

## **Homo- and heterodimerization of bHLH transcription factors balance stemness and bipotential differentiation**

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**Running title:** Da, Emc and Sc balance stemness and differentiation in the fly intestine

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1 **ABSTRACT**

2 Multipotent adult stem cells must balance self-renewal with differentiation into various  
3 mature cell types. How this activity is molecularly regulated is poorly understood. By  
4 using genetic and molecular analyses in vivo, we show that a small network of basic  
5 Helix-Loop-Helix (bHLH) transcription factors controls both stemness and bi-potential  
6 differentiation in the *Drosophila* adult intestine. We find that homodimers of  
7 Daughterless (Da, homolog to mammalian E proteins) maintain the self-renewal of  
8 intestinal stem cells and antagonise the activity of heterodimers of Da and Scute (Sc,  
9 homolog to ASCL and known to promote intestinal secretory differentiation). We find a  
10 novel role for the HLH factor Extramacrochaetae (Emc, homolog to Id proteins),  
11 titrating Da and Sc to promote absorptive differentiation. We further show that Emc  
12 prevents committed absorptive progenitors from de-differentiating, revealing the  
13 plasticity of these cells. This mechanism of interaction partner-switching enables the  
14 active maintenance of stemness, but primes stem cells for differentiation along two  
15 alternative fates. Such regulatory logic could be recapitulated in other bipotent stem  
16 cell systems.

## 1 INTRODUCTION

2 The regulation of stem cell fate decisions hinges on the control of transcription. Central  
3 to this control are gene regulatory networks, whose activity and dynamics steer cells  
4 along particular differentiation pathways (Graf & Enver, 2009; Levine & Davidson,  
5 2005; Moris *et al*, 2016). These pathways can often be construed as a succession of  
6 binary steps that are regulated by the cross antagonism of transcription factor pairs  
7 (Graf & Enver, 2009; Simon *et al*, 2018). However, for multipotent stem cells, several  
8 fate options may be simultaneously available through higher-dimensional switches  
9 (Cinquin & Demongeot, 2002; 2005). To understand the choice between several  
10 alternative fates, knowledge of the interactions between transcriptional regulators is  
11 essential.

12 Intestinal stem cells (ISCs) are a clear example of multipotency in adult tissues. In  
13 *Drosophila* and mammals, ISCs face a triple choice between self-renewal and  
14 differentiation into one of two mature lineages: secretory or absorptive (reviewed in  
15 Jiang & Edgar, 2012; Crosnier *et al*, 2006; Philpott & Winton, 2014). The secretory  
16 lineage comprises several cell types in vertebrates, but only enteroendocrine (EE) cells  
17 in *Drosophila*. Absorptive cells are enterocytes (ECs). In *Drosophila*, ISCs produce  
18 lineage-specific precursors through distinct molecular triggers. High Notch signalling  
19 induces formation of enteroblasts (EBs), which will give rise to ECs. Expression of the  
20 bHLH (basic Helix-Loop-Helix) transcription factors Scute (Sc) or Asense (Ase),  
21 members of the *achaete-scute Complex (AS-C)*, homologs of *ASCL* mammalian genes)  
22 induces the formation of EE precursor cells (pre-EEs) and their transition to mature EE  
23 cells (Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006; Bardin *et al*, 2010; Zeng  
24 & Hou, 2015; Chen *et al*, 2018). This argues for ISCs facing a multiple choice, rather  
25 than a sequence of binary decisions. However, very little is known about the molecular  
26 mechanisms that can simultaneously regulate both self-renewal and bi-potential  
27 differentiation.

28 Transcription factors of the bHLH family control fate in multiple developmental  
29 contexts, from sex determination and mesoderm specification, to neurogenesis and  
30 immune cell type specification (reviewed in Baylies *et al*, 1997; Murre *et al*, 1994;  
31 Murre, 2005; Bertrand *et al*, 2002; Ohtsuka & Kageyama, 2010). Their HLH motif  
32 mediates dimerization, while the preceding region, rich in basic amino acids, allows  
33 DNA binding (Massari & Murre, 2000). bHLH factors are subdivided into several

1 classes, with class I (E proteins) comprising factors such as E2A, E2.2 and HEB  
2 (encoded by *TCF3/4/12*; a single gene, *daughterless (da)*, in *Drosophila*), usually with  
3 broad expression patterns; and class II containing more restrictedly expressed factors  
4 such as MYOD, TWIST and ASCL (MyoD, Twi, and AS-C proteins in *Drosophila*).  
5 Generally, class II factors heterodimerise with a class I member to bind DNA, while  
6 class I factors can either homo- or heterodimerise (Murre *et al*, 1994). The class V  
7 comprises HLH factors lacking the basic domain, known as *Inhibitors of DNA binding*  
8 (Id proteins; a single gene, *extra macrochaetae (emc)*, in *Drosophila*), as  
9 heterodimerisation with them renders dimers unable to bind DNA (Murre *et al*, 1994).  
10 Combinatorial, antagonistic or cooperative interactions confer unique properties to  
11 regulatory networks based on these factors (Amoutzias *et al*, 2008), potentially  
12 including multi-stable dynamical behaviour (Cinquin & Page, 2007; Cinquin &  
13 Demongeot, 2005).

14 Class II bHLH factors are known to regulate differentiation in the metazoan intestine  
15 (reviewed in Philpott & Winton, 2014; Hartenstein *et al*, 2017). In *Drosophila*, Sc and  
16 Ase can initiate EE differentiation (Bardin *et al*, 2010; Amcheslavsky *et al*, 2014; Chen  
17 *et al*, 2018; Guo & Ohlstein, 2015), while other bHLH genes maintain EE function  
18 (*dimmed*, homolog of *NeuroD*; Beebe *et al*, 2015), or promote their functional diversity  
19 (*tap*, homolog of *Neurogenins*; Hartenstein *et al*, 2017). On the other hand, Da is  
20 required for ISC maintenance, as ISCs mutant for *da* differentiate (Bardin *et al*, 2010;  
21 Lan *et al*, 2018). However, which interaction partner binds to Da to maintain stemness  
22 is not known, and how different bHLH factors dimerise to allow differentiation has not  
23 been explored. Here we identify the Da homodimer as the critical bHLH complex  
24 maintaining ISC self-renewal, elucidate the role of the HLH factor Emc in titrating Da  
25 and Sc to promote absorptive differentiation, and find a functional antagonism between  
26 Da:Da and Da:Sc dimers. Our results suggest that a network of bHLH factors acts as a  
27 three-way switch to regulate self-renewal and bipotential differentiation in the adult fly  
28 gut.

29

## 1 RESULTS

### 2 ***emc* is expressed preferentially in the absorptive lineage**

3 bHLH factors are essential for correct differentiation during adult intestinal homeostasis  
4 across metazoans (Philpott & Winton, 2014; Hartenstein *et al*, 2017). This suggests  
5 that *emc*, the only *Drosophila* Id factor (Garrell & Modolell, 1990; Campuzano, 2001),  
6 may have a regulatory function in the adult fly intestine, where it is strongly transcribed  
7 (Chintapalli *et al*, 2007; Brown *et al*, 2014; Dutta *et al*, 2015). We examined its  
8 expression pattern using the homozygous viable protein trap line *emc*<sup>CPTI-002740</sup> (Lowe  
9 *et al*, 2014) and detected Emc in all EBs and most ECs in the posterior midgut (Fig 1A-  
10 B). Some EEs and ISCs showed very low levels of expression (Fig 1C-D). This  
11 expression pattern, with the described role of several bHLH proteins in intestinal  
12 homeostasis, suggested a function of *emc* in promoting EC differentiation.

### 13 **Emc is required for ISC differentiation**

14 We examined the effect of *emc* loss in the adult posterior midgut by generating *emc*  
15 mutant MARCM clones (Lee & Luo, 1999). Clones of the null allele *emc*<sup>AP6</sup> (Ellis, 1994;  
16 Fig S1A) were depleted in differentiated cells and enriched in ISCs, as revealed by  
17 expression of the Notch ligand, Delta (DI) (Ohlstein & Spradling, 2007) (Fig 1E-F, J and  
18 Table S1). Clones for the strong hypomorphic allele *emc*<sup>l</sup> and the predicted  
19 transcriptional null *emc*<sup>LL02590</sup> (Fig S1A) showed similar enrichment in DI<sup>+</sup> ISCs respect  
20 to wild-type clones (Fig 1E, G-J). We then knocked-down *emc* in ISCs and EBs, and  
21 traced their offspring using the *escargot* flip-out (FO) approach ("*esg*<sup>TS</sup>-FO"; Jiang *et al*,  
22 2009; Fig 2A). Loss of *emc* blocked the differentiation of ISCs and EBs, and sometimes  
23 the typically small nests of 1-3 *esg*<sup>+</sup> cells (Ohlstein & Spradling, 2006; de Navascués *et*  
24 *al*, 2012) expanded into larger clusters (Fig 2C). This was suggestive of impairment of  
25 Notch signalling. However, these larger clusters did not display the unchecked growth  
26 and invasive behaviour observed when Notch signalling is deregulated in this tissue  
27 (Micchelli & Perrimon, 2006; Patel *et al*, 2015; see also Fig 4A). Moreover, knockdown  
28 of *emc* in the ISC/EB compartment led to the loss of many *esg*<sup>+</sup> cell nests (identified by  
29 their expression of Hdc, an RNA binding protein expressed like *esg*; Resende *et al*,  
30 2017; Fig S1B) (Fig S1C, compare with Fig 2B). This was due to apoptosis, since co-  
31 expression of the caspase inhibitor p35 and *emc*-specific RNAi prevents the loss of  
32 *esg*<sup>+</sup> cells (Fig S1D). We conclude that *emc* is necessary to allow differentiation of ISCs  
33 and EBs, as well as for their survival.

## 1 ***emc* maintains the commitment of EBs**

2 The increased number of DI<sup>+</sup> cells in *emc* depletion conditions conflicted with *emc*  
3 expression being predominantly in EBs and ECs. To test whether the loss of *emc* in  
4 EBs could induce their expression of DI, we expressed RNAi against *emc* specifically in  
5 EBs using *NRE-Gal4*, *UAS-GFP*, *tub-Gal80<sup>TS</sup>* (Zeng *et al*, 2010). Indeed, *emc*  
6 knockdown led to EBs reacquiring DI expression, often while displaying reduced GFP  
7 signal (Fig 3A-B). This suggested reduced expression of the EB-specific *NRE-Gal4*  
8 and that *emc*-depleted EBs were reverting their identity. To verify this, we used a flip-  
9 out lineage tracing approach to irreversibly label EBs ("*NRE<sup>TS</sup>-FO*"; Fig 3C) and stained  
10 for the mitotic marker phospho-Histone3. In contrast with unmodified EBs, which are  
11 post-mitotic and only produce ECs (Zeng & Hou, 2015; Micchelli & Perrimon, 2006;  
12 Ohlstein & Spradling, 2006), *emc*-depleted EBs undergo cell division and generate DI<sup>+</sup>,  
13 proliferative cells (Fig 3D-E). This suggests that *emc*-deficient EBs can not only  
14 activate DI expression but also de-differentiate into functional ISCs, capable of cell  
15 division and EE differentiation.

## 16 ***emc* acts in parallel to Notch to direct EC differentiation**

17 We next assessed the capacity of *emc* to impose differentiation by overexpressing it  
18 with *esg<sup>TS</sup>-FO*. Indeed, all ISCs and EBs differentiated into ECs (Fig 2E), as previously  
19 reported (Lan *et al*, 2018). This establishes Emc as both necessary and sufficient to  
20 force differentiation into the absorptive fate.

