RESEARCH COMMUNICATION

Figures and figure supplements

Disparate expression specificities coded by a shared Hox-C enhancer

Steve W Miller and James W Posakony

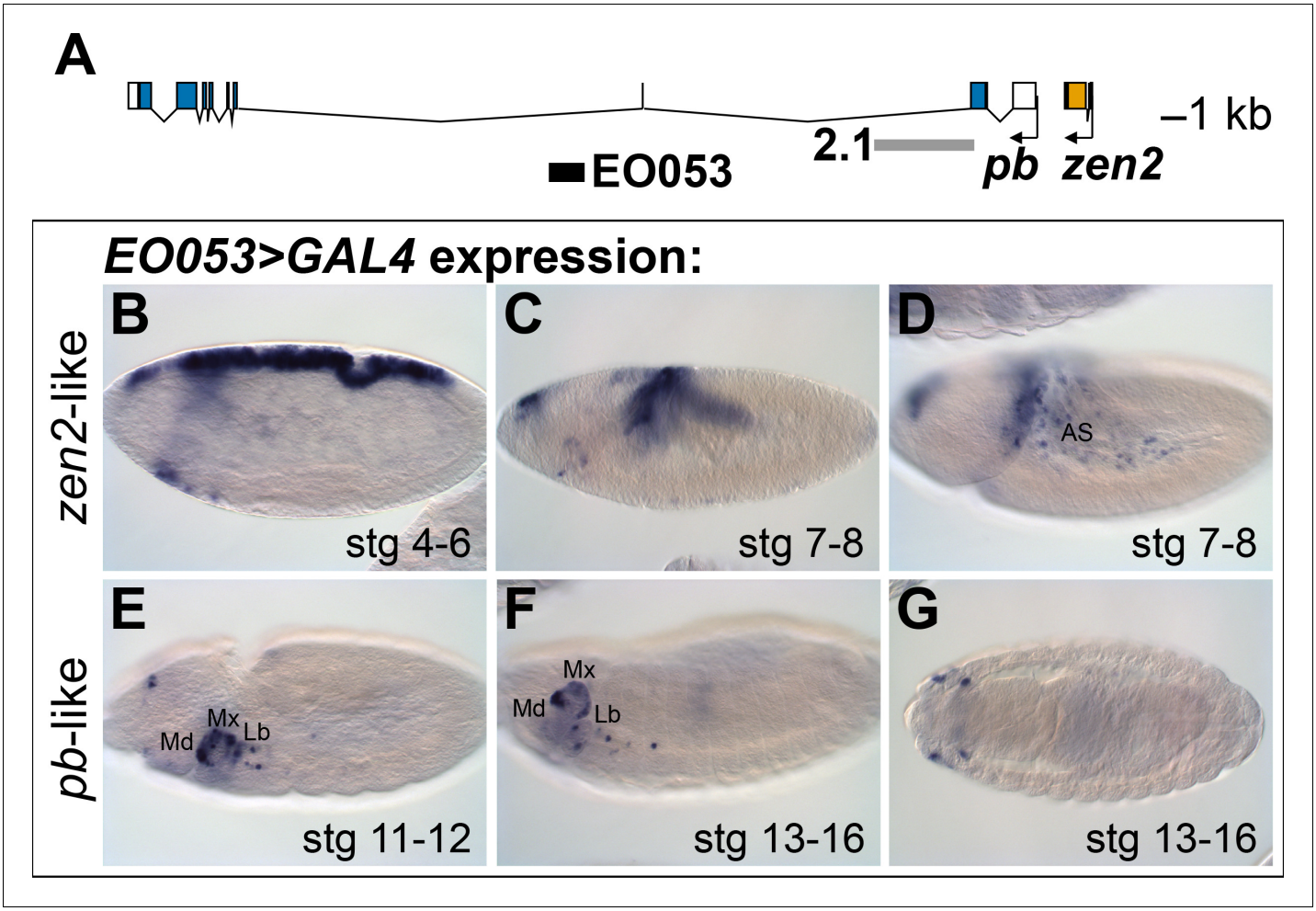

Figure 1. EO053 exhibits both *zen2*-like and *pb*-like expression patterns. (**A**) Diagram of the *pb* (blue) and *zen2* (yellow) genes and the locations of EO053 (black bar) and the 2.1 *pb* regulatory region (grey bar) (***Kapoun and Kaufman, 1995a***). Scale is shown at upper right. (**B-G**) Expression of *GAL4* mRNA by in situ hybridization in *EO053>GAL4* embryos exhibits a pattern reminiscent of *zen2* (***Rushlow et al., 1987***) in early embryonic stages (**B-D**; see also ***Figure 5*** and http://insitu.fruitfly.org/cgi-bin/ex/report.pl?ftype=1&ftext=FBgn0004054) and overlaps expression of *pb* (***Pultz et al., 1988***) in later stages (**E-G**; see also ***Figure 5*** and http://insitu.fruitfly.org/cgi-bin/ex/report.pl?ftype=1&ftext=FBgn0051481). AS: amnioserosa. Md: mandibular segment. Mx: maxillary segment. Lb: labial segment. See also ***Figure 1—figure supplement 1***.

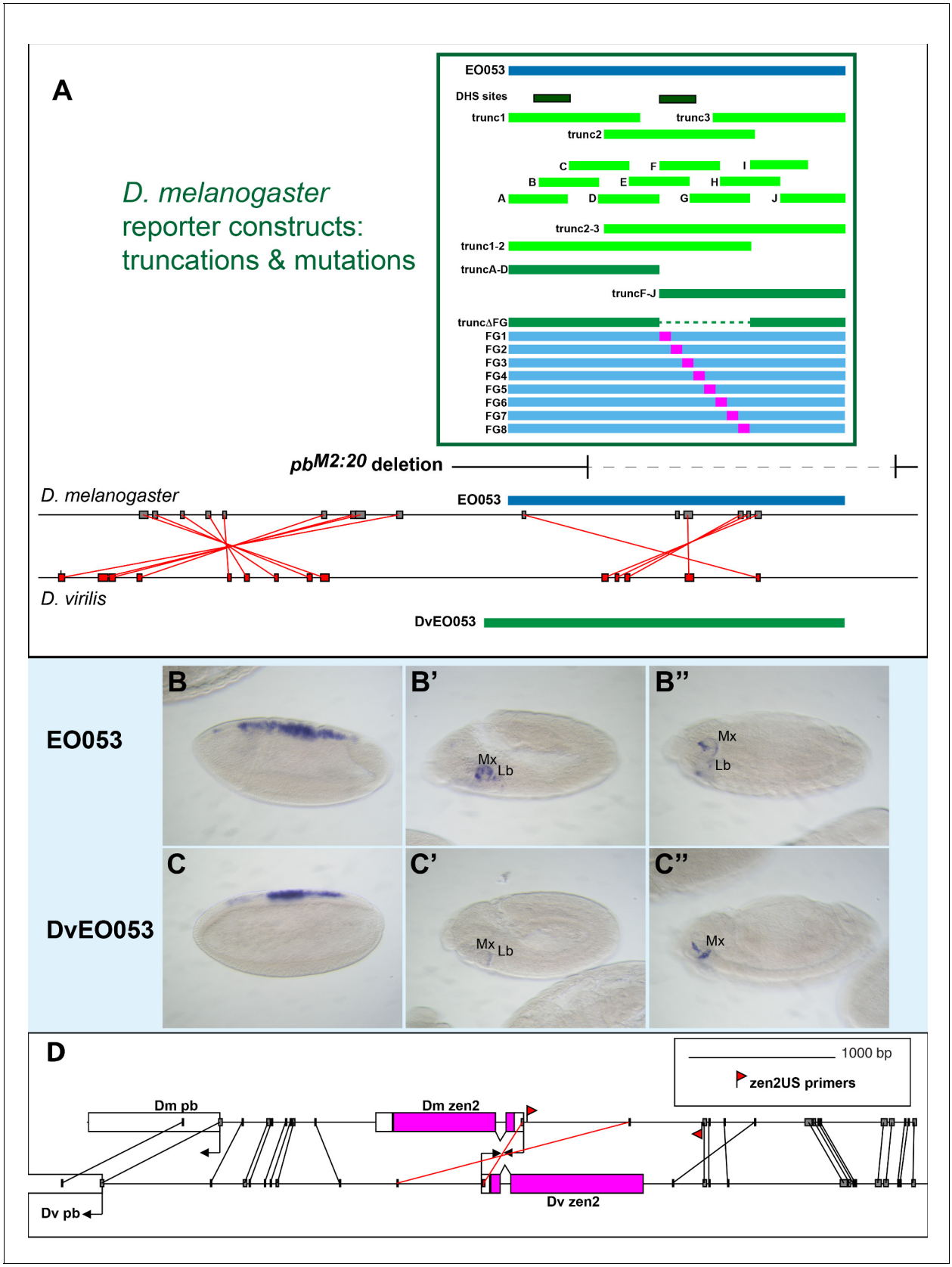

Figure 1—figure supplement 1. Summary of Reporter Fragments. (**A**) Scale diagram indicating sizes and positions of reporter fragments used in this study, relative to EO053 (top). Green-lined box displays DNA segments used in reporter constructs. Below the reporter fragments in the green box, the

Figure 1—figure supplement 1 continued on next page

Figure 1—figure supplement 1 continued

sequence deleted in $pb^{M2:20}$ is indicated by a dotted line. Compare with the boundaries of EO053 either above or below. Below the deletion is comparison of the *D. melanogaster* region and the region orthologous to EO053 in *D. virilis*, with red lines connecting nucleotide stretches identical between the two species (both EO053 and an adjacent region are inverted in *D. virilis* relative to the sequence in *D. melanogaster*). Boxed areas connected by lines are identical sequences (15 bp minimum comparison word size); these can be viewed with reference to *D. melanogaster* reporter fragments above and *D. virilis* sequence and reporter fragment (DvEO053) below. (**B-C''**). *GAL4* mRNA expression driven by the *DvEO053>GAL4* reporter constructs in *D. melanogaster* embryos (**C–C''**), as compared to *D. melanogaster* EO053 (**B–B''**). Shown are stage 5–7 (**B, C**), stage 10 (**B', C'**), and stage 13–16 (**B'', C''**). (**D**) Alignment of the region in *D. melanogaster* and *D. virilis* upstream of the *pb* promoter, containing *zen2* (inverted in *D. virilis* relative to *D. melanogaster*). Grey vertical lines indicate nucleotide stretches identical between the species; red lines indicate sequences both identical and inverted between the species (13 bp minimum comparison word size). Location of primers used to clone zen2US are indicated by red pennants.

