PTEN and LKB1 are differentially required in Gli1-expressing mesenchymal cells to suppress gastrointestinal polyposis

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SUMMARY

PTEN and LKB1 are intimately associated with gastrointestinal tumorigenesis. Mutations of PTEN or LKB1 lead to Cowden syndrome and Peutz-Jeghers syndrome characterized by development of gastrointestinal polyps. However, the cells of origin of these polyps and underlying mechanism remain unclear. Here, we reveal that PTEN or LKB1 deficiency in Gli1\textsuperscript{+} gut mesenchymal cells, but not intestinal epithelium, drives polyp formation histologically resembling polyposis in human patients. Mechanistically, although PTEN and LKB1 converge to regulate mTOR/AKT signaling in various tumor contexts, we find that mTOR is essential for PTEN-deletion-induced polyp formation but is largely dispensable for polyposis induced by mesenchymal LKB1 deficiency. Altogether, our studies identify Gli1-expressing mesenchymal cells as a common cell of origin for polyposis associated with PTEN and LKB1 and reveal their engagement of different downstream pathways in gut mesenchyme to suppress gastrointestinal tumorigenesis.

In brief

PTEN or LKB1 mutations lead to Cowden syndrome or Peutz-Jeghers syndrome characterized by the development of gastrointestinal polyps. Cotton et al. demonstrate that PTEN or LKB1...
deficiency in Gli1+ mesenchymal cells, but not intestinal epithelium, drives polyp formation that faithfully mimics hamartomatous polyposis in human patients.

**Graphical Abstract**

![Graphical Abstract](image)

**INTRODUCTION**

PTEN and LKB1 are intimately associated with gastrointestinal (GI) development and tumorigenesis, including hamartomatous polyposis syndromes (HPSs), the genetic syndromes characterized by the development of GI polyps (Jelsig et al., 2014; Stojcev et al., 2013; Wirtzfeld et al., 2001; Zbuk and Eng, 2007). PTEN hamartoma tumor syndrome (PHTS) and Peutz-Jeghers syndrome (PJS) are two major types of HPSs. PJS is caused by germline mutations of the LKB1 (STK11) gene, while PHTSs (Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome) are associated with mutation or deletion of the tumor-suppressor PTEN (Stojcev et al., 2013; Wirtzfeld et al., 2001; Zbuk and Eng, 2007). Despite variable phenotypical manifestation, affected patients have a significantly increased risk of cancers, including colon cancer, and designing effective therapeutic strategies is clearly needed for these devastating diseases.

The cells of origin of the GI polyps in patients with HPSs remain controversial and have been a matter of debate. Although originally thought to arise from the GI epithelium, two prior studies utilizing the pan-mesenchymal Cre drivers showed that removal of LKB1 from
overall gut mesenchyme promotes polyposis (Katajisto et al., 2008; Ollila et al., 2018). A recent report showed that LKB1 deficiency in T cells can induce the development of GI polyposis (Poffenberger et al., 2018), pointing to a surprising cell lineage that can also give rise to PJS polyps. However, the cells of origin of the polyps linked to PTEN deletion remain unclear. Both the LKB1 and PTEN pathways regulate downstream mTOR signaling (Manning and Cantley, 2007; Papa and Pandolfi, 2019; Saxton and Sabatini, 2017; Shackelford and Shaw, 2009; Shaw, 2009). Although the mTOR-AKT axis plays an important role in many tumor contexts downstream of LKB1 and PTEN, its relative contribution to GI polyposis in PJS and PHTS is still poorly defined.

Here, we used mouse genetics to systemically dissect the cells of origin of GI polyps associated with PJS and PHTS. We demonstrated that Pten deletion in adult gut mesenchyme, but not epithelium, drives polyposis that histologically resembles the polyps in human Cowden syndrome. Our data suggest the Gli1-expressing mesenchymal progenitors as a common origin for GI polyps induced by LKB1 or Pten deficiency. Our work also revealed differential requirement for mTOR/AKT downstream of Pten and LKB1 during polyposis.

RESULTS

Genetic dissection of the cells of origin of GI polyps induced by Pten or LKB1 deficiency