21 This phenotype coincides with that of over-activating the Notch pathway, a known  
22 regulator of ISC fate (Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006; Bardin *et*  
23 *al*, 2010). This suggests that *emc* might be mediating the effects of Notch signalling. To  
24 explore this possibility, we tested whether *emc* could force EC differentiation in the  
25 absence of Notch. Knockdown of *Notch* with *esg<sup>TS</sup>-FO* leads to an invasive expansion  
26 of DI<sup>+</sup> ISC-like and Pros<sup>+</sup> EE-like cells (Micchelli & Perrimon, 2006; Patel *et al*, 2015;  
27 see Fig 4A). When we co-expressed *emc* and *N<sup>RNAi</sup>* together, all ISCs and EBs turned  
28 into ECs (Fig 4B), strongly suggesting that *emc* operates downstream of Notch. This  
29 phenotype, however, could also be due to the excess of Emc quickly driving cells  
30 irreversibly into differentiation, before the knockdown of *Notch* resulted in effective  
31 clearance of Notch protein. To reduce Notch signalling with the kinetics of protein  
32 synthesis, rather than the additive kinetics of RNAi biogenesis, RNA interference, and  
33 Notch decay, we overexpressed Hairless (H), the co-repressor associated to the

1 nuclear effector of N<sup>ICD</sup>, Su(H) (reviewed in Bray & Furriols, 2001), with *esg<sup>TS</sup>-FO*. As  
2 expected, this leads to expansion of DI<sup>+</sup> and Pros<sup>+</sup> cells, with a strong reduction of EC  
3 differentiation (Fig 4C, G; see also Bardin *et al*, 2010). By contrast, co-expression of  
4 *emc* and *H* led to a sharp increase in the levels of EC differentiation (Fig 4D, G). This  
5 further indicates that Emc can induce differentiation in the absence of Notch signalling.

6 Next, we tested whether *emc* mediates the effects of Notch signalling. Activating the  
7 pathway by knocking-down *H* leads to an increase in EC differentiation (Bardin *et al*,  
8 2010; Fig 4E) and to the expression of the NRE reporter in all the progenitor, diploid  
9 cells, including those expressing DI (Fig 4H); this is likely due to an increased baseline  
10 expression of the NRE reporter, as expected in the absence of H (Furriols & Bray,  
11 2001). Simultaneously knocking down *H* and *emc* did not reduce the increase in EC  
12 differentiation induced by overexpression of *H*-specific RNAi, but actually enhanced it.  
13 (Fig 4F-H). Taken together, our data suggest that both Emc and Notch signalling can  
14 induce EC differentiation independently of each other.

### 15 **Emc antagonises self-renewal by disrupting Daughterless homodimers**

16 The molecular function of Emc consists in titrating transcriptional activators. Archetypal  
17 targets of this inactivation are the class I and II bHLH factors such as Da or Sc,  
18 respectively, to which Emc binds directly in vitro and in vivo (Van Doren *et al*, 1991;  
19 Cabrera *et al*, 1994; Alifragis *et al*, 1997; Giot *et al*, 2003; Shokri *et al*, 2019).

20 Therefore, the intestinal phenotypes we observed with the loss of *emc* may be due to  
21 increased activity of Da and its dimerization partners. Indeed, overexpression of *da*  
22 with *esg<sup>TS</sup>-FO* recapitulated precisely the effect of *emc* knockdown in *esg<sup>+</sup>* cell nests.  
23 Some of these nests (labelled by *hdc* expression) were expanded (Fig 5A-B, I), and  
24 many were lost, their survival rescued by co-expression of p35 (Fig 5B and S2C).  
25 Conversely, loss of *da* leads to EC differentiation (Fig S2A-B and 5I; as previously  
26 described (Bardin *et al*, 2010; Lan *et al*, 2018) and mimics the excess of Emc (Fig 2E).  
27 This suggests that the role of Emc is largely to repress one or more Da-containing  
28 bHLH dimers, which in turn maintain stemness and survival. Epistasis experiments  
29 further confirmed this, as the simultaneous loss of Emc and Da led to the differentiation  
30 of ISCs and EBs into ECs (Fig 5E-H).

31 We sought to determine the identity of the Da partners involved in maintaining  
32 stemness. Most *Drosophila* class II bHLH factors (Moore *et al*, 2000) are expressed in  
33 the intestine at very low levels (Fig S2D); several of them are already known not to

1 have an essential role in ISC maintenance (Bardin *et al*, 2010; Beebe *et al*, 2015;  
2 Hartenstein *et al*, 2017). However, Da can form homodimers (Cabrera *et al*, 1994;  
3 Cabrera & Alonso, 1991) with demonstrated or suspected functional relevance in  
4 several contexts, and often antagonised by Emc (Tapanes-Castillo & Baylies, 2004;  
5 Tanaka-Matakatsu *et al*, 2014; D’Rozario *et al*, 2016; Bhattacharya & Baker, 2011;  
6 Andrade-Zapata & Baonza, 2014; Troost *et al*, 2015). To test whether Da:Da dimers  
7 were involved in preventing differentiation, we overexpressed forced Da homodimers  
8 using the *UAS-da:da* tethered construct (Tanaka-Matakatsu *et al*, 2014; Neuhold &  
9 Wold, 1993) with *esg<sup>TS</sup>-FO*. This blocked the differentiation of ISCs and EBs, which  
10 accumulated in larger clusters (Fig 5C-D; Table S2). Expression of Da:Da did not lead  
11 to *esg<sup>+</sup>* cell death (Fig 5D, compare nest density with 5B). This shows that Da  
12 homodimers prevent differentiation; it does not rule out that other Da-containing  
13 complexes are also necessary to maintain self-renewal. To test this, we took  
14 advantage of the *UAS-da<sup>RNAi</sup>* transgene *P{TRiP.JF02488}*; this targets the 5’UTR of the  
15 endogenous *da* transcript, absent in the *UAS-da:da* construct (Fig S2E). Thus, we co-  
16 expressed *da:da* and *da<sup>JF02488</sup>* in ISCs and EBs to evaluate the capacity of Da:Da to  
17 prevent differentiation in the absence of endogenous, monomeric Da. The effects were  
18 identical to the overexpression of *UAS-da:da* alone (Fig S2F, compare with Fig 5D).  
19 This shows that Da can promote ISC self-renewal solely as homodimers, which are  
20 antagonised by Emc to allow EC differentiation. Furthermore, our results suggest that  
21 Da binds to an unknown partner, to form complexes that are titrated by Emc and  
22 whose excess promotes cell death in *esg<sup>+</sup>* cells.

### 23 **Basal levels of Sc in *esg<sup>+</sup>* cells confer ISC properties**

24 While the maintenance of self-renewal corresponds to Da homodimers, the loss of *emc*  
25 mimics best the overexpression of monomeric Da, which increases all Da-containing  
26 complexes. As *emc* loss induces the elevation of DI levels, another Da partner, such as  
27 Scute, could be mediating this effect. Detectable levels of Sc have recently been found  
28 in *esg<sup>+</sup>* cells (Chen *et al*, 2018; Doupé *et al*, 2018), and members of the *AS-C* are  
29 proposed to promote DI expression during neurogenesis (Kunisch *et al*, 1994; Hinz *et*  
30 *al*, 1994). Therefore, we tested whether the increased levels of DI observed upon *emc*  
31 loss (Figs 2C-D) could be caused by elevated activity of Sc or any of the other bHLH  
32 factors belonging to the *AS-C*. We induced *emc* knockdown in MARCM clones, which  
33 were either wild-type or homozygous for *sc<sup>B57</sup>*, a deficiency that removes the entire  
34 *AS-C*, and compared their respective levels of DI expression. Indeed, we observed an



1 average four-fold decrease in DI levels in *emc*-depleted clones when they were  
2 homozygous for *sc*<sup>B57</sup>, indicating that basal Sc levels (and possibly other members of  
3 the AS-C) can boost DI expression in ISCs (Fig 6A-C).

4 These observations prompted us to consider whether Sc could be contributing to  
5 additional stem cell properties in *esg*<sup>+</sup> cells. Expression of Sc with *esg*<sup>TS</sup>-*FO* clearly  
6 leads to increased differentiation into Pros<sup>+</sup> EEs (Bardin *et al*, 2010; Chen *et al*, 2018)  
7 and Fig 6D, H). To uncover a role in inducing stem properties, we overexpressed Sc  
8 with the EB-specific driver *NRE*<sup>TS</sup>-*FO*. Sc-expressing EBs were capable of division, and  
9 their offspring included both Pros<sup>+</sup> and DI<sup>+</sup> expressing cells, indicating the acquisition of  
10 ISC-like properties (Fig 6I, compare with 3D). These functions of Sc depend on the  
11 formation of Sc:Da heterodimers, as the overexpression of Sc in *da*-depleted cells  
12 prevents the induction of EE differentiation and of DI expression (Fig S3E).

### 13 **Sc:Da and Da:Da antagonise each other in the ISC-to-EE transition**

14 We have shown so far that Da homodimers maintain ISC self-renewal, with EC  
15 differentiation simply triggered by the Emc-mediated titration of Da. EE differentiation,  
16 however, requires the transition from the transcriptional program of Da:Da to that of  
17 Da:Sc. Two main scenarios are possible. Sc:Da could initiate a program that silenced,  
18 or was epistatic to, that maintained by Da:Da (“succession”). Alternatively, the relative  
19 strengths of the two programs could determine the fate outcome for the cell  
20 (“antagonism”). To distinguish between these alternatives, we evaluated the ability of  
21 an excess of Da monomer to suppress EE differentiation induced by overexpression of  
22 Sc with *esg*<sup>TS</sup>-*FO*. Overexpression of *sc* in ISCs and EBs leads to a quick expansion of  
23 Pros<sup>+</sup> cells (Fig 6D) (Bardin *et al*, 2010; Chen *et al*, 2018). These were often mitotic  
24 (Fig S3A, D), and the number of GFP<sup>+</sup> cells generated in only 3 days of lineage tracing  
25 was much higher than those generated during normal tissue replacement (Fig 6D,  
26 compare with 2A and 5A). This suggests that some of these cells have been trapped  
27 into a pre-EE state, where cells express Pros and DI and are also capable of mitosis  
28 (Zeng & Hou, 2015; Chen *et al*, 2018). However, the co-expression of Da with Sc  
29 greatly reduced the number of extra Pros<sup>+</sup> DI<sup>-</sup> cells and led to an increase in DI<sup>+</sup> cells  
30 (Fig 6E, H), while maintaining mitotic figures (Fig S3B, D). Under these conditions, the  
31 amount of Sc:Da is expected to be either similar or higher than with the overexpression  
32 of Sc alone (as endogenous *da* is weakly expressed (Bardin *et al*, 2010), likely in  
33 limiting amounts), with a simultaneous increase in Da homodimers. Thus, the reduction

1 of EE differentiation and increase of ISC population is indicative of the antagonism,  
2 rather than the succession, of their respective transcriptional programs. If that was true,  
3 we would expect that the co-expression of tethered Da:Da and Sc would result in even  
4 less EE differentiation, as this construct cannot contribute Da monomers to bind to Sc.  
5 This was the case, resulting in an even higher amount of DI<sup>+</sup> cells at the expense of  
6 Pros<sup>+</sup> cell formation (Fig 6F, H), with a concomitant increase in mitotic figures (Fig S3C,  
7 D).

8 To further test the capacity of Da homodimers to antagonise EE differentiation, we  
9 co-expressed *da:da* and *Notch<sup>RNAi</sup>* with *esg<sup>TS-FO</sup>*, as the reduction of Notch leads to  
10 the formation of abundant Pros<sup>+</sup> EE-like cells (Ohlstein & Spradling, 2006; Bardin *et al*,  
11 2010). Indeed, Da:Da opposed the formation of extra Pros<sup>+</sup> cells under conditions of  
12 *Notch* loss (Fig 6G, compare with Fig 4A). This further shows that Da:Da can oppose  
13 EE differentiation, and suggests that Sc:Da dimers need to overcome the activity of  
14 Da:Da dimers to induce the EE fate.

### 15 **Da:Da promotes ISC identity while Da:Sc confers mixed ISC/EE properties**

16 Our data indicate that Da:Da and Sc:Da activate distinct and antagonistic  
17 transcriptional programs to maintain self-renewal and initiate EE differentiation,  
18 respectively. However, during the development of the peripheral nervous system, both  
19 Da homodimers and Sc:Da heterodimers seem to be able to direct the same cell fate  
20 (sensory organ precursor) (Troost *et al*, 2015). To determine the transcriptional  
21 signature induced by these factors, we performed RNAseq analysis of the  
22 overexpression of either Da, Da:Da or Sc, in ISCs, EBs and ECs simultaneously.

