The POU/Oct Transcription Factor Nubbin Controls the Balance of Intestinal Stem Cell Maintenance and Differentiation by Isoform-Specific Regulation

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SUMMARY

Drosophila POU/Oct transcription factors are required for many developmental processes, but their putative regulation of adult stem cell activity has not been investigated. Here, we show that Nubbin (Nub)/Pdm1, homologous to mammalian OCT1/POU2F1 and related to OCT4/POUSF1, is expressed in gut epithelium progenitor cells. We demonstrate that the nub-encoded protein isoforms, Nub-PB and Nub-PD, play opposite roles in the regulation of intestinal stem cell (ISC) maintenance and differentiation. Depletion of Nub-PB in progenitor cells increased ISC proliferation by derepression of escargot expression. Conversely, loss of Nub-PD reduced ISC proliferation, suggesting that this isoform is necessary for ISC maintenance, analogous to mammalian OCT4/POUSF1 functions. Furthermore, Nub-PB is required in enteroblasts to promote differentiation, and it acts as a tumor suppressor of Notch RNAi-driven hyperplasia. We suggest that a dynamic and well-tuned expression of Nub isoforms in progenitor cells is required for maintaining gut epithelium homeostasis.

INTRODUCTION

Adult stem cells exhibit the ability to replace damaged and aged cells during normal epithelium regeneration as well as in response to injury and cellular stress, thereby maintaining tissue homeostasis. The mammalian adult gut epithelium contains intestinal stem cells (ISCs), which are located in crypts along the small intestine where they frequently proliferate to retain homeostatic status (Barker et al., 2008). Proper ISC activities play a pivotal role in the maintenance of cellular homeostasis in the intestine. Dysfunctional ISC proliferation and/or differentiation in the gut epithelium are associated with chronic diseases and developmental abnormalities, such as inflammatory bowel diseases, dysplasia, and metaplasia (Li and Jasper, 2016). However, the mechanisms that maintain gut homeostasis are still largely unknown. The Drosophila midgut shares many similarities with the mammalian small intestine in terms of organ structure and function. Genetic manipulation of evolutionarily conserved signaling pathways linked to ISC proliferation and differentiation in the Drosophila midgut has become a favorable model for investigations of ISC activities and the underlying mechanisms controlling epithelial regeneration and homeostasis (Apidianakis and Rahme, 2011; Bergman et al., 2017; Liu et al., 2017).

Drosophila ISCs are derived from adult midgut precursors during larval stages, and thereafter reside in the midgut basal epithelium (Michelli, 2012; Michelli and Perimon, 2006). The ISC lineage is controlled by bidirectional Notch signaling in the daughter cells. A daughter cell with a high level of Notch activity becomes an intermediate enteroblast (EB), which further differentiates into an enterocyte (EC). Low degree of Notch activity results in retained ISC identity and, in combination with strong Delta and Prospero expression, the daughter cell is primed to differentiate into a pre-enteroendocrine (pre-EE) cell and further into an enteroendocrine (EE) cell (Biteau and Jasper, 2014; Guo and Ohlstein, 2015; Ohlstein and Spradling, 2006, 2007; Perdigoto et al., 2011; Zeng and Hou, 2015). Establishment and maintenance of the Drosophila gut epithelium require strict control of ISC proliferation and differentiation, and has to be balanced with cell death and delamination of differentiated ECs over time. Disruption of this cellular homeostasis can cause abnormal gut functionalities, such as tumor growth or increased susceptibility to infection (Amcheslavsky et al., 2009; Buchon et al., 2009; Ohlstein and Spradling, 2007; Patel et al., 2015). Both intrinsic and extrinsic signals contribute to maintain normal ISC activities via several evolutionarily conserved signal transduction pathways, such as Notch/Delta, Janus kinase/signal transducer and activator of transcription, Jun N-terminal kinase, epidermal growth factor receptor, bone morphogenetic proteins, Hippo, Slt/Robo, and their downstream transcription factors (Bardin et al., 2010; Biteau et al., 2008; Biteau and Jasper, 2014; Buchon et al., 2010; Dutta et al., 2015; Jiang et al., 2009; Korzelius et al., 2014; Ohlstein and Spradling, 2007; Ren et al., 2010; Tian and Jiang, 2014). Although these studies have endorsed a much better understanding of the processes that promote ISC proliferation, we still have rather limited knowledge about the mechanisms underlying the cellular homeostasis and how ISCs are maintained over a long period of time.
The *nubbin (nub)/POU domain protein 1 (Pdm1)* gene is a member of the class II POU transcription factor family and shares homology with the OCT1/POU2F1 and OCT2/POU2F2 proteins in mammals (Holland et al., 2007; Tantin, 2013). The *nub* gene is also evolutionarily related to the class V POU factor OCT4/POU5F1, which maintains stemness of embryonic stem cells (ESCs) (Niwa et al., 2000), and is one of the crucial pluripotency factors used for reprogramming of differentiated cells to induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). Alternative transcripts have earlier been reported to be expressed from the *nub* gene (Ng et al., 1995), and annotation of the *Drosophila* genome suggested at least two independent transcripts termed *nub-RB* and *nub-RD* (FlyBase: FBgn0085424). Recent experimental evidence has revealed that two protein isoforms, Nub-PB and Nub-PD, are expressed in *Drosophila* (Dantoft et al., 2013; Lindberg et al., 2018). Transcription of the *nub* gene initiates at two major promoters that are separated by more than 30 kbp. The two transcripts *nub-RB* and *nub-RD* are translated into a large (Nub-PB; 103.9 kDa) and a small (Nub-PD; 65.2 kDa) isoform, respectively, with a common C-terminal part comprising the POU-specific (POUS) and POU homeo (POU H) DNA binding domains (Figures 1A and 1B).

