Supplemental Data

Figure S1.

Clones of homozygous *emc* and *da* mutant cells are labeled by the lack of GFP expression (green). Da and Emc proteins are shown in magenta.

(A) Da expression in emc^{AP6} clones in peripodial epithelium of eye-antennal disc (arrows). (B) Da expression in emc^{AP6} clones in wing imaginal disc (arrowa). Da level in *emc* clones is even higher than in the proneural regions adjacent to the dorsoventral wing margin (arrowhead). (C) Da expression in emc^{AP6} clones in peripodial epithelium of wing disc (arrow). (D) Da expression in emc^{AP6} clones in prothoracic leg discs (arrow). (E) Da expression in emc^{AP6} clones in peripodial epithelium of leg disc (arrow).

(F) Emc expression in da^{10} clones in peripodial epithelium of eye-antennal disc (arrow). (G) Emc expression da^{10} cells in wing imaginal discs (arrow). Emc is normally low in proneural regions adjacent to the dorso-ventral margin (arrowhead). (H) Emc expression in da^{10} clones in peripodial epithelium of wing disc (arrow). (I) Emc expression in da^{10} clones in leg disc (arrows). (J) Emc expression in da^{10} clones in peripodial epithelium of leg disc (arrow).

Genotype: (A-E) *ywhsF;* emc^{AP6} FRT80/ [*UbiGFP*] *M*(3)67*C* FRT80; (F-J) *ywhsF;* da^{10} FRT40/ [*UbiGFP*] FRT40.

Figure S2.

(A) Map of *da* genomic region and da^{+} genomic transgene. Fragments tested for Da-dependent enhancer activity are indicated. (B) Fold activation of the luciferase reporter

for each pGL3-enhancer construct upon co-transfection of pRact-*da*, normalized by fold activation for pGL3 vector alone. Reporter activity was strongly enhanced by Fragment 3. For the data shown, enhancer fragments are in reverse orientation to the promoter to rule out the possibility that *da* has internal transcription start sites (the *da* ORF is entirely within the second exon). When Fragment 3 was cloned in the forward orientation, there was no Da dependent transcription stimulation, suggesting an insulator element at the 3' end of Fragment 3. Just such an insulator is predicted by genome-wide studies of insulator-associated protein binding (Negre et al., 2010; Roy et al., 2010). (C) A *da-Gal4* transgene incorporating the *da* transcription start site and 3.2 kb of 5' DNA extending downstream from the HindIII site in m*Rps7* gene (genomic location 2L: 10386953) (Wodarz et al., 1995) gives variegated expression of the UAS-LacZ reporter (green). Differentiation is marked by Elav expression in magenta. Similar variegation was also seen in our pBPeGFPdaw constructs, which also include the *da* promoter without the 3'-end of the gene (main text Figure 2E-H).

Genotypes: (C) *w; UAS-nuclacZ/+; da-Gal4/+*.

Figure S3.

Mutant clones lack either GFP or β-gal (green). Emc or Da proteins are shown in magenta. Yellow arrowheads indicate position of the morphogenetic furrow and yellow arrows indicate morphogenetic furrow in mutant clones.

(A and B) shn^{1B} clones that cannot respond to Dpp. Emc expression is partially maintained in the furrow and Da does not increase so highly. In addition, either shn^{1B} or Mad^{10} clones expressed Emc at a higher level and Da at a slightly lower level than wild

type cells, suggesting Dpp signaling may also contribute to *emc* and *da* expression anterior to the furrow.

(C and D) In arr^{2} mutant clones that cannot respond to Wg, Emc and Da expression remained normal.

(E and F) In arr^{2} shn^{1B} double mutant cells that cannot respond to either Wg or Dpp, Emc and Da expression remained normal inside or outside of the furrow, as in arr^{2} clones (panels C and D).

(G and H) In smo^{3} arr^{2} shn^{1B} triple mutant clones that cannot respond to Hh, Wg and Dpp, Emc is higher and Da is slightly lower than that in adjacent cells, ahead of the morphogenetic furrow. In smo^{3} arr^{2} shn^{1B} triple mutant cells, pre-furrow levels of Emc and Da are maintained in the furrow, as in smo^{3} Mad^{12} clones (Figure 3D and E).

