Intestinal LKB1 loss drives a pre-malignant program along the serrated cancer pathway

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Abstract

Peutz-Jeghers syndrome (PJS) is a familial disorder caused by heterozygous inactivating Liver Kinase B1 (*LKB1)* mutations that promote gastrointestinal polyposis and enhance cancer susceptibility. How *LKB1*-deficiency alters the phenotypical landscape and hierarchical organization of epithelial tissues to mediate increased cancer risk remains poorly understood. Here, we employ small intestinal organoids to investigate these issues for heterozygous and homozygous *Lkb1* epithelial loss. We show that *Lkb1* loss causes an allele dosage-dependent activation of a transcriptional program for tissue repair that is already induced in stem cell populations and mediates alterations in secretory cell type morphology and positioning. Furthermore, this shift towards a regenerative state omits the need for EGF supplementation, thus inducing niche-independent properties. Strikingly, we uncover that heterozygous loss of *Lkb1* is sufficient to push the epithelium into a premalignant program for colorectal carcinogenesis along the serrated pathway, which is further amplified by loss-of-heterozygosity (LOH) or Kras mutations. We conclude that persistent upregulation of a regenerative program due to *LKB1* loss alters cellular hierarchy and induces niche independency, which predisposes PJS epithelium to uncontrolled growth along the serrated pathway.

Introduction

The Peutz-Jeghers syndrome (PJS) is a hereditary genetic disorder that occurs in approximately 1:50.000 - 1:200.000 individuals worldwide (Beggs *et al.*, 2010). PJS patients suffer from a life-long development of benign hamartomatous polyps throughout their gastrointestinal tract for which they need periodical surgical resection. Moreover, patients carry an increased risk of developing early-onset epithelial tumors, particularly within the gastrointestinal (GI) tract (up to 66%), breast (up to 54%), pancreas (up to 36%) and gonads (Giardiello *et al.*, 2000; Hearle *et al.*, 2006; Beggs *et al.*, 2010; Van Lier *et al.*, 2010a), resulting in a cumulative lifetime risk of 93% (Giardiello *et al.*, 2000). Of note, PJS patients are predisposed to malignancies of the small intestine (frequency 13%), a condition that is otherwise very rare (0.03%) (Van Lier *et al.*, 2010a; Tomasetti and Vogelstein, 2015).

Heterozygous inactivating mutations in the tumor suppressor gene Liver Kinase B1 (*LKB1*) were identified as the main cause of PJS (Hemminki *et al.*, 1998; Jenne *et al.*, 1998). Furthermore, *LKB1* mutations are causally linked to a broad range of sporadic cancer cases, including lung carcinoma, melanoma, pancreatic and cervical cancers (Giardiello and Trimbath, 2006; Sanchez-Cespedes, 2007). *LKB1* encodes a serine/threonine kinase implicated in the regulation of a variety of critical cellular processes, including energy metabolism, cell adhesion, DNA methylation and apicobasal polarity (Shackelford and Shaw, 2009; Kline *et al.*, 2013; Pierce *et al.*, 2021). Loss of heterozygosity (LOH) in this gene is regarded as a driving force of cancer development, as over 64% of PJS-derived carcinomas present with inactivation of the second copy of the gene (Entius *et al.*, 2001; De Leng *et al.*, 2003).

Over recent years, modelling of PJS in murine models revealed that *Lkb1* loss in either mesenchymal or T cell lineages induces development of gastro-intestinal polyps with strong resemblance to those observed in PJS patients. (Katajisto *et al.*, 2008; Ollila *et al.*, 2018; Poffenberger *et al.*, 2018; Cotton *et al.*, 2022). These results have shifted focus to non-epithelial roles of *Lkb1* in driving PJS disease development, with an emphasis on processes underlying polyposis. If and how polyp formation in PJS patients links to cancer predisposition however remains a heavily debated issue, as LOH and dysplastic transformation of PJS polyps were reported to be very rare (Shepherd, Bussey and Jass, 1987; Entius *et al.*, 2001; De Leng *et al.*, 2003; Korsse *et al.*, 2013). These observations differ from other polyposis syndromes, such as familial adenomatous polyposis (FAP), where epithelial loss of the underlying germline mutation in the adenomatous polyposis coli gene APC and its role in cancer development has been well described (Fodde, 2002).

Since *LKB1* loss mediates predisposition to carcinoma development in multiple epithelial tissues irrespective of polyp formation, we hypothesized that epithelial-intrinsic alterations may sensitize these tissues to cancer formation. Previous studies indicate that intestinal epithelial loss of *LKB1* does not lead to polyp formation while alterations in secretory cell fate specification were identified (B Y Shorning *et al.*, 2009; Gao *et al.*, 2020; Cotton *et al.*, 2022; Kim *et al.*, 2022). Despite these advancements, how epithelial *LKB1* loss changes the phenotypic landscape of the intestinal epithelium and how these alterations may link to cancer predisposition in PJS patients remains poorly understood.

Here, we model *mono*- and *bi*-allelic inactivation of *Lkb1* in small intestinal organoids to investigate how *Lkb1* loss alters the dynamics of cellular identities and lineage relationships of the intestinal epithelium. We uncover that *Lkb1* loss promotes features of epithelial transformation linked to the serrated pathway for intestinal carcinogenesis. These features include loss of classical adult stem cell populations, activation of transcriptional programs related to regeneration and intestinal-to-gastric cellular transdifferentation, as well as the acquirement of niche-independent growth properties. Our findings argue that *Lkb1* loss mediates epithelium-wide activation of a pre-cancer program akin to sporadic serrated precursor lesions, providing an explanation for the increased cancer risk observed in PJS patients. By linking *Lkb1* loss to a major pathway for colorectal cancer development, these findings pave a path for the development of new strategies for prevention, surveillance and precision treatment of PJS tumors as well as other malignancies harboring *LKB1* inactivating mutations.

Results

Lkb1 bi-allelic loss results in phenotypic heterogeneity and increased self-renewal potential of small intestinal organoids.

To study the role of *Lkb1* loss in the intestinal epithelium, we employed CRISPR/Cas9 technology to generate *Lkb1*-deficient mouse small intestinal organoid lines. To obtain homogenous organoid cultures that are heterozygous or homozygous for *Lkb1* loss, we isolated single organoids from bulk cultures and expanded these as individual clones. After genotyping, we selected one *Lkb1*$^{+/-}$ and two *Lkb1*$^{-/-}$ clonal lines for further characterization (Figure S1A, Figure 1A-B). *Lkb1*$^{+/-}$ organoids were morphologically similar to wild type (WT) organoids, with typical budding structures that reflect a crypt-villus organization (Sato *et al.*, 2009) (Figure 1A). Remarkably, the introduction of homozygous *Lkb1* loss resulted in phenotypic bifurcation, with clonal lines displaying either a budding or a cystic growth morphology (Figure 1A). Notably, from day 3 after passaging, budding *Lkb1*$^{-/-}$ (*Lkb1*$^{bud\ -/-}$) organoids accumulated clusters of dead cells in their periphery (Figure 1A), as indicated by their positive staining for cleaved caspase 3 (Figure S1B). These observations are different from *Lkb1*$^{+/-}$ and WT organoids that mainly accumulate dead cells in their lumen (Figure 1SB) (Sato *et al.*, 2009).

Next, we tested whether *Lkb1* loss alters the ability of intestinal organoids to reconstitute organoids from single cells, as a proxy for their self-renewal capacity. While budding WT and *Lkb1*-mutant organoids lines displayed similar outgrowth rates, cystic *Lkb1*$^{-/-}$ (*Lkb1*$^{cys\ -/-}$) organoids carried a significantly improved capacity to form organoids upon seeding of single cells (Figure 1C). Regardless of these observations, both homozygous mutant morphologies displayed a similar increase in the number of proliferative cells in comparison to heterozygous and wild-type organoids, as shown by EdU incorporation (Figure 1D, E). To rule out possible CRISPR off-target effects, we used an alternative gRNA to introduce *Lkb1* mutations, which resulted in an identical phenotypic heterogeneity of *Lkb1*$^{-/-}$ organoid lines (Figure S1C-E). Moreover, these newly generated *Lkb1*$^{cys\ -/-}$ clones also displayed superior reconstitution potential compared to their homozygous budding counterparts (Figure S1F). These findings reveal that biallelic *Lkb1* loss results in phenotypic heterogeneity within the intestinal epithelium, and that homozygous *Lkb1*-mutant organoids with cystic morphology display an increased recovery potential.

Lkb1 loss alters the number, morphology and positioning of secretory cells.

To investigate whether our *Lkb1*-mutant organoid lines serve as accurate models for intestinal epithelial loss of *Lkb1 in vivo*, we examined alterations in localization and morphology of Paneth (Lyz^{+}) and goblet (Muc2^{+}) cells that were previously reported for the *Lkb1*-mutant mouse intestine (Boris Y. Shorning *et al.*, 2009; Gao *et al.*, 2020). Compared to WT organoids, the localization of Lyz1^{+} Paneth cells in *Lkb1*$^{+/-}$ and *Lkb1*$^{bud\ -/-}$ organoids was not restricted to the bottom of the crypt but displayed a more scattered distribution that reached into the villus domain (Figure 2A). In addition, while Muc2^{+} cells in WT and *Lkb1*$^{+/-}$ organoids were present in modest numbers and always located to the villus region, Muc2^{+} cell numbers were increased in *Lkb1*$^{bud\ -/-}$ organoids and found scattered throughout the crypt-villus axis (Figure 2B). These observations are highly reminiscent of the reported aberrancies in Paneth and Goblet cell numbers and localization in the *Lkb1*-deficient mouse intestine *in vivo* (B Y Shorning *et al.*, 2009). By contrast, *Lkb1*$^{cys\ -/-}$ organoids were devoid of Paneth cells but did allow for the formation of Muc2^{+} cells that are usually lacking in cystic

organoids (Sato, Es, *et al.*, 2011), indicating that secretory lineage specification is also altered within these organoids (Figure 2A-B).

We next investigated how these findings relate to intestinal tissue organization of human PJS patients. While the average number and location of MUC2^{+} cells per intestinal crypt was similar for healthy donors and PJS patients (Figure 2C, Figure S2A-B), LYZ1^{+} Paneth cells were clearly mislocalized within the PJS mucosa (Figure 2D). In the healthy intestine, Paneth cells localized strictly to the crypt base in the healthy intestine, as expected (Barker *et al.*, 2007). In PJS epithelia, however, Paneth cells frequently scattered towards higher regions of the crypt (Figure 2D). Thus, heterozygous loss of *LKB1* in PJS patient-derived intestines leads to mislocalisation of Paneth cells but does not alter goblet cell distribution, similar to our observations in *Lkb1*$^{+/-}$ organoids.

To examine *Lkb1* loss-induced alterations in secretory cell morphology at the ultrastructural level, we employed electron microscopy. In WT intestinal organoids, Paneth cells displayed a typical morphology with prominent apical accumulation of spherically-shaped secretory granules that are uniformly filled with electron-dense content (Figure 2E) (Satoh *et al.*, 1990; Sato *et al.*, 2009). By contrast, secretory granules of *Lkb1*$^{+/-}$ Paneth cells commonly contained an abnormally 'sausage-shaped' electron-dense core (Figure 2E). In *Lkb1*$^{bud-/-}$ organoids, the content of Paneth cell-secretory granules was even more aberrant, displaying a small electron-dense core surrounded by a peripheral electron-lucent halo (Figure 2E). In WT intestinal organoids, columnar-shaped goblet cells were identified with large translucent granules filled with mucus (Figure 2F) (Troughton and Trier, 1969; Bansil and Turner, 2018). In *Lkb1*-deficient clones (*Lkb1*$^{+/-}$ and *Lkb1*$^{bud-/-}$), mucus-secreting cells displayed a more round and flattened morphology compared to WT goblet cells (Figure 2F). In addition, in *Lkb1*$^{cys-/-}$ organoids the content of these mucus-like granules was frequently lost during EM preparation, suggesting an altered mucin composition, which might also explain the limited number of identified Muc2^{+} cells (Figure 2F). Furthermore, previous studies identified roles of *LKB1* in apical brush border formation, using 2D human (cancer) cell lines (Baas *et al.*, 2004). Inspection of our EM images however did not reveal any evidence for loss of these subcellular structures in *Lkb1*-deficient organoids (Figure S2C).

Taken together, our findings reveal that engineering *Lkb1* loss in organoids induces highly similar alterations in secretory cell number, localization and morphology as those observed in *in vivo* mouse models and the intestine of PJS patients. These results thus confirm the suitability and relevance of organoids for modelling epithelial alterations in PJS. Furthermore, our results indicate that *Lkb1* loss-induced alterations in cell lineage specification and positioning are intrinsic to the epithelium and do not rely on interactions with the mesenchyme.

***Lkb1* mono- and bi-allelic loss induces incremental expression of a regenerative gene signature**

To examine how *Lkb1* loss affects epithelial organization, we determined transcriptional changes induced by *Lkb1* loss in organoids using bulk mRNA sequencing. Compared to WT organoids, we observed differential expression of 1298, 2312 and 7816 genes in *Lkb1*$^{+/-}$, *Lkb1*$^{bud\ -/-}$ and *Lkb1*$^{cys\ -/-}$ organoids, respectively (Supplementary Table 1). Using gene set enrichment analysis (GSEA) we first examined the consequences of *Lkb1* loss on well-defined gene signatures derived from individual intestinal cell types (Haber *et al.*, 2017). In agreement with our earlier results, all *Lkb1*-deficient clones showed significant downregulation of gene sets linked to mature Paneth cells, whereas goblet cell gene sets were enriched

within both budding and cystic *Lkb1*$^{-/-}$ organoids in comparison to WT organoids, again suggesting alterations in secretory cell lineage specification (Figure 3A).

Noticeably, the *Lgr5*$^{+}$ adult stem cell signature (Haber *et al.*, 2017) was downregulated in all *Lkb1*-deficient clones (Figure 3A). These data are reminiscent of the previously reported growth state of fetal intestinal spheroids that relies on proliferation of *Lgr5*-negative progenitors (Mustata *et al.*, 2013). Indeed, we uncovered a gradual enrichment of an embryonic spheroid gene signature in *Lkb1*-deficient organoids (Mustata *et al.*, 2013), that correlated with the number of lost *Lkb1* alleles (Figure 3A-B). The transition from adult stem cell to a fetal spheroid signature was most striking for *Lkb1*$^{cys\ -/-}$ organoids compared to *Lkb1*$^{bud\ -/-}$ organoids (Figure 3B). Previous studies indicated that a transient upregulation of a fetal-like intestinal gene signature associates with epithelial remodeling during injury repair and wound healing (Fordham *et al.*, 2013; Nusse *et al.*, 2018; Yui, Azzolin, Maimets, Pedersen, Robert P Fordham, *et al.*, 2018; Wang *et al.*, 2019). In line with these observations, *Lkb1*-deficient organoid clones also exhibited enrichment of gene signatures associated with intestinal regeneration following damage or infection response (Figure 3A). Furthermore, *Lkb1*-deficient clones, as well as control fetal intestinal organoids (E16.5), showed an increased expression of the fetal marker Anxa1, while this marker was not detected in WT adult intestinal organoids (Figure 3C) (Yui, Azzolin, Maimets, Pedersen, Robert P. Fordham, *et al.*, 2018). In addition, a dysplastic area of human PJS patient-derived duodenal epithelial tissue showed patches of cells that stain positive for ANXA1 protein expression, while non-polyp and healthy duodenum epithelial cells remained negative (Figure 3D). Together, these findings indicate that *Lkb1* loss converts the epithelium into a permanent regenerative state. Strikingly, this conversion towards a tissue repair growth state is already apparent upon monoallelic *Lkb1* loss, a representative condition for the PJS intestinal epithelium.

Lkb1-deficient organoids show a Yap-induced regenerative state that promotes intra-epithelial self-sufficiency for Egf.

Over recent years, Yap signaling was placed central to intestinal regeneration (Gregorieff *et al.*, 2015). Additionally, *LKB1* was uncovered previously as a negatively regulator of the Hippo-Yap pathway in an siRNA kinome screen (Mohseni *et al.*, 2014). We therefore wondered if *Lkb1* loss may activate Yap signaling as part of the induced regenerative program in intestinal organoids. Indeed, we identified enhanced expression of a Yap-regulated gene signature linked to intestinal regeneration in *Lkb1*-mutant organoids (Figure 3A) (Gregorieff *et al.*, 2015). Notably, increased Yap target gene expression was already identified in heterozygous *Lkb1*-mutant conditions and progressively increased with loss of both *Lkb1* alleles (Figure S3). Again, *Lkb1*$^{cys\ -/-}$ organoids showed the strongest induction of Yap target genes, in line with their robust expression of a regenerative transcriptional program (Figure S3). Immunofluorescence staining further confirmed an increase in Yap protein expression in both budding and cystic *Lkb1*$^{-/-}$ organoids, and a prominent nuclear localization of Yap, particularly within *Lkb1*$^{cys\ -/-}$ organoids (Figure 4A-B).