To explore the cells of origin of GI polyposis associated with Cowden syndrome and PJS, we crossed the Pten and LKB1 conditional alleles (Bardeesy et al., 2002; Lesche et al., 2002) with a series of mouse Cre driver lines that specifically direct Cre expression in either gut epithelial or mesenchymal compartments (Figure 1). Villin Cre drives Cre recombination throughout the intestinal epithelium from mouse embryonic day 12.5 (E12.5) (Madison et al., 2002), while the inducible Lgr5CreER directs Cre expression in the intestinal epithelial stem cells (Barker et al., 2007). To activate Cre recombination in the gut mesenchyme, we utilized the Nkx3.2Cre and Gli1CreER lines (Ahn and Joyner, 2004; Verzi et al., 2009). Nkx3.2Cre is a pan-GI mesenchyme Cre line that expresses Cre from the lateral plate mesoderm as early as E8.5 (Verzi et al., 2009). Recent reports demonstrate that the Gli1+ cells constitute a population of mesenchymal stem/progenitor cells critical for maintaining the intestinal stem cell niche during gut homeostasis (Degirmenci et al., 2018). These Gli1+ mesenchymal cells are located in the mesenchymal compartment adjacent to the crypts and are positive for mesenchymal markers, including α-smooth muscle actin (SMA) and vimentin (Figure S1) (Degirmenci et al., 2018).

Using these Cre drivers, we removed Pten and LKB1 specifically in the distinct gut epithelial and mesenchymal populations (Figure 1). We found that disruption of Pten and LKB1 function in the mesenchyme, but not the epithelium, drives the formation of GI polyps that histologically resemble the polyps associated with human Cowden syndrome and PJS (Figures 1, 2, and 3). Homozygous deletion of Pten or LKB1 from embryonic gut mesenchyme by the Nkx3.2Cre line led to embryonic or perinatal lethality (Figure 1). Although removing one copy of Pten did not generate apparent gut phenotype (data not shown), heterozygous deletion of LKB1 by both Nkx3.2Cre and Gli1CreER was able to induce polyposis with 100% penetrance (Figure 1). This is consistent with previous reports.
that heterozygous deletion of LKB1 in straight knockout mice was sufficient to generate polyps (Bardeesy et al., 2002). Taken together, our data provided genetic evidence that the GI polyps associated with both Pten and LKB1 deficiency originate from adult gut mesenchyme, and we identified Gli1+ stromal cells as a potential common cell of origin for the polyps in both types of HPS.

**Pten deletion in gut mesenchyme drives polyp formation**

We further characterized the polyps arising from Gli1+ mesenchymal cells carrying Pten deletion. Following tamoxifen (TM) injection at postnatal day 30 (P30), all Gli1CreER;Ptenfl/fl mice developed polyps within 4 months. The formation of numerous polyps was detected throughout the small and large intestines (Figures 2A, 2B, and S2A–S2F) but with a prevalence in the colon that contained more than 70% of the polyps detected throughout the GI tract (Figures 2A, 2B, and S2A). These data are consistent with the colonic predominant distribution of the polyps reported in human patients with Cowden syndrome (Shaco-Levy et al., 2017).

Histological analysis revealed that the intestinal and colon polyps developed in the Gli1CreER;Ptenfl/fl mice closely resembled the polyps reported in the patients with Cowden syndrome (Figure 2C) (Shaco-Levy et al., 2017). Our immunohistochemistry (IHC) analysis showed that Pten deletion led to robust proliferation in the mesenchymal cells within the polyps that were also positive for the mesenchymal markers vimentin and SMA (Figures 2D and 2E). Some intestinal polyps developed epithelial dysplasia with the clear characteristics of adenomas (Figures 2C, S2E, and S2F); however, hamartomatous polyps with disorganized epithelium and strong stromal proliferation were the most common manifestation among these polyps.

The other striking common feature of the polyps detected in the Gli1CreER;Ptenfl/fl mice was the lymphoid follicles within the polyps. We found that more than 75% of the polyps in the colon contained lymphoid follicles (Figures 2B and 2G–2K). These data were also consistent with the findings in Cowden syndrome, in which the lymphoid follicles are a major component of the polyps in these patients (Innella et al., 2021; Shaco-Levy et al., 2017). Our IHC and immunofluorescence (IF) staining further confirmed that these lymphoid follicles were an admixture of CD20-positive cells (B cells) in the germinal centers and CD3-positive cells (T cells) in the periphery (Figures 2G–2J, S2H, and S2I).

To further characterize the polyps induced by mesenchymal Pten deletion, we generated the Gli1CreER;Ptenfl/fl;R26-mT/mG mice. R26-mT/mG is a cell-membrane-targeted, two-color fluorescent Cre-reporter allele that expresses membrane-targeted Tomato (mT) prior to Cre-mediated excision and membrane-targeted GFP (mG) after excision (Muzumdar et al., 2007). Consistent with the mesenchymal origin of Gli1+ cells (Figure S1), we showed that the stroma, but not epithelial cells of the polyps, detected in the Gli1CreER;Ptenfl/fl;R26-mT/mG mice were GFP positive (Figure 2F). Interestingly, we found that the lymphoid follicles within the polyps were largely GFP negative (Figure 2F), suggesting that the lymphocytes in these follicles are unlikely to be derived from Gli1+ mesenchymal cells. Consistent with this view, we found that the lymphoid follicles had much lower levels of phospho-AKT than the adjacent mesenchymal cells within the polyps (Figure S3A).
Because AKT activation is a major signaling event downstream of Pten deletion, our data suggested a paracrine effect of Pten deletion in Gli1+ mesenchymal cells recruiting lymphocyte infiltration into the polyps (Figures S3B and S3C).

