LETTERS

Evidence that stem cells reside in the adult *Drosophila* midgut epithelium

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Adult stem cells maintain organ systems throughout the course of life and facilitate repair after injury or disease[1]. A fundamental property of stem and progenitor cell division is the capacity to retain a proliferative state or generate differentiated daughter cells[2]; however, little is currently known about signals that regulate the balance between these processes. Here, we characterize a proliferating cellular compartment in the adult *Drosophila* midgut. Using genetic mosaic analysis we demonstrate that differentiated cells in the epithelium arise from a common lineage. Furthermore, we show that reduction of Notch signalling leads to an increase in the number of midgut progenitor cells, whereas activation of the Notch pathway leads to a decrease in proliferation. Thus, the midgut progenitor's default state is proliferation, which is inhibited through the Notch signalling pathway. The ability to identify, manipulate and genetically trace cell lineages in the midgut should lead to the discovery of additional genes that regulate stem and progenitor cell biology in the gastrointestinal tract.

The adult *Drosophila* midgut can be identified on the basis of two anatomical landmarks along the anterior–posterior axis of the gastrointestinal tract: the cardia and pylorus (Supplementary Fig. S1). The inner surface of the midgut is lined with a layer of cells that project into the gut lumen. These cells exhibit apical–basal polarity, as staining for F-actin reveals the presence of a distinct striated border on their lumenal surface (Fig. 1a, b, d and Supplementary Fig. S1). This observation is consistent with the suggestion that the midgut is lined by a cellular epithelium[3,4].

Wild-type midguts were stained with 4,6-diamidino-2-phenylindole (DAPI) to reveal the distribution of cell nuclei within the tissue. Nuclei of the midgut display a distinct distribution and fall into two main categories (Fig. 1a–d). The most prominent cells lining the midgut contain large oval nuclei that stain strongly with DAPI (Fig. 1a, b, d). These cells exhibit a region of the nucleus that does not stain with DAPI, giving the nucleus a hollow appearance. This unstained region may correspond to the large nucleolus characteristic of differentiated cells. A second population of cells containing small nuclei can be detected at a basal position within the tissue. The small nuclei are distant from the gut lumen and often lie in close apposition to the two layers of overlying visceral muscle that surround the gut (Fig. 1a–c). On the basis of nuclear size, position and morphology two general populations of midgut cells can, therefore, be distinguished.

Previous studies in *Drosophila* have led to conflicting views over the existence of cell proliferation in the adult gastrointestinal tract[3–6]. Early reports suggested that somatic stem cells were present in the adult because of morphological similarity to certain larval cells and by analogy to different insect species[3–5]. In contrast, ^{3}H-thymidine labelling experiments detected DNA synthesis in the adult *Drosophila* midgut, but no mitotic figures were observed in a large sample analysed[6]. On the basis of these observations, the authors concluded that no somatic cell division occurs during the lifetime of *Drosophila*. To distinguish between these possibilities, we used a series of three independent assays to test whether cell proliferation can be detected in the adult midgut. In the first assay we used genetically marked wild-type cell lineages to identify dividing cells. The production of marked clones after mitotic recombination depends upon subsequent cell division and is, therefore, a direct means to assay proliferation. In these experiments, wild-type lineages were positively marked in adult flies using the MARCM system[7]. Mitotic recombination was induced by heat shock and green fluorescent protein (GFP)-marked clones could be detected in the midgut (Fig. 2b). Similar results were obtained when adults were heat shocked up to 10 days after eclosion (data not shown). This suggests that the ability to generate clones is not transient, and probably persists throughout the entire life of the animal.

