Snai1 regulates cell lineage allocation and stem cell maintenance in the mouse intestinal epithelium

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Abstract

Snail family members regulate epithelial-to-mesenchymal transition (EMT) during invasion of intestinal tumours, but their role in normal intestinal homeostasis is unknown. Studies in breast and skin epithelia indicate that Snail proteins promote an undifferentiated state. Here, we demonstrate that conditional knockout of *Snai1* in the intestinal epithelium results in apoptotic loss of crypt base columnar stem cells and bias towards differentiation of secretory lineages. *In vitro* organoid cultures derived from *Snai1* conditional knockout mice also undergo apoptosis when *Snai1* is deleted. Conversely, ectopic expression of *Snai1* in the intestinal epithelium *in vivo* results in the expansion of the crypt base columnar cell pool and a decrease in secretory enteroendocrine and Paneth cells. Following conditional deletion of *Snai1*, the intestinal epithelium fails to produce a proliferative response following radiation-induced damage indicating a fundamental requirement for *Snai1* in epithelial regeneration. These results demonstrate that *Snai1* is required for regulation of lineage choice, maintenance of CBC stem cells and regeneration of the intestinal epithelium following damage.

Keywords apoptosis; intestinal stem cell; organoid; SerinC3; Snail

Subject Categories Development & Differentiation; Stem Cells; Transcription

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Introduction

Epithelial-to-mesenchymal transition (EMT) is a process whereby polarised epithelial cells undergo conversion to a more motile loosely attached state that is crucial for normal organogenesis, tumour progression and cellular dedifferentiation (De Craene & Berx, 2013). The molecules that initiate the EMT process may therefore be expected to play roles in stem cell biogenesis and maintenance. The Snail family of transcription factors have been characterised as master regulators of EMT with fundamental roles during normal embryogenesis (Barrallo-Gimeno & Nieto, 2005; Murray *et al*, 2007) in addition to promoting tumour invasion (Scheel & Weinberg, 2012; De Craene & Berx, 2013). Studies in cell lines suggest that elevated levels of Snail family proteins can also promote cells to adopt stem cell properties (Mani *et al*, 2008; Guo *et al*, 2012) and drive stem and progenitor cell expansion (Nassour *et al*, 2012; De Craene *et al*, 2014). There is also emerging evidence that Snail proteins are expressed in a variety of vertebrate stem cell populations (Perez-Losada *et al*, 2002; Horvay *et al*, 2011; Nassour *et al*, 2012) and may regulate stem cell activity.

The Snail family of proteins are highly conserved zinc-finger DNA binding proteins first identified in *Drosophila melanogaster* and conserved in many species including mouse and humans (Nieto, 2002) where there are three family members Snai1 (Snail), Snai2 (Slug) and Snai3 (Smuc) (Nieto, 2002). Redundant functional roles have been identified for both Snai1 and Snai2 where significant phenotypes have been identified in compound knockout mouse experiments that remove more than one family member (Murray *et al*, 2007; Pioli *et al*, 2013). In addition to roles in organogenesis, expression of Snail proteins has been associated with several epithelial tumour types, but little is known about the endogenous role of Snail proteins in regulating normal tissue homeostasis in organs. Upregulation of Snail family members has been identified in colorectal

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cancers (Roy *et al*, 2005), but evidence has not been presented demonstrating a role for Snai proteins in the normal intestine.

The mammalian intestinal epithelium is a dynamic tissue that is constantly renewed via a population of intestinal stem cells that also has the capacity to rapidly regenerate following damage (Van Landeghem *et al*, 2012; Clevers, 2013; Barker, 2014). Many years of research on the cellular composition, the recent identification of markers and development of genetic tools has made this tissue an ideal model for studying signals that regulate stem cell behaviour. Crypt base columnar (CBC) intestinal stem cells marked by *Lgr5* reside at the base of crypts where they are interspersed with Paneth cells (Barker *et al*, 2007). *Lgr5*-positive cells have been shown by lineage tracing experiments to reconstitute all cells in the epithelium (Barker *et al*, 2007), and clonal fate mapping strategies suggest this occurs by stochastic outcomes of symmetrical division (Snippert *et al*, 2010). Other markers and regulators of intestinal stem cells that have been described include *Olfm4* (van der Flier *et al*, 2009b), *Bmi1* (Sangiorgi & Capecchi, 2008; Yan *et al*, 2012), *Hopx* (Takeda *et al*, 2011) and *Lrig1* (Powell *et al*, 2012; Wong *et al*, 2012). Paneth cells are located in close proximity to CBC stem cells and supply niche signals including Wnt3, Noggin, Dll4 and EGF (Sato *et al*, 2011; Barker, 2014). A slow cycling reserve population of stem cells that reside just above the Paneth cells (+4 population) have been described that function to replenish the epithelium following injury (Takeda *et al*, 2011; Tian *et al*, 2011; Yan *et al*, 2012). Intestinal stem cells differentiate into a rapidly cycling population of transit amplifying cells that subsequently differentiate into secretory goblet, enteroendocrine, Paneth cells and absorptive enterocytes. Both Wnt and Notch signalling are essential for maintenance of intestinal stem cells in addition to having key roles in regulating cell fate (Pinto *et al*, 2003; VanDussen *et al*, 2012; Horvay & Abud, 2013).

We have recently determined that Snai1 is expressed in the intestinal epithelium where both expression levels and nuclear localisation are dependent upon Wnt signalling (Horvay *et al*, 2011). Snai1 is restricted to the nucleus in both CBC stem cells and transit amplifying progenitor cells within crypts which led us to hypothesise that Snai1, which is a well-known marker and mediator of mesenchymal identity, may have a role in regulating intestinal epithelial cell fate. Using a conditional knockout strategy, we show that deletion of *Snai1* results in the loss of CBC stem cells by apoptosis and an increase in secretory enteroendocrine and Paneth cell numbers. In contrast, elevating levels of Snai1 results in an increase in CBC cells and a decrease in secretory enteroendocrine cell numbers *in vivo*. Our data demonstrate that Snai1 is a key regulator of CBC intestinal stem cells with important roles in regulating intestinal cell survival, proliferation and fate.

Results

Snai1 is the predominant Snail family protein localised within the epithelial layer of the mouse intestine

Snail family members Snai1, Snai2 and Snai3 have been shown to have significant functional redundancy and are found primarily within mesenchymal tissues (Murray *et al*, 2007; Chiang & Ayyanathan, 2013). We therefore examined the expression pattern of all three family members within the mouse intestine. Initially, we

examined the expression of *Snai1*, *Snai2* and *Snai3* using qRT-PCR throughout embryogenesis and postnatal stages (Fig 1A and B) to obtain information regarding which family members were present at what stages in the mouse small intestine and colon. Our analysis revealed expression of both *Snai1* and *Snai2* (Fig 1A and B and Supplementary Fig S1A), but negligible amounts of *Snai3* were present at all stages examined. Further examination of the expression of *Snai2* using LacZ reporter mice (Jiang *et al*, 1998) clearly showed restriction of *Snai2* to the mesenchymal tissue layer at all stages examined (Fig 1 and Supplementary Fig S1). This concurs with studies throughout embryogenesis where *Snai2* is largely restricted to the mesenchyme (Jiang *et al*, 1998; Oram *et al*, 2003). Expression levels were highest throughout embryogenesis and postnatal stages (Fig 1D, F, H and J); however, *Snai2* expression was still detected in adult small intestine (Fig 1K) and adult colon (Supplementary Fig S1D) where a proximal to distal gradient of expression was apparent (Supplementary Fig S1E). Immunohistochemistry using an antibody that detects both Snai1 and Snai2 revealed expression in both the epithelium and mesenchyme (Fig 1C, E, G and I, and Supplementary Fig S1B and C) (Horvay *et al*, 2011). As *Snai2* is not present in the epithelium, signal detected is due to expression of *Snai1*. During embryogenesis, *Snai1* is expressed throughout the epithelium becoming increasingly concentrated between intervillus regions at postnatal stages (Fig 1G). In adult epithelium, strong nuclear localisation is observed in proliferating progenitor and *Lgr5*-positive crypt base columnar stem cells as revealed by co-immunofluorescence staining for *Snai1* and *Lgr5-GFP*-positive CBC cells in crypts from *Lgr5-GFP* mice (Fig 1L–N) (Horvay *et al*, 2011). Expression of *Snai1* in the epithelium was confirmed with an independent Snai1 antibody (Fig 1O and P), and specificity was confirmed by Western blot (Supplementary Fig S1F) and immunohistochemistry on knockout tissue (Fig 3B). This confirmed minimal expression of Snai1 in villi (Fig 1O) and higher expression in crypts (Fig 1P). These data suggest that Snai1 is the only member of the family that is expressed in the epithelial cell layer with the most pronounced nuclear localisation detected in CBC stem cells.

Snai1 regulates lineage allocation in the intestinal epithelium

As *Snai1*^{−/−} animals die early during embryogenesis from gastrulation defects (Carver *et al*, 2001), we undertook a conditional approach to specifically delete *Snai1* from the intestinal epithelium. This was achieved by crossing *Snai1*^{fl/del} (Murray *et al*, 2007) animals with *AhCre* mice in which Cre-recombinase expression, directed by the inducible *CYP1A1* promoter, becomes transcriptionally activated in all cells in intestinal crypts except Paneth cells, following administration of β -naphthoflavone (BNF) over a 4-day regime (Ireland *et al*, 2004) (Fig 2A). We analysed tissue from adult *AhCre Snai1*^{fl/del} mice and *AhCre Snai1*^{+/+} control mice 5 days postinduction as this time point has been demonstrated to achieve efficient gene deletion with floxed alleles (Ireland *et al*, 2004; van der Flier *et al*, 2009b). Specific antibodies and histochemical stains were used to examine the differentiated secretory cell populations present in the small intestine. PAS staining was used to detect and quantify the number of goblet cells per crypt–villus axis (Fig 2B). No difference in goblet cell numbers between control and test animals was observed. Next, we examined enteroendocrine cells

Figure 1. Snai1 is localised to stem and progenitor cells within mouse intestinal crypts, while Snai2 is restricted to the mesenchyme.

