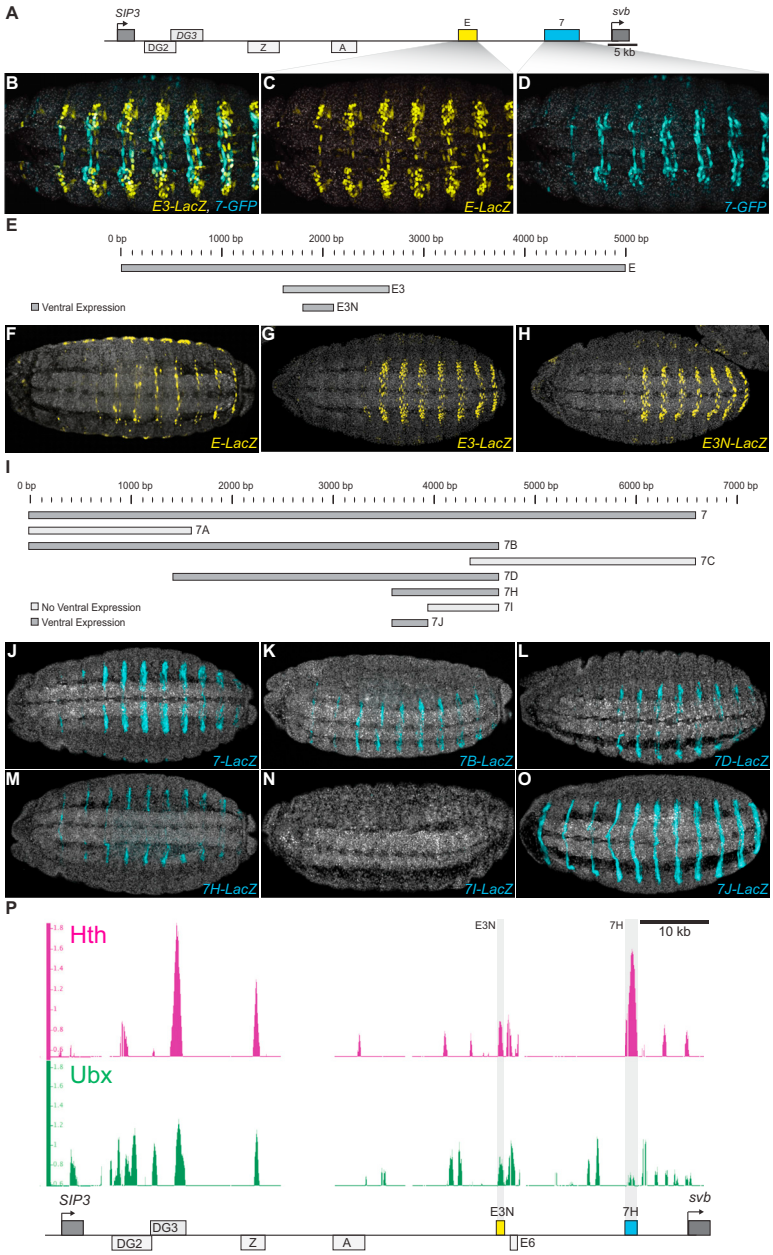

Figure S1. Defining the Minimal Ventral Embryonic *svb* Enhancers, Related to Figure 1 and Table S1

(A) Schematic of the *svb* upstream *cis*-regulatory region, indicating embryonic enhancer elements. The ventral embryonic enhancers *E3N* and *7H* are highlighted in yellow and blue, respectively.

(B–D) Early stage 15 embryo carrying both *E*::*lacZ* and *7*::*GFP* reporter constructs, co-stained with antibodies against β-Galactosidase and GFP (B), demonstrate partially overlapping patterns of expression.

(E) Schematic of a subset of the *E*-derived enhancer fragments tested with transgenic reporter constructs (also see Figure 1). Enhancers indicated with filled boxes drove expression.

(F–H) Early stage 15 embryos carrying three of the *E*-derived enhancer reporter constructs—*E*, *E3*, and *E3N*—stained with an antibody against β-Galactosidase.

(I) Schematic of a subset of the *7* enhancer fragments tested with transgenic reporter constructs. Enhancers indicated with filled boxes drove expression and those with unfilled boxes showed no expression.

