
Figures and figure supplements

Elba, a novel developmentally regulated chromatin boundary factor is a hetero-tripartite DNA binding complex

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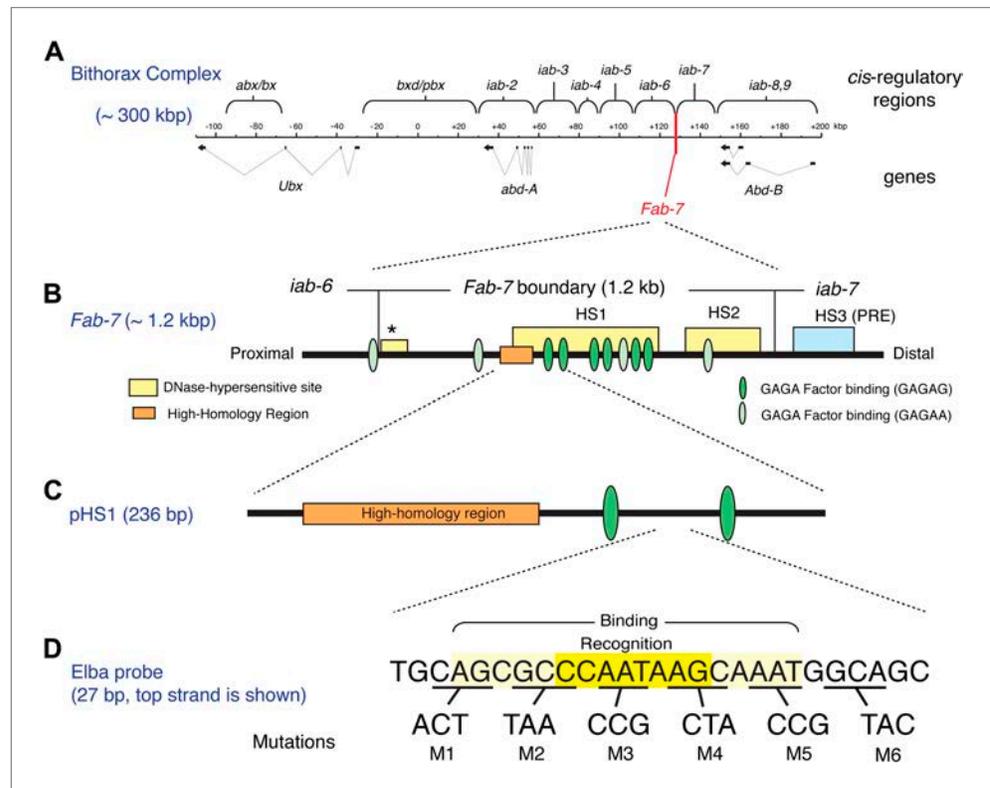


Figure 1. The Bithorax Complex, the *Fab-7* boundary and the Elba recognition element. **(A)** *Drosophila* Bithorax complex (BX-C). BX-C spans ~300 kb and includes three Hox-family genes *Ultrabithorax*, *abdominal-A* and *Abdominal-B*. Parasegment specific expression of these three homeotic genes is generated by a series of functionally autonomous cis-regulatory domains: *abx/bx*, *bxd/pbx* and *iab-2-iab8, 9*. Functional autonomy depends upon boundary elements that lie between each cis-regulatory domain (Maeda and Karch, 2010). One of these boundary elements is *Fab-7*, which is located in between the *iab-6* and *iab-7* cis-regulatory domains. Both in the context of BX-C and in transgene assays, the *Fab-7* boundary can block the action of enhancers/silencers at all stages of development, apparently irrespective of tissue or cell type (Galloni et al., 1993; Hagstrom et al., 1996; Mihaly et al., 1997; Schweinsberg et al., 2004). **(B)** The *Fab-7* boundary spans a sequence of 1.2 kb and consists of two prominent and one minor (*) chromatin specific nuclease hypersensitive regions (shown as yellow boxes). There is a third prominent nuclease hypersensitive region (blue) just distal to the boundary, which corresponds to a Polycomb Response Element (PRE) for the *iab-7* cis-regulatory domain (Maeda and Karch, 2010). The orange box is a ~100 base pair (bp) high-homology region which is conserved among *Drosophila* species (>90%) (Aoki et al., 2008). The ovals are binding sites for Trithorax-like (GAGA factor). **(C)** pHS1 is a 236-bp fragment from the proximal side of HS1 which has enhancer-blocking activity only in early embryos (Schweinsberg and Schedl, 2004). pHS1 includes the high-homology region and two GAGA-binding sites. These two GAGA sites are important for the early boundary activity of *Fab-7*, while GAGA sites elsewhere in *Fab-7* are needed later in development (Schweinsberg et al., 2004). In addition to the GAGA sites, the enhancer-blocking activity of pHS1 in early embryos also depends upon an 8-bp sequence, CCAATAAG, called Elba (Early boundary activity). Mutations in this sequence compromise the blocking activity of a 4x pHS1 multimer, while multimerization of a 27-bp oligo spanning the Elba sequence (8x Elba) [see **(D)**] is sufficient to confer early blocking activity. The Elba sequence is recognized by the stage-specific Elba DNA-binding factor. Elba factor binding is detected in 0–6 hr nuclear extracts, but it is absent in 6–12 hr (and 6–18 hr) nuclear extracts (Aoki et al., 2008). **(D)** Sequence of the 27-bp oligo used as the Elba probe in the EMSA experiments shown in Figures 3A, 4, 5B, and 6. The Elba factor in 0–6 hr nuclear extracts recognizes the 8-bp Elba sequence (shaded by yellow) and requires an additional 5 bp both upstream and downstream for full binding activity (shaded by light yellow). The bases underlined were altered as indicated in the mutant oligos, M1–M6. These mutant oligos were used as cold competitors in Figures 4C and 6C as indicated. For the DNA affinity beads, a 27-bp oligo containing the mutation M3 was used as the mutant Elba sequence.