A, B Temporal expression of *Snail* genes in the intestinal tract in (A) embryonic E12.5 to E18.5 and (B) postnatal P0 to P20 measured by qRT-PCR. Shown are means $\pm$ SD.

C, E, G, I Immunohistochemical staining for Snai1/2 (Abcam ab85931) (C) E14.5 midgut, (E) E14.5 hind gut, (G) postnatal day 14 SI, (I) postnatal day 14 colon. Snai1 immunohistochemistry peptide competition controls are shown as inserts (C', E', G', I'). Scale bars: 20 mm.

D, F, H, J, K X-gal staining for β -galactosidase activity in tissue from *Snai2LacZ* mice stained in whole mount, sectioned and counterstained with nuclear fast red (D) E14.5 midgut, (F) E14.5 hind gut, (H) postnatal day 14 SI, (J) postnatal day 14 colon, (K) adult SI. Controls for X-gal staining are shown as inserts (D', F', H', J').

L, M, N Double immunostaining of small intestinal crypts for Snai1/2 (red) and (M) *Lgr5-GFP* (green); (N) merge shows Snai1 is present in both CBC stem cells and proliferating transit amplifying cells. Scale bars: 10 mm.

O, P Immunohistochemistry for *Snai1* using a different primary antibody (Genetex GTX125918) shows minimal expression of Snai1 in villi. (P) Expression of Snai1 is detected at the base of crypts (P) with strong nuclear expression in CBC stem cells indicated by arrows. Scale bars: 20 mm (O), 10 mm (P).

Q Schematic diagram showing Snai1 expression (red) within different cell types in the crypt. SI = small intestine. See also Supplementary Fig S1.

Figure 2. Loss of *Snai1* predisposes differentiation towards secretory enteroendocrine and Paneth cell fate in small intestinal epithelium.

A To analyse the effect of *Snai1* knockout in the mouse small intestine, *AhCre Snai1^{+/+}* (control) and *AhCre Snai1^{fl/del}* (test) adult mice were injected with β -NF on four consecutive days to induce Cre-mediated recombination. Tissue was harvested for analysis on day 5.

B Periodic Acid Schiff (PAS) staining was used to detect goblet cells in sections of small intestine from control and *Snai1* KO mice. Graph shows number of goblet cells per crypt–villus axes ($n = 3$).

C Immunohistochemistry for synaptophysin was used to detect enteroendocrine cells (marked by arrows). Quantification of enteroendocrine cells per crypt–villus axes in control and *Snai1* KO tissue revealed a significant increase in the number of enteroendocrine cells following *Snai1* loss ($n = 3$, $P = 0.004$).

D Immunohistochemistry for lysozyme was used to detect Paneth cells. Quantification of Paneth cells in control and *Snai1* KO indicates a significant increase in Paneth cell numbers following knockout of *Snai1* ($n = 4$, $P = 0.002$).

E Measurement of villus length (μ m) ($n = 3$, $P = 0.013$) and quantification of enterocytes per crypt–villus unit demonstrate a significant reduction in absorptive enterocytes following *Snai1* loss ($n = 3$, $P = 0.036$).

F Measurement of villus length (μ m) ($n = 3$, $P = 0.003$) 8 days after β -NF induction demonstrates a significant shortening of villi. Bars represent mean $\pm$ SD. Two-tailed Student's t -test was used to assess significance. Scale bars: 20 μ m.

Source data are available online for this figure.

within the intestinal epithelium using immunohistochemistry for synaptophysin (arrows in Fig 2C). An approximate doubling in enteroendocrine cell numbers was observed in *Snai1* knockout tissue ($P = 0.004$). Paneth cells which reside at the base of crypts were detected using anti-lysozyme (Fig 2D). A significant increase in the number of Paneth cells per crypt was detected in knockout animals ($P = 0.002$), but cells were still restricted to the base of crypts (Fig 2D). In order to assess the overall effect on the differentiated compartment of the small intestinal epithelium, villus length was measured and was found to be significantly shorter (Fig 2E) in *Snai1* knockout tissue ($P = 0.013$). To assess whether this was due to a reduction in cell size or a decrease in absorptive enterocyte cell numbers, the number of enterocytes per crypt/villus unit was quantified (Fig 2E) and was significantly reduced ($P = 0.036$). Villus shortening was also observed in tissue from animals 8 days following induction ($P = 0.003$) (Fig 2F). In summary, intestinal *Snai1* knockout mice have an increase in secretory Paneth and enteroendocrine cell numbers accompanied by a decrease in absorptive enterocytes.

Loss of *Snai1* in intestinal epithelium results in reduced cell proliferation and CBC stem cell loss

As *Snai1* family proteins have been implicated in maintaining stem cell populations, we examined the crypt compartment in more detail to determine whether there were any effects on stem and transit amplifying populations. Previously, we have shown that these cells contain the highest levels of nuclear localised *Snai1* in the crypt–villus axis (Horvay et al, 2011). Sections were stained with PCNA to detect proliferating cells. A small but significant decrease in proliferating cells was detected in *Snai1* knockout crypts (Fig 3A) ($P = 0.049$). On closer inspection, PCNA-positive cells (indicated by arrows in Fig 3A) that could be detected between differentiated Paneth cells at the base of crypts in controls were notably reduced in *Snai1* knockout animals. Quantification of proliferating cells at the very base of crypts confirmed that the overall decrease was due to a reduction of cells in this region (Fig 3A) ($P = 0.026$). Quantification of proliferating transit amplifying cells revealed no change in this population (Supplementary Fig S2A).

The loss of PCNA-positive cells at the crypt base suggested that deletion of *Snai1* may affect the CBC stem cell population. To examine this, we looked at the expression of *Lgr5*, a key Wnt-regulated marker of CBC ISCs (Barker et al, 2007), by *in situ* hybridisation (Fig 3B). A conspicuous lack of *Lgr5* expression was detected compared to control tissue (Fig 3B). Therefore, we examined the expression of *Olfm4* which also marks CBC ISCs and is downstream of Notch signalling (van der Flier et al, 2009a,b; VanDussen et al, 2012) and hence would not be predicted to be downregulated by a mutation that specifically affected the output of the Wnt pathway. A lack of *Olfm4* expression in knockout tissue was also apparent while detection of *Cryptin* (used as an *in situ* control for tissue integrity) in adjacent Paneth cells at the bottom of crypts was present in both knockout and control tissue (Supplementary Fig S2B). These results suggest CBC stem cells are lost following deletion of *Snai1* in intestinal crypts. Loss of *Snai1* at day 5 in knockout tissue was verified by immunohistochemistry using a *Snai1* antibody (Fig 3B). To confirm and quantify loss of the CBC stem cell population, we crossed *AhCre Snai1^{fl/fl}* mice to

Lgr5-eGFP mice where CBC cells are marked by GFP expression (Barker et al, 2007) and analysed epithelial preparations by fluorescence-activated cell sorting (FACS). This revealed a significant (~10 times) decrease in the *Lgr5*-positive CBC stem cell population in *Snai1* knockout mice (Fig 3C).

A loss of CBC stem cells could be due to either apoptosis of the stem cell population or induction of stem cell differentiation. Firstly, we examined the pattern of apoptosis within crypts and villi. Activated caspase-3-positive cells are rarely detected in normal adult intestinal epithelium; however, we detected a significant increase in caspase-3-positive cells in both the crypt ($P = 0.0001$) and villus ($P = 0.005$) regions of *Snai1* knockout mice suggesting that the loss of proliferating cells at the base of crypts and decrease in enterocytes in the villus compartment may be explained by induction of apoptosis (Fig 3D).

An alternative explanation for the disappearance of the CBC stem cells may be premature differentiation. To further examine the fate of mutant CBC stem cells, we performed lineage tracing of *Snai1^{-/-}* CBC cells. *Lgr5-CreERT2-GFP* mice (Barker et al, 2007) were crossed with *Rosa26 lacZ* reporter (R26R) mice (Soriano, 1999) and *Snai1^{fl/fl}* mice. *Lgr5-CreERT2-GFP* mice have mosaic expression of CreERT2 and eGFP in CBC stem cells (Barker et al, 2007). Injection of tamoxifen induces recombination in a subset of CBC cells and subsequent expression of *LacZ*. The descendants of CBC cells are also *LacZ* positive and can be traced temporally to follow their fate (Barker et al, 2007). We examined the fate of *Snai1^{-/-}* CBC cells using this strategy. In control mice (*Lgr5-CreERT2-GFP*, R26R, *Snai1^{+/+}*), tamoxifen induction produced *LacZ*-positive progeny in a proportion of crypt–villus units (shown in whole mount (Fig 3E) and in section (Supplementary Fig S2C)). In contrast, lineage tracing analysis in *Lgr5-CreERT2-GFP*, R26R, *Snai1^{fl/fl}* mice revealed that *LacZ*-positive cells were very rarely detected beyond the first day of induction (Fig 3E, Supplementary Fig S2C). If the *Snai1* mutant CBC cells changed fate and differentiated to another cell type, we would expect these progeny to be marked by *LacZ*. This is observed with stem to transit amplifying cell conversion of *Grp78* mutant cells where CBC ISCs relocate up the crypt and adopt a transit amplifying cell fate within 48 h after deletion of *Grp78* (Heijmans et al, 2013). As this was not observed, our results are consistent with *Snai1^{-/-}* CBC cells being lost by apoptosis.