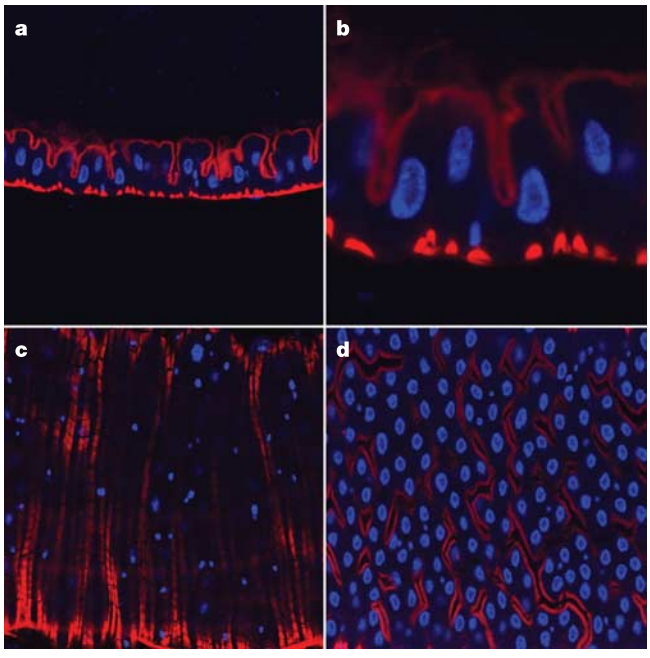

Figure 1 | Cellular organization of the adult *Drosophila* midgut epithelium. **a–d**, Cells with different-sized nuclei occupy distinct positions in the epithelium as seen from two different optical planes (phalloidin, red; DAPI, blue). **a**, A cross-section of the epithelium. The gut lumen is at the top of the image. Magnification is ×80 in each panel, unless otherwise indicated. **b**, High-magnification (×274). Note the small, basally located nucleus adjacent to the surrounding muscle. **c**, A superficial section of the epithelium. Most small nuclei can be detected at this position. **d**, A deeper lumenal section of the epithelium. Most large nuclei can be detected at this position in the epithelium.

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Under the experimental conditions used, the MARCM system produced some background GFP signal that could be detected in control animals (Fig. 2a). To quantify the background signal, we compared the number of GFP-labelled cells in control and experimental animals. A greater than sixfold increase in the number of GFP-labelled cells was detected after heat shock ($n = 10{,}481$ cells). We also used a second independent clone marking method that did not rely on either Gal4 or Gal80 (ref. 8). In these experiments clones were marked by the loss of a ubiquitously expressed GFP and similar results were observed (data not shown). We conclude that a population of actively dividing somatic cells is present in the adult *Drosophila* midgut.

To extend these findings, we conducted 5-bromodeoxyuridine (BrdU) incorporation studies. We observed both large and small BrdU-labelled midgut cells (Fig. 2c, d). Large nuclei adjacent to each other can be differentially labelled, suggesting asynchrony in the timing or extent of DNA synthesis over the course of the labelling period. This is consistent with the notion that the large nuclei are endoreplicating. However, both endoreplication and the canonical cell cycle require new DNA synthesis. To distinguish endoreplicating from dividing cells in the midgut we next stained the tissue with an antibody raised against phospho-histone H3. Careful examination revealed that very low levels of phospho-histone H3 staining could be detected in all cells. However, double staining with DAPI revealed that elevated levels of phospho-histone H3 indicative of mitosis could only be detected among the population of cells with small nuclei (Fig. 2e, f). Thus, cells in the midgut seem to have two distinct cell cycles; whereas both large and small nuclei undergo DNA synthesis, only the cells with small nuclei undergo cell division.

In order to characterize further the small cell population, an expression screen was conducted to identify cell-specific molecular markers. We identified three markers expressed in small cells: *escargot* (*esg*), a transcription factor that belongs to the conserved Snail/Slug family; *prospero* (*pros*), a conserved homodomain transcription factor; and *Su(H)GBE-lacZ*, a transcriptional reporter of the Notch signalling pathway[9]. Simultaneous detection of *esg* expression (*esg-Gal4*, *UAS-GFP*), anti-Pros, *Su(H)GBE-lacZ* expression and DAPI demonstrated that small cells could be subdivided into the following classes on the basis of differential gene expression: *esg*-positive (esg^+), *pros*-positive ($pros^+$), *esg*-negative *pros*-negative ($esg^-\,pros^-$), *esg*-positive