23 Each overexpression experiment gave a distinct signature (Fig 7A, Fig S4A and Table  
24 S3), with Sc inducing modest changes in gene expression, and Da and Da:Da affecting  
25 far more genes (Fig 7B). Interestingly, the three conditions had a modest overlap, and  
26 monomeric Da induced gene expression changes in many genes unaffected by either  
27 Da:Da or Sc, suggesting that it may participate in additional complexes (Fig 7B). We  
28 first considered whether the genes upregulated in the three conditions were located in  
29 the vicinity of regulatory elements containing the conserved binding site of the bHLH  
30 factor, the E-box. Indeed, using the target and enhancer prediction tool *i-cisTarget*  
31 (Herrmann *et al*, 2012; Imrichova *et al*, 2015) we observed that E-boxes were  
32 overrepresented in the predicted regulatory elements of the genes with elevated

1 expression after Da, Da:Da or Sc overexpression (Tables S4, S5 and S6), suggesting  
2 that a good fraction of these genes might be primary targets.

3 Overexpressing Da or Da:Da led to increased expression of many genes known to be  
4 expressed specifically in ISC/EBs and/or required for their maintenance and  
5 proliferation (Table S7) like *spdo*, *polo*, *Cdk1*, *mira*, *HmgD*, *klu*, *zfh1*, *sna*, *ttk* and  
6 multiple components of the Notch pathway (*N*, *Su(H)*, *H*, *neur*, and several members of  
7 the *E(spl)* complex of HLH/BFM transcription factors (*E(spl)-C*), typical targets and  
8 effectors of the pathway); it also led to decreased expression of known mediators of  
9 differentiation into EEs (*pros*, *numb*) or ECs (*Myo31DF*) (Fig 7C-D). By contrast,  
10 overexpression of Sc led to increased expression of genes known to promote EE  
11 differentiation (*ase*, *pros*, *phyl*), as well as of genes expressed specifically in ISCs  
12 and/or being required for ISC function, such as *DI*, *neur*, *spdo*, *polo*, *Cdk1*, *pon*, *sna*,  
13 *mira* and *HmgD* (Fig 7E). This is all in agreement with our phenotypic observations.  
14 However, Da:Da or Da overexpression also induced increased expression of genes  
15 required for differentiation and/or specifically expressed in differentiated cells  
16 (*nub/pdm1*, *ck*) while reducing expression of other ISC and/or EB marker genes (*Smtv*,  
17 *Oatp58Dc*, *Myc*, *bun*, *insc*, *Zip71B*) (Fig 7C-D), which shows that the response of  
18 individual genes is insufficient to characterise the molecular phenotypes of Da:Da, Da  
19 and Sc.

20 To characterise the molecular signature induced by Da:Da, Da and Sc more  
21 systematically, we used Gene Set Enrichment Analysis (GSEA; Subramanian *et al*,  
22 2005) against previously described transcriptional profiles of midgut cell types (Dutta *et al*  
23 *et al*, 2015) and genes reported to be required for normal homeostasis in the *Drosophila*  
24 gut (Zeng *et al*, 2015). Genes upregulated by Da:Da, Da and Sc were enriched in  
25 ISC-specific signatures, in line with the genetic requirements of Da as well as our  
26 observation that Sc contributes to conferring ISC properties (Fig 7F and Fig S4B-C).  
27 Genes regulated by Da and Da:Da also showed depletion of the transcriptional  
28 signature of ECs and genes common to differentiated cells, while Da:Da showed  
29 specifically a reduction in EE-specific genes (Fig 7F and Fig S4D-E). In turn, Sc-  
30 regulated genes showed enrichment in genes specifically expressed in EEs (Fig 7F  
31 and Fig S4F) and required for their differentiation (Fig 7G and Fig S4B-C).

32 We next analysed the transcriptional signatures induced by Da, Da:Da and Sc more  
33 generally, to determine whether they indicated changes in specific cellular processes.

1 Using the Gene List Annotation for Drosophila (GLAD) resource (Hu *et al*, 2015), the  
2 transcriptional signatures of Da:Da, Da and Sc overexpression showed enrichment in  
3 categories involved in regulatory mechanisms (receptors, transcription factors and  
4 DNA binding, GPCRs and signalling pathways, with Notch and EGFR/receptor tyrosine  
5 kinases (RTKs) singled out) (Fig 7H and Fig S4I). Interestingly, they also showed  
6 depletion of genes involved in metabolism, ribosome, mitochondrial function and  
7 oxidative phosphorylation (Fig 7H and Fig S4G-H). This is unlikely to reflect a switch  
8 towards glycolytic metabolism, as most glycolytic genes (including *Pfk*, which codes for  
9 the rate-limiting enzyme) are generally either not affected or mildly downregulated  
10 under these conditions (Table S8). These two features (higher decision-making  
11 pathways and lower metabolism) were confirmed with a GSEA of Gene Ontology terms  
12 (Biological Process), which show an enrichment of terms related to fate specification  
13 and morphogenesis (specifically Notch and RTK pathways), and depletion of metabolic  
14 and mitochondrial-related genes. Interestingly, this analysis also shows an enrichment  
15 of genes regulating cell cycle and cytokinesis (Table S9 and Fig S4J); in the case of  
16 Sc, this is in agreement with its recently described role as a mitotic inducer in the  
17 intestine (Chen *et al*, 2018).

18 These results suggest that Da and Sc, while enhancing largely overlapping  
19 developmental and cell cycle regulatory processes, induce distinct signatures which  
20 actively promote the ISC and EE identities, respectively. However, Sc can also induce  
21 ISC-specific genes. In parallel, Da and Sc promote a state of lower metabolic  
22 signature.

### 23 **Da:Da is antagonised by Notch and maintains self-renewal in parallel to Esg**

24 Esg, a Zinc finger factor of the Snail family, is required to prevent ISC/EB  
25 differentiation, mostly through transcriptionally repressing differentiation genes  
26 (Korzelius *et al*, 2014; Loza-Coll *et al*, 2014). Da:Da seems to have a similar effect,  
27 through the activation of ISC-identity genes. Therefore, we sought to test whether they  
28 may be acting in combination to maintain stemness. Knockdown of *esg* in ISCs and  
29 EBs leads to differentiation, with an overrepresentation of EEs (Fig S5C and 8F), as  
30 expected from the described antagonism between Esg and Sc at the *pros* locus (Li *et*  
31 *al*, 2017). Simultaneous overexpression of *UAS-esg<sup>RNAi</sup>* and *UAS-da:da* prevented  
32 most differentiation, and specifically reduced EE cell production (Fig 8D and 8F),  
33 indicating that Da:Da can maintain ISCs and oppose EE differentiation independently

1 of *esg*. In turn, overexpression of *esg* blocked differentiation irrespective of the  
2 presence of *da* (Fig 8C, F and Fig S5B), indicating that Da:Da and Esg largely act  
3 independently in ISC maintenance. Furthermore, expression of *da* and *esg* are  
4 independent of each other (Fig S5D, E).

5 We next sought to determine whether Da:Da operates as part of a pathway with the  
6 known regulators of ISC fate. Our previous epistatic analysis with *emc* and Notch  
7 signalling suggested that Da:Da is required independently of the Notch pathway.  
8 Indeed, when we knocked-down both *N* and *da* using *esg<sup>TS</sup>-FO*, all ISCs/EBs became  
9 ECs (Fig S5A). Similar results were obtained by simultaneously knocking-down *da* and  
10 inhibiting Notch signalling by overexpressing *H* (Fig 8A, compare with Fig 4C, and Fig  
11 8E). However, expressing *UAS-da:da* prevented the differentiation induced by  
12 increasing Notch signalling by knockdown of *H* (Fig 8B, compare with Fig 4E, and Fig  
13 8E), suggesting that Da:Da is antagonised by Notch signalling. Together, our data  
14 indicate that Da homodimers operate downstream of Notch and in parallel to Esg to  
15 maintain ISC self-renewal.

16

## 1 DISCUSSION

2 Multipotent adult stem cells must be able to change their transcriptional state from  
3 self-renewal to several alternative differentiation programs. bHLH transcription factors  
4 seem well placed to coordinate these changes molecularly; they have been described  
5 to operate synergistically, antagonistically or combinatorially (Amoutzias *et al*, 2008;  
6 Bhattacharya & Baker, 2011; Troost *et al*, 2015; Sallee *et al*, 2017); their capacity for  
7 dimerization allows reduction in molecular noise (Bundschuh *et al*, 2003). Here we  
8 show that a bHLH factor network controls three alternate fates in the adult *Drosophila*  
9 intestine. Class I homodimers (Da:Da) promote the progenitor state of ISCs/EBs.  
10 Progenitor cells will acquire the absorptive fate by titration of Da by the HLH factor  
11 Emc, or the secretory fate by expression of the class II bHLH Sc, which  
12 heterodimerises with Da to initiate EE differentiation. Moreover, Emc is required in EBs  
13 to maintain their committed state, while low levels of Sc seem to contribute to boost the  
14 ISC transcriptional program. Our results argue for a central and conserved role of  
15 bHLH factors in the acquisition and maintenance of multiple fates in the metazoan  
16 intestine.

### 17 **Three fates regulated by a dimerization network**

18 Our data show that Da homodimers maintain ISCs and EBs undifferentiated, but this  
19 situation can be easily swayed towards either EC or EE differentiation by Da:Da  
20 swapping partners with Emc or Sc (Fig 9). Similar networks involving class I, II and V  
21 bHLH factors have been found to regulate the development of the *Drosophila* retina  
22 (Bhattacharya & Baker, 2011) and the peripheral nervous system (PNS) (Cubas *et al*,  
23 1991; Van Doren *et al*, 1991; Troost *et al*, 2015). However, in these cases only one  
24 new fate is accessed (neural), and Da:Da dimers seem to have a relatively ‘accessory’  
25 role. In the retina, Da:Da dampens the titration by Emc of the bHLH proneural factor  
26 Ato (Bhattacharya & Baker, 2011; Li & Baker, 2018). In the PNS, Da:Da cooperate with  
27 Da:Ac/Sc heterodimers to overcome Emc inhibition and induce neural fate, which both  
28 Da:Ac/Sc and Da:Da can induce (Troost *et al*, 2015). By contrast, in the adult midgut  
29 Da:Da and Da:Sc support distinct fates (progenitor and secretory, respectively) while  
30 Emc titrates both dimers to allow EC differentiation, enabling the network to regulate a  
31 three-way fate decision. This is possible because Da:Da and Da:Sc induce specific but  
32 partially overlapping transcriptional profiles (Fig 7) which yet seem to be able to act  
33 antagonistically (Fig 6). By contrast, Emc simply prevents the DNA binding of either Da

1 or Sc (Van Doren *et al*, 1991; Cabrera *et al*, 1994), which suggests that EC is the  
2 default fate when Da:Da dimers do not operate (Fig 5).

3 Interestingly, bHLH transcription factors of classes I-II-V (or E/A/Id) are also active in  
4 the mammalian intestine. Class II bHLH factors promote EE differentiation (reviewed in  
5 Philpott & Winton, 2014) and ISC maintenance (van der Flier *et al*, 2009). However,  
6 class I factors E2a and Heb are specifically expressed in crypt cells in the mouse  
7 intestine (van der Flier *et al*, 2009). Our work suggests that they may function as class I  
8 dimers as well as dimerization partners for class II factors. Meanwhile, Emc homologs  
9 Id2 and Id3 are expressed specifically in the intestinal differentiated cells (Wice, 1998),  
10 suggesting similar functions. By contrast, Id1 is required in ISCs for regeneration  
11 (Zhang *et al*, 2014) and can act as an oncogene (Wice, 1998), which is the opposite  
12 behaviour to that of Emc.