Snai1 is critical for establishment and survival of *in vitro* mouse small intestinal organoids

The discovery of conditions required to establish organoid cultures from isolated crypts or single ISCs that recapitulate the cellular composition of crypts provides an opportunity to assess stem cell function in an *in vitro* system (Sato et al, 2009) and confirm an endogenous requirement for *Snai1* for survival of cells in the epithelium independent of the niche. We attempted to establish organoids from *AhCre Snai1^{fl/del}* mice 24 h post-BNF induction. In contrast to *AhCre Snai1^{+/+}* controls, organoid colonies from *Snai1^{-/-}* tissue are not viable. This is most likely due to loss of functional stem cells required to initiate cultures. Organoid cultures from *AhCre Snai1^{fl/fl}* mice were generated to enable inducible deletion of *Snai1* with BNF following establishment of cultures. Cultures were also generated from *VillinCreERT2 Snai1^{fl/fl}* mice and recombination induced in these cultures by addition of tamoxifen.

Figure 3. Conditional knockout of *Snai1* induces loss of CBC stem cells.

- A** Sections from control and *Snai1* KO small intestine were stained for PCNA to detect proliferating cells (arrows depicting proliferating CBC stem cells at the base of crypts). Quantification of total PCNA-positive cells per crypt and PCNA-positive cells in the base of crypts both revealed a significant reduction in the number of proliferating cells after conditional depletion of *Snai1* ($n = 4$, $P = 0.049$, 0.026). Scale bars: 20 μ m.
- B** The expression of 2 CBC stem cell markers *Olfm4* and *Lgr5* were examined by *in situ* hybridisation in control and *Snai1* KO small intestine. *Lgr5* and *Olfm4* *in situ* hybridisation staining shows a loss of CBC stem cell marker expression after loss of *Snai1* (high magnification inserts depict a representative crypt demarcated by a dotted line). Loss of *Snai1* was confirmed in knockout tissue using immunohistochemistry. Scale bars: 20 μ m.
- C** The analysis of sorted and EGFP-positive cells from *Lgr5*-EGFP-IRES-creERT2 AhCre *Snai1*^{fl/fl} and *Lgr5*-EGFP-IRES-creERT2 AhCre *Snai1*^{+/+} mice 5 days after β NF induction show an ~tenfold reduction in *Lgr5* GFP-positive CBC stem cells.
- D** Immunohistochemistry for active caspase-3 to detect apoptotic cells revealed a significant increase in the number of apoptotic cells in both crypt ($n = 5$, $P = 0.0001$) and villus ($n = 5$, $P = 0.005$) regions in *Snai1*KO tissue (arrows mark active caspase-3-positive cells). Scale bars: 20 μ m.
- E** A lineage tracing strategy was employed to determine the fate of CBC cells that lack *Snai1*. Tissue from *Lgr5*-EGFP-IRES-creERT2 *Snai1*^{+/+} (control) and *Lgr5*-EGFP-IRES-creERT2 *Snai1*^{fl/fl} (*Snai1*KO) that also contained the *ROSA26*LacZ Cre reporter allele was stained with X-Gal 5 days after tamoxifen injection. Quantification of X-Gal-positive foci per cm shows *Snai1* knockout tissue failed to lineage trace indicating that no progeny are produced from *Snai1* knockout cells. Bars represent mean $\pm$ SD ($n = 3$, $P = 0.0001$). Scale bars: 0.50 mm. Two-tailed Student's *t*-test was used to assess significance. See also Supplementary Figure S2.

Source data are available online for this figure.

Successful recombination using these approaches was confirmed by treating organoids containing the *Cre* alleles and a *ROSA26LacZ* *Cre* reporter allele and staining for β -galactosidase activity (Supplementary Fig S3A). The majority of cells within organoids stained blue 24 h after drug treatment indicating that most of the cells had undergone recombination. As the organoids grow in size, they develop a unique structure comprised of buds with *Lgr5*-expressing stem cells present in the bud tips (which could be considered to be inverted crypts). Hence, the number of buds present in an organoid indicates the number of crypt domains present containing stem cells. While control organoids expanded over 4 days in culture and developed many crypt buds, *VillinCreERT2 Snai1^{fl/fl}* organoids treated with tamoxifen failed to grow and regressed with many cells dying compared to controls (Fig 4A and B). When these organoids were reseeded, they also failed to establish new organoids indicating a loss of self-renewal capacity. Similar results were obtained with *AhCre Snai1^{fl/del}* organoids (Supplementary Fig S3B and C). The number of buds per organoid was significantly reduced in knockout organoids, with ~35% exhibiting no buds at all compared to 15% observed in controls (Supplementary Fig S3D) ($n = 3$, $P = 0.002$). The levels of apoptosis in *Snai1*-depleted organoids were examined. In control organoids, a few apoptotic cells are observed in the lumen of organoids as previously reported (Sato *et al*, 2009). In contrast, *Snai1* knockout organoids contained significant apoptosis within the epithelial cell layer that was often localised to buds. When quantified, a highly significant increase in the number of apoptotic organoids was observed ($P = 0.00001$) (Fig 4C). This supports the observations made in animals following *Snai1* deletion where a significant increase in apoptosis is observed in crypts. Collectively, these data indicate that loss of *Snai1* affects the maintenance and function of ISCs.

Depletion of *Snai1* impairs regeneration of the intestinal epithelium following damage induced by radiation

We investigated the phenotype in the intestinal epithelium of *AhCre Snai1^{fl/del}* and *VillinCreERT2 Snai1^{fl/fl}* at longer time points following induction and discovered that the epithelium appeared relatively normal (Fig 5A) 60 days after induction of recombination. Upon closer inspection, it was clear that although the crypts showed a loss of *Snai1* expression 5 days after induction, the epithelium was rapidly replenished from un-recombined cells that were wild-type for *Snai1* (Fig 5A). This phenomenon has been observed with other key regulators of intestinal stem cells including *Ascl2* (van der Flier *et al*, 2009b), *c-myc* (Muncan *et al*, 2006) and *Brg1* (Holik *et al*, 2013) and demonstrates that there is considerable selective pressure to maintain *Snai1* expression in intestinal crypts.

To assess the functional role of *Snai1* in intestinal regeneration, we combined depletion of *Snai1* with exposure to radiation (Fig 5B). *VillinCreERT2 Snai1^{+/+}* (control) and *VillinCreERT2 Snai1^{fl/fl}* (test) mice were irradiated on day 1 and injected with tamoxifen on four consecutive days to induce Cre-mediated recombination. Tissue was harvested on day 5 following injection of BrdU. Control littermates produced the expected proliferative regenerative response (Fig 5C). In contrast, tissue deleted for *Snai1* had a significantly reduced level of BrdU-positive cells (Fig 5C) ($P = 0.00019$). This indicates that *Snai1* is required for robust regeneration of the

epithelium following damage which has also been observed following depletion of *Lgr5*-positive stem cells (Metcalf *et al*, 2014). This is therefore consistent with a key role for *Snai1* in maintaining *Lgr5*-positive CBC cells.

Elevation of *Snai1* expression expands the CBC cell pool and induces an opposite differentiation bias to *Snai1* loss

As our results indicated that *Snai1* has a key role in maintaining the CBC stem cells and *Snai1* protein levels have been shown to be upregulated in epithelial tumours, we decided to investigate the effect of elevating *Snai1* levels on the stem cell population. Mice expressing *Snai1* in the intestine were generated by crossing *AhCre* or *VillinCreERT2* mice to *ROSA^{Snai1}* mice where *Snai1* cDNA is inserted into the *ROSA26* locus and expression is dependent on Cre-mediated excision of a floxed β -geo cassette (Nyabi *et al*, 2009). We analysed tissue from adult *AhCre ROSA26^{Snai1}* mice and *AhCre* control mice 5 days postinduction using the same methodology we employed for the knockout studies. PAS staining was used to identify goblet cells, and immunohistochemistry for synaptophysin and lysozyme was utilised to detect enteroendocrine and Paneth cells, respectively. Analogous to *Snai1* knockout tissue, there was no significant difference in the number of goblet cells detected when *Snai1* levels were elevated (Fig 6A). Interestingly, as observed in *Snai1^{-/-}* tissue, there was a significant difference in both enteroendocrine ($P = 0.003$) and Paneth cell ($P = 0.027$) numbers, but the effect was converse in nature. Instead of an increase in numbers of these two cell types as observed in the knockout, there was a significant decrease in enteroendocrine and Paneth cells in tissue where *Snai1* was over-expressed (Fig 6B and C). This suggests that a critical threshold level of *Snai1* expression is required to maintain the correct balance of cell lineage differentiation. Levels of cell proliferation were also examined in the *AhCre ROSA26^{Snai1}* transgenic animals. A significant increase in crypt cell proliferation was also detected (Fig 6D) ($P = 0.009$). We decided to confirm these observations using *VillinCreERT2 ROSA26^{Snai1}* mice. The phenotype observed in these animals was similar to those generated with the *AhCre* driver. Elevated levels of proliferating PCNA-positive CBC cells were observed at the base of crypts (Fig 6E and F). We examined the expression of several known stem cell markers by qRT-PCR in these mice (Fig 6G). Significant increases in the expression of CBC stem cell markers *Lgr5* ($P = 0.013$) and *Olfm4* ($P = 0.013$) were detected, but *Bmi1* levels were unchanged. Levels of *Cdh1*, a well-characterised target that is directly repressed by *Snai1* (Battle *et al*, 2000; Cano *et al*, 2000), were downregulated as expected ($P = 0.003$). Wild-type CBC stem cells express *Cdh1*/E-cadherin (Snippert *et al*, 2010) suggesting that endogenous levels of *Snai1* are not sufficient to suppress *Cdh1* expression and are counterbalanced by transcriptional activators. It may only be when *Snai1* levels are increased to well above wild-type levels that we observe a decrease in levels of *Cdh1* transcription. CBC stem cells were also quantified using immunohistochemistry for CD44v6 which marks intestinal stem cells (Zeilstra *et al*, 2013), and a significant increase in the number of CD44v6-positive cells localised between Paneth cells at the base of crypts was observed in *AhCre ROSA26^{Snai1}* ($P = 0.00023$) mice compared to controls (Fig 6H).

