Escargot maintains stemness and suppresses differentiation in Drosophila intestinal stem cells

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Abstract

Snail family transcription factors are expressed in various stem cell types, but their function in maintaining stem cell identity is unclear. In the adult Drosophila midgut, the Snail homolog Esg is expressed in intestinal stem cells (ISCs) and their transient undifferentiated daughters, termed enteroblasts (EB). We demonstrate here that loss of esg in these progenitor cells causes their rapid differentiation into enterocytes (EC) or entero-endocrine cells (EE). Conversely, forced expression of Esg in intestinal progenitor cells blocks differentiation, locking ISCs in a stem cell state. Cell type-specific transcriptome analysis combined with Dam-ID binding studies identified Esg as a major repressor of differentiation genes in stem and progenitor cells. One critical target of Esg was found to be the POU-domain transcription factor, Pdm1, which is normally expressed specifically in differentiated ECs. Ectopic expression of Pdm1 in progenitor cells was sufficient to drive their differentiation into ECs. Hence, Esg is a critical stem cell determinant that maintains stemness by repressing differentiation-promoting factors, such as Pdm1.

Keywords Drosophila midgut; enterocyte differentiation; intestinal stem cells; Pdm1; Snail transcription factors

Subject Categories Development & Differentiation; Stem Cells; Transcription

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Introduction

Great progress has been made in understanding how external cues, provided by signaling molecules emanating from stem cell niches, instruct stem cells to either self-renew or differentiate. However, much remains to be learned about the transcriptional programs that define and maintain stem cell identity. Ultimate proof for the potency of transcription factors in controlling stemness comes from work with induced pluripotent stem cells (iPS cells), wherein addition of four factors (Oct4, Sox2, Klf4 and C-Myc) is sufficient to confer stem identity to terminally differentiated cells of many types (Takahashi & Yamanaka, 2006). However, it is less clear how stem cell identities are maintained in vivo.

The Drosophila midgut is an excellent model in which to address how stem cell identity is defined and maintained. Similar to the mammalian small intestine and colon, the fly’s midgut is maintained by intestinal stem cells (ISCs) that divide to give rise to new ISCs and transient cells called enteroblasts (EB) in a near 50/50 ratio. The enteroblast can then differentiate to give rise to either an absorptive enterocyte (EC) or secretory entero-endocrine cell (EE) (Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006). This decision depends on Notch signaling: ISCs produce the Notch ligand Delta, which activates a Notch-dependent transcriptional program in the EB leading to its differentiation into an EC (Ohlstein & Spradling, 2006, 2007; Bardin et al, 2010). Loss of Notch signaling leads to expansion of Delta-positive proliferative stem-like cells as well as postmitotic EEs. Many conserved signaling pathways regulate ISC growth and division during homeostasis and under stress (Jiang & Edgar, 2011; Lucchetta & Ohlstein, 2012). When the midgut is damaged by stresses such as enteric infection, EGFR and Jak/Stat ligands are induced in the ECs and the visceral muscle (VM) surrounding the gut epithelium. These stimulate ISCs to respond to increase their division rate and differentiation speed (Buchon et al, 2009a,b; Jiang et al, 2009, 2011).

The first two studies of Drosophila midgut stem cells described that the progenitor cell compartment (ISCs and EBs) is marked by expression of the zinc-finger transcription factor escargot (Esg). Esg is expressed in both Delta-positive ISCs and transient committed progenitors (EBs) positive for the Notch reporter gene Su(H)-GBE-LacZ. esg mRNA is highly expressed in these cell types, but not in any other cell type of the adult midgut (Supplementary Table S1; Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006; Toledano et al, 2012). However, the function of esg in these progenitor cells...
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has thus far not been reported. The first esg mutant was found in a screen for embryonic lethal mutants and was identified as a Snail-related transcription factor (Whiteley et al., 1992). Escargot, snail and worniu comprise the three Drosophila members of the Snail-related family of zinc-finger transcription factors (Nieto, 2002). Esg is essential for the maintenance of diploidy in larval imaginal disc cells (Hayashi et al., 1993; Fuse et al., 1994), and it plays a critical role in tracheal tube formation through upregulation of the Drosophila E-cadherin, Shotgun (Tanaka-Matakatsu et al., 1996).

Intriguingly, the mammalian Snail family member, Sna1, acts as a transcriptional repressor of E-cadherin expression in mouse and human fibroblasts, and overexpression of Sna1 can trigger an epithelial-mesenchymal transition (EMT) when overexpressed (Batlle et al., 2000; Cano et al., 2000). In addition, the Snail paralog Slug marks a stem cell population in the mouse mammary gland, and mammary epithelial cells (MECs) with high levels of Slug are more clonogenic than Slug-negative MECs (Guo et al., 2012), suggesting a stem cell maintenance function. In fact, there is increasing evidence that cells undergoing EMT gain stem cell-like characteristics (Mani et al., 2008). Hence, Snail family members act as important regulators of both the maintenance function. In fact, there is increasing evidence that cells undergoing EMT gain stem cell-like characteristics (Mani et al., 2008). Hence, Snail family members act as important regulators of both the

Results

Loss of esg causes loss of midgut stem and progenitor cells

To investigate the function of Esg in the adult Drosophila midgut, we used the MARCM technique to generate clones homozygous for the null allele esg<sup>G66B</sup> (Whiteley et al., 1992). With the MARCM method, null mutant clones of esg are generated in ISCs by heat-shock and mutant cells are positively marked by GFP (Lee & Luo, 1999). Subsequently, these MARCM clones were allowed to grow for 4–7 days and stained for the ISC marker Delta (Lee & Luo, 1999). Control clones contained one or more Delta-positive ISCs (Fig 1A, arrows), but esg<sup>G66B</sup> mutant clones contained virtually no Delta-positive ISCs, although ISCs could often be found adjacent to the clones (Fig 1B, arrowhead, quantification in C). Hence, loss of esg led to a loss of Delta-positive ISCs.

