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Dll1 marks early secretory progenitors in gut crypts that can revert to stem cells upon tissue damage

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Abstract

Lgr5 stem cells reside at small intestinal crypt bottoms, generating both the enterocyte and secretory lineage. Entry into the latter epithelial lineage requires silencing of Notch signaling. The Notch ligand Dll1 is strongly up-regulated in a small subset of immediate stem cell daughters. Lineage tracing utilizing a novel *Dll1*$^{GFP\text{-}ires\text{-}CreERT2}$ knock-in mouse reveals that single Dll1^{high} cells generate small, short-lived clones of all four secretory cell types. In culture, sorted Dll1^{high} cells can form long-lived organoids when briefly exposed to Wnt3A. When Dll1 cells are genetically marked prior to tissue damage, significant numbers of stem cell tracing events occur. Lineage specification therefore occurs already in the earliest stem cell daughters through Notch lateral inhibition. Yet, specified secretory progenitors display plasticity and can regain stemness upon tissue damage.

The intestinal epithelium represents a unique model for the study of adult stem cell biology and lineage specification. Not only is it the fastest self-renewing tissue in mammals with a turn-over time of 5 days, it also has a simple, highly repetitive layout. Lgr5^{high} stem cells are intermingled with Paneth cells at the base of the crypt and feed daughter cells into the Transit Amplifying (TA) compartment that fills the remainder of the crypt *(1)*. TA cells undergo approximately 4–5 rounds of rapid cell division, after which they cross the crypt-villus boundary to terminally differentiate into enterocytes or into one of the secretory cell types, i.e goblet cells, tuft cells and enteroendocrine cells *(2-6)*. The Paneth cells represent a fourth secretory cell type that is unusual in its location at crypt bottoms and its relatively long lifespan of 6-8 weeks *(7)*. Paneth cells serve as niche cells, providing Wnt, Notch and EGF signals to stem cells *(8)*. The first binary fate decision of undifferentiated crypt TA cells involves entry into either the enterocyte or the secretory lineage. A simple molecular circuit downstream of the Notch1/2 receptors controls the absorptive/secretory switch *(9)*. Notch signaling activates Hes1 expression in the receiving cell, typically the crypt stem cell or the undifferentiated TA cells *(10-11)*. HES1 in turn represses expression of the Math1

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transcription factor that is crucial for entry into the secretory lineage *(12-14)*. While all TA cells in crypts are capable of converting into secretory cells upon acute blockade of Notch *(11,15,16)*, it is currently not known at what level in the stem cell/TA hierarchy this lineage specification occurs physiologically. Stem cells and the bulk of the TA cells express Notch1 and Notch2 *(9)*, while Delta-like 1 (Dll1) and Delta-like 4 (Dll4) function redundantly as Notch ligands *(17)*.

Dll1 is expressed by rare undifferentiated TA cells that are early descendants of Lgr5 stem cells

While Paneth cells express Dll1 and Dll4, thus activating Notch receptors on adjacent stem cells, it is unclear which cells in the TA compartment express Notch ligands to activate Notch receptors on TA cells. A *Dll1 in situ* hybridization probe brightly marked rare cells 1-2 cell positions above the stem cell/Paneth cell zone (Fig. 1A). Much weaker signals were obtained in individual cells higher up in the crypt and on the villus. This pattern was reminiscent of zebra fish DeltaD in secretory cells of the intestinal tract *(18)*. The rare scattered $Dll1^{high}$ cells in crypts had the morphology of TA cells.

To determine the temporal, hierarchical relationship between the Lgr5 stem cells and these $Dll1^{high}$ cells, we induced lineage tracing in $Lgr5^{GFP\text{-}ires\text{-}CreERT2}$ knock-in mice crossed to the Cre reporter $R26R^{LacZ}$. At various time points post-tamoxifen induction, we analyzed the expression of the stem cell marker gene *Lgr5*, the Cre reporter *LacZ* and *Dll1* by triple color mRNA *in situ* hybridization at single cell resolution *(19)*. We thus noted that 1-2 days after the induction of lineage tracing in stem cells, $Dll1^{high}$ cells were first observed within the marked clone (Fig. 1C versus 1B). This indicated that –as expected- $Dll1^{high}$ cells derive from $Lgr5^{+}$ stem cells. More importantly, the time course revealed that $Dll1^{high}$ cells derive from stem cells within 1-2 cell divisions, given that Lgr5 stem cells divide every 24 hours *(20)*. This was consistent with the physical position of the $Dll1^{high}$ cell, immediately above the stem cell/Paneth cell zone.