Figure 4. Snai1 function is essential for *in vitro* organoid formation and survival.

A *VillinCreERT2 Snai1^{fl/fl}* organoids treated with tamoxifen show arrested growth after 2 and 4 days of treatment compared to controls. Scale bars: 50 μ m.

B Quantification of viable cell growth in *VillinCreERT2 Snai1^{fl/fl}* organoids treated with tamoxifen compared to controls over 4 days. Bars represent mean $\pm$ SD. $n = 3$ experiments per group, $**P = 0.002$.

C Assessment of apoptosis in control and *VillinCreERT2 Snai1^{fl/fl}* organoids after 4 days of growth. Photographs show an overlay of bright-field and fluorescent images with propidium iodide and Hoechst staining. Quantification of apoptotic organoids. Bars represent mean $\pm$ SD. $n = 3$ experiments per group, $**P = 0.00001$. Two-tailed Student's *t*-test was used to assess significance. Scale bars: 50 μ m. See also Supplementary Figure S3.

Growth of organoids that reveals regulation of Snai1 levels is required for the production of Paneth cell-derived Wnt3a

To further examine the phenotypic effects of elevation of Snai1 levels, we established organoid cultures from *VillinCreERT2*

ROSA26Snai1 mice and littermate controls (Fig 7). Following induction with tamoxifen, organoid cultures with elevated Snai1 levels failed to grow (Fig 7A and B) ($P = 0.002$) and displayed a significantly higher level of apoptosis compared to controls (Fig 7C and D) ($P = 0.00003$). This phenotype was different to what we detected

Figure 5. Snai1 function is essential for *in vivo* intestinal regeneration after irradiation treatment.

A The consequences of *Snai1* knockout were analysed over a time course. Immunohistochemistry for Snai1 revealed significant loss of expression at day 5 following induction compared to control. The epithelium is re-populated by wild-type cells as shown on day 60. Scale bars: 20 μ m.

B To analyse the effect of *Snai1* knockout after radiation treatment in the mouse small intestine, *VillinCreERT2 Snai1^{+/+}* (control) and *VillinCreERT2 Snai1^{fl/fl}* (test) adult mice were irradiated on day 1 and injected with tamoxifen on 4 consecutive days to induce Cre-mediated recombination. Tissue was harvested for analysis on day 5.

C Sections from irradiated control and *Snai1* KO small intestine were stained for BrdU to detect proliferating cells. Quantification of BrdU-positive cells per crypt and total number of cells per crypt revealed a significant reduction in both number of proliferating cells and total cells after irradiation and the conditional depletion of *Snai1* ($n = 4$, $P = 0.00019$, 0.014). Bars represent mean $\pm$ SD. Two-tailed Student's *t*-test was used to assess significance. Scale bars: 20 μ m.

Source data are available online for this figure.

in vivo, but we postulated that this may be due to the reduction in Paneth cell numbers that we had observed in our initial analyses of tissue where Snai1 levels were elevated (Fig 6C). Knockout of *Math1* results in depletion of Paneth cells *in vivo* without a significant effect on integrity of the epithelium, but organoids derived from this tissue do not grow unless supplemented with the Paneth cell-secreted growth factor Wnt3a (Durand *et al*, 2012). Similarly, knockout of Wnt3a has little effect on epithelial homeostasis *in vivo*, but organoids derived from mice will not grow unless Wnt3a is added to the medium (Farin *et al*, 2012). This is probably due to compensatory mechanisms *in vivo* which supply essential Wnt signals from other cells. We investigated whether the lack of growth of organoids we observed was due to a reduction in Wnt3a normally secreted from Paneth cells (Fig 7D). Snai1-depleted organoids grown in standard organoid growth conditions showed a significant increase in apoptosis. Addition of Wnt3a to the organoids rescued the apoptotic phenotype (Fig 7D). This supports the hypothesis that

organoids from mice with elevated levels of Snai1 do not survive due to a reduction in Paneth cell numbers and Wnt3a.

Serinc3 is a putative target of Snai1 in intestinal cells

Snail proteins have been shown to directly repress members of the apoptosis inducing BH3-only family of proteins (Wu *et al*, 2005; Franco *et al*, 2010). We used droplet digital PCR (Hindson *et al*, 2013) to analyse expression of *Bbc/PUMA*, *BMF* and *Bcl2f1/BIM* in *Villin-CreERT2* and *Villin-CreERT2 Snai1^{fl/fl}* organoids as all of these genes have previously been shown to be Snail family regulated (Wu *et al*, 2005; Franco *et al*, 2010). We observed no differential expression of any of these genes (Fig 8). To investigate the potential mechanism of *Snai1*-mediated maintenance of the small intestinal CBC ISC population, we performed genome-wide gene expression analysis immediately following *Snai1* loss. We compared *Villin-CreERT2* organoids to *Villin-CreERT2*

Figure 6. Elevated levels of Snai1 result in reduction of secretory enteroendocrine and Paneth cells and an increase of CBC intestinal stem cells.

The effect of elevated levels of *Snai1* was examined using a conditional transgenic mouse model (*Rosa26Snai1*) that permits expression of *Snai1* when exposed to Cre recombinase. *AhCre* (control) and *AhCre Rosa26Snai1* (RS) mice were injected with β -NF for 4 days and tissue harvested for analysis on day 5.

- A** PAS staining was used to detect goblet cells in the small intestine, and no differences in cell numbers were observed in test versus control mice ($n = 4$, NS).
- B** Immunohistochemistry for synaptophysin was used to detect enteroendocrine cells. Quantification of the number of enteroendocrine cells per crypt–villus axes revealed a significant decrease in enteroendocrine cell numbers when *Snai1* levels are elevated ($n = 4$, $P = 0.003$).
- C** Immunohistochemistry for lysozyme was used to detect Paneth cells. Quantification of Paneth cell numbers indicated a significant decrease in Paneth cells in the base of crypts in mice with elevated *Snai1* levels ($n = 5$, $P = 0.027$).
- D, E** Quantification of total PCNA-positive cells per crypt revealed a significant increase when *Snai1* levels were elevated ($n = 4$, $P = 0.009$). This was confirmed in *VillinCreERT2 ROSA26Snai1* mice 5 days after induction with tamoxifen where analysis of (E) PCNA-positive cells in the base of crypts revealed a significant increase in proliferating cells in crypts with elevated *Snai1* levels compared to control tissue ($n = 4$, $P = 0.0005$). Bars represent mean $\pm$ SD.
- F** PCNA immunostaining (green) and DAPI staining (blue) of *VillinCreERT2 ROSA26Snai1* compared to control *VillinCreERT2* littermates. An expansion of proliferating CBC cells (indicated by arrows) is observed at the base of crypts. Scale bars: 10 μ m.
- G** qRT–PCR analysis of stem cell marker expression in the small intestine from *VillinCreERT2 ROSA26Snai1* compared to control *VillinCreERT2* littermates. Bars represent mean $\pm$ SD, $n = 3$ experiments per group, *Olfm4* $P = 0.013$, *Cdh1/E-cadherin* $P = 0.003$, *Lgr5* $P = 0.013$.
- H** CD44v6 immunostaining of tissue from *AhCre* control compared to *AhCre RosaSnai1* tissue. A significant increase in CD44v6-positive cells localised at the base of crypts (depicted by arrows) was observed. Bars represent mean $\pm$ SD, $n = 3$ mice per group, $*P = 0.00023$. Two-tailed Student's *t*-test was used to assess significance. Scale bars: 10 μ m.

Source data are available online for this figure.

Figure 7. Elevated levels of Snai1 in *in vitro* organoids result in reduction of organoid growth and increase in cell death.

A *VillinCreERT2 RosaSnai1* organoids treated with tamoxifen show slower growth after 2 and 4 days of treatment compared to controls. Scale bars: 50 μ m.

B Quantification of viable cells in *VillinCreERT2 RosaSnai1* organoids treated with tamoxifen compared to control growth over 4 days. Bars represent mean $\pm$ SD. $n = 3$ experiments per group, $^{**}P = 0.002$.

C Assessment of apoptosis in control and *VillinCreERT2 RosaSnai1* organoids after 4 days of growth. Photographs show an overlay of bright-field, propidium iodide and Hoechst fluorescence. Scale bars: 50 μ m.

