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ISBN: 9789039363553

Printing: CPI Koninklijke Wöhrmann

Cover Design: Benjamin Tetteh and Paul Tetteh

The research described in this thesis was performed at the Hubrecht Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW), within the framework of the Graduate School of Cancer Genomics and Developmental Biology in Utrecht, the Netherlands

Financial support by the Nederlandse Organisatie voor Wetenschapplijke Onderzoek (NWO).

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**Plasticity of intestinal epithelial cells in regeneration and cancer**

Plasticiteit van darm epitheelcellen in regeneratie en kanker

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 9 juni 2015 des middags te 2.30 uur

door

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geboren op 14 november 1979 te Accra, Ghana

Promotor: Prof. dr. H.C. Clevers

# Dedication

*To my parents*

*To my sweetheart, Haimy*

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# CHAPTER 1

# Plasticity within stem cell hierarchies in mammalian epithelia

1

## Introduction and outline of this thesis

**Adapted from Trends in Cell Biology, February 2015, Vol. 25, No. 2**

*Chapter One*

# Plasticity within stem cell hierarchies in mammalian epithelia

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**Abstract**

**Tissue homeostasis and regeneration are fueled by resident stem cells that have the capacity to self-renew, and to generate all the differentiated cell types that characterize a particular tissue. Classical models of such cellular hierarchies propose that commitment and differentiation occur unidirectionally, with the arrows 'pointing away' from the stem cell. Recent studies, all based on genetic lineage tracing, describe various strategies employed by epithelial stem cell hierarchies to replace damaged or lost cells. While transdifferentiation from one tissue type into another ('metaplasia') appears to be generally forbidden in nonpathological contexts, plasticity within an individual tissue stem cell hierarchy may be much more common than previously appreciated. In this review, we discuss recent examples of such plasticity in selected mammalian epithelia, highlighting the different modes of regeneration and their implications for our understanding of cellular hierarchy and tissue self-renewal.**

## Epithelial tissue homeostasis and regeneration

Cells lost through physiological ageing or as a result of environmental insults must be continuously replaced to preserve the 'cellular status quo' of an organism. This homeostasis is achieved by undifferentiated, self-renewing stem cells that can generate all cell types of the tissue (Figure 1). Some adult tissues, such as the epithelia of intestine, stomach, and skin, are exposed to mechanical wear-and-tear and are continuously self-renewing. Epithelia of other internal organs, such as liver, pancreas, or kidneys, are typically self-renewing at a low rate. Although some general rules may apply across these tissues, each appears to employ uniquely designed stem cell hierarchies and, correspondingly, unique tissue architectures to fulfill specific physiologic demands.

Plasticity refers to the ability of cells to adopt an alternate cellular fate in response to extrinsic or intrinsic factors. When this plasticity involves a differentiated cell changing into another differentiated cell of another lineage within a given tissue, we term this transdifferentiation. Dedifferentiation is a form of plasticity in which a differentiated cell reverts to a less differentiated cell within the same tissue lineage. Of note, this 'working definition' of plasticity excludes epithelial–mesenchymal transitions observed during embryogenesis and tumorigenesis.

Although tissue stem cells are generally viewed as the major drivers of tissue regeneration after damage, endogenous dedifferentiation and transdifferentiation of non- stem cell populations has been observed to play a key role in tissue replacement in planarians, amphibians, fishes, and even some nonepithelial tissues in mammals, (reviewed elsewhere [1–3]). Emerging evidence from mammalian systems implies that differentiated epithelial cells can function as reserve stem cells upon tissue damage, implying that this might be a universal phenomenon adopted by multicellular organisms to replenish lost cells. This has important implications for the definition of what constitutes a stem cell and for our views on cellular hierarchies in homeostasis, regeneration, and in pathologies such as cancer. In this review, we discuss recent examples of endogenous plasticity (without genetic/epigenetic manipulation)

in the epithelia of organs of the gastrointestinal tract, lung, kidney, and adrenal cortex, and highlight different regenerative strategies. Notably, we focus on the intestinal epithelium as an excellent genetic model of plasticity due to the extensive characterization of stem and differentiated cell populations, which enable bona fide cell fate conversions to be established.

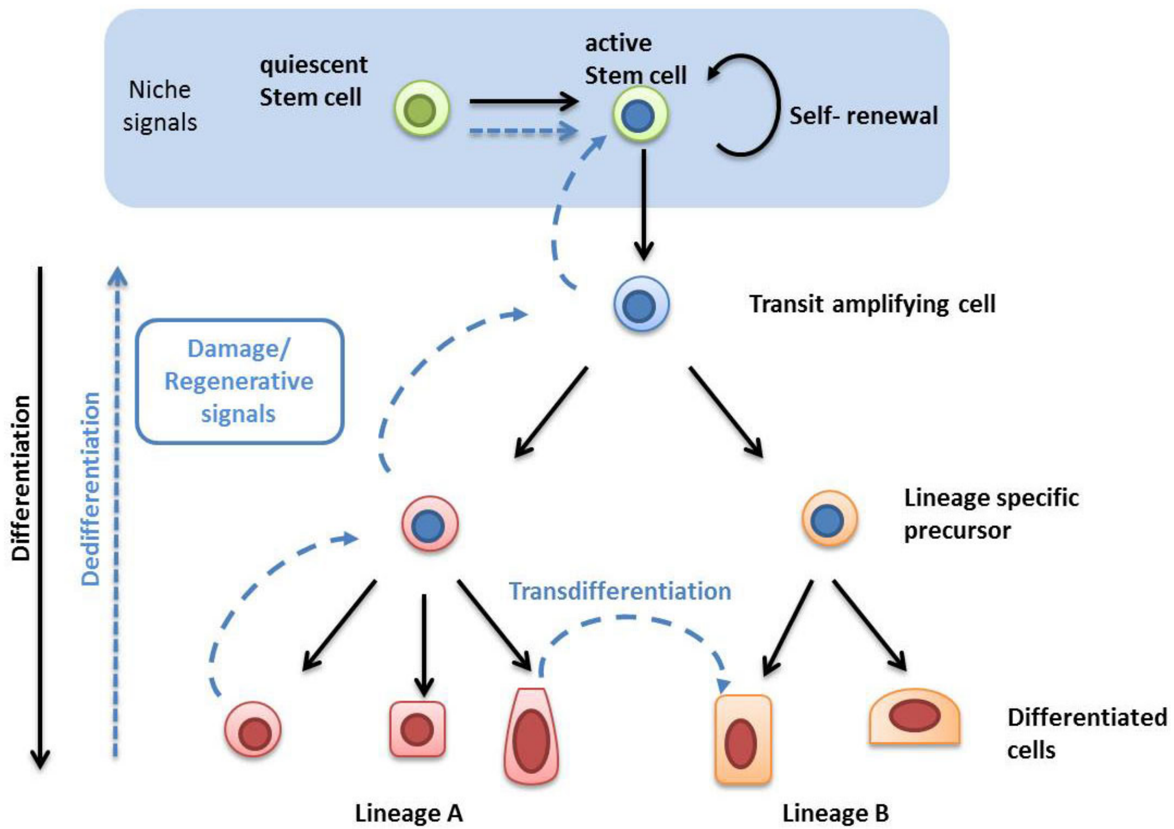


**Figure 1. Stem cell hierarchy in homeostasis and regeneration.** Niche signals drive stem cell self-renewal and differentiation to generate the specialized lineage populations that maintain the tissue during homeostasis. Cellular dynamics during homeostasis is indicated by black arrows. During damage and regeneration (blue arrows), cells can be replenished by mobilization of quiescent stem cells and increased proliferation of surviving stem cells. Alternatively committed cells can dedifferentiate and re-enter the cell cycle. Lost cells can also be replenished via transdifferentiation into another differentiated cell lineage. Proliferating cells are indicated with a blue nucleus, differentiated cells are represented with a red nucleus

### *The intestine as a model of plasticity*

Although the liver and pancreas are much more renowned for regeneration, their suitability as model systems to study epithelial plasticity is laden with controversies surrounding the existence of stem/progenitor cells during homeostasis and regeneration [4–7]. In the single-layered intestine epithelium, however, the localization of all stem cell populations and differentiated cells is known, all cell lineages have been extensively characterized, and multiple mouse models based on stem cell marker genes exist, as well as well-defined injury models that allow the elimination of specific cell types.

**Box 1. Genetic lineage tracing and lineage ablation test to stem cell identity and function**

**Lineage tracing is a technique used to identify stem cells based on their capacity of multi potency and self-renewal. In mice, genetic lineage tracing is performed using the Cre-loxP technique. Here, Cre recombinase is expressed under the control of a cell-type specific promoter. In combination with a second transgene, such as a fluorescent or enzymatic reporter that is inactivated by a loxP- flanked STOP cassette, Cre activity causes recombination between loxP sites, excision of the STOP cassette, and reporter expression, which is also inherited by all of the progeny. Fusion of the Cre enzyme to the tamoxifen-binding domain of the estrogen receptor (ERT) further allows temporal control of recombination activity upon tamoxifen injection.**

**To test the requirement of a particular cell population, a knocking genetic approach of the human DTR has been developed [26]. Mice do not endogenously express DTR, but transgenic expression from a cell-type specific promoter allows selective cell ablation in vivo upon DT administration. DTR lineage ablation after Cre labelling of non-stem cell populations can serve as a powerful assay to reveal the potential of 'reserve' stem cell populations.**

### *Stem cell populations in intestinal homeostasis*

The intestinal epithelium is the fastest self-renewing tissue in mammals. The rapid cellular turnover of the intestinal epithelium is propelled by daily proliferation of stem cells located at crypt bottoms to generate rapidly dividing daughter cells.

(Figure 2A). These in turn differentiate into secretory cells (Paneth cells, goblet cells, and enteroendocrine cells) or absorptive enterocytes, which make up the bulk of the villus epithelium (Figure 2B).

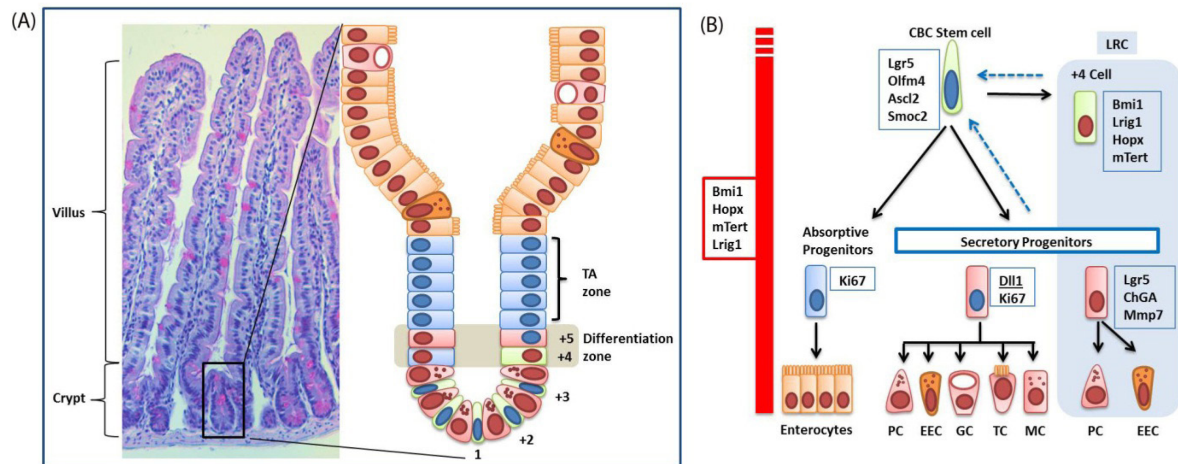


**Figure 2. Current model of the stem cell hierarchies in the intestinal epithelium during homeostasis and regeneration.** (A) The image shows a histological image of the mouse intestine [nuclei, blue hematoxylin, Goblet cells, red periodic acid–Schiff (PAS) staining], and the insert shows a schematic representation of a crypt unit and part of the villus. At the top of the hierarchy are highly proliferative crypt base columnar cells (CBCs) that are intermingled between Paneth cells at the crypt bottom. These divide to generate new CBC stem cells and daughter cells [transit amplifying (TA) cells] that are either absorptive progenitors, which proliferate and then differentiate into enterocytes that make up the bulk of the villus region, or progenitors of secretory cells. (B) The cell lineage relationships of diverse progenitor populations. The expression of markers, as discussed in the text, is indicated in the boxes. In homeostasis, proliferative Lgr5+ CBCs at the crypt base self-renew and spawn a heterogeneous population of daughter cells in the adjacent 'differentiation zone' at cell position +4/+5, where cell lineage decisions take place [see (A)]. This heterogeneous population includes transit amplifying cells (TA), and Dll1+ secretory progenitors with a limited proliferative capacity that give rise to Paneth cells (PC), enteroendocrine cells (EECs), goblet cells (GCs), and tuft cells (TC). Moreover, CBCs give rise to proliferative absorptive progenitors and quiescent label retaining cells (LRC, blue shaded region) that include Lgr5+ secretory precursors of PCs and EECs and an Lgr5- population that may represent the +4 stem cell. Proposed +4 markers such as Bmi1, Lrig1, and mTert have been shown to be ubiquitously expressed in all crypt cells as represented by the red line on the left. Upon damage-induced loss of CBCs, committed progenitors can dedifferentiate (broken blue arrows) to become CBCs and to restore homeostasis.

Cheng and Leblond proposed crypt base columnar (CBC) cells interposed between Paneth cells as the stem cells driving crypt homeostasis [8–10], which was later incorporated in the stem cell zone model of Bjerknes and Cheng [11,12]. The leucine-rich repeat containing G-protein coupled receptor 5 gene (Lgr5) has been identified as a specific marker of CBCs. Genetic lineage tracing (Box 1) in mice harboring a targeted insertion of an expression cassette of green fluorescent protein–internal ribosome entry site–Cre recombinase fused to a mutant human estrogen receptor ligand-binding domain (ERT2) (GFP–IRES–CreERT2) into the Lgr5 locus has revealed that CBCs are bona fide actively cycling, long-lived crypt stem cells that self-re- new and generate progeny comprising all the differentiated cell lineages of the intestinal epithelium [13]. With time, crypts become clonal, as indicated by the pattern of how spontaneous somatic mutations are propagated [14, 15]. Multi-color lineage tracing experiments have allowed the fate of many Lgr5+ stem cell clones to be followed in parallel. In these experiments, it has been shown that clonal evolution follows neutral drift dynamics in which a population of effectively equipotent Lgr5+ stem cells stochastically compete for crypt niche occupancy [16–18]. Furthermore, Lgr5-GFP+ cells sorted by single fluorescence-activated cell sorting (FACS) have the capacity to initiate ex vivo organoid cultures ('mini-guts') in 3D matrigel [19].

An alternative crypt stem cell has been postulated, characterized by its ability to retain DNA labels and occupying the +4 position as counted from the crypt base [20]. More recent lineage tracing studies based on genes, such as polycomb complex protein 1 oncogene (Bmi1), mouse telomerase reverse transcriptase gene (mTert), leucine rich repeat protein 1 gene (Lrig1), and Hop homeobox (Hopx) [21–24] have implied that a quiescent cell population located at the +4 position does indeed play a role in intestinal homeostasis. However, whether these stem cell pools represent distinct or overlapping populations, or how they relate during homeostasis and regeneration, has been the subject of intense debate.

**Plasticity of intestinal stem cell populations**



Several recent papers have addressed how alternative stem cell pools or non-stem cells (identified by several cellular markers; Figure 2B), might be mobilized after perturbation of the crypt stem cell niche. Models employing irradiation and cytotoxic damage with drugs such as 5- fluorouracil (5-FU) and doxorubicin, which induce apoptosis in proliferative cells, have demonstrated a rapid repair and replenishment of crypt units, implying that plasticity of rarely dividing or nonproliferative epithelial cells con- tributes to the regenerative process [25].

Employing a diphtheria toxin receptor (DTR) knockin genetic strategy to selectively delete Lgr5 expressing CBCs in vivo (Box 1), it was noticed that loss of actively dividing Lgr5-expressing CBC stem cells can be accommodated by the small intestine. Combiningthe Lgr5-DTR model with a Bmi1–CreERT2–RosaLacZ model, Bmi1-expressing cells expanded upon the elimination of CBCs to compensate for the loss of the actively cycling stem cell pool. However, following clearance of diphtheria toxin (DT), Lgr5+ cells rapidly reappear in vivo (as well as in mini-gut cultures derived from Bmi1+ cells), suggesting that a non-Lgr5+ stem cell population can convert to actively cycling Lgr5+ stem cells [26]. A parallel experiment involving irradiation of Lgr5–EGFP/Bmi1–CreERT2 models arrived at a similar conclusion. Here, Bmi1-expressing cells expanded upon irradiation to replace the depleted Lgr5+ stem cells [27]. Genetic lineage tracing using a Hopx–CreERT2/ RosaLacZ model (also marking +4 cells) provided further support for the mobilization of a quiescent stem cell pool, in the small intestine upon irradiation [22]. Re-evaluation of the mRNA expression of the purported +4 quiescent stem cell markers showed that they are rather broadly expressed in the crypt including in the Lgr5+ CBCs [28, 29]. Moreover, cells located above Paneth cells at the +4 position also express Lgr5 [30], indicating that lineage tracing may have originated from double Lgr5/Hopx- or Lgr5/Bmi1-expressing CBCs in these experiments. Indeed, a recent study demonstrated the importance of Lgr5- expressing progeny of CBCs in the Lgr5–DTR model during regeneration after DT injection in combination with radiation-induced damage [31]. Under this condition, depletion of Lgr5+ stem cells impaired the regenerative response, demonstrating

that reserve stem cells such as Bmi1+ cells and label retaining cells (LRCs) at the +4 position are unable to mediate efficient tissue repair following irradiation exceeding a critical threshold.



### *Dedifferentiation of committed progenitor cells in the intestine*

Within crypts, the Notch ligand Dll1 is expressed by secretory cell rogenitors at cell positions just above the stem cell zone, which are immediate descendants of Lgr5+ cells [32,33]. Lineage tracing using a Dll1–GFP–IRES– CreERT2 allele has shown that, during homeostasis, these cells generate small, short lived clones that comprise goblet cells, Paneth cells, tuft cells, and enteroendocrine cells. Following ablation of stem cells by irradiation, however, Dll1+ cells yield fully labeled crypt units including absorptive cells, indicating that secretory progenitors can dedifferentiate into stem cells in vivo [34]. This process might occur when Dll1+ cells 'fall back' to replace the deleted stem cells and re-establish contact with Paneth cells that secrete instructive niche signals [35]. The notion that contact with Paneth cells is essential for replacement of lost stem cells is also supported by conditional deletion of b-catenin mouse models. Here, replacement of lost crypt cells was critically dependent on the presence of wild type Paneth cells, while regeneration was significantly compromised upon b-catenin deletion in all crypt cells including Paneth cells [36].

A recent elegant study re-examined the identity and function of LRCs during homeostasis and damage responses, using an inducible histone 2B–yellow fluorescent protein (H2B–YFP) knockin mouse model [37]. Two populations of cells retained the chromatin label: (i) as expected, the long-lived Paneth cells (also corroborated in another study [38]); and (ii) a more heterogeneous population that is scattered in crypts and predominantly localizes around the +3/+4 position. Intriguingly, a fraction of the heterogeneous population was positive for Lgr5, Lrig1 and, prominin-1 (CD133) whereas the bulk expressed Paneth cell and enteroendocrine cell markers and might thus constitute a secretory precursor cell. Strikingly, the expression levels of proposed +4 markers such as Bmi1, mTert, Lrig1, and Hopx were similar in LRCs and CBC stem cells. Notably, YFP–LRCs could

form organoids in vitro when stimulated by Wnt3a, implying that exposure to niche signals can indeed revert them to multipotent stem cells. To study the function of LRCs, an innovative lineage tracing strategy was employed that relied on the cell cycling status rather than on marker genes. This system hinges on a split-Cre recombinase with one split-Cre moiety expressed under the ubiquitous Rosa26 promoter, and the second split-Cre moiety fused to H2B and expressed under the control of a drug-inducible [cytochrome P450 1A (Cyp1a)] promoter. Following H2B–Cre label incorporation into the genome, administration of a dimerizing agent allows temporal control of Cre activity. These experiments have shown that LRCs do not contribute to the stem cell pool during homeostasis. However, upon cytotoxic damage with doxorubicin, LRCs are able to give rise to multipotent stem cells supporting a role as reserve stem cells. Another recent study using a newly generated Ki67-RFP knockin allele has identified a quiescent Lgr5-low crypt population as the early secretory precursors, corroborating this data [39].

These recent studies reconcile the CBC stem cell model and the +4 model [11, 12, 20, and 40]. Actively cycling Lgr5+ CBCs are responsible for the day-to-day generation of new cell lineages. Among the CBC progeny are a subset of Lgr5-low/Bmi1+ quiescent cells that are precursors of Paneth and enteroendocrine cells, and are most likely equivalent to the noncycling +4 cells that can replenish the active stem cell pool upon damage-induced depletion (Figure 3). In effect, a dedicated quiescent, noncommitted stem cell pool in the intestine may not exist, as a committed progeny of Lgr5+ stem cells, including LRCs, can be a source of new stem cells by displaying plasticity. Since individual LRCs are relatively short-lived, they should not be considered stem cells in a strict sense. However, new LRCs are continually generated by the cycling Lgr5$^+$ stem cells. It has also been proposed elsewhere [38] that a pool of LRCs is, therefore, always available to be called into action as facultative reserve stem cells upon tissue damage. Whether fully differentiated intestinal cells such as enterocytes can revert to stem cells, and under which situation this might occur, will be worth investigating.

### *Epigenetic regulation of intestinal plasticity*

Differentiation is generally associated with epigenetic changes, such as DNA methylation and histone methylation and acetylation that regulate access to regulatory sequences for transcriptional activators and repressors [41]. Do niche signals such as Wnt, therefore, play a role in maintaining an epigenetic state that dictates 'stemness' and do epigenetic mechanisms play a role in dedifferentiation? In the intestine the loss of histone deacetylase enzymes (HDAC1/2) disrupts cell lineage commitment [42]. A recent study on histone marks that permit chromatin accessibility surprisingly showed that many intergenic regulatory regions were prominently marked, with no significant differences, between intestinal stem cells and their progeny committed to the secretory and enterocyte lineages [43]. This implies that regulatory regions in intestinal cells are continuously accessible to lineage specifying transcription factor, upstream niche factors, and other environmental factors to alter cell fate via dedifferentiation, or perhaps, transdifferentiation.

DNA methylation is thought to be a relatively stable silencing mark and methylation surrounding transcription start sites (TSS) normally correlates with gene repression [44]. Furthermore DNA methylation is generally most dynamic at regulatory regions outside the TSS, although the functional significance of these dynamics is often unclear. A recent study showed that DNA methylation is static at TSS during small intestine stem cell differentiation, and that the minimal changes observed primarily occurred at enhancer elements [45]. In a parallel study, conditional ablation of the maintenance DNA methylating enzyme Dnmt1 led to crypt expansion, suggesting that global DNA methylation is important for differentiation, and low level DNA methylation changes were identified at regulatory regions close to genes important for stem cell maintenance and differentiation [46]. Although the disparate conclusions could be a result of difference in the analytical methods employed, these methylation changes did not affect histone marks. Given that Dnmt3b knockout (loss of de novo methylation) has no effect in the intestine [47], it remains to be seen what the functional importance is of these minimal local changes during differentiation. Future studies are needed to clarify what level of

methylation change is biologically significant for lineage specification and whether other epigenetic factors such as chromatin remodelers play a role in intestinal plasticity.

1

### *Niche signals during intestinal regeneration*

Niche-derived signaling cues that regulate intestinal homeostasis have been well characterized (reviewed in [48]). Slit2 has been shown to increase stem cell numbers and augments the regenerative response upon damage in an autocrine fashion [49]; however, little is known about exogenous factors that control stem cells and mobilize non- stem cell populations during regeneration. Perhaps best understood is the Drosophila gut where multiple extrinsic signals including nutritional, inflammatory, and physical stress signals have been described to increase stem cell turnover [50, 51]. A role for diet in murine intestinal stem cell regulation, mediated by the niche, has been reported. Here, caloric restriction leads to inhibition of the mammalian target of rapamycin complex 1 (mTORC1) pathway, specifically in Paneth cells, inducing them to release the paracrine factor ADP-ribose, which enhances stem cell function. Furthermore, reduction in caloric intake improves the ex vivo organoid forming efficiency of isolated crypts and in vivo survival and proliferation of intestinal crypts after damage induced by ionizing irradiation [52].

Inflammatory cytokines released upon epithelial barrier damage act as mitogens for the epithelium and directly govern regenerative responses [53]. For example, secretion of interleukin-22 (IL-22) is induced after damage and controlsintestinal repair via activation of signal transducer and activator of transcription 3 (Stat3) signaling [54]. While this pathway facilitates wound healing, its deregulation can cause hyperproliferation and cancer [55]. Not surprisingly, IL-22 signaling activity is tightly controlled by constitutive production of neutralizing IL-22 binding protein (IL22-BP) by colonic dendritic cells [56, 57]. Investigating how signals facilitating plasticity yield normal cells during regeneration or induce neoplastic transformations of differentiated cells will be instrumental for their therapeutic applications.

### Stem cell organization and plasticity in other epithelial organs

Depending on anatomical and physiological constraints, other organs have adopted distinct strategies of self-renewal. In this section, we discuss emerging concepts from recent literature that employ genetic lineage tracing in mice to show how stem cell hierarchies and plasticity are realized in other mammalian epithelial tissues during homeostasis and regeneration.

#### *Dedifferentiation of committed mature airway cells in the lungs*

Mammalian lungs are made up of two distinct domains: the conducting airway tubes, consisting of trachea, bronchi, and bronchioles, and the alveolar spaces where gaseous exchange takes place. Each of these regions harbors unique stem cell types and progenitor cells, which are thought to give rise to the differentiated cells that maintain the functional integrity of the lungs. In the adult mouse trachea, a scattered population of epithelial basal stem cells expressing keratin 5 (K5), keratin 14 (K14), and tumor protein p63, is responsible for cellular turnover. Lineage- tracing experiments of the basal cells using K5–CreERT2 or K14–CreERT2 strains has shown that these cells have the potential to self-renew and generate two differentiated cell types (Clara cells and ciliated cells) during post-natal growth and after injury, placing the basal cells at the top of the cellular hierarchy to generate and repair the tracheal epithelium [58,59].

Recently, it has been demonstrated that differentiated, secretory Clara cells in the trachea can revert into functional stem cells in vivo upon ablation of basal stem cells (Figure 3A). The authors concluded that the presence of basal cells inhibits dedifferentiation of Clara cells during homeostasis. Using an ex vivo co-culturing assay, they could demonstrate that this inhibition is not dependent on a secreted factor. Whether direct cell contact of Clara cells to basal cells inhibits dedifferentiation, or whether acquisition of contact to the underlying basement membrane promotes their dedifferentiation, remains to be seen [60]. The propensity of committed cells to dedifferentiate was inversely correlated to their state of maturity. It will be interesting to ascertain whether this plasticity of long-lived lung cells predisposes them to mutational transformation and is

involved in heterogeneity of lung tumor subtypes and their varied responses to chemotherapy [60].



### *Dedifferentiation of mature proximal tubule epithelial cells in the kidney*

In the adult mammalian kidney, the number of functional nephron units is fixed and it is critical to repair the damaged low turnover epithelium to preserve organ function. Label retaining studies [using 5-bromo-2-deoxyuridine (BrdU) incorporation] have suggested the existence of slow cycling cells in the proximal tubule (PT) that may harbor stem cell potential [61]. Regeneration of the PT epithelium following ischemic reperfusion injury has previously been suggested to involve dedifferentiation and proliferation of all surviving epithelial cells based upon expression of embryonic markers and DNA label retention [62,63]. Recent lineage tracing experiments have corroborated the involvement of dedifferentiated epithelial cells in renal repair. In this study, the researchers used a mouse model where the CreERT2 cassette is under the control of the SLC34a1 gene promoter, which encodes a phosphate transporter that is only expressed in fully differentiated PT epithelial cells [64]. The combina- tion of injury with low dose tamoxifen induction to label single differentiated cells showed that the number of labeled cells increased post-ischemia, that they were highly proliferative, and thus contributed to tube elongation (Figure 3B). Interestingly, in kidneys that had undergone 'saturation labeling' with a high dose of tamoxifen followed by injury, no dilution of the label occurred post-ischemia, implying that no other progenitor population is involved in proximal renal tube repair [65]. It thus appears that dedifferentiation in the kidney may be a quicker and more efficient route to regeneration upon focal damage (such as ischemic reperfusion injury). These results, however, do not exclude the possibility that a distinct progenitor population may exist that is sensitive to ischemic damage [65,66], or that regeneration is by self-duplication of surviving differentiated cells rather than dedifferentiation. Identification of putative markers and lineage tracing procedures in combination with other injury models will be useful in clarifying these issues. Of note, this mechanism is highly reminiscent of that of the liver after hemi-hepatectomy where

fully differentiated hepatocytes rapidly enter the cell cycle to rebuild the missing part of the liver [67, 68].



