Ablated intestinal stem cells recover predominantly by ASCL2-dependent dedifferentiation of their recent progeny

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SUMMARY

After ablation of LGR5$^+$ intestinal stem cells (ISC), new cells reappear quickly. While certain crypt cells clearly dedifferentiate to provide new ISC, the underlying transcriptional and signaling trajectories are unknown. It also remains possible, and a prevalent idea, that quiescent 'reserve' ISC contribute to the restoration. By timing the interval between LGR5$^+$ lineage tracing and lethal injury, we show that dedifferentiation explains nearly all the recovery, with contributions from both absorptive and secretory progenitors. The ISC-restricted transcription factor ASCL2 confers measurable competitive advantage to resting ISC and is essential to restore the ISC compartment. Regenerating cells express *Ascl2* days before *Lgr5* and their capture, including scRNA analyses, revealed transcriptional paths of dedifferentiation. ASCL2 target genes include the Interleukin11 (IL11) receptor *Il11ra1* and recombinant IL11 enhances crypt cell regenerative potential. These findings reveal cell dedifferentiation as the principal means to restore ISC and one ASCL2-regulated signal that enables this adaptive response.

INTRODUCTION

LGR5$^+$ intestinal stem cells (ISC) sustain small intestine and colonic epithelial self-renewal (Barker et al., 2007), but their ablation does not compromise epithelial integrity because other crypt cells soon replenish the LGR5$^+$ compartment (Tian et al., 2011). It remains controversial whether this homeostatic response reflects activation of a 'reserve' pool of quiescent ISC or occurs only by dedifferentiation of LGR5$^+$ cells' recent progeny (Bankaitis et al., 2018; Santos et al., 2018; Yousefi et al., 2017). The parameters and molecular basis of crypt cell plasticity are also largely unknown (de Sousa e Melo and de Sauvage, 2019).

Intestinal crypts and villi rapidly lose S-phase labels as a result of DNA replication and cell attrition. A central argument for 'reserve' ISC is that rare cells in crypt tier 4 retain S-phase tags for many days, signifying the putative stem-cell hallmark of replicative quiescence (Potten, 1998). Cells expressing markers such as *Bmi1*, *Tert*, and *Hopx* also concentrate near tier 4 (Montgomery et al., 2011; Sangiorgi and Capecchi, 2008; Takeda et al., 2011) and LGR5$^+$ cells restored after ISC loss originate in a *Bmi1*$^+$ population (Tian et al., 2011), reinforcing the idea of dedicated 'reserve' *Bmi1*$^+$ ISC. When label-retaining cells (LRC) are followed for >3 weeks, however, S-phase tags shift into Paneth and enteroendocrine (EE) cells, indicating that LRC are secretory (Sec) cell precursors (Buczacki et al., 2013). Moreover, *Bmi1*^{hi} and *Tert*$^+$ populations contain mainly EE cells (Jadhav et al., 2017; Yan et al., 2017) and *Bmi1* mRNA is expressed in many crypt (Itzkovitz et al., 2011; Munoz et al., 2012) and even villus (Ayyaz et al., 2019; San Roman et al., 2015) cells. Therefore, LGR5$^+$ cell recovery from a *Bmi1*$^+$ origin could reflect diverse sources (Barker et al., 2012), including EE cells and possibly rare 'reserve' ISC. Indeed, the idea of 'reserve' LRCs remains popular (Ayyaz et al., 2019; Chaves-Perez et al., 2019).

In response to LGR5$^+$ cell deficits, both Sec and enterocyte (Ent) progenitors (Tetteh et al., 2016; van Es et al., 2012), and occasionally even mature Paneth cells (Jones et al., 2018; Schmitt et al., 2018; Yu et al., 2018), contribute toward a replenished ISC pool. This facility is explained in part by the remarkably similar profiles of active histones in ISC and villus Ent (Kim et al., 2014) and by the observation that areas of open chromatin specific to the Sec lineage reverse readily upon ISC damage (Jadhav et al., 2017); thus, chromatin barriers between crypt cell states are low or easily breached. Although crypt cells *can* dedifferentiate, it remains unclear (a) if they account for the bulk of ISC restoration, (b) which crypt cells harbor this latent potential, and (c) if homeostasis might reflect contributions from both LGR5$^+$ cell derivatives *and* reserve ISC. Here we report that nearly all regeneration after ISC injury occurs by ASCL2-dependent dedifferentiation of recent LGR5$^+$ cell progeny.

The basic-helix-loop-helix transcription factor (TF) gene *Ascl2*, a transcriptional target of Wnt signaling, is restricted to LGR5^{+} basal crypt cells in mice (van der Flier et al., 2009) and humans (Jubb et al., 2006). *Ascl2* deletion by *Ah-Cre* first indicated its requirement for ISC survival *in vivo* (van der Flier et al., 2009), but *Ascl2*$^{-/-}$ organoids later revealed only ~30% growth disadvantage, attributed in part to its co-regulation of Wnt-responsive genes (Schuijers et al., 2015). Using both the original and a new conditional mutant allele, we show that in the absence of ISC injury, ASCL2 confers a modest competitive advantage for cells to occupy the ISC niche. In contrast, ASCL2 is indispensable *in vivo* for crypt cell dedifferentiation after ISC injury. After ISC ablation, ectopic *Ascl2* expression in colonic crypts allowed us to detect and purify actively dedifferentiating cells. mRNA profiles of restorative populations and single cells lacked evidence for a favored +4 cell source and revealed dynamic transcriptional flux in colonocytes and goblet cells, each converging toward ISC. *Bona fide* developmental genes and YAP/TAZ signaling were not appreciable elements of that flux. Coupled evaluation of mRNAs and ASCL2-bound promoters in regenerating cells revealed the Interleukin 11 (IL11) receptor gene *Il11ra1* as a transcriptional target and organoid cultures demonstrated IL11 activity in ISC regeneration. Together, these findings establish crypt cell dedifferentiation as the principal means to restore damaged ISC and reveal one of likely several ASCL2-dependent enabling signals.

RESULTS

Following ablation, ISC regenerate exclusively from daughter crypt cells

To identify the principal source of regenerated ISC, we crossed *Lgr5*$^{GFP\text{-}CreER(T2)}$ (*Lgr5*$^{Gfp\text{-}Cre}$) (Barker et al., 2007) and *R26R*^{tdTom} mice (all strains are listed in the Key Resources Table), then treated adult animals with tamoxifen (TAM), hence permanently labeling the progeny of Lgr5^{+} ISC. Accordingly, after an ISC-ablative dose of γ-irradiation (Metcalfe et al., 2014), ISC restored from Lgr5^{+} cells' recent progeny and the resulting crypts should emit red fluorescence. In contrast, based on the independent origins and replicative quiescence of putative 'reserve' ISC (Li and Clevers, 2010), Lgr5^{+} ISC and the associated crypts arising from 'reserve' ISC will lack that signal (Fig. 1A). The *Lgr5*$^{GFP\text{-}Cre}$ allele is active in a fraction of intestinal crypts (Barker et al., 2007) and 24 h after radiation all crypts lacked GFP (Fig. S1A), as expected. ISC were restored within 6 days, when nearly every recovered crypt in small intestines and ~80% of colonic crypts housed tdTom^{+} cells and GFP^{+} ISC (Fig. 1B-C). Corroborating these rigorous counts of every GFP^{+} crypt by microscopy, flow cytometry revealed tdTom in >99% of duodenal GFP^{+} ISC (Fig. 1D). Thus, regenerated ISC arise almost exclusively from their own recent progeny, with at best

a small contribution from older cells. Because colonic epithelium turns over more slowly than the small intestine (Barker et al., 2007; Cheng and Bjerknes, 1985), the fraction of $GFP^{+}tdTom^{-}$ crypts in the recovered colon likely reflects inefficient labeling of ISC progeny before irradiation.

Dll1^{+} intestinal Sec progenitors can restore ISC damaged by γ-irradiation (van Es et al., 2012) and Ent progenitors contribute when ISC expressing the *Diphtheria* toxin receptor (DTR) are ablated by exposure to DT (Tetteh et al., 2016). Other studies confirm the role of *Atoh1*$^{+}$ Sec cells in low-level baseline ISC production, which increases upon epithelial injury (Ishibashi et al., 2018; Tomic et al., 2018), but recent work suggests that Sec cells may alone replenish damaged colonic ISC (Castillo-Azofeifa et al., 2019). Therefore, we asked if each lineage can restore ISC when the other is depleted and when injury is exquisitely targeted to ISC: by treating *Lgr5*$^{Dtr\text{-}GFP}$ mice (Tian et al., 2011) with DT (Fig. 1E-F). We examined crypt cell dedifferentiation in *Atoh1*$^{-/-}$ intestines, which lack all Sec cells (Shroyer et al., 2007), and in Ent-depleted *Rbpj*$^{-/-}$ intestines (Kim et al., 2014), using *Villin-Cre*$^{ER(T2)}$ mice (el Marjou et al., 2004) to delete *Atoh1* (Shroyer et al., 2007) or *Rbpj* (Han et al., 2002) in all intestinal epithelial cells. The experimental schemes differed because Sec –especially Paneth– cells take up to 4 weeks to disappear after *Atoh1* deletion and mice withstand this loss far longer than they survive after *Rbpj* deletion. In both cases, however, we measured recovery by counting GFP^{+} ISC in crypts 3 days after the last of 4 doses of DT. Neither absence (Fig. 1E) nor substantial enrichment (Figs. 1F and S1B) of Sec cells appreciably impaired full recovery of duodenal or colonic ISC. Modestly limited ISC recovery in *Rbpj*$^{-/-}$ colon reflects either the larger fraction of Sec progenitors normally present in this organ or the poor health of animals, which survive only a few days after *Rbpj* deletion (Kim et al., 2014). Together, these findings indicate that, at least when Sec or Ent cells are missing, the other type dedifferentiates efficiently after ISC ablation.

Limited requirement for ISC-restricted transcription factor ASCL2 in resting ISC functions

After ISC loss, dedifferentiating EE and goblet-cell precursors extensively alter chromatin access and mRNA expression (Jadhav et al., 2017). Because these changes are especially marked and rapid at the *Ascl2* locus (Fig. S1C), which was previously implicated in $Lgr5^{+}$ ISC survival (van der Flier et al., 2009), we generated a new mouse allele, *Ascl2*^{Dfci} (Key Resources Table), which replaces the native coding region with a floxed bicistronic cassette that encodes *mCherry* (mCh) and FLAG epitope-tagged ASCL2 (Fig. S1D). This allele allows detection of $ASCL2^{+}$ cells by fluorescence microscopy or flow cytometry, precipitation of ASCL2 with FLAG antibody, and CRE-mediated gene deletion. mCh signals were confined to the bottoms of all crypts (Fig. S1E), below tier 5 (Fig. 2A), co-localizing precisely with GFP in *Lgr5*$^{GFP\text{-}Cre}$ (Barker et

al., 2007) and *Lgr5*$^{Dtr\text{-}GFP}$ (Tian et al., 2011) mice (Fig. 2B). *Ascl2*^{Dfci} mice thus demonstrate tight *Ascl2* restriction in Lgr5^{+} ISC and can be used to visualize this cell population, especially in the colon, where fluorescence was consistently higher than in small intestine ISC (Fig. S1F).

Ascl2$^{+/Dfci}$ mice were healthy and born in Mendelian proportions, but the targeted allele failed to transmit through the maternal germline, probably because the *Ascl2* locus is imprinted, and we could not achieve homozygosity. We therefore crossed the mice with another *Ascl2*^{Fl} strain, which transmits a floxed allele (whole-gene deletion, hereafter called *Ascl2*^{Umc}, Fig. S1G-H) through both sexes (van der Flier et al., 2009), to derive *Ascl2*$^{Dfci/Umc}$ compound heterozygotes. Additional crosses allowed us to study consequences of ASCL2 loss throughout the intestinal epithelium (with *Villin-Cre*^{ER}) or from some crypts, starting in Lgr5^{+} ISC (with *Lgr5*$^{GFP\text{-}Cre}$). After activation of *Villin-Cre*^{ER} with TAM, both *Ascl2*$^{Dfci/Umc}$ and *Ascl2*$^{Umc/Umc}$ mice displayed no signs of malnutrition or illness, and intestinal histology was overtly normal (Fig. S2A). Immunostains for Ki67 and cleaved Caspase 3 revealed intact crypt cell proliferation (Fig. 2C) and absence of appreciable apoptosis (Fig. S2B). On the *Lgr5*$^{GFP\text{-}Cre}$ background, TAM-treated *Ascl2*-null (mCh^{-} GFP^{+}) and adjacent *Ascl2*-proficient (mCh^{+}GFP^{-}) crypts carried essentially similar ISC numbers (Fig. 2D), a finding confirmed by GFP flow cytometry (Fig. 2E). Moreover, lineage tracing by the *R26R*^{Tom} reporter revealed ostensibly intact ISC activity (Fig. S2C), indicating that ASCL2 is dispensable for resting ISC function.

