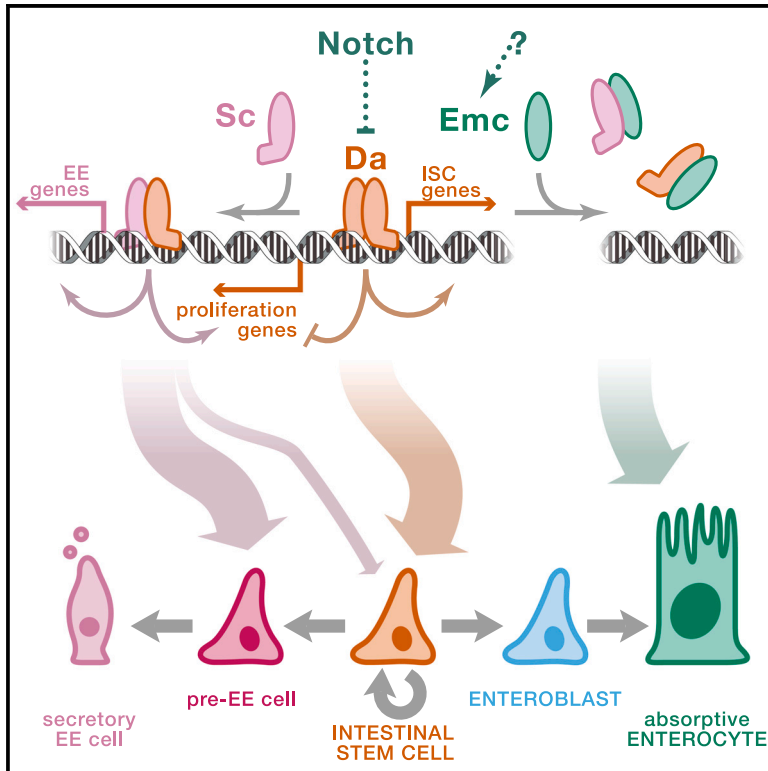


# A bHLH interaction code controls bipotential differentiation and self-renewal in the *Drosophila* gut

## Graphical abstract



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## In brief

Whether tissue stem cells balance self-renewal with multipotent differentiation through successive binary steps or a multiple-option choice is unclear. Puig-Barbe and colleagues find a dimerization network of bHLH factors in the *Drosophila* intestine that provides unified regulation to the choice between stem, absorptive, and secretory fates.

## Highlights

- A network of bHLH factors regulates stemness and bipotent differentiation in the fly gut
- A careful balance between Da, Sc, and Emc determines the fate of intestinal progenitors
- Scute promotes intestinal stem cell fate as well as enteroendocrine differentiation



## Article

# A bHLH interaction code controls bipotential differentiation and self-renewal in the *Drosophila* gut

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<sup>10</sup>J.d.N. dedicates this work to the memory of Juan Modolell and Rosa María Aguilar.

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## SUMMARY

Multipotent adult stem cells balance self-renewal with differentiation into various cell types. How this balance is regulated at the transcriptional level is poorly understood. Here, we show that a network of basic helix-loop-helix (bHLH) transcription factors controls both stemness and bipotential differentiation in the *Drosophila* adult intestine. We find that homodimers of Daughterless (Da), a homolog of mammalian E proteins, maintain self-renewal of intestinal stem cells (ISCs), antagonizing the enteroendocrine fate promoted by heterodimers of Da and Scute (Sc; homolog of ASCL). The HLH factor Extramacrochaetae (Emc; homologous to Id proteins) promotes absorptive differentiation by titrating Da and Sc. Emc prevents the committed absorptive progenitor from dedifferentiating, underscoring the plasticity of these cells. Switching physical interaction partners in this way enables the active maintenance of stemness while priming stem cells for differentiation along two alternative fates. Such regulatory logic is likely operative in other bipotent stem cell systems.

## INTRODUCTION

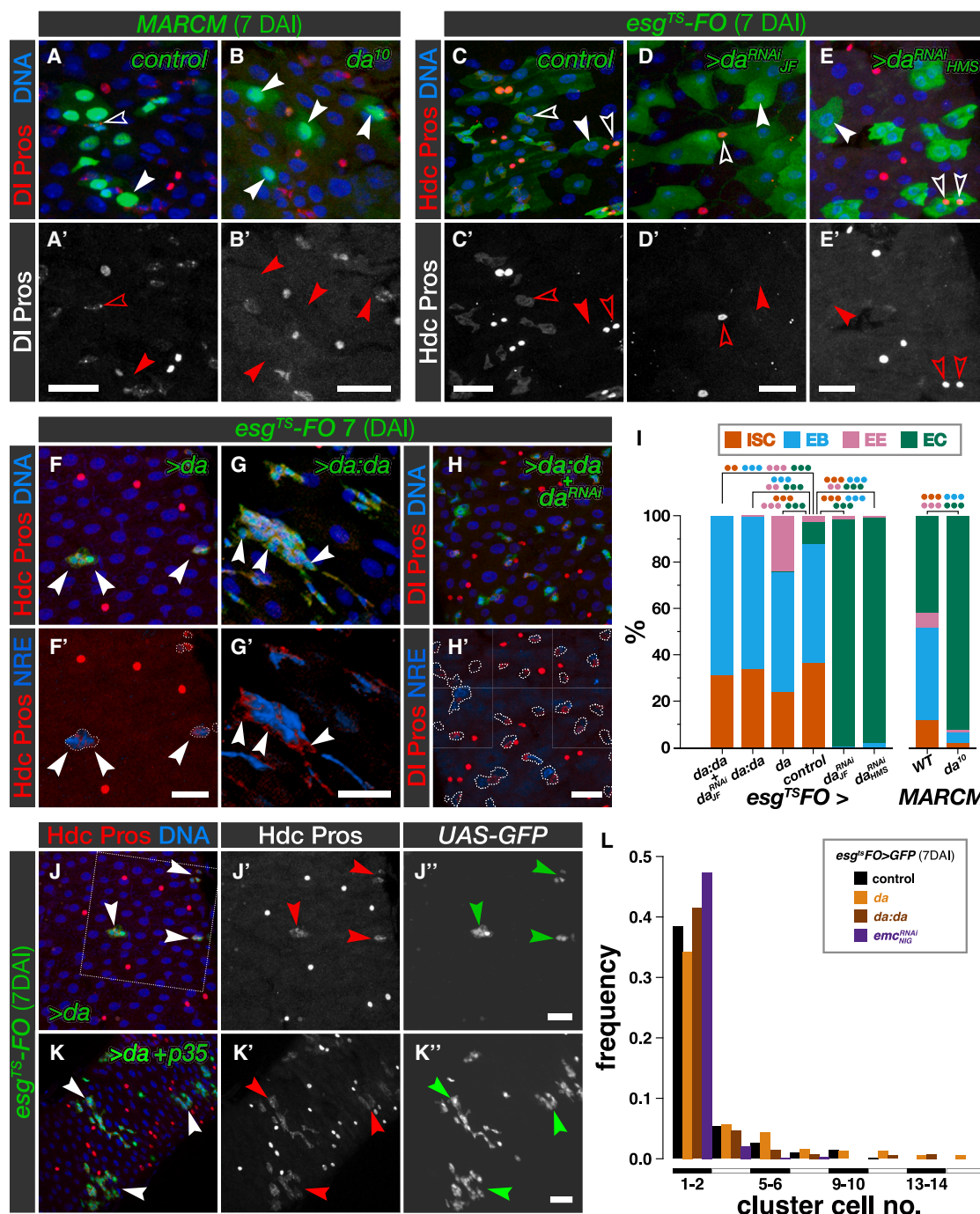
The regulation of stem cell fate decisions hinges on transcriptional control by sequence-specific transcription factors (TFs) forming gene regulatory networks that steer cells along particular differentiation trajectories.<sup>1–3</sup> These trajectories are often considered a succession of binary steps regulated by cross-antagonism between TF pairs.<sup>1,4</sup> However, active multipotent stem cells need to decide between the maintenance of their stem identity and several options of commitment into distinct mature cell fates. To understand how multipotent stem cells make these choices, knowledge of the functional interactions between TFs is essential.<sup>5,6</sup>

Intestinal stem cells (ISCs) are a paradigm of multipotency in adult tissues. ISCs face a choice between self-renewal and differentiation into either the secretory or absorptive cell lineage.<sup>7–9</sup> The intestinal secretory lineage in *Drosophila* consists of enteroendocrine cells (EEs).<sup>10,11</sup> Absorptive cells are called enterocytes (ECs) and differ in morphology and function along the ante-

rior-posterior axis of the gut.<sup>12,13</sup> In *Drosophila*, ISCs produce lineage-specific precursors through distinct molecular triggers. High Notch signaling induces the formation of enteroblasts (EBs), which will give rise to ECs. Expression in ISCs of the bHLH (basic helix-loop-helix) TFs Scute (Sc) and Asense (Ase), members of the *achaete-scute complex* (AS-C; homologs of ASCL mammalian genes), induces the formation of EE precursors (pre-EEs), which quickly turn into EEs.<sup>14–16</sup> It is not clear whether, after division of an ISC, its daughter cells first commit to differentiation before choosing a lineage (two consecutive binary decisions), are already lineage primed before they lose self-renewing ability (binary decisions in inverse order), or choose at the same time between self-renewal and two potential lineages to commit to (single triple decision).<sup>17,18</sup> Little is known about the molecular mechanisms that could allow a triple decision between self-renewal and bipotential differentiation.

The bHLH family of TFs control cell fate in multiple developmental contexts.<sup>19–21</sup> Their HLH motif mediates dimerization, while the preceding region, rich in basic amino acids, allows





(legend continued on next page)

DNA binding.<sup>22</sup> Class I bHLH factors comprise proteins such as E47, E2-2, and HEB (E proteins, encoded by *TCF3/4/12* in mammals; *daughterless* [*da*] in *Drosophila*). Class I bHLH proteins can make dimers within their class (e.g., Da:Da or E47:HEB) but can also heterodimerize with class II bHLH factors. By contrast, class II bHLH factors, such as MYOD or ASCL (MyoD and proteins encoded in the AS-C in *Drosophila*), usually form *trans*-activating complexes only in heterodimers with class I factors.<sup>21</sup> This has been specifically demonstrated for Sc and the other bHLH factors encoded in the AS-C.<sup>23</sup> By contrast, class V bHLH factors are inhibitory: they lack the stretch of basic amino acids preceding the HLH domain, rendering their heterodimers with class I and II unable to bind DNA.<sup>21</sup> Therefore, their mammalian members are named *Inhibitors of DNA binding* (Id proteins). Their only representative in *Drosophila* is *extra macrochaetae* (*emc*); Emc can dimerize with either Da or Sc.<sup>24,25</sup>

Class II bHLH factors regulate differentiation in the metazoan intestine.<sup>9,26</sup> In *Drosophila*, Sc and Ase can initiate EE differentiation,<sup>14,15,27,28</sup> while other bHLH factors maintain EE function (Dimmed, homolog of NeuroD),<sup>29</sup> or promote their functional diversity (Tap, homolog of Neurogenin-3).<sup>9,26</sup> On the other hand, Da is required for ISC maintenance, since ISCs mutant for *da* differentiate.<sup>14</sup> However, the interaction partners of Da to maintain stemness are not known, and how different bHLH factors dimerize to control differentiation has not been explored. Here, we identify the Da homodimer as the critical bHLH complex maintaining ISC self-renewal and find a role of Emc in titrating Da and Sc to promote absorptive differentiation. We show that Da:Da and Da:Sc dimers functionally cooperate to promote ISC fate but act antagonistically for EE differentiation. Our results reveal a network of bHLH factors that forms a three-way switch to regulate self-renewal and bipotential differentiation in the adult fly gut.

## RESULTS

### Da homodimers maintain stemness and prevent differentiation

We quantified the effect of *da* on differentiation in individual null *da*<sup>10</sup> clones using Mosaic Analysis with a Repressible Cell Marker (MARCM; Figures S1A–S1B)<sup>30</sup> and in the entire ISC/EB population by RNAi-mediated knockdown of *da* followed by lineage tracing using the *escargot* Flip-Out approach (“*esg*<sup>TS-FO</sup>”; Figure S1C).<sup>31</sup> Loss of *da* led to almost complete differentiation of cells into ECs, and occasionally into EEs, in *da*<sup>10</sup> mutant clones (Figures 1A–1B, and 1I; Tables S1 and S2) and *esg*<sup>+</sup> cells expressing *da*<sup>RNAi</sup> (Figures 1C–1E and 1I; cell type markers as in Figure S1D). By contrast, in ISCs and EBs that overexpressed *da* with *esg*<sup>TS-FO</sup>, differentiation into ECs was almost completely impaired, while EE differentiation increased (Figures 1F and 1I). Importantly, only a few *esg*<sup>+</sup> cell nests overexpressing *da* sur-

vived, and this was rescued by co-expression of apoptosis inhibitor p35 (Figures 1J and 1K); this further reduced the overall levels of differentiation and seemed to favor the formation of EBs, a few of which became ECs (Figure S1E). While the cell composition of *esg*<sup>TS-FO</sup> > *da* tissue was very different from the wild type, the size of GFP<sup>+</sup> clusters expressing *da* was like that in the controls: most remained below 5 cells, and a few became considerably larger (Figure 1L).

We sought to determine the identity of the Da partners involved in preventing EC differentiation. Since Da can form homodimers to control differentiation and proliferation,<sup>32</sup> we overexpressed forced Da homodimers using a *da:da* tethered construct<sup>33</sup> with *esg*<sup>TS-FO</sup> to test their capacity to block differentiation. The resulting GFP<sup>+</sup> tissue comprised mostly ISCs and EBs, which distributed in clusters of similar size to the wild type (Figures 1G–1I and 1L). To test that Da homodimers were enough to maintain self-renewal without other Da-containing complexes, we expressed Da:Da while removing endogenous Da with the *da*<sup>RNAi</sup> transgene *P{TRIIP.JF02488}*, which does not target the *da:da* construct (Figure S1F). This prevented differentiation entirely (all cells were either ISCs or EBs; Figures 1H and 1I). In addition, we detected no *esg*<sup>+</sup> cell death when the tethered *da:da* construct was overexpressed (compare Figures 1G and 1H with Figure 1J). Together, these results show that Da homodimers promote stemness; additionally, Da probably participates in another complex that induces *esg*<sup>+</sup> cell death.

### Da:Sc and Da:Da antagonize each other in secretory differentiation

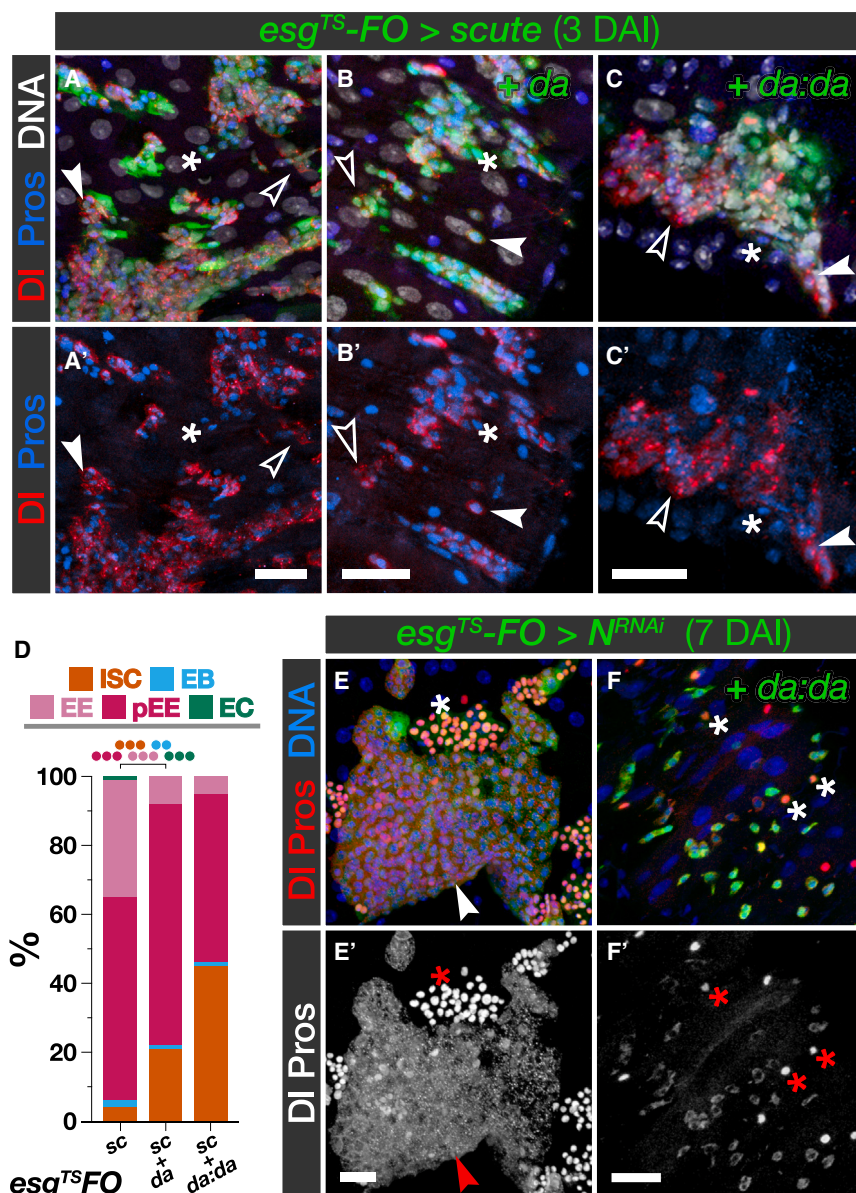
Our results so far indicate that EE differentiation requires the transition from the transcriptional program of Da:Da to that of Da:Sc. This could occur through a “switch,” with Da:Sc targets being epistatic over those of Da:Da, or “antagonism,” whereby the relative strengths of the two programs determine the cell fate. To distinguish between these alternatives, we compared the effects of overexpressing *sc* with *esg*<sup>TS-FO</sup> with those of co-expressing *da* and *sc*. Overexpression of *sc* alone leads to the induction of Pros-positive, DI-negative and DI/Pros-double-positive cells (Figures 2A and S2A), as expected.<sup>14,15</sup> This was likely mediated by Da:Sc, as the co-expression of *sc* and *da*<sup>RNAi</sup> strongly diminished the induction of all Pros-positive cells (Figures S2A–S2C). Many of these cells exhibited the mitotic marker phospho-histone H3S10 (PH3) (Figure S2D) and were probably trapped in a pre-EE state.<sup>15</sup> The co-expression of *da* and *sc* greatly reduced the number of Pros-positive, DI-negative (EE) cells and led to an increase in all DI-positive cells (Figures 2B and 2D) while maintaining mitotic figures (Figures S2E and S2G). Since endogenous *da* is expressed weakly,<sup>14</sup> the overexpression of *da* and *sc* will likely produce more Da:Sc dimers than that of *sc* alone. Therefore, the suppression of the *sc* phenotype by *da+sc* must be due to a higher Da:Da-to-Da:Sc ratio.

(J and K) Expression of *da* with *esg*<sup>TS-FO</sup> (J) results in *esg*<sup>+</sup> cell death, with a few survivors that cannot differentiate; co-expression of *p35* (K) rescues cell death but not differentiation. (J) is a wider field of view than the tissue shown in (F). Arrows: *esg*<sup>+</sup> Hdc<sup>+</sup> ISCs/EBs. Quantified in Figure S1E.

(L) Histograms of GFP<sup>+</sup> cluster sizes for *esg*<sup>TS-FO</sup> driving expression of *da*, *da:da*, *emc*<sup>RNAi</sup>, and their controls. The x axis is truncated at 16 cells, but there were larger clusters in all conditions, except *emc*<sup>RNAi</sup>, at frequencies of 0.3% or lower.

DAI, days after induction. Scale bars: 20 μm. *p* values (binomial regression for individual cell types): ● *p* < 0.05, ●● *p* < 0.01, and ●●● *p* < 0.001. See Tables S1 and S2 for statistical details.