YAP pathway activation is well-known to induce enhanced expression of EGF family ligands, particularly in the context of regeneration (Zhang *et al.*, 2009; Ren *et al.*, 2010; Yang *et al.*, 2012; Gregorieff *et al.*, 2015). We therefore wondered whether *Lkb1*-deficient organoids display altered state of EGF pathway dependency. Noticeably, we found that a combined set of the *Egf* ligand family were upregulated in *Lkb1*-deficient organoids, in a gradual manner along with *Lkb1* allelic loss and alterations in organoid morphology (Figure 5A). Furthermore, these changes were accompanied with an elevated expression of *Egf* receptor

family members (Figure 5B). As survival and growth of healthy homeostatic adult intestinal organoids depends on supplementation with Egf, we investigated whether the enhanced expression of *Egf* family ligands and its receptors in *Lkb1*-deficient organoids mediates niche factor self-sufficiency, by omitting Egf from the culture medium. In contrast to WT organoids, all *Lkb1*-deficient organoids were capable of growing without Egf supplementation (Figure 5C). In support of these findings, we confirmed that organoid survival still depended on EGFR signaling, since *Lkb1*-deficient organoids were sensitive to Gefitinib, a potent EGFR inhibitor (Figure 5D).

Together, these results demonstrate that *Lkb1* loss induces a constitutive Yap-induced regenerative state which drives epithelial self-sufficiency for EGF ligand production. As niche-independent growth properties are considered a hallmark of cancer progression (Fujii *et al.*, 2016), these results provide a potential explanation for a growth advantage of *Lkb1*-deficient epithelial cells and the initiation of tumorigenesis.

***Lkb1*-deficiency mediates reprogramming of intestinal stem cells into a regenerative state.**

To perform an in-depth analysis of how *Lkb1* loss alters intestinal lineage specification and cellular hierarchies, we subjected WT and *Lkb1*-deficient organoids to single cell RNA sequencing. We included an analysis of mouse fetal intestinal organoids, to explore a potential overlap with fetal gene expression. Unsupervised clustering of single cells identified 19 distinct clusters (Figure S4A). A clear separation of cellular profiles derived from $Lkb1^{cys\ -/-}$ organoids, fetal and WT cells was observed, while $Lkb1^{bud\ -/-}$ and $Lkb1^{+/-}$ organoids displayed closer resemblance to WT cells (Figure 6A). The separation of $Lkb1^{cys\ -/-}$ and fetal cell clusters in UMAP indicates that $Lkb1^{cys\ -/-}$ organoids do not fully convert to a fetal state but only show partial expression of a fetal-like gene program (Figure 6A). Furthermore, $Lkb1^{cys\ -/-}$ organoids are composed of a rather homogenous population of cells that are different from all budding organoid clusters. We therefore restricted annotation of intestinal cell types to budding organoid lines, using publicly available intestinal cell type gene signatures (Figure 6C, Figure S4B-I) (Haber *et al.*, 2017). Cell type fraction analysis revealed that $Lkb1^{bud\ -/-}$ organoids comprise an increased fraction of goblet cells compared to WT organoids, confirming alterations in secretory cell differentiation (Figure 6B). Interestingly, the increase in goblet cells was accompanied with a decrease in enterocytes, suggesting that the preferred differentiation trajectory in *Lkb1*-deficient budding organoids is shifted from absorptive towards secretory lineages (Figure 6C). The previously observed morphological aberrancies in secretory lineage specification were confirmed at the transcriptional level, as *Lkb1*-deficient organoids displayed the occurrence of 'hybrid' cells that co-express features of both Paneth and goblet cells (Figure 6D). Moreover, expression of Egf ligands was specifically increased in *Lkb1*-deficient enteroendocrine and goblet secretory cells (Figure 6E) suggesting that these cells might be responsible for the observed niche-independency of *Lkb1*-mutant organoids.

As expected, expression of the fetal and regenerative marker *Anxa1* is highly abundant in both fetal and $Lkb1^{cys\ -/-}$ organoids (Figure 6F). Notably, we found that *Anxa1* expression in $Lkb1^{bud\ -/-}$ organoids is induced mainly within stem and transit amplifying (stem/TA) cell clusters, indicating that this population is the main contributor to the observed epithelial conversion into a regenerative state (Figure 6A, F). To further test this assumption, we performed reclustering of stem/TA cell populations including all $Lkb1^{cys\ -/-}$ cells. Unsupervised clustering indicates distinct clusters for WT, $Lkb1^{+/-}$ and $Lkb1^{bud\ -/-}$ stem/TA cells (Figure S4J-K). To examine the effect of *Lkb1* loss on intestinal stem cells, we performed GSEA on genes differentially

expressed between WT stem cells and *Lkb1*-deficient stem cells (Supplementary Table 2). We observed enrichment of goblet cell gene expression within *Lkb1*-deficient stem cell populations compared to WT organoids (Figure 6G-H). This suggests that the capacity for preferential differentiation towards secretory cells is already present in *Lkb1*-deficient stem cells. Furthermore, we identified an enrichment of four out of six previously reported fetal and regenerative gene signatures, a depletion of the *Lgr5*$^{+}$ stem cell signature and an increased expression of Anxa1 in *Lkb1*-deficient stem cells (Figure 6G, I). These findings strengthen a model in which induction of a regenerative response by *Lkb1* loss originates from the stem cell compartment of the intestinal epithelium.

In conclusion, our results argue that *Lkb1*-deficient stem/TA cells reside in a regenerative state and preferentially differentiate towards secretory rather than absorptive cell lineages. As a result, secretory cells start secreting EGF ligands to drive niche-independent growth of *Lkb1*-deficient epithelium.

Lkb1 loss drives a pre-malignant program along the serrated pathway which is amplified by additional _Kras_ mutations.

Next, we aimed to obtain insight in how transcriptional reprogramming induced by *Lkb1* loss may promote cancer predisposition. Colorectal cancer (CRC) develops mainly via two pathways that involve distinct originating lesions and mutational events (Chen *et al.*, 2021). The classical adenoma-carcinoma sequence is initiated by bi-allelic mutations in the tumor suppressor APC which drives formation of stem cell-enriched adenomatous polyps that collect additional mutations (e.g. in KRAS and p53) to develop into microsatellite-stable CRC characterized by chromosomal instability (CIN) (Fearon and Vogelstein, 1990). Recently, an alternative pathway emerged, in which colorectal cancer arises from serrated precursor polyps that are initiated by BRAF or KRAS mutations and display combined features of fetal gene expression, differentiated cells and gastric metaplasia (Patai *et al.*, 2013; Chen *et al.*, 2021). Whether and how *Lkb1* loss drives entry into one of these pathways for CRC malignant progression is not known.

To investigate this issue, we examined expression of gene sets linked to the conventional and serrated pathways of CRC development in *Lkb1*-deficient organoids (Chen *et al.*, 2021). Strikingly, all *Lkb1*-deficient organoids display significant enrichment of genes that were previously found to mark neoplastic populations of serrated-specific cells (SSC) (Figure 7A). These effects gradually increased with the number of lost *Lkb1* alleles, along with a simultaneous decrease in the expression of adenoma-specific genes (ASC) (Figure 7B). Single cell analysis further revealed that the serrated gene program was selectively increased in *Lkb1*-deficient stem cell populations (Figure S5). Furthermore, *Lkb1*$^{cys\ -/-}$ organoids lose expression of the intestinal transcription factor *Cdx2* and upregulate expression of common gastric markers like *Aqp5*, *Tacstd2* [*Trop2*], *Tff2*, and *Msln* (Figure 7B, Supplementary Table 1), indicating that *Lkb1* loss promotes intestinal-to-gastric cellular transdifferentiation, often referred to as gastric metaplasia (Balbinot *et al.*, 2018; Chen *et al.*, 2021).

To investigate how an *Lkb1*-deficient epithelial state cooperates with common cancer mutations in CRC, we introduced *Apc* or *Kras* mutations in *Lkb1*-mutant organoids. Truncating *Apc* mutations are linked to the classical adenoma sequence, while *Kras* mutations are observed in both pathways (Fearon and Vogelstein, 1990; Patai *et al.*, 2013). As expected, based on earlier reports, introduction of a truncating *Apc* mutation induced cystic morphologies of all organoids (Figure S6A-B) (Sato, Stange, *et al.*, 2011; Dow *et al.*, 2015). Surprisingly, however, introduction of a G12D *Kras* mutation in *Lkb1*-deficient organoids also

consistently mediated transition to a cystic morphology, while single *Kras*-mutant organoids maintained their crypt-villus organization (Figure S6C-D). Functionally, *Kras* mutations conveyed synergistic effects on the ability of single *Lkb1*-deficient cells to reconstitute organoid growth (Figure 7C). By contrast, *Apc* mutations did not add any significant increase to the reconstitution capacity of *Lkb1*$^{-/-}$ organoids (Figure 7D).

To gain insight in how *Apc* or *Kras* mutations altered the phenotypic landscape of *Lkb1*-deficient organoids, we examined transcriptional expression profiles of all organoid lines using bulk mRNA sequencing (Supplementary Table 3). With the introduction of mutant *Apc,* a slight increase was observed in fetal and SSC gene programs in both *Lkb1*$^{+/-}$ and *Lkb1*$^{-/-}$ organoids, but the expression of stem cell and ASC genes remained high for this mutational combination (Figure 7E, Figure S6E). By contrast, introduction of mutant *Kras* in *Lkb1*$^{+/-}$ or *Lkb1*$^{bud\ -/-}$ backgrounds induced a strong shift towards expression of fetal and SSC genes, while stem and ASC genes were downregulated, to a similar extent as observed for *Lkb1*$^{cys\ -/-}$ organoids (Figure 7E, Figure S6E). In *Lkb1*$^{cys\ -/-}$ organoids, introduction of mutant *Kras* did not further enhance the already strong serrated features, while introduction of truncated *Apc* in these organoids clearly suppressed the serrated state. Quantification of these findings by GSEA confirmed a significant amplification of serrated signature by the introduction of *Kras* mutations in both *Lkb1*$^{+/-}$ and *Lkb1*$^{bud\ -/-}$ organoids, and downregulation of the serrated pathway by introduction of mutant Apc (Figure 7F). We conclude that *Lkb1* mutations synergize with mutant *Kras,* but not *Apc,* to drive expression of the pre-malignant serrated gene program.

To investigate the link between *Lkb1* loss and advanced serrated colorectal tumors, we examined the TCGA colorectal cancer database. First, we observed that *LKB1*-mutant tumors are significantly enriched for mutations associated with serrated cancers, including *BRAF*, *RNF43* and *PIK3CA* mutations, while *APC* mutations were significantly enriched in *LKB1* WT tumors (Figure 7G) (Day *et al.*, 2013; Robertson *et al.*, 2019). Mutations found in both classical and serrated adenocarcinomas, such as mutant *KRAS* and *TP53*, were not significantly altered between *LKB1* WT and mutant tumors (Figure 7G). We next scored TCGA colorectal samples for expression of a gene set that was upregulated in all *Lkb1*-deficient organoids. Strikingly, expression of this *Lkb1*-deficient gene signature correlated with a mucinous histological CRC subtype that is often found in serrated adenocarcinomas (Figure 7H) (Tuppurainen *et al.*, 2005). In addition, among the consensus molecular subtypes (CMS), the serrated subtypes CMS1 and CMS4 carry significantly higher expression of the *Lkb1*-deficient gene signature compared to the classical subtype CMS2 (Figure 7I) (Nguyen, Goel and Chung, 2020). A recent study by Joanito *et al.* redefined the CMS classification and found two epithelial intrinsic cell states in CRC samples (Joanito *et al.*, 2022). The iCMS2 state shows more characteristics of classical CRCs, while the iCMS3 state correlates with features of serrated CRCs. Using GSEA, we also found enrichment of iCMS3 transcriptional features in our *Lkb1*-deficient organoids, while the iCMS2 state was downregulated (Figure S6F).

These results indicate that *Lkb1* loss drives cancer predisposition along the serrated pathway of CRC development. *Kras* activating mutations cooperate with *Lkb1* loss by amplifying the serrated state. Furthermore, upon homozygous *Lkb1* loss, a fraction of mutant cells grows out into *Lkb1*$^{cys\ -/-}$ organoids that achieve an advanced serrated state without additional oncogenic mutations. Together, these findings suggest that loss of *LKB1* converts intestinal epithelial cells in a pre-malignant state that primes for progression along the serrated pathway.

Discussion

Over recent years, a variety of *Lkb1*-deficient mouse models have faithfully mimicked intestinal hamartomatous polyp formation as a hallmark for PJS disease. The combined findings have promoted a view in which non-epithelial functions of *LKB1* contribute to PJS disease development (Katajisto *et al.*, 2008; Ollila *et al.*, 2018; Poffenberger *et al.*, 2018; Cotton *et al.*, 2022). Despite this progress, the relationship between *LKB1* loss-induced hamartomatous polyposis and epithelial cancer predisposition has remained highly debated and, moreover, does not explain cancer development in extra-intestinal epithelia (Shepherd, Bussey and Jass, 1987; Entius *et al.*, 2001; De Leng *et al.*, 2003; Van Lier *et al.*, 2010b; Korsse *et al.*, 2013). Furthermore, how heterozygous and homozygous loss of *LKB1* mediates dose-dependent alterations in PJS epithelial tissue organization at the single cell level has not been addressed, leaving the underlying cancer driver mechanisms and pathways obscure.

Here, we employed mouse small intestinal organoids, an epithelium-only culture system, to obtain an in-depth understanding of how *Lkb1* loss affects intestinal epithelial homeostatic cell identities and examine how these alterations drive the epithelium into a pre-malignant state for colorectal cancer formation. By generating both heterozygous and homozygous knockouts of *Lkb1*, we aimed to model aspects of cancer predisposition and development in PJS patients, as over 64% of PJS-derived carcinomas present inactivation of the second copy of the gene (Entius *et al.*, 2001; De Leng *et al.*, 2003). Our findings argue that heterozygous *Lkb1* loss in the intestinal epithelium is sufficient to push cells into a premalignant programme for tumour development along the serrated colorectal cancer pathway, by a mechanism independent of the micro-environment. Furthermore, expression of the serrated premalignant program is amplified upon loss of the second allele. These results thus reveal that heterozygous *Lkb1*-mutant PJS epithelia already have undergone alterations in phenotypic cellular identities in comparison to healthy individuals, and supports the view that loss of the second allele is a driver of cancer formation.

Our organoid-based model further confirms the previously described link between *Lkb1* loss and aberrant secretory cell specification *in vivo* in mice, indicating that our model faithfully reproduces the *in vivo* situation. We observed an increase in goblet cell numbers and mucus formation and identified secretory cells that display 'hybrid' features, based both on morphology and transcriptome analysis. In line with these findings, we show that expression of our newly identified *Lkb1*-deficient gene signature correlates with a mucinous histological subtype of sporadic colorectal cancer cases. Together, these findings indicate a common link between early premalignant alterations in secretory cell type specification and colorectal cancer subtype progression. Similar *Lkb1* loss-induced alterations in secretory cell identities may play a role in other epithelial tissues, as mucinous carcinomas in different organs were frequently reported in PJS patients (Sato *et al.*, 2001; Osoegawa *et al.*, 2011; Collet *et al.*, 2020). If and how aberrant secretory cell specification may facilitate cancer development however remains an issue for further investigation.

Besides the observed alteration in secretory cell identities, transcriptomic analysis revealed that *Lkb1* loss induces an allele dosage-dependent expression of a regenerative epithelial program. Notably, activation of this tissue repair program occurred in regular culture conditions where no intestinal epithelial damage was induced. We conclude that the *Lkb1*-deficient epithelium resides in a persistent regenerative state, a condition that is worsened by loss-of-heterozygosity. A chronic state of regeneration may contribute to an increase in tumour development as exemplified by the increased incidence of colorectal cancer formation in patients suffering from inflammatory bowel disease (Lutgens *et al.*, 2013). As part of the induced

regenerative/YAP signaling-dependent program, the *Lkb1*-deficient epithelium showed an increase in transcriptional expression of the *Egf* receptor family and its ligands, which allowed them to grow in the absence of Egf supplementation, a condition that is not tolerated by healthy small intestinal organoids. These findings thus reveal that the regenerative growth state induced by *Lkb1* loss endows the intestinal epithelium with niche-independent properties, which is regarded as a hallmark of cancer (Hanahan and Weinberg, 2011). Furthermore, these results provide an explanation of the previously reported expanded proliferative zone observed in the intestines of PJS patients (De Leng *et al.*, 2007)

Importantly, we uncovered that *Lkb1* loss predisposes the intestinal epithelium to express a gene program related to the serrated pathway for colorectal cancer formation. In support of these observations, *Lkb1* loss induced common features of early serrated lesions such as gastric metaplasia, induction of a regenerative gene program and depletion of classical adult stem cells (Chen *et al.*, 2021). Furthermore, inspection of a large set of sporadic colorectal cancer cases revealed that *LKB1* mutations frequently co-occur with driver mutations linked to the serrated colorectal cancer pathway. Additionally, we observed elevated expression of our *Lkb1*-deficient gene signature in the serrated colorectal cancer subtypes CMS1 and CMS4. Together, these findings thus suggest that the pathways employed for colorectal cancer development in PJS patients are strikingly different from the well-studied cancer driver events in familial adenomatous polyposis (FAP) patients that develop conventional *APC*-mutant adenocarcinomas (Li *et al.*, 2020).

