Role of LKB1 in stem cell fate determination and tumorigenesis

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“Faber est suae quisque fortunae”

“Each man is the architect of his own fate.”

“每个人都是自己命运的建筑师。”

- Appius Claudius Caecus

Dedicated to my beloved family

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Gao Y*, Yan Y*, Tripathi S, Pentinmikko N, Amaral A, Päivinen P, Domènech-Moreno E, Andersson S, Wong IPL, Clevers H, Katajisto P, Mäkelä TP.

Gastroenterology. 2020 Jan 10. pii: S0016-5085(20)30072-X. doi: 10.1053/j.gastro.2019.12.033. (*Equal contribution)

II N-methylnitrosourea aggravates gastrointestinal polyposis in *Lkb1*$^{+/-}$ mice

Udd L, **Gao Y**, Ristimäki AP, Mäkelä TP.

Carcinogenesis. 2013 Oct;34(10):2409-14. doi: 10.1093/carcin/bgt188. Epub 2013 May 30.

III Stromal Lkb1 deficiency leads to gastrointestinal tumorigenesis involving the IL-11-JAK/STAT3 pathway

Ollila S, Domènech-Moreno E, Laajanen K, Wong IP, Tripathi S, Pentinmikko N, **Gao Y**, Yan Y, Niemelä EH, Wang TC, Viollet B, Leone G, Katajisto P, Vaahtomeri K, Mäkelä TP.

J Clin Invest. 2018 Jan 2;128(1):402-414. doi: 10.1172/JCI93597. Epub 2017 Dec 4.

ABBREVIATIONS

2-DG = 2-deoxy-D-glucose
ß-NF = ß-naphthoflavone
ACC = Acetyl-CoA carboxylase
AMP = Adenosine monophosphate
AMPK = AMP-activated protein kinase
ATP = Adenosine triphosphate
ATOH1 = Atonal bHLH transcription factor 1
Bi-Pro = Bipotent progenitor
COX-2 = Cyclooxygenase-2
DBZ = Dibenzazepine
DCA = Dichloroacetate
Ent-Pro = Enterocyte progenitor
FACS = Fluorescence activated cell sorting
FZD = Frizzled
GI = gastrointestinal
GSEA = Gene set enrichment analysis
GSI = γ-secretase inhibitor
HSC = Haematopoietic stem cell
i.p. = Intraperitoneal
ISC = Intestinal stem cell
KO = knockout
LGR5 = Leucine-rich repeat-containing G-protein coupled receptor 5
LKB1 = Liver Kinase B 1
MEF = Mouse embryonic fibroblast
MNU = N-methylnitrosourea
MSC = Mesenchymal stem cell
OCR = Oxygen consumption rate
QSC = quiescent or reserve stem cell
OXPHOS = Oxidative phosphorylation
PAPG = Pepsinogen-altered pyloric glands
PDH = pyruvate dehydrogenase
PDK = pyruvate dehydrogenase kinase
pIpC = polyinosinic : polycytidylic acid
PJS = Peutz-Jeghers Syndrome
RSPO = R-spondin
Sec-Pro = Secretory progenitor
TA = Transit-amplifying
TAM = Tamoxifen

ABSTRACT

The tumor suppressor kinase, LKB1 (encoded by *STK11*), plays important functions in regulating diverse cell processes, including cell growth, metabolism, and polarity. As a bioenergetic sensor, LKB1 is required for metabolic balancing and maintenance of stem cell homeostasis in the haematopoietic system (Gurumurthy et al. 2010; Gan et al. 2010; Nakada, Saunders, and Morrison 2010) and in muscle (Shan et al. 2014). Intestinal stem cells (ISCs) are regulated by various cues from their niche-derived paracrine signals such as NOTCH and WNT (Clevers and Batlle 2013), and metabolic status (Rodríguez-Colman et al. 2017; Schell et al. 2017). **Study I** of this thesis aimed to investigate whether the metabolic regulator, LKB1, has a role in actively cycling ISCs, and identified it as a critical factor for maintaining ISC homeostasis. Mechanistically, LKB1 represses the transcription of the secretory lineage gatekeeper, *Atoh1*, via pyruvate dehydrogenase kinase 4 (PDK4) in ISCs and restricts ISC differentiation towards secretory cell lineages. These findings define LKB1 as an essential regulator of ISCs, and provide a connection between metabolism and fate determination of ISCs.

Germline mutations inactivating *LKB1* lead to gastrointestinal tumorigenesis in Peutz-Jeghers Syndrome (PJS) patients (Ylikorkala et al. 1999) and mouse models (Rossi et al. 2002; Bardeesy et al. 2002). However, little is known about the cell types and signaling pathways that underlie tumor formation, not much has been learnt about the progression of PJS polyposis neither. The upregulation of Cyclooxygenase-2 (COX-2) is a feature of PJS polyposis (Rossi et al. 2002; H. Takeda et al. 2004), and COX-2 inhibition reduces polyp growth in *Lkb1*$^{+/-}$ mice modelling PJS polyposis (Udd et al. 2004). **Study II** of this thesis evaluated the effect of the mutagenic carcinogen, N-methylnitrosourea (MNU), on gastrointestinal tumorigenesis in *Lkb1*$^{+/-}$ mice and concluded that MNU aggravates Peutz-Jeghers polyposis independently of COX-2. **Study III** of this thesis demonstrated that the loss of *Lkb1* in mesenchymal progenitor or stromal fibroblasts leads to the clonal expansion of stromal cells and to the induction of an inflammatory program involving the IL-11–JAK/STAT3 pathway, which is critical for tumorigenesis. The findings from **Studies II** and **III** provide further understanding of the function of LKB1 in Peutz-Jeghers tumorigenesis, and suggest potential therapeutic avenues for related tumor diseases.

LITERATURE REVIEW

Intestinal Stem Cells (ISCs)

1. Organization of the intestine

The embryonic endoderm is one of the three primary germ layers derived during gastrulation, and goes on to form the intestinal epithelium. The endoderm generates the embryonic gut tube by extensive folding under induction and molecular patterning (Noah, Donahue, and Shroyer 2011). The gut tube gradually divides into three parts, including the foregut, midgut, and hindgut, with the midgut giving rise to the colon and small intestine. Longitudinally (along the rostral-caudal axis), the small intestine can be subdivided into three anatomical segments: the duodenum, the jejunum, and the ileum (Vooijs, Liu, and Kopan 2011; van der Flier and Clevers 2009).

There are four layers in the composition of intestinal tract in all the anatomical segments: the outermost layer of loose connective tissue –termed adventia layer (or serosa)– contains blood vessels, lymphatics and nerves, covered by the visceral peritoneum. The next is smooth muscle layer –termed muscularis propria– composted by a circular muscle layer (inner) and a longitudinal muscle layer (out), executes the peristaltic movements to deliver food down through the gut. Below the muscularis propria there comes the third layer –termed submucosa– a loose connective tissue layer consists larger blood vessels, lymphatics, nerves, and mucous secreting glands. The innermost layer termed mucosa, is made up of three layers –an inner mucous epithelium that is composed of a single layer of lining epithelial cells, the lamina propria that consists loose fibrous connective tissue that provides vascular support for the epithelium, and a thin double layer of smooth muscle called the muscularis mucosa for local movement of the mucosa. The mucosa functions essentially for food digestion, nutrients absorption and pathogen defense (Treuting, Valasek, and Dintzis 2012). The absorptive surface of the small intestine is dramatically increased by numerous luminal protrusions, termed villi, and invaginations into the submucosa, the crypts (Sancho, Batlle, and Clevers 2004).

A cross section at microscopic or histologic view of the intestinal epithelium (Scheme shown in **Figure 1, left**) displays how crypts and villi are organized with various epithelial lineages. The crypts invaginate into the underlying submucosa as a pit in which the ISCs reside at the bottom. These pits also consist of Paneth cells and transit-amplifying progenitors. The villi are finger-like protrusions that project into the lumen to increase the surface area of the intestinal wall for nutrient absorption. Each villus is surrounded by six or more crypts, which continually produce all types of epithelial cells from ISCs to replenish the rapidly renewing intestinal epithelia (van der Flier and Clevers 2009).

2. Intestinal epithelial cell lineages

The intestinal epithelium is the fastest self-renewing tissue in mammals, with a turnover rate of 4–5 days. To replenish the rapidly renewing epithelium, there are new cells constantly generated by the intestinal stem cells ($LGR5^+$ ISCs) residing at the bottom of the crypts (Barker et al. 2007;

van der Flier and Clevers 2009; Watt and Hogan 2000; Clevers 2013). The stem cells divide symmetrically every day and give rise to two equipotential daughter cells, which stochastically adopt either a stem or bipotent progenitor cell fate following the neutral drift of niche factors (Snippert et al. 2010). The bipotent progenitors (Bi-Pro) are the immediate progeny of ISCs in the TA (transit-amplifying) zone (Kim et al. 2016), and they quickly adopt either a secretory progenitor (Sec-Pro) or an absorptive progenitor (enterocyte progenitor, Ent-Pro) fate via lateral inhibition. The TA progenitor pool spends 2–3 days in the crypt and divides approximately every 12–18 hours, or a total of 4–6 times prior to fully differentiating into the various epithelial lineages. This process gives rise to approximately 300 cells per day (Carulli, Samuelson, and Schnell 2014; Marshman, Booth, and Potten 2002) (**Figure 1, right**).

The progenitors, while dividing, become committed to specific lineages and cell types, finally leading to mitotically inactive, fully mature secretory or absorptive cells as they migrate out of the crypt. The secretory cells include mucous-secreting goblet cells, peptide hormone-secreting enteroendocrine cells, Paneth cells, and tuft cells, while the absorptive cells are enterocytes, that take up the majority of the epithelium (**Figure 1, right**) (Carulli, Samuelson, and Schnell 2014; van der Flier and Clevers 2009; Sato et al. 2011). Unlike the other terminally differentiated cells, Paneth cells reside at the bottom of the crypt for 6-8 weeks, providing essential signals for stem cell maintenance (Sato et al. 2011).