**Figure 2. Da:Sc and Da:Da antagonize each other in EE differentiation**

(A–C) Overexpression of *sc* with *esg<sup>TS</sup>-FO* leads to a dramatic increase of pre-EEs and EEs but maintains a population of ISCs (A). The ISC fraction progressively increases by co-expression of *da* (B) and tethered *da:da* (C) at the cost of EE differentiation (B and C) and pre-EE formation (C). (D) Cell composition of GFP<sup>+</sup> tissue from (A)–(C). (E and F) Knockdown of *Notch* with *esg<sup>TS</sup>-FO* results in excess of EEs and ISCs (E); simultaneous overexpression of *da:da* rescues both phenotypes (F). Solid and empty arrowheads: pre-EEs and ISCs, respectively; asterisks: EEs. (E) is reproduced in Figure 7A to aid comparison. DAI, days after induction. Scale bars: 20  $\mu$ m. *p* values (binomial regression):  $\bullet p < 0.05$ ,  $\bullet\bullet p < 0.01$ , and  $\bullet\bullet\bullet p < 0.001$ . See Tables S1 and S2 for statistical details.

(Figures S2D–S2G) and the formation of DI-positive, Pros-negative ISCs (Figures 2A–2D).

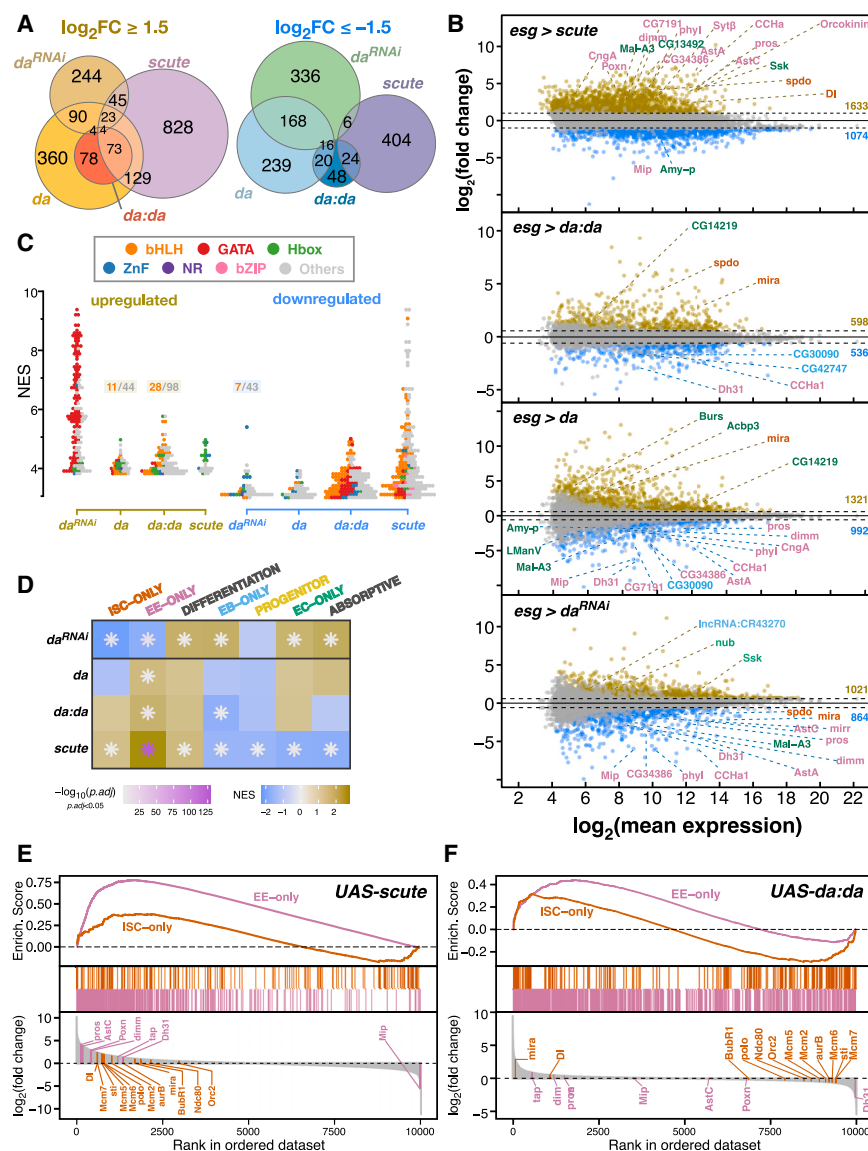
### Both Sc and Da can impart ISC molecular and cellular properties

To understand how Da:Da and Da:Sc activate antagonistic transcriptional programs, we performed mRNA sequencing (mRNA-seq) analysis of purified *esg-Gal4* UAS-GFP ISCs and EBs that overexpressed *da<sup>RNAi</sup>*, *da*, *da:da*, or *sc*. All conditions gave distinct transcriptional signatures (Figures 3A and S3A; Table S3). Interestingly,  $\sim 1/3$  of the genes downregulated upon *da<sup>RNAi</sup>* expression were common to those downregulated upon *da* overexpression (Figure 3A). To explore these transcriptional signatures further, we looked at 57 cell type marker genes for ISCs, EBs, posterior midgut ECs (pECs), and EEs (Table S4) and found that both loss and gain of Da down-

regulate EE-specific genes (Figures 3B and S3C). This may reflect that Da homodimers prevent EE differentiation (Figures 1G–1I and 2B–2D) while Da:Sc dimers induce it<sup>34</sup> (Figures S2A–S2C). Consistent with this, the overexpression of *sc* strongly induced EE-specific genes; it also increased expression of the ISC-specific genes *spdo* and *DI*. Overexpression of *da:da* or *da* alone induced ISC-specific genes (*mira* and *spdo*), while most genes expressed in the absorptive lineage (*myo31DF*, *nub/pdm1*,  $\alpha$ Try,  $\beta$ Try, *E(spl)m $\beta$ -HLH*, *E(spl)m3-HLH*, and other *E(spl)* genes outside the 57-gene panel) were upregulated in *da<sup>RNAi</sup>* overexpression and downregulated in the other conditions (Figures 3B, S3B, and S3C). Thus, the transcriptome analysis supports our histofluorescence observations.

Loss of Notch leads to the formation of masses of Pros-positive, EE-like cells<sup>11,14</sup> (Figure 2E). We further tested whether Da homodimers antagonize EEs differentiation by co-expressing *da:da* and *Notch<sup>RNAi</sup>* with *esg<sup>TS</sup>-FO* and found that Pros-positive cells appeared isolated and in low numbers (Figure 2F). Thus, Da:Da and Da:Sc oppose each other in EE differentiation; intriguingly, they seem to collaborate to induce proliferation

To determine whether the regulated genes were potential direct targets, we scored predicted regulatory elements close



**Figure 3. Da and Sc cooperate to induce the ISC transcriptional signature**

(A) Euler diagram with the sizes of gene sets differentially expressed (at  $|\log_2(\text{fold change})| \geq 1.5$ ) and their approximate intersections upon  $da^{RNAi}$ ,  $da:da$ ,  $da$ , and  $sc$  overexpression.

(B) MA plots for  $esg^{TS} > sc$ ,  $da:da$ ,  $da$ , and  $da^{RNAi}$ , respectively. Cell type markers are shown in colors matching Figure 11.

(C) Normalized enrichment scores (NES) of DNA motifs found in differentially expressed genes ( $|\log_2(FC)| \geq 1.5$ ) in the four conditions analyzed. Dots represent individual motifs and are colored by the transcription factor family that binds them. Some swarms show their bHLH motif fraction.

(D) NES heatmap for cell-type-specific gene sets; colored asterisks indicate significance.

(E and F) Enrichment plots of the transcriptional profiles induced by  $sc$  (E) or  $da:da$  (F) for ISC- and EE-specific genes.  $sc$  induces a clear EE signature. Some ISC genes involved in replication (*Orc2* and *Mcm2*, -5, -6, and -7) and mitosis (*BubR1*, *polo*, *aurB*, *sti*, and *Ndc80*) are repressed by  $da:da$  but activated by  $sc$ .

$da:da$  overexpression (Figure 3D). We looked at individual genes within the regulated, ISC-specific genes. While overexpression of either  $da:da$  or  $sc$  induced regulatory genes such as *DI* or *mira*, they had opposite effects on genes encoding factors involved in DNA replication (*Orc2*, *Mcm2*, *Mcm5*, *Mcm6*, and *Mcm7*) or mitosis (*polo*, *aurB*, *BubR1*, and *Ndc80*): these were repressed by  $da:da$  but induced by  $sc$  (Figures 3E and 3F). This highlights the capacity of  $sc$  to regulate key ISC-specific genes.

We also found differences in broad functional annotations between the overexpression of  $da^{RNAi}$  and that of  $da$ ,

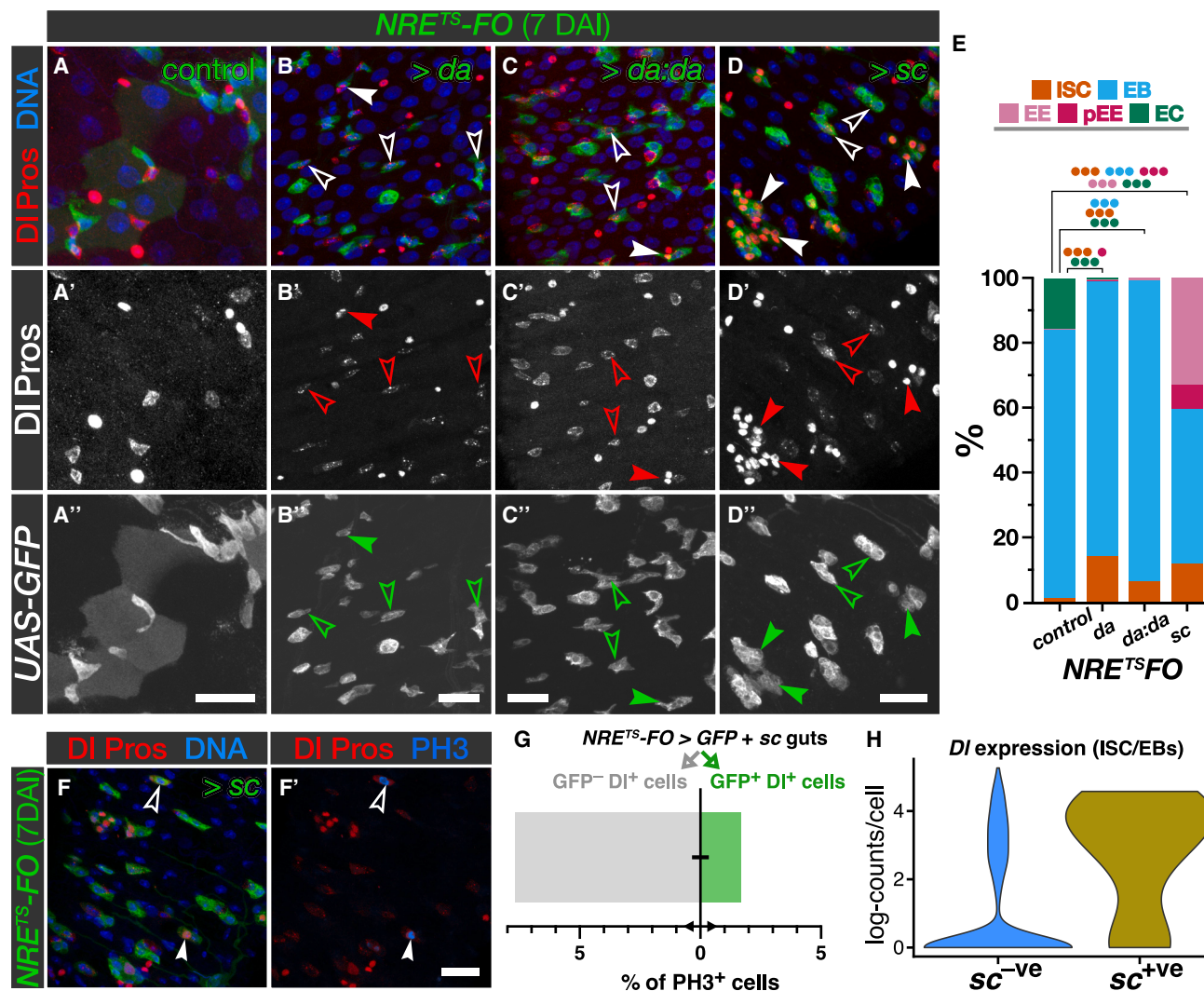
to differentially expressed genes for TF binding motifs. We found E-boxes and other bHLH binding sites over-represented in up-regulated genes upon  $da$  or  $da:da$  overexpression and down-regulated ones upon  $da^{RNAi}$  overexpression (Figure 3C; Table S5). We did not find enrichment in bHLH binding motifs around genes up-regulated by overexpression of  $sc$ , which was unexpected. However, the transcriptional profile induced by  $sc$  corresponds mostly to differentiated EEs (Figures 3C and 3D); as  $sc$  is only expressed transiently to induce EE differentiation, the transcription profile we obtained may be dominated by indirect targets—not necessarily under the control of bHLH factors.

Gene set enrichment analysis<sup>35</sup> (GSEA) against lists of genes significantly expressed in the cell types of the midgut epithelium<sup>36</sup> showed that  $da^{RNAi}$  induced genes expressed in the absorptive lineage while reducing the expression of ISC- and EE-specific genes. Meanwhile,  $sc$  overexpression did the opposite, leading to stronger expression of ISC-specific genes than

$da:da$ , or  $sc$ . While loss of Da induces genes involved in metabolism, biosynthesis, and energy storage and consumption, Da, Da:Da, and Sc reduced the expression signatures of these processes and favored signaling and regulatory genes (Figures S3D and S3E). We conclude that Da:Da and Da:Sc induce distinct signatures that promote the ISC and EE identities, respectively, and repress the active metabolism typical of EC function. However, Da:Da represses ISC-specific genes involved in replication and mitosis, which are up-regulated by Da:Sc.

The transcriptional effects of  $da:da$  and  $sc$  overexpression on ISC-specific genes prompted us to evaluate the capacity of  $sc$ ,  $da$ , or  $da:da$  to impose ISC properties on more differentiated cells. We targeted EBs, which are lineage committed and postmitotic, using the driver *NRE<sup>TS</sup>-FO* (Figure S4A). Wild-type EBs labeled with *NRE<sup>TS</sup>-FO* either remained undifferentiated or became ECs; negligible numbers expressed DI or Pros (Figures 4A and 4E). Driving the expression of  $sc$ ,  $da$ , or  $da:da$  with *NRE<sup>TS</sup>-FO*





**Figure 4. Da:Sc and Da:Da can impose ISC properties on EBs**

(A–D) Expression of *da* (B), *da:da* (C), or *sc* (D) with *NRE<sup>TS</sup>-FO* (see Figure S4A) blocks normal EC differentiation (compare with A) and promotes re-expression of DI (empty arrowheads) and EE differentiation (solid arrowheads). EB-derived EEs occur occasionally upon *da* or *da:da* overexpression but are very frequent upon *sc* overexpression, which also induces the formation of pre-EEs.

(E) Cell composition of GFP<sup>+</sup> tissue from (A)–(D).

(F) Overexpression of *sc* with *NRE<sup>TS</sup>-FO* shows both DI<sup>+</sup> and Pros<sup>+</sup> cells (empty and solid arrowheads, respectively) undergoing mitosis (phospho-H3<sup>+</sup>).

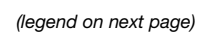
(G) Proportions of mitotic DI<sup>+</sup> cells (Pros<sup>+</sup> or Pros<sup>-</sup>) within and out of the population of cells co-expressing *GFP* and *sc* in *NRE<sup>TS</sup>-FO>GFP + sc* intestines (*n* = 303 cells).

(H) Expression (in logcounts) of *DI* found in ISCs/EBs by scRNA-seq (see Figure S5I), segregated by the expression of *scute* (zero vs. non-zero counts).

DAI, days after induction. Scale bars: 20  $\mu$ m. *p* values (binomial regression): ● *p* < 0.05, ●● *p* < 0.01, and ●●● *p* < 0.001. See Tables S1 and S2 for statistical details.

abolished EC differentiation and led to a significant increase of DI-positive cells (Figures 4B–4E). *sc* overexpression in EBs also induced many pre-EEs and EEs (Figures 4D and 4E). This suggested that *Da*, *Da:Da*, and *Sc* could force EBs to revert to the ISC fate and also, in the case of *Sc*, to the secretory lineage. Incidentally, while *da* overexpression in ISCs/EBs induced cell death (Figures 1J–1K), its overexpression in EBs only with *NRE<sup>TS</sup>-FO* did not. We evaluated whether these induced DI-positive cells could, like ISCs, undergo mitosis. We found mitotic (PH3-positive) cells

within the EB-derived DI-positive cells overexpressing *sc*; their proportion was of the same order as the mitotic, endogenous ISCs within the same tissue (2% vs. 7%; Figures 4F and 4G). To assess whether *sc* may be promoting ISC properties in normal homeostasis, we analyzed published single-cell RNA-seq (scRNA-seq) datasets<sup>37,38</sup> (Figure S4B) and found that cells classified as ISCs/EBs that expressed *sc* had, on average, much higher levels of *DI* (Figure 4H), suggesting that *sc*, apart from inducing EE differentiation, normally promotes the expression of ISC features.





### Emc promotes EC differentiation by titrating Da

Da and Sc functions are often antagonized through direct binding and titration by the HLH class V factor Emc, which prompted us to test *emc* function in intestinal homeostasis. Using the protein-trap line *emc*<sup>CPT1002740</sup>,<sup>39</sup> we found that *emc* was expressed in all cell types of the adult gut but predominantly in EBs and ECs (Figures 5A–5C). scRNA-seq data<sup>37,38</sup> show that *emc* expression is mainly in EBs and ECs of the posterior midgut, with cell type distributions similar to *bona fide* markers of pECs and EBs (Figures S5A–S5H). Consistent with this, *emc* expression decreases along the transcriptional trajectory from ISC/EB to EE and increases in the ISC/EB-to-pEC trajectory (Figures 5D and S5I). Note also how ISCs/EBs expressing *sc*<sup>+</sup> (and higher *DI*) are located, within the ISC/EB cluster, at the beginning of the differentiation trajectories (Figures S5I and 4H). MARCM clones mutant for *emc*<sup>LL02590</sup> (Figure S5J) had fewer differentiated cells and were enriched in DI-positive ISCs (Figures 5E–5F and 5J). Clones for alleles *emc*<sup>1</sup> and *emc*<sup>AP6</sup> (Figure S5J) showed similar enrichment in DI-positive ISCs compared to wild-type clones (Figures 5G–5J). Hypomorphic viable *emc*<sup>LL02590/emc</sup><sup>1</sup> and *emc*<sup>LL02590/emc</sup><sup>P5C</sup> heterozygotes (Figure S5J) showed an increase in ISCs/EBs at the expense of ECs, accompanied by higher levels of DI in ISCs and its ectopic expression in ECs, compared to control *emc*<sup>LL02590/+</sup> guts (Figures 5K–5N). Knockdown of *emc* with *esg*<sup>TS</sup>-FO phenocopied the overexpression of *da*: ISCs/EBs gave rise to labeled clusters of similar size to the controls (Figure 1L) where differentiation was severely impaired (Figures 5O–5Q and 5S). The strength of the effect on differentiation in the *emc*<sup>RNAi</sup> transgenes correlated with their efficacy in depleting Emc protein and inducing “extra macrochaetae” phenotypes (Figures S5K–S5R). The strongest *emc*<sup>RNAi</sup> transgene (*NIG-1007R*) induced, as with *da* overexpression, the loss of many *esg*<sup>+</sup> cell nests by apoptosis. As in the case of *da* overexpression, co-expression of *p35* and *emc*<sup>RNAi</sup> prevented ISC/EB death (Figures S6A and S6B), displayed reduced differentiation with respect to controls, and contained more EBs than *emc*<sup>RNAi</sup> alone (Figure S6A–S6C). We conclude that *emc* is necessary for EC differentiation and the survival of ISCs and EBs. Overexpres-

sion of *emc* with *esg*<sup>TS</sup>-FO resulted in all ISCs/EBs differentiating into ECs (Figures 5R and 5S), as expected.<sup>40</sup> Therefore, Emc is both necessary and sufficient to direct absorptive differentiation.

