Two proteins, one model organism: On the functional characterization of Lkb1 and Ring1b in zebrafish

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The function of Lkb1 in development

To study how Lkb1 function relates to energy metabolism and polarity control at the whole-organism level, we generated lkb1-mutant zebrafish. Homozygous deletion of genes that are essential for embryonic development in mice often do not cause such an early developmental arrest in zebrafish, thus allowing the study of such proteins at later embryonic stages. Although the underlying mechanisms are not fully understood, it is generally appreciated that maternal mRNA and/or protein deposition in the oocyte together with the rapid development of the zebrafish embryo often enables completion of the first developmental stages and delays the developmental arrest. We indeed observed that lkb1 mutants normally completed early embryonic development. In Chapter two, we therefore stated that “Lkb1 is dispensable for embryonic survival”. However, a more correct statement would be that zygotic Lkb1 is dispensable for embryonic survival, as lkb1 mRNA is maternally provided. In addition, because phosphorylation of AMPK can be induced by KCN treatment in unfertilized eggs (Mendelsohn et al., 2008), it is also likely that Lkb1 protein is maternally provided. Maternal Lkb1 has indeed been shown to be essential for early embryonic development in lower organisms. For example, in Caenorhabditis elegans, maternal deletion of the Lkb1 homolog PARtitioning of cytoplasm family member 4 (par-4) causes cell polarity and cell division defects, resulting in an amorphous early embryo without distinct morphology (Kemphues et al., 1988; Morton et al., 1992). Cell polarity defects are also observed upon maternal deletion of Lkb1 in the Drosophila oocyte (Martin and St Johnston, 2003). Although knockdown of maternally provided lkb1 mRNA does not seem to cause an overt early embryonic phenotype in zebrafish (our preliminary results and Marshall et al., 2010), maternally provided Lkb1 protein might be sufficient to mask developmental defects, which should be taken in account in the analysis of Lkb1 function at early embryogenesis.

Zygotic LKB1 is essential for normal embryonic development in higher vertebrates. For instance, Lkb1 deficiency in mice causes a developmental arrest at mid-gestation. Homozygous Lkb1 mice display severe developmental defects, including defects in neural tube closure, abnormal development of the aorta and abnormal vasculature (Jishage et al., 2002; Miyoshi et al., 2002; Ylikorkala et al., 2002).
In addition, conditional deletion of Lkb1 in mice results in differentiation defects of several tissues, including the intestine, pancreas, adipose tissue, muscle, neurons, lymphocytes and germ cells (Udd and Makela, 2011). It was therefore surprising that lkb1 zebrafish mutants were phenotypically indistinguishable from wild-type siblings, even up to day five post fertilization. This suggests that loss of Lkb1 function does not grossly disrupt organogenesis, or, since we did not study every organ in detail, that the defects are relatively mild (as will be discussed for the intestine later on).

In addition to the differentiation defects that arise upon LKB1 deficiency, it is also well established that LKB1 has a critical role in vertebrate neuronal polarization. Lkb1 deletion from the developing cortex impairs axon specification due to the inability to activate the AMPK-related kinases BRSK1 and BRSK2, whereas overexpression of Lkb1 promotes the formation of multiple axons (Barnes et al., 2007). However, a general role for LKB1 in non-neuronal cellular polarization is less clear than would be expected from the studies in lower organisms. Although Lkb1 does control the polarity of pancreatic beta cells and perhaps of hepatic bile ducts, it has not unambiguously been shown that LKB1 controls vertebrate epithelial polarity in vivo (Granot et al., 2009; Woods et al., 2011). It was therefore of interest to address whether Lkb1 has a critical role in cellular polarization in the zebrafish. Our study was focused on addressing a possible role of Lkb1 in the regulation of intestinal polarity, but did not extend to other tissues. Our interest in the role of Lkb1 in zebrafish intestinal development was two-fold. First, the abrupt loss of intestinal folding at 7 dpf represented one of the most severe lkb1 phenotypes and second, because of the hamartomatous polyps in PJS patients, it is of general interest to get a better insight into how Lkb1 functions in the intestinal epithelium.

**Intestinal polarity**

It was already shown in 2004 that forced expression of LKB1 and its binding partner STRADα in the goblet cell-like colorectal cancer LS174T cell line induced complete polarization, including the formation of an apical brush border and sorting of basolateral proteins (Baas et al., 2004). Later, it was proposed that LKB1-induced formation of brush borders, but not other aspects of epithelial polarity, depended upon binding of the kinase MST4 to MO25, another binding partner of LKB1 in LS174T cells (ten Klooster et al., 2009). This caused subsequent translocation of MST4 to the subapical domain, which led to the phosphorylation and activation of the cytoskeletal linker protein Ezrin.