Although ISC survival is compromised in *Ah-Cre;Ascl2*$^{Fl/Fl}$ mice (van der Flier et al., 2009), our findings agree with a subsequent study showing modestly reduced ability of *Villin-Cre;Ascl2*$^{Fl/Fl}$ ISC to form organoids (Schuijers et al., 2015). This difference may reflect unknown effects of ß-naphthoflavone-mediated gene deletion in *Ah-Cre* mice; alternatively, *Ascl2* deletion may have been inefficient in our experiments. To exclude the latter possibility, note that recombination of *Ascl2*^{Dfci} deletes coding exons as well as *mCh* (Fig. S1D) and that PCR can discern parental from CRE-excised alleles (Fig. S1H). mCh signals were substantially reduced after TAM exposure on the *Villin-Cre* background (Fig. S2D), and both mCh^{+}GFP^{+} ISC (Fig. 2F) and *Ascl2* transcripts (Fig. S2E) were much reduced on the *Lgr5*$^{Gfp\text{-}Cre}$ background. Genotyping confirmed significant recombination of targeted *Ascl2* alleles (Fig. S2F). Moreover, *Ascl2*$^{-/-}$ ISC purified by GFP flow cytometry differed from *Ascl2*-proficient ISC, purified by mCh flow cytometry, in expression of ~3,500 mRNAs (>2-fold, P_{adj} <0.01, Fig. S2G and Suppl. Table 1), as expected with global absence of a functional TF. Escape from recombination is therefore small and cannot account for largely intact ISC function days after CRE activation.

ASCL2 requirements in neutral ISC drift and in ISC recovery after injury

Ascl2$^{-/-}$ ISC could, however, be disadvantaged in prolonged competition with the minority of cells lacking biallelic gene deletion. Lgr5^{+} ISC divide symmetrically, with each crypt becoming monoclonal by neutral drift over ~30 days (Lopez-Garcia et al., 2010; Snippert et al., 2010), but cells with a selective advantage prevail over time (Snippert et al., 2014; Vermeulen et al., 2013). Indeed, although GFP^{+} (Cre^{+}) crypts in *Lgr5*^{Cre}*;Ascl2*$^{Fl/Fl}$*;R26R*^{tdTom} mice first expressed tdTom uniformly after CRE activation, progressively larger fractions of these crypts lacked tdTom 1 month and 4 months later (Fig. 3A), implying replacement of *Ascl2*-null cells by ISC with one or both *Ascl2* copies intact. Soon after TAM treatment, unrecombined tdTom^{-} cells were difficult to detect by microscopy, but DNA from GFP^{+} duodenal ISC revealed their presence at 5 days and DNA at 1 month carried similar proportions of parental and recombined *Ascl2* alleles (Fig. 3B). Even at 4 months, rare GFP^{+} crypts housed both tdTom^{+} and tdTom^{-} cells (Fig. 3C), which implies ongoing competition and prolonged delay of monoclonality.

To estimate the selective *Ascl2*$^{-/-}$ ISC disadvantage quantitatively, 2D tissue sections did not accurately define cell ratios in the rare mixed crypts or the ISC fraction that escaped gene recombination. Instead we used the predominantly tdTom^{+} and tdTom^{-} crypt fractions in mice 28 (n=2) and 112 (n=5) days after *Ascl2* excision (Fig. 3A), together with a previous (Lopez-Garcia et al., 2010) population genetics model of ISC dynamics (Fig. 3D and STAR Methods). We considered starting ISC numbers (*N*) of 10 to 14 (Snippert et al., 2010), wild-type replication rates (λ_{WT}) of 0.5/day for duodenal and 0.25/day for colonic ISC (Snippert et al., 2014), and variable numbers of cells rendered *Ascl2*-null (*M*), where *M*/*N* >0.7. We modeled possible *Ascl2*$^{-/-}$ ISC replication rates (λ_{Mut}) by optimizing λ_{Mut} values over 1,000 simulations to minimize the squared distance between predicted and observed tdTom^{+} (presumed Mut) and tdTom^{-} (WT) crypt counts (e.g., Fig. S3A-B). This model recapitulated the data and estimated relative *Ascl2*$^{-/-}$ ISC fitness, $\lambda_{Mut}/\lambda_{WT}$, as ~0.5-0.75 in the duodenum and ~0.33-0.67 in the colon (Figs. 3E and S3C). These estimates were robust over a wide range of parameters, largely insensitive to different ratios of *M*/*N* when *N* >10, to a different duodenal λ_{WT} of 0.3 (Kozar et al., 2013), and whether simulations were fitted to mean or median 112-day crypt counts. The wide range of estimates resulted mainly from uncertainty about the fraction of *Ascl2*$^{-/-}$ ISC (*M*/*N*), as expected, but very small changes in λ_{Mut} moved trajectories across the range of observed 112-day crypt counts, lending confidence in the range of values. Refinement of these provisional estimates will require additional animals, time points, and confident determination of values for *M*.

To determine whether ASCL2 is necessary to restore damaged ISC, we first treated *Ascl2*$^{Umc/Umc}$*;Lgr5*$^{GFP\text{-}Cre}$ mice with TAM to delete *Ascl2*, followed by 10 Gy γ-irradiation to ablate ISC. Whereas control intestines quickly recovered GFP^{+} ISC, absence of ASCL2 prevented this

recovery and animals succumbed to the radiation injury (Fig. 4A). Second, after using DT to kill ISC in *Ascl2*$^{Umc/Umc}$;*Lgr5*$^{Dtr\text{-}GFP}$;*Villin-Cre*$^{ER(T2)}$ mice, we gave TAM to activate CRE and delete *Ascl2* (Fig. 4B). Again, whereas GFP$^+$ ISC were restored within 5 days in control animals, *Ascl2*-null intestines failed to recover in that period and flow cytometry confirmed paucity of GFP$^+$ cells (Fig. 4B). Mutant mice became progressively moribund over the next 3 days, and at euthanasia their intestines showed a persistent Lgr5$^+$ ISC deficit (Fig. 4C), tissue dysmorphology (Fig. 4D), and reduced crypt cell proliferation (Fig. S4A). Whether ISC were ablated by irradiation or DT treatment, *Ascl2*$^{-/-}$ crypts failed to revive in all intestinal regions (Fig. S4B-C). Thus, unlike the subtle role in physiologic neutral drift, ASCL2 is essential for ISC recovery after lethal damage.

Extensive Ascl2 activation in crypt cells before complete dedifferentiation into ISC

As the latter findings suggest that *Ascl2* may act in crypt cell dedifferentiation, we examined *Ascl2*$^{+/Dfci}$;*Lgr5*$^{Dtr\text{-}GFP}$ intestinal crypts before and after treating mice with DT to eliminate mCh$^+$ ISC (Fig. 5A). Abundant mCh$^+$ cells now appeared well above crypt tier 5; over the next 2 days they progressed toward the crypt base (quantified in Fig. 5B) and eventually expressed GFP, i.e., *Lgr5* (Fig. 5A), similar to resting ISC. Thus, progenitor cells that otherwise lack *Ascl2* (see Fig. 2A-B) activate expression as they dedifferentiate. These 'upper' mCh$^+$ cells, readily seen in the regenerating colon (Fig. 5A-B), were difficult to visualize in the small intestine, where *Ascl2* RNA levels in resting ISC are lower than those in the colon (Fig. S1F). Flow cytometry of DT-exposed colonic crypt cells confirmed a sizable mCh$^+$GFP$^-$ cell population, distinct from native resting mCh$^+$GFP$^+$ ISC (Figs. 5C and S5A-B), allowing us to isolate both populations. When cultured in medium containing Wnt, Epidermal Growth Factor, Rspo and Notch ligand (WERN), mCh$^+$GFP$^-$ cells from DT-exposed crypts generated typical colonic organoids (Sasaki et al., 2016; Sato et al., 2009), far in excess of similar structures produced by equal numbers of native mCh$^+$GFP$^+$ colonic ISC (Fig. 5D). 'Upper' cell-derived organoids had an epithelial lining and could be passaged successively *in vitro* (Fig. S5C), indicating that they contain self-renewing ISC. In contrast, mCh$^-$GFP$^-$ cells from DT-treated mice yielded few small spheroids, possibly reflecting ISC potential in some crypt cells that do not yet express *Ascl2*/mCh, and those from untreated mice yielded none (Fig. 5D). These data reveal robust *Ascl2* inactivation in a large fraction of colonic crypt cells destined to dedifferentiate into ISC.

To study ASCL2-dependent crypt dedifferentiation, we purified regenerating mCh$^+$ (Ascl2$^+$) 'upper' cells by flow cytometry and used RNA-seq to compare their bulk RNA profiles with those of uninjured resting ISC (Suppl. Table 1). Out of 485 genes specific to Lgr5$^+$ ISC in the small intestine (Munoz et al., 2012), 176 genes are expressed in resting colonic ISC at appreciable

levels (>10 read per kb per million sequence tags, RPKM) and 401 genes express at any level (>1 RPKM, Fig. S5D). Among these ISC-restricted mRNAs, 149 were appreciably present (>10 RPKM) and 365 were expressed (>1 RPKM) in upper mCh$^+$ cells (Suppl. Table 2), including *Cdca7* and *Smoc2* (Fig. 5E). As a population, upper cells differed from resting ISC in the levels of only 316 mRNAs (q <0.01, Fig. 5F and Suppl. Table 1), implying that although regenerating Ascl2$^+$ cells lack GFP protein (Fig. S5A-B) or *Lgr5* mRNA (Fig. 5E), they are likely well on a path to acquire ISC features. Upper cells were enriched for Sec but not for +4 cell markers (Fig. 5F-G) and did not express mRNA (Fig. 5G) or protein (Fig. S5E) of the 'revival' ISC marker Clu (Ayyaz et al., 2019).

'Enterospheres' expanded in vitro from crypts late in mouse gestation express many RNAs different from those in adult organoids (Fordham et al., 2013; Mustata et al., 2013). In some forms of ISC injury, dedifferentiating adult crypt cells are reported to recapitulate this transcriptional program, represented by *Ly6a*/*Sca1* and triggered in part by YAP/TAZ signaling (Nusse et al., 2018; Yui et al., 2018). To test this general idea in mCh$^+$ colonic 'upper' cells induced upon DT ablation of ISC, we extended an approach originally applied to mouse fetal small bowel endoderm (Banerjee et al., 2018) to isolate colonic EPCAM$^+$ cells at embryonic days (E) 11, 12, 14 and 16. Among the 6,865 fetal transcripts that are reduced or absent in the adult epithelium, mCh$^+$ 'upper' cells reproducibly expressed at most 183 genes (2.67%, Fig. S5F). Enterosphere-specific RNAs overlap minimally with these genes and were expressed comparably in resting and regenerating ISC (Fig. S5G). Key intestinal YAP/TAZ signature genes (Gregorieff et al., 2015) also were not enriched in 'upper' cells (Fig. S5H), whereas Sec marker genes (Fig. 5F) hinted that the population may carry Sec or multipotent progenitors.