To study $Dll1^{+}$ cells, we integrated a GFP-ires-CreERT2 cassette into the start codon of the *Dll1* locus (Suppl. Fig. 1). Heterozygous knock-in mice were healthy and fertile and GFP appeared faithfully expressed as assessed by confocal analysis (Fig. 1D). This analysis showed the presence of rare GFP^{+} cells 1-2 cell cells above the stem cell/Paneth cell zone as well as higher up the crypts and villi (Fig. 1E).

$Dll1^{+}$ cells, obtained by Fluorescence-Activated Cell Sorting (FACS) for GFP and for levels of CD24 expression, were subjected to microarray analysis. This revealed that $Dll1^{GFP+}CD24^{high}$, and $Dll1^{GFP+}CD24^{low}$ cells correspond to Paneth cells, and enteroendocrine/goblet cells, respectively (Fig. 1E and Suppl. Fig. 2). The $Dll1^{GFP+}CD24^{mid}$ cells expressed markers of multiple secretory lineages, suggesting that these cells represent secretory progenitors. Importantly, $Dll1^{GFP+}CD24^{mid}$ cells expressed high levels of Math1 and very low levels of *Notch1*, *Notch2* and *Hes1*, indicative of an inactive Notch signaling pathway (Suppl. Fig. 2). This set them apart from stem cells and the bulk of TA cells that express high levels of *Notch1*, *Notch2* and *Hes*1 and low levels of *Math1 (9,10,14,16)*.

$Dll1^{high}$ precursor cells generate short-lived clones of mixed secretory cell content

We then performed a lineage tracing study by crossing the $Dll1^{GFP\text{-}ires\text{-}CreERT2}$ allele with the Cre reporter allele $R26R^{LacZ}$. Adult mice were subjected to a single tamoxifen pulse and the proximal small intestine was analyzed histologically 12 hrs and, 2, 4, 10 and 122 days

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later. After 12 hrs, single $LacZ^+$ cells occurred mainly around the '+5' position, one cell-diameter removed from the uppermost Paneth cell (Fig. 2A and 2B). We concluded that Cre excision was fortuitously restricted to the cells that express highest *Dll1* mRNA levels as assessed by *in situ* hybridization (Fig. 1B) and not by other GFP-expressing cells (Fig. 1D). On day 2, multiple $LacZ^+$ cells occurred at the top of the crypt and the villus base (average 1.4 $LacZ^+$ cells per tracing crypt/villus unit; range 1-3 $LacZ^+$ cells) (Fig. 2C). On day 4, $LacZ^+$ cells mainly occurred on villi (average 3.2 $LacZ^+$ cells per tracing crypt/villus unit; range 1-7 $LacZ^+$ cells), while occasional $LacZ^+$ Paneth cells were first noted (Fig. 2D). On day 10, only $LacZ^+$ Paneth cells remained (Fig. 2E). On day 122 post-induction, we occasionally detected ribbons of $LacZ^+$ cells (average of 9.8 stem cell derived tracings per duodenum), while we never observed tracing in non-induced mice (not shown).

To determine which cell types became $LacZ^+$, $Dll1^{GFP\text{-}ires\text{-}CreERT2}$-$R26R^{LacZ}$ mice were induced by tamoxifen injections on 3 sequential days. Marker analysis, performed 4 days later, revealed $LacZ^+$ goblet cells (Fig. 3A), Paneth cells (Fig. 3B), tuft cells (Fig. 3C), and enteroendocrine cells (Fig. 3D). Importantly, we never detected $LacZ^+$ enterocytes. We concluded that the $Dll1^{high}$ cells localized at the '+5' position generate short-lived clones exclusively consisting of secretory lineage cells.

Using the multi-color Cre reporter $R26R^{confetti}$ for tracing *(21)*, we found that a single $Dll1^+$ cell could give rise to mixed secretory clones consisting of 2-6 cells. Examples are given in Fig. 3E and 3F.