To confirm this, we depleted esg in ISCs and EBs by RNAi expressed under the control of the conditional, temperature-sensitive esg-Gal4 UAS-GFP tub-Gal80° system (hereafter referred to as esg<sup>RNAi</sup>). In controls, esg-positive progenitor cell nests were evenly dispersed throughout the midgut epithelium (Supplementary Fig S1A and C). Progenitor cell nests are defined by: (i) expression of esg (Supplementary Fig S1A and C); (ii) expression of the Notch ligand Delta in the ISC (Fig 1A); and (iii) increased presence of Drosophila E-cadherin (DE-cadherin, shg), especially at the ISC-EB junction (Supplementary Fig S1C, arrows). Upon 4 days of esg<sup>RNAi</sup> induction, the majority of GFP<sup>+</sup> cells had disappeared from the midgut (Supplementary Fig S1B and D). The loss of small GFP<sup>+</sup> cells was accompanied by loss of strongly DE-cadherin-positive progenitor cell nests (Supplementary Fig S1D–V, arrows). Flow cytometry (Dutta et al., 2013) showed that the GFP-positive progenitor population in control (esg<sup>K+UAS-GFP</sup>) midguts typically accounted for 5% of total cells (Fig 1I). We noted an almost complete disappearance of this GFP-positive population in esg<sup>K+UAS-GFP</sup>, esg<sup>RNAi</sup> animals (Fig 1J). Hence, loss of esg leads to a loss of the stem/progenitor cell population, demonstrating that Esg is required for their maintenance.

If Esg loss depletes ISCs, it should compromise regenerative growth of the intestine. To test this, we used the intestinal lineage-tracing system esg-FlipOut (esg-F/O) (Jiang et al., 2009). This system uses the temperature-inducible expression of a FLPase which will activate a constitutive Act-STOP-Gal4 driver by removing the STOP cassette flanked by FRT sites. This system was used to drive expression of GFP and esg<sup>RNAi</sup> in both progenitor cells and their descendant progeny (ECs and EEs) for 1 week before exposing animals to the pathogenic bacterium Pseudomonas entomophila (P.e.) (see Fig 2F for an overview of the esg-F/O system). Indeed, following depletion of esg, all of the animals died from infection within 4 days, whereas 90% of controls survived to this timepoint (Fig 1D). Animals expressing esg<sup>RNAi</sup> had dramatically shortened, disorganized midguts that had few or no pH3-positive cells compared to control midguts 2 days after P.e. infection (Fig 1E and F, quantification in G). Whereas control midguts showed an almost complete renewal of midgut tissue after 2 days of P.e. infection based on their ubiquitous expression of GFP, esg<sup>RNAi</sup> midguts still had many GFP-negative cells, highlighting the inability of the midgut to self-renew upon infection (Fig 1E and F). Altogether, these data further support that loss of esg leads to loss of the Delta-positive, E-cadherin-positive ISC population in the midgut.

Loss of esg drives stem cells to differentiate

Our results led us to hypothesize that loss of esg resulted in stem cell loss through precocious differentiation, rather than cell death. To test this, we generated esg<sup>G66B</sup> mutant MARCM clones for 4, 7 or 14 days and stained for the EE marker Prospero (Fig 2A and B), or the EC marker nubbin (Pdm1), a POU-domain transcription factor specifically expressed in mature ECs (Lee et al., 2009; Dantoft et al., 2013) (Fig 2C and D). Whereas control clones contained ECs, EEs and also Delta-positive ISCs (Figs 1A and 2A and C), esg<sup>G66B</sup> mutant clones consisted exclusively of differentiated ECs and EEs (Fig 2B and D). Thus, loss of esg leads to precocious differentiation into either EC or EE cell types, with an accompanying loss of Delta-positive ISCs. Similar results were observed using the esg-F/O system to clonally express esg<sup>RNAi</sup>. We quantified the differentiation phenotype that occurred upon loss of esg by using the esg-F/O system to express esg<sup>RNAi</sup> (Fig 2F–H). Strikingly, after 1 day of induction, the esg<sup>RNAi</sup> cell clones consisted mostly of Pdm1-positive ECs (Fig 2H, arrows). Control clones at this timepoint consisted mostly of Pdm1-negative progenitor cells (Fig 2G, quantification in I). Importantly, the total number of GFP-positive cells for each region of interest (ROI) that was analyzed did not differ significantly between control and esg<sup>RNAi</sup> animals, indicating that stem/progenitor cells were not lost through cell death (Fig 2J).

Previous studies demonstrated that loss of esg in larvae leads to a loss of diploidy in abdominal histoblasts (abdominal epithelial
progenitor cells) (Fuse et al., 1994). Hence, we considered the possibility that the main function of Esg in the ISC/EB population might be to restrain polyploidization, a normal aspect of EC differentiation. However, by staining for the entero-endocrine (EE) cell marker Prospero (Pros), we determined that there were also Pros-positive EE cells present in many of the esgG66B mutant clones (Fig 2A and B). EE cells normally differentiate with a 2C DNA content (Zielke et al., 2014). However, we noticed an increase in EE nuclear size inside several of the esgG66B MARCM clones. Quantification of the DNA content of differentiated Pros-positive EEs in wild-type and esgG66B mutant tissue revealed that esgG66B EE cells had, on average, doubled their DNA content compared to WT EE cells (Fig 2E) and thus were mostly in G2. However, these EE cells did not become polyploid like the fully matured EC cells within the clone (compare Fig 2A and B). Thus, Esg acts as a general repressor of cell differentiation, but not as a specific repressor of polyploidization in the ISC/EB compartment.

To precisely delineate the cell type in which Esg is required to repress differentiation, we expressed esgRNAi specifically in EBs using the Su(H)GBE-Gal4 UAS-GFP tub-Gal80ts driver (Su(H)ts), a Notch-responsive transgene combination that is highly active in EBs (Furriols & Bray, 2001; Zeng et al., 2010). While control Su(H)GBE-positive EBs were mostly small, spindle-shaped cells situated...
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Figure 2. Progenitor cells are lost through differentiation upon loss of esg.

A–D esg\(^{-}\) null mutant MARCM clones are solely composed of both Pros\(^{+}\) enteroendocrine (EE) cells (B–B', arrow) and Pdm1\(^{+}\) enterocytes (EC) (D–D', outline, compare with control clones in (A, C)). Scale bars, 50 \(\mu m\).

E Integrated DAPI intensity/nucleus for Pros\(^{+}\) EE cells in esg\(^{-}\) null mutant clones and the surrounding wild-type EE cells. \(n = 320\) for WT, \(n = 35\) for esg\(^{-}\). \(P < 0.0001\), Student’s t-test.

F Diagram outlining the esg-F/O system. (left) At 18\({}°\)C, the system is held inactive by the temperature-sensitive suppressor Gal\(_{80}\)ts. (middle) 1-day shift to 29\({}°\)C inactivates the Gal\(_{80}\)ts, and esg-Gal\(_{4}\) will drive the expression of UAS-GFP and UAS-Flp, thereby activating the Act > STOP > Gal4 cassette. (right) After 6 days, the F/O system will express UAS-GFP in both the progenitor cells and the progeny from these progenitors due to the activated Act-Gal4 driver.