Lee and colleagues showed that energy deprivation-induced activation of AMPK was sufficient to promote polarization in LS174T cells, implicating that LKB1 orchestrates cellular polarity in this cell line at least through two mechanisms (Lee et al., 2007). Furthermore, knockdown of LKB1 in Caco2 enterocyte-like cell line has been shown to inhibit spontaneous polarization (Baas et al., 2004), whereas another study showed that LKB1-depleted Caco2 cells were largely unaffected, even though E-cadherin and villin staining appeared somewhat irregular (Sebbagh et al., 2009).

Although LKB1 regulates epithelial polarity in cell culture systems, it seems that LKB1 does not function as critical regulator of vertebrate intestinal polarity in vivo, since our and other studies indicate that Lkb1 deficiency does not cause overt intestinal polarity defects. The Alan Clarke lab showed that conditional deletion of Lkb1 in the mouse intestine did not cause epithelial polarity defects (Shorning et al., 2009). We demonstrated that Lkb1 deficiency in zebrafish did not lead to gross polarity defects in enterocytes either, since...
several polarity markers were correctly localized and normal apical brush borders, tight junctions and hemidesmosomes were formed. One note of caution is here in place; intestinal polarization in the zebrafish commences at around 2 days post fertilization with the formation of a lumen. Intestinal cells change their morphology at this developmental stage, from cuboid to columnar, nuclei relocate to the basal side of the cell and adherens junctions are formed (Ng et al., 2005). We determined that Lkb1 function is lost in 72 hpf lkb1 mutants, but did not extent our analysis to earlier developmental time points, due to time limitations. Therefore, we cannot formally state that Lkb1 is dispensable for the initiation of intestinal polarization in zebrafish development.

It has recently been suggested that LKB1 may regulate intestinal polarity in mammals in the context of energetic stress (Sebbagh et al., 2011). In Drosophila, loss of Lkb1 indeed disrupts epithelial polarity in follicle cells, only upon energetic stress (Mirouse et al., 2007) and a similar mechanism can be envisaged in the vertebrate intestine. Our study, however, clearly showed that 7 dpf lkb1 mutants are under severe energetic stress, while retaining intestinal polarity, which shows that Lkb1 function in zebrafish is not required for maintenance of intestinal polarity under energetic stress.

The absence of an overt polarity defect upon Lkb1 loss in zebrafish suggests that Lkb1 is not, or perhaps only to a limited extent, involved in the vertebrate intestinal polarization process. On the other hand, Lkb1 may orchestrate intestinal polarization in a redundant fashion. Acquisition and maintenance of cell polarity is critical for normal epithelial physiology and homeostasis and even exerts a tumor suppressive function through prevention of epithelial to mesenchymal transition. It is therefore expected that evolutionary mechanisms developed parallel mechanisms in order to ensure the robustness of the polarization system.

Intestinal differentiation

Preliminary experiments indicated that intestinal differentiation might be affected in lkb1 mutants. Alcian blue stainings, which reveal acid mucins, showed that goblet cells were increased in number and/or in size in 5 and 7 dpf lkb1 mutants (data not shown). Although we did not quantify these findings, similar results have been reported. For example, knockdown of Lkb1 in zebrafish led to a transient increase in goblet cell-specific Rhodamine-conjugated wheat germ agglutinin staining intensity in lkb1 morphants (Marshall et al., 2010). The defect in goblet cell differentiation was studied in more detail in mice (Shorning et al., 2009). Here, it was shown that in mice in which Lkb1 was conditionally deleted in the intestinal epithelium, the average surface of goblet cells in crypts and villi was increased at least three times compared to controls. Moreover, terminal differentiation of goblet cells was also impaired as so-called “intermediate cells”, which bear features of both goblet and Paneth cells, were more frequently observed. The authors showed that delta 1 ligand was downregulated upon Lkb1 deletion and they speculated that impaired Microtubule affinity-regulating kinase 1 (MARK1)-dependent localization of delta 1 ligand would provide a mechanistical explanation. Delta-Notch signaling directs differentiation towards a secretory cell type and loss of deltaD was shown to cause increased intestinal secretory cell numbers in zebrafish (Crosnier et al., 2005). Moreover, given the results from the LS174T cell line, which is a goblet cell-like cell line, it is interesting to speculate that perhaps a defect in goblet cell polarity may underlie the defect in goblet cell differentiation.
The Lkb1 “starvation” phenotype
In Chapter two, we showed that lkb1 mutants display a premature starvation phenotype that is manifested after the embryos have completed yolk absorption and become dependent upon external energy sources. We showed that lkb1 mutants do not feed, rapidly deplete their energy resources and fail to adjust their metabolic rate in response to changes in nutrition status. However, the precise mechanism of how Lkb1 deficiency causes these phenotypes remains elusive. Hereafter, we will discuss possible mechanisms and thoughts relating to the observation that lkb1 mutants do not display feeding behavior and, subsequently, the defects in energy metabolism control that arise upon energetic stress.