Based on the work of Chen and colleagues (Chen *et al.*, 2021) a model was proposed in which different environmental triggers, such as microbial dysbiosis and a subsequent MAPK pathway activation may drive the formation of sessile serrated lesions (IJspeert and Dekker, 2022; Shields *et al.*, 2022). Our findings indicate that different types of insults may induce a shift of the *Lkb1*-mutant epithelium towards full-blown expression of the premalignant serrated program. This is exemplified by introduction of oncogenic mutant *Kras* in *Lkb1*-deficient organoids, which amplified serrated features and synergistically cooperated with *Lkb1* loss for organoid-forming capacity. Alternatively, we found that *Lkb1* loss-of-heterozygosity induced two distinct organoid phenotypes that displayed either a crypt-villus organization or a cystic phenotype with strongly induced serrated features. Since both *Lkb1*$^{-/-}$ organoid types are genomically identical, we hypothesize that an unknown external stressor may induce this switch to a highly serrated cystic phenotype. Although the responsible molecular trigger remains to be identified, our findings indicate that *Lkb1*$^{-/-}$ cells are sensitized to undergo such a phenotypic switch and adopt a pre-malignant growth state. Based on our findings, we conclude that the *Lkb1*-mutant epithelium is primed for the sessile serrated cancer pathway and not the conventional adenoma-to-carcinoma sequence. In line with this reasoning, the introduction of *Apc* mutations in *Lkb1*-deficient organoids did not display synergistic effects on pre-malignant transcriptional features or organoid reconstitution capacity. Together, our findings suggest that the PJS epithelium is sensitized to events that synergistically drive cells into a premalignant serrated state, such as intestinal damage or activating mutations in MAPK pathway components.

PJS patients undergo extensive and life-long monitoring in the clinic, to resect obstructive polyps and screen for (pre)cancerous lesions. Our results argue that monitoring of PJS patients will require specific screening for the occurrence of serrated lesions that potentially may occur in non-polyp regions as well. Serrated lesions are known to be frequently overlooked as their diagnosis relies on the existence of an architecturally distorted serrated crypt that are difficult to identify with regular screening methods (Rosty *et*

al., 2012). Diagnosed serrated lesions may be confirmed by various biomarkers including an increased MUC5AC expression linked to the absence of CDX2 (Chen *et al.*, 2021), or the usage of additional markers identified in this study such as ANXA1.

Taken together, our study reveals that intestinal organoids comprise a valuable model for *LKB1* mutation-induced alterations in epithelial organization and growth in PJS patients. Our findings further establish that induction of a premalignant sessile serrated program is a hallmark of PJS disease and provides an explanation for increased cancer incidence in PJS patients. The presented models may provide a starting point to uncover which and how additional mutations and environmental stressors synergistically cooperate with *LKB1* mutations, and how interactions with stromal and immune components contribute to the initiation of PJS malignant growth of the PJS epithelium. Ultimately, in-depth knowledge on the pathways and processes involved in driving PJS epithelia into a pre-malignant state may help to design strategies that re-establish epithelial homeostasis and reduce the incidence of cancer development in PJS patients.

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Figure legends

Figure 1. *Lkb1*-deficient organoids show heterogeneous morphology and viability.
A) Representative brightfield images of mouse small intestine *Lkb1*-deficient organoid clones. Lower panel images show zoom-in images of boxed area in upper panel images. Scale = 100 µM **B)** Western blot analysis shows loss of Lkb1 expression in heterozygous CRISPR targeted clones and complete loss of Lkb1 protein levels in homozygously targeted clones. **C)** Cell reconstitution assay from single cells performed at day 7 identifies increased organoid forming capacities in *Lkb1*$^{cys-/-}$. One-way ANOVA (Dunnett) was applied for analysis. * = $p < 0.05$. **D)** EdU incorporation experiment marking the proliferative compartments in wild-type (WT) and *Lkb1*-deficient organoids. **E)** Quantification of EdU^{+} cells over the total number of cells (DAPI^{+} cells) of *Lkb1*-deficient organoids, *n*=1000.

Figure 2. *Lkb1* loss alters the number, location and granule composition of secretory cells. A) Representative images of immunostaining for Lysozyme1 (Lyz1) to detect Paneth cells in *Lkb1*-deficient clones. Images are max projections of z-stack. Scale bar = 25 µm. **B)** Immunostaining of Mucin 2 (Muc2) to visualize goblet cells in *Lkb1*-deficient clones. Scale bar =100 µm. **C-D)** Representative images of Mucin 2 **(C)** and Lysozyme 1 **(D)** staining to visualize the localization of goblet and Paneth cells respectively in healthy intestinal epithelium and PJS epithelium. Arrows point towards mislocalized Paneth cells. Scalebar = 100 µm. **E)** Electron microscopy images comparing the ultrastructure of Paneth cells and their secretory granules in wild-type organoids and *Lkb1*-deficient organoids. Scale bar upper panels = 5 µm, scale bar lower panels = 1 µm. **F)** Electron microscopy images comparing the ultrastructure of goblet cells and their secretory granules in wild-type organoids and *Lkb1*-deficient organoids. Scale bar upper panels = 5 µm, scale bar lower panels = 1 µm.

Figure 3. *Lkb1* loss upregulates a regenerative gene signature in the intestinal epithelium. A) *Lkb1*-deficient versus wild-type (WT) organoid derived bulk mRNA-seq sequencing data was analysed by gene set enrichment analysis (GSEA) for cell types (Haber *et al.*, 2017), colitis (Yui, Azzolin, Maimets, Pedersen, Robert P. Fordham, *et al.*, 2018), injury (Qu *et al.*, 2021a), parasite (Nusse *et al.*, 2018), fetal spheroid (Mustata *et al.*, 2013), Yap regeneration (Gregorieff *et al.*, 2015) and revival stem cell (Ayyaz *et al.*, 2019) gene sets. The normalized enrichment score (NES) is depicted on the X-axis and the false discovery rates (FDRs) is indicated by the size of the dots. The color of the lines reflects whether a signature is enriched in WT (red) or *Lkb1*-deficient (blue) organoids. The shape of the dot (open/closed) indicates whether a signature is significantly enriched (FDR < 0.05). **B)** Heatmap of z-score transformed expression values of stem cell genes enriched in WT organoids (top cluster) and fetal genes enriched in *Lkb1*-deficient organoids (bottom cluster). Key marker genes are highlighted. **C)** Immunofluorescent staining to detect expression of the fetal marker Anxa1 in *Lkb1*-deficient clones. Embryonic spheroids from E16.5 mice were used as positive control. **D)** Representative images of immunohistochemistry staining of the fetal marker ANXA1 in healthy intestinal epithelium, PJS normal epithelium and PJS dysplastic tissue. High magnification of the area indicated by black arrows is shown in the upper right corner. Scale bar = 100 µm

Figure 4. *Lkb1*-deficient organoids show a Yap-induced regenerative state.
A) Immunostaining of Yap in wild-type (WT) and *Lkb1*-deficient organoids. Nuclei are visualized by DAPI staining. High magnification of the area indicated by black arrows is shown in the upper right corner. Scale bar = 20 µm. **B)** Quantification of the nuclear fraction of Yap. Dots represent individual organoids. N=5 for WT organoids, N=7 for *Lkb1*$^{+/-}$ organoids, N=7 for *Lkb1*$^{bud\ -/-}$ organoids and N=7 for *Lkb1*$^{cys\ -/-}$. One-way ANOVA was applied for analysis and statistical significance was defined as ** = p < 0.05, ** = p < 0.01 and **** = p < 0.0001.*

Figure 5. *Lkb1*-deficient organoids upregulate EGF family receptors and its ligands for niche independency.
A-B) Average expression of *Egfr* binding ligands **(A)** and *Egf* family receptors **(B)** in wild-type (WT) and *Lkb1*-deficient organoids. One-way ANOVA was applied for analysis and statistical significance was defined as ** = p < 0.05, ** = p < 0.01 and **** = p < 0.0001.* **C)** Representative bright field images of WT and *Lkb1*-deficient organoids grown in the presence (ENR) and absence (NR) of Egf. Organoids were incubated for 7 days in indicated medium and split on day 4. Scale bar = 500 µm. **D)** Representative bright field images of WT and *Lkb1*-deficient organoids cultured in NR and treated with DMSO or 100 nM Gefitinib. Organoids were incubated for 9 days in indicated medium and split on day 6. Scale bar = 500 µm.

Figure 6. *Lkb1*-deficiency redefines intestinal stem cell identity.
A-B) UMAP visualization of scRNA-seq data from wild-type, fetal and *Lkb1*-deficient organoids. Colors indicate the different organoids lines **(A)** and the different cell types **(B)**. **C)** Fraction of cell types for each organoid line. Each color indicates a different cell type. **D)** Scatter plot showing module scores for a Paneth gene signature (Haber *et al.*, 2017) on the y-axis and for a goblet cell signature (Haber *et al.*, 2017) on the x-axis across all cells. Color/shape indicates the origin of the single cell. **E)** Violin plot showing a module score for Egf family ligands within the goblet and enteroendocrine (EEC) cluster. Colors reflect different organoid lines. **F)** Expression of the fetal marker *Anxa1* is overlaid onto the UMAP plot for the different organoid lines. **G)** GSEA was performed for cell types (Haber *et al.*, 2017), colitis (Yui, Azzolin, Maimets, Pedersen, Robert P. Fordham, *et al.*, 2018), embryonic spheroid (Mustata *et al.*, 2013), Yap regeneration (Gregorieff *et al.*, 2015) and revival stem cell (Ayyaz *et al.*, 2019) gene sets. The normalized enrichment score (NES) is depicted on the X-axis and the false discovery rates (FDRs) are indicated by the size of the dots. The color of the lines reflects whether a signature is enriched in WT (red) or *Lkb1*-deficient (blue) organoids. The shape of the dot (open/closed) indicates whether a signature is significantly enriched (FDR < 0.05). **H)** Violin plot showing module scores for a goblet cell gene signature across WT stem cells, *Lkb1*$^{+/-}$ stem cells, *Lkb1*$^{bud\ -/-}$ stem cells and all *Lkb1*$^{cys\ -/-}$ cells. **I)** Violin plot showing expression of the regenerative marker *Anxa1* across WT stem cells, *Lkb1*$^{+/-}$ stem cells, *Lkb1*$^{bud\ -/-}$ stem cells and all *Lkb1*$^{cys\ -/-}$ cells.

Figure 7. *Lkb1* loss drives a pre-malignant program along the serrated pathway which can be amplified by additional *Kras* mutations. A) GSEA was performed for a serrated-specific gene set from (Chen *et al.*, 2021). The normalized enrichment score (NES) is depicted on the X-axis and the false discovery rate (FDR) is indicated by the size of the dots. The shape of the dot (open/closed) indicates whether a signature is significantly enriched (FDR < 0.05). **B)** Heatmap of z-score transformed expression

values of adenoma-specific genes (ASC) enriched in WT organoids (top cluster) and serrated-specific genes (SSC) enriched in *Lkb1*-deficient organoids (bottom cluster). **C)** Cell reconstitution assay from single cells performed at day 7 from trypsinization is shown for WT, *Lkb1*-deficient, *Apc*-deficient and *Apc/Lkb1*-deficient organoids. **D)** Cell reconstitution assay from single cells performed at day 7 from trypsinization is shown for WT, *Lkb1*-deficient, *Kras*-mutant and *Kras*-mutant/*Lkb1*-deficient organoids. **E)** Heatmap of z-score transformed expression values of stem cell genes enriched in control organoids (top cluster) and fetal genes enriched in *Lkb1*-deficient organoids (bottom cluster). **F)** GSEA of a serrated-specific gene set (Chen *et al.*, 2021) was performed on *Lkb1*-deficient and *Apc/Lkb1*-deficient or *Kras*-mutant/*Lkb1*-deficient organoids. The normalized enrichment score (NES) is depicted on the X-axis and the FDR is indicated by the size of the dots. The color of the lines reflect whether a signature is enriched in *Lkb1*-deficient (red) or *Lkb1*-deficient/*Kras*-mutant (blue) organoids. **G)** Percentage of samples mutated for *BRAF, RNF43, PIK3CA, APC, KRAS* or *TP53* in *LKB1* WT and *LKB1*-mutant colorectal tumors according to cBioPortal. **H, I)** Box plots showing log2 Z-scores for the *Lkb1*-deficient signature (genes significantly upregulated in all deficient clones compared to control organoids) over the colon adenocarcinoma TCGA bulk RNA sequencing dataset grouped by pathology classification as mucinous or non-mucinous **(H)** or consensus molecular subtype **(I)**.

Figure S1. *Lkb1* loss induced by different gRNAs results in similar heterogeneous phenotypes.
A) Sequences of *Lkb1*-deficient organoids obtained with gRNA1 showing indels/deletions detected in Exon 1 of the *Lkb1* gene. PAM sequences are underlined. **B)** Immunofluorescent images showing cleaved caspase 3 staining marking apoptotic cells in *Lkb1*-deficient and control organoids. Scale bar = 33 µm. **C)** Sequences of *Lkb1*-deficient organoids obtained with gRNA1 showing indels/deletions detected in Exon 1 of the *Lkb1* gene. PAM sequences are underlined. **D)** Representative images of *Lkb1*-deficient clones showing heterogeneous morphologies. **E)** Western blot analysis of Lkb1 expression in each clone. **F)** Cell reconstitution assay from single cells performed at day 7 from trypsinization.

Figure S2. Goblet cell number and positioning are unaltered in PJS epithelia and *Lkb1*-deficient organoids display proper brush border formation. A-B) The average number **(A)** and position **(B)** of goblet cells healthy and PJS epithelia. Unpaired t-test was applied for analysis. **C)** Electron microscopy images of WT and *Lkb1*-deficient organoids. Arrows indicate brush border formation in indicated organoid lines. A zoom-in of the area indicated by the red arrows is shown in the bottom panel. Scale bar = 2 µm.

Figure S3. Yap target gene expression progressively increases with loss of both *Lkb1* alleles.
Heatmap of z-score transformed expression values of Yap regulated regenerative genes. Key marker genes are highlighted.

Figure S4. Identification of intestinal cell types of WT and *Lkb1*-deficient organoids.
A) UMAP visualization of scRNA-seq data from wild-type, fetal and *Lkb1*-deficient organoids. Colors indicate different clusters as defined by Seurat analysis **B)** Expression of known markers for different cell populations in WT and *Lkb1*-deficient organoids. **C-I)** Module scores for enterocyte **(C)**, enteroendocrine **(D)**, goblet **(E)**, stem **(F)**, transit amplifying (G1) **(G)**, transit amplifying (G2) **(H)** and tuft **(I)** cell gene signatures overlaid on

the UMAP plot of *Lkb1*-deficient and control organoids. **J-K)** Reclustering of stem cell populations from WT, *Lkb1*$^{+/-}$, *Lkb1*$^{-/-\ bud}$ and *Lkb1*$^{-/-\ cys}$ organoids.

Figure S5. Induction of serrated genes in *Lkb1*-deficient organoids is reflected at the single cell level. A) Module score for a serrated-specific (SSC) gene set on the UMAP plot of *Lkb1*-deficient and WT organoids. **B)** Violin plot showing a module score for a serrated-specific gene set across WT adult, WT fetal, *Lkb1*$^{+/-}$, *Lkb1*$^{bud\ -/-}$ and *Lkb1*$^{cys\ -/-}$ organoids. **C)** GSEA was performed for a serrated-specific gene set from (Chen *et al.*, 2021). The normalized enrichment score (NES) is depicted on the Y-axis and the false discovery rate (FDR) is indicated by the size of the dots. Line colors reflects enrichment of the signature in WT or *Lkb1*-deficient stem cells. Dot shape (open/closed) indicates significant enrichment (FDR < 0.05). **D)** Module score for a serrated-specific gene set on the UMAP plot of WT stem cells, *Lkb1*$^{+/-}$ stem cells, *Lkb1*$^{bud\ -/-}$ stem cells and all *Lkb1*$^{cys\ -/-}$ cells. **E)** Violin plot showing module scores of a serrated-specific gene set for WT stem cells, *Lkb1*$^{+/-}$ stem cells, *Lkb1*$^{bud\ -/-}$ stem cells and all *Lkb1*$^{cys\ -/-}$ cells.