G, H esg-F/O clones induced for 1 day show that expression of esg\(^{RNAi}\) leads to a rapid loss of progenitor morphology and the appearance of GFP-positive/Pdm1-positive small EC cells (H, arrows, compare to control in G). Scale bars, 50 \(\mu m\).

I Quantification of GFP-positive/Pdm1-positive cells/ROI in (G, H), \(P < 0.0001\), Student’s t-test.

J Quantification of total number of GFP-positive cells/ROI in (G, H), \(P = 0.097\), Student’s t-test.

Source data are available online for this figure.
adjacent to a Delta-expressing ISC (Supplementary Fig S2A and D arrows), \textit{esg}^{\text{RNAi}} expression transformed most EBs into Pdm1-positive cells with a larger (likely polyploid) nucleus, strongly resembling ECs (Supplementary Fig S2B, arrowheads, quantification in C). GFP expression was also lost from these cells (compare Supplementary Fig S2D with E). Knockdown of \textit{esg} in the EB population for 7 days led to a threefold reduction in the number of \textit{Su(H)} GBE-positive cells as determined by flow cytometry (Supplementary Fig S2F–H). These results indicate that \textit{esg} knockdown in EBs that have received the Notch signal accelerates their differentiation into ECs, suggesting a role for Esg in the maintenance of the transient EB state.

**Enforced Esg expression in ISCs blocks differentiation**

If \textit{esg} loss from progenitor cells results in precocious differentiation, locking Esg expression “on” in this compartment might, conversely, inhibit differentiation. To test this possibility, we used the \textit{esg}-F/O or MARCM systems to clonally express Esg (UAS-esg) in ISCs and EBs. In these cases, Esg expression is driven by the \textit{Actin} 5C or \textit{tabulin} promoters, which are constitutive in all cell types and cannot be switched off. Whereas control MARCM clones consisted of many Pdm1+ ECs and a few Pros+ EE cells (Fig 3A and C, arrows), MARCM clones expressing Esg contained only small, diploid, Pdm1+ and Pros− cells (Fig 3B and D, arrowheads, quantification in E). Similar results were obtained using the \textit{esg}-F/O and \textit{esg}^{\text{TS}} systems to drive UAS-esg expression (data not shown). Upon \textit{P.e.} infection, \textit{esg}^{\text{TS}} animals overexpressing \textit{UAS-esg} in their progenitor cells could still respond by increasing the ISC division rate (Fig 3F). However, an increased mortality was observed upon \textit{P.e.} infection (Fig 1D), most likely because ISCs could not differentiate into ECs, and therefore, the damaged epithelium could not be replenished. Some cells within \textit{esg}-F/O > \textit{UAS-esg} clones were positive for Delta (Fig 3H, arrows, Supplementary Fig S3A, B and E), but other, adjacent cells expressed the Notch-responsive \textit{Su(H)}-GBE-lacZ reporter (Supplementary Fig S3C, D and F). Overexpression of Esg in EBs increased the number of GFP-positive EBs (compare Supplementary Fig S2I with G). This increase in EBs was also seen in \textit{esg}-F/O > \textit{UAS-esg} clones expressing the \textit{Su(H)}-GBE-lacZ reporter (Supplementary Fig S3F). In summary, cells in clones with Esg expression locked “on” retain either ISC or EB identity and do not differentiate.

**Esg represses differentiation genes in progenitor cells**

To gain a comprehensive picture of how Esg controls stem cell identity in the midgut, we generated whole-genome RNA-Seq profiles of sorted Esg-positive cells overexpressing Esg or expressing \textit{esg}^{\text{RNAi}} and compared them to controls, as well as to expression profiles of each normal midgut cell type (Supplementary Table S1) (Dutta et al., 2013) (Materials and Methods and Supplementary Materials and Methods). Upon depletion of \textit{esg} by RNAi, 268 genes were downregulated and 382 genes were significantly upregulated (> twofold change, 95% CI, Fig 4A and Supplementary Table S2). In contrast, very few genes were differentially regulated following overexpression of Esg in \textit{esg}^{\text{TS}} cells (Supplementary Table S2). To gain an overview of the variance between the different samples from the different genotypes used in our analysis, we used principal component analysis (PCA). This showed that the \textit{esg}^{\text{RNAi}} samples distinctly clustered away from control and \textit{UAS-esg} (Fig 4B), whereas control and \textit{UAS-esg} samples clustered nearby each other. Hence, overexpression on Esg in the ISC/EB compartment for 1 day did not lead to significant changes in gene expression, whereas depletion of Esg caused a significant shift in the transcriptional landscape of these progenitor cells. Strikingly, many of the genes that were significantly upregulated following \textit{esg} knockdown were genes normally expressed in ECs and EEs. These included the EC markers \textit{nubbin} (Pdm1), Myo61F (brush border Myosin) and \textit{big bang} (bbg) (Bonnay et al., 2013) as well as the EE markers \textit{prospero}, \textit{tachykinin}, \textit{asense} and \textit{Rab3} (Fig 4A and Supplementary Tables S1 and S2). Several established Notch target genes, such as \textit{E(Spl)} genes and hair, went up upon \textit{esg}^{\text{RNAi}} (Supplementary Table S2). Conversely, many reported stem/progenitor cell-specific genes were downregulated upon \textit{esg}^{\text{RNAi}}. These included \textit{miranda}, Efgfr and its ligand \textit{spitz}, the Jak/Stat receptor \textit{dome/domelss}, \textit{LIMK} and \textit{esg} itself (Jiang et al., 2009, 2011; Bardin et al., 2010; Lourenco et al., 2013) (Fig 4A). Gene Ontology (GO)-term analysis of the genes downregulated upon \textit{esg}^{\text{RNAi}} yielded many significantly enriched GO-terms associated with cell fate specification, cell signalling, and wing disc and tracheal development (Supplementary Table S3). This is consistent with previous work, which demonstrated that Esg plays a crucial role in imaginal disc and tracheal development (Fuse et al., 1994, 1996; Samakovlis et al., 1996; Tanaka-Matakatsu et al., 1996). Few GO-terms were significantly enriched in the upregulated gene set. However, we found a strong overrepresentation of genes involved in septate junction formation (Supplementary Table S3). Both \textit{Snakeskin} (Ssk) and \textit{mesh} are highly upregulated upon \textit{esg}^{\text{RNAi}} (30-fold and 43-fold, respectively). It was shown recently that these smooth septate junction structures are essential in ECs for intestinal barrier function (Izumi et al., 2012; Yanagihashi et al., 2012). Hence, upregulation of septate junction components might be an important early step in EC differentiation, triggered by loss of Esg.

