Transient Scute activation via a self-stimulatory loop directs enteroendocrine cell pair specification from self-renewing intestinal stem cells

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The process through which multiple types of cell-lineage-restricted progenitor cells are specified from multipotent stem cells is unclear. Here we show that, in intestinal stem cell lineages in adult Drosophila, in which the Delta-Notch-signalling-guided progenitor cell differentiation into enterocytes is the default mode, the specification of enteroendocrine cells (EEs) is initiated by transient Scute activation in a process driven by transcriptional self-stimulation combined with a negative feedback regulation between Scute and Notch targets. Scute activation induces asymmetric intestinal stem cell divisions that generate EE progenitor cells. The mitosis-inducing and fate-inducing activities of Scute guide each EE progenitor cell to divide exactly once prior to its terminal differentiation, yielding a pair of EEs. The transient expression of a fate inducer therefore specifies both type and numbers of committed progenitor cells originating from stem cells, which could represent a general mechanism used for diversifying committed progenitor cells from multipotent stem cells.

Homoeostatic renewal of tissues and organs commonly depends on the resident tissue-specific stem cells, which have the ability to self-renew and produce diverse committed progenitor cells that differentiate into multiple cell types. The generation of lineage-restricted progenitor cells can be actively specified by asymmetric cell division, in which cell fate determinants are specifically segregated into one of two stem cell daughter cells. Alternatively, committed progenitor cells can be specified passively; in this case, stem cells differentiate as they physically depart from the niche environment. Examples are Drosophila germline stem cells and stem cells in the mouse small intestine. However, very little is known about the molecular mechanisms by which distinct, lineage-restricted progenitor cells are generated from a common stem cell pool.

Multipotent intestinal stem cells (ISCs) in the Drosophila posterior midgut can differentiate into two distinct cell lineages: absorptive enterocytes (ECs), which have been shown to occur from ~90% of ISC divisions, and secretory enteroendocrine cells (EEs), which occur from ~10% of ISC divisions. A typical ISC division generates a new ISC and a post-mitotic enteroblast (EB), the lineage-committed progenitor cell. ISCs produce the membrane-bound ligand Delta (Dl), which activates its receptor Notch in EBs. Notch activation results in unidirectional differentiation of EBs into ECs. The process of EE specification, however, is much less well understood.

Recent studies have suggested that EEs are directly differentiated from ISCs, implying that the decision of EE specification may occur at the stem cell level in ISCs, but how this occurs remains unclear. A recent study indicates that, in ~90% of cases, EEs are differentiated directly from ISCs in a process that compromises stemness: an ISC divides once, yielding a pair of EEs. This model is in line with the observation that EEs are typically generated in pairs, but it implies that EE generation occurs at the expense of continued existence of ISCs. In view of stem cell maintenance, this seems to be a risky developmental strategy.

Previous studies have demonstrated that the transcription factor Prospero (Pros), which is specifically expressed in EEs, acts as an EE-fate-determination factor. The expression of Pros is promoted by the achaete-scute complex (AS-C) genes, and the expression of AS-C genes is suppressed by the transcriptional repressor Ttk69. Loss of AS-C genes compromises EE generation without affecting EC generation from ISCs. Moreover, overexpressing either Sc or Ase induces increased EE generation from ISCs. However, how Sc is regulated to control EE specification is unclear.

Here, we investigated the regulation of Sc during the process of EE generation, and our results allow us to propose a model through which discrete committed progenitor cell types are generated from stem cells.

### Results

An EE-regeneration model reveals that ISCs self-renew during generation of EE pairs. To better understand the process of EE specification in ISCs, we set up an EE regeneration assay and examined de novo EE regeneration. Conditionally depleting sc in Esg-GAL4 flies by shifting flies to restrictive temperature (RT) (29°C) from pupal stage caused complete EE depletion in the midgut of newly eclosed flies (Fig. 1a and Supplementary Fig. 1), as previously reported. Shifting flies to RT after eclosion also completely disrupted new EE generation, although some EEs produced from the pupal stage remained (Supplementary Fig. 1). Strikingly, three to four days after shifting EE-less flies back to 18°C, EEs marked by Pros, appeared concurrently in many regions of the midgut (Fig. 1b). Interestingly, many cells were co-stained with Dl (membrane) and Pros (nucleus), indicating that EEs arose from ISCs self-renewing at RT.

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and this was further confirmed by separate fluorescent labelling for DI and Pros (Fig. 1b,c). These DI+ Pros+ cells are probably the differentiating EEs described previously in normal midgut6. Similar to what has been observed in normal midgut, ~71% of Pros+ cells appeared in pairs (Fig. 1d). The EE pair is probably generated by cell division of a mother cell, as two EEs in the pair appeared simultaneously and mitotic Pros+ cells could be observed (Fig. 1c).