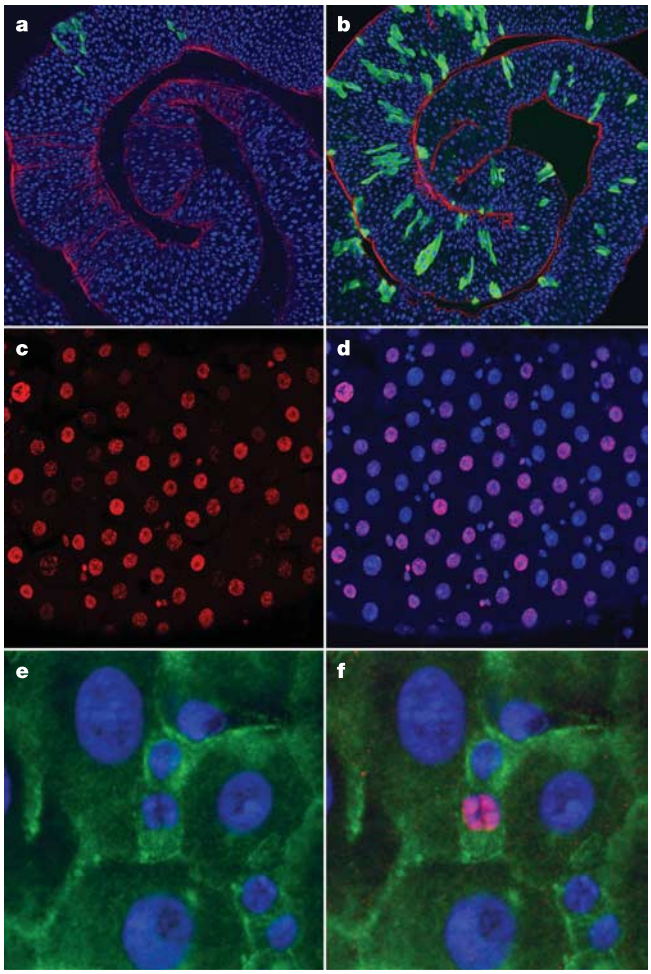

Figure 2 | Cell proliferation in the adult *Drosophila* midgut. **a**, **b**, Domains of cell proliferation are revealed by the presence of positively marked clones (phalloidin, red; anti-GFP, green; DAPI, blue). Original magnification is ×20. **a**, Controls. No heat shock. **b**, Experimental. Heat shock induction results in the generation of marked clones. **c**, **d**, DNA synthesis is detected in both large and small cells after a BrdU pulse. **c**, Anti-BrdU. **d**, Overlay (anti-BrdU, red; DAPI, blue). **e**, **f**, Mitosis is detected in small cells by phospo-histone H3. Original magnification is ×320. **e**, Overlay (*sqh-GFP*, green; DAPI, blue). **f**, Small nucleus with elevated phospho-histone H3 (phospho-histone H3, red; *sqh-GFP*, green; DAPI, blue).