The loss of stem cell and upregulation of differentiation gene expression could be a direct effect of Esg on the promoters of its target genes. Alternatively, these changes might reflect indirect effects of losing Esg, namely transcriptional changes affected as an aspect of differentiation. To distinguish between these possibilities and define direct midgut targets of Esg, we used genome-wide binding data for Esg obtained by \textit{in vivo} Dam-ID (MA Loza-Coll, TD Southall, SL Sandall, AH Brand, DL Jones, submitted). The Dam-ID technique uses a DNA methylase fused to a DNA-binding factor of interest (i.e., Esg) that results in enriched DNA methylation at places in the genome where the Esg-Dam fusion protein binds (van Steensel Southall, SL Sandall, AH Brand, DL Jones, submitted). This \textit{Dam-ID}ing data for Esg obtained by \textit{in vivo} Dam-ID yielded many significantly enriched GO-terms associated with cell fate specification, cell signalling, and wing disc and tracheal development (Fuse et al., 1994, 1996; Samakovlis et al., 1996; Tanaka-Matakatsu et al., 1996). Few GO-terms were significantly enriched in the upregulated gene set. However, we found a strong overrepresentation of genes involved in septate junction formation (Supplementary Table S3). Both \textit{Snakeskin} (Ssk) and \textit{mesh} are highly upregulated upon \textit{esg}^{\text{RNAi}} (30-fold and 43-fold, respectively). It was shown recently that these smooth septate junction structures are essential in ECs for intestinal barrier function (Izumi et al., 2012; Yanagihashi et al., 2012). Hence, upregulation of septate junction components might be an important early step in EC differentiation, triggered by loss of Esg.

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A set of genes that are present in the overlap are likely to be directly regulated by Esg. Esg-Dam binding targets included nubbin (Pdm1), Myo31-DF (MyoIA), the Notch effector gene hairy, the EGF receptor Egfr and the cytokine receptor dome (Domeless). The binding profiles for these target gene loci showed that most have a strong enrichment for Esg-Dam binding at the 5’ UTR of their transcript isoforms, that is, close to the transcription start site (Fig 4C). Hence, Esg acts in progenitor cells to repress differentiation genes and promote stem cell gene expression and in many cases binds the affected target loci.

Figure 3. Overexpression of Esg leads to a block in differentiation.
A–D MARCM FRT82B clones expressing UAS-esg. Control (FRT82B) clones are large and contain many polyploid Pdm1+ ECs 2 weeks after clonal induction (A, arrows), whereas clones expressing UAS-esg only contain small Pdm1- diploid cells (B, arrows). UAS-esg MARCM clones do not contain Pros+ cells (D–D’) compared with control (C–C’, arrows). Scale bars, 50 μm.
E Quantification of clones in (A, B). GFP-positive/Pdm1-positive cells within each clone were counted 2 weeks after clonal induction. n = 22 clones for control (FRT80B), n = 46 clones for UAS-esg FRT80B, P < 0.0001, Mann–Whitney U-test.
F Quantification of mitoses/midgut in control, esgRNAi and UAS-esg animals.
G, H Both control FRT80B (G–G’) and UAS-esg FRT80B (H–H’) MARCM clones contain Delta-positive ISCs. Scale bars, 50 μm.
Source data are available online for this figure.
Ectopic expression of Esg in enterocytes triggers hallmarks of de-differentiation

Our observations support a model in which stem and progenitor cells require Esg to maintain their identity, whereas Esg function needs to be extinguished for the de-repression of differentiation genes in order for differentiation to proceed. To further test this model, we ectopically expressed Esg in differentiated ECs using the EC-specific, temperature-inducible driver MyoIA-Gal4, UAS-GFP, tub-Gal80ts (MyoIAts). MyoIA encodes a gut-specific myosin that is a component of the apical brush border and is found only in differentiated enterocytes. Forced expression of Esg in ECs had dramatic effects on intestinal homeostasis. First, UAS-GFP expression driven by MyoIA-Gal4 was lost from ECs within 24 h of Esg induction (Fig 5A–C). Furthermore, these midguts displayed an increased mitotic index (Supplementary Fig S4A and C, quantification in G) and increased expression of Upd3 cytokines (Fig 5F and Supplementary Fig S4E and F), which occurs as a regenerative response to EC loss (Buchon et al., 2009a; Jiang et al., 2009). Close inspection revealed that the loss of GFP expression was not, however, solely due to a loss of GFP-positive ECs. Indeed, many large polyploid EC-like cells with reduced or undetectable GFP expression were observed (Fig 5A–C).
remained in the gut for 1–3 days following induction of Esg in ECs. Co-expression of the apoptosis inhibitor dIAP with Esg could partially rescue EC loss and the concomitant mitotic response. However, the level of GFP expression in MyoIA\textsuperscript{esg} > UAS-esg UAS-dIAP animals still decreased dramatically following 24 hours of transgene induction (Fig 5C, arrows). These GFP-negative ECs in MyoIA\textsuperscript{esg} > UAS-esg UAS-dIAP animals appeared to be normally structured and similar in shape with wild-type ECs (Fig 5A and C, arrows).

Since MyoIA and Pdm1 were identified as Esg-DamID targets (Fig 4C), we hypothesized that ectopic expression of Esg in ECs might repress these differentiation markers. To further test this, we performed RNA-Seq on whole midguts that expressed either Esg alone or in combination with dIAP from the MyoIA\textsuperscript{esg} driver for 24 hours. In both cases, Esg repressed the expression of a myriad of differentiation genes, including known EC markers such as MyoIA and Pdm1 (Fig 5D and G). Various genes involved in midgut immune recognition (PGRP-SC2, PGRP-LE, bbg) and smooth septate junction formation (mesh, cora, Ssk) were also significantly downregulated (Fig 5D and G, and Supplementary Tables S4 and S5) (Izumi et al, 2012; Bonnay et al, 2013; Buchon et al, 2013). Furthermore, most digestive enzymes (Trypsins, Amylases, Lysozymes) as well as transport proteins were significantly downregulated. GO-term enrichment analysis showed significant (P < 0.05) enrichment for GO-terms such as peptidase activity (e.g., \( \alpha, \beta, \gamma \)-Trypsin, 8.5% in MyoIA\textsuperscript{esg} > UAS-esg, > twofold down genes VS 4.3% in the genome), as well as a high enrichment for genes with transmembrane activity (12.3 Versus 3.2%) and hydrolyase activity (e.g., Mal-A1, Mal-A8, LysB, 23.7 versus 14.1%) (Supplementary Table S5). On the other hand, upregulated genes included many cell cycle genes as well as the stem cell marker gene Delta (Fig 5D, and cell cycle-related GO-terms were highly enriched in the upregulated gene set, in line with the increased mitotic activity in these guts at this timepoint (Supplementary Fig S4G). Interestingly, many genes that went down significantly upon Esg overexpression in ECs were upregulated in esg\textsuperscript{esg\textsuperscript{RNAi}} RNA samples (Fig 5G). We confirmed repression of the EC marker Pdm1 and upregulation of Upd3 by qRT-PCR (Fig 5E and F). Thus, ectopic expression of Esg in ECs results in transcriptional repression of EC-specific differentiation genes such as MyoIA and Pdm1 as well as a general loss of expression for genes important in the metabolic functions of the absorptive enterocytes.