We recently reported that Nub-PD is a transcriptional repressor of immune and stress response genes in the gut (Dantoft et al., 2013), and that loss of such repression leads to gut bacterial dysbiosis and short life span (Dantoft et al., 2016). Nub/Pdm1 immunostaining is robust in mature midgut ECs (Beebe et al., 2010; Lee et al., 2009; Mathur et al., 2010), and it was suggested that Nub-PD acts as a differentiation factor by repressing *escargot* (*esg*) expression (Korzelius et al., 2014). However, neither has a possible role of Nub-PB in regulation of intestinal stem cell activity been investigated, nor have the discrete roles and possible interplay between Nub-PB and Nub-PD in gut epithelium regeneration been analyzed.

Here, we report that Nub proteins are not only expressed in adult midgut ECs, but also present in progenitor cells (ISC + EB), and are necessary for maintaining cellular homeostasis during normal gut epithelium regeneration. Most importantly, we show that the two transcription factor isoforms, Nub-PB and Nub-PD, play opposite roles in regulating ISC maintenance and proliferation in the posterior midgut. Loss of Nub-PB leads to hyperplasia, as progenitor cells fail to differentiate and instead continue to proliferate. In contrast, Nub-PD mutant clones fail to proliferate, indicating that Nub-PD is necessary for the maintenance of ISCs. We suggest that this represents a mechanism to regulate adult stem cell activity with two transcription factor isoforms acting in opposite manners, one as a differentiation factor, the other as a stemness factor.

**Figure 1. Midgut Length in Different nub Mutants during Adult Stages**
(A) Schematic structure of the *Drosophila nub* gene. The *nub* gene contains seven exons, as depicted with boxes, and transcription is initiated at two separate promoters (arrows). Exons and introns (solid black line) are drawn to scale, except for the large intron between exon 2 and 3 (dashed line), which is reduced in size.
(B) Organization of Nub-PB and Nub-PD proteins encoded by the *nub* gene. Note that Nub-PB protein contains three unique exons (1, 2, and 3) while Nub-PD protein contains one unique exon (4). The shared three exons (5, 6, and 7) contain the coding sequence for the DNA-binding POUS and POUH domains (orange). Filled black triangle indicates the *nub-RB gal4* insertion site and black bold line shows the position of the *nub-RD-gal4* enhancer line. Two *nub* mutants, *nub*; 412 transposable element insertion site indicated by a dashed triangle in (A) and *nub* (an EMS-induced null mutant for both Nub-PB and Nub-PD; Yeo et al., 1995) are used in this work.
(C) Midguts from 21-day-old control (top), *nub*/+ (middle), and *nub*/*nub* (bottom) flies.
(D) Measurement of midgut lengths in 5-day-old (circle) and 21-day-old (triangle) flies. Statistical significance was calculated using two-way ANOVA, Tukey’s multiple comparisons test, **p < 0.01, ****p < 0.0001. n = 13–21 guts. Error bars represent SEM.
RESULTS

Loss of Nub-PD Restricts Midgut Length during Adult Life

To investigate whether nub is involved in regulation of adult gut homeostasis, the adult midgut length was measured in two different nub mutant fly strains, nub\(^{1}\) and nub\(^{E37}\). The nub\(^{1}\) mutation eliminates expression of the Nub-PD isoform, while neither isoform is produced in nub\(^{E37}\) (Ng et al., 1995; Yeo et al., 1995). Dissected midguts of 21-day-old nub\(^{1}\) flies were clearly shorter than midguts of control flies (Figures 1C and 1D), indicating that Nub-PD is involved in development or regeneration of the adult midgut. To distinguish between defects during larval/pupal development from regeneration failure in adults, the midgut length was measured in young flies (5 days after eclosion). As expected, midguts derived from controls were shorter at day 5 compared with day 21 (Figure 1D), demonstrating that the midgut length normally increases between days 5 and 21. However, the midgut length of nub\(^{1}\) flies did not increase with age (Figure 1D). This indicates that the short midguts of nub\(^{1}\) flies at day 21 are not caused by early developmental effects, but rather due to failure in maintaining cellular homeostasis in the midgut. The nub\(^{E37}\) mutant is lethal during the pupal stage, while heteroallelic nub\(^{1}\)/nub\(^{E37}\) flies are viable. We used this combination to analyze whether Nub-PB is also involved in regulation of midgut length. Interestingly, loss of one copy of Nub-PB in the Nub-PD mutant background partly restored the midgut length at day 21 (Figure 1D), indicating that Nub-PB and Nub-PD may confer opposite roles in maintenance of midgut epithelial regeneration. Taken together, the changes in adult midgut length of different nub mutants suggest that both Nub isoforms are contributing to cellular homeostasis in the adult midgut.