**LKB1 deficiency in Gli1+ stromal cells induces PJS polyposis**

We found that heterozygous deletion of LKB1 using the two different mesenchymal Cre lines (Nkx3.2\textsuperscript{Cre} and Gli1\textsuperscript{CreER}) was sufficient to induce GI polyposis with 100% penetrance (Figure 1). Although the pan-mesenchymal Nkx3.2\textsuperscript{Cre} induces Cre recombination and LKB1 deficiency from the embryonic stage, our data showed that disruption of LKB1 function in adult Gli1+ mesenchymal progenitors could effectively drive polyp formation that faithfully mimics hamartomatous polyposis associated with PJS (Figures 3A–3C). These LKB1-deficient polyps were observed at the junction of the stomach and the small intestine, displaying an arborizing smooth-muscle core and histologically indistinguishable from polyps observed in LKB1\textsuperscript{+/−} mice and prior published models (Figures 3B and 3C) (Bardeesy et al., 2002; Katajisto et al., 2008; Ollila et al., 2018). IHC analysis also showed that the stromal cells in these polyps were proliferative, expressing the mesenchymal markers SMA, desmin, and vimentin, although they were negative for CD20 (Figure 3G). We also performed the genetic fate-mapping experiment by crossing the Gli1\textsuperscript{CreER};LKB1\textsuperscript{fl/+} mice with the R26-mT/mG allele. Following TM injection, we found that the robustly expanding stroma, but not the epithelium in the polyps, was filled with GFP-positive cells (Figures 3D–3F), further supporting the idea that adult Gli1-expressing mesenchymal cells can serve as a cell of origin for GI polyps associated with LKB1 deficiency.

In contrast to the widespread polyp formation in the small intestine and colon of the Gli1\textsuperscript{CreER};Pten\textsuperscript{fl/fl} mice, the polyps were predominantly developed at the junction of the small intestine and stomach in the Gli1\textsuperscript{CreER};LKB1\textsuperscript{fl/+} mice (Figures 2A, 2B, and 3A–3C). These polyp distribution patterns are also reminiscent of polyp formation in Cowden syndrome and PJS. Further analysis of the polyps revealed the increased proliferation in both the epithelial and mesenchymal compartments of the polyps from Gli1\textsuperscript{CreER};Pten\textsuperscript{fl/fl} and Gli1\textsuperscript{CreER};LKB1\textsuperscript{fl/+} mice (Figures 2D, 3G, and S5A). Because Pten or LKB1 loss was restricted to the stromal cells, it suggested potential mesenchymal-epithelial interaction. To test this idea, we analyzed the status of Wnt pathway activity in the intestinal and colon polyps developed in both mouse models. We found that both types of mesenchyme-originated polyps had more elevated expression levels of CD44, a bona fide Wnt pathway target in the gut, in polyp epithelium than that in control epithelium (Figures S4A and S4B). However, nuclear β-catenin accumulation was not detected in the polyps of both mouse models (Figures S4A and S4B), and we did not detect up-regulation of intestinal stem cells in polyp epithelium, measured by IHC of the intestinal stem cell marker Olfm4 (Figure S5C), further supporting the notion that these polyps are distinct from the canonical polyps or adenomas. In addition, our analysis revealed the differentiation defects in the epithelium of the polyps were associated with Pten or LKB1 deficiency. We found up-regulation of Sox4 (Wnt target and progenitor marker) and the absence of lysozyme (Paneth cells) and Alcian blue staining (Goblet cells) in the polyp epithelium adjacent to the mesenchyme (Figures S5B, S5D, and S5E). Together, these data suggested that Pten or...
LKB1 deficiency in Gli1+ mesenchymal progenitors within the intestinal stem cell niche may affect mesenchymal-epithelial reciprocal interaction during polyposis.

**mTOR is differentially required for polyposis induced by Pten or LKB1 deletion**

Pten and LKB1 are thought to converge to regulate mTOR-AKT (Manning and Cantley, 2007; Papa and Pandolfi, 2019; Saxton and Sabatini, 2017; Shaw, 2009). Thus, we examined the status of mTOR and AKT activity in the hamartomatous polyps using IHC and immunoblot analysis of phospho-AKT and phospho-S6 (Figures 4A and 4B). We found that the polyps induced by Pten deletion in Gli1Crem;Ptenfl/fl mice displayed significantly higher activity of mTOR and AKT than the PJS polyps from Gli1Crem;LKB1fl/+ mice (Figures 4A and 4B), suggesting that the mTOR-AKT axis is differentially regulated during polyposis associated with Pten or LKB1 deficiency.