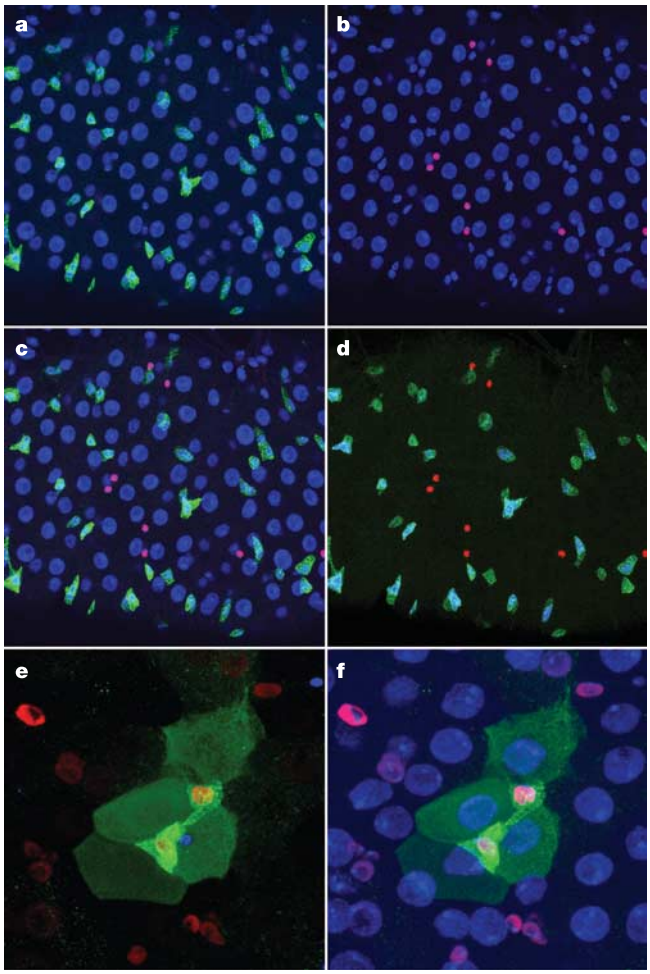

Figure 3 | Cells of the midgut arise from a common lineage. **a**–**d**, Midgut cells can be distinguished on the basis of differential gene expression. **a**, esg^+ cells (anti-GFP, green; DAPI, blue). **b**, $pros^+$ cells (anti-Pros, red; DAPI, blue). **c**, esg^+ and $pros^+$ cells mark distinct populations (anti-Pros, red; anti-GFP, green; DAPI, blue). **d**, *Su(H)GBE-lacZ* expression subdivides the esg^+ cells into two subpopulations (anti-Pros, red; anti-GFP, green; *Su(H)GBE-lacZ*, blue). **e**, **f**, The cells of the midgut are related by lineage. A positively marked MARCM clone is revealed by anti-GFP staining (green). Original magnification is ×160. **e**, Overlay (*esg-lacZ*, red; anti-GFP, green; anti-Pros, blue). **f**, Overlay (*esg-lacZ*, red; anti-GFP, green; DAPI, blue).

Su(H)GBE-lacZ-positive (esg^+ $Su(H)GBE\text{-}lacZ^+$) and *esg*-positive *Su(H)GBE-lacZ*-negative (esg^+ $Su(H)GBE\text{-}lacZ^-$) (Fig. 3a–d). esg^+ and $pros^+$ expression define distinct cell populations (Fig. 3c), whereas *Su(H)GBE-lacZ* expression subdivides the esg^+ class into esg^+ $Su(H)GBE\text{-}lacZ^+$ and esg^+ $Su(H)GBE\text{-}lacZ^-$ subpopulations (Fig. 3d). Quantification reveals that each cell type is present in the midgut in different proportions (Table 1).

The ability to distinguish different cell types using molecular markers enabled us to determine the cell lineage relationships in this tissue. If the large and small nuclei are lineally distinct then marked clones should be restricted to one or the other cell type. However, if a common stem cell progenitor exists in the adult midgut, then marked lineages should contain both large and small nuclei within a clone. To distinguish between these possibilities we generated positively marked MARCM clones and labelled nuclei using DAPI. Lineage analysis shows that marked clones generated in the adult contain both large and small nuclei (Figs 2b and 3f). In addition, both *esg* expression and anti-Pros-labelled cells could be detected within the clones (Fig. 3e). These lineage-tracing experiments suggest that a stem cell progenitor exists and is sufficient to generate the distinct cell types of the adult midgut. We refer to this cell as the adult intestinal stem cell (ISC).

esg expression in diploid cells has been shown to be necessary for the maintenance of diploidy[10]. In addition, the distribution of *esg* messenger RNA has been used as a marker for male germline stem cells[11]. Together, these observations raise the hypothesis that *esg* expression may also mark a population of progenitors in the midgut. We therefore asked whether *esg* expression correlates with markers of cell proliferation. Simultaneous staining with anti-BrdU and DAPI reveals that *esg*-expressing cells are among the population of cells that are also positively labelled by BrdU (Supplementary Fig. S2a, b). To ask whether *esg*-expressing cells also undergo cell division, the midgut was double stained to detect both *esg* expression and phospho-histone H3. High levels of phospho-histone H3 can be detected specifically in *esg*-expressing cells (Supplementary Fig. S2c). Our results demonstrate that *esg* expression marks a population of proliferating progenitor cells in the midgut.