Nub Is Expressed in Adult Midgut and in Cell Lines

Adult midgut ECs are recognized as large cells with polyploid nuclei, expressing MyoI\(A\) and marked by Nub antibody staining (Beebe et al., 2010; Jiang et al., 2009; Lee et al., 2009; Mathur et al., 2010). To reveal the specific expression of nub-RB and nub-RD transcripts, spatial expression of promoter-specific nub-RB and nub-RD reporter lines were analyzed. For analysis of nub-RB expression, a MiMIC-transgene (nub\(^{M05\_126}\)) (Venken et al., 2011) (Figure 1A) was converted into a Gal4-expressing reporter and combined with UAS-mCherry. This reporter conferred robust staining throughout the adult midgut, overlapping with MyoI\(A\)-lacZ-positive ECs (Figures 2A–2A\(^{\prime}\) and S1A–S1A\(^{\prime}\)). For nub-RD expression, a number of Vienna tiles enhancer lines (Kwon et al., 2014) were analyzed and the VT6452 Gal4 line, which carries a 2.2-kb fragment including part of the first nub-RD exon and downstream intron sequences (Figure 1A), revealed strong reporter gene expression in adult midgut (Figures 2B–2B\(^{\prime}\) and S1B–S1B\(^{\prime}\)). This demonstrates that both Nub-PB and Nub-PD are expressed in anterior and posterior midgut ECs. In addition, some cells with nub-RB- and nub-RD-driven mCherry expression were found to overlap with EBs, marked by the Notch activity reporter GBE-Su(H)-nlsGFP (Figures 2C–2D\(^{\prime}\)), indicating that Nub-PB and Nub-PD are also expressed in EBs. However, reporter lines are not optimal for following protein dynamics. Instead, immunostaining was used to analyze the cellular distribution of Nub, after first validating antibody specificity in nub\(^{E37}\) mutant clones (Figures S2B–S2B\(^{\prime}\)). Interestingly, we observed Nub immunostaining in the nucleus of cells expressing esg-gal4 driven GFP (Figures 2E–2E\(^{\prime}\)) and esg-lacZ-positive cells (Figures 2F–2F\(^{\prime}\)), indicating that Nub protein is expressed in progenitor cells. However, not all progenitor cells stained with Nub antibody and staining was not uniform throughout the nucleus, suggesting a dynamic nature of Nub proteins and post-translational modifications. Interestingly, mammalian OCT1/POU2F1 display a dynamic behavior, with regulated sub-nuclear localization and post-translational modifications in response to cellular stress, which was suggested to correlate with changes in transcriptional activity (Boubriak et al., 2017; Kang et al., 2013; Malhas et al., 2009). To increase the number of progenitor cells we aimed at analyzing midguts undergoing frequent regeneration. For this, oral infections were performed with Erwinia carotovora carotovora 15 (Ec15) to induce ISC proliferation (Buchon et al., 2010). Importantly, the number of Nub-positive esg-lacZ-marked cells (Figures 2G–2G\(^{\prime}\)) increased upon Ecc15 infection, validating that Nub is expressed in progenitor cells during stimulated regenerative conditions. To further analyze Nub expression in dividing cells, we analyzed Drosophila malignant blood neoplasm-2 (mbn-2) cells in culture. Interestingly, Nub immunostaining was observed specifically in cells during mitosis. Although weak Nub immunostaining was evident throughout the cytosol of the dividing cells, it was highly concentrated at the midbody during anaphase (Figure 2H\(^{\prime}\)), overlapping with microtubuli, marked by β-tubulin immunostaining (Figures 2H and 2H\(^{\prime}\)). For additional confirmation, we analyzed Nub immunostaining in the Fly-specific fluorescent ubiquitination-based cell-cycle indicator cell line, which enables specific labeling of different cell phases during the cell cycle (Zielke et al., 2014). Cells in G1 and S phases are labeled by GFP and RFP, respectively, while cells in G2/M phases express both GFP and RFP and thus appear yellow (Figure S1D). Nub antibody-stained cells (Figure S1C\(^{\prime}\)) were expressing both GFP and RFP (Figures S1C–S1C\(^{\prime}\)) markers, demonstrating that Nub protein was present in these cells during the

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G2/M phase. Taken together, antibody staining confirmed that Nub is expressed in actively dividing cells.

