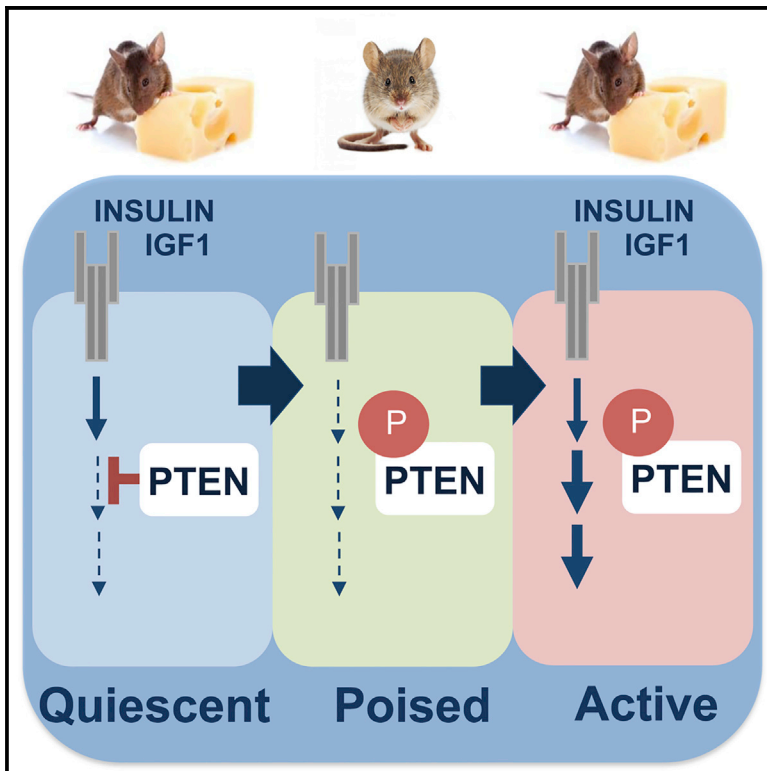# Cell Reports

Dormant Intestinal Stem Cells Are Regulated by PTEN and Nutritional Status

Graphical Abstract

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In Brief

Richmond et al. show that PTEN is required for dormant intestinal stem cell (d-ISC) maintenance and regeneration after intestinal injury. Changes in nutrient status (fasting) lead to transient PTEN inhibition, rendering d-ISCs functionally poised to contribute to regeneration upon re-feeding via cell-autonomous activation of the insulin/growth factor→PI3K→AKT→mTORC1 pathway.

Highlights

- Dormant stem cells (d-ISCs) contribute to intestinal regeneration after fasting

- Decreased nutrients lead to transient PTEN inhibition and increased d-ISC numbers

- Cell-autonomous activation of PI3K→AKT→mTORC1 signaling mediates d-ISC response

- PTEN is essential for d-ISC maintenance and intestinal regeneration

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Dormant Intestinal Stem Cells Are Regulated by PTEN and Nutritional Status

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SUMMARY

The cellular and molecular mechanisms underlying adaptive changes to physiological stress within the intestinal epithelium remain poorly understood. Here, we show that PTEN, a negative regulator of the PI3K→AKT→mTORC1-signaling pathway, is an important regulator of dormant intestinal stem cells (d-ISCs). Acute nutrient deprivation leads to transient PTEN phosphorylation within d-ISCs and a corresponding increase in their number. This release of PTEN inhibition renders d-ISCs functionally poised to contribute to the regenerative response during re-feeding via cell-autonomous activation of the PI3K→AKT→mTORC1 pathway. Consistent with its role in mediating cell survival, PTEN is required for d-ISC maintenance at baseline, and intestines lacking PTEN have diminished regenerative capacity after irradiation. Our results highlight a PTEN-dependent mechanism for d-ISC maintenance and further demonstrate the role of d-ISCs in the intestinal response to stress.

INTRODUCTION

The physiological response to fasting has had enormous selective pressure throughout evolution and has been linked to disease prevention and improved clinical outcomes for many conditions (Longo and Mattson, 2014). Additionally, clinicians have increasingly recognized the value of providing even small-volume enteral feeds to maintain and augment the recovery of the critically ill patient by preserving bowel mucosal integrity, thereby helping to prevent enteral-systemic bacterial translocation. Despite its relevance to human health, surprisingly little is known regarding how fasting affects metabolically dynamic and expensive tissues such as the gut. Some insight comes from animals that feed sporadically, such as snakes and hibernating mammals, which exhibit an astonishing capacity to modulate the intestinal mucosa in response to the presence or absence of enteral nutrition (Dunel-Erb et al., 2001; Secor et al., 1994). Similarly, humans and rodents that undergo periods of fasting or receive all their nutrition intravenously experience marked intestinal atrophy (Chappell et al., 2003). These findings underscore the requirement of luminal nutrition for intestinal maintenance and highlight an important energy-conserving mechanism. Under fed conditions, energy consumed by the intestinal mucosa represents ~15% of the total basal metabolic rate (Aiello and Wheeler, 1995), and after an extended fast, up to 25% of newly available calories are committed to restoring the intestinal mucosa (Secor et al., 1994), emphasizing the critical importance of intestinal homeostasis to the body. The cellular and molecular mechanisms underlying intestinal adaptation to fasting and re-feeding, however, remain poorly characterized. We propose that this process is regulated in part at the level of intestinal stem cells (ISCs).