After carefully counting the total number of DI+ cells in the epithelium before and after EE appearance, we found that the number of DI+ cells remained constant, although DI expression was downregulated during EE appearance (Fig. 1b,c). In addition, there was always a DI+ or DIlo cell next to each newly formed EE or EE pair (Fig. 1b). These observations suggest that, instead of directly differentiating into an EE as proposed previously9, an ISC actually divides first to generate a new EE progenitor cell (EEP), which then undergoes one round of cell division before its terminal differentiation, yielding an EE pair (Fig. 1f). Interestingly, like in normal midgut10, the two EEs in the newly generated EE pair produce different peptide hormones (Supplementary Fig. 2). The EEP is reminiscent of the EE mother cell in the pupal midgut, which is also capable of cell division prior to terminal differentiation9.

**Sc is dynamically expressed in ISCs.** Among AS-C complex genes, sc is both necessary and sufficient for EE specification12,13. It is therefore important to understand how sc expression is regulated. Using the CRISPR-Cas9 technique, we generated a Sc-GFP knock-in line in which green fluorescent protein (GFP) was fused to 3’ of the Sc coding region (Fig. 2a). The expression pattern of Sc-GFP in larval imaginal discs was virtually identical to the reported sc mRNA and protein expression patterns (Supplementary Fig. 3). In adult midgut, albeit variable in numbers among different midguts, Sc-GFP (GFP fluorescence)-expressing cells were observed only in a subset of DI+ cells (Fig. 2b,c). By quantification, on average, ~15% of DI+ cells had distinguishable Sc-GFP expression in both posterior (15.6 ± 2.0% n = 32 guts) and anterior (15.5 ± 5.9%, n = 12 guts) midguts. Immunostaining with anti-GFP, which greatly amplified the Sc-GFP signal, revealed that Sc-GFP could be observed in virtually all DI+ cells (Supplementary Fig. 3). Therefore, Sc is dynamically expressed in ISCs, with a weak expression level in most ISCs, and increased expression levels in a small subset of ISCs.

**Transient Sc activation induces EE generation from ISCs.** From 23 (upstream and downstream) glass multiple reporter (GMR) enhancer-GAL4 lines generated for sc (ref. 14) (Fig. 2d), we identified one line, GMR14C12, that drove UAS-GFP expression in some diploid cells in the midgut epithelium (Fig. 2e,f). The density, distribution and individual variability of the GFP+ cells were largely similar to those of Sc-GFP+ cells, suggesting that this reporter line is driven by the enhancer element for Sc expression in the midgut. Co-staining with cell markers revealed that GMR14C12>GFP was mainly expressed in 15 ± 0.69% DI+ cells (n = 26 guts), but residual GFP was also often present in the newly formed EEs in the same cell nest (Fig. 2g,h). Interestingly, the GFP+ DI+ cells commonly showed relatively low levels of DI expression compared to the GFP− DI+ cells (Fig. 2g,h). Interestingly, ISCs that are producing EEs are known to be associated with low levels of DI expression7. We also compared the expression pattern between Sc-GFP and GMR14C12-GAL4, UAS-RFP (red fluorescent protein) (GMR14C12>RFP). Similarly, GMR14C12>RFP+ cells were found in small cell nests of two to three cells. About half of them contain Sc-GFP+ cells (Supplementary Fig. 4), suggesting that the GMR14C12>RFP reporter only partially recapitulates Sc-GFP expression in ISCs. This is probably because Sc is expressed transiently and the expression of GMR14C12>RFP is relatively delayed due to the binary expression system used and/or extra time required for RFP maturation. Similar to the GMR14C12>GFP cell nest, many GMR14C12>RFP cell nests also contained newly formed EEs (Supplementary Fig. 4). The residual GMR14C12>RFP or GMR14C12>GFP expression found only in the newly formed EEs but rarely in any ECs indicates that the ISCs with GMR14C12-GAL4 expression only generate EEs. To test this hypothesis, we performed cell lineage tracing studies (Fig. 2i) with flies carrying GMR14C12-GAL4, Tub-Gal80°;
Fig. 2 | Transient Sc expression precedes EE generation from ISC. a, Diagram showing genomic information for C-terminal insertion of enhanced green fluorescent protein (EGFP) into the sc gene region. b,c, Expression of Sc-GFP (green), DI (white) and Pros (red) in midgut of five- to seven-day-old flies. Note that in each microscopic field of view, the number of GFP+ cells (arrowheads) could vary significantly from a few (b) to a dozen (c) cells, possibly reflective of the local proliferation rate. d, Diagram showing the genomic regions of GMR-GAL4 lines screened for sc midgut enhancer. e,f, Expression of GMR14C12>GFP (green), DI (red on membrane) and Pros (red in nucleus) in midgut of five- to seven-day-old flies. The number of GFP+ cell nests varies significantly in different fields of view. GFP was also present in newly formed EEs in the same cell nest (inset). g, Classification of GMR14C12>GFP+ cell clusters: EE only (blue column), ISC/EEP+EE (green), ISC/EEP cluster without EE (red) and EC only (grey). h, Quantitative analysis of Dll+ (red) and Dll- (green) cells within GMR14C12>GFP+ cell clusters. i, Diagram showing the strategy for the short- and long-term tracing for GMR14C12-GAL4. j, Expression of lineage marker (green), DI (red on membrane) and Pros (red in nucleus) in the midgut of GMR14C12-GAL4 flies tracing for 2 days (j) and 8 days (k). l, Classification of marked clones in lineage tracing analysis: ISC+EE (red), ISC+EC (blue) and ISC clone containing both EE and EC (green). n indicates GFP cell nests, Dll+ GFP cells and clones, respectively, as indicated. Samples in g and h were pooled from 32 animals, and samples in l were pooled from four experiments. Experiments were repeated three to five times independently, with similar results. Scale bars, 30 μm.
**Notch mutant ISCs have increased Sc expression and only give rise to EEs.** It has been shown that loss of *Notch* (N) in ISCs causes the formation of DI+ and Pros+ cell tumours. We found that the majority of DI+ cells in the tumour showed increased expression of Sc-GFP (Fig. 3a). Interestingly, Sc-GFP protein level was transiently downregulated at metaphase of mitosis (Fig. 3a’). In agreement with a recent study, the EE-like tumour cells were post-mitotic cells, in contrast to the ISC-like tumour cells (Fig. 3b,c). In addition, BrdU pulse and chase experiments revealed that DI+ cells could be readily labelled with BrdU, but by chase, the BrdU signal in DI+ cells quickly disappeared and reappeared in Pros+ cells (Fig. 3d,e), suggesting that the clustered Pros+ cells are derived from DI+ cells. Consistent with this notion, there are many DI+ Pros+ cells at the boundary between DI+ and Pros+ cell clusters (Fig. 3f), and these are probably the cells in the process of EE differentiation. The DI+ cells in N mutant tumour are therefore unipotent: they only give rise to EEs (Fig. 3g). This property of N mutant DI+ cells further supports the notion that increased Sc expression primes ISCs to produce EE progenies.