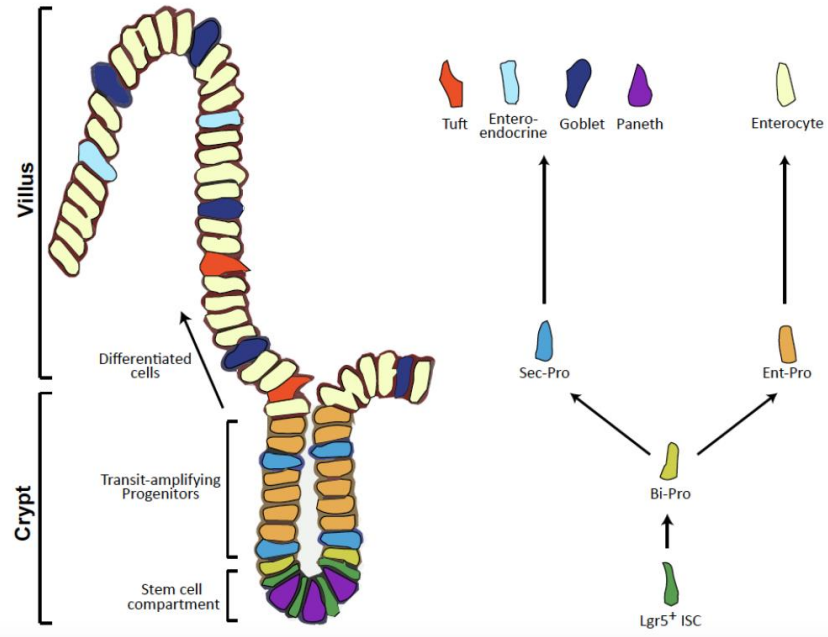

Figure 1. Organization of the intestinal epithelium. The intestinal crypt consists of intestinal stem cells, Paneth cells, and Transit-amplifying Progenitors (TA progenitors). The intestinal villi consist of terminally differentiated enterocytes, goblet cells, enteroendocrine cells, tuft cells. The LGR5+ ISCs keep self-renewing and give rise to the Bipotent-Progenitors (Bi-Pro), which then decide to

become either Secretory-Progenitors (Sec-Pro) or Enterocyte-Progenitors (Ent-Pro) depending on the signals received. Although Wnt signals promote secretory differentiation and absorptive differentiation is induced by active Notch, there is interplay between the two signaling pathways to regulate the intestinal epithelial differentiation. The Bi-Pro will take the route to become Sec-Pro when the secretory lineage gatekeeper, ATOH1, gets induced. They further differentiate to various mature secretory cell types depending on the regulation of secretory lineage genes that are downstream of ATOH1. The enterocytes are generated by the Ent-Pro. (Gerbe et al. 2011)

3. LGR5⁺ intestinal stem cells

The definition of stem cells may vary with the concepts of research interests and the system under analysis. The intestinal epithelium, that holds the most rapid renewing rate among the adult steady-state renewing tissues, a definition was formulated by Potten & Loeffler in 1990 and described with following terms: relatively undifferentiated, proliferative cells that maintain their numbers (self-renew), while at the same time producing a range of differentiated progeny that may continue to divide (potentiality of generating multiple lineages) (C.S. Potten and Loeffler 1990).

Prior to the genetic identification of stem cells with specific markers, there was a debate over the location and identity of ISCs. Based on an early DNA-label-retention study, the cycling "+4 cells", that locate at approximately four cell positions immediately above the base of the crypt, were proposed as the ISCs (Christopher S. Potten, Booth, and Pritchard 1997), and this has been widely recognized for a long time thereafter. An alternative view proposed the crypt base columnar (CBC) cells, that locate at the base of the crypt intermingled with the Paneth cells, as the true ISCs (Carulli, Samuelson, and Schnell 2014).

The first genetic identification of ISCs was described in the landmark paper published in 2007 by Nick Barker and Hans Clevers (Barker et al. 2007). *Lgr5*, a G-protein coupled receptor and a facultative component of the Wnt receptor complex that acts as a receptor for R-spondin1 (RSPO1) (De Lau et al. 2011), was found to be a specific marker of CBC cells (Barker et al. 2007). By generating *Lgr5*$^{EGFP\text{-}IRES\text{-}creERT2/+}$ mice, the Clevers group functionally demonstrated that LGR5+ cells are stem cells which are capable of producing all of the mature cell types in the intestinal epithelium (Barker et al. 2007). This conclusion was achieved through a lineage tracing strategy (with a *Rosa26R-LacZ reporter* in their setting) , using a technique that allows permanent activation of a reporter gene in a cell and all of its progeny and is therefore considered as the gold standard for defining a stem cell *in vivo* (Bjerknes and Cheng 2002). In addition, isolated LGR5+ cells were subsequently shown to possess the capacity to produce intestine-like tissue grown in perpetuity *in vitro*, termed intestinal organoids (enteroids), providing another line of evidence of stem-like function (Sato et al. 2009).

Despite the fact that the LGR5+ CBC cells had been established as the cycling stem cells, there are many additional studies supporting the idea of another stem cell population that resides in the approximately +4 position. Studies utilizing immunostaining and lineage tracing identified a number of putative markers of this population including *Bmi1* (Sangiorgi and Capecchi 2008), *Hpox* (N. Takeda et al. 2011), *Lrig1* (Powell et al. 2012; Wong et al. 2012), and *mTert* (Breault et al. 2008; Montgomery et al. 2011). Similar to the *Lgr5* study, the +4 lineage tracing experiments demonstrated that the +4 cells are a quiescent, or reserve stem cell (QSC) population that divides

more slowly but can be activated to both produce all of the differentiated intestinal cell types, in response to depletion of LGR5+ cells and drive regeneration after injury (Breault et al. 2008; Powell et al. 2012; Montgomery et al. 2011; Yan et al. 2012; N. Takeda et al. 2011; Tian et al. 2011).

Although there is abundant lineage tracing data supporting the idea of those +4 genes that mark the QSCs, whether these cells are truly an independent stem cell population still remains as a debate. The argument mostly arises from studies that find those QSC markers also express in cycling stem cells (**Table 1**) (Muñoz et al. 2012; Itzkovitz et al. 2012; Carulli, Samuelson, and Schnell 2014).

Table 1. Intestinal stem cell markers

Gene	Expressed in	Reference
Lgr5	CBC cells and +4 cells (low)	(Barker et al. 2007)
Ascl2	CBC cells	(van der Flier, van Gijn, et al. 2009)
Smoc2	CBC cells	(Muñoz et al. 2012)
Olfm4	CBC cells	(van der Flier, Haegebarth, et al. 2009)
Rnf43	CBC cells	(Koo et al. 2012)
Znrf3	CBC cells	(Koo et al. 2012)
Troy (Tnfrsf19)	CBC cells	(Fafilek et al. 2013)
Prom1	CBC cells and +4 cells	(Snippert et al. 2009)
Sox9	CBC cells and +4 cells	(Gracz, Ramalingam, and Magness 2010; Van Landeghem et al. 2012; Roche et al. 2015)
Msi1	CBC cells and +4 cells	(Christopher S. Potten et al. 2003)
Lrig1	CBC cells and +4 cells	(Powell et al. 2012; Wong et al. 2012; Muñoz et al. 2012)
Bmi1	CBC cells and +4 cells	(Sangiorgi and Capecchi 2008; Tian et al. 2011; Itzkovitz et al. 2012; Muñoz et al. 2012)
mTert	CBC cells and +4 cells	(Breault et al. 2008; Montgomery et al. 2011; Itzkovitz et al. 2012; Muñoz et al. 2012)
Hopx	CBC cells and +4 cells	(N. Takeda et al. 2011; Muñoz et al. 2012)

Location-wise, Paneth cells are in close association with the LGR5+ ISCs, with over 80% of the LGR5+ ISC surface area in contact with neighboring Paneth cells (Sato et al. 2011). Unlike other terminally differentiated cell types that migrate up the villi and shed off from the tip into the lumen with a life span of about 3–5 days, Paneth cells migrate down towards the crypt base, where they persist for approximately 6-8 weeks (Sato et al. 2011). Paneth cells express signaling molecules such as WNT3, EGF, TGFα, and DLL4, which are essential for the maintenance of ISCs. Together

with the important finding from the Clevers group that co-culturing the isolated LGR5+ ISCs together with CD24+ Paneth cells dramatically improves the organoid formation capacity of the ISCs (Sato et al. 2011), the Paneth cells are believed to serve as niche for LGR5+ ISCs.

4. Regulation of stemness and differentiation

The LGR5+ ISCs have the capacity of both self-renewal and multipotency—the properties that define stem cells. How ISCs make the decision to self-renew or differentiate is incompletely understood. In the current view, there are four major signaling pathways coordinatively regulating this process at the crypt base in a cell-extrinsic manner, meaning that the growth factors that are present are secreted by neighboring Paneth cells or the surrounding mesenchyme: Wnt, Notch, Bmp and Egf (Clevers and Batlle 2013). The most well-studied pathways that are critical to the development and maintenance of the intestinal epithelium are the Wnt and Notch pathways.

Wnt signaling is required for ISC and progenitor cell proliferation and is also implicated in regulating aspects of cell differentiation, likely through cross-talk with the Notch signaling pathway (Crosnier, Stamataki, and Lewis 2006). Genetic ablation of Wnt signaling disrupts normal homeostasis and leads to loss of the crypts (Fevr et al. 2007). Wnt signaling is activated by Wnt ligands, which are secreted from Paneth cells (Kim and Shivdasani 2011) or alternatively from the mesenchymal compartment (Farin, Van Es, and Clevers 2012), and bind to the Frizzled (FZD) receptor on Wnt-responding ISCs. The E3 ubiquitin ligases, RNF43/ZNRF3, suppress Wnt signaling by targeting FZD receptors for lysosomal degradation (Koo et al. 2012). *Lgr5* and its homologs, *Lgr4* and *Lgr6*, constitute the receptors for R-spondins (RSPO), and the Lgr5/RSPO complex reinforces Wnt signaling by neutralizing RNF43/ZNRF3 E3 ligases (De Lau et al. 2011; de Lau et al. 2014). Activated Wnt stabilizes β-catenin through a cascade of events and leads to the accumulation and nuclear translocation of β-catenin, which forms a complex with TCF/LEF and other co-activators (Martin-Orozco et al. 2019). This enhances transcriptional activation of TCF/LEF target genes such as *Lgr5* and *c-Myc* to maintain stemness and drive proliferation (Sato and Clevers 2013).

The Notch signaling pathway is also essential in controlling lineage specification in the intestinal epithelium. Active Notch signaling in proliferative stem and progenitor cells leads to differentiation towards absorptive lineages while blocked Notch signaling results in secretory lineage differentiation (Van Es et al. 2005). The Notch ligands, DLL1+DLL4+, secreted from Paneth cells trigger signaling in neighboring ISCs with Notch receptors NOTCH1/2, thus keeping them from secretory differentiation (Pellegrinet et al. 2011). The activated Notch signaling in ISCs leads to γ–secretase-mediated proteolytic release of the Notch intracellular domain (NICD) to the nucleus. NICD binds to the transcription factor recombination signal-binding protein J kappa (Rbpj, also known as CBF-1 or CSL) to activate transcription of the Notch target gene, *Hes1*, which represses *Atoh1* expression and promotes an absorptive rather than secretory cell fate (Jensen et al. 2000; Clevers 2013). Thus, the absorptive/secretory cell fate switch of ISCs and their immediate progeny (bi-potential progenitors) (Kim et al. 2016) following their exit from the crypt base due to neutral competition (Snippert et al. 2010) seems to be controlled through Notch by lateral inhibition (Artavanis-Tsakonas, Rand, and Lake 1999).

