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

Katja Horvay¹, Thierry Jardé¹, Franca Casagranda², Victoria M Perreau², Katharina Haigh³,⁴, Christian M Nefgzer¹,⁵, Reyhan Akhtar¹, Thomas Gridley⁶, Geert Berx⁴,⁷, Jody J Haigh³,⁴, Nick Barker⁸, Jose M Polo¹,⁵, Gary R Hime²,⁸† & Helen E Abud¹,⁸**

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 Snai2, the intestinal epithelium fails to produce a proliferative response following radiation-induced damage indicating a fundamental requirement for Snai2 in epithelial regeneration. These results demonstrate that Snai2 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

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 Snail (Snai1), 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...
cancers (Roy et al., 2005), but evidence has not been presented demonstrating a role for Snail 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, Dil4 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 vivo lineage tracing experiments to reconstitute all cells in the epithelium 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 (Fig1K) 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) with studies throughout embryogenesis where Snai2 is restricted to the mesenchyme (Jiang et al., 1998; VanDussen 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, Dil4 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

Snai 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 & Ayyana-than, 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 (Fig1K) 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) with studies throughout embryogenesis where Snai2 is restricted to the mesenchyme (Jiang et al., 1998; VanDussen 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, Dil4 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.

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 Snai1fl/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 Snai1fl/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.** Snail1 is localised to stem and progenitor cells within mouse intestinal crypts, while Snail2 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 ± SD.

C, E, G, I Immunohistochemical staining for Snail1/2 (Abcam ab85931) (C) E14.5 midgut, (E) E14.5 hind gut, (G) postnatal day 14 SI, (I) postnatal day 14 colon. Snail1 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 Snail2LacZ 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 Snail1/2 (red) and (M) Lgr5-GFP (green); (N) merge shows Snail1 is present in both CBC stem cells and proliferating transit amplifying cells. Scale bars: 10 mm.

O, P Immunohistochemistry for Snail1 using a different primary antibody (Genetex GTX125918) shows minimal expression of Snail1 in villi. (P) Expression of Snail1 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 Snail1 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 (μm) (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 (μm) (n = 3, P = 0.003) 8 days after β-NF induction demonstrates a significant shortening of villi. Bars represent mean ± SD. Two-tailed Student’s t-test was used to assess significance. Scale bars: 20 μm.

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 Snail 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 Snail1 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 *Cryp* tin (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 *AhrCre Snai1fl/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 *Snai1fl/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−/−* 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 *Gpr78* mutant cells where CBC ISC numbers relocate up the crypt and adopt a transit amplifying cell fate within 48 h after deletion of *Gpr78* (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 *AhrCre Snai1fl/del* mice 24 h post-BNF induction. In contrast to *AhrCre 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 *AhrCre Snai1fl/R* mice were generated to enable inducible deletion of Snai1 with BNF following establishment of cultures. Cultures were also generated from *VillinCreERT2 Snai1fl/R* mice and recombination induced in these cultures by addition of tamoxifen.
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 μm.

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 μm.

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 INJ 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 = 0.0005) regions in Snai1KO tissue (arrows mark active caspase-3-positive cells). Scale bars: 20 μm.

E. A lineage tracing strategy was employed to determine the fate of CBC cells that lack Snai1. Tissue from Lgr5-EGFP-ireS-CreERT2 Snai1^{fl/fl} (Snai1KO) that also contained the ROSA26Loa-Z 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 ± 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 Snai1fl/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 Snai1fl/fl 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 ISC.

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

We investigated the phenotype in the intestinal epithelium of AhCre Snai1fl/fl and VillinCreERT2 Snai1fl/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 deletion of Snai1 with exposure to radiation (Fig 5B). VillinCreERT2 Snai1+/− (control) and VillinCreERT2 Snai1fl/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 (Metaclle 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 ROSASnai1 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 ROSA26Snai1 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 knock- out 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 ROSA26Snai1 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 ROSA26Snai1 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 (Baille 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 ROSA26Snai1 \((P = 0.00023)\) mice compared to controls (Fig 6H).
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...
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 Snai1fl/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...
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 with elevated Snai1 levels compared to control tissue (n=4, P=0.0005). Bars represent mean ± 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 μm.

G qRT–PCR analysis of stem cell marker expression in the small intestine from VillinCreERT2 ROSA26Snai1 compared to control VillinCreERT2 littermates. Bars represent mean ± SD. n = 3 experiments per group, Olfm4 P = 0.013, Cdh1/β-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 ± SD. n = 3 mice per group, *P = 0.00023. Two-tailed Student’s t-test was used to assess significance. Scale bars: 10 μm.

