92.

Furuchi, T., Masuko, K., Nishimune, Y., Obinata, M., and Matsui, Y. (1996). Inhibition of testicular germ cell apoptosis and differentiation in mice misexpressing Bcl-2 in spermatogonia. Development *122*, 1703–1709.

Gisselbrecht, S.S., Palagi, A., Kurland, J.V., Rogers, J.M., Ozadam, H., Zhan, Y., Dekker, J., and Bulyk, M.L. (2020). Transcriptional silencers in Drosophila

serve a dual role as transcriptional enhancers in alternate cellular contexts. Mol. Cell *77*, 324–337.e8.

Gompel, N., Prud'homme, B., Wittkopp, P.J., Kassner, V.A., and Carroll, S.B. (2005). Chance caught on the wing: cis-regulatory evolution and the origin of pigment patterns in Drosophila. Nature *433*, 481–487.

Graur, D., Zheng, Y., Price, N., Azevedo, R.B., Zufall, R.A., and Elhaik, E. (2013). On the immortality of television sets: "function" in the human genome according to the evolution-free gospel of ENCODE. Genome Biol. Evol. *5*, 578–590.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. *59*, 307–321.

Guo, Y., Gu, X., Sheng, Z., Wang, Y., Luo, C., Liu, R., Qu, H., Shu, D., Wen, J., Crooijmans, R.P., et al. (2016). A complex structural variation on chromosome 27 leads to the ectopic expression of HOXB8 and the muffs and beard phenotype in chickens. PLoS Genet. *12*, e1006071.

Jeong, S., Rokas, A., and Carroll, S.B. (2006). Regulation of body pigmentation by the Abdominal-B Hox protein and its gain and loss in Drosophila evolution. Cell *125*, 1387–1399.

Jeong, S., Rebeiz, M., Andolfatto, P., Werner, T., True, J., and Carroll, S.B. (2008). The evolution of gene regulation underlies a morphological difference between two Drosophila sister species. Cell *132*, 783–793.

Kellis, M., Wold, B., Snyder, M.P., Bernstein, B.E., Kundaje, A., Marinov, G.K., Ward, L.D., Birney, E., Crawford, G.E., Dekker, J., et al. (2014). Defining functional DNA elements in the human genome. Proc. Natl. Acad. Sci. USA *111*, 6131–6138.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S.L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. *14*, R36.

Koshikawa, S., Giorgianni, M.W., Vaccaro, K., Kassner, V.A., Yoder, J.H., Werner, T., and Carroll, S.B. (2015). Gain of cis-regulatory activities underlies novel domains of wingless gene expression in Drosophila. Proc. Natl. Acad. Sci. USA *112*, 7524–7529.

Kvon, E.Z., Kamneva, O.K., Melo, U.S., Barozzi, I., Osterwalder, M., Mannion, B.J., Tissières, V., Pickle, C.S., Plajzer-Frick, I., and Lee, E.A. (2016). Progressive loss of function in a limb enhancer during snake evolution. Cell *167*, 633–642.e11.

Lee, T.I., and Young, R.A. (2013). Transcriptional regulation and its misregulation in disease. Cell *152*, 1237–1251.

Li, L.M., and Arnosti, D.N. (2011). Long- and short-range transcriptional repressors induce distinct chromatin states on repressed genes. Curr. Biol. *21*, 406–412.

Ludwig, M.Z., Palsson, A., Alekseeva, E., Bergman, C.M., Nathan, J., and Kreitman, M. (2005). Functional evolution of a cis-regulatory module. PLoS Biol. *3*, e93.

Mooers, A.Ø., and Schluter, D. (1999). Reconstructing ancestor states with maximum likelihood: support for one-and two-rate models. Syst. Biol. *48*, 623–633.

Morgan, B.A., Izpisúa-Belmonte, J.-C., Duboule, D., and Tabin, C.J. (1992). Targeted misexpression of Hox-4.6 in the avian limb bud causes apparent homeotic transformations. Nature *358*, 236–239.