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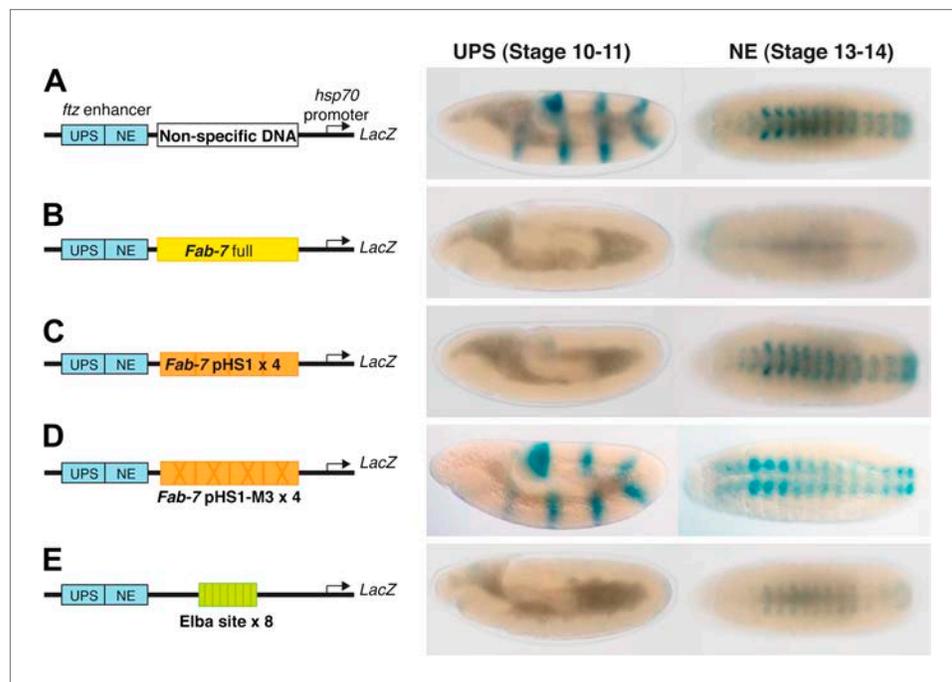


Figure 1—figure supplement 1. Enhancer blocking activity of Fab-7, pHS1×4 and the Elba×8 multimer. In this enhancer blocking assay, putative boundary elements are placed in between two *fushi tarazu* (*ftz*) enhancers (UPS and NE) and an *hsp70:LacZ* reporter. The UPS enhancer is active in early embryos and drives LacZ expression a seven stripe pair-rule pattern. The NE enhancer is active later in development and drives LacZ expression in the central nervous system. (A) When a ‘non-specific’ DNA sequence is placed between the *ftz* enhancers and the *hsp70:LacZ* reporter, no blocking is observed. In early embryos (stage 10–11 in this assay), the *ftz* UPS enhancers drive LacZ expression in seven stripes. In older embryos (stage 13–14) the NE enhancer drives LacZ expression in the CNS. (B) When *Fab-7* is placed between the *ftz* enhancers and the reporter, it blocks both enhancers from activating the *hsp70* promoter and there is little if any LacZ expression in either early or late embryos. (C) When pHS1×4 (four copies of the 236-bp pHS1; see **Figure 1**) is placed between the enhancers and the reporter, it blocks the UPS enhancer from activating stripe expression. However, boundary activity is stage specific and pHS1×4 does not block the NE from activating LacZ expression in the CNS in older embryos (**Aoki et al., 2008**). (D) When mutations (M3 in **Figure 1**) that disrupt Elba binding in nuclear extracts are introduced into the Elba sequence of pHS1, the enhancer blocking activity of the mutated pHS1×4 is compromised. In this case, both UPS stripe and NE CNS LacZ expression is observed (**Aoki et al., 2008**). (E) When a 27-bp oligo containing the Elba binding sequence is multimerized (Elba×8) and placed in between the *ftz* enhancers and the reporter, it blocks UPS driven LacZ stripe expression. It also causes a reduction in the extent of NE activation. This effect is position dependent. The blocking activity seen at this stage with the 8× multimer is likely due to the presence of some residual Elba activity in older embryos as seen in EMSA experiments with 6–12 hr nuclear extracts (**Aoki et al., 2008**).

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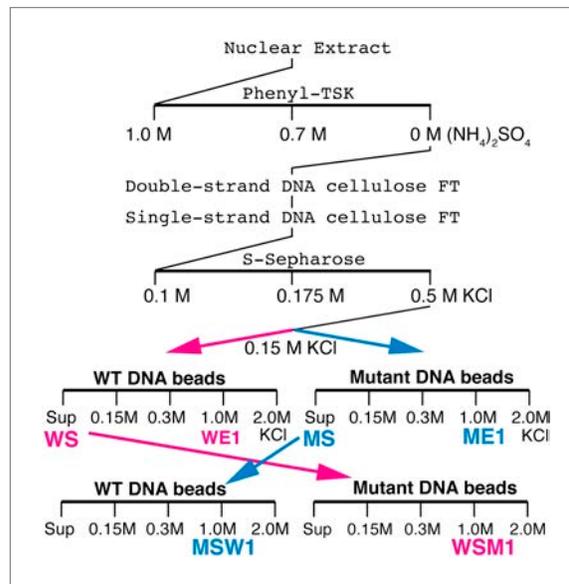


Figure 2. Elba purification scheme. Details provided in 'Materials and methods'.