Transcriptional features and trajectories of crypt cell dedifferentiation

To resolve the mCh$^+$ 'upper' population, we used 10X Genomics scRNA-seq to study single cells collected 2 days after the last DT dose (day 8 in the experimental scheme, Fig. 5A). After excluding cells with low information content or a high fraction of mitochondrial RNAs (Fig. S6A and STAR Methods), 3,254 cells were informative. Individual cells expressed *Ascl2* but little to no *Lgr5* mRNA (Fig. 6A), consistent with mCh$^+$GFP$^-$ fluorescence (Fig. S5A-B). ISC-restricted genes such as *Cdca7* and *Smoc2* (Suppl. Table 2) further delineated distinct ISC-like cells (Fig. 6A). Proliferative activity was largely confined to this population, while +4 cell markers were absent (e.g., *Clu*) or expressed broadly (e.g., *Bmi1*) (Fig. 6B). Projection of cell-specific markers on a Uniform Manifold Approximation and Projection (UMAP) plot identified *Fabp2*$^+$ *Car1*$^+$ colonocytes, abundant *Muc2*$^+$ *Tff3*$^+$ goblet cells (Fig. 6C), and a tiny fraction of *Chga*$^+$ *Pyy*$^+$ EE

cells (Fig. S6B). Notably, 556 cells expressed appreciable levels of ISC genes as well as colonocyte or goblet cell markers (Fig. 6D), implying that they represent a *bona fide* transition along a spectrum of mature and dedifferentiated states. Within that spectrum, genes from enterosphere/fetal (Fordham et al., 2013; Mustata et al., 2013) and YAP/TAZ signaling (Gregorieff et al., 2015; Yui et al., 2018) modules, including genes common to two (*Ly6d*) or all three (*Ly6c1* and *Ly6a*/*Sca1*) modules did not concentrate in the transitional cells or show a consistent pattern; individual transcripts were largely absent or broadly expressed (Fig. S6C).

To derive pseudotime trajectories based on cell-to-cell differences in RNA expression, we used the Monocle algorithm (Trapnell et al., 2014), designating high expression of ISC markers as the cellular end-point. This unsupervised approach identified a continuum of cell states, with abundant goblet cells and fewer colonocytes showing distinct trajectories (Fig. 6E). Positioning of transitional cells and their corresponding mature precursors along the pseudotime axis highlighted the distinct properties. Colonocyte markers declined more rapidly in pseudotime than goblet cell markers; conversely, ISC markers appeared more gradually in dedifferentiating goblet cells than in colonocytes (Fig. 6F). These data reveal both cell types as sources of regenerating colonic ISC, but do not distinguish whether goblet cells are the principal source, as proposed (Castillo-Azofeifa et al., 2019), or were captured more readily than colonocytes.

ACSL2 target genes such as Il11ra1 *facilitate ISC regeneration*

To identify transcriptional targets of ectopic ASCL2 in regenerating mCh^+ 'upper' cells, we used the 3xFLAG epitope in $ASCL2^{DFCI}$ (Fig. S1D) to precipitate TF-bound genomic regions, and identified 4,943 binding sites (Suppl. Table 3). Because ISC restoration likely depends on multiple target genes and transcriptional target assignment to distant enhancers is inherently ambiguous, we focused on genes with promoter binding (n=3,530) and altered expression in mCh^+ crypt cells (n=316). Most mRNA changes were gains (Fig. 5F) and correlated with higher ASCL2 binding at promoters in 'upper' cells than in resting ISC (Fig. 7A). Four candidate target genes encode cell surface receptors that we could assess in functional assays: *Fgf18* and *Nov*, which act respectively in Fibroblast Growth Factor and Notch signaling; the Wnt receptor *Fzd9*; and the Interleukin-11 (IL-11) receptor gene *Il11ra1* (Figs. 7A and S7A). Neither FZD9 Ab nor recombinant (r) FGF18 or rNOV affected organoid formation by mCh^+ 'upper' crypt cells (Fig. S7B – though we are unsure if rNOV is active in vitro or if FZD9 Ab neutralizes the receptor).

Il11ra1 is a noteworthy candidate because IL-11 protects animals from various forms of ISC ablation (Du et al., 1994; Liu et al., 1996; Potten, 1996). *Il11ra1* transcripts were significantly increased in every bulk isolate of mCh^+ 'upper' cells, compared to resting colonic $Lgr5^+$ ISC (Fig.

7B) and well represented in scRNA analysis of regenerating ISC, including transitional cells and especially in the goblet cell fraction (Fig. 7C). Flow cytometry for surface IL11RA1 revealed a small fraction of IL11RA$^+$ 'upper' mCh$^+$ cells, compared to none in resting mCh$^+$ ISC (Fig. S7C). IL11 signals transduce via STAT3 (Jenkins et al., 2007) and we detected phosphorylated STAT3 in rare 'upper' cells, but never in the absence of crypt ablation (Fig. 7D). Importantly, rIL-11 had a reproducible, dose-dependent effect in augmenting organoid formation (Fig. 7E), which did not occur with other targeted perturbations (Fig. S7B). This augmentation occurred only in upper-cell, not in resting ISC cultures (Fig. 7E), and organoids enhanced in response to IL-11 could be passaged serially (Figs. 7F and S7D), indicating that they contain colonic ISC. Although other signals likely also contribute, these findings collectively implicate *Il11ra1* as one functional target of ASCL2 control in ISC regeneration.

DISCUSSION

Both principal cell types derived from Lgr5$^+$ ISC (Tetteh et al., 2016; van Es et al., 2012) and even rare Paneth cells (Jones et al., 2018; Schmitt et al., 2018; Yu et al., 2018) help restore crypt function after ISC damage, but reports to date leave open the possibility of contributions from 'reserve' ISC (Bankaitis et al., 2018; de Sousa e Melo and de Sauvage, 2019). Substantial crypt recovery after Lgr5$^+$ ISC ablation originates in *Bmi1*^{Cre}-expressing cells (Tian et al., 2011), and although Bmi^{hi} cells and S-phase LRC located above the Paneth/ISC zone (Potten, 1998; Sangiorgi and Capecchi, 2008; Yan et al., 2012) contain mainly Paneth and EE cell precursors (Buczacki et al., 2013; Jadhav et al., 2017; Yan et al., 2017), these populations could in principle include putative 'reserve' stem cells. Using *Lgr5*^{Cre}-derived cell labels, we show that nearly all crypt regeneration after lethal damage to Lgr5$^+$ ISC originates in their own recent progeny, i.e., by dedifferentiation and not by recruiting 'reserve' ISC. Moreover, neither 'reserve' nor recently reported 'revival' ISC (Ayyaz et al., 2019) exist in numbers sufficient to account for extensive ISC restoration at the observed speed, and our bulk and single-cell RNA profiles of regenerating colonic crypt cells lacked enrichment of +4 cell markers. *Bmi1*^{Cre}-derived crypt recovery after ISC ablation (Tian et al., 2011) likely reflects *Bmi1* expression in a large fraction of crypt cells (Itzkovitz et al., 2011; Munoz et al., 2012), whereas GFP expression in *Bmi1*^{Gfp} mice is fortuitously restricted to EE cells enriched in the +4 tier (Buczacki et al., 2013; Jadhav et al., 2017; Yan et al., 2017).

Several observations indicate that diverse crypt progenitors are able to dedifferentiate. First, although we did not strictly compare regenerative efficiencies of Sec and Ent cells, each lineage

readily restores ISC when the other is absent. Second, after lethal ISC injury *Ascl2* (mCh) appears in high colonic crypt tiers, which normally lack expression, and *Ascl2*$^{+}$ cells move into the ISC zone, where they later express *Lgr5* (GFP). ASCL2 is required for crypt regeneration and this ectopic, likely homeostatic, *Ascl2* expression is not confined to rare cells but evident in a large fraction of GFP^{-} crypt cells that we could purify. The short time between administration of DT and appearance of ectopic mCh^{+} cells suggests that *Ascl2* was activated *de novo* in diverse crypt cells, not in clones expanded from rare 'reserve' cells. Third, we previously observed extensive changes in mRNA levels and ATAC signals as purified EE and goblet cell precursors were dedifferentiating (Jadhav et al., 2017); such signals would be detected only if they occur in a sizable fraction of cells. Fourth, scRNA-seq analysis of mCh^{+} 'upper' cells showed both goblet cell and colonocyte streams converging onto restored ISC, with seemingly distinct temporal dynamics. Competitive repopulation studies may in the future reveal if restorative contributions from Sec and Ent cells are quantitatively different and occur at different speeds.

ISC-depleted colonic crypts express *Ascl2* (mCh) only 8-9 cell tiers higher than normal. It is therefore unclear if regenerative potential is restricted to progenitors or extends to terminally differentiated cells. In the small intestine, Paneth cell dedifferentiation (Jones et al., 2018; Schmitt et al., 2018; Yu et al., 2018) and ectopic crypt formation along Bone Morphogenetic Protein-inhibited villi (Batts et al., 2006; Haramis et al., 2004) hint that some mature epithelial cells may harbor that capacity. YAP/TAZ signaling and a developmental transcriptional program were recently implicated in ISC regeneration (Gregorieff et al., 2015; Nusse et al., 2018; Yui et al., 2018). Our findings suggest that these conclusions may reflect unique properties of cultured late fetal ISC rather than epithelial genesis *per se*. *Ascl2* expression in mCh^{+} 'upper' cells is not confined to actively cycling single cells (Fig. 6A-B), but our experiments do not distinguish whether it is required for crypt cells to dedifferentiate or to expand. Resting ISC are uniquely sensitive to ~10 Gy γ-irradiation, while other crypt cells survive that dose (Potten, 1998). After ISC ablation, however, regenerating crypt cells become exquisitely radiosensitive (Metcalfe et al., 2014). We propose that consequences of ASCL2 activity, present in resting ISC and initiated early during progenitor dedifferentiation, may underlie this sensitivity.

Various immune signals are implicated in the regenerative response (Biton et al., 2018; Zhou et al., 2013). IL-11 in particular, possibly from a myofibroblast source (Bamba et al., 2003), aids in intestinal mucosal recovery from bowel resection (Liu et al., 1996), radiation (Potten, 1996), and other ablative injuries (Du et al., 1994). It is, however, unclear which crypt cells respond to IL-11 and whether this ability is constitutive or induced upon ISC attrition. We find that resting ISC express little *Il11ra1* and levels are elevated in regenerating *Ascl2*$^{+}$ crypt cells.

Moreover, unlike native colonic ISC, regenerating cells respond to rIL-11 with enhanced spheroid formation *in vitro*. ISC-depleted crypts are thus uniquely sensitive to IL-11, which augments dedifferentiation and is probably one of several immune and non-immune signals that help restore ISC. As a TF essential to the process, ASCL2 helps orchestrate that robust homeostatic response.

AUTHOR CONTRIBUTIONS

K.M. and R.A.S. conceived and designed the studies; K.M. performed experiments; U.J. and S.M. performed computational analyses; U.J. and A.C. contributed ISC and fetal colonic gene expression data; J.D. and F.M. simulated computer models for ISC fitness; K.W. supervised scRNA experiments; J.v.E. and H.C. provided *Ascl2*^{Umc} mice; K.M., U.J., and R.A.S. interpreted results; K.M. and R.A.S. drafted and edited the manuscript, with input from all authors.

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REFERENCES

Anders, S., Pyl, P.T., and Huber, W. (2015). HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics *31*, 166-169.

Ayyaz, A., Kumar, S., Sangiorgi, B., Ghoshal, B., Gosio, J., Ouladan, S., Fink, M., Barutcu, S., Trcka, D., Shen, J.*, et al.* (2019). Single-cell transcriptomes of the regenerating intestine reveal a revival stem cell. Nature *569*, 121-125.

Bamba, S., Andoh, A., Yasui, H., Makino, J., Kim, S., and Fujiyama, Y. (2003). Regulation of IL-11 expression in intestinal myofibroblasts: role of c-Jun AP-1- and MAPK-dependent pathways. Am J Physiol Gastrointest Liver Physiol *285*, G529-238.

Banerjee, K.K., Saxena, M., Kumar, N., Chen, L., Cavazza, A., Toke, N.H., O'Neill, N.K., Madha, S., Jadhav, U., Verzi, M.P.*, et al.* (2018). Enhancer, transcriptional, and cell fate plasticity precedes intestinal determination during endoderm development. Genes Dev *32*, 1430-1442.

Bankaitis, E.D., Ha, A., Kuo, C.J., and Magness, S.T. (2018). Reserve Stem Cells in Intestinal Homeostasis and Injury. Gastroenterology *155*, 1348-1361.

Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, 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 stem cell of the intestinal crypt: Strategies and pitfalls. Cell Stem Cell *11*, 452-460.

Batts, L.E., Polk, D.B., Dubois, R.N., and Kulessa, H. (2006). Bmp signaling is required for intestinal growth and morphogenesis. Dev Dyn *235*, 1563-1570.

Biton, M., Haber, A.L., Rogel, N., Burgin, G., Beyaz, S., Schnell, A., Ashenberg, O., Su, C.W., Smillie, C., Shekhar, K.*, et al.* (2018). T Helper Cell Cytokines Modulate Intestinal Stem Cell Renewal and Differentiation. Cell *175*, 1307-1320 e1322.

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.

