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Distinct levels of Notch activity for commitment and terminal differentiation of stem cells in the adult fly intestine

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SUMMARY

Tight regulation of self-renewal and differentiation of adult stem cells ensures that tissues are properly maintained. In the *Drosophila* intestine, both commitment, i.e. exit from self-renewal, and terminal differentiation are controlled by Notch signaling. Here, we show that distinct requirements for Notch activity exist: commitment requires high Notch activity, whereas terminal differentiation can occur with lower Notch activity. We identified the gene *GDP-mannose 4,6-dehydratase* (*Gmd*), a modulator of Notch signaling, as being required for commitment but dispensable for terminal differentiation. *Gmd* loss resulted in aberrant, self-renewing stem cell divisions that generated extra ISC-like cells defective in Notch reporter activation, as well as wild-type-like cell divisions that produced properly terminally differentiated cells. Lowering Notch signaling using additional genetic means, we provided further evidence that commitment has a higher Notch signaling requirement than terminal differentiation. Our work suggests that a commitment requirement for high-level Notch activity safeguards the stem cells from loss through differentiation, revealing a novel role for the importance of Notch signaling levels in this system.

KEY WORDS: Commitment choice, Adult stem cells, Fucose modification

INTRODUCTION

Stem cells are defined by their ability to self-renew, their uncommitted state and yet their capacity to generate committed progeny that will ultimately undergo terminal differentiation. During embryogenesis, an orchestrated commitment of stem cells underlies the formation of the germ layers and adult structures (Arnold and Robertson, 2009; Hemberger et al., 2009). In adult tissues, however, a homeostatic state is reached where stem cell numbers are in relative equilibrium with their terminally differentiated progeny (Blanpain et al., 2007; Li and Clevers, 2010). Thus, within adult tissues, the properties of stem cells including self-renewal and commitment status need to be safeguarded and coupled to the needs of the tissue to provide new differentiated cells. Consequently, the commitment choice of stem cells is crucial for tissue homeostasis: this decision is often irreversible and will limit the number of stem cells in the tissue.

The adult *Drosophila* midgut harbors multipotent intestinal stem cells (ISCs) and is a powerful model system with which to investigate basic mechanisms of maintenance, proliferation, commitment and terminal differentiation (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Long-term maintenance and ISC proliferation are controlled in part by a surrounding layer of muscle cells that secretes ligands activating Wingless and Jak/Stat in ISCs (Lin et al., 2008; Lin et al., 2010). Surrounding terminally differentiated cells also regulate proliferation in response to tissue cell death, nutrition, oxidative stress and aging via the Jak/Stat, Jnk, InR, EGFR and Hippo signaling pathways (Amcheslavsky et al., 2009; Buchon et al., 2009a; Buchon et al., 2009b; Chatterjee and Ip, 2009; Jiang et al., 2009; Buchon et al., 2010; Karpowicz et al., 2010; Liu et al., 2010; McLeod et al., 2010; Ren et al., 2010; Shaw et al., 2010; Staley and Irvine, 2010; Biteau and Jasper, 2011; Jiang et al., 2011). In addition, Hippo signaling, as well as inhibition of Wingless signaling by Adenomatous polyposis coli, are required cell autonomously in ISCs to limit proliferation (Lee et al., 2009; Karpowicz et al., 2010; Shaw et al., 2010). Furthermore, permissive signals such as Insulin also influence ISC proliferation (Biteau et al., 2008; Amcheslavsky et al., 2009). These signals seem to primarily act on proliferation rate of the ISCs or the number of dividing ISCs at any given time.

Cell fate acquisition governing self-renewal of the ISC and daughter cell terminal differentiation is controlled largely by Notch signaling occurring between the two ISC daughter cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Each ISC divides to self-renew and to produce a daughter cell (enteroblast; EB) that does not divide but will terminally differentiate into one of two types of cells: an enteroendocrine cell (ee) expressing Prospero (Pros) or an enterocyte cell (EC) expressing Pdm1 and undergoing endoreplication to become a large epithelial cell (Fig. 1A) (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Lee et al., 2009). The Notch ligand Delta is specifically expressed in ISCs (Ohlstein and Spradling, 2007). Upon division of the ISC, expression of the Notch ligand Delta is progressively restricted to one of the two daughter cells. This cell keeps the ISC fate and activates the Notch receptor in its sister cell, the EB, thereby promoting expression of Notch target genes that are important for differentiation (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007; Bardin et al., 2010). Loss of Notch signaling results in increased numbers of stem cells, increased numbers of ees and a loss of ECs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). The Jak/Stat pathway is also involved in promoting the differentiation (Lin et al., 2010; Beebe et al., 2010).

Specification of ee and EC daughter terminal cell fates has been proposed to be regulated by differential Notch signaling, whereby low signaling promotes ee fate and high signaling promotes EC fate (Ohlstein and Spradling, 2007). This model is supported by the

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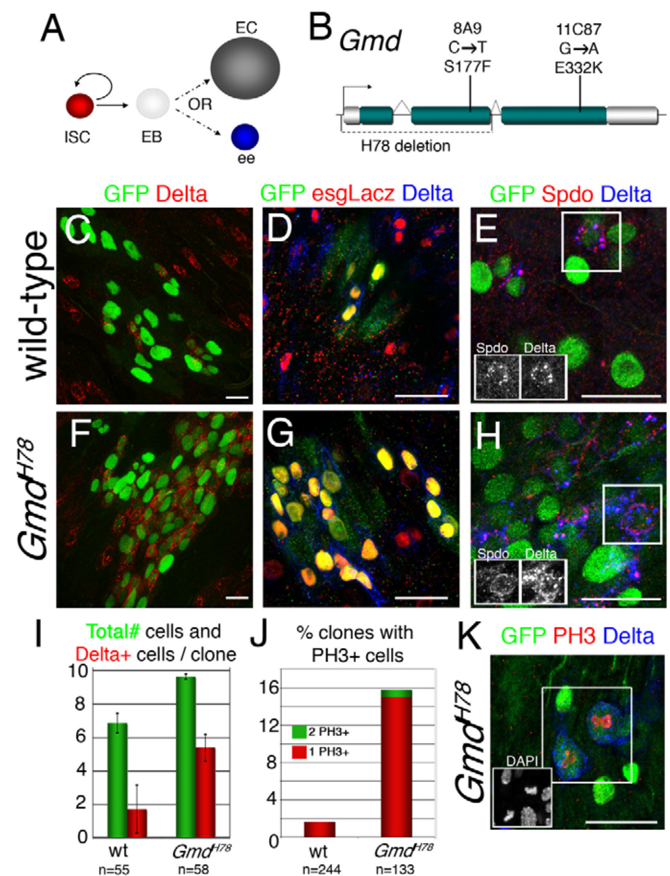

Fig. 1. Loss of *GDP-mannose 4,6-dehydratase* (*Gmd*) specifically increased stem cells numbers. (**A**) The intestinal stem cell (ISC) self-renews and produces a post-mitotic enteroblast daughter cell (EB) that subsequently terminally differentiates into either an enterocyte (EC, gray) or an enteroendocrine cell (ee, blue). (**B**) Structure of *Gmd* mutants isolated in EMS screen (Gmd^{8A9}=S177F; Gmd^{11C87}=E332K) and the H78 deletion (Sasamura et al., 2007). (**C**) Wild-type clones at 10 days AHS (GFP+). (**D**) All Delta+ cells in wild-type clones (10 days AHS) expressed esg-*lacZ* (100%; *n*=74 cells). (**E**) In wild-type clones 10 days AHS, most Delta+ cells were Spdo+ (89.2%; *n*=65 cells). (**F**) Gmd^{H78} mutant clones at 10 days AHS (GFP+) contain increased numbers of Delta+ cells. (**G**) All Delta+ Gmd^{H78} cells expressed esg*lacZ*+ (100%; *n*=235 cells). (**H**) Most of the Delta+ Gmd^{H78} cells were Spdo+ (95.6%; *n*=412). (**I**) Gmd^{H78} mutant stem cell clones (5 d) contained more cells (in green) and more Delta+ cells (in red) than did wild-type stem cell clones. Data are mean±s.e.m. (**J**) More Gmd^{H78} clones (5 d) contained dividing cells (phosopho-Histone H3, PH3+) than wild-type; and two PH3+ cells were see in Gmd^{H78} but not wild-type clones. (**K**) Example of a Gmd^{H78} clone (5 d) containing two dividing cells (PH3+), DAPI in inset. Scale bars: 10 μm.

correlation of Delta protein levels in the ISC with the fate of its recently specified daughter cell, as well as the *Notch* mutant phenotype (Ohlstein and Spradling, 2007). Thus, it is clear that Notch signaling promotes terminal differentiation and needs to be kept off in the ISC for the fate of this cell to be maintained via preventing differentiation (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007; Bardin et al., 2010). However, whether Notch regulates the commitment process of the ISC daughter cells independent of its role in regulating terminal differentiation is not known.