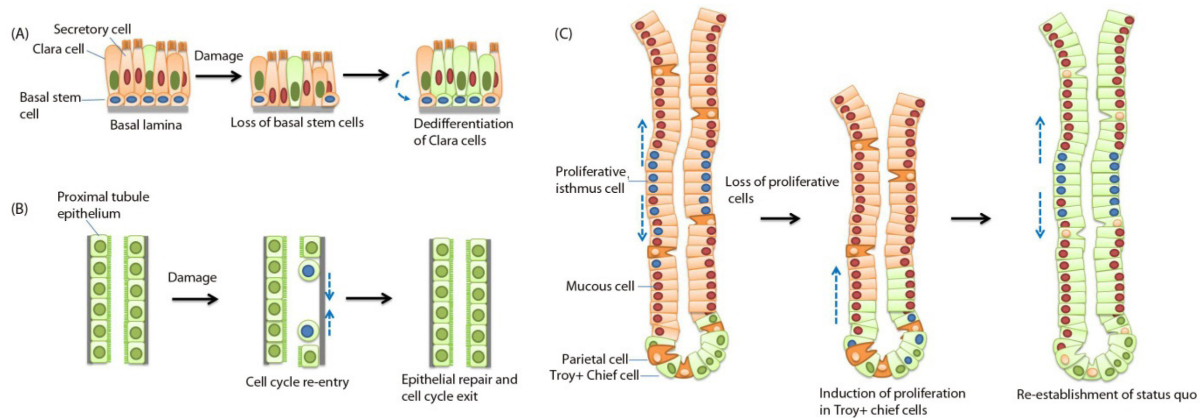


**Figure 3. Dedifferentiation of epithelial cells during regeneration.** (A) Basal stem cells generate differentiated Clara and secretory cells of the trachea during homeostasis. Damage-induced loss of basal stem cells leads to dedifferentiation of Clara cells to repair the lung epithelium; (B) Differentiated proximal tubule epithelial cells of the kidney become proliferative upon damage, generating new cells to repair the epithelium; (C) Troy+ differentiated chief cells populate the base of the stomach corpus epithelium. Upon damage-induced loss of proliferative isthmus cells, Troy+ chief cells become proliferative and are able to replenish proliferative isthmus cells as well as parietal cells, mucous cells, and neuroendocrine cells. Green colour marks plastic cell populations and their offspring during regeneration. Proliferative cells are signified with blue nuclei; differentiated cells have a red nucleus.

### *Dedifferentiation of mature chief cells in the stomach*

In the stomach, region-specific stem cell populations have been identified. In the distal pyloric antrum (also known as the pylorus), genetic lineage tracing using the Lgr5– CreERT2 mice has shown that cells at the base of the stomach glands give rise to all the differentiated lineages [69]. Similar to the intestine, these pyloric Lgr5+ cells are actively cycling, self-renewing every 3–4 days, are multi- potent, have a Wnt target gene expression signature, and can generate self-renewing gastric organoids in vitro. The existence of rare progenitor cells, marked by a villin transgene, can be found in the isthmus and base of stomach glands and under homeostatic conditions, appear quiescent. However, upon induction by inflammatory damage, notably by interferon gamma, they become proliferative and regenerate the entire gastric epithelium [70]. It is currently unclear what the nature of these

cells is, but they may represent the stomach version of intestinal LRCs. Independent genetic lineage tracing experiments have also identified a Sox2-expressing stem cell population composed of actively proliferating and quiescent subpopulations, just above the base of the pylorus glands [71]. However, it is unclear how these are functionally related to Lgr5+ pyloric stem cells.

In the corpus, Trefoil factor 2 protein gene TFF2+ cells located in the proliferative isthmus region have been identified as multipotent progenitor cells using genetic lineage tracing [72]. Using a Troy–GFP–IRES–CreERT2 model, a fully differentiated cell population with stem cell potential was recently identified [73]. Troy (also known as Tnfrsf19) is a Wnt target gene that is also expressed in Lgr5+ stem cells in the small intestine and colon [74]. In the corpus, however, Troy is expressed by a subpopulation of chief cells and parietal cells located at the gland base. During homeostasis, Troy+ chief cells fulfill all requirements of differentiated cells. Interestingly, upon removal of proliferative isthmus cells induced by 5-FU treatment, Troy+ chief cells re-enter the cell cycle to replenish the lost cells [73] (Figure 3C). In support of their stem cell potential, single isolated Troy+ chief cells can generate stomach organoids ex vivo that contain mucous cells of the neck and pit. Therefore, differentiated, quiescent Troy+ chief cells can turn into stem cells to regenerate damaged gastric glands of the corpus upon loss of the proliferative stem cell pool. It appears that this plasticity of Troy+ chief cells may be regulated by Wnt signaling, because these cells express multiple Wnt target genes. However, the exact source of Wnt ligands remains to be identified.

### *Transdifferentiation of mature hepatocytes in the liver*

The liver is undeniably the doyen of regeneration, where differentiated cells can function as facultative stem cells after injury [75]. Although lineage tracing techniques with putative marker genes have been employed to characterize various progenitor populations during homeostasis and regeneration, controversies still exist about the specificity of the cell fate, and the existence and functional characterization of liver stem cells (reviewed elsewhere [76,77]). A recent lineage tracing study using a hepatocyte specific adenovirus expressing a Cre

transgenic model crossed to a Rosa–YFP reporter line [68] confirmed a previous study [78], showing that, upon toxin injuries and bile duct ligation, mature hepatocytes transdifferentiate into differentiated biliary epithelial cells (Figure 4A), most likely through an a typical ductal cell intermediate, under the influence of Notch signaling to replenish lost cells. This process was thought to occur via a stepwise cascade of induction of biliary markers and morphological changes; although it is unclear if the hepatocytes undergo proliferation [68]. A follow-up study using the same adenovirus Cre/reporter model corroborated previous observations that, upon partial hepatectomy and toxin injuries, hepatocytes can also undergo self-duplication to generate new hepatocytes [7]. Identification of niches and markers that will allow lineage tracing of stem cell activity during homeostasis will be useful in clarifying cell lineage specification and plasticity in the liver.

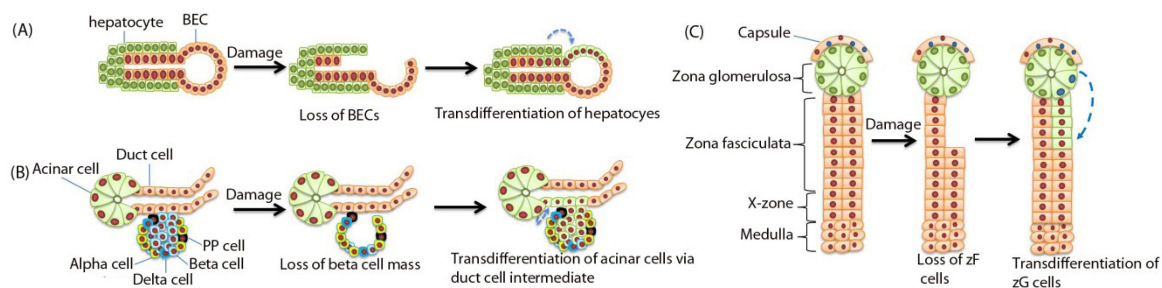


**Figure 4. Transdifferentiation of epithelial cells during regeneration.** (A) Hepatocytes in the liver transdifferentiate upon loss of biliary epithelial cells (BEC); (B) acinar cells of the exocrine pancreas transdifferentiate upon ablation of beta cells, which make up the bulk of the islet of Langerhans, possibly through a duct cell intermediate; (C) In homeostasis of adrenal cortex epithelium, proliferative cells in the capsule generate differentiated cells of zona glomerulosa (zG) and zona fasciculata (zF). Upon loss of zF cells, zG cells transdifferentiate to replenish lost zF cells concomitantly with cell cycle re-entry and cell migration. Green color marks plastic cell populations and their offspring during regeneration. Proliferative cells are signified with blue nuclei; differentiated cells have a red nucleus.

### *Transdifferentiation of acinar cells in the pancreas*

The pancreas epithelium has a low turnover and exerts both exocrine and endocrine secretory functions. The exocrine glands consist of differentiated acinar and ductal cells, whereas the endocrine function is mediated by differentiated epithelial cells in the islets of Langerhans, which consists of glucagon secreting alpha cells, insulin secreting beta cells, somatostatin secreting

delta cells, and polypeptide producing (PP) cells. Lineage tracing of alpha [79], beta [80], duct [81], and acinar cells [82] in the adult pancreas suggests that these cells self-duplicate without the need for a dedicated stem/progenitor cell population during homeostasis. Although controversies still surround the existence of pancreas stem cells and facultative progenitors, in vivo lineage tracing experiments have shown that transdifferentiation is utilized by the pancreas during regeneration [83]. Due to lack of space, we only highlight one recent in vivo study (read [4] for a detailed review). Combining acinar cell specific lineage tracing with pharmacological ablation of beta cells, it was shown that acinar cells can transdifferentiate into beta cells, most likely through a duct cell intermediate and regain of embryonic multipotency [84] (Figure 4B). Although the fraction of mature beta cells generated by this exocrine to endocrine interconversion was small, it widens the therapeutic options for de novo generation of beta cells in diseases such as diabetes.

### *Transdifferentiation in the adrenal cortex*

The adrenal cortex constitutes the outer layer of the capsule-surrounded adrenal gland and physiologically secretes steroid hormones. Anatomically, it is demarcated into an outermost zona glomerulosa (zG) layer, a middle zona fasciculate (zF), and an innermost reticularis (Figure 4B). In rodents, a poorly understood X zone lies between the reticularis and the medulla. The zG and zF comprise differentiated epithelial cells secreting mineralocorticoids (aldosterone) and glucocorticoids (such as corticosterone and cortisol), respectively. Progenitor cells located in the capsule and subcapsular region, and expressing Gli1 and Sonic Hedgehog (Shh), contribute to the differentiated cell lineages in the zG and zF of the adrenal cortex [85], which has a slow turnover of approximately 12 weeks. Another population of progenitors in the X zone has also been reported to be involved in generating differentiated cells of the adrenal cortex [86].

Recently, lineage-tracing studies using a zG-specific CreERT2 line have demonstrated that postnatal adrenocortical zonation involves direct lineage conversion of zG cells into zF cells during homeostasis [87] (Figure 4C). This transdifferentiation is also

observed during adrenal regeneration in older animals upon dexamethasone-induced ablation of zF cells. However, in the absence of zG cells, specification of zF cells was unperturbed implying that the adrenal cortex is highly plastic, with a preferred transdifferentiation mode of regeneration and alternate modes to generate zF cells. It will be interesting to deter- mine in the future if progenitors in the capsule or X zone, or other differentiated progenitor cells in the cortex cell or medulla, represent alternative sources for zF cells during regeneration.

## Concluding remarks

It is evident that multiple epithelial tissues in both fast- renewing as well as slow-renewing organs employ committed cells as reserve stem cells upon damage. Although there is much debate about the existence of distinct quiescent stem cells acting as reserve stems cells upon damage, it appears they may not be needed in the epithelial tissues discussed above, because differentiated cells can perform that function. In fact, it may not be easy to distinguish noncycling committed cells from genuine quiescent stem cells, unless lineage tracing strategies can be designed. It remains to be seen if the facultative employment of differentiated cells as reserve stem cells during regeneration represents a universal phenomenon in all epithelial tissues. Tissue-specific regeneration strategies that focus on defining the right conditions to 'awaken' the plasticity potential of differentiated cells (be it in vivo or in vitro) might be a valuable alternative to stem cell-focused strategies.


## Acknowledgements

The authors thank I. Heo, O. Basak, L. Kaaij, and H. Gehart for critical reading of the manuscript of this review. P.W.T. was supported by a Netherlands Organization for Scientific Research (NWO) personal grant. H.F.F. was supported by an EMBO long-term fellowship.

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## OUTLINE OF THE THESIS

Intestinal stem cells exhibit plasticity during homeostasis to generate all the differentiated cell types that maintain the functional integrity of the intestine. Whether all the differentiated cells in the intestine also possess such plasticity, and under which conditions, is the subject of intense investigation. In this thesis I report on the generation of novel mouse models to examine the plasticity of specific differentiated cell types in intestinal regeneration and cancer.

Committed secretory progenitors resident in the intestinal crypt, and that give rise Paneth cells, enteroendocrine cells, and goblet cells have been shown to revert to stem cells upon irradiation or cytotoxic induced loss of the regular actively cycling Lgr5+ stem cell pool. We reasoned that the numerous absorptive progenitors in crypts that give rise to enterocytes might also exhibit such plasticity and contribute to intestinal regeneration. To this effect, I generated a novel enterocyte specific cre line. In chapter 2 of this thesis, I describe the generation and characterization of the enterocyte specific cre line. In combination with stem cell injury models, I report the involvement of committed enterocyte progenitors in intestinal regeneration.

Recent studies have reported small intestinal cancer initiation due to plasticity of mutated differentiated villus cells using Cre mouse models. However it is unclear which specific differentiated cells are responsible for the tumor initiation as models used in these studies have ubiquitous cre expression in all differentiated cells. Because enterocytes form the bulk of the villus, they have been assumed to be tumor initiating cells in these studies. In chapter 3, we examine the tumor initiating propensity of differentiated enterocytes in vivo and ex vivo using our novel enterocyte specific Cre line. Contrary to previous reports, we do not observe tumorigenic plasticity of enterocytes upon mutations of the Apc and K-ras genes. We also address the role of the stem cell niche in tumor formation from enterocyte progenitors.

In chapter 4, we describe the generation and characterization of an inducible colon specific Cre model for colorectal cancer research. Cre expression in this model is observed in most epithelial cells in the cecum, and restricted to differentiated colonoic epithelial cells in the proximal colon. We show that Apc mutations in differentiated colonocytes in the proximal colon does not yield adenomas however combined Apc/K-ras mutations makes them plastic, transforming them into tumor initiating cells with cancer stem cell characteristics.

In chapter 5, I describe the generation of a floxed allele for Amica1, a stem cell specific transmembrane molecule, and show preliminary results upon intestinal specific deletion of this gene.

In chapter 6, I describe the generation of conditional alleles for Zfp12 and Rbak, novel transcriptional regulators expressed in Lgr5+ stem cells

In chapter 7, I give a summarizing discussion of the work presented in this thesis.

# CHAPTER 2

# Replacement of lost Lgr5+ stem cells through plasticity of their enterocyte-lineage daughters

2

Submitted to Cell Stem Cell (in revision)

**Replacement of lost Lgr5+ stem cells through plasticity of their enterocyte-lineage daughters**

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**Summary**

Intestinal crypts display robust regeneration upon injury. The relatively rare secretory precursors can replace lost stem cells, but it is unknown if the abundant enterocyte progenitors also have this capacity. Alkaline phosphate intestinal (Alpi) is uniquely expressed in the enterocyte lineage: modestly in enterocyte crypt precursors and very high in mature enterocytes on the villus. We created an Alpi-Creert2 knock-in allele for lineage tracing. Marked clones consist entirely of enterocytes and are all lost from villus tips within days. To test plasticity of enterocytes upon stem cell loss, we crossed Alpi-Creert2; R26R-LacZ mice with Lgr5-DTR mice. Lineage tracing was induced before or during ablation of Lgr5-expressing stem cells using diphtheria toxin (DT). We observed numerous long-lived crypt-villus 'ribbons', indicative of dedifferentiation of enterocyte precursors into Lgr5+ stems. We conclude that the highly proliferative, short-lived enterocyte precursors serve as a large reservoir of potential stem cells during crypt regeneration.

## Introduction

In mammals, the intestinal epithelium is the fastest self-renewing tissue (Clevers, 2013). The rapid cellular turnover of the single-layered intestinal epithelium is powered by proliferation in the crypts of Lieberkühn to generate differentiated villus cells. Actively cycling crypt base-resident Lgr5+ stem cells (Barker et al., 2007) generate precursors of secretory cells and of enterocytes, that divide while moving upwards terminally differentiating into either goblet cells and enteroendocrine cells or into nutrient-absorbing enterocytes. The atypical Paneth cells belong to the secretory lineage, yet reside at crypt bottoms, are long-lived and contribute to the stem cell niche (Sato et al., 2011). The crypts display a remarkable regenerative capacity following DNA and cytotoxic damage (Withers, 1971) or for instance surgical resection (Bernal et al., 2005).

Although surviving stem cells play a critical role in this regenerative process (van der Flier et al., 2009a), it has been proposed that a quiescent stem cell population residing at the +4 position and expressing markers such as Bmi1, mTert, Lrig1 and Hopx (Sangiorgi and Capecchi, 2008)(Takeda et al., 2011)(Powell et al., 2012)(Montgomery et al., 2010) may function as reserve stem cells upon depletion of the actively cycling stem cell pool. As an alternative mechanism, intestinal regeneration may be driven by dedifferentiation of committed progeny. As implied by a recent study, radiation-sensitive cells occupying cell position 6 and above can replenish loss of Lgr5+ stem cells (Metcalfe et al., 2013). During homeostasis, secretory progenitors derived from Lgr5+ stem cells and expressing Dll1 generate short-lived clones comprising of Paneth cells, goblet cells, enteroendocrine cells and tuft cells. Lineage tracing followed by irradiation in Dll1-GFP-IRES-Creert2 mice indicated that Dll1+ cells dedifferentiated to stem cells in vivo to replenish lost stem cells, generating long-lived stem cell-driven crypt-villus ribbons (van Es et al., 2012).

Recently, an elegant study corroborated the involvement of secretory precursors in intestinal regeneration. Buczaki et al observed that a quiescent label-retain cell (LRC) population predominantly populates the +4 position and express Lgr5 as well as the proposed markers of +4 cells such as Bmi1, mTert, HopX and Lrig1. During homeostasis, these LRCs (which derive from Lgr5+ stem cells) serve as short-lived precursors of Paneth and enteroendocrine cells. However, upon loss of proliferative crypt

cells induced by cytotoxic damage with doxorubicin, these LRCs dedifferentiate to Lgr5+ stem cells (Buczacki et al., 2013).

**Results**

**Enterocyte marker, Alpi is not expressed in Lgr5+ CBC stem cells**



We sought to establish if the most abundant and most proliferative cell type in the crypt, the enterocyte precursor, could display similar plasticity. Previous gene expression data sets involving microarray analysis of FACS-sorted crypt populations (Muñoz et al., 2012) revealed that the enterocyte differentiation marker alkaline phosphatase intestinal (Alpi) was absent in Lgr5high cells, but showed low yet detectable levels in Lgr5 low early offspring (Figure 1A). Alpi is highly expressed in mature small intestinal enterocytes and encodes the alkaline phosphatase enzyme involved in lipid absorption, pH regulation, attenuating bacterial invasion via detoxification of lipopolysaccharide, intestinal inflammatory response, and modulation of gut microbiota. Of note, Alpi-/- mice are viable and fertile (Narisawa et al., 2003).

In situ hybridization analysis confirmed high-level expression in villus enterocytes (Figure 1B), and low but detectable expression in cells in the upper crypt. Single molecule FISH analysis for Alpi and Lgr5 confirmed that Alpi transcripts in the crypts do not co-localize with Lgr5 transcripts at the crypt bottom implying that Alpi is not expressed in Lgr5 stem cells (Figure 1 C-F). Alpi transcripts were observed in most cells from cell position +6 or +7 upwards to the top of the crypt (Figure 1D), coinciding with the Ki67+ (supplementary figure 4) transit-amplifying (TA) cells (Itzkovitz et al., 2012), implying that Alpi+ enterocyte progenitors are proliferative and make up the bulk of the TA zone. Furthermore, single molecule FISH for Alpi and Dll1 showed that Alpi+ transcripts do not localize with Dll1+ transcripts located at the +4/+5 position from the crypt base (supplementary figure 5) implying that Alpi+ progenitors are distinct from Dll1+ secretory progenitors.

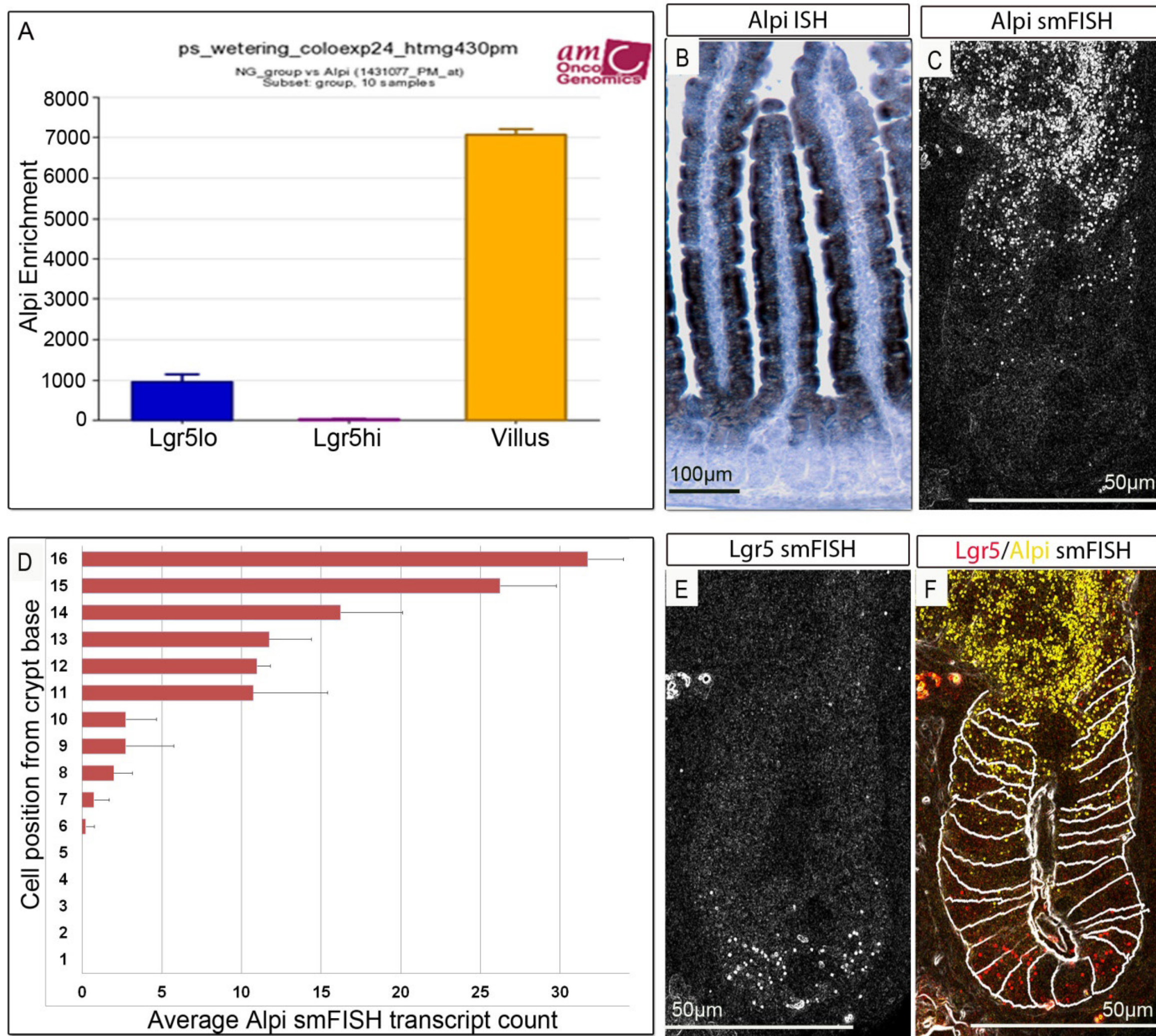


**Figure 1. Expression of Alpi in intestinal crypts-** A) Microarray enrichment of Alpi in Lgr5 low, Lgr5 high and villus fractions; Alpi is not expressed in Lgr5 high cells; B) In situ hybridization of Alpi in the intestinal crypt shows that Alpi is not expressed in lower crypt cells (magnification: x20). C-F) Single molecule FISH for Alpi (C) ;D) Alpi transcripts can be detected from position +6 and progressively increasing to the top of the crypt; (E) single molecule FISH for Lgr5; F) Lgr5 transcripts (red dots) localize at crypt bottom and do not overlap with Alpi transcripts (yellow dots).

## Generation of an inducible enterocyte specific Cre line

To generate an enterocyte specific Cre-line, we inserted an internal ribosome entry site (IRES)-CreERT2 cassette at the stop codon located in the last exon of the Alpi gene (suppl. Figure 1A). This strategy employs the endogenous poly A signal and the 3'UTR of the Alpi gene. To characterize Cre activity of the Alpi-CreER allele, mice were crossed to R26R-LacZ reporter mice where the lacZ gene is under the control of the ubiquitous

R26 locus (Soriano, 1999). Eight-to-twelve-week old mice were injected with a single dose of tamoxifen (TAM), and sacrificed at various time points for LacZ analysis. As expected, LacZ+ cells were observed exclusively in enterocytes in the villus domains of the proximal small intestine (Figure 2A-D) but never in villus goblet cells or enteroendocrine cells (supplementary figure 2A and B). At the earliest time point, LacZ+ cells were detected in the upper crypt but never in the bottom half (Figure 2A). Nearly all LacZ+ cells disappeared within 6 days with occasional single labelled cells at the top of the villus. Importantly, no labeled cells (be it single cells or clones) were detected 28 days after tamoxifen injection (Figure 2F). No LacZ staining was ever observed in non-induced mice.



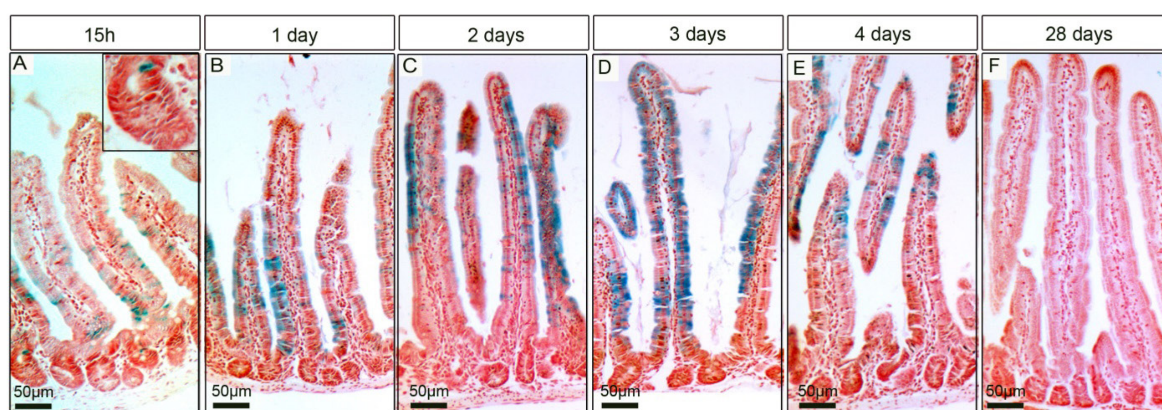


**Figure 2. Histological analysis of LacZ activity in Alpi-CreER KI.** Mice were induced with 5mg/kg 4OH-tamoxifen (TAM) and then sacrificed after A) 15h, B) 1 day, C) 2days, D) 3days, E) 4days and F) 28 days. After 15 hours, majority of the cells labelled were located in the villus. Occasionally, labelled cells were observed in the neck of the crypt or the upper crypt level. Of note, no labelled cells were detected at the bottom of the crypts. By Day2 labelled cells had almost reached the villus tip. By Day3 labelled cells had reached the tips. By Day 4 labelled cells were observed only in the upper half of the villus implying that most of the labelled cells had completed their life cycle, being shed in the lumen. No labelled cells by day 28 were observed implying that Alpi is not expressed in stem cells (magnification: x10).

## Alpi+ enterocytes dedifferentiate into stem cells upon depletion of Lgr5+ stem cells

We next investigated whether absorptive Alpi-expressing enterocytes were capable of conversion into Lgr5+ crypt stem cells. To do so, we crossed Alpi-CreER ; R26R-LacZ mice with Lgr5-DTR-GFP mice (Tian et al., 2011). In these mice, injection of diphtheria toxin (DT) will cause depletion of $Lgr5^+$ stem cells as well as $Lgr5^+$ quiescent secretory progenitors residing around the +4 position (Buczacki et al., 2013). Alpi-CreER ; R26R-LacZ ; Lgr5-DTR+/- mice were treated simultaneously with TAM and DT and their duodenums were analyzed by LacZ staining 14 days post-injection. As

controls, we used Alpi-CreER ; R26R-LacZ mice also treated with TAM and DT. Whole mount X-gal staining and subsequent histological analysis of Alpi-CreER ; R26R-LacZ ; Lgr5-DTR+/- mice revealed many contiguous ribbons of LacZ$^{+}$ cells emanating from crypt bottoms and extending up towards adjacent villi (Figure 3C) (500-600 ribbons per mouse). Significantly, no tracing events could be observed in the control mice (Figures 3A and 3B). Similar results were obtained when Cre expression was induced one day before DT administration, albeit at a somewhat lower frequency (200-300 tracing events per mouse; Figure 3E).