Genotypes: (A and B) *ywhsF;* FRT42 shn^{1B}/ FRT42 [*UbiGFP*] *M(2)56F*; (C and D) *ywhsF;* FRT42 arr^{2}/ FRT42 [*UbiGFP*] *M(2)56F;* (E and F) *ywhsF;* FRT42 arr^{2} shn^{1B}/ FRT42 [*UbiGFP*] *M(2)56F;* (G and H) *ywhsF;* smo^{3} FRT42 arr^{2} shn^{1B}/ smo^{D16} FRT42 P{smo^{+}} [*UbiGFP*] *M(2)56F.*

Figure S4.

Homozygous mutant clones are marked by the absence of GFP expression (green). Elav expression (blue) labels all neurons. (A) Runt expression (red) marks R8 cells from column 1 onwards and R7 cells from column 8 or 9 onwards (Kaminker et al., 2002). Ahead of the morphogenetic furrow, loss of *emc* leads to ectopic neuronal cells (arrows) that express Elav but not Runt. (B) da^{3} emc^{AP6} double mutant clones lack ectopic neural

differentiation ahead of the furrow (arrowhead) as well as neural differentiation behind the furrow (arrow).

Genotype: (A) *ywhsF; emc*^{AP6} FRT80/ [*UbiGFP*] *M(3)*67*C* FRT80; (B) *ywhsF; da*^{3}*; P{da*$^{+}$*, w*$^{+}$*}*68A4 [*UbiGFP*] FRT80/ *emc*^{AP6} FRT80.

Figure S5.

(A and B) Flip-on clones expressing *emc* from *emc*^{EP3620} line were marked by GFP expression (green). (A) The *sca* gene is expressed in the clusters of 6-10 cells at the anterior of the morphogenetic furrow ('intermediate groups') that elevate Ato, and later in the isolated R8 precursor cells that also express Ato. A lower, uniform level of secreted Sca protein is taken up by other nearby cells (Lee et al., 1996). When *emc* expression was maintained, Sca expression (magenta) was lower, so that the intermediate groups no longer stood out above the background of secreted Sca protein (white arrows), in contrast to the situation outside the clone (yellow arrows).

(B) Spalt-major (Salm) is a transcription factor expressed in R7 cells (white arrowhead) and in non-neuronal cone cells (white arrow) (Domingos et al., 2004). Maintaining Emc expression delayed onset of Salm expression (red) in R7 cells and cone cells by 2 columns (yellow arrowhead and arrow, respectively). Salm is also present transiently in R3 and R4 cells, closer to the furrow (Domingos et al., 2004).

(C) Prothoracic leg disc expressing UAS-GFP under the control of en-Gal4. Emc protein (red); Da protein (blue).

(D, E) Posterior views of the adult prothoracic femur (D) and tibia (E). The posterior compartments bear 40 ± 1.6 and 31.9 ± 1.0 sensory bristles, respectively (n = 16).

(F) Prothoracic leg disc from *en-Gal4 UAS-GFP emc*^{EP3620} at 23°C. Emc protein (red); Da protein (blue). Note that Emc and Da levels are not noticeably changed in the posterior compartment, except that SOP's are reduced.

(G, H) Posterior views of the adult prothoracic femur (G) and tibia (H) from *en-Gal4 UAS-GFP emc*^{EP3620} at 23°C. The posterior compartments bear only 17.7 ± 2.3 bristles on the femur (G) and 4.8 ±1.4 bristles on the tibia (H). N = 24.

(I) Clones over expressing Da are marked by GFP expression (green). Da overexpression occasionally led to ectopic neuronal differentiation (Elav: magenta) ahead of the furrow (yellow arrow).

(J) Clones ectopically expressing Sc and GFP (green) in the notum region of the late 3rd instar wing disc. No Elav-expressing neurons are seen (magenta).

(K) Clones over-expressing Da and GFP likewise lack ectopic neural differentiation in the notum (Elav: magenta).

(L) Clones co-expressing GFP, Sc and Da induced ectopic neural differentiation in the notum (Elav: magenta). Sens expression in the developing macrochaetae was used to identify wing discs of comparable developmental stage for panels (J-L) (not shown).