Ng, S.H., Shankar, S., Shikichi, Y., Akasaka, K., Mori, K., and Yew, J.Y. (2014). Pheromone evolution and sexual behavior in Drosophila are shaped by male sensory exploitation of other males. Proc. Natl. Acad. Sci. USA *111*, 3056–3061.

Ng, W.C., Chin, J.S., Tan, K.J., and Yew, J.Y. (2015). The fatty acid elongase Bond is essential for Drosophila sex pheromone synthesis and male fertility. Nat. Commun. *6*, 8263.

Ni, J.Q., Liu, L.P., Binari, R., Hardy, R., Shim, H.S., Cavallaro, A., Booker, M., Pfeiffer, B.D., Markstein, M., Wang, H., et al. (2009). A Drosophila resource of transgenic RNAi lines for neurogenetics. Genetics *182*, 1089–1100.

Payne, J.L., and Wagner, A. (2014). The robustness and evolvability of transcription factor binding sites. Science *343*, 875–877.

Preger-Ben Noon, E., Davis, F.P., and Stern, D.L. (2016). Evolved repression overcomes enhancer robustness. Dev. Cell *39*, 572–584.

Rebeiz, M., and Tsiantis, M. (2017). Enhancer evolution and the origins of morphological novelty. Curr. Opin. Genet. Dev. *45*, 115–123.

Rebeiz, M., Jikomes, N., Kassner, V.A., and Carroll, S.B. (2011). Evolutionary origin of a novel gene expression pattern through co-option of the latent activities of existing regulatory sequences. Proc. Natl. Acad. Sci. USA *108*, 10036–10043.

Revell, L.J. (2013). Two new graphical methods for mapping trait evolution on phylogenies. Methods Ecol. Evol. *4*, 754–759.

Roy, S., Ernst, J., Kharchenko, P.V., Kheradpour, P., Negre, N., Eaton, M.L., Landolin, J.M., Bristow, C.A., Ma, L., Lin, M.F., et al.; modENCODE Consortium (2010). Identification of functional elements and regulatory circuits by Drosophila modENCODE. Science *330*, 1787–1797.

Small, S., and Arnosti, D.N. (2020). Transcriptional Enhancers in *Drosophila*. Genetics *216*, 1–26.

Small, S., Blair, A., and Levine, M. (1992). Regulation of even-skipped stripe 2 in the Drosophila embryo. EMBO J. *11*, 4047–4057.

Spitz, F., and Furlong, E.E. (2012). Transcription factors: from enhancer binding to developmental control. Nat. Rev. Genet. *13*, 613–626.

Stewart, A.J., Hannenhalli, S., and Plotkin, J.B. (2012). Why transcription factor binding sites are ten nucleotides long. Genetics *192*, 973–985.

Struffi, P., Corado, M., Kaplan, L., Yu, D., Rushlow, C., and Small, S. (2011). Combinatorial activation and concentration-dependent repression of the Drosophila even skipped stripe 3+7 enhancer. Development *138*, 4291–4299.

Sumiyama, K., and Saitou, N. (2011). Loss-of-function mutation in a repressor module of human-specifically activated enhancer HACNS1. Mol. Biol. Evol. *28*, 3005–3007.

Szafer-Glusman, E., Giansanti, M.G., Nishihama, R., Bolival, B., Pringle, J., Gatti, M., and Fuller, M.T. (2008). A role for very-long-chain fatty acids in furrow ingression during cytokinesis in Drosophila spermatocytes. Curr. Biol. *18*, 1426–1431.

Thomas, G.W.C., Dohmen, E., Hughes, D.S.T., Murali, S.C., Poelchau, M., Glastad, K., Anstead, C.A., Ayoub, N.A., Batterham, P., Bellair, M., et al. (2020). Gene content evolution in the arthropods. Genome Biol. *21*, 15.

Thurmond, J., Goodman, J.L., Strelets, V.B., Attrill, H., Gramates, L.S., Marygold, S.J., Matthews, B.B., Millburn, G., Antonazzo, G., Trovisco, V., et al.; FlyBase Consortium (2019). FlyBase 2.0: the next generation. Nucleic Acids Res. *47*, D759–D765.