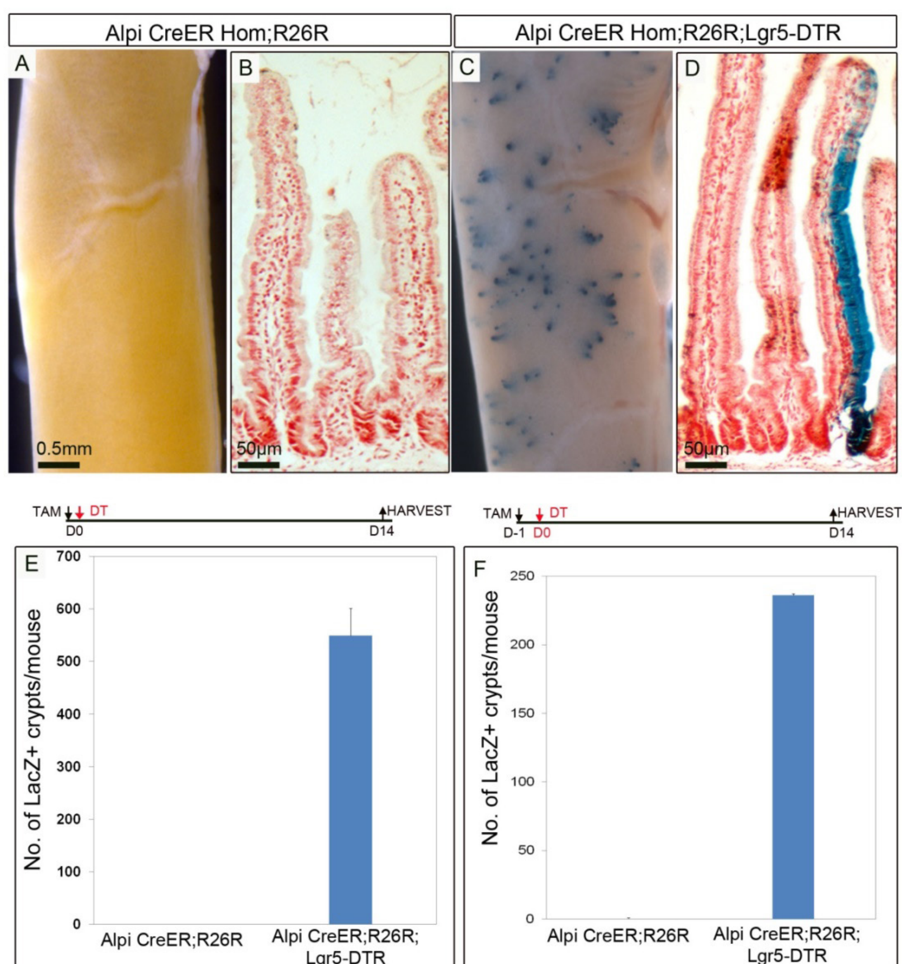


**Figure 3. Alpi+ enterocytes dedifferentiate upon depletion of Lgr5 stem cells.** Alpi-CreER Hom;R26R (control) and Alpi-CreER Hom;R26R;Lgr5-DTR mice were given a single injection each of 5mg/kg TAM and 50µg/kg DT and harvested after 2 weeks. X-gal stained proximal intestine A) whole mount and B) histological section showing no staining in control group;C and D) whole mount staining and histological section of X-gal stained proximal intestine showing beta-galactosidase + crypt/villus units in stem cell depleted group; Quantification of B-gal+ crypt/villus crypts in control and stem cell deleted group upon simultaneous TAM/DT injection (E) and TAM injection one day before DT treatment (F). Beta-galactosidase staining signifying Alpi+ progenitor dedifferentiation events was higher with simultaneous TAM/DT injection.

Contiguous LacZ+ ribbons were still detected in crypt villus units in Alpi-CreER ; R26R-LacZ ; Lgr5-DTR-GFP mice after 1 month positive for GFP expressed by Lgr5+ stem cells, and contained Paneth, enteroendocrine, goblet cells and tuft cells (not shown) of the secretory lineage (Figure 4D-F) implying that dedifferentiated Alpi+ cells exhibited the Lgr5+ stem cell characteristics of self-renewal and multipotency. Of note, the number of tracing events from dedifferentiated enterocytes was significantly diminished (average of 10 tracing events/ mouse) upon simultaneous injection of AlpiCreER; R26R-LacZ mice with the antiproliferative drug, 5-fluorouracil (5-FU) and TAM (supplementary figure 3) supporting the notion that proliferative progenitors are responsible for the bulk of the observed rescue.



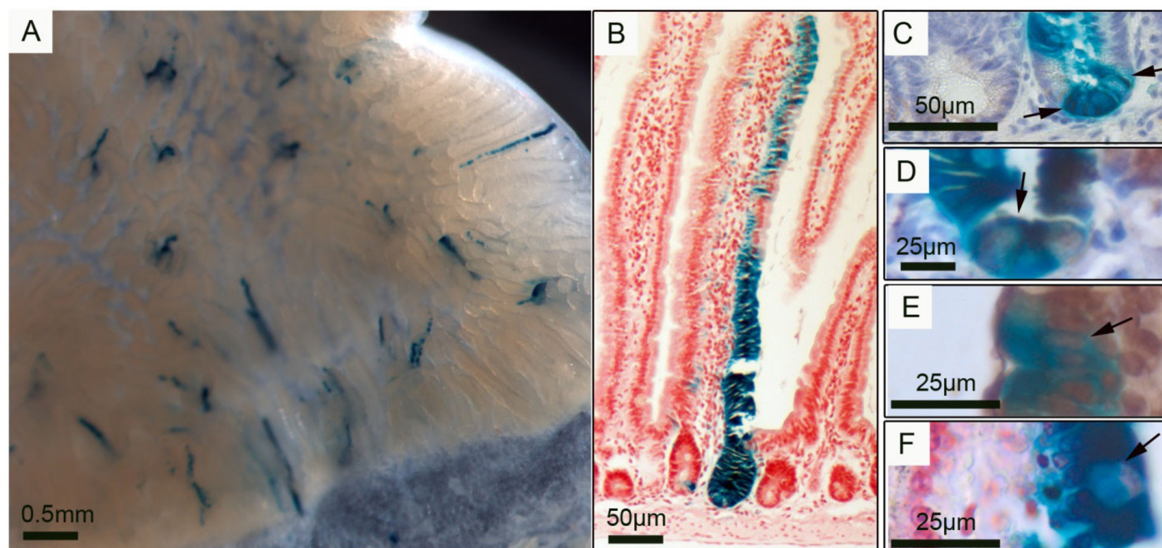


**Figure 4. Dedifferentiated Alpi+ cells are long-lived and multipotent.** Whole mount staining (A) and histological section (B) of beta-galactosidase + crypt villus units after long term analysis (3 months) signifying self-renewal of Alpi+ dedifferentiated cells; C) Co-staining of beta-galactosidase with anti-GFP antibody showing dedifferentiated Alpi+ cells give rise to Lgr5+/GFP+ stem cells (depicted with arrows). D-F Secretory cells derived from dedifferentiatd Alpi+ cells (depicted with arrows); Co-staining of beta-galactosidase with secretory cell markers in Alpi-CreER Hom;R26R; Lgr5-DTR mice dosed with a single injection of both TAM/DT shows that dedifferentiated Alpi+ cells give rise to Lyz+ Paneth cells (D), ChrgA+ enteroendocrine cells (E), and Muc2+ goblet cells (F)

## Discussion

Combined with previous studies, our current observations underscore the extent of plasticity of crypt progenitors. Previous reports have demonstrated that cycling secretory progenitors (van Es et al., 2012) as

well as quiescent secretory precursors (Buczacki et al., 2013) can revert to a multipotent state upon loss of resident Lgr5 stem cells. We now find that a population that makes up the bulk of the crypt above the stem/Paneth cell niche, also displays similar plasticity. The classical view of an adult stem cell hierarchy such as defined for hematopoietic stem cells appears not to apply to the crypt. Rather, crypts are populated by multiple committed progenitors that can revert to a stem cell phenotype when exposed to the niche at the crypt bottom. Similar mechanisms of daughter cell plasticity is emerging in other epithelial systems, i.e. the dedifferentiation of committed mature airway cells in the lung (Tata et al., 2013), and of Troy+ chief cells in the gastric corpus (Stange et al., 2013). A plausible explanation for the observed enterocyte plasticity could rest in a permissive epigenetic state in enterocyte precursors. Recent studies on DNA methylation and histone marks in intestinal crypt/villus cells revealed the virtual absence of differences between Lgr5+ stem cells and committed enterocytes precursors (Kim et al., 2014)(Kaaij et al., 2013), in striking contrast to the situation in the hematopoietic stem cell hierarchy (Hogart et al., 2012) (Hodges et al., 2011) (Ji et al., 2010), implying that the chromatin in enterocytes is permissive for rapid reprogramming into Lgr5+ stem cells during regeneration. At least in crypts, 'stemness' does not appear to be an intrinsic, 'hard-wired' property of rare stem cells, but can be imposed on any progenitor by the niche.

**Acknowledgements**

The authors express their sincere gratitude to Stieneke van den Brink, Nobuo Sasaki, Anna van Oudenaarden, Kay Wiebrands and Lennart Kester for excellent technical assistance.

PWT was supported by a Netherlands Organization for Scientific Research (NWO) personal grant.

**Conflict of interest declaration**

The authors declare no competing financial interests.

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## Experimental procedures

### *Generation of mice*

Lgr5-DTR-GFP mice have been previously described (Tian et al., 2011).

Knock-in construct for generation of Alpi-CreER mice was assembled according to the diagram in **supplementary figure 1A.** Oligonucleotides used for targeting arms are given in Supplementary table 1A and 1B. 100ug of the targeting construct was linearized and transfected (800V; 3F) into embryonic stem (ES) cells derived from 129/Ola-derived IB10 strain. Recombined ES clones expressing the neomycin gene were selected in G418 (200g/ml) supplemented medium.

Southern blot analysis **(supplementary figure 1B)** with a probe upstream of the targeted region confirmed precise homologous recombination in approximately 5 in 100 ES clones. Southern blot probe oligonucleotides are given in Supplementary table.

Two independent positive clones were injected into C57BL/6 blastocysts according to standard procedures. The neomycin selection cassette flanked by FRT sites, was excised in vivo by crossing the mice with FLP1 mice.

Heterozygous and homozygous mice were retrieved at the expected Mendelian ratios at birth, and adult transgenic animals showed no discernible abnormality, with comparable life span and fertility compared to wild type litter mates.

Rosa26-LacZ Cre reporter mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). 8-14 week old mice were injected intraperitoneally with 5mg/kg of Tamoxifen and 50ug/kg Diphtheria toxin. All procedures were performed in compliance with local animal welfare laws, guidelines and procedures.

### X-gal staining

Proximal intestines isolated from mice were fixed for 2 hours on ice with fix solution (1% paraformaldehyde (PFA), 0.2% glutaraldehyde, and 0.02% NP40 in PBSO), and washed twice for 15min in PBSO. This was followed by overnight staining in the dark with 1mg/ml X-gal in PBSO solution containing 5mmol/L potassium-hexacynoferrate III, 5mmol/L potassium-hexacynoferrate (IV) trihydrate, 2mmol/L magnesium chloride, 0.02% NP40 and 0.1% sodium deoxycholate. Subsequently, tissues were washed twice in PBSO and whole mount analyzed for LacZ positivity followed by overnight fixation in 4% PFA, and paraffin embedded using standard

procedures. 4-8um tissue sections were counterstained with neutral red. Three mice per each experimental group were used for analysis.

**Immunohistochemistry**

Mice tissues were fixed in 4% PFA overnight, paraffin embedded and sectioned at 4-10um. Immunohistochemistry was carried out as previous described (Barker et al., 2007) using the following antibodies for immunostaining: anti-mucin 2 (Santa Cruz, sc-15334; 1:250) , rabbit anti-ChrgA (Millipore; 1:4000), anti-GFP (Abcam; 1:100) and anti-lysozyme (Dako, 1:1,500, A009902).



**In situ hybridization**

In situ hybridization probe targeting Alpi was generated by PCR from whole intestine cDNA using the oligonucleotides given in Supplementary Table 1, with the antisense primer tethered to T3 promoter sequence. Tissue preparation and hybridization procedures were as previously described (van der Flier et al., 2009b).

Microarray analysis was performed with data uploaded on the ‘R2: microarray analysis and visualization platform (http://r2.amc.nl).

**Single molecule FISH**

Probe library for Alpi were designed and constructed as previously described. Library consisted of 48 probes of length 20 base pairs, complementary to the coding sequence of Alpi. Lgr5-Cy5 probe was a kind gift from Anna van Oudenaarden. Tissue processing and hybridization procedures were according to protocol described in (Lyubimova et al., 2013). Briefly, hybridizations were carried out overnight with Lgr5 labelled Cy5 probe and Alexa 594 labeled Alpi probe. DAPI dye was added to washing buffer followed by counterstaining with Phalloidin. Images were taken with a Leica MM-AF fluorescence microscope equipped with a 100X oil-immersion objective and a Princeton Instruments camera using Metamorph software (Molecular Devices). Image-plane pixel dimensions was 0.13um. Quantification of transcripts in 10 crypts,was carried out on 20 stacks with a Z spacing of 0.3um. Image processing was done with ImageJ software, using the variance filter for image enhancement.

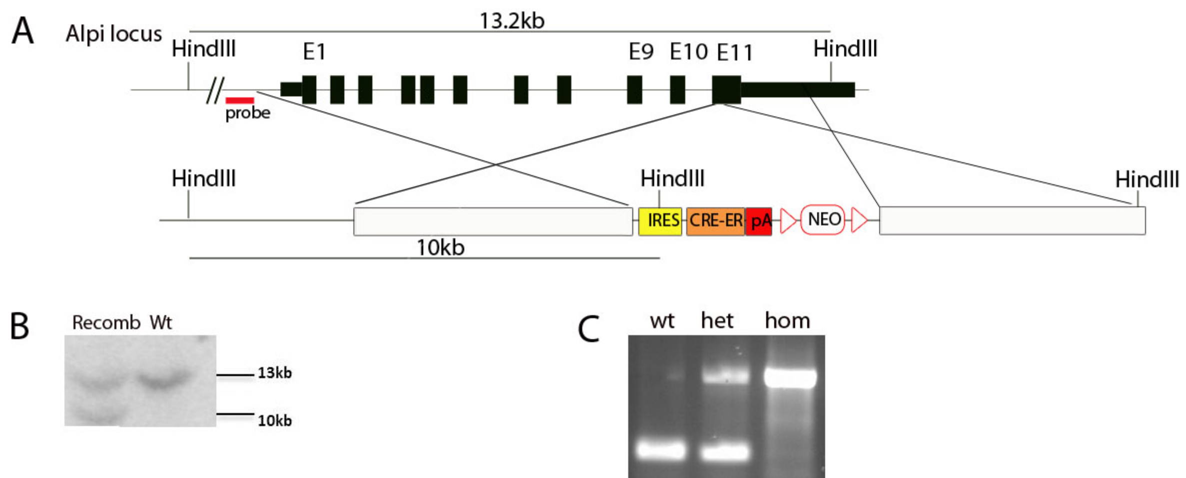
### Organoid Culture

Mouse organoids were established and maintained from isolated crypts of the proximal small intestine as previously described (Sato et al., 2009). To detect B-gal in organoids cultured for more than 3 days, 4-hydroxytamoxifen (4-OHT; Sigma; 10nmol/L) was added to the culture medium for 10hours. B-gal staining was performed in organoids still in matrigel, as described above.

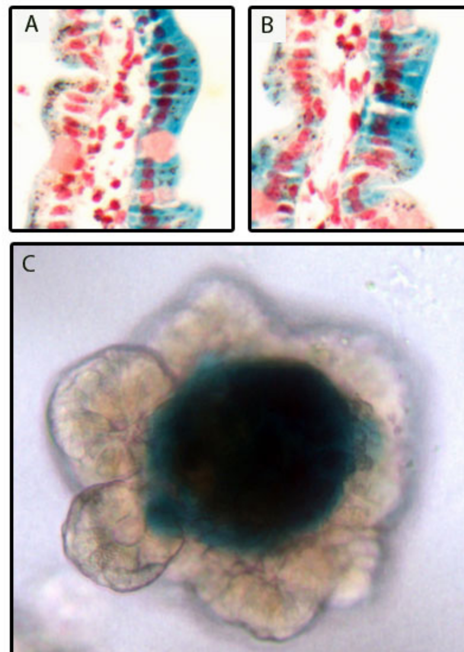


**Supplementary Table 1. List of oligonucleotides**

| | | |
|---|---|---|
| A | **Amplification of 5'arm** | |
| | Alpi 5arm forward | CCAACACTGGGATTATGGGCA |
| | Alpi 5arm rev | TCAGGACACCACCATGGCTGT |
| | Product size | 3889bp |
| | | |
| B | **Amplification of 5'arm** | |
| | Alpi 3arm fwd | TGATGGTCAGGTCCTCCGCTG |
| | Alpi 3arm rev | AGACAGCTACAGTGTACTTCG |
| | Product size | 4000bp |
| | | |
| C | **Alpi-CreER genotyping primers** | |
| | Alpi.wt.geno.fwd.5 | ACTGTCCTGCTGTCCTTGC |
| | Alpi.wt.geno.rev.5 | CCGGAACTGATTGGGCTCAG |
| | Cre geno fwd 3 | gctggacgcccaccgcctac |
| | Wild type band | 193bp |
| | Knockin band | 560bp |
| | | |
| D | **Alpi in situ hybridization primers** | |
| | fwd | **GAGCCCGGTATGTTTGGAAC** |
| | rev | ***AATTAACCCTCACTAAAG*****GGAACTGATTGGGCT-CAGGAAATC** |
| | Product size | 500bp |
| | | |
| E | **Southern probe primers** | |
| | Alpi_SP_fwd | TCTGGAGTTAGGCCTGTAGCCA |
| | Alpi_SP_rev | ACCACTGCCCATATATACTATGC |
| | Product size | 310bp |
| | | |




***Supplementary Figure 1: Targeting strategy and genotyping.*** *A) Targeting strategy of Alpi-CreER KI. Oligonucleotides for amplifying arms from ES cell genomic DNA are supplied in supplementary table 1A and 1B; B) Southern blot of successfully targeted ES cell clone (double band) and non-recombined wild type (wt) ES clone (single band); C)Genotyping PCR for Alpi-CreER KI mice. Genotyping primers and DNA band sizes are supplied in supplementary table 1A.*




***Supplementary Figure 2: Specificity of Alpi-CreER KI****. Alpi expression is restricted to villus enterocytes and not expressed in other villus cells such as A) enteroendocrine cells and B) goblet cells. C) Alpi expression is restricted to the villus domain of organoid cultures generated from isolated crypts from Alpi-CreER Hom mice.*

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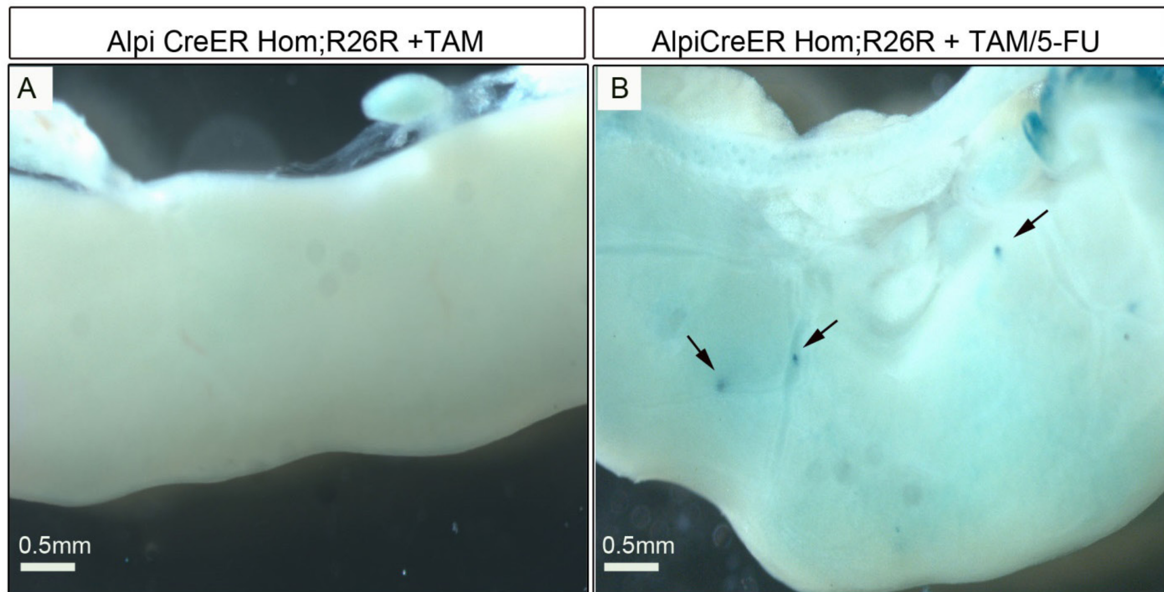
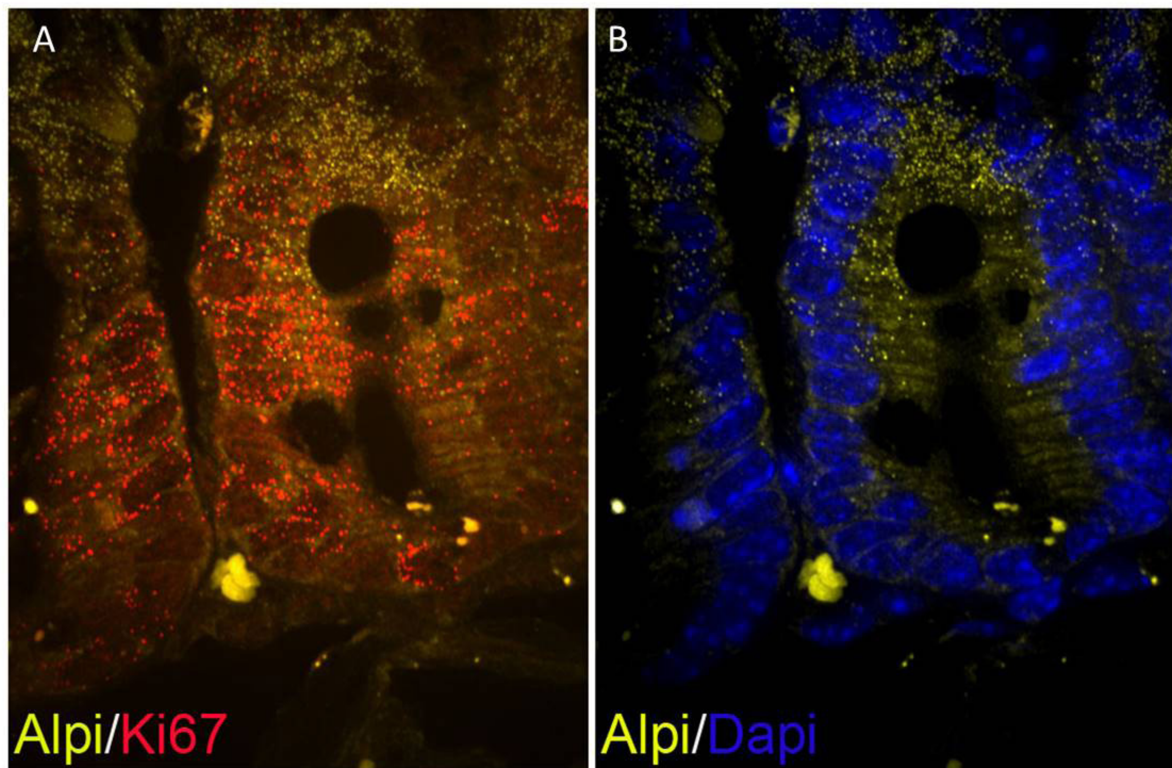
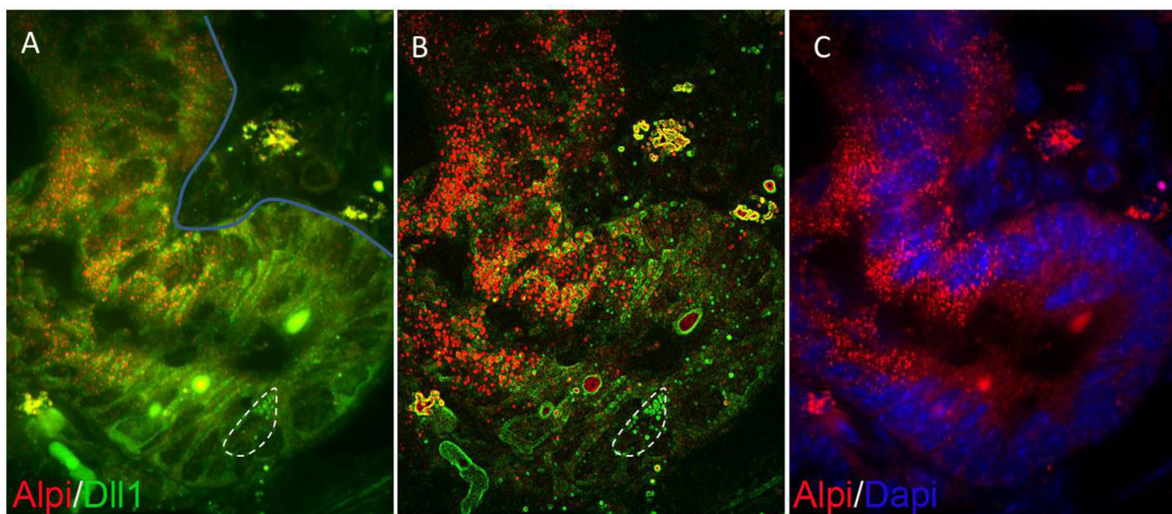



***Supplementary Figure 3: Dedifferentiation of Alpi + enterocytes upon 5-FU treatment.*** *Alpi-CreER;R26R mice were given either A) single dose of 5mg/kg TAM (control) or B) TAM and 150mg/kg 5-fluorouracil (5-FU) and intestines harvested after 1 week for lacZ activity. Dedifferentiation capacity of proliferative Alpi+ enterocyte progenitors was significantly impaired, yielding an average of 10 X-gal stained crypts/mouse (depicted by black arrows).*




**Supplementary figure 4. Alpi/Ki67 single molecule FISH.** Single molecule FISH for Alpi transcripts (yellow) colocalize with transcripts for Ki67 (red) implying Alpi+ enterocyte progenitors in the crypts are proliferative.




**Supplementary figure 5. Alpi/ Dll1 single molecule FISH**. Single molecule FISH for Alpi transcripts (red) do not colocalize with transcripts for Dll1 (red) implying Alpi+ enterocyte progenitors in crypts are distinct from Dll1+ secretory progenitors (encircled with white dashed lines.

# CHAPTER THREE

# A critical role for the niche in intestinal tumorigenesis from enterocyte progenitors

3

Manuscript in preparation

Chapter Three

# A critical role for the niche in intestinal tumorigenesis from enterocyte progenitors

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3

## Abstract

*Identification of the cell of origin of cancers in general is crucial in developing better diagnostic and therapeutic interventions that detect these cancers at an early stage in cancer progression, and specifically target them for elimination to improve patient survival. Mouse models of small intestinal cancers have established stem cells in the proliferative crypt compartment as tumor initiating cells. Various cell types reside in the villus. Although recent studies have reported tumor initiation from villus cells it is unclear which particular cell types in the villus are transformed by oncogenic mutations due to the limitations of the mouse models employed. Using a novel enterocyte specific Cre model (Alpi-CreER), we investigate the tumor initiating ability of enterocytes in the proximal small intestine upon Apc-Kras mutations. Neither Apc nor Apc/Kras combined mutations in either crypt resident enterocyte progenitors or mature enterocytes in the villus gave rise to tumors in mice. When mutations were combined with loss of Lgr5 cells in mice, only enterocyte progenitors with both Apc/Kras mutations form tumors. In vitro, only isolated crypts with combined Apc/Kras mutations formed tumor organoids, which can be cultured for long term without organoid growth factors. These results demonstrate that enterocyte progenitors with combined Apc/Kras mutations need the right niche to form tumors, and suggest the convergence of a conducive niche, differentiation state, and appropriate mutations to transform non-stem cells into tumor initiating cells.*

## Introduction

Intestinal cancer is one of the most common causes of cancer associated morbidity worldwide. The current model of intestinal cancer posits that tumor initiation and progression occurs when epithelial cells acquire a series of activating and inactivating mutations in various oncogenes and tumor suppressors respectively (Fearon, 2011). The initiating event of intestinal carcinogenesis is most commonly caused by activating mutations in the Wnt pathway (i.e. in APC or CTNNB (β-catenin)), followed by a progressive series of mutations in genes involved in the RAS, TP53, and TGF signaling pathways (Puglisi, 2013).



### *Small intestinal model of intestinal cancer*

Pioneering research into the molecular underpinnings of intestinal cancer has relied on mouse models that study cancer initiation and progression in the small intestine (Johnson and Fleet, 2012). However small intestinal cancer is rare in humans, where the disease is predominantly restricted to the colon, despite the similarity of homeostatic cues and renewal dynamics between the small intestine and colon. Although small intestinal cancer models are limited in mimicking human colon cancers in terms of tumor location, microenvironmental regulation and metastatic potential, these models have nonetheless been instrumental in shedding light on the cellular and mutational origins of intestinal cancer (Fessler et al., 2013).

### *Intestinal cancer cell or origin*

The small intestine is compartmentalized into a proliferative crypt zone and a differentiated villus zone. Multipotent Lgr5+ CBC stem cells located at the crypt bottom divide daily to spawn a heterogeneous population of transit amplifying daughter cells which include bipotent progenitors of differentiated secretory and absorptive cells. Secretory progenitors then generate Paneth cells, Goblet cells, Enteroendocrine cells and Tuft cells which migrate upwards into the villus, with the exception of Paneth cells which remain in the crypts, in close contact with Lgr5+ CBC stem cells. Proliferative absorptive progenitors give rise to mature enterocytes which also migrate upward, and form the bulk of the villus compartment.