Genotypes: (A-B) *ywhsF; emc*^{EP3620}*/ act>CD2>Gal4, UAS-GFP*; (C-E) *en-Gal4, UAS-GFP/+*; (F-H) *en-Gal4, UAS-GFP/+*; *emc*^{EP3620}*/+*; (I and K) *ywhsF; UAS-da/+; act>CD2>Gal4, UAS-GFP/+*; (J) *ywhsF; UAS-sc/+; act>CD2>Gal4, UAS-GFP/+*; (L) *ywhsF; UAS-da/ UAS-sc; act>CD2>Gal4, UAS-GFP/+*.

Supplemental Experimental Procedure

Drosophila husbandry and strains

All flies were raised at 25°C except otherwise where specified. For non-Minute genotypes, larvae were subjected to 1 hour heat shock at 37°C, 60 ± 12 hr after egg laying, to induce FLP-mediated mitotic recombination. For Minute genotypes, heat shock was 84 ± 12 hours after egg laying. The MARCM technique was used to obtain *da* over-expressing clones in either *M*/+ or +/+ backgrounds (Lee and Luo, 1999). 'Flip-on' clones were generated by applying heat shock for 30 minutes at 37°C after 60 ± 12 hours of egg laying. Larvae were usually dissected ~72 hours after heat shock. Flies were raised at 29°C when generating flip-on clones expressing *emc* from emc^{EP3620} or MARCM clones over-expressing *da*, and at 23°C when expressing *emc* from emc^{EP3620} using *en-Gal4*.

The following transgenic and mutant strains were used: w^{1118}; emc^{AP6} (Ellis, 1994); da^{10} (Caudy et al., 1988); da^{2} and da^{3} (Cronmiller and Cline, 1987); *act>CD2>Gal4, UAS-GFP* (Pignoni and Zipursky, 1997; Neufeld et al., 1998); [*Ubi-GFP*] *M(3)*67*C* FRT80 (Janody et al., 2004); UAS-*da* (Hinz et al., 1994); smo^{3} (Chen and Struhl, 1998); shn^{1B} (Arora et al., 1995); top^{CO} (Price et al., 1989); mam^{10} (Lehmann, 1983); Mad^{10} (Sekelsky et al., 1995); Mad^{12} (Raftery et al., 1995; Sekelsky et al., 1995); arr^{2} (Wehrli et al., 2000); ato^{1} (Jarman et al., 1994); emc^{EP3620} (Abdelilah-Seyfried et al., 2000); [*arm-LacZ*] FRT80 (Vincent et al., 1994); UAS-*ato* (Jarman et al., 1994); UAS-*sc* (Parras et al.,

1996); *da-Gal4* (Wodarz et al., 1995); [*Ubi-GFP*] FRT40; FRT82 [*tub-Gal80*] (Lee and Luo, 1999); *GMR-Gal4* (Freeman, 1996).

Construction of *da*$^{+}$ genomic rescue transgenes on chromosomes 1 and 2

The *da*$^{+}$ transgene was designed based on previous transgenes that no longer exist (Brand and Campos-Ortega, 1990; Smith and Cronmiller, 2001). An 8.8kb genomic region from the EcoRI site in *cdc2* gene to the KpnI site in the *CG5362* gene was sub-cloned from cosmid clone 100A9 (Siden-Kiamos et al., 1990) into pW8 (Klemenz et al., 1987).

DNA for injection was prepared using QIAfilter Plasmid Midi Kit (Qiagen) and sent to Model System Genomics, Duke University for generation of transgenic flies. Independent transgenic lines were obtained by P-element integration into 1st and 2nd chromosome. One copy of either transgene was able to rescue da^{10}/da^{3} or da^{3}/da^{3} flies to normal fertile adults.

Antibodies

Antibody labeling were performed as described previously (Firth et al., 2006; Bhattacharya and Baker, 2009). Primary antibodies used were: mouse anti-βGal (mAb40-1a) (1:100), rat anti-ELAV (7E8A10) (1:50), mouse anti-ELAV (9F8A9) (1:100), mouse anti-Ro (1:25), all from DSHB, mouse anti-Da (1:200) (Cronmiller and Cummings, 1993), rabbit anti-Emc (1:8000) (a gift from Y.N. Jan) (Brown et al., 1995), guinea pig anti-Sens (1:500) (Nolo et al., 2000), rabbit anti-Ato (1:50000) (Jarman et al., 1994),

rabbit anti-Salm (1:50) (Kuhnlein et al., 1994); mouse anti-Sca (Rd2) (1:50) (Lee et al., 1996), guinea pig anti-Runt (1:1500) (Duffy et al., 1991), anti-GFP (1:500) (Invitrogen). Secondary antibodies used were multi-labeling antibodies from Jackson ImmunoResearch Laboratories.

