Apc inactivation, but not obesity, synergizes with Pten deficiency to drive intestinal stem cell-derived tumorigenesis

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Abstract

Obesity is a major risk factor for colorectal cancer and can accelerate Lgr5+ intestinal stem cell (ISC)-derived tumorigenesis after the inactivation of Apc. However, whether non-canonical pathways involving PI3K-Akt signaling in ISCs can lead to tumor formation, and if this can be further exacerbated by obesity is unknown. Despite the synergy between Pten and Apc inactivation in epithelial cells on intestinal tumor formation, their combined role in Lgr5+-ISCs, which are the most rapidly dividing ISC population in the intestine, is unknown. Lgr5+-GFP mice were provided low-fat diet (LFD) or high-fat diet (HFD) for 8 months, and the transcriptome was evaluated in Lgr5+-ISCs. For tumor studies, Lgr5+-GFP and Lgr5+-GFP–Ptenfloxflox mice were tamoxifen treated to inactivate Pten in ISCs and provided LFD or HFD until 14–15 months of age. Finally, various combinations of Lgr5+-ISC-specific, Apc- and Pten-deleted mice were generated and evaluated for histopathology and survival. HFD did not overtly alter Akt signaling in ISCs, but did increase other metabolic pathways. Pten deficiency, but not HFD, increased BrdU-positive cells in the small intestine (P < 0.05). However, combining Pten and Apc deficiency synergistically increased proliferative markers, tumor pathology and mortality, in a dose-dependent fashion (P < 0.05). In summary, we show that HFD alone fails to drive Akt signaling in ISCs and that Pten deficiency is dispensable as a tumor suppressor in Lgr5+-ISCs. However, combining Pten and Apc deficiency in ISCs synergistically increases proliferation, tumor formation and mortality. Thus, aberrant Wnt/β-catenin, rather than PI3K-Akt signaling, is requisite for obesity to drive Lgr5+ ISC-derived tumorigenesis.
Introduction

Evidence suggests that intestinal stem cell (ISC) populations can serve as the origin of tumor development. Indeed, increased Wnt/β-catenin signaling in Lgr5+, Bmi1+ or Lrig1+ ISCs, as well as Ah-cre cells in the transit amplifying (TA) compartment, have been shown to rapidly promote tumorigenesis (Sangiovanni & Capechi 2008, Barker et al. 2009, Powell et al. 2014). The phosphoinositide-3-kinase (PI3K)-Akt pathway, which is commonly deregulated in human colorectal cancer, has been causally linked to tumor development in rodents, independent of canonical Wnt signaling, via PIK3CA mutations (Leystra et al. 2012) or Pten inactivation in the intestinal epithelium (Byun et al. 2011). Furthermore, constitutive activation of PI3K or loss of Pten in the intestinal epithelium can synergize with Apc inactivation to dramatically increase intestinal tumor development (Shao et al. 2007, Marsh et al. 2008, Langlois et al. 2009, Deming et al. 2014). In small intestine, PTEN is most robustly expressed in the villus, but levels are also detectable in the crypt base, where Lgr5+-ISCs reside (Byun et al. 2011). However, to what extent the PI3K-Akt pathway may be involved in driving Lgr5+-ISC-derived tumorigenesis, either independently, or in cooperation with dysregulated Wnt/β-catenin signaling, is unknown.

Obesity and diet strongly regulate CRC risk and progression (Kim et al. 2006, Pischon et al. 2006, Cheskin & Prosser 2007, Giovannucci & Michaud 2007, Bardou et al. 2013, Schlesinger et al. 2015) as well as intestinal tumor development (Gravaghi et al. 2008, Hata et al. 2011, Pettan-Brewer et al. 2011, Day et al. 2013, Huffman et al. 2013, Beyaz et al. 2016) in humans and rodent models, respectively. Even prior to tumor initiation, obesity appears to ‘prime’ the normal intestinal epithelium toward tumor development, by promoting proliferation of ISCs and hypertrophy of the epithelium (Mao et al. 2013), while also altering the epigenomic landscape of the colonic epithelium in a manner resembling cancer progression (Li et al. 2014). Obesity has also been shown to alter the systemic and local microenvironment in the gut, including the microbiome (Cani et al. 2008), which can create a pro-inflammatory environment in the colon to increase oxidative stress, genome instability and potential risk of CRC (O’Callaghan et al. 2009, Pindyala et al. 2011).

More recently, diet-induced obesity was found to increase the number and function of Lgr5+ ISCs, while also promoting stemness and tumorigenicity of progenitor cells after inactivation of Apc (Beyaz et al. 2016). Although obesity can clearly instigate processes related to increased tumor risk, as well as accelerate intestinal tumorigenesis after loss of Apc in ISCs and epithelium (Gravaghi et al. 2008, Hata et al. 2011, Pettan-Brewer et al. 2011, Day et al. 2013, Huffman et al. 2013, Beyaz et al. 2016), whether obesity can promote Lgr5+-ISC-derived tumorigenesis via non-canonical mechanisms involving PI3K signaling (Huang & Chen 2009, Vucenik & Stains 2012) has not been investigated. Here, we demonstrate that although obesity can modify the transcriptome of Lgr5+-ISCs and expression of specific metabolic pathways, it fails to alter genes related to the Akt signaling and other proliferative pathways in Lgr5+-ISCs. Further, inactivation of Pten in Lgr5+ ISCs, either alone, or in combination with obesity, is insufficient to drive intestinal pathology and adenoma development in mice. However, we show Pten deletion in Lgr5+-ISCs can synergize with Apc loss to increase tumor multiplicity and worsen survival, demonstrating a previously unappreciated role for enhanced Akt-PI3K signaling, in cooperation with Apc deficiency, to drive Lgr5+-ISC-derived tumorigenesis.

