Electronic Supplementary Material (ESI) for Integrative Biology.

Supplemental Methods

Construct design and transgenic fly line creation

Reconstituted enhancers were designed using footprinted binding sites from RedFly [1] separated by spacer sequences generated by SiteOut [2]. We screened against sites for *bicoid, hunchback, zelda, Krüppel, giant, caudal, tailless, Dichaete, Stat92E, knirps,* and *nubbin*. Enhancer sequences were synthesized commercially and cloned into the pBΦY vector between the NotI and BglII restriction sites using Gibson assembly [3]. The pBΦY vector contains the *even-skipped* basal promoter driving *lacZ*, as well as Amp^R and *mini-white* cassettes and an attB site for site-specific genome integration using the PhiC31 system [4,5]. Constructs were injected into w^{118} flies containing the attP2 integration site using commercial services provided by BestGene. Successful transformants were screened for the presence of *mini-white* and homozygosed.

In situ hybridization and imaging

In situ hybridization was performed as previously described [6]. Briefly, embryos were collected for 0-4 hours at 25˚C and fixed in heptane and 5% paraformaldehyde for 25 minutes. We then incubated the embryos for 2 days with digoxigenin (DIG)-labeled probes for *fushi tarazu* (*ftz*) and dinitrophenol (DNP)-labeled probes for *lacZ* and *huckebein* (*hkb*). *hkb* is expressed in nuclei at the poles of the blastoderm embryo, and *hkb* expression is used as a co-stain to normalize expression levels between different transgenic lines [7]. Probes were then detected following sequential incubation with commercial anti-DIG or anti-DNP antibodies conjugated to horseradish peroxidase from PerkinElmer, as well as a color reaction with coumarin-tyramide or Cy3-tyramide, respectively. Nuclei were labeled using SYTOX Green from Life Technologies, and embryos were mounted in DePex from Electron Microscopy Sciences. Stage 5 embryos were sorted into one of six time classes using the extent of membrane invagination as a morphological marker. Data presented in the main text was taken from the third cohort (9-25% membrane invagination). Three-dimensional images of individual embryos were then obtained using two-photon laser scanning microscopy. Data from embryos that did not contain a hkb co-stain is also available (see Supplemental Data below).

Image processing and analysis

Image stacks from individual embryos were converted into pointcloud files containing the xyz coordinates and expression levels of *ftz* or *lacZ/hkb* for each nucleus [6]. Individual pointclouds were averaged together into a gene expression atlas using the software described in [8]. Normalized *lacZ* levels were calculated for individual embryos using the *hkb* co-staining method [7]. Line traces were plotted using the extractpattern function contained in the PointCloud toolbox (http://bdtnp.lbl.gov/Fly-Net/bioimaging.jsp?w=analysis) which calculates the mean expression level in 100 bins along the anterior-posterior axis for 16 strips around the circumference of the embryo. We used the average of the 4th and 5th strip for our analysis.

Binding site predictions

Binding sites for *caudal* were predicted using a position weight matrix generated by bacterial 1-hybrid methods [9] and the PATSER software using a p-value cutoff of 0.01 [10]. Binding sites were visualized using the inSite software (http://www.cs.utah.edu/~miriah/insite/).

Enhancer Sequences

The following sequences correspond to the wild-type and reconstituted versions of *eve2* that were assayed in reporter constructs. Lowercase letters indicate wild-type sequence, while uppercase letters indicate designed spacer sequences. One base pair in a footprinted *bicoid* site was altered in both reconstituted constructs; this base pair is indicated in bold.

eve2

ggttacccggtactgcataacaatggaacccgaaccgtaactgggacagatcgaaaagctggcctggtttctcgctgtgtgtgccgtgt
taatccgtttgccatcagcgagattattagtcaattgcagttgcagcgtttcgctttcgtcctcgtttcactttcgagttagactttattgcagcat
cttgaacaatcgtcgcagtttggtaacacgctgtgccatactttcatttagacggaatcgagggaccctggactataatcgcacaacga
gaccgggttgcgaagtcagggcattccgccgatctagccatcgccatcttctgcgggcgtttgtttgtttgtttgctgggattagccaaggg
cttgacttggaatccaatcccgatccctagcccgatcccaatcccaatcccaatcccttgtccttttcattagaaagtcataaaaacacat
aataatgatgtcgaagggattagggg

reconstituted eve2 – spacer set 1

ACTGGACAGTttacccggtacCCGACTGCGTTACTATGGCGACTAtaactgggacaCACATCAAACAC
CATctggtttCACGGTCGATACTAAGTgttaatccgttAGATGATGgcgagattattagtcaattgcagttgcTTGAG
ATCTTTTAATTCTGTTGAATGGGATCCTAAACgactttattgcagcatcttgaacaatcgtcgcagtttggtaacac
gctCATTACTTAGACGCACCagacggaatcgagggaccctggactataatcgcTCCGAATTaccgggttgcGGT
ACGTTTCTCATTCTGTGACCGGAGGCCTATGTCTGGTATCATGTAGGAgtttgtttgtttgctgggattag
ccaagggcttgaAGATGGGtccaatcccgatccctagcccgatcccaatcccaatcccaatccctTATTTACTTAGACTg
aaagtcataaaaacacataataTCTTTG**A**cgaagggattaggCCTAACTACA

reconstituted eve2 – spacer set 2

ACTCGTATACttacccggtacAAACTGTGTTGGGGATTTCTCGTAtaactgggacaGGAAACGACGTA
CAGctggtttGACAATGTAAGATAAATgttaatccgttACAACTCCgcgagattattagtcaattgcagttgcGTGCA
TGTTCACTCGCTGTTTATCTATACGAATAGAAgactttattgcagcatcttgaacaatcgtcgcagtttggtaacac
gctAGGGGTTGTGCTGTACTagacggaatcgagggaccctggactataatcgcTAGTCAATaccgggttgcTGA
GCGAAATACACGTTGTCGTCAGTACTGATTTGTACTAGGCATATGCTCgtttgtttgtttgctgggattag
ccaagggcttgaGTGTTGAtccaatcccgatccctagcccgatcccaatcccaatcccaatccctGTTTAGCAGCGAAG
gaaagtcataaaaacacataataGCTAGT**A**cgaagggattaggCATACTACAC

Supplemental Data

Cellular resolution expression data from wild-type and reconstituted *eve2* enhancer reporter constructs is available at figshare.com: https://figshare.com/articles/Cellular_resolution_data_from_wild_type_and_reconstituted_eve_stripe_2_enhancer_reporter_constructs/2948377

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