After longer induction of Esg expression in ECs, midguts shortened and animal viability declined. Whereas MyoIA\textsuperscript{esg} > UAS-esg midguts lost gut structure and integrity after 5 days of induction (Supplementary Fig S4I), midguts from MyoIA\textsuperscript{esg} > UAS-esg UAS-dIAP animals retained a comparatively normal morphology (Supplementary Fig S4J) and had extended long-term survival, albeit still well below that of controls (Supplementary Fig S4K). Thus, ectopic expression of Esg in differentiated ECs had a detrimental effect on gut homeostasis, which was only partially relieved by inhibition of the apoptotic pathway.

To further distinguish direct effects of Esg on gene transcription from indirect effects, we cross-compared the datasets of Esg-Dam binding targets, genes upregulated upon esg\textsuperscript{esg\textsuperscript{RNAi}} and genes downregulated in whole midguts upon MyoIA\textsuperscript{esg} > UAS-esg expression. Genes that are direct targets of Esg would be expected: 1) to show increased expression upon loss of Esg, 2) to show reduced expression upon overexpression of Esg and 3) to have binding sites for Esg in their promoters. We found 76 genes in this category (Fig 5H and Supplementary Table S6). These genes showed > twofold upregulation upon esg knockdown, > twofold downregulation upon UAS-esg expression and had 1 or more EBRs. Hence, we consider these 76 genes high-confidence Esg targets. This set included Pdm1, the smooth septate junction component Snakeskin (Ssk), the transcription factor bowl and the cell cycle regulator tribbles (Trbl) (Supplementary Table S6), suggesting that Esg represses diverse aspects of the differentiation process.

**Overexpression of nubbin/Pdm1 triggers loss of esg expression and EC differentiation**

Loss of esg causes a strong upregulation of Pdm1 in progenitor cells. Furthermore, our transcriptome analysis data strongly suggest that Esg directly downregulates Pdm1 expression. Therefore, we investigated whether ectopic expression of Pdm1 in the stem/progenitor compartment would be sufficient to cause a loss of stemness and EC differentiation. Expression of a UAS-Pdm1 construct with esg\textsuperscript{esg\textsuperscript{RNAi}} triggered a rapid loss of Delta\textsuperscript{+} ISCs and increased the ploidy of the remaining GFP\textsuperscript{+} cells, which resemble larger, developing ECs (Fig 6A and B, quantification of Delta\textsuperscript{+} cells in H). These cells also express the EC-specific MyoIA-lacZ reporter gene (Fig 6D–F, arrows). The total number of GFP\textsuperscript{+} cells/ROI in UAS-Pdm1 midguts did not significantly change at this timepoint compared to the control (data not shown). We confirmed the apparent increased ploidy upon Pdm1 overexpression by calculating the integrated DAPI intensity/nucleus for GFP\textsuperscript{+} cells in both genotypes. Control esg\textsuperscript{–} progenitor cells resided mainly in G2 and G1, consistent with recent reports on cell cycle phasing in the adult midgut (Zielke et al, 2014). Overexpression of Pdm1 caused a depletion of both the G1 and G2 peaks, with the vast majority of cells having an 8C DNA content (Fig 6E). Thus, based on the increased ploidy and morphological features, we conclude that Pdm1 overexpression results in EC differentiation. To confirm loss of stem/progenitor identity upon ectopic Pdm1 expression, we combined UAS-Pdm1 expression with the progenitor-specific mira-GFP reporter and used an esg\textsuperscript{esg\textsuperscript{RNAi}} driver strain (Fig 6F and G). Whereas control animals have high levels of both mira-GFP and esg promoter-driven UAS-myrRFP (Fig 6F–F'), Pdm1 expression resulted in a rapid loss of mira-GFP/esg\textsuperscript{+} double-positive progenitor cell nests (Fig 6G–G'). Thus, expression of Pdm1 is sufficient to trigger a loss of esg expression and stem cell identity and results in a rapid initiation of EC differentiation.

**Activated Notch overrides ectopic Esg**

Notch signaling is a key regulator of differentiation in the *Drosophila* ISC lineage. (Ohlstein & Spradling, 2006, 2007; Perdigoto & Bardin, 2013). To determine the epistatic relationship between Notch and Esg, we tested whether a constitutively active intracellular domain of the Notch receptor (N\textsuperscript{Ntra}) could override the block to differentiation that results from enforced Esg expression. We used esg-F/O to clonally express UAS-N\textsuperscript{Ntra}, UAS-esg or the combination of both and then scored the appearance of differentiated ECs in the clones. As previously reported (Ohlstein & Spradling, 2007), N\textsuperscript{Ntra} rapidly drove differentiation of the ISCs and EBs into ECs (Fig 7B–B', arrows). Conversely, UAS-esg expression caused the formation of
Figure 5. Overexpression of Esg in differentiated enterocytes leads to a loss of EC-specific differentiation gene expression.

A–C MyoIAts > UAS-esg and MyoIAts > UAS-esg; UAS-dIAP animals show a loss of GFP after 24 h of induction, even in ECs with a wild-type structure (B–B' and C–C', arrows, see A–A' for control). Cells are co-stained with Armadillo (β-catenin) staining (red), which outlines the cell borders. Scale bars, 50 μm.

D Scatter plot showing the genes differentially expressed between MyoIAts > UAS-esg and control.

E, F qRT-PCR analysis for the enterocyte marker Pdm1 (E) and the Unpaired 3 (Upd3) cytokine (F).

G Expression heatmap of representative EC differentiation genes from MyoIAts > UAS-esg and esgts > esgRNAi RNA-Seq samples.

H Venn diagram showing the overlaps between Esg-Dam midgut target genes, genes upregulated upon esgts > esgRNAi expression and genes downregulated in MyoIAts > UAS-esg midguts. The P-value for the overlap was calculated using the hypergeometric probability function.
small, diploid cells that were Pdm1-negative (Fig 7C–C’, arrow and Fig 3B–D). Combined expression of UAS-Nintra and UAS-esg led to the appearance of large polyploid cells that had low levels of Pdm1 (Fig 7D–D’, arrows). Based on their morphology and condensed nuclear DNA, it is likely that these cells were ECs undergoing apoptosis. Hence, Notch activation can at least partially override the block to differentiation that occurs when Esg is overexpressed in progenitor cells.