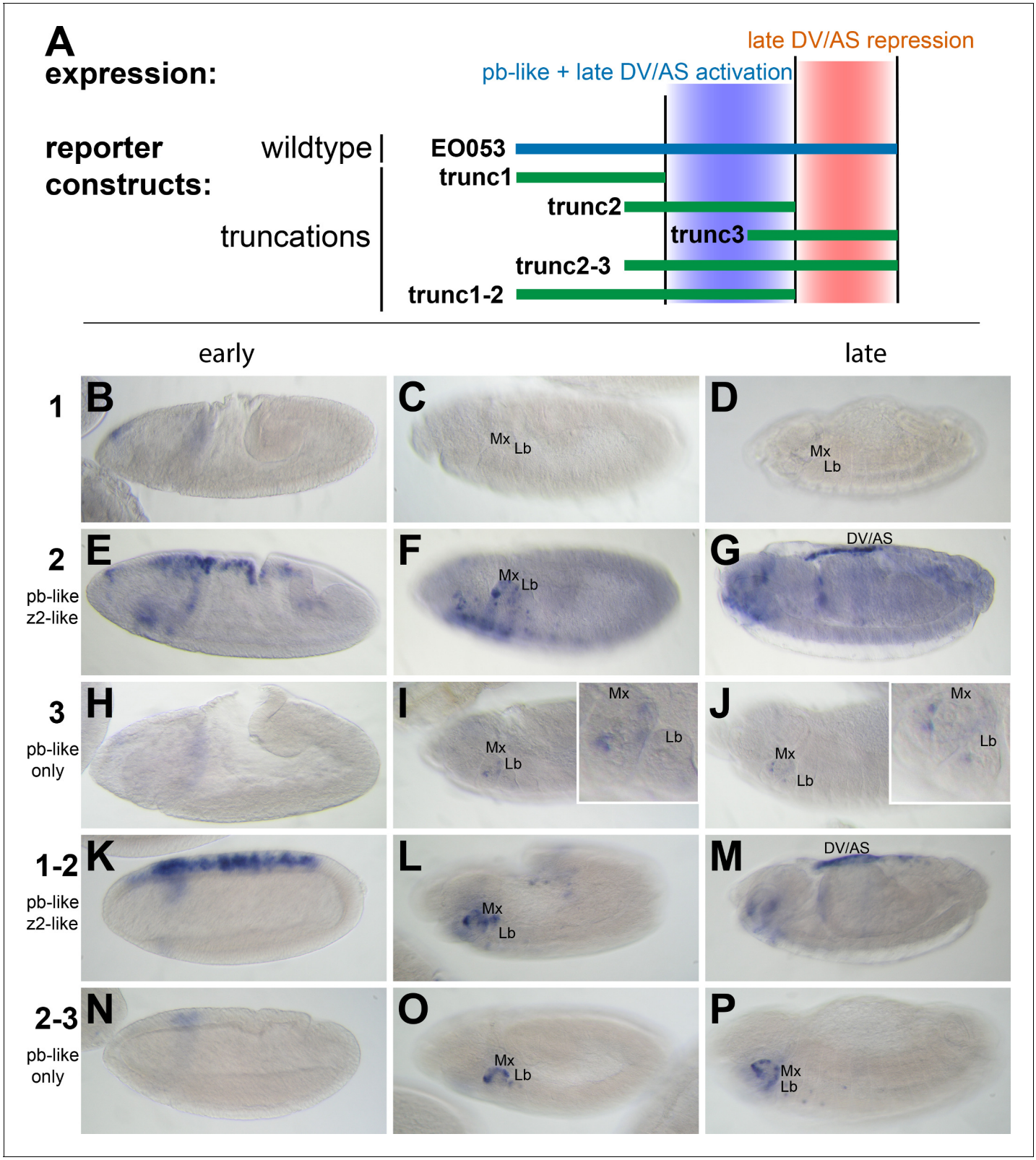

Figure 2. *pb*-like expression driven by EO053 can be localized to a central region of the enhancer. (**A**) Diagram indicating the boundaries of five truncations of EO053 (green bars) and localized expression specificities deduced from reporter assays. While *pb*-like expression can be localized to a subregion of EO053, the *zen2*-like expression cannot. 'DV/AS'=dorsal vessel/amnioserosa. (**B-P**) Expression of *GAL4* mRNA by in situ hybridization in transgenic reporter lines described in panel **A**. (**B, E, H, K, N**) *GAL4* expression in early embryos (stg 5–8), noting the *zen2*-like pattern in **E** and **K** only.

Figure 2 continued on next page

Figure 2 continued

E represents a rare embryo with early dorsal expression, and only during stage 6. (**C, F, I, L, O**) Segment labels as in ***Figure 1***. *GAL4* expression in stage 10–12 embryos, noting *pb*-like expression in panels **F, I, L, and O**. (**D, G, J, M, P**) *GAL4* expression in stage 13–16 embryos. Two constructs that both contain the trunc2 region but lack the remaining 3' portion of EO053 express ectopic *GAL4* in the DV/AS region (**G, M**). Insets in **I and J** represent zoomed-in sections highlighting the low signal in the maxillary and labial segments found with the trunc3 construct. See ***Figure 1—figure supplement 1*** for a diagram of these and all constructs used in this study.

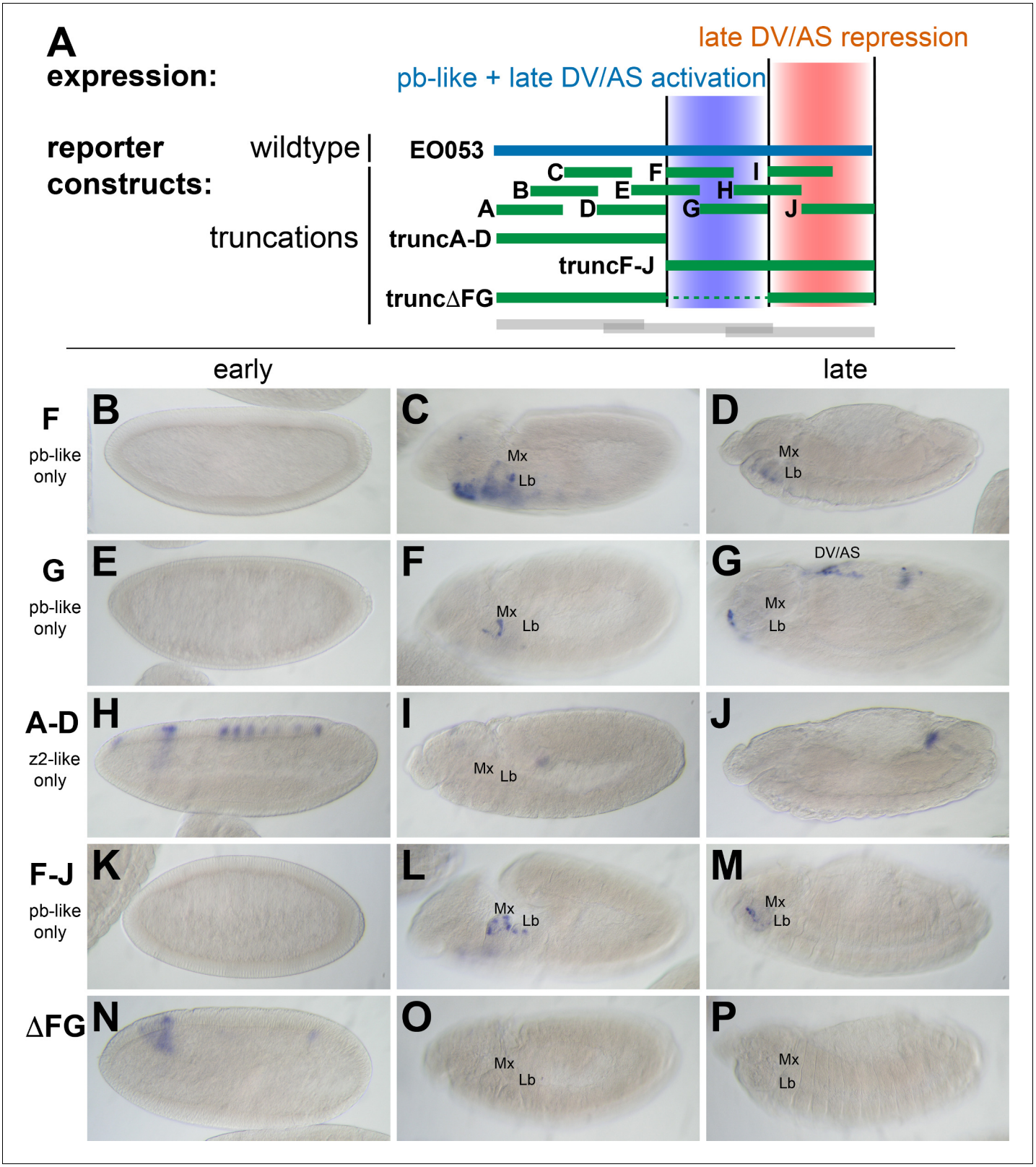

Figure 3. *zen2*-like expression driven by EO053 requires the central region of the enhancer. (**A**) Diagram indicating relative locations of the second set of constructs representing truncated versions of EO053 (green bars). Boundaries of the constructs shown in ***Figure 2*** are indicated for comparison (grey bars). (**B-P**) Expression of *GAL4* mRNA by in situ hybridization in a subset of transgenic reporter lines described in **A** (See ***Figure 3—figure supplement 1*** for images of truncA – truncJ). DV/AS: dorsal vessel/amnioserosa; segment labels as in previous figures. (**B, E, H, K, N**) *GAL4* expression in early

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Figure 3 continued

embryos (stg 5–8), noting the striped *zen2*-like pattern in **H** only. (**C, F, I, L, O**) *GAL4* expression in stage 10–12 embryos, noting *pb*-like expression in panels **C, F, and L**. (**D, G, J, M, P**) *GAL4* expression in stage 13–16 embryos. truncG overlaps trunc2 region but lacks the remaining 3' portion of EO053 and expresses ectopic *GAL4* in the DV/AS region (**G**). (**N-P**) truncΔFG, which lacks regions **F** through **G**, fails to express *GAL4* in either *pb*- or *zen2*-like patterns. See also ***Figure 1—figure supplement 1***.

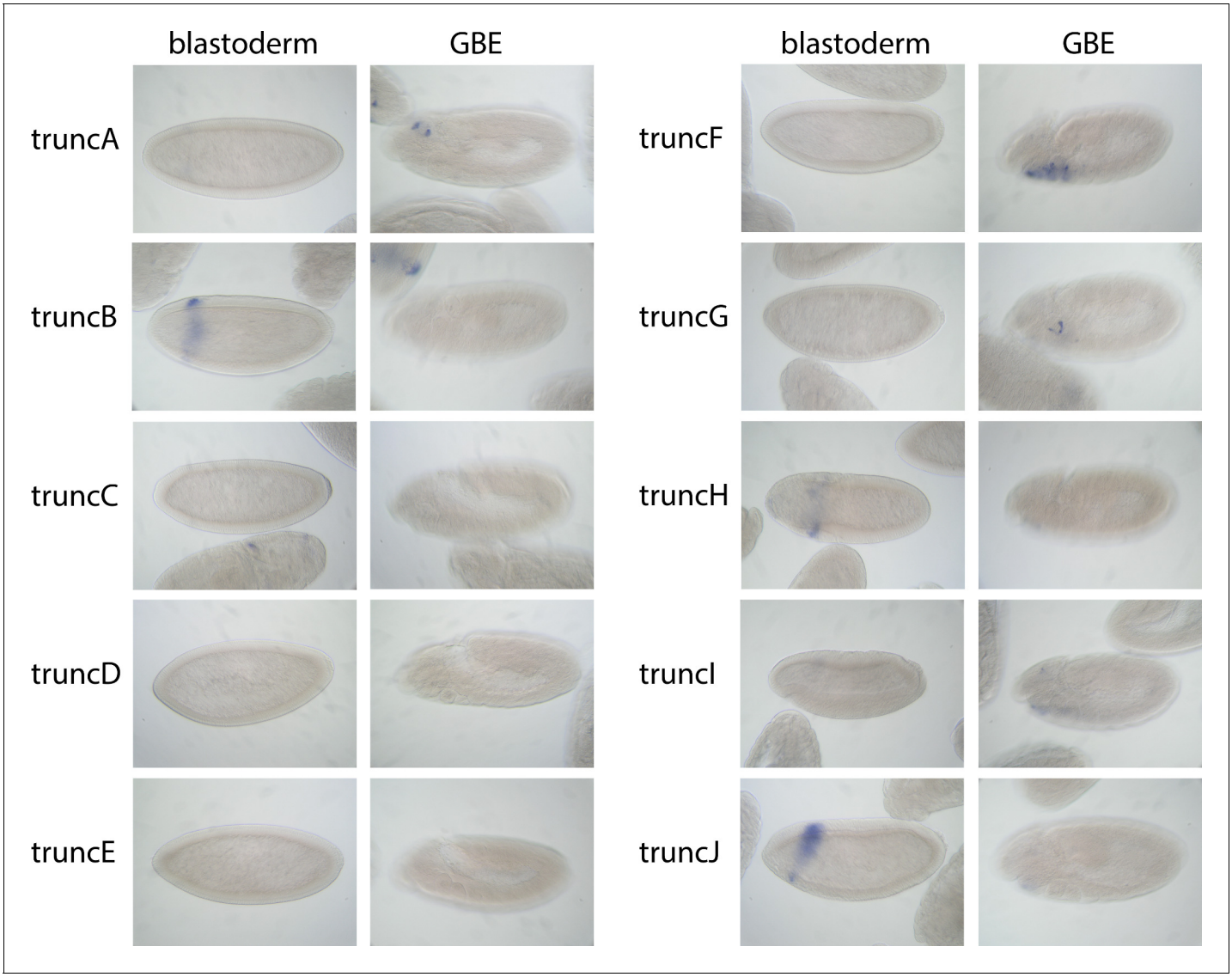

Figure 3—figure supplement 1. Embryo images of truncA – truncJ expression patterns. See also ***Figure 3***. Representative images from either stg 5–7 ('blastoderm') or germ band-extended embryos ('GBE'; stg. 10) containing the indicated *EO053*>GAL4* reporter constructs and probed for *GAL4* mRNA expression by in situ hybridization. Note absence of any embryos expressing a *zen2*-like pattern at the early stages; only truncF and truncG express *GAL4* in the *pb*-like territory in GBE embryos.