Here, we show that the self-renewal versus commitment choice can be altered without affecting the proper specification of the differentiated cells. In a genetic screen, we identified the gene *GDP-mannose 4,6-dehydratase* (*Gmd*) as being specifically required to limit the number of uncommitted ISCs, yet *Gmd* was dispensable for terminal differentiation. *Gmd* has previously been shown to be required for GDP-fucose production in flies and to affect Fringe-dependent Notch signaling via Notch receptor fucosylation by the fucosyltransferase Ofut1 (for reviews, see Jafar-Nejad et al., 2010; Takeuchi and Haltiwanger, 2010). We found that in the intestine *Gmd* acts in a *fringe*-independent but *Ofut1*-dependent manner to influence commitment, probably by promoting high-level Notch signaling through fucose modification. Furthermore, the decision to commit and not to self-renew has a higher requirement for Notch signaling than does terminal differentiation into either cell type. A requirement for high-level Notch signal for commitment provides a potential mechanism by which the ISCs could be protected against loss by weak signals that could otherwise drive terminal differentiation.

MATERIALS AND METHODS

Drosophila stocks and clonal analysis

Adult flies were kept in freshly yeasted tubes, changed every 2 days and kept at 25°C unless otherwise noted. The mosaic analysis with repressible cell marker (MARCM) technique (Lee and Luo, 1999) was used using stocks previously described (Bardin et al., 2010). MARCM was combined with the *lacZ* lineage labeling technique (Harrison and Perrimon, 1993) using the following genotypes: *y w P[hs-FLP] P[pTub-GAL4] P[UASnlsGFP] / yw*; *X-15-29[FRT lacZ]60B FRT40A / X-15-33 [tub FRT]60B FRT40A P[pTub-GAL80]* ('wild-type MARCM + *lacZ* lineage') and *y w P[hs-FLP] P[pTub-GAL4] P[UASnlsGFP] / yw; X-15-29[FRT lacZ]60B FRT40A* Gmd^{H78} */ X-15-33 [tub FRT]60B FRT40A P[pTub-GAL80]* ('*Gmd* MARCM + *lacZ* lineage').

For the GFP and *lacZ* double-labeled clones, flies were heat shocked 3 days after eclosion (10 minutes, 36.5°C) and again 5 days later (25 minutes, 36.5°C). Upon a single heat shock, 7.8% of wild-type clones were double positive, whereas 12.8% of the *Gmd* MARCM + *lacZ* clones were double positive. Thus, while occurring at low frequency, double labeling of an ISC can occur with only one heat shock event. Therefore, we scored only double-labeled *lacZ*+ clones clearly inside a larger GFP+ clones.

The following alleles and fly stocks were used in this study: Gmd^{8A9} and Gmd^{11C87} (this study), Gmd^{H78} (Sasamura et al., 2007), $fringe^{13}$ (Irvine and Wieschaus, 1994), $Ofut1^{4R6}$ (Sasamura et al., 2003), *UAS-Notch*^{cdc10} [Notch ICD (Ncdc10); a truncated active version of intracellular Notch (Brennan et al., 1999)], UAS-OFUT1R245A (Okajima et al., 2005), $kuzbanian^{ES24}$ (Li and Baker, 2001), $rumi^{44}$ (Acar et al., 2008), $Su(H)^{\Delta 47}$ (Morel and Schweisguth, 2000), Su(H)GBE-*lacZ* (Furriols and Bray, 2001). UAS-*Notch* RNAi (VDRC #1112), *esgGAL4 tubGAL80ts UAS-GFP* (Jiang et al., 2009) and *esglacZ* (Yagi and Hayashi, 1997).

Quantification and statistical analysis

Only posterior midgut tissue was analyzed. in Fig. 1I, Delta+ cells per clone at 5 days AHS were scored in 'stem cell clones', i.e. clones containing two or more cells. in Fig. 1J, PH3+ cells per clone were scored 5 days AHS. In Fig. 1D,H and Fig. 5G, cells were assessed in clonal tissue, because, at 10 days, AHS individual clones could not accurately be distinguished. In Fig. 1D,H, all Delta+, esglacZ+ and Delta+esglacZ+ cells in clonal tissue were scored. In, Fig. 1E,H and Fig. S1 in the supplementary material, all Delta+, Spdo+ and Delta+Spdo+ cells in clonal tissue were scored. At least three representative clonal regions were counted per posterior midgut of three different flies for all of the above quantifications.

The density of ees and ECs in Fig. 2M and Fig. 5H was quantified by counting the number of Pros+ cells and ECs (DAPI+ nuclei with diameter ≥7 μm), and measuring clone area using ImageJ. A 10-day time point was used to quantify terminal differentiation to ensure clones were at homeostasis.

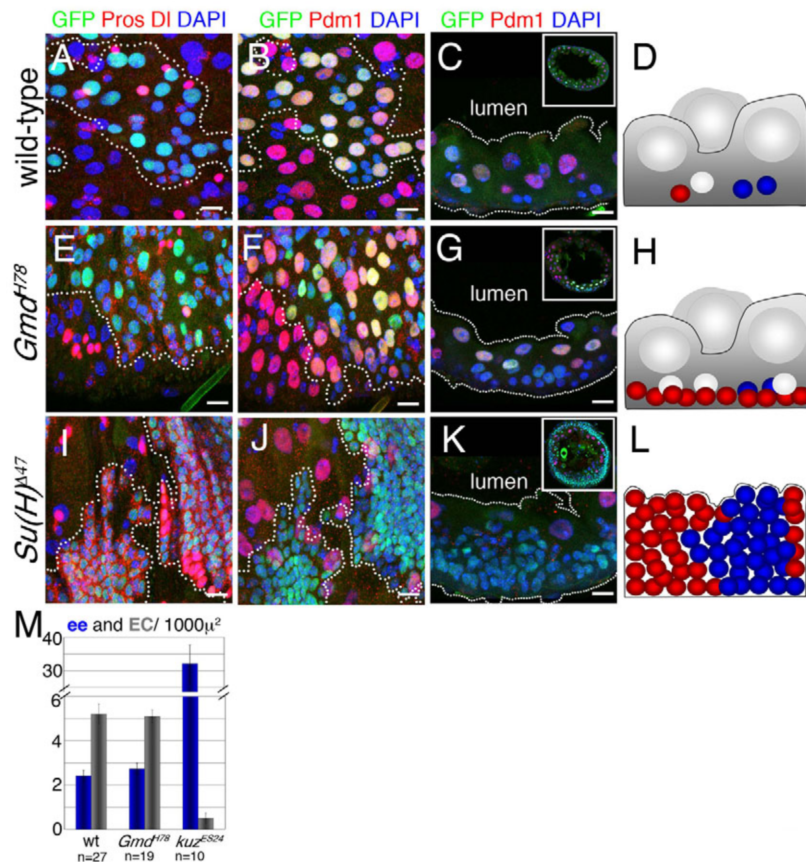

Fig. 2. Terminal differentiation of enteroendocrine cells and enterocytes is unaffected in *Gmd*^{H78} mutant clones. (**A**,**E**,**I**) Projections of basal-most layers containing ees (Prospero+, nuclear red) of wild-type (A), *Gmd*^{H78} (E) and *Su(H)*$^{\Delta47}$mutant clones (I; clones outlined in white, GFP+) at 10 days AHS. (**B**,**F**,**J**) Projections of apical planes where ECs are found (marked by Pdm1 and large nuclear size) of the same clones as in A,E,I of wild-type (B), *Gmd*^{H78} (F) and *Su(H)*$^{\Delta47}$(J) mutant clones. All Pdm1+ cells in J are below and outside the clonal tissue. (**C**,**G**,**K**) Tissue organization seen in cross-section, insets show low magnification cross-section view. (**D**,**H**,**L**) Cartoon of cross-section of wild type (D), *Gmd*^{H78} mutant (H) and canonical Notch mutant *Su(H)*$^{\Delta47}$ (L). ISCs are represented in red, EBs in white, ees in blue and ECs in gray. (**M**) Densities of Pros+ ees (blue) and large nuclear size ECs (gray) in wild type, *Gmd*^{H78} mutant clones and the canonical Notch signaling component *kuzbanian* (*kuz*^{ES24}) at 10 days AHS. Data are mean±s.e.m. Scale bars: 10 μm.

The same data set used in Fig. 2 for wild-type controls and *Gmd* mutants is presented again in Fig. 5 for reference purposes.

Fig. 3B-G, a 4-day time point after the second heat shock was used to allow cells within clones time to undergo terminal differentiation. Lineages with greater than two cells and containing at least one Delta+ cell were included in the analysis, and the cell composition of each clone was determined. ISCs were identified as Delta+ cells, ees as Pros+, ECs as cells with a DAPI+ nuclei with diameter ≥7 μm, and EBs as Delta– cells less than 7 μm nuclear diameter.

Fig. 5J-P, ISCs (Delta+), ees (Pros+) and ECs (nucleus with diameter ≥7 μm), the total number of cells were counted and area of the field was measured in three representative fields per RNAi line and temperature.

Fig. 6D-F,H, the pixel intensity of Su(H)GBE-*lacZ* signal was assessed using intensity profiling from ImageJ. In Fig. 6E, a *z*-test of proportions was used in which significant differences have ε>1.96 (95% confidence). The difference in proportion of Delta– βgal-high cells between *Gmd* mutant and wild-type cells was not significant (ε=0.22). By contrast, the difference in proportion of Delta+ βgal-high cells was very significant (ε=3.0). In Fig. 6F, the total number of Delta+ cells per clone at 5 days AHS was assessed.