If Emc promoted EC differentiation by titrating Da and Sc, then *emc* function should depend on *da*. We tested this by generating MARCM clones mutant for *da* and expressing *emc*<sup>RNAi</sup> or mutant for *emc* and expressing *da*<sup>RNAi</sup>. Both conditions led to EC differentiation like in *da*<sup>10</sup> clones (compare Figures 6A–6C with Figure 6D). Considering our previous results where *sc* promoted ISC characteristics (Figures 3 and 4) and that ISCs express Sc,<sup>15,41</sup> we tested whether the effects of *emc* loss depended on *sc* or any other member of the AS-C. We induced MARCM clones for *Df(1)sc*<sup>B57</sup> (a deletion of the AS-C)<sup>42</sup> expressing *emc*<sup>RNAi</sup> and compared them with clones that only expressed *emc*<sup>RNAi</sup>. Differentiation was impaired in both conditions (Figures 6F and 6G), but DI expression was 4-fold lower in the absence of the AS-C (Figure 6H). We conclude that Emc promotes EC differentiation and dampens DI expression by preventing the formation of Da:Da and Da:Sc dimers, respectively.

### *emc* is required for the commitment of EBs

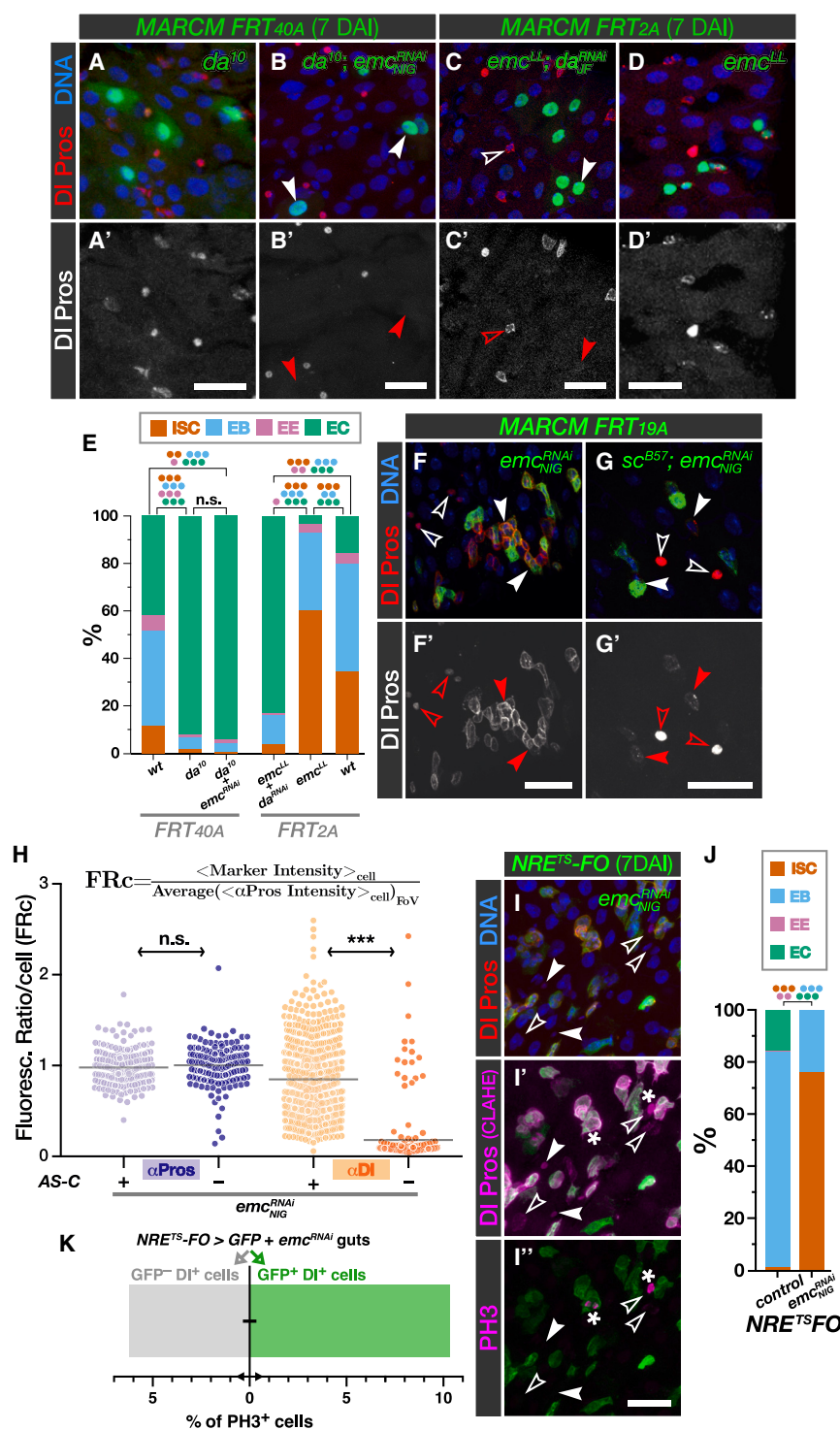
The requirement of *emc* for differentiation prompted us to investigate whether it was necessary to maintain the commitment of EBs as absorptive progenitors. We used the EB-specific driver *NRE*<sup>TS</sup>-Gal4, which never co-expresses with DI in the wild type, to express *emc*<sup>RNAi</sup>. This led to DI expression in *NRE*<sup>+</sup> cells (Figures S6C and S6D), suggesting that *emc*-depleted EBs may revert to ISCs. We verified this with *NRE*<sup>TS</sup>-FO driving *emc*<sup>RNAi</sup> expression and observed that *emc*-depleted EBs give rise to DI-positive cells (Figures 6I and 6J) that undergo mitosis (PH3 positive) at a similar frequency to neighboring normal ISCs (Figures 6I and K). This suggests that *emc* is necessary for the commitment of EBs.

### Notch and *emc* converge independently on Da to regulate ISC fate

Notch signaling is key for ISC differentiation and absorptive fate acquisition.<sup>10,11</sup> We found that simultaneous knockdown of *N*

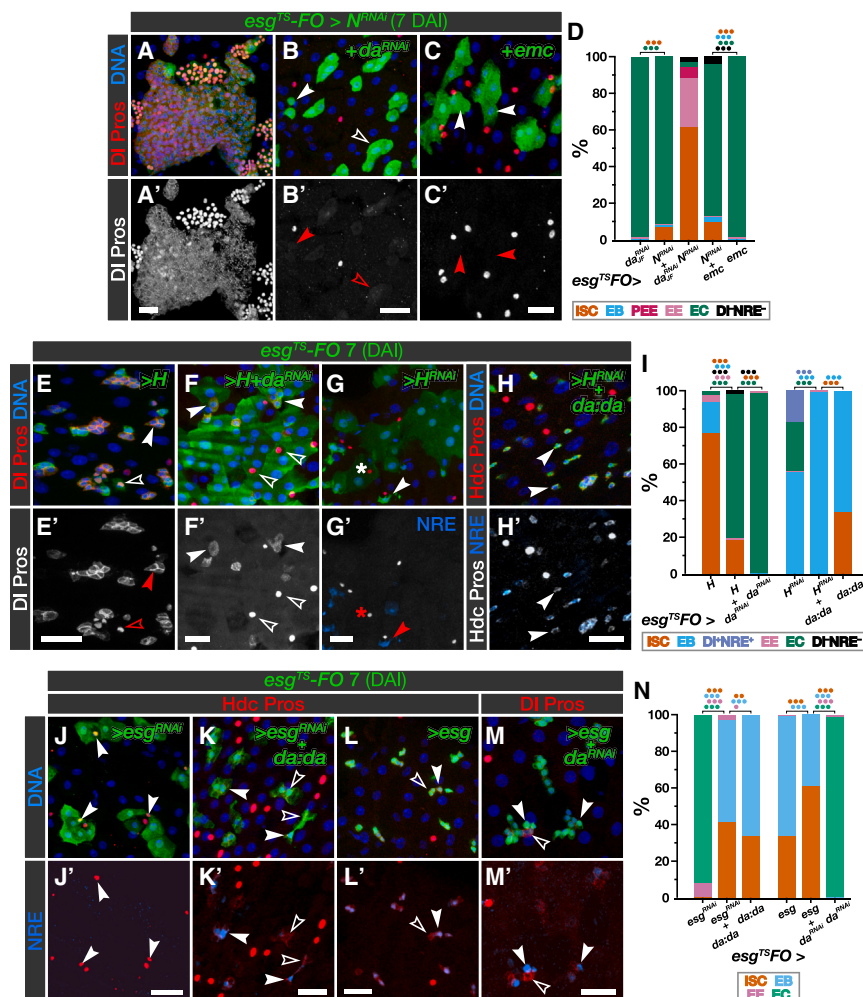
### Figure 5. *emc* is necessary and sufficient to induce EC differentiation

(A–C) Expression of Emc-GFP in the homozygous viable protein-trap insertion *emc*<sup>CPT1002740</sup>. Emc is expressed in EBs (*NRE-lacZ*<sup>+</sup>, arrowheads) (A) and some ECs (*myoIA-lacZ*<sup>+</sup>, solid arrowheads) but not all (empty arrowheads) (B). Some ISCs (DI<sup>+</sup>, solid arrowheads) and EEs (Pros<sup>+</sup>, solid asterisks) express Emc but many do not (empty arrowheads and asterisks) (C).  
(D) *emc* expression along the pseudotime trajectories from ISCs/EBs into EEs and ECs (Figure S5I) decreases toward the secretory fate and increases toward the absorptive one. The continuous line represents mean values; shading represents its standard error.  
(E–I) Cells in MARCM clones for *emc*<sup>LL02590</sup> (F), *emc*<sup>AP6</sup> (H), and *emc*<sup>1</sup> (I) are enriched in ISCs (solid arrowheads) and EBs (empty arrowheads) compared to controls (E and G). *FRT*<sub>80B</sub> and *FRT*<sub>2A</sub> are two independent insertions for inducing MARCM clones in chromosomal arm 3L.  
(J) Cell composition of clones from (E)–(I).  
(K–M) Midguts of viable hypomorphic mutants *emc*<sup>LL02590/1</sup> (L) and *emc*<sup>LL02590/P5C</sup> (M) contain more ISCs, pre-EBs (L, asterisk), and EEs and less ECs than heterozygous controls (K). In these mutants, DI expression is elevated in some ISCs (L and M, empty arrowheads) and ectopic in some ECs (L and M, solid arrowheads).  
(N) Cell composition in genotypes from (K)–(M).  
(O–Q) Expressing *emc*<sup>RNAi</sup> with *esg*<sup>TS</sup>-FO using transgenes *NIG-1007R* (P) or *KK108316* and *JF02300* together (Q) increases the proportion of DI<sup>+</sup> cells (solid arrowheads) and EEs (empty arrowheads) and reduces that of ECs (asterisks) compared to controls (O). *KK108316* and *JF02300* together produced DI<sup>+</sup>, *NRE-lacZ*<sup>+</sup> cells, possibly dedifferentiating EBs (see Figures 6I and 6J) or cells with mixed ISC/EB identity or marker expression. *emc*<sup>RNAi</sup> also increases DI expression (P and Q), especially with *NIG-1007R* (P).  
(R) Overexpression of *emc* with *esg*<sup>TS</sup>-FO forces differentiation into ECs.  
(S) Cell composition in genotypes from (O)–(R).  
DAI, days after induction. Scale bars: 20 μm. *p* values (binomial regression): ● *p* < 0.05, ●● *p* < 0.01, and ●●● *p* < 0.001. See Tables S1 and S2 for statistical details. (F), (P), and (R) are reproduced in Figures 6D, S7G, and S7C, respectively, to aid comparison.



and *da* with *esg*<sup>TS-FO</sup> prevented the tumorous expansion of DI-positive and Pros-positive cells typically found with *NRE*<sup>RNAi</sup> alone<sup>10,43</sup> and forced most cells to differentiate as ECs (Figures 7A, 7B, and 7D). This extent of EC differentiation in the absence of Notch has not been seen before<sup>10,11,14,34,44,45</sup> and strongly suggests that the Notch pathway could be regu-

EC differentiation and the expression of *NRE-lacZ* in all diploid cells (Figures 7G and 7I).<sup>14</sup> The co-expression of *da:da* with *H*<sup>RNAi</sup> prevents all EC differentiation, with all *esg*<sup>+</sup> cells becoming *NRE-lacZ*<sup>+</sup> (Figures 7H and 7I). Thus, *da* is epistatic over Notch signaling, suggesting that at least part of the function of Notch to induce differentiation is to repress Da. Next,



*p* values (binomial regression for individual cell types): ● *p* < 0.05, ●● *p* < 0.01, and ●●● *p* < 0.001. See Tables S1 and S2 for statistical details. DAI, days after induction. Scale bars: 20 μm. (E) and (G) are reproduced in Figures S7A and S7E, respectively, to aid comparison.

we asked whether this function was exerted through the induction of *emc*.

*emc* transcription is regulated by Notch signaling in multiple tissues,<sup>47–51</sup> and we found that co-expressing *emc* and *N<sup>RNAi</sup>* with *esg<sup>TS</sup>-FO* leads to EC differentiation (Figures 7C and 7D). Therefore, Notch could be inducing EC differentiation by activating *Emc*, which in turn would titrate *Da*. *emc* expression induces EC differentiation rapidly,<sup>52</sup> so these *esg<sup>TS</sup>-FO>emc+N<sup>RNAi</sup>* ECs could have differentiated before *N* protein was depleted. To inhibit Notch signaling faster, we co-expressed *H* with *emc* using *esg<sup>TS</sup>-FO*. This led to an increase in EC differentiation compared to expression of *H* alone but far from the near-complete EC differentiation observed when only expressing *emc* (Figures S7A–S7D) or expressing *H* with *da<sup>RNAi</sup>* (compare with Figures 7F and 7I). This suggests that *Emc* can induce EC differentiation in the absence of Notch signaling but not efficiently. Moreover, expressing *H<sup>RNAi</sup>* and *emc<sup>RNAi</sup>* with *esg<sup>TS</sup>-FO* did not prevent the excess EC differentiation observed upon expression of *H<sup>RNAi</sup>* alone; this contrasts with the capacity of *emc<sup>RNAi</sup>* to

block differentiation entirely (Figures S7D–S7G). Therefore, *Emc* is dispensable for Notch to induce EC differentiation. Moreover, clonal expression of the Notch intracellular domain does not induce *emc* expression in any cell type of the midgut epithelium (Figure S7H). We conclude that *emc* and Notch signaling act independently to promote the absorptive fate.

### Da:Da and Esg block differentiation independently

*Esg*, a TF of the *Snail* family, regulates ISCs by preventing ISC/EB differentiation.<sup>44,45</sup> As *Da:Da* dimers have the same capacity, we considered whether *Esg* and *Da* act together to maintain stemness. Expression of *esg<sup>RNAi</sup>* with *esg<sup>TS</sup>-FO* led to differentiation into ECs and EEs (Figures 7J and 7N) as expected.<sup>34,44,45</sup> The simultaneous overexpression of *esg<sup>RNAi</sup>* and *da:da* blocked EC differentiation and significantly reduced the formation of EEs (Figures 7K and N). In turn, the expression of *esg* with *esg<sup>TS</sup>-FO* blocked differentiation, irrespective of whether *da<sup>RNAi</sup>* was also expressed (Figures 7L–7N). This independence seems mirrored in their downregulated gene sets: almost half the *Esg* repression

### Figure 7. *da* is epistatic to Notch signaling and acts in parallel with *esg*

(A–C) Expressing *N<sup>RNAi</sup>* together with *da<sup>RNAi</sup>* (B) or *emc* (C) using *esg<sup>TS</sup>-FO* leads to EC differentiation, abolishing the tumorous expansion of *DI<sup>+</sup>* and *Pros<sup>+</sup>* cells with *N<sup>RNAi</sup>* alone (A). Solid arrowheads: polyploid ECs; empty arrowheads: *DI<sup>+</sup>* diploid cells (EBs or early ECs). (A) is reproduced from Figure 2E to aid comparison.

(D) Cell composition of GFP<sup>+</sup> tissue from (A)–(C); data from Figures 1I and S5 are replicated here to aid comparison.

(E and F) Expressing *H* with *esg<sup>TS</sup>-FO* (E) blocks EC differentiation and leads to accumulation of ISCs. This is rescued by simultaneous expression of *da<sup>RNAi</sup>* (F). Solid arrowheads: ISCs; empty arrowheads: EEs.

(G and H) Expressing *H<sup>RNAi</sup>* with *esg<sup>TS</sup>-FO* (G) induces EC differentiation, some EE differentiation (asterisk), and the expression of the *NRE-lacZ* reporter in all *esg<sup>+</sup>* cells (solid arrowhead). Simultaneous expression of *da:da* (H) blocks the formation of ECs, as in *esg<sup>TS</sup>-FO>da:da* alone, and all cells become *NRE-lacZ<sup>+</sup>* (solid arrowheads).

(I) Cell composition of GFP<sup>+</sup> tissue from (E)–(H); data from Figure 1I are replicated here to aid comparison.

(J and K) Knockdown of *esg* with *esg<sup>TS</sup>-FO* (J) induces differentiation of all cells into ECs and EEs (solid arrowheads); this is prevented by simultaneous expression of *da:da* (K), which blocks differentiation of ISCs (empty arrowheads) and EBs (solid arrowheads) to the same levels as in *esg<sup>TS</sup>-FO>da:da* alone (see N).

(L and M) Expression of *esg* with *esg<sup>TS</sup>-FO* (L) blocks differentiation regardless of whether *da* is simultaneously knocked down (M). Empty arrowheads: ISCs; solid arrowheads: EBs.

(N) Cell composition of GFP<sup>+</sup> tissue from (J)–(M); data from Figure 1I are replicated here to aid comparison.



targets<sup>44</sup> overlap with genes downregulated by misexpression of *da* or *da:da* or upregulated by misexpression of *da<sup>RNAi</sup>*, including genes essential for EC function like *nub/pdm1*, *ssk*, and *Tsp2A* (Figure S7I). Finally, we observed that the expression of neither *da* nor *esg* was affected by the overexpression of the other (Figures S7J and S7K). We conclude that *da* and *esg* contribute to stemness independently of each other.

## DISCUSSION

We demonstrate a central role for a bHLH factor code in the acquisition and maintenance of three alternative cell fates in the adult *Drosophila* intestine. Class I homodimers (Da:Da) maintain the progenitor state of ISCs/EB, with changes in dimerization partners governing the fate transitions. Sequestration of Da with class V HLH factor Emc into Da:Emc dimers incapable of DNA binding induces progenitor cells to acquire the absorptive fate, while the formation of Da:Sc dimers by binding of Da with class II bHLH Sc initiates EE differentiation (Figures 2 and S2).<sup>14,16</sup> Moreover, Emc is required in EBs to maintain their committed state, while low levels of Sc boost aspects of the ISC transcriptional state.