**Sc functions as both a mitogenic factor and a cell-fate inducer.** To further understand the function of sc, we conditionally induced sc expression in ISCs and monitored the cellular events in a time-lapse. Transient sc induction caused a rapid cell division response (Fig. 4a,b), revealing that Sc is a potent inducer of cell division. As Sc is known as an EE fate regulator by inducing Pros expression, we examined whether continuous expression of sc could cause ISC differentiation into EE. Continuous sc expression for a week resulted in dramatic accumulation of Pros+ EEs in the epithelium (Fig. 4c and Supplementary Fig. 5). Cell lineage tracing studies confirmed that sc-overexpressing ISCs only generate EEs (Supplementary Fig. 5). After continuous sc overexpression in ISCs for 3 weeks, we began to observe regional ISC loss at the anterior midgut (Fig. 4d). Some residual Esg>GFP cells co-expressed Pros, indicating that the ISC loss is due to differentiation into EEs. Therefore, although short-time expression of sc is not, prolonged activation of sc is detrimental to ISC self-renewal/maintenance.

Pros is also known as a potent cell cycle inhibitor. Indeed, in contrast to the effect of Sc, conditionally inducing Pros expression immediately caused ISCs to exit the cell cycle (Fig. 4b). Sc is thus both a mitogenic factor and an EE-cell-fate inducer, whereas Pros is both a cell cycle inhibitor and an EE-cell-fate determination factor. These properties underline a precisely regulated circuitry that directs formation of a pair of EEs from each EEP, which will be described below.

**Process of sc-overexpression-induced EE specification in ISCs.** To understand how EEs are generated from ISCs, we examined the cellular events in a time window (24–72 h post sc induction) when new EEs just begin to be generated. To do this, we expressed a UAS-RedStinger reporter in the overexpression system. RedStinger is relatively stable and can serve as a lineage marker to trace the progeny of the originally marked ISCs. The number of cell divisions of the initially labelled ISCs could be deduced based on the total number of cells present within each clone. The first cell division following sc overexpression occurred in ISCs (PH3+ in a one-cell clone, Fig. 5a), and during mitosis, Pros expression was marginally detectable on the ISC membrane (Fig. 5a). Differential Pros expression was first observed in cells at telophase, where only one of the two daughter cells retained membrane and cytoplasmic Pros expression (Fig. 5b,f), indicating that the cell division is asymmetric. By contrast, DI+ was still evenly expressed in the two daughter cells (Fig. 5f). The DI+ Pros+ daughter is probably the EEP, which seems to enter mitosis rapidly, as the second cell division (one PH3+ cell in a
two-cell clone, Fig. 5c) following sc overexpression always happened in these cells, before the original ISC re-enters mitosis (in a three-cell clone, Fig. 5e). During mitosis, EEPs began to have strong and punctate nuclear Pros expression (Fig. 5c,d,g), indicative of the initiation of EE commitment.

Collectively, these observations suggest a stepwise model for EE regeneration from ISCs: transient activation of sc expression induces asymmetric cell division, which generates a new ISC and an EEP. Sc also induces Pros expression, but the nuclear translocation and transcriptional activity of Pros can only occur in EEPs, not ISCs. Residual Sc activity in the newly formed EEP then induces one round of cell division before the accumulation of nuclear Pros that causes the cell cycle exit and EE commitment. In this manner, precisely a pair of EEs is generated from each EEP (Fig. 5h).