Immunofluorescence

The fixation protocol has been described previously (Lin et al., 2008) and was used except for Fig. 1E,H, Fig. 2, Fig. 6G and Fig. S1 in the supplementary material, where the fixation protocol described by Bardin et al. (Bardin et al., 2010) was used. The following antibodies were used: anti-Delta ECD C594.9B [ascites, 1/2000, Developmental Studies Hybridoma Bank (DSHB)]; anti-Notch ECD C458.2H (ascites, 1/100, DSHB); anti-Ofut1 (1/2000, K. Irvine, Rutgers, NJ, USA); anti-Pros (1/10; DSHB); anti-Pros (1/2000; Y. N. Jan, UCSF, CA, USA); anti-βGal (1/500; Biogenesis); anti-Pdm1 (1/1000; W. Chia, National University of Singapore); anti-GFP (1/1000, Molecular Probes); anti-HRP (1/1000; J.-R. Martin, CNRS, France); anti-PH3 (1/2000; Upstate); anti-Sanpodo (1/2000; preabsorbed; J. Skeath, Washington University, St Louis, MO, USA); and anti-GFP (Abcam).

RESULTS

Gmd regulates ISC commitment but is dispensable for terminal differentiation

Through an EMS-based genetic screen (C.P., F.S. and A.B., unpublished), we identified two alleles of the gene *Gmd* (*GDP-mannose 4,6-dehydratase*; Fig. 1B) required for the synthesis of GDP-fucose, the loss of which led to an increased number and density of diploid cells, potentially stem cells. The two alleles we isolated (Fig. 1B) showed the same phenotype as a previously characterized null allele *Gmd*^{H78}, removing a large region of the N terminus of *Gmd* (Sasamura et al., 2007). All further analysis was carried out with the *Gmd*^{H78} null allele.

We assessed the fate of the extra diploid cells in the *Gmd* mutant tissue using several markers of and/or properties of ISC and EB cells: Delta, Su(H)GBE-*lacZ*, clone size, the number of dividing cells/clone, escargot*lacZ* (esg*lacZ*) and Sanpodo, a novel marker of the ISC. We first examined the expression of the ISC marker Delta (Ohlstein and Spradling, 2007). Wild-type control clones contained 2.3 (±0.5 s.e.m.) Delta+ cells at 5 days after heat shock (AHS), suggesting that in our staining conditions Delta was often present in the ISC and its sister cell (Fig. 1I). However, *Gmd* mutant clones had more Delta+ cells per clone (5.4±0.8 s.e.m.; Fig. 1I). The phenotype was even more severe by 10 days AHS (Fig. 1C,F). Although some small cells and differentiating cells did express the Su(H)GBE-*lacZ* Notch reporter (Furriols and Bray, 2001) (as presented and quantified in detail below; see Fig. 6), activation of which is thought to be required for the differentiation process, the majority of the Delta+ *Gmd* cells were negative, suggesting that these cells were stem cells. Wild-type clones contained 6.9 cells±0.6 s.e.m. per clone at 5 days AHS. *Gmd* clones, however, contained more cells per clone (9.6

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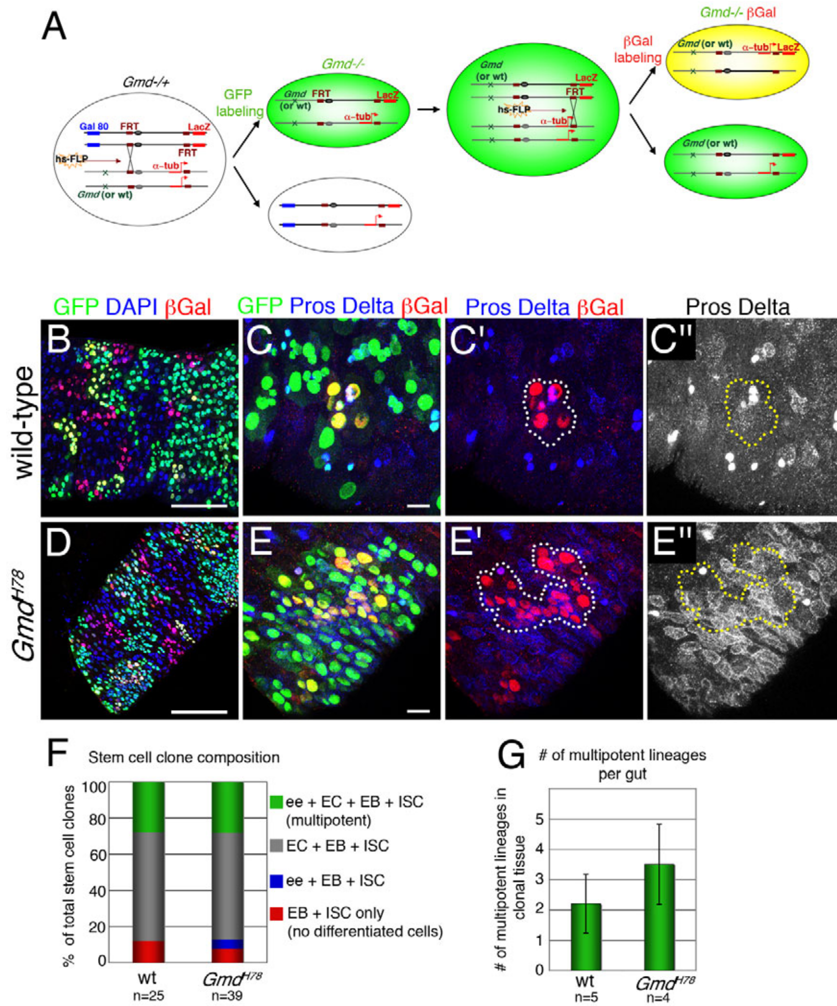

Fig. 3. Extra stem cells produced in *Gmd* are multipotent. (**A**) Lineage-marking strategy: heat shock-induced recombination created wild-type or $Gmd^{-/-}$ MARCM clones expressing GFP (green) and βgal-marked lineages (red); a second heat shock after 5 days induced a second set of recombination events. (**B-E″**) βgal+ lineages in wild-type and Gmd^{H78} described in A. βgal lineage within GFP clone is outlined. Both wild-type and Gmd^{H78} mutant ISC lineages produced ees and ECs; however, Gmd^{H78} lineages also had increased numbers of Delta+ cells. Scale bars: 50 μm in C,E; 10 μm in D,F. (**F**) Composition of Gmd^{H78} and wild-type βgal+ lineages at 4 days. Lineages scored for cell type: 'multipotent lineages' contained at least one Delta+ cell, at least one Pros+ ee and at least one polyploid EC. (**G**) Multipotent βgal+ lineages in Gmd^{H78} and wild-type clones at 4 days from F plotted per midgut. Though increased in Gmd^{H78}, the difference was not statistically significant with this sample size. Data are mean±s.e.m.

cells±1.4 s.e.m.; Fig. 1I; see Fig. S2D in the supplementary material), consistent with an increase in either ISC proliferation rate or ISC number. The effect on clone size was exacerbated by 3 weeks AHS: guts were almost entirely composed of *Gmd* mutant tissue, suggesting that mutant clones had a growth advantage over both heterozygous and wild-type cells (see Fig. S2 in the supplementary material). As the ISC is the only dividing cell type (Ohlstein and Spradling, 2006), markers of mitosis such as Phospho Histone H3 (PH3) can be used to assess the capacity of supernumerary Delta+ cells to act like ISCs. Consistent with a low basal rate of proliferation (Jiang et al., 2009), few wild-type clones contained one PH3+ cell (1.6%; *n*=244 clones) and none contained more than one. By contrast, the number of *Gmd* clones containing one or more PH3+ cell was elevated (15.8%; *n*=133; Fig. 1J,K). Strikingly, we could also identify clones containing more than one dividing cell, indicating an increased number of cells per clone with ISC-like properties in *Gmd* mutant clones (Fig. 1K). In addition, all Delta+ diploid cells in *Gmd* mutant clones express esg*lacZ* a marker of both ISCs and EBs (100%; *n*=235 cells; Fig. 1D,G) (Micchelli and Perrimon, 2006). Finally, we identified Sanpodo (Spdo) as a new marker for the ISC (O'Connor-Giles and Skeath, 2003). Spdo was largely co-expressed with Delta in wild-type intestines (91% of the Delta+ cells were Spdo+, *n*=168; Fig. 1E; see full characterization in Fig. S1 in the supplementary material). We found that *Gmd* Delta+ cells co-expressed Spdo (95.6% of the Delta+ *Gmd* mutant cells were Spdo+, *n*=412; Fig. 1H; see Fig. S1 in the supplementary material). Altogether, these data suggest that loss of *Gmd* results in an increased number of ISCs.

We then assessed terminal differentiation in *Gmd* mutants. *Gmd* tissue had wild-type densities of Pros+ ees and large polyploid ECs (Fig. 2A,B,E,F,M). In addition, the overall tissue morphology appeared largely unaffected (Fig. 2C,D,G,H). The *Gmd* phenotype was distinct from those previously reported for loss of Notch signaling components that strongly affected terminal differentiation and led to increased numbers of Pros+ ees and complete loss of ECs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007; Bardin et al., 2010) as shown here for *Su(H)* (Fig. 2I,J) and *kuzbanian* (Fig. 2M). Additionally, loss of canonical Notch components led to a multi-layering of ISCs and ees, which was not observed in *Gmd* mutant tissue (Fig. 2C,D,G,H,K,L). We conclude that Gmd is required for proper commitment of the ISC.

