Boisclair Lachance et al. Supplemental Methods

CRISPR/Cas9-mediated generation of *eve*^{MHEWT} and *eve*^{MHEmut2,3} alleles*:* A 2.1 kb fragment that includes the MHE was amplified from genomic DNA made from *vas-Cas9* flies, subcloned into the HindIII/XbaI sites of pBluescript and confirmed by sequencing. Mutagenesis of the NGG sequence of the two PAM sites and ETS sites 2 and 3 was performed via Quick-change (Stratagene; primers listed in Supplementary material). The upstream PAM site was mutated to add a Fsp1 site and the downstream PAM site was mutated to add a BamH1 site. A 1300bp fragment including the mutated upstream PAM site and the MHE sequence (WT or mut2,3) was amplified using primer pair AB and a 815 bp homology fragment that included the downstream mutated PAM site was amplified with primer pair CD. The transformation marker 3xPax-RFP with PiggyBac (PB) transposase arms was amplified from the pHD-Scarless vector (generated by O'Connor-Giles lab, DGRC, #1364) using primer pair EF; a Kpn1 site introduced at the end of the PB arm was used to confirm orientation. The origin of replication and the Ampicillin resistance gene were isolated from pHD-Scarless as a 2434 bp SnaB1-Nru1 restriction fragment. The four purified fragments PCR AB, the PCR EF, the PCR CD and the digested SnaB1-NruI fragment were assembled according the Gibson assembly tool (NEB, version 1.12.15, see Supplemental Fig. S4) and transformed into XL1-Blue electrocompetent *E. coli*. The eye marker 3XPax-RFP was removed by piggyBac excision by crossing to Wg^{Sp}/ *Cyo,Tub>PBac.*

Crosses: Flies were cultured at 25°C on standard cornmeal molasses agar medium unless otherwise stated.*Twi-Gal4>UAS-GFP* marked 2nd and 3rd chromosome balancers were used to genotype Stage 11 embryos. *pnt* null embryos were from a *pnt*$^{\Delta 88}$*/TTG stock. yan* null embryos were from either a cross of *yan*^{E443}*/CTG X yan*^{E833}*/CTG* or the *yan*^{E443}*/CTG* stock.

For overexpression studies, UAS-*Yan*^{ACT}/+; *twi*-GAL4/TM6B,tubGAL80 or *twi*-GAL4,UAS-PntP1/TM6B,tubGAL80 were crossed to the relevant pJR20 transgenes and UAS-*Yan*^{ACT}/+; *twi*-GAL4/pJR20 or *twi*-GAL4,UAS-PntP1/pJR20 embryos were identified based on the respective reduction or increase in expression of endogenous Eve.

To obtain embryos of the genotypes indicated in Fig. 5 and 6 the following recombinant chromosomes were generated: (1) *Pnt-GFP*, *eve*^{MHEWT} (2) *Pnt*-GFP, *eve*^{MHEmut2,3} (3) *yan*^{E443}, *eve*^{MHEWT} (2) *yan*^{E443}, *eve*^{MHEmut2,3}. *eve*^{MHEWT} and *eve*^{MHEmut2,3} were also crossed to *aos*^{D7}/TTG or *w*^{1118} flies. For temperature stress assays, staged embryos from timed collections were hand-picked, transferred to vials and incubated at either 25 ^{o}C or 30^{o}C until adults eclosed.

Embryo staining: Embryos were harvested from apple juice plates, dechorionated in 50% bleach for 3 minutes at room temperature solution (RT), fixed in a 1:1 mix of 4% formaldehyde:heptane for 20 minutes at RT, wash 3X in methanol, 3X in PBT (1xPBS, 0.1%-TritonX-100), blocked in PNT (1xPBS, 0.1% Triton X-100, 1% normal goat serum, with 0.02% sodium azide), incubated overnight at 4°C with primary antibodies, washed 3x 20 minutes in PBT, incubated in secondary antibody for 2 hours at RT, washed 3x20 minutes in PBT and mounted in 90 µl of 10% 1M Tris pH8.0, 90% glycerol, 0.5% w/v of n-propyl gallate.

Imaging: For imaging, fixed, stained and appropriately oriented Stage 11 embryos were identified by morphology and when necessary, genotyped by lack of GFP expression from the *twist-Gal4,UAS-GFP* marked balancers. Serial 0.45µm slices were taken through GFP or Eve+ clusters and maximum projections generated with the z-planes containing all 10 GFP or Eve+ clusters, respectively. Relative intensity was determined by measuring the average pixel intensity of each individual cluster and the average pixel intensity of a region of the embryo in close proximity to each cluster (background pixel intensity). This background intensity was then subtracted from the average cluster intensity. To compare between conditions, each cluster

measurement was normalized to the average cluster intensity of the relevant control imaged in the same session. For cell counts, Eve positive cells were counted by going through Z-stack projections of the slices where these cells reside.

Gel shift. Briefly, late log phase cultures were induced with 0.8mM IPTG for 2hrs at 18^{o}C. Pelleted cells were resuspended in lysis buffer (100mM Tris pH8.0, 50mM NaCl, 1 mM EDTA, 1mM DTT plus Complete Mini protease inhibitor cocktail (Roche)). Clarified lysates were incubated with Glutathione-Sepharose 4B (GE Healthcare 17075601) for 2 hours at 4°C. Beads were washed 3X with TEV buffer (100mM Tris pH8.0, 50mM NaCl, 1mM DTT), incubated with TEV protease overnight 4°C.

ChIP-qPCR. qPCR was performed using the QuantiTech SYBR Green PCR kit (Qiagen) with primers specific to the D1, D2, MHE *eve* CRMs and to a previously characterized *aos* CRM (Webber et al., Genes and Dev, 2013). A standard curve for each primer pair was generated using serial dilutions of genomic DNA. Occupancy values were calculated by determining the ratio of immunoprecipitated DNA in the respective ChIP sample over that in the input sample (% Input). These values were then normalized to levels of occupancy at a negative control region previously utilized in Webber et al., Genetics, 2013), which was defined as 1.