The swim bladder
A thought that comes to mind is how the absence of feeding behavior relates to the lkb1 starvation phenotype. If lkb1 mutants would eat, or if it was possible to force-feed the mutants, would there still be a phenotype? Or is Lkb1 dispensable for larval development under such conditions of non-energetic stress?

One mechanism by which feeding behavior and food intake could be impaired in lkb1 mutants is their defect in swim bladder development. The majority of lkb1 mutants do not inflate their swim bladder, whereas in some mutants the swim bladder is hyperinflated, which leads to the larvae floating at the surface. Of note, defective swim bladder development is in fact the first phenotypical difference between wild-types and lkb1 mutants. Defective swim bladder development is a very common defect in zebrafish mutants that interferes with food intake and correlates with larval lethality (Driever et al., 1996; Kim et al., 2011; McCune and Carlson, 2004). In Chapter two, we showed that lkb1 mutants do not display any structural abnormalities in the jaw or esophagus that would prohibit feeding. Because we also showed that lkb1 mutants can absorb and process lipids normally, a normal swim bladder might alleviate the absence of feeding behavior in lkb1 mutants.

Appetite control and food intake
The lack of feeding behavior and food intake may prove to be a mere secondary defect due to defective swim bladder development. However, Lkb1 deficiency might also be more directly involved in this process.

In vertebrates, it is well established that the hypothalamus is the primary centre for appetite control through integration of signals that are derived from the gastrointestinal tract (e.g. the orexigenic peptide hormone ghrelin) and adipose tissue (e.g. the anorexigenic peptide hormone leptin) (Schwartz et al., 2000). In a simplified view, feeding behavior is primarily controlled by two distinct sets of neurons in the hypothalamus: neuropeptide Y / agouti-related protein-expressing neurons (NPY/AgRP neurons) and pro-opiomelanocortin neurons (POMC neurons). Feeding is induced by increased activity of NPY/AgRP neurons, whereas feeding is inhibited by increased activity of POMC neurons.

One molecular mechanism by which the Lkb1 “starvation” phenotype
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Both LKB1 and AMPK have been implicated in the hypothalamic modulation of appetite control. Several studies show that AMPK acts as an important player in this process. In agreement with its function as cellular energy sensor, hypothalamic AMPK activity is increased upon fasting and decreased after refeeding (Minokoshi et al., 2004). Moreover, pharmacological activation of hypothalamic AMPK increases food intake, whereas deletion of the AMPKα2 subunit in POMC neurons impairs the ability to sense a reduction in extracellular glucose levels (Andersson et al., 2004; Claret et al., 2007; Claret et al., 2011). One molecular mechanism by which
hypothalamic AMPK activation stimulates food intake has recently been reported (Yang et al., 2011). The authors proposed that binding of ghrelin to its receptor causes an increase in intracellular calcium concentration in presynaptic neurons that act upstream of NPY/AgRP neurons. In response to the increased calcium concentration, AMPK is phosphorylated in a CaMKKβ-dependent manner, which ultimately results in the release of neurotransmitters onto NPY/AgRP neurons, thereby triggering an increase in promoting appetite.

The role of LKB1 in the modulation of appetite control is less studied. Nevertheless, it has been shown that Lkb1 deletion both in pancreatic beta-cells and in a diverse set of hypothalamic neurons, caused diminished food intake and a reduced body weight under fed conditions (Sun et al., 2011). The authors argued that this phenotype was caused by disruption of the Lkb1 function in the hypothalamus, because mice in which Lkb1 was specifically deleted from the pancreas did not show a change in body weight. Another recent study demonstrated that specific deletion of Lkb1 in POMC neurons did not affect POMC neuron glucose sensing, food intake or body weight, but did alter peripheral glucose homeostasis (Clarret et al., 2011). Together, these studies imply that although both LKB1 and AMPK function in the hypothalamus to control aspects of appetite control, their functions are, at least partially, distinct.

