INTRODUCTION

Epithelial stem cells are multipotent cells with self-renewal capacity that ensure normal epithelial renewal and tissue regeneration in response to injury (e.g., radiation). The intestinal epithelium is highly proliferative, being renewed every few days in the mouse (Bjerknes and Cheng, 1999; Wong et al., 1999; Barker et al., 2012). Two main intestinal stem cell (ISC) pools orchestrate maintenance of the intestinal homeostatic epithelium (Beumer and Clevers, 2016), first, an actively proliferative Lgr5+ crypt base columnar cell population (CBC) is driven by the activity of the canonical Wnt pathway (Barker et al., 2007; Kretzschmar and Clevers, 2017). CBCs are also characterized by the expression of Ascl2, Offm4 (van der Flier et al., 2009), and Smoc2 (Munoz et al., 2012). Second, a slower cycling reserve crypt stem cell population is located around the +4 position above the crypt base and lacks regulation by the canonical WNT signaling pathway (Sangiorgi and Capecchi, 2008). Specifically, reserve ISC are marked by CreER insertions into the Bmi1 (Sangiorgi and Capecchi, 2008) or Hopx loci (Takeda et al., 2011), as well as by a Tert-CreER transgene mouse (Montgomery et al., 2011). Reserve ISCs were originally associated with label-retention capacities (Potten et al., 1978). The identity and function of intestinal label-retaining cells (LRCs) remain to be fully understood, but recent work shows that intestinal LRCs are secretory precursors of Paneth and enteroendocrine cells, located in the crypt and express Lgr5 (Buczacki et al., 2013). Subsequent work showed the label-retaining secretory precursor cells to be a distinct population from the reserve ISCs labeled by CreER knockin reporters (Li et al., 2016).

While a body of work has illuminated the distinct nature of these two populations, certain controversies persist. For example, in contrast to Bmi1-CreER+ cells, Bmi1-GFP+ cells may represent an enteroendocrine progenitor cell population (Jadhav et al., 2017). Furthermore, the heterogeneity of these populations makes interpretation of genetic labeling challenging at times. For example, the RNA binding protein Mex3a marks a subpopulation of Lgr5+ cells displaying characteristics consistent with reserve-like stem cells (Barriga et al., 2017). Other alleles can broadly mark several cell types; for example, Lrig1 marks Lgr5+ cells (Wong et al., 2012) and reserve ISCs (Powell et al., 2012). However, the populations marked by Lrig1 can vary greatly depending on whether the readout is endogenous mRNA, protein (which may be antibody dependent), or reporter alleles (Poulin et al., 2014; Powell et al., 2012; Wong et al., 2012). The Sox9-CreER allele also marks reserve ISCs and CBCs (Roche et al., 2015). The transcripts of certain reserve stem cell markers are expressed in other crypt cells, notably CBCs, thereby complicating analysis (Li et al., 2014; Munoz et al., 2012; Grun et al., 2015). Nevertheless, single-cell profiling has revealed that Bmi1-CreER+ cells and Hopx-CreER+ cells...
are transcriptionally distinct from Lgr5-CreER+ cells and generate CBCs under homeostatic conditions (Li et al., 2014). Also, it has been demonstrated that Bmi1-CreER-labeled cells could replenish Lgr5+ stem cell population after diphtheria toxin (DT)-mediated ablation (Tian et al., 2011). Hopx-CreER-labeled cells could also give rise to CBCs (Takeda et al., 2011).

Interestingly, Lgr5+ cells are sensitive to DNA damage and largely ablated with high-dose irradiation (Yan et al., 2012; Hua et al., 2012; Metcalfe et al., 2014; Tao et al., 2015), whereas Bmi1-CreER cells (Yan et al., 2012), Hopx-CreER cells (Yousef et al., 2016), and Lrig1-CreER cells (Powell et al., 2012) are resistant to high-dose radiation injury. Following radiation, reserve ISC can give rise to CBCs (Montgomery et al., 2011; Yan et al., 2012; Yousefi et al., 2016). Although Lgr5+ cells are sensitive to injury, ablation of Lgr5+ cells comitment with or following radiation results in failed regeneration, suggesting that de novo generation of new Lgr5+ cells is required for efficient tissue repair (Metcalfe et al., 2014). Interestingly, despite the existence of Wnt-negative, injury-resistant reserve ISCs that contribute to intestinal epithelial regeneration, evidence exists for plasticity in more differentiated intestinal cells. For example, DilI+ secretory progenitor cells can revert to a stem cell state and give rise to Lgr5+ cells (van Es et al., 2012). More recently, Asfaha et al. (2015) identified Krt19+/Lgr5- radio-resistant and cancer-initiating cells in the small intestine located above the crypt base. Similarly, alkaline-phosphatase-positive transit-amplifying cells can regenerate CBCs after their genetic ablation with Lgr5-DTR; however, it remains unknown whether this mechanism is employed in DNA damaging injury under physiological conditions, which might be expected to also ablate the very rapidly cycling transit-amplifying cells (Tetteh et al., 2016).

In the present study, we describe the role of Keratin 15 (Krt15)-labeled cells in the mouse small intestinal epithelium. Krt15-labeled cells were characterized initially as stem cells in the hair follicle bulge contributing to wound healing and the development of squamous papilloma (Morris et al., 2004; Ito et al., 2005; Li et al., 2013). We described recently a long-lived Krt15+ progenitor cell population in the mouse esophageal epithelium (Giroux et al., 2017). Herein, we identify and describe a long-lived Krt15+ cell population in the small intestinal crypt using genetic lineage tracing in mice. Krt15+ crypt cells give rise to all the intestinal lineages and have self-renewal capacity. Radio-resistant Krt15+ cells contribute to tissue regeneration after radiation-mediated injury. Interestingly, Apc loss in Krt15+ cells leads to adenoma and adenocarcinoma formation in the small intestine, as well as occasional adenoma formation in the colon, demonstrating the tumor-initiating potential of these cells.