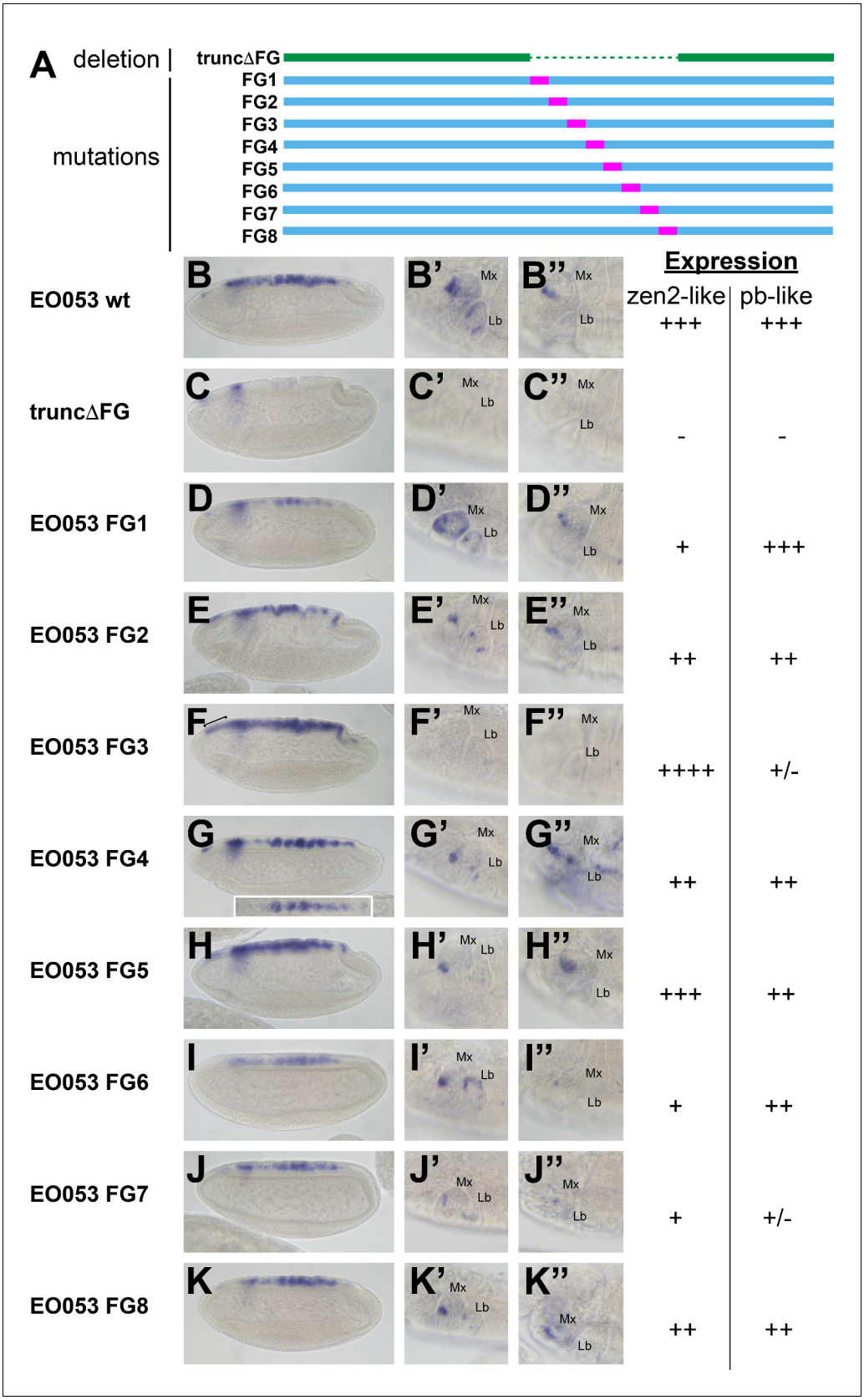

Figure 4. Mutation of specific nucleotide segments in the FG region of EO053 can affect either *pb*-like or *zen2*-like expression. (**A**) Diagram of a series of 47-nt non-complementary transversion mutants generated within the FG region (FG1 – FG8, blue, with mutated segments shown in pink), and the

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same region deleted in the truncΔFG construct (green). (**B-K**) *GAL4* mRNA expression in early (stage 4–6) embryos. *zen2*-like expression is absent in truncΔFG (**C**); reduced in FG1 (**D**), FG2 (**E**), FG4 (**G**), FG6 (**I**), FG7 (**J**), and FG8 (**K**); and expanded anteriorly in FG3 (**F**: bracket). Inset in **G** is a dorsal view of an embryo exemplifying the pseudo-stripe pattern of *GAL4* expression along the anteroposterior axis driven by the FG4 mutant reporter. (**B'-K''**) *GAL4* mRNA expression in maxillary and labial segments of stage 10–12 embryos (**B'–K'**) and stage 13–16 embryos (**B''–K''**). Segment labels as in previous figures. *pb*-like expression is absent in truncΔFG (**C', C''**) and strongly reduced in FG3 (**F', F''**) and FG7 (**J', J''**). Qualitative scoring of reporter strength is represented to the right of the images for each line. See also ***Figure 1—figure supplement 1***.

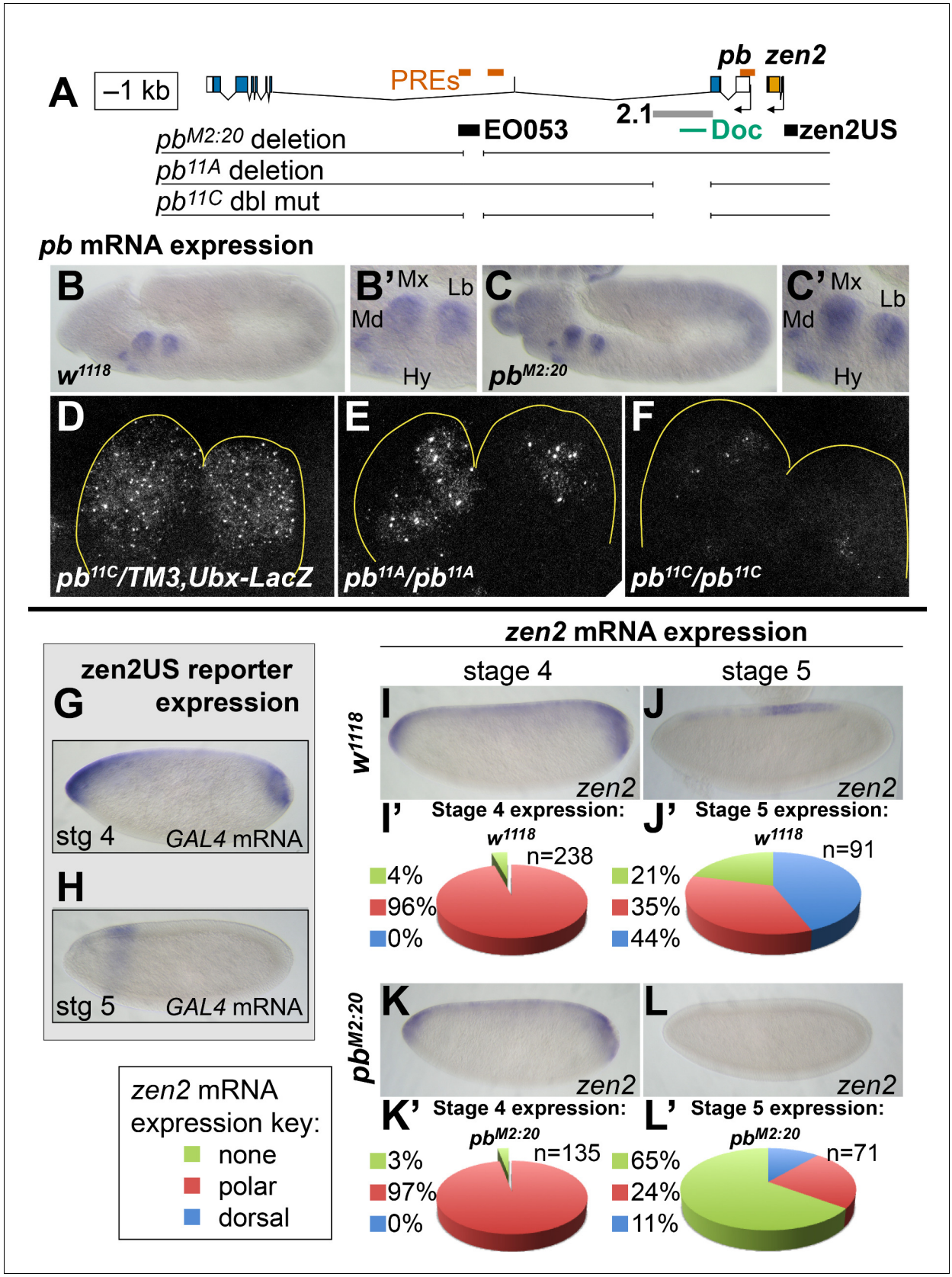

Figure 5. EO053 cooperates with other enhancers to regulate mRNA accumulation from both *pb* and *zen2*. (**A**) Diagram of the *pb-zen2* region, noting the locations of putative Polycomb Response Elements ('PREs', red; see Discussion) (***Nègre et al., 2011***); EO053 and zen2US enhancer regions (black)

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Figure 5 continued

and the *pb* 2.1 regulatory region (grey) (***Kapoun and Kaufman, 1995a***); and a Doc type transposon (***Vaury et al., 1994***) in the 5′ end of *pb* intron 2. Below the diagram of the genomic region are shown CRISPR/Cas9-generated deletions overlapping EO053 only ($pb^{M2:20}$), the 2.1 enhancer only (pb^{11A} or the identical pb^{11D} seen in ***Figure 5—figure supplement 3*** and ***Figure 5—figure supplement 1***), and both EO053 and 2.1 enhancers (pb^{11C} or the identical pb^{11E} seen in ***Figure 5—figure supplement 3*** and ***Figure 5—figure supplement 1***). (**B-F**) Effects of enhancer deletion on *pb* expression. (**B**) *pb* mRNA expression in a w^{1118} embryo at stage 11–12. (**B′**) Zoom-in of the *pb* in situ signal in the mandibular (Md), maxillary (Mx), labial (Lb) segments, and hypopharyngeal lobe (Hy). (**C**) *pb* mRNA expression in a $pb^{M2:20}$ embryo at stage 11–12. (**C′**) Zoom-in of the *pb* in situ signal, with labeling as in **B′**. See also ***Figure 5—figure supplement 2***. (**D-F**) Confocal maximum projection of *pb* mRNA detected through fluorescent in situ hybridization (FISH) in the maxillary and labial segments of embryos of the indicated genotypes. (**D**) pb^{11C}/*TM3,Ubx-LacZ* stage 11–12 embryo. (**E**) pb^{11A}/pb^{11A} stage 11–12 embryo, noting dramatically reduced signal area relative to **D**. (**F**) pb^{11C}/pb^{11C} stage 11–12 embryo exhibiting signal area reduced relative to **D and E**. See also ***Figure 5—figure supplement 3*** – 5. (**G, H**) Expression of *GAL4* directed by the reporter zen2US. Embryos containing *zen2US>GAL4* have detectable *GAL4* mRNA expression at stage 4 (**G**) and lack *GAL4* expression during stage 5 (**H**). (**I-L′**) Effect of the $pb^{M2:20}$ deletion on *zen2* expression. *zen2* mRNA expression at either stage 4 (**I,K**) or stage 5 (**J,L**) in w^{1118} embryos (**I,J**) or $pb^{M2:20}$ embryos (**K,L**). (**I′,J′,K′,L′**) Pie-chart representation of *zen2* mRNA expression pattern as resembling zen2US (polar, red), EO053 (dorsal, blue), or absent (none, green).