The genetic framework in lkb1 zebrafish mutants is clearly different from the mouse models described above, since lkb1 is deleted in the whole organism. Therefore, it can be envisaged that hypothalamic modulation of food intake and appetite control is deregulated to a greater extent in Lkb1-deficient zebrafish larvae than in mice in which Lkb1 is deleted in only a subset of hypothalamic neurons. It would be of interest to address this point and obtain more insight into the possible hypothalamic functions of Lkb1.

What is the underlying mechanism of premature starvation in lkb1 mutants?

Regardless of the molecular mechanism that underlies the lack of feeding behavior and food intake, our study demonstrated that lkb1 mutants exhibit prematurely hallmarks of starvation, at the organismal, organ, as well as at the cellular and molecular level. However, the outstanding question of which Lkb1 substrates are critically involved in this premature starvation phenotype remains unresolved.

The role of AMPK in the lkb1 “starvation” phenotype

Because of its function as cellular energy sensor, we considered impaired AMPK activation as the prime mechanism by which loss of Lkb1 could result in defective energy metabolism control. AMPK was indeed, as expected, not phosphorylated at detectable levels in lkb1 mutants at 7 dpf, but neither in food-deprived wild-type larvae at this developmental stage. This latter result was unexpected and begs the question whether the loss of AMPK is causally involved in the perturbed metabolism control under energy-limiting conditions in lkb1 mutants.

Because AMPK is such a central player in the sensing of energetic stress, it was to be expected that two days of food deprivation, which did result in decreased hepatic glycogen and low level steatosis in 7 dpf food-deprived wild-type larvae, would promote AMPK phosphorylation. It is plausible that AMPK phosphorylation is only moderately increased in wild-type larvae in response to two days of food deprivation and that, because of technical limitations, low levels of AMPK phosphorylation were not detected. It is also possible that AMPK is only robustly phosphorylated in a subset of tissues and since we used whole embryos as input...
material, AMPK phosphorylation may be masked. Together, we do not exclude that impaired AMPK phosphorylation contributes to the premature starvation phenotype in lkb1 mutants.

It remains therefore of interest to address whether impaired AMPK phosphorylation is causing the premature starvation phenotype. In the course of experiments, we tried to activate AMPK through pharmacological means, including incubation of larvae in AICAR, metformin, phenphormin, berberine, H$_2$O$_2$ and osmotic stress. Although these compounds have been shown to require LKB1 for AMPK phosphorylation in certain cell types (Sakamoto et al., 2005; Shaw et al., 2004b; Xie et al., 2008), several other reports have shown that these pharmacological compounds and treatments can result in AMPK phosphorylation also in an LKB1-independent manner (Emerling et al., 2009; Imai et al., 2006; Sun et al., 2007; Turner et al., 2008; Xie et al., 2006). However, because these treatments did not cause AMPK phosphorylation in 7 dpf wild-type larvae and did not rescue the lkb1 phenotype, we were unable to address whether impaired AMPK phosphorylation causes premature starvation.

To address directly the role of AMPK in the starvation response in a genetic manner, zebrafish carrying mutations in the catalytic subunits of AMPK would be an excellent genetic tool. Interestingly, such mutants may become available within 2012 through the Zebrafish Mutation Project, which aims to create a knockout allele in every protein coding gene in the zebrafish genome (http://www.sanger.ac.uk/Projects/D_rerio/zmp/). Mutant alleles in prkaa1 and prkaa2, the genes encoding AMPKa1 and AMPKa2, respectively, have been identified. Both mutations are expected to disrupt the function of the respective AMPK protein. The mutation in prkaa1 results in a premature stop codon, whereas the mutation in prkaa2 affects a critical splicing site. Thus, generation and analysis of prkaa1/prkaa2 double mutants would reveal whether or to which extent loss of AMPK phosphorylation is implicated in the lkb1 phenotype.

**TORC1 signaling in zebrafish**

It is well established that LKB1, via AMPK, can inhibit TOR Complex 1 (TORC1) signaling in order to ensure a general downregulation of anabolic processes and upregulation of catabolic processes upon energetic stress (Gwinn et al., 2008; Inoki et al., 2003; Shaw et al., 2004a). Loss of Lkb1 has indeed been associated with increased activation of TORC1 signaling (Corradetti et al., 2004; Shackelford et al., 2009; Shaw et al., 2004a). Moreover, inhibition of TORC1 signaling by rapamycin reduces polyp burden and polyp size in Lkb1+/− mice, which demonstrates the physiological relevance of the LKB1-AMPK-TORC1 axis (Robinson et al., 2009; Wei et al., 2009).