However, the esg^+ cell population can be divided on the basis of *Su(H)GBE-lacZ* expression (Table 1). To distinguish functionally the two esg^+ populations, we examined the consequences of altering Notch signalling in the adult midgut. We first tested the effect of globally reducing Notch signalling using the conditional *Notch* temperature-sensitive (N^{ts}) mutant. N^{ts} flies were first crossed to an allelic series that included N^{55e11}, $N^{264.47}$, N^{ts1} and $N^{nd.1}$. We found that the strongest loss of function combinations (N^{ts}/N^{55e11} and $N^{ts}/N^{264.47}$) failed to generate viable adult flies even at the permissive temperature, often dying as pharate adults (data not shown). N^{ts}/N^{ts} flies produced viable adults at the permissive temperature with midguts similar to wild type (11 out of 12 midguts; Fig. 4a). N^{ts}/N^{ts} flies shifted to the non-permissive temperature led to a mild increase in the number of small cells (8 out of 12 midguts; Fig. 4b). The weakest allelic combination, $N^{ts}/N^{nd.1}$, also produced viable adults at the permissive temperature but showed no detectable phenotype when shifted to the non-permissive temperature (data not shown).

We next sought to test the requirement of *N* only in esg^+ progenitor cells. To obtain both spatial and temporal control over transgene expression in *esg*-expressing cells, we combined the temperature-sensitive Gal80 inhibitor, $Gal80^{ts}$ (ref. 12), with the *esg-Gal4* transcriptional activator. To verify that the $Gal80^{ts}$ transgene functions in the midgut, we first characterized the temporal and spatial induction of a *UAS-GFP* transgene. Adult *esg-Gal4,UAS-GFP*, $tub\text{-}Gal80^{ts}$ flies grown at the permissive temperature showed no detectable GFP expression in their midguts (Supplementary Fig. S3a, b). In contrast, when these flies were shifted to the non-permissive temperature they showed high levels of GFP expression that were detectable after 1 day and maximal by 2 days (Supplementary Fig. S3c).

The requirement of Notch was then tested in esg^+ cells using a $UAS\text{-}N^{RNAi}$ transgene[13], to reduce Notch signalling. In control experiments, $UAS\text{-}N^{RNAi}$; *esg-Gal4,UAS-GFP*, $tub\text{-}Gal80^{ts}$ flies grown at the permissive temperature appeared to have wild-type midguts and showed no detectable GFP expression, suggesting that