We previously showed that Apc deletion within Lgr5+ stem cells using an Lgr5-CreER leads to formation of macroadenomas however mutation in transit amplifying cells and villus cells using Ah-Cre mouse model did not yield macroadenomas arguing for a stem cell origin of small intestinal cancers (Barker et al., 2008). In support, CD133 CreER and Bmi1-CreER

models which also mark stem cell populations in the crypts demonstrated adenoma formation upon Apc deletion and beta catenin hyperactivation respectively (Zhu et al., 2008)(Sangiorgi and Capecchi, 2008).



Two recent studies suggest that differentiated villus cells can give rise to tumors. Schwitalla et al. reported that β-catenin/NFkB activation (inflammation) transformed villus epithelial cells into aberrant crypt foci in vivo. Furthermore β-catenin/Kras mutations using a mouse model with Cre-expression limited to non-Lgr5+ stem cells (Xbp1-Cre mouse model) led to adenoma formation in villus cells. In addition, ex vivo data suggested that villus cells with mutations of Apc/Kras mutations can form organoids in contrast to villi with Apc mutation alone, which cannot (Schwitalla et al., 2013). The BMP antagonist, Grem1 is normally expressed in non-epithelial myofibroblasts surrounding the crypts. Using the Villin1 promoter to express Grem1 in epithelial cells, it was recently reported that Grem1 overexpression in differentiated villus epithelial cells resulted in adenomas resembling human HMPS tumor type (Davis et al., 2015). However it is unclear which specific differentiated cells initiated the tumors as the mouse models employed have ubiquitous Cre expression in all non-Lgr5 expressing cells. In this study we investigated the tumor initiating propensity of enterocytes upon Apc and Kras mutations using Alpi-CreER mouse model where Cre expression is restricted to proliferative enterocyte progenitors in the upper crypts, and mature enterocytes in the villi of the proximal small intestine.

## Materials and Methods

### Mice experiments

The generation and characterization of Alpi-CreER in which the tamoxifen inducible Cre recombinase is expressed under the control of the enterocyte specific Alpi-locus has been described in Chapter 2 of this thesis. Alpi-CreER mice were bred with Apcfl/fl and KrasLSL G12D mice to generate Alpi-CreER/Apc Hom/Kras Het mice or Alpi-CreER/Apc Hom mice. 6-14week old mice were used for all experiments. A single dose of 5mg/ml Tamoxifen was injected intraperitoneally to activate Cre mediated mutation of Apc and/or Kras in Alpi+ cells. A total of 5 mice were injected for each experimental group. As controls, we used Alpi-CreER mice given similar doses of tamoxifen.

**Immunohistochemistry**

Freshly isolated intestines were flushed and fixed with formalin (4% formaldehyde in phosphate-buffered saline [PBS]) overnight (O/N) at room temperature (RT). The formalin was removed, and the intestines were washed once in PBS at RT. The intestines were then transferred to a tissue cassette and dehydrated by serial immersion in 20-fold volumes of 70%, 96%, and 100% ethanol (EtOH) for 2 h each at RT. Excess ethanol was removed by incubation in xylene for 1.5 h at room temperature, and the cassettes were then immersed in liquid paraffin (58°C) overnight. Paraffin blocks were prepared using standard methods. Tissue sections of 4 μm were dewaxed by immersion in xylene (2 times, 5 min) and hydrated by serial immersion in 100% EtOH (2 times, 1 min), 96% EtOH (2 times, 1 min), 70% EtOH (2 times, 1 min), and distilled water (2 times, 1 min). Endogenous peroxidase activity was blocked by immersing the slides in peroxidase blocking buffer (0.040 M citric acid, 0.121 M disodium hydrogen phosphate, 0.030 M sodium azide, 1.5% hydrogen peroxide) for 15 min at room temperature. Antigen retrieval was performed, and blocking buffer (1% bovine serum albumin [BSA] in PBS) was added to the slides for 30 min at room temperature. Primary antibodies were then added, and the slides were incubated as detailed below. The slides were then rinsed in PBS, and secondary antibody was added (polymer horseradish peroxidase-labeled anti-mouse or -rabbit antibody; Envision) for 30 min at room temperature. Slides were again washed in PBS, and bound peroxidase was detected by adding diaminobenzidine (DAB) substrate for 10 min at room temperature. Slides were then washed 2 times in PBS, and nuclei were counterstained with Mayer's hematoxylin for 2 min, followed by two rinses in distilled water. Sections were dehydrated by serial immersion for 1 min each in 50% EtOH and 60% EtOH, followed by 2 min each in 70% EtOH, 96% EtOH, 100% EtOH, and xylene. Slides were mounted in Pertex mounting medium, and a coverslip was placed over the tissue section. Antigen retrieval was performed by boiling samples for 20 min in 10 mM sodium citrate buffer (pH 6.0). Antibody used was anti β-catenin; (1:100, Transduction lab). Slides were counterstained with hematoxylin and mounted.

Intestine was cut open along the length. Villi were removed by scraping with a sterile microscope glass slide and separated into two parts. Whole

villi were washed in cold PBS (without Ca/Mg) and seeded in matrigel with either organoid medium (ENR) or Wnt supplemented organoid medium (WENR).

For making single cells from villi, scraped villi were transferred to 30ml of ice cold PBS + 5mM EDTA in a falcon tube and incubated for 30min at 4°C with rolling. Tube was then centrifu ged at 650rpm for 5 minutes and the supernatant carefully removed. Villi were then resuspended in 1ml SMEM calcium free medium (Gibco 11380) and mixed with 1ml of SMEM with 1mg/ml Trypsin (Sigma T1426) followed by mixing by pipetting. DNAse (final concentration of 1u/ul) was then added to the villi suspension and incubated 10min at 37°C with intermittent shaking and checking under a microscope for single cells. Suspension was then centrifuged for 5 minutes at 650rpm, supernatant discarded, and seeded in matrigel and subsequently cultured with either ENR or WENR media. Rho Kinase inhibitor was added to the culture media in all cases



## Results

We have recently generated a novel enterocyte specific cre line (Alpi-Creert2), described elsewhere. Briefly, the Cre-ERT2 fusion gene encoding the Cre recombinase protein is expressed exclusively in Alpi+ enterocyte progenitors located in the upper reaches of the small intestinal crypts, and in mature enterocytes in the villi. Activation of cre activity by tamoxifen injection allows deletion/activation of floxed genes specifically in enterocytes.

In line with previous studies where deletion of floxed Apc in non-stem cells does not lead to adenoma formation, no adenomas were detected in Alpi-CreER-Apc floxed mice 2 and 28 days after tamoxifen injection. Nuclear β-catenin staining is a hallmark of Wnt signaling hyperactivation; early time point analysis of beta catenin expression, two days after tamoxifen injection did not show nuclear accumulation of beta catenin in either enterocyte progenitors or mature villus enterocytes.

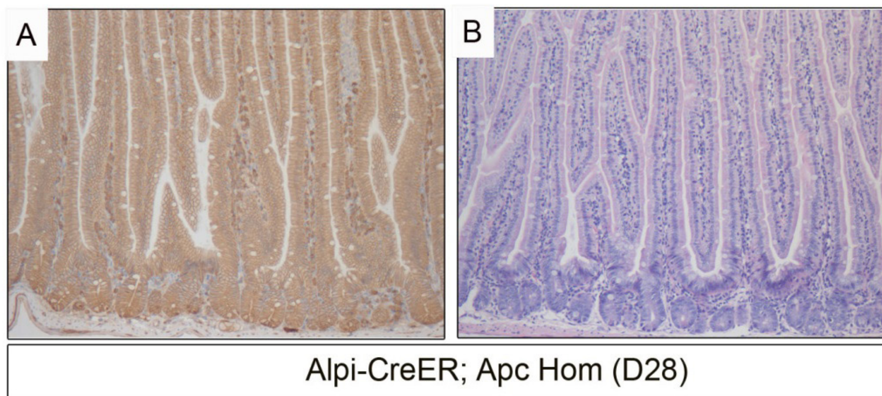




**Figure 1. Apc deletion in enterocytes.** Nuclear β-catenin staining A) and H and E staining of Alpi Hom/Apc Hom mouse injected with a single dose of tamoxifen and sacrificed after 28days showing no adenoma formation or morphological aberrations in the proximal small intestine

Oncogenic Kras synergistically enhances Wnt hyperactivation upon Apc deletion and thus tumor progression in the intestine (Janssen et al., 2006). We generated Alpi-CreER-Apc Hom/Kras Het mice to mutate both Apc and Kras specifically in enterocytes. Surprisingly, combined mutations of both APC and KRAS in enterocytes did not cause nuclear accumulation of β-catenin (Figure 2A) nor yield any adenomas in vivo two weeks and 28days after tamoxifen injection (Figure 2B and 2C).

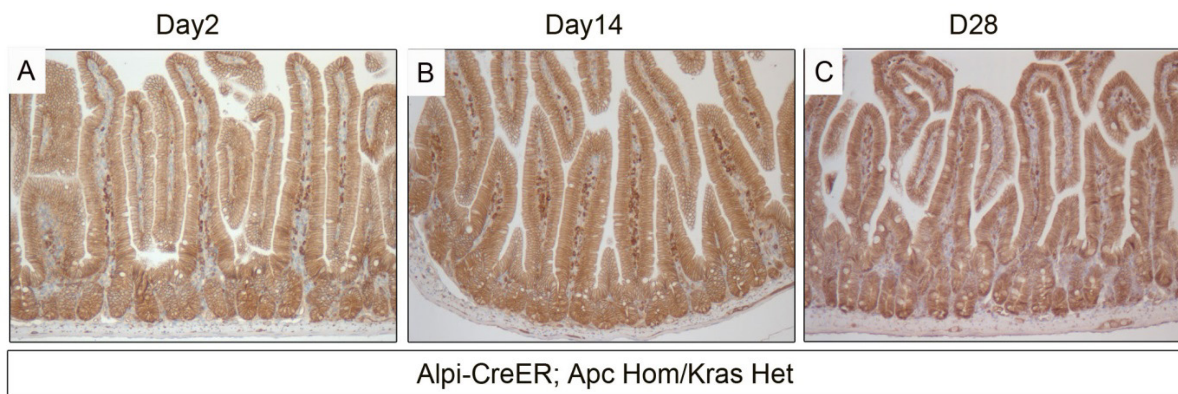


**Figure 2. Apc and Kras mutation in enterocytes.** Alpi-Cre-ER Hom; Apc Hom/Kras Het animals were injected with a single dose of 5mg/ml and sacrificed at various times . A) No accumulation of nuclear β-catenin two days (A). No adenomas were observed at later time points 14 days (B) and 28 days (C).

We next investigated whether mutated enterocytes have the capacity to dedifferentiate ex vivo and exhibit cancer stem cell properties. Crypts and villi were isolated from Alpi-CreER Hom/ Apc Hom and Alpi-CreER

Hom/Apc Hom/Kras Het compound mutants 2 days after tamoxifen injection and cultured in organoid growth medium (ENR) (Figure 3A). Isolated crypts from Apc deleted enterocyte progenitors did not form spherical organoids (Figure 3C) characteristic of hyperactive Wnt triggered cells. However, a significant number of spherical organoids could be observed in Apc/Kras mutated crypts (Figures 3D and 3F). These tumor organoids could be cultured for weeks independent of EGF, Noggin and Rspondin required for normal organoids (Figures 4A-D and G). Additionally, Alpi+ tumor organoids did not require n-Acetyl cysteine and B27 supplement (Figures 4E and F), essential antioxidant components for normal organoid culture. Villi from both Apc mutated (not shown) and Apc/Kras mutated mice failed to generate spheroid organoids regardless of whether they were cultured as whole villi or single villus cells (Figures 3F and 3G).



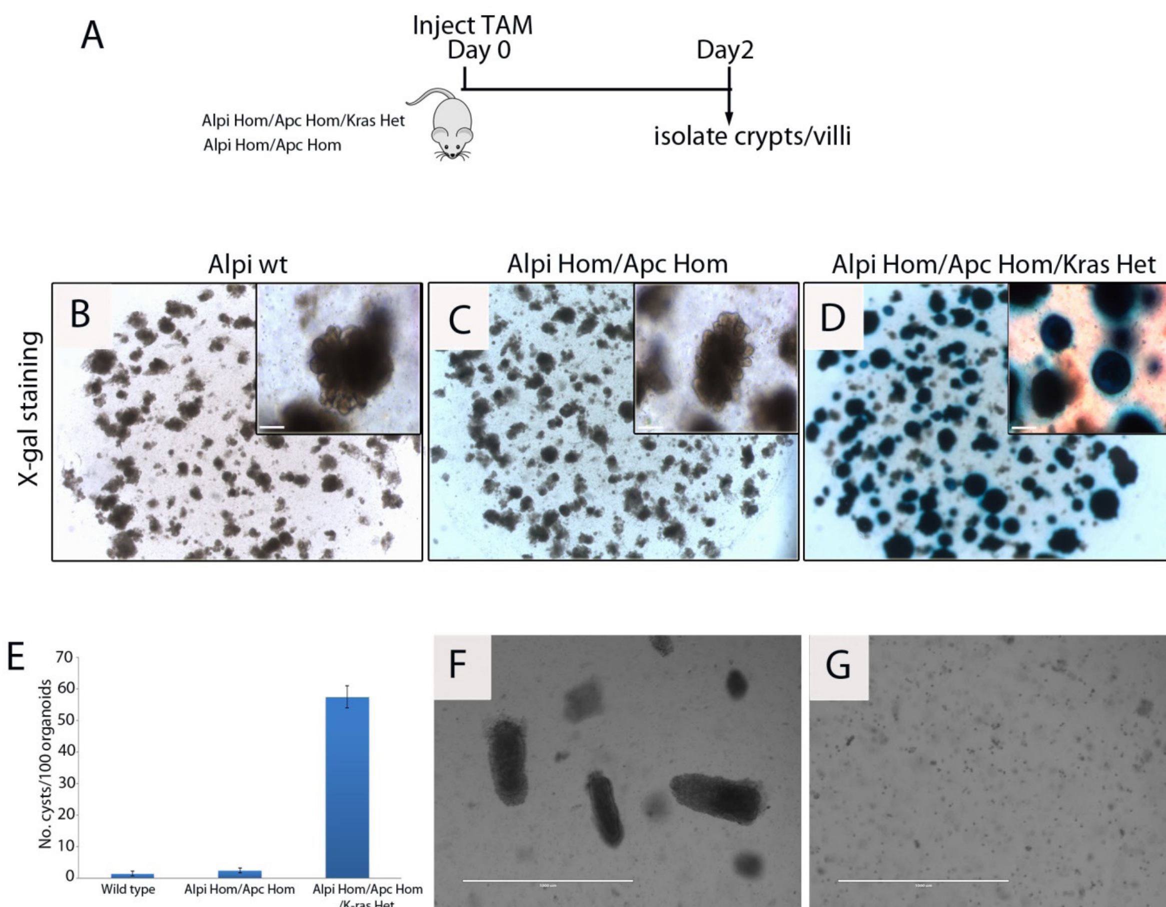


**Figure 3. Alpi+ crypt cells form tumor organoids in vitro.** A) Scheme to mutate and isolate Alpi+ cells for in vitro organoid assay. Alpi Hom/Apc Hom/Kras het; R26R and Alpi Hom/Apc Hom R26R mice were injected with a single dose of tamoxifen and sacrificed after 2 days for crypt and villi isolation. Controls were wild type mice. (B-C) Organoid formation from isolated Alpi+ crypt cells; Alpi Hom/Apc Hom crypts formed

normal organoids (B) comparable to wild type organoids A) whereas Alpi Hom/Apc/Kras crypts formed spheroid tumor organoids that were LacZ+ showing their enterocyte origin. E) Spheroid organoid forming efficiency of mutated Alpi+ crypt cells; 50% of Alpi Hom/Apc Hom/Kras Het crypts formed spherical organoids characteristic of tumor organoids at 1 passage after isolation. F and G) Isolated villi from Alpi Hom/Apc Hom/Kras were isolated and embedded into matrigel whole (F) or single cells (G) but failed to grow into spherical tumor oganoids after 1 passage

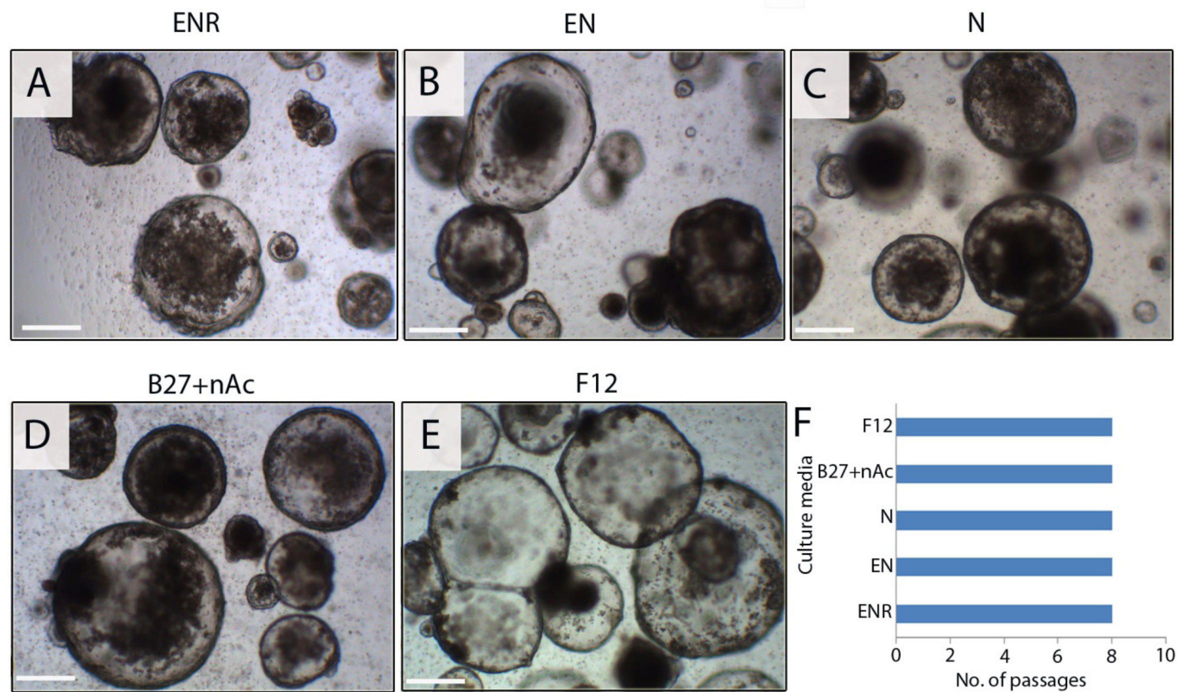


**Figure 4. Long term culture of Apc/Kras mutated Alpi + tumor spheroids independent of organoid growth factors**. A) Spherical organoids cultured with EGF(E), Noggin (N), and Rspondin (R). B and C. Alpi tumor organoids cultured without EGF and Rspondin. D) Alpi tumor organoids cultured without EGF, Noggin and Rspondin. E) Alpi tumor organoids cultured in Ad F12 medium (containing Hepes, Pen strep, and Glutamax) without B27 supplement and n-acetyl cysteine (nAc) which protect against oxidative damage. F) Passage or Alpi tumor organoids in various culture media showing no growth advantage over organoids grown in F12 medium.

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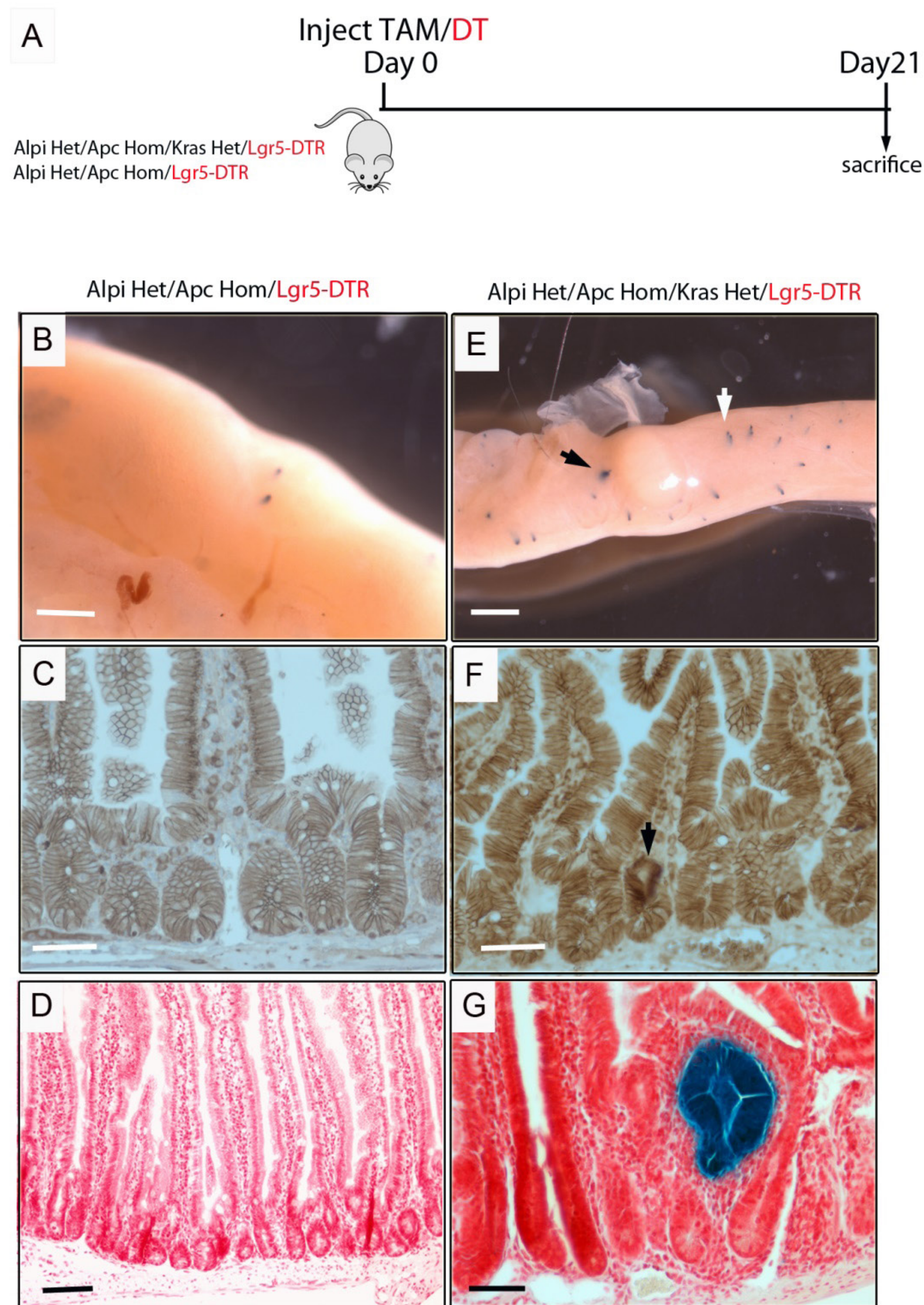


**Figure 5. Tumorigenesis of Alp+ crypt cells upon loss of Lgr5+ stem cells.** A) Experimental strategy. Mice were injected with a single dose of tamoxifen (TAM) and diphtheria toxin (DT), and sacrificed after 21days for analysis. B-D. Apc mutated Alpi+ cells did not form tumors upon loss of Lgr5+ stem cells. Whole mount staining showed lacZ+ crypts stained blue and characteristic of normal stem cell tracing (B). No nuclear β-catenin staining was observed (C). D) Neutral red staining showed occasional LacZ+ cells (not shown). E; Whole mount staining of Alpi Het/Apc Hom/Kras Het mouse showed characteristic stem cell LacZ+ crypts (white arrow) and occasional larger staining patches likely representing tumors (black arrow). F) Rare β-catenin stained aberrant crypt foci (black arrow) were observed in Apc/Kras mutated mouse. LacZ+ microadenoma within crypts of Alpi Het/Apc Hom/Kras mouse.

To investigate whether mutated enterocyte progenitors can generate tumors in vivo when they get access to the stem cell niche, Alpi-CreER/Apc Hom;Lgr5-DTR Het and Alpi-CreER/Apc Hom/Kras Het;Lgr5-DTR Het mice were generated by breeding, and injected with diphtheria toxin (DT) and tamoxifen (TAM) (Figure 5A). In these mice, TAM will induce mutations in enterocyte progenitors, whereas DT will delete Lgr5+ stem cells creating an 'empty niche' which can be filled by mutated Alpi+ enterocyte progenitors. No adenomas or polyps were observed in Alpi-CreER/Apc Hom;Lgr5-DTR Het mice injected with a single dose of TAM/DT (Figure 5B-D). However, adenomas were observed in Alpi-CreER/Apc Hom/Kras Het;Lgr5-DTR Het mice (Figure 5E-G). Of note, the frequency of these adenomas in the proximal small intestine 21 days post-induction was very low with smaller sizes as compared to previously reported tumors from Lgr5+ stem cells.



**Discussion**

Proliferative crypt resident enterocyte progenitors derived from Lgr5+ stem cells give rise to short lived differentiated enterocytes that comprise the bulk of the villus. Using a novel enterocyte specific cre line, Alpi-CreER, we queried intestinal tumorigenesis from enterocyte progenitors and mature cells in vivo and in vitro upon tamoxifen induced Apc and K-ras mutations. Apc mutation in enterocytes failed to form tumors both in vivo and in vitro, in agreement with previous studies where Apc deletion in non-stem cells did not form tumors (Barker et al., 2008)(Schwitalla et al., 2013). No tumors are observed upon Apc deletion in Dclk1+ differentiated tuft cells scattered along the crypt-villus axis (Nakanishi et al., 2013) (Westphalen et al., 2014) and crypt localized Paneth cells (Johan van Es, personal communication) further supporting the stem cell origin of Apc mutations driving intestinal tumorigenesis. Of note, Dclk1+ cells readily gave rise to intestinal cancers when Apc loss was combined with an inflammatory stimulus (DSS colitis) regardless of whether the inflammatory stimulus was given immediately after tamoxifen injection or 3 months after tamoxifen injection (Westphalen et al., 2014) implying that long lived mutated differentiated cells can be transformed into tumor initiating cells by an inflammatory microenvironment.

K-ras mutations in Lgr5+ stem cells accelerates their proliferation rates and gives them unequal competitive advantage over normal stem cells for niche occupancy and clonal expansion (Snippert et al., 2013). Additionally, K-ras mutations cooperate with Apc deletion to enhance and sustain Wnt hyperactivation in cancer cells (Janssen et al., 2006).

Alpi-CreER mice with Apc/Kras mutations did not show any adenomas arising from mature villus enterocytes in vivo. Also, there was no

discernible alteration in crypt-villus architecture and function suggesting that hyperactivated Wnt signaling in enterocytes is insufficient to transform them into tumor initiating cells. These results are in sharp contrast to the findings by Schwitalla et al and Davis et al, and could be attributed to the differences in models employed and different villus cell populations being mutated. For instance, the Xbp1 Cre model employed by (Schwitalla et al., 2013) expresses the cre protein ubiquitously in all non Lrg5+ stem cells including enterocytes, Paneth cells, goblet cells, tuft cells, and perhaps yet unidentified rare cell types. As such tumors from this model may have originated from non-enterocyte villus cells. As differentiated short lived enterocytes in our Alpi-CreER model failed to generate tumors upon Apc/Kras mutations, it is likely that the villus tumors observed by (Schwitalla et al., 2013) upon combined Wnt hyperactivation and inflammation originated from long-lived Dclk1+ tuft cells as has been speculated elsewhere (Westphalen et al., 2014). Similarly, the Villin promoter used to overexpress Grem1 in epithelial cells is not only restricted to enterocytes and it is likely that the tumors observed by (Davis et al., 2015) were also from non-enterocyte villus cells. Future studies that use mouse models with cre expression restricted to specific villus cell types will clarify the involvement of differentiated goblet cells or enterondocrine cells in cancer initiation following Apc/Kras mutations.

Surprisingly, we did not observe tumors in vivo from Apc/Kras mutated enterocyte progenitors even when we increased the dosage of tamoxifen injection in Alpi-CreER Apc Hom/Kras Het animals to hit more Alpi+ cells. One reason for this can be a result of mutated Alpi+ cells being higher up in the crypt and thus denied access to the stem cell niche at the bottom of the crypt which might support cancer stem cells. However, depletion of normal stem cells to pave way for Apc/Kras mutated Alpi+ cells to access the normal stem cell niche yielded fewer adenomas than reported from Lgr5+ stem cells. This suggests that the normal stem cell niche in the proximal small intestine may not be supportive of transformation of mutated enterocyte progenitors. Additionally, fewer observable adenomas may be due in part to the lower cre expression and recombination efficiency in heterozygous Alpi-CreER mice used for this experiment. Interestingly, Alpi+ Apc/Kras mutated crypt cells from both homozygous and heterozygous Alpi-CreER mice formed spherical tumor organoids in ex vivo 3-D matrigel cultures without growth factors normally required for normal stem cells. Although Matrigel derived from mouse sarcoma cells mimics the basement membrane extracellular matrix that supports intestinal epithelial cells in vivo, it contains a host of growth factors that are known to support tumor growth (Benton et al., 2014). Thus matrigel might be more

representative of a cancer niche that can support Apc/Kras mutated enterocyte progenitors.