Our study did not reveal a similar function for Lkb1 in the regulation of TORC1 signaling during embryogenesis and the defect in energy metabolism control upon energetic stress may be largely TORC1 independent. Although somewhat surprising, it is not without precedent. LKB1 deficiency in the hematopoietic system has also been shown to perturb energy metabolism control in a largely mTORC1 independent fashion (Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010).

The lack of increased TORC1 signaling in lkb1 mutants before the onset of energetic stress indicates that loss of LKB1 does not intrinsically promote increased TORC1 signaling during embryogenesis and the defect in energy metabolism control upon energetic stress may be largely TORC1 independent. Although somewhat surprising, it is not without precedent. LKB1 deficiency in the hematopoietic system has also been shown to perturb energy metabolism control in a largely mTORC1 independent fashion (Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010).
that lkb1 mutants fail to downregulate TORC1 signaling in response to energetic stress. Another possibility is that TORC1 signaling remains active due to the extreme energetic stress that lkb1 mutants experience. It seems counterintuitive that severe energetic stress promotes TORC1 signaling, but it is, in fact, a described phenomenon (Anand and Gruppuso, 2005; Yu et al., 2010). A mechanism by which prolonged energetic stress promotes increased TORC1 signaling has been proposed a few years ago (Anand and Gruppuso, 2005). In this study, the authors suggested that the observed increase in TORC1 signaling upon prolonged starvation might be caused by an increase in circulating branched amino acids. It is known that circulating branched amino acid concentrations increase upon fasting due to skeletal muscle catabolism (Adibi, 1976; Hutson and Harper, 1981) and that branched amino acids, including leucine, potently induce TORC1 signaling (Kim and Guan, 2011). Although correlation does not always mean causation, this view has been supported by a more recent publication. In this study, the authors showed that autophagy, a conserved catabolic process critical for survival upon energetic stress, is essential for reactivation of TORC1 signaling upon prolonged starvation (Yu et al., 2010). During autophagy, a membrane is formed around a specific targeted region of the cytoplasm, generating an autophagosome. Fusion with a lysosome, forming an autolysosome, then promotes lysosomal degradation of cytoplasmic components in order to reuse and replenish nutrients. This renewed availability of nutrients then supposedly triggers the reactivation of TORC1 signaling. In light of these findings, we favor the view that the TORC1 status in 7 dpf lkb1 mutants reflects a response to starvation, as seen in 11 dpf starved wild types, but that this happens prematurely.

A possible role of AMPK-related kinases in the premature starvation phenotype

Our study focused on a possible role for AMPK and TORC1 signaling in causing the premature starvation phenotype because of their well established function in the regulation of energy metabolism. Nevertheless, this does not necessarily mean that AMPK is the sole physiologically relevant substrate that exerts the effects of LKB1 function on metabolism, since LKB1 also phosphorylates 12 AMPK-related kinases (Lizcano et al., 2004). Of particular interest are NUAK2 and SIK2, because both have been reported to be activated upon energetic stress (Du et al., 2008; Lefebvre and Rosen, 2005). A detailed disquisition goes beyond the scope of this discussion, but the few studies that addressed NUAK2 and SIK2 function indeed showed a role for these proteins in energy metabolism control (Du et al., 2008; Ichinoseki-Sekine et al., 2009; Tsuchihara et al., 2008).

Finally, LKB1 has also been shown to interact with a number of other proteins, including LKB1 interacting protein 1 (LIP1), brahma-related gene 1 (BRG1), Phosphatase and tensin homolog (PTEN) as well as transcription factors (Marignani et al., 2001; Mehenni et al., 2005; Setogawa et al., 2006; Smith et al., 2001; Upadhyay et al., 2006). Although this further highlights the multifunctional role of LKB1, it is currently unclear whether these interactions are of physiological relevance under energetic stress.

Concluding remarks

In this part of the thesis, we revealed that Lkb1 functions as critical regulator of metabolism control upon energy limiting conditions at the organismal level in vertebrates. Although our study confirms and highlights the conserved role of Lkb1 in metabolism control, further studies are required to identify the critical Lkb1 substrates and precise molecular mechanisms underlying the lkb1
phenotype.

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