Quantitative RT-PCR (qPCR)

Total RNA was isolated either from 3rd instar eye imaginal discs, from which antennal discs had been removed, or from HEK293T cells 48hr after transfection, using TriZol (Invitrogen) per the manufacturer's protocol, followed by RQ1-DNase (Promega) treatment. Total cDNA was synthesized using oligo$(dT)_{20}$ primer and SuperScript III First-Strand Synthesis System, according to the Invitrogen protocol. qPCR was performed using gene specific primers and SYBR Green PCR Master Mix (Applied Biosystems) either in a SmartCycler System (Cepheid) for *Drosophila* RNA or in a 7900HT FAST Real-Time PCR System (Applied Biosystems) for human RNA. Melting curves were analyzed for purity of the product. For *Drosophila* eye discs, *gal4* transcript was used as an internal control to normalize RNA template levels. For 293T cells, *act*β transcript was used as an internal control for normalization.

Primers used against the first exon of *da* to specifically amplify the endogenous transcript:

Forward primer 5' GTGGCTCAACGTCAACACTC 3'

Reverse primer 5' TACAAGTGCATCGGCTCATC 3'

Primers used to amplify *emc* transcript:

Forward primer 5' ACCAACTCAGCAGCACACAC 3'

Reverse primer 5’ TGCCTGGAAATGAACTTTCTC 3’

Primers used to amplify *gal4* transcript:

Forward primer 5’ ACCAATTGCCTCCTCTAACG 3’

Reverse primer 5’ GTCCAACCAGGTGACAGTGG 3’

Primers used to amplify *act*β transcript (Wang et al., 2008):

Forward primer 5’ CCCAGCCATGTACGTTGCTA 3’

Reverse primer 5’ TCACCGGAGTCCATCACGAT 3’

Primers used to amplify *E2a* transcript:

Forward primer 5’ CACCAGCACGAGCGTATG 3’

Reverse primer 5’ GAGAAGGAGGATGCAGATGG 3’

Primers used to amplify *HEB* transcript (PrimerBank):

Forward primer 5’ CAACCACACTGCCAGGAAC 3’

Reverse primer 5’ GCCTACTGATAACTCTGGAACTGG 3’

Primers used to amplify *E2-2* transcript:

Forward primer 5’ TGAGAACCTGCAAGACACG 3’

Reverse primer 5’ GGTGTCAGGTCCTCATCGTC 3’

Primers used to amplify *Id1* transcript (PrimerBank):

Forward primer 5’ CTGCTCTACGACATGAACGG 3’

Reverse primer 5’ GAAGGTCCCTGATGTAGTCGAT 3’

Primers used to amplify *Id2* transcript (PrimerBank):

Forward primer 5’ GACCCGATGAGCCTGCTATAC 3’

Reverse primer 5’ AATAGTGGGATGCGAGTCCAG 3’

Primers used to amplify *Id3* transcript (PrimerBank):

Forward primer 5’ CATCGACTACATTCTCGACCTG 3’

Reverse primer 5’ TCCTTTTGTCGTTGGAGATGAC 3’

Primers used to amplify *Id4* transcript (PrimerBank):

Forward primer 5’ TCCCGCCCAACAAGAAAGTC 3’

Reverse primer 5’ CCAGGATGTAGTCGATAACGTG 3’