RESULTS

Krt15 Marks Proliferating Cells in the Small Intestinal Crypt

Krt15+ stem/progenitor cells were described originally in the bulge of the hair follicle (Morris et al., 2004) and, more recently, within the basal compartment of the esophageal squamous epithelium (Giroux et al., 2017). Taken together, this suggests an important role for Krt15+ cells in the maintenance of squamous epithelia and appendages. In contrast to the multi-layered squamous epithelium in the skin and esophagus, a single layer of the columnar epithelium is present in the mammary gland, pancreas, and intestine. We hypothesized that Krt15+ cells in the small intestine might annotate a unique subpopulation of cells with properties that contribute to tissue maintenance and regeneration.

First, we noted that endogenous K15 protein is detected in some crypt cells and occasionally in cells within the villi (Figure 1A) of the small intestine. K15 protein can be detected in all small intestinal segments with slightly higher expression in the ileum. We confirmed these results using a mouse expressing a progesterone receptor (PR)-fused Cre recombinase downstream of Krt15 promoter (Krt15-CrePR1 mouse) (Morris et al., 2004) crossed with a reporter mouse expressing the mTomato-LSL-mGFP construct from the Rosa26 locus (R26<sup>mT/mG</sup>) (Muzumdar et al., 2007). A single injection of the PR agonist, RU486, was used for Cre induction and mice were sacrificed 24 hr later to visualize Krt15+ (GFP+) cells. Krt15+ cells were observed in some crypts and occasionally in the villi of the small intestine (Figure 1B). In the villi, 10% of the GFP+ cells also stained positive for Alcian blue, a goblet cell marker, and 3% also expressed chromogranin A (CHGA), an enteroendocrine cell marker (Figures S1A and S1B), suggesting that the majority of Krt15+ cells in the villi are enterocytes. Since the Krt15+ villi cells could result from migration of a recombination event in the top of the crypt, we injected Krt15-CrePR1;R26<sup>mT/mG</sup> mice with a single dose of RU486 and mice were sacrificed 8 hr later to minimize the potential for migration of labeled cells. We observed that most labeled cells were located in the lower third of the villi, thereby suggesting that recombination happened in the crypt or at the crypt/villi junction (Figure S1C).

Interestingly, approximately 40% of Krt15-labeled cells in the crypt are localized in the stem cell compartment (i.e., +4 position and below) (Figures 1C and S1D). We confirmed that the GFP+ cells located between positions +1 and +4 from the crypt base are not Paneth cells by lysozyme staining (Figures 1D and 1E). GFP can also be detected in some Ki-67+ transit-amplifying cells as a result of possible stem cell division and/or residual Krt15 promoter activity.
in these cells (Figures 1D and 1E). Interestingly, the +5 to +10 region may contain progenitor/stem cells as illustrated by Krt19 genetic labeling of multipotent and self-renewing cells (Asfaha et al., 2015). In order to evaluate the crypt cell types that express Krt15, we used Lgr5-EGFP mice. Krt15 expression is enriched in Lgr5+ cells compared with unsorted crypt cell samples (Figure 1F). Finally, small intestines were harvested from Krt15-CrePR1;R26mT/mG mice sacrificed 24 hr after Cre recombination. OLFM4, another marker of Lgr5+ stem cells, expression was evaluated. OLFM4 co-expressing GFP+ cells were observed in the bottom of the crypt (Figure 1G). In addition, we investigated the potential overlap between Krt15+ cells and +4 reserve ISCs. Bmi1 and Hopx expression is significantly lower in Krt15+ cells versus Krt15− cells (Figure S1E). These results suggest that Krt15-labeled cells in the crypt overlap with CBCs, as well as transit-amplifying cells.

**Krt15 Marks Long-Lived Multipotent Crypt Cells with Self-Renewal Capacity**

We next performed lineage-tracing experiments using Krt15-CrePR1;R26mT/mG mice to investigate the self-renewing capacity of Krt15+ crypt cells. Cre recombination was induced by daily injection of RU486 for 5 days (Figure 2A). Krt15-derived cells completely labeled the small intestinal crypt-villus axis by 14 days. In the early time points, we rarely observed clusters of cells at the crypt base, but found them mostly in the transit-amplifying zone, which suggests that the Krt15+ cells present in that region might be at the origin of the long-term tracing events as was
Figure 2. Krt15 Marks Long-Lived Cells in the Small Intestinal Crypt as Well as Some Cells in the Villi

(A and B) Six- to 8-week-old Krt15-CrePR1;R26mT/mG mice were injected daily with 0.5 mg RU486 (PR agonist) to induce Cre recombination for 5 consecutive days and sacrificed at different time points (days post-recombination = D). (B) Krt15+ cells were visualized by GFP immunofluorescence (n = 4–7 mice/time point).

(C and D) Krt15-CrePR1;R26Conf mice were injected twice a day with 1 mg RU486 for 10 consecutive days to induce recombination. Mice were sacrificed 2 months following recombination (n = 4 mice). (D) Representative images of YFP+ and RFP+ Krt15-derived clones visualized by immunofluorescence. Scale bars, 50 μm. See also Figures S2 and S3.
observed with Krt19+/Lgr5− cells (Asfaha et al., 2015). Labeling persists beyond 6 months, thereby suggesting that some Krt15+ crypt cells are long-lived stem cells (Figures 2B and S2A). Notably, 7% of the crypts were labeled 1 day after the last RU186 injection and 3% after 2 months (Figure S2B). Co-localization of GFP with Ki-67, Alcian blue, chromogranin A, and lysozyme demonstrate that Krt15+ cells can give rise to proliferative, transit-amplifying cells, goblet cells, enteroendocrine cells, and Paneth cells, respectively (Figures S3A–S3D). Krt15-CrePR1 mice were also crossed with RosaConfetti mice. Every Krt15-labeled ribbon observed was monochromatic, suggesting that the progeny of Krt15+ cells may arise from monoclonal units (Figures 2C and 2D). Interestingly, Krt15 marked clones in the colon epithelium as well (Figure S3E). Taken together, these results indicate that Krt15 can mark long-lived and multipotent crypt stem cells.