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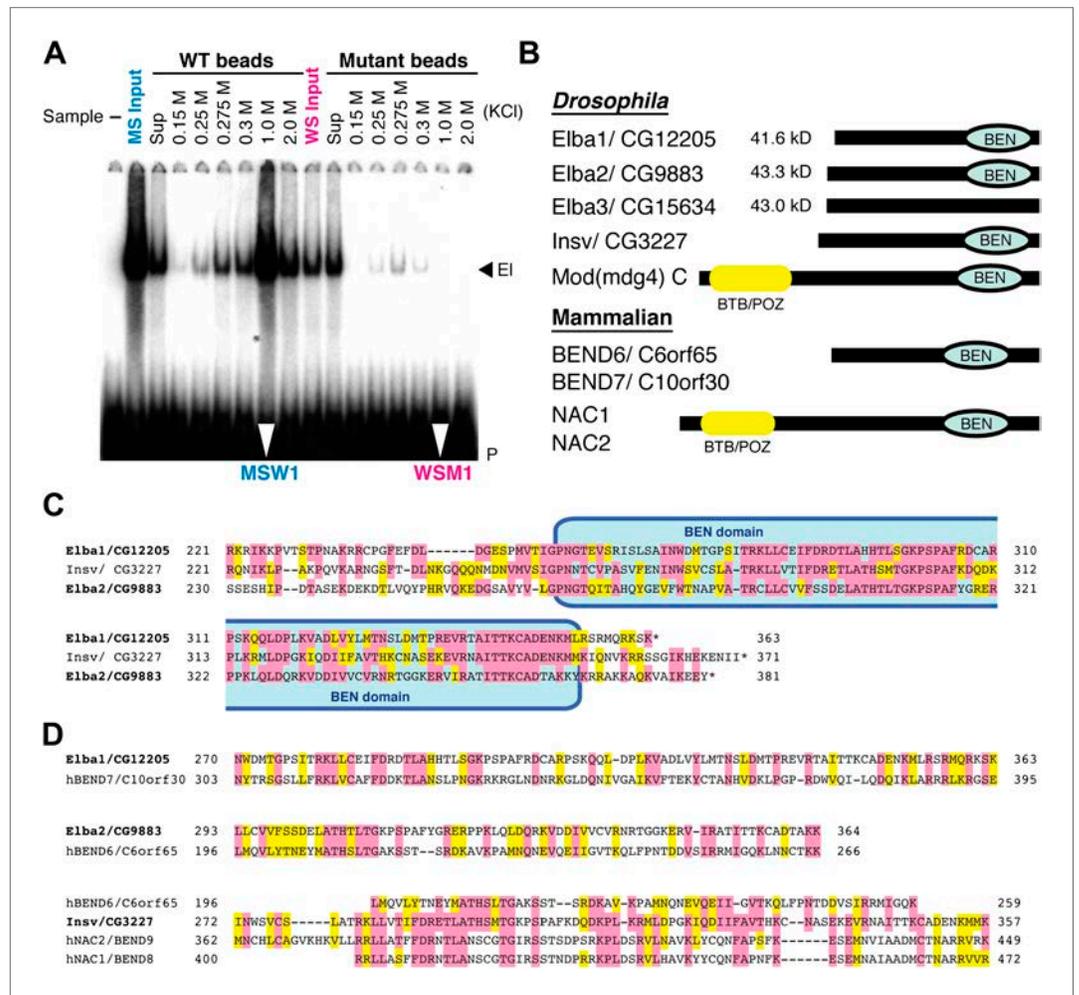


Figure 3. EMSA and Elba factor proteins. **(A)** EMSA of fractions from the cross-affinity purification. The ³²P-labeled Elba probe was incubated with fractions as indicated and subjected to 4% acrylamide-gel electrophoresis. El: Elba shift. P: probe. **(B)** Schematic of the Elba factors and BEN domain-containing (green) orthologs. BTB/POZ (yellow) domain is absent from Elba factors but is present in related proteins. **(C)** Sequence alignment of the C-terminal half of the *Drosophila* Elba1, Elba2 and a third *Drosophila* Ben protein Insv (Insensitive). The sequences of the three proteins were aligned according to the results of NCBI (National Center for Biotechnology Information) blast search (bl2seq). The amino acid residues conserved in more than two proteins are shaded with red. The residues that have similarities with each other are shaded with yellow. The predicted BEN domain region is boxed with pale blue. **(D)** Sequence alignment of *Drosophila* and mammalian (human) orthologs of BEN domain proteins. The C-terminal sequences of Elba1, Elba2 and Insv were subjected to blast search with human databases. Within the BEN domain sequences, the closest human ortholog of Elba1 is BEND7 (BEN domain-containing 7)/C10orf30 (29% identical, 46% positive), whereas for Elba2 the closest ortholog is BEND6/C6orf65 (29% identical, 53% positive). The BEN domain of Insv is most similar to three proteins: NAC2 (Nucleus accumbens-associated protein 2, NACC2)/BEND9, BEND6 and NAC1 (Nucleus accumbens-associated protein 1, NACC1)/BEND9. The amino acid residues conserved with each *Drosophila* protein are shaded with red. The residues that have similarities with each *Drosophila* protein are shaded with yellow.