Butler, A., Hoffman, P., Smibert, P., Papalexi, E., and Satija, R. (2018). Integrating single-cell transcriptomic data across different conditions, technologies, and species. Nat Biotechnol *36*, 411-420.

Castillo-Azofeifa, D., Fazio, E.N., Nattiv, R., Good, H.J., Wald, T., Pest, M.A., de Sauvage, F.J., Klein, O.D., and Asfaha, S. (2019). Atoh1(+) secretory progenitors possess renewal capacity independent of Lgr5(+) cells during colonic regeneration. EMBO J *38*.

Chaves-Perez, A., Yilmaz, M., Perna, C., de la Rosa, S., and Djouder, N. (2019). URI is required to maintain intestinal architecture during ionizing radiation. Science *364*, eaaq1165.

Cheng, H., and Bjerknes, M. (1985). Whole population cell kinetics and postnatal development of the mouse intestinal epithelium. Anat Rec *211*, 420-426.

Corces, M.R., Trevino, A.E., Hamilton, E.G., Greenside, P.G., Sinnott-Armstrong, N.A., Vesuna, S., Satpathy, A.T., Rubin, A.J., Montine, K.S., Wu, B.*, et al.* (2017). An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues. Nat Methods *14*, 959-962.

Cornwell, M., Vangala, M., Taing, L., Herbert, Z., Koster, J., Li, B., Sun, H., Li, T., Zhang, J., Qiu, X.*, et al.* (2018). VIPER: Visualization Pipeline for RNA-seq, a Snakemake workflow for efficient and complete RNA-seq analysis. BMC Bioinformatics *19*, 135.

de Sousa e Melo, F., and de Sauvage, F.J. (2019). Cellular plasticity in intestinal homeostasis and disease. Cell Stem Cell *24*, 54-64.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics *29*, 15-21.

Du, X.X., Doerschuk, C.M., Orazi, A., and Williams, D.A. (1994). A bone marrow stromal-derived growth factor, interleukin-11, stimulates recovery of small intestinal mucosal cells after cytoablative therapy. Blood *83*, 33-37.

el Marjou, F., Janssen, K.P., Chang, B.H., Li, M., Hindie, V., Chan, L., Louvard, D., Chambon, P., Metzger, D., and Robine, S. (2004). Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis *39*, 186-193.

Fordham, R.P., Yui, S., Hannan, N.R., Soendergaard, C., Madgwick, A., Schweiger, P.J., Nielsen, O.H., Vallier, L., Pedersen, R.A., Nakamura, T.*, et al.* (2013). Transplantation of expanded fetal intestinal progenitors contributes to colon regeneration after injury. Cell Stem Cell *13*, 734-744.

Gregorieff, A., Liu, Y., Inanlou, M.R., Khomchuk, Y., and Wrana, J.L. (2015). Yap-dependent reprogramming of Lgr5(+) stem cells drives intestinal regeneration and cancer. Nature *526*, 715-718.

Han, H., Tanigaki, K., Yamamoto, N., Kuroda, K., Yoshimoto, M., Nakahata, T., Ikuta, K., and Honjo, T. (2002). Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. Int Immunol *14*, 637-645.

Haramis, A.P., Begthel, H., van den Born, M., van Es, J., Jonkheer, S., Offerhaus, G.J., and Clevers, H. (2004). De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. Science *303*, 1684-1686.

Ishibashi, F., Shimizu, H., Nakata, T., Fujii, S., Suzuki, K., Kawamoto, A., Anzai, S., Kuno, R., Nagata, S., Ito, G.*, et al.* (2018). Contribution of ATOH1(+) Cells to the Homeostasis, Repair, and Tumorigenesis of the Colonic Epithelium. Stem Cell Reports *10*, 27-42.

Itzkovitz, S., Lyubimova, A., Blat, I.C., Maynard, M., van Es, J., Lees, J., Jacks, T., Clevers, H., and van Oudenaarden, A. (2011). Single-molecule transcript counting of stem-cell markers in the mouse intestine. Nat Cell Biol *14*, 106-114.

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 secretory precursors into Lgr5+ intestinal stem cells. Cell Stem Cell *21*, 65-77.

Jenkins, B.J., Roberts, A.W., Greenhill, C.J., Najdovska, M., Lundgren-May, T., Robb, L., Grail, D., and M., E. (2007). Pathologic consequences of STAT3 hyperactivation by IL-6 and IL-11 during hematopoiesis and lymphopoiesis. Blood *109*, 2380-2388.

Jones, J.C., Brindley, C.D., Elder, N.H., Myers, M.G., Jr., Rajala, M.W., Dekaney, C.M., McNamee, E.N., Frey, M.R., Shroyer, N.F., and Dempsey, P.J. (2018). Cellular plasticity of Defa4(Cre)-expressing Paneth cells in response to Notch activation and intestinal injury. Cell Mol Gastroenterol Hepatol *7*, 533-554.

Jubb, A.M., Chalasani, S., Frantz, G.D., Smits, R., Grabsch, H.I., Kavi, V., Maughan, N.J., Hillan, K.J., Quirke, P., and Koeppen, H. (2006). Achaete-scute like 2 (ascl2) is a target of Wnt signalling and is upregulated in intestinal neoplasia. Oncogene *25*, 3445-3457.

Kim, T.H., Li, F., Ferreiro-Neira, I., Ho, L.L., Luyten, A., Nalapareddy, K., Long, H., Verzi, M., and Shivdasani, R.A. (2014). Broadly permissive intestinal chromatin underlies lateral inhibition and cell plasticity. Nature *506*, 511-515.

Kozar, S., Morrissey, E., Nicholson, A.M., van der Heijden, M., Zecchini, H.I., Kemp, R., Tavare, S., Vermeulen, L., and Winton, D.J. (2013). Continuous clonal labeling reveals small numbers of functional stem cells in intestinal crypts and adenomas. Cell Stem Cell *13*, 626-633.

Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nat Methods *9*, 357-359.

Li, L., and Clevers, H. (2010). Coexistence of quiescent and active adult stem cells in mammals. Science *327*, 542-545.

Liu, Q., Du, X.X., Schindel, D.T., Yang, Z.X., Rescorla, F.J., Williams, D.A., and Grosfeld, J.L. (1996). Trophic effects of interleukin-11 in rats with experimental short bowel syndrome. J Pediatr Surg *31*, 1047-1050.

Lopez-Garcia, C., Klein, A.M., Simons, B.D., and Winton, D.J. (2010). Intestinal stem cell replacement follows a pattern of neutral drift. Science *330*, 822-825.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome biology *15*, 550.

Mao, Q., Wang, L., Goodison, S., and Sun, Y. (2015). Dimensionality Reduction Via Graph Structure Learning. Proc 21st ACM SIGKDD Intl Conf Knowledge Disc Data Mining *KKD 15*, 765-774. (doi 10.1145/2783258.2783309).

Metcalfe, C., Kljavin, 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., Kranendonk, M.E., Henderson, D.E., Baffour-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.

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.

Mustata, R.C., Vasile, G., Fernandez-Vallone, V., Strollo, S., Lefort, A., Libert, F., Monteyne, D., Perez-Morga, D., Vassart, G., and Garcia, M.I. (2013). Identification of Lgr5-independent spheroid-generating progenitors of the mouse fetal intestinal epithelium. Cell Rep *5*, 421-432.

Nusse, Y.M., Savage, A.K., Marangoni, P., Rosendahl-Huber, A.K.M., Landman, T.A., de Sauvage, F.J., Locksley, R.M., and Klein, O.D. (2018). Parasitic helminths induce fetal-like reversion in the intestinal stem cell niche. Nature *559*, 109-113.

Ootani, A., Li, X., Sangiorgi, E., Ho, Q.T., Ueno, H., Toda, S., Sugihara, H., Fujimoto, K., Weissman, I.L., Capecchi, M.R.*, et al.* (2009). Sustained in vitro intestinal epithelial culture within a Wnt-dependent stem cell niche. Nat Med *15*, 701-706.

Pinello, L., Xu, J., Orkin, S.H., and Yuan, G.C. (2014). Analysis of chromatin-state plasticity identifies cell-type-specific regulators of H3K27me3 patterns. Proc Natl Acad Sci USA *111*, E344-353.

Potten, C.S. (1996). Protection of the small intestinal clonogenic stem cells from radiation-induced damage by pretreatment with interleukin 11 also increases murine survival time. Stem Cells *14*, 452-459.

Potten, C.S. (1998). Stem cells in gastrointestinal epithelium: numbers, characteristics and death. Philos Trans R Soc Lond B Biol Sci *353*, 821-830.

R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria http://wwwR-projectorg/.

Ramirez, F., Ryan, D.P., Gruning, B., Bhardwaj, V., Kilpert, F., Richter, A.S., Heyne, S., Dundar, F., and Manke, T. (2016). deepTools2: a next generation web server for deep-sequencing data analysis. Nucleic Acids Res *44*, W160-165.

Robinson, J.T., Thorvaldsdottir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., and Mesirov, J.P. (2011). Integrative genomics viewer. Nat Biotechnol *29*, 24-26.

San Roman, A.K., Tovaglieri, A., Breault, D.T., and Shivdasani, R.A. (2015). Distinct processes and transcriptional targets underlie CDX2 requirements in intestinal stem cells and differentiated villus cells. Stem Cell Reports *5*, 673-681.

Sangiorgi, E., and Capecchi, M.R. (2008). Bmi1 is expressed in vivo in intestinal stem cells. Nat Genet *40*, 915-920.

Santos, A.J.M., Lo, Y.H., Mah, A.T., and Kuo, C.J. (2018). The intestinal stem cell niche: Homeostasis and adaptations. Trends Cell Biol *28*, 1062-1078.

Sasaki, N., Sachs, N., Wiebrands, K., Ellenbroek, S.I., Fumagalli, A., Lyubimova, A., Begthel, H., van den Born, M., van Es, J.H., Karthaus, W.R.*, et al.* (2016). Reg4+ deep crypt secretory cells function as epithelial niche for Lgr5+ stem cells in colon. Proc Natl Acad Sci USA *113*, E5399-5407.

Sato, T., Vries, R.G., Snippert, H.J., van de Wetering, M., Barker, N., Stange, D.E., van Es, J.H., Abo, A., Kujala, P., Peters, P.J.*, et al.* (2009). Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature *459*, 262-265.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B.*, et al.* (2012). Fiji: an open-source platform for biological-image analysis. Nat Methods *9*, 676-682.

Schmitt, M., Schewe, M., Sacchetti, A., Feijtel, D., van de Geer, W.S., Teeuwssen, M., Sleddens, H.F., Joosten, R., van Royen, M.E., van de Werken, H.J.G.*, et al.* (2018). Paneth cells respond to inflammation and contribute to tissue regeneration by acquiring stem-like features through SCF/c-Kit signaling. Cell Rep *24*, 2312-2328 e2317.

Schuijers, J., Junker, J.P., Mokry, M., Hatzis, P., Koo, B.K., Sasselli, V., van der Flier, L.G., Cuppen, E., van Oudenaarden, A., and Clevers, H. (2015). Ascl2 acts as an R-spondin/Wnt-responsive switch to control stemness in intestinal crypts. Cell Stem Cell *16*, 158-170.

Shen, L., Shao, N.Y., Liu, X., Maze, I., Feng, J., and Nestler, E.J. (2013). diffReps: detecting differential chromatin modification sites from ChIP-seq data with biological replicates. PLoS One *8*, e65598.

Shroyer, N.F., Helmrath, M.A., Wang, V.Y., Antalffy, B., Henning, S.J., and Zoghbi, H.Y. (2007). Intestine-specific ablation of mouse atonal homolog 1 (Math1) reveals a role in cellular homeostasis. Gastroenterology *132*, 2478-2488.

Snippert, H.J., Schepers, A.G., van Es, J.H., Simons, B.D., and Clevers, H. (2014). Biased competition between Lgr5 intestinal stem cells driven by oncogenic mutations induces clonal expansion. EMBO Rep *15*, 62-69.

Snippert, H.J., van der Flier, L.G., Sato, T., van Es, J.H., van den Born, M., Kroon-Veenboer, C., Barker, N., Klein, A.M., van Rheenen, J., Simons, B.D.*, et al.* (2010). Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. Cell *143*, 134-144.

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.

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*.

Tian, H., Biehs, 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.