To genetically test mTOR function during polyposis, we used the conditional mTOR allele (Risson et al., 2009) to generate Gli1Crem;Ptenfl/fl;mTORfl/fl and Gli1Crem;mTORfl/fl mice. Removal of mTOR by Gli1Crem did not appear to affect normal GI homeostasis and overall animal health (Figure 4D; data not shown). However, mTOR deletion inhibited polyposis induced by mesenchymal Pten deletion. Following TM injection, the Gli1Crem;Ptenfl/fl;mTORfl/fl mice developed much fewer polyps and had significantly prolonged survival compared with Gli1Crem;Ptenfl/fl mice (Figures 4C–4H). These data demonstrated that the mTOR-AKT axis is absolutely required for polyposis induced by mesenchymal Pten deletion.

However, unlike mTOR requirement in the Gli1Crem;Ptenfl/fl mice, the mice carrying the concurrent deletion of both LKB1 and mTOR (Gli1Crem;LKB1fl/+;mTORfl/fl) still robustly developed hamartomatous polyps at the GI junction (Figures 4I and 4J). Furthermore, mTOR deletion did not affect the growth of the polyps and did not prolong the survival of the mutant animals (Figures 4K–4N). Together, these data from our genetic mouse models revealed the differential requirement for mTOR during polyposis and suggested that the mTOR pathway plays an essential role in polyposis induced by Pten deletion but does not appear to have major contribution to PJS polyp development.

**DISCUSSION**

The cells of origin for the polyps developed in HPSs, including PJS, Cowden syndrome, and juvenile polyposis syndrome (JPS), remain elusive. Although they are originally thought to arise from the GI epithelial cells, recent reports suggest possible alternate non-epithelial origins in PJS polyps. In these studies, disrupting LKB1 function in the gut pan-mesenchymal compartments, or in the T cell lineage, induces the development of GI polyps (Katajisto et al., 2008; Ollila et al., 2018; Poffenberger et al., 2018). Our comprehensive genetic analyses rendered further support for the mesenchymal origin of PJS polyps. More importantly, our studies revealed that mesenchymal, but not epithelial, deletion of Pten drives the formation of polyps that are histologically similar to the polyps observed in Cowden syndrome.
Prior studies of epithelial loss of Pten have reported no significant change of intestinal proliferation and differentiation upon Pten deletion, while intestinal polyps or tumors were only detected in a subset of old animals at the age of more than 12 months (Byun et al., 2011; Marsh Durban et al., 2014). It remains possible that polyp formation in these old animals is due to additional genetic mutations accumulated during animal aging. Furthermore, the polyp types or tumors detected in these aged animals do not exhibit the characteristics of the polyps in Cowden syndrome (Byun et al., 2011; Marsh Durban et al., 2014). In contrast, we found that Pten deletion in Gli1+ mesenchymal cells results in robust colon polyp formation with 100% penetrance, faithfully mimicking polyposis observed in Cowden syndrome, including the characteristic lymphoid follicles in the majority of the polyps (Innella et al., 2021; Shaco-Levy et al., 2017). Thus, our study established a faithful genetic model of polyposis of human Cowden syndrome. In addition, our study suggests an intriguing possibility that the Gli+ gut mesenchymal cells may serve as a common cell of origin for hamartomatous polyps associated with HPSs, and it is interesting to determine whether the polyps developed in JPS can also arise from the Gli1-expressing cells.

Gli1+ mesenchymal cells are recently identified as a critical population of gut stromal progenitors within the intestinal stem cell niche (Degirmenci et al., 2018). These cells express Gli1, the component and target of Hedgehog signaling, and other mesenchymal markers, such as vimentin, SMA, and PDGFRα, having the characteristics of active stromal progenitors capable of further differentiation into different lineages (Degirmenci et al., 2018). However, Gli1+ mesenchymal cells do not express T cell markers and have not been reported to be able to generate T cells (Degirmenci et al., 2018); thus, it is unlikely that Pten- or LKB1-related hamartomatous polyps arise from T cells in our Gli1CreER models. Further supporting this notion, we found that lymphoid follicles in Gli1CreER;Ptenfl/fl polyps are not derived from Gli1+ cells. Gli1+ cells play an essential role in maintaining the intestinal stem cells through secretion of Wnt ligands and agonists (Degirmenci et al., 2018).