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

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 μm.

B Quantification of viable cells in VillinCreERT2 RosaSnai1 organoids treated with tamoxifen compared to control growth over 4 days. Bars represent mean ± 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 μm.

D Quantification of apoptotic organoids with and without Wnt3A treatment shown as mean ± SD. n = 3 experiments per group; **P = 0.00003. Two-tailed Student’s t-test was used to assess significance.
Snai1\textsuperscript{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\textsuperscript{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 VillinCreERT\textsuperscript{2} Snai1\textsuperscript{+/+} (control) and VillinCreERT\textsuperscript{2} Snai1\textsuperscript{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 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.

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 Bcl2/11/Bim after conditional Snai1 loss in in vitro organoid culture. VillinCreERT\textsuperscript{2} Snai1\textsuperscript{+/+} (control) and VillinCreERT\textsuperscript{2} Snai1\textsuperscript{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. VillinCreERT\textsuperscript{2} Snai1\textsuperscript{+/+} (control) and VillinCreERT\textsuperscript{2} Snai1\textsuperscript{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.

**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 gland morphogenesis and mammosphere formation (Nassour et al., 2008; 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 (Metcalfe 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 (Bosсоласко 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; Matsumo 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<sup>fl/fl</sup> mice (Murray et al, 2006, 2007) or Lgr5-CreERT2-GFP (Barker et al, 2007)/Snai1<sup>fl/fl</sup> 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 PGK<sub>neo</sub> cassette) to AhCre mice. Expression of Cre recombining 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<sup>fl/fl</sup> mice for Snai1 loss of function experiments using <i>in vitro</i> organoid culture and also with RosaSnai1 for gain of function experiments (Nyabi et al, 2009). For lineage tracing, Snai1<sup>fl/fl</sup> 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<sup>fl/fl</sup>) mice were injected with 200 µl 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<sup>fl/fl</sup>) 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 RNaseasy 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 µg 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 µg) using the iScript Advanced cDNA synthesis kit for RT–qPCR (Bio-Rad cat no. 170-8842). The ddPCR mix consisted of 2× ddPCR master mix (Bio-Rad cat no. 186-3010), 20× primers and probe mix (IDT assays final 1× concentration of 500 nM primers and 250 nM probe; TaqMan assay final 1× concentration of 900 nM primers and 250 nM probe) (see Supplementary Table S2 for assay details) and template (variable volume) in a final volume of 25 µl. 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 × 10 min; 40 cycles × (94°C × 30 s, 60°C × 60 s); 98°C × 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 <i>in situ</i> 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 µm. Immunohistochemistry, β-galactosidase staining and <i>in situ</i> 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 <i>t</i>-test in GraphPad Prism6 and <i>P</i>-test to compare variance.

Intestinal crypt cell purification for FACS and <i>in vitro</i> 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 µg/ml) (Sigma-Aldrich) for 20 min at 37°C and filtered through a 70-µm 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, Snai1fl/fl and Villin-Cre, Snai1fl/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 PrestoBlue 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 10x objective, monochromator, motorised XY stage, CO2 and temperature-controlled atmospheric chamber, and running LAS AF software (Leica Microsystems, Manheim, 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 lumi package of R Bioconductor. ANOVA of normalised probe intensities values was performed in Partek Genomic SuiteTM software, version 6.6. ANOVA was used to calculate significance of variation in normalised expression values between sample groups; fold change of gene expression analysis

ChiP and qPCR assays

SW480 cells (Leibovitz et al., 1976) were transiently transfected with pEGFP-C2-Sna1WT (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 glyceline (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 ± 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-Sna1 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-Sna1 binding to DNA (please refer to Supplementary Experimental Procedures for primer sequences).

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

Acknowledgements

We thank staff at Monash Animal Services, Helen Lescesen and Emma Murphy for assistance with genotyping. The authors acknowledge the facilities and scientific and technical assistance of Monash Micro Imaging and Histology platform at Monash University, Victoria, Australia. This work was supported by National Health and Medical Research Council (NH&MR) Australia, project grant (509158) to H.E.A. and G.R.H. and (1011187) to H.E.A. and N.B. G.B.’s work was funded by grants from VIB, the geconcerteerde onderzoeksacties of Ghent University, the stichting tegen kanker and the EU-FP7 Framework Program TU(MIC) 2008-201662. T.C. was funded by NIH Grant HD034883.

Author contributions

HA, KHo and GH designed experiments, interpreted data and wrote the manuscript. NB, JH, GB, TG 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


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


**Function of Snai1 in the mouse small intestine** Katja Horvay et al