### Three cell fates regulated by a dimerization network

Networks involving class I, II, and V bHLH factors regulate the development of the *Drosophila* retina<sup>53</sup> and peripheral nervous system.<sup>25,54,55</sup> However, in these cases, the choice is between only two alternative fates (neural vs. epidermal), with Da homodimers promoting the same fate as heterodimers between Da and a class II bHLH proneural factor.<sup>53,55,56</sup> By contrast, in the adult midgut, Da:Da and Da:Sc promote distinct fates (progenitor and secretory, respectively) through distinct transcriptional programs, while Emc titrates both dimers to allow EC differentiation. This integrated mechanism suggests that the balance between ISC self-renewal and absorptive or secretory differentiation rests on a triple choice rather than two consecutive binary decisions. An equivalent network could operate in similar stem cell systems, and it is tempting to speculate on this possibility in the mammalian intestine, where the relevant factors (mouse *da* homologs *E2a* and *Heb*, *emc* homolog *Id1*, and *sc* homolog *Ascl1*) are expressed in the crypts of Lieberkühn and have roles in fate determination.<sup>57–59</sup>

### A balance of factors regulates ISC fate

We refer to the Da/Emc/Sc network as a bHLH code due to its modularity, but it does not behave as a Boolean switch; the relative abundance of the different components seems critical. Da, the centerpiece of the network, forms homodimers that maintain stemness and prevent EC and EE differentiation (Figures 1 and 2), but it is also an essential partner for Sc in EE differentiation (Figure S2). The activity of Da homodimers in promoting stemness is also nuanced, as, in parallel to activating some ISC genes and repressing some EE/EC ones, Da:Da downregulates proliferation effector proteins, in agreement with its previously reported anti-proliferative activity.<sup>60</sup> Strikingly, an excess of Da monomers (whether induced directly by overexpression or indirectly by loss of Emc) promotes apoptosis (Figures 1 and S6), while the excess of Sc or tethered Da:Da does not. It follows

that Da also participates in an apoptosis-promoting complex yet to be identified. By contrast, the activity of Emc is determinant but not intrinsically instructive: through titration of Da and Sc, cells have to differentiate but lack the capacity to initiate EE differentiation, so they differentiate into ECs by default. However, Emc loss in EBs induces expression of ISC features, possibly through the increased activity of Sc (Figure 6), and prevents Da from inducing apoptosis of ISC/EBs (Figure S6). This makes Emc also an essential factor in maintaining the progenitor population. Sc, which is normally expressed at low levels in ISCs,<sup>15,41</sup> triggers EE differentiation when expressed over a threshold.<sup>14,15,27</sup> We find that Sc boosts the expression of essential ISC genes, such as *DI* or those involved in proliferation. Moreover, Sc seems to induce fate reversal in EBs. Therefore, Sc also boosts the ISC function, which sits well with the role of its homolog *Acs12* in maintaining mammalian ISCs.<sup>57</sup>

We conclude that a dynamic balance of Da, Emc, and Sc is required for homeostasis and that this bHLH code is not composed of “master” but rather contextual regulators. This pushes the question toward the regulation of their protein concentrations and/or functions across intestinal cell types and over time. The specific role of Notch signaling in regulating Da and the identity of *emc* regulators is bound to be informative.

In line with this view, another essential regulator of ISC fate, *Esg*, operates in parallel to the bHLH network. Excess of either *Esg* or Da can compensate for the absence of the other to keep ISCs/EBs undifferentiated, suggesting that at physiological levels, when either is essential for stemness (Figure 1),<sup>44</sup> they have some additive effect. This may be reflected in the significant overlap of their downregulated genes (Figure 7), which includes the EC fate inducer *nub/pdm1*. On the other hand, *Esg* seems to have a more specific role than Da:Da in preventing EE differentiation and lacks its anti-proliferative activity,<sup>44,45</sup> suggesting that they also govern non-shared aspects of ISC identity and function. These non-redundant layers of regulation likely allow fine-tuning of the ISC function.

### Multiple factors maintain EB commitment

Notch signaling induces the formation of EBs,<sup>10,11</sup> which, under normal conditions, give rise to mature ECs without further division (Figure 4).<sup>16,61,62</sup> EBs are relatively long lived,<sup>63</sup> and though they are recognized by expression of the *NRE*, once formed, they do not need Notch to maintain their commitment.<sup>64</sup> However, we found that they require Emc, as its depletion in EBs gave rise to *DI*-expressing proliferative cells—likely a reversal of fate toward ISCs. Qualitatively similar effects resulted from misexpressing *da*, *da:da*, or *sc* in EBs. We interpret that combined baseline levels of Da and Sc in EBs may lead them to revert their fate into ISCs if not titrated by Emc. EB-to-ISC reversion is also prevented by TF *Sox21a*<sup>65</sup> and the global co-repressor *Groucho*.<sup>66</sup> EB-to-EE *trans*-differentiation has also been observed when either *Ttk69* or *Klu* was depleted or *Phyllopod* overexpressed in EBs.<sup>61,62,67</sup> These observations underscore the plasticity of the EB and resemble the behavior of EC precursors in the mammalian intestine, which can dedifferentiate and repopulate the intestinal crypt during regeneration.<sup>68</sup>



### Limitations of the study

Our interpretation of the functional relationships between *Da*, *Sc*, and *Emc* are based on misexpression tools with no control over resulting stoichiometry. Future studies should address this limitation with newly developed tools with more precise control.<sup>69–71</sup> Our analysis of *emc* function involved the *emc<sup>RNAi</sup>* transgene *NIG-1007R*, which is far more effective at depleting *Emc* than either *KK108316* or *JF02300* or both combined (Figure S5). Several *emc* loss-of-function conditions affecting large amounts of tissue induce increased *DI* expression (Figure 5), but with *NIG-1007R*, this effect is much higher and not observed in MARCM *emc* null clones. This may point to non-cell-autonomous, suppressing effects on *DI* expression that are only inactivated when the whole tissue or the whole progenitor population is affected. We are confident that *NIG-1007R* elicits genuine *emc* loss phenotypes because: the strength of the increase in *DI* levels correlates with the strength of the *emc<sup>RNAi</sup>* transgenes (Figures 5 and S6); *NIG-1007R* phenocopies the overexpression of *da*, including the induction of apoptosis (which *KK108316* and *JF02300* cannot recapitulate; Figures 1, 5, and S5); and we can fully rescue the increase in *DI* expression induced by *NIG-1007R* by simultaneous loss of *sc* (Figure 6). While our tenet does not hinge on this observation, it deserves future attention.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Joaquín de Navascués ([j.denavascues@essex.ac.uk](mailto:j.denavascues@essex.ac.uk)).

#### Materials availability

*Drosophila* strains generated in this study are available upon request. Requests for *Drosophila* strains should be directed to and will be fulfilled by the lead contact.

#### Data and code availability

- RNA-seq data have been deposited at GEO and are publicly available as of June 7, 2023. The accession number is in the [key resources table](#). Microscopy data reported in this paper will be shared by the lead contact upon request.
- scRNA-seq data were generated by Hung et al.<sup>37</sup> and Li et al.<sup>38</sup> and obtained from GEO and Array Express, respectively. The accession numbers are in the [key resources table](#).
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

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### AUTHOR CONTRIBUTIONS

Conceptualization, J.d.N.; data curation, J.d.N., A.P.-B., and J.K.; formal analysis, J.d.N., A.P.-B., and V.D.N.; funding acquisition, J.d.N., J.K., B.A.E., and P.V.-W.; investigation, A.P.-B., J.d.N., H.M., J.K., S.D., and S.A.; methodology, J.d.N., J.K., and P.V.-W.; project administration, J.d.N.; resources, J.d.N., J.K., and B.A.E.; supervision, J.d.N., J.K., B.A.E., and P.V.-W.; validation, J.K. and S.D.; visualization, J.d.N., A.P.-B., and V.D.N.; writing – original draft, J.d.N.; writing – review & editing, J.d.N., A.P.-B., P.V.-W., J.K., and B.A.E.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

### STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- [KEY RESOURCES TABLE](#)
- [EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS](#)
- [METHOD DETAILS](#)
  - Transgene and clonal induction
  - Immunohistochemistry
  - Cell counts
  - RNA-seq
  - RNA-seq and scRNA-seq analysis
  - Quantification of Delta expression
  - Segmentation and quantification in Pros<sup>+</sup> cells
  - Segmentation and quantification in DI<sup>+</sup> cells
- [QUANTIFICATION AND STATISTICAL ANALYSIS](#)

### SUPPLEMENTAL INFORMATION

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## STAR★METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Mouse monoclonal anti-Delta, extracellular domain, c594.9b (1:50)	Developmental Studies Hybridoma Bank (DSHB)	RRID: AB_528194
Mouse monoclonal anti-Prospero, MR1A (1:200)	DSBH	RRID: AB_528440
Mouse monoclonal anti-Headcase, HDC U33 (1:100)	DSHB	RRID: AB_10659722
Mouse monoclonal anti-Armadillo, N2 7A1 (1:100)	DSHB	RRID: AB_528089
Rabbit polyclonal anti-Prospero (1:1000)	Vaessin et al. <sup>72</sup>	N/A
Guinea pig polyclonal anti-Emc (1:1000)	Antonio Baonza (CSIC, Spain)	N/A
Rabbit polyclonal anti-Phospho-Histone H3 (Ser10) (1:200)	Cell Signaling	Cat# 9701, RRID: AB_331535
Rabbit polyclonal anti-GFP (1:2000)	Abcam	Cat# ab6556, RRID: AB_305564
Chicken polyclonal anti-GFP (1:3000)	Abcam	Cat# ab13970, RRID: AB_300798
Chicken polyclonal anti-beta Galactosidase (1:2000)	Abcam	Cat# ab9361, RRID: AB_307210
Rabbit polyclonal anti-beta Galactosidase (1:10000)	ThermoFisher Scientific	Cat# A11132, RRID: AB_221539
Goat anti-Mouse IgG, Alexa Fluor 568-conjugated (1:500)	ThermoFisher Scientific	Cat# A-11031, RRID: AB_144696
Donkey anti-Mouse IgG, Alexa Fluor 594-conjugated (1:500)	ThermoFisher Scientific	Cat# A-21203, RRID: AB_2535789
Goat anti-Mouse IgG, Alexa Fluor 633-conjugated (1:500)	ThermoFisher Scientific	Cat# A-21050, RRID: AB_2535718
Goat anti-Rabbit IgG, Alexa Fluor 488-conjugated	ThermoFisher Scientific	Cat# A-11008, RRID: AB_143165
Donkey anti-Rabbit IgG, Alexa Fluor 594-conjugated	ThermoFisher Scientific	Cat# A-21207, RRID: AB_141637
Goat anti-Rabbit IgG, Alexa Fluor 633-conjugated	ThermoFisher Scientific	Cat# A-21070, RRID: AB_2535731
Goat anti-Guinea pig IgG, Alexa Fluor 546-conjugated	ThermoFisher Scientific	Cat# A-11074, RRID: AB_2534118
Goat anti-Chicken IgY, Alexa Fluor 488-conjugated	ThermoFisher Scientific	Cat# A-11039, RRID: AB_2534096
Goat anti-Chicken IgY, Alexa Fluor 594-conjugated	ThermoFisher Scientific	Cat# A-11042, RRID: AB_2534099
Goat anti-Chicken IgY, Alexa Fluor 633-conjugated	ThermoFisher Scientific	Cat# A-21103, RRID: AB_2535756
<b>Chemicals, peptides, and recombinant proteins</b>		
Phosphate buffered saline tablets	Sigma-Aldrich	Cat# P4417
Triton X-100	Sigma-Aldrich	Cat# T8787
Bovine Serum Albumin fraction V	Roche	Cat# 10735108001
Formaldehyde solution	Sigma-Aldrich	Cat# F8775
Hoechst 33342 (used at 2 $\mu$ g/mL)	Sigma-Aldrich	Cat# B2261
N-propyl-gallate	Sigma-Aldrich	Cat# 02370
Glycerol (spectrophotometric grade)	Sigma-Aldrich	Cat# G9012

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Critical commercial assays</b>		
PureLink RNA mini preps	ThermoFisher Scientific	Cat# 12183020
Illumina polyA library preparation and NovaSeq PE sequencing	Genewiz/Azenta Life Sciences	N/A
<b>Deposited data</b>		
RNA-seq	This work	GEO:GSE234019
Single-cell RNA-seq	Hung et al. <sup>37</sup>	GEO:GSE120537
Single-cell RNA-seq	Li et al. <sup>38</sup>	ArrayExpress:E-MTAB-10519, ArrayExpress:E-MTAB-10628
<b>Experimental models: Organisms/strains</b>		
<i>D. melanogaster</i> : Su(H)GBE-lacZ; esg-Gal4, UAS-GFP, tubP-Gal80 <sup>ts</sup> /CyO; UAS-FLP, Act5C-FRT-CD2-FRT-Gal4/TM6C (esg <sup>ts</sup> -FO driver)	Jiang et al. <sup>31</sup>	N/A
<i>D. melanogaster</i> : y, w; Su(H)GBE-Gal4, UAS-GFP, tubP-Gal80 <sup>ts</sup> /CyO; UAS-FLP, Act5C-FRT-CD2-FRT-Gal4/TM6B (NRE <sup>ts</sup> -FO driver)	Zeng et al. <sup>73</sup>	Derived from RRID:BDSC_83377
<i>D. melanogaster</i> : y, w; Su(H)GBE-Gal4/CyO; UAS-GFP, tubP-Gal80 <sup>ts</sup> /TM6B (NRE <sup>ts</sup> driver)	Zeng et al. <sup>73</sup>	Derived from RRID:BDSC_83377
<i>D. melanogaster</i> : UAS-da	Sonsoles Campuzano (CSIC, Spain)	N/A
<i>D. melanogaster</i> : UAS-sc	Sonsoles Campuzano	N/A
<i>D. melanogaster</i> : emc <sup>EP3620</sup> (UAS-emc gene trap)	Sonsoles Campuzano	N/A
<i>D. melanogaster</i> : UAS-p35	Sonsoles Campuzano	N/A
<i>D. melanogaster</i> : UAS-da:da	Sangbin Park (Stanford University, USA)	N/A
<i>D. melanogaster</i> : UAS-N <sup>ICD</sup>	Alfonso Martínez Arias (UPF, Spain)	N/A
<i>D. melanogaster</i> : UAS-N <sup>RNAi</sup>	Presente et al. <sup>74</sup>	N/A
<i>D. melanogaster</i> : UAS-H	Allison Bardin (Curie Institute, France)	N/A
<i>D. melanogaster</i> : UAS-esg	Korzelius et al. <sup>44</sup>	N/A
<i>D. melanogaster</i> : UAS-Dcr-2	Bloomington <i>Drosophila</i> Stock Center (BDSC)	RRID:BDSC_24646
<i>D. melanogaster</i> : y sc; UAS-da <sup>RNAi</sup> HMS01851	BDSC	RRID:BDSC_38382
<i>D. melanogaster</i> : y v;; UAS-da <sup>RNAi</sup> JF02488	BDSC	RRID:BDSC_29326
<i>D. melanogaster</i> : UAS-emc <sup>RNAi</sup> 1007R-2	National Institute of Genetics (Japan)	Stock# 1007R-2
<i>D. melanogaster</i> : UAS-emc <sup>RNAi</sup> KK108316	Vienna <i>Drosophila</i> Resource Center	Stock# 100587
<i>D. melanogaster</i> : UAS-emc <sup>RNAi</sup> JF02300	BDSC	RRID:BDSC_26738
<i>D. melanogaster</i> : UAS-H <sup>RNAi</sup> JF02624	BDSC	RRID:BDSC_27315
<i>D. melanogaster</i> : UAS-esg <sup>RNAi</sup> HMS00025	BDSC	RRID:BDSC_34063
<i>D. melanogaster</i> : emc <sup>CPT1002740</sup>	Kyoto <i>Drosophila</i> Stock Center (DGRC)	Stock# 115317
<i>D. melanogaster</i> : Myo1A-lacZ	Jiang et al. <sup>31</sup>	RRID:BDSC_24646
<i>D. melanogaster</i> : da-GFP.FPTB	BDSC	RRID:BDSC_55836
<i>D. melanogaster</i> : y w hs-Flp <sub>1,22</sub> ; Act5C-FRT-y <sup>+</sup> -FRT-Gal4, UAS-lacZ <sub>20b</sub>	BDSC (modified)	RRID:BDSC_4410
<i>D. melanogaster</i> : y w hs-Flp <sub>1,22</sub> tub-Gal4 UAS-GFP; tub-Gal80 FRT40A/CyO (MARCM FRT40A)	Allison Bardin	N/A

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>D. melanogaster</i> : y w <i>hs-Flp</i> <sup>1.22</sup> <i>tub-Gal4</i> UAS-GFP; <i>tub-Gal80</i> FRT80B/TM6B (MARCM FRT80B)	Sonsoles Campuzano (Centro de Biología Molecular, Spain)	N/A
<i>D. melanogaster</i> : y w <i>hs-Flp</i> <sup>1.22</sup> <i>tub-Gal4</i> UAS-GFP; <i>tub-Gal80</i> FRT2A/TM6B (MARCM FRT2A)	Sonsoles Campuzano	N/A
<i>D. melanogaster</i> : w <i>hs-Flp</i> <i>tub-Gal80</i> FRT19A; <i>tub-Gal4</i> UAS-GFP/CyO (MARCM FRT19A)	Shinya Yamamoto (Baylor College, USA)	N/A
<i>D. melanogaster</i> : w; FRT40A	BDSC	RRID:BDSC_1646
<i>D. melanogaster</i> : w;; FRT80B	BDSC	RRID:BDSC_1620
<i>D. melanogaster</i> : y w;; FRT2A	BDSC	RRID:BDSC_1997
<i>D. melanogaster</i> : y w FRT19A	BDSC	RRID:BDSC_1709
<i>D. melanogaster</i> : w; <i>Df(2L)da</i> <sup>10</sup> , FRT40A/In(2LR)Gla, Bc	BDSC	RRID:BDSC_5531
<i>D. melanogaster</i> : w;; <i>emc</i> <sup>AP6</sup> FRT80B/TM6B	BDSC	RRID:BDSC_36544
<i>D. melanogaster</i> : w;; <i>emc</i> <sup>1</sup> FRT80B/TM2	BDSC	RRID:BDSC_5532
<i>D. melanogaster</i> : <i>emc</i> <sup>LL02590</sup> FRT2A FRT82B/TM6C	DGRC	Stock# 140642
<i>D. melanogaster</i> : <i>Df(1)sc</i> <sup>B57</sup> w FRT19A/FM7g	Allison Bardin	N/A
<b>Software and algorithms</b>		
Rstudio	Posit Software PBC	<a href="http://www.rstudio.com">http://www.rstudio.com</a>
Illustrator CS6	Adobe Inc.	N/A
Photoshop CS6	Adobe Inc.	N/A
Affinity Designer 2	Serif (Europe) Ltd.	<a href="https://affinity.serif.com">https://affinity.serif.com</a>
FIJI	Schindelin et al. <sup>75</sup>	<a href="https://fiji.sc">https://fiji.sc</a>
Analysis scripts	this work	<a href="https://doi.org/10.5281/zenodo.8116966">https://doi.org/10.5281/zenodo.8116966</a>
<b>Other</b>		
Flygutseq	Dutta et al. <sup>76</sup>	<a href="https://flygutseq.buchonlab.com">https://flygutseq.buchonlab.com</a>
Flybase	Öztürk-Çolak et al. <sup>77</sup>	<a href="https://flybase.org">https://flybase.org</a>

## EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

*Drosophila melanogaster* experimental subjects were adult mated females, aged for ~2 weeks. Subject females were housed with males at a ratio of 1:2 to 3:4 males to females, to allow mating, at a density of 5–9 flies/cm<sup>2</sup> of food surface and 2–3 flies/cm<sup>3</sup> of vial volume. Food was made from organic yellow maize flour (80 g/L), inactivated yeast powder (30 g/L), brewer's dextrose (80 g/L), agar (6.67 g/L), cooked at 95°C before adding propionic acid (0.5%) and tegosept (0.005%). Vials were kept at 18°C, 25°C or 29°C in a 12h:12h light/dark cycle. Breeding vials were flipped twice a week at 25°C and once at 18°C. Vials with adult experimental subjects were flipped every other day.

## METHOD DETAILS

### Transgene and clonal induction

For experiments using Gal80<sup>TS</sup>, adult flies were aged to gut maturity (4–7 days) at 18°C, then transferred to 29°C. For induction of MARCM and flip-out clones, 4–7 days old flies were treated at 37°C for 60 or 15 min, respectively. Flies were aged for 7 days after induction treatment before dissection, unless otherwise indicated (see Table S2). Fly strains are listed in the key resources table. All RNAi transgenes were co-expressed with UAS-*Dcr-2*.