Figure S6. *Lkb1* loss drives expression of a pre-malignant program along the serrated pathway which can be amplified by additional *Kras* mutations. A) Representative images of *Apc* and *Apc/Lkb1*-deficient organoids. **B)** Representative images of *Kras*-mutant and *Kras*-mutant/*Lkb1*-deficient organoids. **C)** Heatmap of z-score transformed expression values of stem cell genes enriched in control organoids (top cluster) and fetal genes enriched in *Lkb1*-deficient organoids (bottom cluster). **D)** GSEA of iCMS2 and iCMS3 gene signatures (Joanito *et al.*, 2022) was performed on *Lkb1*-deficient organoids compared to control organoids. The normalized enrichment score (NES) is depicted on the X-axis and the FDR is indicated by the size of the dots. Line color reflects signature enrichment in *Lkb1*-deficient (red) or *Lkb1*-deficient/*Kras*-mutant (blue) organoids. Dot shape (open/closed) indicates significant enrichment (FDR < 0.05).

Materials and Methods

Organoid culture

Small intestinal mouse organoids were established from crypts isolated from the small intestine of wild-type mice, as previously described (Sato *et al.*, 2009). Basal culture medium for mouse organoids contained advanced DMEM/F12 (Gibco) supplemented with 1% Penicillin-Streptomycin (Sigma-Aldrich), 10 mM HEPES (Gibco), 1% Ultraglutamine 1 (Lonza), 1% B27 (Sigma-Aldrich), 0.5% N2 (Sigma-Aldrich) and 1 mM N-acetylcysteine (Sigma- Aldrich). Basal culture medium was further supplemented with murine recombinant EGF (Peprotech), 2.5% R-spondin1 conditioned medium (homemade) and 10% Noggin conditioned medium (homemade) to obtain the complete ENR (EGF-Noggin-Rspondin) medium. Organoids were grown in Matrigel (Corning) or BME (Cultrex) and passaged every 6-8 days as previously described (Sato *et al.*, 2009); their medium was refreshed every 2-3 days. For growth factor withdrawal experiments, EGF was removed from organoid culture medium and organoids were monitored for a total of 16 days. Bright field images were acquired with the Invitrogen™ EVOS™ FL Color Imaging System (Thermo Fisher Scientific).

Vectors and sgRNAs

The pSpCas9(BB)-2A-Puro plasmid was obtained from Addgene (48139). Single guide RNAs (sgRNAs) and are listed in **Table S4**. Cloning of sgRNAs into the The pSpCas9(BB)-2A-Puro plasmid was performed according to previously published protocols (Ran *et al.*, 2013) and vector sequences conformed by Sanger sequencing.

Organoid electroporation

For electroporation, mouse organoids were expanded in ENR medium without antibiotics and supplemented with 50% Wnt3a-conditioned medium (homemade) and 1mM Nicotinamide (Sigma-Aldrich). Electroporation of organoids was performed as previously described using a NEPA21 (NEPAGENE) electroporator (Fujii *et al.*, 2015). To obtain *Lkb1*-deficient organoids, a total of 20 µg of DNA (10 µg gRNA, 7.2 µg of pPB-CAG-rtTA_Hygromycin and 2.8 µg PiggyBac transposase) was used. To obtain *Apc* and *Kras* mutant organoids a total of 10 µg DNA (gRNA and gRNA with oligonucleotide respectively) was used. 100 µg/mL Hygromycin B was used to select *Lkb1*-deficient organoids and Noggin and Egf were omitted from the culture medium to select for *Apc* and *Kras* mutant organoids respectively. After selection, single organoids were manually picked for clonal expansion and then genotyped. Genomic DNA was isolated using the DNA micro kit (Qiagen) according to the manufacturer's instructions and PCR amplification was performed using the GoTaq Flexi DNA polymerase kit (Promega). Organoid genotype was confirmed by Sanger sequencing. Primers are listed in **Table S5.**

Protein extraction and Western blotting

Organoids were washed in cold PBS and collected in cell recovery solution (Corning) according to the manufacturer's instructions. Whole cell lysates were obtained by direct lysis in RIPA buffer (50 mM Tris- HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% Na-Deoxycholate, 1% NP-40) containing complete protease inhibitors (Sigma-Aldrich) for 30 minutes on ice. Proteins were run on SDS–PAGE gels and transferred to

PVDF membranes, followed by blocking with Odyssey blocking buffer (LI-COR). The antibodies used are described in **Table S6**. Blots were imaged with the Amersham Typhoon (GE Healthcare Life Sciences).

Immunofluorescence

Organoids were seeded in 15-well Ibidi chambers after passaging and fixed 3-4 days later in 4% Paraformaldehyde (Sigma-Aldrich) for 60 minutes at room temperature. After fixation organoids were washed with cold PBS and permeabilized with PBD0.2T (48,5 mL PBS, 1 mL 10%Triton, 0,5mL DMSO and 0,5 g BSA) for 20 minutes at room temperature, after which they were incubated with primary antibodies diluted in PBD0.2T buffer at 4°C overnight. Washes were performed PBD0.2T buffer and secondary antibodies incubated 2-3 hours at room temperature. All antibodies are described in **Table S7**. Quantification of the nuclear fraction of Yap was analyzed using the JACoP Fiji plugin. EdU incorporation assays were performed by culturing organoids in ENR medium containing 10 µM Edu for 6 hours to cells, after which single organoids were harvested, fixed in 4% PFA and EdU-labeling was performed according to manufacturer instructions (Click-iT EdU Cell Proliferation Kit for Imaging, Thermo Fisher). Images were acquired with a Zeiss LSM700 or LSM880 confocal microscope.

Patient tissue samples and counting

Patient material was collected from the pathology archives of Dutch hospitals (UMC). In total five PJS patients were included in this study. Five normal controls were randomly selected. In 10 microscopically normal looking crypts $MUC2^+$ cells were counted from the bottom toward to the top of the crypt. Only longitudinally oriented crypts that were completely visible on the slide, were counted. Cell positions were defined as the bottom of the crypt is cell position one (1), going upward to cell position +2, +3, and so forth till cell position 30. Within the part of the crypt between cell position 1 and 30, the number of positively labelled cells were counted. Also the total cell position was determined by adding up each cell position of a labelled cell and average cell position of the labelled cells was determined by adding up each cell position of a labelled cell and dividing the sum by the total number of labelled cells counted.

Immunohistochemistry

Fresh mouse small intestinal organoids were fixed in 4% Formaldehyde for 1 hour followed by dehydration in 25%, 50% and 70% ethanol and embedding in paraffin. Briefly, 4µm FFPE organoid or PJS patient tissue sections were obtained, deparaffinised, blocked in 0.3% H2O2 in methanol for 20 minutes. Antigen retrieval was performed in Tris/EDTA buffer (10 mM/1 mM; pH 9.0) or Sodium-citrate buffer (0,01 M/pH6.0) for 20 minutes at 100°C. Nonspecific binding sites were blocked using Protein Block Serum free (DAKO) for 10 minutes, followed by primary antibody incubation. Antibody binding was visualized using the using BrightVision Goat anti Rabbit / Poly-AP and BrightVision Goat anti Mouse / Poly-HRP (VWR/ImmunoLogic), with DAB as chromogen (Sigma-Aldrich). Sections were counterstained with Haematoxylin or neutral red and mounted using Pertex. Primary antibodies are described in **Table S8**.

Electron microscopy

Organoids in Matrigel were fixed in a solution of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C. On the following day, organoids were washed twice in 0.1 M

phosphate buffer (pH 7.4) and subsequently post-fixed with 1% Osmium tetroxide for 2 hours at 4°C. Organoids were then stained with 0.5% uranyl acetate 1 hour at 4°C in the dark. Matrigel droplets were transferred into glass vials for dehydration with increasing acetone concentrations according to the following protocol: 70% overnight at 4°C, 90% for 15 minutes at RT, 96% for 15 minutes at RT, and 100% 3 times for 30 minutes each at RT. After dehydration, samples were embedded in EPON resin. Ultrathin sections of 65 nm were obtained with a Leica UC7 microtome and stained with uranyl and lead citrate. Electron microscopy was performed in the Cell Microscopy Core (CMC) of the UMC Utrecht. Sections were imaged with a JEOL JEM-1010 transmission electron microscope (TEM).

Cell viability assay

For the organoid reconstitution assays, organoids were dissociated to single cells after incubation with TrypLE (Life Technologies) for 5 min at 37°C. Dissociated organoids were passed through a 40 µm strainer and counted. Cells were seeded at a density of 5000 cells/10µl Matrigel and cultured in ENR medium with 10 µM Y-27632 (Sigma-Aldrich) for 7 days. Organoids were then lysed with CellTiter-Glo® (Promega) according to the manufacturer's protocol. ATP produced by viable cells was measured by a Berthold luminometer Centro LB960.

Bulk RNA sequencing and data analysis

Organoids were lysed in RLT lysis buffer (Qiagen) 3-5 days after passaging. Biological triplicates were employed. RNA was obtained using the Qiagen QiaSymphony SP (Qiagen) according to the manufacturer's protocol. Single-end RNASeq reads were processed using the UMCU RNASeq pipeline (https://github.com/UMCUGenetics/RNASeq, v2.3.0) with default settings. Read quality was assessed by FastQC (0.11.4) followed by splice-aware alignment against the mouse reference genome (GRCm38) with STAR (2.4.2a). RNA expression quantification was performed with htseq-count (0.6.0) in reverse-stranded mode. Differential gene expression analysis was performed with the DESeq2 (1.22.2) R package. In each comparison, genes were selected with an absolute fold-change > 2 and alpha P > 0.05. Data clustering and generation of heatmaps was performed with pheatmap. Gene Set Enrichment Analysis was performed using GSEA (v4.2.0, Windows App) and visualized using ggplot2. Intestinal cell-type specific gene sets for GSEA were obtained from Extended Figure Table 3 of Haber et al., 2017 (Haber *et al.*, 2017). The other datasets employed in GSEA were derived from previously published studies (Mustata *et al.*, 2013; Gregorieff *et al.*, 2015; Nusse *et al.*, 2018; Yui, Azzolin, Maimets, Pedersen, Robert P. Fordham, *et al.*, 2018; Ayyaz *et al.*, 2019; Chen *et al.*, 2021; Qu *et al.*, 2021b). Human gene sets were converted to their respective mouse homologs using biomaRt (2.50.3) in R where necessary.

Single cell RNA sequencing

Sample preparation and sequencing: Single cells from organoid were harvested using TrypLE incubation at 37 °C, four days after passaging. Cells were filtered through a 40 µm nylon cell strainer (Falcon). scRNA-seq was performed according to the Sort-seq protocol (Muraro *et al.*, 2016). Libraries were sequenced on an Illumina NextSeq500 at paired-end 60- and 26-bp read length and 75,000 reads per cell.

Mapping and filtering: The Sharq pipeline was used to process the sequencing data (Candelli *et al.*, 2018). Mapping was performed using STAR (version 2.6.1), on the Genome Reference Consortium Mouse built 38

(GRCm38). Read assignment was with feature Counts version 1.5.2 using a gene annotation based on GENCODE version M14.

External RNA controls (ERCCS) and transcripts mapping to the mitochondrial genome were removed from all cells. Low quality cell barcodes having either less than 800 unique transcripts, 200 genes 33.3% mitochondrial transcripts or over 4-fold non-exonic to exonic reads were excluded, as well as barcodes with over 100,000 unique transcripts.

scRNA-seq analysis: Unique transcript counts were normalized using sctransform (Hafemeister and Satija, 2019) and analyzed using the Seurat R package (version 4.1.0) (Butler *et al.*, 2018). From the top 3000 variable genes, genes associated with cell cycle phase, dissociation stress (heat shock and chaperone proteins according to GO:0006986), sex (Xist, Tsix, and Y chromosome-specific genes), and activity (ribosomal protein genes according to GO:0022626) were removed from the list of variable genes to avoid biases in cell clustering, as described before (Hanemaaijer *et al.*, 2021). The first 20 principal components were used both to calculate dimensionality reduction using UMAP and to perform clustering with a resolution of 1, k.param = 10 and the Louvain algorithm.

Supplementary information

Gene	Sequence
Lkb1 gRNA1	AGCTTGGCGCGTTTGCGGCG
Lkb1 gRNA2	TGAGGATCTTGACCGCCCTG
Apc	TGTCTGGCTCCGGTAAGTGA
Kras	CTTGTGGTGGTTGGAGCTGG

Table S4: gRNA sequences

Table S5: Genotyping primers

Gene	Forward primer	Reverse Primer
Lkb1 gRNA1	GGGGAAAATCAAAAGTGAAGAA	CCCTCTAGCTGCGTAAACAAAC
Lkb1 gRNA2	CTCCACCGAGGTAATCTACCAGCCG	TCTAACCGTTTTCTCTTCTCATTTCC
Apc	ATAGAAACAGCACTGACCCAAATTTCA	AGGCCTCTTTGCTTAGAGCTTTCATAA
Kras	TGGCTCCAACACAGATGTTC	GGATGGCATCTTGGACCTTA

Table S6: Western Blot antibodies

Antibody	Dilution	Supplier	Cat. number
Anti-LKB1	1:1000	Santa Cruz	sc-32245
Anti-GAPDH	1:8000	Millipore	CB1001
Anti-mouse Alexa 800	1:5000	LI-COR	926-32211
Anti-rabbit Alexa 680	1:5000	LI-COR	925-68070

Table S7: Immunostaining antibodies

Antibody	Dilution	Supplier	Cat. number
Anti-Muc2	1:200 (IF) 1:1000 (IHC)	Santa Cruz	sc-515032
Anti-Lysozyme1	1:200 (IF) 1:2000 (IHC)	Dako	A0099
Anti-ANXA1	1:200 (IF) 1:1000 (IHC)	Atlas Antibodies	HPA011271
Anti-YAP	1:200 (IF)	Santa Cruz	sc-101199
Anti-Cleasved-Caspase-3	1:200 (IF)	Cell Signalling	9661
Anti-rabbit Alexa Fluor 488	1:300 (IF)	Invitrogen	A11034
Anti-mouse Alexa 488	1:300 (IF)	Invitrogen	A11029
DAPI	1:1000 (IF)	Sigma	D9542
Click-iT™ EdU Alexa Fluor™	n.a.	Invitrogen	C10424

647			
Alexa Fluor™ 647 Phalloidin	1:500 (IF)	Invitrogen	A22287

Data availability

The scRNA-seq and bulk RNA-seq data generated in this study will be uploaded to Gene Expression Omnibus (GEO) upon publication. If needed earlier, data can be requested via the corresponding author.

Author contributions

S.F.P, H.M., A.M. and M.M.M., conceived and designed the experiments. S.F.P, H.M., A.M., D.X., F.H.M.M., C.J. and A.R.K performed the experiments. S.F.P, H.M., A.M., J.Y.V., J.S., T.M., T.C., R.S., J.R., F.C.P.H., G.J.A.O. and M.M.M. analyzed the data. W.W.J.L. and G.J.A.O. provided patient material. S.F.P, H.M., A.M., J.Y.V. and M.M.M. wrote the manuscript, which was reviewed by all authors.

Disclosure and competing interest statement

M.M.M. is an inventor on patents related to membrane protein degradation; she is co-founder and shareholder of Laigo Bio. J.R. is co-founder of Cyclomics BV., a genomics company.