Gmd mutant ISCs retain multipotency

These data suggested that increased numbers of ISC-like cells were produced in *Gmd* mutant clones, whereas at the same time producing the proper cellular density of terminally differentiated cells. Two models could explain this: (1) *Gmd* mutant tissue contains a single population of stem cells that is capable of dividing in two modes either to produce one stem cell and one differentiated cell, or to produce two stem cells; (2) two populations of stem cells

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exist within *Gmd* mutant tissue, one population consisting of 'normal' ISCs that self-renew and produce a differentiated daughter, and another 'aberrant' population, no longer multipotent, producing only stem cells upon division. To distinguish between these possibilities, we conducted lineage analysis within *Gmd* mutant tissue. If two distinct populations exist, we should observe lineages containing only Delta+ cells. If a single population of ISCs exists, most lineages would contain both terminally differentiated cells and extra Delta+ cells. We combined two previously established methods of lineage analysis: the MARCM technique in which induced mitotic recombination leads to loss of *Gmd* activity (or control) and GFP expression (Lee and Luo, 1999). We also generated clones of cells expressing a *lacZ* marker following the recombination of the *tubulin* promoter with the *lacZ* open reading frame (Harrison and Perrimon, 1993) (Fig. 3A). Important for our analysis were the previous observations that recombination between the *tubulin* promoter and *lac*Z gene, present in trans on homologous chromosomes, occurs almost exclusively in mitotically dividing cells (Harrison and Perrimon, 1993; Fox et al., 2009).

We selected only βGal+ lineages that were entirely GFP+ and surrounded by GFP+ cells, suggesting that the βGal+ lineage was induced after the MARCM recombination event. A 4-day time point was used for analysis to allow time for lineages to produce differentiated cells while avoiding caveats of clone fusion after 4 days. As shown above, *Gmd* lineages had extra Delta+ cells when compared with wild-type lineages (Fig. 3C,E). In order to assess the potency of the ISCs, we determined the cell types present in each lineage: ISCs marked by Delta, ees marked by Pros, ECs identified by a large nucleus. Diploid unmarked cells were interpreted as EBs. Importantly, no *Gmd* lineages (*n*=39) contained only Delta+ cells, excluding the existence of a second, unipotent population of *Gmd* mutant ISCs. Furthermore, we found that 57.7% of wild-type (*n*=25) and 71.0% of *Gmd* lineages (*n*=39) contained only one of the two differentiated cell types, either ee or EC (Fig. 3F). This is consistent the fact that our analysis was carried out 4 days after the second heat shock and that probably not enough time had elapsed for a single ISC to produce both differentiated cell types. Importantly, both 28% of wild-type (*n*=25) and *Gmd* lineages (*n*=39) were multipotent ('multipotent' defined as containing one or more Delta+ cell, at least one ee and at least one EC; Fig. 3B-F). When analyzed per gut, there were more multipotent lineages in *Gmd* (Fig. 3G). These data suggested that loss of *Gmd* results in extra ISC-like cells that are a single multipotent population, capable of producing either two stem cells or one stem cell and one cell that terminally differentiated.

Loss of fucosyltransferase activity of *Ofut1* mimics loss of *Gmd*

Gmd is required for the biosynthesis of GDP-fucose that is used as a substrate of fucosyltransferases to modify lipids, glycans and proteins. Consistent with this, the GDP-fucose-dependent epitope recognized by the horseradish perioxidase (HRP) antibody (Seppo et al., 2003) was lost in clones of *Gmd* in a cell-autonomous manner (Fig. 4A,A′). Thus, in the midgut, GDP-fucose production requires *Gmd*, as in other contexts, and is not provided via gap junctions from neighboring cells as proposed in the wing epithelial cells (Sasamura et al., 2007; Okajima et al., 2008).

Gmd is required for the *O-fucosyltransferase-1* (*Ofut1*)-dependent modification of Notch (Okajima et al., 2005; Sasamura et al., 2007). Consistent with Notch being the relevant fucosylation target in the adult midgut and with *Gmd* acting upstream of activated Notch, we found that the expression of activated Notch suppressed the formation of supernumerary Delta+ ISC-like cells in the *Gmd* mutant and resulted in terminal differentiation into EC cells (Fig. 4B,C).

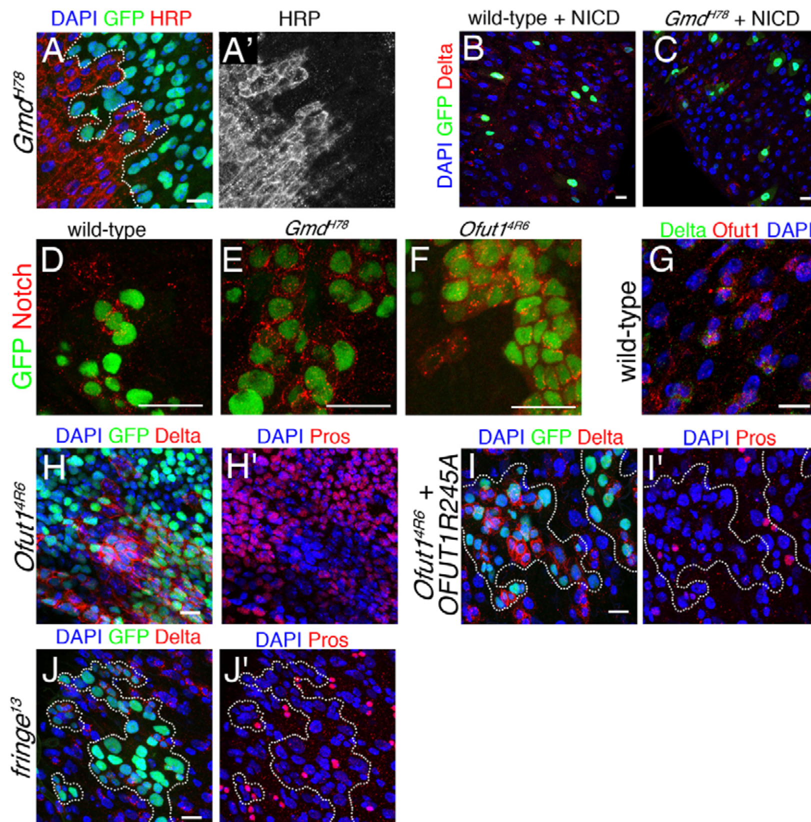

Fig. 4. Ofut1 glycosyltransferase activity but not that of Fringe is required to limit the number of stem cell-like cells. (**A**,**A′**) Immunofluroescence of the fucosylation-dependent epitope recognized by anti-horseradish perioxidase antibodies (HRP) in *Gmd*^{H78} mutant clones (GFP+) and heterozygous (wild-type) tissue (GFP–). (**B**,**C**) Expression of the activated nuclear Notch intra cellular domain (NICD) in wild-type or *Gmd*^{H78} MARCM clones (GFP+). (**D-F**) The localization of Notch in wild-type (D), *Gmd*^{H78} (E) and *Ofut1*^{4R6} (F) mutant tissue. There were an increased number of cells expressing Notch in *Gmd* mutant tissue consistent with the previous observation that ISCs and EBs express Notch (Ohlstein and Spradling, 2006). (**G**) Ofut1 was detected primarily in small nuclei cells. (**H**,**H′**) Effect on Delta+ cells (H) and terminal differentiation of ee (Pros+) and EC cells (large nuclei; H′) upon loss of *Ofut1*^{4R6} (GFP+) removing chaperone and catalytic activity. (**I**,**I′**) Effect of loss of catalytic activity alone (*Ofut1*^{4R6} with rescued chaperone activity by expression UAS-OFUT1R245A) on Delta+ cells (I) and terminal differentiation (ees, Pros+; and ECs, large nuclei in I′). (**J**,**J′**) Delta+ cells and terminally differentiated ee and EC cells (ee, Pros+; ECs large nuclei) in *fringe*^{13} clones. Scale bars: 10 μm.

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Ofut1 has two distinct molecular activities: a protein fucosyltransferase activity that modifies Notch EGF repeats with fucose and a chaperone function. The fucosyltransferase function is required for further modification of Notch by the N-acetylglucosaminyltransferase Fringe, whereas the chaperone activity promotes proper folding and trafficking of Notch from the ER to the cell surface and is essential for Notch signaling (Okajima et al., 2005; Okajima et al., 2008; Vodovar and Schweisguth, 2008) (see also Fig. 6F). As fucosyltransferase activity of Ofut1 is dispensable for trafficking of Notch from the ER to the cell surface, *Gmd* loss should not affect Notch localization. Indeed, Notch localization appeared unaffected in *Gmd* cells (Fig. 4D-F) (Okajima et al., 2005; Okajima et al., 2008; Vodovar and Schweisguth, 2008). Ofut1 protein was expressed preferentially in small diploid cells in the intestine (Fig. 4G).

We next examined whether the Ofut1 fucosyltransferase activity was required to limit the ISC pool. To separate chaperone and transferase functions, we compared *Ofut1^{4R6}* clones (null lacking both activities) and null clones expressing OFUT1R245A mutant protein retaining chaperone activity but lacking fucosyltransferase activity (Okajima et al., 2005; Okajima et al., 2008). Concurrent loss of chaperone function and transferase activity affected both Delta+ cell specification and terminal differentiation (Fig. 4H,H′). However, the specific loss of fucosyltransferase activity resulted in an increase in Delta+ ISC-like cells, without affecting the specification of differentiated Pros+ ees or large ECs, like loss of *Gmd* (Fig. 4I,I′). We conclude that the role of *Gmd* in the midgut is to produce GDP-fucose that then serves as a substrate for Ofut1 fucosyltransferase.

