supplemental material for:

Embryonic *even skipped*-dependent muscle and heart cell fates are required for normal adult activity, heart function, and lifespan

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Materials and Methods

Transgenes and antibodies

The complete *eve* transgenes that are capable of rescuing all aspects of normal function in *eve* null mutants[1], the eme-deficient *eve* rescue constructs and the eme-GAL4 construct[2], the transgenes carrying modified version of Eve[3], the *UAS-τLacZ* transgene[4] used in combination with *eme-Gal4* to mark cells that normally express *eve* in the mesodermal lineages, the reporter UAS construct used to test the ability of Gal4-Eve to actively repress transcription in the mesoderm (eme-UAS-*lacZ*)[5], and the transgenes used to label the *eve*-expressing neurons (RP2 and aCC) that innervate DA1 and DO1 (RN2-Gal4 and UAS-GFP)[5] were all described previously. The eme-Gal4-Eve construct used to express the Gal4-Eve fusion protein in the mesoderm consists of two tandem copies of the eme fragment (+5.7 to +6.6 kb relative to the *eve* transcription start site) upstream of the same *eve* promoter-Gal4-Eve coding region fragment described previously[5]. Transgenic lines were established as described previously[6,7].

Antibodies used were anti-β-galactosidase (β-gal, 1:1000, ICN), anti-GFP (1:200) (Roche), anti-Eve[8] (1:10,000), anti-Krüppel[9] (Kr, 1:500), and anti-fasciclin II 1D4[10] (1:10). Antibody staining was visualized using horseradish peroxidase and DAB[11], either with or without Ni. In situ hybridization was performed as described previously[12].

Evx expression

RT-PCR for *Evx1* or *Evx2* was performed using RNA isolated from 12.5dpc embryonic mouse heart, an established rat epicardial cell line (EMC), and chicken

epicardial explants, with gene- and species-specific primers. Wilm's Tumor (*WT1*) gene expression served as an epicardial-specific marker[13,14], and *Nkx2.5* expression as a myocardial-specific marker. Epicardial Explant Assay: primary chick epicardial cell monolayers from HH21 embryonic chick hearts were grown on three-dimensional gels containing 1% rat tail collagen type I (BD Biosciences), as previously described[15]. After 72 hours, epicardial cells from 10 different explants were pooled and used for RNA extraction using TriZol Reagent (Invitrogen). The hearts from the explants devoid of epicardial cells were also recovered and RNA was extracted.

Pericardial cell counts

Third instar larvae were dissected in AHL (adult hemolymph) media[16] to expose hearts, then stained for 15 seconds with Neutral Red for visualization of pericardial (PC) cells. Larval hearts were observed and photographed on a stereo dissecting scope at 20x magnification. The total number of PC cells was averaged over the number of larval segments counted.

Heart rate measurement

Transparent white pre-pupae were viewed with an inverted compound microscope on a temperature-controlled stage set at 25^{o}C, while adults were viewed on a stereoscope with a temperature-controlled stage set at 25^{o}C. Each animal was allowed 2 min. to acclimate before counting. Since heart contractions are externally visible without dissection, the number of heart beats in a 15 second period was determined and charted as beats/second for pupae and Hz (cycles/second) for adults.

Heartbeat for each individual was counted five times with a 15 second interval, with the average of the five reported as the individual value.

Cardiac stress-induced failure rate measurement

Adult fly hearts were paced to a heart rate of 6 Hz for 30 seconds via an external current using a square wave stimulator, then following cessation of treatment, the percentage of flies undergoing cardiac arrest or fibrillation was charted[17,18].

Flight assay and lifespan measurement

For flight assays, flies were gently tapped into a 72 inch-long tube lined with flypaper. The flypaper was divided into numbered quadrants, with the highest quadrant 4, the lowest 1. Flies that fell through the tube without getting stuck on the flypaper were scored as 0. The average score for the total sample of each genotype was reported as the flight index.