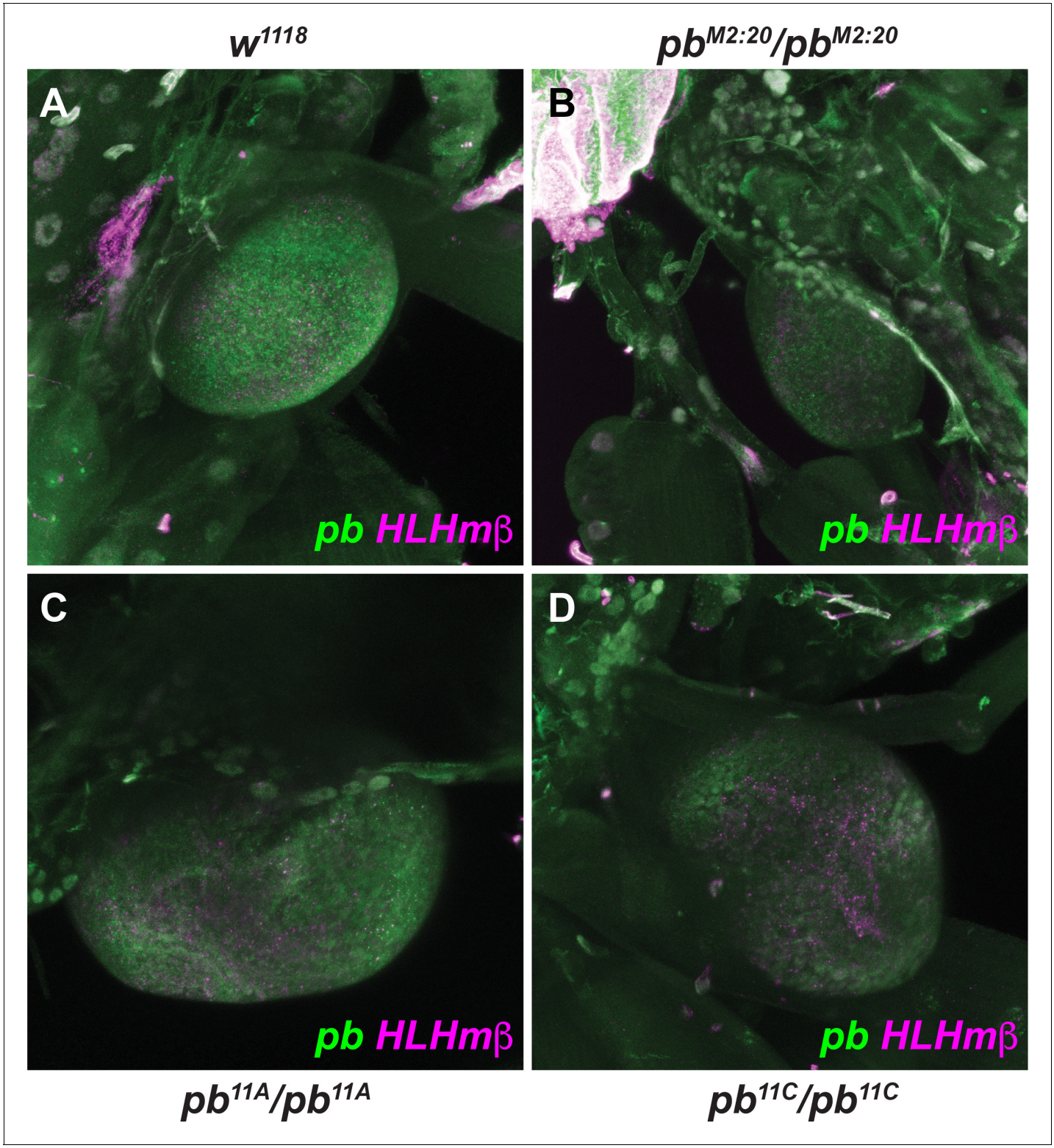
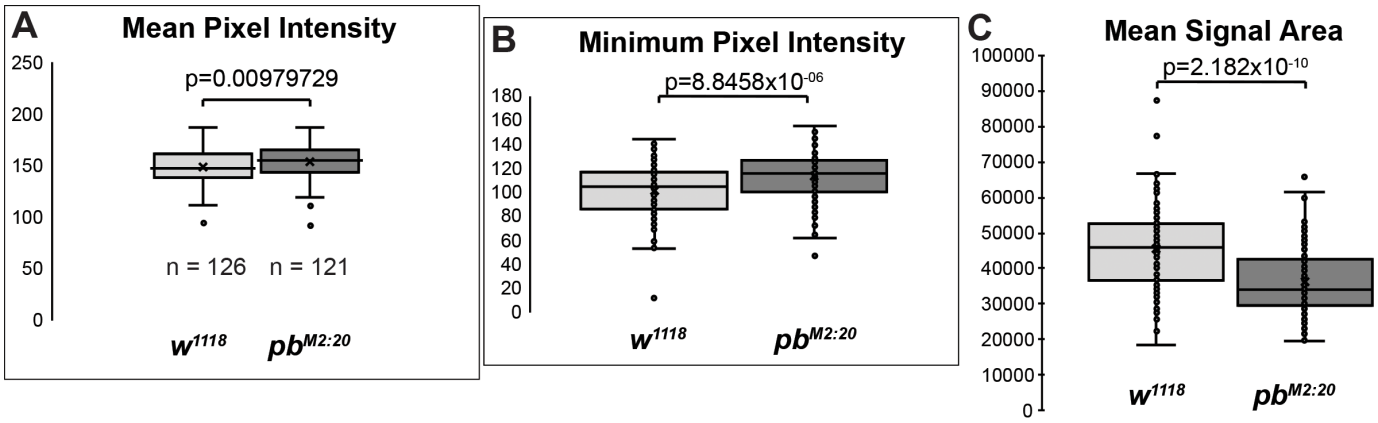
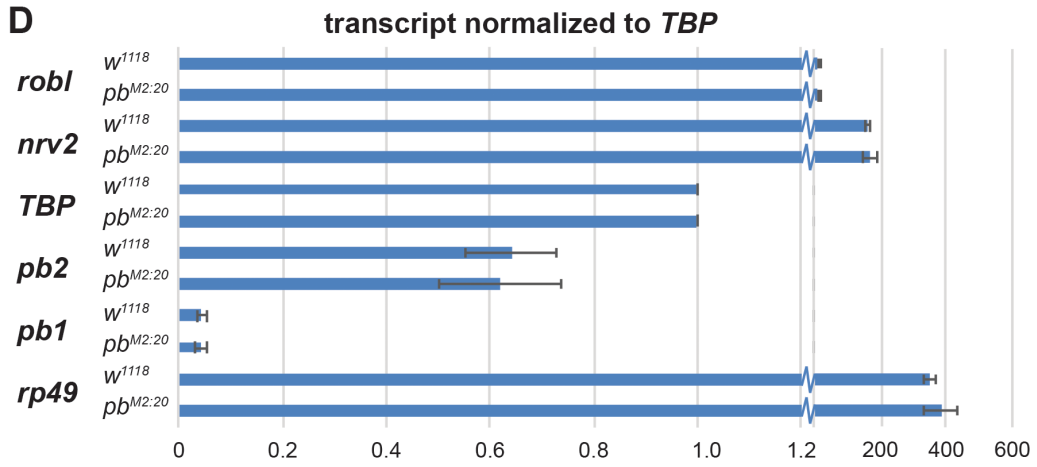


Figure 5—figure supplement 1. Fluorescent detection of *pb* mRNA in 3rd-instar labial discs. Fluorescent detection of *pb* mRNA (green) and *HLHmβ* mRNA (magenta) following in situ hybridization in 3rd-instar labial discs from w^{1118}, $pb^{M2:20}/pb^{M2:20}$ (EO053 deletion), pb^{11A}/pb^{11A} (region 2.1 deletion), or pb^{11C}/pb^{11C} (region 2.1, EO053 double deletion) larvae. Both *pb* and *HLHmβ* are detectable in multiple cytoplasmic dots throughout the labial discs in both **A and B**. In **C**, while *HLHmβ* expression remains similar, discrete *pb* cytoplasmic dots are only present in a subset of the disc above the broader background signal. In **D**, *HLHmβ* expression is again similar to **A-C**, but no discrete *pb* cytoplasmic dots are visible above background.





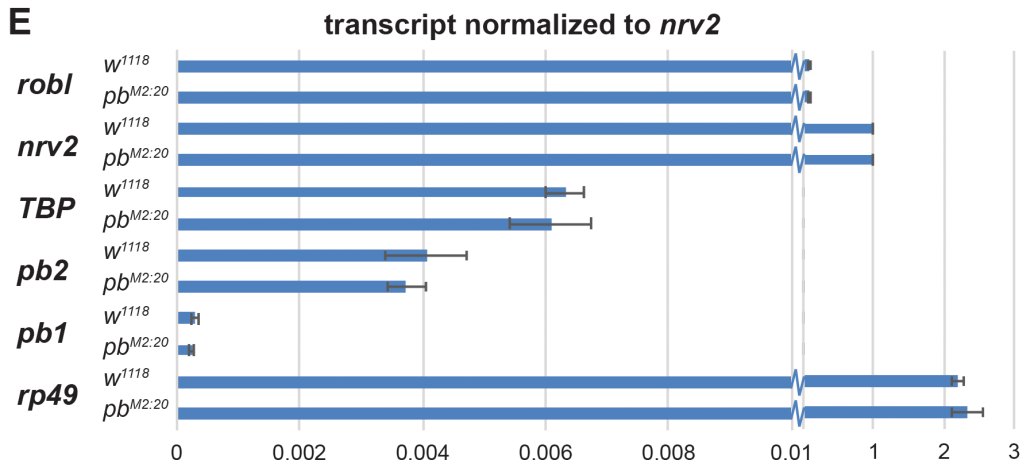


Figure 5—figure supplement 2. Quantification of *pb* expression in $pb^{M2:20}$ mutant embryos. (**A–C**) Analysis of histochemical detection of *pb* mRNA in germ band-extended w^{1118} and $pb^{M2:20}$ embryo images from ***Figure 5B–C***, measuring the mean (**A**) and minimum (**B**) pixel intensity within the area of visible in situ hybridization signal. The alkaline phosphatase reaction deposits a blue product; less product results in a higher pixel intensity. (**C**) Analysis of the mean area of visible in situ hybridization signal in w^{1118} and $pb^{M2:20}$ germ band-extended embryo images with representatives shown in ***Figure 5B–C***. The datasets were subject to a Student's t-test, with the p value reported in each panel. (**D,E**) Quantitative PCR detection of first-strand

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cDNA from *pb* (using two different target regions) and the control genes *robl*, *nrv2*, *TBP*, and *rp49* in staged embryo collections from either w^{1118} or $pb^{M2:20}$ embryos. Relative expression is normalized to either *TBP* expression (**D**) or *nrv2* expression (**E**). No significant difference in *pb* expression is detected between w^{1118} or $pb^{M2:20}$ in either normalization.