$Dll1^{high}$ precursor cells build long-lived organoids when exposed to Wnt

To examine the potential stemness of $Dll1^+$ cells *in vitro*, we sorted and cultured $Dll1^{GFP+}CD24^{high}$, $Dll1^{GFP+}CD24^{mid}$ and $Dll1^{GFP+}CD24^{low}$ cells. Under standard crypt culture conditions (EGF, Noggin and R-spondin1 in Matrigel; *(22)*), none of the $Dll1^+$ FACS-sorted subpopulations generated organoid structures (Fig. 4A), in sharp contrast to sorted $Lgr5^+$ cells *(22)*. However, addition of Wnt-3A enabled $Dll1^+CD24^{mid}$ cells, but not $Dll1^+CD24^{high}$ or $Dll1^+CD24^{low}$ cells, to give rise to organoid structures. Single $Dll1^{GFP+}$/ $CD24^{mid}$ cells derived from $Dll1^{GFP\text{-}ires\text{-}CreERT2}$_$Lgr5^{LacZ}$ small intestinal crypts and cultured in the presence of Wnt3A, showed the induction of $Lgr5^{LacZ+}$ stem cells at the bottom of crypt equivalents (Fig. 4B). Moreover, the presence and location of $Dll1^{GFP+}$ secretory cells in these organoids mimicked the *in vivo* situation (Fig. 4C). Taken together, $Dll1^+CD24^{\mathbf{mid}}$ secretory progenitor cells could regain stemness upon Wnt stimulation in culture.

$Dll1^{high}$ precursor cells revert to stem cells upon tissue damage

$Dll1^{high}$ precursor cells generate short-lived secretory clones, but rarely generate the "signature" long-lived ribbons that $Lgr5^{high}$ stem cells will *(1)*. The intestine has a unique capacity to regenerate upon extensive damage. We wondered if the $Dll1^{high}$ fated precursor cells would be capable of replacing lost stem cells upon extensive radiation damage. We therefore induced Cre expression with tamoxifen in the $Dll1^{GFP\text{-}ires\text{-}CreERT2}$-$R26R^{LacZ}$ mice, one day prior to sublethal irradiation (6.0 Gy gamma at day 0). This dosage of irradiation causes strong reduction of $Lgr5^+$ stem cells and major apoptosis in the intestinal crypt (not shown, and *1*). The duodenum was subsequently analyzed by LacZ staining at day 28 post irradiation. At this time point, we normally only observe $Dll1^+$ cell-derived $LacZ^+$ Paneth cells at crypt bottoms. As demonstrated by sectioning (Fig. 4D), stem cell tracing events shown as ribbons of cells emanated from the crypt bottoms which ran up the side of adjacent villi (average of 96.1 stem cell derived tracings per duodenum per mouse)(Fig. 4E). These contiguous ribbons consisted of secretory as well as absorptive cells (i.e. enterocytes). These data unequivocally demonstrated that $Dll1^{high}$ precursor cells can revert to stem cells upon

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tissue damage. In our control, i.e. tamoxifen induction at day 0 without radiation, we observed occasional complete LacZ^{+} ribbons which shows that this phenomenon also occasional takes place in a normal intestine (average 9.8 stem cell derived tracings per duodenum per mouse, resp) (Fig. 4E). In our control, i.e. tamoxifen injection at day -14 followed by radiation on day 0, we observed similar number of tracings (average 8.4 stem cell derived tracings per duodenum per mouse) showing that this is a phenomenon which is not caused by Dll1 induction in the intestinal stem cells (Fig. 4E).

In this study, we have investigated *Dll1*-expressing crypt cells from the perspective of the stem cell hierarchy of the small intestinal epithelium. The cells expressing highest levels of *Dll1* typically reside one cell diameter above the top Paneth cell, a position we refer to as "+5". Lineage tracing using a CreERT2-expressing *Lgr5* allele demonstrates that Dll1^{high} cells are immediate descendants of Lgr5^{+} stem cells. Mice generated to carry a novel allele of *Dll1* (by insertion of a cassette containing GFP and CreERT2 into the first coding exon) allowed lineage tracing of Dll1^{high} cells, which were thus shown to generate small, short-lived clones that uniquely consist of cell types of the secretory lineage. Moreover, we found that the Dll1^{+} cells can revert to stem cells, *in vitro* when provided with exogenous Wnt signals, and *in vivo* upon tissue damage.