Loss of Nub-PB in Progenitor Cells Leads to Increased esg Expression and ISC Proliferation

Next, we aimed at characterizing the roles of the two Nub protein isoforms in the regulation of ISC activity and differentiation. While Nub-PD has been shown to confer proliferation in embryonic neuroblasts (Bahrampour et al., 2017), nothing is known about the function of Nub-PB. We used an RNAi line directed specifically against nub-RB in combination with the esgts flip-out (F/O) system (Jiang et al., 2009) to analyze whether Nub-PB is involved in turnover of posterior midgut epithelial cells. In midguts of control flies, esg-gal4 drives UAS-GFP expression in progenitor cells (ISC + EB) and, upon flip-out, small clusters of daughter cells were labeled by actin-gal4-driven GFP (Figure 3A). Overexpression of nub-RB for 7 days eliminated all GFP-marked cells (Figure 3B), indicating that no new progenitor cells were generated. In contrast, depletion of nub-RB promoted the growth of hyperplastic masses of GFP-positive cells (Figure 3C), indicating increased ISC proliferation and rapid epithelial turnover. To confirm this, we compared the number of mitotic cells by

Figure 2. Localization of Nub in Adult Posterior Midgut and the mbn-2 Cell Line

(A–D”) Midguts expressing nub-RB > mCherry (A’–A” and C’–C”; red) and nub-RD > mCherry (B’–B” and D’–D”; red), combined with EC-specific reporter (A, A’ and B, B”; green) or an EB-specific Notch reporter (C, C” and D, D”; green) in 5-day-old female posterior midguts. White arrows indicate cells positive for both GBE reporter (C–D”) and nub-RB reporter (C–C”) or nub-RD reporter (D–D”).

(E–F”) Nub antibody staining (E’, E” and F’, F”; red) in 5-day-old female midguts with progenitor cells marked with esgts > GFP (E, E”; green) and esg-lacZ (F, F”; green). White arrows indicate cells positive for both Nub and esg-reporters.

(G–H”) Nub antibody staining (G’, G” and H’, H”; red) in 5-day-old esg-lacZ (green) female midguts upon Ecc15 infection for 12 hr. Note the increased number of Nub/esg-positive cells (white arrows) after infection.

See also Figures S1 and S2.
phospho-histone 3 (PH3) labeling following overexpression or downregulation of nub-RB in progenitor cells. The number of PH3-positive cells was significantly decreased upon nub-RB overexpression (Figures 3E and 3H) compared with control (Figures 3D and 3H). In contrast, nub-RB downregulation resulted in hyper-proliferation (Figures 3F and 3H). These results indicate that the role of Nub-PB is to block proliferation and to drive differentiation in the midgut. Recently, Esg has been reported to act as a stemness factor in adult midgut, as reducing esg expression in stem cells decreased their proliferative capacity (Korzelius et al., 2014). To examine whether esg is involved in nub-RB depletion-induced hyper-proliferation, we downregulated both nub-RB and esg in progenitor cells.
Figure 4. Nub-PB Functions in EBs to Control Differentiation

(A–B) Prospero (Pros) antibody staining (red) in the anterior midgut of control (A’–A”) and after nub-RB downregulation (B’–B”) in progenitor cells (ISC + EB, GFP-labeled) for 21 days.

(C–D) PH3 antibody staining (red) in the anterior midgut of control (C’–C”), and after nub-RB downregulation (D’ and D”) in EBs (GFP-labeled) for 21 days. Note that more mitotic cells are present upon nub-RB downregulation, white arrows in (D’–D”).

(E and F) Downregulation of nub-RB specifically in ISCs (GFP-labeled) using esg⁰;GBE-Gal80 in anterior midgut for 21 days. Scale bars, 20 µm in (A)–(F).

(legend continued on next page)
and measured the rate of ISC proliferation. Importantly, this abolished the hyper-proliferative phenotype (Figures 3G and 3H), suggesting that esg is required for nub-RB depletion-induced ISC hyper-proliferation. In addition, nub-RB overexpression caused a significant decrease in esg expression (Figure 3I), whereas nub-RB downregulation promoted increased esg transcript levels (Figure 3I). This suggests that Nub-PB inhibits ISC proliferation in newly formed daughter cells, by direct or indirect repression of esg expression, promoting them to exit the cell cycle and begin differentiation.