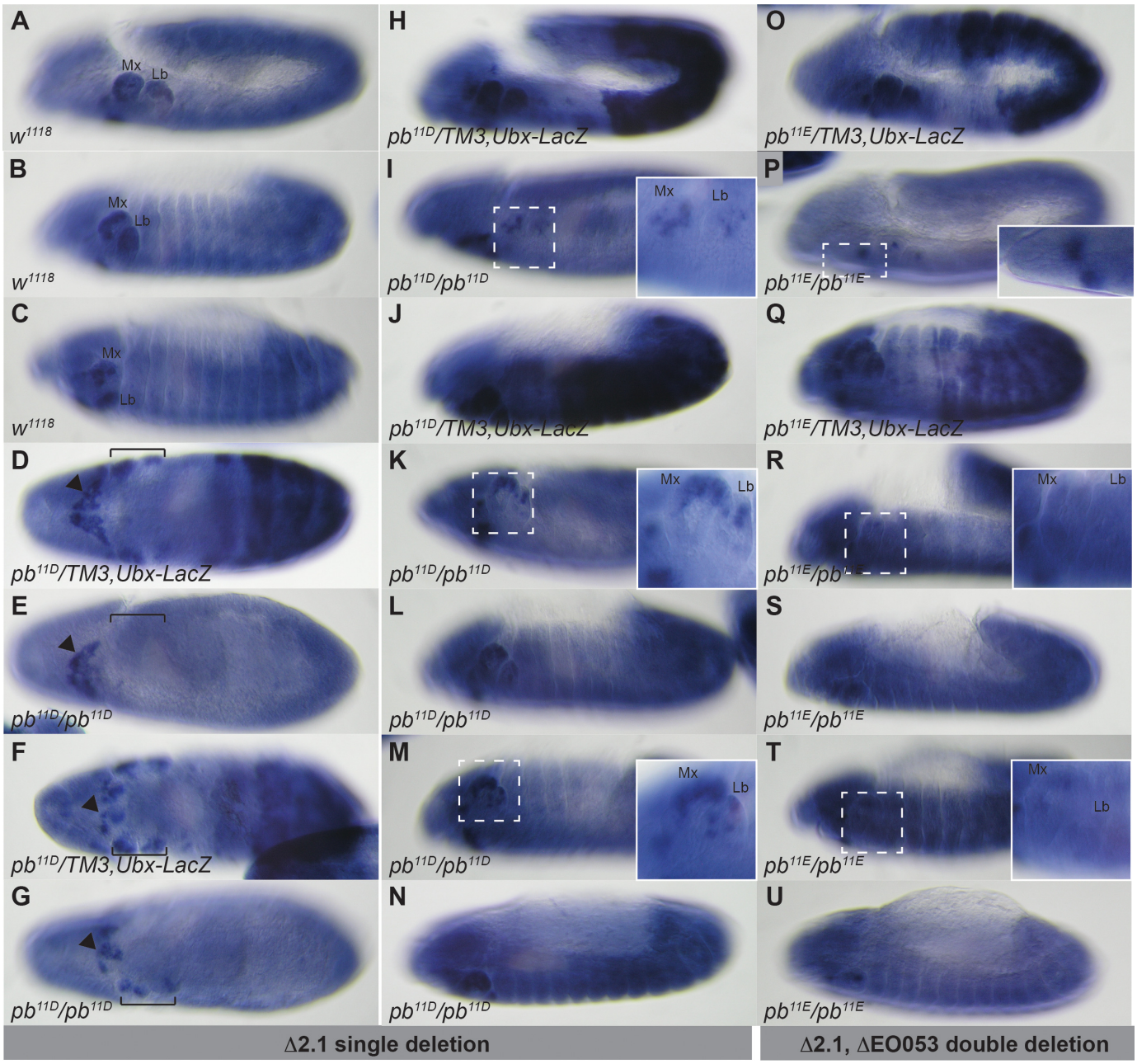

Figure 5—figure supplement 3. Region 2.1 and EO053 cooperate to drive *pb* expression. Histochemical detection of *pb* and *LacZ* mRNA expression following in situ hybridization in embryos of the indicated genotypes and stages. (**A–C**). w^{1118} embryos at stage 11 (**A**), stage 12 (**B**), and stage 13 (**C**). (**D–N**) Region 2.1-deleted embryos, either as balanced heterozygotes identified by abdominal *LacZ* expression (**D, F, H, J**) or homozygotes lacking *LacZ* (**E, G, I, K, L, M, N**). (**D,E**) Ventral view of *pb* expression in maxillary and labial lobes (bracket) that is present in pb^{11D}/*TM3,Ubx-LacZ* (**D**) and absent in pb^{11D}/pb^{11D} embryos at stage 9–10 (**E**), while expression in the hypopharyngeal lobe is similar for both genotypes (arrowhead). (**F,G**) By stage 11, expression in the maxillary and labial lobes (bracket) is detectable in both pb^{11D}/*TM3,Ubx-LacZ* (**F**) and pb^{11D}/pb^{11D} (**G**) embryos, but visibly reduced in pb^{11D}/pb^{11D} (**G**). As in **D,E**, similar hypopharyngeal lobe expression is detectible in both genotypes (arrowhead). (**H**) Laterial view of stage 11 embryos, illustrating *pb* expression in pb^{11D}/*TM3,LacZ* similar to w^{1118} (compare with **A**). (**I**) pb^{11D} homozygous stage 11 embryos have detectable *pb* expression in the maxillary and labial lobes (see inset for higher magnificiation), but in a noticeably reduced territory. (**J-N**) Stage 12 pb^{11D}/*TM3,Ubx-LacZ* embryos have *pb* expression comparable to w^{1118} (**J**, compare with **B**), while stage 12–13 pb^{11D} homozygous embryo show similar reduction in expression area relative to w^{1118} and pb^{11D}/*TM3,Ubx-LacZ*, (see also insets in **K and M** for zoom of maxillary and labial lobes, as well as ***Figure 5—figure supplement 4B,C***). (**O-U**) Embryos from a double deletion of region 2.1 and EO053 (pb^{11E}), either as balanced heterozygotes (**O,Q**), or homozygotes (**P,R–U**).

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Detection of *pb* mRNA in pb^{11E} heterozygous embryos at stage 11 (**O**) or stage 12 (**Q**) is comparable to both w^{1118} (**A**,**B**) and pb^{11D}/*TM3,Ubx-LacZ* (**H**, **J**; See also ***Figure 5—figure supplement 4A,D***). (**P**) *pb* mRNA expression is noticeably reduced in stage 11 pb^{11E}/pb^{11E} homozygous embryos, while expression in the hyopharyngeal lobe is unaltered (inset). (**R-U**) Representative stage 12–13 pb^{11E}/pb^{11E} embryos also demonstrating reduced *pb* mRNA detection. Compare insets in **R** and **T** with **K** and **M**, respectively, and also refer to ***Figure 5—figure supplement 4E,F***.

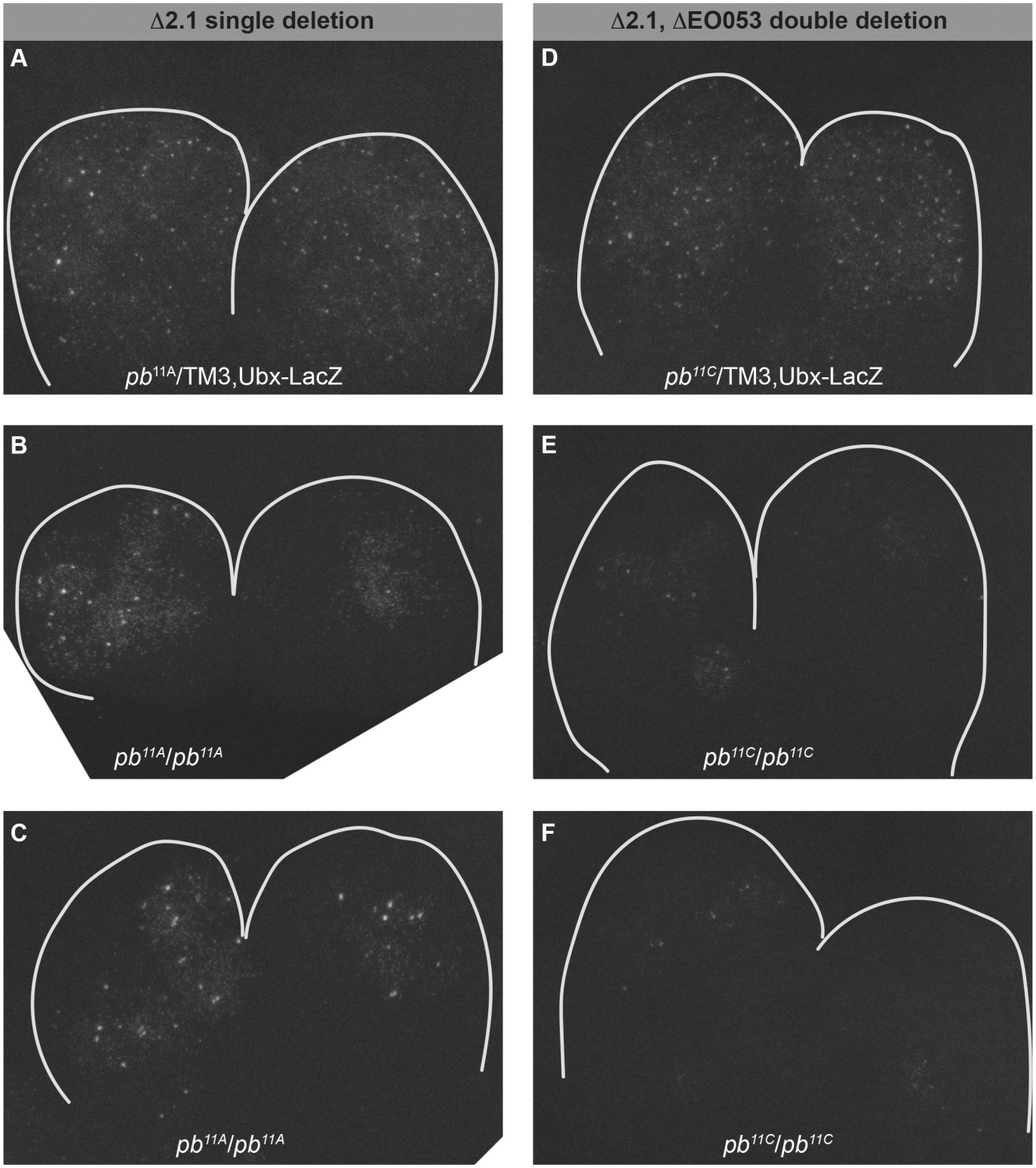

Figure 5—figure supplement 4. Fluorescent detection of *pb* mRNA in the maxillary and labial lobes in region 2.1 single deletions and 2.1, EO053 double deletions. (**A–C**) Fluorescent detection of *pb* mRNA expression following in situ hybridization in maxillary and labial lobes from stage 11 embryos with only region 2.1 deleted (pb^{11A}), either in heterozygous pb^{11A}/*TM3,Ubx-LacZ* embryos (**A**), or two representative pb^{11A}/pb^{11A} homozygous embryos (**B,C**), illustrating the noticeable reduction in expression area (compare with **A**, but also note similarity with ***Figure 5—figure supplement 3I***

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inset). (**D-E**) *pb* mRNA expression in maxillary and labial lobes from embryos with a double deletion in region 2.1 and EO053 (pb^{11C}), either in heterozygous pb^{11C}/*TM3,Ubx-LacZ* embryos (**D**), or two representative pb^{11C}/pb^{11C} homozygous embryos (**E,F**), noting markedly reduced expression area relative to both the heterozygous genotype (**D**) and the region 2.1 single deletion homozygotes (**B,C**). Note also the comparison with ***Figure 5—figure supplement 3P***.

