Expanded View Figures

**Figure EV1.**
Figure EV1. *sub* is required in ISCs/EBs and sub loss does not affect differentiation.

A Expression of E6 enhancer in the posterior midgut, as monitored by X-Gal staining of E6-LacZ reporter line.
B ISCs midguts expressing YFP alone (control), or expressing sub-RNAi. Samples were stained for YFP (yellow) and DAPI (blue); the quantification is shown in Fig 1E.
C *Su(H)* midguts expressing GFP alone (control), or expressing sub-RNAi. Samples were stained for GFP (green), Prospero (red), and DAPI (blue). The graph shows quantification of the number of Prospero-positive cells (EEs). See also Fig 1E.
D Voilà midguts expressing GFP alone (control), or expressing sub-RNAi. Samples were stained for GFP (green), Prospero (red), and DAPI (blue); lower panels show separate channels. The graph displays quantification of the average number of Prospero-positive cells (EEs) in control conditions, or upon sub-RNAi treatment.
E Schematic representation of the RedDM lineage tracing system (Antonello et al, 2015) in which esg drives expression of both mCD8::GFP (green) and H2B::RFP (red). esg cells are labeled by cytoplasmic GFP and nuclear RFP, while cells of their differentiated progeny only maintain the very stable H2B-RFP. Pictures show posterior midguts in control conditions, or upon expression of sub-RNAi. Samples were stained for GFP (green), RFP (red), and DAPI (blue). For each genotype, merge picture is shown at left and the red channel at right. Bottom panels show magnified views; arrow show differentiated cells (RFP-positive/GFP-negative). Graphs show quantification of the number of GFP-positive (precursors) and the percentage of GFP-negative RFP-positive (differentiated) cells.

Data information: Boxes extend from the 25th to 75th percentiles, whiskers from min to max, the line in each box is plotted at the median; data were collected from three independent replicates. P values from Mann–Whitney tests are ns > 0.05, **** < 0.0001. Scale bars are 20 µm.

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Figure EV2. Pri and Ubr3 are required for the maintenance of progenitor cells.

A ISCs midguts expressing YFP alone (control), or expressing pri-RNAi. Samples were stained for YFP (yellow) and DAPI (blue); quantification is shown in Fig 1E.
B esg midguts expressing GFP alone (control), or expressing two RNAi lines that target non-overlapping regions of the Ecdysone receptor (EcR) mRNA. Samples were stained for GFP (green), Prospero (red), and DAPI (blue); quantification is shown in Fig 2E.
C ISCs midguts expressing YFP alone (control), or expressing UAS-pri, EcR-DN, and EcR-DN+ pri. Samples were stained for YFP (green), Prospero (red), and DAPI (blue). The graph shows quantification of the number of YFP-positive cells for each genotype.
D ISCs midguts expressing YFP alone (control), or expressing Ubr3-RNAi, and Ubr3-RNAi+ OvoB. Samples were stained for YFP (yellow) and DAPI (blue). The graph shows quantification of the number of YFP-positive cells for each genotype.

Data information: Boxes extend from the 25th to 75th percentiles, whiskers from min to max, the line in each box is plotted at the median; data were collected from three independent replicates. P values from one-way ANOVA are: ns > 0.05, * < 0.05, *** < 0.001. Graphs are drawn with a log (10) Y scale. Scale bars are 20 µm.
Figure EV3. Svb acts as a transcriptional activator in ISC/EB cells.

A, A’ esg<sup>a</sup> midguts expressing GFP alone (control), or expressing OvoB, and Svb<sup>ACT</sup> + pri. Samples were stained for GFP (green) and DAPI (blue). The graph (A’) plots the number of GFP-positive cells in each genotype.

B esg<sup>a</sup> midguts expressing GFP alone (control) or expressing Svb<sup>ACT</sup>. Samples were stained for GFP (green) and DAPI (blue).

C ISC<sup>a</sup> midguts expressing YFP alone (control), or expressing OvoB, and Svb<sup>ACT</sup> + pri. Samples were stained for YFP (yellow) and DAPI (blue). The graph shows quantification of the number of YFP-positive cells in ISC<sup>a</sup> midguts expressing YFP alone (control), or expressing OvoB, Svb<sup>ACT</sup> + pri, and pri.