5. ATOH1, an intestinal secretory cell gatekeeper

Atonal bHLH Transcription Factor 1 (ATOH1, also known as MATH1) belongs to the basic helix-loop-helix (bHLH) family of transcription factors and is required for all secretory cell lineage differentiation (Q. Yang et al. 2001; Shroyer et al. 2007). ATOH1 reinforces its own expression and directly regulates *Dll1* and *Dll4* to control lateral inhibition, thus enabling Notch-dependent *Atoh1* repression in neighboring cells (Kim et al. 2014). Furthermore, it induces a number of its downstream secretory lineage genes: Growth factor independent 1 transcriptional repressor (*GFI1*) for goblet and Paneth cell differentiation (Shroyer et al. 2007) and Neurogenin-3 (*NEUROG3*) for enteroendocrine cell differentiation (Jenny et al. 2002).

In ISCs, ATOH1 is repressed at a low level (by Paneth cells) and in enterocytes as well via lateral inhibition by the canonical Delta-Notch-RBPjk-Hes1 signaling cascade (Jan and Jan 1994). Genetic ablation of the Notch effector, RBP-J (Van Es et al. 2005; Kim and Shivdasani 2011), or pharmacological inhibition using γ-secretase inhibitor (GSI) Dibenzazepine (DBZ) (Milano et al. 2004; Van Es et al. 2005; van Es et al. 2010) both lead to Notch inhibition and result in the loss of ISCs (van Es et al. 2010) and an increase in all intestinal secretory lineages in the crypts (Kim and Shivdasani 2011; Van Es et al. 2005; van Es et al. 2010), and this is mediated by induced *Atoh1* expression (Kim and Shivdasani 2011; van Es et al. 2010). Consistently, transgenic expression of *Atoh1* in the epithelium resembles Notch inhibition and converts ISCs and progenitors to secretory cells both *in vivo* (VanDussen and Samuelson 2010) and *ex vivo* (Koo et al. 2012). There is an interplay between Notch and Wnt signaling, or, more precisely, Notch activity is required for maintaining the correct balance of Wnt signaling in the crypts, which regulates *Atoh1* expression in a cell-extrinsic manner and allows for simultaneous maintenance of ISCs, proliferation, and differentiation (Tian et al. 2015).

6. Metabolism in stem cell fate determination

While the self-renewal and differentiation of ISCs is largely controlled by coordinated regulation of well-characterized Notch and Wnt signaling pathways, emerging evidence highlights the contribution of energy metabolism to the regulation of ISC function (Rodríguez-Colman et al. 2017; Schell et al. 2017; Beyaz et al. 2016). In Rodríguez-Colman et al., the authors examined the metabolic identity of LGR5+ CBCs and Paneth cells in supporting optimal stem cell function, and identify mitochondria and reactive oxygen species signaling as driving cellular differentiation (Rodríguez-Colman et al. 2017). In Schell et al., it was reported that intestinal stem cell function and proliferation can be controlled by mitochondrial pyruvate metabolism (Schell et al. 2017). Another observation described in Beyaz et al. demonstrated that high-fat diet (HFD)-induced obesity enhances stemness, as evidenced by augmented numbers and function of LGR5+ intestinal stem cells of the mammalian intestine (Beyaz et al. 2016).

The LKB1 kinase

1. LKB1 as a kinase

The *STK11* gene encodes Liver Kinase B1 (LKB1), a widely expressed serine/threonine kinase, and it was also established as a gene that can cause sporadic tumorigenesis due to somatic impaired encoded protein kinase activity (Ylikorkala et al. 1999)

LKB1 phosphorylates and activates its fourteen downstream intracellular kinases, including the well-known AMP-activated protein kinase α1 (AMPKα1) and AMPKα2, NUAK1/2, SIK1–3, SNRK, BRSK1/2, and MARK1–4. Through activation of these kinases, LKB1 regulates multiple signaling pathways functioning in essential cell processes, including protein synthesis, cell proliferation, metabolism, and polarity (Katajisto et al. 2007).

Full kinase activity can be reached only when LKB1 forms a trimeric complex with two other proteins, the pseudokinase STRAD and the scaffolding protein MO25, which also stabilize the LKB1 protein and anchor it in the cytoplasm, where it can mediate its known cellular functions through phosphorylating the downstream substrates (Baas A et al. 2003; Boudeau J et al. 2003).

2. LKB1 as a metabolic regulator

AMP-activated protein kinase (AMPK) was the first identified and best characterized substrate for the LKB1 kinase (Hawley et al. 2003; Woods et al. 2003; Shaw, Kosmatka, et al. 2004). When there is a lowered energy potential inside the cell, reflected in high AMP/ATP ration, the activating allosteric changes induced by AMP allows the Thr172 in AMPK to be phosphorylated by upstream kinase LKB1 (Xiao et al. 2013). For AMPK to be active, the heterotrimeric AMPK complex consisting of an α catalytic subunit (AMPKα) and two regulatory subunits (AMPKß and AMPKγ), needs to bind AMP in order to allow the activating phosphorylation by LKB1 (Hawley et al. 2003).

AMPK activation leads to a switch from energy consumption to energy generation, represented as a restoration of energy balance by catabolic-process promotion and anabolic-process inhibition. The reprograming of cellular metabolism by AMPK is conducted through the phosphorylation of its downstream substrates with cascade events. The promoted catabolic processes include, for instance, fatty acid uptake, glucose uptake, glycolysis, and autophagy. And the inhibition of energy consuming anabolic processes mainly include fatty acid synthesis suppression via acetyl-CoA carboxylase (ACC), as well as the downregulation of mTORC1-dependent translation, cell growth and proliferation (Hardie and Alessi 2013; Shorning and Clarke 2016; Garcia and Shaw 2017). Therefore AMPK functions as the cellular energy sensor, and LKB1, as the upstream kinase of AMPK, plays important role as a metabolic regulator.

3. Lkb1 in adult stem cells

Although recent study reported that ISCs exhibit high mitochondrial activity and oxidative phosphorylation (Rodríguez-Colman et al. 2017), however, in contrast to the ISCs, many other

adult stem cells including the haematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) reside in a hypoxic niche and reliant on anaerobic glycolysis (Ito and Suda 2014).

Lkb1 is critical for the maintenance of stem cell homeostasis in the haematopoietic system (for HSCs) (Gurumurthy et al. 2010; Gan et al. 2010; Nakada, Saunders, and Morrison 2010) and in muscle (Shan et al. 2014). In both cases, Lkb1 loss disrupts stem cell quiescence and lifts an initial temporary cell proliferation; but eventually, the former ends up with a depletion of HSCs, and the latter undergoes a defective differentiation of muscle progenitors. And the reason for both cases is mitochondrial defects and altered energy metabolism due to the loss of Lkb1.

LKB1 in Peutz-Jeghers Syndrome

Peutz-Jeghers syndrome (PJS) is a rare hereditary autosomal dominant cancer predisposition disease (Eng, Hampel, and Chapelle 2001), characterized by dark blue or dark brown mucocutaneous pigmentation as an early sign and multiple benign hamartomatous polyps developed in gastrointestinal (GI) tract as the main symptoms, as well as increased risk of various types of malignant cancer (Van Lier et al. 2011). The cause of PJS is germline mutations that inactivate *STK11* encoding the LKB1 tumor suppressor kinase (Aretz et al. 2005; Hemminki et al. 1998).

About half of the PJS patients display small intestinal obstruction or intussusception at their early age due to benign gastrointestinal polyps, which have to treated with repeated surgical removal (Utsunomiya et al. 1975). Besides the small intestine (64%) where polyps most commonly occur, there is also involvement with the colon (53%), stomach (49%), and rectum (32%), and rare cases have also been found in the upper and lower respiratory tract and bladder. The presenting symptoms in PJS patients include abdominal pain, rectal bleeding, anemia, small intestinal intussusception, bowel obstruction, and rectal prolapse of polyps. PJS is associated with increased risk of malignancy, and the mortality in PJS patients is mainly due to various malignancies rather than polyposis (Zbuk and Eng 2007; Shorning and Clarke 2016). A follow-up study restricted to PJS patients with LKB1 germline mutations demonstrated that about 81% of these patients developed any type of cancer by the age of 70. And the most commonly observed cancer is colorectal, followed by breast, small bowel, gastric and pancreatic malignancies (Lim et al. 2004).

The PJS polyps have no defining features on endoscopy, however, they display extensive stromal compartment with an elongated arborizing smooth muscle core microscopically. And this prominent smooth muscle core serves as the defining attribute of a PJS polyp (Estrada and Spjut 1983). Haploid loss of LKB1 is sufficient for polyp formation in *Lkb1*$^{+/-}$ mice, which develop gastrointestinal polyps that are histologically indistinguishable from PJS polyps, therefore *Lkb1*$^{+/-}$ mice are used to model the PJS polyposis (Rossi et al. 2002) (**Table 2**). Mice with haploid loss of LKB1 that is restricted to stromal smooth muscle cells also develop polyps, however, with lower penetrance and smaller size compared to *Lkb1*$^{+/-}$ mice in which both the GI stroma and epithelium are heterozygous for *Lkb1* loss (Katajisto et al. 2008).

The upregulated expression of COX-2, an enzyme eminently attribute to control the inflammatory reaction, has been determined as a feature of the hamartomatous polyposis in both PJS patients and *Lkb1*$^{+/-}$ mice (Rossi et al. 2002; de Leng et al. 2003). Knockout of *Cox-2*, or inhibition of COX-2 with celecoxib, can partially suppress PJS polyposis by reducing the size but not the number of tumors (Udd et al. 2004).

Recent studies have identified increased inflammatory signaling upon *Lkb1* loss in different cell and tissue contexts, such as in T cells (MacIver et al. 2011), macrophages (Liu et al. 2015), skeletal muscle (Chen et al. 2016), and lung cancer (Koyama et al. 2016), indicating *Lkb1* as a suppressor of inflammatory pathways. Taking into account the role of COX-2 in PJS polyposis, it would be

highly interesting to investigate the potential role of inflammatory pathways in PJS pathogenesis, which currently remains unknown.

Decrease or disappearance of the pepsinogen expression in pyloric mucosa has been regarded as a marker of preneoplasia in the stomach in rat, during the early stages of *N*-methyl-N′-nitro-N-nitrosoguanidine (MNNG)-induced gastric carcinogenesis before distinct morphological lesions arise (Furihata et al. 1975). Decreased pepsinogen expression is also observed in adenomas and carcinomas (Tatematsu et al. 1987). Such alteration of pepsinogen expression (pepsinogen-altered pyloric glands, PAPG) can be detected immunohistochemically in normal-looking pyloric mucosa. The mutagenic carcinogen N-Methylnitrosourea (MNU), a DNA-alkylating agent, can induce gastric carcinogenesis in several mouse strains, where PAPG have been found to be induced dose-dependently by MNU-treatment, and correlate with a higher proliferative activity than the normal counterpart epithelium (Yamamoto et al. 1997, 2002). Therefore, PAPG may be a precursor generally representing the preneoplastic lesion for gastric chemical carcinogenesis in rodents. In *Lkb1*$^{+/-}$ mice, stomach glands display aberrant or incomplete differentiation as detected by pepsinogen C staining, which shows an increased frequency of PAPG (Udd et al. 2010).