Materials and methods

Animals

C57BL/6/J.129S4-Pten<sup>tm11Hwq</sup>/J mice (Pten<sup>lox/−</sup>-stock#006440), C57BL/6/J.129P2-Lgr5<sup>tm1(R2T2)Clfj</sup>/J mice (Lgr5+;GFP; stock#008875) and C57BL/6;129S6-Gt(Rosa)26Sortm14(CAG-tlTomato)Hze mice (Rosa26<sup>GFP</sup>; stock#007914) were all obtained from Jackson Laboratories. Apc<sup>S580S/S580S</sup> mics on a C57BL/6 background were a kind gift of Dr Leonard Augustinlicht (Peregrina et al. 2015). To generate Pten deficiency and/or Apc deficiency in Lgr5+ ISCs, Lgr5+-GFP–Apc<sup>S580S/−</sup>, Pten<sup>lox/−</sup>- mice were bred with Apc<sup>S580S/−</sup>, Pten<sup>lox/−</sup>- animals to generate Lgr5+-GFP (Control), Lgr5+-GFP–Apc<sup>S580S/−</sup> (Apc Het), Lgr5+-GFP Pten<sup>lox/</sup>- (Pten KO), Lgr5+-GFP–Apc<sup>S580S/−</sup>, Pten<sup>lox/</sup>- (Apc KO) and Lgr5+-GFP–Apc<sup>S580S/−</sup>, Pten<sup>lox/</sup>- (Apc KO–Pten KO) mice. Mice were genotyped as described (Barker et al. 2009, Byun et al. 2011, Peregrina et al. 2015), and males were weaned at 3 weeks of age and provided a purified low-fat diet (D12450H; Research Diets Inc, New Brunswick, NJ, USA). Animals were maintained under standard temperature and photoperiod as described (Huffman et al. 2013). All experimental procedures were approved by the Einstein Institutional Animal Care and Use Committee.
**Experiment 1: Obesity and Lgr5+ intestinal stem cells**

At weaning, male Lgr5+-GFP mice were placed on a defined, purified ingredient LFD (3.85 kcal/g; D12450H). At 7–8 weeks of age, animals were randomized to remain on LFD \((n=6)\) or switched to a more energy-dense sucrose-matched HFD feeding \((n=6)\) consisting of 45% Kcal from fat withlard as the predominant fat source in lieu of corn starch and maltodextrin, but all other components remained constant \((4.73 \text{ kcal/g}; \text{D12451; Research Diets Inc})\) until ~8 months of age. At the end of the study, body weight was recorded, and animals were killed after a brief 3- to 4-h fast for blood collection, and isolation of Lgr5+–ISCs from the small intestine by FACS, as described below, for RNA sequencing analysis.

**Experiment 2: Pten deficiency and obesity**

In order to determine the role of Pten deficiency on Lgr5+-ISC-derived tumorigenesis under low-fat or high-fat-fed conditions, 3-months-old male Lgr5+-GFP (Control) and Lgr5+-GFP–Pten\(^{flox/flox}\) (Pten KO) mice were injected intraperitoneally (i.p.) with 1mg tamoxifen (TAM) on two consecutive days to induce Cre recombinase in Lgr5+-ISCs. The efficacy of the TAM protocol to induce Cre recombination was confirmed in Lgr5+-GFP–Rosa reporter mice, as shown in Supplementary Fig. 2A and B (see on supplementary data given at the end of this article). Animals were then placed on either a purified LFD or sucrose-matched HFD and monitored for up to 12 months after injection (15 months of age) for specimen collection and histopathology.

**Experiment 3: Pten and Apc deficiency**

It was reported that complete inactivation of Apc in Lgr5+-ISCs led to rapid onset of intestinal adenomas and related mortality \((\text{Holik et al. 2014})\), whereas heterozygous deletion of Apc in Lgr5+ ISCs leads to significant pathology within 6 months of induction \((\text{Peregrina et al. 2015})\). In order to determine if Pten loss in Lgr5+-ISCs can synergize with Apc inactivation, six groups of mice were generated (Lgr5+-GFP (control), Lgr5+-GFP–Apc\(^{580S/−}\) (Apc Het), Lgr5+-GFP–Pten\(^{flox/flox}\) (Pten KO), Lgr5+-GFP–Apc\(^{580S/−}\)–Pten\(^{flox/flox}\) (Apc het–Pten KO) Lgr5+-GFP–Apc\(^{580S/580S}\) (Apc KO), Lgr5+-GFP–Apc\(^{580S/580S}\)–Pten\(^{flox/flox}\) (Apc KO–Pten KO)). Animals were injected with 1 mg TAM on two consecutive days at 3–5 months of age as described previously and immediately placed on a purified diet (D12450H). Animals were then monitored for up to 4 months prior to killing, for tissue collection and histopathology \((n=4–13/group)\) and/or survival \((n=9–13/group)\). Mice were removed prior to 4 months after induction if >25% weight loss was observed within a 1-week period, combined with signs of sickness and lethargy that suggested the animal was unlikely to survive an additional 24–48 h longer, and this was considered the time of death pending necropsy.