Two regulatory feedback loops control transient Sc activation in ISCs. It is intriguing how this transient activation of Sce is achieved. A feedback mechanism via Slit-Robo2 signalling has been implicated in regulating EE generation22, but we found that the Slit-Robo2 signalling does not affect Sc expression in ISCs (Supplementary Fig. 3). Previous studies on early Drosophila development have suggested reciprocal regulatory relationships between AS-C genes and the enhancer of split complex (E(spl)) genes, which are known as the Notch target genes23-27. To test whether similar mechanisms occur in ISCs, we screened a number of lacZ, GAL4 or GFP reporters or candidate reporters for individual E(spl) genes28, m3>Gal4 and m8-lacZ showed patterns similar to the general Notch activation reporter NRE-lacZ, which is specifically expressed in ECs. Interestingly, m8-lacZ showed a very weak, but similar expression pattern to Sc (Fig. 6a-c): it was weakly detectable in a small subset of diploid cells. Moreover, m8-lacZ cells were largely overlapped with GMR14C12>GFP+ cells. In addition, the majority of E(spl)m8-lacZ ISCs were Dlllow (Fig. 6b), again similar to the profile of GMR14C12>GFP+ cells (Fig. 2h). This indicates a direct regulatory relationship between Sc and E(spl)m8. Indeed, transient overexpression of sc in ISCs led to robust upregulation of m8-lacZ expression in all progenitor cells (Fig. 6c-e). In addition, ectopic expression of sc in ECs using MyoIA-GAL4 also led to ectopic m8-lacZ expression in ECs (Fig. 6f). Notably, co-expressing Notch-RNAi did not prevent the upregulation of m8-lacZ expression caused by sc overexpression (Fig. 6g,h), suggesting that E(spl)m8 expression is independent of Notch activity in ISCs.

To further characterize the regulatory relationship between sc and E(spl) genes, we transiently overexpressed sc in ISCs by Dl-GAL4 and sorted out Dl-GFP+ cells for RNA-seq analysis. From this analysis, we found that, in addition to m8, many other E(spl) genes, such as m4, m6, m7, my and m6, were upregulated as well (>100-fold) upon sc overexpression (Fig. 6i). The analysis also revealed that sc overexpression strongly induced the expression of multiple cell cycle-promoting genes, such as stg, CycA, CycB and CycE (Fig. 6i), further supporting that Sc is a potent mitogenic factor. By analysing previously reported ChIP-seq data for Sc in ISCs29, we found that Sc could bind to the enhancer regions of multiple E(spl) genes, including my, m6, m7 and m8 (Supplementary Fig. 6), suggesting that Sc directly regulates their transcription. In the enhancer sequence for m8, we identified two consensus E-box sites (CANNTG) for proneural factors, named here E-box #1 (E1) and E-box #2 (E2) (Supplementary Fig. 6). Mutation of E1 severely dampened lacZ reporter expression in response to sc induction, while mutation of E2 only mildly (if at all) reduced the reporter expression (Supplementary Fig. 6), suggesting that the E1 site is a major Sc-binding site in the E(spl)m8 enhancer.

To study the effect of E(spl) genes on sc, we found that transient overexpression of E(spl)m8 rapidly eliminated sc-GFP expression in all ISCs (Fig. 7a,b), and also rapidly suppressed Dl expression in ISCs (Fig. 7a,b). This explains the decreased Dl expression in ISCs during EE regeneration as well as in GMR14C12>GFP+ cells and m8-lacZ+ cells. To determine whether or not E(spl)m8 suppresses sc expression directly, we expressed E(spl)m8-Dam fusion gene in
ISCs, and performed DNA adenine methyltransferase identification (DamID) analysis. Interestingly, we observed a strong and specific enrichment peak at the GMR14C12 region (Fig. 7e), suggesting that E(spl)m8 is able to directly bind to the enhancer of sc in ISCs. The direct two-way regulation between Sc and E(spl)m8 further supports the notion that Sc and E(spl)m8 (plus other E(spl) proteins) form a negative feedback regulatory loop in ISCs, which may explain the transient activation pattern of Sc in ISCs.

To test the functional requirement of E(spl) genes in suppressing sc expression, we generated MARCM clones mutant for Df(3R) E(spl)b32.2 gro+, a deletion and transgene combination in which all of the E(spl) genes have been removed18. Consistent with previous