Intestinal adaptation to fasting involves dramatic alterations in cell number, cycling frequency, and cell turnover, suggesting fundamental changes in stem/progenitor cell function (Dunel-Erb et al., 2001). In mice, a diverse array of ISC markers has been described and used to characterize this functionally heterogeneous population (Carlone and Breault, 2012; Goodell et al., 2015; Ritsma et al., 2014; Takeda et al., 2011; Tao et al., 2015; Yan et al., 2012). The spectrum of ISCs includes rapidly cycling crypt base columnar ISCs (CBC ISCs) marked by *Lgr5* expression and slowly cycling, relatively dormant ISCs (d-ISCs) marked by *mTert* (telomerase), *Bmi1*, *Lrig1*, *HopX*, and *Dclk1* (Barker et al., 2007; Montgomery et al., 2011; Powell et al., 2012; Sangiorgi and Capecchi, 2008; Takeda et al., 2011; Westphalen et al., 2014). CBC ISCs play a dominant role during daily intestinal maintenance and are sensitive to intestinal stress and injury (Barker et al., 2007; Carlone and Breault, 2012; Metcalfe et al., 2014; Ritsma et al., 2014). In contrast, d-ISCs, located in the "+4" supra-Paneth position, are resistant to stress and are activated upon injury to restore homeostasis (Metcalfe et al., 2014; Montgomery et al., 2011; Powell et al., 2012; Ritsma et al., 2014; Sangiorgi and Capecchi, 2008; Takeda et al., 2011; Tian et al., 2011). Adding additional complexity, recent data suggest a level of cellular plasticity within the ISC population, thereby allowing for inter-conversion between compartments (Goodell et al., 2015; Muñoz et al., 2012; Ritsma et al.,

2014; Takeda et al., 2011). Whereas there are between 12 and 16 CBC ISCs in a single small intestinal crypt (Lopez-Garcia et al., 2010; Snippert et al., 2010), only one to two d-ISCs are typically present, underscoring their reserve role in intestinal maintenance (Breault et al., 2008; Powell et al., 2012; Sangiorgi and Capecchi, 2008; Takeda et al., 2011). To date, most studies looking at the stress response of ISCs have focused on radiation-induced injury (Kirsch et al., 2010; Potten, 2004; Roche et al., 2015), a potent but pathological insult. In contrast, few studies have examined the ISC response to more subtle yet common physiological stressors such as acute nutrient deprivation.

To date, studies investigating the role of nutrients in ISC regulation have yielded conflicting results. In *Drosophila*, ISCs are largely unaffected by acute nutrient deprivation but upon re-feeding are activated to proliferate in response to canonical insulin signaling (Choi et al., 2011; O'Brien et al., 2011). In contrast, long-term caloric restriction in mice leads to activation of CBC ISC self-renewal, mediated indirectly by decreased mTORC1 signaling in neighboring Paneth cells (Yilmaz et al., 2012). How d-ISCs respond to changes in nutrient levels either during an acute fast or upon re-feeding remains unknown. Given that these cells are intrinsically stress responsive, it will be of particular interest to delineate how acute changes in nutrient levels specifically affect this quiescent population.

The molecular mechanisms underlying the regulation of d-ISC quiescence and activation remain poorly understood. PTEN (phosphatase and tensin homolog), a negative regulator of the PI3K→AKT→mTORC1-signaling pathway, is an essential regulator of numerous cellular processes including survival, proliferation, and energy metabolism. In addition, PTEN has been implicated in the maintenance of quiescent cell numbers (Di Cristofano and Pandolfi, 2000; Shen et al., 2007; Song et al., 2012); however, its complete loss has been associated with cellular senescence, providing a block to unrestrained cellular growth (Chen et al., 2005; Papa et al., 2014; Song et al., 2012). PTEN is also a known negative regulator of genes associated with ISC self-renewal and maintenance, including *mTert* (telomerase reverse transcriptase) (Kang et al., 1999; Kyo and Inoue, 2002; You et al., 2007; Zhou et al., 2006). In the intestine, PTEN is an important regulator of homeostasis (Langlois et al., 2009) with loss-of-function mutations giving rise to the PTEN hamartoma tumor syndrome (Hobert and Eng, 2009). In addition, PTEN is negatively regulated by phosphorylation (Ross and Gericke, 2009; Vazquez et al., 2000, 2001), and both PTEN and its inactive isoform, phospho-PTEN (pPTEN), have been shown to mark a discrete population of DNA label-retaining cells in the +4 crypt position (He et al., 2004).

Here, we show that the physiological stress of fasting leads to the transient inhibition of PTEN in *mTert*-expressing d-ISCs and a corresponding increase in their number. In addition, fasting renders d-ISCs functionally poised to contribute to the regenerative response during re-feeding. This response is mediated by cell-autonomous activation of the PI3K→AKT→mTORC1 pathway, made possible by the inhibition of PTEN. Consistent with its role as a regulator of cell survival, we also show that PTEN is required for d-ISC maintenance at baseline and that intestines lacking PTEN have diminished regenerative capacity after irradiation.
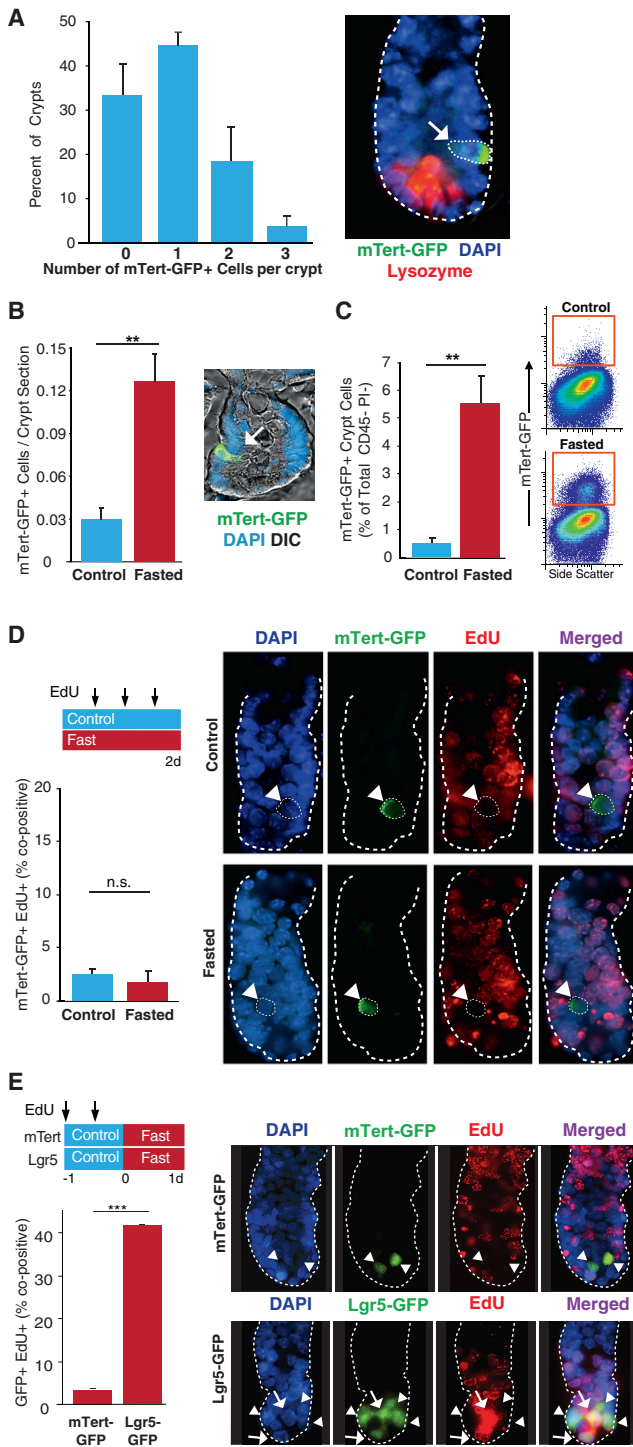
RESULTS