**Plasma insulin and glucose determination**

Whole blood was collected from Lgr5+-GFP mice on LFD or HFD after a 3- to 4-h fast into K2-EDTA collection tubes \((\text{Sarstedt AG & Co, Numbrecht, Germany})\), and immediately centrifuged \((1500g; 4°C, 15\text{ min})\) to separate plasma from red blood cells. Plasma Insulin levels were measured by a rat/mouse ELISA \((\text{EMD Millipore})\) with rat insulin standards using a spectrophotometer \((\text{Biorad iMark plate reader})\) following the manufacturer’s instructions. Plasma glucose was determined via the glucose oxidase method with an Analox GM7 analyzer \((\text{Analox Inst., USA Inc, Lunenberg, MA, USA})\), as described previously \((\text{Muzumdar et al. 2009, Einstein et al. 2010, Huffman et al. 2016})\).

**Intestinal histopathology**

For evaluation of epithelial cell proliferation and migration in the small intestine, random mice were injected i.p. with 100mg/kg BrdU \((\text{Sigma})\) 24h prior to killing. At necropsy, the entire intestine was quickly excised, surrounding mesenteric fat was removed and the gut was divided into duodenum, jejunum, ileum and colon, as previously described \((\text{Huffman et al. 2013})\). Each segment was opened longitudinally, rinsed in ice-cold phosphate-buffered saline and carefully flattened for examination of tumor multiplicity with the aid of a dissecting magnifying lens. Macroadenomas \((>0.5\text{ mm diameter})\), when present, were counted in each segment of intestinal tissue and recorded. Tissue was subsequently rolled and fixed overnight in 10% neutral-buffered formalin at 4°C for staging as a Swiss roll. Specimens were then processed through a series of alcohols and xylenes and embedded in paraffin. Hematoxylin & eosin (H&E)–stained sections \((5\text{ μm})\), from each segment of small intestine, capturing the entire proximal to distal length, were subsequently evaluated by a pathologist (A P B), who was blinded to the experimental groups, for histological changes following consensus recommendations for assessing intestinal pathology and tumors in rodents \((\text{Boivin et al. 2003})\).
3D organoid assay

Crypts were isolated from the small intestine of LFD- and HFD-fed mice (n=4 group) as described elsewhere (Yilmaz et al. 2012). Isolated crypts were washed with ADF medium, centrifuged at 300g for 5 min, resuspended in ADF medium and counted on a hemocytometer. Approximately 250 crypts were then resuspended in 25 µL of matrigel, transferred to a 48-well plate to solidify at 37°C for 30 min and overlaid with 250 µL of culture medium (ADF 1x, Pen/Strep 1x, HEPES 1x, Glutamax 1x, N2 1x, B27 1x, N-acetyl-l-cysteine 1 µM, oogin 100 ng/mL, EGF 50 ng/mL, Rock inhibitor 10 µM, and R-Spondin 500 ng/mL) and maintained at 37°C. Fresh medium was applied every 3 days and the number and area of budding crypts on day 5 and organoid formation on day 9 were assessed with a light microscope and normalized to the beginning number of counted crypts and expressed as organoids per crypt.

Immunohistochemistry

Immunostaining was performed as previously described (Huffman et al. 2007, 2008). In brief, intestinal sections were subjected to antigen retrieval (Citrate buffer pH 6) using a pressure cooker on high steam for 10 min. After rehydration, slides were treated with 3.0% H₂O₂ for 5 min to quench endogenous peroxidase activity, subjected to an avidin–biotin blocking step (Vector Labs SP-2001) and subsequently blocked with pre-immune goat or rabbit serum (1%) for 20 min. Sections were then incubated with an antibody against Ki67 (1:400; cat#12202) pAktSer473 (1:50; cat#4060), phospho- (Ser/Thr) Akt substrate (1:500; cat#9611), β-catenin (1:100; cat#8480) and anti-BrdU antibody (1:200; cat#5292) from Cell Signaling. A negative control was included in the same run using a subset of slides by omitting primary antibody from the staining procedure. Sections were then incubated with the appropriate biotinylated secondary antibody for 20 min, followed by a streptavidin–HRP detection system (Vector) and application of 3,3′-diaminobenzidine (DAB) for visualization of the antigen–antibody complex (ScyTek). Digital files of all slides were then acquired with a PerkinElmer P250 High-Capacity Slide Scanner and positively stained cells were analyzed using QuantCenter Software.