Ungar, A.R., Kelly, G.M., and Moon, R.T. (1995). Wnt4 affects morphogenesis when misexpressed in the zebrafish embryo. Mech. Dev. *52*, 153–164.

Williams, T.M., Selegue, J.E., Werner, T., Gompel, N., Kopp, A., and Carroll, S.B. (2008). The regulation and evolution of a genetic switch controlling sexually dimorphic traits in Drosophila. Cell *134*, 610–623.

Wittkopp, P.J., and Kalay, G. (2011). Cis-regulatory elements: molecular mechanisms and evolutionary processes underlying divergence. Nat. Rev. Genet. *13*, 59–69.

Xin, Y., Le Poul, Y., Ling, L., Museridze, M., Mühling, B., Jaenichen, R., Osipova, E., and Gompel, N. (2020). Enhancer evolutionary co-option through shared chromatin accessibility input. Proc. Natl. Acad. Sci. USA *117*, 20636–20644.

Yew, J.Y., Dreisewerd, K., Luftmann, H., Müthing, J., Pohlentz, G., and Kravitz, E.A. (2009). A new male sex pheromone and novel cuticular cues for chemical communication in Drosophila. Curr. Biol. *19*, 1245–1254.

Zaret, K.S., and Mango, S.E. (2016). Pioneer transcription factors, chromatin dynamics, and cell fate control. Curr. Opin. Genet. Dev. *37*, 76–81.

Zhang, S.-D., and Odenwald, W.F. (1995). Misexpression of the white (w) gene triggers male-male courtship in Drosophila. Proc. Natl. Acad. Sci. USA *92*, 5525–5529.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
AscI	NEB	Cat# R0558L
SbfI	NEB	Cat# R0642L
TRIzol Reagent	Invitrogen	Cat# 15596026
Critical commercial assays		
TruSeq RNA Library Prep Kit v2	Illumina	Cat# RS-122-2001
Deposited data		
Transcriptomic profiles obtained from 8-day old *D. melanogaster* male ejaculatory bulbs.	This paper	GEO: GSE185053
The concatenated alignment and output tree files	This paper	10.5281/zenodo.5238168
Experimental models: Organisms/strains		
D. melanogaster: Canton-S	Laboratory of Sean B. Carroll Lab	N/A
D. melanogaster: Xout	Williams et al., 2008, Laboratory of Sean B. Carroll Lab	N/A
D. simulans: w^{501}	National Drosophila Species Stock Center (Cornell University)	14021-0251.195
D. yakuba	National Drosophila Species Stock Center (Cornell University)	14021-0261.00
D. erecta	National Drosophila Species Stock Center (Cornell University)	14021-0224.01
D. ananassae	National Drosophila Species Stock Center (Cornell University)	14024-0371.13
D. pseudoobscura	National Drosophila Species Stock Center (Cornell University)	14011-0121.94
D. subobscura	National Drosophila Species Stock Center (Cornell University)	14011-0131.05
D. nebulosi	National Drosophila Species Stock Center (Cornell University)	14030-0761.00
D. sturtevanti	National Drosophila Species Stock Center (Cornell University)	14043-0871.16
D. willistoni	National Drosophila Species Stock Center (Cornell University)	14030-0811.24
D. immigrans	National Drosophila Species Stock Center (Cornell University)	15111-1731.03
D. nasuta	National Drosophila Species Stock Center (Cornell University)	15112-1781.00
D. Americana	National Drosophila Species Stock Center (Cornell University)	15010-0951.00
D. virilis	National Drosophila Species Stock Center (Cornell University)	15010-1051.87
D. mercatorum	National Drosophila Species Stock Center (Cornell University)	15082-1521.38
D. mojavensis	National Drosophila Species Stock Center (Cornell University)	15081-1352.22
S. latifasciaeformis	National Drosophila Species Stock Center (Cornell University)	11030-0061.01