D Quantification of apoptotic organoids with and without Wnt3A treatment shown as mean $\pm$ SD. $n = 3$ experiments per group, $^{**}P = 0.00003$. Two-tailed Student's *t*-test was used to assess significance.

Figure 8. Snai1 does not regulate BH3-only protein expression in the intestinal epithelium but regulates *Serinc3* expression.

A–C Droplet digital PCR analysis of *Bmf*, *Bbc3/Puma* and *Bcl2l 11/Bim* after conditional *Snai1* loss in *in vitro* organoid culture. *VillinCreERT2 Snai1^{+/+}* (control) and *VillinCreERT2 Snai1^{fl/fl}* SI organoids were treated for 24 h with tamoxifen. No differences in gene expression were observed ($n = 3$).
D qRT–PCR analysis of *Serinc3* downregulation after conditional *Snai1* loss in *in vitro* organoid culture. *VillinCreERT2 Snai1^{+/+}* (control) and *VillinCreERT2 Snai1^{fl/fl}* SI organoids were treated for 24 ($P = 0.0001$) and 72 h ($P = 0.006$) with tamoxifen. Bars represent mean $\pm$ SD. $n = 3$ mice per group.
E SW480 cells, which transiently express GFP–SNAI1, were used to detect direct binding of SNAI1 to the promoters of known target genes *CDH1*, *IL8* and a novel target *SERINC3* revealed by microarray analysis. An unrelated DNA region was used as a negative control. Anti-GFP antibody was used to immunoprecipitate GFP-tagged SNAI1, while IgG control antibody was used as a negative control. The results are given as relative enrichment compared to input material and are representative of three independent experiments.

Snai1^{fl/fl} organoids after both had been treated with tamoxifen. Our analysis was conducted 24 h following *Snai1* deletion to detect changes in gene expression immediately after *Snai1* loss but before complete loss of the respective CBC stem cells. RNA was extracted from epithelial preparation of control and test mice. Samples were labelled and hybridised to an Illumina MouseWG-6_V2 array (Supplementary Table S1). Differential gene expression between normal and *Snai1*-deficient organoid preparations was validated by both droplet digital PCR and qRT–PCR. *Serinc3*, previously identified as having a protective role against apoptosis in a cell culture study (Bossolasco *et al*, 2006), showed differential regulation in all tests at the 24-h time point. qRT–PCR analysis of *Serinc3* in *VillinCreERT2 Snai1^{+/+}* (control) and *VillinCreERT2 Snai1^{fl/fl}* SI organoids treated for 24 and 72 h with tamoxifen demonstrated that *Serinc3* expression is downregulated by 24 h postinduction ($P = 0.001$), and this is maintained after 72 h ($P = 0.006$) (Fig 8D). No other changes in genes implicated in regulation of apoptosis were identified in this assay or with a

commercial PCR array of apoptosis-related genes. We determined whether *Snai1* could associate with the *Serinc3* promoter by chromatin immunoprecipitation (ChIP) in an intestinal cell line. We transiently expressed GFP–SNAI1 in SW480 cells and immunoprecipitated sonicated DNA fragments with an anti-GFP antibody (Fig 8E) compared to an IgG control. We detected binding of SNAI1 to promoters of known target genes *CDH1*, *IL8* as well as *SERINC3*. These data support the hypothesis that *Serinc3* may be a direct target of *Snai1* activity whose expression is directly activated upon expression of *Snai1*. *Serinc3* has been implicated in regulating cell survival (Bossolasco *et al*, 2006) and may act to maintain survival of ISCs.

Discussion

The relationship between intrinsic tissue stem cells responsible for normal organ homeostasis and cancer stem cells within solid

tumours derived from the same tissue is not clear. *Snail* family proteins have been extensively studied for their role in promoting EMT, tumour progression and invasion of epithelial tumours (Scheel & Weinberg, 2012; De Craene & Berx, 2013) but have been primarily associated with mesenchymal function in normal tissue. More recently, evidence has been accumulating to support a role for *Snail* proteins in enhancing “stemness”. Elevating *Snai1* levels in mammary epithelial cell lines promotes acquisition of many properties characteristic of stem cells (Mani et al, 2008), and co-expression of *Snai2* and *Sox9* can induce differentiated mammary cells to adopt a mammary stem cell state (Guo et al, 2012). Expression of *Snai1* also promotes epidermal stem and progenitor cell expansion (De Craene et al, 2014). In this study, we investigated the role of *Snai1* in regulating stem cell function in a normal epithelial tissue, the intestinal tract of the mouse.

Analysis of which *Snail* family members were expressed during normal intestinal homeostasis determined that *Snai1* is the only member of the family detected in epithelial crypts. Interestingly, in the mammary gland, *Snai2* (*Slug*) not *Snai1* is expressed in the basal epithelial cell compartment and is required for normal mammary gland morphogenesis and mammosphere formation (Nassour et al, 2012; Mani et al, 2008; Scheel & Weinberg, 2012). The function of *Snai1* in the intestinal stem cell compartment was investigated by conditional knockout. Our data indicate that loss of *Snai1* results in loss of CBC stem cells and a differentiation bias towards producing more secretory enteroendocrine and Paneth cells. Loss of stem cells was associated with an increase in apoptosis in crypts, which was confirmed in organoid culture, and a failure of *Snai1*^{-/-} ISCs to lineage trace showing that ISCs are lost by apoptosis. Multiple experimental lines of evidence support a role for *Snai1* in maintaining CBC ISCs. Three different inducible Cre alleles were utilised to knockout *Snai1* in the intestine (*AhCre*, *Villin-CreERT2* and *Lgr5-CreERT2*). All three approaches resulted in the loss of *Lgr5*-expressing CBC stem cells. Functional loss of CBC cells was analysed by the ability of *Snai1*^{-/-} tissue to establish and promote growth of organoids *in vitro*. When *Snai1* was deleted from the intact epithelium, organoids could not be established; however, when *Snai1* knockout was induced subsequent to establishment of cultures, organoids could form, but a decrease in growth and a significant increase in apoptosis were observed. This suggests a key role for *Snai1* in the maintenance of self-renewal within the stem cell compartment. This is an evolutionary conserved function as two studies in *Drosophila* have recently shown that *Escargot* (one of three *Snail* family members in *Drosophila*) is required for maintenance of ISCs in the *Drosophila* midgut (Korzeliuss et al, 2014; Loza-Coll et al, 2014). *Ascl2*, which like *Snai1* is also downstream of Wnt signalling and required for maintenance of intestinal CBC stem cells (van der Flier et al, 2009b), produces a similar phenotype with an increase in apoptosis when depleted from intestinal tissue.

Analysis of the consequences of depletion of *Snai1* at longer time points revealed that the intestinal epithelium was re-populated by un-recombined cells wild-type for *Snai1*. Several weeks after *Snai1* depletion, the epithelium appeared completely normal. This has been observed when several other key stem cell regulatory genes are conditionally knocked out in intestinal crypts (Muncan et al, 2006; van der Flier et al, 2009b; Holik et al, 2013) and demonstrates the strong selective pressure for maintenance of *Snai1* in the intestinal epithelium.

Lgr5-positive CBC stem cells are required for effective regeneration following radiation-induced epithelial damage (Metcalf et al, 2014). Knockout of *Snai1* concurrently with radiation treatment demonstrated a reduction in the proliferative regeneration of the epithelial layer. As our previous experiments demonstrate CBC stem cells are lost following loss of *Snai1*, this is most probably due to a reduction of CBC cells available to drive regeneration.

Snai1 expression has been linked to enhanced cellular survival in mouse embryos and cell lines (Vega et al, 2004) and in the context of chemoresistance in tumours where *Snai1* expression can enhance resistance of cells to stress-induced apoptosis (Lim et al, 2013). *Snai1* expression in keratinocytes can also enhance survival following stress (De Craene et al, 2014). Our expression and ChIP analysis identified *Serinc3* as a potential target of *Snai1* in mouse intestinal tissue. *Serinc3* has been implicated in regulating cell survival and hence may be involved in regulating the survival of intestinal stem cells downstream of *Snai1* (Bossolasco et al, 2006).

Snai1 knockout animals also showed a lineage differentiation bias where an increase in secretory Paneth and enteroendocrine cells was accompanied by a decrease in absorptive enterocytes. The opposite phenotype was observed in *Snai1* transgenic mice where a decrease in Paneth and enteroendocrine cells was observed. Inhibition of Notch signalling using a number of different experimental strategies results in dramatic phenotypes of secretory hyperplasia and loss of CBC stem cells (Milano et al, 2004; van Es et al, 2005; Fre et al, 2005; Riccio et al, 2008; Wu et al, 2010; Pellegrinet et al, 2011; Ueo et al, 2012; VanDussen et al, 2011). Direct regulation of *Snai1* by Notch has also been demonstrated (Sahlgren et al, 2008) (Timmerman et al, 2004; Matsuno et al, 2012). Although some features of deregulation of Notch signalling were present, *Snai1* knockout did not have the same dramatic enhancement of secretory cells. For example, there were no observed changes in goblet cell numbers. Taken together, these data suggest *Snai1* may be one of the targets that act downstream of both Notch and Wnt to regulate maintenance of crypt CBC cells and cell fate decisions. Recent studies of *Escargot* function in the *Drosophila* midgut also indicate that it regulates an absorptive versus secretory cell fate decision (Loza-Coll et al, 2014).