Flow cytometry

Lgr5+–GFP expression cells were isolated from LFD- and HFD-fed mice (n=6 group) following established methods (36, 76). In brief, cells from isolated crypts were disassociated and subjected to Flow Cytometry (BD LSR II flow cytometer, Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed with FlowJo software (Tree Star). Cells were gated to select for only Lgr5+–GFP expressing ISCs and were sorted directly into TRIzol LS to ensure good RNA quality, prior to phenol/chloroform extraction. We routinely isolated ~200,000–300,000 Lgr5-GFP expressing ISCs per animal, and purity was confirmed by gene expression of Lgr5 in GFP expressing cells vs GFPneg cells using established primers (Munoz et al. 2012).

Total RNA library preparation and sequencing

RNA sequencing libraries were prepared using the KAPA Stranded Rnaseq Kit with RiboErase (Kapa Biosystems) in accordance with the manufacturer’s instructions. Briefly, 100 ng of total RNA was used for ribosomal depletion and fragmentation. Depleted RNA underwent first- and second-strand cDNA synthesis and cDNA was adenylated, ligated to Illumina sequencing adapters and amplified by PCR (10 cycles). Final libraries were evaluated using fluorescent-based assays, including PicoGreen (Life Technologies), Qubit Fluorometer (Invitrogen) and Fragment Analyzer (Advanced Analytics) or BioAnalyzer (Agilent 2100). Deep sequencing (~70 M reads) was then performed on samples using an Illumina HiSeq2500 sequencer (v4 chemistry, v2 chemistry for Rapid Run) using 2×50-bp cycles.

Expression analysis

Reads were aligned to the mouse reference 10 mm using STAR aligner (v2.4.2a) (Dobin et al. 2013). Quantification of genes annotated in Gencode vM5 was performed using featureCounts (v1.4.3) and quantification of transcripts using Kalisto (doi:10.1038/nbt.3519). QC was collected with Picard (v1.83) and RSeQC (Wang et al. 2012) (http://broadinstitute.github.io/picard/). Normalization of feature counts was done using the DESeq2 package, version 1.10.1 (doi:10.1101/002832). Gene set enrichment was performed on genes found differentially expressed between HFD and LFD with a false discovery rate (FDR) of 5%. Traditional gene set enrichment analysis does not take into account the physical characteristics of the gene and has been shown to be biased by factors such as the length of the gene (Geelker et al. 2013). To address this, we used the Bioconductor package GoSeq (Young et al. 2010) developed to control for variability of length of genes to assess enriched pathways based on the KEGG database (Kyoto Encyclopedia of Genes and Genome). In addition,
one sample from the LFD group was determined to be a statistical outlier by PCA and was excluded from the analysis.

**Statistics**

Parametric data were analyzed by independent sample t-tests or two-way ANOVA, and longitudinal measures were assessed by repeated-measures ANOVA and planned contrasts were performed with Bonferroni adjustment when appropriate. Data were log transformed when necessary to ensure the normality of distribution. Non-parametric data were analyzed by the Kruskal–Wallis test and followed up with Mann–Whitney U tests when appropriate. Survival analysis was performed using the Kaplan–Meier procedure and log-rank test. All analyses were performed using SPSS (SPSS). Experiments were designed to achieve 80% power to detect a mean difference of −1.8 (n=6) to −1.5 (n=8 per group) with a standard deviation of 1.0 and alpha of 0.05. For RNAseq, the average expression and standard deviation for each group with n=5–6 samples per group for LFD and HFD respectively enabled >80% power to detect at least one group expression difference >2 fold-change at an FDR=0.05. A P<0.05 was considered statistically significant for all analyses.

**Results**

**HFD upregulates fatty acid metabolism, but not Akt pathway genes in Lgr5+-ISCs**

It was previously established that Akt-related genes are upregulated in the colonic mucosa in obese, tumor-prone mice (Pfalzer et al. 2016). To determine if the Akt pathway is upregulated in Lgr5+-ISCs with obesity in normal mice, we performed RNAseq on isolated Lgr5+ ISCs from LFD- and HFD-fed animals. HFD mice were nearly 50% heavier than LFD mice (Fig. 1A; P<0.01), were hyperinsulinemic (Fig. 1B; P<0.01) and hyperglycemic (Fig. 1C; P<0.001). However, employing an ex vivo 3D intestinal organoid assay, previously used to demonstrate increased ISC proliferation by caloric restriction, rapamycin (Yilmaz et al. 2012), as well as obesity (Beyaz et al. 2016), we failed to observe any effect of HFD on ISC proliferation, as compared to LFD controls (Fig. 1D). Lgr5+-ISCs were next isolated by FACS, and purity was confirmed by qPCR (Fig. 1D and E). Transcriptome analysis by RNAseq on Lgr5+-ISCs detected 798 differentially regulated genes between LFD and HFD (adjusted P value ≤0.05; **Supplementary Table 1** and Fig. 1F and G). However, the magnitude of differences between differentially regulated genes between LFD and HFD were mostly limited and predominantly downregulated in HFD animals (Fig. 1G). To further characterize the overlap between our genes of interest and the Akt pathway, we ran a supervised analysis looking at the level of expression and distribution of P values in our samples for genes annotated as part of the Akt pathway in KEGG database (Fig. 1H). Enrichment for a low P value will indicate the correlation between diet and Akt pathway. Enrichment was quantified using the π1 statistic (Storey & Tibshirani 2003). Both, the clustering approach and the π1 statistic confirmed the lack of enrichment for Akt pathway with a π1 score equal to 0 and a random clustering of the samples (Fig. 1H). Likewise, no difference was observed for cancer, MAPK or Wnt pathways between groups (**Supplementary Fig. 1**). However, the unbiased analysis implicated several other pathways as differentially regulated, including an upregulation in fatty acid metabolism and PPAR signaling (Fig. 1I), with the latter consistent with a recent report (Beyaz et al. 2016).