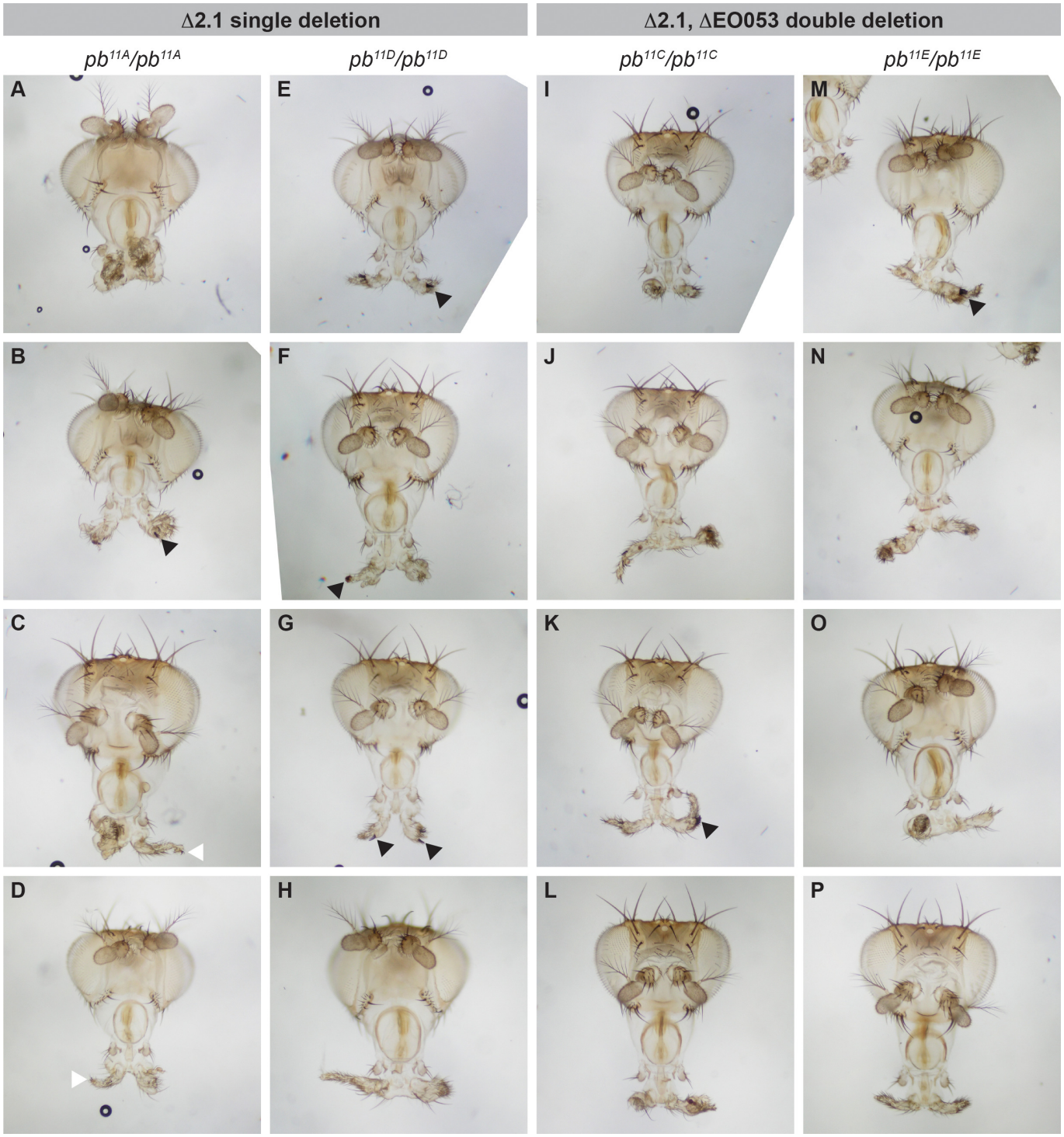

Figure 5—figure supplement 5. Deletion of region 2.1 is sufficient to cause a proboscis-to-leg transformation. Representative adult heads collected from flies homozygous either for a region 2.1 deletion (pb^{11A}, (**A–H**) or for a double-deletion of region 2.1 and EO053 (pb^{11C}, (**I–P**). Black arrowheads denote the presence of male sex combs, while white arrowheads point to terminal claws.

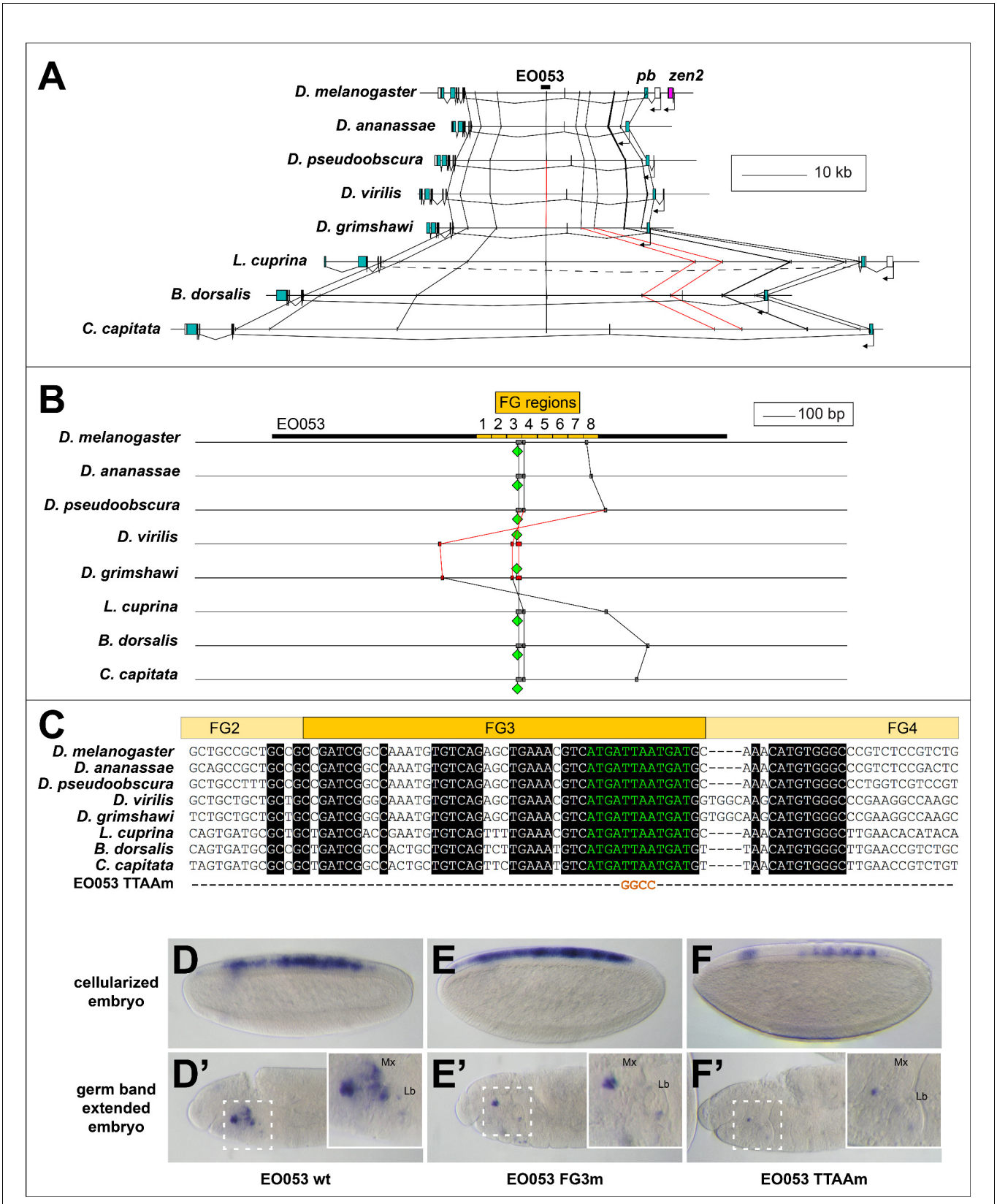

Figure 6. Conservation of EO053 sequences within the Schizophora. (**A**) Gene diagrams of *pb* from select Schizophoran flies with available genome sequence data. Coding exons of *pb* in each species are colored blue-green, based upon existing genome annotations, and the locations of EO053 and

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Figure 6 continued

zen2 in *D. melanogaster* are also noted. Vertical lines between species diagrams connect 14 bp or greater identical sequence blocks present in all eight species. Red lines (e.g., connected to the corresponding EO053 regions in *D. virilis* and *D. grimshawi*) represent sequences inverted relative to *D. melanogaster* (see also ***Figure 1—figure supplement 1***). Dashed line in *L. cuprina* diagram joins two separate coding regions annotated as belonging to *pb*, due to the presence of coding sequences for a YPWM motif (right-most exons) and a homeodomain (left-most exons). (**B**) Diagram of EO053 sequence conservation within select Schizophoran flies. *D. melanogaster* EO053 span is indicated by the thick black line and yellow boxes represent the boundaries of FG regions mutated in ***Figure 4***. Grey or red boxes connected between species represent 8 bp or greater identical sequence blocks present in all eight species. Green diamonds denote the location and orientation of the conserved 'EO053 motif' sequence shown in green in panel **C**. (**C**) Alignment of the region including FG3 from select Schizophoran flies, indicating additional sequences conserved in this region in these species (see also ***Figure 6—figure supplements 1–9***). Line below the alignment indicates the nucleotides mutated in the 'TTAAm' reporter construct shown in F. (**D-F'**) GAL4 expression in embryos carrying mutant FG3 region reporter constructs in either early embryos (**D–F**) or germ band extended embryos (**D'–F'**). (**D, D'**) Wildtype EO053 reporter. (**E, E'**) Noncomplementary transversion FG3 mutant reporter. (**F, F'**) 'TTAAm' reporter, mutating the four nucleotides indicated in **C**. Insets in **D'-F'** show higher-magnification images of the maxillary and labial segments.