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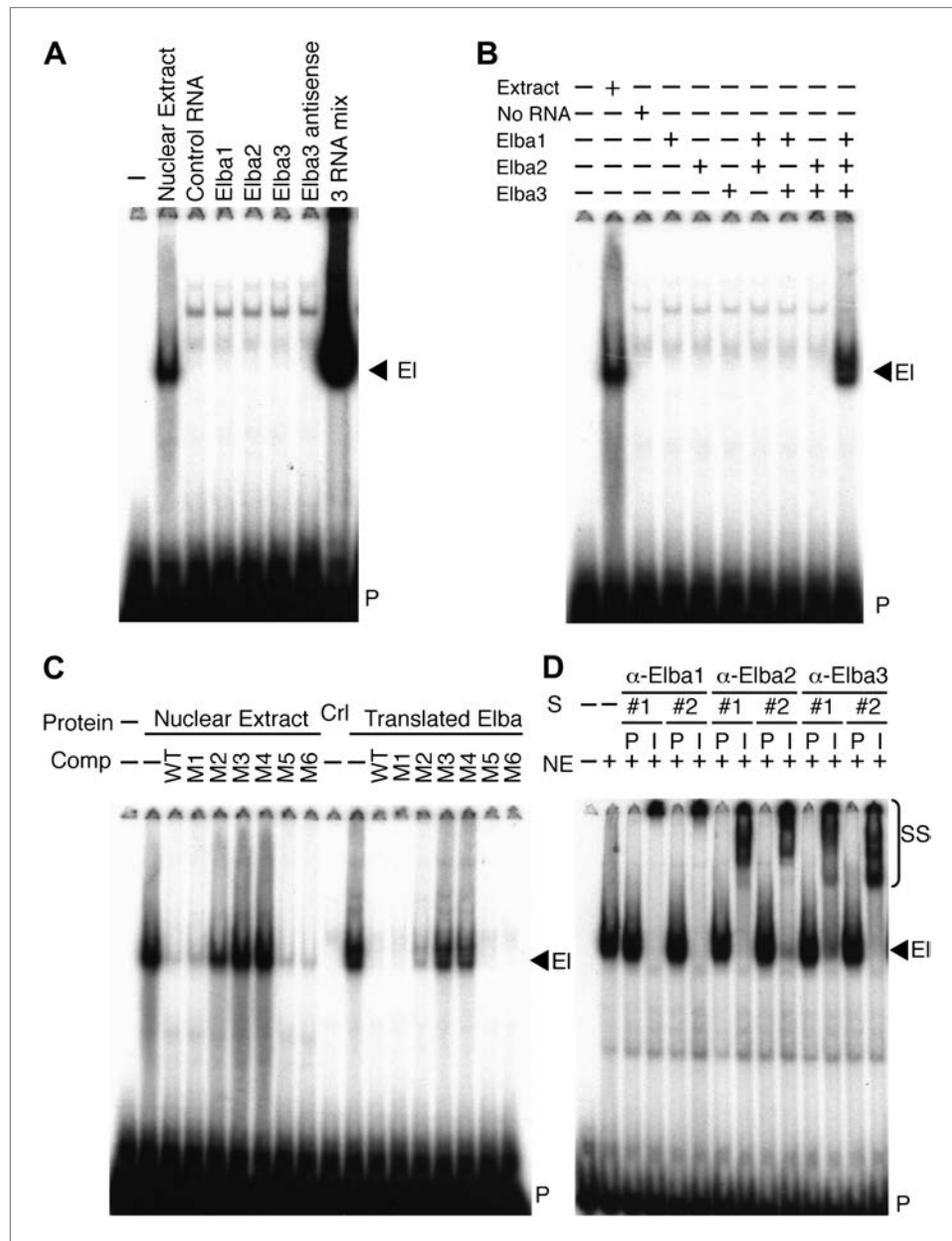


Figure 4. Elba is a hetero-tripartite complex. **(A)** and **(B)** All three Elba proteins are required to reconstitute DNA binding activity. In vitro translated proteins either singly or in combination as indicated were incubated with the Elba probe. El: Elba shift. P: probe. **(C)** Reconstituted Elba has the same sequence specificity as nuclear Elba. Reconstituted and nuclear extract shifts with or without (minus) a 100-fold excess of competitor (Comp) as indicated above each lane. WT: wild-type probe. M1–M6: mutant probes (see **Figure 1D**). Ctl: no-RNA control. **(D)** Nuclear Elba factor has all three Elba proteins. Nuclear extracts (NE) incubated with preimmune (P) or immune (I) polyclonal rabbit serum as indicated. #1, #2: serum from different rabbits. SS: antibody supershifts. El: Elba shift. P: free probe.

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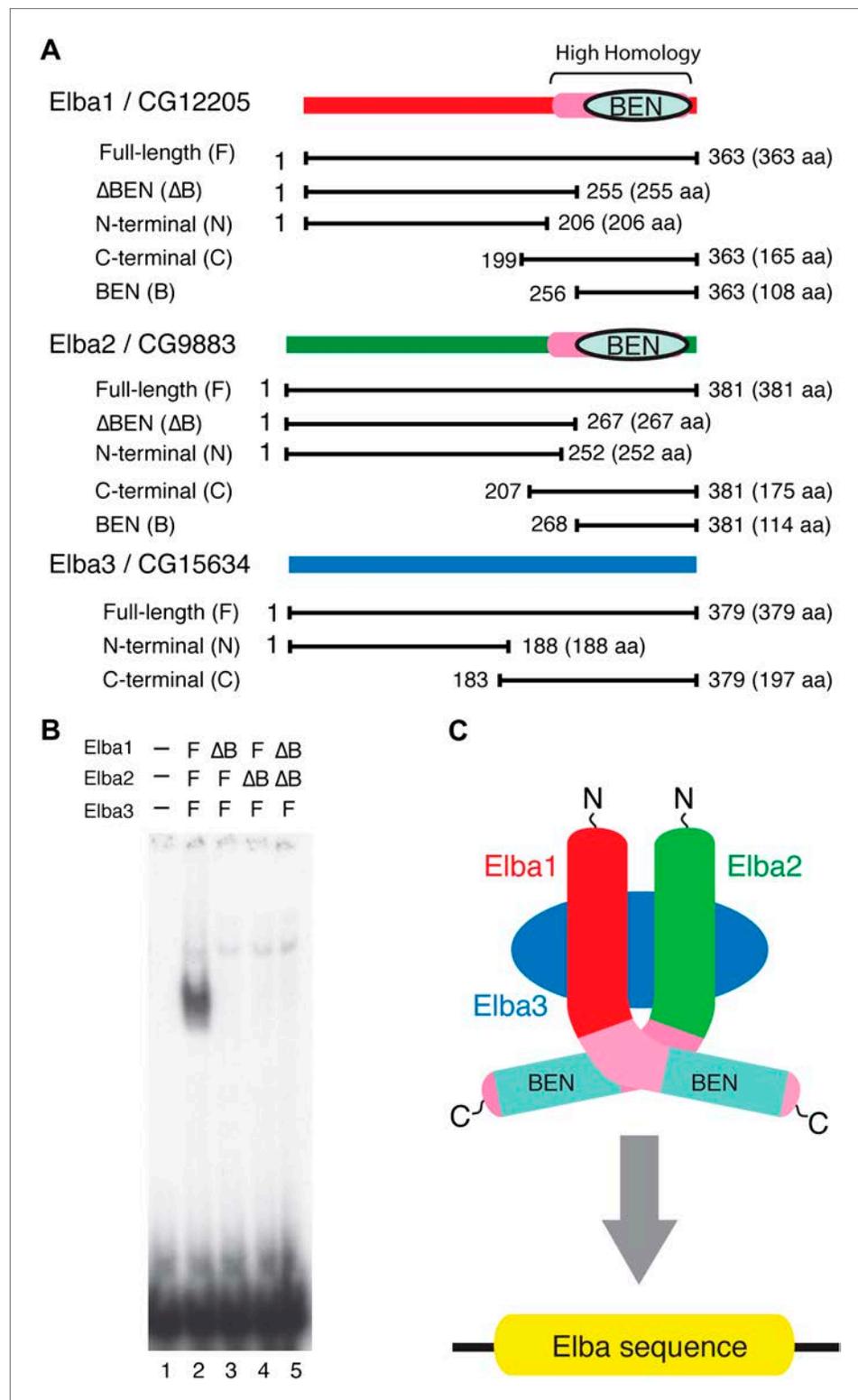