Fringe is not required to limit ISC self-renewal

Fucosylated residues of Notch can also be further modified by the glycosyltransferase Fringe in some developmental contexts, a modification that modulates ligand binding (Panin et al., 1997; Bruckner et al., 2000; Moloney et al., 2000). Indeed, the only known function of *Gmd* in *Drosophila* is in Fringe-dependent Notch signaling (Okajima et al., 2005; Sasamura et al., 2007; Okajima et al., 2008). Here, we found that ISC specification and terminal differentiation in *fringe* clones were indistinguishable from wild-type clones (Fig. 4J,J′). Therefore, Ofut1-mediated fucosylation affects a process in the intestine that does not require *fringe*. Thus, loss of *Gmd* probably affects Notch signaling independently of Fringe, possibly by adding fucose on Notch that may influence the interaction of Notch with Delta. Indeed, work in mammalian cells has demonstrated that fucosylation of Notch increases its capacity to signal through increased binding to Delta (Moloney et al., 2000; Chen et al., 2001; Stahl et al., 2008).

High-level Notch signaling is important for the self-renewal/commitment choice

Our data suggested two possibilities: (1) GDP-fucose modification is specifically required for commitment to occur, perhaps by altering the nature of the signal; or (2) GDP-fucose modification acts to make Notch signaling more efficient, promoting high signaling levels, which, in turn, are important for commitment of the ISC. To test this possibility, we used several genetic contexts to modulate Notch activity. Rumi is a glycosyltransferase that conjugates glucose on to the extracellular domain of Notch. The requirement for Rumi in Notch signaling events is dependent on temperature (Acar et al., 2008). At 18°C, *rumi^{44}* cells have nearly wild-type Notch activity, whereas at 28°C they exhibit little Notch signaling activity (Acar et al., 2008). In the intestine, *rumi^{44}* mutant clones were indistinguishable from wild-type control clones at 18°C, suggesting that both ISC and differentiated cell specification occurred normally (Fig. 5A,A′,G-I). However, at 21°C and 22°C, ISC commitment, but not terminal differentiation, was affected: *rumi^{44}* mutant clones had increased numbers of Delta+ cells but the ratio of differentiated ees to ECs was unaffected, as in *Gmd^{H78}* clones (Fig. 5B-C′,G-I). Thus, the self-renewal/commitment choice appeared to be specifically affected, whereas the ee/EC terminal differentiation choice was not detectably altered. At 23°C, there were many diploid Delta+ cells when compared with wild-type controls and a very mild increase in the ratio of ees to ECs. Nevertheless, most ees appeared as pairs, as in wild type, and ECs were normally specified (Fig. 5D,D′,G-I). At 25 and 28°C, both ISC commitment and terminal differentiation were strongly affected: a large increase in Delta+ cells was detected and the ratio of ees to ECs was strongly increased, resulting from increased numbers of Pros+ ees. At 28°C, large polyploid ECs were no longer observed (Fig. 5E-I).

Furthermore we used RNAi directed against *Notch* to test further the effect that lowering the levels of Notch signaling had on the commitment decision. Using the TARGET system (McGuire et al., 2004) in which the GAL4 inhibitor GAL80 can be controlled by temperature, we assessed the phenotype induced by reducing the Notch receptor (Fig. 5J-P). In control intestines, 12.9% of cells were Delta+ versus 34.4% upon reduction of *Notch* activity at 22°C, whereas terminal differentiation was unaffected (Fig. 5J,K,N). Consistent with an expansion of the number of ISCs, there was a mild increase in the density of both ees and ECs upon *Notch* silencing at 22°C, as in *rumi* mutants at 21 and 22°C, although the ratio of the two differentiated cell types was not affected (Fig. 5J,K,O,P). At 25 and 28°C, we observed both a dramatic increase in number of ISC-like cells (Delta+ cells) and ees (Pros+ cells), and concomitant loss of ECs (large nuclei; Fig. 5L-P). Together, these data strongly suggest that the self-renewal/commitment decision requires a high level of Notch activity, whereas the ee/EC terminal differentiation choice can occur properly with reduced levels of Notch activity.

Gmd is required for Notch signaling to be reliably activated in daughter cells

We next assessed nuclear Notch activity in *Gmd* mutant cells using the reporter Su(H)GBE-*lacZ*. Consistent with its previously described expression in EB cells in response to Notch signaling (Micchelli and Perrimon, 2006), Su(H)GBE-*lacZ* was expressed in wild-type clones and absent in *kuzbanian* clones lacking Notch activation (Fig. 6A-A‴,C-C‴). We found that Su(H)GBE-*lacZ* was activated in cells within *Gmd* mutant clones, indicating that GDP-fucose is not essential for Notch signaling in the gut (Fig. 6B-B‴).

The Su(H)GBE-*lacZ* signal intensity (βGal levels) was quantified in cells with small nuclei (3-5 μm) corresponding to ISCs, EBs, undifferentiated ECs and ees (Fig. 6D). First, *Gmd* and wild-type clones contained similar percentages of Delta negative (Delta–) small cells with high βGal levels (Fig. 6E, graph 1). These cells probably correspond to differentiating EBs. This result is consistent with our finding in Fig. 2 that differentiation was unaltered in *Gmd* mutants. Second, consistent with our data in Fig. 1, we found that *Gmd* clones contained more Delta+ ‘βGal low’ cells, probably corresponding to ISCs (5.75±0.89 s.e.m., *n*=24 clones) than wild type (1.93±0.24 s.e.m., *n*=29 clones) at 5 days AHS (Fig. 6F). Third, we observed a threefold increase in a population of small Delta+ cells that were ‘βGal high’ in *Gmd* mutant clones (26.6%, *n*=188 *Gmd* mutant cells; 8.2%, *n*=61 wild-

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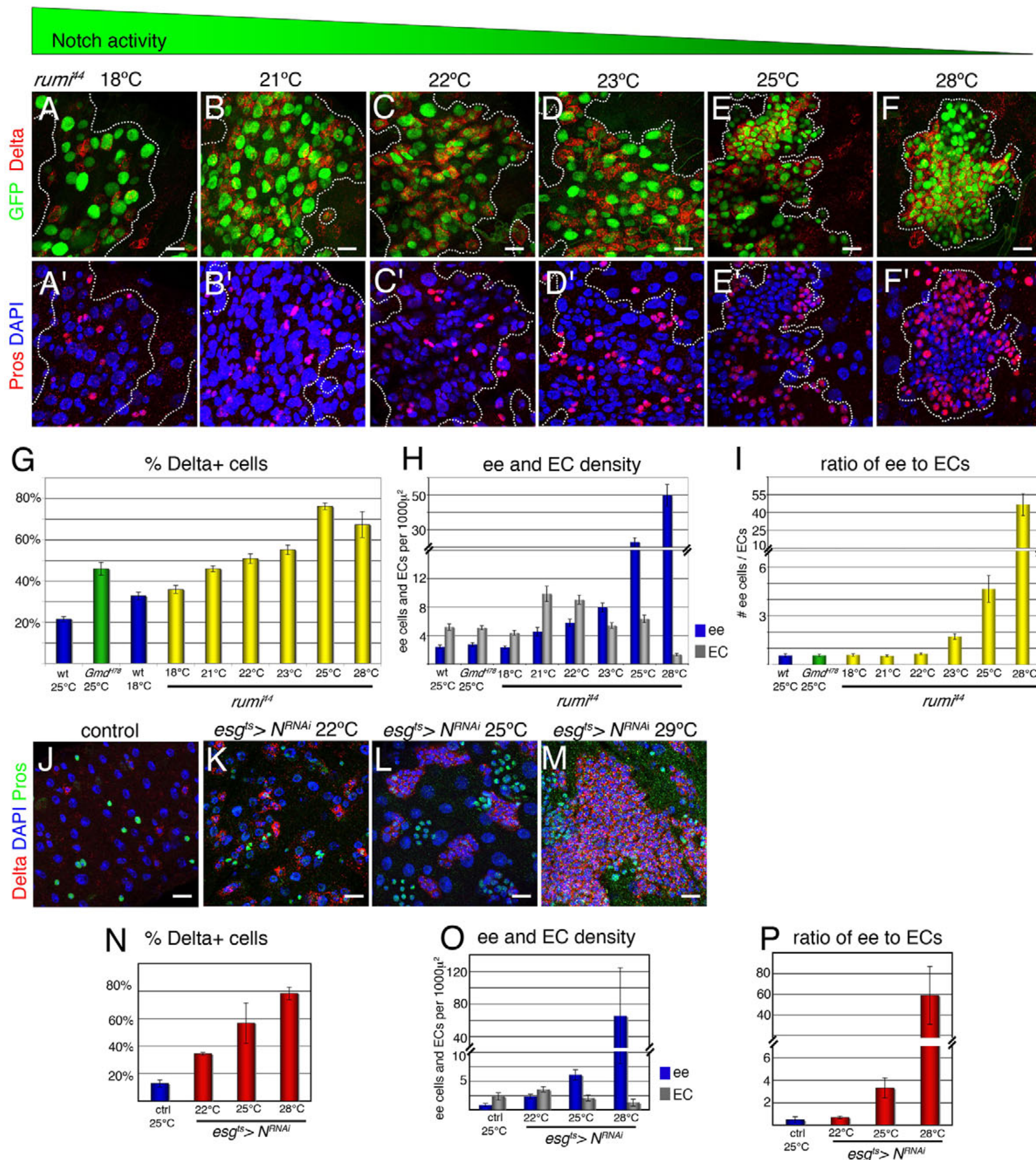