Our data demonstrate that *Snai1* is not only required for maintenance of CBC ISCs but can also act instructively as the use of a transgenic approach to elevate *Snai1* levels enhanced proliferation and increased the proliferative CBC cells in intestinal crypts *in vivo*. *Snai1* expression has been linked to inhibition of differentiation in the mammary gland (Guo et al, 2012) and skin where elevated levels of *Snai1* in keratinocyte stem cells promote proliferation and expand the progenitor pool (De Craene et al, 2014). Interestingly, when *Snai1*-expressing intestinal epithelium was placed in organoid culture, a different phenotype was observed as the organoids failed to grow and displayed an increase in apoptosis. This could be rescued by addition of Wnt3a which is normally secreted by Paneth cells (Farin et al, 2012). These observations suggest that the *Snai1*-induced reduction in Paneth cells was not adequate to support the growth of CBC stem cells in organoid cultures and that *Snai1* expression is not sufficient to promote survival when Wnt3a levels are reduced.

This study has implications for defining the role of *Snai1* in intestinal tumorigenesis where it may function to enhance cell survival,

promote invasion and enhance stemness in the propagation of tumours.

Materials and Methods

Ethics statement

All animals were handled in strict accordance with good animal practice as defined by the National Health and Medical Research Council (Australia) Code of Practice for the Care and Use of Animals for Experimental Purposes, and experimental procedures were approved by the Monash University Animal Research Platform Animal Ethics Committee and the Anatomy and Neuroscience, Pathology, Pharmacology and Physiology Animal Ethics Committee of the University of Melbourne.

Mice

Animals were housed in conventional animal house conditions at Monash Animal Services, Clayton, Australia. Conditional deletion of *Snai1* in the intestinal epithelium was achieved by crossing *Snai1^{fl/del}* mice (Murray *et al*, 2006, 2007) or *Lgr5-CreERT2-GFP* (Barker *et al*, 2007)/*Snai1^{fl/fl}* mice to *AhCre* mice (obtained from A. Clarke, Cardiff, UK) (Ireland *et al*, 2004) or *VillinCreERT* mice (El Marjou *et al*, 2004). Elevated expression of *Snai1* was achieved by crossing *RosaSnai1* mice (Nyabi *et al*, 2009) (where expression of *Snai1* depends on Cre-mediated excision of a floxed *PGKneo* cassette) to *AhCre* mice. Expression of Cre recombinase was induced by giving control (*AhCre*) and test mice four daily intraperitoneal injections of β -naphthoflavone (β -NF) (80 mg/kg) or control (*VillinCreERT*) and test mice four daily injections of tamoxifen (100 mg/kg). On day 5, the mice were killed and intestinal tissue harvested for analysis. Groups of at least three animals of each genotype utilising littermate controls were used for induction experiments and analysis of phenotypes. *Villin-CreERT2* mice (El Marjou *et al*, 2004) were crossed with *Snai1^{fl/fl}* mice for *Snai1* loss of function experiments using *in vitro* organoid culture and also with *RosaSnai1* for gain of function experiments (Nyabi *et al*, 2009). For lineage tracing, *Snai1^{fl/fl}* mice were crossed with *Lgr5-CreERT2-GFP* and *Rosa26 lacZ* reporter (R26R) mice (Soriano, 1999). Control (*Lgr5-CreERT2-GFP* R26R) and test (*Lgr5-CreERT2-GFP* R26R *Snai1^{fl/fl}*) mice were injected with 200 μ l tamoxifen (Sigma-Aldrich) dissolved in sunflower oil (10 mg/ml) and tissues dissected on day 7. To assess cell proliferation, mice were injected with BrdU (Sigma-Aldrich) dissolved in PBS (50 mg/kg) 2 h before dissection. Control (*VillinCreERT2*) and test mice (*VillinCreERT2 Snai1^{fl/fl}*) underwent 12 Gy irradiation treatment.

Quantitative RT-PCR analysis

Total RNA was isolated from whole dissected intestinal tissue from different embryonic or postnatal stages using TRIzol Reagent (Invitrogen). Further RNA cleanup and DNase I digestion was performed using the RNeasy kit (Qiagen) and cDNA prepared using Superscript III Reverse Transcriptase (Invitrogen) using the random hexamer method. Quantitative PCR was performed using Platinum Taq Polymerase (Invitrogen) and SYBR Green (Fisher) using a

RotorGene 3000 cycler (Corbett Research). Expression was normalised relative to β -actin and B2M RNA expression.

Quantitative reverse transcriptase PCR from organoids was performed using 1 μ g total RNA isolated using Qiagen RNeasy kit and transcribed into cDNA utilising the Qiagen Quantitect Reverse Transcription Kit. qPCR was performed on a LightCycler 480 II (Roche) using the LightCycler 480 SYBR Green I Master (Roche).

Droplet digital PCR

cDNA was generated from RNA (1 μ g) using the iScript Advanced cDNA synthesis kit for RT-qPCR (Bio-Rad cat no. 170-8842). The ddPCR mix consisted of 2 $\times$ ddPCR master mix (Bio-Rad cat no. 186-3010), 20 $\times$ primers and probe mix (IDT assays final 1 $\times$ concentration of 500 nM primers and 250 nM probe; TaqMan assay final 1 $\times$ concentration of 900 mM primers and 250 mM probe) (see Supplementary Table S2 for assay details) and template (variable volume) in a final volume of 25 μ l. The ddPCR mix and droplet generation oil were loaded into an eight-channel droplet generator cartridge. Droplets formed were transferred to a 96-well PCR plate; a 2-step thermocycling protocol [95°C $\times$ 10 min; 40 cycles $\times$ [(94°C $\times$ 30 s, 60°C $\times$ 60 s); 98°C $\times$ 10 min, ramp rate set at 2.5°C/s]] was carried out in a thermal cycler (Bio-Rad C1000 Touch). The PCR plate was then transferred to the QX100 Droplet Reader (Bio-Rad) which automatically reads the droplet fluorescence from each well of the plate. Analysis of the ddPCR data was performed with QuantaSoft analysis software (Bio-Rad).

Histology, histochemistry, immunohistochemistry, β -galactosidase staining and *in situ* hybridisation

Intestinal tissue for immunohistochemistry and Periodic Acid Schiff (PAS) staining was collected, washed in PBS, fixed overnight in 4% PFA at 4°C, processed, embedded in paraffin and sectioned at 5 or 8 μ m. Immunohistochemistry, β -galactosidase staining and *in situ* hybridisation staining were performed using standard techniques. More detailed information is available in Supplementary Experimental Procedures. Morphometric analysis and cell counts were performed using the ImagePro software counting 20 crypt/villus units per tissue section. Statistical significance was determined using the two-tailed Student's *t*-test in GraphPad Prism6 and *F*-test to compare variance.

Intestinal crypt cell purification for FACS and *in vitro* organoid experiments

Mouse small intestinal tracts were isolated and cut open longitudinally, and villi were removed. After 30-min incubation in PBS/EDTA (5 mM) at 4°C, free crypts were harvested by centrifugation. For FACS experiments, crypts were incubated in DMEM/10% FCS for 45 min at 37°C, dissociated in TrypLE Express (Life Technologies) and DNase I (800 μ g/ml) (Sigma-Aldrich) for 20 min at 37°C and filtered through a 70- μ m cell strainer before sorting using Cytopeia Influx cell sorter (BD).

Organoid culture was conducted as previously described (Shorning *et al*, 2012; Jardé *et al*, 2013). Briefly, isolated small intestinal tracts were opened longitudinally and chopped into 5-mm pieces. Crypts were released from tissue fragments by incubation for

30 min at 4°C in 2 mM EDTA. Isolated crypts were mixed with 50 µl of growth factor-reduced Matrigel (BD Biosciences), seeded in 24-well plates, and 500 µl of crypt culture medium was overlaid [DMEM/F12 supplemented with N2, B27, penicillin/streptomycin, glutamax, 10 mM HEPES, fungizone, 50 ng/ml EGF (Peprotech), 100 ng/ml Noggin (Peprotech) and 600 ng/ml R-spondin 1 (R&D Systems)]. Intestinal organoids were maintained in a 37°C humidified atmosphere under 5% CO₂ and passaged every week.

For *in vitro* regeneration experiments, organoids were mechanically passaged and 150 crypt fragments were seeded per 24-well plate well. *Snai1* gene deletion was induced by treating *Ah-Cre*, *Snai1^{fl/fl}* and *Villin-Cre*, *Snai1^{fl/fl}* organoids with 10 ng/ml BNF (Sigma) or 0.5 µM tamoxifen (Sigma), respectively, in the culture medium. Organoids were incubated in culture medium with 50% Wnt3a-conditioned L cell medium for Wnt3a treatment.

For assessment of cell growth, organoids were exposed to Presto-Blue cell viability reagent (Invitrogen) in the culture medium for 45 min at 37°C according to the manufacturer's instructions at the time points specified.

For assessment of apoptosis, organoids were incubated with 100 µl/ml Hoechst 33342 (Sigma) and 100 µl/ml propidium iodide (Sigma) for 30 min. Organoids were imaged using a Leica AF6000LX live cell microscope equipped with a 10× objective, monochromator, motorised XY stage, CO₂ and temperature-controlled atmospheric chamber, and running LAS AF software (Leica Microsystems, Mannheim, Germany).