(J–O) Early stage 15 embryos carrying six of the *7*-derived enhancer reporter constructs—*7*, *7B*, *7D*, *7H*, *7I*, and *7J*—stained with an antibody against β-Galactosidase. Further reduction of *7H* to *7J* led to additional anterior expression (O).

(P) Log2 ratio enrichment profiles for Ubx and Hth across the *svb* upstream *cis*-regulatory region, with the minimal *E3N* and *7H* enhancers indicated in yellow and blue, respectively.

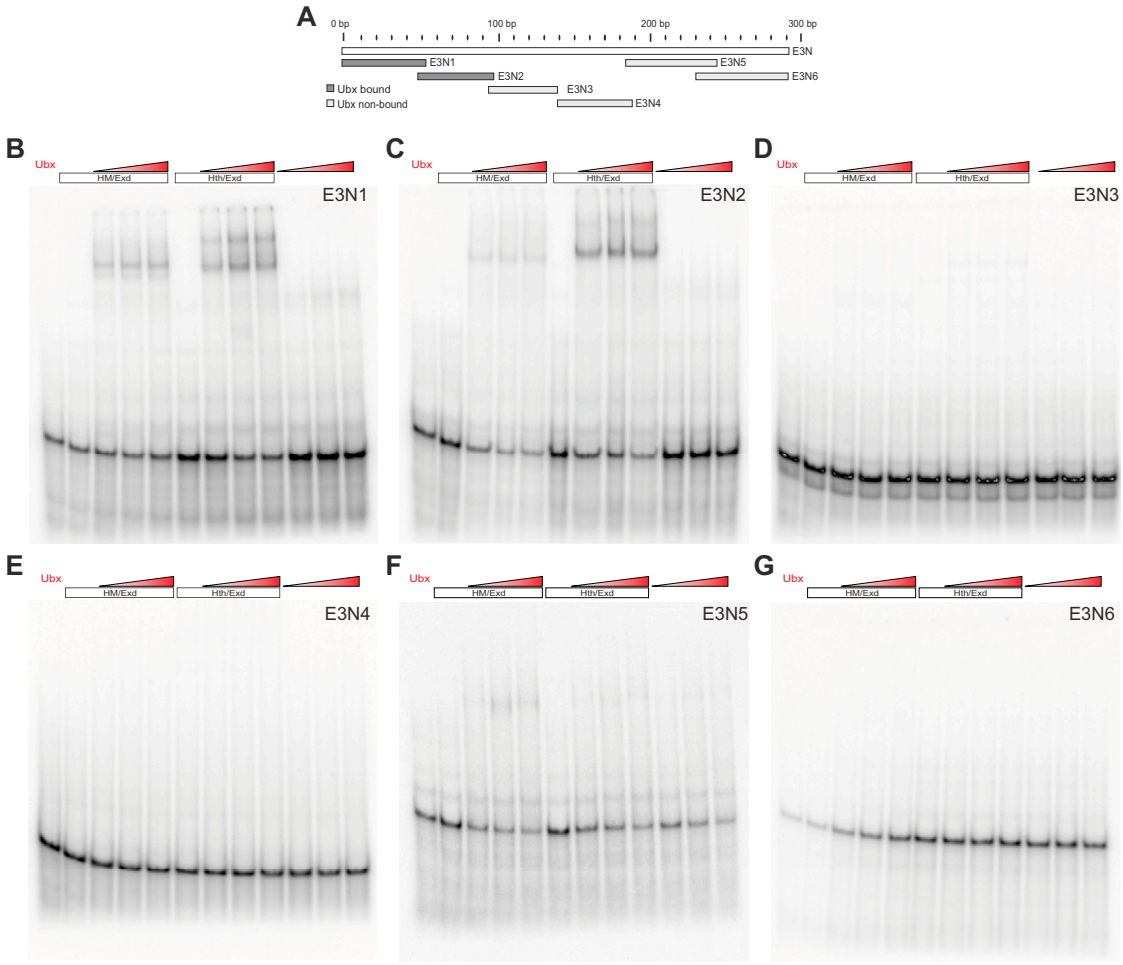

Figure S2. Identification of Regions of the *E3N* Enhancer that Bind Ubx-Exd In Vitro, Related to Figure 2 and Table S1

(A) A schematic of the regions tested for the ability to bind Ubx-Exd assayed with EMSAs.

(B–G) Ubx-Hth^{FL}-Exd and Ubx-Hth^{HM}-Exd bound fragments *E3N1* (B) and *E3N2* (C), as demonstrated with EMSAs. There was also very weak binding of Ubx-Hth^{FL}-Exd and Ubx-Hth^{HM}-Exd to fragment *E3N5* (F).