**Fig. 5 | Process of sc-overexpression-induced EE generation from ISCs.** **a–e.** Expression of Dl>RedStinger (red), PH3 (green) and Pros (white) during sc-overexpression-induced mitosis. The order of cell division can be deduced based on the mitotic marker PH3 and the number of RedStinger cells in a single cluster. a, Pros showed marginal expression on membrane at anaphase of the first cell division, which occurs in ISCs. b, At telophase of the first cell division, one of the two daughter cells began to show cytoplasmic Pros accumulation. c, d, The second cell division always occurred in the Pros+ daughter cell. During mitosis, Pros began to accumulate in the nucleus with a punctate pattern. e, The third cell division occurred again in ISCs. f, Expression of Dl>RedStinger (red), PH3 (green) and Dl (white) in ISCs during sc-overexpression-induced mitosis. Dl did not show differential level during asymmetric cell division. g, Quantitative analysis of Pros subcellular localization in mitotic cells during the first (ISC), second (EEP) and third (ISC) cell divisions: no detectable expression (grey), nuclear expression (red) and membrane expression (blue). n represents RedStinger clones, as indicated. h, Graphic model for the process of EE generation from ISCs with continuous sc expression. sc induction in ISCs promotes asymmetric cell division that generates EEPs, which begin to show punctate nuclear Pros expression. Each EEP immediately divides once before terminal differentiation, yielding a pair of EEs. Experiments were repeated three to five times independently, with similar results. Scale bars, 5 μm.
Fig. 6 | Sc induces E(spl) genes in ISCs. **a**, Expression of GMR14C12>GFP (green), E(spl)m8-lacZ (red) and Pros (white) in midgut of adult flies. **b**, Quantitative analysis of cell proportion of E(spl)m8-lacZ/GMR14C12>GFP+ (left), GMR14C12>GFP+/E(spl)m8-lacZ+ (middle), DI+//E(spl)m8-lacZ+ cells (right). *n* represents total cells (pooled from 24 animals), as indicated. **c, d**, Expression of DI-GFP (green), E(spl)m8-lacZ (red), DI (white on membrane) and Pros (white in nuclear) in ctrl (**c**) and sc-overexpressing midguts (**d**). Overexpression of sc in ISCs led to robust upregulation of m8-lacZ expression in all progenitor cells. Note that for the lacZ staining images in **c** and **d**, the same exposure time was used for comparison. **e**, Quantitative analysis of cell proportion of E(spl)m8-lacZ+ cells in ctrl (**c**) and sc-overexpressing midgut (**d**). The population of E(spl)m8-lacZ+ cells was classified into two types: E(spl)m8high (red) and E(spl)m8low (yellow). *n* represents ISCs (pooled from 25 animals), as indicated. **f**, Expression of E(spl)m8-lacZ (red), DI (green on membrane) and Pros (green in nuclear) with sc overexpression in ECs by Myo1A-GAL4. Ectopic expression of m8-lacZ expression was found in ECs. **g, h**, Expression of DI>GFP (green), E(spl)m8-lacZ (red), DI (white on membrane) and Pros (white in nucleus) with co-expression of sc and Notch-RNAi for two days (**g**) and five days (**h**). Co-expressing Notch-RNAi did not prevent the upregulation of m8-lacZ expression caused by sc overexpression. Note that sc overexpression prevented N-RNAi-induced tumour development by promoting EE differentiation. **i**, Scatter plot showing the comparison of gene expression profiles of DI>GFP+ cells in WT and sc-overexpressing midgut. Pink dots depict genes with twofold and higher changes, including decreased genes (left) and increased genes (right). Both WT and sc overexpression datasets have three biological replicates. Experiments were repeated three to five times independently, with similar results. Scale bars, 20 μm.
Fig. 7 | Regulatory feedback loops control Sc expression in ISCs. a, b, Expression of Esg > RFP (red), Sc-GFP (green), DI (white on membrane) and Pros (white in nucleus) in midguts of WT (a) and m8-overexpressing midgut (b). Overexpression of m8 rapidly reduced sc-GFP and DI expression in all ISCs (b). c, d, Expression of DI > GFP (green), GMR14C12-lacZ (white) and Pros (red) in the midgut of WT (c) and sc-overexpressed midgut (d). GMR14C12-lacZ was nearly undetectable in normal midgut epithelium. Overexpression of sc in ISCs led to GMR14C12-lacZ expression in progenitor cells and newly formed EEs. Experiments were repeated three to five times independently, with similar results.

e, DamID analysis for E(spl)m8 and ChIP-seq analysis for Sc in ISCs revealed binding activities for both E(spl)m8 and Sc at the GMR14C12 region. f, Graphic model to describe how ECs and EEs are respectively generated from ISCs. In the ISC lineages of adult Drosophila midgut, Delta-Notch-signalling-guided EC generation from ISCs acts as the default mode, while transient expression of Sc triggers EE generation from ISCs. Oscillatory expression of Sc in ISCs is achieved by transcriptional self-stimulation combined with negative feedback regulation between Sc and E(spl) proteins and other Notch targets. During generation of EEs, increased Sc expression induces asymmetric cell division that generates a new ISC and an EEP. Because Sc functions both as a mitogenic factor and a cell-fate inducer, residual Sc activity in the newly formed EEP is then able to induce one round of cell division before accumulation of nuclear Pros causes the cell cycle exit and EE differentiation. In this manner, precisely a pair of EEs is generated from each EEP. Scale bars, 30 μm (a, b, d), 20 μm (c).
observations\(^1\), the mutant clones contained increased number of Dl\(^+\) cells, and further properly differentiated ECs, but not EEs (Supplementary Fig. 3). We found that there was no obvious upregulation of sc-GFP expression in any of the ISC-like cells within the Df(3R) E(spl) b32.2 gro + mutant clones (25 clones examined, corresponding to 416 Dl\(^+\) cells). One likely possibility is that, in addition to the E(spl) genes, there are other Notch downstream target genes that function to promote EC differentiation, and that participate in negative feedback regulation with sc. Conceivably, the loss of E(spl) genes may possibly cause compensatory upregulation of such unidentified genes in a manner that prevents the activation of AS-C genes, and that primes all of the undifferentiated cells to adopt an EC fate.