For lifespan measurements, 250 male flies of each genotype were divided into groups of 15 flies each and given fresh food every other day. The number of dead flies was recorded each time fresh food was supplied. Flies were fed on standard yeast glucose food and raised at 25^{o}C.

Results

Other functional deficits

Since flies do not depend on circulation for acute oxygen supply, it is possible for flies with drastically compromised heart function to live and reproduce. However,

normal circulation may be needed for distribution of hormones and other physiologically important factors[19-21]. Indeed, *eve* meso$^-$ flies are visibly sluggish, and reproduce slowly and inefficiently (data not shown). To quantify this perceived lack of vigor, we assayed meso$^-$ flies for their ability to engage in the high-energy activity of flying. *eve* wild-type rescued flies behaved identically to wild-type flies in a simple flight assay (Fig. 5C). However, heterozygous *eve* meso$^-$ flies scored 25% lower than wild-type, the same as flies carrying the *Cy* mutation, which causes curled wings and thus impedes flying ability (Fig. 5C and data not shown). The two *eve* meso$^-$ lines tested each scored about 50% lower than wild-type flies and other controls (Fig. 5C), and lower than *Cy* flies, which are considered almost flightless[22]. This suggests that they suffer from more than just a deficiency in the anatomical flight apparatus.

References

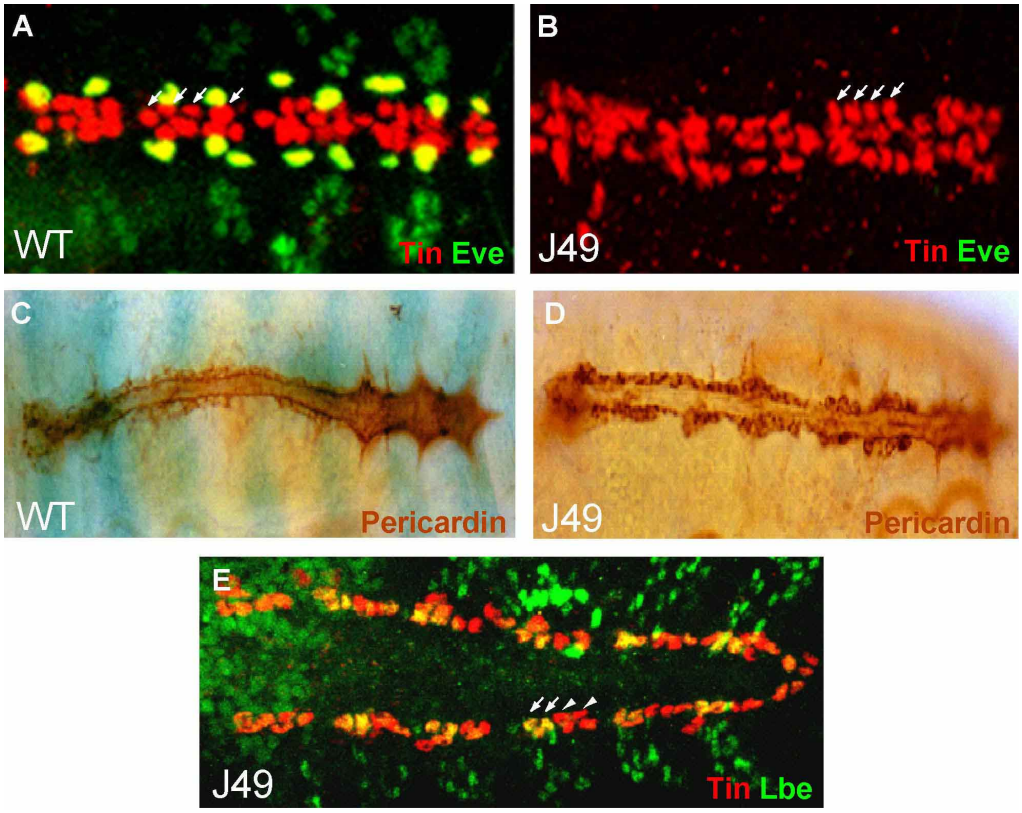
1. Fujioka M, Emi-Sarker Y, Yusibova GL, Goto T, Jaynes JB. Analysis of an *even-skipped* rescue transgene reveals both composite and discrete neuronal and early blastoderm enhancers, and multi-stripe positioning by gap gene repressor gradients. *Development* 1999;126:2527-2538.
2. Han Z, Fujioka M, Su M, Liu M, Jaynes JB, Bodmer R. Transcriptional integration of competence modulated by mutual repression generates cell-type specificity within the cardiogenic mesoderm. *Dev. Biol.* 2002;252:225-240.
3. Fujioka M, Yusibova GL, Patel NH, Brown SJ, Jaynes JB. The repressor activity of Even-skipped is highly conserved, and is sufficient to activate *engrailed* and to regulate both the spacing and stability of parasegment boundaries. *Development* 2002;129:4411-4421.
4. Hidalgo A, Urban J, Brand AH. Targeted ablation of glia disrupts axon tract formation in the *Drosophila* CNS. *Development* 1995;121:3703-3712.
5. Fujioka M, Lear BC, Landgraf M, Yusibova GL, Zhou J, Riley KM, Patel NH, Jaynes JB. Even-skipped, acting as a repressor, regulates axonal projections in Drosophila. *Development* 2003;130:5385-5400.
6. Fujioka M, Jaynes JB, Bejsovec A, Weir M. Production of transgenic *Drosophila*. *Methods Mol. Biol.* 2000;136:353-363.
7. Rubin GM, Spradling AC. Genetic transformation of *Drosophila* with transposable element vectors. *Science* 1982;218:348-353.
8. Frasch M, Hoey T, Rushlow C, Doyle H, Levine M. Characterization and localization of the even-skipped protein of *Drosophila*. *EMBO J.* 1987;6:749-759.