Esg is required for the growth of ISC-derived tumors induced by NotchRNAi

Loss of Notch leads to a rapid overproliferation of stem-like cells that express high levels of Delta, have a high mitotic index and no longer differentiate into ECs. Large numbers of EE cells are also produced (Supplementary Fig S5; Ohlstein & Spradling, 2007; Perdigoto et al, 2011). These Notch mutant ISC-derived tumors rapidly supplant differentiated cells in the intestinal epithelium and eventually kill the host (P. Patel and B.A.E. unpublished observations). We investigated the requirement for Esg in these stem cell tumors by creating esgG66B null mutant MARCM clones that also expressed NotchRNAi. Many of these esgG66B NotchRNAi clones had a phenotype similar to esgG66B single mutant clones, namely that the clones consisted of only a few cells and these were differentiated ECs and EEs. However, infrequent Delta-positive stem cell overgrowths were also observed (Fig 7H). Despite these rare overgrowths, midguts from esgG66B NotchRNAi animals still had much lower mitotic indices than seen in NotchRNAi controls, and the overgrowths in these midguts
Figure 7. Esg is required for NotchRNAi-induced stem cell overgrowths in the midgut.

A Control esg-FO clones 2 days after clonal induction. Few cells have differentiated into mature Pdm1-positive ECs (arrow).
B Overexpression of the constitutively active Notch intracellular domain (N\textsuperscript{extra}) results in EC differentiation in most of the esg-FO clones (arrows).
C UAS-esg-expressing esg-FO clones consist of small clusters of diploid cells, negative for Pdm1 (arrow).
D UAS-esg UAS-N\textsuperscript{extra} expression results in an extensive loss of GFP-positive cells with few large polyploid GFP-positive cells with an aberrant nuclear morphology (arrows).
E Control FRT\textsuperscript{40}A clones consist mainly of large differentiated ECs, with occasionally a mitotic ISC (arrow).
F esg\textsuperscript{G66B} clones consist mainly of differentiated cells (also see Fig 1).
G Clonal expression of NotchRNAi generates large overgrowths, consisting of highly mitotic Delta-positive cells (arrows, quantification in I).
H esg\textsuperscript{G66B},NotchRNAi clones occasionally form overgrowths resembling NotchRNAi clones, but these are much smaller and less mitotic as the NotchRNAi-only tumors.
I Quantification of mitotic cells/midgut 7 days AHS for the genotypes presented in (E–H). P < 0.0001, Student’s t-test with Welch’s correction.
J Quantification of the percentage of mitotic cells/clonal area unit (1,000 \textmu m) for NotchRNAi and esg\textsuperscript{G66B},NotchRNAi stem cell clones. n = 11 clones for NotchRNAi clones and n = 18 clones for esg\textsuperscript{G66B}NotchRNAi clones.
K Kaplan–Meier survival curves for the different genotypes. n = 3 × 20 animals/genotype for each of the genotypes.

Data information: Scale bars, 50 \textmu m.
Source data are available online for this figure.
were much smaller than overgrowths in NotchRNAi midguts (Fig 7I and Supplementary Fig S5A and B). Quantification of the number of mitoses per clonal area unit in cell overgrowths from NotchRNAi and esgG66B NotchRNAi animals showed a bimodal distribution, such that many overgrowths in esgG66B NotchRNAi animals contained no mitotic cells whereas others had as many mitoses as clones expressing NotchRNAi alone (Fig 7J). The percentage of Pros-positive EE cells in esgG66B NotchRNAi clones was significantly higher than in NotchRNAi clones (Supplementary Fig SSC and D, quantification in E), suggesting that loss of Esg in NotchRNAi clones biases precocious differentiation toward the EE fate. The EE bias in these clones is in line with results from Loza-Coll et al, which show that loss of Esg results in a bias toward EE differentiation (MA Loza-Coll, TD Southall, SL Sandall, AH Brand, DL Jones, submitted).

Long-term survival assays showed that the extensive overproliferation of ISC-derived tumors in NotchRNAi animals led to lethality (Fig 7K). However, this loss of viability was nearly completely rescued in esgG66B NotchRNAi animals (Fig 7K), supporting our hypothesis that Esg is required to maintain the proliferative potential of ISCs. In summary, activated Notch can overcome the action of Esg to induce differentiation. However, Esg is required in stem cell-derived tumors to maintain stem cell characteristics such as proliferative capability, and to prevent differentiation toward the EE fate.

**Discussion**

Stem cell identity is controlled by both extrinsic cues from the niche and cell-intrinsic transcriptional programs. Thus far, most studies of the *Drosophila* midgut have focused on the niche-derived signals that control midgut stem cell self-renewal (Jiang & Edgar, 2011; Lucchetta & Ohlststein, 2012). Here, we demonstrate a cell-intrinsic role for the Snail family transcription factor, Escargot, in controlling ISC self-renewal and differentiation. Loss of Esg leads to a rapid loss of all stem/progenitor cells in the midgut, due to their differentiation, whereas Esg overexpression keeps these cells permanently in an undifferentiated state. The dramatic effects of manipulating Esg levels support a central role for this Snail family member in controlling stem cell identity in the fly intestine.

**Esg promotes self-renewal by repressing differentiation gene expression**

Our transcriptomics analysis indicated that Esg acts as a transcriptional repressor of a large diverse set of differentiation genes. These targets include transcription factors specific to ECs and EEs (Pdm1, Prospero) and genes used in digestion, immunity and cytoarchitectural specialization (Figs 4 and 5 and Supplementary Tables S2, S4 and S6). Interestingly, one of these transcription factors, Pdm1, plays an important role in EC differentiation: ectopic expression of Pdm1 in progenitor cells was sufficient to trigger EC differentiation, partially mimicking the esg loss of function phenotype. The rapid loss of the Esg-expressing cell population upon Pdm1 overexpression suggests that Pdm1 might repress Esg expression, perhaps directly (Fig 6). In this case, Esg and Pdm1 together would constitute a negative feedback switch that governs EC differentiation (see Fig 8).