**Pten inactivation alone, or combined with obesity, is insufficient to drive Lgr5+-ISC-derived tumorigenesis**

We next attempted to determine whether Pten serves as an obligate tumor suppressor in Lgr5+-ISCs and if tumorigenesis could be further augmented by obesity in Pten KO mice. Control and Pten KO male animals were i.p. injected with TAM at 3 months of age and placed on either a purified sucrose-matched LFD or a 45% HFD and monitored for up to 12 months after injection (14–15 months of age). As expected, control and KO mice on HFD were heavier than their LFD-fed counterparts (Fig. 2A; P<0.001), whereas Pten deficiency in ISCs per se had no effect on body weight. Intestinal histopathology analysis in these mice revealed that neither HFD nor Pten deficiency per se in Lgr5+-ISCs profoundly altered gut pathology (**Table 1**). Of note, a reduction in multifocal crypt hyperplasia was observed in Pten KO mice on HFD (P<0.05), but the pathologic relevance of this alteration is unknown as this histologic change was unrelated to the atypical form commonly linked to dysplasia and pre-neoplastic lesions. Sporadic dysplastic foci in the small intestine were identified in 2 LFD and HFD Pten KO mice, respectively, along with one instance of carcinoma and colonic dysplasia in a HFD-fed KO animal. However, the frequency of these alterations did not reach significance, whereas no macroadenomas were observed in these mice. Further, analysis of BrdU labeling in duodenum
revealed a significant effect of Pten KO (P < 0.001) and diet (P < 0.05) on proliferation, but no significant Pten×diet interaction was observed (Fig. 2B). Elsewhere, a main effect for Pten KO was observed in jejunum (P < 0.01) and ileum (P < 0.001), but diet had no effect on the number of BrdU-positive cells. An analysis of pAkt-positive staining revealed a significant main effect for Pten inactivation only in duodenum (P = 0.05), but no effect of diet or its interaction was observed, nor was any effect observed in other intestinal segments (Fig. 2C).

**Pten deficiency synergizes with Apc inactivation in Lgr5+ ISCs to drive tumorigenesis**

Despite the inability of Pten inactivation per se to drive ISC-derived tumorigenesis, Pten inactivation appears to
influence ISC proliferation rates. Thus, we next determined if Pten inactivation can have additive or synergistic effects with Apc deficiency on ISC-derived tumor development. To test this possibility, 6 unique models were generated, and all animals were TAM treated at 3–5 months of age and followed for up to 4 months for effects on tumor development and survival. Pten inactivation or Apc haploinsufficiency alone did not result in the development of pathology within 4 months (Table 1), whereas Apc Het–Pten KO mice developed a synergistic rise in dysplastic foci (P<0.05) and macroadenoma formation (Fig. 3A and Table 2). Although mild pathology was observed in Apc KO mice, tumor multiplicity and pathology were markedly increased in Apc KO–Pten KO animals (Fig. 3A and Table 2; P<0.05), with significant range in tumor multiplicity, which is a typical observation of aggressive, Apc-deficient tumor models (Gravaghi et al. 2008, Taketo & Edelmann 2009, Huffman et al. 2013). Furthermore, no deaths were observed in WT, Pten KO, Apc Het or Apc KO mice over 16 weeks, with the latter observation contrary to prior reports of rapid mortality in Lgr5+-specific, Apc KO mice (Holik et al. 2014). Meanwhile, approximately 40% of Apc Het–Pten KO mice did not survive up to 16 weeks, whereas 100% of Apc KO–Pten KO mice did not survive beyond 13 weeks after TAM induction.

**Pten and Apc deficiency selectively increases proliferative markers in intestine**

In order to determine the independent and combined effect of Apc and Pten inactivation in ISCs on proliferative markers in the intestine, we next stained for Ki67, β-catenin and pAkt. In duodenum, a significant main effect of Apc deletion on Ki-67-positive cells was found

Table 1 | Histopathology of the gastrointestinal tract in control and Lgr5+ stem cell specific Pten knockout mice fed either a low-fat or high-fat diet.