To investigate the self-renewing capacity of Krt15+ crypt cells, we used Krt15-CrePR1;Rosa26SL-tdTomato (Krt15-CrePR1;R26tom) mice. A single injection of RU486 was used to induce recombination and mice were sacrificed 24 hr later. Crypts were isolated and grown as 3D organoids. Krt15+ (Tomato+) cells were detected in 3D organoid crypts and these cells expanded to form lineage-traced ribbons as observed in vivo (Figure 3A). Interestingly, following 3D organoid passing, we observed organoids consisting entirely of Tomato+ cells (Figures 3A and 3B). The multipotency of Krt15+ crypt cells was confirmed in these 3D organoids with Tomato+ proliferative cells, goblet cells, enteroendocrine cells, and Paneth cells (Figure 3C). Furthermore, single crypt cell suspensions were prepared from Krt15-CrePR1;R26tom mice 24 hr following Cre recombination. Krt15+ cells were able to grow as single cell cultures, suggesting that Krt15+ crypt cells may display “stemness” features. The organoid formation efficiency of Krt15+ crypt cells is 0.01%, which is less than the 6% efficiency of Lgr5+ cells originally reported (Sato et al., 2011), but similar to the 0.01%–1.2% efficiency reported by other groups (Xian et al., 2017; Qi et al., 2017). Of note, these differences in organoid formation capacity of Krt15+ and Lgr5+ cells may not correlate with distinct properties in vivo. Taken together, these results demonstrate that Krt15 marks some long-lived and multipotent crypt cells with self-renewing capacity, characteristics consistent with stem cells.

Krt15+ Cells Are Radio-Resistant and Expand in Response to High-Dose Radiation

Reserve stem cells are believed to be radio-resistant and contribute to tissue regeneration following high-dose radiation (Sangiorgi and Capecchi, 2008; Yan et al., 2012; Takeda et al., 2011; Yousefi et al., 2016). Interestingly, Krt15 mRNA expression is increased in small intestinal cells following irradiation (Figure S4A). Therefore, we investigated the contribution of Krt15+ cells to tissue regeneration following high-dose γ-irradiation. First, we used Krt15-CrePR1;R26mTomG mice to determine if Krt15+ cells are resistant to high-dose radiation. Following CRE recombination, mice were subjected to whole-body γ-irradiation (12 Gy) (Figure 4A). After irradiation, Krt15+ cells were present in the highly proliferative crypts that underwent regeneration and designated as microcolonies (Figure 4B). Five days following irradiation, the percentage of GFP+ cells per crypt was increased in the irradiated mice when compared with non-irradiated mice (Figure 4C). Furthermore, Krt15+ crypt cells were more proliferative in irradiated animals versus non-irradiated mice (Figures 4D and 4E). These results are consistent with an expansion of Krt15+ cells in response to irradiation. Finally, we induced CRE recombination in Krt15-CrePR1;R26mTomG mice following whole-body irradiation and noticed that Krt15+ cells expanded in response to injury in these conditions as well (Figures S4B and S4C). Thus, we demonstrate that Krt15-labeled cells are radio-resistant and expand in response to high-dose irradiation.

Due to the multiple roles of keratins in cell migration and proliferation, we sought to characterize the potential role of K15 protein itself in tissue regeneration following injury. We subjected Krt15+/+ and Krt15−/− mice to whole-body γ-irradiation (12 Gy) and sacrificed them 5 days later (Figure 5A). Regeneration of the ileum from Krt15−/− mice was impaired following irradiation (Figure 5B). First, fewer residual crypts were observed in the ileum from Krt15−/− compared with Krt15+/+ irradiated mice (Figure 5C). Second, crypt length and villus height were both significantly reduced in Krt15-deficient mice when compared with Krt15+/+ mice (Figures 5D and 5E). Third, crypt regenerative capacity was determined using bromodeoxyuridine (BrDU) incorporation. Crypts containing >10 BrdU+ cells were considered as microcolonies. Krt15 deficiency significantly reduced the percentage of microcolonies present in the ileum 5 days following irradiation (Figures 5F and 5G). Finally, the percentage of BrdU+ cells per crypt was lower in Krt15−/− versus Krt15+/+ ileum. These results suggest a possible role for K15 protein in intestinal tissue regeneration. We appreciate this is likely different from the properties of the Krt15+ cells.

ApC Loss in Krt15+ Cells Leads to Adenoma and Adenocarcinoma Formation in the Small Intestine and Colon

Tumor-initiating capacity is an important feature of ISCs. Lgr5+, Lrig1+, and Krt19+/Lgr5− populations have all been described to contain a cell of origin for intestinal tumorigenesis following ApC loss (Barker et al., 2009; Powell et al., 2012; Asfaha et al., 2015). Also, Bmi1-CreER+ cells can form adenomas following induction of a stable form of
β-catenin in these cells (Sangiorgi and Capecchi, 2008). In order to determine if Krt15+ cells can initiate tumor formation in the intestine, we bred Apcfl/fl mice with the Krt15-CrePR1;R26mT/mG mice. Cre recombination was induced using daily injection of 0.5 mg RU486 for 5 consecutive days. Mice were sacrificed 6 months after Cre recombination or at prior time points if severe weight loss necessitated sacrifice (Figure 6A). Gross lesions were observed in the

