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Supplemental information

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

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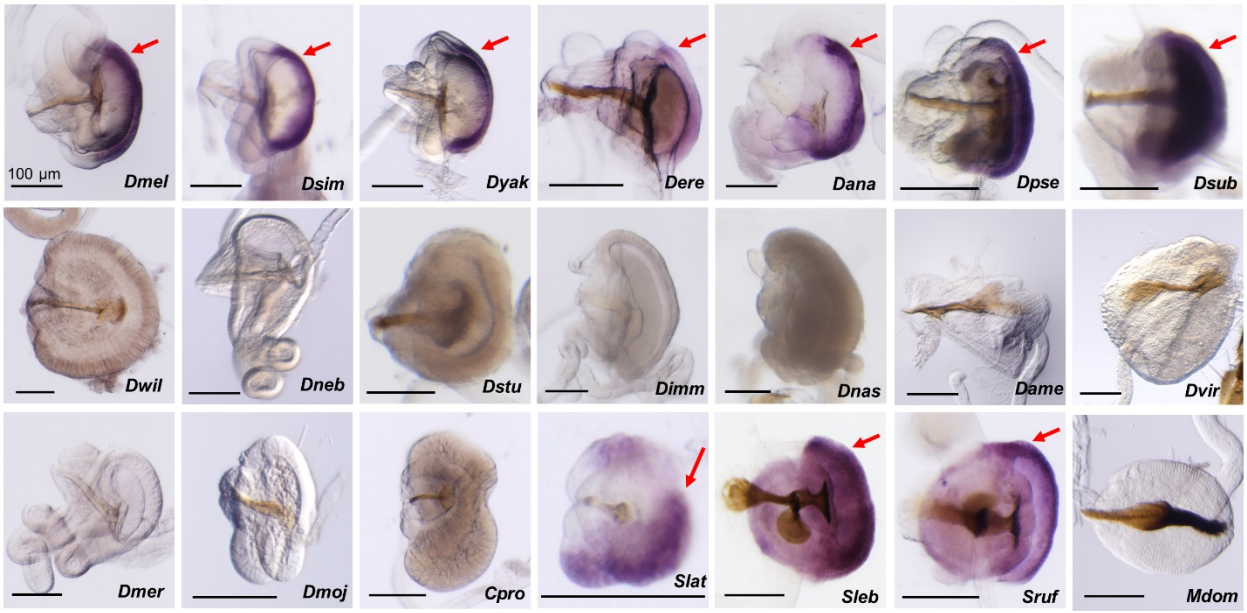

Figure S1. *In situ* hybridization of *bond* in the ejaculatory bulb (EB) in 21 species. *In situ* hybridization of *bond* to the EB of various *Drosophila* and other closely related species showed EB expression of *bond* in *D. melanogaster*, *D. simulans*, *D. erecta*, *D. yakuba*, *D. ananassae*, *D. pseudoobscura*, and *D. subobscura*, *Scaptodrosophila* species, *S. latifasciaeformis*, *S. lebanonensis*, and *S. rufifrons,* but not in other species tested. Arrows indicate *bond* expression in the EB determined by *in situ* hybridization. *Dmel = Drosophila melanogaster*, *Dsim = D. simulans, Dyak= D. yakuba, Dere = D. erecta, Dana = D. ananassae, Dpse = D. pseudoobscura, Dsub = D. subobscura, Dneb = D. nebulosa, Dstu = D. sturtevanti, Dimm = D. immigrans, Dnas = D. nasuta, Dame = D. americana, Dvir = D. virilis, Dmer = D. mercatorum, Dmoj = D. mojavensis, Cpro = Chymomyza procnemis, Slat = Scaptodrosphila latifasciaeformis, Sleb = S. lebanonensis, Sruf = S. rufifrons, and Mdom = Musca domestica.* The EBs of the *Scaptodrosophila*, *Chymomyza* and *Musca* species are morphologically different from the EBs of the *Drosophila* species. The EB of *M. domestica* is previously referred to as an ejaculatory sac. (Related to Figure 1B).