Figure 5. Elba protein deletions and functional organization of the Elba complex. **(A)** Elba proteins and deletion mutants. The bars under each diagram indicate the sequences retained in the mutant protein. The numbers correspond to the amino acid residues at the N and C terminal ends of the protein. The letter in parentheses is used to designate proteins added to the lanes in panel B and in **Figure 6**. Each protein was expressed either with an N-terminal FLAG tag or with an N-terminal FLAG tag plus a GST tag. The FLAG tag was used to determine the **Figure 5**. *Continued on next page*

Figure 5. Continued

relative amount of each protein so that the input of the translated proteins in the gel shift experiments could be adjusted. **(B)** BEN domain is required for the DNA-binding activity of Elba. EMSA experiments were performed as described in **Figure 4** by translating the full-length (F) or Δ BEN (Δ B) mutant Elba subunits in vitro and by mixing each as indicated above the lanes. **(C)** A schematic structure of proposed model for the Elba complex.

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Figure 6. Continued

Elba1C:Elba2C shift is indicated by closed arrowhead. Position of the Elba1C:Elba2C FLAG supershift is indicated by open arrowhead. Proteins added to each lane including FLAG and GST antibodies are indicated above the lane. Note that the same shift was detected in lane 16 of (A) when the X-ray film was exposed for a longer period of time. (E) Supershifts of the GST-Elba1F:GST-Elba2F generated by the addition of Elba3. Proteins corresponding to the full length Elba3 (F), or the N-terminal (N) and C-terminal (C) halves of Elba3 were added to an EMSA reaction mix containing the GST-Elba1:GST-Elba2 dimer. Proposed structures of the native Elba complex, the artificial GST-dependent dimer by GST-Elba1:GST-Elba2 and GST-Elba1C:GST-Elba2C are shown in **Figure 6—figure supplement 1–3**, respectively.

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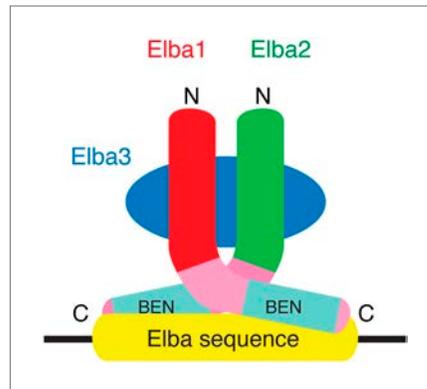


Figure 6—figure supplement 1. A schematic model for the tripartite Elba complex. Elba3 links Elba1 and Elba2 through sequences in their N-terminus. This brings the C-terminal halves of Elba1 and Elba2 together to form a 'DNA binding pocket' which binds to the asymmetric Elba recognition sequence.

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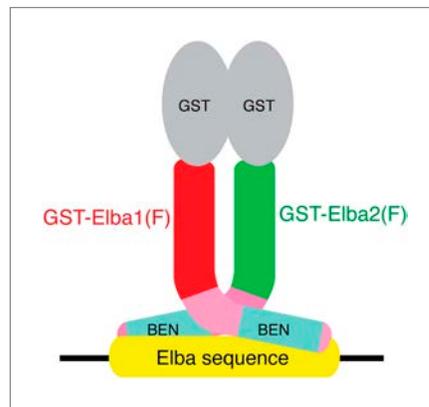


Figure 6—figure supplement 2. The artificial GST-Elba1:GST-Elba2 heterodimer binds to the asymmetric Elba recognition sequence. The N-terminal GST moiety mediates dimerization of Elba1 and Elba2, mimicking the coupling of these two proteins by Elba3.

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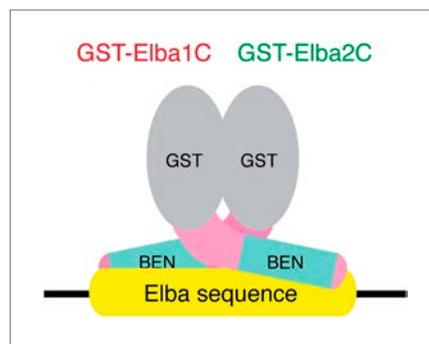


Figure 6—figure supplement 3. The artificial heterodimer of GST-Elba1C:GST-Elba2C binds to the asymmetric Elba recognition sequence. The C-terminal 165–175 residues of Elba1 and Elba2 retain DNA-binding activity with the same sequence-specificity as hetero-tripartite Elba complex when they are dimerized by GST moieties.