Fasting Increases d-ISC Numbers

We have previously identified *mTert* expression as a marker for d-ISCs under baseline fed conditions (Montgomery et al., 2011). Using a high-resolution, three-dimensional imaging technique that allows for the analysis of endogenous GFP fluorescence in freshly isolated intestinal crypts, we were able to identify at least one *mTert*-GFP[+] cell in up to two-thirds of all crypts (Figures 1A and S1A), supporting the physiologically relevant role of these cells as dormant ISCs.

To assess the effect of acute nutrient deprivation on d-ISCs, *mTert*-GFP mice were fasted for 48 hr with ad libitum access to water. This led to a 20% weight loss, a reduction in villus height and villus and crypt cell number, decreased crypt cell proliferation, and increased crypt cell apoptosis (Figures S1B–S1H). Despite this, we observed a dramatic *increase* in the number of *mTert*-GFP^{+} cells in response to fasting (Figures 1B and 1C). To determine whether this increase was due to enhanced cell proliferation, EdU-labeling studies were performed. Analysis of 364 *mTert*-GFP^{+} cells from 250 crypts showed no increase in the low number (2%) of co-positive *mTert*-GFP[+] EdU^{+} cells between fed and fasted mice (Figure 1D), indicating that the marked increase in the number of *mTert*-GFP^{+} cells in response to fasting occurred via a proliferation-independent mechanism.

It has also been suggested that CBC ISCs express *mTert* at high levels (Muñoz et al., 2012), raising the possibility that the increase in *mTert*[+] ISCs after fasting may be due to a specific increase in *mTert* expression in the CBC ISC population. To investigate this, we examined the position of *mTert*-GFP^{+} cells within the crypts of fed and fasted mice. If fasting induced CBC ISCs to express *mTert*, then the mean distribution of GFP^{+} cells should shift from the supra-Paneth (+4) position (Montgomery et al., 2011) toward the base of the crypt where CBC ISCs are located (Barker et al., 2007). However, analysis of fasted *mTert*-GFP mice revealed no change in the distribution of GFP^{+} cells within the crypt, with the peak incidence still at the +4 position (Figure S1I). To further confirm that fasting did not induce *mTert*-GFP expression within CBC ISCs, *mTert*-GFP mice were treated with two doses of EdU 12 hr apart prior to fasting. Because CBC ISCs are rapidly cycling, we reasoned that increased levels of EdU[+] *mTert*-GFP^{+} cells should be detected if *mTert* expression was induced within these cells upon fasting. Instead, we observed that less than 4% of fasted *mTert*-GFP^{+} cells co-expressed EdU, consistent with the general quiescent nature of these cells (Figure 1E; Montgomery et al., 2011). As expected, direct analysis of CBC ISCs, using *Lgr5*-GFP-ires-CreER (*Lgr5*-GFP) reporter mice, showed a significant proportion of fasted *Lgr5*-GFP^{+} cells remained EdU[+], consistent with their rapid cycling (Figure 1E; Barker et al., 2007). In addition, there was no significant change in the total number of *Lgr5*-GFP^{+} cells with fasting (Figure S1J); however, there was a moderate decrease in the overall fraction that was actively cycling (Figure S1K). Together, these results confirm that the increased number of *mTert*-GFP^{+} cells in response to fasting occurs independently of the CBC ISC population.