The following scenario can be scripted. In the stem cell zone, the Dll1^{+} Dll4^{+} Paneth cells trigger Notch1 and Notch2 on stem cells, thus keeping the stem cells from terminal differentiation into the secretory lineage *(9,17)*. Each day, each of the 15 stem cells divides and consequently 15 daughter cells exit the Paneth/stem cell zone. These daughters pass through the '+5' position, thereby losing direct access to Delta ligands. Stochastically, some of these cells lose Notch expression, and strongly up regulate Dll1 expression, thereby setting their own secretory fate. Simultaneously, such a cell can present Dll1 to 6-8 neighboring TA cells. These TA cells will thus maintain an active Notch pathway and will stay fated towards the enterocyte lineage. Thus, the enterocyte/secretory switch appears to be controlled through Notch by lateral inhibition. Many examples illustrate this classical mechanism, which typically operates to induce opposite cell fates within fields of initially identical cells *(23)*

In addition, we identified another characteristic of the Dll1^{+} cells. *In vitro* as well as *in vivo,* these early "fated" progenitors in the intestinal epithelium can revert to stem cells, presumably by regaining proximity to Paneth cells, the source of Wnt, Notch and EGF signals *(8)*. Although not probed here, it appears highly like that the bulk of the TA cells which are underway to become enterocytes rather than secretory cells, can similarly de-differentiate into stem cells. This process of dedifferentiation, or plasticity, is not uncommon. For instance in the Drosophila gonad, both female and male germ cells can regain stem cell identity after initiation of differentiation *(24-25)*. Thus, stemness in the intestine may be regarded as a cellular 'state' determined by location, rather than a cellular 'fate' determined by history. This plasticity may have implications for views on the occurrence and role of cancer stem cells, as have been described in intestinal cancer *(reviewed in 26,27)*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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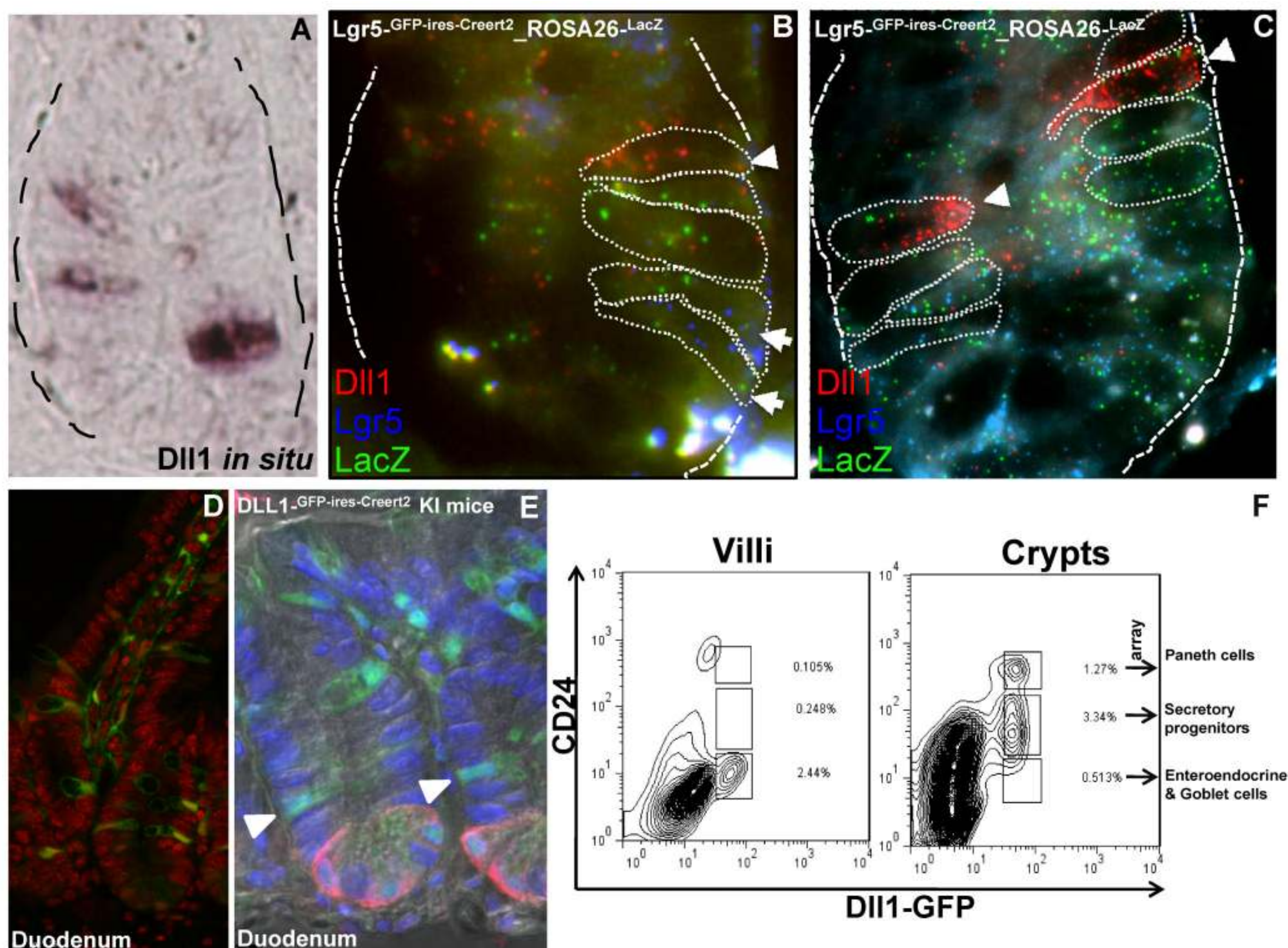