Table 2. Lkb1 knockout mouse models discussed in this thesis

* heterozygous (het), homozygous (hmz)

Genotype	Tissue involved	Phenotype (with Reference)
***Lkb1*$^{+/-}$**	all tissues het	PJS GI polyposis (Bardeesy et al. 2002; Jishage et al. 2002; Miyoshi et al. 2002), polyp COX-2 upregulated (Rossi et al. 2002; H. Takeda et al. 2004), Wnt-signaling deregulated (Lai et al. 2011), polyp mTOR signaling upregulated (Shaw, Bardeesy, et al. 2004), Osteoblastosis and benign osteogenic tumors (Robinson et al. 2008), well-differentiated endometrial adenocarcinomas in 50% of females (Contreras et al. 2008), Hepatocellular carcinomas in 70% of males (Nakau et al. 2002).
***Lkb1*$^{fl/+}$*;TagIn*$^{SM\text{-}CreERT2}$** **+ i.p. TAM**	smooth muscle cells het	PJS GI polyposis, polyp TGFß signaling deregulated (Katajisto et al. 2008).
***Lkb1*$^{fl/fl}$*;TagIn*$^{SM\text{-}CreERT2}$** **+ i.p. TAM**	smooth muscle cells hmz	PJS GI polyposis, stronger tumorigenicity than *Lkb1*$^{fl/+}$*;TagIn*$^{SM\text{-}CreERT2}$ + i.p. TAM mice (Katajisto et al. 2008).
Lkb1*$^{fl/fl}$*;Ah-Cre **+ i.p. ß-NF**	many tissues hmz, at least prostate, liver, GI epithelium, others hypomorph	Altered differentiation in GI epithelium with the appearance of intermediate cells and increase in angiotensin related signaling molecules, especially Ang II and renin, also defective Par-1 signaling implicated (Shorning et al. 2009, 2012).
Lkb1*$^{fl/fl}$*;Mx1-Cre **+pIpC induction**	variable knockout effect, in most tissues	Pancytopenia in 24-35 days, bonemarrow failure, lethality within 120 days due to pancytopenia, an initial and temporary proliferation increase in HSCs, but a rapid depletion thereafter (Gurumurthy et al. 2010; Nakada, Saunders, and Morrison 2010).

Lkb1$^{fl/fl}$;Ubc-CreERT2 **+ i.p. TAM**	all tissues	HSC phenotype as *Lkb1$^{fl/fl}$;Mx1-Cre* above (Nakada, Saunders, and Morrison 2010).
Lkb1$^{fl/fl}$;Rosa26-CreERT2 **+ i.p. TAM**	all tissues	Severe pancytopenia within 1 week, lethality within 30 days, first days after ablation HSC proliferate, then die through apoptosis, WT mice reconstituted with Lkb1 KO bonemarrow die within 60 days (Gan et al. 2010; Gurumurthy et al. 2010).
Lkb1$^{fl/fl}$;MyoD^{Cre}	embryonic myogenic progenitors	Premature death, due to severe myopathy characterized by central nucleated myofibers, reduced mobility, growth retardation (Shan et al. 2014).
Lkb1$^{fl/fl}$;Pax7^{CreER} **+ TAM**	myosatellite cells, also known as satellite cells or muscle stem cells	Lkb1 null satellite cells lose their regenerative capacity cell-autonomously, fail to maintain quiescence in noninjured resting muscles and exhibit accelerated proliferation but reduced differentiation kinetics (Shan et al. 2014).

AIMS OF THE STUDY

I To investigate the potential role of Lkb1 in intestinal stem cell fate determination by using genetically engineered mouse models and their derived three-dimensional organoid cultures with stem-cell-specific deletion of Lkb1, as well as LKB1-depleted human cancer cell lines.

II To evaluate the effect of MNU treatment on gastrointestinal tumorigenesis in *Lkb1*$^{+/-}$ mice modelling Peutz-Jeghers polyposis, and to assess the efficiency of COX-2 inhibition in this setting.

III To identify the mechanism by which Lkb1 mutations lead to Peutz-Jeghers Syndrome tumorigenesis by using genetically engineered mouse models, organoid cultures, and mouse embryonic fibroblast (MEF) cells.

MATERIALS AND METHODS

1. Materials

The materials used in this study are listed below with references. More detailed information can be found in the original publications referred to with Roman numerals.

Mouse line

Name	Description	Source/reference	Used in
Lgr5*EGFP-IRES-creERT2/+*	Heterozygous mice harbor an *Lgr5-EGFP-IRES-creERT2* "knock-in" allele that expresses EGFP and CreERT2 fusion protein. When bred with mice containing a *loxP*-flanked sequence of interest, Cre-mediated recombination will result in deletion of the floxed sequences in the *Lgr5*-expressing cells of the offspring upon the induction with Tamoxifen.	Clevers lab (Barker et al., 2007)	I, III
Lkb1*flox/flox*	Homozygous mice harbour conditional alleles flanking exons 3-6 of *Lkb1* (*Stk11*). When bred with mice containing the Cre recombinase-expressing allele, *Lkb1* is deleted.	DePinho lab (Bardeesy et al., 2002)	I, III
Rosa26*LSL-TdTomato/+*	Also referred to as Ai14 mice. These mice harbor a Cre reporter allele designed to have a *loxP*-flanked STOP cassette preventing transcription of a CAG promoter-driven red fluorescent protein variant (tdTomato) – all inserted into the *Gt(ROSA)26Sor* locus. When bred with mice containing the Cre-recombinase-expressing allele, the reporter expresses robust tdTomato fluorescence.	The Jackson Laboratory (Madisen et al., 2010)	I, III
CD1	Outbred mice derived from a group of outbred Swiss mice developed at the Anti-Cancer Center in Lausanne, Switzerland. The CD-1 IGS mice are generally used for genetics, toxicology, pharmacology, and aging research.	The Jackson Laboratory	II
C57BL/6J	Also known as Black 6, B6, B6J, C57 Black. C57BL/6J mice are the first strain to have its genome sequenced, and the most widely used inbred mice for	The Jackson Laboratory	II

	transgenic and knockout model development (the wild-type C57BL/6J mice are a good control), obesity and immunological studies as well. This strain is a permissive background for maximal expression of most mutations, though it is refractory to many tumors.		
Lkb1$^{+/-}$	Mice heterozygous for *Lkb1* that develop hamartomatous polyps that are indistinguishable from the human polyps, thereby serving as a disease model.	Mäkelä lab (Ylikorkala et al. 2001)	I,II, III
Fsp1-Cre	Also known as *S1004A-Cre*. These transgenic mice express Cre recombinase under the control of the mouse *S100a4*, S100 calcium binding protein A4, promoter. Cre recombinase expression is detected specifically in stromal fibroblasts of tissues such as the prostate, forestomach and mammary gland.	Leone lab (Trimboli et al. 2008)	III
Twist2-Cre (Dermo1-Cre)	These *Twist2-Cre* (*Dermo1-Cre*) mutant mice harbor a Cre recombinase knock-in allele that also abolishes endogenous twist homolog 2 (*Twist2*) gene function. Heterozygotes are viable and fertile, while homozygotes (*Twist2*$^{-/-}$) die a few days after birth. Cre recombinase activity is reported in the mesoderm as early as embryonic day 9.5, as well as in mesodermal tissues such as branchial arches and somites, and in condensed mesenchyme-derived chondrocytes and osteoblasts.	The Jackson Laboratory (Yu et al. 2003)	III
Rosa26R-LacZ reporter	The targeted mutant mice carry a *loxP*-flanked neo cassette upstream of a β-galactosidase (*lacZ*) sequence. Removal of the neo cassette by Cre recombination results in lacZ expression in Cre-expressing tissues of the offspring.	The Jackson Laboratory (Soriano et al. 1999)	III
Rosa26R-mTmG reporter	Also known as mT/mG. *Rosa26R-mTmG* is a cell membrane-targeted, two-color fluorescent Cre-reporter allele. Prior to Cre recombination, cell membrane-localized tdTomato (mT) fluorescence expression is widespread in cells/tissues. Cre-recombinase-expressing cells (and future cell lineages derived from these cells) have cell-membrane-localized	The Jackson Laboratory (Muzumdar et al. 2007)	III

	EGFP (mG) fluorescence expression replacing the red fluorescence.		
Rosa26R-Confetti reporter	Also known as R26R-Brainbow2.1 , Rosa26-CAG-Brainbow2.1/Confetti. Homozygous R26R-Confetti mice are viable and fertile. The R26R-Confetti conditional allele contains the Brainbow 2.1 cassette, which has a ubiquitous CAG promoter followed by a *loxP*-flanked NeoR-cassette whose polyadenylation sequence terminates transcription of the downstream 4 fluorescent reporter genes, encoding for a nuclear-localized green fluorescent protein (hrGFPII), a cytoplasmic yellow fluorescent protein (mYFP), a cytoplasmic red fluorescent protein (tdimer2(12)) and a membrane-tethered cyan fluorescent protein (mCerulean) fluorescent proteins, positioned in two tandems. No fluorescence is expressed prior to Cre activation. Cre-mediated recombination simultaneously excises the NeoR-cassette and leads to the production of certain fluorescent proteins depending on which parts of the Brainbow 2.1 construct are excised.	The Jackson Laboratory (Snippert et al. 2010)	III

Cell lines

Name	Description	Source/reference	Used in
HEK293FT	Human embryonic kidney	ATCC	I
Ls174t	Human colorectal adenocarcinoma	ATCC	I

Antibodies

Name	Description	Source/reference	Used in
anti-GAPDH	Rabbit monoclonal, WB	Cell Signaling, #2118	I, III
anti-Vinculin	Mouse monoclonal, WB	Sigma, V9131	I
anti-LKB1	Mouse monoclonal, WB	Abcam, ab15095	I, III
anti-cleaved Notch1	Rabbit monoclonal, WB	Cell Signaling, #4147	I

anti-Phospho-S6	Rabbit polyclonal, WB	Cell Signaling, #2211	I
anti-S6	Rabbit monoclonal, WB	Cell Signaling, #2217	I
anti-Phospho-Acetyl-CoA Carboxylase	Rabbit polyclonal, WB	Cell Signaling, #3661	I
anti-Acetyl-CoA Carboxylase	Rabbit polyclonal, WB	Cell Signaling, #3662	I
anti-PDK4	Mouse monoclonal, WB	Abcam, ab110336	I
anti–STAT3-phosphoY705	Rabbit monoclonal, WB	Cell Signaling, #9145	III
anti-STAT3	Rabbit polyclonal, WB	Cell Signaling, #9132	III
anti-ERK1/2-phosphoT202/204	Rabbit monoclonal, WB	Cell Signaling, #4695	III
anti-ERK1	Rabbit polyclonal, WB	Santa Cruz Biotechnology, sc-94	III
anti-GFP	Goat polyclonal, IF and IHC	Abcam, ab5450	I, III
anti-RFP	Rabbit polyclonal, IF and IHC	Rockland, 600-401-379	I, III
anti-LKB1	Rabbit monoclonal, IHC	Cell Signaling, #13031	I
anti-PDK4	Rabbit polyclonal, IHC	Abcam, ab71240	I
anti-pepsinogen C	Rat, IHC	(Furihata et al., 1973)	II
anti-p53	Mouse monoclonal, IHC	Invitrogen, 13-4100	II
anti-Ki-67	Rat monoclonal, IHC	DAKO, M7249	II, III
anti-Fsp1	Rabbit Polyclonal, IHC	DAKO, A5114	III
anti-αSMA	Mouse monoclonal, IHC	Sigma, A2547	III
anti-vimentin	Rabbit monoclonal, IHC	Abcam, ab92547	III
anti-Ki67	Rabbit polyclonal, IHC	Abcam, ab15580	III
anti-CD45	Rabbit polyclonal, IF	Abcam, ab10588	III
anti-CD24	Rat monoclonal, Pacific blue conjugated, FACS	Biolegend, 101820	I
anti-EPCAM	Rat monoclonal, APC conjugated, FACS	eBioscience, 17-5791-82	I