### 13 **A balance of bHLH factors regulates intestinal stem cell fate**

14 Our observations indicate that additional work on the mechanisms that regulate the  
15 transcription of both *emc* and *sc* would be very informative. The *cis*-regulatory region of  
16 *emc* seems to be complex and spreads across a large genomic region (Li & Baker,  
17 2019), so it is likely that is subject to multiple regulatory inputs. An obvious candidate  
18 for regulation of *emc* transcription is Notch signalling, as shown in other contexts  
19 (Baonza *et al*, 2000; Baonza & Freeman, 2001; Adam & Montell, 2004; Bhattacharya &  
20 Baker, 2009; Spratford & Kumar, 2015), and considering the importance of Notch for  
21 EB establishment and EC differentiation (Micchelli & Perrimon, 2006; Ohlstein &  
22 Spradling, 2006; Bardin *et al*, 2010). However, our epistasis analyses suggest that  
23 Emc and Notch signalling can induce EC differentiation independently of each other,  
24 observation that makes Notch an unlikely regulator of *emc* in the midgut. A major  
25 regulator of Sc is the transcriptional repressor Ttk69 (Wang *et al*, 2015), whose stability  
26 is in turn regulated by the E3 ubiquitin ligase Sina and its adaptor Phyl (Yin & Xi, 2018).  
27 Therefore, Phyl expression could be the trigger to allow the accumulation of Sc and  
28 initiate EE differentiation, but how *phyl* is regulated is not known yet. On the other  
29 hand, Chen *et al.* (2018) observed that Sc activates itself as well as *E(spl)m8*, a  
30 member of the *Enhancer of split Complex* (which comprises several transcription  
31 factors, many of which are often both targets and effectors of the Notch pathway;  
32 reviewed in Delidakis *et al*, 2014). As *E(spl)m8* is able to repress Sc, the authors  
33 proposed that a cell-autonomous oscillator involving Sc and *E(spl)-C* members would

1 create pulses of Sc expression which would set the pace of EE production (Chen *et al*,  
2 2018). However, while we too observe induction of *E(spl)-C* genes upon Sc forced  
3 expression, so we do as a result of Da:Da and Da overexpression (Fig 7). This  
4 suggests that the regulation of *E(spl)-C* (and therefore of *sc*) may be more  
5 complicated. Additional work will be necessary to determine the sequence and timing  
6 of regulatory inputs that initiate fate-inducing levels of Sc or Emc.

7 Moreover, the Emc/Da/Sc network clearly does not work as a simple boolean switch.  
8 The three genes are expressed, and seem to be functionally relevant, in both ISCs and  
9 EBs (Bardin *et al*, 2010; Chen *et al*, 2018; Doupé *et al*, 2018; this work). This argues  
10 against a mechanism that simply initiates Emc or Sc expression to induce the EC or  
11 EE fates. Moreover, the functions of Emc and Sc seem to be more complex than acting  
12 as mere fate switches. Adequate levels of Emc are required in the EB, as its depletion  
13 induces de-differentiation into ISCs (Fig 3), but its overexpression accelerates EB  
14 terminal differentiation into ECs (Fig 2). Intriguingly, it has recently been proposed that  
15 the Emc homolog Id1 protects pluripotency specifically during the peri-implantation  
16 transition (Malaguti *et al*, 2019), which suggests that Emc could be part of a timing  
17 mechanism in the transition between ISC and EC. As for Sc, our data suggests that  
18 low levels of expression in ISCs (Chen *et al*, 2018; Doupé *et al*, 2018) induce the  
19 expression of ISC-specific genes (Fig 7) without eliciting a conversion into pre-EE cell.  
20 Higher levels would be required to initiate EE differentiation. Therefore, the amounts of  
21 Da, Sc and Emc must be maintained in balance. In the case of Emc, this is likely to be  
22 achieved through post-transcriptional mechanisms. During neurogenesis, Emc is  
23 stabilised when bound to Da, and degrades when Da frees itself to bind a proneural  
24 factor (Bhattacharya & Baker, 2011; Li & Baker, 2018). This may be the reason why  
25 Emc shows higher levels of accumulation in EBs and ECs (Fig 1), where Da is  
26 expressed but other bHLH factors are at minimal levels.

### 27 **Da and Sc cooperate to endow cells with ISC properties**

28 Our data shows a critical requirement for Da:Da in ISC maintenance, but also that Sc  
29 can contribute to enhance ISC features (proliferation, expression of DI). This is likely an  
30 active promotion of ISC fate, rather than just prevention of differentiation, as our  
31 transcriptomic analysis shows an enrichment of the ISC transcriptional signature when  
32 overexpressing either Da:Da, Da or Sc, together with a depletion of differentiation  
33 genes (except for EE-specific genes and Sc). This active role is further supported by



1 the capacity of Sc to induce EBs to de-differentiate into ISCs and re- or  
2 transdifferentiate into EEs (Fig 6). The transcriptional changes induced by Sc, Da and  
3 Da:Da expression also show an enrichment in receptors and ligands of signalling  
4 pathways (Fig 7). This is in agreement with the recent proposal that ISCs are sources  
5 of autocrine signals required for their own maintenance (Doupé *et al*, 2018). We also  
6 find evidence that mitochondrial function is reduced by expression of Sc, Da and  
7 Da:Da (Fig 7), which fits well the observation that ISCs display an ‘empty mitochondria’  
8 phenotype under the electron microscope (Hung *et al*, 2018). Together, this suggests  
9 that low levels of Sc:Da dimers contribute non-critically to the maintenance of the ISC  
10 identity and function, and we surmise that Emc expression in EBs might be required to  
11 dampen this Sc activity.

## 12 **Regulation of the committed state of the enteroblast**

13 Under normal conditions, EBs will only give rise to mature ECs without further division  
14 (Zeng & Hou, 2015; Wang *et al*, 2015; Yin & Xi, 2018). Depletion of Emc specifically in  
15 EBs coupled to lineage tracing results in cells proliferating and expressing DI (Fig 3),  
16 which likely represents a de-differentiation process. This may partially reflect the need  
17 to control Sc activity in the EB, as the expression of Sc can induce the same effects  
18 (Fig 6). Intriguingly, EB-specific depletion of Ttk69 or Klu or overexpression of Phyl  
19 seem to induce trans-differentiation, as these conditions produce Pros<sup>+</sup> cells (Wang *et al*  
20 *et al*, 2015; Yin & Xi, 2018). This difference could be because Emc is also influencing the  
21 activity of multiple Da-containing complexes in the EB, or because of the activity of  
22 additional targets of Klu and the Phyl/Ttk69 pathway than Sc. The reacquisition of  
23 proliferation and Delta expression is also controlled by other factors, such as Sox21a  
24 (Zhai *et al*, 2015), or the global co-repressor Gro, acting downstream of Notch (Guo *et al*  
25 *et al*, 2019) (though surprisingly Notch itself does not seem to be required to maintain the  
26 EB; Siudeja *et al*, 2015). Together with our observations, this indicates a previously  
27 unappreciated plasticity of the EB, which is consistent with its similarity to the ISC in  
28 transcriptome (Hung *et al*, 2018) and chromatin accessibility landscape (Aughey *et al*,  
29 2018). Importantly, this resembles the behaviour of EC precursors in the mammalian  
30 intestine, which can dedifferentiate and repopulate the intestinal crypt during  
31 regeneration (Tetteh *et al*, 2016).

## 1 MATERIALS AND METHODS

### 2 *Drosophila* culture and genetics

3 Flies were raised and maintained on standard cornmeal/yeast medium at 25°C unless  
4 specifically indicated. Experiments were conducted in mated females, collected daily  
5 and maintained in fresh vials with added yeast until 4-7 days old. For experiments  
6 using Gal80<sup>TS</sup>, flies were reared and aged to gut maturity (~7 days) at 18°C, then  
7 transferred to 29 °C. For induction of MARCM and flip-out clones, 4-7 days old flies  
8 were treated at 37°C for 60 or 15 min, respectively. Flies were aged for 7 days after  
9 induction treatment before dissection, unless otherwise indicated. See Appendix for fly  
10 strains.

### 11 Immunohistofluorescence, confocal microscopy and image analysis

12 Stainings were performed as in Bardin *et al*, (2010). See Appendix for details on  
13 primary and secondary antibodies. DNA dye was Hoechst (Sigma-Aldrich B2261 used  
14 1:5000 from a stock solution at 10 mg/ml). Mounting medium was 4% N-propyl-galate,  
15 80% glycerol. Confocal stacks were obtained in a Zeiss LSM 710 with an EC Plan-  
16 Neofluar 40X oil immersion objective (numerical aperture 1.3). All stack positions were  
17 acquired in the posterior midgut. Typically, three positions along the anterior-posterior  
18 axis of the posterior midgut were acquired for each organ. In MARCM clone  
19 experiments, stacks were acquired from all clones in the posterior midgut.  
20 Quantification of anti-DI staining is detailed in Images and figures were assembled  
21 using Adobe Photoshop and Illustrator CS6.

### 22 Cell counts and statistics

23 For evaluating the proportion of cell types in GFP<sup>+</sup> clones or labelled tissue, confocal  
24 stacks were maximum-intensity projected using FIJI/ImageJ (Schindelin *et al*, 2012),  
25 cells of the relevant types were counted with the Cell Counter plugin. In the  
26 experiments overexpressing *UAS-sc* and *UAS-da:da*, the associated increase in  
27 proliferation generated large, highly densely populated cell clusters which could not be  
28 counted with single-cell precision. Therefore, for this genotype we estimated the  
29 proportion of each cell population in each field of view separately. See the Appendix for  
30 details on cell type identification.

31

1 Statistical tests were performed in Prism 8 or in R 3.5.1 (R Core Team, 2018) using  
2 RStudio 1.1.383. Statistical significance of changes in proportions of cell types was  
3 assessed by binomial logistic regression in basic R. We used Firth's bias reduced  
4 logistic regression (package *logistf*, Heinze and Ploner, 2013) in experiments with zero  
5 observations in one or more of the genotypes considered, to avoid the nonsensical  
6 results arising from the 'complete separation' of data (Albert & Anderson, 1984).

## 7 **RNA-seq**

8 Flies bearing either *UAS-da:da*, *UAS-da* or *UAS-sc* as well as *esg-Gal4*, *Myo1A-Gal4*  
9 and *tub-Gal80<sup>TS</sup>* were reared at 18°C until 4-7 days old, transferred to 29°C for 24h and  
10 their posterior midguts dissected, collected in lysis buffer and frozen at -80°C, then  
11 thawed and extracted for total RNA. Libraries from three biological replicates per  
12 condition were prepared and ~26 million reads per library were generated using  
13 Illumina technology. See the Appendix for additional details.

14

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15    **AUTHOR CONTRIBUTIONS**

16    Conception of the study and supervision: JdN. Data acquisition, analysis and  
17    interpretation of data: AP and JdN. Experimental design; writing, revising and approval  
18    of the manuscript: JdN and AP.

19    **CONFLICT OF INTEREST**

20    The authors declare no competing interest.

## 1 **FIGURE LEGENDS**

### 2 **Figure 1. *emc* is expressed in the absorptive lineage and is required for** 3 **differentiation.**

4 **A-D.** Projected confocal stacks showing endogenous Emc:GFP expression. Emc is  
5 expressed in EBs (marked with *NRE-lacZ*, arrowheads in A, A') and most ECs (positive  
6 for *myoIA-lacZ*, solid arrowheads in B, B') but not all (empty arrowhead in B, B'). *emc*  
7 expression can also be detected in some ISCs (DI<sup>+</sup>) and EEs (Pros<sup>+</sup>) (solid arrowheads  
8 and asterisks, respectively, in C-D') but not all (empty arrowheads and asterisks in C-  
9 D').

10 **E-I.** Differentiation is impaired in *emc* mutant clones. Control clones display more  
11 polyploid DI<sup>-</sup>/Pros<sup>-</sup> ECs (asterisks in E, H) than clones homozygous of for the mutant  
12 alleles *emc*<sup>AP6</sup> (F), *emc*<sup>1</sup> (G) and *emc*<sup>LL02590</sup> (I), which are enriched in DI<sup>+</sup> ISCs (solid  
13 arrowheads) and diploid DI<sup>-</sup>/Pros<sup>-</sup> EBs (empty arrowheads) with respect to controls.  
14 **J.** Stacked bars plots summarising the cell type composition of clones by genotype.  
15 Numbers (cells/clones) are: 194/106, 213/118, 310/117, 112/95 and 208/200 for control  
16 *FRT2A*, *emc*<sup>LL02590</sup>, control *FRT80B*, *emc*<sup>1</sup>, and *emc*<sup>AP6</sup> respectively. p-values are  
17 <0.05, <0.01 and <0.001 for one, two or three asterisks (see Tables S1 and S2 for  
18 further details).