Fig. 5. Modulation of Notch signaling reveals a high-level signaling requirement for efficient commitment/exit from self-renewal. (**A-F′**) Modulation of Notch activity in $rumi^{44}$ clones (GFP+) at 18-28°C. Delta+ cells and terminally differentiated cells (ees, Pros+; ECs large nuclei) at an age equivalent of 10 days at 25°C, at this time point clones were often fused. (**G**) Quantification of Delta+ cells as a percentage of total cells in clone tissue. Wild-type clones as controls are shown both at 18°C and 25°C; the percentage of Delta+ cells is slightly higher at 18°C than at 25°C; *n*=43 (clonal regions), wild-type 25°C; *n*=13, Gmd^{H78}; *n*=11, wild-type 18°C; *n*=11, $rumi^{44}$ 18°C; *n*=16, $rumi^{44}$ 21°C; *n*=23, $rumi^{44}$ 22°C; *n*=14, $rumi^{44}$ 23°C; *n*=15, $rumi^{44}$ 25°C; *n*=9, $rumi^{44}$ 28°C. (**H**) Quantification of the density of large nuclei ECs and Pros+ ees in wild-type, Gmd^{H78} and $rumi^{44}$ mutant clones at the indicated temperatures. (**I**) The ratio of ees to ECs in wild-type control and $rumi^{44}$ mutant clones at the indicated temperatures. For H and I, *n*=27 (clonal regions), wild-type 25°C; *n*=19, Gmd^{H78} 25°C; *n*=15, $rumi^{44}$ 18°C; *n*=14, $rumi^{44}$ 21°C; *n*=18, $rumi^{44}$ 22°C; *n*=24, $rumi^{44}$ 23°C; *n*=19 $rumi^{44}$ 25°C; *n*=9, $rumi^{44}$ 28°C. (**J**) Control esg^{ts}> at 25°C (equivalent results at 22°C and 28°C, data not shown). (**K-M**) esg^{ts} >*Notch RNAi* at 22°C (K), 25°C (L) and 28°C (M). (**N**) Percentage of Delta+ cells in total cells per 100 μm^2 field at indicated temperatures of esg^{ts} >*Notch RNAi.* (**O**) Density of Pros+ ees and large nuclei EC cells at indicated temperatures of esg^{ts} >*Notch RNAi*. (**P**) The ratio of ees to ECs (from O) of esg^{ts} >*Notch RNAi*. Data are mean±s.e.m. Scale bars: 10 μm in A-F,J-M.

type cells; Fig. 6E, graph 2, 6F). These cells co-expressed Delta, an ISC marker, and Su(H)GBE-*lacZ*, an EB marker, and may represent cells that have not yet accumulated high enough levels of Notch activity to exit self-renewal and differentiate. Interestingly, we found that 23.5% of dividing *Gmd* cells (*n*=34 cells) expressed detectable levels of Su(H)GBE-*lacZ* (Fig. 6G,G′). These cells were also found to express Delta (*n*=10 cells; Fig. 6G,H,I). Dividing cells expressing Su(H)GBE-*lacZ* were not observed in wild-type

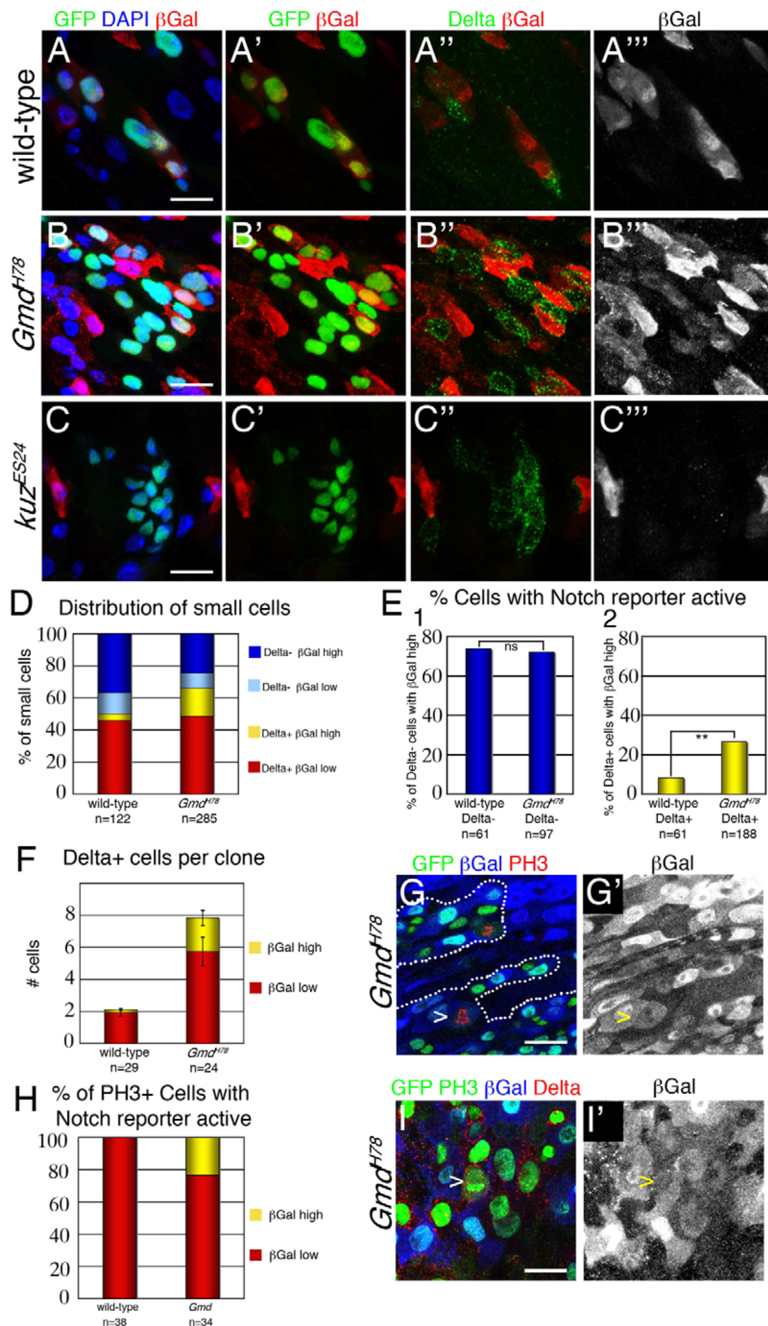

Fig. 6. Notch reporter activation in *Gmd*^{H78} mutant clones. (**A-C‴**) The Notch signaling transcriptional reporter [Su(H)GBE-LacZ; βGal+] was activated in cells of wild-type and *Gmd* mutant clones, but not that of the canonical Notch signaling component *kuzbanian* (*kuz*). Clones marked by nuclear GFP. (**D**) The relative percentage of the total population of small cells (3-5 μm nuclei) that were Delta-βGal high (dark blue), Delta- βGal low (light blue), Delta+ βGal high (yellow) and Delta+βGal low (red) in wild-type and *Gmd*^{H78} mutants. (**E**) The percentage of cells that were βgal high (dark blue) of the Delta– cells (1). The difference between wild-type and *Gmd*^{H78} was not significant (n.s.). (2) The percentage of cells that were βgal high (yellow) of the Delta+ cells. The difference between wild-type and *Gmd*^{H78} was significant. **P*<0.01. (**F**) The number of Delta+ βgal low cells (red) per clone was greater in *Gmd*^{H78} than in wild-type clones at 5 days. In addition, *Gmd*^{H78} contained more Delta+ βgal high (yellow) per clone. Data are mean±s.e.m. (**G**) Dividing *Gmd*^{H78} cells could be detected with Su(H)GBE-*lacZ* activity (arrowhead). (**H**) PH3+ cells, scored for βGal high or low in wild type and *Gmd*^{H78}. (**I**) A dividing Su(H)GBE-*lacZ*+ Delta+ *Gmd*^{H78}, arrowhead. Scale bars: 10 μm.

intestines (*n*=38, Fig. 6H; *n*=11, Fig. 6I, data not shown). Thus, a majority of dividing *Gmd* mutant cells are Delta+Su(H)GBE-*lacZ*– (76.5%) and a subset are Delta+Su(H)GBE-*lacZ*+ (23.5%). Together, these data suggest that *Gmd* and high-level Notch activation are required for 'reliable' activation of Notch signaling after every ISC cell division, allowing one ISC daughter to undergo commitment.

DISCUSSION

Our results show that the self-renewal/commitment decision has a specific requirement for high Notch signaling levels. Using loss of *Gmd* activity, loss of Ofut1 catalytic activity, *rumi* mutants at 21 and 22°C and RNAi against the Notch receptor at 22°C, we could uncouple self-renewal/commitment from the ee/EC terminal differentiation decision. We propose that after ISC division, one of the two ISC daughter cells first receives a high, GDP-fucose-dependent, Notch commitment signal to exit the self-renewal program and that terminal differentiation into ee or EC can occur via lower Notch activity (Fig. 7). We hypothesize that a subset of cells lacking *Gmd* could activate Notch signaling to some extent but not to high enough levels to commit. These cells would therefore express detectable Su(H)GBE-*lacZ* though fail to commit, and would re-enter self-renewal and cell division. It has also been proposed that the EC fate, but not the ee fate, is dependent on Notch signaling (Micchelli and Perrimon, 2006; Beebe et al., 2010). Our data could also be consistent with this model, although our data suggest that precise control of commitment, requiring high-level Notch signaling, is at least indirectly important for specification of both terminal differentiated cell types, as failure to commit produces supernumerary multipotent ISCs.