<table>
<thead>
<tr>
<th></th>
<th>Low-fat diet</th>
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<th>High-fat diet</th>
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<tbody>
<tr>
<td></td>
<td>Control (n=8)</td>
<td>Pten KO (n=9)</td>
<td>Control (n=14)</td>
</tr>
<tr>
<td>Hyperplasia, crypt epithelial (focal)†</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0.14 ± 0.14</td>
</tr>
<tr>
<td>Hyperplasia, crypt epithelial (multifocal)†</td>
<td>1.28 ± 0.18a</td>
<td>1.27 ± 0.25a</td>
<td>1.07 ± 0.47a</td>
</tr>
<tr>
<td>Dysplastic foci‡</td>
<td>0 ± 0</td>
<td>0.11 ± 0.11</td>
<td>0.28 ± 0.13</td>
</tr>
<tr>
<td>Macroadenomas#</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Carcinomas‡</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Dysplasia, colon</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
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</tbody>
</table>

Data are means ± S.E.M. Non-parametric data were analyzed by the Kruskal–Wallis non-parametric test planned contrast performed by Mann–Whitney U test. A significant effect was observed for multifocal crypt hyperplasia (chi-square = 10.2; P = 0.017), with a significant reduction in Pten KO mice on HFD. Different letters denote a significant difference between groups, P<0.05.

†Value based upon post-mortem analysis of total tumor multiplicity throughout the intestinal tract. Also shown in Fig. 2A. *Value based on the pathologic severity using a 1–4 scale, with 4 being most severe. †Value indicates the number of identified dysplastic foci per section.
Figure 3
Pt en deletion synergizes with Apc deficiency in Lgr5+-ISCs to promote tumorigenesis and worsen survival. Two-way ANOVA for tumor multiplicity revealed a significant main effect for Pt en (P < 0.001), Apc Het (P < 0.01), Apc KO (P < 0.01), Pt en × Apc Het (P = 0.01) and Pt en × Apc KO interaction (P = 0.001). At necropsy, macroadenoma formation in the small intestine was absent in control and Pt en KO mice, whereas a modest number of tumors were observed in Apc Het and Apc KO animals. However, post hoc analyses confirmed that tumor multiplicity was significantly increased when combining Apc and Pt en deficiency in a dose-dependent manner, such that Apc Het–Pt en KO mice had increased tumor number, with the greatest evidence of tumor formation in Apc KO–Pt en KO animals (P < 0.05) (con (n = 6), Pt en KO (n = 8), Apc het (n = 13), Apc Het–Pt en KO (n = 11), Apc KO (n = 6), Apc KO–Pt en KO (n = 4)) (A). Likewise, although no death was observed in Apc Het or Apc KO mice, significant death began to be observed in Apc Het–Pt en KO animals within 3 months (60% survival to 4 months), whereas a severe increase in mortality was observed in Apc KO–Pt en KO within 1 months, leading to 100% mortality within 3 months of TAM injection (n = 9–13 group, P < 0.01) (Con (n = 6), Pt en KO (n = 9), Apc het (n = 15), Apc Het–Pt en KO (n = 13), Apc KO (n = 10) and Apc KO–Pt en KO (n = 9)) (B). For macroadenomas, different letters denote a significant difference between groups with Bonferroni adjustment (P < 0.05).

Discussion
There has been an intense interest in both the origin and natural history of colorectal cancer onset and progression. Loss of Apc has been viewed as an important initiating, was combined with Apc haploinsufficiency or deficiency in duodenum and jejunum (Fig. 4C, P < 0.05). This was confirmed by immunostaining for Akt substrate in intestinal segments, which was lower in controls, but strong staining was present in Apc Het–Pt en KO and Apc KO–Pt en KO mice (Supplementary Fig. 3, n = 3 per group).

Table 2  Histopathology of the gastrointestinal tract in control and Lgr5+ stem cell specific Apc and Pt en inactivation.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 6)</th>
<th>Pt en KO (n = 8)</th>
<th>Apc Het (n = 13)</th>
<th>Apc Het–Pt en KO (n = 11)</th>
<th>Apc KO (n = 6)</th>
<th>Apc KO–Pt en KO (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperplasia, crypt epithelial (focal)†</td>
<td>0 ± 0</td>
<td>0.38 ± 0.38</td>
<td>0.23 ± 0.21</td>
<td>0.29 ± 0.20</td>
<td>1.50 ± 0.50</td>
<td>0 ± 0</td>
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<tr>
<td>Hyperplasia, crypt epithelial (multifocal)†</td>
<td>0.67 ± 0.18b</td>
<td>0.50 ± 0.27b</td>
<td>0.61 ± 0.17b</td>
<td>0.60 ± 0a</td>
<td>0.67 ± 0.49b</td>
<td>2.5 ± 0.65c</td>
</tr>
<tr>
<td>Dysplastic foci</td>
<td>0 ± 0</td>
<td>0 ± 0a</td>
<td>0 ± 0a</td>
<td>0 ± 0a</td>
<td>0 ± 0a</td>
<td>0 ± 0a</td>
</tr>
<tr>
<td>Macroadenomas</td>
<td>0 ± 0</td>
<td>0 ± 0a</td>
<td>0 ± 0a</td>
<td>0 ± 0a</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Carcinomas</td>
<td>0 ± 0</td>
<td>0 ± 0a</td>
<td>0 ± 0a</td>
<td>0 ± 0a</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Dysplasia, colon</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0.08 ± 0.08</td>
<td>0.13 ± 0.0a</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

Data are means ± s.e.m. Non-parametric data were analyzed by the Kruskal–Wallis non-parametric test planned contrast performed by Mann–Whitney U. A significant effect was observed for multifocal crypt hyperplasia (chi-square = 17.8; P = 0.003), dysplastic foci (chi-square = 16.7; P = 0.005) and macroadenoma (chi-square = 27.8; P < 0.001). Different letters denote a significant difference between groups, P < 0.05.