Gene expression analysis

Total RNA prepared as per the quantitative RT-PCR protocol was checked for quality on a Bioanalyzer, labelled and hybridised to an Illumina MouseWG-6_V2 array at the Australian Genome Research Facility. Raw signal intensity values were subjected to variance stabilisation transformation including background correction, log2 transformation and variance stabilisation using the lumiR package of R Bioconductor. ANOVA of normalised probe intensities values was performed in Partek Genomic Suite™ software, version 6.6. ANOVA was used to calculate significance of variation in normalised expression values between sample groups; fold change of gene expressions was calculated as mean ratio. The microarray data from this publication have been submitted to the GEO repository database (<http://www.ncbi.nlm.nih.gov/geo/>) and assigned the identifier (GSE65005).

ChIP and qPCR assays

SW480 cells (Leibovitz *et al.*, 1976) were transiently transfected with pEGFP-C2-Snai1WT (Addgene plasmid 16225) (Zhou *et al.*, 2004) using Lipofectamine 2000 reagent according to Life Technologies protocols. 1×10^7 SW480 cells were fixed in 1% formaldehyde for 10 min at room temperature, quenched with glycine (final concentration 0.125 M) for 10 min at room temperature, washed twice with cold PBS and lysed for 20 min on ice (1% SDS, 10 mM EDTA pH 8, 50 mM Tris-HCl pH 8.1, Roche protease inhibitor mix). Samples were sonicated for 15 s 20 times using a Bioruptor (Diagenode, Philadelphia, PA). Supernatants were pre-cleared with Protein-A/G Dynabeads (Invitrogen), and 10% input was collected. Immunoprecipitations were performed using

anti-GFP antibody, and DNA-GFP-Snai1 complexes were pulled down using Protein-A/G Dynabeads (Invitrogen). DNA was purified using QIAquick PCR purification columns (QIAGEN, Maryland) after overnight reverse cross-linking at 65°C. Quantitative PCR (Roche Lightcycler 480 SYBR green I master mix) was used to validate GFP-Snai1 binding to DNA (please refer to Supplementary Experimental Procedures for primer sequences).

Supplementary information for this article is available online: <http://emboj.embopress.org>

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Author contributions

HA, KHO and GH designed experiments, interpreted data and wrote the manuscript. NB, JH, GB, TC and VP contributed to experimental design and data interpretation. KHO, TJ, FC, RA, KHA and CN performed experiments and interpreted data.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegbarth A, Korving J, Begthel H, Peters PJ, Clevers H (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 449: 1003–1007
- Barker N (2014) Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration. *Nat Rev Mol Cell Biol* 15: 19–33
- Barrallo-Gimeno A, Nieto MA (2005) The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development* 132: 3151–3161
- Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, Garcia De Herreros A (2000) The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol* 2: 84–89
- Bossolasco M, Veillette F, Bertrand R, Mes-Masson AM (2006) Human TDE1, a TDE1/TMS family member, inhibits apoptosis in vitro and stimulates in vivo tumorigenesis. *Oncogene* 25: 4549–4558
- Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA (2000) The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* 2: 76–83
- Carver EA, Jiang R, Lan Y, Oram KF, Gridley T (2001) The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. *Mol Cell Biol* 21: 8184–8188

- Chiang C, Ayyanathan K (2013) Snail/Gfi-1 (SNAG) family zinc finger proteins in transcription regulation, chromatin dynamics, cell signaling, development, and disease. *Cytokine Growth Factor Rev* 24: 123–131
- Clevers H (2013) The intestinal crypt, a prototype stem cell compartment. *Cell* 154: 274–284
- De Craene B, Bex G (2013) Regulatory networks defining EMT during cancer initiation and progression. *Nat Rev Cancer* 13: 97–110
- De Craene B, Denecker G, Vermassen P, Taminiau J, Mauch C, Derore A, Jonkers J, Fuchs E, Bex G (2014) Epidermal Snail expression drives skin cancer initiation and progression through enhanced cytoprotection, epidermal stem/progenitor cell expansion and enhanced metastatic potential. *Cell Death Differ* 21: 310–320
- Durand A, Donahue B, Peignon G, Letourneur F, Cagnard N, Slomianny C, Perret C, Shroyer NF, Romagnolo B (2012) Functional intestinal stem cells after Paneth cell ablation induced by the loss of transcription factor Math1 (Atoh1). *Proc Natl Acad Sci USA* 109: 8965–8970
- El Marjou F, Janssen K-P, Hung-Junn Chang B, Li M, Hindie V, Chan L, Louvard D, Chambon P, Metzger D, Robine S (2004) Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. *Genesis* 39: 186–193
- van Es JH, van Gijn ME, Riccio O, van den Born M, Vooijs M, Begthel H, Cozijnsen M, Robine S, Winton DJ, Radtke F, Clevers H (2005) Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* 435: 959–963
- Farin HF, Van Es JH, Clevers H (2012) Redundant sources of Wnt regulate intestinal stem cells and promote formation of paneth cells. *Gastroenterology* 143: 1518–1529
- van der Flier LG, Haegebarth A, Stange DE, van de Wetering M, Clevers H (2009a) OLFM4 is a robust marker for stem cells in human intestine and marks a subset of colorectal cancer cells. *Gastroenterology* 137: 15–17
- van der Flier LG, van Gijn ME, Hatzis P, Kujala P, Haegebarth A, Stange DE, Begthel H, van den Born M, Guryev V, Oving I, van Es JH, Barker N, Peters PJ, van de Wetering M, Clevers H (2009b) Transcription factor achaete scute-like 2 controls intestinal stem cell fate. *Cell* 136: 903–912
- Franco DL, Mainze J, Vega S, Sancho P, Murillo MM, de Frutos CA, Del Castillo G, Lopez-Blau C, Fabregat I, Nieto MA (2010) Snail1 suppresses TGF-beta-induced apoptosis and is sufficient to trigger EMT in hepatocytes. *J Cell Sci* 123: 3467–3477
- Fre S, Huyghe M, Mourikis P, Robine S, Louvard D, Artavanis-Tsakonas S (2005) Notch signals control the fate of immature progenitor cells in the intestine. *Nature* 435: 964–968
- Guo W, Keckesova Z, Donaher JL, Shibue T, Tischler V, Reinhardt F, Itzkovitz S, Noske A, Zurrer-Hardi U, Bell G, Tam WL, Mani SA, van Oudenaarden A, Weinberg RA (2012) Slug and Sox9 cooperatively determine the mammary stem cell state. *Cell* 148: 1015–1028
- Heijmans J, van Lidde de Jude JF, Koo BK, Rosekrans SL, Wielenga MC, van de Wetering M, Ferrante M, Lee AS, Onderwater JJ, Paton JC, Paton AW, Mommaas AM, Kodach LL, Hardwick JC, Hommes DW, Clevers H, Muncan V, van den Brink GR (2013) ER stress causes rapid loss of intestinal epithelial stemness through activation of the unfolded protein response. *Cell Rep* 3: 1128–1139
- Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ, Vessella RL, Tewari M (2013) Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nat Methods* 10: 1003–1005
- Holik AZ, Krzysztyniak J, Young M, Richardson K, Jarde T, Chambon P, Shorning BY, Clarke AR (2013) Brg1 is required for stem cell maintenance in the murine intestinal epithelium in a tissue-specific manner. *Stem Cells* 31: 2457–2466
- Horvay K, Casagrande F, Gany A, Hime GR, Abud HE (2011) Wnt signaling regulates Snai1 expression and cellular localization in the mouse intestinal epithelial stem cell niche. *Stem Cells Dev* 20: 737–745
- Horvay K, Abud HE (2013) Regulation of intestinal stem cells by Wnt and Notch signalling. *Adv Exp Med Biol* 786: 175–186
- Ireland H, Kemp R, Houghton C, Howard L, Clarke AR, Sansom OJ, Winton DJ (2004) Inducible Cre-mediated control of gene expression in the murine gastrointestinal tract: effect of loss of beta-catenin. *Gastroenterology* 126: 1236–1246
- Jardé T, Evans RJ, McQuillan KL, Parry L, Feng GJ, Alvares B, Clarke AR, Dale TC (2013) In vivo and in vitro models for the therapeutic targeting of Wnt signaling using a Tet-OΔN89β-catenin system. *Oncogene* 32: 883–893
- Jiang R, Lan Y, Norton CR, Sundberg JP, Gridley T (1998) The Slug gene is not essential for mesoderm or neural crest development in mice. *Dev Biol* 198: 277–285
- Korzeliuss J, Naumann SK, Loza-Coll MA, Chan JS, Dutta D, Oberheim J, Glasser C, Southall TD, Brand AH, Jones DL, Edgar BA (2014) Escargot maintains stemness and suppresses differentiation in Drosophila intestinal stem cells. *EMBO J* 33: 2967–2982
- Leibovitz A, Stinson JC, McCombs WB III, McCoy CE, Mazur KC, Mabry ND (1976) Classification of human colorectal adenocarcinoma cell lines. *Cancer Res* 36: 4562–4569.
- Lim S, Becker A, Zimmer A, Lu J, Buettner R, Kirfel J (2013) SNAI1-mediated epithelial-mesenchymal transition confers chemoresistance and cellular plasticity by regulating genes involved in cell death and stem cell maintenance. *PLoS ONE* 8: e66558
- Loza-Coll MA, Southall TD, Sandall SL, Brand AH, Jones DL (2014) Regulation of Drosophila intestinal stem cell maintenance and differentiation by the transcription factor Escargot. *EMBO J* 33: 2983–2996
- Mani SA, Guo W, Liao M-J, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Briskin C, Yang J, Weinberg RA (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 133: 704–715
- Matsuno Y, Coelho AL, Jaraí G, Westwick J, Hogaboam CM (2012) Notch signaling mediates TGF-beta1-induced epithelial-mesenchymal transition through the induction of Snai1. *Int J Biochem Cell Biol* 44: 776–789
- Metcalfe C, Kljavin NM, Ybarra R, de Sauvage FJ (2014) Lgr5⁺ stem cells are indispensable for radiation-induced intestinal regeneration. *Cell Stem Cell* 14: 149–159
- Milano J, McKay J, Dagenais C, Foster-Brown L, Pognan F, Gadiant R, Jacobs RT, Zacco A, Greenberg B, Ciaccio PJ (2004) Modulation of notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. *Toxicol Sci* 82: 341–358
- Muncan V, Sansom OJ, Tertoolen L, Pheasant TJ, Begthel H, Sancho E, Cole AM, Gregorieff A, de Alboran IM, Clevers H, Clarke AR (2006) Rapid loss of intestinal crypts upon conditional deletion of the Wnt/Tcf-4 target gene c-Myc. *Mol Cell Biol* 26: 8418–8426
- Murray SA, Carver EA, Gridley T (2006) Generation of a Snail1 (Snai1) conditional null allele. *Genesis* 44: 7–11
- Murray SA, Oram KF, Gridley T (2007) Multiple functions of Snail family genes during palate development in mice. *Development* 134: 1789–1797
- Nassour M, Idoux-Gillet Y, Selmi A, Come C, Faraldo ML, Deugnier MA, Savagner P (2012) Slug controls stem/progenitor cell growth dynamics during mammary gland morphogenesis. *PLoS ONE* 7: e53498
- Nieto MA (2002) The snail superfamily of zinc-finger transcription factors. *Nat Rev Mol Cell Biol* 3: 155–166