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
S. lebanonensis	National Drosophila Species Stock Center (Cornell University)	11010-0011.00
S. rufifrons	National Drosophila Species Stock Center (Cornell University)	11040-0071.00
C. procnemis	National Drosophila Species Stock Center (Cornell University)	20000-2631.01
M. domestica	Josh's Frogs (Owosso, MI)	B06XJ8DQ2X
D. melanogaster: Various GFP reporter constructs	This study	N/A
D. melanogaster: Various UAS-RNAi lines	Bloomington Drosophila Stock Center	See Table S2
D. melanogaster: EB-GAL4	Gift from Dr. Phillip Daborn (The University of Melbourne)	N/A
Oligonucleotides		
Primers: Generation of GFP reporter constructs	This paper	See Table S3
Primers: Generation of *in situ* hybridization probes	This paper	See Table S3
Recombinant DNA		
GFP reporter vector *pS3aG*	Williams et al., 2008, Laboratory of Sean B. Carroll Lab	N/A
Software and algorithms		
MUSCLE	Edgar, 2004	http://www.drive5.com/muscle
PhyML 3.0	Guindon et al., 2010	http://www.atgc-montpellier.fr/phyml/
'Phytools' package	Revell, 2013	https://cran.r-project.org/web/packages/phytools/index.html
TopHat v2.0.9	Kim et al., 2013	https://ccb.jhu.edu/software/tophat/index.shtml

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Henry Chung (hwchung@msu.edu)

Materials availability

All constructs and transgenic fly lines generated in this study are available from the Lead Contact upon request.

Data and code availability

The RNA-seq datasets reported in this study has been deposited at NCBI GEO and is publicly available as of the date of publication. Accession number is listed in the Key resources table. The concatenated alignment and output tree files used to generate the phylogenetic tree are downloadable from Zenodo (Accession number is listed in the Key resources table).

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The Canton-S strain was used as the wild-type *D. melanogaster* strain. Fifteen other *Drosophila* species (D. simulans, D. yakuba, D erecta, D. ananassae, D. pseudoobscura, D. subobscura, D. nebulosa, D. sturtevanti, D. willistoni, D. immigrans, D. nasuta, D. americana, D. virilis, D. mercatorum, and D. mojavensis), three Scapto*Drosophila* species (S. latifasciaeformis, S. lebanonensis, and S. rufifrons) and one Chymomyza species (C. procnemis) were obtained from the National *Drosophila* Species Stock Center at Cornell

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University. The housefly (Musca domestica) was obtained from Josh's Frogs (Owosso, Michigan, USA). The EB-GAL4 *D. melanogaster* line drives GFP in the entire EB and is a gift from Dr. Phillip Daborn (University of Melbourne, Australia). UAS::RNAi transgenic flies from the Transgenic RNAi Project (TRiP) (Ni et al., 2009) were obtained from the Bloomington *Drosophila* Stock Center. The *D. melanogaster* Xout line was used in the production of transgenic lines carrying GFP reporter constructs (Williams et al., 2008). All flies were maintained at room temperature on standard *Drosophila* food (Bloomington formulation, Genesee Scientific). *D. melanogaster* GAL4/UAS-RNAi experiments were performed at 25°C.

METHOD DETAILS

Generation of GFP reporter constructs and transgenic flies

All GFP reporter constructs were generated by PCR amplification of the genomic fragments from different *Drosophila* species and cloned into the GFP reporter vector *pS3aG* via the *AscI* and *SbfI* site (All primers listed in Table S3). The initial screen to locate the *D. melanogaster* EB enhancer focused on four different regions based on bond-PB transcript: i) 5′ of the gene (3R: 22547847..22550679), intron 1 (3R: 22550873..22554636), intron 3 (3R: 22555014..22555385) and the 3′ non-coding region (3R: 22556495..22558341). All constructs were injected into the *D. melanogaster Xout* line and integrated into the genome using the *PhiC31* integrase system.