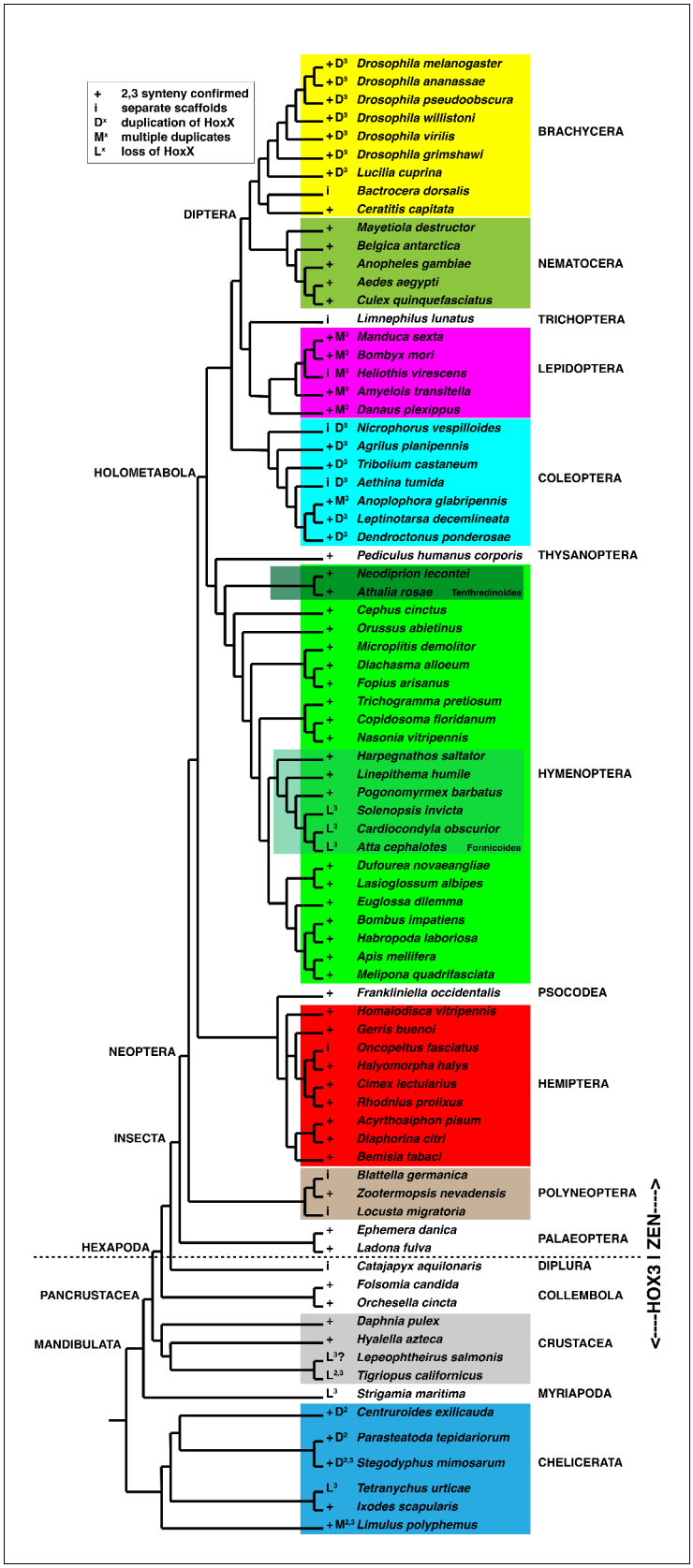

Figure 6—figure supplement 1. Synteny of *pb* and *zen2* orthologs across Arthropoda. Best to view high-quality image and zoom in and out as needed. Simplified Arthropod phylogeny indicating functional classification of

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Figure 6—figure supplement 1 continued

Hox3 ortholog and observed synteny of *pb* and *zen* orthologs. Cladogram based upon annotation in Ensembl Metazoa (https://metazoa.ensembl.org/), Ant Genomes Portal (http://hymenopteragenome.org/ant_genomes/) (***Elsik et al., 2016***), and others (***Beckenbach, 2012***; ***Branstetter et al., 2017***; ***Mao et al., 2015***; ***Misof et al., 2014***; ***Munro et al., 2011***; ***Oosterbroek and Courtney, 1995***; ***Pu et al., 2017***; ***Peters et al., 2017***; ***Regier et al., 2010***; ***Regier et al., 2013***; ***Song et al., 2016***). '+" next to each species name indicates confirmed synteny of *pb* and *Hox3* orthologs; 'i' indicates that *pb* and *Dfd* orthologs are found on distinct scaffolds and absence of *Hox3* on *pb* scaffold ('incomplete information'). 'D' indicates duplication of either *pb* (D^2) and/or *Hox3* (D^3), while 'M' indicates multiplication (more than two paralogs). 'L' indicates loss of either *pb* (L^2) and/or *Hox3* (L^3). The transition from Hox-like expression of *Hox3* to extraembryonic expression following the divergence of the Collembola and Insecta within Hexapoda (***Hughes et al., 2004***; ***Papillon and Telford, 2007***) is indicated with a dashed line. Colored boxes identify clades relevant to ***Figure 6—figure supplements 2–9***.

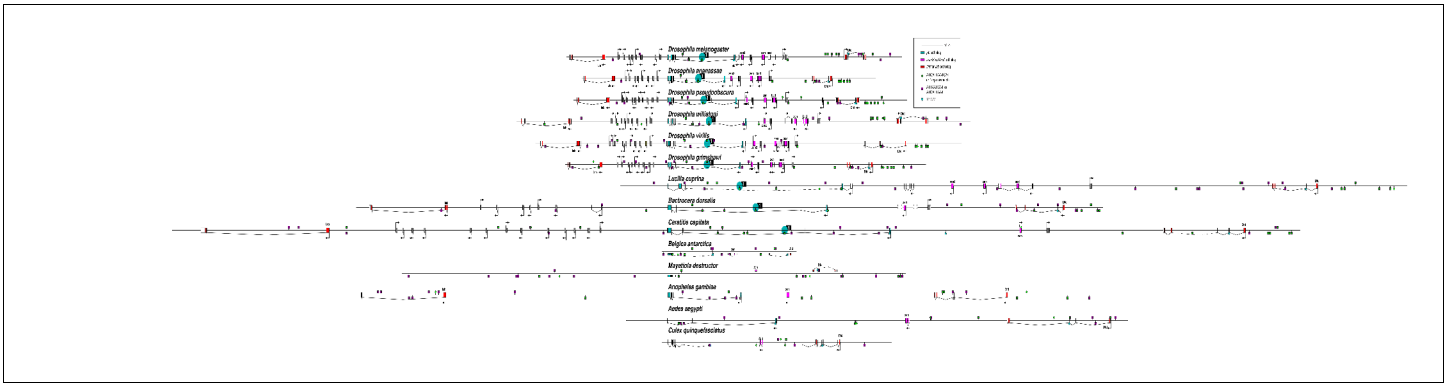

Figure 6—figure supplement 2. Instances of motifs similar to the EO053 conserved Hox-like motif in the *pb* region across Arthropods. Best to view high-quality images and zoom in and out as needed. Visualization of the *pb* region from the 80 species listed in ***Figure 6—figure supplement 1***. Refer to ***Figure 6—figure supplement 1*** for phylogenetic relationships between species. Each diagram is aligned with the *pb* ortholog (blue-green) underneath each species name, with *labial* to the left (red, if present), and *Hox3* (magenta, if present) and *Dfd* (red, if present) to the right of each *pb* ortholog. Intron/exon structure shown is according to existing genome annotations; dashed lines represent approximate inferred splicing. Open brackets represent boundaries of distinct scaffolds. Locations of sequences matching (12/12 or 11/12) the sequence ATCATTAATCAT are indicated by green diamonds (see ***Figure 6***), while similar sequences (exact matches to either ATCATTAAT or ATTAATCAT) are indicated by dark purple squares. Where green diamonds and purple squares are coincident, the *Hox*-like ATTAAT core remains intact. Specific *Hox*-like motifs that show patterns of conservation within clades are indicated by numbered ovals; these specific sequences are aligned in the accompanying ***Supplementary file 1***. The Schizophoran EO053 motif (1; see ***Figure 6***) is not conserved in other Diptera.

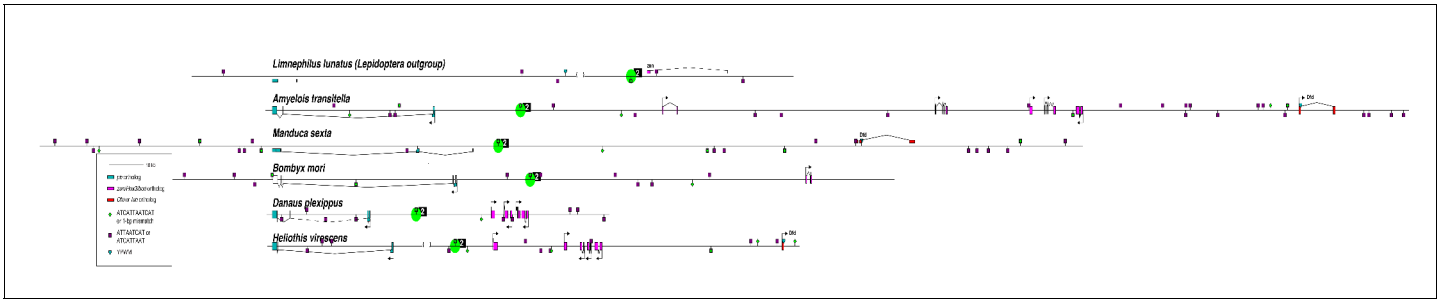

Figure 6—figure supplement 3. A motif upstream of *pb* (2) is conserved in Lepidoptera. Note that for most species many of the *Shx* (*zen* homolog) gene duplications are not shown (***Ferguson et al., 2014***).

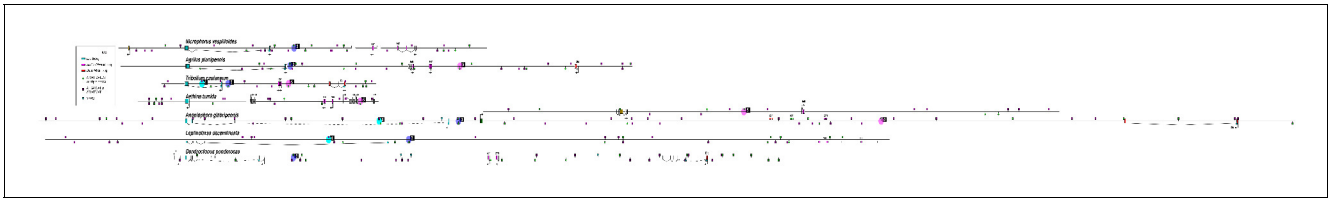

Figure 6—figure supplement 4. Several motif instances (3, 4, 5) show conservation within the Coleoptera. The scaffold containing the additional isolated *zen* paralog in *Anoplophora glabripennis* is shown above the *zen* paralogs syntenic to *pb*.