### Immunohistofluorescence

For antibody staining, adult guts were dissected in ice-cold PBS (maximum 10 min). Tissues were fixed in PBS-formaldehyde 4% (15 min at room temperature, RT), then in methanol (15 min RT). Methanol was washed off with three rinses in PBS-Triton X100 0.1% (PBT), followed by blocking and permeabilisation in PBT-BSA 2% (PBTB; three times, 15 min each). Tissues were incubated overnight at RT in primary antibody solution (diluted in PBTB to their final concentration; see key resources table). Primary antibody was washed off with three rinses and three 15-min incubations in PBT at RT. Tissue was incubated in PBT-secondary antibody solution (2 h at RT), then rinsed three times and incubated twice in PBT (15 min each); then once more in PBS. Tissue was equilibrated in mounting medium (4:1 glycerol:PBS with 4% w/v N-propyl-gallate) 4 h at RT or overnight at 4°C. After mounting, 3D confocal imaging was performed in a Zeiss LSM 710 with an EC Plan-Neofluar 40X oil immersion objective (numerical aperture 1.3). Three fields of view (typically 213 μm × 213 μm) along the anterior-posterior axis of each of (at least) three posterior midguts (regions R4-R5)<sup>12</sup> were

imaged. In MARCM clone experiments, stacks were acquired from all clones found in each posterior midgut. Figures were assembled using FIJI and Adobe Photoshop/Illustrator CS6 or Affinity Designer 2.

### Cell counts

For evaluating the proportion of cell types in GFP-labelled tissue, confocal stacks were maximum-intensity projected using FIJI and cells of the relevant types were counted manually with the Cell Counter plugin. Details of markers used can be found in Figure S1D. In the experiments co-expressing *sc* and *da:da*, the associated increase in proliferation generated large, highly densely populated cell clusters which could not be counted with single-cell precision. Therefore, for this genotype we estimated the proportion of each cell population in each field of view separately and then estimated the aggregated proportions.

### RNA-seq

Flies bearing either *UAS-da*, *UAS-da<sup>RNAi</sup>* (TRiP.JF02092), *UAS-da:da* or *UAS-sc* as well as *esg-Gal4*, *UAS-GFP* and *tub-Gal80<sup>TS</sup>* were reared at 18°C until 4–7 days old, transferred to 29°C for 2 days and their midguts dissected, then processed as described.<sup>76</sup> Libraries from three biological replicates per condition (except one condition, with two) were prepared in two batches and ~37 million reads (either 50 or 300bp long, for each batch respectively) were generated per library using Illumina technology. See key resources table for additional details.

### RNA-seq and scRNA-seq analysis

Fastq read files, with adaptors pre-trimmed by the sequencing provider, were mapped to release 6.28 of the *Drosophila melanogaster* genome using *STAR* and *bamtools*<sup>78,79</sup> and assigned to genes with *featureCounts*.<sup>80</sup> Differential gene expression, gene set and DNA motif enrichment analyses were performed with the key R packages *DESeq2*,<sup>81</sup> *limma*,<sup>82</sup> *fgsea*<sup>83</sup> and *RcisTarget*.<sup>84</sup> We performed GSEA<sup>35</sup> against the Gene List Annotation for *Drosophila* (GLAD),<sup>85</sup> the Kyoto Encyclopedia of Genes and Genomes (KEGG)<sup>86</sup> and gene lists from Dutta et al.<sup>76</sup> scRNAseq data were obtained from GEO and <https://flycellatlas.org>. To correct for variability arising from technical and biological effects, we used the *IntegrateData* function in *Seurat* v4.<sup>87,88</sup> The *Slingshot* library<sup>89</sup> was used for the trajectory analysis, which focused on ISC/EB cells as the initial state, pEC and EE cells, resulting in the identification of two distinct trajectories. The analysis code is in GitHub ([https://github.com/jdenavascues/bHLH\\_code\\_midgut](https://github.com/jdenavascues/bHLH_code_midgut)) and archived at Zenodo (<https://doi.org/10.5281/zenodo.8116966>).

### Quantification of Delta expression

We took advantage of using simultaneously anti-DI and anti-Pros mouse monoclonals, detected with the same anti-mouse secondary antibody, and of the robustness and reproducibility of the anti-Pros signal across conditions. This allows to use nuclear Pros staining outside the GFP-labelled MARCM clones as a normalisation reference, as the variation of DI/Pros intensity ratio between samples is caused by the relative differences in DI antigen. DI<sup>+</sup> cells within clones and Pros<sup>+</sup> cells outside the clones were segmented so that we could take a value of fluorescence intensity per cell per marker, and normalised these values respect the average intensity of Pros per cell in that field of view.

### Segmentation and quantification in Pros<sup>+</sup> cells

The positions of all cells were recorded in FIJI using CellCounter. A median filter was applied to the Pros/DI channel to remove small features while preserving edges. A binary mask was created using Otsu thresholding<sup>90</sup> of the filtered image. This mask captured most of the Pros<sup>+</sup> nuclei but missed some with lower expression. To segment these, we used the manually determined XY positions of the Pros<sup>+</sup> cells to add a 3-pixel diameter disk for each Pros<sup>+</sup> cell absent in the original mask. Fused nuclei were separated by marker-controlled watershed transformation.<sup>91</sup> Pros expression for each nucleus was determined as the average pixel intensity value of the Pros signal channel in each segmented nucleus.

### Segmentation and quantification in DI<sup>+</sup> cells

Clones were detected by thresholding the GFP signal using the minimum cross-entropy method.<sup>92</sup> This missed a few cells, which were added to the mask using a similar approach to the Pros<sup>+</sup> nuclei, and the mask was consolidated by morphological filling and binary closing. Individual DI<sup>+</sup> cells within the clone were identified by marker-controlled watershed segmentation, using the manually determined positions of the cells as markers. DI expression for each cell was determined as the average pixel intensity of the DI signal for each segmented object, normalised by the average Pros expression for that field of view.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical tests were performed in R. Change in cell type composition was assessed by binomial logistic regression for each individual cell type. In experiments with zero observations in one cell type, we used Firth's bias-reduced logistic regression (package *logistf*)<sup>93</sup> to avoid the nonsensical results arising from the 'complete separation' of data.<sup>94</sup> All statistical tests and *p*-values of significance are specified in the corresponding figure legend; numbers of subjects are described either in the figure legend or Table S1 and Table S3. RNA-seq and scRNA-seq analyses are described in detail in their specific section and the code repository indicated there.



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**Supplemental information**

**A bHLH interaction code  
controls bipotential differentiation  
and self-renewal in the *Drosophila* gut**

**Aleix Puig-Barbe, Svenja Dettmann, Vinícius Dias Nirello, Helen Moor, Sina Azami, Bruce A. Edgar, Patrick Varga-Weisz, Jerome Korzelius, and Joaquín de Navascués**

## SUPPLEMENTARY FIGURE LEGENDS

### Figure S1. Da monomers induce ISC/EB death by apoptosis, Related to Figure 1.

**A-B.** MARCM method: (A) the FRT-FLP system is used to induce recombination in G2, which during mitosis (M) can result in co-segregation of sister chromatid arms. (B) After induction, some ISCs generate sparse clones, allowing lineage tracing. **C.** The *esg<sup>TS</sup>-FO* system allows simultaneous manipulation and tracing of ISCs/EBs. Top row: *esg-Gal4*, *UAS-GFP* is expressed specifically in ISCs/EBs. Bottom row: after induction, *Actin5C-Gal4* is reconstituted irreversibly in ISCs/EBs; they and their offspring will maintain GFP expression regardless of fate changes. **D.** Cell type markers used: ISCs are *DI<sup>+</sup>* or *Hdc<sup>+</sup>/NRE-lacZ<sup>-</sup>*; EEs, *Pros<sup>+</sup>*; preEEs, *DI<sup>+</sup>/Pros<sup>+</sup>*; EBs, *NRE-lacZ<sup>+</sup>/Hdc<sup>+</sup>*; and ECs are *myoIA-lacZ<sup>+</sup>* and polyploid. **E.** Cell composition of GFP<sup>+</sup> tissue from **Figure 1J-K**. *p*-values (binomial regression for individual cell types): ● < 0.05; ●● < 0.01; ●●● < 0.001. See **Tables S1-2** for statistical details. **F.** Sequences used to generate *da<sup>RNAi</sup>* and *da:da* transgenes; the *da<sup>10</sup>* allele deletes the entire *da* locus.

### Figure S2. Da:Sc and Da:Da collaborate to induce proliferation, Related to Figure 2.

**A-B.** *sc* expression with *esg<sup>TS</sup>-FO* (A) leads to formation of EEs and preEEs (A, empty asterisks and arrowheads, respectively) at the expense of EBs (A-B, solid arrowheads). Simultaneous knockdown of *da* (B) suppresses EE and preEE formation. Solid asterisks: extant EEs. **C.** Cell composition of GFP<sup>+</sup> tissue from A-B. **D-F.** Mitotic events (phospho-Histone 3, PH3) observed upon overexpression of *sc* (D), *sc* and *da* (E) or *sc* and tethered *da:da* (F) with *esg<sup>TS</sup>-FO*. **G.** Quantification of mitosis in whole posterior midguts of wild-type and conditions in A-C. Long and short horizontal bars represent the mean values ± SEM, respectively. Numbers are 15, 10, 11 and 9 guts for wild-type, *sc*, *sc+da* and *sc+da:da*, respectively. *p*-values (Kruskall-Wallis/Dunn tests): \* < 0.05; \*\* < 0.01; \*\*\* < 0.001. DAI: days after induction. Scale bars: 20 μm.

### Figure S3. Da:Da and Da:Sc cooperate to induce the ISC transcriptional signature, Related to Figure 3.

**A.** PCA plot of RNAseq samples after log-normalisation and batch correction. PC1 segregates the overexpression of *sc* from the other conditions. PC2 is driven by the sequence *da<sup>RNAi</sup>* < control < *da* < *da:da*. **B-C.** Log<sub>2</sub>FC heatmap for the *E(spl)*-Complex genes (B) and 57 genes specific in expression or function for a midgut cell type (coloured as in **Figure 1I**) (C), for *sc*, *da<sup>RNAi</sup>*, *da* and *da:da* RNAseq data. Non-HLH

*E(spl)*-C genes are Bearded Family Members (BFM). **D-E.** Normalised enrichment scores (NES) heatmaps for GLAD gene sets (D) or KEGG pathways (E). Coloured asterisks in heatmaps indicate significance as per each column map. Column order follows hierarchical clustering.

**Figure S4. Sc confers ISC properties to EBs, Related to Figure 4.**

**A.** The *NRE<sup>TS</sup>*-*FO* system allows simultaneous manipulation and tracing of EBs. Top row: *NRE-Gal4*, *UAS-GFP* is expressed specifically in EBs. Bottom row: after induction, *Actin5C-Gal4* is reconstituted irreversibly in EBs; they and their offspring will maintain GFP expression regardless of fate changes. **B.** t-distributed stochastic neighbor embedding (t-SNE) representation of two integrated scRNAseq datasets from the adult intestine. All the annotated cell types are identified, and ISCs and EBs are distinguished from each other by the combined expression of *DI*, *sna*, *polo* and *cnn* in ISCs, and *klu*, *E(spl)m3-HLH*, *E(spl)mα-BFM* and *E(spl)mβ-HLH* in EBs.

**Figure S5. *emc* is expressed in EBs/ECs and characterization of *emc<sup>RNAi</sup>* transgenes, Related to Figure 5.**

**A-H.** Gene expression (in logcounts) by cell type, assessed with scRNAseq. *emc* (A) is expressed predominantly in EBs and posterior midgut ECs (pEC), with specificity similar to or higher than established EC markers *LManV*, *γ Try*, *β Try* and *myoIA* (B-E) and EB markers *E(spl)m3-HLH*, *klu* and *E(spl)mβ-HLH* (F-H). **I.** PCA plot with the transcriptional trajectories from ISCs/EBs into EEs or pECs derived from scRNAseq data. *sc<sup>+</sup>* and *sc<sup>-</sup>* ISCs/EBs from **Figure 4H** are indicated in black and yellow, respectively. **J.** Molecular nature of alleles *emc<sup>LL02590</sup>*, *emc<sup>1</sup>*, *emc<sup>AP6</sup>* and *emc<sup>P5C</sup>*, and protein-trap *emc<sup>CPT1002740</sup>*. **K-N.** Characterization of the efficiency of *emc<sup>RNAi</sup>* transgenes *KK108316* and *JF02300* together (L) or *NIG-1007R* (M) expressed with *hh-Gal4*. Fold change in anti-Emc detection (N) is calculated as the ratio of nuclear staining in the anterior (GFP<sup>-</sup>, left) wing compartment versus the affected posterior (GFP<sup>+</sup>, right) compartment, normalised across individual discs by the average anterior/posterior ratio in the wild-type controls (K). Each data point represents mean sample value, with short bars depicting SEM, and red horizontal bars, mean values per genetic condition. **O-R.** Nota of flies bearing the *MS248* driver (O) and individual *emc<sup>RNAi</sup>* transgenes, as indicated (P-R), showing increasingly strong *extra macrochaetae* phenotype.

**Figure S6. *emc* is required to prevent *esg<sup>+</sup>* cell death and to repress *DI* in EBs, Related to Figure 6.**

**A-B.** Expressing *emc*<sup>RNAi</sup><sub>NIG</sub> with *esg*<sup>TS</sup>-FO leads to loss of ISCs/EBs (A); this is prevented by co-expression with *p35* (B). Arrowheads: *esg*<sup>+</sup> cells. **C.** Cell composition of GFP<sup>+</sup> tissue from A-B. *p*-values (binomial regression for individual cell types): ● < 0.05; ●● < 0.01; ●●● < 0.001. See **Tables S1-2** for statistical details. **D-E.** The *NRE-Gal4* (D) driver is expressed in EBs at high (solid arrowheads) and lower levels (empty arrowheads), but never in DI<sup>+</sup> cells. In *NRE-Gal4, UAS-emc*<sup>RNAi</sup> intestines (E), many EBs co-express DI (empty arrowheads). DAI: days after induction. Scale bars: 20μm.

**Figure S7. *emc* and Notch work in parallel and there is no *da-esg* cross-regulation, Related to Figure 7.**

**A-C.** The co-expression of *H* and *emc* (B) increases EC differentiation (solid arrowheads) compared to *H* overexpression (A), but not as extensively as with *emc* (C). **D.** Cell composition of GFP<sup>+</sup> tissue from A-C and E-G; data from **Figures 7I, 5S** are replicated here to aid comparison. *p*-values (binomial regression for individual cell types): ● < 0.05; ●● < 0.01; ●●● < 0.001. See **Tables S1-2** for statistical details. **E-G.** The simultaneous knock-down of *H* and *emc* (F) rescues the loss of ISCs (empty arrowheads) and EEs (asterisk) observed with *H*<sup>RNAi</sup> alone (E), but results in even further EC differentiation, and does not produce as many ISCs as with *emc*<sup>RNAi</sup> alone (G). **H.** Clonal expression with *Act5C-FO* of *N<sup>ICD</sup>*, the cleavage-activated Notch reporter (blue) does not modify the expression of the GFP protein-trap line *emc*<sup>CPT1002740</sup>. Solid arrowheads: ECs; empty arrowheads: EEs; asterisks: ISCs. **I.** Modified Euler diagram showing the overlap between gene sets repressed directly by *Esg* (white) with those either repressed by overexpression of *da* (blue) or *da:da* (yellow) or de-repressed by expression of *da*<sup>RNAi</sup> (red), all three at  $|\log_2(\text{FC})| \geq 1$ . Areas approximate set sizes and their intersections. Overlaps between *da*-regulated sets are not depicted. *p*-values for overlap sizes ( $\text{Esg} \cap \text{Da}$ ,  $\text{Esg} \cap \text{Da:Da}$  and  $\text{Esg} \cap \text{da}^{\text{RNAi}}$ ; hypergeometric test with Bonferroni correction) are: \*\*\* < 1e-36, \* < 0.01, \* < 0.05. **J-K.** Clonal expression with *Act5C-FO* of *esg* (J; blue) or *da:da* (K, green) does not modify the expression of the recombineering reporter *da:GFP* or the *lacZ* enhancer trap insertion *esg*<sup>k00606</sup>, respectively. Solid arrowheads: *da* or *esg*-mis-expressing cells in J, K, respectively; empty arrowheads: non-mis-expressing cells. DAI: days after induction. Scale bars: 20μm. Panels A, C, E, G are reproduced from **Figures, 7E, 5R, 7G, 5P**, respectively, to aid comparison.



Figure S1

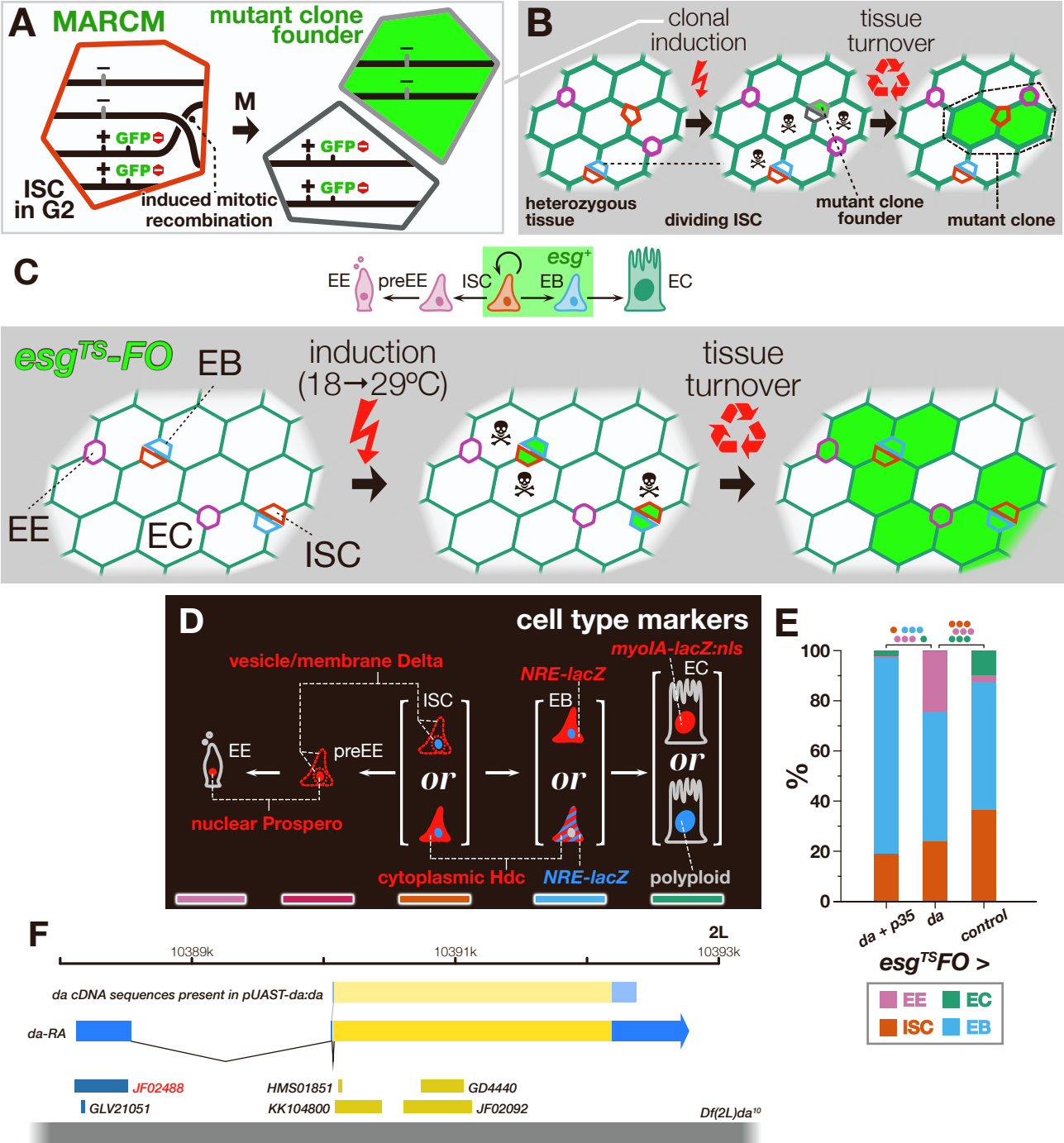
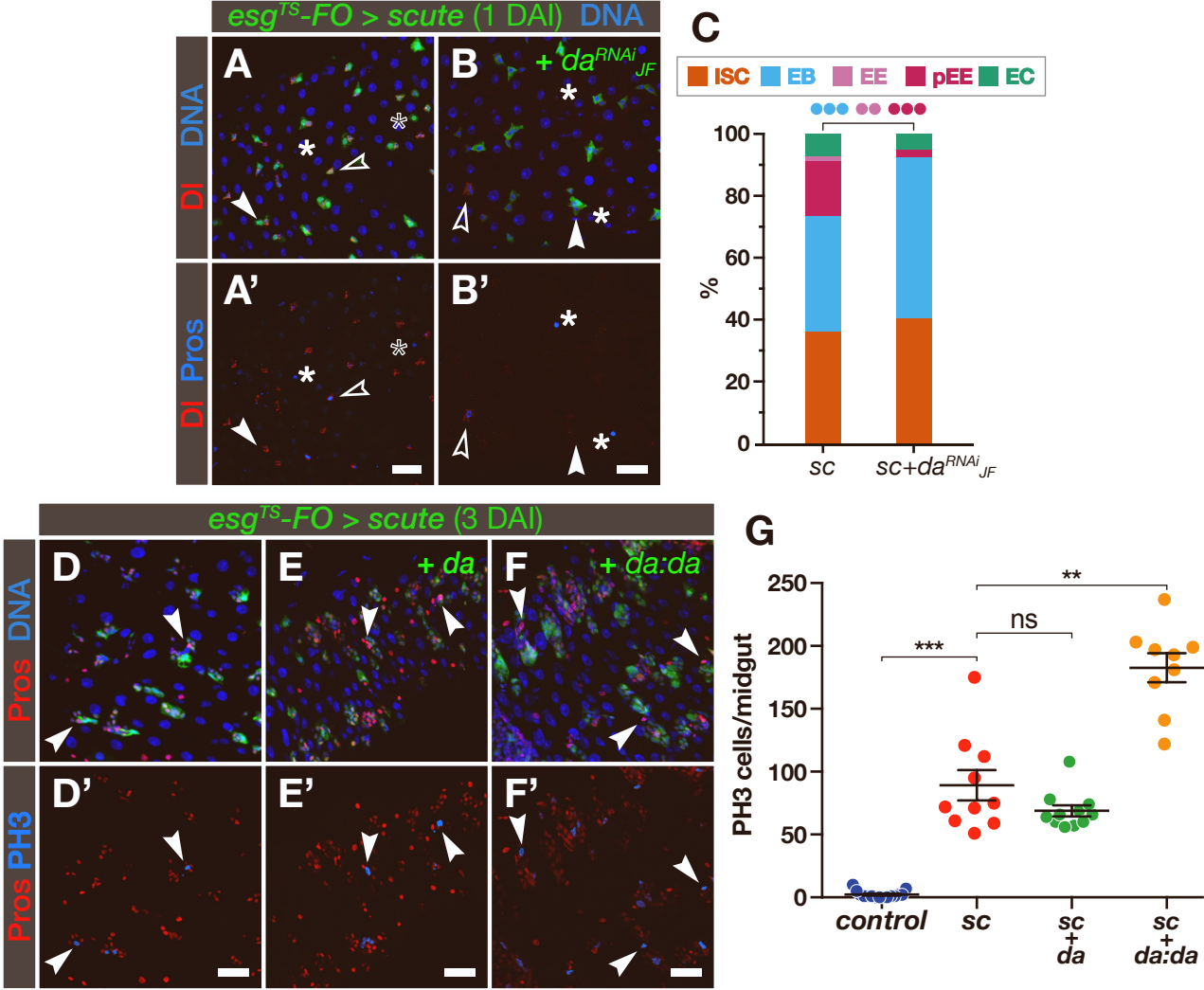