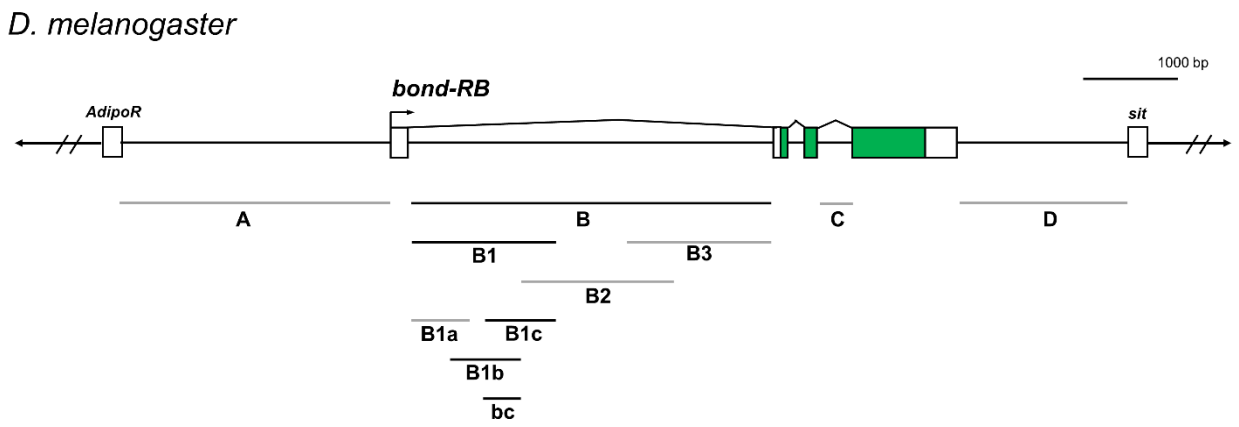

Figure S2. The large intron of *bond* contains sequences that can drive GFP expression in the EB semicircular wall epithelium (*swe*) in *D. melanogaster*. Overlapping fragments from the non-coding region around the *D. melanogaster* bond locus were screened for *cis*-regulatory sequences that were able to drive GFP reporter protein expression in the EB *swe*. Black lines indicate fragments able to drive GFP expression in the EB *swe*. Grey lines indicate fragments not able to drive GFP in the EB *swe*. (Related to Figure 2, 3A)

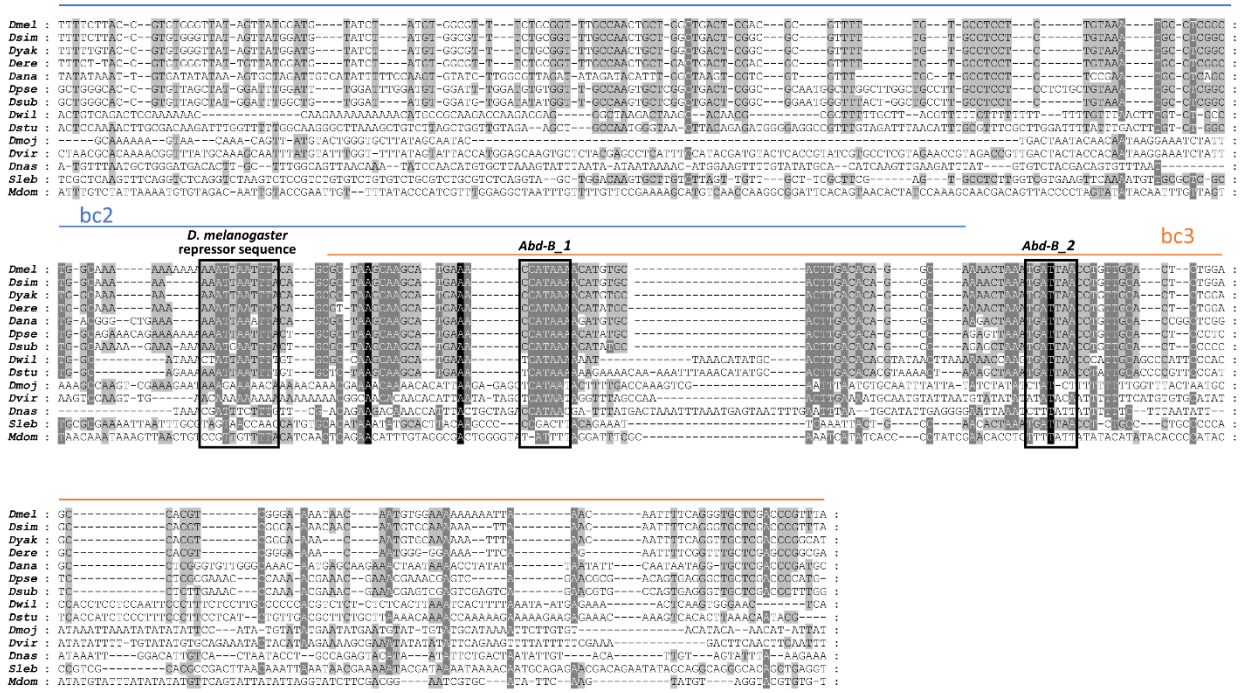

Figure S3. Alignment of the *bc23* region. The 11bp repressor sequences and the two putative Abd-B binding sites in *D. melanogaster* are shown. (Related to Figure 4D, 5C-F)