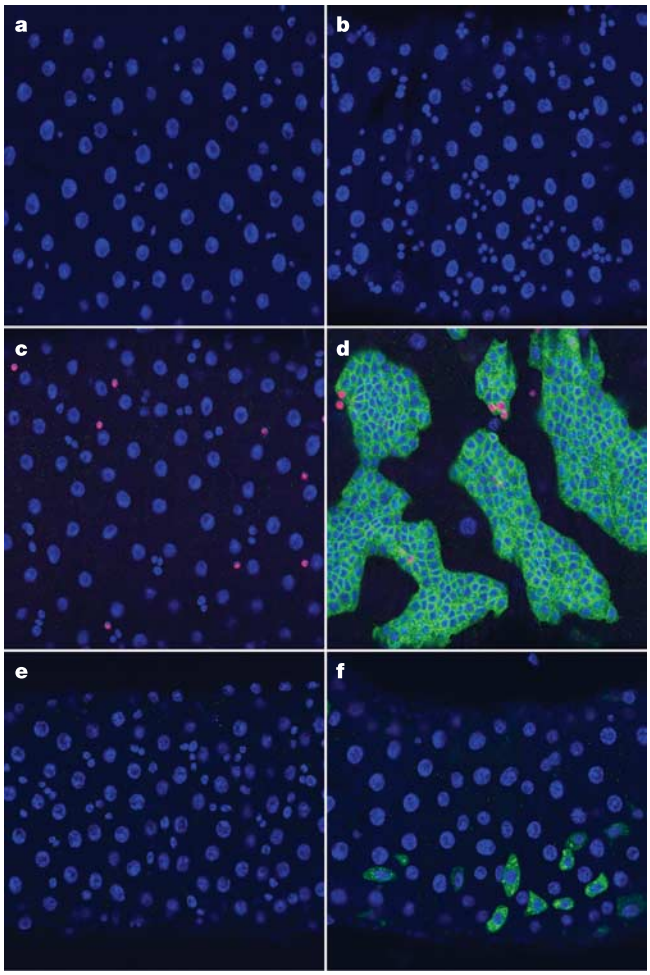

Figure 4 | Notch pathway regulates the number of midgut progenitors. **a**, **b**, Notch is required to restrict the number of small cells. **a**, Controls. N^{ts} flies grown at the permissive temperature (DAPI, blue). **b**, Experimental. N^{ts} flies shifted to the non-permissive temperature (DAPI, blue). Note the increase in small nuclei. **c**, **d**, Notch is required in esg^+ progenitors to restrict their number (anti-Pros, red; anti-GFP, green; DAPI, blue). **c**, Controls. $UAS\text{-}N^{RNAi}$; *esg-Gal4,UAS-GFP*, $tub\text{-}Gal80^{ts}$ flies grown at the permissive temperature. **d**, Experimental. $UAS\text{-}N^{RNAi}$; *esg-Gal4,UAS-GFP*, $tub\text{-}Gal80^{ts}$ flies shifted to the non-permissive temperature. **e**, **f**, Notch is sufficient to promote the early differentiation of epithelial cells (anti-GFP, green; DAPI, blue). **e**, Controls. $UAS\text{-}N^{intra}$; *esg-Gal4,UAS-GFP*, $tub\text{-}Gal80^{ts}$ flies grown at the permissive temperature. **f**, Experimental. $UAS\text{-}N^{intra}$; *esg-Gal4,UAS-GFP*, $tub\text{-}Gal80^{ts}$ flies shifted to the non-permissive temperature.

Table 1 | Distribution of small cell types in the adult *Drosophila* midgut

Cell type	Percentage of total in wild type*
esg^+	36
$pros^+$	5
esg^- $pros^-$	1
esg^+ $Su(H)GBE\text{-}lacZ^+$	18
esg^+ $Su(H)GBE\text{-}lacZ^-$	18

*$n = 242$.

under these conditions *UAS* transgenes are efficiently suppressed (0 out of 20 midguts; Fig. 4c). In contrast, *UAS-N^{RNAi}*; *esg-Gal4,UAS-GFP, tub-Gal80^{ts}* flies shifted to the non-permissive temperature showed an increase in the number of small cells (19 out of 20 midguts; Fig. 4d). Notably, the presence of *esg-Gal4*, *UAS-GFP* in this experiment enabled us to determine that the increased number of small cells were also *esg*$^+$. When these guts were co-stained with anti-Pros antibody we observed ectopic small cells that also expressed *pros*, and these cells were often associated with lower levels of *esg* expression (Fig. 4d). Taken together our experiments suggest that Notch signalling in *esg*$^+$ cells is necessary to restrict proliferation.

We also tested the effect of Notch activation in *esg*$^+$ cells using *N^{intra}*, a constitutively active form of Notch. In control experiments, *esg-Gal4,UAS-GFP, tub-Gal80^{ts}*; *UAS-N^{intra}* flies grown at the permissive appeared to have wild-type midguts and showed no detectable GFP expression (0 out of 23 midguts; Fig. 4e). In contrast, *esg-Gal4,UAS-GFP, tub-Gal80^{ts}*; *UAS-N^{intra}* flies shifted to the non-permissive temperature showed a decrease in phospho-histone H3 staining compared to controls that were not shifted (mitotic index = phospho-histone H3 positive cells/adult midgut: mitotic index experimental = 0.1, $n = 17$; mitotic index controls = 16, $n = 15$). In addition, although some *esg*$^+$ cells appear to be wild type, we observed a region-specific decrease in the levels of *esg* expression and a concomitant increase in nuclear size similar to that of midgut epithelial cells (Fig. 4f). These observations demonstrate that Notch activation is sufficient to limit proliferation of *esg*$^+$ cells and suggests that Notch may also be sufficient to promote early steps of epithelial cell differentiation.