Figure 3. Krt15 Lineage-Labeled Cells Have Clonogenic Potential
(A–D) Krt15-CrePR1;R26Tom mice were injected with 0.5 mg RU486 for Cre induction and sacrificed 24 hr later. 3D organoids were grown from isolated small intestinal (ileum) crypts (P0) and passaged once (P1) (n = 4 mice).
(A and B) Tomato+ (Krt15-derived) cells (A) were visualized on live 3D organoids as well as (B) by IF on sections of fixed organoids.
(C) Representative co-staining of Tomato with Ki-67 (proliferative cell marker), Mucin2 (MUC2, goblet cell marker), Chromogranin A (CHGA, enteroendocrine cell marker), and lysozyme (LYS, Paneth cell marker). Arrows mark co-localization.
(D) Single suspensions of Tomato+ cells were sorted and seeded in Matrigel and grown for 10 days. Representative pictures of 3D organoids growing from Krt15+ cells at different time points following seeding (D = days) (n = 4 mice, 4 experimental replicates/mouse). Arrows mark spheres that are forming.
Scale bars, 50 μm.
intestinal tract of Krt15-CrePR1;Apcfl/fl;R26mT/mG mice but not in control mice (n = 8 mice) (Figure 6B). All mice developed lesions in the small intestine while colon lesions were observed in 37% of the mice (Figure 6C). Mice developed between 4 and 33 tumors measuring on average 3.17 mm. Most observed lesions were adenomas, but invasive adenocarcinomas were occasionally observed. Interestingly, half of the Krt15-CrePR1;Apcfl/fl;R26mT/mG mice developed at least one adenocarcinoma (Figures 6D and 6E). The lesions were highly proliferative, as demonstrated by a large number of Ki-67+ cells (Figure 6F) and had also evidence of nuclear β-catenin staining (Figure 6G). Tp53 mutations are observed at later stages of colon cancer and typically lead to nuclear accumulation of p53 (Fearon and Vogelstein, 1990). We observed nuclear p53 staining in some tumor regions (Figure 6H). These results demonstrate that tumor-initiating cells exist within the Krt15+ population, and lesions derived from Krt15+ cells can even progress to invasive adenocarcinoma.

**DISCUSSION**

We report that some Krt15+ lineage-labeled small intestinal crypt cells have self-renewal capacity, give rise to all differentiated cell lineages, and participate in tissue regeneration following high-dose radiation injury. In combination with Apc loss, Krt15+ cells can give rise to adenomas and even adenocarcinomas. While the spatial localization of Krt15+ cells in intestinal crypts is not limited to CBCs or +4 ISCs, our results suggest that Krt15+ could annotate a subpopulation of Lgr5+ cells. Krt15+ cells are predominantly detected at the crypt base where Lgr5+ stem cells reside (Barker et al., 2007), while the Bmi1+ or Hopx+ reserve stem cells are localized at the +4 position above the crypt base (Sangiorgi and Capecchi, 2008; Takeda et al., 2011). Sorted Lgr5+ cells also harbor higher Krt15 mRNA expression and some Krt15-labeled cells also express OLFM4. These results suggest at least partial overlap between Krt15+ cells and Lgr5+ cells. Analysis of
published datasets also suggests upregulation of Krt15 transcript in Lgr5+ cells versus Lgr5− cells (1.00842 versus 0.0258, p = 0.052) (Yan et al., 2012). Some Krt15+ cells are located above the +4 position, in the transit-amplifying zone similar to Krt19+/Lgr5−/C0 stem-like cells (Asfaha et al., 2015). This suggests heterogeneity throughout the Krt15+ crypt population. Nevertheless, we observed multipotency and self-renewing capacity in Krt15+ cells, indicating that at least some Krt15+ crypt cells have properties consistent with stem cells or long-lived progenitor cells.

Lgr5+ CBCs are thought to be highly sensitive to high-dose radiation, while the reserve stem cells are resistant to such an insult (Hua et al., 2012; Metcalfe et al., 2014; Yan et al., 2012; Tao et al., 2015). Herein, we demonstrate that intestinal Krt15+ crypt cells are resistant to high-dose radiation. Due to the possible overlap between Krt15+ cells and Lgr5+ cells, Krt15 could mark a subpopulation of Lgr5+ cells that could be resistant to radiation. Although radiation ablates the vast majority of Lgr5+ cells, a small fraction of Lgr5+ cells persists (Yan et al., 2012; Tao et al., 2015; Hua et al., 2012; Metcalfe et al., 2014) and these might be marked by Krt15 promoter activity. Recently, a subpopulation of Lgr5+ cells was described as expressing a high level of Mex3a, suggesting heterogeneity in the Lgr5+ cell population (Barriga et al., 2017). However, Mex3a is not differentially expressed between Krt15+ and Krt15− cells (data not shown). Furthermore, analysis of published datasets revealed no enrichment of Krt15 in previously described Lgr5+ subpopulations of cells (Barriga et al., 2017; Yan et al., 2012; Munoz et al., 2012). Krt15+ cells located above the +4 position might also be radio-resistant, as has been described for the similarly located Krt19+/Lgr5− cells (Asfaha et al., 2015).

Inadequate regeneration following radiation was observed in the Krt15-deficient mice, suggesting a possible role for the K15 protein. Keratins confer structural support in epithelial cells and are also involved in sensing cues from the microenvironment. Keratins form a strong complex with integrins and other extracellular-matrix-associated proteins. It is believed that the stem cell niche often

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**Figure 5. Krt15 Loss Impairs Intestinal Tissue Regeneration Following Gamma-Irradiation**