Our expression analysis also raised the possibility that Esg activates progenitor cell-specific genes in ISCs and EBs. These include the EGF signaling components Cbl, spitz, argos and Egr as well as the Jak/Stat receptor *domeless*. Both EGFR and Jak/STAT pathways are crucial for ISC growth and maintenance, and receptivity to these signals is downregulated in differentiated ECs and EEs (Jiang & Edgar, 2009; Jiang et al, 2011; J. Xiang, D. Dutta and B.A. Edgar, unpublished observations). While Snail family members are best understood as repressors, the Esg paralog Snail has been reported to function as a context-dependent transcriptional activator (Rembold et al, 2014), suggesting that an activating role for Esg is also plausible. The function of Esg as either an activator or repressor is likely determined by co-factors and/or other transcription factors acting on the same promoters that are expressed in the ISC and EB population. In the *Drosophila* embryo, Snail cooperates with Twist at
distinct promoters to activate EMT gene expression during meso-
derm formation (Rembold et al., 2014). Snail2 can bind to Sox9 to
activate expression from its own promoter during chick neural crest
formation (Sakai et al., 2006). In its role as a repressor, Esg binds
the co-repressor CtBP to maintain somatic Cyst stem cells and hub
cells in the Drosophila male testis (Leatherman & Dinardo, 2008; Voog et al., 2014). Future work to unravel the complete transcriptio-
nal network within which Esg functions to maintain the stem/
progenitor state should prove to be very interesting.

Esg, Notch and Stat form a transcriptional circuit that
governs differentiation

Our data support a model in which Esg acts in a circuit with Delta-
Notch signaling to control the switch from stem/progenitor identity
to differentiated cell identities (Fig 8). In its simplest form, this
circuit might be a bistable switch in which Esg and Notch mutually
inhibited each other, with Esg being “on” and dominant in progeni-
tor cells and Notch signaling “on” and dominant in their differenti-
ated progeny, the enterocytes. However, the constant presence of a
substantial population of intermediate progenitor cells, the entero-
blasts (EBs), which express both Esg and Notch reporter genes, indi-
cates that a simple bistable switch is not an accurate conception.
Indeed, EBs, defined here as cells positive for both Esg and the
Notch reporter Su(H)Gbe-LacZ, can persist for many days in the
absence of ISC division. Thus, the EB transition state is metastable.
In this transition state, Notch is apparently active, but secondary
downstream targets that directly affect differentiation, such as
Pdm1, brush border Myosin and smooth septate junction proteins,
remain repressed (Supplementary Table S1). Since these genes are
rapidly activated following depletion of Esg, we suggest that their
repression is most likely mediated by Esg binding.

We can offer two potential explanations for the longevity of the
EB transition state. First, we suggest that the repression of esg
transcription by Notch is indirect and that this delays esg silencing
(Fig 8). Silencing of Esg is not likely to be mediated by the Notch-
regulated transcription factor Su(H) (a transcriptional activator) but
by downstream repressors that act only after enterocyte or endo-
crine differentiation has begun. Pdm1 in ECs and Prospero in EBs
are presently the most obvious candidates. Both are specifically
induced coincident with Esg silencing, in ECs and EEs, respectively,
and Dam-ID assays suggest that Pros has binding sites in the esg
locus (Choksi et al., 2006). Our finding that overexpression of Pdm1
caused the rapid differentiation of Esg+ stem/progenitor cells
supports the notion that Pdm1 could directly repress Esg expression
to control EC differentiation. Furthermore, nubbin/Pdm1 was found to
restrict expression of Notch target genes in the Drosophila
larval wing disc (Neumann & Cohen, 1998). Hence, Pdm1 likely
triggers EC differentiation by downregulating both Esg and the
expression of Notch target genes in the EB. Therefore, Notch is only
transiently active in EBs but fully off in mature ECs with high levels
of Pdm1.

While a delay circuit that controls the silencing of Esg is likely,
theoretically it cannot explain how Esg+ EBs can persist for such
long periods during times of low gut epithelial turnover and then
rapidly differentiate during gut regeneration. Hence, we speculate
that a second input signal acts in combination with Notch-
dependent factor(s) to silence Esg (Fig 8). This second signal is
likely to be a downstream effector of the growth factor signaling
network that also drives ISC division and gut epithelial renewal
(Jiang & Edgar, 2011; Lucchetta & Ohiostein, 2012). Of the transcrip-
tional effectors involved in maintaining gut homeostasis, the most
obvious candidate as an indirect mediator of esg repression is
Stat92E, which is activated by the highly stress-dependent cyto-
kines, Upd2 and Upd3. Tellingly, the cytokine receptor, Dome, Janus Kinase (hop) and Stat92E are all required for EB maturation
into ECs (Jiang & Edgar, 2009; Beebe et al, 2010; Lin et al, 2010). If
the silencing of esg was dependent upon both Notch and Stat92E,
and Delta-Notch signaling was irreversible once resolved; then, the
Notch+ Esg+ EB transition state should in principle be stable in
conditions of low Jak/Stat signaling, as is observed during periods
of midgut quiescence. It needs to be noted, however, that ISCs and
EBs maintain appreciable levels of Stat-reporter gene expression even
during relative quiescence, and so, in this model, it would be
Stat activity above some threshold that would combine with Notch
signaling to trigger differentiation. Since Jak/Stat signaling also
triggers ISC division, a surge in cytokine signaling could coordinately
trigger both the differentiation of older EBs and the production of
new ones in this model (Fig 8), thus explaining how a significant
EB population is maintained even as stem cell activity waxes and
wanes.

A central role for Snail family members in regulating stemness
and differentiation

Snail family transcription factors have been described as regulators
of epithelial-to-mesenchyme transitions (EMT) that occur during
development, wound healing and cancer metastasis (Thiery et al.,
2009). In some contexts, notably metastasis, EMT is believed to
accompany the acquisition of stem-like properties (Mani et al.,
2008). Although Esg itself has not been reported to regulate EMT, its
paralog in flies (Sna) and homologs in mammals (Snai1, Snai2) do
promote EMT (Thiery et al., 2009). Interestingly, our RNA-seq exper-
iments showed that not only Esg, but Snail, Woundii and the Zeb
family members Zfh1 and Zfh2 were all expressed in intestinal stem
cells and downregulated in ECs and EEs (Supplementary Table S1).
Thus, these EMT-linked transcription factors may work together to
affect different aspects of midgut homeostasis and ISC differentia-
tion. Indeed, Esg-positive ISCs and EBs are morphologically more
similar to mesenchymal cells than they are epithelial, whereas Esg-
negative EEs and ECs have the pronounced apical-basal polarity
typical of epithelial cells. Esg+ cells often make striking lateral
projections, suggestive of dynamic behavior, and they have the
capacity to multilayer when their differentiation is blocked or they
are forced to overproliferate. Furthermore, a number of epithelial-
class genes are repressed in Esg+ progenitors and activated upon EC
and/or EE differentiation (Supplementary Table S1). These include
genes encoding the apico-lateral cortical Lgl-Dlg-Scrib-Crb complex,
septate junction proteins (e.g., Ssk, Cora, Mesh) and polarity factors
including Par3 and Par6. Strikingly, Scrib and Ssk both have
Esg-binding sites in their promoters, and their expression is highly
regulated by Esg (Fig 5 and Supplementary Tables S2, S4 and S6).
However, some gene targets that are central to EMT in mamma-
lian cells show opposite trends in the fly’s ISC lineage. For
instance, Esg+ progenitors express significant levels of integrins,
and E-cadherin—typically lost during EMT—is highly upregulated.
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Esg's role in ISC maintenance nicely parallels the functions of other Snail family members in Drosophila and mammals. For instance, in Drosophila neuroblasts (neural stem cells), the Snail family member Worniu promotes self-renewal and represses neuronal differentiation (Lai et al., 2012). In mice, Snail family members have been associated with the regulation of the stem cell state in both normal and pathological conditions (reviewed in Scheel & Weinberg, 2012). For instance, mammary stem cells require the Snail family member Slug to retain their MaSC identity (Guo et al., 2012). Mouse Snai1 also represses the transition from the stem cell-like mitotically cycling trophoblast precursor cell to the endoreplicating trophoblast giant cell during rodent placental development (Nakayama et al., 1998). This process, which also requires a mitotic-to-endocyte switch upon differentiation, is strikingly similar to the role we describe here for Esg in EC differentiation and its role during imaginal disc development (Fuse et al., 1994).