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Figure 1

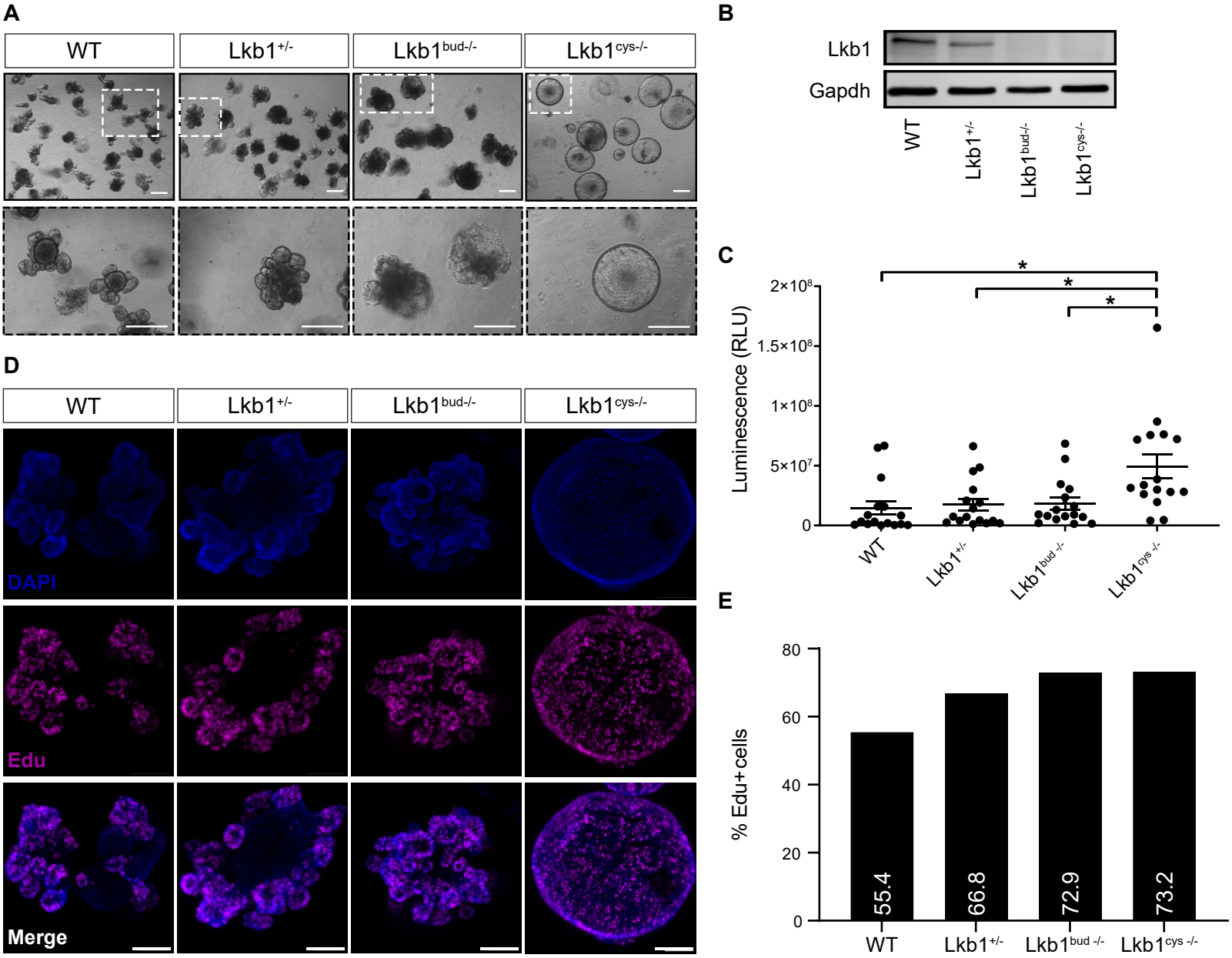
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Figure 2

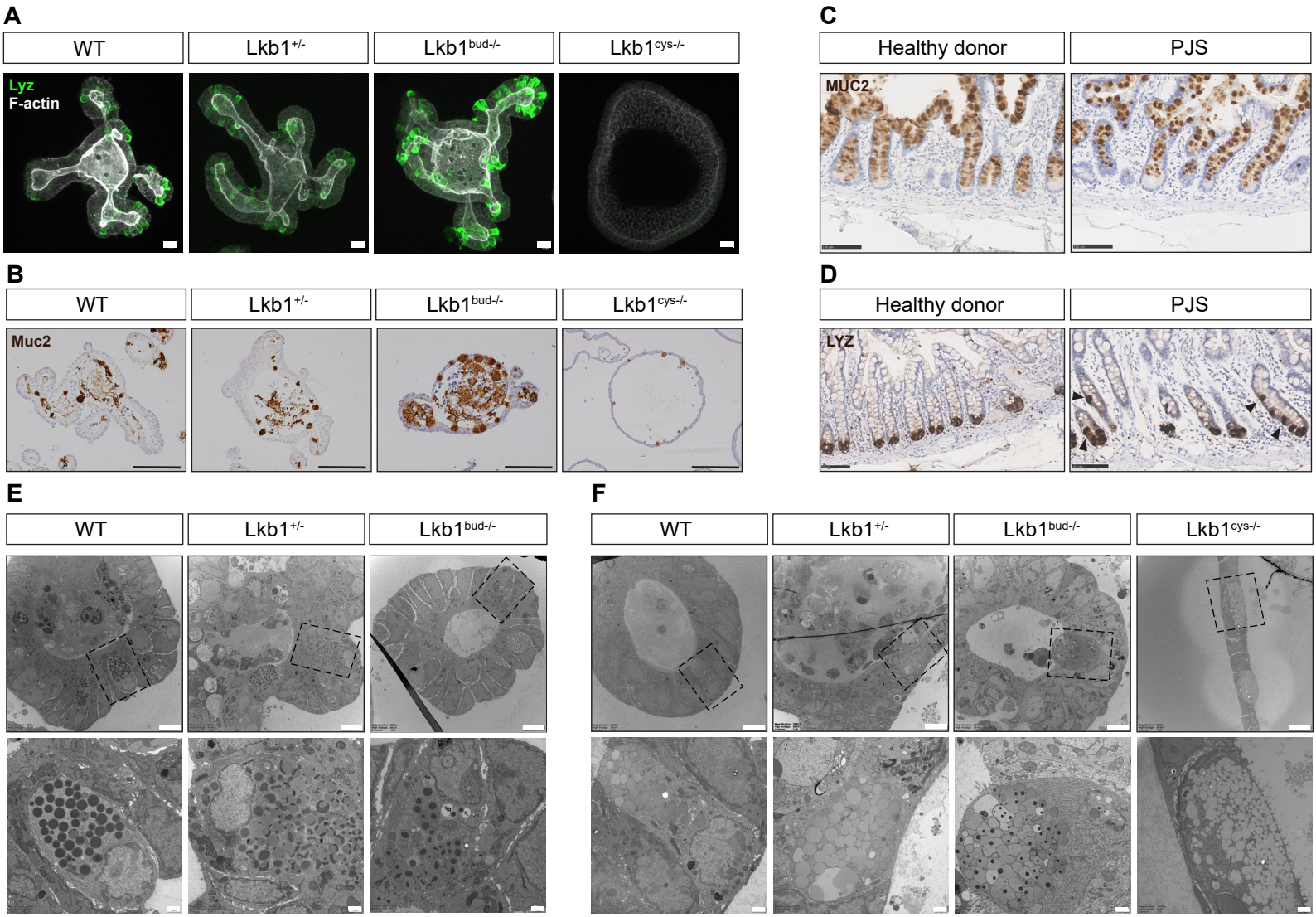
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Figure 3

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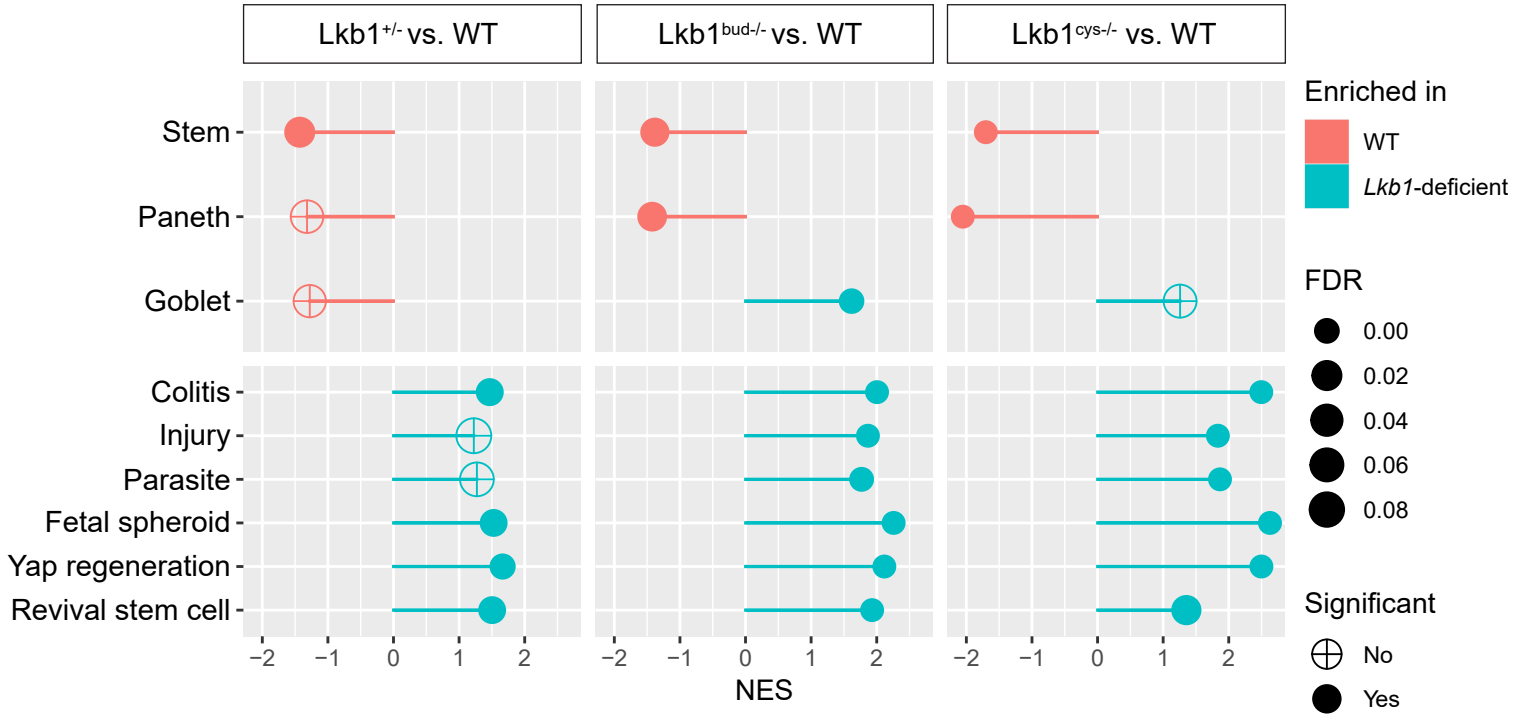

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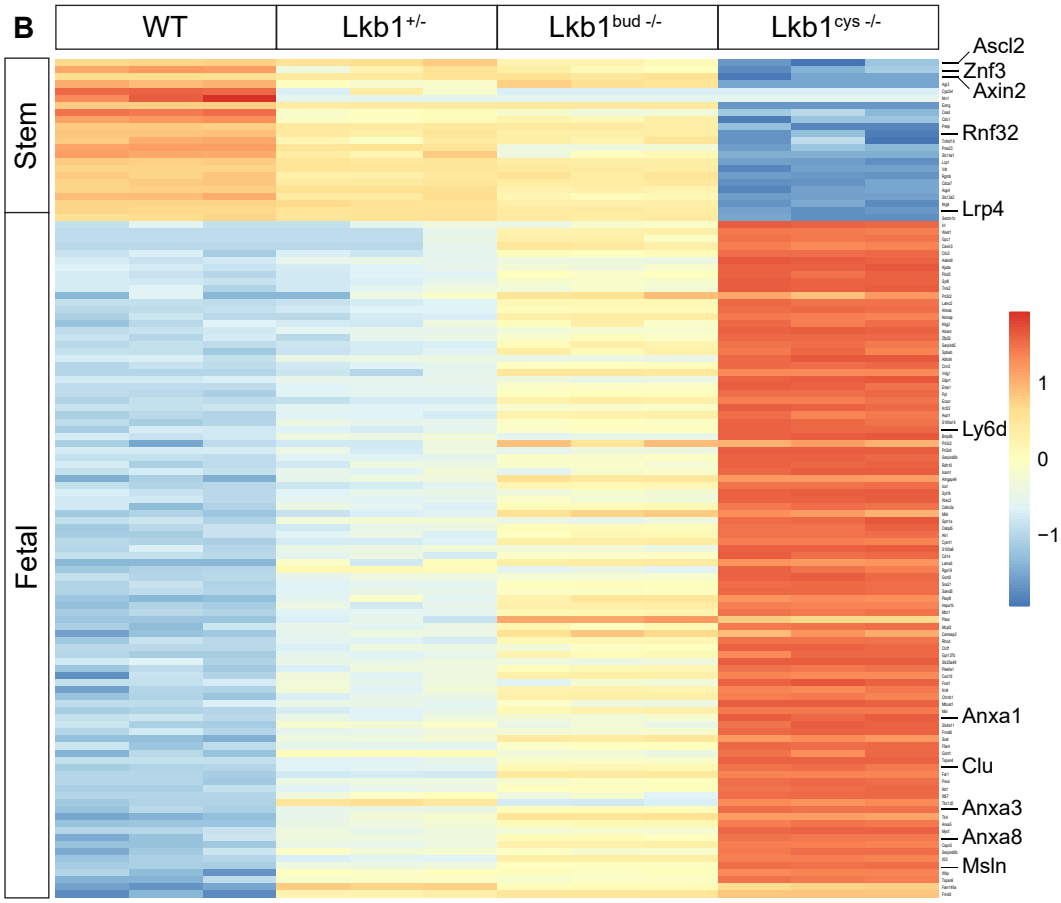

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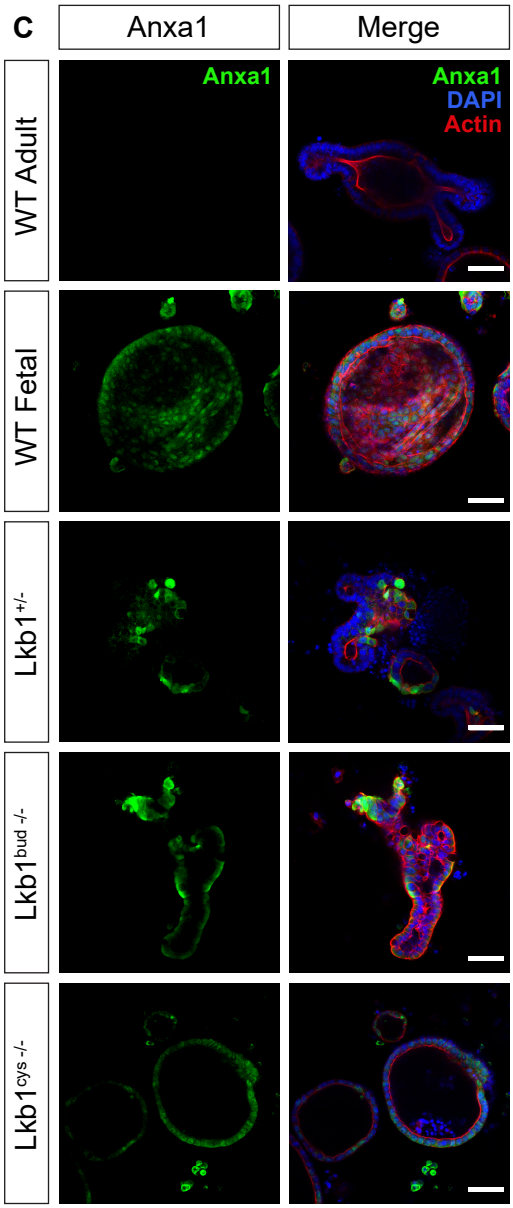

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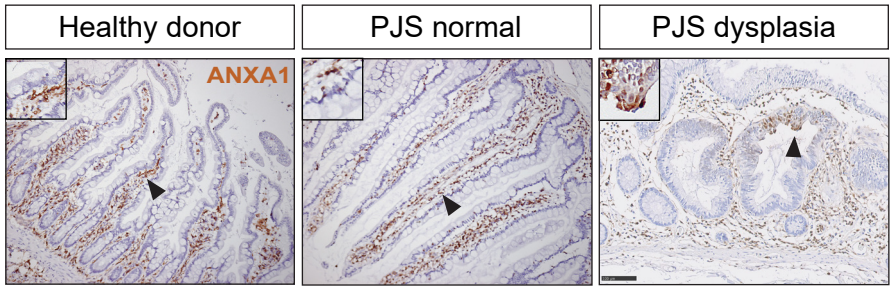
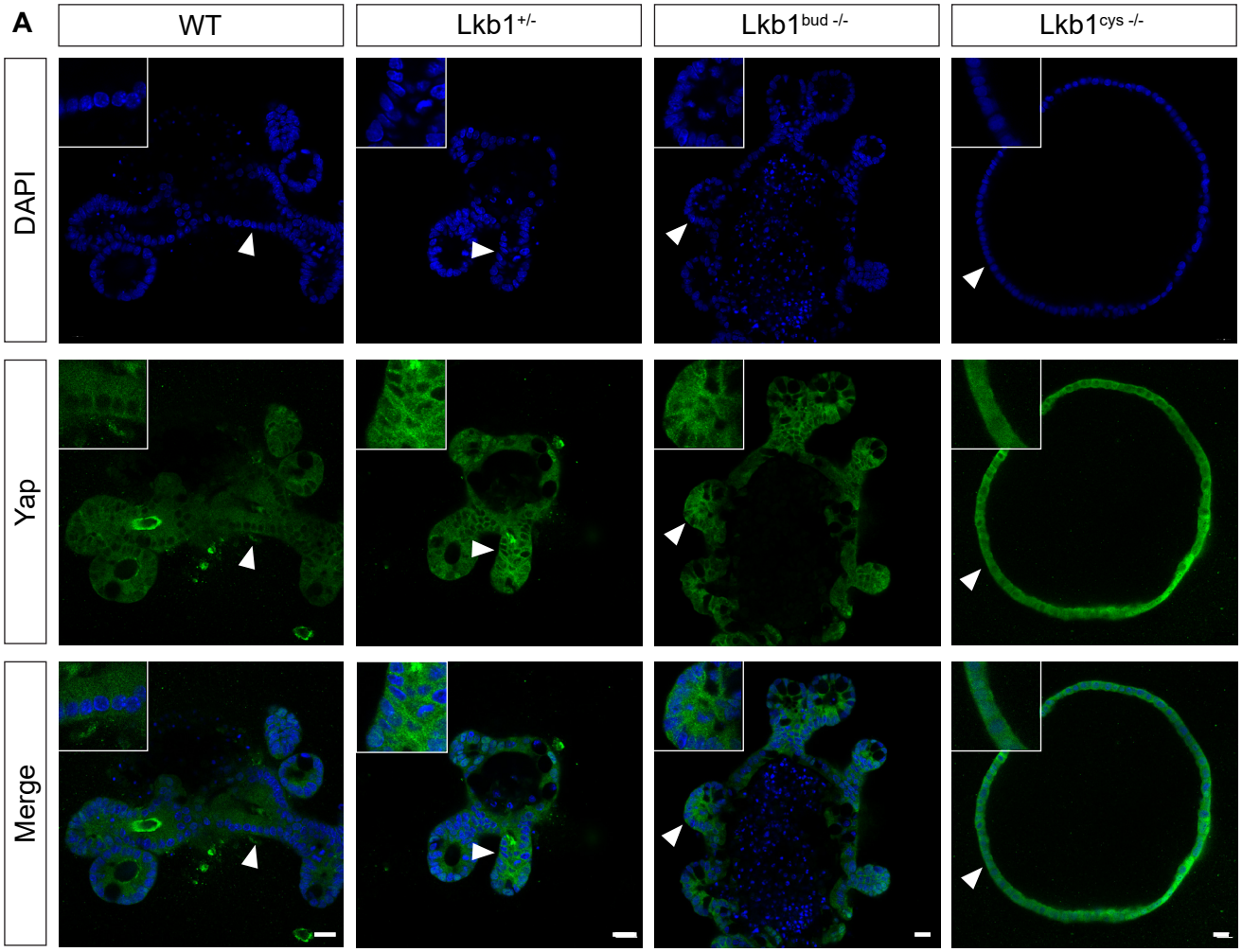