19 Scale bar: 20µm in all panels.

### 20 **Figure 2. *emc* is necessary for differentiation and sufficient for selection of the** 21 **EC fate.**

22 **A.** The *esg*<sup>TS</sup>-*FO* method allows the temporal control of expression in progenitor cells  
23 (*esg*<sup>+</sup> ISCs and EBs) and their lineage tracing to access their fate potential. *UAS-GFP*  
24 and *UAS-FLP* are expressed specifically in ISCs and EBs after induction by  
25 temperature switch. This leads to the progenitor-restricted expression of the  
26 (potentially) ubiquitous *Actin5C-Gal4* by excision of a transcriptional terminator.  
27 *Actin5C-Gal4* maintains *UAS-GFP* expression in the ISC/EB lineage when  
28 differentiation occurs and *esg* expression stops.

29 **B-E.** Emc is required for differentiation and sufficient for induce the EC fate. ISCs and  
30 EBs in control guts produce both EC (DI<sup>-</sup>/Pros<sup>-</sup>) and EE (DI<sup>-</sup>/Pros<sup>+</sup>) GFP<sup>+</sup> cells (B, note  
31 the difference in intensity levels between DI and Pros, respectively solid and empty  
32 arrowheads), while differentiation is reduced when ISCs/EBs express *emc*-specific  
33 RNAi, shown by increased number of DI<sup>+</sup>/GFP<sup>+</sup> cells (C, D). Reduction of *emc* also  
34 induces increased DI expression: compare DI-expressing with Pros-expressing cells

1 (respectively solid and empty arrowheads in C, D, compare with those similarly marked  
2 in B). Forced expression of *emc* in ISCs/EBs forces differentiation into ECs (E).  
3 **F.** Stacked bars plots summarising the cell type composition of *esg<sup>TS</sup>-FO* tissue.  
4 Numbers (total GFP<sup>+</sup> cells / fields of view) are 1046/15, 104/21, 832/15 and 812/10 for  
5 control, *UAS-emc<sup>RNAi</sup><sub>NIG</sub>*, *UAS-emc<sup>RNAi</sup><sub>KK+HMS</sub>*, and *UAS-emc*, respectively. p-values are  
6 <0.05, <0.01 and <0.001 for one, two or three asterisks (see Tables S1 and S2).  
7 Scale bar: 20µm in all panels.

8 **Figure 3. *emc* is required for maintenance of the EB committed state.**

9 **A-B.** The *NRE-Gal4* driver is expressed in EBs at different levels in normal intestines  
10 (solid and empty arrowheads in A for higher and lower-expressing EBs), but never  
11 co-express DI. When *emc* is depleted with *NRE-Gal4*, many EBs co-express DI (empty  
12 arrowheads, B) but not all (solid arrowheads, B). Also, EEs develop close to  
13 *emc*-depleted EBs (arrow, B).

14 **C.** Use of *NRE<sup>TS</sup>-FO* allows the simultaneous manipulation and tracing of the EB  
15 lineage. *NRE-Gal4*, *UAS-GFP* is expressed specifically in EBs after induction and  
16 activates specifically in EBs the (potentially) ubiquitous *Actin5C-Gal4*, which in turn will  
17 maintain *UAS-GFP* expression if differentiation or decommitment occur.

18 **D-E.** Loss of *emc* in EBs leads to their decommitment. Activation of *NRE<sup>TS</sup>-FO* in  
19 control guts leads exclusively to the labelling of EBs and ECs, with no co-expression of  
20 GFP and DI or Pros (solid and empty arrowheads, respectively, in D; some ISCs and  
21 EEs in the micrograph seem inside the GFP territory due to the Z-projection of the  
22 confocal stack). By contrast, in *NRE<sup>TS</sup>-FO* guts where *emc* is depleted, EB-derived  
23 cells express DI and are capable of mitosis, as judged by phospho-Histone 3 staining  
24 (arrowhead in E).

25 Scale bar: 20µm in all panels.

26 **Figure 4. *emc* acts in parallel to Notch signalling.**

27 **A-D.** *emc* can direct EC differentiation in the absence of Notch signalling. EC  
28 differentiation is abolished or strongly impaired with knockdown of *N* (A) or  
29 overexpression of *H* (C), respectively, with accumulation of DI<sup>+</sup> or Pros<sup>+</sup> cells (solid and  
30 empty arrowheads, respectively, in A, C). Simultaneous overexpression of *emc*  
31 induces EC differentiation in both cases (arrowheads in B, D), but to a lesser an extent  
32 in a background of *H* overexpression (asterisks in D, compare with B).

33 **E-F.** Notch signalling can direct EC differentiation in the absence of *emc*. Loss of EC  
34 differentiation caused by depletion of *emc* (see panel H and Fig. 2C-D, F) is rescued by

1 simultaneous knockdown of *H* (F). Depletion of *H* can induce EC differentiation on its  
2 own (E).  
3 **G-H.** Stacked bars plots summarising the cell type composition of *esg<sup>TS</sup>-FO* tissue.  
4 Numbers (total GFP<sup>+</sup> cells / fields of view) are 1810/21 and 1292/27 for *UAS-H* and  
5 *UAS-H+UAS-emc*, respectively (G) and 984/15 and 126/16 for *UAS-H<sup>RNAi</sup><sub>HMS</sub>* and  
6 *UAS-H<sup>RNAi</sup><sub>HMS</sub>+UAS-emc<sup>RNAi</sup><sub>NIG</sub>*, respectively (H). p-values are <0.05, <0.01 and <0.001  
7 for one, two or three asterisks (see Tables S1 and S2).  
8 Scale bar: 20µm in all panels.

9 **Figure 5. Da homodimers prevent differentiation and are antagonised by Emc.**

10 **A-D.** Da homodimers maintain ISCs/EBs undifferentiated. Solid arrowheads: ISCs/EBs;  
11 empty arrowheads: ECs/EEs. Overexpression of Da with *esg<sup>TS</sup>-FO* prevents formation  
12 of ECs, though it allows EE differentiation (B, compare with A). Expression of a forced  
13 Da:Da dimer in *esg<sup>+</sup>* cells prevents all differentiation, as evaluated with Hdc/NRE-lacZ  
14 (C) or DI (D).

15 **E-H.** *da* is epistatic over *emc*. Solid arrowheads: ECs; empty arrowheads: DI<sup>+</sup> ISCs.  
16 MARCM clones mutant for *da<sup>10</sup>* differentiate as ECs (E). This phenotype is  
17 recapitulated when these clones simultaneously express *emc*-specific RNAi (F).  
18 Likewise, impaired differentiation in *emc<sup>LL02590</sup>* clones (G) is rescued by simultaneous  
19 knockdown of *da*, which leads to EC formation (H).

20 **I.** Stacked bars plots summarising the cell type composition of *esg<sup>TS</sup>-FO* tissue.  
21 Numbers (total GFP<sup>+</sup> cells / fields of view) are 1135/15, 549/14, 693/13, 803/15 and  
22 1086/19 for control, *UAS-da*, *UAS-da:da*, *UAS-da<sup>RNAi</sup><sub>JF</sub>* and *UAS-da<sup>RNAi</sup><sub>HMS</sub>*,  
23 respectively. P-values are <0.05, <0.01 and <0.001 for one, two or three asterisks (see  
24 Tables S1 and S2).  
25 Scale bar: 20µm in all panels.

26 **Figure 6. Sc:Da and Da:Da antagonise each other in EE formation but collaborate**  
27 **in ISC maintenance.**

28 **A-C.** Emc antagonises Sc in inducing DI expression. Loss of the *AS-C* using the *sc<sup>B57</sup>*  
29 deficiency reduces the increase in DI expression observed with knockdown of *emc* (B,  
30 compare with A). DI levels are quantified by comparison with those of Pros (see  
31 Appendix). Numbers (DI<sup>+</sup> cells/Pros<sup>+</sup> cells) are 210/146 and 718/210 for  
32 *UAS-emc<sup>RNAi</sup><sub>NIG</sub>* and *UAS-emc<sup>RNAi</sup><sub>NIG</sub>, Df(1)sc<sup>B57</sup>*, respectively. P-values are <0.05,  
33 <0.01 and <0.001 for one, two or three asterisks (Mann-Whitney test).

1 **D-F.** Antagonism/collaboration between Sc and Da. Solid arrowheads: DI<sup>+</sup>/Pros<sup>+</sup>  
2 pre-EEs; empty arrowheads: DI<sup>+</sup> ISCs; asterisks: Pros<sup>+</sup> EEs. Overexpression of Sc  
3 leads to a dramatic increase of DI<sup>+</sup>/Pros<sup>+</sup> pre-EEs and Pros<sup>+</sup> EEs, but maintains a  
4 population of DI<sup>+</sup> ISCs (D). The population of ISCs progressively increases by  
5 co-expression of monomeric Da (E) and dimeric Da:Da (F), at the cost of EE  
6 differentiation (E, F) and pre-EE formation (F). Quantified in H.

7 **G.** Da:Da antagonises EE formation. While reduction of Notch signalling leads to an  
8 excess of EEs, simultaneous overexpression of Da:Da and *N*-specific RNAi results in  
9 very few Pros<sup>+</sup> cells (solid arrowheads, compare with Fig 4A).

10 **H.** Stacked bars plots summarising the cell type composition of *esg<sup>TS</sup>-FO* tissue.  
11 Numbers (total GFP<sup>+</sup> cells / fields of view) are 1241/6, 2131/10 and not determined/11  
12 for *UAS-sc*, *UAS-sc*, *UAS-da*, and *UAS-sc*, *UAS-da:da*, respectively. p-values are  
13 <0.05, <0.01 and <0.001 for one, two or three asterisks (see Tables S1 and S2).

14 **I.** Sc can induce decommitment of EBs. Expression of Sc with *NRE<sup>TS</sup>-FO* leads to  
15 reacquisition of ISC properties (DI expression, mitosis assessed with phospho-Histone  
16 3, empty arrowhead) and pre-EE formation (Pros expression and mitosis, solid  
17 arrowhead).

18 Scale bar: 20µm in all panels.

19 **Figure 7. Da and Sc cooperate to induce expression of the ISC signature.**

20 **A-B.** Da:Da, Da and Sc induce specific but overlapping transcriptional signatures.  
21 Hierarchical clustering of differentially expressed genes (fold change > 4) (A) shows  
22 groups of genes specifically increased or reduced in Da, Da:Da and Sc respect to the  
23 wild-type. UpSetR plots (B) indicate the degree of overlap between these signatures,  
24 which is highest between Da and Da:Da.

25 **C-E.** MA plots (log<sub>2</sub> fold change vs mean expression in transcripts per million, tpm) for  
26 the overexpression of Da:Da, Da and Sc, respectively. Some genes of interest are  
27 identified because of their role or expression pattern.

28 **F-H.** Heatmaps of gene set enrichment scores for sets of genes that are: expressed in  
29 specific cell types (Dutta *et al*, 2015) (F), known to be functionally relevant in different  
30 aspects of intestinal homeostasis (Zeng *et al*, 2015) (G) or annotated because of their  
31 broad molecular function in *Drosophila* (Hu *et al*, 2015). Black asterisks indicate the  
32 enrichment scores with p-values < 0.05 in that heatmap. White crosses indicate the  
33 enrichment scores with p-values > 0.05 in that heatmap; the rest have p-values < 0.05.

34



1 **Figure 8. *da* is epistatic over Notch signalling but acts in parallel with *esg*.**

2 Solid arrowheads: ISCs ( $Hdc^+/NRE^-$  or  $DI^+/NRE^-$ ); empty arrowheads: EEs ( $Pros^+$ ).

3 **A-B.** *da* is epistatic over Notch signalling. Loss of *da* leads to EC and EE differentiation  
4 when Notch signalling is impaired by overexpression of *H* (A), while overexpression of  
5 *da:da* maintains cell undifferentiated even when Notch signalling is exacerbated by  
6 knockdown of *H* (B).

7 **C-D.** *da* and *esg* function in parallel to prevent differentiation. Overexpression of *esg*  
8 can prevent differentiation and compensate for the loss of *da* (C), while overexpression  
9 of *da:da* can similarly compensate for the loss of *esg*, including prevention of EE  
10 formation (D).