Taken together, our data demonstrate that transformation of Alpi+ cells in crypts and villi by Apc mutation or Apc/Kras mutations is an inefficient route to intestinal tumorigenesis even when mutated enterocytes are lodged in the normal stem cell niche. As such, it is plausible to suggest that cancer niche requirements for tumor initiation might be different for Lgr5+ cancer stem cells and mutated enterocyte progenitors.



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# 4

CHAPTER FOUR

# Differentiated colonic epithelial cells as cells of origin of colon cancer

4

Manuscript in preparation

4

## Differentiated colonic epithelial cells as cells of origin of colon cancer

**Paul W. Tetteh**, Harry Begthel , Maaike van den Born, Jeroen Korving, , Folkert Morsink, Henner F. Farin, Johan van Es, Johan Offerhaus, and Hans Clevers

### Abstract

**Animal models for human colon cancer can be useful for studying the mechanism of colon cancer development and for testing cancer prevention and treatment approaches. Current mouse models often significantly differ from human colon cancer, e.g. in intestinal location. We aimed to develop a colon-specific inducible mouse model which can faithfully recapitulate human colon cancer initiation and progression. Carbonic anhydrase I (Car1) is a gene expressed in colonic epithelial cells.**

**We generated a novel colon specific inducible Cre Knock-In mouse (Car1-Creert2 KI) with broad Cre activity in crypt bottom epithelial cells and differentiated cells of the cecum. Cre expression in the colon proper was however restricted to short lived differentiated epithelial cells in mid crypt to luminal regions of the proximal colon, and never observed in crypt bottom stem cells. Deletion of Apc tumor suppressor gene using the Car1-Creert2 KI caused tumor formation in the cecum but did not yield adenomas in the proximal colon. However combined mutation of both Apc and Kras yielded microadenomas in both the cecum and proximal colon which progressed to macroadenomas with significant morbidity and cancer stem cell characteristics. Importantly no adenomas were observed in the small intestine.**

**Our results indicate that differentiated colonic epithelial cells in the proximal colon require mutations in both Wnt and K-Ras signaling to transform them into tumor initiating cancer stem cell-like cells and have important implications on our understanding of cell plasticity and cancer.**

## Introduction

The mucosa of the large intestine which consists of the cecum, the colon, and rectum, contains simple columnar epithelium and a lamina propria, which form crypts of Lieberkühn. Unlike the small intestine, the large intestine is bereft of villi.

The colon epithelium is a constantly self-renewing tissue. The epithelium contains actively cycling Lgr5+ stem cells at the crypt bottom, proliferative transit amplify cells that give rise to mature cells such as mucous secreting goblet cells, and short lived absorptive enterocytes with apical microvilli (distinct from small intestine enterocytes) at the luminal end. Scattered in the large intestinal crypts are Chromogranin A+ enteroendocrine cells and tuft cells. M cells are also located in the Peyer's patches of the large intestine.



Tissue homeostasis in the normal colonic crypt relies on a balance between proliferation, differentiation and apoptosis. Deregulation of this balance may cause diseases such as cancer. Anatomically, the colon, which forms part of the large intestine, is delineated into the ascending colon, the transverse colon, the descending colon and the sigmoid colon.

Human colorectal cancer is the second leading cause of cancer related death in the Western world (Siegel et al., 2014). Generally, disease progression from benign adenomas induced by Wnt pathway mutations in colonic epithelial cells to invasive carcinomas involves sequential accumulation of activating mutations in RAS oncogenes, inactivating activating mutations in the SMAD family of tumor suppressor genes, and inactivating p53 gene mutations (Fearon, 2011). Colorectal cancer (CRC) is a heterogeneous disease with proximal (ascending and transverse sections) and distal (descending and sigmoid sections) CRCs showing multiple biological and clinical differences (Gervaz et al., 2004).

Controlled in vivo studies in genetic mouse models based on the Cre-loxP system offer an important avenue to model the molecular aetiology of CRC development via timely mutation of oncogenes, and tumor suppressor genes, and to test potential preventive and therapeutic interventions (Becher and Holland, 2006)"abstract":"Mouse models of human cancer are valuable tools for cancer research. Although xenografts and genetically engineered models (GEMs(Johnson and Fleet, 2012). Here, Cre recombinase expressed under the control of a cell-type specific promotor is combined with a second transgene such as a tumor suppressor gene or oncogene flanked by loxP sites, such that Cre activity can cause recombination between loxP sites to induce cancer initiating mutations in these mice.

An excellent mouse model for CRC should have Cre expression and hence mutated genes and tumors, restricted to the colonic epithelial cells of adult mice to closely mimic the disease development observed in humans. A major limitation of current Cre models is the occurrence of tumors in the small intestine which is anatomically distinct from the colon and rarely presents with tumors in humans, and also tumors in other organs.

Carbonic anhydrase I (Car1) is a gene expressed in normal colon epithelial cells as well as colorectal tumors (Sowden et al., 1993)(Bekku et al., 2000) that encodes a metalloenzyme involved in hydration of carbon dioxide, pH balance and anion exchange. Recently, a promoter/enhancer from the mouse Car1 gene was used to generate a Cre-expressing transgenic mouse (Xue et al., 2010). Although Cre-expression was indeed limited to the colon and not the small intestine, the usefulness of this model is diminished in that Cre is also expressed during embryonic development.



To overcome this limitation, we aimed to generate a tamoxifen inducible Cre-ERT2 transgene expressed from the Car1 promoter that will allow for temporal control of Cre-activity (Feil et al., 2009). Here, we characterize cre activity in this model and test the efficacy of this model for colon cancer research by mutating the major genes involved in colorectal cancer initiation and progression in Car1 expressing colonic epithelial cells of adult mice.

## Materials and Methods

### Mouse Experiments

The generation of Car1-CreER knock-in mice is described below. Car1-CreER mice were crossed with Rosa 26-LacZ reporter mice. Car1-CreER; R26R mice were used for lineage tracing experiments. Car1-CreER were bred to Apc Hom; Kras Het mice to generate Car1-CreER; Apc Hom, Car1-CreER/ Apc Hom/Kras Het and Car1-CreER/Kras Het mice for tumor formation experiments. A total of 3 mice were used for each time point analyzed for each experimental group.

### X-gal staining

Proximal intestines isolated from mice were fixed for 2 hours on ice with fix solution (1% paraformaldehyde (PFA), 0.2% glutaraldehyde, and 0.02% NP40 in PBSO), and washed twice for 15min in PBSO. This was followed by overnight staining in the dark with 1mg/ml X-gal in PBSO solution containing 5mmol/L potassium-hexacynoferrate III, 5mmol/L potassium-hexacynoferrate (IV) trihydrate, 2mmol/L magnesium chloride, 0.02% NP40 and 0.1% sodium deoxycholate. Subsequently, tissues were washed

twice in PBSO and whole mount analyzed for LacZ positivity followed by overnight fixation in 4% PFA, and paraffin embedded using standard procedures. 4-8um tissue sections were counterstained with neutral red. Three mice per each experimental group were used for analysis.

**Immunohistochemistry**

Freshly isolated intestines were flushed and fixed with formalin (4% formaldehyde in phosphate-buffered saline [PBS]) overnight (O/N) at room temperature (RT). The formalin was removed, and the intestines were washed once in PBS at RT. The intestines were then transferred to a tissue cassette and dehydrated by serial immersion in 20-fold volumes of 70%, 96%, and 100% ethanol (EtOH) for 2 h each at RT. Excess ethanol was removed by incubation in xylene for 1.5 h at room temperature, and the cassettes were then immersed in liquid paraffin (58°C) overnight. Paraffin blocks were prepared using standard methods. Tissue sections of 4 µm were dewaxed by immersion in xylene (2 times, 5 min) and hydrated by serial immersion in 100% EtOH (2 times, 1 min), 96% EtOH (2 times, 1 min), 70% EtOH (2 times, 1 min), and distilled water (2 times, 1 min). Endogenous peroxidase activity was blocked by immersing the slides in peroxidase blocking buffer (0.040 M citric acid, 0.121 M disodium hydrogen phosphate, 0.030 M sodium azide, 1.5% hydrogen peroxide) for 15 min at room temperature. Antigen retrieval was performed (see details below for each antibody), and blocking buffer (1% bovine serum albumin [BSA] in PBS) was added to the slides for 30 min at room temperature. Primary antibodies were then added, and the slides were incubated as detailed below. The slides were then rinsed in PBS, and secondary antibody was added (polymer horseradish peroxidase-labeled anti-mouse or -rabbit antibody; Envision) for 30 min at room temperature. Slides were again washed in PBS, and bound peroxidase was detected by adding diaminobenzidine (DAB) substrate for 10 min at room temperature. Slides were then washed 2 times in PBS, and nuclei were counterstained with Mayer's hematoxylin for 2 min, followed by two rinses in distilled water. Sections were dehydrated by serial immersion for 1 min each in 50% EtOH and 60% EtOH, followed by 2 min each in 70% EtOH, 96% EtOH, 100% EtOH, and xylene. Slides were mounted in Pertex mounting medium, and a coverslip was placed over the tissue section. Antigen retrieval was performed by boiling samples for 20 min in 10 mM sodium citrate buffer (pH 6.0). Antibodies used were mouse anti-Ki67 (1:100 dilution; Novocastra), mouse monoclonal ant- β-catenin (1:100 dilution; Transduction lab); rabbit polyclonal anti-estrogen receptor (ER) (ab27595; Abcam), mouse monoclonal anti Msi1 antibody (Msi1-clone14H1, MBL),

goat polyclonal anti-chromogranin A(ChrgA) (1:500 dilution, sc-1488, Santa Cruz), mouse anti-EphB2 antibody. Incubation of antibodies was performed for 1h at room temperature for antibodies directed against Ki67, ChrgA, and β-catenin. In all cases, reagent from the Envision$^{+}$ kit (Dako) was used as a secondary reagent. Stainings were developed with DAB. Slides were counterstained with hematoxylin and mounted.



## Results

### Generation and characterization of Car1 CreER KI

Various studies have analysed the expression of Car1 in a host of tissues and organs. These studies have established that in the gastrointestinal tract, Car1 is expressed in the colon and not the small intestine (Kivelä et al., 2005)(Sowden et al., 1993)(Bekku et al., 2000), with expression higher in the proximal colon (Gervaz et al., 2004). We confirmed the expression of Car1 by mRNA in situ hybridization with a probe specific for the Car1 gene. Comparison of Car1 mRNA expression with that of the colonic stem cell marker gene by in situ hybridization confirmed Car1 localization in differentiated colonic epithelial cells (Figure 1A), with Lgr5 localized to the stem cells at the crypt bottom (Figure 1B).

To generate an inducible colon specific Cre-line, we inserted an internal ribosome entry site (IRES)-CreERT2 cassette at the stop codon located in the last exon of the Car1 gene (Supplementary Figure 1A). This strategy employs the endogenous poly A signal and the 3’UTR of the Car1 gene. To indirectly visualize expression of the Cre protein, we performed immunohistochemical staining on paraffin embedded intestinal tissue slides with an antibody against the Estrogen receptor (ER), fused to the Cre-protein. ER antibody staining depicted Cre expression faithfully mirrored endogenous Car1 expression (Figure 1C and D).

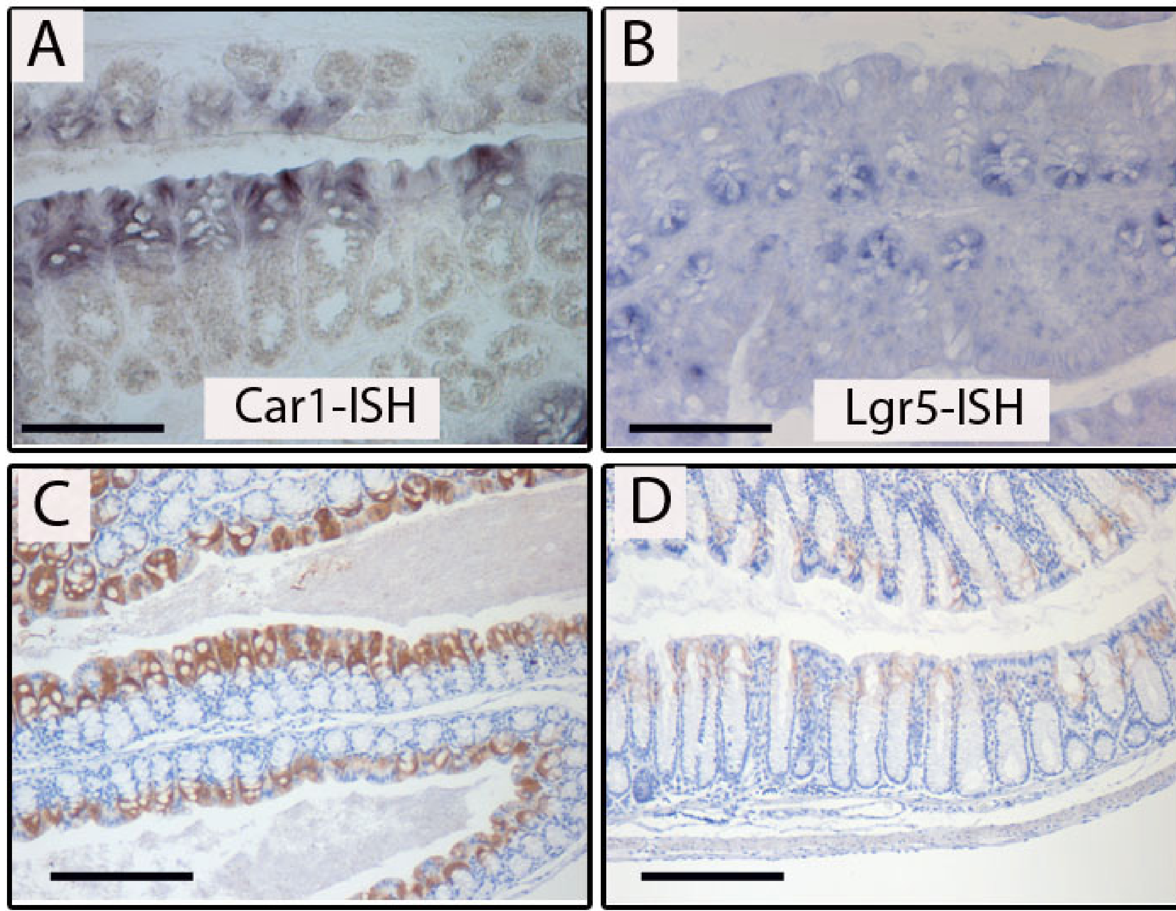




**Figure 1 Car1 expression in colon and Cre expression in Car1-CreER KI.** Comparison of Car1 (A) mRNA expression to stem cell marker gene, Lgr5(B) in the proximal colon. Car1 is localized to differentiated cells at the top of colonic crypt whereas Lgr5 is found in stem cells at the bottom of the crypt.; C and D) ER antibody staining showing expression of Cre-ER fusion from the Car1 promoter in the Car1-CreER KI. Cre-ER expression is higher in the proximal colon (C) than in the distal colon (D).

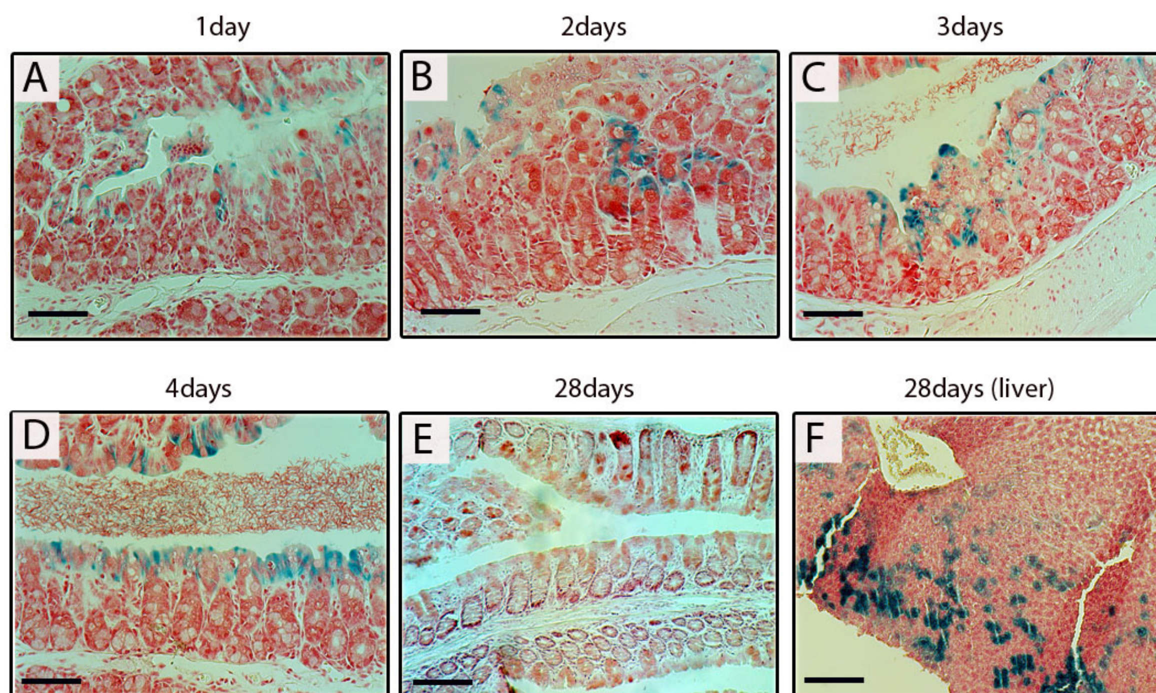


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**Figure 2 Lineage tracing Car1-CreER; Rosa LacZ mice.** Car1-CreER; Rosa lacZ miice were induced with 5mg/kg 4OH-tamoxifen (TAM) and then sacrificed after A) 1day, B) 2 day, C) 3days, D) 4days, and 28 days E) days and F).

To further characterize Cre activity of the Car1-CreER allele and trace the behavior of Car1+ cells, mice were crossed to R26R-LacZ reporter mice where the lacZ gene is under the control of the ubiquitous R26 locus (Soriano, 1999). Eight-to-twelve-week old mice were injected with a single dose of tamoxifen (TAM), and sacrificed at various time points (3mice per time point) for LacZ analysis. β-galactosidase+ cells were observed exclusively in differentiated colonic epithelial cells of the proximal colon. At the earliest time point, LacZ+ cells were detected in the upper crypt (Figure 2 A-D) but never in the bottom half where proliferative cells and stem cells reside. No beta gal+ cells were observed after 8 days (data not shown) or 28 days (Figure 2E) implying Car1 is expressed by short-lived differentiated colonic cells of the proximal colon.

Analysis of Car1-Cre activity in the cecum showed β-gal+ cells and ER-antibody stained cells not only in differentiated cells but also at the crypt bottom suggesting broad expression of Car1 in both stem cells and differentiated cells of the cecum (Supplementary Figure 2). β-gal+ cells were also detected in long lived hepatocytes in the liver, which have been reported to express Car1 (Figure 2F) (Xue et al., 2010).

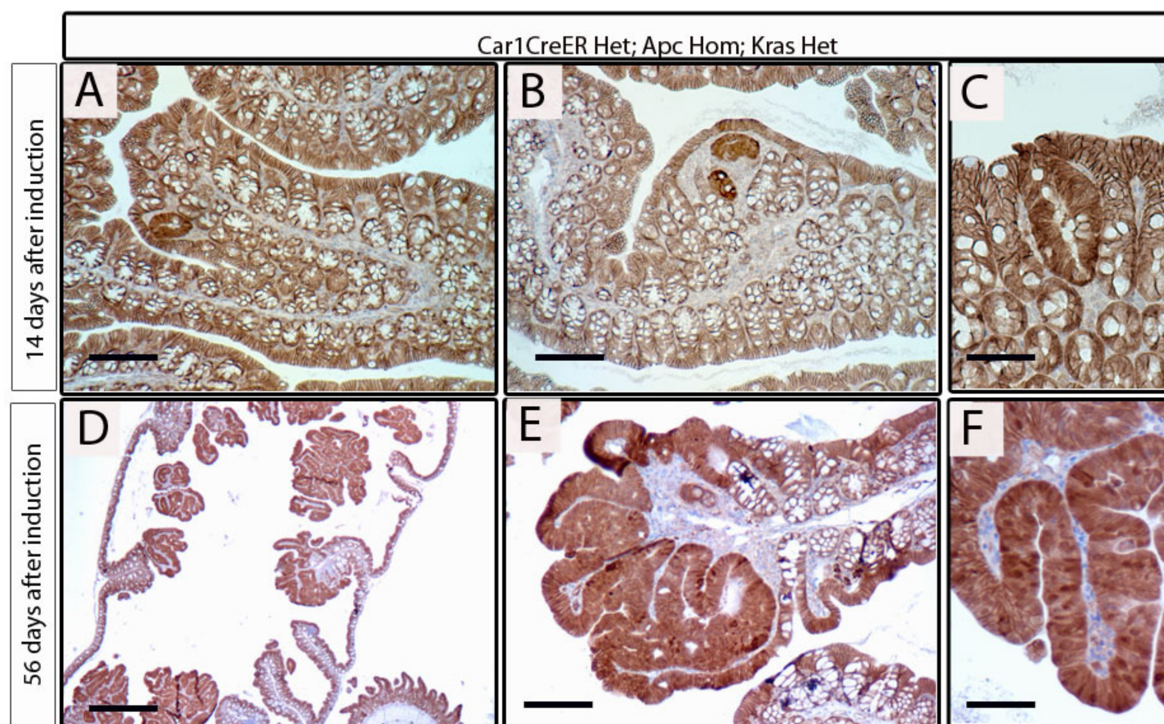


**Figure 3 Adenoma formation upon Apc and Kras mutations in in Car1 expressing colonic epithelial cells.** A single dose of TAM was injected int Car1-CreER Het/Apc-Hom/Kras Het mice, which were sacrificed after 14 and 56 days. B-catenin nuclear staining was used to identify adenomas



Mutations in the Apc gene are key initiating events in colorectal cancer initiation (Fodde et al., 2001)although it is not completely understood at the molecular level. As truncations of the Adenomatous Polyposis Coli (APC . To determine the expediency of the Car1-CreER KI for colon cancer research, we asked whether Car1+ differentiated colonic epithelial cells can function as tumor initiating cells upon Apc deletion. To address this question we crossed the Apc floxed our Car1-CreER KI. The presence of tumors was assessed by nuclear accumulation of β-catenin, a hallmark of hyperactive Wnt signaling. As expected, based on previous studies in small intestine tumorigenesis, Apc mutation in differentiated Car1+ cells yielded microadenomas which failed to progress to macroadenomas 7 days and 14 post TAM induction (supplementary figure 3), and also 28 days, and 2 months post-induction(not shown). In the cecum, however, Apc deletion yielded aggressive adenomas in the cecum (Supplementary Figure 4) presumably because crypt bottom stem cells in the cecum expressing Cre were induced to initiate tumors.

We next asked whether additional mutation in the KRAS gene, another commonly mutated gene in colorectal cancer, can enhance the tumorigenic potential of differentiated Car1+ cells in the proximal colon. Significant

microadenomas could be observed in Car1-CreER; Apc Hom,/Kras Het mice two weeks post TAM induction (Figure 3 A -C). β-catenin antibody staining of mice 56days after TAM injection showed that these microadenomas had progressed into large adenomas originating from differentiated Car1 expressing cells in the proximal colon, and growing toward the luminal side of the intestine (figure 3 D-E). We observed 5 times more Apc/Kras adenomas from homozygous Car1-CreER mice than heterozygous mice. Of note, no adenomas were ever observed in the small intestine, the distal colon and the liver (figure 4A-C).



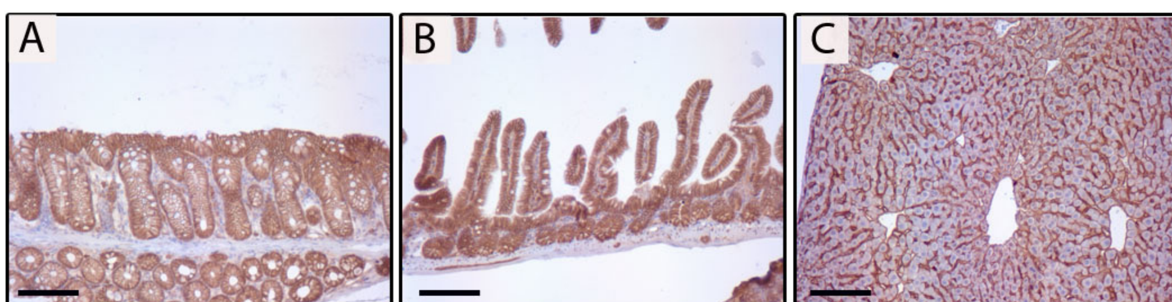


**Figure 4 Restricted tumor formation in proximal colon.** Histological analysis of tumor formation in A) distal colon, B) small intestine, and C) liver by β-catenin nuclear localization, in Car1-CreER-Apc Hom/Kras het mice 56 days after tamoxifen injction . No adenomas were observed in these locations

Histopathological analysis of hematoxylin and eosin (HE) stained sections of tumors from Car1 positive cells revealed that they resembled conventional adenomas observed in colorectal cancer patients (Supplementary figure 5); Proximal colon tumors from Car1-CreER; Apc Hom/Kras het mice had recognizable tubular structures and severely disturbed architechture. Glands within the tumors were lined with a stratified epithelium that showed increased nuclear/cytoplasmic ratio, hyperchromatism, prominent nucleoli, and numerous abnormal mitotic figures. Car1+ tumors were also highly proliferative, and poorly differentiated (Figure 5 A-F). Histological analysis of cancer stem cell marker genes in these proximal Apc/Kras tumors showed high expression of Lgr5, Msi1 and EphB2 in tumors originating from differentiated Car1+ cells (Figure 6 A-C).

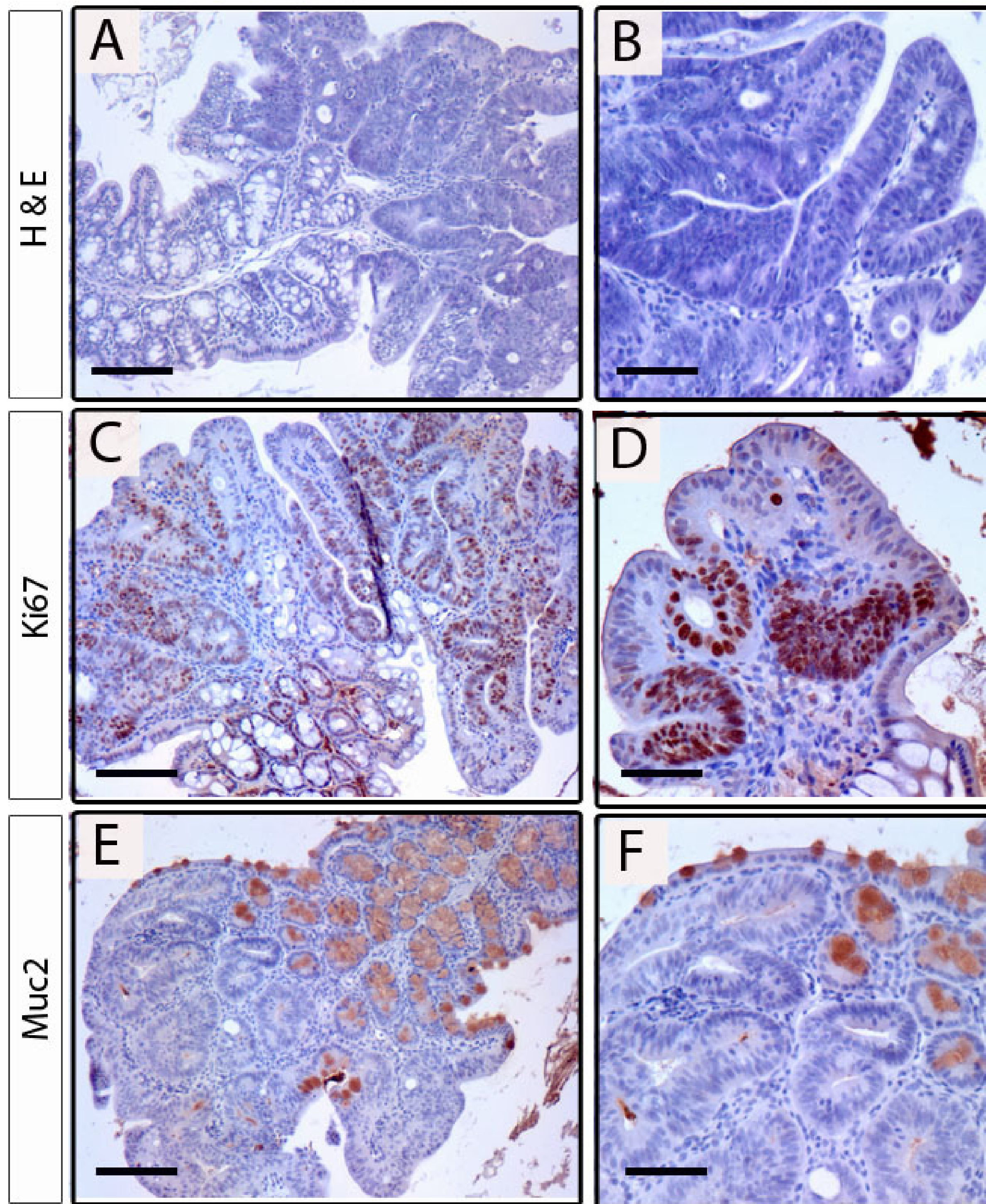




**Figure 5 Histological analysis of Car1-CreER; Apc Hom; Kras Het proximal colon tumors.** A) and B) Hematoxylin and Eosin (H&E) staining showing tubular adenomas in Apc/Kras tumors; C and D) Tumors were highly proliferative as shown by antibody staining of the proliferation marker, Ki67; E and F) Proximal colon tumors were poorly differentiated as shown by the absence of differentiated Muc2+ goblet cells, and Chromogranin A+ enterondocrine cells (not shown).