Figure S2



### Figure S3

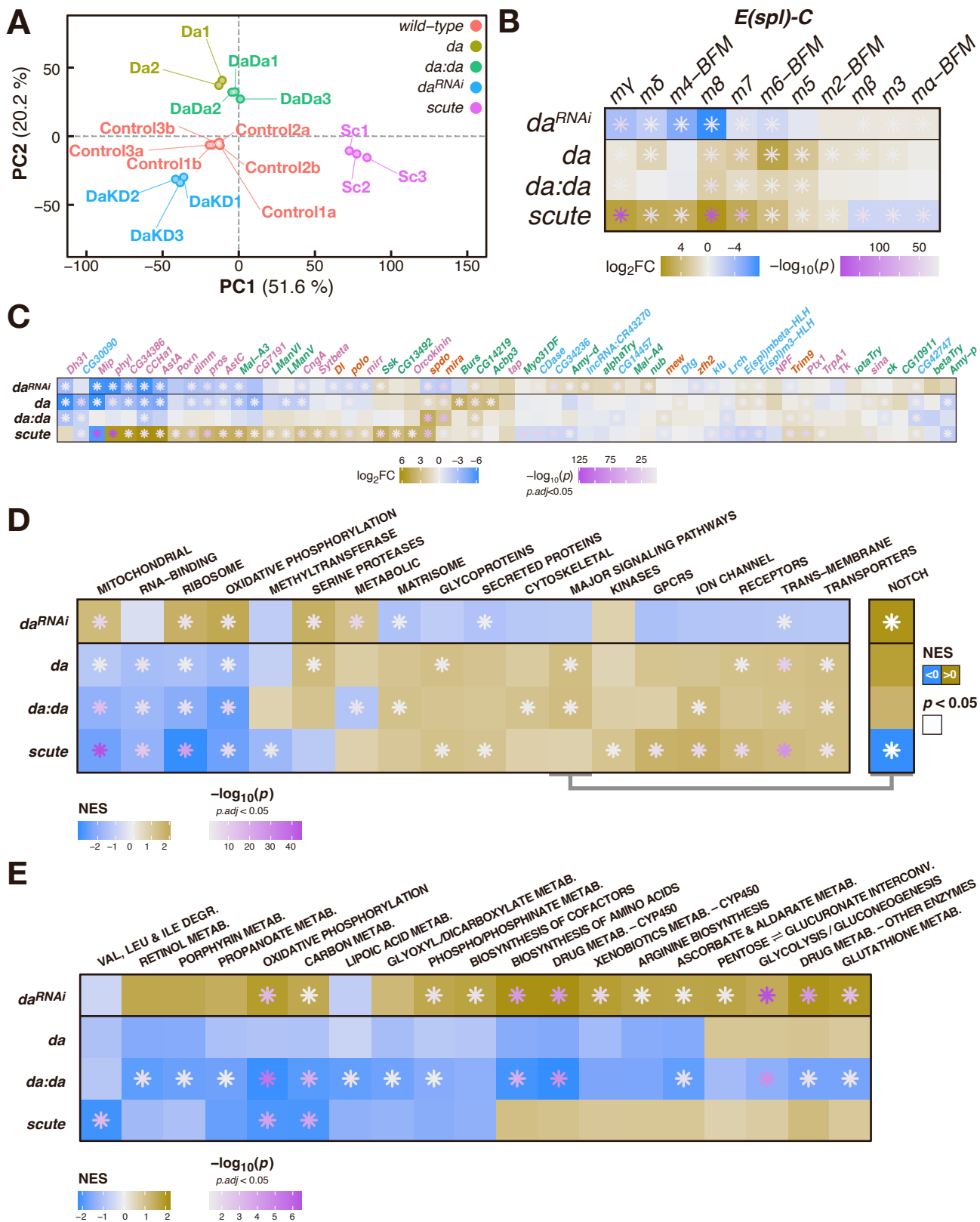


Figure S4

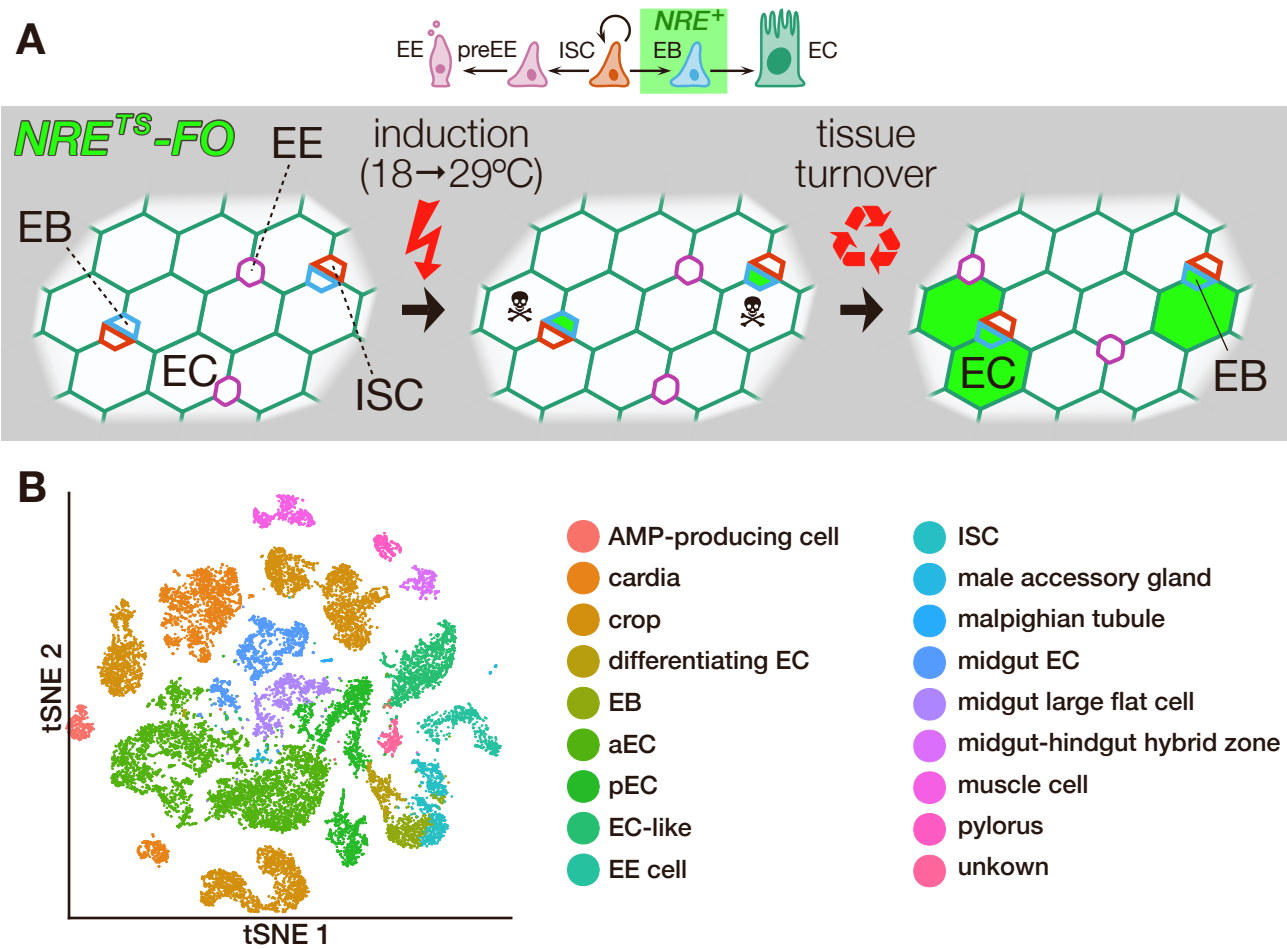




Figure S5

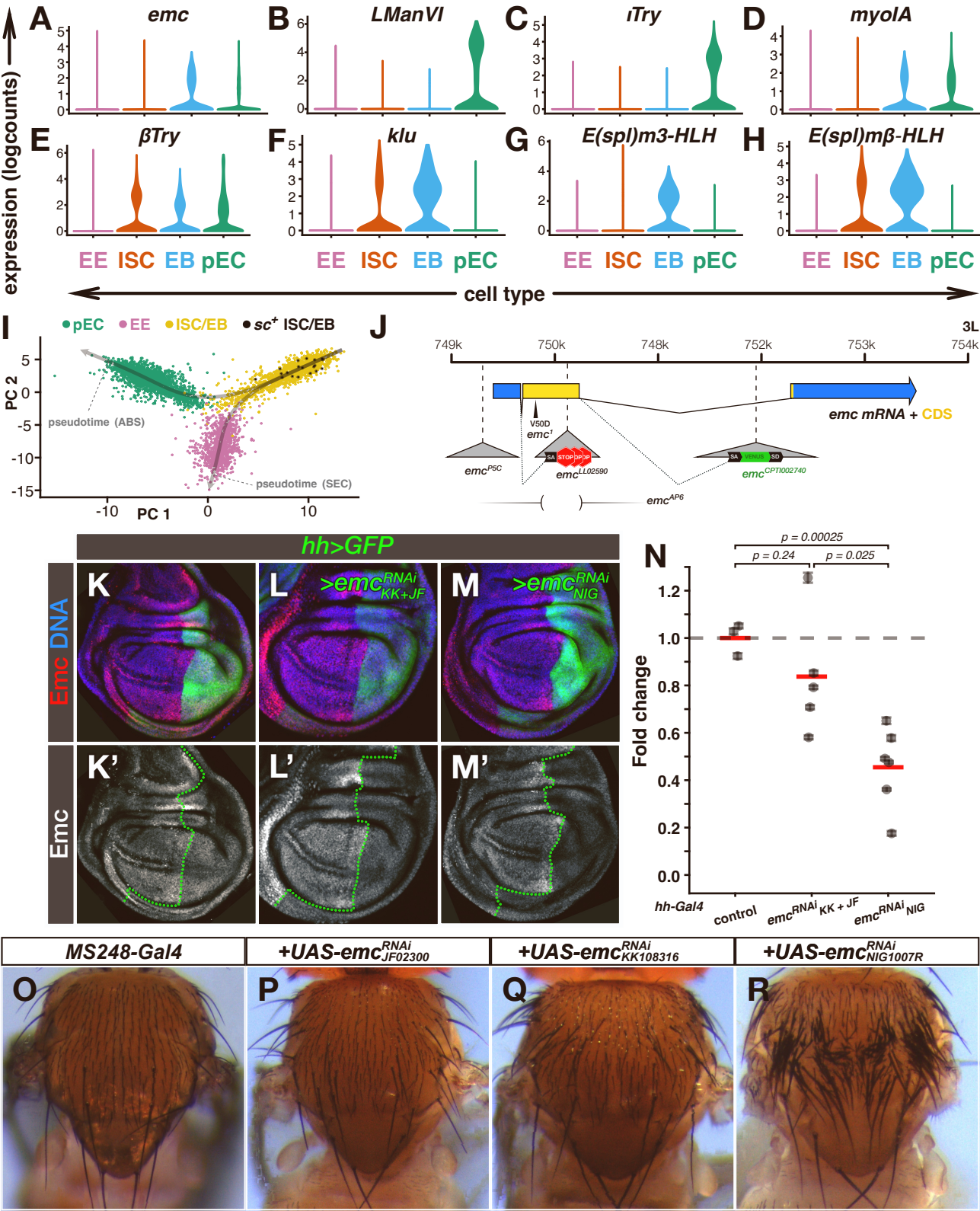


Figure S6

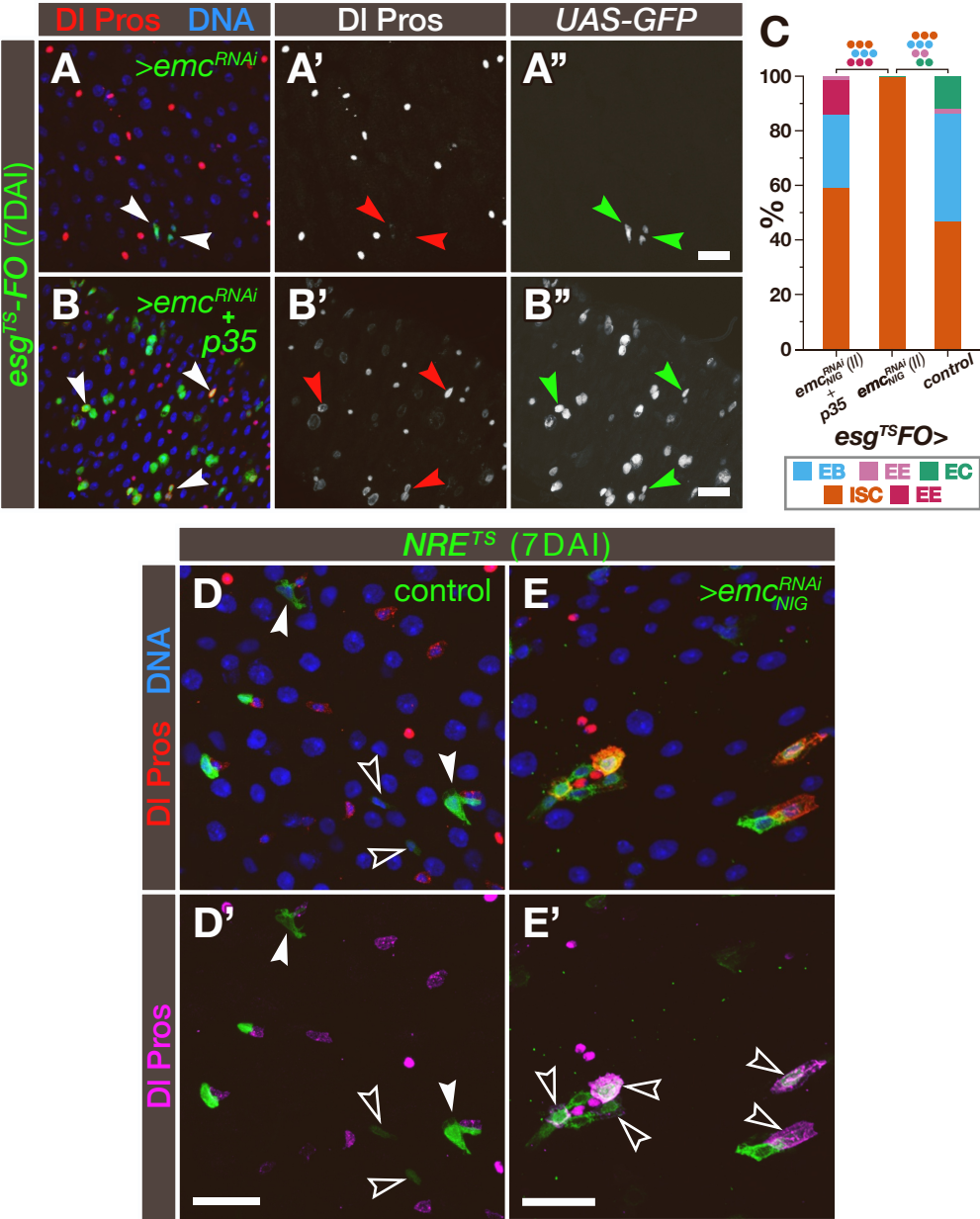
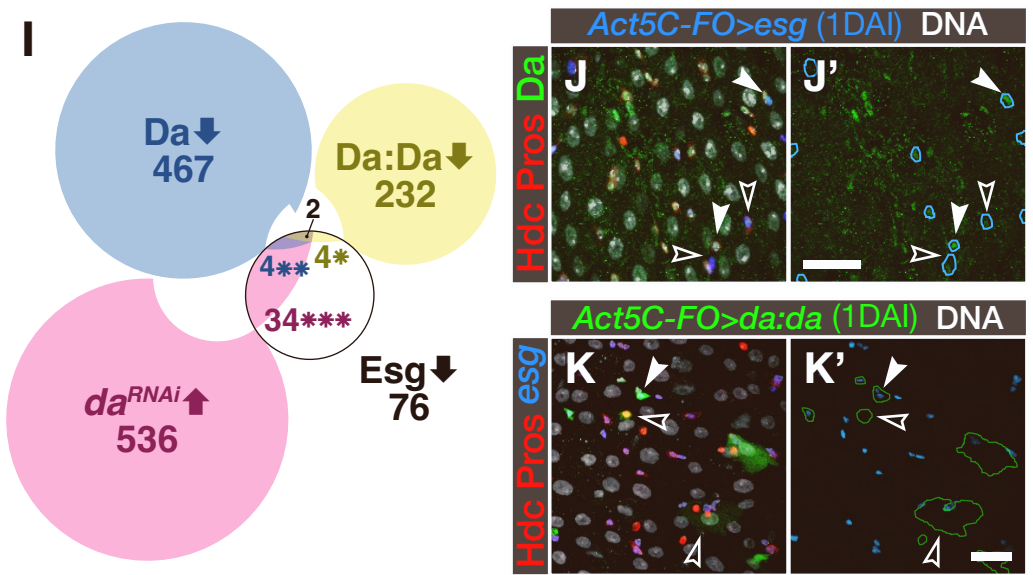
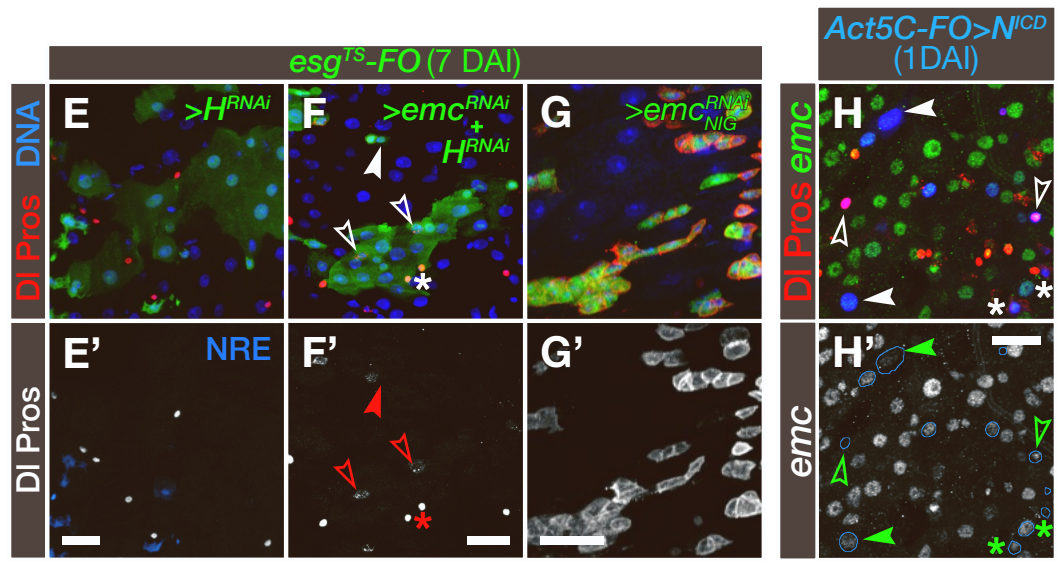
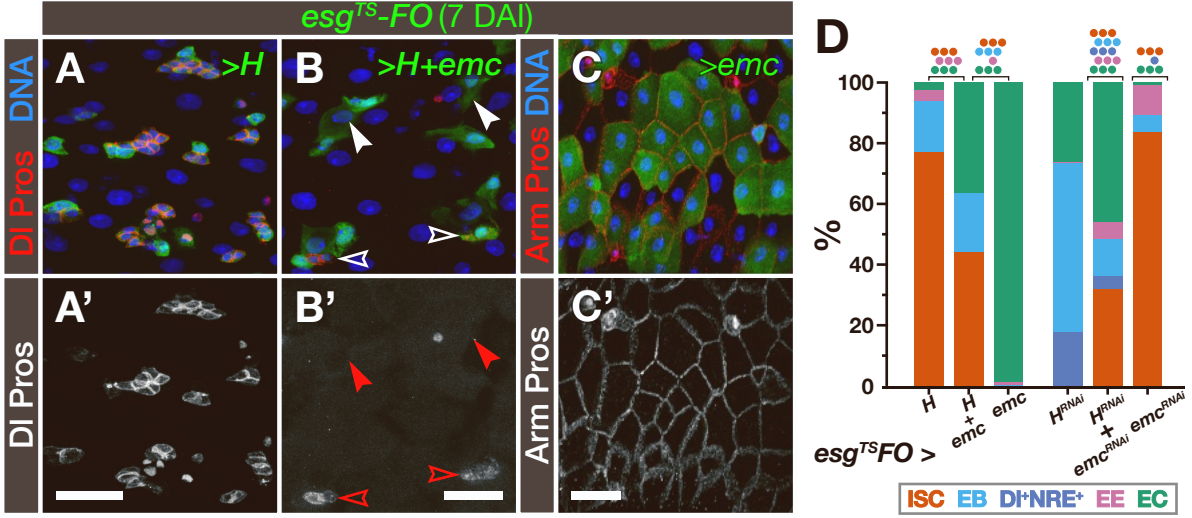