(A–H) Six- to 8-week-old Krt15+/+ and Krt15−/− mice were subjected to 12 Gy whole-body γ-irradiation and sacrificed 5 days later. Tissue injury and regeneration were compared in ileal sections. (B) Representative H&E staining from ileal sections. (C) Residual crypts by high-power field (HPF) images were quantified, graph represents mean ± SEM (n = 3–4 mice/group, 25 HPFs/mouse were analyzed. *Represents statistical significance with p < 0.05 using Student’s t test). (D and E) Crypt length (D) and villi height (E) were measured in ileal sections, graph represents mean ± SEM (n = 3–4 mice/group, 50 crypts and villus/mouse were measured. *Represents statistical significance with p < 0.05 using Student’s t test). (F-H) Mice were injected with BrdU 1.5 hr prior to sacrifice and BrdU+ cells were detected by IHC. (G) The percentage of microcolonies (crypt with >10 BrdU+ cells/total number of crypts by HPF) was quantified, graph represents mean ± SEM (n = 3–4 mice/group, 25 HPFs/mouse were analyzed. *Represents statistical significance with p < 0.05 using Student’s t test). (H) The percentage of BrdU+ cells/crypt was quantified, graph represents mean ± SEM (n = 3–4 mice/group, 50 crypts/mouse were analyzed. *Represents statistical significance with p < 0.05 using Student’s t test). Scale bars, 50 μm.
involves extrinsic cells or extracellular proteins or molecules that will modify the immediate environment of stem cells. Interestingly, integrin signaling has been shown to be essential for ISC maintenance and proliferation in Drosophila (Lin et al., 2013; You et al., 2014). It has been shown that keratins can interact with several kinases and other cytoplasmic proteins to regulate cell proliferation and cell metabolism (Bragulla and Homberger, 2009). For example, K19 interacts with β-catenin/RAC1 complex in breast cancer cells inducing nuclear translocation of β-catenin (Saha et al., 2017). K8 and K18 interact with TNFR2 affecting nuclear factor κB signaling (Caulin et al., 2000) and K17 regulates the AKT/mTOR pathway by interacting with 14-3-3, resulting in protein synthesis and cell growth upregulation (Kim et al., 2006). Herein, we demonstrate that mice lacking Krt15 recover more slowly in response to high-dose radiation, thereby suggesting that K15 protein could also play a role in tissue regeneration.

Several studies have been conducted to identify the cell of origin of intestinal cancers. Apc loss in Lgr5-CreER+ cells (Barker et al., 2009; Asfaha et al., 2015), Lrig1-CreER+ cells (Powell et al., 2012, 2014), and Krt19-CreER+ cells (Asfaha et al., 2015) lead to adenoma formation. Adenomas are also observed following induction of stable β-catenin in Bmi1-CreER+ cells (Sangiorgi and Capecchi, 2008). None of these studies have reported progression to adenocarcinoma. Herein, we observed that mice harboring Apc loss in Krt15-CrePR1+ cells develop adenomas and invasive adenocarcinomas. These results suggest that Krt15+ cells are tumor-initiating cells, spanning benign to malignant lesions. Loss of Apc in Krt15+ cells leads to more severe lesions than what was reported for Lgr5+, Lrig1+, or Krt19+ cells. Mice presenting Apc loss in Krt15+ cells survive longer in contrast to other reported mice, which could mean that the latter mice do not survive long enough to display adenocarcinomas. Alternatively, this could suggest different cells of origin for adenomas and adenocarcinomas. Interestingly, in inherited colon cancer syndromes, one may see variable presentation of adenomatous polyps or hamartomatous polyps with or without progression to colon cancer (Rustgi, 2007) and this may underlie the potential heterogeneity of cells of origin that may be implied in the various mouse models. Finally, extensive adenoma formation like observed in mice carrying loss of Apc in Lgr5+ cells could require culling before cancer progression.

Figure 6. Krt15+ Cells Are Tumor-Initiating Cells in the Intestine
(A) Krt15-CrePR1;Apcfl/fl;R26mT/mG mice were injected daily with 0.5 mg RU486 for 5 consecutive days to induce Cre recombination and mice were sacrificed when sick or 150 days following Cre induction (n = 8 mice).
(B) Representative image of small intestinal tumors observed in Krt15-CrePR1;Apcfl/fl;R26mT/mG mice. Scale bar, 1 mm.
(C) Incidence of lesions in small intestine (SI) and colon of Krt15-CrePR1;Apcfl/fl;R26mT/mG mice (n = 8 mice).
(D) Incidence of adenomas and adenocarcinomas in Krt15-CrePR1;Apcfl/fl;R26mT/mG mice (n = 8 mice).
(E–H) Representative histology of flat adenoma and invasive adenocarcinoma observed in Krt15-CrePR1;Apcfl/fl;R26mT/mG mice. (F) Ki-67, (G) β-catenin, and (H) p53 staining of small intestinal tumors observed in Krt15-CrePR1;Apcfl/fl;R26mT/mG mice. Scale bar, 50 μm.
The lesions formed by Krt15+ cells most likely originate from the cells located at the crypt base and/or those located in the transit-amplifying zone. We also cannot exclude the possibility that Krt15+ villi cells might have given rise to the lesions since it has been demonstrated that cells in the villi can initiate tumorigenesis (Davis et al., 2015). In that model, aberrant expression of the BMP antagonist, GREM1, led to formation of intestinal tumors histologically similar to polyps observed in hereditary mixed polyposis syndrome. Nevertheless, Krt15-CrePR1;Apcfl/fl mice might represent a new mouse model for intestinal cancer as a foundation for understanding the interrelationship between normal intestinal cells and malignant transformation.

Overall, we demonstrate that Krt15+ crypt cells are self-renewing, multipotent, radio-resistant, and tumor initiating in concert with Apc loss. Krt15+ cells could represent a radio-resistant subpopulation of Lgr5+ cells. RNA profiling of Krt15+ esophageal long-lived progenitor cells revealed enrichment for gene sets associated with Wnt/β-catenin pathway and DNA repair (Giroux et al., 2017). It is possible, if not likely, that the Wnt/β-catenin pathway is involved in the proliferative capacity of Krt15+ intestinal crypt cells and their tumor-initiating capacities. Furthermore, Krt15+ cells could also display higher DNA repair capacity to ensure genomic stability in response to radiation by example.