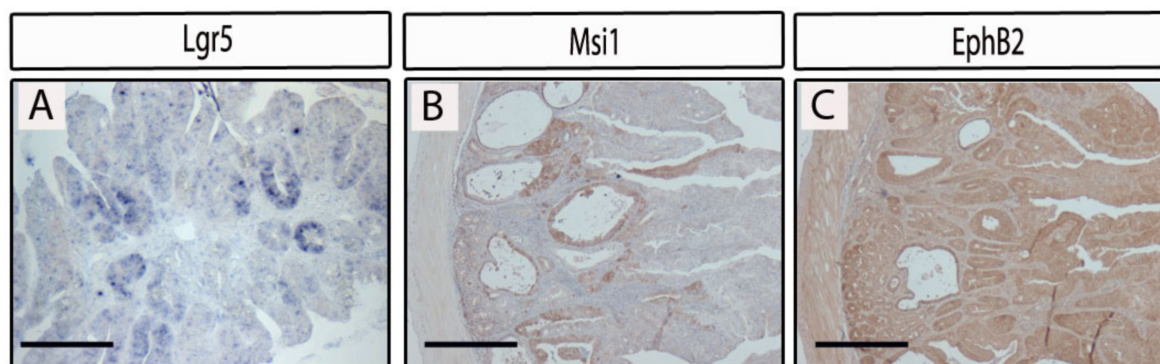


**Figure 6 Expression of Cancer stem cell markers in Car1-CreER; Apc Hom; Kras Het tumors.** A) Lgr5 in situ hybridization on Apc/Kras proximal colon tumors in Car1-CreER mice showed restricted expression of the stem cell marker within tumors. B) Antibody Staining of the musashi1 (Msi1) stem cell marker in proximal colon tumors; C) EphB2, colon stem cell marker, is expressed within proximal colon tumors.



## Discussion

In this study, we report the generation of an inducible colon specific Car1-CreER KI line in which Cre expression is driven by the Carbonic anhydrase 1 gene, which allows temporal control of Cre activity. Intestinal cre expression in this model is restricted to almost all epithelial cells in the cecum, mostly differentiated cells in the proximal colon, and lack of Cre activity in the small intestine. The differential pattern of cre expression in the cecum (in all epithelial cells) and proximal colon (differentiated epithelial cells) makes the Car1-CreER mouse an excellent model to study large bowel tumor dynamics arising from stem cells and differentiated cells.

A limitation of this Car1-CreER model for colon research is the absence of cre activity in the stem cells of the proximal colon and in epithelial cells of the distal colon and rectum however this is according to the endogenous expression of the Car1 promoter. Recently, Xue et al reported the generation of a Car1-Cre (CAC) transgenic line also with Cre expression restricted to the colon (Xue et al., 2010). Although similar, spatiotemporal differences of Cre expression exist between both models. A limitation of the CAC transgene is that Cre expression is constitutively active and persists throughout the embryonic stage and adulthood, making it unsuitable to study adult onset biological processes which is more relevant for human colorectal cancer. Whereas Cre expression in the Car1-CreER KI is restricted to differentiated colonic cells of the proximal colon, cre expression in the CAC transgene was observed in all the epithelial cell types in the proximal, distal and rectal sections of the colon. A reason for the apparent discrepancy of Cre expression in the two models may be due to

the different targeting strategies employed. In our Car1-CreER KI, the ires-creERT2 cassette is inserted at the 3'UTR of the Car1 gene without disrupting expression of the Car1 gene, thus Cre-expression follows endogenous expression of the Car1 gene. In contrast, the targeting construct is in the CAC transgene is inserted immediately downstream of the Car1 promoter which leads to random integration of the DNA targeting construct thus each founder can show different expression of Cre due to the chromosomal context of integration.

Plasticity refers to the ability of a cell to change its fate in response to external or internal cues. Plasticity via dedifferentation of committed secretory (van Es et al., 2012) (Buczacki et al., 2013) and absorptive progenitors (Tetteh et al, unpublished) into intestinal stem cells occurs during regeneration to replenish loss of Lgr5+ stem cells. Various types of cell plasticity have been observed in the initiation and progression of solid tumors be it epithelial mesenchymal transitions, transformation of a normal stem cell into a cancer stem cell, or dedifferentiation of differentiated cells either within or without a tumor into tumor initiating cells with cancer stem cell properties.



Evidence of the involvement of short lived differentiated cells in colonic cancer initiation has not been previously reported. The unique expression of Cre in differentiated epithelial cells of the proximal colon in our Car1-CreER model permitted us to interrogate this question. Although loss of Apc in short lived Car1+ cells led to nuclear accumulation of β-catenin and formation of microadenomas, these failed to progress into large adenomas. However, Apc deletion and oncogenic Kras hyperactivation transforms Car1+ cells into tumor initiating cells with cancer stem cell characteristics. Of note, these tumors develop without the introduction of an inflammatory stimulus, and neither developed in the small intestine nor metastasize to the liver. These results suggest that colorectal cancer initiation does not only initiate from stem cells but also from short lived differentiated epithelial cells. Tumorigenic plasticity in differentiated cells could be due to the combination of multiple oncogenic mutations inducing genetic and epigenetic alterations aided by a conducive microenvironment to nurture cancer stem cells. Recent xenograft transplantation studies of human cancer cells bearing Apc and Kras mutations into mice have shown that oncogenic K-ras enhances tumor progression and metastasis by activating cancer stem cell properties in colorectal cancers harboring Apc mutations (Moon et al., 2014)respectively. However, little is known about the role these mutations play in cancer stem cells (CSCs. Stabilization of mutant K-ras by Apc loss may lead to further aberrant activation of Wnt signaling (Jeong et al., 2012)we report a distinct mechanism for regulating Ras activity through phosphorylation-mediated degradation and describe the role of this second regulatory mechanism in the suppression of cellular transformation and tumors induced by Ras mutations. We found that negative regulators of Wnt/β-catenin signaling contributed to the polyubiquitin-dependent degradation of Ras after its phosphorylation by glycogen synthase kinase 3β (GSK3β inducing plasticity of diffferentiated Car1+ cells into cancer stem cells. Further

evidence of the role of aberrant Wnt signaling in promoting the cancer stem cell phenotype was shown recently by Vermulen et al who showed that heterogenous β-catenin activity observed in colorectal cancers depended on the level of Wnt activation. Moreover, myofibroblast secreted factors such as HGF significantly modulated Wnt signaling activity and the CSC phenotype via activation of –c-Met, Akt and β-catenin (Vermeulen et al., 2010). Interestingly, HGF also induced CSC phenotype in differentiated cell lines suggesting that the tumor microenvironment might regulate tumorigenic plasticity of differentiated colonic epithelial cells (Vermeulen et al., 2010) such as short lived Car1+ cells.

The metastatic potential of tumors arising from mutated Car1+ cells upon acquisition of additional mutations of SMAD4 and p53 is currently the subject of intense investigation.

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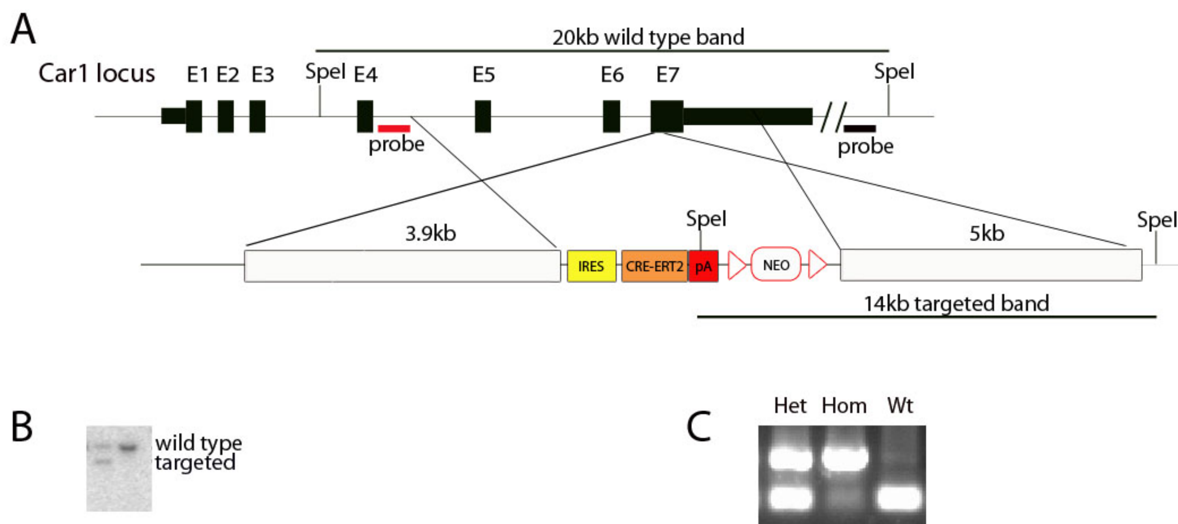
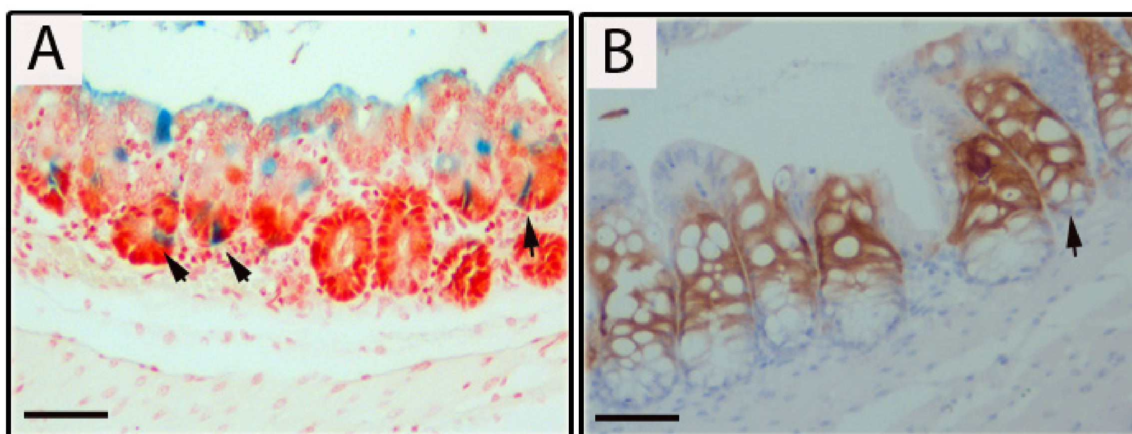
## Supplementary Figures

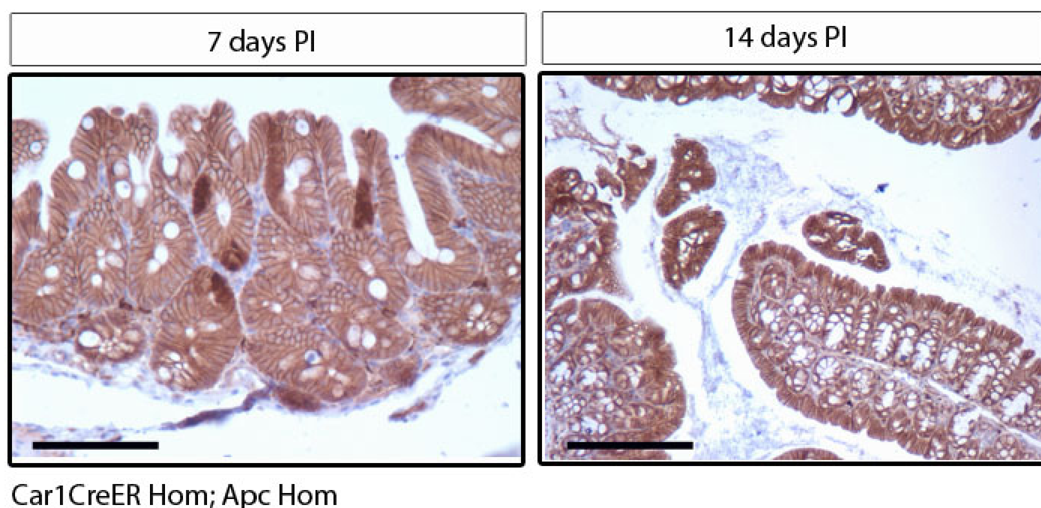



**Supplementary figure 1 Targeting strategy and generation of Car1-CreER KI.** A) Ires CreERT2 targeting construct used to target Car1 locus. B) Southern blotting showing successfully targeted ES cells. C) Genotyping of Car1-CreER knock-in mice



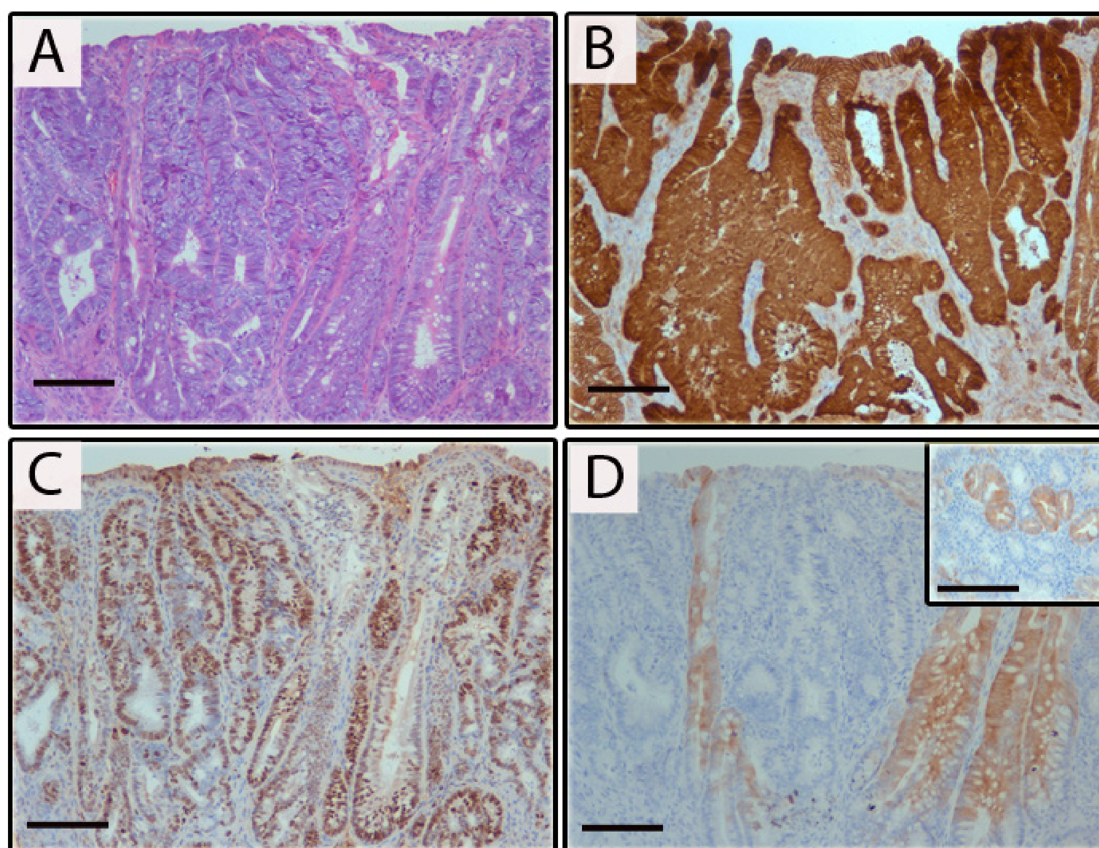



**Supplementary Figure 2. Cre expression in the cecum of Car1-CreER KI.** A) Car1-CreER mouse was injected with a single dose of tamoxifen for 3 successive days and sacrificed one day after the last injection. X-gal staining showed labelled crypt bottom cells (black arrows) of cecum suggesting stem cells in the cecum express Car1. B) Estrogen receptor (ER) antibody staining of cecum 2 months after single dose of tamoxifen shows cre expression in crypt bottom cells (stem cells) of cecum.

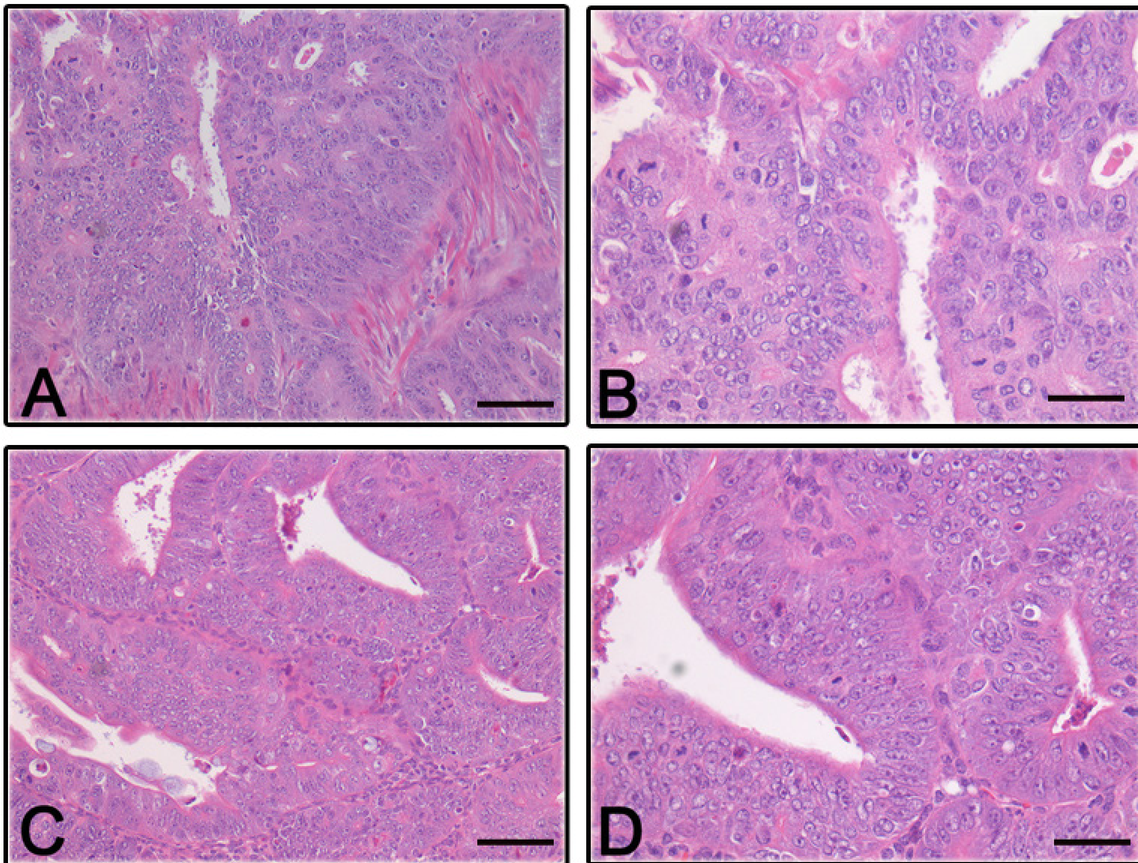





**Supplementary Figure 3 Lack of adenomas upon Apc deletion in proximal colon Car1+ cells.** β-catenin nuclear accumulation 7 days after Apc deletion in Car1 cells were not visible after 14days and l month(not shown).




**Supplementary Figure 4 Adenomas in cecum upon Apc deletion.** Car1-CreER Het; Apc Hom mouse was injected with a single dose of tamoxifen, sacrificed after 2 months and analysed.A) H and E staining showings tubular adenomas in cecum; B) Nuclear β-catenin staining showing Wnt hyperactivation in cecal adenomas; C) Ki67 staining showing cecal adenomas were highly proliferative; D) ER antibody staining showing Cre expression is restricted in cecal adenomas suggesting restricted expression in cancer cells fueling cancer progression (insert).






**Supplementary Figure 5. Comparison of proximal colon tumors from Car1 CreER; Apc Hom/Kras Het mouse and human patient.** A and B) adenomas from Car1-CreER;Apc Hom/Kras Het mouse 56days post tamoxifen induction; C and D) Conventional proximal colon adenomas from elderly patient with Apc and PTEN mutations.

# CHAPTER FIVE

# The role of Amica1 gene in intestinal stem cells

5

## The role of Amica1 Gene in Intestinal stem cells

**Paul W. Tetteh,** Harry Begthel , Maaike van den Born, Jeroen Korving, Henner F. Farin, Johan van Es, and Hans Clevers

### Abstract

**Stem cell specific transmembrane molecules may be important in their interaction with their niche. We have identified Amica1 as a transmembrane molecule in Lgr5+ intestinal stem cells. Here we describe the generation of a conditional floxed allele for the Amica1 gene and show preliminary results upon intestinal specific deletion of this gene.**

## Introduction

The mouse small intestinal epithelium is an archetypical model of mammalian stem cell biology and tissue self-renewal. It is organized into pit-like recessions called crypts, and fingerlike protrusions into the lumen called villi. Proliferative columnar basal cells (CBCs) resident at the crypt bottom, and that express the Wnt target gene Lgr5, have been described as bonafide stem cells that maintain the intestinal epithelial cell populations (Barker et al., 2007). We have previously shown that Lgr5+ stem cells self-renew daily and generate immediate daughter cells that exit the stem cell zone into the transit amplifying zone. Stem cell daughters in the TA zone undergo a series of short divisions whilst moving up towards the villus, to generate secretory progenitors and numerous absorptive progenitors. Absorptive progenitors differentiate at the crypt-villus junction into non-proliferative mature enterocytes that move towards the villus tip. Secretory progenitors are further specified into mature goblet cells, tuft cells, enteroendocrine cells, and Paneth cells, all of which move up the villus with the exception of the Paneth cells that escape the upward drift to reside at the crypt bottom in close proximity to Lgr5+ stem cells. We have also previously showed that localization of Paneth cells to crypt bottom is dependent on Wnt signaling mediated expression of EphB2/EphB3 receptors and their ligand, ephrin-B1. Knock out of the EphB3 gene leads to upward migration of Paneth cells into the villus (Batlle et al., 2002). Paneth cells constitute the epithelial component of the niche ensemble that supports stem cell growth by providing essential cues such as Wnt, Noggin, and EGF (Sato et al., 2011). Secreted factors from the mesenchyme at the crypt base and the underlying basement membrane also contribute to stem cell maintenance but it is unclear how stem cells are anchored in the niche.



We hypothesized that specific adhesion molecules might be involved in the close association between stem cells and Paneth cells. Recently, we have described transcriptome profiles of Lgr5+ small intestinal stem cells and their immediate daughter cells by GFP based sorting of epithelial cells from isolated crypts of Lgr5-EGFP-ires-CreERT2 mice (Muñoz et al., 2012). When expression of adhesion molecule and transmembrane receptors was verified by in situ hybridization, the Adhesion molecule interacting with CXADR antigen (Amica1) emerged as a stem cell specific adhesion molecule localized to the crypt bottom.

AMICA1, also known as Junction adhesion molecule like (JAML), is a 65 kDa, type I transmembrane glycoprotein that belongs to the junctional adhesion molecule (JAM) subset of the immunoglobulin superfamily. JAM family

proteins contribute to intercellular connections within epithelial and endothelial cell layers and mediate their interactions with various hemopoietic cells (Moog-Lutz, 2003). The mouse AMICA cDNA encodes a 379 amino acid (aa) precursor that includes a 20 aa signal sequence, a 261 aa extracellular domain (ECD) with two Iglike domains, a 21 aa transmembrane segment, and a 77 aa cytoplasmic domain. In contrast to other JAM family proteins, AMICA does not contain a cytoplasmic PDZ binding motif (Moog-Lutz, 2003).Amica1 is expressed on the surface of granulocytes and monocytes and is upregulated during the differentiation of myeloid leukemia cells. Amica1 mediates adhesion of monocytes to endothelial cells and neutrophil migration across epithelial cell monolayers through its interaction with its cognate receptor, Cxadr in epithelial tight junctions (Zen et al., 2005) (Verdino et al., 2010). Disruption of Cxadr in the intestine did not yield any discernible phenotype after 18months analysis (Pazirandeh et al., 2011) however a possibility has been raised for Amica1-Amica1 interactions or Amica interactions with other receptors due to the promiscuity of immunoglobulin superfamily receptor binding (Moog-Lutz, 2003).



This Amica1-Cxadr interaction has been reported to activate PI3K signaling in epidermal cells during wound healing in the skin (Verdino et al., 2010).

To gain insight into the role of Amica1 in the intestine, we generated a conditional Amica1 allele. We show preliminary data on the function of Amica1 in adult mice homeostasis after intestine specific deletion using the inducible Villin-CreER transgene

## Materials and Methods

### Generation of conditional Amica1 floxed allele

The murine Amica1 gene encoding a single transcript, contains 11 exons, with the start codon located in exon 3, the first extra cellular Immunogloblin domain (D1) encoded by exon 4, the second extra cellular Ig domain (D2) encoded by exon 6, the transmembrane domain encoded by exon 8, and the intracellular domain encoded by exon 9. Removal of exon 3 introduces a frame shift which results in a non-sense protein. For this reason, we introduced one loxP site in the untranslated region upstream of the ATG start codon in exon 3. A second loxP site was introduced in the untranslated region between exon 3 and exon 4.

Amica1loxP mice were generated via homologous recombination in embryonic stem (ES) cells Both the 4.5kb and 2.5kb targeting arms were generated by high fidelity PCRs from mouse genomic DNA isolated from 129/Ola- derived IB10 ES cells and subsequently cloned into a PL451

plasmid vector. 100ug of the targeting construct was linearized and electroporated (800V, 3F) into male 129/Ola-derived IB10 ES cells. Recombinant ES cell clones expressing the neomycin gene were selected in medium supplemented with 250ug/ml G418. Approximately 300 recombinant ES cell clones were screened by Southern blotting, out of which 8% were homologously recombined. Two positive clones were selected and injected into C57BL/6 mouse-derived blastocysts using standard procedures. Both clones gave germ line transmission. The neomycin selection cassette was flanked by Frt recombination sites and excised *in vivo* by crossing the mice with the general FLP deleter strain (Jackson Laboratories).

**Immunohistochemistry**

Freshly isolated intestines were flushed and fixed with formalin (4% formaldehyde in phosphate-buffered saline [PBS]) overnight (O/N) at room temperature (RT). The formalin was removed, and the intestines were washed once in PBS at RT. The intestines were then transferred to a tissue cassette and dehydrated by serial immersion in 20-fold volumes of 70%, 96%, and 100% ethanol (EtOH) for 2 h each at RT. Excess ethanol was removed by incubation in xylene for 1.5 h at room temperature, and the cassettes were then immersed in liquid paraffin (58°C) overnight. Paraffin blocks were prepared using standard methods. Tissue sections of 4 μm were dewaxed by immersion in xylene (2 times, 5 min) and hydrated by serial immersion in 100% EtOH (2 times, 1 min), 96% EtOH (2 times, 1 min), 70% EtOH (2 times, 1 min), and distilled water (2 times, 1 min). Endogenous peroxidase activity was blocked by immersing the slides in peroxidase blocking buffer (0.040 M citric acid, 0.121 M disodium hydrogen phosphate, 0.030 M sodium azide, 1.5% hydrogen peroxide) for 15 min at room temperature. Antigen retrieval was performed (see details below for each antibody), and blocking buffer (1% bovine serum albumin [BSA] in PBS) was added to the slides for 30 min at room temperature. Primary antibodies were then added, and the slides were incubated as detailed below. The slides were then rinsed in PBS, and secondary antibody was added (polymer horseradish peroxidase-labeled anti-mouse or -rabbit antibody; Envision) for 30 min at room temperature. Slides were again washed in PBS, and bound peroxidase was detected by adding diaminobenzidine (DAB) substrate for 10 min at room temperature. Slides were then washed 2 times in PBS, and nuclei were counterstained with Mayer's hematoxylin for 2 min, followed by two rinses in distilled water. Sections were dehydrated by serial immersion for 1 min each in 50% EtOH and 60% EtOH, followed by 2 min each in 70% EtOH, 96% EtOH, 100%

EtOH, and xylene. Slides were mounted in Pertex mounting medium, and a coverslip was placed over the tissue section. Antigen retrieval was performed by boiling samples for 20 min in 10 mM sodium citrate buffer (pH 6.0). Antibodies used were mouse anti-Ki67 (1:100 dilution; Novocastra), rabbit anti-synaptophysin (1:200 dilution; Dako), rabbit anti-lysozyme (1:1,750 dilution; Dako),. Incubation of antibodies was performed for 1h at room temperature for antibodies directed against Ki67, ChrgA, β-catenin, and lysozyme. In all cases, reagent from the Envision$^{+}$ kit (Dako) was used as a secondary reagent. Stainings were developed with DAB. Slides were counterstained with hematoxylin and mounted.