11 **E-F.** Stacked bars plots summarising the cell type composition of *esg<sup>TS</sup>-FO* tissue.

12 Numbers (total GFP<sup>+</sup> cells / fields of view) are 1510/21, 1810/21, 613/8 and 984/15 for

13 *UAS-H*, *UAS-H+UAS-da<sup>RNAi</sup><sub>JF</sub>*, *UAS-H<sup>RNAi</sup><sub>HMS</sub>* and *UAS-H<sup>RNAi</sup><sub>HMS</sub>+UAS-da:da*,

14 respectively (E) and 850/9, 1236/12, 628/18 and 939/19 for for *UAS-esg*

15 *UAS-esg+UAS-da<sup>RNAi</sup><sub>JF</sub>*, *UAS-esg<sup>RNAi</sup><sub>HMS</sub>* and *UAS-esg<sup>RNAi</sup><sub>HMS</sub>+UAS-da:da*, respectively

16 (F). p-values are <0.05, <0.01 and <0.001 for one, two or three asterisks (see Tables

17 S1 and S2 for further details). Scale bar: 20µm in all panels.

18 **Fig 9. Sc, Da and Emc regulate self-renewal and bipotential differentiation in the**  
19 **intestine.**

20 Orange arrows: Da homodimers maintain ISCs and EBs undifferentiated, antagonising  
21 EC differentiation and pre-EE formation by inducing expression of ISC-specific genes.

22 Pink arrows: An increase of Sc expression induces formation of Da:Sc heterodimers,  
23 which steer the transcriptional program towards pre-EE formation; Sc can also induce  
24 expression of the ISC-specific signature, potentially leading to EB decommitment if its

25 activity is not prevented by titration by Emc. Green arrows: Emc increased expression  
26 in EBs maintains commitment for EC differentiation and can direct terminal EC

27 differentiation by interruption of Da:Da and Da:Sc transcriptional activities. This occurs  
28 in parallel to Esg prevention of differentiation and Notch signalling being upstream and  
29 opposing Da:Da to induce EC differentiation.

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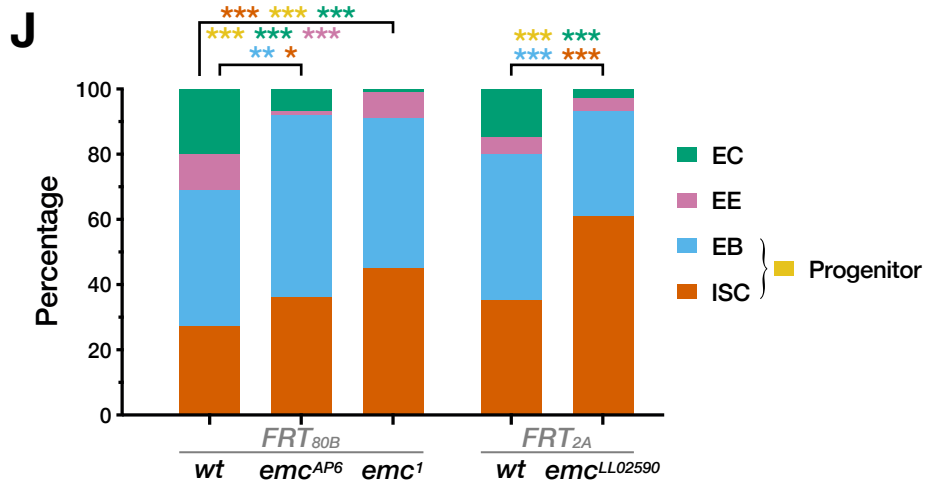
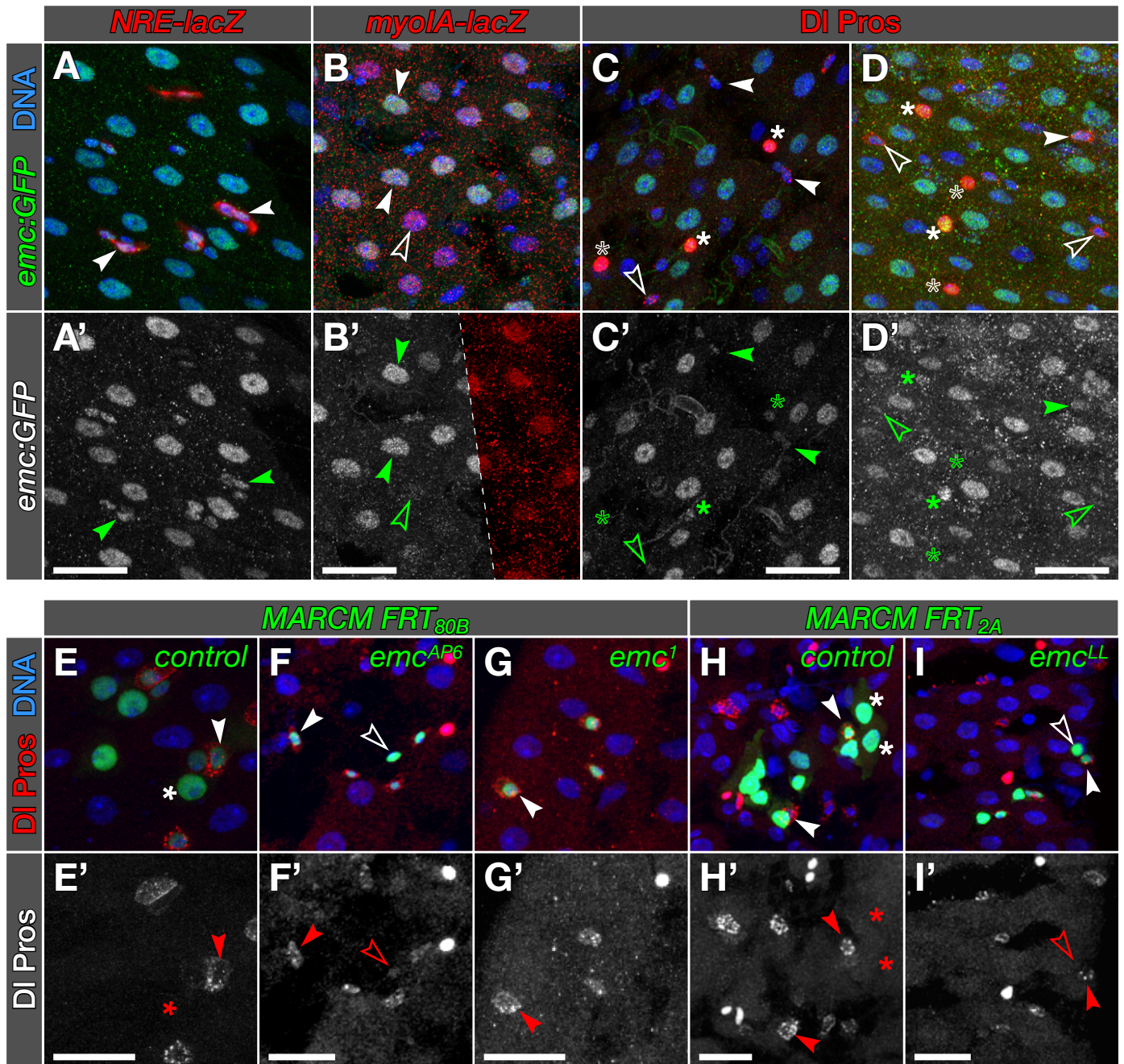
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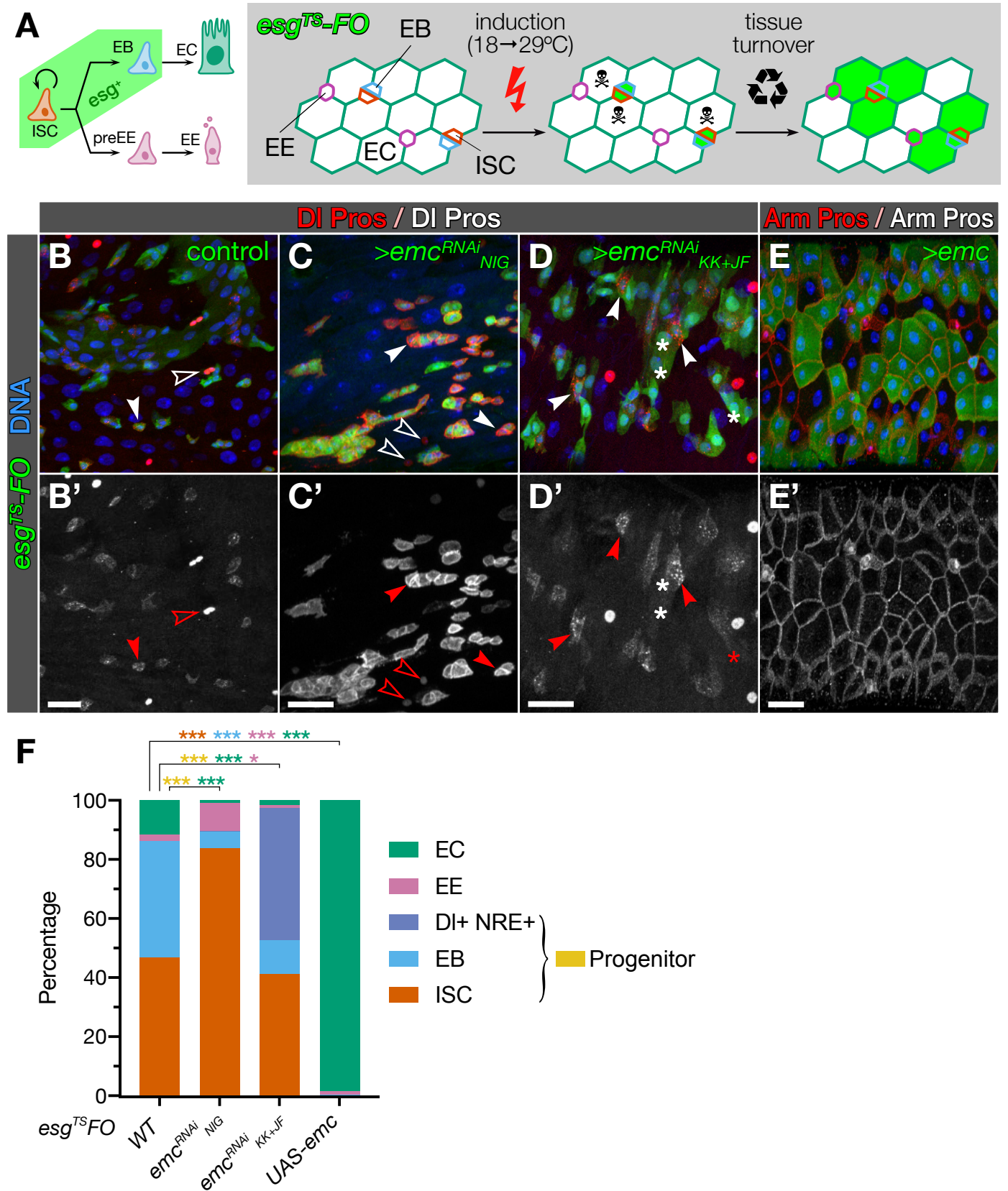
**Figure 1**