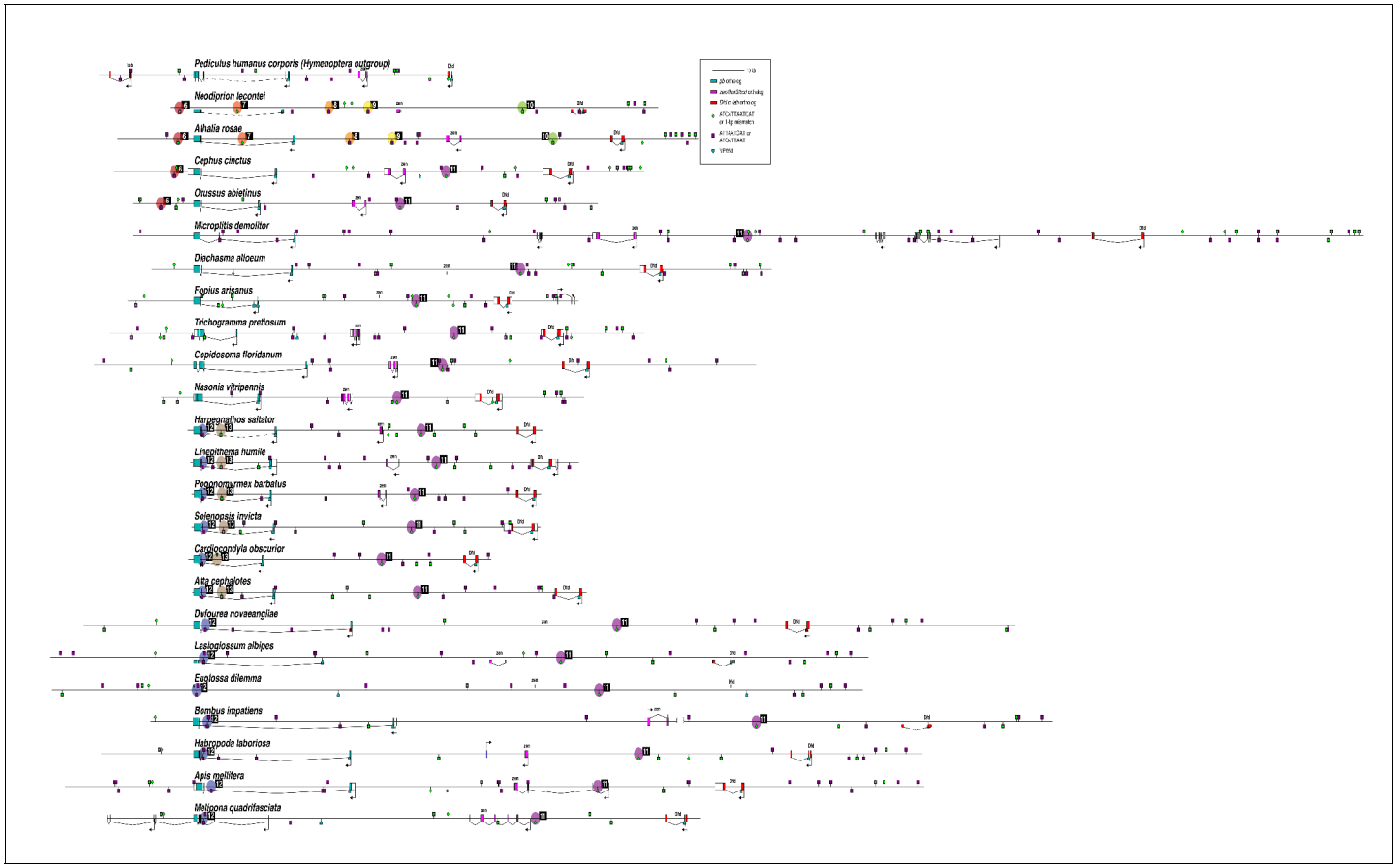

Figure 6—figure supplement 5. Conservation of several motifs (6-13) within Hymenoptera.

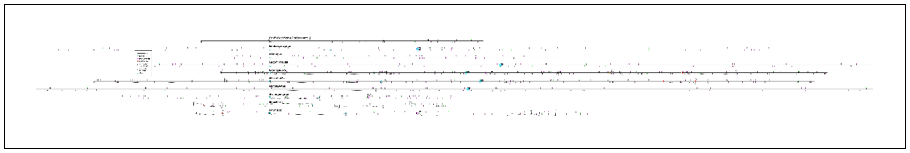

Figure 6—figure supplement 6. A single motif instance (14) appears conserved within some of the Hemiptera, excluding Sternorrhyncha.

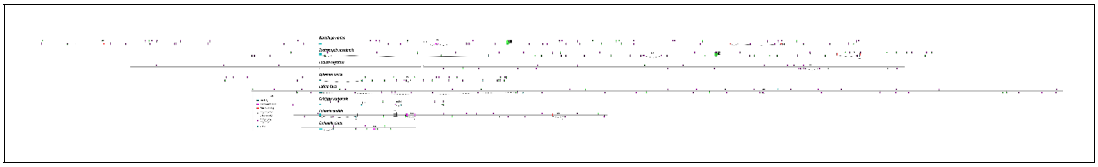

Figure 6—figure supplement 7. Diverse basal Hexapods include a motif (15) found in both the termite and cockroach.

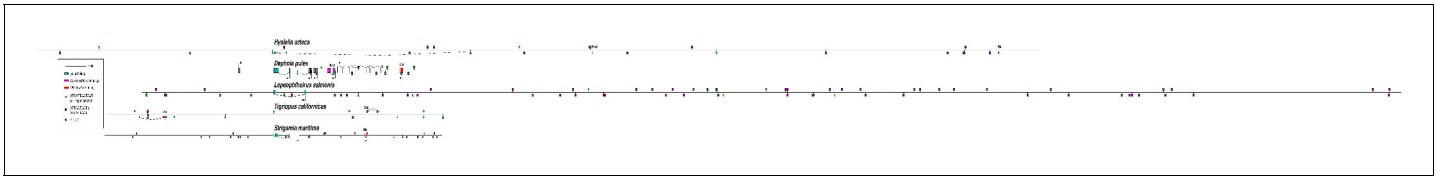

Figure 6—figure supplement 8. Crustacea and Myriapoda lack motif conservation and exhibit loss of *pb* and/or *Hox3* orthologs.

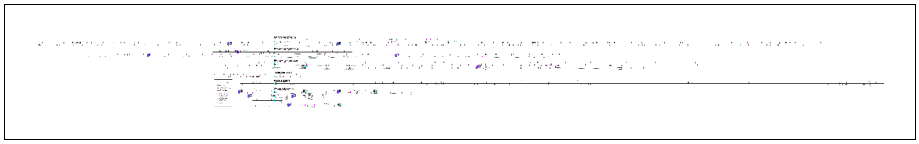

Figure 6—figure supplement 9. Some Chelicerates contain similar motifs (16, 17) near the duplicated *pb*/*Hox3* genes. Where duplicated, paralogs are represented on separate lines.

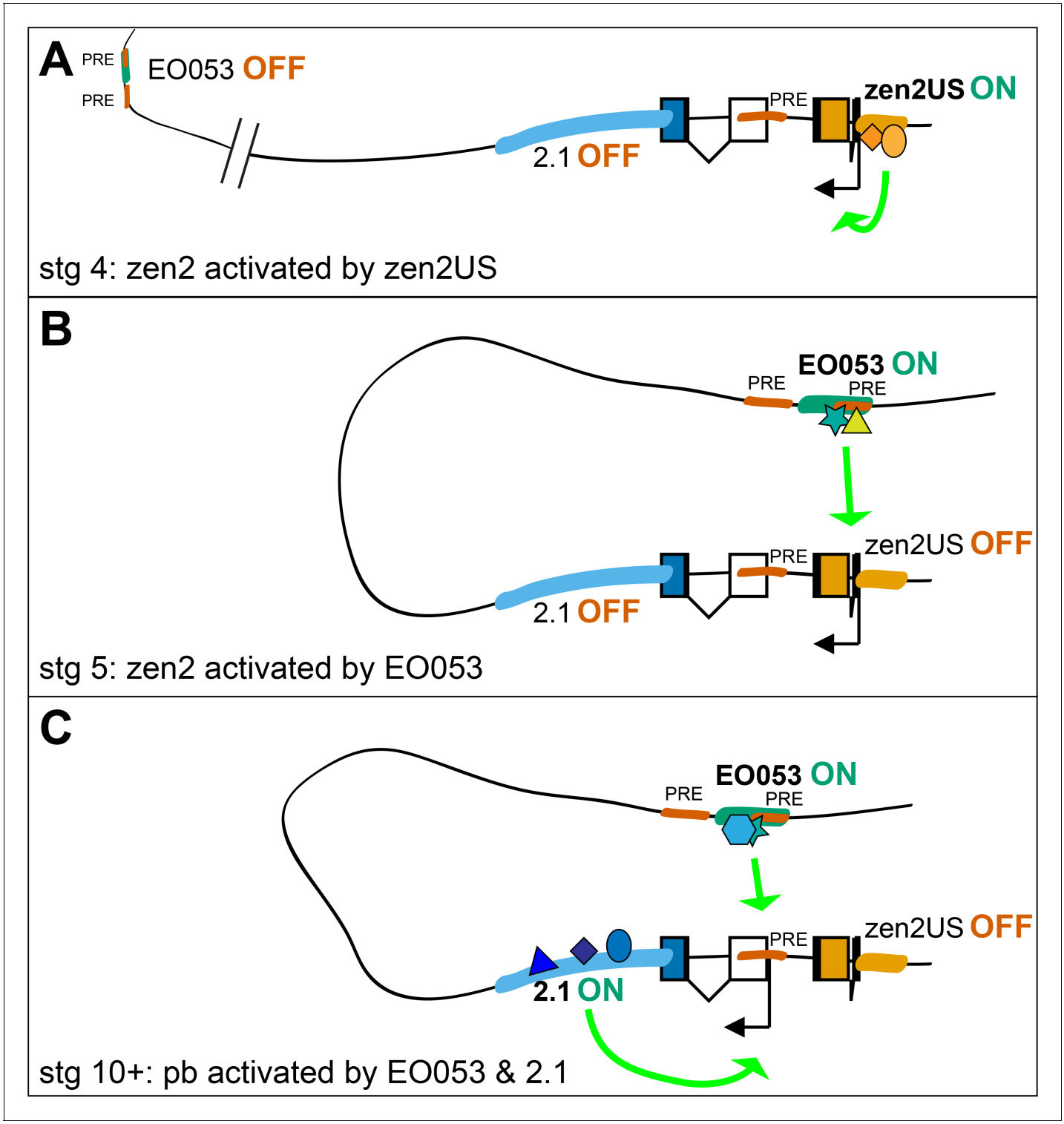

Figure 7. Possible model for EO053 regulation of both *zen2* and *pb*. (**A-C**) Diagram of the dynamic activities of EO053 during development. Black arrows indicate active transcription of either *zen2* (orange exons) or *pb* (white and blue exons), and green arrows signify active enhancers regulating transcription of either promoter. (**A**) At stage 4 in the dorsal blastoderm and at both anterior and posterior poles, *zen2* expression is initiated by the upstream enhancer, zen2US. (**B**) As zen2US loses activity in stage 5, expression of *zen2* instead becomes dependent upon EO053 in the dorsal blastoderm, potentially mediated by chromatin looping. (**C**) Later, in the developing head primordium, EO053 assists region 2.1 in directing *pb* expression, which may be mediated by interactions involving factors bound to nearby PREs (red in **A-C**; see also gene diagram in ***Figure 5***).

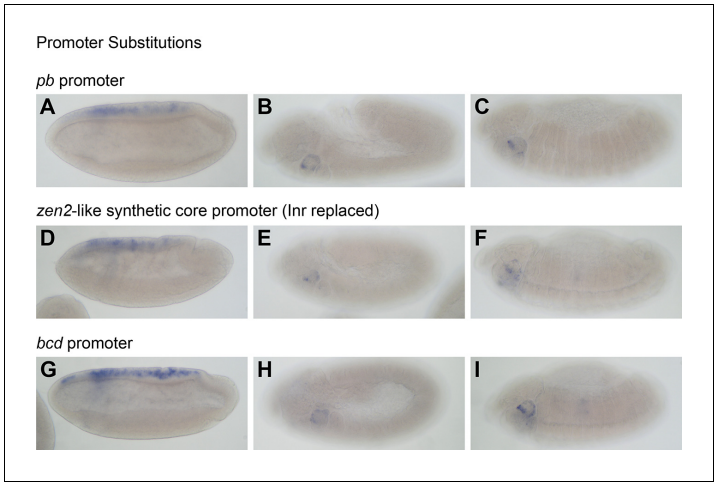

Figure 7—figure supplement 1. Modifying the reporter promoter does not affect expression pattern driven by EO053. See also ***Supplementary file 3*** for promoter sequences. Replacement of the *Drosophila* Synthetic Core Promoter (DSCP) in the reporter vector pBPGUw with either the *pb* promoter (**A–C**), a replacement of the DSCP Initiator sequence with that from *zen2* (**D–F**), or the promoter from *bcd* (**G–I**). *GAL4* mRNA expression from all constructs is shown during the early phase of *zen2*-like expression (**A, D, G**) or during later stages of *pb*-like expression (**B, E, H**: stage 11–12; **C, F, I**: stage 13–16).