Tomic, G., Morrissey, E., Kozar, S., Ben-Moshe, S., Hoyle, A., Azzarelli, R., Kemp, R., Chilamakuri, C.S.R., Itzkovitz, S., Philpott, A.*, et al.* (2018). Phospho-regulation of ATOH1 Is required for plasticity of secretory progenitors and tissue regeneration. Cell Stem Cell *23*, 436-443 e437.

Trapnell, C., Cacchiarelli, D., Grimsby, J., Pokharel, P., Li, S., Morse, M., Lennon, N.J., Livak, K.J., Mikkelsen, T.S., and Rinn, J.L. (2014). The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. Nat Biotechnol *32*, 381-386.

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, I.*, et al.* (2009). Transcription factor achaete scute-like 2 controls intestinal stem cell fate. Cell *136*, 903-912.

van Es, J.H., Sato, 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.

Vermeulen, L., Morrissey, E., van der Heijden, M., Nicholson, A.M., Sottoriva, A., Buczacki, S., Kemp, R., Tavare, S., and Winton, D.J. (2013). Defining stem cell dynamics in models of intestinal tumor initiation. Science *342*, 995-998.

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.

Yan, K.S., Gevaert, O., Zheng, G.X.Y., Anchang, B., Probert, C.S., Larkin, K.A., Davies, P.S., Cheng, Z.F., Kaddis, J.S., Han, A.*, et al.* (2017). Intestinal enteroendocrine lineage cells possess homeostatic and injury-inducible stem cell activity. Cell Stem Cell *21*, 78-90.

Yousefi, M., Li, L., and Lengner, C.J. (2017). Hierarchy and plasticity in the intestinal stem cell compartment. Trends Cell Biol *27*, 753-764.

Yu, S., Tong, K., Zhao, Y., Balasubramanian, I., Yap, G.S., Ferraris, R.P., Bonder, E.M., Verzi, M.P., and Gao, N. (2018). Paneth cell multipotency induced by Notch activation following injury. Cell Stem Cell *23*, 46-59 e45.

Yui, S., Azzolin, L., Maimets, M., Pedersen, M.T., Fordham, R.P., Hansen, S.L., Larsen, H.L., Guiu, J., Alves, M.R.P., Rundsten, C.F.*, et al.* (2018). YAP/TAZ-Dependent Reprogramming of Colonic Epithelium Links ECM Remodeling to Tissue Regeneration. Cell Stem Cell *22*, 35-49.

Zhang, Y., Liu, T., Meyer, C.A., Eeckhoute, J., Johnson, D.S., Bernstein, B.E., Nussbaum, C., Myers, R.M., Brown, M., Li, W.*, et al.* (2008). Model-based analysis of ChIP-Seq (MACS). Genome biology *9*, R137.

Zhou, W.J., Geng, Z.H., Spence, J.R., and Geng, J.G. (2013). Induction of intestinal stem cells by R-spondin 1 and Slit2 augments chemoradioprotection. Nature *501*, 107-111.

FIGURE LEGENDS

Figure 1. ISC regeneration by enterocyte and secretory progenitors.

A) Schema for ISC regeneration in *Lgr5$^{GFP\text{-}Cre\text{-}ER(T2)}$;R26R^{tdTom}* mice. Lgr5$^+$ ISC were ablated by γ-irradiation 4 days after induction of Cre to track tdTom$^+$ progeny 6 days thereafter. If ISC regenerate from those progeny, then freshly restored GFP$^+$ ISC will carry the tdTom label. If ISC are restored from a separate reserve pool, those ISC will express GFP but not tdTom.

B-C) Representative micrographs (B) and quantitation (C) of crypts containing restored tdTom$^+$ and tdTom$^-$GFP$^+$ ISC in the duodenum and colon 6 days after γ-irradiation. Scale bars, 50 μm. Bar graphs represent the tdTom$^+$ fraction (means ±SD) in all (hundreds in each of N=5 mice) GFP$^+$ crypts.

D) Two-color (GFP and tdTom) flow cytometry of duodenal crypt cells isolated from TAM-treated *Lgr5$^{GFP\text{-}Cre}$;R26R^{tdTom}* mice 6 days after γ-irradiation (N=2 animals). tdTom$^+$ cells represent GFP$^+$ ISC (>99% express tdTom) and their labeled GFP$^-$ progeny.

E-F) Experimental schemes and results of ISC restoration in DT-treated *Lgr5$^{Dtr\text{-}GFP}$* mice by Ent cells when the Sec lineage is absent (*Atoh1$^{-/-}$*, **E**) or by Sec cells when the Ent lineage is depleted (*Rbpj$^{-/-}$*, **F**). Tamoxifen (TAM) was administered to delete genes and DT was given to ablate ISC. Every GFP$^+$ crypt was counted on the indicated days in *Atoh1$^{-/-}$* (N=6) or *Rbpj$^{-/-}$* (N=4) intestines and equal numbers of controls. Within the plots, boxes demarcate quartiles 1 and 3, bars represent median values, whiskers represent 1.5 times the inter-quartile range, and differences were assessed using Student's t-test. Dotted white lines in representative micrographs outline selected crypts. Scale bars, 50 μm.

See also Figure S1.

Figure 2. *Ascl2* expression and perturbation.

A) mCh$^+$ cell positions in *Ascl2^{Dfci}* mouse intestines relative to the bottom of colonic crypts (tier 0; positions 1 and 1', 2 and 2', etc. correspond to higher tiers). Results are quantified below as means ±SD (N=4 mice).

B) Confocal micrographs of mCh and GFP co-localization in the duodenum and colon of strains with mosaic (*Lgr5$^{GFP\text{-}Cre}$*) and non-mosaic (*Lgr5$^{Dtr\text{-}GFP}$*) GFP$^+$ ISC. Results represent findings in at least 5 mice of each genotype. Scale bars, 50 μm. Dotted white lines outline single crypts.

C) Duodenal crypt cell proliferation was largely intact after *Ascl2* deletion by *Villin-Cre$^{ER\text{-}T2}$* (N=3 mice of each genotype).

D) Counts of all GFP$^+$ crypts in every microscopic field in *Ascl2*$^{+/Fl}$ and *Ascl2*$^{Fl/Fl}$ intestines (N=5 mice each) on the *Lgr5*$^{GFP\text{-}Cre}$ background, 7 days after the first TAM dose. Differences in C and D were assessed by Student's t-test.
E) Flow cytometry confirmed persistence of duodenal GFP$^+$ ISC in *Lgr5*$^{GFP\text{-}Cre}$*;Ascl2*$^{Fl/Fl}$ mice 7 days after TAM treatment. Data are shown for 1 pair from N=2 mice of each genotype.
F) In Cre-activated crypts, *Lgr*$^{GFP\text{-}Cre}$*;Ascl2*$^{Fl/Fl}$ mice lacked mCh expression, which was readily evident in neighboring Cre$^-$ (GFP$^-$) crypts. Dashes outline single crypts, scale bar =50 μm.
See also Figures S1 and S2.

Figure 3. Reduced fitness of *Ascl2*$^{-/-}$ ISC.
A) Early (5 days) and late (28 days and 112 days) crypt composition after *Ascl2* deletion in *Lgr5*$^{GFP\text{-}Cre}$*;R26R*^{tdTom} intestines. All GFP$^+$ crypts on this mosaic background showed tdTom expression 5 days after the first dose of TAM, but the proportion of tdTom$^+$ GFP$^+$ crypts (red arrow) was reduced by 28 days (N=2 animals) and substantially so by 112 days (N=5 mice), when large fractions of duodenal and colonic GFP$^+$ ISC lacked tdTom (green arrow). Bar graphs depict mean counts $\pm$SD and differences were evaluated by Student's t-test. Thus, Lgr5$^+$ cells that escaped *Ascl2* and *R26R* recombination (tdTom$^-$) are selected over *Ascl2*-null (tdTom$^+$) ISC. Scale bars, 50 μm.
B) Genotyping of GFP$^+$ ISC purified by flow cytometry 5 days and 28 days after TAM treatment shows presence of recombined (*Ascl2*-null) and parental (unrecombined, 'escaper') alleles. The genotyping strategy is shown in Fig. S1G-H.
C) High-magnification micrographs showing the presence of tdTom$^-$ (green arrowheads) and tdTom$^+$ (yellow arrowheads) GFP$^+$ ISC in the same crypt. Such mixed crypts were rare (<15 per animal). tdTom$^+$GFP$^-$ cells are Paneth cells arising from tdTom$^+$ ISC that previously contributed to this representative crypt.
D) Population genetics model for ISC dynamics (see STAR Methods). *N* number of ISC are arranged on the cycle graph, representing crypts that contain infrequent tdTom$^-$ 'escaper' ISC (0, wild-type) amid mostly recombined tdTom$^+$ ISC (1, *Ascl2*$^{-/-}$). During each time step of the stochastic model, we first sample an exponential waiting time for each ISC with rates λ_{WT} and λ_{Mut} for WT and mutant ISC, respectively. The smallest waiting time defines which cell divides first. Thereafter, one resulting daughter takes the parent's spot, while the other usurps one of the two neighboring cells, each with probability 0.5. This cycle repeats over many cell divisions until crypts carry predominantly tdTom$^+$ or tdTom$^-$ ISC, i.e., fixation, which was evident in mice examined 28 and 112 days after gene excision (Fig. 3A).

E) Density plot of estimated *Ascl2*$^{-/-}$ fitness (λ_{Mut}) values relative to wild-type duodenal or colonic ISC (λ_{WT}), determined using simulations for different parameter regimes (see Figure S3C).
See also Figures S1 and S3.

Figure 4. Ascl2 requirement in ISC restoration after injury.
A) Test of *Ascl2* requirements in ISC regeneration after 10 Gy γ-irradiation of *Lgr5*$^{GFP\text{-}Cre}$ mice. GFP^{+} ISC failed to regenerate in the absence of ASCL2. Graph depicts survival of unirradiated mice with *Ascl2*$^{-/-}$ intestines and mice with *Ascl2*$^{+/+}$ or *Ascl2*$^{-/-}$ intestines after irradiation (N=5 animals per cohort). Differences were assessed by the log-rank test.
B) *Ascl2* requirement for ISC restoration after *Lgr5*$^{Dtr\text{-}GFP}$ mice were treated with DT. In mice harvested on day 11 of the study, all GFP^{+} crypts were counted in every microscopic field in experimental (*Ascl2*$^{-/-}$) and two groups of control (*Ascl2*$^{+/+}$ and no DT treatment) intestines (N=4 mice per cohort) harvested on day 11. Boxes: quartiles 1 and 3, bars: median values, whiskers: 1.5 times the inter-quartile range. Differences assessed using Student's t-test. Fluorescence micrographs representing 4 experimental pairs and flow cytometry data representing 1 of N=2 experimental pairs demonstrate the paucity of duodenal GFP^{+} ISC in *Ascl2*$^{-/-}$ intestines.
C-D) Representative fluorescence micrographs (**C**, dotted white lines outline selected crypts) and histology (**D**, hematoxylin & eosin stain) from intestines harvested from 2 mice on day 14, showing absence of GFP^{+} crypt base cells and distorted tissue morphology, which was patchy. Scale bars, 50 μm.
See also Figure S4.

Figure 5. *Ascl2* expression in colonic non-stem crypt cells after ISC ablation.
A) *Lgr5*$^{Dtr\text{-}GFP}$ ISC were ablated by administering DT, followed by colon harvests on days 7, 8, 9, and 10. mCh first appeared on day 8 in cells located well above the ISC zone, in positions never occupied by mCh^{+} cells in the absence of ISC injury (see Fig. 2A-B). Each subsequent day, mCh^{+} cells appeared in positions closer to the crypt base, and by day 10, many of these cells acquired GFP expression (arrowhead in inset micrograph). Scale bars, 50 μm.
B) Illustrative tier positions and manual counts of mCh^{+} cells (red) relative to the crypt bottom on days 8 (N=5 mice) and 10 (N=3 mice). The green line (from Fig. 2A) depicts mCh^{+} cell positions in untreated animals (N=4 mice).
C) Flow cytometry confirmed that mCh^{+} crypt cells on day 8 lack GFP and give higher mCh signals than resting ISC (additional examples and comparative statistics shown in Fig. S5B).
D) Organoid formation *in vitro* by single GFP^{-} cells (mCh^{+} or mCh^{-}) captured by flow cytometry from the same animals (N=3) on day 8. mCh^{+} cells formed organoids more efficiently than mCh^{-}

cells or native stem cells (mCh^+GFP^+ from uninjured mice). Relative organoid numbers (yellow, red bars) are expressed in relation to those cultured from resting ISC (green bar).
E-G) Differential gene expression in uninjured (resting) colonic ISC and regenerating day 8 mCh^+ 'upper' crypt cells, determined by DEseq2 analysis of RNA-seq data. Overall differences (>$log_2$1.5-fold, q <0.05) were limited to 316 genes, most of which increased in regenerating cells (**F**). Reads per kb per million sequence tags (RPKM) values from replicate samples show that the regenerating 'upper' cell population lacked *Lgr5* but expressed many ISC-specific mRNAs (**E**) and that markers attributed to +4 'reserve' ISC were absent or equally expressed in resting and regenerating colonic ISC (**G**). *Gapdh*, *Tbp* confirm proper normalization of RNA-seq data.
See also Figure S5.