Figure S7



**Table S1. Binomial regression summary statistics, Related to STAR Methods.**

Figures	Allele/transgene	Reference genotype	Time (*)	Cell type tested	Effect	Odds Ratio	p-value (†)
<b>11</b>	<i>FRT<sub>40A</sub> da<sup>10</sup></i>	<i>FRT<sub>40A</sub> control</i>	7 DAI	ISC	↓↓↓	0.15	8e-08
				EB	↓↓↓	0.07	1.2e-28
				EE	↓↓↓	0.15	8.1e-05
				EC	↑↑↑	17	1.1e-47
	<i>esg<sup>TS-FO</sup> &gt; da<sup>RNAi</sup><sub>JF</sub></i>	<i>esg<sup>TS-FO</sup> control</i>	7 DAI	ISC	↓↓↓ <sup>(f)</sup>	1.1e-03	0
				EB	↓↓↓	2.4e-03	1.9e-17
				EE	—	0.61	0.14
				EC	↑↑↑	490	9.7e-109
	<i>esg<sup>TS-FO</sup> &gt; da<sup>RNAi</sup><sub>HMS</sub></i>	<i>esg<sup>TS-FO</sup> control</i>	7 DAI	ISC	↓↓↓ <sup>(f)</sup>	8e-04	0
				EB	↓↓↓	0.016	1e-63
				EE	↓↓	0.38	6e-03
				EC	↑↑↑	340	4.2e-164
	<i>esg<sup>TS-FO</sup> &gt; da</i>	<i>esg<sup>TS-FO</sup> control</i>	7 DAI	ISC	↓↓↓	0.55	2.6e-07
				EB	—	1	0.81
				EE	↑↑↑	12	8.4e-32
				EC	↓↓↓	0.017	4.8e-05
	<i>esg<sup>TS-FO</sup> &gt; da:da</i>	<i>esg<sup>TS-FO</sup> control</i>	7 DAI	ISC	—	0.89	0.22
				EB	↑↑↑	1.9	6.3e-10
				EE	↓↓	0.16	2.6e-03
				EC	↓↓↓ <sup>(f)</sup>	6.7e-03	0
	<i>esg<sup>TS-FO</sup> &gt; da:da + da<sup>RNAi</sup><sub>JF</sub></i>	<i>esg<sup>TS-FO</sup> control</i>	7 DAI	ISC	↓↓	0.8	6.1e-03
				EB	↑↑↑	2.1	5.6e-20
				EE	↓↓↓ <sup>(f)</sup>	0.012	2.5e-12
				EC	↓↓↓ <sup>(f)</sup>	3e-03	0



<b>S1E</b>	<i>esg<sup>TS</sup>-FO</i> > <i>da</i> + <i>p35</i>	<i>esg<sup>TS</sup>-FO</i> > <i>da</i>	7 DAI	ISC	↓	0.76	0.019
				EB	↑↑↑	3.3	1.8e-30
				EE	↓↓↓	0.018	1.6e-30
				EC	↑	12	0.016
<b>2D</b>	<i>esg<sup>TS</sup>-FO</i> > <i>sc</i> + <i>da</i>	<i>esg<sup>TS</sup>-FO</i> > <i>sc</i>	3 DAI	ISC	↑↑↑	6.3	2.8e-32
				EB	↓↓	0.44	1.8e-03
				EE	↓↓↓	0.18	6.9e-67
				EC	↓↓↓	0.1	2.7e-04
				PEE	↑↑↑	1.6	3e-11
<b>S2C</b>	<i>esg<sup>TS</sup>-FO</i> > <i>sc</i> + <i>da<sup>RNAi</sup><sub>JF</sub></i>	<i>esg<sup>TS</sup>-FO</i> > <i>sc</i>	1 DAI	ISC	—	1.2	0.16
				EB	↑↑↑	1.8	5.6e-08
				EE	↓↓	0.13	8e-03
				EC	—	0.76	0.24
				PEE	↓↓↓	0.11	2e-18
<b>4E</b>	<i>NRE<sup>TS</sup>-FO</i> > <i>da</i>	<i>NRE<sup>TS</sup>-FO</i> control	7 DAI	ISC	↑↑↑	12	2.5e-19
				EB	—	1.2	0.19
				EE	—	0.13	0.064
				EC	↓↓↓	0.028	1.1e-22
				PEE	↑ <sup>(f)</sup>	10	0.028
	<i>NRE<sup>TS</sup>-FO</i> > <i>da:da</i>	<i>NRE<sup>TS</sup>-FO</i> control	7 DAI	ISC	↑↑↑	5.1	7.8e-07
				EB	↑↑↑	2.7	4.8e-07
				EE	—	1.4	0.68
				EC	↓↓↓ <sup>(f)</sup>	5.9e-03	0
	<i>NRE<sup>TS</sup>-FO</i> > <i>sc</i>	<i>NRE<sup>TS</sup>-FO</i> control	7 DAI	ISC	↑↑↑	9.9	7e-15
				EB	↓↓↓	0.19	9.6e-48
				EE	↑↑↑	100	5.2e-24
				EC	↓↓↓ <sup>(f)</sup>	4.2e-03	0
				PEE	↑↑↑ <sup>(f)</sup>	170	0

<b>5J</b>	<i>FRT</i> <sub>2A</sub> <i>emc</i> <sup>LL02590</sup>	<i>FRT</i> <sub>2A</sub> control	7 DAI	ISC	↑↑↑	2.9	2.2e-07
				EB	↓↓	0.58	7.5e-03
				EE	—	0.8	0.66
				EC	↓↓↓	0.19	1e-04
	<i>FRT</i> <sub>80B</sub> <i>emc</i> <sup>AP6</sup>	<i>FRT</i> <sub>80B</sub> control	7 DAI	ISC	↑	1.5	0.027
				EB	↑↑	1.8	1.8e-03
				EE	↓↓↓	0.12	4.7e-04
				EC	↓↓↓	0.28	4.8e-05
	<i>FRT</i> <sub>80B</sub> <i>emc</i> <sup>1</sup>	<i>FRT</i> <sub>80B</sub> control	7 DAI	ISC	↑↑↑	2.2	4.4e-04
				EB	—	1.2	0.45
				EE	—	0.71	0.38
				EC	↓↓↓	0.035	9.8e-04
<b>5N</b>	<i>emc</i> <sup>LL02590</sup> / <i>emc</i> <sup>1</sup>	<i>emc</i> <sup>LL02590</sup> / +	11-14 DAE	ISC	↑↑	1.3	1.4e-03
				EB	↑	1.2	0.017
				EE	↑↑↑	1.6	8.3e-06
				EC	↓↓↓	0.64	4.4e-16
				PEE	— ( <i>f</i> )	8.4	0.079
				DI* EC	↑↑↑	58	5.7e-05
	<i>emc</i> <sup>LL02590</sup> / <i>emc</i> <sup>P5C</sup>	<i>emc</i> <sup>LL02590</sup> / +	11-14 DAE	ISC	↑↑↑	1.4	1.7e-07
				EB	↑↑↑	1.3	2.2e-04
				EE	—	1.1	0.35
				EC	↓↓↓	0.45	1.4e-48
				PEE	— ( <i>f</i> )	3.5	0.41
				DI* EC	↑↑↑	320	8.8e-09
<b>5S</b>	<i>esg</i> <sup>TS-FO</sup> > <i>emc</i> <sup>RNAi</sup> <sub>NIG</sub> (III)	<i>esg</i> <sup>TS-FO</sup> control	7 DAI	ISC	↑↑↑	8.9	9.4e-16
				EB	↓↓↓	0.059	2.4e-11
				EE	↑↑↑	3.9	3.3e-04
				EC	↓	0.09	0.017



	<i>esg<sup>TS</sup>-FO</i> > <i>emc<sup>RNAi<sub>NIG</sub></sup></i> (II)	<i>esg<sup>TS</sup>-FO</i> control	7 DAI	ISC	↑↑↑	350	5.3e-09
				EB	↓↓↓ <sup>(f)</sup>	2.4e-03	0
				EE	↓↓ <sup>(f)</sup>	0.09	8.6e-03
				EC	↓↓	0.046	2.2e-03
	<i>esg<sup>TS</sup>-FO</i> > <i>emc<sup>RNAi<sub>KK+JF</sub></sup></i>	<i>esg<sup>TS</sup>-FO</i> control	7 DAI	ISC	↑	1.2	0.033
				EB	↓↓↓	0.12	4.9e-64
				EE	↓↓	0.31	5.9e-03
				EC	↓↓↓	0.16	1.3e-10
				DI*NRE <sup>+</sup>	↑↑↑ <sup>(f)</sup>	1850	0
	<i>esg<sup>TS</sup>-FO</i> > <i>emc</i>	<i>esg<sup>TS</sup>-FO</i> control	7 DAI	ISC	↓↓↓ <sup>(f)</sup>	1.1e-03	0
				EB	↓↓↓	2.4e-3	1.7e-17
				EE	↓↓	0.14	1e-03
				EC	↑↑↑	1500	6.3e-57
6E	<i>FRT<sub>40A</sub> da<sup>10</sup></i> + <i>emc<sup>RNAi<sub>NIG</sub></sup></i>	<i>FRT<sub>40A</sub></i> control	7 DAI	ISC	↓↓	0.054	4e-03
				EB	↓↓↓	0.058	8.5e-10
				EE	↓	0.21	0.035
				EC	↑↑↑	23	1.5e-16
	<i>FRT<sub>40A</sub> da<sup>10</sup></i> + <i>emc<sup>RNAi<sub>NIG</sub></sup></i>	<i>FRT<sub>40A</sub> da<sup>10</sup></i>	7 DAI	ISC	—	0.36	0.33
				EB	—	0.78	0.62
				EE	—	1.6	0.66
				EC	—	1.3	0.47
	<i>FRT<sub>2A</sub> emc<sup>LL02590</sup></i> + <i>da<sup>RNAi<sub>JF</sub></sup></i>	<i>FRT<sub>2A</sub></i> control	7 DAI	ISC	↓↓↓	0.077	4.3e-18
				EB	↓↓↓	0.17	8.4e-18
				EE	↓↓	0.15	4.9e-03
				EC	↑↑↑	27	1.2e-43

	<i>FRT</i> <sub>2A</sub> <i>emc</i> <sup>LL02590</sup> + <i>da</i> <sup>RNAi</sup> <sub>JF</sub>	<i>FRT</i> <sub>2A</sub> <i>emc</i> <sup>LL02590</sup>	7 DAI	ISC	↓↓↓	0.026	7.4e-36
				EB	↓↓↓	0.29	3.3e-09
				EE	↓	0.19	0.014
				EC	↑↑↑	150	1.3e-34
6J	<i>NRE</i> <sup>TS</sup> -FO > <i>emc</i> <sup>RNAi</sup> <sub>NIG</sub>	<i>NRE</i> <sup>TS</sup> -FO control	7 DAI	ISC	↑↑↑	240	1.8e-87
				EB	↓↓↓	0.065	1.5e-162
				EE	↓↓ <sup>(f)</sup>	0.058	6.2e-03
				EC	↓↓↓	6.7e-03	2.2e-12
S6C	<i>esg</i> <sup>TS</sup> -FO > <i>emc</i> <sup>RNAi</sup> <sub>NIG</sub> (II) + p35	<i>esg</i> <sup>TS</sup> -FO > <i>emc</i> <sup>RNAi</sup> <sub>NIG</sub> (II)	7 DAI	ISC	↓↓↓	0.0073	9.2e-07
				EB	↑↑↑ <sup>(f)</sup>	146	0
				EE	— <sup>(f)</sup>	5.6	0.11
				EC	— <sup>(f)</sup>	0.063	0.063
				PEE	↑↑↑ <sup>(f)</sup>	60	1.1e-11
7D	<i>esg</i> <sup>TS</sup> -FO > <i>N</i> <sup>RNAi</sup> + <i>da</i> <sup>RNAi</sup> <sub>JF</sub>	<i>esg</i> <sup>TS</sup> -FO > <i>da</i> <sup>RNAi</sup> <sub>JF</sub>	7 DAI	ISC	↑↑↑ <sup>(f)</sup>	130	0
				EB	—	2.2	0.3
				EE	—	0.51	0.076
				EC	↓↓↓	0.20	3e-09
	<i>esg</i> <sup>TS</sup> -FO > <i>N</i> <sup>RNAi</sup> + <i>emc</i>	<i>esg</i> <sup>TS</sup> -FO > <i>emc</i>	7 DAI	ISC	↑↑↑ <sup>(f)</sup>	175	0
				EB	↑↑↑	12	0.00076
				EE	—	2.3	0.22
				EC	↓↓↓	0.030	1.3e-14
				DI-NRE-	↑↑↑ <sup>(f)</sup>	67	7.1e-11
7I	<i>esg</i> <sup>TS</sup> -FO > <i>H</i>	<i>esg</i> <sup>TS</sup> -FO > <i>H</i> + <i>da</i> <sup>RNAi</sup> <sub>JF</sub>	7 DAI	ISC	↑↑↑	16	2.5e-216
				EB	↑↑↑	310	1e-08
				EE	↑↑↑	3.2	1.5e-05
				EC	↓↓↓	5e-03	6.8e-173
				DI-NRE-	↓↓↓ <sup>(f)</sup>	0.012	6.4e-13

	$esg^{TS-FO} > da^{RNAi}_{JF}$	$esg^{TS-FO} > H + da^{RNAi}_{JF}$	7 DAI	ISC	↓↓↓ <sup>(f)</sup>	2.8e-03	0
				EB	—	3.8	0.28
				EE	—	1.4	0.4
				EC	↑↑↑	15	1.3e-23
				DI*NRE <sup>-</sup>	↓↓↓ <sup>(f)</sup>	0.026	2.8e-07
	$esg^{TS-FO} > H^{RNAi}_{HMS}$	$esg^{TS-FO} > H^{RNAi}_{HMS} + da:da$	7 DAI	EB	↓↓↓	0.01	6.8e-24
				EE	—	0.62	0.45
				EC	↑↑↑ <sup>(f)</sup>	430	0
				DI*NRE <sup>+</sup>	↑↑↑ <sup>(f)</sup>	260	0
	$esg^{TS-FO} > da:da$	$esg^{TS-FO} > H^{RNAi}_{HMS} + da:da$	7 DAI	ISC	↑↑↑ <sup>(f)</sup>	620	0
				EB	↓↓↓	0.016	1.1e-19
				EE	—	0.53	0.38
<b>7N</b>	$esg^{TS-FO} > esg^{RNAi}_{HMS}$	$esg^{TS-FO} > esg^{RNAi}_{HMS} + da:da$	7 DAI	ISC	↓↓↓	2.3e-03	1.2e-09
				EB	↓↓↓	2.4e-03	2.6e-17
				EE	↑↑↑	4.4	2.8e-07
				EC	↑↑↑ <sup>(f)</sup>	21500	0
	$esg^{TS-FO} > da:da$	$esg^{TS-FO} > esg^{RNAi}_{HMS} + da:da$	7 DAI	ISC	↓↓	0.72	1.4e-03
				EB	↑↑↑	1.5	1.8e-04
				EE	↓	0.24	0.021
	$esg^{TS-FO} > esg$	$esg^{TS-FO} > esg + da^{RNAi}_{JF}$	7 DAI	ISC	↓↓↓	0.32	4.2e-34
				EB	↑↑↑	3.1	7.7e-34
				EE	— <sup>(f)</sup>	4.4	0.33
	$esg^{TS-FO} > da^{RNAi}_{JF}$	$esg^{TS-FO} > esg + da^{RNAi}_{JF}$	7 DAI	ISC	↓↓↓ <sup>(f)</sup>	3.9e-04	0
				EB	↓↓↓	4e-03	7.9e-15
				EE	↑↑↑ <sup>(f)</sup>	42	3.6e-06
				EC	↑↑↑ <sup>(f)</sup>	126000	0

<b>S7D</b>	$esg^{TS-FO} > H$	$esg^{TS-FO} > H + emc$	7 DAI	ISC	↑↑↑	4.4	1.5e-76
				EB	—	0.86	0.12
				EE	↑↑↑ <sup>(f)</sup>	100	1.1e-16
				EC	↓↓↓	0.031	1.9e-76
	$esg^{TS-FO} > emc$	$esg^{TS-FO} > H + emc$	7 DAI	ISC	↓↓↓ <sup>(f)</sup>	7.8e-04	0
				EB	↓↓↓	0.01	1.4e-10
				EE	↑ <sup>(f)</sup>	11	0.042
				EC	↑↑↑	280	1.3e-35
	$esg^{TS-FO} > H^{RNAi_{HMS}}$	$esg^{TS-FO} > H^{RNAi_{HMS}} + emc^{RNAi_{NIG}}$	7 DAI	ISC	↓↓↓ <sup>(f)</sup>	1.1e-03	0
				EB	↑↑↑	9.3	2.9e-15
				EE	↓↓↓	0.087	3.8e-05
				EC	↓↓↓	0.41	5e-06
				DI*NRE <sup>+</sup>	↑↑↑	4.3	6.3e-04
	$esg^{TS-FO} > emc^{RNAi_{NIG}}$ (III)	$esg^{TS-FO} > H^{RNAi_{HMS}} + emc^{RNAi_{NIG}}$	7 DAI	ISC	↑↑↑	11	2.2e-13
				EB	—	0.45	0.12
				EE	—	1.8	0.25
				EC	↓↓↓	0.011	1.2e-05
				DI*NRE <sup>+</sup>	↓ <sup>(f)</sup>	0.089	0.023

\* DAI, days after transgene or clone induction. DAE, days after emergence.