Nub-PB Functions in EBs to Regulate Differentiation
To clarify in which cell types Nub-PB normally acts to regulate proliferation and differentiation, we depleted nub-RB expression by RNAi either in ISC s (esg\textsuperscript{S}:Su(H)\textsubscript{GBE-Gal80}, in EBs (Su(H)\textsubscript{GBE-Gal80\textsuperscript{ts}}) (hereafter referred to esg\textsuperscript{S}:\textsubscript{GBE-Gal80 and GBE\textsuperscript{ts}}, respectively), or in both (esg\textsuperscript{S}). Reduction of nub-RB expression in ISC + EB cells with esg-Gal4 for 21 days led to pronounced accumulation of non-differentiated esg-positive GFP clusters in the anterior and posterior midgut (Figures 4A–4B\textsuperscript{ts} and S3A–B\textsuperscript{ts}). This suggests that depletion of nub-RB in esg-positive cells is associated with loss of progenitor cell differentiation. This was confirmed by the absence of differentiated EE cells in the same region, using Prospero (Pros) antibody staining (Figures 4B–4B\textsuperscript{ts}), indicating that both EC and EE lineage differentiation is defect. In the adult midgut, EBs are transient non-proliferative cells, which either differentiate rapidly after completing cell division and cytokinesis, or remain as EBs with the capacity to differentiate later (Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006). To investigate the role of Nub-PB in EBs, nub-RB was downregulated specifically in EBs by using GBE\textsuperscript{ts}. Strikingly, this also promoted strong accumulation of GFP-labeled cell clusters in the anterior and posterior midgut (Figures 4C–4D\textsuperscript{ts} and S3C–S3D\textsuperscript{ts}). Furthermore, there were more PH3-positive cells in the anterior midgut after nub-RB downregulation in EBs (Figures 4C\textsuperscript{ts}–4D\textsuperscript{ts} and 4G), suggesting that EB-specific nub-RB depletion increases ISC proliferation. In contrast, we did not observe any changes in cell proliferation or differentiation upon nub-RB downregulation in ISC s (Figures 4E, 4F, 4H, and S3E–S3F\textsuperscript{ts}), indicating that Nub-PB does not play a direct role in regulating stem cell activity. In summary, Nub-PB appears to specifically function in EBs to block proliferation and promote differentiation.

Overexpression of Nub-PB Rescues a Notch RNAi-Mediated Differentiation Defect
Notch signaling is a core pathway for controlling ISC fate specification and its inhibition leads to both ISC and EE tumor formation in the posterior midgut (Ohlstein and Spradling, 2007). As nub-RB RNAi in progenitor cells results in accumulation of non-differentiated progenitor-like cells, we next investigated whether Nub-PB is involved in Notch RNAi-mediated differentiation defects. As expected, downregulation of Notch in progenitor cells for 2 or 5 days resulted in both ISC and Pros-positive EE tumor formation (Figures 5A, 5B, 5E, and 5F), confirming that Notch signaling is required for both ISC proliferation and differentiation (Michelli and Perrimon, 2006; Ohlstein and Spradling, 2007). Overexpression of nub-RB in progenitor cells for 2 days reduced the number of stem cells (Figure 5C) and erased almost all esg-positive GFP progenitor cells after 5 days (Figure 5G), approving that overexpression of nub-RB blocks stem cell proliferation (Figure 3). Importantly, downregulation of Notch did not cause ISC and EE tumor formation when combined with nub-RB overexpression for 2 or 5 days (Figures 5D and 5H), indicating that high expression levels of Nub-PB in progenitor cells rescues the Notch RNAi-mediated differentiation defect, including EE tumor formation. It has been shown that esg is required for Notch RNAi-induced tumor formation (Korzelius et al., 2014), and our data suggest that overexpression of nub-RB repressed esg expression (Figure 3). Taken together, these results show that Nub-PB acts as a tumor suppressor in Notch RNAi background, possibly by repression of esg expression, either directly or indirectly.

Nub-PD Is Required for Both Basal and Infection-Induced ISC Proliferation and Regulates ISC Fate in an Opposite Manner Compared with Nub-PB
To analyze the role of Nub-PD in regulation of ISC proliferation we measured the mitotic activity in midguts of the Nub-PD mutant nub\textsuperscript{1} by using PH3 antibody staining. The number of PH3-positive cells was significantly reduced in nub\textsuperscript{1} posterior midgut compared with control (Figures 6A–6C), indicating a low mitotic activity in Nub-PD mutant guts. Moreover, stem cell-specific Delta antibody staining confirmed reduced numbers of ISC s in nub\textsuperscript{1} midguts (Figures 6A–6C). It was also evident that nub\textsuperscript{1} midguts display aberrant epithelium cell arrangements, with no or weak Armadillo staining (a cell adhesion marker) in

(G) Quantification of PH3-positive mitotic cells in the R2 region from control guts (C\textsuperscript{ts}) (n = 10) and nub-RB downregulation guts (D\textsuperscript{ts}) (n = 10).