Fig. 1. Dll1 is expressed by rare undifferentiated TA cells that are early descendants of Lgr5 stem cells

(**A**) *In situ* hybridization performed on small intestine, suggesting the presence of rare *Dll1* mRNA expressing cells above the Paneth cell zone. (**B-C**) Three color single molecule FISH (LacZ – TMR (green), Dll1 – cy5 (red), and Lgr5 – Alexa594 (blue)) on the intestine of Lgr5$^{\text{GFP-ires-CreERT2}}$-R26R$^{\text{LacZ}}$ mice 1 day (**B**) and 2 days (**C**) after cre induction. On day 1, the Lgr5^{+} cells (arrows), but not the Dll1$^{\text{high}}$ precursor (arrow heads), expressed LacZ, while on day 2 also the Dll1$^{\text{high}}$ precursor expressed LacZ (arrow heads). Rarely did we see Dll1$^{\text{high}}$ precursors being LacZ^{+} on day 1. (**D**) Confocal GFP imaging of Dll1$^{\text{GFP-ires-CreERT2}}$ duodenum visualizes Dll1-GFP expressing cells. (**E**) Confocal GFP imaging of Dll1$^{\text{GFP-ires-CreERT2}}$ duodenum, counterstained for the Paneth cell marker lysozyme. GFP marked cells occur 1-2 cell diameters (arrowheads)(so-called '+5 position'), and higher, above the stem cell/Paneth cell zone. (**F**) Purified villi or crypts are dissociated into single cells, and analyzed for Dll1-GFP and CD24-PE by flow cytometry. The dot plots are gated on viable single cells using negative propidium iodide staining and Pulse width/FSC parameters. Discrete populations in Dll1$^{\text{GFP+}}$ cells are depicted. Subsequent microarray analysis revealed that Dll1$^{\text{GFP+}}$/CD24$^{\text{mid}}$, Dll1$^{\text{GFP+}}$/CD24$^{\text{high}}$ and Dll1$^{\text{GFP+}}$/CD24$^{\text{low}}$ cells are Paneth cells, secretory progenitors and goblet/enteroendocrine cells, respectively.