Figure 6. Transcriptomics of ISC restoration at single-cell resolution.
A) Uniform manifold Approximation and Projection (UMAP) plots from RNA analysis of single mCh^+ regenerating 'upper' cells, showing the paucity of Lgr5 expression compared to *Ascl2* and other ISC markers, including. In nearest-neighbor depiction, $Lgr5^-$ ISC-like cells cluster together. Expression scales are different for each marker.
B) Within the same UMAP-specified cell groups: Top – Distributions of actively cycling and non-replicating cells. Bottom – Expression of +4 'reserve' ISC marker genes *Bmi1* and *Clu*.
C) Projection of colonocyte (*Fabp2*, *Car1*) and goblet cell (*Tff3*, *Muc2*) markers on the same UMAP plot reveals that non-cycling mCh^+ 'upper' cells are similar to mature epithelial cells.
D) Hundreds of cells that cluster at the junctions of mature and ISC-like cells co-express ISC and either colonocyte (e.g., *Fabp2*) or goblet cell (e.g., *Muc2*) markers. In the dedifferentiation context, we therefore consider them *bona fide* transitional (Trans) cells.
E) Analysis of scRNA-seq data in Monocle v2.12.0 (Trapnell et al., 2014). Left: Depiction of cells along the defined trajectory, color-coded according to categories defined in UMAP analysis by expression of cell-specific marker genes. Inset: ISC, goblet cells, and colonocytes projected separately on the trajectory. Right: Cells are color-coded according to their imputed pseudotime, with high ISC marker-expressing cells as the destination.
F) Colonocyte (*Car1*, *Fabp2*), ISC (*Cdca7*), and goblet cell (*Spdef*, *Muc2*) marker expression in mature and transitional (Trans) plotted along the above-defined pseudotime axis. Fall of mature cell, and rise of ISC, markers occurs faster in dedifferentiating colonocytes than in goblet cells. In this interpretation, Trans colonocytes are fewer than Trans goblet cells because the latter dedifferentiate over a longer period.
See also Figure S6.

Figure 7. Transcriptional targets of ASCL2 in ISC regeneration.

A) Fraction of ASCL2^{Flag} ChIP-seq peaks (Suppl. Table 3) present <1 kb (designated promoters) or >1 kb (presumptive enhancers) from TSSs in regenerating Ascl2^{+} cells. The graph shows the relation between 316 genes differentially expressed in 'upper' cells (compared to resting ISC – Suppl. Table 1) and relative ASCL2^{Flag} ChIP-seq signals at their respective promoters in the two populations. Dashed lines point to candidate ASCL2 target genes that encode signaling factors.

B) Integrated Genome Viewer (IGV) tracks of RNA-seq and ASCL2^{Flag} ChIP-seq data from uninjured colonic ISC and regenerating day 8 mCh^{+} crypt cells, showing differential ASCL2 occupancy at the *Il11ra1* promoter and reproducibly increased *Il11ra1* mRNA in the latter cells.

C) Projection of *Il11ra1* mRNA levels onto the tSNE plot from single regenerating cells (Fig. 6), showing its broad distribution and particular enrichment in the goblet cell fraction.

D) Phosphorylated (p) STAT3 immunofluorescence, showing its presence in rare crypts ($\leq$10 per colon, N=5 mice) after ISC ablation, compared to absence of pSTAT3 (0 crypts/colon, N=5 mice) in the absence of ISC injury.

E) Response of GFP^{-} mCh^{+} 'upper' cells, mCh^{-} crypt cells post-ISC ablation, and uninjured mCh^{+} resting ISC to recombinant IL-11 in organoid formation (N=4 mice). Scale bars, 1 mm. Bars in the graph represent mean ($\pm$SD) ratios of organoids generated with or without rIL-11. Relative organoid numbers in IL11-treated cultures are expressed in relation to those that each population yielded in WENR medium without IL11.

F) Representative structures derived at the first passage of mCh^{+} 'upper' crypt cells initially cultured with rIL-11, indicating that they contained ISC.

See also Figure S7.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. ISC ablation by γ-irradiation and generation of *Ascl2^{Dfci}* mice. (Related to Figs. 1 and 2)

A) Ablation of native Lgr5/GFP^{+} ISC after 10 Gy γ-irradiation. GFP^{+} cells are absent at 24 h.

B) Representative micrograph of crypt recovery in ISC-depleted *Rbpj$^{-/-}$* small intestine.

C) Soon after DT-mediated ablation of ISC in *Lgr5$^{Dtr\text{-}GFP}$* mice, CD69^{+}CD274^{+} goblet (GOB)-cell precursors open sites in the *Ascl2* locus (ATAC-seq) and activate transcription (RNA-seq).

D) Targeting strategy for the *Ascl2^{Dfci}* allele. We replaced the coding region in all 3 exons (Ex) with *LoxP* cassettes flanking an *Ascl2* cDNA with a 5' triple-FLAG epitope and a 3' *mCherry* cDNA separated from *Ascl2* by a P2A cleavage sequence. A Neomycin selection cassette contained 2 *Frt* sites and was excised by crossing with mice that expressed FLP recombinase.

E) Fluorescence micrograph revealing mCh expression at the bottom of all duodenal crypts.

F) mCh fluorescence in *Ascl2^{Dfci}* mice is higher in colonic than in small intestine crypts.

G) Design of the *Ascl2^{Umc}* allele (van der Flier et al., 2009). Positions of genotyping primers are shown.

H) Sizes of fragments expected in PCR genotyping for *Dfci* and *Umc* alleles before (Unrecomb.) and after (Recombined) Cre-mediated excision of the floxed cassette.

Figure S2. Limited *Ascl2* dependence in resting intestinal epithelium. (Related to Fig. 2)

A) One week after treatment of *Ascl2$^{Fl/Fl}$;Villin-Cre$^{ER(T2)}$* mice with tamoxifen (TAM), histology of resting *Ascl2$^{-/-}$* intestines was normal and indistinguishable from *Ascl2$^{+/+}$* controls. N=3 mice of each genotype.

B) Absence of appreciable ISC apoptosis in *Ascl2$^{Fl/Fl}$;Lgr5$^{GFP\text{-}Cre}$* mouse intestines. Irradiated mouse intestine provided a positive control for cleaved Caspase3 immunostaining.

C) Lineage tracing by the *R26R^{Tom}* reporter in *Ascl2$^{Fl/Fl}$*;*Lgr5$^{GFP\text{-}Cre}$*;*R26^{tdTomato}* mice using the indicated regimen of TAM treatment (Cre activation). Tissue harvest on successive days revealed intact ISC activity, reflected in colonization of villi by cells derived from *Ascl2$^{-/-}$* ISC. N=5 mice of each genotype.

D) Flow cytometry confirmed paucity of duodenal mCh^{+} ISC in *Villin-Cre;Ascl2$^{Fl/Fl}$* mice 7 days after TAM treatment. N=3 mice of each genotype; data are shown for 1 pair.

E) IGV tracks of RNA-seq data, showing absence of transcripts from the deleted *Ascl2* exons in Lgr5^{+} ISC isolated from 2 independent animals.

F) PCR genotyping for *Ascl2* locus recombination shows efficient Villin-Cre$^{ER\text{-}T2}$ deletion of the floxed cassette in both alleles, *Dfci* and *Umc*. Genotyping strategy illustrated in Fig. S1D, G-H.

G) FACS plots from isolation of mCh$^+$ wild-type (top) and GFP$^+$ *Ascl2*$^{-/-}$ colonic ISC (bottom, from *Ascl2*$^{Fl/Fl}$;*Lgr5*$^{GFP\text{-}Cre}$ mice) and volcano plot of 3,502 differentially expressed genes (>2-fold, *q* <0.01, Suppl. Table 1). N=2 mice of each genotype. IGV tracks from RNA-seq data of selected examples are shown to the right.

Figure S3. Mathematical modeling for relative *Ascl2*$^{-/-}$ ISC fitness. (Related to Fig. 3)
A,B) Example plots from simulations of duodenal (A) and colonic (B) crypt dynamics (STAR methods), comparing fractions of crypts with predominantly tdTom$^+$ ISC (GFP$^+$ cells) in the simulations with those observed in mice. X-axis represents time in days, y-axis represents the fraction of crypts with predominantly tdTom$^+$ ISC. Vertical lines at 5, 28, and 112 days denote the days on which crypts were counted. Red (duodenum) and purple (colon) dots represent data from each mouse, triangles represent the means of these data points. Trajectories show the evolution over time (averaged over 1,000 simulations) of the fraction of predominantly tdTom$^+$ crypts under different definitions of *M*/*N* predominance (fractions of *Ascl2*$^{-/-}$ mutant ISC). Values tested range from over 50% to over 90% (for fitting values, we required >70%). Average point trajectories were stable when comparing different sets of 1,000 simulations each.
C) Table summarizing relative fitness estimates for *Ascl2*$^{-/-}$ ISC in different parameter regimes. *N*, number of ISC on the cycle graph; *M*, number of initial tdTom$^+$ (*Ascl2*$^{-/-}$, mutant) ISC; λ_{WT}, growth rate of WT (tdTom$^-$); λ_{Mut}, growth rate of mutant (predominantly tdTom$^+$) ISC. For some simulations we chose *M* from a distribution rather than fixing it deterministically, where d_1 =11 with probability (P) 0.9 and 12 with P 0.1; d_2 = 10 with P 0.4, 11 with P 0.4, and 12 with P 0.2; and d_3 = 10 with P 0.3, 11 with P 0.6, and 12 with P 0.1.

Figure S4. *Ascl2* is required to restore ISC after lethal injuries. (Related to Fig. 4)
A) Crypt cell proliferation (Ki67 immunostaining) and additional example of abnormal tissue morphology (hematoxylin stain), representing *Ascl2*$^{Fl/Fl}$;*Lgr5*$^{Dtr\text{-}GFP}$;*Villin-Cre* mouse intestines (N=2) harvested 14 days after the start of ISC ablation. Scale bars, 50 μm. Ki67$^+$ cells were enumerated in 200 individual crypts and differences were assessed by Student's t-test.
B-C) Lack of ISC regeneration in *Ascl2*$^{-/-}$ crypts in all regions of the intestine. *Ascl2*$^{Fl/Fl}$ was introduced separately on the *Lgr5*$^{GFP\text{-}Cre}$ (**B**) or *Lgr5*$^{Dtr\text{-}GFP}$;*Villin-Cre*$^{ER(T2)}$ (**C**) background, followed by CRE activation, ablation of native ISC using γ-irradiation (**B**) or DT (**C**), and tissue harvest. Restored GFP$^+$ ISC were absent in *Ascl2*-null crypts. N=4 (**B**) or 5 (**C**) mice and equal numbers of each control. Dotted white lines outline selected crypts.

Figure S5. Properties of regenerating ASCL2$^+$ crypt 'upper' cells. (Related to Fig. 5)

A-B) Representative flow cytometry plots showing that resting uninjured colonic ISC express both mCh and GFP, whereas regenerating 'upper' cells express mCh but not GFP. The graph (B) quantifies these differences using Student's t-test.
C) Structures generated by mCh^+ GFP^- regenerating crypt cells are epithelial and could be passaged, implying that they contain ISC. Organoids originating in FACS-sorted single 'upper' mCh^+ cells were cultured for 10 days (hematoxylin & eosin stains revealed an epithelial lining), then mechanistically disaggregated, and transferred to new WENR cultures.
D) Most of the 485 genes previously shown by strict criteria to be enriched in small intestine ISC (Munoz et al, 2012) are also expressed in colonic $Lgr5^+$ ISC.
E) Absence of CLU immunostaining (green signals) in *Ascl2*$^{Dfci/Umc}$*;Lgr5*$^{Dtr\text{-}GFP}$*;Villin-Cre*$^{ER(T2)}$ mouse intestines 8 days after the start of ISC ablation by DT, when dedifferentiating mCh^+ cells were evident in upper crypt tiers. Normal mouse stomach serves as a positive control.
F) Genes expressed during colonic epithelial development (E, mouse embryonic day) and down-regulated or silenced in the adult tissue are not materially reactivated in regenerating mCh^+ colonic 'upper' cells.
G) The 183 fetal transcripts detected in regenerating cells overlap minimally with enterosphere-enriched genes. Moreover, the latter genes are comparably expressed in isolated $Lgr5^+$ resting and $Ascl2^+$ regenerating cells.
H) RPKM values from replicate samples show that resting ISC and regenerating cell populations express nearly equal levels of marker genes attributed to YAP/TAZ signaling (Gregorieff et al., 2015).