Growth factors and supplements in organoid culture

Name	Description	Source/reference	Used in
EGF	Recombinant full-length mouse EGF protein, untagged, for organoid culture.	ThermoFisher Scientific, *E.coli* derived, PMG8041	I, III
Noggin	Recombinant full-length mouse Noggin protein, for organoid culture.	Peprotech, *E.coli* derived, 250-38	I, III
R-spondin1	Recombinant truncated mouse R-spondin protein (Ser21-Gly209), for organoid culture.	R&D, *E.coli* derived, 3474- RS-050	I, III

N2 supplement	A chemically defined, serum-free supplement based on Bottenstein's N-1 formulation.	ThermoFisher Scientific, 17502048	I, III
B27 supplement	A serum-free supplement ideal for the cultivation of neural progenitor and stem cells, either as neurospheres in suspension or in adherent monolayer culture, without inducing differentiation.	ThermoFisher Scientific, 12587010	I, III
IL-6	Recombinant Human IL-6 Protein.	R&D, *E. coli* derived, 206-IL-01	III
IL-11	Recombinant Murine IL-11 Protein.	PeproTech, *E. coli* derived, 220-11	III
Y-27632 dihydrochloride	Rho-associated protein kinase (ROCK) inhibitor.	Sigma, Y0503	I, III
N-Acetyl-L-cysteine (NAC)	NAC has been used as a component of intestinal basal medium for the culture of mouse intestinal stem cells and also as a component of expansion medium.	A9165	I, III

Plasmids

Name	Description	Source/reference	Used in
pLKO.1-puro-shNT	pLKO.1 lentiviral vector for expression non-targeting shRNA, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), SHC002	I, III
pLKO.1-puro-shLKB1_8	pLKO.1 lentiviral vector for expression shRNA targeting human *LKB1*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000000408	I
pLKO.1-puro-shLKB1_9	pLKO.1 lentiviral vector for expression shRNA targeting human *LKB1*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000000409	I
pLKO.1-puro-shLKB1_11	pLKO.1 lentiviral vector for expression shRNA targeting human *LKB1*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000000411	I
pLKO.1-puro-shLKB1_13	pLKO.1 lentiviral vector for expression shRNA targeting human *LKB1*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000000413	I

pLKO.1-puro-shPRKAA1_7	pLKO.1 lentiviral vector for expression shRNA targeting human *PRKAA1*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000000857	I
pLKO.1-puro-shPRKAA1_8	pLKO.1 lentiviral vector for expression shRNA targeting human *PRKAA1*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000000858	I
pLKO.1-puro-shPRKAA2_8	pLKO.1 lentiviral vector for expression shRNA targeting human *PRKAA2*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000002168	I
pLKO.1-puro-shPRKAA2_9	pLKO.1 lentiviral vector for expression shRNA targeting human *PRKAA2*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000002169	I
pLKO.1-puro-shATOH1_5	pLKO.1 lentiviral vector for expression shRNA targeting human *ATOH1*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000013585	I
pLKO.1-hygro-shNT	pLKO.1 lentiviral vector for expression non-targeting shRNA, with Hygromycin antibiotic resistant gene for selection stable cell line.	Mäkelä lab based on SHC002	I
pLKO.1-hygro-shATOH1_5	pLKO.1 lentiviral vector for expression shRNA targeting human *ATOH1*, with Hygromycin antibiotic resistant gene for selection stable cell line.	Mäkelä lab based on TRCN0000013585	I
pLKO.1-puro-Nuak1	pLKO.1 lentiviral vector for expression shRNA targeting mouse *Nuak1*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000024112	III
pLKO.1-puro-Nuak2	pLKO.1 lentiviral vector for expression shRNA targeting mouse *Nuak2*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000024271	III
pLKO.1-puro-Mark1	pLKO.1 lentiviral vector for expression shRNA targeting mouse *Mark1*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000024173	III
pLKO.1-puro-Mark2	pLKO.1 lentiviral vector for expression shRNA targeting mouse *Mark2*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000023988	III
pLKO.1-puro-Mark3	pLKO.1 lentiviral vector for expression shRNA targeting mouse *Mark3*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000024107	III

pLKO.1-puro-Mark4	pLKO.1 lentiviral vector for expression shRNA targeting mouse *Mark4*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000024281	III
pLKO.1-puro-Sik1	pLKO.1 lentiviral vector for expression shRNA targeting mouse *Sik1*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000024098	III
pLKO.1-puro-Sik2	pLKO.1 lentiviral vector for expression shRNA targeting mouse *Sik2*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000024288	III
pLKO.1-puro-Sik3	pLKO.1 lentiviral vector for expression shRNA targeting mouse *Sik3*, with Puromycin antibiotic resistant gene for selection stable cell line.	The RNAi Consortium (Moffat et al., 2006), TRCN0000079132	III

siRNAs

Name	Description	Source/reference	Used in
siNT pool	Non-targeting siRNA pool	Dharmacon, D-001819-10	I
siCTNNB1 pool	siRNA pool targeting human *CTNNB1*	Dharmacon, L-003482-05	I
siPDK4 pool	siRNA pool targeting human *PDK4*	Dharmacon, L-005025-05	I

Gene expression arrays

Name	Source/reference	Used in
TRI Reagent	MRC, TR118	I, III
NucleoSpin RNA Plus	Machery-Nagel, 740984	I
RNeasy Plus Mini Kit	Qiagen, 74136	I, III
RNA*later*™ Stabilization Solution	Ambion, AM7020	III
TaqMan™ Reverse Transcription Reagents	ThermoFisher Scientific, N8080234	I, III
KAPA SYBR® FAST qPCR Kits (ABI Prism) from Roche	Roche, KK4617	I, III

Other Major Reagents

Name	Source/reference	Used in
TAM (tamoxifen)	Sigma, T5648	I, III
4-OHT ((Z)-4-hydroxytamoxifen)	Sigma, H7904	I
DAPT	Sigma, D5942	I
AICAR	Tocris Bioscience, A611700	I
DCA (Dichloroacetate)	Sigma, 347795	I
2-DG (2-deoxy-D-glucose)	Sigma, D6134	I
Phloretin	Sigma, P7912	I
UK-5099	R&D, 4186	I
Lactate	Sigma, L7022	I
Pyruvate	Sigma, P2256	I
MG132	Calbiochem, 474791	I
CHIR (CHIR99021)	Sigma, SML1046	I
AR (AR-A014418)	Sigma, A3230	I
LiCl (Lithium Chloride)	Sigma, L9650	I
Curcumin	Enzo LifeSci, ALX-350-M010	I
VPA (Valproic acid)	Sigma, PHR1061	I
TSA (Trichostatin A)	Sigma, T8552	I
BT3 (Butyrolatone 3)	Enzo LifeSci, ALX-270-411-M005	I
CCCP (Carbonyl cyanide 3-chlorophenylhydrazone)	Sigma-Aldrich, C2759	I
Rotenone	Sigma-Aldrich, 45656	I
Antimycin A	Sigma-Aldrich, A8674	I
A-769662	Abcam, ab120335	I
Alcian Blue	Sigma, B8438	I
Target Retrieval Solution, pH 9	DAKO, S236784-2	I, III
Target Retrieval Solution, Citrate pH 6.1	DAKO, S169984-2	I, II, III
SignalStain DAB Substrate Kit	Cell Signaling, #8059	I, II, III
Celecoxib	Searle-Pharmacia, Pfizer	II
N-methyl-N-nitrosourea	Sigma-Aldrich	II
Tissue-Tek® O.C.T.™ Compound	SAKURA, 4583	III
Ruxolitinib	Incyte, INCB18424	III
Mouse IL-11 ELISA Kit	Sigma, RAB0251	III

2. Methods

The methods are listed below alphabetically, and the details are described in original publications.

Method/Protocol	Used and described in
Adenovirus transduction	III
Alcian blue staining	I
DNA and RNA transfection	I, III
ELISA	III
Flow cytometry analysis and sorting	I, III
Frozen sections	III
Generation of mouse embryonic fibroblasts (MEFs)	III
Generation of shRNA constructs	I, III
Gene Set Enrichment Analysis (GSEA)	I, III
Image acquisition and analysis	I, II, III
Immunofluorescence analysis and quantification	I, III
Immunohistochemistry	I, II, III
Intestinal crypt and stem cell isolation	I
Intestinal organoid culture	I, III
Intraperitoneal (IP) injection	I, III
Lentivirus production and transduction	I, III
Mammalian cell culture	I
Mouse breeding and genotyping	I, II, III
Mouse dissection	I, II, III
Mouse husbandry and handling	I, II, III
Quantitative real-time PCR (qRT-PCR)	I, III
RNA sequencing, data acquisition and analysis	I, III
Statistics	I, II, III
Tissue processing, embedding and sectioning	I, II, III
Total RNA extraction	I, III
Western blotting (WB)	I, III
X-gal staining	III

RESULTS AND DISCUSSION

LKB1 links energy metabolism to ATOH1-mediated ISC fate determination (I)

RESULTS (I)

1. Lkb1 loss in LGR5⁺ ISCs leads to crypt-specific phenotypes

Experimental mouse models were generated from previously established mouse strains including *Lgr5*$_{EGFP\text{-}IRES\text{-}creERT2/+}$ mice (Barker et al. 2007), *Lkb1*$_{flox}$ (*Lkb1*$_{fl}$) mice (Bardeesy et al. 2002), and *Rosa26*$_{LSL\text{-}TdTomato}$ mice (Madisen et al. 2010). Eight to twelve week-old *Lgr5*$_{EGFP\text{-}IRES\text{-}creERT2/+}$; *Lkb1*$_{flox/flox}$; *Rosa26*$_{LSL\text{-}TdTomato/+}$ mice were intraperitoneally injected with a single dose of Tamoxifen in order to both achieve inactivation of *Lkb1* alleles in LGR5$_{+}$ ISCs and allow lineage tracing with TdTomato expression (*Lkb1*$_{Lgr5\text{-}KO}$) (Model explained in **Figure 2**). The control group consisted of *Lgr5*$_{EGFP\text{-}IRES\text{-}creERT2/+}$; *Rosa26*$_{LSL\text{-}TdTomato/+}$ mice that received the same Tamoxifen treatment. A highly efficient recombination rate (close to 100%) in ISCs (Lgr5$_{Hi}$) was detected by flow cytometry analysis of TdTomato expression from those mice five days post Tamoxifen induction, and further confirmed with LKB1 protein loss in TdTomato$_{+}$ crypts by immunohistochemistry. At the same time point, however, Paneth cell recombination was not yet noted at a significant level.