Taken together, our characterization of the adult *Drosophila* midgut suggests that a population of adult stem cells resides within this tissue. We note that this general conclusion of our study is consistent with an accompanying paper[14]. Our analysis of the Notch signalling pathway in *esg*$^+$ cells suggests that *esg*$^+$ *Su(H)GBE-lacZ*$^-$ cells mark a population of dividing progenitors and that Notch is necessary and sufficient to regulate proliferation. We propose a model in which *esg*$^+$ *Su(H)GBE-lacZ*$^-$ progenitors generate at least two different types of daughter cells depending on the level of Notch activation (Supplementary Fig. S4a–c). Under conditions of reduced Notch function we observed an expansion of both *esg*$^+$ progenitor cells and *pros*$^+$ cells. These observations suggest that *esg*$^+$ cells give rise to *pros*$^+$ cells in a Notch-independent manner. Under conditions of Notch activation we observed a decrease in the proliferation and promotion of epithelial cell fate differentiation, while the number of *pros*$^+$ cells remained unaffected (data not shown).

Several lines of evidence suggest that *pros*$^+$ cells correspond to gut enteroendocrine cells. Previous studies show that *prox1*, the vertebrate *pros* homologue, is associated with post-mitotic cells and early steps of differentiation in the central nervous system[15]. Furthermore, in *Drosophila*, *pros* is thought to be a pan-neural selector gene that is both necessary and sufficient to terminate cell proliferation[16]. Finally, although vertebrate enteroendocrine cells arise from endodermal origins they are known to express neural-specific markers[17]. Therefore, *pros*$^+$ cells probably define a population of enteroendocrine cells in the midgut.

Studies of stem cell compartments in *Drosophila* have led to the characterization of two types of progenitor cells in the germ line[18,19]. The first is referred to as the germline stem cell and is sufficient to give rise to the respective cells of either the male or female germ line. The second type of progenitor cell described is called the cystoblast in female germ line and gonialblast in the male germ line. Although the cystoblast and gonialblast both have the capacity to generate the differentiated cells of their respective tissues, they are thought to be more restricted in their fate than the germline stem cells. On this basis, we suggest that an analogous progenitor may also exist in the adult *Drosophila* midgut; we refer to this cell as the enteroblast (EB). The population of *esg*$^+$ *Su(H)GBE-lacZ*$^-$ progenitor cells, which we have described, display characteristics of both the ISC and the EB; therefore, additional experiments will be necessary to distinguish unambiguously these alternatives.

METHODS

Fly culture and strains. Flies were maintained on standard media. Crosses were cultured at either 18 or 25 °C in humidity controlled incubators on a 12 h light/dark cycle. In the experiments described here only female flies were analysed. The following fly strains were used: *Oregon^R* (wild type); *esg-Gal4* (Hayashi laboratory); *UAS-mCD8GFP*; *esg^{K606}/Cyo* (*esg-lacZ*); *y,w, hsflp^{122}*; *FRT^{40A}*, *ubiq GFP/Cyo; y,w; FRT^{40A} y +*; *y,w,UAS-GFP, hsflp*; *tubulin-Gal4*; *FRT^{82B}*, *tubulin Gal80/TM6B*; *y,w,hsflp*; *FRT^{82B}*, *hs pi-Myc*; *y,w,hsflp*; *esg^{K606}/Cyo*; *FRT^{82B}*, *hs pi-Myc*; *sqh-GFP*; P(*tubP-Gal80^{ts}*)*9,w/FM7c*; *Su(H)GBE-lacZ* (Bray laboratory); *N^{55e11}* (null allele of *N*); *N$^{264.47}$*; *N^{ts1}* (a temperature-sensitive allele of *N*); and *N$^{nd.1}$*; *w, UAS-N^{RNAi}*; *w*; *UAS-N^{intra}* (constitutively active form, deleted extracellular domain). All stocks were obtained from the Bloomington stock centre unless otherwise indicated.