PTEN Regulates the Fed-Fasting Transition in d-ISCs

To further investigate the mechanism underlying the increased number of *mTert*-GFP⁺ d-ISCs with fasting, we examined the role of PTEN within these quiescent cells. Because PTEN and its inactive isoform, pPTEN, are expressed in slowly cycling +4 crypt cells (He et al., 2004; Montgomery et al., 2011), we reasoned that they may also regulate d-ISCs. Analysis of pPTEN immunostaining in intestinal crypts revealed a general *decrease* in the total number of pPTEN-expressing crypt cells with fasting (Figure 2A). In contrast, analysis of pPTEN staining specifically within the *mTert*-GFP⁺ population revealed a dramatic *increase* in pPTEN co-staining during fasting, with >60% of all *mTert*-GFP⁺ cells co-expressing pPTEN, a >5-fold increase from baseline (Figures 2B and S3A). Together, these results demonstrate selective inactivation of PTEN within the *mTert*-GFP⁺ d-ISC population. In addition, given that PTEN is a known negative regulator of telomerase (Kang et al., 1999; Kyo and Inoue, 2002; You et al., 2007; Zhou et al., 2006), inactivation of PTEN likely leads to de-repression of the *mTert* locus, accounting for the increased frequency of *mTert*-GFP cells with fasting. Finally, these results reveal that fasting alters PTEN status in d-ISCs, rendering them functionally poised for lineage contribution upon re-feeding (Figure 2C model).

d-ISCs Are Functionally Poised to Contribute to Regeneration During Re-feeding

Remarkably, re-feeding for 24 hr was sufficient to reverse the morphological changes seen with fasting, underscoring the enormous regenerative capacity of this tissue (Figures S1B–S1F). To assess whether poised d-ISCs contribute to this regeneration, we performed lineage-tracing studies using *mTert*-CreER::R26R(LacZ) reporter mice to specifically label d-ISCs and their progeny (Montgomery et al., 2011). Mice received a single dose of tamoxifen and were either allowed to feed ad libitum for 7 days or were fasted for 48 hr then re-fed for the next 5 days. All marked crypt cells in the proximal 10 cm of intestine (∼200,000 crypts) were analyzed using our quantitative whole-mount LacZ-staining assay (Figure S2; Montgomery et al., 2011). Consistent with the fasting-induced increase in *mTert*-GFP⁺ cells (Figures 1B, 1C, and S3B), we observed a 4-fold increase in the number of LacZ-marked crypts
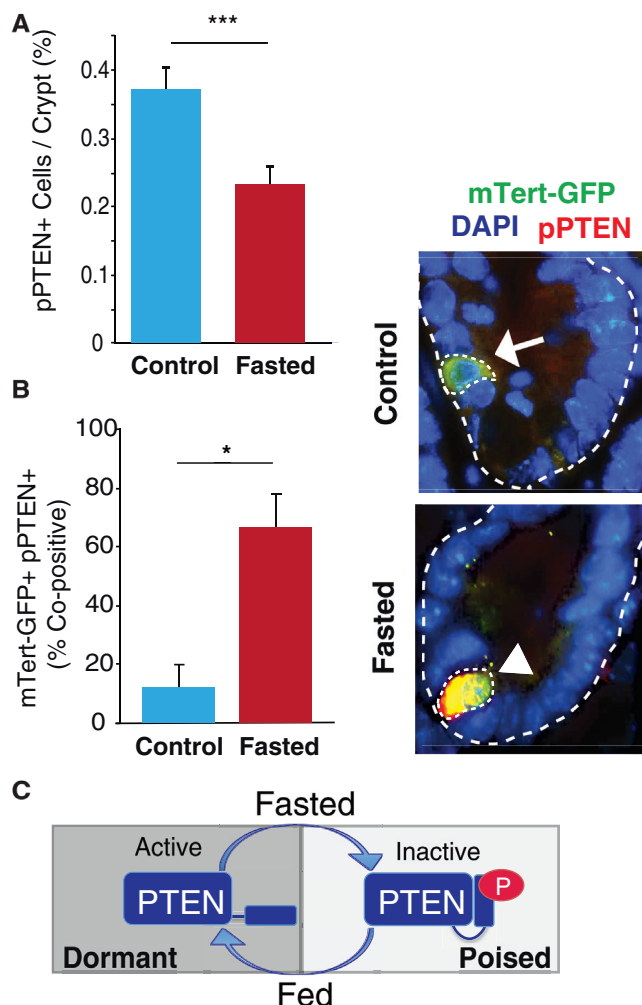

(arrow) identified by immunofluorescent analysis, 60×, is shown. n = 3 and 6.

(C) Percentage of *mTert*-GFP⁺ cells per total CD45⁻PI⁻ crypt cells from control (fed) and fasted mice. Representative FACS plots showing GFP⁺ cells in boxes are shown. n = 3 and 3.

(D) Percentage of *mTert*-GFP⁺ cells co-expressing EdU in isolated crypts from control (fed) and fasted mice. Schematic of treatment regimen is shown. Representative optical sections showing non-cycling *mTert*-GFP⁺ cells (arrowheads) in both control (fed) and fasted crypts in DAPI, *mTert*-GFP, EdU (Click-iT), and merged channels, 63×, are shown. n = 3 and 3.

(E) Percentage of *mTert*-GFP⁺ and *Lgr5*-GFP⁺ cells co-expressing EdU in isolated crypts from fasted mice. Schematic of treatment regimen is shown. Representative optical sections of isolated crypts from *mTert*-GFP and *Lgr5*-GFP mice showing actively cycling (arrows) and non-cycling (arrowheads) ISCs in DAPI, endogenous GFP, EdU (Click-iT), and merged channels, 60×, are shown. n = 3 and 3.

Mean ± SEM; Student's t test; **p < 0.01; ***p < 0.001; n.s., not significant.

Figure 1. d-ISCs Increase in Number During Fasting

(A) Percentage of individual intestinal crypts from control (fed) *mTert*-GFP mice containing 0–3 labeled cells. n = 3 animals. Representative image of isolated crypt showing endogenous *mTert*-GFP expression in the "+4" supra-Paneth position (arrow) is shown. Lysozyme (red) immunostaining identifies Paneth cells, 20×.

(B) Number of *mTert*-GFP⁺ d-ISCs per crypt section from control (fed) and fasted mice. Representative image from a control mouse showing GFP⁺ cell

Figure 2. Fasting Leads to PTEN Inactivation in d-ISCs

(A) Percentage of total phospho-PTEN (pPTEN)+ cells per crypt in control (fed) and fasted mice. n = 5 and 3.