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## Figure 2

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## Figure 3

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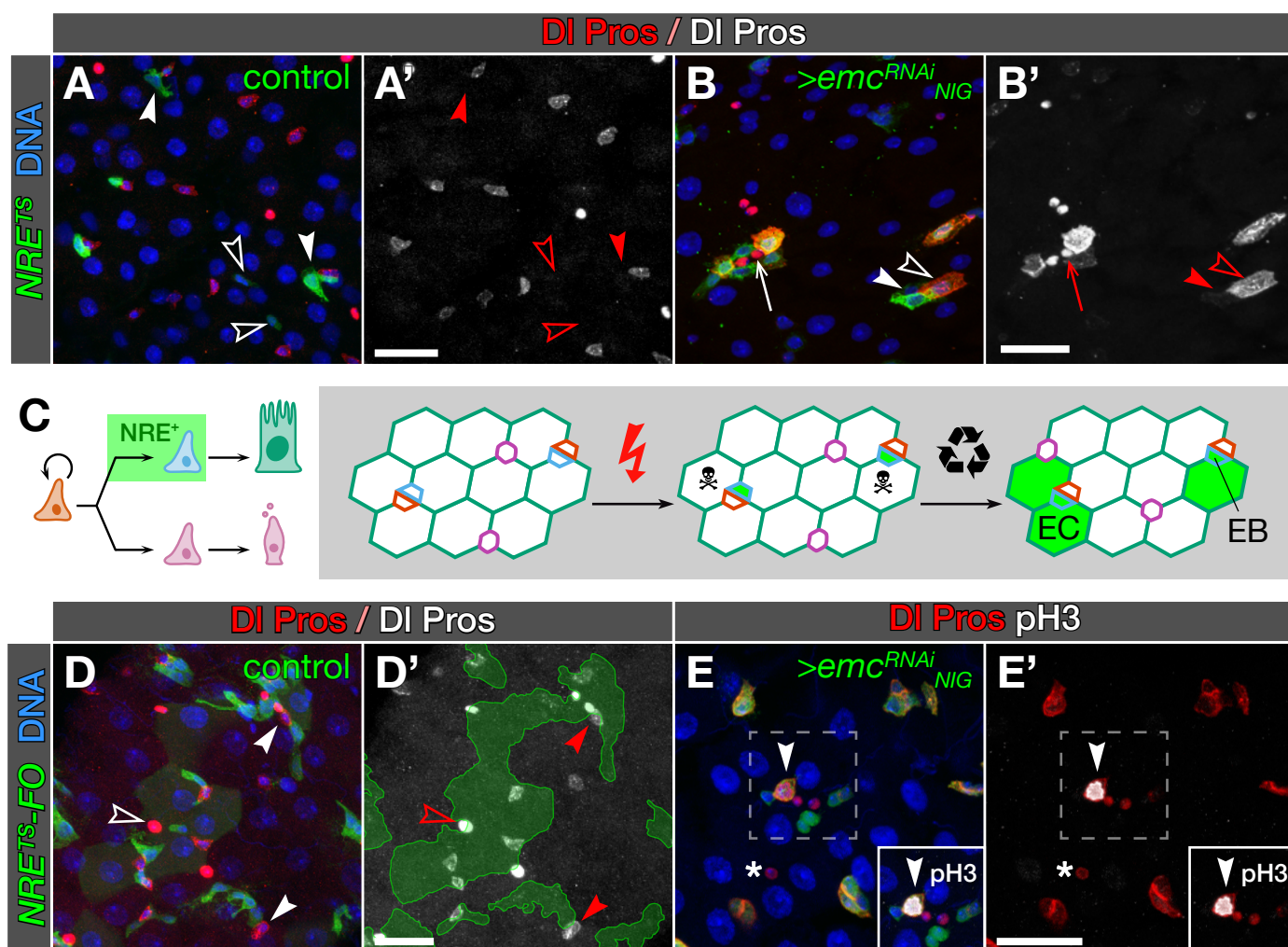
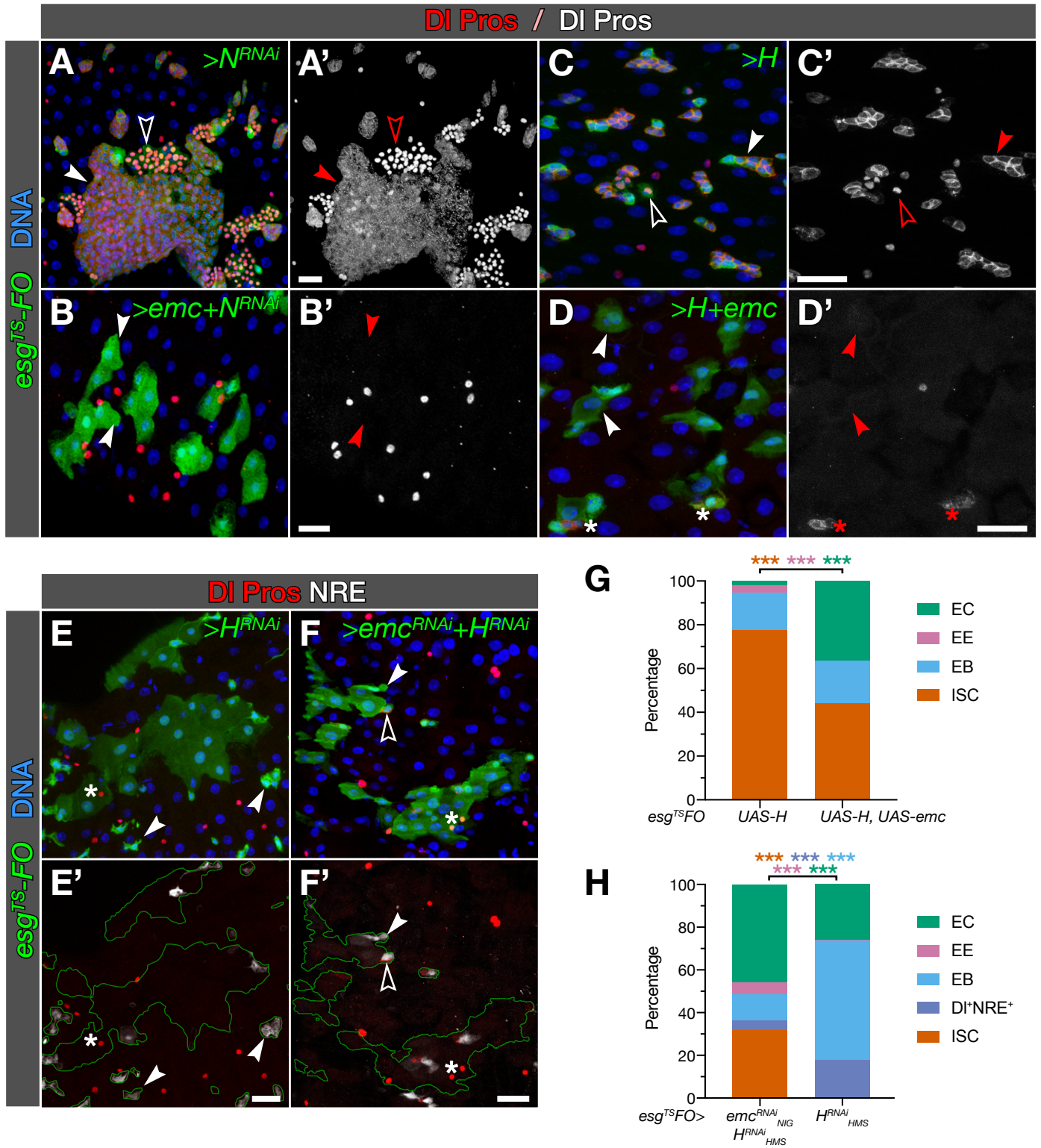


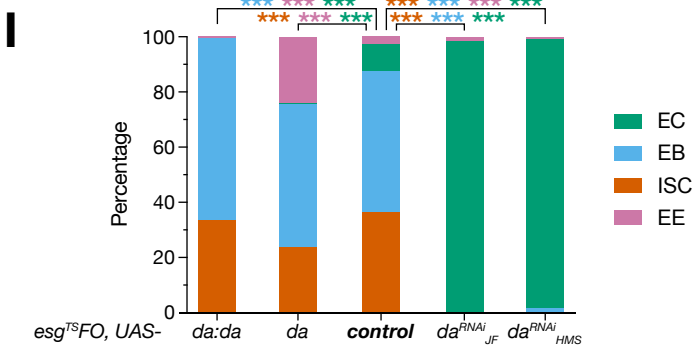
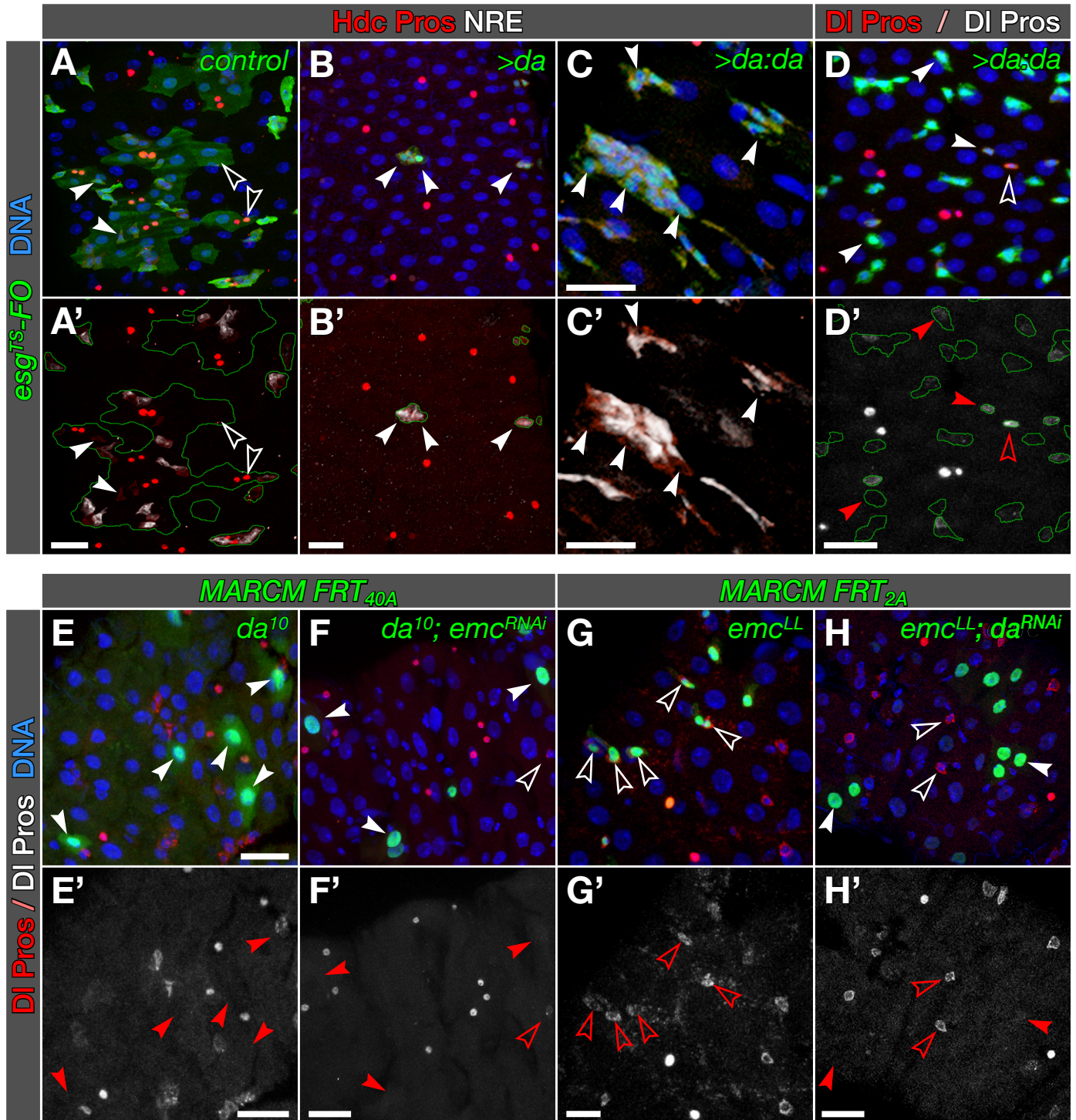
Figure 4