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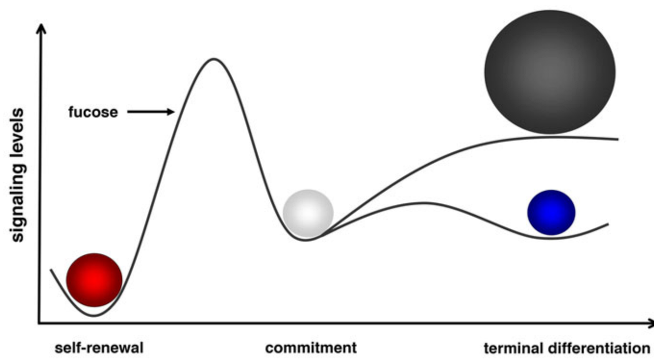

Fig. 7. Model for high-level signal to exit self-renewal. A high-level Notch signaling barrier needs to be crossed in order for ISCs (red) to exit the self-renewing state and become committed (white). Inability to reach this high level either by loss of *Gmd* or reduction of Notch signaling levels results in ISC divisions producing two ISCs. It is likely that attaining high level signaling must be achieved within a given time window or cell cycle state. However, terminal differentiation of committed EBs into ee (blue) or EC (dark gray) cells can occur with lower levels of Notch signaling and does not require *Gmd*. A requirement for high-level signaling may ensure that the stem cell pool is protected and not lost through weak differentiation signals.

Our finding that the reliable commitment of one ISC daughter at each cell division relies on high-level Notch receptor activation is somewhat surprising in light of previous work suggesting that different levels of Notch signaling dictate the terminal fate of EBs (Ohlstein and Spradling, 2007). How then can a level-based Notch signaling be used to instruct the terminal differentiation status of the same cell that has received a high level of Notch signaling to exit self-renewal? We speculate that regulation of Delta ligand levels in the ISC after the commitment decision may then control further activation of Notch transcriptional output, thereby promoting terminal differentiation choice. This would be consistent with both our findings and those Ohlstein and Spradling, who observed a correlation of Delta levels with recent daughter terminal cell fate (Ohlstein and Spradling, 2007).

The *Drosophila* intestine represents an important system in which to understand the interplay between cell fate decisions and tissue homeostasis. It is currently unclear when and how the ee and EC terminal differentiation fate choices are made. There are three possibilities.

(1) The state of the ISC prior to its cell division (such as expression levels of Delta or another factor) could dictate the outcome of the daughter terminal differentiation decision occurring after division. Thus, the choice between ee and EC fate of the daughter cell terminal has already been made at the time of cell division.

(2) Alternatively, the state of the ISC may be plastic and have the capacity to be altered after cell division (perhaps via levels of Delta) to impact the terminal fate of its neighboring sister cells. In this model, the terminal differentiation decision is not pre-made, but can be modulated in the ISC after cell division.

(3) Finally, it is possible that the presumptive EB is the recipient of signals both from the ISC (like Delta) but also parallel input that could communicate the needs of the tissue necessary to decide between ee and EC fates.

Our findings do not fit well with a model in which levels of Delta ligand in the ISC prior to division would dictate daughter terminal cell fate. Indeed, it is not clear how a low level of Delta ligand [promoting ee cell specification (Ohlstein and Spradling, 2007)] would provide high enough levels of Notch activation for commitment to occur. A model postulating that the ISC is plastic in state and could be modified to accommodate the needs of the tissue and dictate the terminal differentiation of its sister cell would best fit our data and that of Ohlstein and Spradling (Ohlstein and Spradling, 2007). Importantly, our findings that the self-renewal/commitment step can be affected without impacting the terminal differentiation process or the relative abundance of the ee and EC cell types suggest that commitment does not simply arise from activation of the terminal differentiation process, at least in the genetic contexts of our experiments.

We note that our findings raise the possibility that stem cell numbers in the intestine may be controlled through modulation of Notch signaling levels, which could affect self-renewal without affecting terminal differentiation. Understanding how each step of cell fate is acquired in the relatively simple *Drosophila* ISC system may provide significant insight in to more complicated mammalian lineages that may use similar strategies in which stem cells can choose between three or more cell fates.

Interestingly, the 'mistakes' in commitment seen either in the *Gmd* mutant or in the *rumi* mutant at 21 and 22°C did not occur at each and every ISC division, but the EB cells, when produced, could appropriately terminally differentiate. What might be the source of this variability and is it inherent to the commitment decision? One possibility could be stochastic cell-to-cell variability [such as in gene transcription, chromosomal domain replication, protein expression, signaling network and/or cell cycle kinetics (Colman-Lerner et al., 2005; Chang et al., 2008; Spencer et al., 2009; Gilbert, 2010; Raj et al., 2010)]. Thus, in a stochastic manner, individual daughter cells with weakened Notch pathway activity maybe able to reach levels of pathway output required for commitment or not depending other components of the Notch signaling network. Could the commitment decision be inherently more susceptible variation to signaling levels? One possibility is that the timing of the commitment decision is made during a shorter time window, perhaps in the G1 phase of the cell cycle (Mummery et al., 1987; Orford and Scadden, 2008; Singh and Dalton, 2009; Lange and Calegari, 2010), than that of the terminal differentiation decision, necessitating signaling modulators to boost signaling that are not required for terminal fate decisions with a longer time window.

A requirement of high-level signaling for commitment choices could represent a strategy whereby stem cells must first cross a high signaling barrier for commitment to occur prior to the terminal differentiation process and could provide insurance that the stem cell pool is not lost. In some instances, it may be preferable to gamble having too many stem cells in a tissue, possibly putting the tissue in jeopardy for development of a cancer-like state, rather than risk the depletion of tissue specific stem cells that may have more immediate detrimental consequences on the tissue. Consequently, commitment decisions may need specific modifiers of signaling networks that may not be required for generalized function of these signaling networks. It will be important to determine whether this model is a more general feature of stem-daughter cell decisions.

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Competing interests statement
The authors declare no competing financial interests.