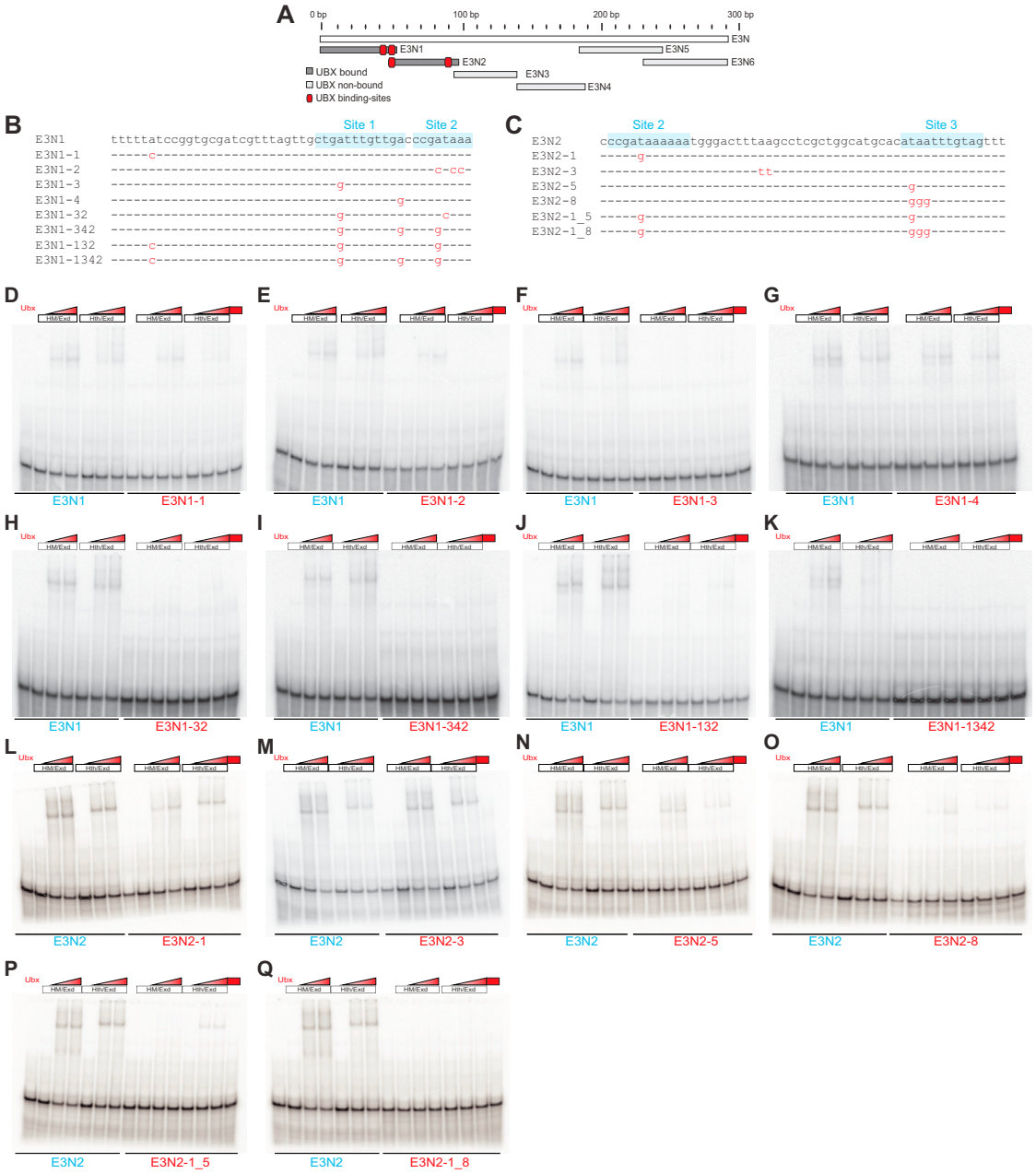

Figure S3. *E3N* Contains a Cluster of Three Ubx-Exd Binding Sites, Related to Figure 2 and Table S2

(A) A schematic of the regions tested for the ability to bind Ubx-Exd assayed with EMSAs.