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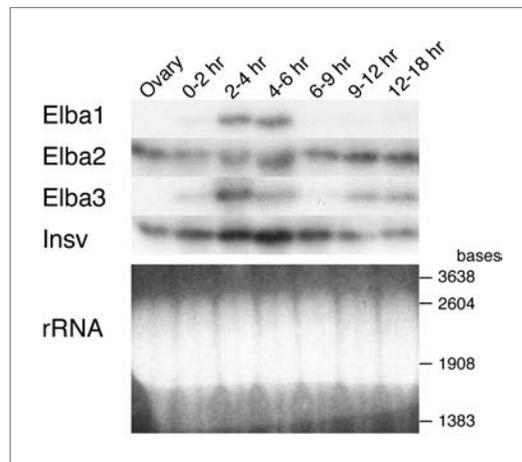


Figure 7. Expression of mRNAs encoding Elba factors and Insv. Total RNA from ovaries and staged embryos as indicated were probed with cDNAs encoding each protein. Shown on the bottom is the ethidium bromide staining of a gel for the Northern blotting as a loading control. The positions of the RNA markers are indicated on the right.

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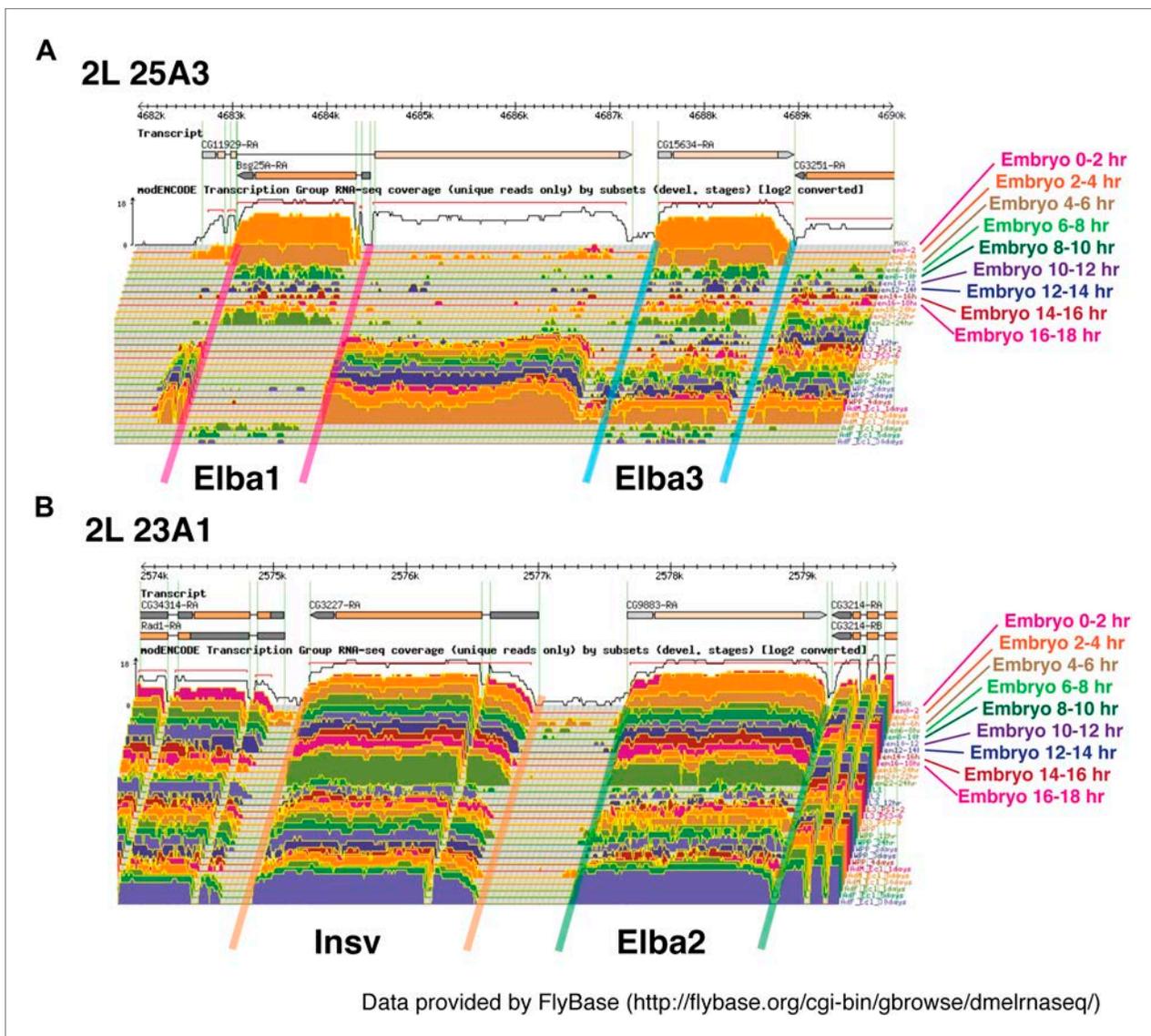