- Nyabi O, Naessens M, Haigh K, Gembarska A, Goossens S, Maetens M, De Clercq S, Drogat B, Haenebalcke L, Bartunkova S, De Vos I, De Craene B, Karimi M, Berx G, Nagy A, Hilson P, Marine JC, Haigh JJ (2009) Efficient mouse transgenesis using Gateway-compatible ROSA26 locus targeting vectors and F1 hybrid ES cells. *Nucleic Acids Res* 37: e55
- Oram KF, Carver EA, Gridley T (2003) Slug expression during organogenesis in mice. *Anat Rec A Discov Mol Cell Evol Biol* 271: 189–191
- Pellegrinet L, Rodilla V, Liu Z, Chen S, Koch U, Espinosa L, Kaestner KH, Kopan R, Lewis J, Radtke F (2011) Dll1- and dll4-mediated notch signaling are required for homeostasis of intestinal stem cells. *Gastroenterology* 140: 1230–1240 e1231-1237
- Perez-Losada J, Sanchez-Martin M, Rodriguez-Garcia A, Sanchez ML, Orfao A, Flores T, Sanchez-Garcia I (2002) Zinc-finger transcription factor Slug contributes to the function of the stem cell factor c-kit signaling pathway. *Blood* 100: 1274–1286
- Pinto D, Gregorieff A, Begthel H, Clevers H (2003) Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. *Genes Dev* 17: 1709–1713
- Pioli PD, Dahlem TJ, Weis JJ, Weis JH (2013) Deletion of Snai2 and Snai3 results in impaired physical development compounded by lymphocyte deficiency. *PLoS ONE* 8: e69216
- Powell AE, Wang Y, Li Y, Poulin EJ, Means AL, Washington MK, Higginbotham JN, Juchheim A, Prasad N, Levy SE, Guo Y, Shyr Y, Aronow BJ, Haigis KM, Franklin JL, Coffey RJ (2012) The pan-ErbB negative regulator Lrig1 is an intestinal stem cell marker that functions as a tumor suppressor. *Cell* 149: 146–158
- Riccio O, van Gijn ME, Bezdek AC, Pellegrinet L, van Es JH, Zimmer-Strobl U, Strobl LJ, Honjo T, Clevers H, Radtke F (2008) Loss of intestinal crypt progenitor cells owing to inactivation of both Notch1 and Notch2 is accompanied by derepression of CDK inhibitors p27Kip1 and p57Kip2. *EMBO Rep* 9: 377–383
- Roy HK, Smyrk TC, Koetsier J, Victor TA, Wali RK (2005) The transcriptional repressor SNAIL is overexpressed in human colon cancer. *Dig Dis Sci* 50: 42–46
- Sahlgren C, Gustafsson MV, Jin S, Poellinger L, Lendahl U (2008) Notch signaling mediates hypoxia-induced tumor cell migration and invasion. *Proc Natl Acad Sci USA* 105: 6392–6397
- Sangiorgi E, Capecchi MR (2008) Bmi1 is expressed in vivo in intestinal stem cells. *Nat Genet* 40: 915–920
- Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ, Clevers H (2009) Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 459: 262–265
- Sato T, van Es JH, Snippert HJ, Stange DE, Vries RG, van den Born M, Barker N, Shroyer NF, van de Wetering M, Clevers H (2011) Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature* 469: 415–418
- Scheel C, Weinberg RA (2012) Cancer stem cells and epithelial-mesenchymal transition: concepts and molecular links. *Semin Cancer Biol* 22: 396–403
- Shorning BY, Jardé T, McCarthy A, Ashworth A, de Leng WW, Offerhaus GJ, Resta N, Dale T, Clarke AR (2012) Intestinal renin-angiotensin system is stimulated after deletion of Lkb1. *Gut* 61: 202–213
- Snippert HJ, van der Flier LG, Sato T, van Es JH, van den Born M, Kroon-Veenboer C, Barker N, Klein AM, van Rheenen J, Simons BD, Clevers H (2010) Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. *Cell* 143: 134–144
- Soriano P (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 21: 70–71
- Takeda N, Jain R, LeBoeuf MR, Wang Q, Lu MM, Epstein JA (2011) Interconversion between intestinal stem cell populations in distinct niches. *Science* 334: 1420–1424
- Tian H, Biehs B, Warming S, Leong KG, Rangell L, Klein OD, de Sauvage FJ (2011) A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. *Nature* 478: 255–259
- Timmerman LA, Grego-Bessa J, Raya A, Bertran E, Perez-Pomares JM, Diez J, Aranda S, Palomo S, McCormick F, Izpisua-Belmonte JC, de la Pompa JL (2004) Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. *Genes Dev* 18: 99–115
- Ueo T, Imayoshi I, Kobayashi T, Ohtsuka T, Seno H, Nakase H, Chiba T, Kageyama R (2012) The role of Hes genes in intestinal development, homeostasis and tumor formation. *Development* 139: 1071–1082
- Van Landeghem L, Santoro MA, Krebs AE, Mah AT, Dehmer JJ, Gracz AD, Scull BP, McNaughton K, Magness ST, Lund PK (2012) Activation of two distinct Sox9-EGFP-expressing intestinal stem cell populations during crypt regeneration after irradiation. *Am J Physiol Gastrointest Liver Physiol* 302: G1111–G1132
- VanDussen KL, Carulli AJ, Keeley TM, Patel SR, Puthoff BJ, Magness ST, Tran IT, Maillard I, Siebel C, Kolterud A, Grosse AS, Gumucio DL, Ernst SA, Tsai YH, Dempsey PJ, Samuelson LC (2012) Notch signaling modulates proliferation and differentiation of intestinal crypt base columnar stem cells. *Development* 139: 488–497
- Vega S, Morales AV, Ocana OH, Valdes F, Fabregat I, Nieto MA (2004) Snail blocks the cell cycle and confers resistance to cell death. *Genes Dev* 18: 1131–1143
- Wong VW, Stange DE, Page ME, Buczacik S, Wabik A, Itami S, van de Wetering M, Poulsom R, Wright NA, Trotter MW, Watt FM, Winton DJ, Clevers H, Jensen KB (2012) Lrig1 controls intestinal stem-cell homeostasis by negative regulation of ErbB signalling. *Nat Cell Biol* 14: 401–408
- Wu WS, Heinrichs S, Xu D, Garrison SP, Zambetti GP, Adams JM, Look AT (2005) Slug antagonizes p53-mediated apoptosis of hematopoietic progenitors by repressing puma. *Cell* 123: 641–653
- Wu Y, Cain-Hom C, Choy L, Hagenbeek TJ, de Leon GP, Chen Y, Finkle D, Venook R, Wu X, Ridgway J, Schahin-Reed D, Dow GJ, Shelton A, Stawicki S, Watts RJ, Zhang J, Choy R, Howard P, Kadyk L, Yan M et al (2010) Therapeutic antibody targeting of individual Notch receptors. *Nature* 464: 1052–1057
- Yan KS, Chia LA, Li X, Ootani A, Su J, Lee JY, Su N, Luo Y, Heilshorn SC, Amieva MR, Sangiorgi E, Capecchi MR, Kuo CJ (2012) The intestinal stem cell markers Bmi1 and Lgr5 identify two functionally distinct populations. *Proc Natl Acad Sci USA* 109: 466–471
- Zeilstra J, Joosten SP, van Andel H, Tolg C, Berns A, Snoek M, van de Wetering M, Spaargaren M, Clevers H, Pals ST (2013) Stem cell CD44v isoforms promote intestinal cancer formation in Apc(min) mice downstream of Wnt signaling. *Oncogene* 33: 665–667
- Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, Hung MC (2004) Dual regulation of Snail by GSK-3 β -mediated phosphorylation in control of epithelial-mesenchymal transition. *Nat Cell Biol* 6: 931–940