(B and C) The aligned *E3N1* and *E3N2* sequences from wild-type and mutated sequences. Dashes indicate non-altered sequence. Red letters denote mutated sites. Blue boxes highlight Ubx-Exd binding sites.

(D–Q) Ubx-Hth^{FL}-Exd and Ubx-Hth^{HM}-Exd EMSAs for each enhancer fragment outlined in (B) and (C). Mutation of the putative Ubx-Exd binding sites shown in (B) and (C) led to decreased binding of Ubx-Exd.

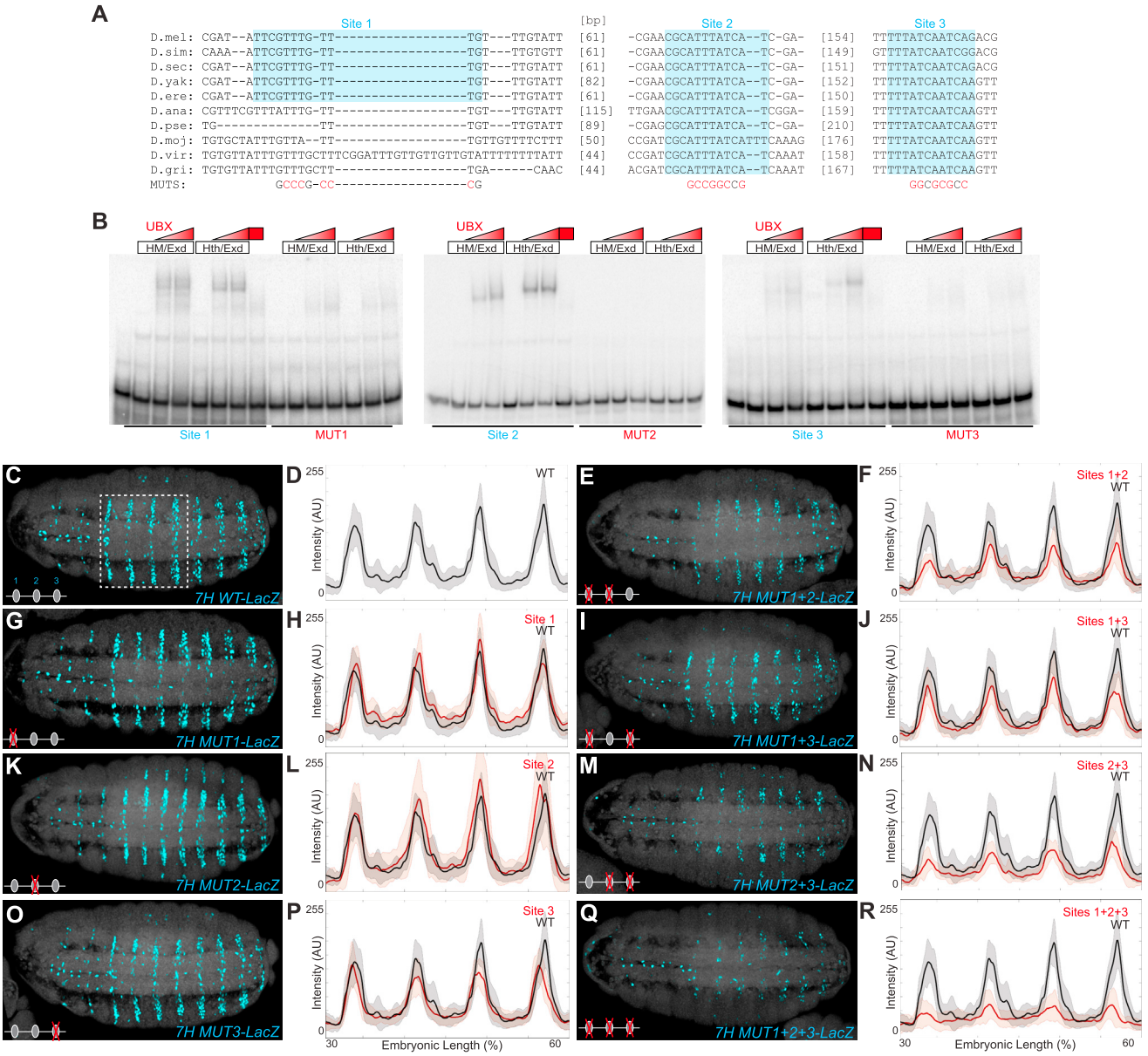

Figure S4. The *svb 7H* Enhancer Contains a Cluster of Ubx-Exd Binding Sites, Related to Figure 2 and Tables S1 and S2

(A) A multiple sequence alignment for selected regions of the *7H*-enhancer, with the three Ubx-Exd sites labeled and highlighted. Dashes indicate gaps in the aligned sequence. Bracketed numbers indicate the number of base pairs between the aligned sequences shown. Mutations of the Ubx-Exd binding-sites are shown (MUTS).