†Value based on post-mor tem analysis of total tumor multiplicity throughout the intestinal tract. Also shown in Fig. 2A. Value based on the pathologic severity using a 1–4 scale, with 4 being most severe. Value indicates the number of identified dysplastic foci per section.
and often required event in disease pathogenesis, with additional mutations in p53, Kras and the PI3K-Akt pathway among others, as necessary to promoting advanced disease (Huels & Sansom 2015). Further, studies have demonstrated that Apc inactivation in several ISC populations can give rise to tumors (Sangiorgi & Capecchi 2008, Barker et al. 2009, Powell et al. 2014), supporting the 'bottom-up' model of tumorigenesis, whereby dysplastic cells originate from ISCs in the crypt base rather than the villus. However, to what extent mutations affecting non-canonical signaling pathways such as the PI3K-Akt pathway among others, as necessary to promoting advanced disease, is insufficient to drive tumorigenesis, suggesting that PTEN is dispensable as a tumor suppressor in these cells. This is in partial agreement with another report showing that Kras activation per se in Lgr5+-ISCs could result in hyperplasia, but failed to induce dysplasia or adenoma development (Feng et al. 2011). Given that Pten-knockout animals were not examined until 14–15 months of age, nearly 12 months after Cre induction, it is unlikely that the lack of observed transformation in these mice was

Here, we demonstrate that in the absence of Apc mutations, inactivation of Pten per se in Lgr5+-ISCs, either alone, or in combination with obesity, does not substantially alter intestinal homeostasis and is insufficient to drive tumorigenesis, suggesting that PTEN is dispensable as a tumor suppressor in these cells. This is in partial agreement with another report showing that Kras activation per se in Lgr5+-ISCs could result in hyperplasia, but failed to induce dysplasia or adenoma development (Feng et al. 2011). Given that Pten-knockout animals were not examined until 14–15 months of age, nearly 12 months after Cre induction, it is unlikely that the lack of observed transformation in these mice was
confounded by the reportedly long latency of disease onset in *Pten*-deficient animals (Knobbe et al. 2008). Likewise, RNAseq confirmed high expression of PTEN in Lgr5+--ISCs (not shown), whereas insulin receptor, IGF-1 receptor expression, as well as insulin receptor substrates 1 and 2 were also present in these cells, suggesting that the insulin/IGF-1 signaling pathway and PTEN may be integral to cellular function in Lgr5+-ISCs.

Genetic and epigenetic alterations that lead to dysregulated PI3K–Akt signaling, including those affecting PTEN function, have been reported in human colon cancers (Liao et al. 2012, Ogino et al. 2014). However, in animal models, the effect of *Pten* inactivation in intestinal homeostasis has been somewhat controversial. Some reports observed that loss of *Pten* in the gut (He et al. 2007, Byun et al. 2011, Yu et al. 2014) or whole body (Di Cristofano et al. 1998), is sufficient to drive intestinal pathology and tumorigenesis, but others have failed to observe any such effect of *Pten* loss alone on disease pathogenesis (Marsh et al. 2008, Langlois et al. 2009). The reason for these discrepant reports involving *Pten*-inactivating mutations is not entirely clear, but may involve the diversity in promoters (Rosa, Ah-cre, Vil-cre, Vil-cre/ERT2), genetic backgrounds and time of follow-up (5, 50 days and 1 year) used. For instance, using the Vil-cre mouse to delete *Pten* throughout the gut epithelium with 1-year follow-up (Langlois et al. 2009), resulted in hypertrophy and proliferation of the mucosa, but in contrast to prior reports (Di Cristofano et al. 1998), no evidence of tumors were detected. This contrary finding was speculated to potentially involve *Pten* deletion being restricted to the epithelium and not the stroma. However, a later report using the same Vil-cre promoter and follow-up period, but different genetic background, reported intestinal tumors in 19% of mice (Byun et al. 2011). If intestinal tumors can in fact arise from ISCs after an inactivation mutation in *Pten*, our results suggest that Lgr5+--ISCs are unlikely to be the site of origin. However, we cannot rule out an effect of *Pten* loss in other epithelial cells, stromal cells as well as other ISC populations (Bmi1+, Lrig1+) or progenitor cells (TA cells) to instigate tumorigenesis.