This is consistent with our observation of mis-regulation of the mesenchymal-epithelial crosstalk (Figures S4 and S5) leading to Wnt pathway activation in the polyp epithelium through mesenchymal LKB1 or Pten deletion, which may also provide a potential molecular mechanism underlying the polyp-to-tumor transition.

mTOR/AKT signaling has long been considered a major downstream event underlying the tumor-suppressing function of Pten and LKB1 (Manning and Cantley, 2007; Papa and Pandolfi, 2019; Saxton and Sabatini, 2017; Shackelford and Shaw, 2009; Shackelford et al., 2009; Shaw, 2009). In this study, we identified a requirement for mTOR in hamartomatous polyps induced by Pten deletion but found that it is largely dispensable for polyposis suppressed by mesenchymal LKB1. This differential requirement highlights the importance of oncogenic contexts that may dictate downstream signaling specificity and function output of these pathways. In addition to AMPK-mTOR regulation, LKB1 is known to regulate other downstream pathways including Hippo/YAP (Mohseni et al., 2014; Nguyen et al., 2013). Two recent reports suggest that IL/STAT signaling may also play critical roles in polyposis associated with LKB1 deficiency (Ollila et al., 2018; Poffenberger et al., 2018). Clearly, further elucidation of the interplay among these critical signaling pathways will lead to the design of more effective therapeutic approaches for PJS and Cowden syndrome.
Limitations of the study

Our mouse genetic study revealed that mTOR is required for PTEN-deletion-induced polyp development but is largely dispensable for polyposis induced by LKB1 deficiency. However, a limitation of our study is that we were not able to examine the activity of mTOR or AKT signaling in both epithelial and mesenchymal compartments of the polyps developed in human Cowden syndrome and PJS. In addition, our study demonstrated that deletion of PTEN or LKB1 in intestinal mesenchyme initiates polyp formation and identified Gli1+ mesenchymal cells as a common origin for polyps associated with PTEN or LKB1 deficiency. However, we could not rule out the possibility that epithelial PTEN or LKB1 activity may contribute to later progression of polyp growth, and it remains possible that other mesenchymal populations or a subset of Gli1+ cells in the GI tract can also serve as the cells of origin for hamartomatous polyps.

STAR METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Junhao Mao (Junhao.mao@umassmed.edu).

Materials availability—This study did not generate new unique reagents.

Data and code availability

- The data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice—All animals use protocols were reviewed and approved by The University of Massachusetts Medical School Institutional Animal Care and Use Committee. Gli1CreER (Ahn and Joyner, 2004), Gli1LacZ (Bai et al., 2002), VillinCre (Madison et al., 2002), Lgr5CreER (Barker et al., 2007), Ptenfl (Lesche et al., 2002), mTORfl (Risson et al., 2009), R26mT/mG (Muzumdar et al., 2007) mice were obtained from the Jackson laboratory. The LKB1flox (Bardeesy et al., 2002) mice were obtained from NCI mouse repository. Nkx3.2Cre (Verzi et al., 2009) mice were kindly provided by Drs. RA Shivdasani and WE Zimmer. Cre activation of the inducible Cre lines was achieved by one-time intraperitoneal injection of 120mg/kg Tamoxifen (Sigma) at the age of one month old. Both male and female mice with appropriate genotypes were used in our study.

METHODS DETAILS

Tissue collection and histology—Following euthanasia, mouse intestinal or polyp tissue was dissected and fixed in 10% Neutral Buffered Formalin (NBF) at 4°C overnight. For paraffin sections, tissue was dehydrated, embedded in paraffin, and sectioned at 6 μm.
For frozen sections, tissue was dehydrated in 30% sucrose overnight at 4°C, embedded in OCT, and sectioned at 12 μm. Paraffin sections were stained using standard hematoxylin & eosin reagents. For intestinal epithelium and mesenchyme isolation, mouse small intestinal tissues at different prenatal stages are dissected and washed in cold PBS, before transferring to PBS containing 3 mM EDTA for rotation at 4°C for 30 mins. After vigorous shaking for 2 mins, the epithelial tissues are collected in the supernatant, while the remaining mesenchymal tissues are washed and incubated with the digestion buffer containing Collagenase XI and Dispase at 37°C for 30min, and the samples are then subjected to Western blot analysis.