(B) Ubx-Hth^{FL}-Exd and Ubx-Hth^{HM}-Exd bound each of the three sites, as demonstrated with EMSAs. This binding was reduced when the sites were mutated (MUT).

(C, G, K, O, E, I, M, and Q) Early stage 15 embryos carrying *7H*::*lacZ* reporter constructs stained with an antibody against β-Galactosidase, with Ubx sites mutated as indicated in (A).

(D, H, L, P, F, J, N, and R) Plots showing profiles of average expression in the region outlined in (C) for the corresponding genotype (n = 10 for each genotype). In all plots, the black lines denote WT embryos and the red lines denote the MUT line indicated. Shaded bounding areas indicate ± 1 s.d. AU, arbitrary units of fluorescence intensity.

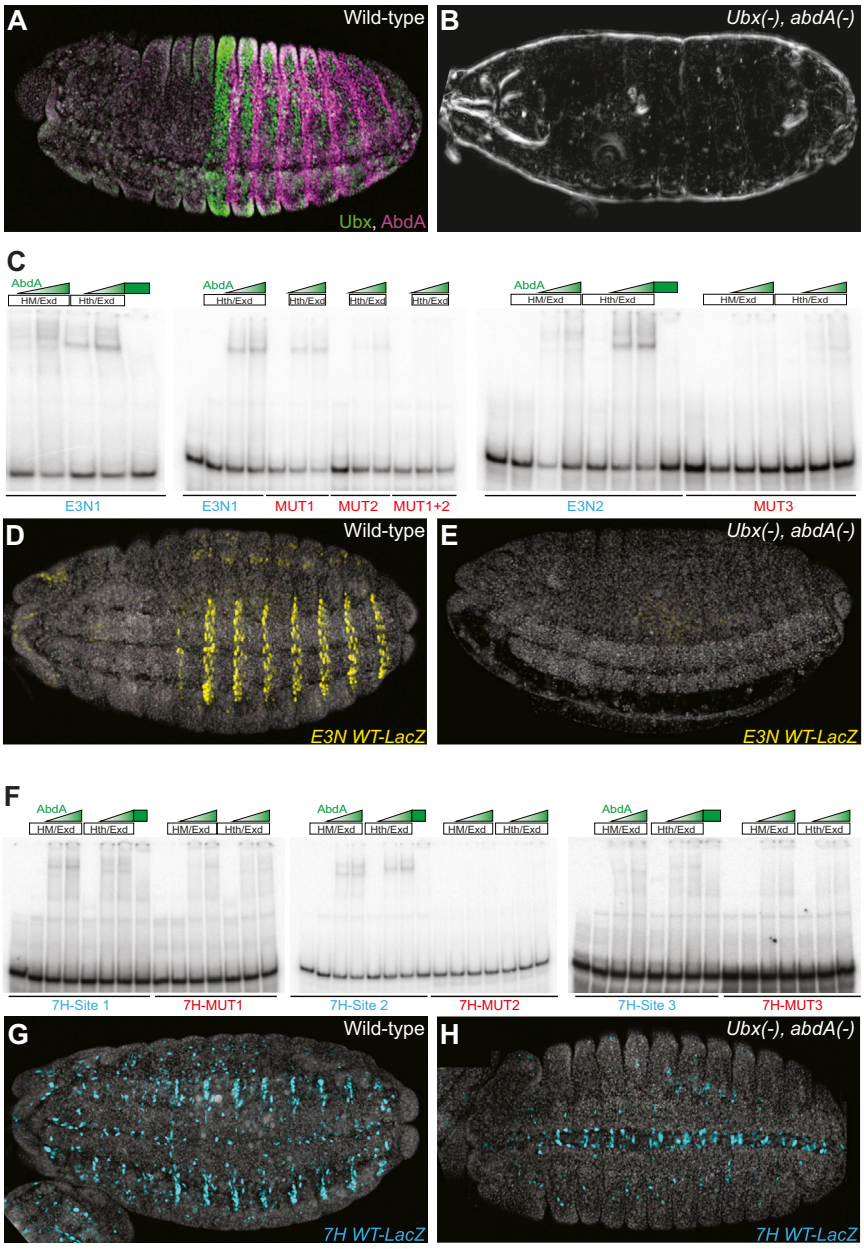

Figure S5. AbdominalA Binds to the Ubx Binding Sites in the *7H* and *E3N* Enhancers, Related to Figure 2 and Tables S1 and S2

(A) Early stage 15 embryos stained with an antibody against AbdA and Ubx.