9. Kosman D, Small S, Reinitz J. Rapid preparation of a panel of polyclonal antibodies to *Drosophila* segmentation proteins. *Dev. Genes Evol.* 1998;208:290-294.
10. Vactor DV, Sink H, Fambrough D, Tsoo R, Goodman CS. Genes that control neuromuscular specificity in *Drosophila*. *Cell* 1993;73:1137-1153.
11. Patel NH. Imaging neuronal subsets and other cell types in whole mount *Drosophila* embryos and larvae using antibody probes. In: (Goldstein LSB, Fyrberg E, eds.) *Methods Cell Biol.* New York: Academic Press; 1994:445-487.
12. Tautz D, Pfeifle C. A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. *Chromosoma* 1989;98:81-85.
13. Perez-Pomares JM, Phelps A, Sedmerova M, Carmona R, Gonzalez-Iriarte M, Munoz-Chapuli R, Wessels A. Experimental studies on the spatiotemporal expression of WT1 and RALDH2 in the embryonic avian heart: a model for the regulation of myocardial and valvuloseptal development by epicardially derived cells (EPDCs). *Dev. Biol.* 2002;247:307-326.
14. Moore AW, McInnes L, Kreidberg J, Hastie ND, Schedl A. YAC complementation shows a requirement for Wt1 in the development of epicardium, adrenal gland and throughout nephrogenesis. *Development* 1999;126:1845-1857.
15. Dettman RW, Denetclaw W, Jr., Ordahl CP, Bristow J. Common epicardial origin of coronary vascular smooth muscle, perivascular fibroblasts, and intermyocardial fibroblasts in the avian heart. *Dev. Biol.* 1998;193:169-181.
16. Wang J, Zugates CT, Liang IH, Lee CH, Lee T. *Drosophila* Dscam is required for divergent segregation of sister branches and suppresses ectopic bifurcation of axons. *Neuron* 2002;33:559-571.