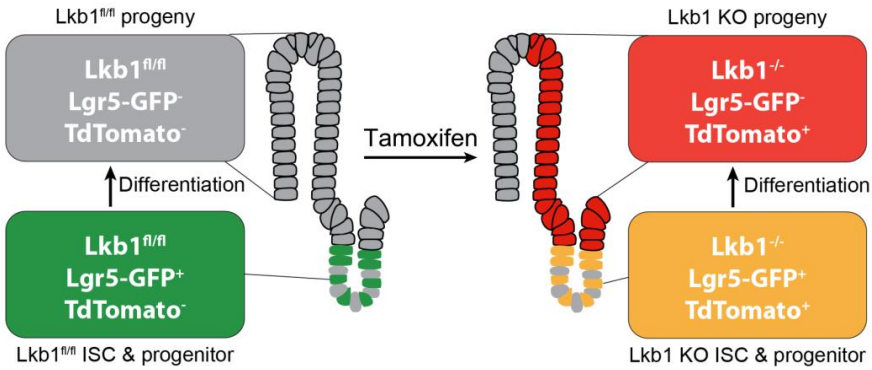

Figure 2. Deletion of *Lkb1* in LGR5$_{+}$ ISCs and lineage tracing to mark *Lkb1*-deleted (*Lkb1 KO*) ISCs and progeny. In *Lgr5*$_{EGFP\text{-}IRES\text{-}creERT2/+}$; *Lkb1*$_{flox/flox}$; *Rosa26*$_{LSL\text{-}TdTomato/+}$ mice. **(Left)** *Lkb1*$_{fl}$ alleles are not recombined in the absence of Tamoxifen, therefore, functional *Lkb1* is expressed in both ISCs and their progeny. ISCs express Lgr5-GFP (in green) and are not labeled with TdTomato. **(Right)** Tamoxifen treatment activates *Cre* recombinase, which irreversibly induces both recombination of the *Lkb1*$_{fl}$ alleles and activation of the TdTomato reporter. Therefore, *Lkb1* deletion and TdTomato lineage tracing first happen in ISCs and are inherited by their progeny upon differentiation. The recombined ISCs express both GFP and TdTomato (in yellow) and recombined progeny only express TdTomato (in red).

The GFP and TdTomato expression analyzed from the *Lkb1*$_{Lgr5\text{-}KO}$ small intestine (up to 600 days post Tamoxifen induction) and from the organoid formation assay (Sato et al. 2009) from *Lkb1*$_{Lgr5\text{-}KO}$ crypts demonstrated the long-term maintenance of *Lkb1*-depleted intestinal stem cells and their capacity of producing progeny.

Despite the long term maintenance of some *Lkb1*-deficient ISCs, we also noted comparable reductions in both the total GFP^{+} crypt number (47%, based on immunofluorescence staining quantification) and total GFP^{+} LGR5^{+} ISC number (49%, based on flow cytometry analysis) after five days in *Lkb1*$_{Lgr5\text{-}KO}$ mice compared to control. In addition, TdTomato detection was more often found to be restricted to the villi in these mice, suggesting that ISCs in some of the *Lkb1*-deleted crypts had differentiated. No further decrease of *Lkb1*$_{Lgr5\text{-}KO}$ ISCs was noted at later time points (up to 600 days).

Taken together, *Lkb1* deletion leads acutely to a reduction in a subset of ISCs in a crypt-specific manner, while in other crypts, *Lkb1*-deficient stem cells are maintained and functional in the long term.

2. Transcriptomic analysis reveals Atoh1 induction and secretory progenitor signature in Lkb1$^{Lgr5\text{-}KO}$ ISCs

A FACS-based LGR5^{+} ISC purification method established by the Clevers lab (Sato et al. 2009) and RNA sequencing (RNA-seq) technology provide the opportunity to decipher the molecular changes following Lkb1 deletion in ISCs (Lgr5^{Hi}) and progenitors (Lgr5^{Low}). The transcriptomes of both Lgr5^{Hi} and Lgr5^{Low} populations five days after *Lkb1* loss were compared with control samples. To improve the previously established wild-type (WT) ISC signature and progenitor signature (Muñoz et al. 2012), we took the union of 733 significantly altered transcripts between Hi and Low populations in our study, and 1056 transcripts identified by re-analyzing the microarray data from Muñoz et al. (Muñoz et al. 2012) displaying a strong similarity with our study. The union generated a robust 464-gene ISC signature (ISC_464) and a 538-gene progenitor signature (Progenitor_538). While several of the well-characterized ISC/quiescent/+4 markers, such as *Lgr5*, *Olfm4*, *Bmi1*, *mTert*, *Hopx*, and *Lrig1* were not significantly altered in *Lkb1*$_{Lgr5\text{-}KO}$ ISCs (*Lkb1*$_{Lgr5\text{-}KO}$_Hi) compared to WT controls, gene set enrichment analysis (GSEA) with the ISC_464 and Progenitor_538 signatures suggested a shift of the *Lkb1*-deleted ISCs toward the progenitor lineage.

To further characterize the *Lkb1*$_{Lgr5\text{-}KO}$_Hi cells, we compared their signature to the signatures of secretory progenitors (Sec-Pro) and enterocyte progenitors (Ent-Pro). Remarkably, the *Lkb1*$_{Lgr5\text{-}KO}$_Hi cells displayed significant similarity with three different Sec-Pro signatures generated from existing datasets (Van Es et al. 2012; Basak et al. 2014; Kim et al. 2014). By contrast, no significant similarity was noted between the *Lkb1*$_{Lgr5\text{-}KO}$_Hi cells and an Ent-Pro signature obtained from *Atoh1*-deleted cells (Kim et al. 2014). When comparing the *Lkb1*$_{Lgr5\text{-}KO}$_Low cells, the similarity with the Sec-Pro signatures was even more pronounced, but some similarity was also noted with Ent-Pro. These results indicate at the molecular level that *Lkb1* loss in ISCs leads to a predominance of secretory characteristics.

Leading edge genes from the comparison of *Lkb1*$_{Lgr5\text{-}KO}$_Hi cells with Sec-Pro signatures contained a number of secretory markers including the master regulator of secretory cell lineage, *Atoh1* (Q. Yang et al. 2001; Shroyer et al. 2007), and its downstream targets, *Gfi1* (Shroyer et al. 2005) and *Neurog3* (Jenny et al. 2002). The same analysis also identified *Atoh1* and several secretory markers in *Lkb1*$_{Lgr5\text{-}KO}$_Low cells. Although the *Atoh1* level in *Lkb1*$_{Lgr5\text{-}KO}$_Hi cells was significantly higher than in WT_Hi cells, the level of induction was lower than noted between WT_Hi and WT_Low, consistent with the retained stem cell capacity of the *Lkb1*$_{Lgr5\text{-}KO}$ cells (Kim et al. 2016). It is also notable that the *Lkb1*$_{Lgr5\text{-}KO}$_Hi signature is similar to an *Atoh1*$_{TdTomato}$*Lgr5*$^{+}$ signature (Ishibashi et al. 2018).

The enrichment of secretory genes noted in *Lkb1*-deleted Hi and Low cells was associated with an increase of mucin-secreting cells (Alcian blue positive) in *Lkb1*-deleted crypts and villi marked by TdTomato, consistent with increased and perturbed secretory cell differentiation noted following *Lkb1* loss in differentiated lineages (Shorning et al. 2009).

3. Induction of the secretory phenotype in Lkb1-deficient cells is dependent on Atoh1

shRNA-mediated depletion of *LKB1* in Ls174t cells—which have high *LGR5* (Barker et al. 2007), low *ATOH1* (Kazanjian et al. 2010), and functional *LKB1*—leads to increased expression of *ATOH1* mRNA and a secretory phenotype that is evidenced by increased mucin production, which well-recapitulates the *in vivo* phenotype. When *ATOH1* is depleted in addition to *LKB1*, the cells lack mucin production. These results indicate that LKB1 suppresses the *ATOH1*-mediated secretory phenotype.

4. Lkb1 represses ATOH1 independently of Wnt and Notch signaling

Atoh1 has been identified to be regulated in ISCs by the niche factors, Wnt (Shi et al. 2010; Tian et al. 2015) and Notch (Van Es et al. 2005; Kazanjian et al. 2010). Our GSEA analysis of RNA-seq data did not demonstrate significant changes in Wnt and Notch signaling (besides *Atoh1*) in *Lkb1*-deleted ISCs.

The notion that *Lkb1* regulates *Atoh1* independently of Wnt signaling was also supported by the observation that *LKB1* represses *ATOH1* in Ls174t cells with a constitutively active ß-catenin and Wnt pathway (Li et al. 2012). To investigate this directly, we depleted ß-catenin in Ls174t cells, where *ATOH1* expression was decreased in consistence with an earlier report (Shi et al. 2010), and observed that *LKB1* depletion induces *ATOH1* both in control and ß-catenin-depleted conditions.

LKB1 knockdown in Ls174t cells did not affect Notch activation as measured by cleaved Notch 1, consistent with the GSEA results from ISCs. To further investigate the interrelationship of *Lkb1* and the Notch pathway in *Atoh1* induction, we analyzed the *ATOH1* and Notch target, *HES1*, following *LKB1* knockdown or Notch inhibition by 20μM γ–secretase inhibitor DAPT, which efficiently blocks Notch activity. Consistent with results from the ISC RNA-seq analysis, *LKB1* knockdown did not affect *HES1* or its downregulation by DAPT. As expected, Notch inhibition induced *ATOH1*, similar to the *LKB1* knockdown. Importantly, the combination of *LKB1*

knockdown and Notch inhibition led to significant further induction of *ATOH1*. The independent regulation of *Atoh1* by *Lkb1* and Notch inhibition was also supported by a further reduction of ISCs in DAPT-treated *Lkb1*$_{Lgr5\text{-}KO}$ organoids.

Collectively, the cell line studies demonstrated that ß-catenin knockdown did not override the effect of *Lkb1*, and that Notch inhibition showed a synergistic effect with *Lkb1* in both the cell line and *ex vivo* organoid models. Together with the RNA-seq analysis, we concluded that *Lkb1* represses *Atoh1* independently of Wnt and Notch signaling.