Figure 4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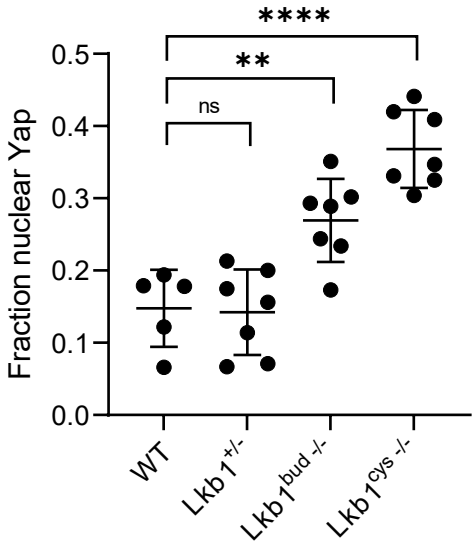
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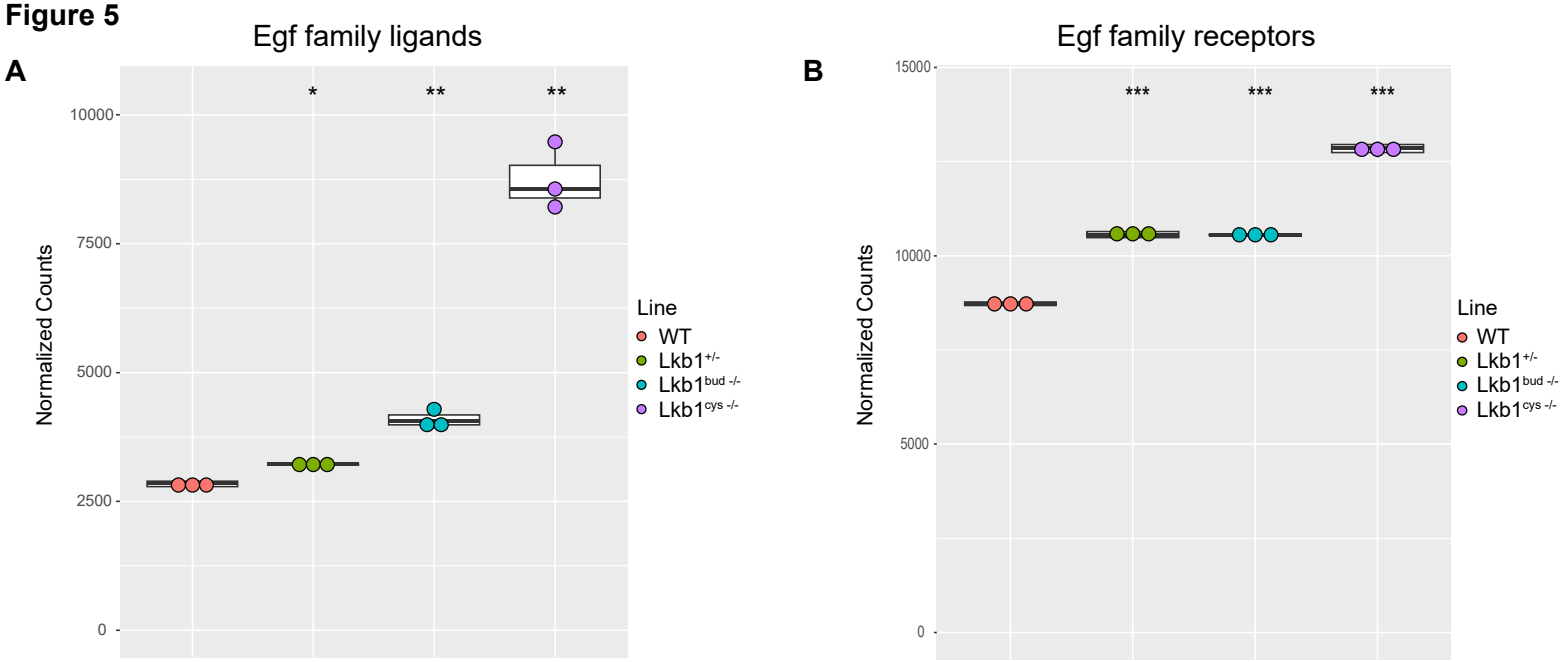
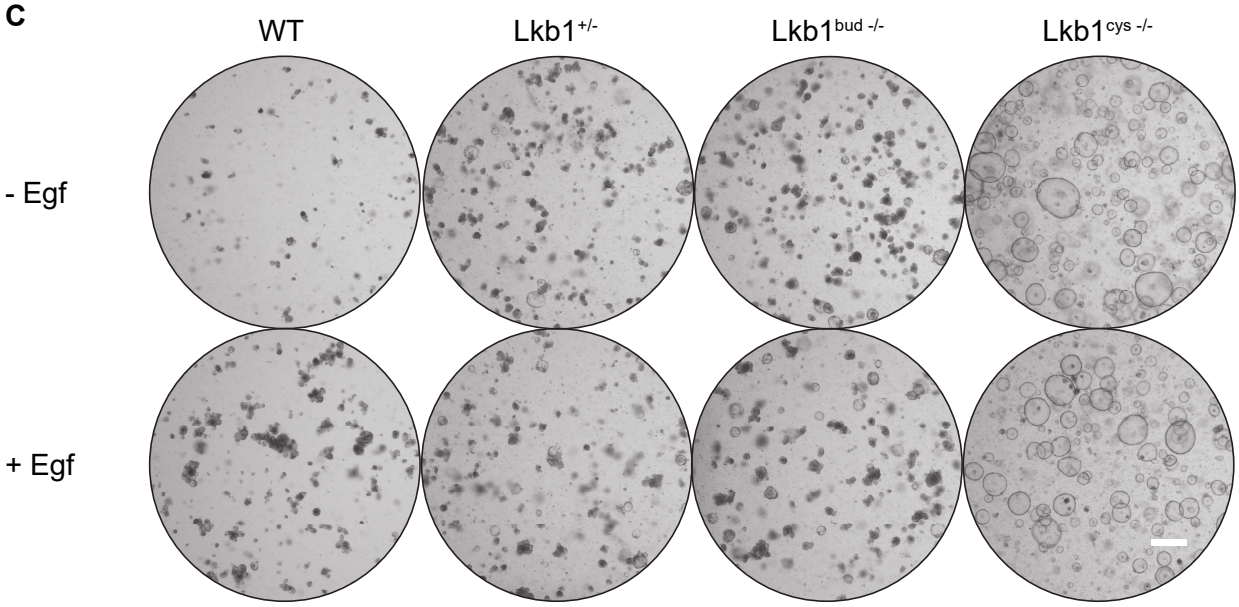
Lkb1bud -/-
Lkb1cys -/-

Figure 5


A
Egf family ligands
Normalized Counts
10000
7500
5000
2500
0
*
**
**
Line
WT
Lkb1+/-
Lkb1bud -/-
Lkb1cys -/-
B
Egf family receptors
Normalized Counts
15000
10000
5000
0
***
***
***
Line
WT
Lkb1+/-
Lkb1bud -/-
Lkb1cys -/-


C
WT
Lkb1+/-
Lkb1bud -/-
Lkb1cys -/-
- Egf
+ Egf

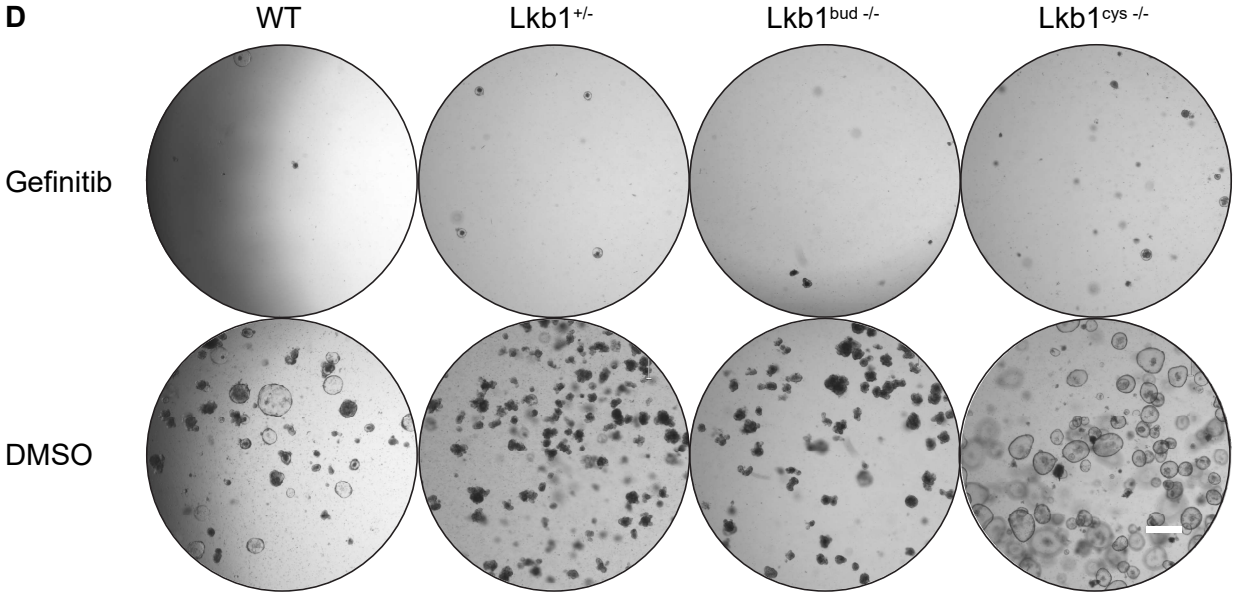

D
WT
Lkb1+/-
Lkb1bud -/-
Lkb1cys -/-
Gefinitib
DMSO

Figure 6

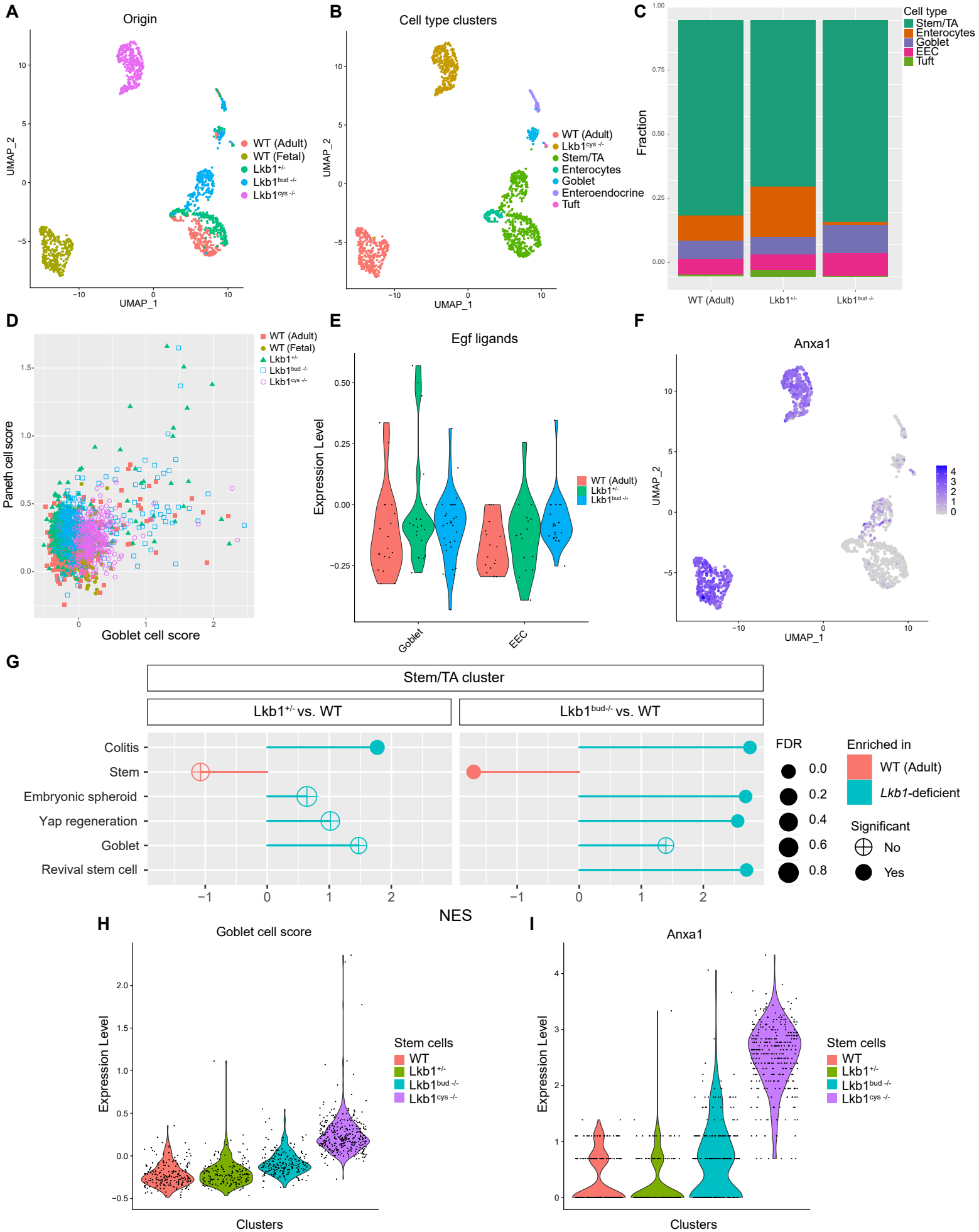

A
Origin
UMAP_2
UMAP_1
WT (Adult)
WT (Fetal)
Lkb1+/-
Lkb1bud -/-
Lkb1cys -/-
B
Cell type clusters
UMAP_2
UMAP_1
WT (Adult)
Lkb1cys -/-
Stem/TA
Enterocytes
Goblet
Enteroendocrine
Tuft
C
Cell type
Stem/TA
Enterocytes
Goblet
EEC
Tuft
Fraction
WT (Adult)
Lkb1+/-
Lkb1bud -/-
D
WT (Adult)
WT (Fetal)
Lkb1+/-
Lkb1bud -/-
Lkb1cys -/-
Paneth cell score
Goblet cell score
E
Egf ligands
Expression Level
WT (Adult)
Lkb1+/-
Lkb1bud -/-
Goblet
EEC
F
Anxa1
UMAP_2
UMAP_1
G
Stem/TA cluster
Lkb1+/- vs. WT
Lkb1bud-/- vs. WT
Colitis
Stem
Embryonic spheroid
Yap regeneration
Goblet
Revival stem cell
NES
FDR
0.0
0.2
0.4
0.6
0.8
Enriched in
WT (Adult)
Lkb1-deficient
Significant
No
Yes
H
Goblet cell score
Expression Level
Clusters
Stem cells
WT
Lkb1+/-
Lkb1bud -/-
Lkb1cys -/-
I
Anxa1
Expression Level
Clusters
Stem cells
WT
Lkb1+/-
Lkb1bud -/-
Lkb1cys -/-