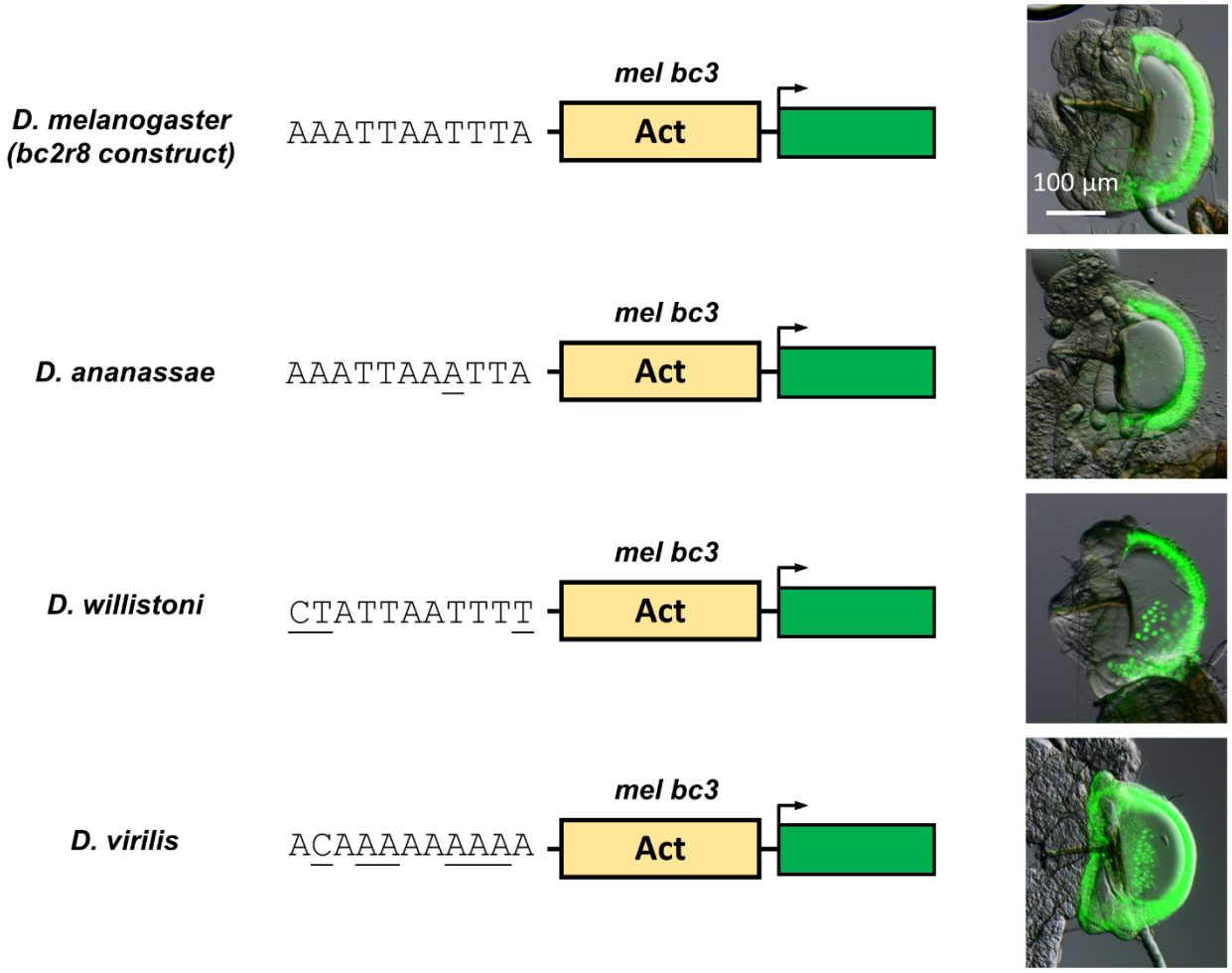

Figure S4. Testing 11bp sequences from other *Drosophila* species for ability for spatial repression in the EB. The 11bp sequences from *D. ananassae* and *D. willistoni* at the same location as the *D. melanogaster* 11bp repressor sequences are able to repress expression in the EB *hb* and *hwe*. The 11bp sequences from *D. virilis* did not repress expression in these sites. (Related to Figure 4D)

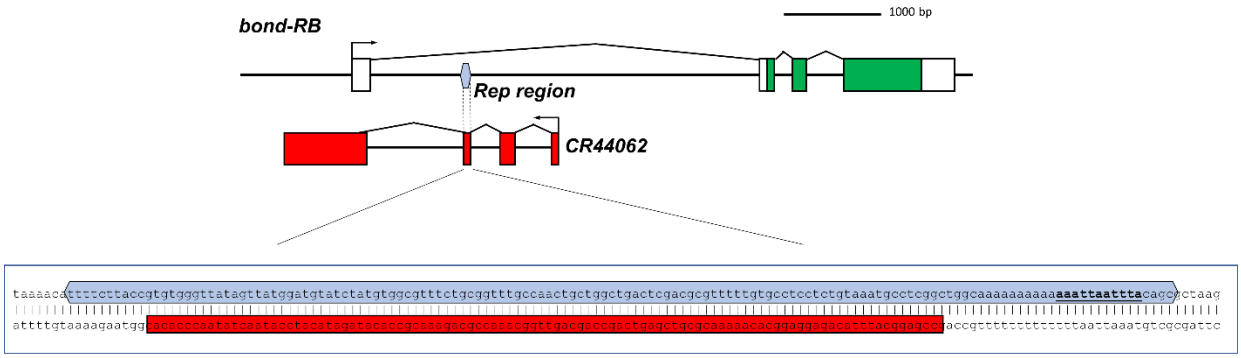

Figure S5. Schematic showing positions of Rep region (blue) of EB swe enhancer and the antisense non-coding RNA *CR44062* relative to the bond locus. The Rep region overlaps with the exon of *CR44062*. (Related to Figure 4D)