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References

Acar, M., Jafar-Nejad, H., Takeuchi, H., Rajan, A., Ibrani, D., Rana, N. A., Pan, H., Haltiwanger, R. S. and Bellen, H. J. (2008). Rumi is a CAP10 domain glycosyltransferase that modifies Notch and is required for Notch signaling. *Cell* **132**, 247-258.
Amcheslavsky, A., Jiang, J. and Ip, Y. T. (2009). Tissue damage-induced intestinal stem cell division in *Drosophila*. *Cell Stem Cell* **4**, 49-61.
Arnold, S. J. and Robertson, E. J. (2009). Making a commitment: cell lineage allocation and axis patterning in the early mouse embryo. *Nat. Rev. Mol. Cell Biol.* **10**, 91-103.
Bardin, A. J., Perdigoto, C. N., Southall, T. D., Brand, A. H. and Schweisguth, F. (2010). Transcriptional control of stem cell maintenance in the *Drosophila* intestine. *Development* **137**, 715-724.
Beebe, K., Lee, W. C. and Micchelli, C. A. (2010). JAK/STAT signaling coordinates stem cell proliferation and multilineage differentiation in the *Drosophila* intestinal stem cell lineage. *Dev. Biol.* **338**, 28-37.
Biteau, B. and Jasper, H. (2011). EGF signaling regulates the proliferation of intestinal stem cells in Drosophila. *Development* **138**, 1045-1055.
Biteau, B., Hochmuth, C. E. and Jasper, H. (2008). JNK activity in somatic stem cells causes loss of tissue homeostasis in the aging *Drosophila* gut. *Cell Stem Cell* **3**, 442-455.
Blanpain, C., Horsley, V. and Fuchs, E. (2007). Epithelial stem cells: turning over new leaves. *Cell* **128**, 445-458.
Brennan, K., Tateson, R., Lieber, T., Couso, J. P., Zecchini, V. and Arias, A. M. (1999). The abruptex mutations of notch disrupt the establishment of proneural clusters in *Drosophila*. *Dev. Biol.* **216**, 230-242.
Bruckner, K., Perez, L., Clausen, H. and Cohen, S. (2000). Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. *Nature* **406**, 411-415.
Buchon, N., Broderick, N. A., Chakrabarti, S. and Lemaitre, B. (2009a). Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in *Drosophila*. *Genes Dev.* **23**, 2333-2344.
Buchon, N., Broderick, N. A., Poidevin, M., Pradervand, S. and Lemaitre, B. (2009b). *Drosophila* intestinal response to bacterial infection: activation of host defense and stem cell proliferation. *Cell Host Microbe* **5**, 200-211.
Buchon, N., Broderick, N. A., Kuraishi, T. and Lemaitre, B. (2010). *Drosophila* EGFR pathway coordinates stem cell proliferation and gut remodeling following infection. *BMC Biol.* **8**, 152.
Chang, H. H., Hemberg, M., Barahona, M., Ingber, D. E. and Huang, S. (2008). Transcriptome-wide noise controls lineage choice in mammalian progenitor cells. *Nature* **453**, 544-547.
Chatterjee, M. and Ip, Y. T. (2009). Pathogenic stimulation of intestinal stem cell response in *Drosophila*. *J. Cell. Physiol.* **220**, 664-671.
Chen, J., Moloney, D. J. and Stanley, P. (2001). Fringe modulation of Jagged1-induced Notch signaling requires the action of beta 4galactosyltransferase-1. *Proc. Natl. Acad. Sci. USA* **98**, 13716-13721.
Colman-Lerner, A., Gordon, A., Serra, E., Chin, T., Resnekov, O., Endy, D., Pesce, C. G. and Brent, R. (2005). Regulated cell-to-cell variation in a cell-fate decision system. *Nature* **437**, 699-706.
Fox, D. T., Morris, L. X., Nystul, T. and Spradling, A. C. (2009). Lineage analysis of stem cells (January 31, 2009). In *StemBook* (ed. The Stem Cell Research Community, StemBook), doi/10. 3824/stembook.1.33.1.
Furriols, M. and Bray, S. (2001). A model Notch response element detects Suppressor of Hairless-dependent molecular switch. *Curr. Biol.* **11**, 60-64.
Gilbert, D. M. (2010). Cell fate transitions and the replication timing decision point. *J. Cell Biol.* **191**, 899-903.
Harrison, D. A. and Perrimon, N. (1993). Simple and efficient generation of marked clones in *Drosophila*. *Curr. Biol.* **3**, 424-433.
Hemberger, M., Dean, W. and Reik, W. (2009). Epigenetic dynamics of stem cells and cell lineage commitment: digging Waddington's canal. *Nat. Rev. Mol. Cell Biol.* **10**, 526-537.
Irvine, K. D. and Wieschaus, E. (1994). fringe, a Boundary-specific signaling molecule, mediates interactions between dorsal and ventral cells during *Drosophila* wing development. *Cell* **79**, 595-606.
Jafar-Nejad, H., Leonardi, J. and Fernandez-Valdivia, R. (2010). Role of glycans and glycosyltransferases in the regulation of Notch signaling. *Glycobiology* **20**, 931-949.
Jiang, H., Patel, P. H., Kohlmaier, A., Grenley, M. O., McEwen, D. G. and Edgar, B. A. (2009). Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the *Drosophila* midgut. *Cell* **137**, 1343-1355.
Jiang, H., Grenley, M. O., Bravo, M. J., Blumhagen, R. Z. and Edgar, B. A. (2011). EGFR/Ras/MAPK signaling mediates adult midgut epithelial homeostasis and regeneration in *Drosophila*. *Cell Stem Cell* **8**, 84-95.
Karpowicz, P., Perez, J. and Perrimon, N. (2010). The Hippo tumor suppressor pathway regulates intestinal stem cell regeneration. *Development* **137**, 4135-4145.
Lange, C. and Calegari, F. (2010). Cdks and cyclins link G(1) length and differentiation of embryonic, neural and hematopoietic stem cells. *Cell Cycle* **9**, 1893-1900.
Lee, T. and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451-461.
Lee, W. C., Beebe, K., Sudmeier, L. and Micchelli, C. A. (2009). Adenomatous polyposis coli regulates *Drosophila* intestinal stem cell proliferation. *Development* **136**, 2255-2264.
Li, L. and Clevers, H. (2010). Coexistence of quiescent and active adult stem cells in mammals. *Science* **327**, 542-545.
Li, Y. and Baker, N. E. (2001). Proneural enhancement by Notch overcomes Suppressor-of-Hairless repressor function in the developing *Drosophila* eye. *Curr. Biol.* **11**, 330-338.
Lin, G., Xu, N. and Xi, R. (2008). Paracrine Wingless signalling controls self-renewal of *Drosophila* intestinal stem cells. *Nature* **455**, 1119-1123.
Lin, G., Xu, N. and Xi, R. (2010). Paracrine unpaired signaling through the JAK/STAT pathway controls self-renewal and lineage differentiation of *Drosophila* intestinal stem cells. *J. Mol. Cell Biol.* **2**, 37-49.
Liu, W., Singh, S. R. and Hou, S. X. (2010). JAK-STAT is restrained by Notch to control cell proliferation of the *Drosophila* intestinal stem cells. *J. Cell. Biochem.* **109**, 992-999.
McGuire, S. E., Mao, Z. and Davis, R. L. (2004). Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in *Drosophila*. *Sci. STKE* **220**, pl6.
McLeod, C. J., Wang, L., Wong, C. and Jones, D. L. (2010). Stem cell dynamics in response to nutrient availability. *Curr. Biol.* **20**, 2100-2105.
Micchelli, C. A. and Perrimon, N. (2006). Evidence that stem cells reside in the adult *Drosophila* midgut epithelium. *Nature* **439**, 475-479.
Moloney, D. J., Panin, V. M., Johnston, S. H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K. D., Haltiwanger, R. S. et al. (2000). Fringe is a glycosyltransferase that modifies Notch. *Nature* **406**, 369-375.
Morel, V. and Schweisguth, F. (2000). Repression by suppressor of hairless and activation by Notch are required to define a single row of single-minded expressing cells in the *Drosophila* embryo. *Genes Dev.* **14**, 377-388.
Mummery, C. L., van den Brink, C. E. and de Laat, S. W. (1987). Commitment to differentiation induced by retinoic acid in P19 embryonal carcinoma cells is cell cycle dependent. *Dev. Biol.* **121**, 10-19.
O'Connor-Giles, K. M. and Skeath, J. B. (2003). Numb inhibits membrane localization of Sanpodo, a four-pass transmembrane protein, to promote asymmetric divisions in *Drosophila*. *Dev. Cell* **5**, 231-243.
Ohlstein, B. and Spradling, A. (2006). The adult *Drosophila* posterior midgut is maintained by pluripotent stem cells. *Nature* **439**, 470-474.
Ohlstein, B. and Spradling, A. (2007). Multipotent *Drosophila* intestinal stem cells specify daughter cell fates by differential notch signaling. *Science* **315**, 988-992.
Okajima, T., Xu, A., Lei, L. and Irvine, K. D. (2005). Chaperone activity of protein O-fucosyltransferase 1 promotes notch receptor folding. *Science* **307**, 1599-1603.
Okajima, T., Reddy, B., Matsuda, T. and Irvine, K. D. (2008). Contributions of chaperone and glycosyltransferase activities of O-fucosyltransferase 1 to Notch signaling. *BMC Biol.* **6**, 1.
Orford, K. W. and Scadden, D. T. (2008). Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. *Nat. Rev. Genet.* **9**, 115-128.
Panin, V. M., Papayannopoulos, V., Wilson, R. and Irvine, K. D. (1997). Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-912.
Raj, A., Rifkin, S. A., Andersen, E. and van Oudenaarden, A. (2010). Variability in gene expression underlies incomplete penetrance. *Nature* **463**, 913-918.
Ren, F., Wang, B., Yue, T., Yun, E. Y., Ip, Y. T. and Jiang, J. (2010). Hippo signaling regulates *Drosophila* intestine stem cell proliferation through multiple pathways. *Proc. Natl. Acad. Sci. USA* **107**, 21064-21069.
Sasamura, T., Sasaki, N., Miyashita, F., Nakao, S., Ishikawa, H. O., Ito, M., Kitagawa, M., Harigaya, K., Spana, E., Bilder, D. et al. (2003). neurotic, a novel maternal neurogenic gene, encodes an O-fucosyltransferase that is essential for Notch-Delta interactions. *Development* **130**, 4785-4795.
Sasamura, T., Ishikawa, H. O., Sasaki, N., Higashi, S., Kanai, M., Nakao, S., Ayukawa, T., Aigaki, T., Noda, K., Miyoshi, E. et al. (2007). The O-fucosyltransferase O-fut1 is an extracellular component that is essential for the

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constitutive endocytic trafficking of Notch in *Drosophila*. *Development* **134**, 1347-1356.

Seppo, A., Matani, P., Sharrow, M. and Tiemeyer, M. (2003). Induction of neuron-specific glycosylation by Tollo/Toll-8, a *Drosophila* Toll-like receptor expressed in non-neural cells. *Development* **130**, 1439-1448.

Shaw, R. L., Kohlmaier, A., Polesello, C., Veelken, C., Edgar, B. A. and Tapon, N. (2010). The Hippo pathway regulates intestinal stem cell proliferation during *Drosophila* adult midgut regeneration. *Development* **137**, 4147-4158.

Singh, A. M. and Dalton, S. (2009). The cell cycle and Myc intersect with mechanisms that regulate pluripotency and reprogramming. *Cell Stem Cell* **5**, 141-149.

Spencer, S. L., Gaudet, S., Albeck, J. G., Burke, J. M. and Sorger, P. K. (2009). Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis. *Nature* **459**, 428-432.

Stahl, M., Uemura, K., Ge, C., Shi, S., Tashima, Y. and Stanley, P. (2008). Roles of Pofut1 and O-fucose in mammalian Notch signaling. *J. Biol. Chem.* **283**, 13638-13651.

Staley, B. K. and Irvine, K. D. (2010). Warts and Yorkie mediate intestinal regeneration by influencing stem cell proliferation. *Curr. Biol.* **20**, 1580-1587.

Takeuchi, H. and Haltiwanger, R. S. (2010). Role of glycosylation of Notch in development. *Semin. Cell Dev. Biol.* **21**, 638-645.

Vodovar, N. and Schweisguth, F. (2008). Functions of O-fucosyltransferase in Notch trafficking and signaling: towards the end of a controversy? *J. Biol.* **7**, 7.

Yagi, Y. and Hayashi, S. (1997). Role of the *Drosophila* EGF receptor in determination of the dorsoventral domains of escargot expression during primary neurogenesis. *Genes Cells* **2**, 41-53.

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