Mosaic analysis. For MARCM clones, fly crosses were established and cultured at 18 °C to generate adults of the genotype *y,w,UAS*-GFP, *hsflp*; *tubulin-Gal4*/+; *FRT^{82B}*, *tubulin Gal80*/*FRT^{82B}*, *hs pi-Myc* or *y,w,UAS*-GFP, *hsflp*; *tubulin-Gal4*/*esg^{K606}*; *FRT^{82B}*, *tubulin Gal80*/*FRT^{82B}*, *hs pi-Myc*. Negatively marked clone crosses were established and cultured at 25 °C to generate adults of the genotype *y,w, hsflp^{122}*/*y, w*; *FRT^{40A}*, *ubiq GFP*/*FRT^{40A} y +*. Equal numbers of adult flies were then divided into experimental and control groups. Experimental animals were subjected to between one and three 37 °C heat shocks for clone induction. After heat shock, MARCM clones were grown at 18 °C to minimize background signal from the Gal4/UAS system. Control flies did not receive heat shock and were maintained at 18 °C. Midguts were examined between 7–27 days after clone induction.

Temperature shift experiments. Crosses were established and cultured at 18 °C, the permissive temperature, until adulthood. The progeny were divided into two equal pools; controls were cultured at 18 °C and the experimental group was shifted to 29 °C. Flies were shifted to 29 °C for the following periods of time: *N^{ts}* (15–18 days); *UAS-N^{RNAi}*; *esg-Gal4,UAS-GFP, tub-Gal80^{ts}* (17–19 days); *esg-Gal4,UAS-GFP, tub-Gal80^{ts}*; *UAS-N^{intra}* (7–17 days). Cultures were transferred onto fresh food augmented with yeast paste every 2–3 days during the experimental period.

BrdU labelling. BrdU staining was performed using standard methods and modified as follows. Adult flies were cultured on standard fly media vials augmented with 200 µl of 6 mg ml^{-1} BrdU plus 20% sucrose. Flies were subsequently cultured at 25 °C and transferred to new media every 2 days for between 4–8 days to achieve maximal labelling.

Histology. Adult flies were dissected in *Drosophila* Ringer's solution. The entire gastrointestinal tract was removed and fixed in ×0.5 PBS (Sigma) plus 4% EM grade formaldehyde (Polysciences) for 30 min. Samples were washed in ×1 PBS plus 0.1% Triton X-100 (PBST) for 2 h, then incubated with primary antibodies over night. Primary antibodies were removed and samples were washed in PBST for 2 h. Secondary antibodies were incubated for 3 h. Secondary antibodies were removed and samples washed in PBST for 2 h. Mounting media containing DAPI (Vectashield) was added and the samples were allowed to clear for 1 h before mounting. All steps completed at 4 °C, with no mechanical agitation.

Antisera. The following primary antibodies were used: goat anti-GFP (Molecular Probes) 1:2,000; rabbit anti-β-gal (Cappel) 1:2,000; rabbit anti-phospho-histone H3 (Upstate Biotechnology) 1:1,000; mouse anti-tubulin (Sigma) 1:1,000; mouse anti-BrdU (Becton Dickson) 1:100; anti-Pros (Developmental Studies Hybridoma Bank) 1:40. The following secondary antibodies were used: donkey anti-goat Alexa 488 (Molecular Probes) 1:500; donkey anti-goat Alexa 594 (Molecular Probes) 1:500; donkey anti-goat Alexa 633 (Molecular Probes) 1:500; donkey anti-rabbit rhodamine RRX (Jackson ImmunoLabs) 1:250; donkey anti-mouse Cy5 (Jackson ImmunoLabs) 1:250. We used the following dyes and mounting media: Alexa 546- and Alexa 594-conjugated phalloidin (Molecular Probes) 1:500; Vectashield plus DAPI (Vector).

Microscopy and imaging. Fluorescent samples were examined on a Zeiss Axioskop 2*mot*plus upright microscope. Confocal images were collected using a Leica TCS SP2 AOBS confocal system. Images were processed and assembled in Photoshop CS.

Nomenclature. In consensus with the accompanying paper[14], we have agreed to use the terms intestinal stem cell (ISC) and enteroblast (EB) to refer to progenitor cells of the adult *Drosophila* midgut epithelium.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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