(H) Quantification of GFP-labeled stem cell numbers in the R2 region in control guts (E) (n = 5) and after nub-RB downregulation (F) (n = 6). Statistical significance was calculated using unpaired t test. **p < 0.01, ns, not significant. Error bars in each graph represent SEM. See also Figure S3.
most ECs compared with control (Figures S4D and S4E), suggesting defects in the regeneration process. However, the EE lineage was not changed in nub1 midguts, as the proportion of Pros-positive EE cells was comparable in control and nub1 midguts (Figures S4D–S4F). Next, we investigated the mitotic activity in nub1 midguts in response to Ecc15 infection. As expected, fewer PH3-positive cells were found in nub1 uninfected posterior midguts compared with controls (Figures 6D, 6F, and 6H), confirming the low level of ISC proliferative rate in nub1 midguts. Importantly, Ecc15 infection for 12 hr triggered ISC division both in control and nub1 midguts (Figures 6E, 6G, and H), but the number of PH3+ cells in nub1-infected midguts was significantly lower compared with infected control midguts (Figure 6H), suggesting that Nub-PD is required for full activation of ISC proliferation upon Ecc15 infection. Taken together, Nub-PD is necessary for accurate rate of ISC proliferation both in basal and infection-induced conditions.

To investigate the interplay between Nub-PB and Nub-PD during cell fate determination we used MARCM system to track mitotic cell fates during development (Lee and Luo, 2001). MARCM was performed in Nub-PD mutant (nub1) and Nub-PB/Nub-PD mutant (nubE37) midguts to examine how Nub isoforms determine ISC fate. As shown in Figure 6, control clones (Figure 6I) became multicellular with an average number of 15–30 cells in each clone (Figure 6L), while nub1 clones (Figure 6J) were always single-cell clones, confirming that Nub-PD is required for ISC proliferation. Strikingly, the nubE37 clones (Figure 6K), which lack both Nub-PD and Nub-PB, were multicellular and had similar cell numbers as the control (Figure 6L), indicating that, upon concomitant loss of both Nub-PB and Nub-PD, ISC proliferation is rescued. In addition, we observed many single-cell clones in the posterior midgut after overexpression of nub-RB both in controls (Figures S5B and S5J) and in nubE37 background (Figures S5E and S5J), suggesting that high levels of Nub-PB expression in progenitor cells indeed block ISC proliferation, which corroborates the results of Figure 3. Surprisingly, we did not observe any multicellular clones in the posterior midgut upon nub-RD overexpression in control background (Figures S5C and S5J) or in combination with the nubE37 background (Figures S5F and S5J), indicating that Nub-PD overexpression is not sufficient for activating ISC proliferation. Rather it blocked proliferation upon overexpression, a result that will be further discussed below.

The fact that the nubE37 MARCM clones resembled control clones with normal proliferation rate was surprising, and we decided to investigate this further with an independent approach, using a nub-RB/RD IR line, in which both nub-RB and nub-RD are targeted for degradation. In contrast to the hyper-proliferation caused by nub-RB IR (Figures S5G and S5H), expression of nub-RB/RD IR in progenitor cells rescued the hyper-proliferation (Figure S5I), demonstrating that the hyper-proliferation is Nub-PD dependent, and that

Figure 5. Overexpression of nub-RB Blocks Notch RNAi-Driven Differentiation Defects
Pros antibody staining (red) in 2-day-old (A–D) and 5-day-old (E–H) posterior midguts. Downregulation of notch in progenitor cells, driven by esgIR, led to both ISC + EB (green) and EE (red) tumor-like cell formation (B and F) in posterior midguts compared with controls (A and E). nub-RB overexpression combined with notch IR in progenitor cells inhibits tumor formation both in 2-day-old (D) and 5-day-old (H) posterior midguts. Scale bars, 20 μm.
concomitant loss of Nub-PB and Nub-PD restores cellular homeostasis. Thus, these results strongly indicate that Nub-PB and Nub-PD regulate ISC fate in opposite direction and suggest that the primary role of Nub-PD is to inhibit Nub-PB-driven differentiation, thereby ensuring ISC maintenance.

**DISCUSSION**

In the past few years, Nub/Pdm1 has been used as a marker of mature ECs in the *Drosophila* midgut (Beebe et al., 2010; Lee et al., 2009; Mathur et al., 2010), but a direct role in epithelium regeneration has not been fully investigated.
Here, we found that Nub isoforms are not only highly expressed in differentiated ECs, but also present in progenitor cells in the midgut. Nub immunostaining was not evident in all progenitor cells but specifically observed during mitosis and in a subset of EBs. This may either reflect that Nub protein concentration is very dynamic during the cell cycle or that antibodies directed against Nub are unable to detect its antigen during other phases of the cell cycle due to post-translational modifications and/or protein-protein interactions and complex formations. In addition to these immunostaining results, several global RNA sequence datasets of sorted midgut cells, such as the Flygut-seq (http://flygutseq.buchonlab.com/data) and (Zhai et al., 2015) have indicated expression of the nub gene in ISCs and EBs.