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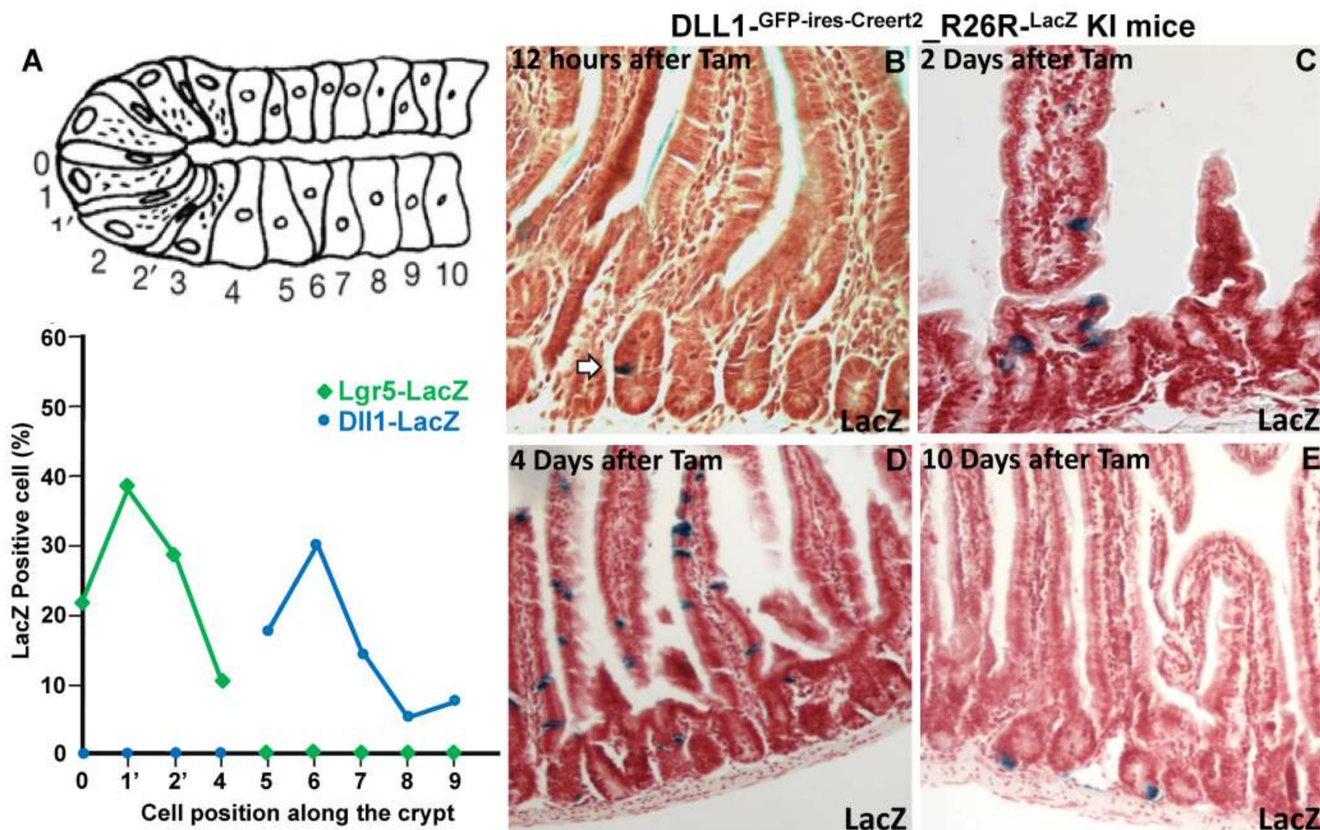

Fig. 2. Lineage tracing of Dll1$^{GFP\text{-}ires\text{-}CreERT2}$ × R26R^{LacZ} knock-in intestine

(A) Frequency at which LacZ cells first appeared at 12 hrs post-tamoxifen induction at specific positions relative to the crypt bottom (blue line). Quantitative data on the position of LacZ^{+} cells observed upon tamoxifen induction in Lgr5$^{GFP\text{-}ires\text{-}CreERT2}$ × R26R^{LacZ} mice are from (*1*) (green line). Histological analysis of R26R^{LacZ} activity in Dll1$^{GFP\text{-}ires\text{-}CreERT2}$ × R26R^{LacZ} on 12 hours(**B**), 2days (**C**), 4days (**D**) and 10days (**E**) post Cre-induction revealed that single marked cells grew into short-lived clones of scattered cells.