**Immunohistochemistry, immunofluorescence and β-galactosidase staining—**
For immunohistochemistry (IHC), sections were deparaffinized and rehydrated before undergoing heat-induced antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) for 30 minutes. Slides were blocked for endogenous peroxidase for 20 minutes, then blocked for 1 hour in 5% BSA, 1% goat serum, 0.1% Tween-20 buffer in PBS, and incubated overnight at 4°C in primary antibody diluted in blocking buffer or SignalStain® Antibody Diluent (Cell Signaling). Slides were incubated in biotinylated secondary antibodies for 1 hour at room temperature and signal was detected using the Vectastain Elite ABC kit (Vector Laboratories). For β-galactosidase staining, frozen sections were cut at 12μm intervals and subjected to standard β-galactosidase staining. For immunofluorescence (IF), cells or tissue sections were fixed by 4% paraformaldehyde for 5 minutes, blocked for 1 hour and incubated overnight at 4°C in primary antibody diluted in blocking buffer. Slides were then incubated for 1 hour at room temperature in Alexa Fluor-conjugated secondary antibodies (Invitrogen) at 1:500 dilution in blocking buffer and mounted using mounting media with DAPI (EMS). Primary antibodies used for IHC/IF were: Vimentin (Cell Signaling), CD20 (Abcam), CD3 (Abcam), Ki67 (Abcam), phospho-AKT S473 (Cell Signaling), phospho-S6 (Cell Signaling), β-catenin (BD Biosciences), CD44 (eBioscience), β-galactosidase (Abcam), Desmin (ThermoFisher), and α-smooth muscle actin (SMA) (Abcam).

**Western blot analysis—** Freshly isolated mouse tissue or cultured HEK293T cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, phosphatase inhibitor cocktail, complete EDTA-free protease inhibitors cocktail) for 30 min at 4°C. The supernatants of the extracts were then used for Western blot following the protocols described previously (Cotton et al., 2017) and the primary antibodies used in these assays were listed as follows: phospho-AKT S473 (Cell Signaling), AKT (Cell Signaling) and GAPDH (Bethyl). HRP-conjugated Secondary antibodies were obtained from Jackson Laboratories.

**QUANTIFICATION AND STATISTICAL ANALYSIS**
No statistical method was used to predetermine sample size. The experiments were not randomized. For biochemical experiments we performed the experiments at least three independent times. Experiments for which we showed representative images were performed successfully at least 3 independent times. No samples or animal were excluded from the analysis. The investigators were not blinded to allocation during experiments and outcome
assessment. Student’s t test was used to generate p values (* = p value ≤0.05; ** = p value ≤0.01). The variance was similar between groups that we compared.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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• PTEN deletion in gut mesenchyme but not epithelium drives polyposis in Cowden syndrome
• Gli1+ gut mesenchymal cells are a common origin of polyps with PTEN or LKB1 deficiency
• PTEN and LKB1 engage distinct pathways in gut mesenchyme to suppress tumorigenesis
• mTOR/AKT is required for polyp formation induced by mesenchymal PTEN deletion
### Figure 1. Genetic dissection of the cells of origin of gastrointestinal polyps associated with Pten or LKB1 deficiency

| Genotype           | Cre expression                  | Polyp formation               |
|--------------------|---------------------------------|------------------------------|
| Villin<sup>Cre</sup>;<Pten<sup>fl/fl</sup> | Intestinal epithelium (from E12.5) | No polyp* (0/12)            |
| Lgr<sup>5</sup>;<Pten<sup>fl/fl</sup> | Intestinal stem cells (from P30)   | No polyp* (0/10)             |
| Nkx3.2<sup>Cre</sup>;Pten<sup>fl/fl</sup> | Gut mesenchyme (from E8.5)   | Embryonic lethal              |
| GliI<sup>CreER</sup>;Pten<sup>fl/fl</sup> | Gut stromal progenitors (from P30) | Hamartomatous polyp** (16/16) |
| Villin<sup>Cre</sup>;LKB1<sup>fl/fl</sup> | Intestinal epithelium (from E12.5) | No polyp*** (0/15)          |
| Lgr<sup>5</sup>;<LKB1<sup>fl/fl</sup> | Intestinal stem cells (from P30)   | No polyp*** (0/10)           |
| Nkx3.2<sup>Cre</sup>;LKB1<sup>fl/fl</sup> | Gut mesenchyme (from E8.5)   | Perinatal lethal              |
| Nkx3.2<sup>Cre</sup>;LKB1<sup>fl/+</sup> | Gut mesenchyme (from E8.5)   | Hamartomatous polyp*** (8/8)  |
| GliI<sup>CreER</sup>;LKB1<sup>fl/+</sup> | Gut stromal progenitors (from P30) | Hamartomatous polyp*** (25/25) |

*No polyp detected at the age of 8 months or 8 months following tamoxifen injection. **Polyp detected within 4 months following Cre activation. ***Polyposis monitored for 12 months following Cre activation.

See also Figure S1.
Figure 2. Mesenchymal Pten deletion drives polyposis

(A and B) Polyposis in the colon of Gli1CreER;Ptenfl/fl mice following tamoxifen injection.
(A) Whole amount image shows numerous colon polyps detected at 6 months after tamoxifen injection. Scale bar, 10 mm.
(B) Representative histological image of the colon of Gli1CreER;Ptenfl/fl mice at 6 months after tamoxifen injection. Scale bar, 2 mm.
(C) Representative histological images of wild-type intestine and colon (control) and polyps developed in the intestine and colon of \( \text{Gli1}^{\text{CreER};\text{Pten}^{+/+}} \) mice. Note that large lymphoid follicles are detected in a subset of the polyps. Scale bars, 50 μm.