Figure S6. Single-cell transcriptomics of colonic crypt cell dedifferentiation. (Related to Fig. 6)
A) Cell density plots for stringency filters applied to scRNA-seq data, which restricted our further analysis to 3,254 informative cells: >4,000 unique molecular identifiers (UMIs) per cell, >1,000 genes detected per cell, and mitochondrial ratio <0.12 per cell.
B) Projection of *Chga* and *Pyy* expression levels onto the UMAP plot identified a tiny population of EE cells.
C) Evaluation at single-cell resolution of ideas that stem cell restoration recapitulates YAP/TAZ-mediated fetal transcription. Expression levels of key signature genes from published studies, including those common to 2 or more studies, are projected onto the UMAP plot of mCh^+ 'upper' cells. UMAP layout is shown on the top right, with transitional (Trans) cell populations colored.

Figure S7. Candidate ASCL2 target genes nominated by differential mRNA expression and promoter ASCL2 occupancy in regenerating mCh$^+$ 'upper' cells. (Related to Fig. 7)
A) In addition to *Il11ra1* (Fig. 7), *Fgf18*, *Nov*, and *Fzd9* transcript levels were increased and their promoters showed differential ASCL2 occupancy in regenerating mCh$^+$ crypt cells. IGV tracks for *Tbp* mRNA shows good normalization across samples.
B) Addition of recombinant NOV or FGF18 to crypt cell cultures initiated by single mCh$^+$ 'upper' cells did not enhance organoid formation (N=3 experiments each). Relative organoid numbers in factor-supplemented cultures are expressed in relation to those that cells yielded in WENR medium without additional factors. Differences were evaluated by Student's t-test.
C) Fractions of IL11RA$^+$ regenerating mCh$^+$ 'upper' cells (red) or resting colonic *Lgr5*$^{Dtr\text{-}GFP}$ ISC (green), determined by flow cytometry with IL11RA antibody in 3 independent isolates of each cell population.
D) Representative structures derived at the second passage of mCh$^+$ 'upper' crypt cells initially cultured in WENR + rIL-11, indicating that ISC were expanded in the original culture.

STAR METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Requests for reagents should be directed to and will be fulfilled by the Lead Contact, Ramesh Shivdasani (ramesh_shivdasani@dfci.harvard.edu), after execution of a suitable Materials Transfer Agreement.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Animals. All mouse strains (*Lgr5*$^{GFP\text{-}Cre}$, *Lgr5*$^{Dtr\text{-}GFP}$, *R26R*^{tdTomato}, *Villin-Cre*$^{ER\text{-}T2}$, *Atoh1*^{Fl}, *Rbpj*^{Fl}, *Ascl2*^{Dfci}, *Ascl2*^{Umc}, and *Apc*^{Fl}) were maintained on a predominantly C57Bl/6 background. Mouse sources and citations are provided in the Key Resources Table. Animals were housed under specific pathogen-free conditions in 12-hour light/dark cycles at 23 ± 1^{o}C and humidity 55 ± 15%. Food and water were provided *ad libitum*. Animals were weaned 21 to 28 days after birth and handled and euthanized according to procedures approved by the Animal Care and Use Committee of the Dana-Farber Cancer Institute. Mice were at least 8 weeks old at the time of experiments and cell isolations. Mice of the both sexes were used in most experiments, with littermate controls.

To generate *Ascl2*^{Dfci} mice, the two *Ascl2* homology arms and 3XFLAG-tagged *Ascl2* cDNA were cloned by high-fidelity PCR from C57BL/6 genomic DNA. mCherry cDNA was extracted from the plasmid pEF1alpha-mCherry (Takara, 631969) and all fragments were moved into the targeting vector (Fig. S1D). The targeting construct was verified by DNA sequencing, linearized, and electroporated into JM8.N4 male embryonic stem cells (ESC), which were selected in medium containing G418 and gancyclovir. Recombinant ESC clones were identified by long-range PCR across the *Ascl2* locus and injected into C57BL/6-albino blastocysts. Founder males, identified by PCR analysis of tail DNA, were bred to *B6.Cg-Tg(Pgk1-Flpo)10*^{Sykr}*/J* females (*FLPo-10*, Jackson Laboratories stock #011065) to delete the *Neo*^{R} cassette. As we expanded the *Ascl2*^{Dfci} colony by crossing with *Lgr5*$^{GFP\text{-}Cre}$, *Lgr5*$^{Dtr\text{-}GFP}$, or *Villin-Cre*$^{ER\text{-}T2}$ mice, mice carrying *FLPo-10* were excluded and we verified the whole *Ascl2*^{Dfci} locus by sequencing genomic DNA. Except where stated otherwise (e.g., Fig. 4), *Ascl2*$^{Fl/Fl}$ and *Ascl2*$^{-/-}$ refer to any combination of the *Ascl2*^{Dfci} and *Ascl2*^{Umc} alleles.

METHOD DETAILS

Mouse treatments. To delete floxed *Atoh1* alleles, we administered 1 mg tamoxifen (Sigma-Aldrich, T5648; stock solutions prepared in cornflower oil) by intraperitoneal (IP) injection daily for 5 consecutive days. To activate $CRE^{ER\text{-}T2}$ in $Lgr5^{GFP\text{-}Cre}$;$R26R^{tdTomato}$ mice, we administered 2 mg tamoxifen IP on days 1 and 2. To delete floxed *Rbpj*, *Ascl2* and/or *Apc* alleles, we injected 2 mg tamoxifen IP on the 1st and 2nd days and 1 mg tamoxifen on the 3rd day. $Lgr5^{GFP\text{-}Cre}$; $R26R^{tdTomato}$ and $Ascl2^{Fl/Fl}$;$Lgr5^{GFP\text{-}Cre}$ mice received 10 Gy whole body γ-irradiation (^{137}Cs source) 96 h or 48 h, respectively, after the first dose of tamoxifen. To ablate ISC in $Lgr5^{Dtr\text{-}GFP}$ mice, we administered Diphtheria toxin (Sigma-Aldrich, 50 µg/kg) by IP injection 4 times on alternate days.

Detection of fluorescent cells in tissues. To detect mCh^+, GFP^+, and $tdTomato^+$ cells, we fixed mouse small and large intestines in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4°C overnight with gentle agitation, washed the specimens 3 times in PBS at 4°C, and rotated them overnight at 4°C in 30% sucrose in PBS. Tissues were embedded in OCT compound (Tissue-Tek, 4583) and stored at -80°C. Tissue sections (8 µm) adhered onto glass slides were washed in PBS and mounted in medium containing 4′,6-diamidino-2-phenyl-indole (DAPI – Vector Laboratories, H-1200). Fluorescent cells were visualized and counted using a spinning disc confocal microscope (Yokogawa). Images were analyzed by Fiji software (Schindelin et al., 2012). In experiments with $GFP^+Tomato^+$ cells, GFP^+ cells were counted first and then examined for $Tomato^+$ in the red channel to determine if they were double positive.

Immunofluorescence. Intestinal or gastric tissue sections (5-7 µm thick) were incubated overnight with CLU (R&D Systems Inc, AF2747, 1:40), phospho-STAT3 (Cell Signaling, 9145, 1:100) or cleaved Caspase-3 (Cell Signaling, 9664, 1:100) antibodies (Ab) at 4°C, followed by Alexa Fluor 488- or Alexa Fluor 594-conjugated anti-mouse (Invitrogen, A-21202, 1:2,000) or anti-rabbit IgG (Invitrogen, A-11034 or A-11037, 1:2000) for 1 h at room temperature. To determine fractions of $IL11RA^+$ mCh^+ cells, we mixed IL11RA antibody (R&D Systems, AF490) with Alexa Fluor 488-conjugated anti-goat IgG (Invitrogen, A-11055) on ice for 1 h and incubated with crypt single-cell suspensions for 30 min on ice. Cells were washed 5 times with cold PBS and analyzed on a Sony SH800z flow cytometer.

Colonic cell isolation. Immediately after euthanasia of mice by CO_2 inhalation, the proximal colon was harvested and ~2-mm slices were cut with a razor blade. These slices were washed briefly in in, then rotated in 10 mM EDTA solution prepared in Dulbecco's Modified Eagle/F-12 medium (DMEM/F-12, Life Technologies, 12634010) conditioned by growth of Wnt3a (70%) and Rspo1 (30%) secreting cells for 30 min at room temperature with change of solution every 10 min. Wnt3a was generated from L-Wnt-3A cells (ATCC, CRL-2647) and Rspo1 from HA-R-Spondin1-Fc293T cells (Ootani et al., 2009). Crypt epithelium released by this treatment was washed in DMEM (Corning, 17-205-CV), then digested for 30 min at 37°C in a mixture of 2.5 mL Accumax (Innovative Cell Technologies, AM105), 2.5 mL Dispase (Stem Cell Technologies, 07913), 0.5 mL TrypLE (Thermo Fisher, A1217702), 1.5 mL Wnt3a- and 0.5 mL Rspo1-conditioned media, washed in PBS, and passed through 35 µm filters (Falcon, 352235) to prepare single-cell suspensions. Live mCh^+ or GFP^+ cells were sorted on a Sony SH800z flow cytometer, using far-red dye (Life Technologies, L10120) or SYTOX Blue (Life Technologies, S34857) to exclude dead cells. Data were analyzed using FlowJo software (BD Biosciences).

Culture and analysis of colonic organoids. Single colonic epithelial cells isolated by flow cytometry were cultured as described (Sato et al., 2009) in Matrigel droplets (BD Biosciences, 356231) supplemented with 1 µM Jagged-1 peptide (AnaSpec, 61298) and covered with 0.5 mL Wnt3a (70%) and R-spo1 (30%) conditioned medium supplemented with antibiotics, N-2 and B-27 supplements and rEGF as described (Sato et al., 2009). In various experiments, 10 µg/ml NOV (R&D Systems, 1976-NV-050), 10 ng/ml FGF18 (R&D Systems, 8988-F18-050), or IL-11 (Peprotech, 220-11, 0.1 µg, 0.2 µg or 0.4 µg) was added to the culture medium. For passaging, organoids were disaggregated manually by pipetting, then transferred to fresh Matrigel droplets for further culture as above. Organoids were visualized using a Celigo S Imaging Cytometer (Nexcelom) to capture at least 5 focal planes, then counted on the resulting images, ensuring that each structure was counted only once. For histology, organoids were fixed for 15 min in 4% PFA in PBS at room temperature, while still in Matrigel, then incubated in 5 U Dispase (Stem Cell Technologies, 07913) at 37°C for 30 min to remove Matrigel, and fixed overnight at 4°C in 4% PFA in PBS. Organoids were then embedded in OCT compound (Tissue-Tek, 4583), frozen at -80°C, and 8 µm sections were cut for staining with hematoxylin and eosin.

RNA isolation and sequencing. For studies on bulk cell populations, mCh^+ or GFP^+ cells were isolated by flow cytometry (Sony SH800z Cell Sorter) directly into Trizol reagent (ThermoFisher, 15596026). RNA was purified as recommended by the manufacturer, treated with Turbo DNase I (Life Technologies, AM1907) to eliminate DNA contaminants, and purified over RNeasy

columns (Qiagen, 74104). Total RNA (5 to 10 ng) was used to prepare libraries with SMARTer Universal Low Input RNA kits (Takara Bio, 634940). Libraries were sequenced on a NextSeq 500 instrument (Illumina) to obtain 75-bp single-end reads. For scRNA-seq, we purified mCh^+ ($Ascl2^+$) 'upper' cells from mouse colons 8 days after ISC ablation, using flow cytometry. About 10^4 cells were loaded onto Chromium Chip B using the 3' GEM Library & Gel Bead Kit v3 (10X Genomics), followed by reverse transcription, cDNA amplification, and library preparation according to the manufacturer's recommendations. Libraries were sequenced on a HiSeq4000 instrument (Illumina).