Sc has been reported to transcriptionally self-stimulate itself, which acts as an essential mechanism for progenitor protein accumulation during sensory organ development\(^25,26\). To test whether this also occurs in ISCs, we constructed LacZ reporter for sc using the GMR14C12 enhancer fragment. GMR14C12-lacZ was barely detectable in WT midgut epithelium. However, overexpression of sc (2 days at RT) by D1-GAL4 effectively induced lacZ reporter expression in many diploid cells in the epithelium, including ISCs, presumptive EEs, and newly formed EEs (Fig. 7c,d). The Sc ChIP-seq data analysis also revealed two Sc binding peaks within the GMR14C12 region (Fig. 7e). Together, these data suggest that Sc is able to stimulate its own transcription directly by binding to the sc enhancer, and this self-stimulation may allow Sc to gradually accumulate to a relatively high level before being shut down via feedback regulation.

Collectively, these data suggest a model to explain the dynamic Sc expression in ISCs. Sc is usually expressed at low levels, and E(spl) genes are not expressed. Through transcriptional self-stimulation, Sc gradually builds up and eventually reaches a level that is sufficient to induce EE generation. Concomitantly, the high level of Sc also induces E(spl) genes. These induced E(spl) proteins and other unidentified Notch targets then function as a negative-feedback mechanism that rapidly turns Sc expression back to the baseline level. The expression of E(spl) genes is consequently downregulated, and the expression of both E(spl) proteins and Sc then returns to the default states (Fig. 7f).

The production of EEs from pupal ISCs (pISCs) occurs at ~48–72 h after pupal formation (APF) (Supplementary Fig. 7)\(^32,33\), and we found that transient expression of Sc directs EE generation in pupal ISCs as well.

**Discussion**

This study suggests a simple model to describe how ECs and EEs are specified from ISCs. Delta-Notch-signalling-guided EC generation from ISCs acts as the default mode, while transient Sc expression triggers EE generation from ISCs (Fig. 7f).

The finding that a cell-fate inducer is expressed in stem cells to induce progenitor cell specification is unexpected, as expression of a cell-fate inducer could potentially compromise stem cell maintenance by promoting differentiation. As we have demonstrated, overexpressing sc in ISCs for a prolonged time is able to deplete ISCs by forcing their differentiation. Under normal conditions, however, Sc expression is only transiently increased. In addition, although Sc is sufficient to induce Pros expression in ISCs, Pros protein is only marginally detectable, strictly localized to the cell membrane and/or cytoplasm of ISCs. This indicates that there are additional mechanisms through which Pros activation is prevented in ISCs, such as by destabilizing Pros or preventing Pros from entering the nucleus, mechanisms that are utilized in neural stem cell lineages\(^36,37\). Therefore, transient expression of a cell-fate inducer is a seemingly risky but rather safeguarded mechanism by which lineage-committed progenitors are generated from stem cells.

Sc is dynamically expressed in ISCs, and our study suggests that this dynamic expression pattern is driven by a coordinated action of two feedback loops: a transcriptionally self-stimulatory loop of Sc and a negative feedback loop between Sc and E(spl) proteins. As the depletion of the entire E(spl) genes does not lead to continuous activation of Sc, it is likely that other Notch downstream targets or other mechanisms could also participate in this negative feedback loop. For instance, a Sina-Phyl-Ttk69 complex has recently been implicated in a feedback loop with Sc to regulate EE differentiation\(^30\). Given that the negative feedback is a common mechanism underlying biochemical oscillations\(^38\), these feedback interplays could provide a mechanism that drives oscillatory expression of Sc in ISCs. This hypothesis is attractive, because Sc oscillation could potentially serve as an internal timer for periodic production of EEs from ISCs. Future cellular and molecular analysis combined with in vivo live imaging should help to further test and refine this oscillation model, and to understand whether and how this internal timer is regulated with environmental cues.

Lineage-committed progenitor cells usually transiently amplify themselves prior to terminal differentiation. For instance, the transit-amplifying progenitor cells in mammalian small intestine usually divide four to five times before terminal differentiation\(^39\). Similarly, each ganglion mother cell in Drosophila neuroblasts divides exactly once prior to terminal differentiation, which yields a pair of neurons or glial cells\(^40\), akin to the EE pair generation from an EEP reported here. We find that Sc is both a mitogenic factor and a cell-fate inducer. Pros, on the other hand, is a known inhibitor of cell division and a cell differentiation factor\(^41\). Because of these properties, the net result of the diminishing and accumulating activity of Sc and Pros, respectively, guides each EEP to divide exactly once prior to terminal differentiation, yielding an EE pair (Fig. 7f). Our study therefore also demonstrates a mechanism that determines the number of transient amplifications of committed progenitor cells.