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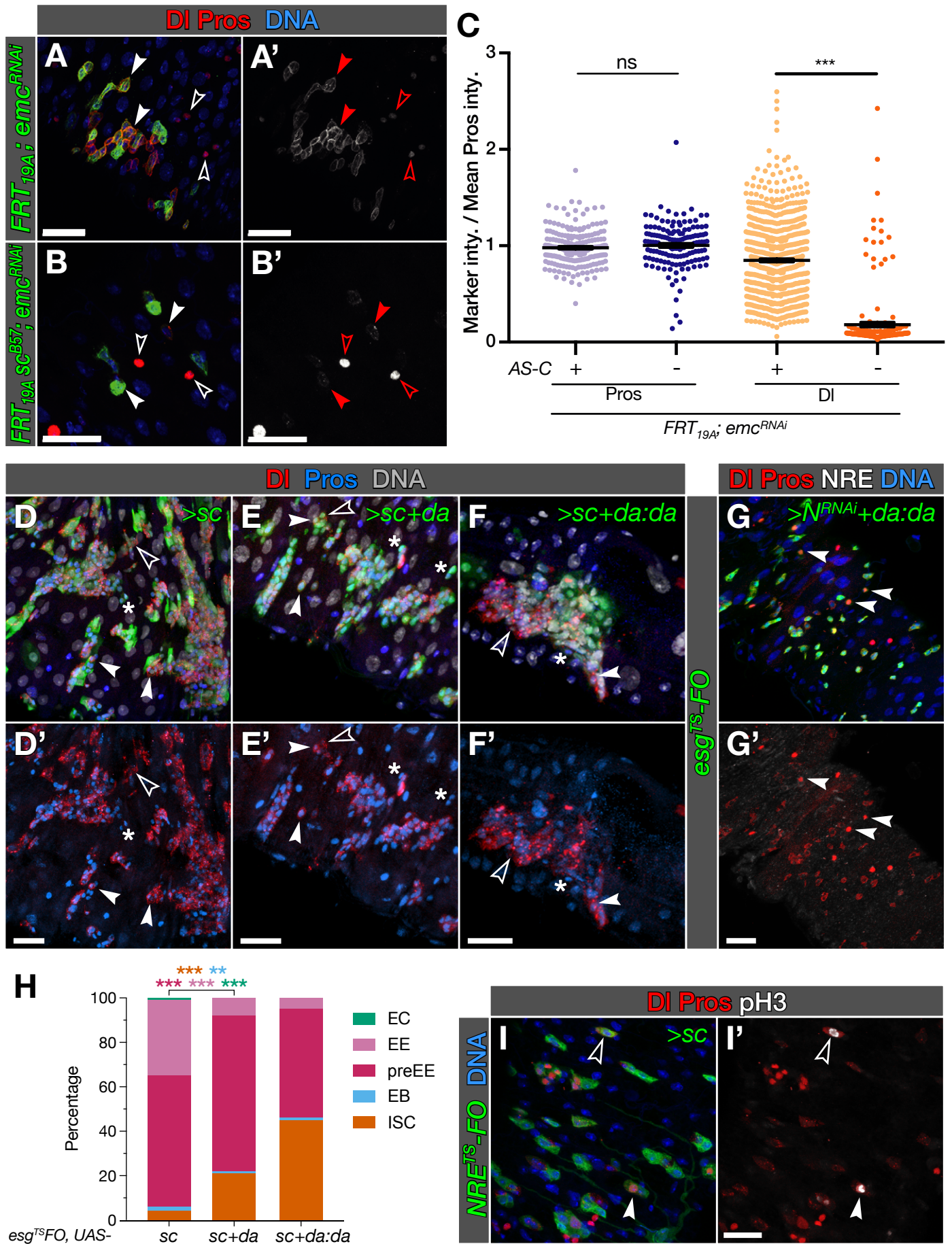
**Figure 5**

Puig and de Navascués, 2019



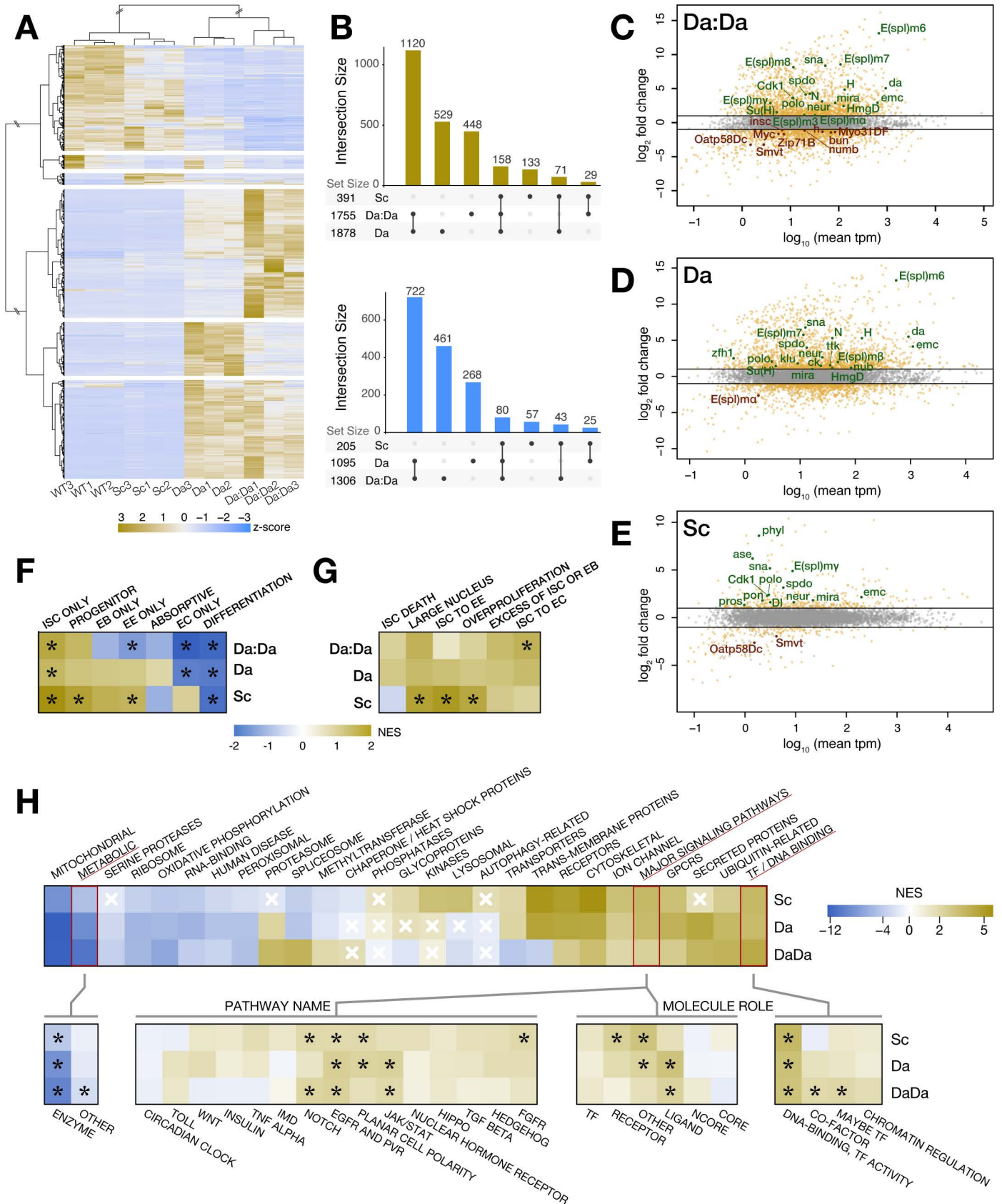
**Figure 6**

Puig and de Navascués, 2019



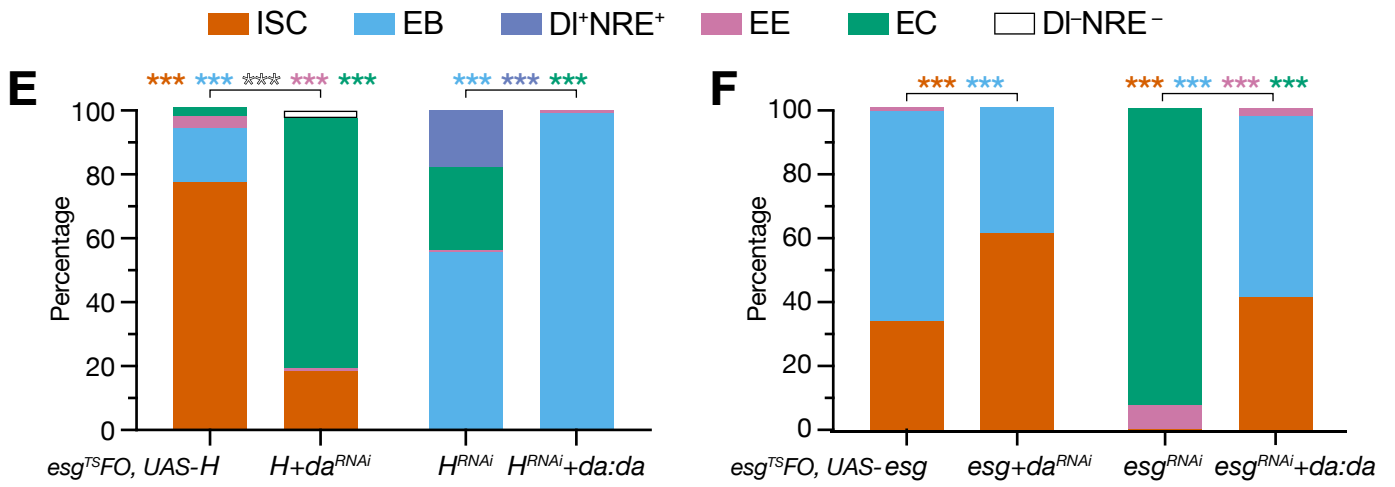
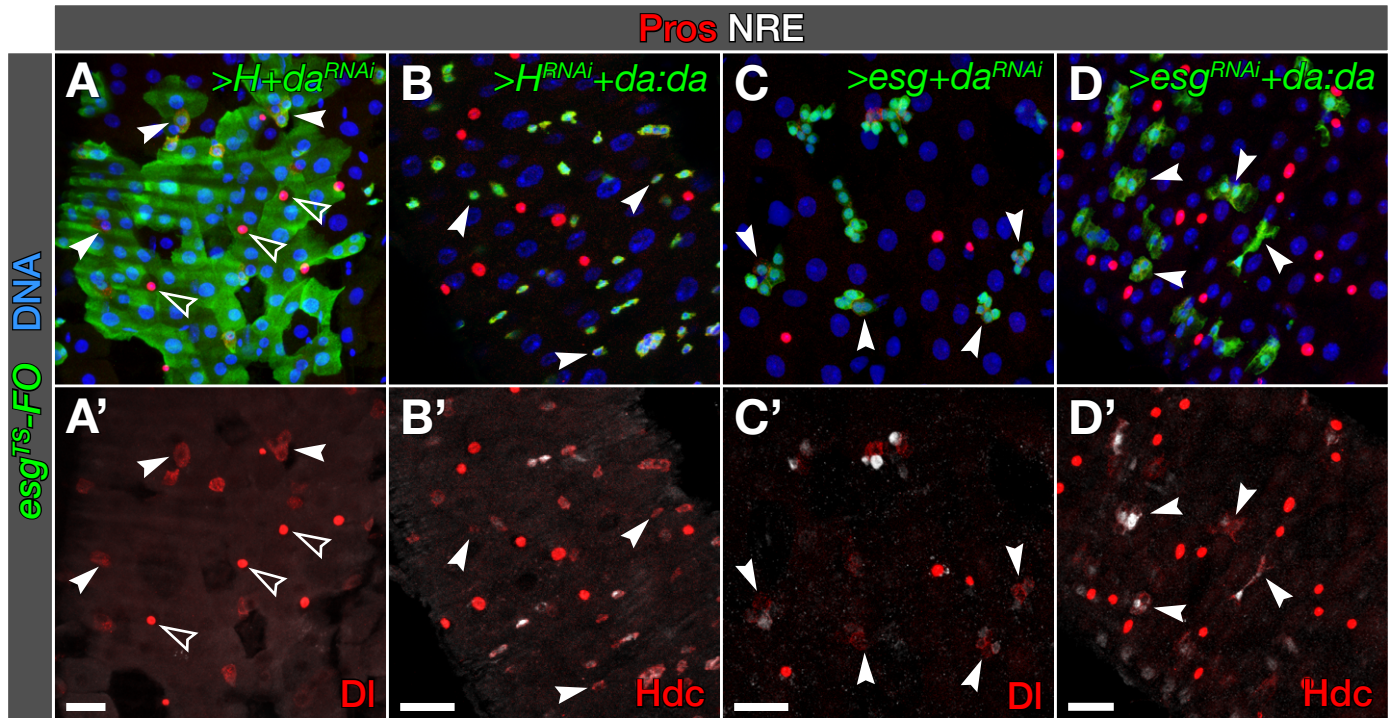
## Figure 7

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**Figure 8**

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**Figure 9**

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