5. AMPK is not required for ATOH1 repression by LKB1

Urged by recent studies indicating the critical role of cellular metabolism in regulating ISC functions (Rodríguez-Colman et al. 2017; Schell et al. 2017; Mattila et al. 2018), we became interested in asking whether metabolism contributes to *ATOH1* repression by LKB1, thereby influencing the secretory differentiation. One of the important downstream substrates of LKB1 is AMPK, which functions as an energy sensor that regulates multiple metabolic pathways (Hardie and Alessi 2013). Therefore, we investigated whether the *ATOH1* induction following LKB1 loss is mediated by AMPK.

The allosteric activator, A769662, can activate AMPK in the absence of LKB1 (Göransson et al. 2007), thus providing the possibility to restore AMPK activation in *LKB1*-depleted cells. *LKB1* deletion indeed eliminated AMPK activity in Ls174T cells, as measured by the level of ACC phosphorylation, and this could be restored by A769662 treatment without affecting *ATOH1* induction. In addition, we also found that neither AMPKalpha1 (*PRKAA1*) nor alpha2 (*PRKAA2*) depletion was sufficient to induce *ATOH1* expression. These results suggest that AMPK is not required for *ATOH1* repression by LKB1.

6. The PDK inhibitor, DCA, attenuates ATOH1 induction and alleviates impaired respiration in LKB1-deficient cells

To look for new ways in which *ATOH1* might be regulated by *LKB1*, we screened the following compounds in Ls174t cells by adding them respectively to the cells in culture to see which compound would modulate the *ATOH1* induction upon *LKB1* depletion. The compounds include those that modulate glycolysis and oxidative phosphorylation (Rodríguez-Colman et al. 2017; Schell et al. 2017), and also those that regulate *ATOH1* by targeting i) proteasome degradation (Peignon et al. 2011); ii) GSK3ß (Peignon et al. 2011; Yin et al. 2014); iii) histone acetyl-transferases (Stojanova, Kwan, and Segil 2015); or iv) histone deacetylases (Yin et al. 2014; Stojanova, Kwan, and Segil 2015). Interestingly, the only compound identified to modulate *ATOH1* induction following *LKB1* depletion was the pyruvate dehydrogenase kinase (PDK) inhibitor, dichloroacetate (DCA).

PDK inhibits pyruvate dehydrogenase (PDH) and thereby oxidative phosphorylation (OXPHOS) and thus cellular respiration. Thus, DCA works to inhibit aerobic glycolysis and promote OXPHOS. We measured the oxygen consumption rate (OCR) following DCA treatment, and

found that DCA can partially restore the impaired basal and maximal respiration in *LKB1*-deficient cells.

We went back to our RNA-seq data , an unbiased analysis of pathways deregulated in *Lkb1*-deleted ISCs and progenitors revealed a significant enrichment in an OXPHOS signature. Interestingly, the top-ranked gene in $Lkb1_{Lgr5\text{-}KO}$ ISCs was *Pdk4* in this analysis, whereas several other OXPHOS genes were more significantly altered later in the $Lkb1_{Lgr5\text{-}KO}$ progenitors. *Pdk4* was the only PDK (the PDK family includes PDK1, PDK2, PDK3, and PDK4) induced by *Lkb1* deletion in ISCs and this was recapitulated in Ls174t cells. Importantly, *PDK4* depletion attenuated the induction of *ATOH1* in *LKB1*-depleted Ls174t cells, indicating a critical role of PDK4 in mediating the repression of *ATOH1* by LKB1.

Taken together, these results demonstrated that LKB1 specifically represses PDK4, and thereby maintains normal OXPHOS. It also provided evidence that the repression of *ATOH1* by LKB1 is mediated by suppressing PDK4.

DISCUSSION (I)

Our findings reveal the important role of *Lkb1* in ISC homeostasis. The specific deletion of *Lkb1* in LGR5+ cells generates ISCs with elevated *Atoh1*, yet without a noticeable effect on stem cell functions in some of the targeted crypts. The ISCs with a high level of *Atoh1* share molecular similarities with DLL1+ secretory progenitors (Van Es et al. 2012) and recently described ATOH1+ secretory lineage intestinal epithelial cells (Ishibashi et al. 2018). Those secretory progenitors can revert to ISCs following tissue damage, therefore it would be interesting to investigate whether this is associated with LKB1 activation.

However, in other crypts *Lkb1* deletion leads to a rapid loss of ISCs prior to any noticeable targeting of the niche Paneth cells indicating that *Lkb1* loss disrupts homeostasis in these ISCs in a cell-intrinsic manner. The two ISC fates following *Lkb1* loss suggest crypt-specific differences in ISCs as identified by Snippert et al. (Snippert et al. 2010). It is tempting to speculate that the observed heterogeneity of ISC *Atoh1* levels (Kim et al. 2016) would underlie the two fates noted in *Lkb1* deficient ISCs, considering the critical role that *Atoh1* levels play in ISC secretory differentiation. In this case, *Atoh1* levels would be regulated in a crypt-specific manner.

The altered ISC homeostasis was not noted in $Lkb1_{Lgr5\text{-}Het}$ mice (data not shown), suggesting that the phenotype is not penetrant in PJS models, where *Lkb1* acts as a haploinsufficient tumor suppressor (Rossi et al. 2002), and where tumorigenesis is driven by stromal cells (Katajisto et al. 2008; Ollila et al. 2018). However, loss-of-heterozygosity (LOH) analysis identified regions with complete loss of LKB1 in some PJS patient polyps (Hemminki et al. 1997), a phenomenon that has not been noted in the mouse models. Consistent with our results, these regions display a secretory lineage enrichment based on a high proportion of goblet-like cells, and have been suggested to represent progression of PJS tumors (Hemminki et al. 1997). Our *Lgr5-Lkb1-KO* model provides an opportunity to explore this further.

Atoh1 has been coined the gatekeeper of secretory differentiation based on its induction in ISCs through regulation by Notch (van Es et al. 2010; Kim and Shivdasani 2011) and Wnt (Tian et al. 2015) signaling. An earlier pan epithelium deletion of *Lkb1* (Shorning et al. 2009) noted an enrichment of secretory cell lineages, which was correlated with increased Notch signaling without specification of the cell type(s) involved. The ISC-specific *Lkb1* deletion used here enabled the distinction of cell-intrinsic signaling changes in ISCs and identified *Atoh1* induction as an early change that is independent of Notch signaling.

Our findings demonstrated that the loss of the metabolic sensor, *Lkb1*, in ISCs leads to dysregulated expression of OXPHOS-associated genes, including a marked induction of *Pdk4*, an observation that is consistent with increasing evidence that cellular metabolism has a major impact on adult stem cell (including ISCs) function (Rodríguez-Colman et al. 2017). Further evidence from our *in vitro* data indicates compromised OXPHOS in *LKB1*-depleted cells, which can be partially restored by PDK inhibition. This observation, together with the observation that the spare

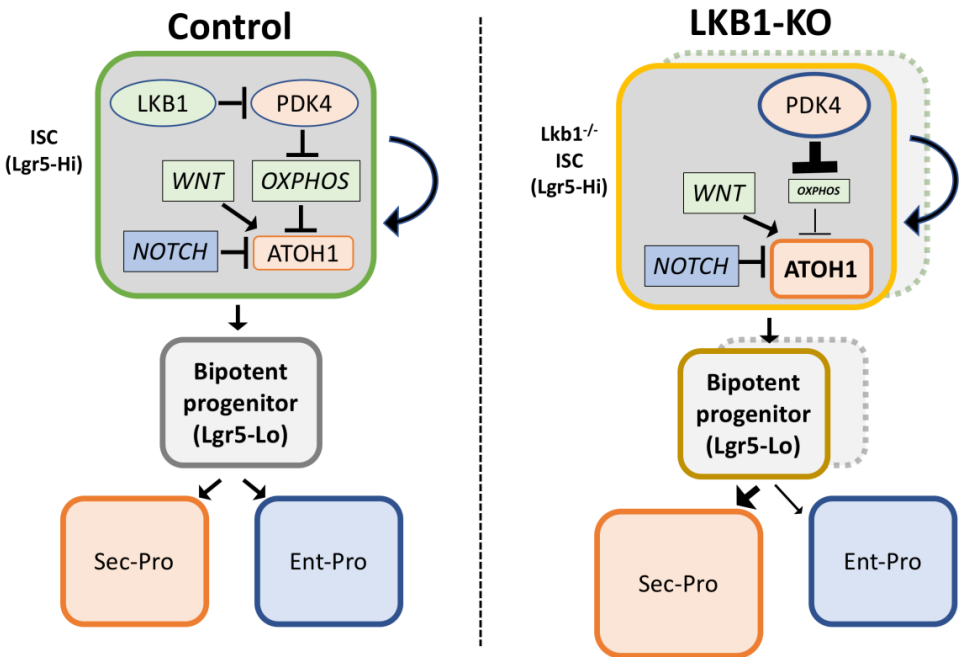

Figure 3. Lkb1 maintains LGR5+ ISC homeostasis by repressing *Atoh1*-mediated secretory cell differentiation, via Pdk4-regulated energy metabolism. (Left) In presence, *Lkb1* contributes to the repression of *Atoh1* to maintain ISCs homeostasis by restricting *Atoh1*-mediated secretory cell differentiation and maintains normal cell respiration. **(Right)** *Lkb1* loss induces *Atoh1* in ISCs, a process which is mediated by elevated *Pdk4* and reduced OXPHOS, and this leads to the acquisition of a Sec-Pro signature while still maintaining the stem cell identity and functions, and thereby an increase in the differentiation specifically towards secretory cell lineages.

respiratory capacity of mitochondria also gets compromised following LKB1 loss, suggests that there are also other alterations in mitochondria in addition to their functional regulation, and provides a possible explanation as to why the basal respiration in *LKB1*-deficient cells cannot be completely rescued by PDK inhibition. Taking into account the previous observation that the induction of *ATOH1* following *LKB1* depletion is dependent on PDK4 raises the possibility that *Lkb1* represses *Pdk4* in order to restrict *Atoh*1-mediated secretory cell differentiation in ISCs (model explained in **Figure 3**). In several other systems where *Lkb1* is required for stem cell survival and function, including regulatory T cells (K. Yang et al. 2017; Wu et al. 2017; He et al. 2017) and HSCs (Gurumurthy et al. 2010; Nakada, Saunders, and Morrison 2010; Gan et al. 2010), impaired OXPHOS following *Lkb1* loss has been also noted. Thus, our findings suggest that LKB1-regulated energy metabolism also plays an important role in stem cell fate determination in addition to its crucial impact on cell survival.

MNU treatment provokes *Lkb1*$^{+/-}$ polyposis independently of COX-2 (II)

RESULTS (II)

1. MNU treatment promotes Lkb1$^{+/-}$ *polyposis*

MNU treatment led to a decrease in survival of *Lkb1*$^{+/-}$ mice compared to wildtype mice with the same treatment. The gastric polyp burden was dramatically increased compared to untreated *Lkb1*$^{+/-}$ mice. This increase was due to both an increase in polyp size, through enhanced epithelial proliferation, and an increase in polyp number, including the number of small polyps. These results suggest that MNU treatment promotes both the initiation and development of PJS polyposis.