EXPERIMENTAL PROCEDURES

Mouse Experimental Design
Krt15-CrePR1 (Morris et al., 2004) and Krt15−/− mice were provided by Dr. George Cotsarelis (University of Pennsylvania). Rosa26Tomato/CreERT2 (R26Tom/C0) mice (Muzumdar et al., 2007) were purchased from Jackson Laboratories. Rosa26Confetti (R26conf) mice were provided by Dr. Ben Stanger (University of Pennsylvania). Rosa26LSL-tdTomato (R26Tom) mice were provided by Dr. Christopher Lengner (University of Pennsylvania). Apcfl/fl mice were obtained from the NCI Mouse Repository (Kuraguchi et al., 2006). All experiments were performed with 6- to 10-week-old mice. Intrapertioneal injection of PR agonist, RU486, was performed to induce Cre recombination. The Institutional Animal Care and Use Committee of the University of Pennsylvania approved all animal studies. Detailed information regarding mouse experimental design and treatment are in the Supplemental Information.

Irradiation
Six- to eight-week-old mice were subjected to 12 Gy whole-body gamma-irradiation (GammaCell 40 Cesium 137 Irradiation Unit). Mice were sacrificed 5 days later, and the small intestines were harvested and fixed for histology. Surviving crypts, crypt length, and villi height were measured on H&E-stained ileum sections. Twenty-five high-magnification fields were analyzed for each mouse and a minimum of 100 crypts or villi were measured for each mouse.

Single-Cell Isolation
Crypt single cells were isolated as described previously (Hamilton et al., 2015). Briefly, the small intestine was opened longitudinally and washed with cold PBS. Tissue was incubated 20 min on ice in PBS-EDTA-DTT. Tissues were then incubated in PBS-EDTA at 37°C. Crypts were isolated from the epithelial fraction using a 70-μm filter. Crypts were then dissociated to a single-cell suspension using PBS-dispase at 37°C.

Crypt Isolation and 3D Organoid Culture
Crypts were isolated from the mouse small intestinal ileum. Tissues were chilled in Ca2+-Mg2+-free Hank’s balanced salt solution (CMF-HBSS) with 1 mM N-acetyl-cysteine (NAC). Tissues were then incubated in CMF-HBSS/1 mM NAC/10 mM EDTA for 45 min at 4°C. Epithelial cells were then dissociated through vortexing/resting cycles. Crypts were separated from epithelial dissociation with a 70-μm filter. Crypts were pelleted and resuspended in Basal Media (Advanced DMEM/F12, 2 mM Glutamax, 10 mM HEPES, 1 mM penicillin/streptomycin, 5 μM CHIR99021 [Cayman Chemical], 1 mM NAC, 1× N2 Supplement [Gibco], and 1× B27 Supplement [Gibco]). Approximately 500 crypts were then embedded in 80% Matrigel/20% ENR (Basal Media; 50 ng/ml recombinant mouse epidermal growth factor [R&D Systems] and 1% Noggin/R-Spondin conditioned media). Enteroids were cultured in ENR.

Fluorescence-Activated Cell Sorting
Crypt single-cell suspension was prepared previously from Krt15-CrePR1;R26Tom small intestines as described above. The fluorescence-activated cell sorting sorter Jazz (BD Biosciences) was used to sort Tomato+ and Tomato-crypt cells. Sorting was conducted at the University of Pennsylvania Flow Cytometry and Cell Sorting Facility. Cells were sorted in Advanced DMEM/F12 media supplemented with 1× penicillin-streptomycin, 1× GlutaMAX, 1× HEPES, and 10 μM Rock inhibitor Y27632. Cells were then used for 3D organoid formation or RNA extraction.

Details on mouse experimental design, immunohistochemistry and immunofluorescence, RNA extraction, qPCR, and statistical analyses are available in Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.04.022.

AUTHOR CONTRIBUTIONS

V.G., K.E.H., C.J.L., and A.K.R. designed the study. V.G., J.S., and P.C. performed the experiments. B.R. maintained the mouse colony. E.P.W. assisted with statistical analysis. A.J.K.-S. assisted with histology analysis. V.G. and A.K.R. wrote the manuscript. J.S. is currently affiliated with University Medicine Greifswald (Department of Medicine).

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REFERENCES

Asfaha, S., Hayakawa, Y., Muley, A., Stokes, S., Graham, T.A., Ericksen, R.E., Westphalen, C.B., von Burstin, J., Mastracci, T.L., Worthley, D.L., et al. (2015). Krt15+/- or Lgr5-/- cells are radiosensitive cancer-initiating stem cells in the colon and intestine. Cell Stem Cell 16, 627–638.

Barker, N., Ridgway, R.A., van Es, J.H., van de Wetering, M., Begthel, H., van den Born, M., Danenberg, E., Clarke, A.R., San-Som, O.J., and Clevers, H. (2009). Crypt stem cells as the cells-of-origin of intestinal cancer. Nature 457, 608–611.

Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijn, M., Haegebarth, A., Korting, J., Begthel, H., Peters, P.J., et al. (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449, 1003–1007.

Barker, N., van Oudenaarden, A., and Clevers, H. (2012). Identifying the fcy receptor for the intestinal crypt: strategies and pitfalls. Cell Stem Cell 11, 452–460.

Barriga, F.M., Montagni, E., Mana, M., Mendez-Lago, M., Hernandez-Momblona, X., Sevilla, M., Maunstrae-Adkins, A., Rodriguez-Esteban, G., Buczacki, S.J.A., Gut, M., et al. (2017). Mex3a marks a slowly dividing subpopulation of Lgr5+ intestinal stem cells. Cell Stem Cell 20, 801–816.e7.

Beumer, J., and Clevers, H. (2016). Regulation and plasticity of intestinal stem cells during homeostasis and regeneration. Development 143, 3639–3649.

Bjerke, M., and Cheng, H. (1999). Clonal analysis of mouse intestinal epithelial progenitors. Gastroenterology 116, 7–14.

Bragulla, H.H., and Homberger, D.G. (2009). Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia. J. Anat. 214, 516–559.