Figure 7

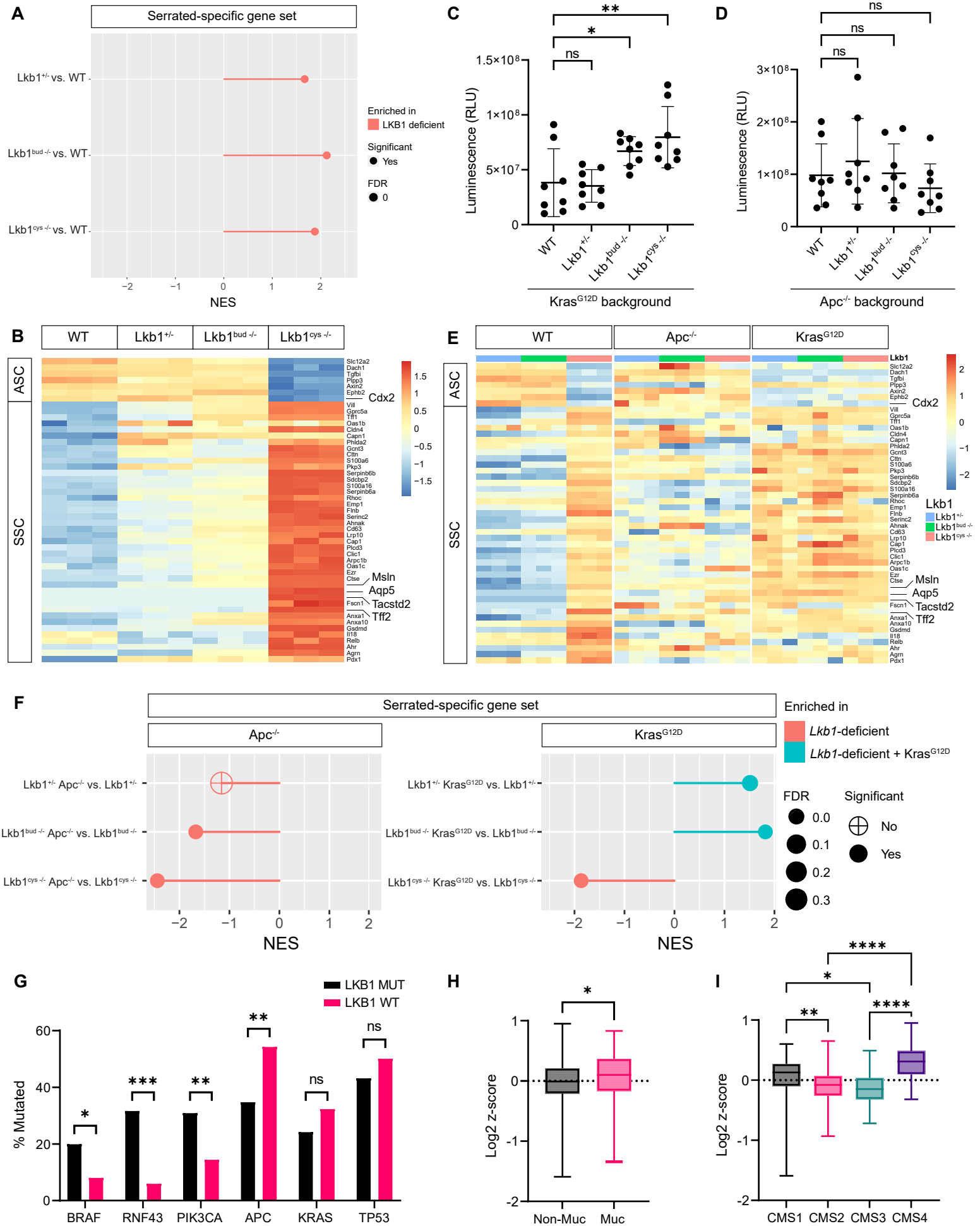

A
Serrated-specific gene set
Lkb1+/- vs. WT
Lkb1bud -/- vs. WT
Lkb1cys -/- vs. WT
Enriched in
LKB1 deficient
Significant
Yes
FDR
0
NES
B
WT
Lkb1+/-
Lkb1bud -/-
Lkb1cys -/-
ASC
SSC
Cdx2
Msln
Aqp5
Tacstd2
Tff2
C
Luminescence (RLU)
KrasG12D background
D
Apc-/- background
E
Apc-/-
KrasG12D
Lkb1
F
Serrated-specific gene set
Apc-/-
KrasG12D
Lkb1+/- Apc-/- vs. Lkb1+/-
Lkb1bud -/- Apc-/- vs. Lkb1bud -/-
Lkb1cys -/- Apc-/- vs. Lkb1cys -/-
Lkb1+/- KrasG12D vs. Lkb1+/-
Lkb1bud -/- KrasG12D vs. Lkb1bud -/-
Lkb1cys -/- KrasG12D vs. Lkb1cys -/-
Enriched in
Lkb1-deficient
Lkb1-deficient + KrasG12D
FDR
Significant
No
Yes
G
LKB1 MUT
LKB1 WT
% Mutated
BRAF
RNF43
PIK3CA
APC
KRAS
TP53
H
Log2 z-score
Non-Muc
Muc
I
CMS1
CMS2
CMS3
CMS4

Figure S1

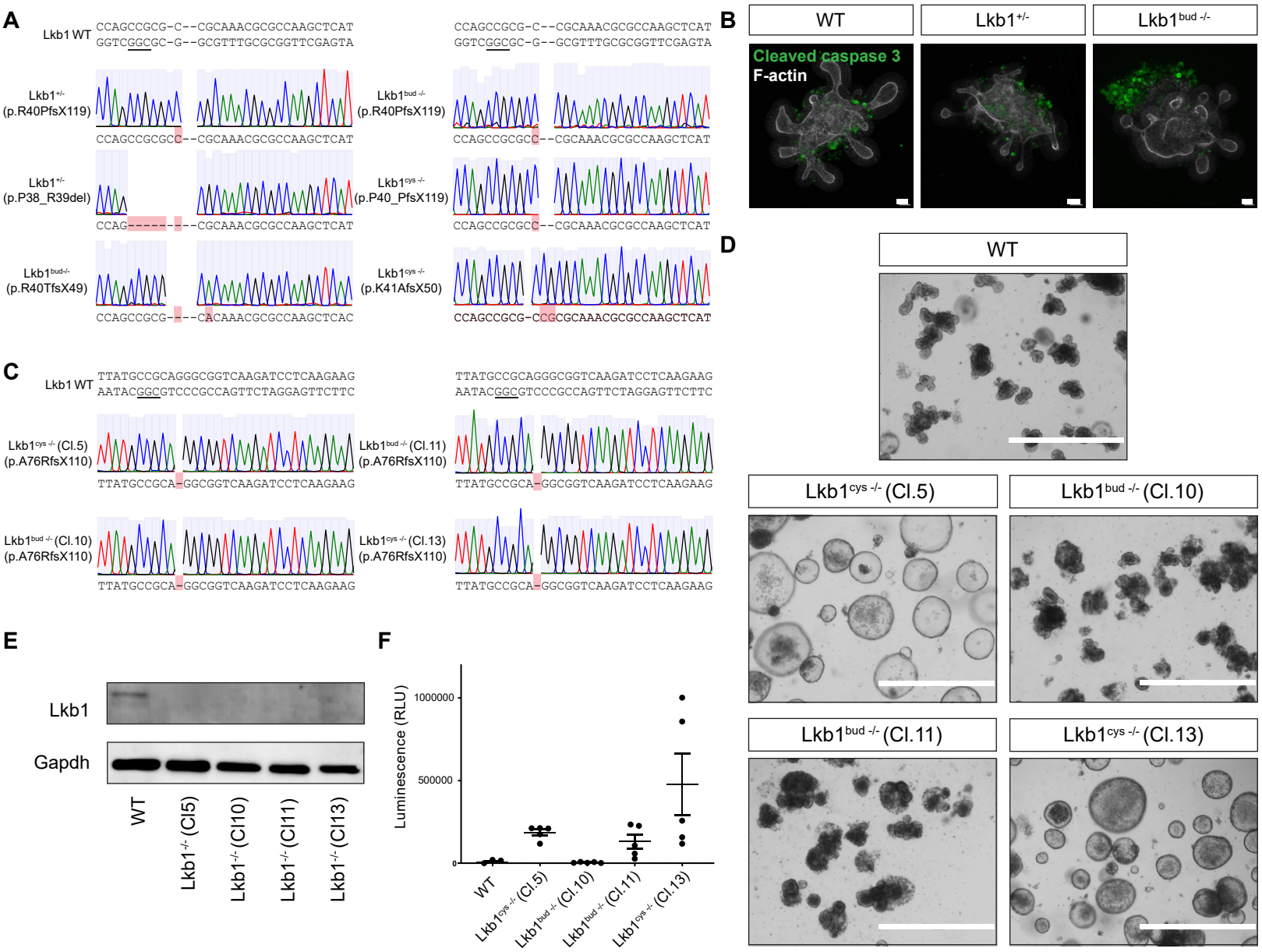

A
Lkb1 WT
CCAGCCGCG-C--CGCAAACGCGCCAAGCTCAT
GGTCGGCGC-G--GCGTTTGCGCGGTTCGAGTA
CCAGCCGCG-C--CGCAAACGCGCCAAGCTCAT
GGTCGGCGC-G--GCGTTTGCGCGGTTCGAGTA
Lkb1+/- (p.R40PfsX119)
CCAGCCGCGCC--CGCAAACGCGCCAAGCTCAT
Lkb1bud -/- (p.R40PfsX119)
CCAGCCGCGCC--CGCAAACGCGCCAAGCTCAT
Lkb1+/- (p.P38_R39del)
CCAG-------- --CGCAAACGCGCCAAGCTCAT
Lkb1cys -/- (p.P40_PfsX119)
CCAGCCGCGCC--CGCAAACGCGCCAAGCTCAT
Lkb1bud-/- (p.R40TfsX49)
CCAGCCGCG---CACAAACGCGCCAAGCTCAC
Lkb1cys -/- (p.K41AfsX50)
CCAGCCGCG-CCGCGCAAACGCGCCAAGCTCAT
B
WT
Lkb1+/-
Lkb1bud -/-
Cleaved caspase 3
F-actin
C
Lkb1 WT
TTATGCCGCAGGGCGGTCAAGATCCTCAAGAAG
AATACGGCGTCCCGCCAGTTCTAGGAGTTCTTC
TTATGCCGCAGGGCGGTCAAGATCCTCAAGAAG
AATACGGCGTCCCGCCAGTTCTAGGAGTTCTTC
Lkb1cys -/- (Cl.5) (p.A76RfsX110)
TTATGCCGCA-GGCGGTCAAGATCCTCAAGAAG
Lkb1bud -/- (Cl.11) (p.A76RfsX110)
TTATGCCGCA-GGCGGTCAAGATCCTCAAGAAG
Lkb1bud -/- (Cl.10) (p.A76RfsX110)
TTATGCCGCA-GGCGGTCAAGATCCTCAAGAAG
Lkb1cys -/- (Cl.13) (p.A76RfsX110)
TTATGCCGCA-GGCGGTCAAGATCCTCAAGAAG
D
WT
Lkb1cys -/- (Cl.5)
Lkb1bud -/- (Cl.10)
Lkb1bud -/- (Cl.11)
Lkb1cys -/- (Cl.13)
E
Lkb1
Gapdh
WT
Lkb1-/- (Cl5)
Lkb1-/- (Cl10)
Lkb1-/- (Cl11)
Lkb1-/- (Cl13)
F
Luminescence (RLU)
1000000
500000
0
WT
Lkb1cys -/- (Cl.5)
Lkb1bud -/- (Cl.10)
Lkb1bud -/- (Cl.11)
Lkb1cys -/- (Cl.13)

Figure S2

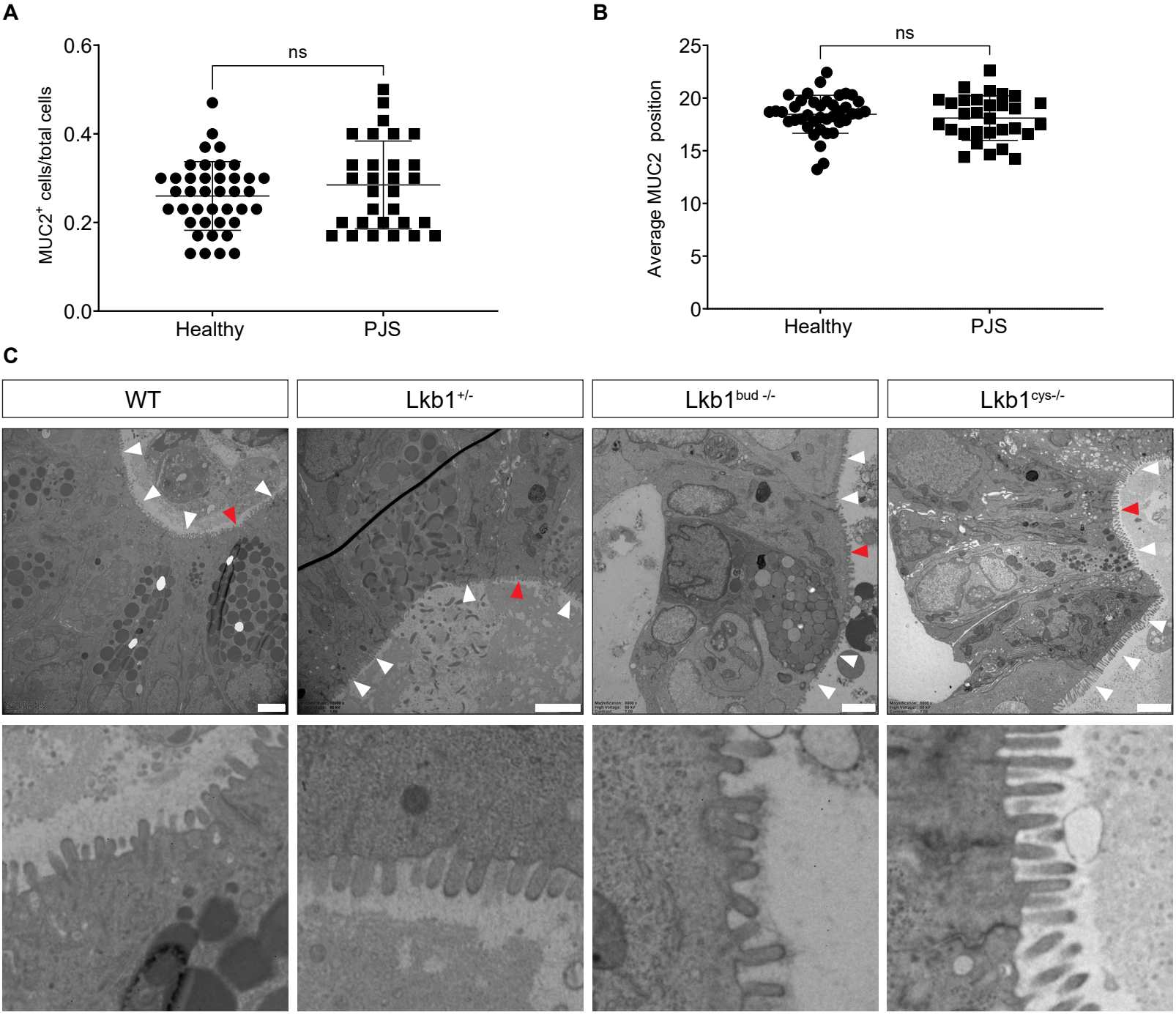

A
0.6
0.4
0.2
0.0
MUC2+ cells/total cells
ns
Healthy
PJS
B
25
20
15
10
5
0
Average MUC2 position
ns
Healthy
PJS
C
WT
Lkb1+/-
Lkb1bud -/-
Lkb1cys-/-