In summary, our study demonstrates that the transient expression of a fate inducer specifies both the type and the cell division number of committed progenitor cells from multipotent stem cells. Recent studies on progenitor cells in mammalian tissues have found that the expression of fate-inducing transcription factors is frequently detected in self-renewing stem cells\(^40,41\), indicating that this seemingly risky but safely guarded mechanism reported here could represent a general principle for producing several distinct types of committed progenitor cell from the same pool of multipotent stem cells.

**Methods**

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41556-017-0020-0.

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**References**


Articles


Methods

Fly stocks. Female flies aged 4–7 days were selected for the experiments, unless otherwise noted. Animal care and use followed the institutional guidelines of the National Institute of Biological Sciences (NIBS), Beijing, and the authors affirm that the study is compliant with all relevant ethical regulations regarding animal research.

The following stocks were used in this study: UAS-sc-RNAi (BDS, #26206); Esg-GAL4/UAS-GFP (gift from S. Hayashi); UAS-RedStinger (BDS, #8547); Df-GAL4 (gift from X. Zeng and S. Hou); GMR14C12-GAL4 (BDSC, #48607); UAS-RNAi (BDS, #26095); UAS-Notch-RNAi (BDS, #7078); UAs-Gsc (BDS, #26683); lea (BDS, #3102); m8-lacZ (BDS, #26786); UAs-E(spl)m8-DamID (gift from F. Schweisguth); Df(3R) E(spl) b32.2 gro+.

The Scute-GFP knock-in line was generated by Cas9-mediated gene knock-in using a previously described protocol5. The GMR14C12-LacZ reporter line was generated by inserting the GMR14C12 DNA fragment into the multiple cloning site of CAPLZ vector, followed by DpnI-mediated transformation. The m8-lacZ reporter construct was generated by inserting the enhancer region of E(spl)m8 into the multiple cloning site of C4PLZ vector. The E1 or E2 site mutant m8-nlacz reporter constructs were generated by site-directed mutagenesis, and in both cases, the E-box sequence CANNNTG was mutated to AANNGG.

Immunostaining. Midgut samples were fixed and immunostained as previously described5. Briefly, midguts were dissected into cold Grace's media, and fixed in a mixture of 4% formaldehyde in PBS and N-heptane in an equal volume for 30 min. The lower phase of the mixture was then replaced with methanol, followed by vigorous shaking. Both samples were then washed once with methanol for 5 min, followed by gradual rehydration in PBT (PBS with 0.1% Triton X-100). For staining with anti-GFP antibody, the fixation was done by incubating midguts with 4% formaldehyde in PBS for 30 min. Samples were then incubated in block (5% normal goat serum in PBT) for 1 h. Samples were stained with primary antibodies overnight at 4°C, washed with PBT, and incubated with secondary antibodies for 2 h or overnight at 4°C. Samples were washed with PBT and mounted in 70% glycerol. The primary antibodies used in this study include mouse anti-DI (Developmental Studies Hybridoma Bank, Iowa (DSHB), 1:100 dilution); mouse anti-Pros (DSHB, 1:300); rabbit anti-phospho-Histone H3 antibody (Cell Signaling Technology, 1:500); rabbit anti-GFP (Cell Signaling Technology, 1:200); rabbit polyclonal anti-lacZ antibody (Cappel, 1:8,000); rabbit anti-Pros (gift from Y.-N. Jan, 1:1,000); mouse anti-Allatostatin A (DSHB, 1:300); rabbit anti-tachykinin antibody (gift from D. Nassel, 1:300); rat anti-Brdu (Abcam, 1:200); and mouse anti-Cut (DSHB, 1:20). The secondary antibodies used in the study were goat anti-rabbit, anti-rat or anti-mouse IgGcs-conjugated to Alexa (568 or Cy5) (Molecular Probes, 1:300). Images were acquired on a Zeiss LSM510 or LSM880 inverted confocal microscope. All images were assembled in Adobe Photoshop and Illustrator.

Binary GAL4/UAS system and mosaic analysis. The temporal and regional gene expression targeting (TARGET) system, which utilizes the binary GAL4/UAS system and a temperature-sensitive GAL80, was used for spatial and temporal control of transgene expression in fly midgut16. Crosses were carried out at 18°C unless otherwise stated. Two- to three-day-old female progenies were then cultured at 29°C with regular corn meal without BrdU supplement and cultured for an additional 4 days before experiment. The RNA-seq data that support the findings of this study are available in the Life Sciences Reporting Summary.
References

Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

1. **Experimental design**

   1. **Sample size**
      
      Describe how sample size was determined.
      
      No statistics was used to predetermine sample size. Sample sizes were determined based on either our previous experiences or the published studies with similar experimental designs.

   2. **Data exclusions**
      
      Describe any data exclusions.
      