2. Histological analysis in MNU-treated mice revealed no malignant transformation

Histological analysis of gastric mucosa revealed no change in polyp morphology under the MNU treatment, except only one case of dysplasia, among all the 18 *Lkb1*$^{+/-}$ and 41 wildtype mice that include in total 104 gastric *Lkb1*$^{+/-}$ polyps and 11 wildtype gastric lesions.

p53 is induced in response to DNA damage (Kastan et al. 1991). Mutated p53 was found in 5 of 15 analyzed gastric *Lkb1*$^{+/-}$ polyps upon MNU treatment by mutant conformation-specific PAb240 antibody. However, there was no correlation between the inactivated p53 and the detected dysplasia case.

3. MNU treatment does not affect gastric gland differentiation in Lkb1$^{+/-}$ *mucosa*

Previous work in our lab demonstrated that stomach glands display aberrant or incomplete differentiation in *Lkb1*$^{+/-}$ mouse, as detected by pepsinogen C staining showing increased frequency of PAPG (Udd et al. 2010). However, in *Lkb1*$^{+/-}$ mice there was no further increase of PAPG above the basal level upon MNU treatment, though wildtype mice showed a pattern of increased PAPG with MNU treatment. These results suggest that the mechanism underlying PAPG in *Lkb1*$^{+/-}$ mice is different than carcinogen-induced PAPG in wildtype mice, and could rather be a sign of nascent polyposis.

4. COX-2 inhibition does not suppress MNU-induced Lkb1$^{+/-}$ *polyposis acceleration*

In *Lkb1*$^{+/-}$ mice, the COX-2 inhibitor, celecoxib, suppresses polyposis (Udd et al. 2004). Celecoxib administration slightly but not significantly decreased both the mortality and the gastric polyp burden in *Lkb1*$^{+/-}$ mice that had received MNU treatment. The results demonstrated that COX-2 inhibition might not be sufficient to suppress the provoked PJS polyposis induced by carcinogen exposure.

DISCUSSION (II)

Treatment with the mutagenic carcinogen MNU could generate additional mutations, therefore provide us an opportunity to evaluate the effect of mutagenesis on Peutz-Jeghers polyposis in *Lkb1*$^{+/-}$ mice. We observed that MNU treatment accelerated polyposis by increasing both the size and number of polyps in *Lkb1*$^{+/-}$ mice, suggesting that additional mutations may promote both the initiation and subsequent growth of *Lkb1*$^{+/-}$ polyps. However, MNU treatment showed no effect on defective gastric gland differentiation (assessed by PAPG), which has been considered as an early sign of polyp initiation (Udd et al. 2010). Thus, it would be interesting to look for the reason behind increased polyp initiation in a future study.

Another observation was that MNU treatment accelerated *Lkb1*$^{+/-}$ polyposis without causing malignant transformation in the polyps. This result indicates that mutations leading to malignant transformation are not favored in the *Lkb1*$^{+/-}$ stomach, consistent with a previous report that Lkb1 loss drives benign hyperproliferation that is resistant to malignant transformation (Bardeesy et al. 2002).

The notion we had from our previous study is that COX-2 promotes the growth of polyps rather than their initiation (Udd et al. 2004). In this study, when the *Lkb1*$^{+/-}$ polyp initiation had been provoked by mutagenesis that also affected other genes, COX-2 inhibition with celecoxib had no particular effect on limiting *Lkb1*$^{+/-}$ polyp growth. The result suggests that celecoxib alone is not efficient in suppressing PJS polyposis when the polyposis has been accelerated by additional mutations. This would probably also explain why celecoxib represses gastric polyposis only in a subset of PJS patients (Udd et al. 2004).

Stromal Lkb1 deficiency leads to gastrointestinal tumorigenesis involving an induced inflammatory program (III)

RESULTS (III)

Two alternative strategies were used to achieve stromal *Lkb1* deletion: the *Twist2-Cre* (also known as *Dermo1-Cre*) allele targeting mesenchymal progenitor cells, and the fibroblast-specific protein-Cre (*Fsp1-Cre*, also known as S100A-Cre) allele targeting fibroblasts.

We first studied the heterozygous *Lkb1* deletion in stroma by crossing *Twist2-Cre* and *Fsp1-Cre* mice with $Lkb1^{flox}$ mice to generate *Twist2-Cre;*$Lkb1^{fl/+}$ ($Lkb1^{TwKO/+}$) and *Fsp1-Cre;*$Lkb1^{fl/+}$ ($Lkb1^{FspKO/+}$) mice. $Lkb1^{TwKO/+}$ mice showed full penetrance in polyp formation, in addition to comparable survival and tumor burden to previously reported $Lkb1^{+/-}$ mice (Rossi et al. 2002). Lineage tracing (*Rosa26R-mTmG*) analysis confirmed exclusive stromal recombination in $Lkb1^{TwKO/+}$ mice. These results demonstrated that heterozygous *Lkb1* loss in stroma is sufficient to drive PJS polyposis. $Lkb1^{FspKO/+}$ mice showed lower tumorigenic potential with very limited polyp formation (both the penetration and tumor burden), and this is probably due to low recombination frequency.

We next studied homozygous *Lkb1* deletion in stroma by generating *Fsp1-Cre;*$Lkb1^{fl/fl}$ ($Lkb1^{FspKO/FspKO}$) mice (the *Twist2-Cre;*$Lkb1^{fl/fl}$ is embryonic lethal). The $Lkb1^{FspKO/FspKO}$ mice rapidly developed polyps with full penetrance already at four months of age, and the polyps displayed dramatically expanded *Lkb1*-deficient stroma, as detected by the reporter (*Rosa26R-LacZ*). Further study with the $Lkb1^{FspKO/FspKO}$*;Rosa26R-Confetti* tumors demonstrated an oligoclonal origin of the tumor stroma. By contrast, the analysis in polyps of $Lkb1^{+/-}$ mice carrying the *Lgr5-EGFP-IRES-ERT2* allele with $Rosa26^{LSL\text{-}TdTomato/+}$reporter demonstrated that the epithelial compartment in polyps did not display clonal growth. Together with the evidence from histological analysis showing stromal cells with characteristics of activated and contractile myofibroblasts, and epithelial cells with increased proliferation, these results indicate that PJS polyps form from clonally expanding stromal myofibroblasts and reactively hyperproliferating epithelia.

Transcriptome analysis (RNA-seq) from $Lkb1^{FspKO/FspKO}$ polyps revealed prominent inflammatory hallmarks, as also seen from the analysis of human PJS polyp transcriptomes. One of the top hits was JAK/STAT signaling in both cases, consistent with the observation from the polyps showing activated JAK/STAT3 signaling detected by Western blotting. Notably, histological staining revealed that the JAK/STAT3 pathway was activated in the epithelial compartment of the polyp. Next, we identified IL-11 secreted from fibroblasts as the potential activator of JAK/STAT3 signaling in polyps. Finally we found that pharmacological inhibition of JAKs with the JAK1/2 inhibitor, Ruxolitinib (Quintás-Cardama et al. 2010) (which is already clinically approved for myeloproliferative diseases), can reduce the PJS polyp development in mice.

DISCUSSION (III)

Our work demonstrated that mesenchymal-progenitor or stromal-fibroblast–specific deletion of *Lkb1* is sufficient for PJS polyposis. Taking into account the previous observations that *Lkb1* loss from smooth muscle cells leads to the development of PJS-type polyps (Katajisto et al. 2008), and that epithelial deletion failed to induce polyp formation (Shorning et al. 2009, 2012), we conclude that PJS polyposis is a stromal disease. Interestingly, the observations in both this study and a previous study (Katajisto et al. 2008) indicate that homozygous deletion of *Lkb1* in stroma enhances tumorigenicity while heterozygous deletion is sufficient for polyposis. Thus, it is tempting to focus future LOH studies in PJS on the stromal compartment of tumor.

Mechanistically, *Lkb1* loss in gastric stromal cells leads to clonal expansion of stromal cells and activation of an inflammatory program involving the IL-11–JAK/STAT3 pathway, which promotes the expansion of stroma and overgrowth of epithelia. Together with the study of *Lkb1* in T cells by Poffenberger et al. (Poffenberger et al. 2018), a critical role of inflammatory signals in tumorigeneses has been revealed, that they can profoundly alter the microenvironment and fuel tumor formation. In addition, the findings from both our work and Poffenberger et al. also suggest that JAK inhibitors may be potential new therapeutic modalities for GI and other tumors arising in PJS patients for which there are few treatment options.

CONCLUSIONS

To conclude the work described in this thesis:

Study I revealed an essential role of LKB1 in the maintenance of intestinal stem cell homeostasis. *Lkb1* loss induces the master transcription factor *Atoh1* and its associated secretory progenitor signature in LGR5+ ISCs, therefore perturbs the LGR5+ ISC homeostasis by shifting towards the secretory differentiation. The induction of *Atoh1* is mediated by increased PDK4 and suppressed OXPHOS, independent of well-established Wnt or Notch signaling, and does not require AMPK. The results identify a novel mechanism in ISCs for cell fate determination involving metabolic regulation; establish a critical role of *Lkb1*-regulatd energy metabolism in stem cell fate determination; identify a novel mechanism repressing *Atoh1*, the master transcription factor of secretory fate determination in the ISC, involving suppression of PDK4 and OXPHOS by LKB1. These findings are of general interest in terms of the novelty of the identified biology of the intestine homeostasis, and provide a connection between metabolism and the fate determination of ISCs.

In Study II, we treated *Lkb1*+/- mice with carcinogen MNU to induce additional mutations, and this is to mimic progression in PJS tumorigenesis. We observed that MNU treatment can promote *Lkb1*+/- polyposis demonstrated by reduced survival and increased tumor burden, but was not accompanied by consistent substantial histological changes in those polyps, supporting the notion that LKB1 loss drives benign growth but resistant to malignant transformation (Bardeesy et al. 2002). Focal area of mutated *p53* was detected in MNU-treated *Lkb1*+/- polyps, but unlinked to malignant progression in this setting. COX-2 inhibition with celecoxib is not sufficient to prevent the *Lkb1*+/- polyposis accelerated by MNU treatment. Study III used various stromal-*Lkb1*-deletion mouse models to decipher the originating cell type and molecular mechanism underlying the *Lkb1*+/- polyposis. Loss of *Lkb1* in stroma leads to clonal expansion of stromal cells and full penetrance of gastric polyp formation, strongly supporting a stromal-deriving of PJS polyps. The activation of an inflammatory program involving the JAK/STAT signaling was noted as a major alteration in PJS and mouse model polyps in this study. In addition, treatment with the JAK1/2 inhibitor ruxolitinib dramatically decreased polyposis in *Lkb1*-dificent mice. Therefore, this study demonstrates that PJS polyposis is a stromal-deriving disease mediated by induced inflammatory program that involves the IL-11–JAK/STAT3 pathway. Collectively, the findings in II and III help us to further understanding PJS, and provide potential therapeutic opportunities.

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