Figure S3

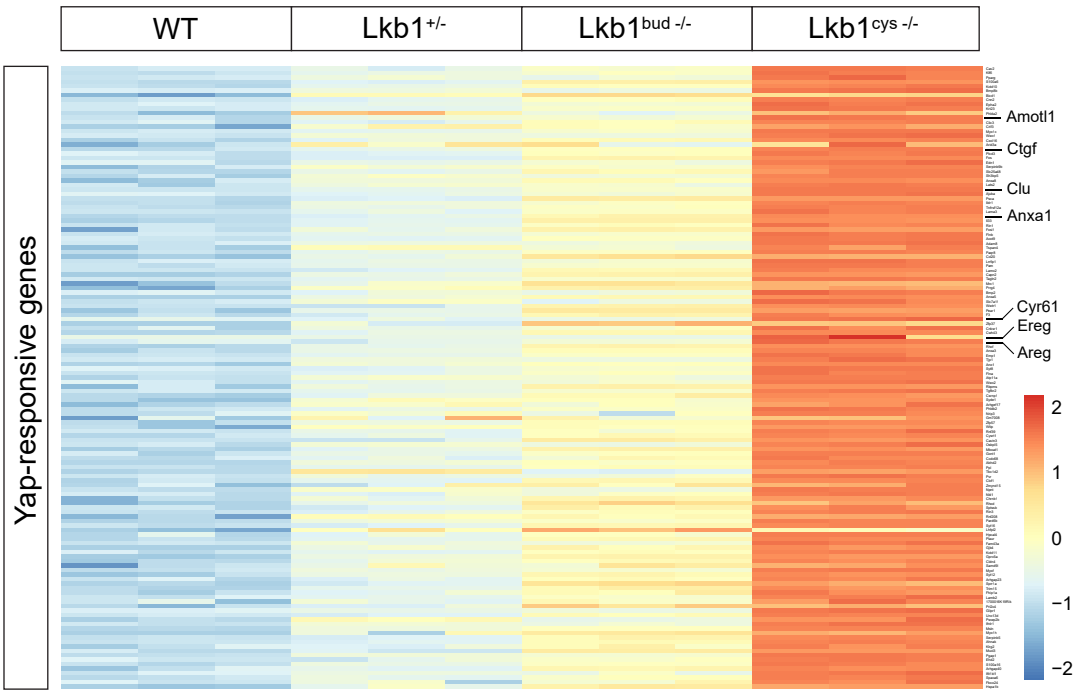

WT
Lkb1+/-
Lkb1bud -/-
Lkb1cys -/-
Yap-responsive genes
Amotl1
Ctgf
Clu
Anxa1
Cyr61
Ereg
Areg
2
1
0
−1
−2

Figure S4

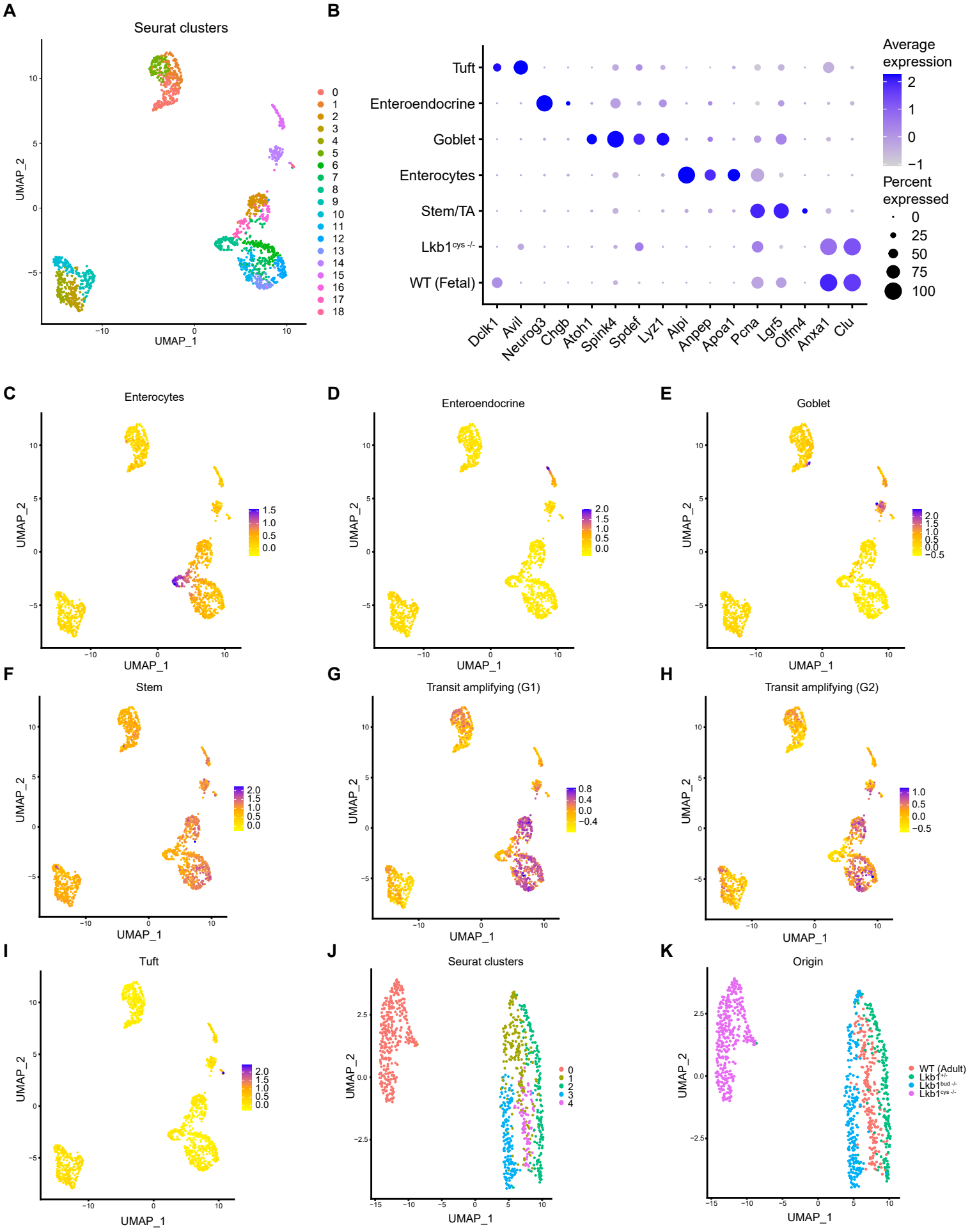

A
Seurat clusters
UMAP_1
UMAP_2
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
B
Tuft
Enteroendocrine
Goblet
Enterocytes
Stem/TA
Lkb1cys -/-
WT (Fetal)
Dclk1
Avil
Neurog3
Chgb
Atoh1
Spink4
Spdef
Lyz1
Alpi
Anpep
Apoa1
Pcna
Lgr5
Olfm4
Anxa1
Clu
Average expression
Percent expressed
C
Enterocytes
D
Enteroendocrine
E
Goblet
F
Stem
G
Transit amplifying (G1)
H
Transit amplifying (G2)
I
Tuft
J
Seurat clusters
K
Origin
WT (Adult)
Lkb1+/-
Lkb1bud -/-
Lkb1cys -/-

Figure S5

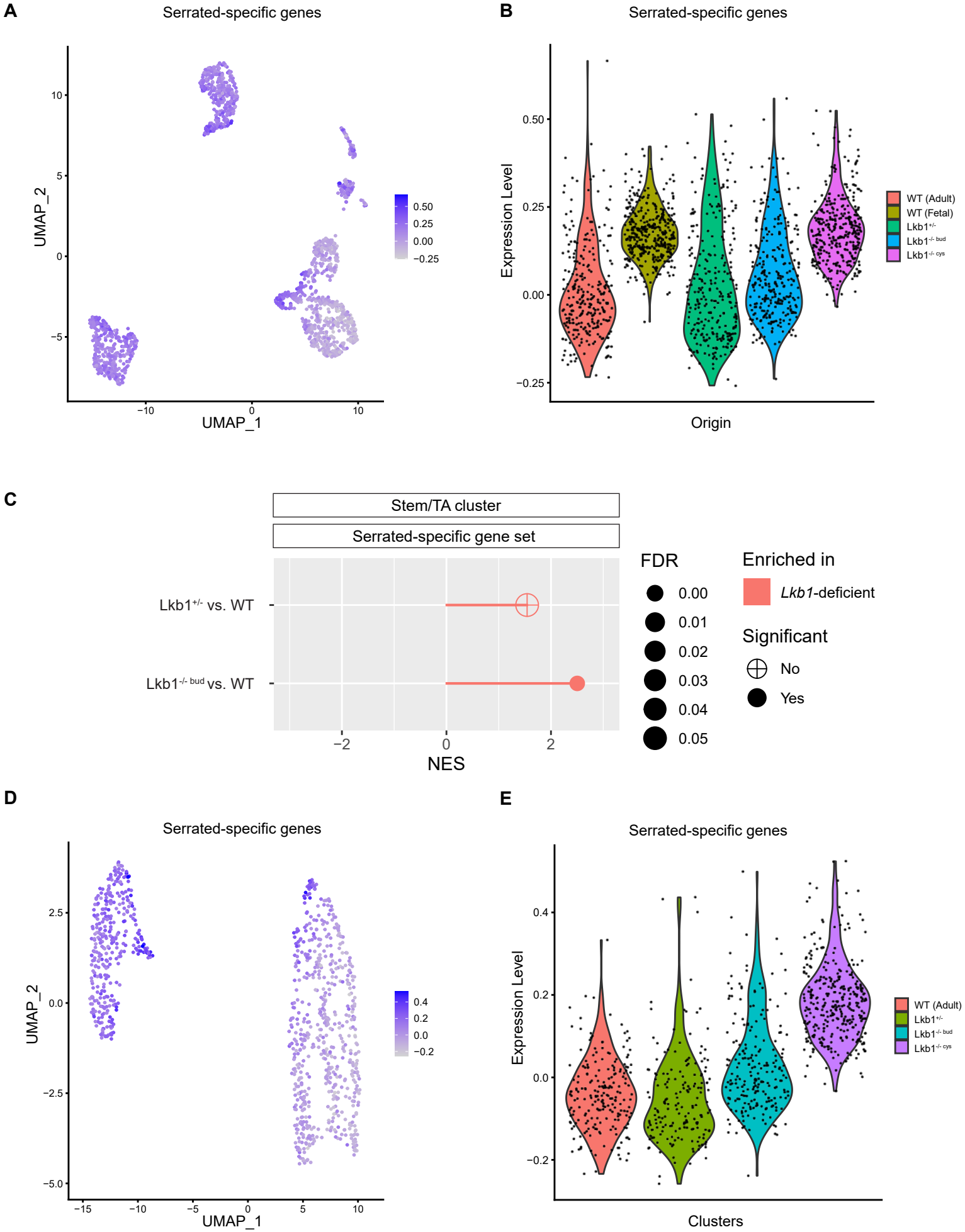
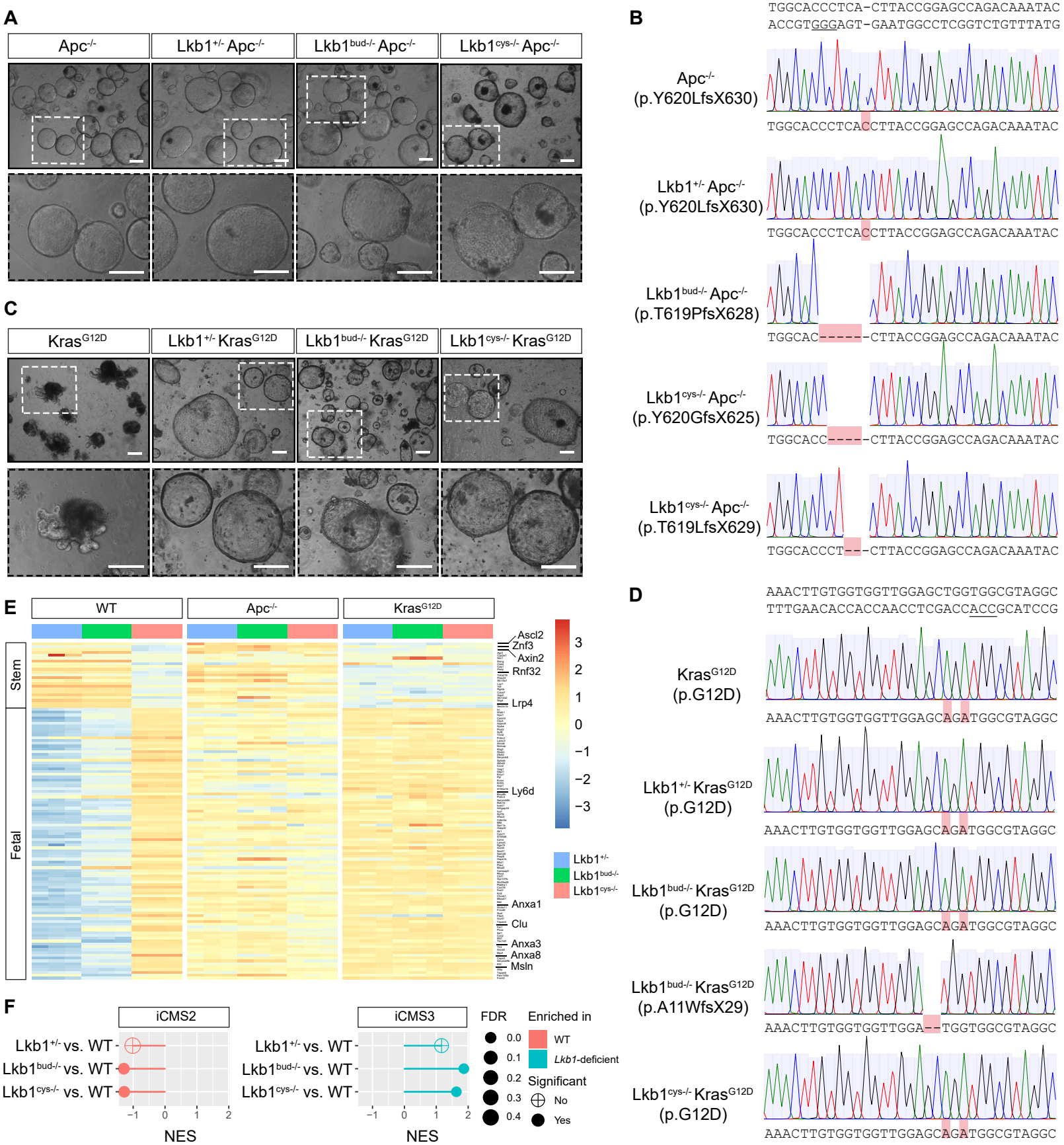

A
Serrated-specific genes
UMAP_2
UMAP_1
0.50
0.25
0.00
−0.25
B
Serrated-specific genes
Expression Level
Origin
WT (Adult)
WT (Fetal)
Lkb1+/-
Lkb1-/- bud
Lkb1-/- cys
C
Stem/TA cluster
Serrated-specific gene set
Lkb1+/- vs. WT
Lkb1-/- bud vs. WT
NES
FDR
0.00
0.01
0.02
0.03
0.04
0.05
Enriched in
Lkb1-deficient
Significant
No
Yes
D
Serrated-specific genes
UMAP_2
UMAP_1
0.4
0.2
0.0
−0.2
E
Serrated-specific genes
Expression Level
Clusters
WT (Adult)
Lkb1+/-
Lkb1-/- bud
Lkb1-/- cys

Figure S6


A
Apc-/-
Lkb1+/- Apc-/-
Lkb1bud-/- Apc-/-
Lkb1cys-/- Apc-/-
B
TGGCACCCTCA-CTTACCGGAGCCAGACAAATAC
ACCGTGGGAGT-GAATGGCCTCGGTCTGTTTATG
Apc-/- (p.Y620LfsX630)
TGGCACCCTCACCTTACCGGAGCCAGACAAATAC
Lkb1+/- Apc-/- (p.Y620LfsX630)
TGGCACCCTCACCTTACCGGAGCCAGACAAATAC
Lkb1bud-/- Apc-/- (p.T619PfsX628)
TGGCAC------CTTACCGGAGCCAGACAAATAC
Lkb1cys-/- Apc-/- (p.Y620GfsX625)
TGGCACC-----CTTACCGGAGCCAGACAAATAC
Lkb1cys-/- Apc-/- (p.T619LfsX629)
TGGCACCCT---CTTACCGGAGCCAGACAAATAC
C
KrasG12D
Lkb1+/- KrasG12D
Lkb1bud-/- KrasG12D
Lkb1cys-/- KrasG12D
D
AAACTTGTGGTGGTTGGAGCTGGTGGCGTAGGC
TTTGAACACCACCAACCTCGACCACCGCATCCG
KrasG12D (p.G12D)
AAACTTGTGGTGGTTGGAGCAGATGGCGTAGGC
Lkb1+/- KrasG12D (p.G12D)
AAACTTGTGGTGGTTGGAGCAGATGGCGTAGGC
Lkb1bud-/- KrasG12D (p.G12D)
AAACTTGTGGTGGTTGGAGCAGATGGCGTAGGC
Lkb1bud-/- KrasG12D (p.A11WfsX29)
AAACTTGTGGTGGTTGGA--TGGTGGCGTAGGC
Lkb1cys-/- KrasG12D (p.G12D)
AAACTTGTGGTGGTTGGAGCAGATGGCGTAGGC
E
WT
Apc-/-
KrasG12D
Stem
Fetal
Ascl2
Znf3
Axin2
Rnf32
Lrp4
Ly6d
Anxa1
Clu
Anxa3
Anxa8
Msln
3
2
1
0
−1
−2
−3
Lkb1+/-
Lkb1bud-/-
Lkb1cys-/-
F
iCMS2
iCMS3
Lkb1+/- vs. WT
Lkb1bud-/- vs. WT
Lkb1cys-/- vs. WT
−1
0
1
2
NES
FDR
0.0
0.1
0.2
0.3
0.4
Enriched in
WT
Lkb1-deficient
Significant
No
Yes