      No data was excluded.

   3. **Replication**
      
      Describe whether the experimental findings were reliably reproduced.
      
      All experiments were repeated at least three times unless otherwise indicated, and all attempts at replication were successful. For DamID experiments, one replicate was performed in which the relative enrichment peaks were determined by log (fold change) between Sc-Dam-expressed (experiment) and Dam-expressed (control) samples.

   4. **Randomization**
      
      Describe how samples/organisms/participants were allocated into experimental groups.
      
      No randomization was applied.

   5. **Blinding**
      
      Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
      
      Investigators were not blinded to group allocation.

   6. **Statistical parameters**
      
      For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

      - **n/a Confirmed**

        - [ ] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
        - [ ] A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
        - [ ] A statement indicating how many times each experiment was replicated
        - [ ] The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
        - [ ] A description of any assumptions or corrections, such as an adjustment for multiple comparisons
        - [ ] The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
        - [ ] A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
        - [ ] Clearly defined error bars

      See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Prism 6, STAR (v020201), Cufflinks (v2.2.1), Bowtie (version 1.1.2), MACS (version 1.4.1)

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All materials are readily available from authors or standard commercial sources

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

mouse anti-DI (DSHB, 1:100 dilution); mouse anti-Pros (DSHB, 1:300); mouse anti-phospho-Histone H3 antibody (Cell Signaling Technology, 1:500); rabbit anti-GFP (Cell Signaling Technology, 1:200); rabbit polyclonal anti-lacZ antibody (Cappel, 1:6000); rabbit anti-Pros (gift from Yuh-Nung Jan, 1:1000); mouse anti-Allatostatin A (DSHB, 1:300); rabbit anti-Tachykinin antibody (a gift from Dick Nassel, 1:300); rat anti-BrdU (Abcam, 1:200); mouse anti-Cut (DSHB, 1:20).

Secondary antibodies used in this study: goat anti-rabbit or anti-mouse IgGs-conjugated to Alexa (568 or Cy5) (Molecular Probes, 1:300). The commercial antibodies were validated based on the information on the manufacturers' instructions. The gift antibodies were validated based on the reported patterns.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

No cell lines were used in the manuscript.

b. Describe the method of cell line authentication used.

No cell lines were used in the manuscript.

c. Report whether the cell lines were tested for mycoplasma contamination.

No cell lines were used in the manuscript.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Female flies of 4-7 days old were selected for the experiments, unless otherwise noted. Duration of the experiment is dependent on each experimental design. The following fly lines were used in this study: UAS-sc-RNAi (BDSC, #26206); esg-GAL4, UAS-GFP (gift from Shigeo Hayashi); UAS-RedStinger (BDSC, #8547); DI-GAL4 (gift from Xiankun Zeng and Steven Hou); GMR14C12-GAL4 (BDSC, #48607); UAS-RFP; UAS-Notch-RNAi (BDSC, #7078); UAS-Sn (BDSC, #26687); lea2 (BDSC, #3102); m8-lacZ (BDSC, #26786); UAS-E(spl)m8-DamID (gift from Francois Schweisguth); Df(3R) E(spl) b32.2 gro+. The Scute-GFP knock-in line was generated by Cas9-mediated gene knock-in using previously described protocol. The GMR14C12-LacZ reporter line was generated by inserting the GMR14C12 DNA fragment into the multiple cloning site of C4PLZ vector, followed by P-element-mediated transformation. The m8-ncZ reporter construct was generated by inserting the enhancer region of E(spl)m8 into the multiple cloning site of C4PLZ vector. The E1 or E2 site mutant m8-ncZ reporter constructs were generated by site-directed mutagenesis, and in both cases, the E-box sequence “CANNTG” was mutated to “AANNGG”.

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June 2017
Policy information about studies involving human research participants

12. Description of human research participants
   Describe the covariate-relevant population characteristics of the human research participants.
   This study did not involve human research participants.
ChIP-seq Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

▶ **Data deposition**

1. For all ChIP-seq data:
   - a. Confirm that both raw and final processed data have been deposited in a public database such as GEO.
   - b. Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

2. Provide all necessary reviewer access links. The entry may remain private before publication.

3. Provide a list of all files available in the database submission.
   - Ctrl_Dam.fastq.gz; m8_Dam.fastq.gz; Ctrl_Dam.bw; m8_Dam.bw

4. If available, provide a link to an anonymized genome browser session (e.g. UCSC).
   - N.A.

▶ **Methodological details**

5. Describe the experimental replicates.
   - No experimental replicates.

6. Describe the sequencing depth for each experiment.
   - 10x coverage for Ctrl_Dam and 16x coverage for m8_Dam

7. Describe the antibodies used for the ChIP-seq experiments.
   - A DamID method was used, which does not need antibodies.

8. Describe the peak calling parameters.
   - Default parameters

9. Describe the methods used to ensure data quality.
   - The cutoff of peakcalling is pvalue<1e-5.

10. Describe the software used to collect and analyze the ChIP-seq data.
    - Bowtie(v1.1.2) and MACS(v1.4.2)