17. Wessells RJ, Bodmer R. Screening assays for heart function mutants in *Drosophila*. *Biotechniques* 2004;37:58-64.
18. Paternostro G, Vignola C, Bartsch DU, Omens JH, McCulloch AD, Reed JC. Age-associated cardiac dysfunction in *Drosophila melanogaster*. *Circ. Res.* 2001;88:1053-1058.
19. Rizki T. The circulatory system and associated cells and tissues. In: (Ashburner M, Wright TRF, eds.) *The Genetics and Biology of Drosophila*. London: Academic Press; 1978:1839-1845.
20. Wiggelsworth V. Principles of Insect Physiology. New York: Methuen; 1965.
21. Hertel W, Pass G. An evolutionary treatment of the morphology and physiology of circulatory organs in insects. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 2002;133:555-575.
22. Chiarodo A, Reing CM, Jr., Saranchak H. On neurogenetic relations in *Drosophila melanogaster*. *J. Exp. Zool.* 1971;178:325-329.
23. Yarnitzky T, Volk T. Laminin is required for heart, somatic muscles, and gut development in the *Drosophila* embryo. *Dev. Biol.* 1995;169:609-618.

Supplemental Figure 1: (A, B) Tinman-positive myocardial cells form normally in *eve* meso$^-$ embryos. Stage 16 wild-type (A) and mutant (B) embryos stained with antibodies against Tinman (red) and Eve protein (green). Note the absence of Eve in the mutant and the regular arrangement of four Tinman-positive myocardial cells (white arrows) in each hemisegment of both wild-type and mutant embryos. (C, D) Pericardial cells marked by the secreted collagen protein Pericardin (Mab 3^{23}) align similarly along the myocardial cells in heterozygous (C, blue stripes indicate presence of balancer chromosome) and homozygous *eve* meso$^-$ embryos (D). E) Tinman (red) and Lbe (green) myocardial cells are normally patterned in stage 15 *eve* meso$^-$ embryos: two Lbe/Tinman double-labeled cells (in yellow marked by arrows) are located anteriorly to two Tinman-only labeled cells (in red marked by arrowheads) in each hemisegment.

Supplemental Fig. 1



Supplemental Fig. 2. Eve acts as a repressor in the mesoderm. The genetic situation that applies to C-E is illustrated in A: The *J49 eve* meso$^-$ rescue transgene is present (two copies) in an *eve* null background, along with a complete rescue transgene driving expression of an altered Eve protein. B-E) 3rd instar larvae dissected to reveal the dorsal musculature (anterior to the left, dorsal up): B) *eve* null mutant carrying two copies of a complete rescue transgene (86K); C, D) the genetic situation shown in A, with only the Eve HD (with its highly conserved immediate flanking residues) expressed in the mesoderm (EveH); E) as in C, D, but with the Eve HD region fused with the En repressor domain (EnRD). Note that in B, the normal arrangement of muscles is present (compare to Fig. 3A), while in C, DA1 is missing; in D, DA1 is missing and there is an extra muscle adjacent to DO2, which is positioned somewhat abnormally, as is DO1, and in E, the normal pattern is restored. The red and green bars beside each micrograph in B-E mark the edges of the dorsal acute (DA) and dorsal oblique (DO) muscles, respectively, and the diagrams below illustrate the positions of muscles in the DA and DO focal planes in the same colors. The embryo shown in G carries a transgene, diagrammed in F (upper half) in which the eme enhancer drives GFP expression, and which also contains a Gal4 binding site (UAS). Note reporter expression in the mesodermal *eve* pattern in G (stage 11; anti-GFP immunostaining). The embryo in H carries a transgene in which a Gal4-Eve fusion protein containing the Gal4 DNA binding domain and the Eve repressor regions is driven by eme (F, lower half) in addition to the reporter transgene present in G. Note the essentially complete repression of the reporter (compare H to G).

Supplemental Fig. 2