More interesting yet, mouse Snai1 is specifically expressed and required for stem cell maintenance in the crypts of the mouse intestine and expands the stem cell population when overexpressed (Horvay et al., 2011; Horvay et al., personal communication). However, few studies highlight the target genes responsible for the function of Snail family members in stem cell maintenance. One example is from mouse muscle progenitors (myoblasts), where Snai1 and Snai2 repress expression from MyoD target promoters and this is required to maintain their progenitor state (Soleimani et al., 2012). The work we present here shows that Esg affects many aspects of the differentiation process and that it can form a transcriptional switch with one of the targets it represses (Pdm1) to balance aspects of the differentiation process and that it can form a transcriptional switch with one of the targets it represses (Pdm1) to balance aspects of the differentiation process.

Materials and Methods

Fly genetics

See Supplementary Materials and Methods for fly stocks used in this study and a detailed description of clonal analysis and quantification methods.

Immunohistochemistry and microscopy

Drosophila female adult intestines were dissected in phosphate-buffered saline (PBS) and fixed for 30 min at room temperature (RT) in 4% paraformaldehyde. Antibody incubations were performed in 2.5% BSA, 0.01% Triton X-100 and 10% normal goat serum. Washes were performed using PBS containing 0.15% Triton X-100 or 0.1% Tween-20 (for anti-dCad2), respectively. Samples were mounted in Vectashield (Vector Laboratories). Antibodies: chicken anti-GFP (Life Technologies/Molecular probes, 1:500); mouse anti-Pdm1 (kindly provided by Steve Cohen, 1:20); mouse anti-Delta extracellular domain (Developmental Studies Hybridoma Bank (DSHB), 1:50); mouse anti-Prospero (DSHB, 1:50); mouse anti-Armadillo (DSHB, 1:50) rabbit anti-phospho-Histone 3 (Upstate Biotechnology, 1:1,000); rat anti-dCad2 (DSHB, 1:20); mouse anti-β-galactosidase (Sigma, 1:100). Images were acquired on a Leica TCS SP5II inverted confocal microscope, equipped with a HCX Plan APO 40×/1.30 oil-immersion objective and processed with Fiji/ImageJ and Adobe Photoshop. Scale bars are 50 μm in all images, unless otherwise indicated.

Flow Cytometry and RNA-Seq

For transcriptome profiling of sorted progenitor cells, esgDNAs were transfected using either UAS-GFP alone, esgRNAi or UAS-esg were shifted for 24 h at 29°C. Midgut dissociation and FACS were performed as described previously (Dutta et al., 2013). Raw RNA-Seq data were submitted to ArrayExpress (accession E-MTAB-2915). A detailed description of the FACS/RNA-Seq procedure and bioinformatic data analysis can be found in the Supplementary Materials and Methods.

qRT-PCR

MyoIAqRT-PCR was used to drive transgene expression for 24 h at 29°C. 25 adult female intestines per genotype were dissected, and midguts were homogenized in TRizol (Life Technologies). RNA was isolated by phenol/chloroform extraction, followed by purification of the RNA-containing fractions using RNAeasy columns supplied with the RNAeasy Mini Kit (Qiagen). 250 ng of total RNA was used for cDNA synthesis reactions using the QuantiTect Reverse Transcription Kit (Qiagen). qPCR was performed on a LightCycler 480 II (Roche) using the LightCycler Universal Probes and Probes Master (Roche). Each assay was performed in triplicate on four biological repeats. Expression levels of targets analyzed were calculated relative to GAPDH expression, using the ΔΔCt method. Primer sequences can be found in the Supplementary Materials and Methods.

Dam-ID

In transgenic flies that expressed a fusion of Esg and the bacterial DNA methylase Dam, genomic DNA was extracted from whole midguts, and the methylated regions were fluorescently labeled by PCR amplification and hybridized to whole-genome tiling arrays. Approximately 50 female flies expressing the Dam:Esg fusion (or control flies expressing Dam alone) were dissected 5-6 days after eclosion, and genomic DNA (gDNA) was isolated and labeled with minor modifications to the protocol in Choksi et al. (2006). Triplicate samples of labeled gDNA were hybridized with a dye-swap to NimbleGen 2.1M Whole-Genome tiling arrays (Roche), and the fluorescence intensity ratios between Esg:Dam and Dam controls were analyzed to identify EBRs using ad hoc Perl scripts with minor modifications to those reported by Southall and Brand (Southall & Brand, 2009). Briefly, a gene was identified as a putative Esg target if an EBR was mapped within ± 5 kb of its sequence. EBRs are strings of at least 8 consecutive tiling array probes with a log2 (Dam:Esg/Dam intensity ratio) above a threshold that was adjusted for each dataset to correct for differences in background binding and thus generate similar EBR size distributions (overall FDR < 0.01). The putative Esg targets considered in this study had an associated EBR in at least 2 out of the 3 datasets, and only EBRs between 8 and 12 probes in
length were considered (MA Loza-Coll, TD Southall, SL Sandall, AH Brand, DL Jones, submitted).

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

JK designed, performed and analyzed experiments and wrote the manuscript. SKN, JSKC, MALC, TDS, AHB, JO and DD performed and analyzed experiments. DD and CG performed the bio-informatics data analysis. DLJ and BAE analyzed experiments and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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