Analysis of *da* enhancers in vivo

pBPeGFPdaw (Figure 2D, main text) was made from pBPGUw (Pfeiffer et al., 2008). The *Gal4-hsp70* 3’ UTR cassette of pBPGUw was replaced by *eGFP-NLS-SV40* 3’ UTR from the pStinger vector (Barolo et al., 2000), using 5’ *KpnI* and 3’ *SpeI* sites to get pBPeGFPUw. To replace the *Drosophila* synthetic core promoter (Pfeiffer et al., 2008) with 542bp of *da* proximal promoter region, genomic DNA (2L: 10387806-10388342) was PCR amplified from the da^{+} genomic rescue plasmid using Platinum Taq DNA polymerase high fidelity (Invitrogen), cloned into PCR 2.1- TOPO (Invitrogen), and confirmed by sequencing. The *da* promoter fragment was recovered by EcoRI digestion and cloned by blunt end ligation into pBPeGFPUw digested with *FseI* and *KpnI* after repair with Klenow to get pBPeGFPdaw. Fragment 3 (Figure 2D), obtained by *BglII* digestion of cosmid 100A9 (Siden-Kiamos et al., 1990), was repaired using Platinum Taq DNA polymerase high fidelity (Invitrogen), cloned into pCP8/GW/TOPO (Invitrogen), and confirmed by sequencing. Fragment 3 was transferred into pBPeGFPdaw in the reverse orientation using the in-vitro LR reaction using Gateway LR Clonase II Enzyme Mix (Invitrogen) to replace the cell lethal *ccd*B gene positioned within Gateway cloning sites. To make the *da* promoter only construct, i.e pBPeGFPdaw vector without the *ccd*B

gene, Fragment 3 was removed from the pCP8/GW/TOPO clone by *EcoRI* digestion before the LR reaction. DNA for injection was prepared using QIAfilter Plasmid Midi Kit (Qiagen). DNA constructs were microinjected into *yw* {*nos-PhiC31 integ,* y^+}*/Y;* {y^+*attP2}* (Bischof et al., 2007) embryos by Model System Genomics (Duke University). Single males derived from these embryos were crossed to *w; Sb/ TM6B* females and individual F1 males with w^+ eye color selected. This step removed the integrase source. Individual males were crossed to *w; Sb/ TM6B* females to establish balanced stocks.

Cell culture, transient transfection, luciferase and β−galectosidase assays

Drosophila Schneider S2 cells were cultured at 25°C in Schneider's Drosophila Medium (GIBCO) supplemented with 10% heat inactivated fetal bovine serum and Penicillin-Streptomycin (Invitrogen). Transient transfection of approx. 1.8 X 10^5 cells/0.7 ml in 24 well plates were performed using Effectene Transfection Reagent (Qiagen), according to the manufacturer's protocol. All plasmids were purified using QIAprep Spin Plasmid Miniprep Kit (Qiagen), according to the manufacturer's protocol. *da* was over-expressed by adding 10 ng of pRact-*da*. 25 ng of *hs-lacZ* construct was added per well for normalization of transfection efficiency. Total amount of DNA added to each well was adjusted to 200 ng using empty pRactHAdh (Swevers et al., 1996) vector. Luciferase activities were assyed using Luciferase Assay System (Promega), according to the manufacturer's protocol. Luminescence was measured using a Turner TD-20/20 luminometer.

HEK 293T cells were cultured at 37°C in DME medium supplemented with 10% fetal bovine serum, Penicillin-Streptomycin (Invitrogen), 1% Pyruvate, 1% L-Glutamate and 0.1% β-Mercaptoethanol. Transient transfection of approx. 6 X 10^6 cells in 10-cm plates was performed using Lipofectamine2000 reagent (Invitrogen), according to the manufacturer's protocol. All plasmids were purified using QIAfilter Plasmid Midi Kit (Qiagen), according to the manufacturer's protocol. Human E47 was over-expressed by transfecting 300 ng of pcDNA3-hE47 (Jen et al., 1992). 1.5 μg of pMX-GFP construct was added to monitor transfection efficiency, which was typically 70-80%. The total amount of DNA added to each plate was adjusted to 10 μg using empty pcDNA3 vector (Invitrogen).

Cloning of *da* enhancer fragments into pGL3-Promoter vector

Coordinates of fragments cloned into pGL3-promoter vector (Promega):

Fragment 1: From 5' HincII (at genome location 10386986) to 3' HincII site (at genome location 10388342).

Fragment 2: From 5' SalI (at genome location 10388340) to 3' BglII site (at genome location 10390048).

Fragment 3: From 5' BglII (at genome location 10390048) to 3' BglII site (at genome location 10392573).

Fragment 4: From 5' XhoI (at genome location 10391544) to 3' NheI site (at genome location 10393810).

All genomic locations mentioned are according to release=r5.32.

Standard cloning methods were used to clone all above fragments in both orientations at multiple cloning site of pGL3-Promoter vector (Promega) upstream of SV40 minimal promoter.

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