(B) Larval cuticle prep of the indicated genotype.

(C) AbdA-Hth^{FL}-Exd and AbdA-Hth^{HM}-Exd bound each of the Ubx-Exd sites in the *E3N* enhancer (see Figure 2), as demonstrated with EMSAs. This binding was decreased when the sites were mutated (as per Figure 2).

(D and E) Early stage 15 embryos carrying *E3N*::*lacz* reporter constructs, stained with an antibody against β-Galactosidase, with genotypes indicated.

(F) AbdA-Hth^{FL}-Exd and AbdA-Hth^{HM}-Exd bound each of the Ubx-Exd sites in the *7H* enhancer (see Figure S4), as demonstrated with EMSAs. This binding was decreased when the sites were mutated (as per Figure S4).

(G and H) Early stage 15 embryos carrying *7H*::*lacZ* reporter constructs, stained with an antibody against β-Galactosidase, with genotypes indicated.

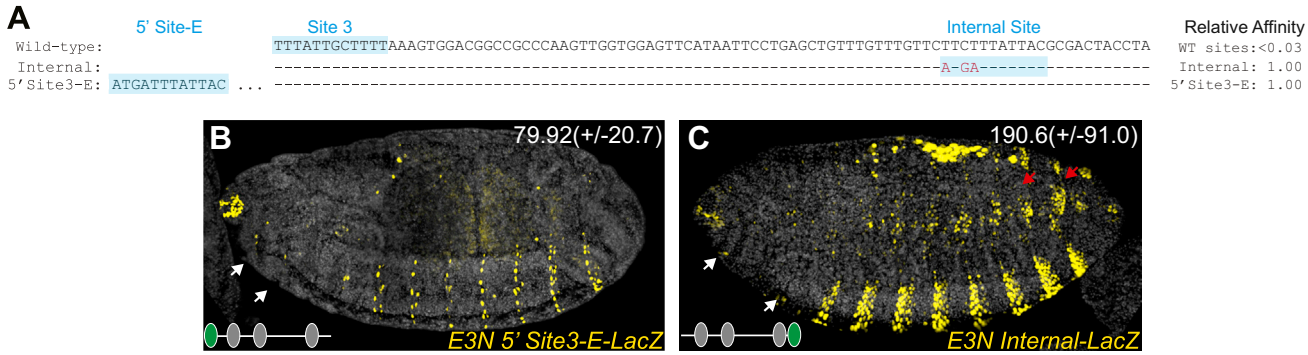

Figure S6. Addition of a High Affinity Site Results in Ectopic Expression, Related to Figure 4 and Table S1

(A) A sequence alignment of the wild-type and mutated versions of the *E3N* region, showing the modified sequences in red.

(B and C) Early stage 16 embryos carrying *E3N*::*lacZ* reporter constructs stained with an antibody against β-Galactosidase, with an ectopic highest-affinity Ubx-Exd site added either 5′ (B) or internally (C) to the *E3N* enhancer. The numbers shown in the top right of each panel indicate the average level of expression in the region outlined in Figure 6I for the corresponding genotypes (n = 10 for each genotype), measured in arbitrary units of fluorescence intensity. Numbers in parantheses indicate ± 1 s.d. White arrows denote expression in domains anterior to segment A1 (A,B). Red arrows indicate ectopic dorsal and lateral expression (B).