Buczacki, S.J., Zecchini, H.I., Nicholson, A.M., Russell, R., Vermeulen, L., Kemp, R., and Winton, D.J. (2013). Intestinal label-retaining cells are secretory precursors expressing Lgr5. Nature 495, 65–69.

Caulin, C., Ware, C.F., Magin, T.M., and Oshima, R.G. (2000). Keratin-dependent, epithelial resistance to tumor necrosis factor-induced apoptosis. J. Cell Biol. 149, 17–22.

Davis, H., Irshad, S., Bansal, M., Rafferty, H., Boitsova, T., Bardella, C., Jaeger, E., Lewis, A., Freeman-Mills, L., Giner, F.C., et al. (2015). Aberrant epithelial GREM1 expression initiates colonic tumorigenesis from cells outside the stem cell niche. Nat. Med. 21, 62–70.

Fearon, E.R., and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. Cell 61, 759–767.

Giroux, V., Lento, A.A., Islam, M., Pitarrresi, J.R., Kharbanda, A., Hamilton, K.E., Whelan, K.A., Long, A., Rhoades, B., Tang, Q., et al. (2017). Long-lived keratin 15+ esophageal progenitor cells contribute to homeostasis and regeneration. J. Clin. Invest. 127, 2378–2391.

Grun, D., Lyubimova, A., Kester, L., Wiebrands, K., Basak, O., Sasaki, N., Clevers, H., and van Oudenaarden, A. (2015). Single-cell messenger RNA sequencing reveals rare intestinal cell types. Nature 525, 251–255.

Hamilton, K.E., Crissey, M.A., Lynch, J.P., and Rustgi, A.K. (2015). Culturing adult stem cells from mouse small intestinal crypts. Cold Spring Harb. Protoc. 2015, 354–358.

Hua, G., Thin, T.H., Feldman, R., Haimovitz-Friedman, A., Clevers, H., Fukaz, Z., and Kolesnick, R. (2012). Crypt base columnar stem cells in small intestines of mice are radiosensitive. Gastroenterology 143, 1266–1276.

Ito, M., Liu, Y., Yang, Z., Nguyen, J., Liang, F., Morris, R.J., and Cotsarelis, G. (2005). Stem cells in the hair follicle bulb contribute to wound repair but not to homeostasis of the epidermis. Nat. Med. 11, 1351–1354.

Jadhav, U., Saxena, M., O’Neill, N.K., Saadatpour, A., Yuan, G.C., Herbert, Z., Murata, K., and Shivdasani, R.A. (2017). Dynamic reorganization of chromatin accessibility signatures during dedifferentiation of secreatory precursors into Lgr5+ intestinal stem cells. Cell Stem Cell 21, 65–77.e5.

Kim, S., Wong, P., and Coulombe, P.A. (2006). A keratin cytokerin-14 mutant protein regulates protein synthesis and epithelial cell growth. Nature 441, 362–365.

Kretzschmar, K., and Clevers, H. (2017). Wnt/beta-catenin signaling in adult mammalian epithelial stem cells. Dev. Biol. 428, 273–282.

Kuraguchi, M., Wang, X.P., Bronson, R.T., Rothenberg, R., Ohene-Baah, N.Y., Lund, J.J., Kucherlapati, M., Maas, R.L., and Kucherlapati, R. (2006). Adenomatous polyposis coli (APC) is required for normal development of skin and thymus. PLoS Genet. 2, e146.

Li, N., Nakauka-Ddamba, A., Tobias, J., Jensen, S.T., and Lengner, C.J. (2016). Mouse label-retainng cells are molecularly and functionally distinct from reserve intestinal stem cells. Gastroenterology 151, 298–310.e7.