### *In situ* hybridization

8-µm-thick intestinal sections were rehydrated as described above. Afterward, the sections were treated with 0.2 N hydrochloric acid for 15min and proteinase K treated for 20min at 37°C. Slides were then post-fixed with freshly prepared 4%PFA for 10, washed with PBS, and then demethylated with acetic anhydride (2 times for 5min each), followed by pre-hybridization for 2hours at 70°C. Hybridization with RNA probes was performed in a humid chamber with 500 ng/ml freshly prepared digoxigenin (DIG)-labeled RNA probes



### *Generation of RNA probes*

In situ hybridization probes for Amica, and Cxadr were generated by in vitro transcription (David and Wedlich, 2001) from PCR amplified templates from whole intestine cDNA using the following oligonucleotides:

Olfm4 (image clone 1078130) Probes for Amica was transcribed from PCR generated template DNA amplified from whole intestine cDNA with the following primers: Amica-5'GACATCTCCCGCAATGATGG; 3'***AATTAACCCTCACTAAAGGG***TTCCAACAAATCACACCTATTTGG. Sections were incubated for 48-72hours at 70°C. The slides were washed, and incubated with the secondary anti-DIG antibody (Roche) at 4°C overnight. Sections were subsequently washed and developed using Nitro Blue Tetrazolium chloride–5-bromo-4-chloro-3-indolylphosphate.

## Results and Discussion

### Amica1 expression in the intestine

We have previously defined intestinal stem cell gene signature by microarray analysis after FACS isolation of Lgr5+ stem cells (Muñoz et al., 2012). Based on this transcriptional profile, we identified Amica1 as a transmembrane molecule enriched in intestinal stem cells. To confirm the microarray data, we performed RNA in situ hybridization with a probe specific for Amica1 transcripts. Analysis of these results showed that the expression pattern of Amica1 in the small intestine is strikingly similar to that Lgr5 and Olfm4, established markers for CBC stem cells ((Barker et al., 2007)(van der Flier et al., 2009). Amica1 is expressed only in the bottom of the crypts, in the slender CBC stem cells but not in granulated Paneth cells (figure 1A). This expression was higher in crypts in the jejunum and ileum as compared to the duodenum. Low level expression of Amica1 mRNA was also detected in the proximal colon crypt bottom. We did not detect Amica1 mRNA expression in Wnt signaling mutated tumors from Apc$^{min}$ mice suggesting Amica1 might be important for normal stem cells and dispensable for cancer stem cells.

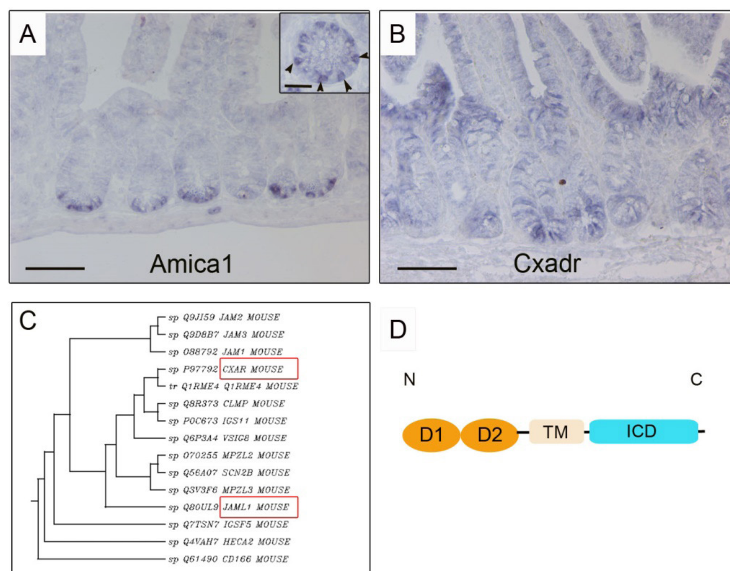



**Figure1. Amica1(JAML) gene and binding partner in intestine**. A) In situ hybridization showing Amica1 mRNA localization to CBC stem cells. B) mRNA localization of Cxadr, a cognate receptor for Amica1. Cxadr is ubiquitously expressed in all epithelial cells with a high expression in crypt stem cells and Paneth cells, as well as crypt villus junction epithelia C) Amica1 (JAML) and its binding partner Cxadr (Cxar) and other related family members. D) Protein domains of Amica1; two extra cellular domains, D1 and D2, a transmembrane domain ™, and in intracellular domain (ICD).

To study the role of Amica1 in the adult murine intestine, we crossed the Amica1$^{loxP}$ animals with the Villin Cre-ERT2 mouse (El Marjou et al., 2004). In these mice, the promoter of the Villin1 gene which is expressed in all intestinal epithelial cells, drives expression of Cre recombinase, the

function of which is induced upon tamoxifen injection.

Amica1$^{loxP/loxP}$/Villin CreERT2 (mutant) was injected with the maximal dose of tamoxifen. and sacrificed 5 days after the last tamoxifen injection. As control, we used wild type mouse with same tamoxifen regimen. Deletion of Amica1$^{loxP}$ allele was confirmed by RT-PCR and in situ hybridization.

In situ hybridization for Amica1 transcript indicated that a significant deletion of the Amica1 gene in the intestine as visualized by reduction of Amica1 mRNA in mutant as compared to control (Figure 3A and B). There was some residual Amica1 expression in mutant crypt as the recombination using the Villin Cre-ER reporter is not 100% efficient. Further analysis of Amica1 deletion by Southern blotting will be more informative on the extent of cre mediated recombination.



In situ hybridization analysis for effect of Amica1 deletion on intestinal stem cells a loss of Olfm4+ cells in mutant crypts suggesting that this gene might be important for stem cell maintenance. Analysis of proliferation by immunostaining of Ki67 showed a loss of proliferative crypts in the mutant with large patches of the intestine lacking proliferative crypts. Additionally remaining proliferative cells in mutant crypts were disorganized with occasional clumps of Ki67+ cells.

Deletion of Amica1 did not affect the numbers of lysozyme+ Paneth cells in mutant crypts. However occasional lysozyme positive cells could be observed on villi.

Mucin2+ Goblet cells are normally interspersed along the crypt-villus axis. In Amica1 mutant, there was a reduction in the number of Muc2+ goblet cells especially in the crypts.

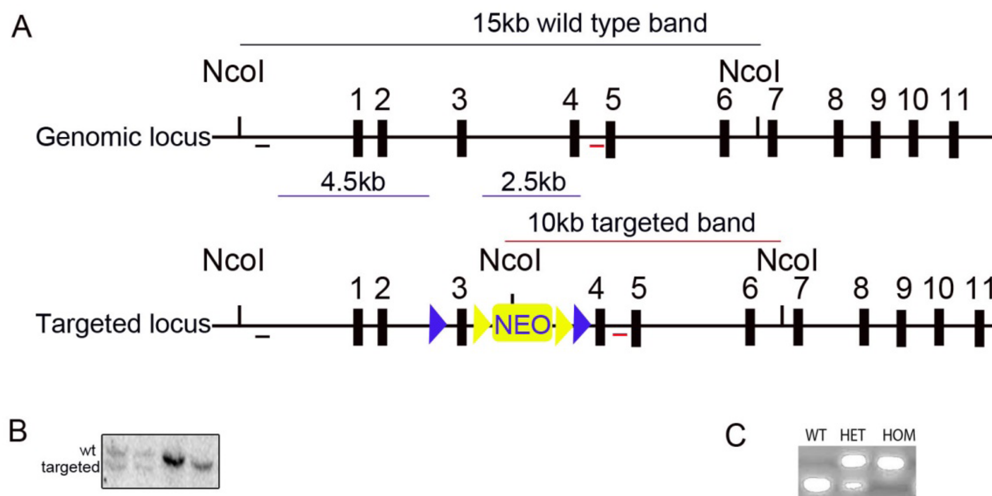



Figure 2. **Generation of Amica1 floxed allele. A)** Targeting strategy for Amica1. B) Southern blot of targeted ES cell clone for Amica1. C) Genotyping of wild type, heterozygous and homozygous Amica1$^{loxP}$ mice.



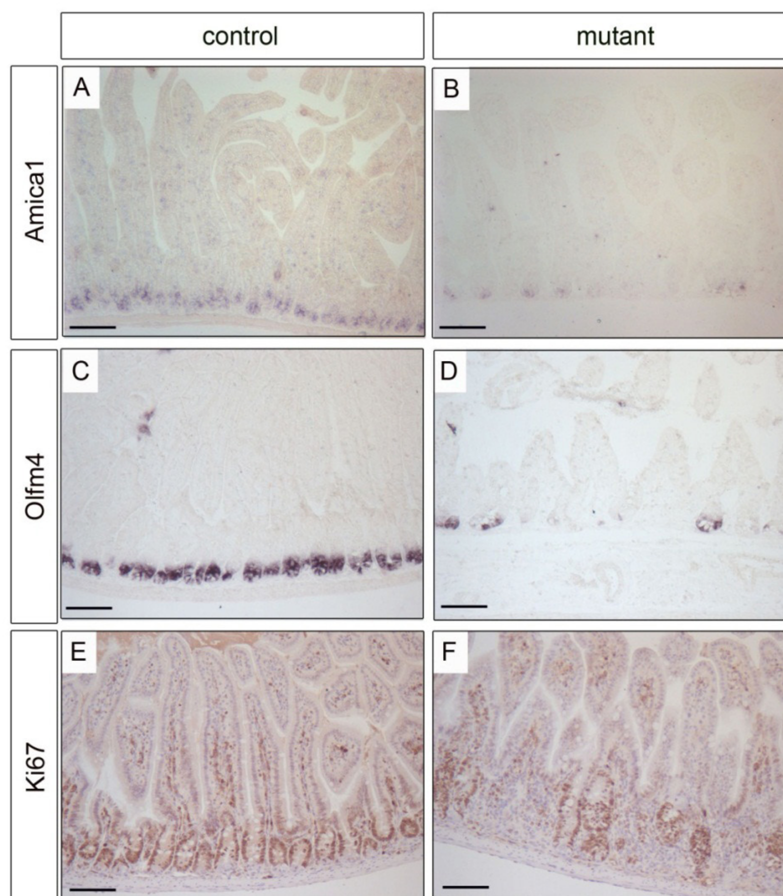



Figure 3. Effect of Amica1 deletion on stem cells and proliferation. A and B) ISH showing loss of Amica1 mRNA expression upon tamoxifen induced recombination; C and D) loss of Olfm4+ stem cells in mutant crypts; E and F) loss of Ki67+ cells in crypts of Amica1 mutant.

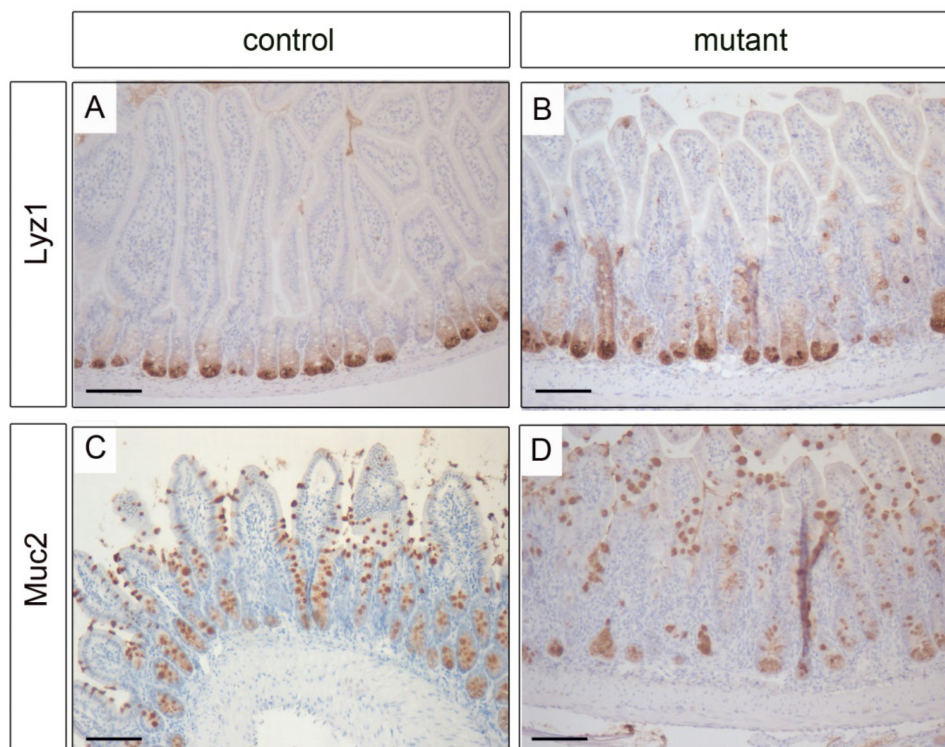



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**Figure 4. Amica1 deletion and secretory cells.** A and B) No effect of Amica 1 loss on Lysozyme (Lyz1)+ Paneth cells; C and D) Muc2+ goblet cell numbers were not affected upon deletion of Amica1 in mutant.

These preliminary data suggest that loss of Amica1 may affect maintenance of intestinal stem cells. Ongoing experiments are focused on analyzing more mice, and also at later time points to definitively determine a function for Amica1 in intestinal stem cells. Of interest will also be to determine the mode of Amica1 function in the intestine; whether it interacts with its cognate receptor, Cxadr in the intestinal crypts, or whether it interacts with receptors on Paneth cell or in the underlying basement membrane, or whether it is involved in intracellular signaling in stem cells.

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# CHAPTER SIX

# Role of ZFP12 and RBAK transcription factors in intestinal stem cells

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## Role of ZFP12 and RBAK transcription factors in intestinal stem cells

**Paul W. Tetteh,** Harry Begthel , Maaike van den Born, Jeroen Korving, Inha Heo, Henner F. Farin, Johan van Es, and Hans Clevers

The small intestinal epithelium is the champion of self-renewal in mammals, with the fastest turnover rate of 4 to 5 days. This high turnover is powered by proliferative crypt stem cells that divide daily to generate the differentiated cells of the villus, the functional unit of the intestine. The major driving force for intestinal stem cell proliferation, self-renewal and differentiation is the Wnt signaling pathway. Briefly, binding of Wnt ligands to Wnt receptors in crypt stem cells induce nuclear localization of β-catenin to bind Tcf4 transcription factor which in turn orchestrates the transcription of Wnt target genes necessary for stem cell maintenance and function. Mice with intestinal specific deletion of Tcf4 lack proliferative crypts during late gestation (Korinek et al., 1998) and also in adult stages (Es et al., 2012). Conditional deletion of β-catenin or transgenic over-expression of secreted Wnt inhibitor Dickkopf-1 results in disappearance of proliferative crypts in adult mice. Aberrant activation of the Wnt signaling pathway leads to neoplastic transformation of the intestine (Korinek et al., 1997)(Morin et al., 1997). Other signaling pathways such as the Notch (Noah and Shroyer, 2013) and BMP pathways (Haramis et al., 2004) also play a role in maintaining intestinal stem cells.



We have previously shown that columnar base cells (CBCs) wedged between Paneth cells at the crypt bottom and expressing the Wnt target gene Lgr5, are bonafide stem cells with self-renewal and multipotency capabilities, giving rise to all the various cell lineages that constitute the small intestine (Barker et al., 2007). Furthermore, Lgr5+ stem cells can be isolated from the intestinal epithelium and grown in 3-dimensional cultures in vitro, attesting to their stemness. These organoid cultures recapitulate the intestinal epithelium in terms of architecture and proper positioning of all the cell lineages, and require the Wnt agonist Rspondin1, EGF, and the BMP antagonist, Noggin for their maintenance (Sato et al., 2009).

The difference between stem cell renewal and differentiation into specific cell lineages can be regulated by specific transcription factors. We have defined the murine intestinal stem cell gene signature via transcriptome analysis of sorted Lgr5+ stem cells and their daughter cells (Muñoz et al., 2012). Using these gene profiles, we have identified novel transcription factors that are unique to Lgr5+ stem cells. One of these genes is the zinc finger protein 12 (ZFP12) transcription factor.

Zinc finger protein 12 (ZFP12) is a KRAB domain containing transcription factor that belongs to the C2H2-associated box-zinc finger protein (KRAB-ZFP) family, one of the largest families of potential transcriptional regulators (Lemon and Tjian, 2000). The functions currently known for members of the KRAB-containing protein family include transcriptional repression of RNA polymerase I, II, and III promoters, binding and splicing of RNA, and control of nucleolus function (Zhao et al., 2006). The C2H2 type zinc-finger motifs participate in the formation of amino acid loops (Pavletich and Pabo, 1991) whereas the KRAB motif can physically interact with RING proteins known as KAP1/TIF1b and KRIP-1, respectively, to mediate the silencing function and the repressor effect of KRAB transcription factors (Ganss et al., 2002) may extend over a distance. Interestingly, RBAK (Retinoblastoma associated Kruppel-like factor), a paralogue of ZFP12, has a similar mRNA expression in intestinal crypts and villi.

Our objective in this study is to gain insight into the role of Zfp12 and Rbak transcriptional factors in adult intestinal stem cells by generating and analyzing conditional Zfp12 and/or Rbak mutant mice.

## Materials and Methods

### Generation of ZFP 12 conditional allele

The Zfp12 gene is situated on chromosome 5 of the mouse genome with 5 exons encoding a 75kDa protein consisting of a conserved KRAB domain and 15 C2H2 type zinc fingers. The 654 amino acids that generate the Zfp12 protein are all located in its largest exon, exon 5. Coincidentally the closely related Rbak is also located on chromosome 5 with a similar (75%) exome and protein coding sequence. Both genes are about 85kb apart, with another unrelated gene, Spdyb in-between.

### In situ hybridization

8-μm-thick intestinal sections were rehydrated as described above. Afterward, the sections were treated with 0.2 N hydrochloric acid for 15min and proteinase K treated for 20min at 37°C. Slides were then post-fixed with freshly prepared 4%PFA for 10, washed with PBS, and then demethylated with acetic anhydride (2 times for 5min each), followed by pre-hybridization for 2hours at 70°C. Hybridization with RNA probes was performed in a humid chamber with 500 ng/ml freshly prepared digoxigenin (DIG)-labeled RNA probe of Zfp12 and Rbak. Sections were incubated for 48-72hours at 70°C. The slides were washed, and incubated with the secondary anti-DIG antibody (Roche) at 4°C overnight. Sections

were subsequently washed and developed using Nitro Blue Tetrazolium chloride–5-bromo-4-chloro-3-indolylphosphate.

Microarray analysis was performed with data uploaded on the 'R2: microarray analysis and visualization platform (http://r2.amc.nl)'



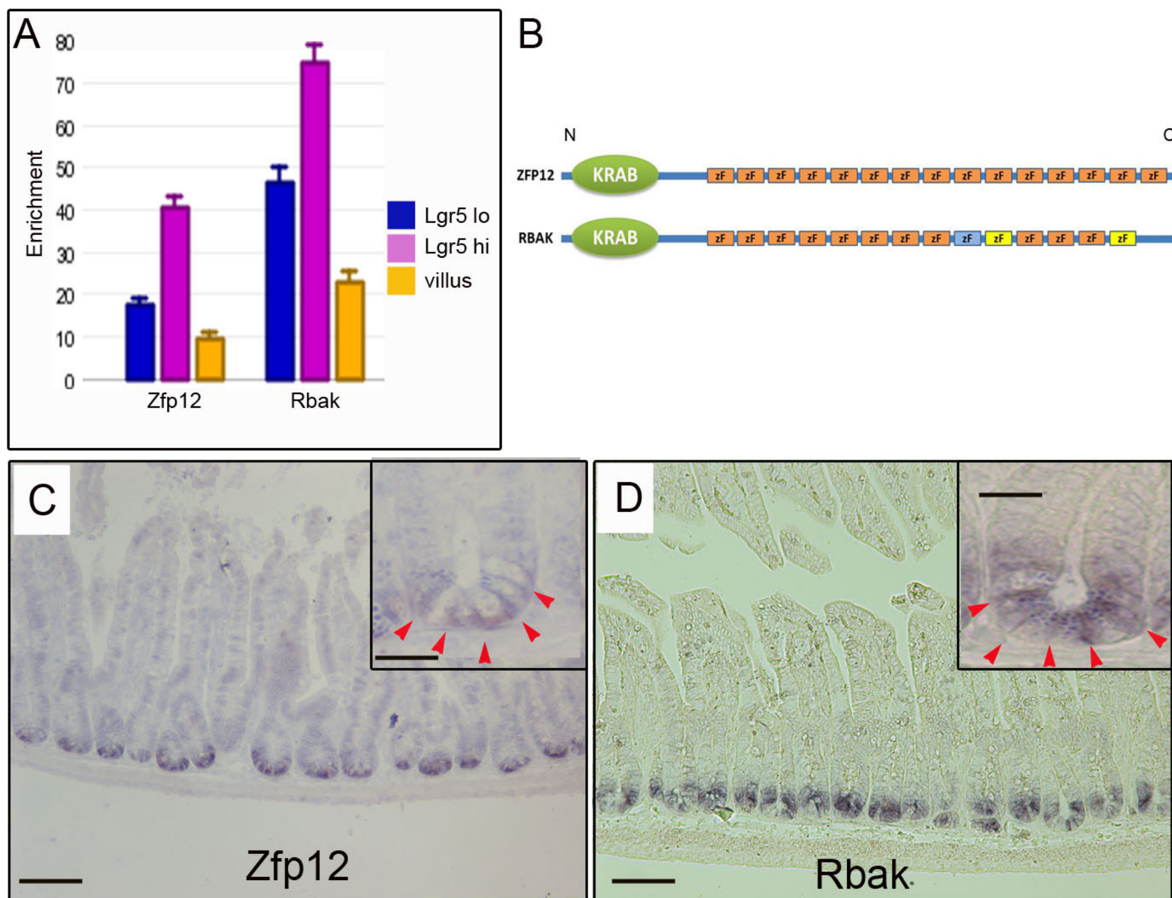


**Figure1. Zfp12 and Rbak genes.** A) Microarray analysis of Zfp12 and Rbak showing enrichment of both genes in Lgr5 high stem cells and Lgr5 low cells as compared to villus fractions. B) Protein domains of Zfp12 and Rbak Both have a conserved KRAB domain and C2H2 type zinc fingers in tandem. C) In situ hybridization showing expression of Zfp12 in CBC stem cells.D) In situ hybridization for Rbak showing expression in CBC stem cells.

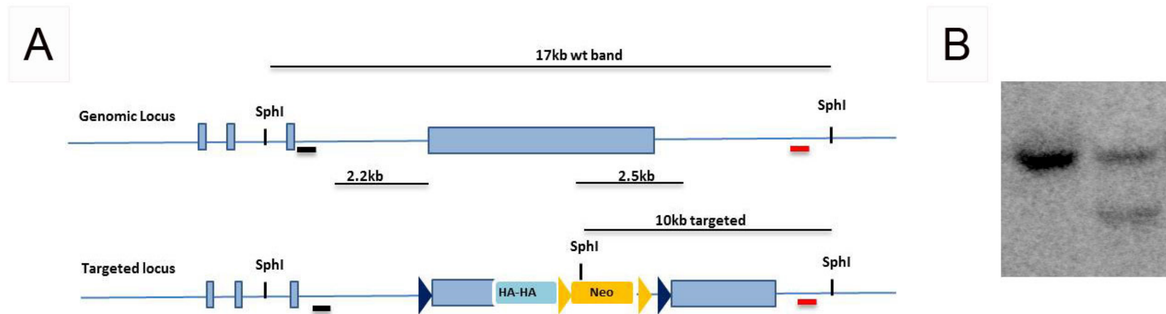


**Figure 2. Conditional Zfp12 allele**. A)Targeting strategy for Zfp12$^{loxP}$-2HA allele. B) Southern blot showing targeted ES cell clone (double band) and wild type (single band) control.

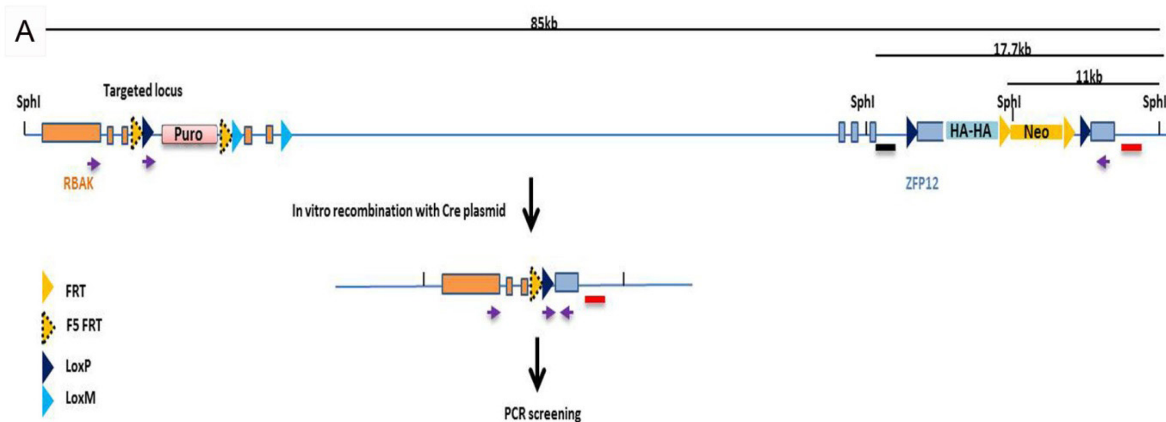


**Figure 3 Targeting strategy for conditional double knockout allele for Rbak$^{loxM}$-Zfp$^{loxP}$**

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## Results and Discussion

### Zfp12 and Rbak are enriched in intestinal stem cells

The identity, location, function and behavior, of a particular cell type within a tissue depends in part on the activated genes and gene regulatory networks present in the cell, which can also be used to characterize the cell type in question. We have previously identified CBCs at the crypt base as genuine stem cells with self-renewal and multipotency characteristics based on the unique expression of the Wnt target gene, Lgr5, in these cells. Generation of Lgr5-GFP-CreERT2 transgenic mice have permitted interrogation and observation of stem cell activity of Lgr5+ stem cells by in vivo lineage tracing and establishment of long term ex vivo organoids from sorted Lgr5+ crypt cells. Sorted GFP+ cells from these mice have also been used to generate gene expression profile for Lgr5+ intestinal stem cells commonly referred to as Lgr5+ high cells (van der Flier et al., 2009a) (Muñoz et al., 2012). These profiles have revealed an extensive array of transcripts enriched in CBCs. One of these stem cell enriched genes is ZFP12 gene belonging to the KRAB box family of transcriptional regulators.

Incidentally, Rbak, a closely related family member of Zfp12 is also enriched in Lgr5 high cells (Figure 1A). RNA in situ hybridization experiments of mouse small intestine confirmed a highly specific localization of both Zfp12 and Rbak in intestinal crypt CBCs. Expression of Rbak appeared to be broader than Zfp12, encompassing early stem cell daughter cells in the transit amplifying (TA) compartment (Figure 1B).

No definitive in vivo function has been ascribed to Zfp12. In vitro, small intestine organoids require EGF, Rspo1 and Noggin for long term culture. Withdrawal of noggin, a BMP inhibitor, from the culture medium leads to death of organoids. However, small intestine organoids in which Zfp12 had been over-expressed showed long term survivability upon noggin withdrawal (Schuijers et al, unpublished) suggesting Zfp12 might be critical for stem intestinal stem cell maintenance.

Withdrawal of noggin from in vitro small intestine organoids leads to death of organoids. Preliminary in vitro experiments in our lab suggests that small intestine organoids in which Zfp12 had been over-expressed showed long term survivability upon noggin withdrawal.



In order to study the function of Zfp12, we have generated a conditional allele for this gene by inserting loxP sites on either side of exon 5 which contains the protein coding sequence. Crossing the Zfp12loxP allele with tissue specific Cre reporter mice will enable the function of Zfp12 to be determined after cre mediated deletion of its protein coding region. We have crossed the Zfp12loxP allele to the intestine specific Villin-CreERT2. Future experiments with Villin-CreERT2-Zfp12loxP/loxP mice will help ascertain the function of Zfp12 in the adult intestine. As Zfp12 might function as a transcriptional repressor, unravelling the genes it interacts with in intestinal stem cells will will give a better understanding of its function if its expression expression in the intestine is critical for stem cell biology. Preempting this scenario, we have we have inserted a double HA tag downstream in the 3'UTR to generate a ZFP12-2HA fusion protein which can be used for chromatin-immunoprecipitation experiments from isolated crypt cells to enable identification of Zfp12 interacting partners. Due to the similar expression of the Zfp12 paralogue, Rbak in intestinal stem cells, it will be expedient to delete Rbak as well to avoid functional redundancy although it is also likely they may have divergent roles in intestinal stem cells. In vitro studies in fibroblasts have reported RBAK interacts with and transcriptionally represses the activity of the retinoblastoma (RB) tumor suppressor gene (Skapek et al., 2000) RBAK also interacts with the

androgen receptor and increases its transcriptional activity (Hofman et al., 2003).

As both Zfp12 and Rbak occur on the same chromosome and separated by a large 85kb region with a gene of unknown function in-between we re-targeted $Zfp12^{loxP}$ ES cells with a targeting construct for Rbak ($Rbak^{loxM}$) where the first two exons of Rbak have been floxed with loxM sites. This variant lox sequence will not recombine with loxP sites around Zfp12, and will allow conditional deletion of both genes as long as they have been targeted in cis on the same allele (Figure 2A).

Future experiments with both $Zfp12^{loxP}$ and $Rbak^{loxM}$ conditional alleles will be useful in determining a role of novel transcriptional regulators in intestinal stem cell biology.