(D) Immunohistochemistry of Ki67 in control intestine and colon and polyps from \( \text{Gli1}^{\text{CreER};\text{Pten}^{+/+}} \) mice. Scale bars, 50 μm.

(E) Percentage of Ki67-positive cells in the mesenchyme of control intestine and colon and polyps from \( \text{Gli1}^{\text{CreER};\text{Pten}^{+/+}} \) mice. \( n = 3 \) animals per group, 8–10 fields per animal, \(* * p < 0.01\).

(F) Immunohistochemistry of vimentin and smooth muscle actin (SMA) in intestinal and colon polyps induced by mesenchymal Pten deletion. Scale bars, 50 μm.

(G) Induced Cre activity representing Pten-deleted mesenchymal cells as depicted by GFP signal in \( \text{Gli1}^{\text{CreER};\text{Pten}^{+/+};\text{R26-mT/mG}} \) colon polyps. The white dash line demarcates the boundary between epithelium and mesenchyme. Ep, epithelium; Me, mesenchyme. Note that GFP is detected in stromal cells but largely absent in lymphoid follicle. Scale bars, 50 μm.

(H–J) Immunohistochemistry of CD20 and CD3 in colon polyps developed in \( \text{Gli1}^{\text{CreER};\text{Pten}^{+/+}} \) mice.

(H) Scale bar, 2 mm.

(I and J) Scale bars, 50 mm.

(K) Immunofluorescence staining of CD20 and CD3 in a colon polyp from \( \text{Gli1}^{\text{CreER};\text{Pten}^{+/+}} \) mice. Scale bars, 50 μm.

(L) Percentage of intestinal and colon polyps with lymphoid follicles in \( \text{Gli1}^{\text{CreER};\text{Pten}^{+/+}} \) mice. Error bars represent mean ± standard deviation. \( N = 6 \) per group per anatomical location.

See also Figures S2–S5.
Figure 3. LKB1 deficiency in Gli1+ mesenchymal cells induces polyp formation

(A–C) Representative histological images of the junction of small intestine and stomach in wild-type mice (control) and polyps developed in Gli1CreER;Lkb1fl/+ mice at 4 months and 12 months following tamoxifen injection.

(A and B) Scale bar, 50 μm.

(C) Scale bars, 2.5 mm.
(D–F) Induced Cre activity in polyps from $Gli1^{Cre-ER};LKB1^{fl/+};R26-mT/mG$ mice representing LKB1-deficient mesenchymal cells as depicted by GFP signal. Ep, epithelium; Me, mesenchyme. Scale bars, 50 μm.

(G) Immunohistochemistry of Ki67, SMA, desmin, vimentin, and CD20 in polyps from $Gli1^{CreER};Lkb1^{fl/+}$ mice. Scale bars, 50 μm.

See also Figures S4 and S5.
Figure 4. Differential requirement for mTOR during polyposis associated with Pten or LKB1 deficiency

(A) Immunohistochemistry of phospho-AKT (pAKT) and phospho-S6 (pS6) in polyps carrying LKB1 or Pten deficiency showing differential activation of AKT and S6 activity in polyp mesenchyme. Arrows point to the mesenchymal compartment of the polyps. Scale bars, 50 μm.

(B) Immunoblot analysis of pAKT levels in control intestine and polyps with Pten or LKB1 deficiency.
(C–F) Representative histological images of the colon samples from Gli1\textsuperscript{CreER};Pten\textsuperscript{fl/fl}, Gli1\textsuperscript{CreER};mTOR\textsuperscript{fl/fl}, and Gli1\textsuperscript{CreER};Pten\textsuperscript{fl/fl};mTOR\textsuperscript{fl/fl} mice. Scale bars, 2 mm.

(G) The numbers of colon polyps detected in Gli1\textsuperscript{CreER};Pten\textsuperscript{fl/fl}, Gli1\textsuperscript{CreER};mTOR\textsuperscript{fl/fl}, and Gli1\textsuperscript{CreER};Pten\textsuperscript{fl/fl};mTOR\textsuperscript{fl/fl} mice. n = 6 per group, **p < 0.01.

(H) Survival analysis of Gli1\textsuperscript{CreER};Pten\textsuperscript{fl/fl} Gli1\textsuperscript{CreER};mTOR\textsuperscript{fl/fl} and Gli1\textsuperscript{CreER};Pten\textsuperscript{fl/fl};mTOR\textsuperscript{fl/fl} mice. n = 17 per group, **p < 0.01.