Li, N., Yousefi, M., Nakauka-Ddamba, A., Jain, R., Tobias, J., Epstein, J.A., Jensen, S.T., and Lengner, C.J. (2014). Single-cell analysis of proxy reporter allele-marked epithelial cells establishes intestinal stem cell hierarchy. Stem Cell Reports 3, 876–891.
Drosophila. Integration of intestinal stem cells in (2013). Integrin signaling is required for maintenance and proliferation of intestinal stem cells in *Drosophila*. Dev. Biol. 377, 177–187. Metcalfe, C., Klijavin, N.M., Ybarra, R., and de Sauvage, F.J. (2014). Lgr5+ stem cells are indispensable for radiation-induced intestinal regeneration. Cell Stem Cell 14, 149–159. Montgomery, R.K., Carlone, D.L., Richmond, C.A., Farilla, L., Krannendorn, M.E., Henderson, D.E., Balfour-Awuah, N.Y., Ambruzs, D.M., Fogli, L.K., Algra, S., et al. (2011). Mouse telomerase reverse transcriptase (mTert) expression marks slowly cycling intestinal stem cells. Proc. Natl. Acad. Sci. USA 108, 179–184. Morris, R.J., Liu, Y., Marles, L., Yang, Z., Trempus, C., Li, S., Lin, J.S., Sawicki, J.A., and Cotsarelis, G. (2004). Capturing and profiling adult hair follicle stem cells. Nat. Biotechnol. 22, 411–417. Munoz, J., Stange, D.E., Schepers, A.G., van de Wetering, M., Koo, B.K., Itzkovitz, S., Volckmann, R., Kung, K.S., Koster, J., Radulescu, S., et al. (2012). The Lgr5 intestinal stem cell signature: robust expression of proposed quiescent ‘+4’ cell markers. EMBO J. 31, 3079–3091. Muzumdar, M.D., Tasic, B., Miyamichi, K., Li, L., and Luo, L. (2007). A global double-fluorescent cre reporter mouse. Genesis 45, 593–605. Potten, C.S., Hume, W.J., Reid, P., and Cairns, J. (1978). The segregation of DNA in epithelial stem cells. Cell 15, 899–906. Poulin, E.J., Powell, A.E., Wang, Y., Li, Y., Franklin, J.I., and Coffey, R.J. (2014). Using a new Lrig1 reporter mouse to assess differences between two Lrig1 antibodies in the intestine. Stem Cell Res. 13, 422–430. Powell, A.E., Vlacich, G., Zhao, Z.Y., McKinley, E.T., Washington, M.K., Manning, H.C., and Coffey, R.J. (2014). Inducible loss of one Apc allele in Lrig1-expressing progenitor cells results in multiple distal colonic tumors with features of familial adenomatous polyposis. Am. J. Physiol. Gastrointest. Liver Physiol. 307, G16–G23. Powell, A.E., Wang, Y., Li, Y., Poulin, E.J., Means, A.L., Washington, M.K., Higginbotham, J.N., Luchheimer, A., Prasad, N., Levy, S.E., et al. (2012). The pan-ErbB negative regulator Lrig1 is an intestinal stem cell marker that functions as a tumor suppressor. Cell 149, 146–158. Qi, Z., Li, Y., Zhao, B., Xu, C., Liu, Y., Li, H., Zhang, B., Wang, X., Yang, X., Xie, W., et al. (2017). BMP restricts stemness of intestinal Lgr5(+) stem cells by directly suppressing their signature genes. Nat. Commun. 8, 13824. Roche, K.C., Gracz, A.D., Liu, X.F., Newton, V., Akiyama, H., and Magness, S.T. (2015). SOX9 maintains reserve stem cells and preserves radioresistance in mouse small intestine. Gastroenterology 149, 1553–1563.e10. Rustgi, A.K. (2007). The genetics of hereditary colon cancer. Genes Dev. 21, 2525–2538. Saha, S.K., Choi, H.Y., Kim, B.W., Dayem, A.A., Yang, G.M., Kim, K.S., Yin, Y.F., and Cho, S.G. (2017). KRT19 directly interacts with beta-catenin/RAC1 complex to regulate NUMB-dependent NOTCH signaling pathway and breast cancer properties. Oncogene 36, 332–349. Sangiorgi, E., and Capecchi, M.R. (2008). Bmi1 is expressed in vivo in intestinal stem cells. Nat. Genet. 40, 915–920. Sato, T., van Es, J.H., Snippert, H.J., Stange, D.E., Vries, R.G., van den Born, M., Barker, N., Shroyer, N.E., van de Watering, M., and Clevers, H. (2011). Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature 469, 415–418. Takeda, N., Jain, R., LeBoeuf, M.R., Wang, Q., Lu, M.M., and Epstein, J.A. (2011). Interconversion between intestinal stem cell populations in distinct niches. Science 334, 1420–1424. Tao, S., Tang, D., Morita, Y., Sperka, T., Omrani, O., Lechel, A., Sakk, V., Kraus, J., Kestler, H.A., Kuhl, M., et al. (2015). Wnt activity and basal niche position sensitize intestinal stem and progenitor cells to DNA damage. EMBO J. 34, 624–640. Tetteh, P.W., Basak, O., Farin, H.F., Wiebrands, K., Kretzschmar, K., Begthel, H., van den Born, M., Korving, J., de Sauvage, F., van Es, J.H., et al. (2016). Replacement of lost Lgr5-positive stem cells through plasticity of their enterocyte-lineage daughters. Cell Stem Cell 18, 203–213. Tian, H., Biels, B., Warming, S., Leong, K.G., Rangell, L., Klein, O.D., and de Sauvage, F.J. (2011). A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. Nature 478, 255–259. van der Flier, L.G., van Gijn, M.E., Hatzis, P., Kujala, P., Haegebarth, A., Stange, D.E., Begthel, H., van den Born, M., Guryev, V., Oving, L., et al. (2009). Transcription factor achaete scute-like 2 controls intestinal stem cell fate. Cell 136, 903–912. van Es, J.H., Sat0, T., van de Wetering, M., Lyubimova, A., Nee, A.N., Gregorieff, A., Sasaki, N., Zeinstra, L., van den Born, M., Korving, J., et al. (2012). Dll1+ secretory progenitor cells revert to stem cells upon crypt damage. Nat. Cell Biol. 14, 1099–1104. Wong, M.H., Stappenbeck, T.S., and Gordon, J.I. (1999). Living and commuting in intestinal crypts. Gastroenterology 116, 208–210. Wong, V.W., Stange, D.E., Page, M.E., Bucczaki, S., Wabik, A., Itami, S., van de Wetering, M., Poulsom, R., Wright, N.A., Trotter, M.W., et al. (2012). Lrig1 controls intestinal stem-cell homeostasis by negative regulation of ErbB signalling. Nat. Cell Biol. 14, 401–408. Xian, L., Georgess, D., Huso, T., Cope, L., Belton, A., Chang, Y.T., Kuang, W., Gu, Q., Zhang, X., Senger, S., et al. (2017). HMGAI amplifies Wnt signalling and expands the intestinal stem cell compartment and paneth cell niche. Nat. Commun. 8, 15008. Yan, K.S., Chia, L.A., Li, X., Ootani, A., Su, J., Lee, J.Y., Su, N., Luo, Y., Heilshorn, S.C., Amieva, M.R., et al. (2012). The intestinal stem cell markers Bmi1 and Lgr5 identify two functionally distinct populations. Proc. Natl. Acad. Sci. USA 109, 466–471. You, J., Zhang, Y., Li, Z., Lou, Z., Jin, L., and Lin, X. (2014). *Drosophila* perlecan regulates intestinal stem cell activity via cell-matrix attachment. Stem Cell Reports 2, 761–769. Yousefi, M., Li, N., Nakauka-Ddamba, A., Wang, S., Davidow, K., Schoenberger, J., Yu, Z., Jensen, S.T., Kharas, M.G., and Lengner, C.J. (2016). Msi RNA-binding proteins control reserve intestinal stem cell quiescence. J. Cell Biol. 215, 401–413.