Figure 7—figure supplement 1. Loci of *Elba* proteins and the expression patterns of their mRNAs during the development. **(A)** modENCODE expression profiles of mRNAs encoding *Elba1* and *Elba3*. As indicated in the diagram, *elba1* (CG12205) and *elba3* (CG15634) are transcribed in opposite directions and are separated from each other by sequences encoding much of the CG11929 open reading frame <http://flybase.org/reports/FBgn0000227.html/>, <http://flybase.org/reports/FBgn0031621.html/>. As seen in the Northern blots, there is little or no maternal deposition of *elba1* or *elba3* mRNAs. These two genes are not expressed until the mid-blastula transition and the highest levels of *elba1* and *elba3* mRNAs are found in 2–4 hr embryos. Relatively high levels of both mRNAs are found in 4–6 hr embryos and then both largely but not completely disappear during the remainder of embryogenesis. In our Northern blot experiments transcript levels also drop dramatically after 6 hr, but in older embryos both *elba1* and *elba3* are detected again. However, it is likely that the amounts of *elba1* and *elba3* in these later stages are overestimated in our experiments. Our gels were overloaded, and transfer of the two *elba* mRNAs (which migrate very near the bottom of the rRNA band) in the 2–4 hr samples does not appear to be as efficient as it is at later stages when their levels are greatly reduced. The presence of only quite low levels of *elba1* and *elba3* mRNAs at these later stages would be consistent with the modENCODE data. **(B)** modENCODE expression profiles of mRNAs encoding *Elba2* and *Insv*. Like *elba1* and *elba3*, *elba2*/CG9883 and *insensitive*/CG3227 are closely linked and transcribed in opposite orientations. As seen in our Northern blot experiments, the modENCODE temporal expression patterns of the mRNAs encoding these two proteins are quite different from that of *elba1* and *elba3* <http://flybase.org/reports/FBgn0031434.html/>, <http://flybase.org/reports/FBgn0031435.html/>. Both are maternally deposited and high levels are present throughout embryogenesis. Also unlike *elba1* and *elba3*, both transcripts are expressed at later stages of development.

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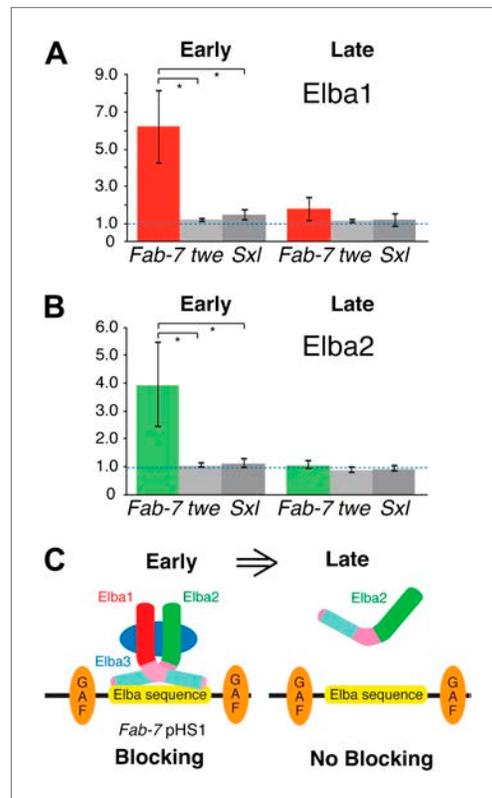


Figure 8. Elba proteins are bound to *Fab-7* in early but not late embryos. **(A)** and **(B)** Elba1 and 2 ChIPs. Early (2–5 hr) or late (9–12 hr) embryos were cross-linked and after processing immunoprecipitated with Elba1 or Elba2 antibodies, or pre-immune serum. Sequences from the pHS1 region of *Fab-7* or control *twe* and *Sex-lethal* sequences were detected by qPCR. The y-axis shows the average immune/preimmune ratio. * $p < 0.05$. **(C)** Model showing binding and enhancer blocking by the Elba complex in early but not late embryos. GAF: GAGA factor and its binding sites.

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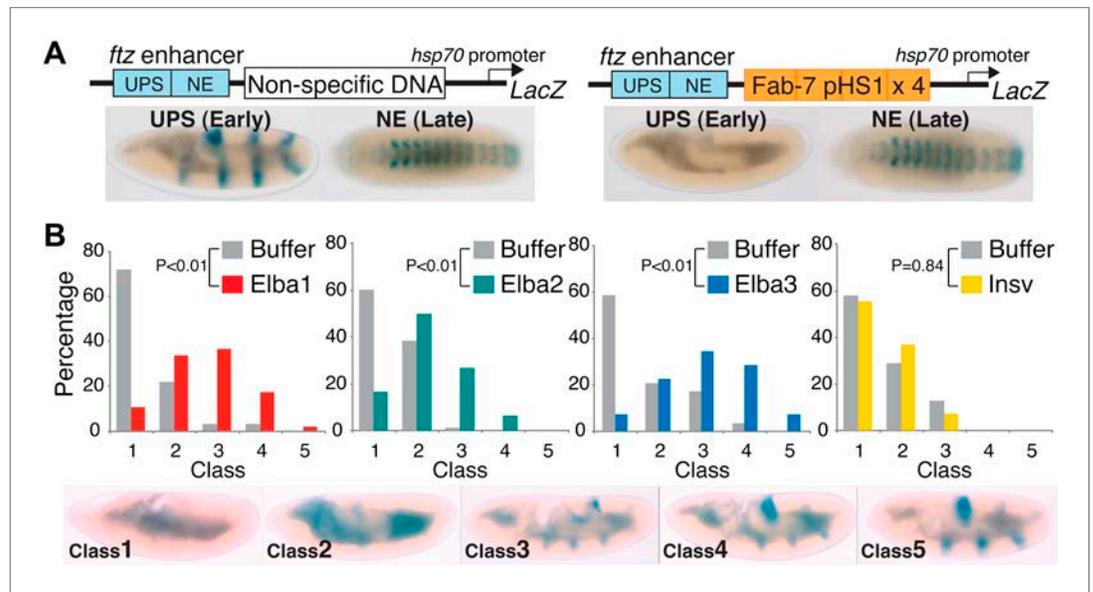


Figure 9. Hetero-tripartite Elba complex mediates early boundary activity. **(A)** Top: *fushi tarazu* (*ftz*) enhancers drives stripe expression (UPS) in stage 10–11 embryos and central nervous system expression (NE) in stage 13–14 embryos. Bottom: Four copies of *Fab-7* pHS1 (which contains the Elba sequence) blocks the UPS, but not the NE enhancer. *elba1-3* and *insv* dsRNAs or buffer alone were injected into embryos transgenic for the *ftz-4* × pHS1-LacZ enhancer blocking reporter and blocking activity examined. In each experiment ~100 embryos were photographed and categorized into Class 1–5 as indicated. Graphs show the percentage of embryos in each class for RNAi and the buffer control in that experiment. Three independent injection experiments were done for each protein. All gave similar results and only one is shown here. p values from the t-tests are shown in the graphs.