(I–L) Whole amount (I and J) and histological (K and L) images of the polyps detected at the junction of small intestine and stomach in Gli1\textsuperscript{CreER};Lkb1\textsuperscript{fl/+} and Gli1\textsuperscript{CreER};Lkb1\textsuperscript{fl/+};mTOR\textsuperscript{fl/fl} mice. Scale bars, 2.5 mm.

(M and N) Immunohistochemistry of Ki67 in the polyps from Gli1\textsuperscript{CreER};Lkb1\textsuperscript{fl/+} and Gli1\textsuperscript{CreER};Lkb1\textsuperscript{fl/+};mTOR\textsuperscript{fl/fl} mice. Scale bars, 50 μm.

(O) Quantification of Ki67 staining in the polyp mesenchyme from Gli1\textsuperscript{CreER};Lkb1\textsuperscript{fl/+} and Gli1\textsuperscript{CreER};Lkb1\textsuperscript{fl/+};mTOR\textsuperscript{fl/fl} mice. n = 3 animals per group, 8–10 fields per animal.

(P) Survival analysis of the control, Gli1\textsuperscript{CreER};LKB1\textsuperscript{fl/+}, and Gli1\textsuperscript{CreER};LKB1\textsuperscript{fl/+};mTOR\textsuperscript{fl/fl} mice. n = 5 per group. Scale bars, 50 μm.
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Rabbit anti-Ki67    | Abcam  | Cat# ab15580, RRID: AB_443209 |
| Rabbit anti-Vimentin| Cell Signaling | Cat# 5741, RRID: AB_10695459 |
| Rabbit anti-CD20    | Abcam  | Cat# ab64088, RRID:AB_1139386 |
| Rabbit anti-CD3     | Abcam  | Cat# ab16669, RRID:AB_443425 |
| Rabbit anti-Phospho-AKT | Cell Signaling | Cat# 4060, RRID:AB_2315049 |
| Rabbit anti-Phospho-S6 | Cell Signaling | Cat# 5364, RRID:AB_10694233 |
| Rabbit anti-Sox9    | Abcam  | Cat# ab185230, RRID: AB_2715497 |
| Rabbit anti-Olfm4   | Cell Signaling | Cat# 14369, RRID:AB_2798465 |
| Mouse anti-Lysozyme| Novus  | Cat# NBP1–95509, RRID: AB_11037059 |
| Mouse anti-β-Catenin| BD Biosciences | Cat# 610153, RRID:AB_397554 |
| Rabbit anti-Desmin  | Abcam  | Cat# ab32362, RRID:AB_731901 |
| Rabbit anti-a-smooth muscle actin | Abcam | Cat# ab7817, RRID:AB_262054 |
| Rabbit anti-AKT     | Cell Signaling | Cat# 4685, RRID:AB_2225340 |
| Rat anti-CD44       | Thermo Fisher Scientific | Cat# 14–0441-82 RRID: AB_467246, |
| Rabbit anti-GAPDH   | Bethyl | Cat# A300–641A |
| Anti-Rabbit HRP     | Cell Signaling | Cat# 7074S, RRID:AB_2099233 |
| Anti-Mouse HRP      | Cell Signaling | Cat# 7076S, RRID:AB_330924 |
| Alexa Fluor 568, goat anti-rabbit | Thermo Fisher Scientific | Cat# A-11011, RRID:AB_143157 |
| Alexa Fluor 633, goat anti-rabbit | Thermo Fisher Scientific | Cat# A-21070, RRID:AB_2535731 |

Chemicals, peptides, and recombinant proteins

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Laemmli (SDS-Sample Buffer, Reducing, 6X) | Boston BioProducts | Cat# BP-111R |
| Trizol reagent      | Invitrogen | Cat# 15596026 |
| Tomoxifen           | Sigma  | Cat# T5648 |

Critical commercial assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Vectastain Elite ABC kit | Vector Lab | Cat# PK-6011 |

Experimental models: Organisms/strains

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse: VillinCre    | (Madison et al., 2002) | JAX# 024842 |
| Mouse: Gli1CreER    | (Ahn and Joyner, 2004) | JAX# 007913 |
| Mouse: Lgr5CreER    | (Barker et al., 2007) | JAX# 008875 |
| Mouse: Lkb1flox     | (Bardeesy et al., 2002) | NCI Mouse Repository: 01XN2 |
| Mouse: Gli1LacZ     | (Bai et al., 2002) | JAX# 008211 |
| Mouse: Nk3.2Cre     | (Verzi et al., 2009) | N/A |
| Mouse: Ptenflox     | (Lesche et al., 2002) | JAX# 006440 |
| Mouse: mTORflox     | (Risson et al., 2009) | JAX# 011009 |
| Mouse: R26Mt-mG     | (Muzumdar et al., 2007) | JAX# 014141 |