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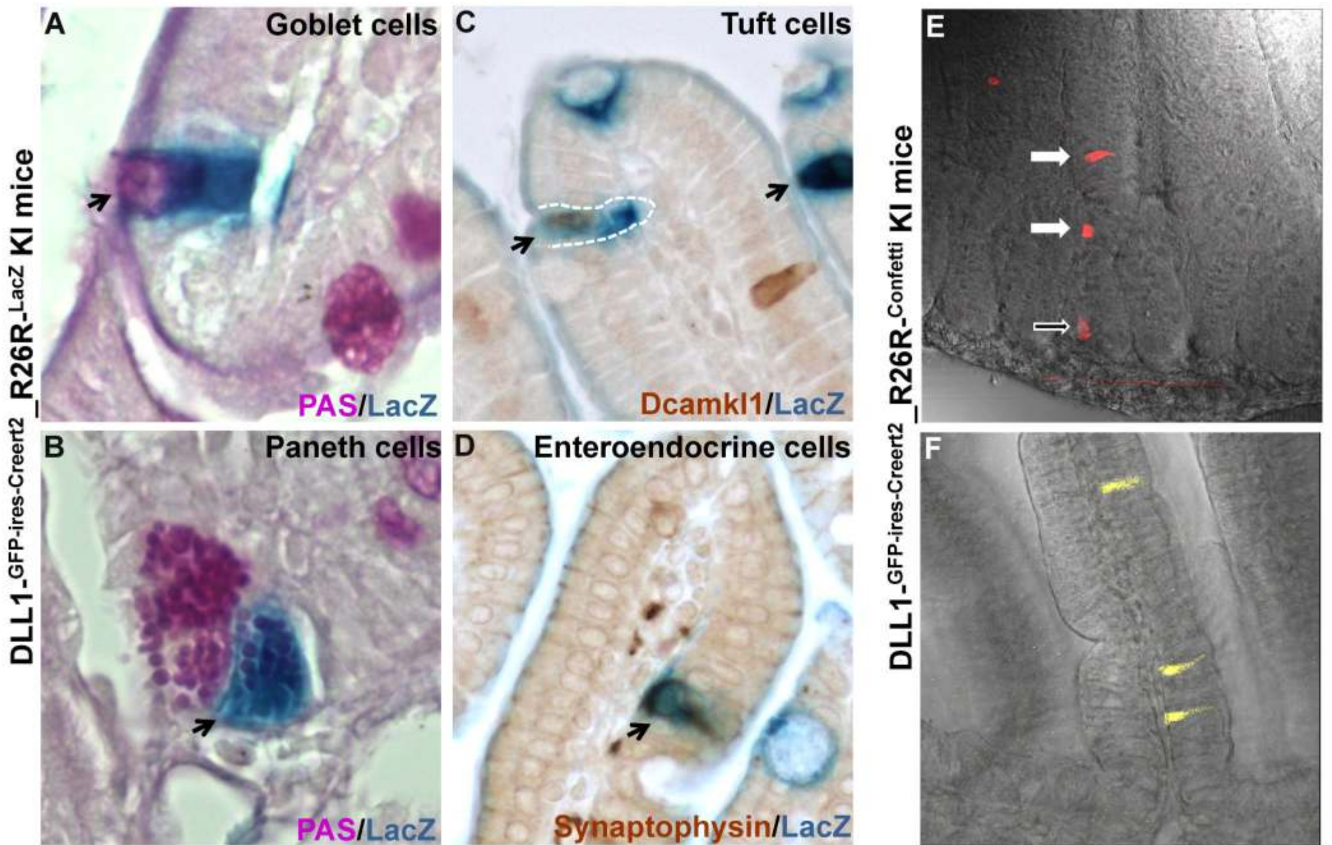

Fig. 3. Intestinal Dll1$^+$ cells are secretory lineage precursors

Double-labeling of LacZ-stained intestine with PAS demonstrates the presence of goblet cells (**A**, arrow) and Paneth cells (**B**, arrow) in induced LacZ$^+$ (blue) clones. Double-labeling with synaptophysin demonstrates the presence of enteroendocrine cells (**C**, arrow), while double-labeling with Dcamkl1 demonstrates the presence of Tuft cells (**D**, arrows) within the induced LacZ$^+$ clones. **E/F** Two examples of confocal analysis of Dll1$^{GFP\text{-}ires\text{-}CreERT2}$ × R26R^{confetti} reporter mice shows that a single Dll1$^+$ precursor cell gives rise to a Paneth (black arrow) cell and 2 goblet cells (white arrows) (**E**) or multiple goblet cells (**F**).