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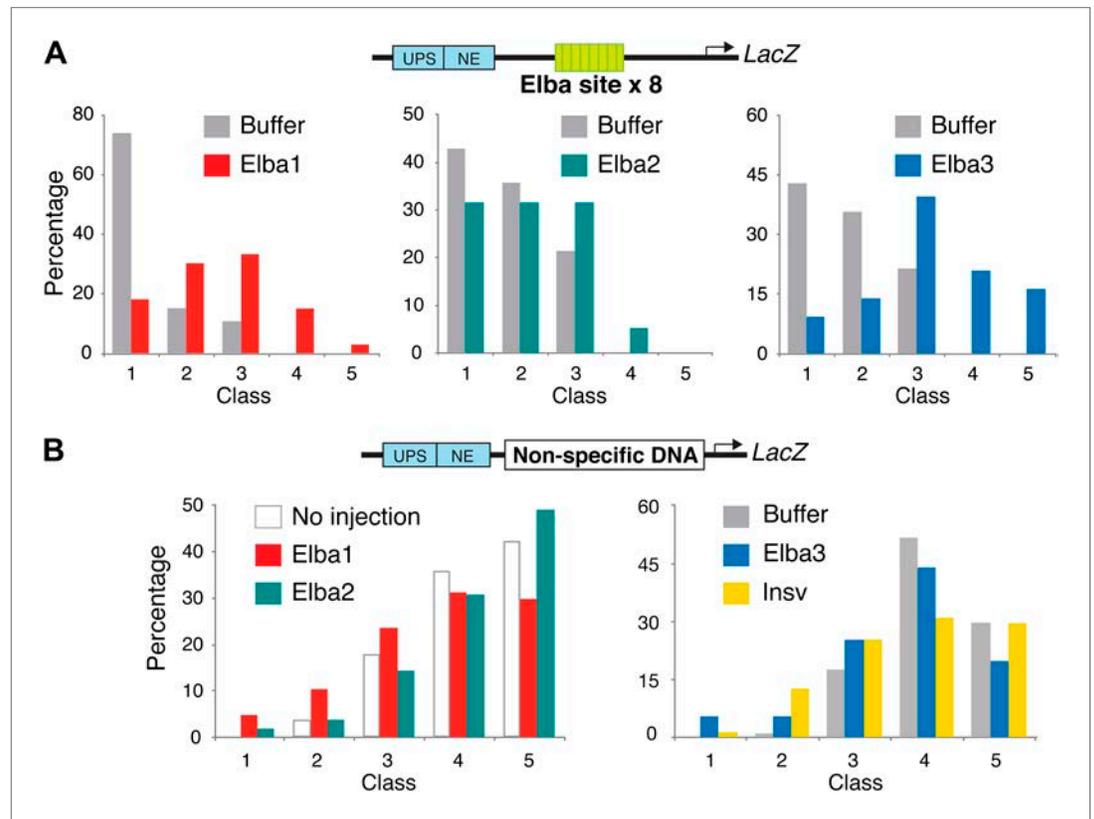


Figure 9—figure supplement 1. Double-stranded RNA injection into embryos transgenic for the Elba \times 8 multimer reporter and a reporter containing non-specific DNA in the blocking position. **(A)** Embryos transgenic for the *ftz*-Elba \times 8 (multimer)-*Hsp70*:*LacZ* reporter (see **Figure 1—figure supplement 1**) were injected with dsRNAs specific for each of the *elba* genes. The injected embryos were allowed to develop until stage 10–11 and then stained for *LacZ* expression. In each experiment, buffer-injected control embryos were prepared and processed in parallel with the dsRNA injected embryos. Embryos were photographed and then categorized into Class 1–5 according to the intensity of *ftz* UPS-enhancer stripes. (**Figure 9** shows representative examples of each class.) The graphs show the percentage of embryos (y-axis) that fell into each class (x-axis) in the *elba* dsRNAi and the corresponding control buffer injection. As was observed in the pHS1 \times 4 injection experiments, the blocking activity of the Elba \times 8 multimer is compromised by injection of dsRNA for each of *elba* gene. The loss of blocking activity can be seen by comparing the class distribution for the dsRNA injection with the class distribution for the buffer control. (Note that the *elba2* and *elba3* dsRNA injections shown here were part of the same injection experiment and have the same buffer injected control.) The dsRNA injections for the two mid-blastula transition genes *elba1* and *elba3* have a greater effect on the blocking activity of the Elba \times 8 multimer than the dsRNA injections for *elba2*. This was observed in other Elba \times 8 multimer injection experiments and was also seen for the pHS1 \times 4 reporter (see **Figure 9**). The smaller reduction in blocking activity observed with *elba2* dsRNA injections is most likely due to the presence of substantial amounts of maternal *elba2* mRNA. **(B)** *elba* and *insv* dsRNA injections do not affect *LacZ* expression from a control reporter which has a non-specific DNA sequence in the blocking position. The expressions of the UPS *LacZ* stripes in dsRNA and in the buffer injected or no injection controls give a similar class distribution.

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