Chromatin immunoprecipitation (ChIP-seq). ChIP for FLAG-tagged ASCL2 on the typically small number of resting or regenerating ISC (<20,000 colonic ISC or mCh^+ 'upper' cells from *Ascl2*^{Dfci} mice) required special methods, including Tn5 transposition to isolate bound fragments. As a negative control, we performed ChIP on an equal number of GFP^+ colonic ISC (no FLAG epitope, from *Lgr5*$^{Dtr\text{-}GFP}$ mice). Cells were fixed in 1% formaldehyde in PBS for 10 min, followed by quenching with 0.125 M Glycine for 10 min, both at room temperature. Fixed cells were washed in cold lysis buffer (10 mM Tris HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% (v/v) Igepal CA-630) and incubated for 30 min on ice in ATAC-RSB^{++} buffer (10 mM Tris Hcl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% Igepal CA-630, 0.1% Tween-20, 0.01% Digitonin) (Corces et al., 2017). Cells were then washed twice in cold lysis buffer and incubated with Tn5 in 50 µL Transposition mix (Illumina, FC-121-1030) for 30 min at 37°C with agitation at 1,000 RPM (Thermomixer, Eppendorf). Reactions were stopped by adding 150 µL SDS buffer (50 mM Tris HCl pH 8, 10 mM EDTA, 1.35% SDS) on ice for 1 h. SDS was then diluted by adding 750 µL 2xChIP buffer (100 mM Tris HCl pH 8, 0.3 M NaCl, 2 mM EDTA, 1mM EGTA, 1% N-lauroylsarcosine, 2% TritonX-100) and 550 µL water. After rotation for 2 h at 4°C, cell debris were removed by centrifugation and 5 µg FLAG antibody M2 (Sigma-Aldrich, F1804) was added to the supernatant, with overnight rotation at 4°C. Antibody-associated chromatin was isolated with Protein G Dynabeads (Thermo Fisher, Q32854, 50 µL) for 4 h at 4°C, washed twice each with RIPA-LS (10 mM Tris HCl pH 8, 140 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.1% Na-Deoxycholate, 1% TritonX-100), RIPA-HS (10 mM Tris HCl pH 8, 500 mM NaCl, 1 mM EDTA pH, 0.1% SDS, 0.1% Na-Deoxycholate, 1% TritonX-100), RIPA-LiCl (10 mM Tris HCl pH 8, 250 mM LiCl, 1 mM EDTA, 0.1% Na-Deoxycholate, 0.5% Igepal CA-630), and TE buffer (10 mM Tris pH 8, 1 mM EDTA).

Immunoprecipitated chromatin was eluted in 100 µL elution buffer (50 mM Tris HCl pH 8, 10 mM EDTA, 1% SDS) and cross-links were reversed overnight at 65°C, followed by treatment

with 0.2 mg/mL RNase A in 100 µl TE buffer for 2 h at 37°C and with 0.2 mg/mL Proteinase K and 300 mM $CaCl_2$ for 1 h at 37°C. DNA was extracted and precipitated with phenol, chloroform, isopropanol, Na acetate, and linear acrylamide as a carrier. DNA was washed with 70% ethanol, resuspended in water, amplified over 16 cycles using high-fidelity 2X PCR Master Mix, and purified over columns (Qiagen, 28004). After removal of primer dimers (<100 bp) using AMPure beads (Beckman Coulter, A63881), the libraries were sequenced on a NextSeq 500 instrument (Illumina) to obtain 75-bp single-end reads.

QUANTIFICATION AND STATISTICAL ANALYSIS

General. For reproducible quantitation of crypts with GFP^+tdTom^+ or $GFP^+tdTomato^-$ cells, we assessed 5 independent wild-type intestines (Figs. 1C and 3A), 5 *Ascl2*$^{-/-}$ intestines at 112 days and 2 *Ascl2*$^{-/-}$ intestines at 28 days (Fig. 3A). Bar graphs in these panels represent mean ±SD of crypts containing GFP^+ ISC. To examine ISC maintenance and restoration, crypts with GFP^+ cells were counted in every microscopic field from 6 (Fig. 1E), 4 (Figs. 1F), 5 (Fig. 2D and Fig. 5B, 8 days) or 3 (Fig. 5B, 10 days) independent control and experimental (each) animals. All samples were inspected randomly and, when feasible, blindly. Within the dot plots (Figs. 1E-F, 2D and 4B), boxes demarcate quartiles 1 and 3, bars represent median values, whiskers represent 1.5 times the inter-quartile range, and differences were assessed using Student's t-test. We assessed post-irradiation lifespans in 5 independent *Ascl2*$^{-/-}$ and equal numbers of control mice (Fig. 4A), using the log-rank test in GraphPad Prism 7 to generate survival curves. ISC positions in colonic crypts were assessed in 4 independent mice (Fig. 2A) and 5 or 3 independent animals treated with DT for 8 or 10 days, respectively (Fig. 5B). Organoid cultures from single crypt cells were generated from a minimum of 3 mice for each condition (Figs. 5D, 7E, and S7B). Relative organoid numbers are expressed in relation to those that cells yielded in the corresponding control conditions; differences were evaluated by Student's t-test and error bars in graphs that depict relative organoid numbers (ratios) represent SDs. IL11RA expression was examined by flow cytometry in cells isolated from N=3 independent mice of each genotype.

Computational analyses. Raw reads from RNA- and ChIP-seq were aligned to the mouse genome (Mm10, Genome Reference Consortium GRCm38) using STAR aligner v2.53a (Dobin et al., 2013) for RNA or Bowtie2 (Langmead and Salzberg, 2012) for ChIP data. RNA-seq data quality was assessed using VIPER (Cornwell et al., 2018) and transcript levels were expressed as read counts using HTSeq (Anders et al., 2015). Sex chromosome-encoded and ribosomal

genes were removed for comparative analyses. Data were normalized, and sample variability assessed by principal component analysis in DEseq2 (Love et al., 2014). Expression values were represented as reads per kilobase per million sequence tags (RPKM) and differential expression was defined in DEseq2 using the indicated Log_2 fold-changes, false discovery rates (FDRs) and basemeans. Volcano plots of differentially expressed RNAs were generated using the ggplot2 package in R.

For ChIP-seq, we used MACS (Zhang et al., 2008) to align signals in raw (bam) files, then filtered to remove PCR duplicates and reads that aligned to multiple locations. Signals across samples were quantile normalized with Haystack (Pinello et al., 2014), using 50-bp windows across the genome. Sites bound to FLAG-ASCL2 in regenerating colonic crypt cells (enriched over the background in resting FLAG-negative *Lgr5*$^{Dtr\text{-}Egfp}$ ISC) were identified using diffReps (Shen et al., 2013), with z-score cut-off 5, window size 200 bp, step size 20, and p-value 0.0001. Raw signals from individual samples of a given cell type were converted to signal files (bigWig) using deepTools v2.1.0 (Ramirez et al., 2016). To impute candidate ASCL2 targets (Fig. 7A), we considered genes differentially expressed between resting ISC and mCh^+ 'upper' cells (q <10^{-5}, 2X change, n=316) and differential promoter ChIP-seq signals in regenerating cells compared to resting ISC. RNA and ChIP signals at individual loci were visualized using the Integrated Genomics Viewer v2.3 (Robinson et al., 2011).

Single-cell RNA-seq. A library with 190,971,192 raw reads was aligned to the GRCm38/mm10 mouse genome, and Cell Ranger v3.0.2 (10X Genomics) was used to estimate unique molecular identifiers (UMIs). Raw aligned features were loaded and processed using the Seurat v3.0.2 package (Butler et al., 2018) in R version 3.6.1 (Team, 2013). We retained only cells with $\geq$4,000 UMIs, $\geq$1,200 unique genes, <20% mitochondrial gene contribution, and expression novelty (log_{10} Genes per UMI) >0.8 (Fig. S5D). These 3,254 cells gave information on 14,737 genes.

Normalization, reduction of dimensionality, and clustering: Data were normalized in Seurat and variable genes were detected using Variance Stabilization transformation, followed by scaling and principal component (PC) analysis. The top 10 PCs were used to construct a Shared Nearest Neighbor graph and determine cell clusters, followed by reduction of dimensionality using the Uniform Manifold Approximation and Projection (UMAP) technique (McInnes et al, arXiv:1802.03426. Clusters co-expressing ISC (e.g. *Cdca7)* and colonocyte (e.g., *Fabp2*) or goblet cell (e.g., *Muc2*) markers were designated as transitional cells (Fig. 6C), while clusters with high lone expression of ISC or lineage markers were designated accordingly. Total

normalized counts for groups of known marker genes were used to determine cell cycle progression (*Plk1, Rrm2, Ccna2, Ccnb1, Ccnb2, Cdk1, Cdc25c, Cdca2, Cdc20, Ccne2, Cdc6, Cdc45*) or inhibition (*Cdkn2b, Cdkn2a, Cdkn1a, Cdkn1c*) (Fig. 6B).Normalized count data were used to determine relative expression levels of genes in defined cell populations (Fig. 6C – violin plots generated using the ggplot2 package in R).

Pseudotime analysis (Fig. 6D): Filtered data from Seurat were modeled in Monocle v2.12.0 (Trapnell et al., 2014) using the negative binomial distribution. After calculating size factors and dispersion using default parameters, differential genes were detected in the above-defined cell types using Seurat (cut-off q <0.01). Dimensionality reduction and trajectory reconstruction were done using the advanced nonlinear reconstruction algorithm DDRTree to determine two components (Mao et al., 2015). The branch of the trajectory plot with the most ISC was used as the end-state to determine pseudotime for all cells, then dedifferentiation dynamics of cell groups were analyzed using pseudotime and lineage marker expression.

Mathematical modeling to quantify relative fitness of *Ascl2*$^{-/-}$ ISC. We designed a simple mathematical model of ISC dynamics (Lopez-Garcia et al., 2010), based on a Moran model on the cycle graph with a fixed number (N) of ISC arranged in a ring (Fig. 3D). M is the initial number of *Ascl2*-recombined (mutant, tdTom$^+$) ISC and the remaining cells, $N-M$, escaped recombination (tdTom$^-$). Cells are placed uniformly at random on the cycle graph, so that each initial configuration is equally likely. We sampled an exponential waiting time for each ISC (i.e., each vertex in the graph), with rates λ_{WT} and λ_{Mut} for WT and mutant ISC, respectively (each defined to 2 decimal places), and the smallest waiting time determines which cell divides first. One daughter then takes the parent's spot and the other replaces one or the other flanking ISC, each with probability 0.5. We updated the configuration of cells and repeated this process until fixation, i.e., when all ISC were tdTom$^+$ or tdTom$^-$. Because 2D tissue sections miss infrequent tdTom$^-$ ISC (which must be there), we considered tdTom$^+$ crypts to be 'predominantly' tdTom$^+$, making predominance precise as a parameter of the model.

We implemented exact stochastic simulations of this process over a range of parameter regimes, fixing N between 10 and 14 (Snippert et al., 2010) for each regime; replacing this number with a distribution did not change the results significantly. We also explored a range of values for M, fixing it deterministically or sampling it according to various plausible distributions, and set λ_{WT} to 0.25 and 0.5 divisions/day in the colon and duodenum, respectively (Snippert et al., 2014). We then estimated λ_{Mut} by optimizing over λ_{Mut} values to fit the model to the data. Each set of parameters produced a plot of the form shown in Fig. S3A-B. At the start, all crypts

are predominantly tdTom^{+} (mutant) and the point trajectories show that fraction decreasing over time as wild-type ISC gradually exert their advantage. The lines eventually flatten and converge as crypts fixate as predominantly tdTom^{+} or tdTom^{-}. Raising mutant fitness would pull all point trajectories upward, so the optimization procedure amounts to identifying the λ_{Mut} value that minimizes the squared difference between predicted and observed results, for a fixed set of plausible parameters. We obtained an estimate for *Ascl2*$^{-/-}$ ISC disadvantage for each regime (examples in Fig. S3C).

DATA AND SOFTWARE AVAILABILITY

All RNA, ChIP, and ATAC data are deposited in the Gene Expression Omnibus (GEO), under accession number GSE130822.
Editors and reviewers may access the data using the token **cnutmmwulhchdgh**.