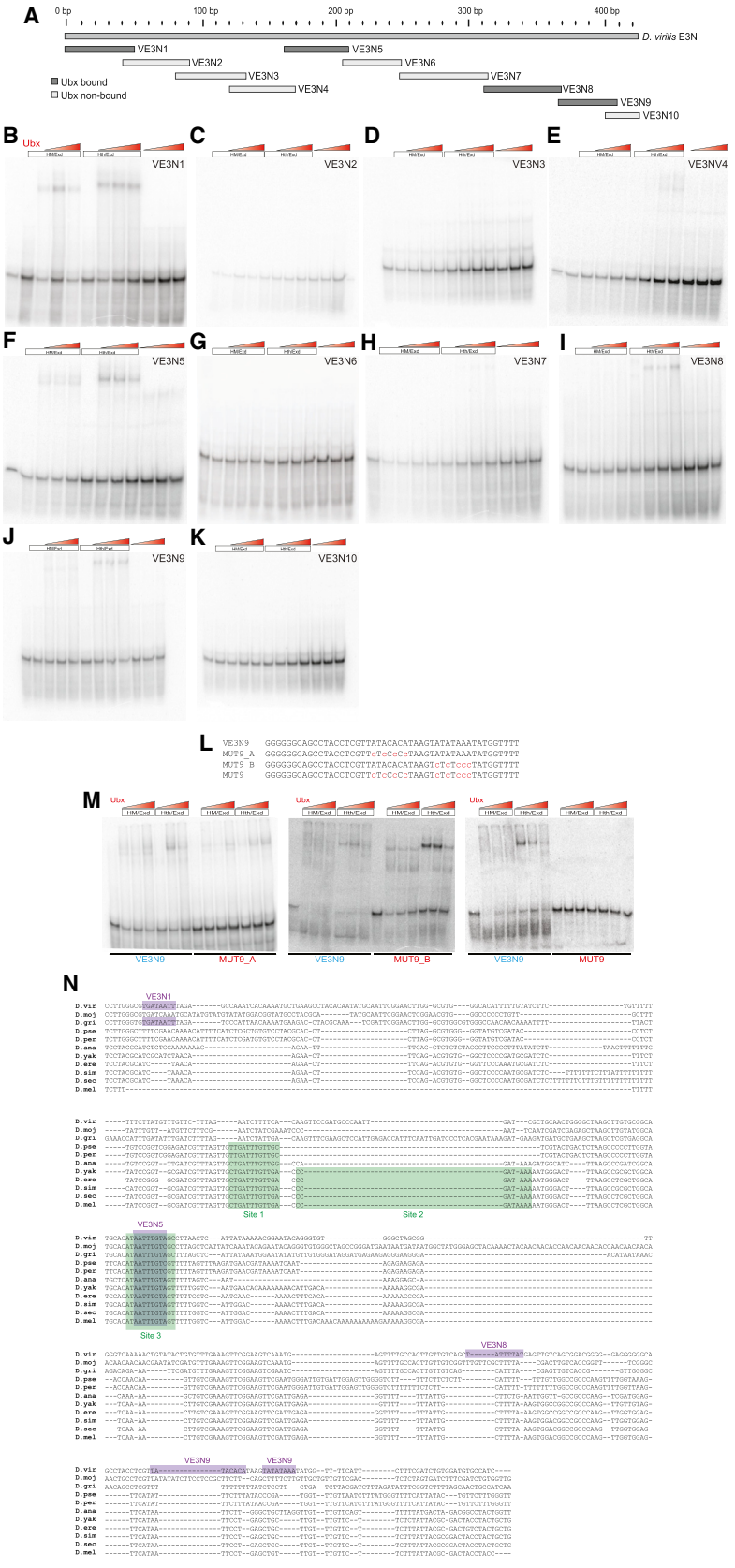

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Figure S7. Identification of Regions of the *D. virilis E3N* Enhancer that Bind Ubx-Exd In Vitro, Related to Figure 7 and Table S2

(A) A schematic of the regions tested for the ability to bind Ubx-Exd, assayed via EMSAs. Filled boxes indicate regions that bound Ubx-Exd.

(B–G) Ubx-Hth^{FL}-Exd and Ubx-Hth^{HM}-Exd bound fragments *VE3N1* (B), *VE3N5* (F), *VE3N*I (I), and *VE3N9* (J), as demonstrated with EMSAs.

(L) A sequence alignment of the wild-type and mutated versions of the *VE3N9* region, showing the mutated putative Ubx-Exd binding sites in red.

(M) EMSAs demonstrated that both putative Ubx-Exd binding sites must be mutated to eliminate binding of Ubx-Hth^{HM}-Exd or Ubx-Hth^{FL}-Exd to *VE3N9*.

(N) DNA sequence alignment of the *E3N* enhancer region. Shaded boxes indicate regions that bound Ubx-Exd in *D. melanogaster* (green) and *D. virilis* (purple).