<sup>†</sup> ↑, ↑↑, or ↑↑↑ indicate  $p < 0.05$ ,  $p < 0.01$  or  $p < 0.001$ , respectively, for increased cell type frequency; ↓ is used instead of ↑ to indicate significance for decreased cell type frequency.

<sup>f</sup> Cases where Firth's bias reduced logistic regression was used. See Methods.

Table S2. Cell type proportions in the genotypes analysed, Related to STAR Methods.

Figure	Genotype	Time		Cells		Cell Types (%)							
		Days	since (*)	Total	within (†)	ISCs	EBs	EEs	ECs	PEEs	DI <sup>+</sup> NRE <sup>+</sup>	DI <sup>-</sup> NRE <sup>-</sup>	DI <sup>+</sup> ECs
1A	<i>FRT40A control</i>	7	induction	443	12	12.0	39.7	6.5	41.8	0.0	0.0	0.0	0.0
1B	<i>FRT40A da10</i>	7	induction	495	54	2.0	4.6	1.0	92.3	0.0	0.0	0.0	0.0
1C	<i>esg<sup>TS</sup>-FO control</i>	7	induction	1135	15	36.5	51.1	2.6	9.8	0.0	0.0	0.0	0.0
1D	<i>esg<sup>TS</sup>-FO &gt; da<sup>RNAi</sup><sub>JF</sub></i>	7	induction	803	15	0.0	0.2	1.6	98.1	0.0	0.0	0.0	0.0
1E	<i>esg<sup>TS</sup>-FO &gt; da<sup>RNAi</sup><sub>HMS</sub></i>	7	induction	1086	19	0.0	1.7	1.0	97.3	0.0	0.0	0.0	0.0
1F	<i>esg<sup>TS</sup>-FO &gt; da</i>	7	induction	549	14	23.9	51.7	24.2	0.2	0.0	0.0	0.0	0.0
1G	<i>esg<sup>TS</sup>-FO &gt; da:da</i>	7	induction	693	13	33.6	65.9	0.4	0.0	0.0	0.0	0.0	0.0
1H	<i>esg<sup>TS</sup>-FO &gt; da:da + da<sup>RNAi</sup><sub>JF</sub></i>	7	induction	1578	18	31.4	68.6	0.0	0.0	0.0	0.0	0.0	0.0
1K	<i>esg<sup>TS</sup>-FO &gt; da + p35</i>	7	induction	1554	21	19.2	78.2	0.6	2.1	0.0	0.0	0.0	0.0
2A	<i>esg<sup>TS</sup>-FO &gt; sc</i>	3	induction	1241	6	3.9	2.7	33.8	1.4	58.3	0.0	0.0	0.0
2B	<i>esg<sup>TS</sup>-FO &gt; sc + da</i>	3	induction	2131	10	20.5	1.2	8.6	0.1	69.6	0.0	0.0	0.0
2C	<i>esg<sup>TS</sup>-FO &gt; sc + da:da (#)</i>	3	induction	100	0	49.0	1.0	5.0	0.0	45.0	0.0	0.0	0.0
S2A	<i>esg<sup>TS</sup>-FO &gt; sc</i>	1	induction	553	11	36.3	37.4	1.8	6.5	17.9	0.0	0.0	0.0
S2B	<i>esg<sup>TS</sup>-FO &gt; sc + da<sup>RNAi</sup><sub>JF</sub></i>	1	induction	853	17	40.1	52.3	0.2	5.0	2.3	0.0	0.0	0.0
4A	<i>NRE<sup>TS</sup>-FO control</i>	7	induction	1032	17	1.4	82.7	0.5	15.5	0.0	0.0	0.0	0.0
4B	<i>NRE<sup>TS</sup>-FO &gt; da</i>	2	induction	1563	20	14.4	84.6	0.1	0.5	0.4	0.0	0.0	0.0
4C	<i>NRE<sup>TS</sup>-FO &gt; da:da</i>	2	induction	460	4	6.5	92.8	0.7	0.0	0.0	0.0	0.0	0.0
4D	<i>NRE<sup>TS</sup>-FO &gt; sc</i>	7	induction	649	7	12.0	47.8	32.8	0.0	7.4	0.0	0.0	0.0
5E	<i>FRT2A control</i>	7	induction	194	106	34.5	45.4	4.6	15.5	0.0	0.0	0.0	0.0
5F	<i>FRT2A emc<sup>LL02590</sup></i>	7	induction	213	117	60.6	32.4	3.8	3.3	0.0	0.0	0.0	0.0
5G	<i>FRT80B control</i>	7	induction	310	117	26.5	42.3	11.0	20.3	0.0	0.0	0.0	0.0
5H	<i>FRT80B emc<sup>AP6</sup></i>	7	induction	208	110	35.6	56.2	1.4	6.7	0.0	0.0	0.0	0.0
5I	<i>FRT80B emc<sup>1</sup></i>	7	induction	112	95	44.6	46.4	8.0	0.9	0.0	0.0	0.0	0.0
5K	<i>emc<sup>LL02590</sup> / +</i>	11-14	emergence	2964	10	16.6	17.9	5.6	59.8	0.0	0.0	0.0	0.0
5L	<i>emc<sup>LL02590</sup> / emc<sup>1</sup></i>	11-14	emergence	2488	12	20.0	20.5	8.7	48.8	3.0	0.0	0.0	1.9



<b>5M</b>	<i>emc</i> <sup>LL02590</sup> / <i>emc</i> <sup>P5C</sup>	11-14	emergence	2582	10	22.2	21.9	6.2	40.0	1.0	0.0	0.0	9.7
<b>5P</b>	<i>esg</i> <sup>TS-FO</sup> > <i>emc</i> <sup>RNAi</sup> <sub>NIG</sub> (III)	7	induction	104	22	83.7	5.8	9.6	1.0	0.0	0.0	0.0	0.0
<b>5P</b>	<i>esg</i> <sup>TS-FO</sup> > <i>emc</i> <sup>RNAi</sup> <sub>NIG</sub> (II)	7	induction	202	9	99.5	0.0	0.0	0.5	0.0	0.0	0.0	0.0
<b>5Q</b>	<i>esg</i> <sup>TS-FO</sup> > <i>emc</i> <sup>RNAi</sup> <sub>KK+JF</sub>	7	induction	832	15	41.2	11.3	0.8	1.7	0.0	45.0	0.0	0.0
<b>5R</b>	<i>esg</i> <sup>TS-FO</sup> > <i>emc</i>	7	induction	812	10	0.0	0.2	0.4	99.4	0.0	0.0	0.0	0.0
<b>6B</b>	<i>FRT40A da</i> <sup>10</sup> + <i>emc</i> <sup>RNAi</sup> <sub>NIG</sub>	7	induction	137	23	0.7	3.6	1.5	94.2	0.0	0.0	0.0	0.0
<b>6C</b>	<i>FRT2A emc</i> <sup>LL02590</sup> + <i>da</i> <sup>RNAi</sup> <sub>JF</sub>	7	induction	412	35	3.9	12.1	0.7	83.3	0.0	0.0	0.0	0.0
<b>6I</b>	<i>NRE</i> <sup>TS-FO</sup> > <i>emc</i> <sup>RNAi</sup> <sub>NIG</sub>	7	induction	1628	16	76.4	23.5	0.0	0.1	0.0	0.0	0.0	0.0
<b>S6B</b>	<i>esg</i> <sup>TS-FO</sup> > <i>emc</i> <sup>RNAi</sup> <sub>NIG</sub> (II) + <i>p35</i>	7	induction	1072	30	59.3	26.5	1.3	0.0	12.9	0.0	0.0	0.0
<b>7A</b>	<i>esg</i> <sup>TS-FO</sup> > <i>N<sup>RNAi</sup></i>	7	induction	891	2	62.0	0.0	26.4	3.5	5.5	0.0	2.7	0.0
<b>7B</b>	<i>esg</i> <sup>TS-FO</sup> > <i>N<sup>RNAi</sup></i> + <i>da</i> <sup>RNAi</sup> <sub>JF</sub>	7	induction	1808	33	7.4	0.6	0.8	91.2	0.0	0.0	0.0	0.0
<b>7C</b>	<i>esg</i> <sup>TS-FO</sup> > <i>N<sup>RNAi</sup></i> + <i>emc</i>	7	induction	949	15	9.7	2.8	0.8	82.7	0.0	0.0	3.9	0.0
<b>7E</b>	<i>esg</i> <sup>TS-FO</sup> > <i>H</i>	7	induction	1810	21	77.5	17.0	3.7	1.8	0.0	0.0	0.0	0.0
<b>7F</b>	<i>esg</i> <sup>TS-FO</sup> > <i>H</i> + <i>da</i> <sup>RNAi</sup> <sub>JF</sub>	7	induction	1510	21	18.2	0.1	1.2	78.2	0.0	0.0	2.3	0.0
<b>7G</b>	<i>esg</i> <sup>TS-FO</sup> > <i>H<sup>RNAi</sup></i> <sub>HMS</sub>	7	induction	984	15	0.0	55.7	0.5	26.1	0.0	17.7	0.0	0.0
<b>7H</b>	<i>esg</i> <sup>TS-FO</sup> > <i>H<sup>RNAi</sup></i> <sub>HMS</sub> + <i>da:da</i>	7	induction	613	8	0.0	99.2	0.8	0.0	0.0	0.0	0.0	0.0
<b>7J</b>	<i>esg</i> <sup>TS-FO</sup> > <i>esg</i> <sup>RNAi</sup> <sub>HMS</sub>	7	induction	628	18	0.2	0.3	7.5	92.0	0.0	0.0	0.0	0.0
<b>7K</b>	<i>esg</i> <sup>TS-FO</sup> > <i>esg</i> <sup>RNAi</sup> <sub>HMS</sub> + <i>da:da</i>	7	induction	939	19	41.4	56.8	1.8	0.0	0.0	0.0	0.0	0.0
<b>7L</b>	<i>esg</i> <sup>TS-FO</sup> > <i>esg</i>	7	induction	850	9	34.1	65.8	0.1	0.0	0.0	0.0	0.0	0.0
<b>7M</b>	<i>esg</i> <sup>TS-FO</sup> > <i>esg</i> + <i>da</i> <sup>RNAi</sup> <sub>JF</sub>	7	induction	1236	12	61.7	38.3	0.0	0.0	0.0	0.0	0.0	0.0
<b>S7B</b>	<i>esg</i> <sup>TS-FO</sup> > <i>H</i> + <i>emc</i>	7	induction	1292	27	44.2	19.2	0.0	36.6	0.0	0.0	0.0	0.0
<b>S7F</b>	<i>esg</i> <sup>TS-FO</sup> > <i>H<sup>RNAi</sup></i> <sub>HMS</sub> + <i>emc</i> <sup>RNAi</sup> <sub>NIG</sub>	7	induction	126	16	31.7	11.9	5.6	46.0	0.0	4.8	0.0	0.0

\* Induction consists of a temperature switch for Flip-Out genotypes and a heat shock of 60 min at 37°C for MARCM genotypes.

† This column refers to the number of MARCM clones (for *FRT* genotypes) or fields of view (for *esg*<sup>TS-FO</sup> or zygotic mutant genotypes) from where the total cells were obtained.

‡ Cell type proportions are estimated (see methods).

**Table S4. Cell type-exclusive markers<sup>‡</sup>, Related to Figure 3.**

Cell type	Gene symbol	Reference (function)	Reference (expression)	FCA <sup>§</sup> marker
EB	<i>klu</i>	Korzelius et al. <sup>S1</sup>	Korzelius et al. <sup>S1</sup> ; Hung et al. <sup>S2</sup>	TRUE
EB	<i>E(spl)mbeta-HLH</i>	Lu and Li <sup>S3</sup>	Lu and Li <sup>S3</sup>	TRUE
EB	<i>CG42747</i>	NA	Li et al. <sup>S4</sup>	TRUE
EB	<i>Dtg</i>	NA	Li et al. <sup>S4</sup>	TRUE
EB	<i>CG14457</i>	NA	Li et al. <sup>S4</sup>	TRUE
EB	<i>CG30090</i>	NA	Li et al. <sup>S4</sup>	TRUE
EB	<i>CG34236</i>	NA	Li et al. <sup>S4</sup>	TRUE
EB	<i>lncRNA:CR43270</i>	NA	Li et al. <sup>S4</sup>	TRUE
EB	<i>CDase</i>	NA	Li et al. <sup>S4</sup>	TRUE
EB	<i>E(spl)m3-HLH</i>	NA	Li et al. <sup>S4</sup>	FALSE
EB	<i>Lrch</i>	NA	Li et al. <sup>S4</sup>	FALSE
pEC	<i>alphaTry</i>	NA	Hung et al. <sup>S2</sup>	TRUE
pEC	<i>Amy-d</i>	NA	Hung et al. <sup>S2</sup>	TRUE
pEC	<i>Amy-p</i>	NA	Hung et al. <sup>S2</sup>	TRUE
pEC	<i>betaTry</i>	NA	Hung et al. <sup>S2</sup>	TRUE
pEC	<i>ck</i>	NA	Chen et al. <sup>S5</sup>	FALSE
pEC	<i>LManV</i>	NA	Hung et al. <sup>S2</sup>	FALSE
pEC	<i>LManVI</i>	NA	Hung et al. <sup>S2</sup>	TRUE
pEC	<i>Myo31DF</i>	NA	Hung et al. <sup>S2</sup> ; Jiang et al. <sup>S6</sup>	TRUE
pEC	<i>nub</i>	Dantoft et al. <sup>S7</sup>	Hung et al. <sup>S2</sup> ; Lee et al. <sup>S8</sup>	TRUE
pEC	<i>Ssk</i>	Chen et al. <sup>S9</sup>	Hung et al. <sup>S2</sup>	TRUE
pEC	<i>iotaTry</i>	NA	Hung et al. <sup>S2</sup>	TRUE
pEC	<i>CG14219</i>	NA	Li et al. <sup>S4</sup>	TRUE
pEC	<i>CG13492</i>	NA	Li et al. <sup>S4</sup>	TRUE
pEC	<i>CG10911</i>	NA	Li et al. <sup>S4</sup>	TRUE
pEC	<i>Mal-A4</i>	NA	Li et al. <sup>S4</sup>	TRUE
pEC	<i>Acbp3</i>	NA	Li et al. <sup>S4</sup>	TRUE
pEC	<i>Burs</i>	NA	Li et al. <sup>S4</sup>	TRUE
pEC	<i>Mal-A3</i>	NA	Li et al. <sup>S4</sup>	TRUE
EE	<i>AstA</i>	NA	Ohlstein and Spradling <sup>S10</sup> ; Hung et al. <sup>S2</sup>	TRUE
EE	<i>Mip</i>	NA	Beehler-Evans and Micchelli <sup>S11</sup>	FALSE
EE	<i>AstC</i>	NA	Ohlstein and Spradling <sup>S10</sup> ; Hung et al. <sup>S2</sup>	TRUE
EE	<i>Dh31</i>	NA	Beehler-Evans and Micchelli <sup>S11</sup> ; Chen et al. <sup>S12</sup>	FALSE
EE	<i>dimm</i>	Beebe et al. <sup>S13</sup>	Hung et al. <sup>S2</sup> ; Beebe et al. <sup>S13</sup>	TRUE
EE	<i>mirr</i>	NA	Guo et al. <sup>S14</sup>	FALSE
EE	<i>NPF</i>	NA	Chen et al. <sup>S12</sup> ; Hung et al. <sup>S2</sup>	TRUE
EE	<i>phyl</i>	Yin and Xi <sup>S15</sup>	NA	FALSE
EE	<i>Poxn</i>	NA	Dutta et al. <sup>S16</sup>	FALSE
EE	<i>Pros</i>	Biteau and Jasper <sup>S17</sup>	Ohlstein and Spradling <sup>S10</sup> ; Micchelli and Perrimon <sup>S18</sup>	FALSE

EE	<i>Ptx1</i>	NA	Guo et al. <sup>S14</sup>	FALSE
EE	<i>sina</i>	Yin and Xi <sup>S15</sup>	NA	FALSE
EE	<i>tap</i>	Hartenstein et al. <sup>S19</sup>	Hartenstein et al. <sup>S19</sup>	FALSE
EE	<i>Tk</i>	NA	Ohlstein and Spradling <sup>S10</sup> ; Hung et al. <sup>S2</sup>	TRUE
EE	<i>CG34386</i>	NA	Li et al. <sup>S4</sup>	TRUE
EE	<i>TrpA1</i>	NA	Li et al. <sup>S4</sup>	TRUE
EE	<i>Orcokinin</i>	NA	Chen et al. <sup>S12</sup>	TRUE
EE	<i>CG7191</i>	NA	Li et al. <sup>S4</sup>	TRUE
EE	<i>CngA</i>	NA	Li et al. <sup>S4</sup>	TRUE
EE	<i>CCHa1</i>	NA	Li et al. <sup>S4</sup>	TRUE
EE	<i>Sytbeta</i>	NA	Li et al. <sup>S4</sup>	TRUE
ISC	<i>DI</i>	Ohlstein and Spradling <sup>S20</sup>	Ohlstein and Spradling <sup>S20</sup>	TRUE
ISC	<i>mira</i>	NA	Bardin et al. <sup>S21</sup>	FALSE
ISC	<i>polo</i>	NA	Amcheslavsky et al. <sup>S22,23</sup>	FALSE
ISC	<i>spdo</i>	NA	Perdigoto et al. <sup>S24</sup>	FALSE
ISC	<i>zfh2</i>	Rojas Villa et al. <sup>S25</sup>	Doupé et al. <sup>S26</sup> ; Dutta et al. <sup>S16</sup>	TRUE
ISC	<i>mew</i>	NA	Li et al. <sup>S4</sup>	TRUE
ISC	<i>Trim9</i>	NA	Li et al. <sup>S4</sup>	TRUE

# 'Exclusive markers' refers to those that are not shared by other cell types (e.g. *esg* and *hdc* are used as markers for both ISCs and EBs—these and others are non-exclusive with respect to any cell type and not included here).

§ FCA: Fly Cell Atlas. TRUE/FALSE indicate whether this gene was used in the Fly Cell Atlas as a marker for the corresponding cell type.

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