The observation that Nub immunostaining is enriched in the midbody of dividing cells may reflect a role of Nub either during the mitosis itself or directly after, in early G1 phase. In mammalian cells, OCT1/POU2F1 is phosphorylated in early mitosis and then concentrated in the midbody during cytokinesis, and was suggested to take part in regulation of mitosis (Kang et al., 2011). Importantly, both depletion and overexpression of OCT1/POU2F1 interfered with normal mitosis. Moreover, OCT4/POU5F1 is phosphorylated at the onset of mitosis and then dephosphorylated after exiting M phase. This cycle is necessary for its role in resetting the transcription machinery and to maintain ESC pluripotency (Shin et al., 2016). It is therefore plausible that the presence of OCT/POU proteins in mitotic cells, their post-translational modifications, and their role as transcriptional regulators in early G1 phase is important for regulation of normal stem cell division and for re-entering another round of the cell cycle.

Mammalian Oct/Pou genes are also producing several isoforms, but knowledge about their specific roles is relatively limited (Zhao, 2013). However, it was recently reported that different isoforms made from the human Oct1/Pou2f1 gene control expression of distinct sets of genes (Pankratova et al., 2016). Interestingly, these OCT1 isoforms are produced from transcripts initiated at independent transcriptional start sites, giving rise to three OCT1 isoforms with different N termini but identical DNA-binding domains, resembling how the nub gene is organized. The genes for Oct2/Pou2f1 and Oct4/Pou2f1 also produce several isoforms via alternative splicing. These different isoforms have been suggested to regulate neural differentiation, pluripotency, and some have been linked to malignancy (Zhao, 2013).

Here, we show that Drosophila Nub isoforms are involved both in the maintenance of the stemness of ISCs and in the differentiation of the EB to EC lineage. The role of Nub-PD as a stemness factor was demonstrated by the loss of ISC proliferation in whole midguts and in MARCM clones (Figure 6). However, overexpression of nub-RD did not activate cell division in the MARCM clones (Figure S5), but rather promoted differentiation. This result corroborates the findings of Korzelius et al., (2014), who reported that overexpression of Nub-PD in gut progenitor cells forced differentiation into ECs. One possibility is that the concentration of Nub-PD in ISCs is critical, and that overloading the cell with Nub-PD causes cell-cycle exit and differentiation. Importantly, the dosage of OCT4 is critical for maintaining stem cell self-renewal of ESCs, and both up- and downregulation of OCT4 induce differentiation into alternative lineages (Niwa et al., 2000). Similarly, accurate concentration of OCT1 was critical for normal mitotic progression (Kang et al., 2011). In the Drosophila embryonic nervous system, misexpression of Nub-PD results in neuroblast proliferation (Bahrampour et al., 2017; Bhat and Apsel, 2004; Bhat et al., 1995). It is therefore evident that Nub-PD has the capacity to stimulate neurogenic stem cells to proliferate.

A recent paper reported that one of the Snail family transcription factors, Esg, could directly bind to the promoter region of the nub gene and repress its expression, and it was suggested that such repression is necessary to maintain ISC identity (Korzelius et al., 2014). However, the study did not consider the two isoforms encoded by the nub gene and only focused on Nub-PD. Furthermore, it was suggested that Esg and Nub might have bidirectional regulatory roles in progenitor cells in different conditions (Korzelius et al., 2014). Here we show that overexpression of nub-RB in progenitor cells blocks ISC proliferation, possibly by downregulation of esg expression, either directly or indirectly. In correlation with this, downregulation of nub-RB by RNAi in progenitor cells elevated the expression level of esg and activation of ISC proliferation (Figure 3). Moreover, the hyper-proliferation phenotype induced by nub-RB RNAi was abolished when esg was depleted simultaneously. It is therefore likely that Nub-PB is controlling esg expression in progenitor cells, thereby prohibiting ISC proliferation. Moreover, nub-RB RNAi results indicate that Nub-PB is normally repressing proliferation in EBs. As a consequence, the increase in PH3 labeling upon EB-specific nub-RB RNAi is most likely a result of both ISC daughter cells maintaining self-renewal capacity in the absence of Nub-PB and sustained esg expression.