D Snapshot view of ChIPseq signal in embryonic cells (Menoret et al, 2013), showing in vivo binding of Svb on the singed (sn) locus that encodes Fascin. The snE<sup>1</sup> enhancer (purple) contains two Svb-binding sites and is directly activated by Svb<sup>ACT</sup>. Pictures at right show expression in the posterior midgut of wild-type snE<sup>1</sup> (snE<sup>1</sup>-wt), and a variant of it that contains mutation of the two Svb-binding sites (snE<sup>1</sup>-Svb-mt). Samples were stained for β-Gal activity (cyan blue).

Data information: Boxes extend from the 25<sup>th</sup> to 75<sup>th</sup> percentiles, whiskers from min to max, the horizontal line is plotted at the median; data were collected from three independent replicates. P values from one-way ANOVA are ns > 0.05, ** < 0.01, *** < 0.001, **** < 0.0001. Scale bars are 20 µm.
Identification of transcription factors required for the activity of E3N and 9CJ2 sub enhancers in the embryo.

A. Drawing of the E3N sub enhancer, with position of putative binding sites for Pnt (red) and TCF (green) factors, and evolution of DNA sequences across Drosophila species. Nucleotides in red represent point mutations introduced to disrupt either Pnt- or TCF-binding sites.

B. Consequences of knocking out Pnt- or TCF-binding sites on expression of the E3N sub enhancer in the embryonic epidermis. Pictures show ventral views of stage-15 embryos. Scale bar is 50 µm.

C. Trichome rescue assays (Crocker et al., 2015) showing the influence of TCF-binding sites on E3N function. Picture show cuticle preparations of wild-type and sub-mutant embryos, focusing on the ventral region of A6 segments. sub mutants display strong reduction in the number of trichomes, remaining ones being highly abnormal. Consistent with its expression pattern, E3N driving sub cDNA (E3N-wt::svb) rescues formation of the anterior-most trichome row (arrow). Knocking out TCF-binding sites (E3N-TCF-mt::svb) disrupts rescuing ability of the E3N enhancer. The graph plots the number of trichomes in the anterior-most row. Boxes extend from the 25th to 75th percentiles, whiskers from min to max, the horizontal line in each box is plotted at the median; data were collected from three independent replicates. P values from one-way ANOVA are ns > 0.5, **** < 0.0001. Scale bar is 15 µm.

D. Drawing of the 9CJ2 sub enhancer, with position of putative binding sites for Pdm-1 (orange) and evolutionary conservation of the DNA sequence. Nucleotides in red show mutations that have been introduced to disrupt Pdm-binding sites.

E. Consequences of knocking down Pdm-1-binding sites on expression of the 9CJ2 sub enhancer in the embryonic epidermis. Pictures show lateral (left), ventral (middle), and dorsal (right) views of stage-15 embryos. Scale bar are 50 µm.

Data information: Dmel, Drosophila melanogaster; Dsim, Drosophila simulans; Dyac, Drosophila yacuba; Dere, Drosophila erecta; Dfic Drosophila ficusphila; Dtak, Drosophila takahashii; Dana, Drosophila ananassae.
Figure EV4.
Figure EV5. Svb acts as a transcriptional repressor in differentiated enterocytes.

A  MyoIAts midguts expressing GFP alone (control), or expressing svb-RNAi and SvbREP. Samples were stained for GFP (green) and DAPI (blue). Lower panel shows staining for cleaved DCP1 (red). Scale bar is 20 µm.

B  MyoIAts midguts expressing GFP alone (control), or expressing Ubr3-RNAI and SvbREP. Samples were stained for GFP (green) and DAPI (blue). Scale bar is 20 µm.

C  Cross sections of control MyoIAts midguts (expressing GFP and mCherry-RNAI, top row), or SvbREP (bottom row). Samples were stained for F-actin (white), DAPI (blue), and Coracle (red), Tsp2a (yellow) or Scribble (red). Scale bar is 5 µm.