(B) Percentage of *mTert*-GFP+ crypt cells co-expressing pPTEN in control (fed) and fasted mice. Representative optical sections showing *mTert*-GFP+ (arrow) and pPTEN+ *mTert*-GFP+ (arrowhead) cells, 60×, are shown. n = 6 and 3.

(C) Schematic illustration showing the dynamic regulation of PTEN by phosphorylation in the fasted and fed states.

Mean ± SEM; Student's t test; *p < 0.05; ***p < 0.001.

and villi in fasted mice (Figures 3A and S3C), underscoring their contribution to tissue regeneration. By contrast, lineage-tracing analysis of CBC ISCs using *Lgr5*-GFP-ires-CreER::R26R(LacZ) mice revealed a 40% decrease in LacZ-marked crypts after fasting (Figure 3B). Taken together, these data show that, with fasting, d-ISCs become functionally poised to contribute to the intestinal lineage upon re-feeding.

d-ISCs Demonstrate Cell-Autonomous Induction of mTORC1 Signaling Upon Re-feeding

Because the transition from fasting to re-feeding results in a dramatic induction in circulating insulin levels and canonical PI3K→AKT→mTORC1 signaling, we next sought to investi-
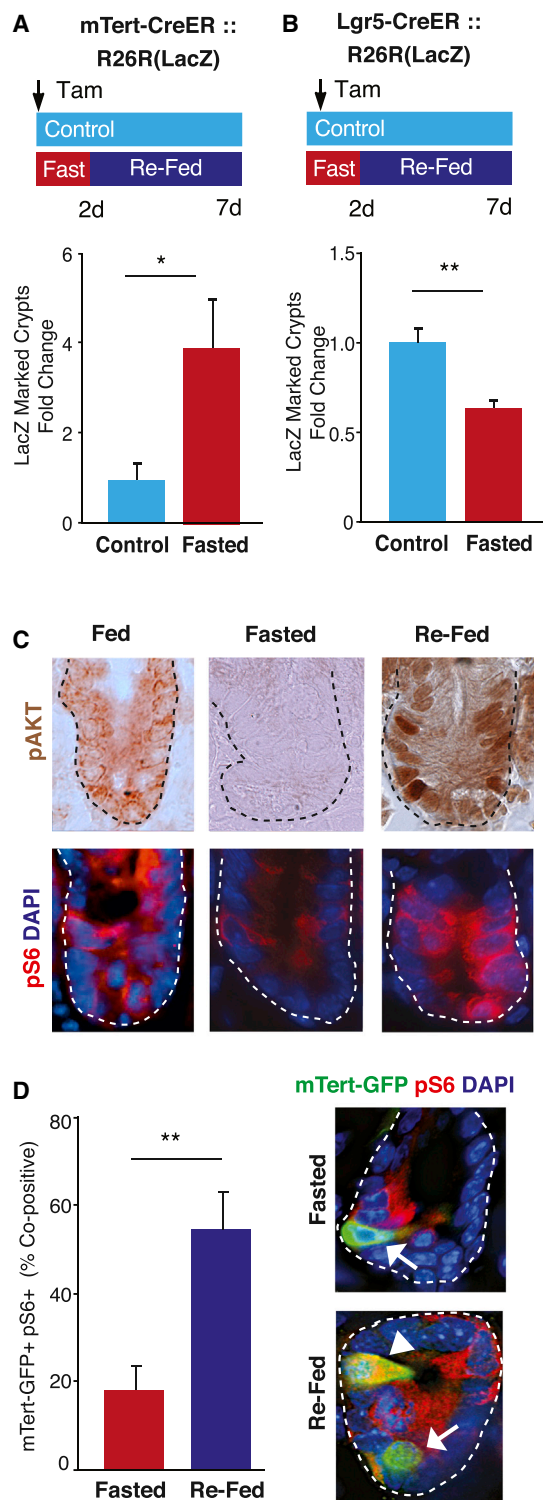
gate the role this pathway plays in the d-ISC response to the return of nutrients. Initially, we confirmed the effect of fasting and re-feeding on the phosphorylation of AKT and S6, a marker for mTORC1 signaling, in intestinal crypts. We detected modest diffuse staining of both phospho-AKT (pAKT) and phospho-S6 (pS6) in fed crypts, a very low level of both in fasted crypts, and a marked induction of both upon re-feeding (Figure 3C). Next, we performed co-immunofluorescence for *mTert*-GFP and pS6 to determine whether re-feeding activated this pathway specifically in d-ISCs. In both the fed and fasted states, we observed an overall low level of d-ISC co-staining (GFP+ pS6+; Figure 3D). With re-feeding, however, a significant induction in the fraction (55%) of dual positive (GFP+ pS6+) cells was detected (Figure 3D), demonstrating that d-ISCs undergo cell-autonomous induction of mTORC1 signaling upon re-feeding. This is in contrast to CBC ISCs, in which mTORC1 signaling in adjacent Paneth cells regulates the response to calorie intake during long-term caloric restriction (Yilmaz et al., 2012). Combined, the above data show that the PI3K→AKT→mTORC1 pathway is altered in d-ISCs upon re-feeding, indicating it functions in regulating lineage contribution. Activation of this pathway in d-ISCs was presumably due to the persistence of inactive PTEN (pPTEN+) within these cells in the re-fed state (compare Figure S3D with Figure 2B).

A Subpopulation of d-ISCs Is Sensitive to the Re-fed Milieu

During our analysis of mTORC1 signaling in re-fed d-ISCs, we noted that re-feeding led to an apparent decrease in the overall number of *mTert*-GFP+ cells. To quantify this, we performed flow cytometric analysis on isolated crypt cells from *mTert*-GFP mice fasted for 48 hr and then re-fed for 24 hr, which showed a return to baseline levels of *mTert*-GFP+ cells (Figure 4A). In an effort to understand the mechanism(s) underlying this reduction, we assessed their rate of apoptosis using co-immunostaining for *mTert*-GFP and activated caspase-3 at 10 and 24 hr after re-feeding. Collectively, nearly 30% of *mTert*-GFP+ cells were found to be entering apoptosis at these time points, which is particularly notable given the absence of apoptosis in *mTert*-GFP+ cells from either fed or fasted mice (Figure 4B). These results demonstrate that a subpopulation of d-ISCs are sensitive to the re-fed milieu and suggest a potential negative role for active PI3K→AKT signaling in these cells.