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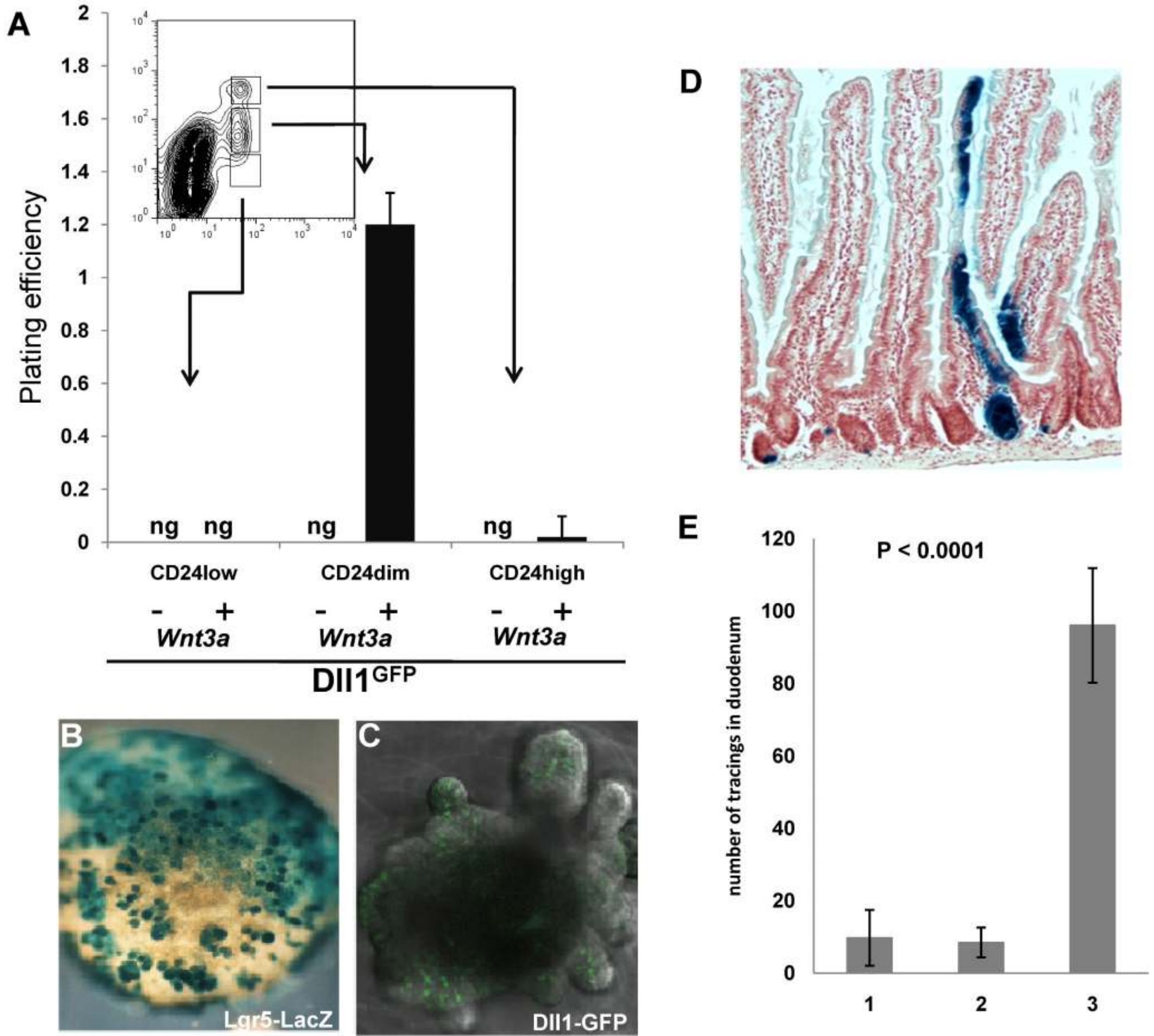

Fig. 4. Dll1 precursors can convert to intestinal stem cells

(A) *Dll1*$^{GFP\text{-}ires\text{-}CreERT2}$ crypts are dissociated into single cells. Single FACS purified cells (Dll1^{GFP+}/CD24^{low}, Dll1^{GFP+}/CD24^{mid} and Dll1^{GFP+}/CD24^{high} cells) are cultured +/− Wnt3A and the frequency of organoid formation is determined 10 days after culture. (**B-C**)**.** Organoids grown from single Dll1^{GFP+}/CD24^{mid} cells derived from *Dll1*$^{GFP\text{-}ires\text{-}CreERT2}$/*Lgr5*^{LacZ} small intestinal crypts, cultured in the presence of Wnt3A. The expression of Lgr5^{LacZ} is analyzed by X-gal staining 14 days after culture. LacZ expression is only seen at the bottom of budding structures. **(C)** The expression of Dll1^{GFP} staining of cultured organoids analyzed by confocal imaging. Dll1^{GFP} expression resembles *in vivo* presence of Dll1^{+} cells. Staining (**D**) and quantitative analysis (**E**) of LacZ activity in the duodenum of the Dll1$^{GFP\text{-}ires\text{-}CreERT2}$ × R26R^{LacZ} mice upon cre induction followed by radiation 1 day later (exp. group 3). This analysis reveals the formation of numerous LacZ^{+} stem cell ribbons (exp group 3, n = 96.1). Tamoxifen induction at day 0 without radiation (exp. group 1; n= 9.8) and tamoxifen injection at day −14 followed by radiation on day 0 (exp. group 2,

n=8.4), resulted in rare background LacZ$^+$ ribbons. The values are given as mean +/− standard error of the mean (s.e.m.), derived from the analyses of 8 different mice for each group.

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