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# CHAPTER 7
# Summerizing Discussion

# SUMMERIZING DISCUSSION

**Paul W. Tetteh**

Cells lost as a result of genetic and environmental insults, and the physiological ageing process must be continuously replaced to sustain the life of the organism. The maintenance of this cellular homeostasis is marshalled by tissue resident stem cells that have the capacity to self-renew, and differentiate to generate all the cell types that characterize a particular tissue. How do tissues cope with insults that cause loss of the stem cell pool that maintain homeostasis? It has been assumed that quiescent stem cells pools exist that are utilized to supply cells upon loss of homeostatic stem cells. Many lineage tracing studies have focused on identifying and functionally characterizing these quiescent stem cells. In the small intestine, Lgr5+ stem cells at the crypt bottom maintain homeostasis of the epithelial cell populations. Alternatively, cells located at the +4 position from the crypt base and expressing markers such as Bmi1, Hopx and mTERT have been proposed as reserve stem cells that generate all the cell lineages upon loss of the Lgr5+ stem cell population. Various studies have questioned the existence of a distinct +4 stem cell pool as proposed markers are broadly expressed in all crypt cells. Additionally secretory progenitor daughter cells of Lgr5+ stem which are located in the same vicinity as these +4 cells have been shown to dedifferentiate upon loss of stem cells suggesting there may be a heterogenous population that replenish loss of Lgr5+ stem cells.



In chapter 2 of this thesis, the generation and characterization of a novel inducible enterocyte specific Cre line was described. Using this model in combination with the Lgr5-DTR model, the plasticity of committed enterocyte progenitors was investigated. Similar to secretory progenitors, enterocyte progenitors are also plastic during regeneration. It raises the question, are distinct quiescent stem cell pools needed by the intestine, if committed progeny can be called into action? Or that if indeed distinct bonafide quiescent stem cell pools exist, they are insufficient to ensure complete and total regeneration thus committed progeny are needed to complement the regenerative process. A limitation of the current study is the restricted expression of cre from the Alpi locus to the proximal small intestine thus we could not explore enterocyte plasticity in the distal small intestine. Whether regional specific differences exist in terms of pools of differentiated cells employed will be interesting to investigate further.

The marked plasticity of differentiated intestinal epithelial cells has implications for our understanding of cellular hierarchy and regeneration in the intestine. Whilst the existence of a distinct quiescent stem cell pool as reserve stem cells might hold true for other systems, it appears such a strategy might not be necessary in the small intestine to replenish cell loss.

Another implication of this finding is that 'stemness'of progenitors in the intestinal crypt are not hardwired but heavily dependent on niche association (Tetteh et al.)(Barker, 2014). Intestinal stem cell identity in the crypts is regulated during homeostasis by the niche which comprises Paneth cells, stromal cells in the underlying mesenchyme, and extracellular matrix proteins. These niche components and the essential signals they supply (such as Wnt, EGF and Notch ligands) are concentrated at the crypt base, diminish up the crypt and absent in the villus. Stem cells that are in closest proximity to the crypt base are endowed with a constant supply of these signals to maintain their 'stemness'. In the experiments described in chapter 2, deletion of Lgr5+ stem cells creates a temporary void at the crypt base which can be competed for by various committed progenitor cell populations. Because these progenitors are highly plastic, exposure to the crypt base niche environment transforms them into multipotent stem cells.

Plasticity of committed epithelial cells can be exploited for endogenous regeneration of the intestine such as replenishment of intestinal stem cells in cancer patients undergoing radiation chemotherapy. A thorough understanding of the molecular mechanisms behind intestinal plasticity will facilitate the development of improved therapeutic adjuvants that coerce committed cells to dedifferentiate, to potentiate the recovery process.

The requirements for a normal cell to be transformed into a cancer initiating cells include the nature of the oncogenic mutation, the differentiation state of the cell, and the longevity of the cell which allow mutations to accumulate and the microenvironment or niche where the cell resides. The cancer niche plays important role in maintaining cancer stem cells, tumor initiation and protection against chemotherapeutic agents. One of the major hallmarks of cancer cells is the uncontrolled cell division and proliferation as such mutated proliferative cells are more prone to initiate tumors. Highly proliferative long-lived Lgr5+ stem cells with Apc mutations have been shown to be transformed into tumor initiating cancer stem cells without additional niche requirements(Barker et al., 2007). Here, the Lgr5+

cancer stem cells presumably ‘hijack’ the normal stem cell niche for their maintenance and tumor progression. Although not probed in these experiments, it is possible for these Lgr5+ cancer stem cells to also create their own niche by secreting signaling factors that modulate their microenvironment to support cancer stem cells in fuelling tumor formation and progression.

Short-lived committed enterocytes progenitors are proliferative however they do not form tumors both in vivo and in vitro upon Apc mutation. This suggests that the differentiation state or proliferative state of enterocyte progenitors is not amenable to tumorigenic transformation upon deletion of Apc. Of note, Apc deletion in long-lived tuft cells which are scattered along the crypt villus axis also do not give rise to tumors (Westphalen et al., 2014) supporting the notion that Apc mutations require a bonafide stem cell cellular machinery to induce tumor formation.

Apc mutated cancer cells with additional Kras mutation have enhanced Wnt signaling to promote cancer formation and progression (Janssen et al., 2006). It is intriguing that short lived differentiated Alpi+ enterocytes with Apc and K-ras mutations fail to develop adenomas in the proximal small intestine, whereas short lived differentiated Car1+ cells with these mutations are transformed into tumor initiating cells with progressive adenomas in the proximal colon. However, Apc/Kras mutated enterocytes from crypts form tumor organoids in ex vivo matrigel 3-D cultures but rarely form tumors in vivo even when exposed to the normal stem cell niche upon loss of Lgr5+ stem cells. It is possible that matrigel contains additional factors that are supportive of cancer cell growth.Worth investigating will be which factors in matrigel are crucial elements for a cancer niche.

These results suggest that the normal stem cell niche may be insufficient to support plasticity of Apc/Kras mutated enterocyte progenitors into cancer initiating cancer stem cells. Thus tumor initiation from mutated enterocytes may have different cancer niche requirements in comparison to mutated Lgr5+ stem cells.

In view of the fact that human intestinal cancers which these mice model is more frequent in the colon and rarely in the small intestine, it is tempting to speculate that this could be a consequence of the colonic crypt microenvironment being more conducive for mutated differentiated epithelial cells to flourish. It is tempting to speculate that differentiated

goblet cells, enteroendocrine cells and tuft cells in the colon may also give rise to tumors upon Apc/Kras mutations. Worth investigating will be whether differences in micro-environment or inherent genetic fitness of these dissimilar differentiated epithelial cell types accounts for the disparate susceptibility to tumor initiating mutations. Derivation of cancer stem cells in colon cancer does not only emanate from mutations in normal Lgr5+ colonic stem cells, but also from plasticity of differentiated Car1+ cells. It is unlikely that anti-cancer therapeutics targeting cancer stem cells exclusively may yield the desired clinical outcome as plasticity of differentiated Car1+ cells can be employed by the tumor to replenish the CSC population lost as a result of the therapy.

In conclusion, plasticity of differentiated cells is employed in intestinal regeneration or exploited by oncogenic mutations thus regeneration and cancer represent two sides of the same coin; changing cell fate for better or for worse.

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# Addendum

**Nederlandse Samenvatting**

**Acknowledgements**

**Curriculum vitae**

**List of Publications**

## Nederlandse Samenvatting

Stamcellen in volwassen weefsels zijn cellen met een lange levensduur die het vermogen van zelfvernieuwing bezitten. Ze kunnen de verschillende gedifferentieerde celpopulaties van het orgaan voortbrengen die daardoor zijn functie behoudt. Wanneer gedifferentieerde cellen verloren gaan door veroudering of letsel, brengen stamcellen nieuwe cellen voort om de verloren gegane cellen te vervangen. DNA veranderingen (mutaties) in stamcellen kunnen ervoor zorgen dat zij transformeren naar kanker-initiërende stamcellen.

Eén van de meest betrouwbare methoden om stemcellen te markeren en hun dochtercellen te volgen is met behulp van genetische "lineage tracing" in combinatie met de Cre-loxP technologie. Bij deze technologie worden genen in een specifiek celtype gemodificeerd om een reporter (zoals GFP, LacZ of Cre-ER recombinase) tot expressie te brengen, wat doorgegeven kan worden aan de dochtercellen tijdens de celdeling. Het injecteren van tamoxifen induceert Cre activiteit en reporterexpressie zodat de afstammelingen van de cel getraceerd kunnen worden in ruimte en in tijd. In de dunne darm zijn *base columnar cells* geïdentificeerd en gekarakteriseerd die in de crypten het marker gen Lgr5 tot expressie brengen. Deze stamcellen delen dagelijks om de gedifferentieerde epitheel celpopulaties (secreterende cellen (goblet cellen, Paneth cellen, enteroendocrine cellen), en absorberende cellen (enterocyten)) in stand te houden. Lgr5 markeert ook stamcellen in de dikke darmcrypten, waar zij gedifferentieerde darmepitheelcellen voortbrengen. Daarnaast zijn deze Lgr5+ stamcellen betrokken bij de initiatie van darmkanker. Lang werd gedacht dat cel identiteit vaststaat, en dat differentiatie in één richting verloopt (van een stamcel naar een gedifferentieerde cel). Echter, er is steeds meer bewijs dat gedifferentieerde cellen hun lot kunnen veranderen en zich kunnen gedragen als normale stamcellen of kanker-initiërende cellen. Dit vermogen van cellen om hun lot of identiteit te veranderen wordt "plasticiteit" genoemd.

In dit proefschrift beschrijf ik de ontwikkeling van nieuwe muismodellen om de plasticiteit van gedifferentieerde epitheliale celpopulaties in darmregeneratie en kanker-initiatie te onderzoeken.

Er is vermeld dat secretoire cel progenitoren plasticiteit vertonen bij verlies van Lgr5 stamcellen . Het is echter onbekend of absorberende enterocyten voorlopercellen een rol in darmregeneratie spelen. Enterocyte voorlopercellen zijn dochtercellen van crypte Lgr5 + stamcellen, die leiden

tot volwassen enterocyten die migreren naar de villus, functioneren in de voedselopname, en worden afgestoten in het darmlumen. Deze enterocyten hebben een zeer korte levensduur van 4 tot 5 dagen. Enterocyte voorlopercellen in de crypten en volwassen enterocyten in de villus hebben een unieke expressie van het alkalische fosfatase gen, Alpi.

Hoofdstuk 2 beschrijft de generatie van een Alpi-CreER muismodel waar de Alpi-gen is ontworpen om ook het Cre-recombinase eiwit uit te drukken, specifiek in enterocyten. Lineage tracing experimenten met deze muis in combinatie met Rosa gen reporter muizen tonen aan dat Alpi+ cellen een korte levensduur hebben, en zich niet gedragen zoals stamcellen tijdens homeostase. Om te bepalen of Alpi+ enterocyten een rol spelen in intestinale regeneratie, kruisden we de Alpi-créer/Rosa-LacZ muizen met het Lgr5-DTR-model. In dit DTR model zijn alle Lgr5+ stamcellen ontworpen om de difterie toxine receptor tot expressie te brengen; binding van difterie toxine geïnjecteerd in muizen resulteert in het doden van alle Lgr5 + cellen. Na injectie van tamoxifen en difterie toxine in Alpi-CreER/Rosa-LacZ/Lgr5-DTR muizen konden stamcel lineage tracings worden waargenomen, echter dergelijke traces werden niet waargenomen bij muizen zonder verlies van Lgr5 stamcellen. Uit analyse van deze stamcellen traces bleek dat zij alle verschillende celtypen die in de darm gevonden kunnen worden bevatten. Deze resultaten demonstreren dat Alpi+ stamcellen plasticiteit vertonen om verloren Lgr5+ stamcellen in vivo te regenereren. Verdere studies toonden aan dat Alpi+ cellen zich ook gedragen als normale stamcellen in vitro.



Cellulaire plasticiteit speelt ook een belangrijke rol in tumorvorming. Mutaties in stamcellen of gedifferentieerde cellen maken ze plastisch, waardoor ze getransformeerd worden tot kanker initiërende cellen. Identificatie van cellen die de vorming van kanker veroorzaken is cruciaal voor therapie gericht op het stoppen van progressie van kanker. Momenteel zijn er twee gedachtegangen over de oorsprong van kleine darmkankers; het 'bottom down' model stelt dat stamcellen in de proliferatieve crypte regio de kanker initiëren cellen zijn, terwijl het 'top down' model stelt dat gemuteerde gedifferentieerde cellen de oorsprong van darmkanker zijn. Eerdere studies in ons laboratorium ter ondersteuning van het 'bottom-up' model bleek dat Lgr5+ stamcellen met een APC mutatie kanker vormen in zowel de dunne darm als de dikke darm. Andere groepen hebben tumorvorming gemeld van dunne darm gedifferentieerde epitheelcellen ter ondersteuning van het 'bottom up'

model, maar het is duidelijk uit deze studies welke specifieke groep gedifferentieerde cellen de tumorvorming initiëren vanwege beperkingen van de gebruikte muizenmodellen. Met behulp van ons nieuwe enterocyt specifieke Cre-muis-model hebben we onderzocht of gemuteerde gedifferentieerde enterocyten tumoren kunnen vormen.

Mijn resultaten in hoofdstuk 3 laten zien dat enterocyten in muizen met APC mutaties of beide APC en K-ras mutaties geen tumoren in crypten of villi ontwikkelen. Wanneer gemuteerde enterocyten uit de muizencrypten worden genomen en gekweekt worden in organoïde kweekmedium, kunnen slechts enterocyten van crypten met APC en K-ras mutaties tumor organoids vormen die gekweekt kunnen worden gedurende lange tijd, onafhankelijk van groeifactoren voor normale intestinale epitheelcellen. Wanneer bovendien kankerverwekkende mutaties gecombineerd worden met verlies van Lgr5 cellen in muizen, kunnen alleen enterocyten met APC en K-ras mutaties tumoren vormen. Deze resultaten suggereren dat noch volledig gedifferentieerde enterocyten van de villi noch de enterocytvoorlopers in de crypten tumoren vormen bij de verkrijging van beide APC en K-ras mutaties. Echter, APC/K-ras gemuteerde enterocytvoorlopers in crypten vormen tumoren als zij toegang hebben tot de stamcelniche na verlies van Lgr5+ stamcellen, of indien gekweekt in vitro in organoïde groeimedium. In totaal benadrukken deze resultaten het belang van de stamcelniche voor enterocytvoorlopers om omgezet te worden in tumor-initiërende cellen.

Bij mensen is darmkanker in de dunne darm zeldzaam, het treedt meestal op in de dikke darm. Er is gesuggereerd dat verschillen in celtypen, micro-omgeving en blootstelling aan carcinogenen tussen de dunne darm en de dikke darm invloed hebben op tumorvorming. Muismodellen zijn belangrijk voor de bestudering van de ontwikkeling van kanker, en ook om therapieën die gericht zijn op het terugdringen van de progressie van kanker te testen. Een huidige beperking van darmkanker muismodellen is dat tumoren meestal ontstaan in de dunne darm en in andere organen, waardoor het moeilijk is om kanker te onderzoeken in het kader van de dikke darm. In hoofdstuk 4 beschrijf ik het genereren van een nieuwe muis lijn, Car1-CreER, met cre expressie beperkt tot de dikke darm en niet de dunne darm. Dit model is bruikbaar voor het muteren van kankergenen specifiek voor de dikke darm, op een gecontroleerde manier. Interessant genoeg is Cre expressie in de Car1-CreER muis gezien in beide stamcellen en gedifferentieerde cellen in de blinde darm, en beperkt tot

gedifferentieerde epitheelcellen van de proximale dikke darm, waardoor we plasticiteit van deze cellen kunnen onderzoeken bij de verkrijging van genetische mutaties van APC en K-ras. Interessant genoeg ontwikkelen Car1 + cellen met APC mutaties tumoren alleen in de blindedarm, terwijl Apc/K-ras mutaties leiden tot tumoren in de blindedarm en de proximale dikke darm. Tumoren van deze gedifferentieerde Car1+ cellen in de proximale dikke darm waren zeer proliferatief en brachten stam markers tot expressie, wat suggereert dat de mutaties ze plastisch maken, en ze transformeert naar kankerstamcellen. Deze resultaten suggereren dat in de proximale dikke darm, zowel de top-down en bottom-up modellen goed zijn voor tumorinitiatie.

Stamcel specifieke genen zijn cruciaal voor het behoud van de identiteit en functie van volwassen stamcellen. De functie van vele genen specifiek tot expressie gebracht in darm stammen blijven echter onbekend. Amica1 is een intestinale stamcelspecifiek transmembraan molecuul. Om de functie van Amica1 te bepalen in darmstamcellen, genereerde ik een Amica1 floxed muismodel om dit gen specifiek te verwijderen in de darm. Een ander darmstamcel specifiek gen met onbekende functie is het Zfp12 transcriptie repressor gen.Om de rol van dit gen in de darm te onderzoeken, beschrijf ik in hoofdstuk 6 het genereren van een muismodel specifiek voor de deletie van dit gen in de darm.



Ter conlusie, dit proefschrift onthult nieuwe inzichten in de plasticiteit van gedifferentieerde epitheliale cellen in darmregeneratie en kanker. In de dunne darm lijkt celidentiteit in de crypte niet vast te liggen. Cellen zoals enterocytvoorlopers kunnen hun lot veranderen naar normale stamcellen als ze de toegang hebben tot de stamcelzone of niche. De niche speelt dus een cruciale rol bij bepaling of een cryptecel een stamcel of een gedifferentieerde cel is. Gedifferentieerde cellen in de dikke darm worden gemakkelijker omgezet in kanker initiërende cellen dan gedifferentieerde cellen in de dunne darm. Misschien is dit een belangrijke factor om de verhoogde incidentie van darmkanker in de dikke darm te verklaren aangezien hier meer cellen gevoelig zijn voor kanker inducerende mutaties. Interventiestrategieën voor het doden van darmkankercellen moeten zich niet alleen richten op kankerstamcellen maar ook gedifferentieerde cellen die ook aanleiding tot kanker kunnen geven.

**Acknowledgements**

I recall my first visit to Utrecht in the summer of 2010 with 5 other prospective PhD students as part of the Cancer Genomics and Developmental Biology (CGDB) PhD rotation program. Back then, I had the opportunity of visiting 4 distinguished labs in the UMC and the Hubrecht Institute, and the 'rare luxury' of selecting a lab of my preference for my doctoral research. I'm grateful to the CGDB committee, the Netherlands Organization for Scientific Research (NWO) and the Royal Netherlands Academy of Sciences (KNAW), under whose auspices my doctoral research was funded.

Working in the Clevers lab as a PhD student is very challenging and it was no surprise that none of the PhD students in my rotation program opted to work in the Clevers lab regardless of its excellent reputation. Dear Hans Clevers, I was the most daring one of the PhD rotation students to choose to work in your lab because I wanted to learn from the best no matter the risk involved. Thank you for the opportunity to work under you. Your personality and achievements will always remain a source of inspiration in my scientific career. Your tutelage has been instrumental in shaping my research focus and interests and the lessons I've learnt in your lab will be with me for the rest of my life. Shidaa agbo!

To Henner Farin. I would not have gotten this far without your assistance. Thank you for your supervision and all the things you taught me about doing science and life in general. Though I was sometimes slow in learning I appreciate your patience to explain things to me over and over again. Shidaa agbo!

Johan van Es, I will like to thank you for all your help with my mouse experiments. Your comments and suggestions always challenged me to critically think about my research questions and experimental set-ups. How true the saying in the lab, '' if you can address all of Johan's questions, you can pass any reviewer's scrutiny'. I appreciate the sacrifices you made to help me with some of the difficult experiments. Shidaa agbo!

Maaike van den Born, I am highly indebted to you for your assistance in taking care of my mice, and also helping out with all my experiments. Your suggestions were also timely to enhance my experiments. Forgive me for sometimes submitting my genotyping results very late. Thank you very much. Shidaa agbo!

Harry Begthel and Jeroen Korving. I am jealous of your many years of experience in the Histology lab. I am grateful that you both taught me how

to section tissues (though I never managed to make them as perfect as you do). I am more appreciative of the fact that you took time of your heavy work schedules to cut sections for me and help with the histological staining. And to Jeroen, thank you for also helping with the blastocyst injections. Less I forget, thanks for the enjoyable Dutch lessons. Shidaa agbo!

Dear Stieneke van den Brink, Thanks for helping with all the cell culture media preparation. If anyone asks me in the future who taught me how to do ES cell culture so excellently, I will say, Stieneke taught me☺ Shidaa agbo! Karin Hammer, thank you for your supportive role as well. I remember the time you taught me how to use the automated pipette. Thank you for making my stay in the Clevers lab a memorable one.

Dear Wim de Lau, knowing you as a scientist and as a person made my stay in the lab comforting. I appreciate your concerns, input into my experiments, your encouragements when things didn't go well, the occasional jokes we shared together (especially about Africa), not forgetting the lively discussions. Thank you! Shidaa agbo!

To Marc van de Wetering, thank you for being a role model. I remember the times you joined us for the Basket burgers; I learned a lot by just listening to your comments garnered from your years of experience in science. Thank you for all the little helps with explaining stuff when I needed to ask you something. Shidaa agbo!



Nobuo Sasaki, how can I forget you? I think you were the person in the lab I had the most interaction with, both on a scientific and personal level. ありがとう!

I appreciate the advice you gave me (especially about planning and thinking ahead into the future), the motivation to think critically about my experiments and conclusions, the lively discussions on a broad range of subjects, and the encouragement to continue pursuing a career in science.

To Norman Sachs! I'm very grateful for your useful suggestions and comments on my experiments, not forgetting the sacrifices you had to made to help me with some of my experiments. I remember when you once had to come to the lab early in the morning just to help me. Vielen Dank!

To Jarno Jarno Drost, Bedankt voor alles je heb gedaan voor mij met betrekking tot mij experimenten.

Dear Kai Kretzschmar, Although I've known you for a very short time, I've enjoyed our friendship since you joined the lab. I'm grateful for all the help with injecting mice (in the dark), cryosectioning and microscopy, as well as

constructive comments and suggestions on experiments, not forgetting our usual conversations on you know what…Vielen dank!

Helmuth Gerhart, I enjoyed the many discussions we had over biology and many other topics. Thanks for all the helpful comments and suggestions which gave me a lot of insight for my experiments. Vielen dank!

Thank you Yi Chen, for offering help when I needed it.

To Oded Kopper, Shalom! Thank you for explaining things to me whenever I asked you, and generously giving me primers here and there. I enjoyed the conversations we had over you know what… הדות

Onur Basak, It's been great knowing you. What impressed me the most about you was your broad knowledge about science; you were always reliable when I had difficult scientific questions and situations. Teşekkür ederim!

Rob Vries, without your input into my experiments and welfare, I wouldn't have made it this far. Thanks for being an excellent project manager. Dank je wel!

To Sylvia Boj, Thanks for all the useful comments you gave me for my experiments. The way you conducted and presented your research was always an inspiration to me. Gracias!

Dear Inha Heo, Thank you for your contribution to the completion of my PhD. I benefited very much from our discussions, your suggestions and help with organoid culture. 감사합니다

To Benedetta Artegiani. Thanks for helping me with the basics of CRISPR/Cas9 gene editing, and the useful discussions we had concerning science.

I will like to pay tribute to the former postdocs in the Clevers lab who helped me in various ways: the Bon-Kyong Koos, the Meritxell Huchs, the Vivian Lis, the Gerald Schwanks, the Sina Bartfelds, the Valentina Sassellis. I nearly forgot, Nick Barker and Toshiro Sato, thank you for all the help in answering questions, giving advice, and just being an inspiration. The way you all did science was always an inspiration! I'll never forget the concern you showed for my experiments when things were not going smooth, even calling me aside to discuss my experiments. Thank you all for sharing your time, knowledge, expertise and experience with me.

To my senior PhD students in the Clevers lab, your contribution has immensely impacted on my person and my research. I say a big thank you to Hugo Snippert, Arnout Schepers, Jurian Schuijers, and Wouter Karthaus. I learnt a lot from you.

To Laura Zeinstra, Benaissa El Haddouti, Carla Kroon-Veenboer, thank you for the various helps with reagents, plasmids, primers and mouse genotyping.

To all the members of the Alexander van Oudenaard group, especially Anna van Oudenaaarden, Kay Wiebrand and Lennart Kester, Thank for accommodating me, and helping with smFISH among other things.

Stefan van der Elst, Many thanks for FACS help and all the tips.

Thanks to Arianna Fumagalli for mice and fruitful discussions on colon cancers.

A big thank you to Prof. G.J.A. Offerhaus, Folkert Morsink and Miangela Lacle for help with patient tumor samples and histology.

To all the members of the Hub, who shared my office with me the last few months of my course, Joyce, Sepideh, Ana, Sridevi, Tamana, Jinyi, Karin, Ida and Marvin, thank you for showing concern and the occasional interesting chatter which was a relief from all the stress after long hours of experiments.



Dear Janny van Eldik-Bonouvrie, I really do not know how to thank for all the assistance you offered me. Your administrative skills and practical help with many things related to my welfare really made my life in the Netherlands very easy. Dankjewel! Shidaa agbo!

I will like to acknowledge all the supporting staff of the Hubrecht both past and present for their contribution to the successful completion of my thesis; To Susan Maas, Sophia Yamini, Rosali Plemp van Duiveland, Ilonka Agterberg, Yvonne Schenke, Roco Gobel from the Personnel Department, thank you for your immense support in various ways to facilitate my stay in the Netherlands. To Romke, Rob, Jules, Richard and Elroy, I say thank you for all the help you offered me. To Thea Timmermans and all the other receptionists, thank you for your work at the reception desk helping with all my letters and parcels.

To all my mentors in Ghana and Germany, thank you for your encouragement, support and motivation to pursue a PhD.

To all the saints in the Netherlands and Germany, I couldn't have come this far without your support and prayers. Because of you, the Netherlands and Europe will always be in my heart. God bless you with His abundant grace!

To all and sundry who have helped me one way or the other during the course of my PhD, please forgive me if I did not mention your name. Thank you!

To my parents, Da and Ma, I really do not know how to thank you. You sacrificed your all for us to give us the best education. You motivated and inspired us to be the best or be among the best. I wouldn't have gotten this far without you. Your investment has not been in vain. May He grant you long life to enjoy Him and the fruit of your labor.

To my sweetest heart, Haimy, thank you for your love, care, support and understanding. You have been more than accommodating especially in the final leg of my course. I couldn't have finished without you. አመሰግናለሁ

*In Him are hidden all the treasures of wisdom and knowledge!*

## Curriculum vitae

Paul Winston Tetteh was born in Accra, Ghana, on 14th November 1979. He completed his primary and junior secondary school education at the Accra Ridge Church School from 1986-1995. Paul then attended and completed his senior secondary school education at the Presbyterian Boys' Secondary School in Legon, Accra, from 1996-1998. In 2000, Paul was admitted to the University of Ghana, where he obtained a bachelor's degree in Zoology in 2004. His bachelor's thesis' dissertation was on molecular identification of sibling species of *Anopheles funestus* from different ecological regions in Northern Ghana, which research was carried out at the Noguchi Memorial Institute for Medical Research in Accra. Paul did his National Service as a Teaching assistant at the Zoology Department of the University of Ghana, combined with part-time lecturing as a Biology tutor at his Alma mater in 2005. After completing an MPhil in Physiology at the University of Ghana Medical School in 2007, working on the role of oxidative stress in preeclampsia in Ghanaian pregnant women, Paul was awarded a scholarship to further his education in Germany. From 2008 to 2010, Paul completed a Master of Bioscience in Developmental Biology program at the University of Heidelberg. In September 2010, Paul moved to the Netherlands for his doctoral research in the lab of Hans Clevers at the Hubrecht Institute, affiliated with the University of Utrecht, having been awarded an NWO personal grant.

**List of Publications**

- **miR-137 inhibits the invasion of melanoma cells through downregulation of multiple oncogenic target genes.**

  Luo C, **Tetteh PW**, Merz PR, Dickes E, Abukiwan A, Hotz-Wagenblatt A, Holland-Cunz S, Sinnberg T, Schittek B, Schadendorf D, Diederichs S, Eichmüller SB.

  J Invest Dermatol. 2013 Mar;133(3):768-75. doi: 10.1038/jid.2012.357. Epub 2012 Nov 15.

- **Plasticity within stem cell hierarchies in mammalian epithelia.**

  **Tetteh PW**, Farin HF, Clevers H.

  Trends Cell Biol. 2015 Feb;25(2):100-8. doi: 10.1016/j.tcb.2014.09.003. Epub 2014 Oct 9. Review.

**Unpublished work**

- **Replacement of lost Lgr5+ stem cells through plasticity of their enterocyte-lineage daughters**

  **Paul W. Tetteh,** Henner F. Farin, Harry Begthel, Maaike van den Born, Jeroen Korving, Frederic de Sauvage, Johan H. van Es and Hans Clevers (Submitted, In revision)

- **A critical role for the niche in intestinal tumorigenesis from enterocyte progenitors**

  **Paul W. Tetteh,** Henner F. Farin, Harry Begthel, Maaike van den Born, Jeroen Korving, Frederic de Sauvage, Johan H. van Es and Hans Clevers (In Preparation)

- **Differentiated colonic epithelial cells as cells of origin of colon cancer**

  **Paul W. Tetteh,** Harry Begthel , Maaike van den Born, Jeroen Korving, Folkert Morsink, Henner F. Farin, Johan van Es, Johan Offerhaus, and Hans Clevers (In Preparation)