PTEN Is Essential for Dormant ISC Maintenance and Intestinal Regeneration

To further investigate both the potential sensitivity of d-ISCs to active PI3K→AKT signaling and the more-general role of PTEN in d-ISC maintenance, we next asked how d-ISCs would respond to permanent PTEN loss under control fed conditions. We generated *mTert*-Cre::R26R(LacZ)::PTEN^fl/fl mice to conditionally delete PTEN in d-ISCs. In this system, tamoxifen-mediated nuclear translocation of CreER within *mTert*-expressing d-ISCs leads to concurrent *activation* of the R26R(LacZ) reporter allele and *deletion* of the floxed PTEN allele. Remarkably, whole-mount LacZ analysis revealed near complete loss of lineage marking in these mice as compared to

A mTert-CreER ::
R26R(LacZ)

↓ Tam

Control

Fast | Re-Fed

2d | 7d

B Lgr5-CreER ::
R26R(LacZ)

↓ Tam

Control

Fast | Re-Fed

2d | 7d

C

| Fed | Fasted | Re-Fed |

pAKT

pS6 DAPI

D

mTert-GFP pS6 DAPI

Fasted

Re-Fed

control mice with functionally intact PTEN (*mTert*-Cre::R26R (LacZ)::PTEN$^{+/+}$) 1 week after tamoxifen administration (Figure 4C). Additional analysis at shorter time points revealed that loss of d-ISCs after PTEN deletion was evident as early as 2 days following tamoxifen induction, indicating a rapid and progressive depletion of these cells in fed animals (Figure 4D). Consistent with this, no LacZ-marked crypts were detected when lineage-tracing studies were performed with mice constitutively haploinsufficient for PTEN (*mTert*-Cre::R26R (LacZ)::PTEN$^{+/null}$; Figure 4C). These results show that PTEN is required for maintenance of the d-ISC population in the baseline fed state.

To functionally assess the impact of d-ISC loss after loss of PTEN, we utilized high-dose radiation (16 Gy), a model that obliterates CBC ISCs (Barker et al., 2012; Marshman et al., 2001; Potten, 2004; Roche et al., 2015) while activating d-ISCs (Marshman et al., 2001; Montgomery et al., 2011; Potten, 2004; Powell et al., 2012; Roche et al., 2015; Yan et al., 2012). PTEN$^{+/+}$ and PTEN$^{+/null}$ mice were irradiated, and intestines were collected 96 hr later, during the peak of the regenerative response (Potten, 2004). To quantitate radiation-dependent damage to the stem cell compartment, intestinal sections were scored using the intestine-specific microcolony assay (Withers and Elkind, 1970). This analysis revealed an ∼40% decrease in both crypt number and crypt depth in irradiated PTEN$^{+/null}$ mice as compared to controls (Figures 4E–4G), suggesting decreased stem cell function in these mice during recovery from injury. Combined, these data underscore the critical role of PTEN in d-ISC maintenance and intestinal regeneration.

d-ISCs Are Sensitive to PI3K Signaling and the Fed Milieu

Because PI3K→AKT signaling is negatively regulated by PTEN, we next investigated whether the loss of d-ISCs after conditional PTEN deletion was due to increased activity of this signaling pathway. *mTert*-CreER::R26R(LacZ)::PTEN$^{fl/fl}$ mice were treated with tamoxifen and the selective class I PI3 kinase inhibitor, NVP-BKM120, and analyzed after 2 days. Whole-mount LacZ analysis revealed that inhibition of PI3K signaling restored the number of LacZ-marked crypts despite the deletion of PTEN (Figure 4H). These results indicate that the loss of d-ISCs following PTEN deletion is due, in part, to increased PI3K→AKT signaling and indicate that d-ISCs are particularly sensitive to activation of this pathway.
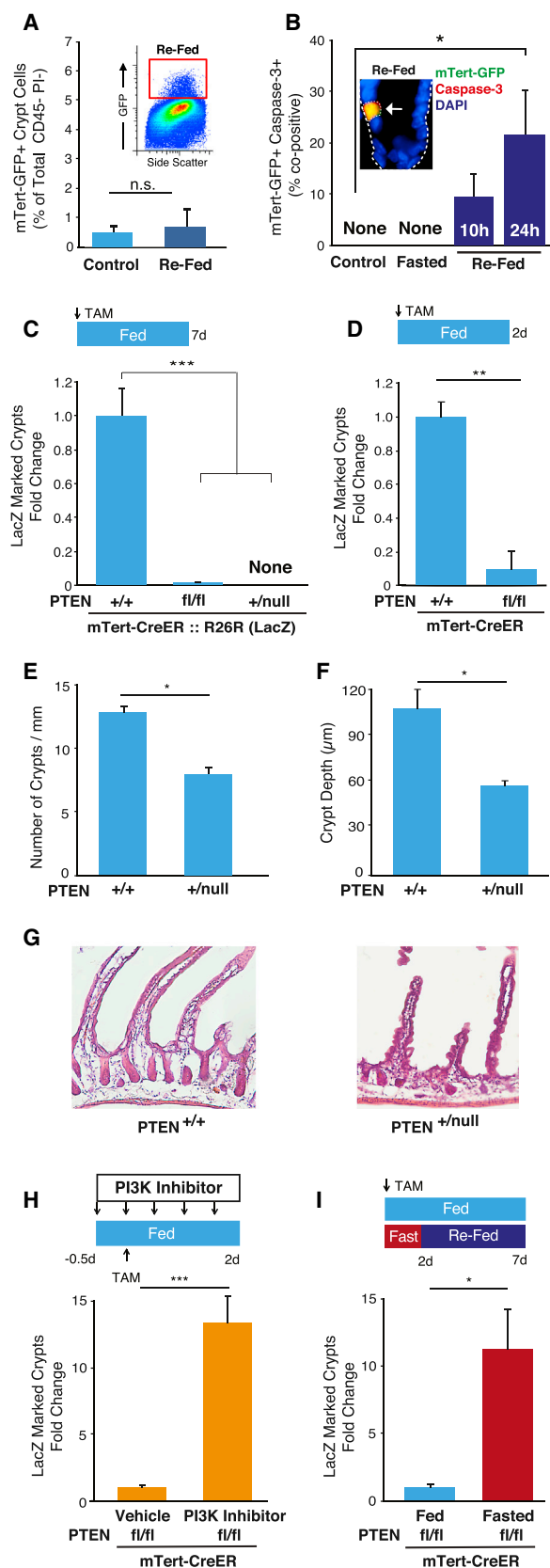
Finally, given the low level of PI3K signaling with fasting (Figure 3C), we investigated whether fasting could rescue the