Obesity *per se* has also been shown to increase intestinal proliferation (Mao et al. 2013), and data from our laboratory and others have shown that HFD can exacerbate tumorigenesis in several *Apc* models (Gravaghi et al. 2008, Hata et al. 2011, Pettan-Brewer et al. 2011, Day et al. 2013, Huffman et al. 2013), including *Apc*-deficient Lgr5+--ISCs (Beyaz et al. 2016). However, even when combined with obesity, *Pten* deficiency *per se* in Lgr5--ISCs, in the absence of *Apc* mutations, is insufficient to drive adenoma formation in these mice, even up to 15 months of age. Because spontaneous intestinal tumor development in normal mice is exceedingly rare, these data by no means suggest that obesity *per se* is not a risk factor for tumor development, but rather that additional mutations besides *Pten* (i.e. *Apc*) in ISCs are required for obesity-induced transformation and tumor progression in mice. Indeed, overwhelming evidence in humans has implicated obesity, and particularly visceral obesity, as a strong regulator of CRC risk and progression in humans (Kim et al. 2006, Pischon et al. 2006, Cheskin & Prosser 2007, Giovannucci & Michaud 2007, Bardou et al. 2013, Schlesinger et al. 2015), including greater risk of CRC-related mortality (Calle et al. 2003).

Interestingly, in contrast to prior reports (Mao et al. 2013, Beyaz et al. 2016), we surprisingly did not observe any increase in ISC proliferation by obesity, as determined by intestinal organoid assay or BrdU-labeling studies, although caution should be exercised for results involving the latter assay, due to a limited sample size for control HFD mice. In addition, a transcriptome analysis of Lgr5+--ISCs from LFD- and HFD-fed mice did not detect any effect on growth and proliferation-related pathways (Akt, MAPK, Cancer and Wnt). Instead, the major pathways affected by obesity in these cells involved fatty acid metabolism, propanoate metabolism and PPAR signaling among others, with the latter observation consistent with a prior report implicating PPAR-delta in the effects of HFD on Lgr5+--ISCs (Beyaz et al. 2016).

A definitive explanation for some discordant results between our model and prior models is not entirely clear, but it is noteworthy that our study utilized a well-matched, purified and defined control companion diet for comparison, whereas many other studies have utilized standard rodent chow-based diets as a control-feeding regimen. Although mice maintained on standard chow do remain leaner than their HFD counterparts, mouse chow also harbors a poorly defined concentration of macronutrients and micronutrients, as well as elevated fiber and vitamin D, and the amount of these components can vary greatly among batches. Many of these constituents have important biologic activities, particularly in the gut, including effects on growth and differentiation pathways, the microbiome (Desai et al. 2016) and tumorigenesis (Augenlicht 2014). Thus, the inherent contribution of obesity in reports using chow as a control, particularly in the gut, should be interpreted
with caution, given the stark differences in dietary composition between these formulas.

Given the evidence that Pten and Apc can synergize in the intestinal epithelium to promote tumorigenesis, we next performed a gene dosage experiment in Lgr5+-ISCs by combining Pten deficiency with either Apc heterozygous or homozygous deletion. We observed a dose-dependent, synergistic rise in tumors along with accelerated mortality in double knockout mice. Indeed, although significant pathology and lethality were noted in combined Apc heterozygosity and Pten loss, observed effects on intestinal pathology and survival were far more extensive when combined with complete Apc inactivation in Lgr5+-ISCs. These findings are somewhat in agreement with other models that have found that inactivation of Apc or Pten throughout the intestinal epithelium augments Wnt/β-catenin-driven tumor formation (He et al. 2007), though our results suggest far greater dysregulation when both genes are completely inactivated. The rapidity of disease onset and death in mice lacking Pten and Apc only in Lgr5+-ISCs was comparable to those reported by combined deficiency throughout the gut epithelium (Marsh et al. 2008), suggesting that Lgr5+-ISCs are a key site of tumor initiation by combined dysregulation of Wnt/β-catenin and PI3K signaling. Likewise, the necessity for combined Pten and Apc loss to augment Akt activation was also consistent with prior observations in the intestine (Marsh et al. 2008). Collectively, these data confirm that PTEN alone is dispensable as a tumor suppressor in Lgr5+-ISCs when Apc is present, whereas PTEN plays a tumor-suppressive role when Apc is lost in Lgr5+-ISCs.

In summary, we show that Pten loss per se in Lgr5+-ISCs is not required either as a tumor suppressor or for maintaining intestinal homeostasis when Apc is functional, even when combined with obesity. Further, obesity leads to modest alterations in the Lgr5+-ISC transcriptome and augments fatty acid-related pathways in Lgr5+-ISCs, but does not alter Akt signaling-related genes in these cells. In contrast, Pten loss per se in Lgr5+-ISCs, but not diet, explained alterations to intestinal proliferation and Akt signaling. Although Apc inactivation was required to induce tumorigenesis in the intestine, disease severity and mortality were synergistically increased when this was further combined with Pten deficiency in Lgr5+-ISCs. Thus, these data demonstrate that Lgr5+-ISCs are an important site of Pten and Apc deficiency and establish the importance of PTEN in the control of PI3K/Akt signaling in these cells to prevent accelerated disease progression by canonical pathways involved in intestinal tumorigenesis.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-16-0536.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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