In situ hybridization

Ejaculatory bulbs (EBs) from three-day old male adult flies were dissected in Phosphate-Buffered Saline (PBS). *In situ* hybridization was performed with RNA probes as described previously (Chung et al., 2009). Probes for bond *in situ* hybridization were synthesized from cDNA using species-specific primers (Table S3). The *D. melanogaster* probe was used for *in situ* hybridization to *D. melanogaster, D. simulans, D. yakuba, and D. erecta*. *D. ananassae* probe was used for *D. ananassae. D. pseudoobscura* probe was used for *D. pseudoobscura* and *D. subobscura. D. willistoni* probe was used for *D. willistoni, D. sturtevanti,* and *D. nebulosa. S. lebanonensis* probe was used to *S. lebanonensis* and *S. rufifrons*. *C. procnemis* probe was used for *C. procnemis* and *S. latifasiaeformis*. *M. domestica* probe was used for *M. domestica*.

Phylogenetic analyses

A multilocus dataset of 17 genes from 21 species was used for phylogenetic reconstruction. The genes included the 15 nuclear markers *Amyrel* (*Amyrel*), *Distal-less* (*Dll*), *Dopa decarboxylase* (*Ddc*), *ebony* (*e*), *engrailed* (*en*), *even-skipped* (*eve*), *hedgehog* (*hh*), *Notum* (*Notum*), *patched* (*ptc*), *wingless* (*wg*), *28S ribosomal RNA* (*28S*), *Alcohol dehydrogenase* (*Adh*), *Glycerol-3-phosphate dehydrogenase* (*Gpdh*), *Superoxide dismutase* (*Sod*), *Xanthine dehydrogenase* (*Xdh*), and the two mitochondrial markers *cytochrome oxidase subunit 1* (*COI*) and *cytochrome oxidase subunit 2* (*COII*). Nucleotide sequences of the 20 drosophilid species were retrieved from the DrosoPhyla project (Finet et al., 2021), and nucleotide sequences of *M. domestica* were collected from the NCBI database. Alignments for each individual gene were generated using MUSCLE (Edgar, 2004) with default parameters. Unreliably aligned positions were excluded using trimAl with parameters -gt 0.5 and -st 0.001 (Capella-Gutiérrez et al., 2009). In-house Python scripts were used to concatenate the aligned sequences (Finet et al., 2021). Maximum-likelihood searches were performed using PhyML 3.0 (Guindon et al., 2010) under the GTR+Γ_4+I model, and 100 bootstrap replicates were conducted for support estimation.

RNA sequencing and analysis

RNA from the EBs of approximately 200 eight-day old Canton-S *D. melanogaster* males was extracted using TRIzol Reagent according to manufacturer's instructions. Indexed RNA-Seq libraries were prepared from ~1 μg of total RNA using the TruSeq RNA Library Prep Kit v2 (Illumina) according to manufacturer's protocol. RNA quality and concentration were measured on an Agilent 2100 Bioanalyzer (Thermo Scientific). Paired end sequencing was performed on an NGS Illumina Hiseq 2000 with a 20 M read depth (75bp X 2; AITBiotech; Singapore). FastQ files were aligned to the Dmr6.05 *Drosophila melanogaster* reference genome (2012, r5.48) using TopHat v2.0.9 (Kim et al., 2013).

RNAi screen

Based on the expression level of the transcription factors (TFs) in EB and predicting TF binding sites of the *bc3* fragment, 100 candidate TFs were used for the RNAi screen. Males from each *UAS::RNAi* line were crossed with virgin females of the *bc3::GFP*; *EB-GAL4;* + fly line. The EBs of three-day old males from the resulting crosses were dissected and imaged for GFP expression.

Imaging

All *in situ* hybridization and GFP images were captured using the Nikon SMZ18 dissecting stereo microscope system. For GFP images, EBs were dissected from three-day old males in 1 × PBS and mounted on slides with glycerol mountant [80% (vol/vol in water) glycerol, 0.1 M Tris (pH 8.0)].

QUANTIFICATION AND STATISTICAL ANALYSIS

To determine the evolution of *bond* expression in EB across the phylogeny, we reconstructed its ancestral state using the method with the ‘Phytools’ package in R (Revell, 2013). The maximum likelihood approach was used for discrete characters, based on the equal-rate model (Mooers and Schluter, 1999).