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(B) Quantitative whole-mount analysis showing change in number of lineage-marked LacZ$^+$ crypts from *Lgr5*-Cre::R26R(LacZ) control (fed) versus fasted/re-fed mice. Schematic of treatment regimen is shown above. n = 4 and 4.

(C) Phospho-AKT (pAKT) and phospho-S6 (pS6) immunostaining in fed, fasted (48 hr), and re-fed (10 hr) crypt sections. Representative images showing pAKT (DAB immunohistochemistry) and pS6 (red immunofluorescence), 60×, are shown. n = 3 and 3.

(D) Percentage of *mTert*-GFP$^+$ crypt cells that co-express pS6 in fasted (48 hr) and re-fed (10 hr) mice. Representative optical sections of crypt sections showing *mTert*-GFP$^+$ pS6$^-$ cells (arrows) and an *mTert*-GFP$^+$ pS6$^+$ cell (arrowhead) in DAPI, *mTert*-GFP, and pS6 merged channels, 60×, are shown. n = 3 and 3.

Mean ± SEM; Student's t test; *p < 0.05; **p < 0.01.

Figure 3. d-ISCs Show Increased Lineage Contribution After Fasting/Re-feeding and Activation of mTORC1 Signaling

(A) Quantitative whole-mount analysis showing change in number of lineage-marked LacZ$^+$ crypts from *mTert*-Cre::R26R(LacZ) control (fed) versus fasted/re-fed mice. Schematic of treatment regimen is shown above. n = 6 and 9.

Figure 4. PTEN Is Necessary for d-ISC Maintenance and Intestinal Regeneration but Is Dispensable when PI3K Signaling Is Low

(A) Percentage of *mTert*-GFP$^+$ cells per total CD45$^-$PI$^-$ crypt cells from control (fed) and re-fed (24 hr) mice. Representative re-fed FACS plot showing GFP$^+$ cells in box is shown. n = 3 and 3.

(B) Percentage of *mTert*-GFP$^+$ crypt cells co-expressing activated caspase-3 in control (fed; 0/39 GFP$^+$ cells), fasted (0/139 GFP$^+$ cells), and re-fed (10 and 24 hr) mice. Representative optical section showing a GFP$^+$-activated caspase-3$^+$ cell (arrow) upon re-feeding, 60×, is shown. n = 3, 4, 4, and 5.

(C) Quantitative whole-mount analysis showing change in lineage-marked LacZ$^+$ crypts from *mTert*-Cre::R26R(LacZ) mice that were PTEN$^{+/+}$, PTEN$^{fl/fl}$, or PTEN$^{+/null}$ 7 days after tamoxifen treatment. Schematic of treatment regimen is shown above. n = 24, 19, and 7.

(D) Change in lineage-marked LacZ$^+$ crypts from *mTert*-Cre::R26R (LacZ)::PTEN$^{+/+}$ and *mTert*-Cre::R26R(LacZ)::PTEN$^{fl/fl}$ mice 2 days after tamoxifen treatment. Schematic of treatment regimen is shown above. n = 4 and 5.

(E and F) Number of intestinal crypts per mm of intestine (E) and crypt depth (F) in PTEN$^{+/+}$ (control) and PTEN$^{+/null}$ mice 96 hr after 16 Gy irradiation; n = 3 and 4.

(G) Representative H&E image of intestines, 20×, is shown.

(H) Change in LacZ-marked crypts from *mTert*-Cre::R26R(LacZ)::PTEN$^{fl/fl}$ mice treated with vehicle or PI3K inhibitor (NVP-BKM120). Schematic of treatment regimen is shown above. n = 5 and 4.

(I) Change in LacZ-marked crypts from *mTert*-Cre::R26R(LacZ)::PTEN$^{fl/fl}$ mice under fed or fasted conditions. Schematic of treatment regimen is shown above. n = 7 and 9.

Mean ± SEM; Student's t test was used to compare groups of two. One-way ANOVA with Bonferroni post hoc analysis was used to compare groups of three or more; *p < 0.05; **p < 0.01; ***p < 0.001.

PTEN-dependent loss of d-ISCs. Whole-mount LacZ analysis of tamoxifen-treated fasted *mTert*-CreER::R26R(LacZ)::PTEN$^{fl/fl}$ mice showed an increase in LacZ-marked crypts (Figure 4I) to levels comparable to wild-type PTEN mice. Genomic PCR analysis confirmed deletion of the floxed PTEN allele in LacZ-labeled cells (Figure S3E). Taken together, our results indicate that PTEN is required for the maintenance of dormant ISCs when PI3K/AKT signaling is active but is dispensable when PI3K→AKT signaling is low.