We propose a model for how Nub-PB and Nub-PD may regulate ISC proliferation and differentiation (Figure 7). In this model, Nub-PB acts as a repressor of esg expression in progenitor cells, promoting differentiation of one of the daughter cells to an EB. Nub-PD, which counteracts the activity of Nub-PB, will thereby inhibit differentiation and allow stem cells to continue to divide, i.e., to maintain stemness. In the absence of Nub-PD (nub4 mutant clones),
Nub-PB will push both daughter cells to exit the cell cycle and promote rapid differentiation, hence, the stem cells will be lost and no more cell divisions will occur. In contrast, depletion of Nub-PB (nub-RB IR) will lead to a continuously high level of esg expression and enable both daughter cells to continue to divide, promoting increased number of ISCs. In this model, the primary role of Nub-PD is to repress the activity of Nub-PB on its target genes, meaning that midguts lacking both Nub-PB and Nub-PD would have a relatively normal cell cycle and rate of proliferation, as well as upon nubE37 mutant, as well as upon nub-RB/RD RNAi in progenitor cells. This may imply that the nub gene itself is dispensable for ISC proliferation during basal conditions. However, the Drosophila pdm2 gene, which is a nub paralog, has been shown to confer a redundant role during neuroblast cell proliferation and specification in the embryo (Bhat et al., 1995). It is therefore possible that Pdm2 can compensate for the loss of nub and maintain normal ISC proliferation rate. In contrast, unbalanced expression of the two Nub protein isoforms, either as a result of overexpression or RNAI, interfered with cell proliferation in all variants tested. Thus, well-tuned expression of the two Nub protein isoforms is necessary for normal ISC proliferation and differentiation. Moreover, it was recently reported that Nub-PB and Nub-PD regulate the expression of immune genes in gut ECs in an antagonistic manner (Lindberg et al., 2018), pointing to the possibility that Nub-PB and Nub-PD play important roles in controlling epithelial renewal in the gut upon infection. This is supported by the fact that in nub1 mutants, ISC proliferation was considerably reduced (Figures 6A–6H), suggesting that Nub-PD is also required for infection-induced stem cell division.

In a recent study, Shen et al. (2017) found that mammalian POU2F1/OCT1 and POU5F1/OCT4 occupy both overlapping and independent sets of target genes in ESCs and their daughters. Importantly, concomitant with the loss of OCT4 binding, OCT1 occupancy increased during differentiation (Shen et al., 2017). This implies that POU/OCT factors may act in succession to regulate developmental processes based on sequential binding to common targets. Phylogenetic analyses have indicated that Drosophila and other invertebrates lack POU5 factors, i.e., direct homologs of OCT4/POU5F1, while Nub is most closely related to mammalian OCT1/POUF1 and OCT2/POUF2 (Gold et al., 2014). Importantly, though, it was shown using high-throughput SELEX experiments that the DNA binding specificity is highly conserved between mouse/human OCT4, OCT1, and Drosophila Nub (Nitta et al., 2015). This suggests that Nub proteins are in fact functionally analogous to OCT4 and OCT1 in sustaining stem cell renewal capacity, and thereby also restoring multipotency (OCT4 and Nub-PD), and that Nub-PB acts analogous to OCT1 in supporting differentiation of specific cell lineages. It is well established that mammalian POU/OCT factors act as crucial regulators of stemness, multipotency, and
differentiation. This work suggests that these properties of POU/OCT factors are evolutionarily ancient, operating in organisms as divergent as Drosophila and mammals.

EXPERIMENTAL PROCEDURES

Immunofluorescence Staining and Imaging
All crosses were set up at 18°C, then F1 progenies were kept at 18°C for 3–7 days before transferring to 29°C for initiation of GAL4 activity. Otherwise, flies were reared at 25°C.

Female intestines were dissected in PBS, pH 7.4 (PBS) and fixed in 4% paraformaldehyde for 1 hr at room temperature (RT), then briefly washed in PBST (PBS with 0.1% Triton X-100) and blocked with PBST+ 0.5% normal goat serum for 30 min at RT. The tissues were incubated with primary antibodies at 4°C overnight on a shaker. Next day, samples were washed in PBST (4 × 15 min) and incubated with secondary antibodies at RT for 2 hr on a shaker. Finally, samples were mounted in DABCO (Sigma) on a glass slide after staining with DAPI (Sigma). Images were taken by LSM 780 (Zeiss) or LSM 500 (Zeiss) and processed with Adobe Photoshop CS6 and Adobe Illustrator CS6.

RT-qPCR
Total RNA was extracted from 20 to 30 adult females’ intestines with TRIzol (Bioline). cDNA synthesis and PCR were done as described previously (Dantoft et al., 2013). TaqMan Gene Expression Assays (Applied Biosystems) were used to quantify the given gene expression (TaqMan probes: esg: Dm01841264_s1; Applied Biosystems); all experiments were analyzed in biological triplicates and the measured mRNA expression level were normalized relative to those of RpL32.

Statistical Analysis
Statistical significances were calculated using either unpaired t test in pairwise comparisons or one- or two-way ANOVA combined with Tukey’s post hoc test in multiple comparisons as shown in related figures. Graphical plotting was done in GraphPad Prism 6.0.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.03.014.

AUTHOR CONTRIBUTIONS

Conceptualization, X.T., Y.Z., B.N., and Y.E.; Methodology and Investigation, X.T., Y.Z., B.N., and Y.E.; Data Analysis, X.T. and Y.Z.; Writing – Original Draft, X.T. and Y.E. with input from all authors; Writing – Review & Editing, X.T., Y.Z., B.N., and Y.E.; Funding Acquisition, Y.E.

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