DISCUSSION

Our data support a model (Figure 5) where fasting leads to the inhibition of PTEN and activation of *mTert* expression in d-ISCs, rendering them functionally poised to contribute to intestinal regeneration. Repression of PTEN in these cells subsequently allows for cell-autonomous activation of the PI3K→AKT→mTORC1-signaling pathway upon the return of enteral nutrition. With re-feeding, these activated d-ISCs undergo one of three fates: (1) lineage contribution to intestinal renewal as active stem cells; (2) initiation of programmed cell death; or (3) return to dormancy. Together, these events contribute to the homeostatic response and ensure that d-ISC numbers return to a pre-fasting baseline.

The notion of stem cell activation refers to the transition of a cell from a resting (dormant) state to a metabolically active state whereby cells become functionally poised. The mechanisms underlying stem cell activation, however, remain largely unknown. Recently, Rando and colleagues (Rodgers et al., 2014) described the "G$_{alert}$ state" of stem cell activation, a transitional stage in the
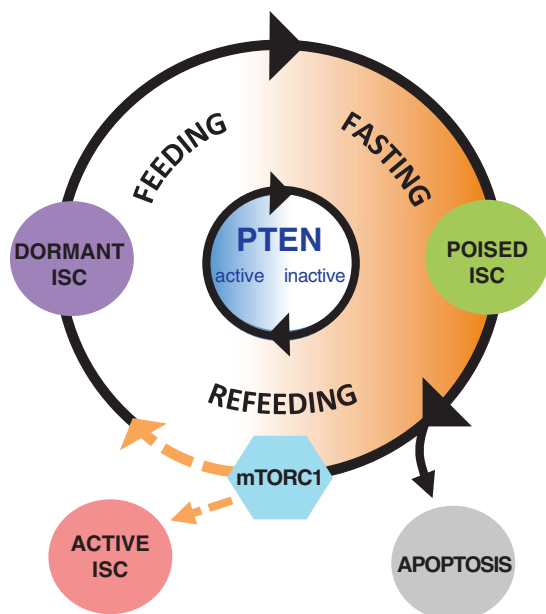
Figure 5. Model of d-ISC Regulation by PTEN and Nutrient Status
Model in which fasting leads to the inhibition of PTEN and subsequent activation of *mTert* expression in d-ISCs, rendering them functionally poised to contribute to intestinal regeneration.

cell cycle between G_0 and G_1. In their model, dormant muscle stem cells enter G_{alert} in response to a remote tissue injury; then, depending on the physiological need, these cells either contribute directly to the muscle lineage or return to the dormant state. Our data indicate that d-ISCs behave in a similar fashion in response to the stress of fasting and re-feeding. Moreover, resolution of the G_{alert} state in both dormant muscle stem cells (Rodgers et al., 2014) and d-ISCs is mediated by an mTORC1-dependent mechanism. The extent to which other dormant stem cell populations utilize the G_{alert} state remains to be determined.

The mechanism(s) responsible for maintaining dormant ISC numbers are complex and include factors such as PTEN, which plays a central role in the regulation of numerous cellular processes including survival, proliferation, and energy metabolism (Song et al., 2012). PTEN is best known for its ability to suppress the PI3K→AKT pathway through its lipid phosphatase activity (Song et al., 2012), although increasing evidence also points to an expanding role for phosphatase-independent effects of PTEN on cellular regulation including functions within the nucleus (Lee et al., 1999; Song et al., 2011, 2012; Trotman et al., 2007). Here, we show that PTEN is required for maintenance of d-ISC numbers under baseline (fed) conditions, that loss of PTEN results in the rapid loss of d-ISCs, and that PTEN is critical for intestinal regeneration after severe injury. Interestingly, both fasting and inhibition of PI3K→AKT signaling prevent the PTEN-mediated loss of d-ISCs, which implies that PTEN is required for the maintenance of dormant ISCs when PI3K→AKT signaling is active but is dispensable when PI3K→AKT signaling is low. A similar response to the loss of PTEN has also been shown for dormant hematopoietic stem

cells (d-HSCs), which initially demonstrate cell-cycle activation and enhanced lineage contribution followed by premature exhaustion and stem cell loss (Yilmaz et al., 2006). This effect has been linked, at least in part, to an enhanced rate of protein synthesis within d-HSCs following loss of PTEN (Signer et al., 2014). Analogous results have been shown in the prostate, where complete loss of PTEN leads to a "fail-safe" mechanism of cellular senescence, thus protecting the organism from unrestrained cellular growth (Chen et al., 2005; Papa et al., 2014; Song et al., 2012).

Whereas, in most cells, active PI3K→AKT signaling is essential for growth and survival, our studies imply that this pathway may have deleterious effects in specific cellular contexts. The evolution of these cellular and molecular mechanisms may represent a conserved strategy to cope with physiological stress in metabolically expensive tissues. A better understanding of these mechanisms will contribute to improved strategies to promote tissue regeneration.

EXPERIMENTAL PROCEDURES

Detailed methods and raw data are available in Supplemental Information.

Statistical Analysis
Two-tailed Student's t test was used to compare groups of two, and one-way ANOVA with Bonferroni post hoc analysis was used to compare groups of three or more. Statistical significance was set at $p < 0.05$ unless otherwise noted.

Study Approval
All animal procedures were approved by the Boston Children's Hospital institutional animal care and use committee. IACUC.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.11.035.

AUTHOR CONTRIBUTIONS

C.A.R., R.K.M., D.L.C., and D.T.B. designed the experiments. C.A.R., M.S.S., L.T.D., D.C.T., H.T., D.M.A., L.J., B.B.W., H.D.R., R.K.M., and A.T. carried out the experiments. C.A.R. and D.T.B. analyzed the data, and C.A.R., D.L.C., and D.T.B. wrote and edited the manuscript.

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