The role of the tumor suppressor Lkb1 in energy homeostatis

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Chapter 7

Summary and discussion

Metabolism in health and disease

Metabolism is an essential process that regulates energy homeostasis at the cellular and organismal level, from stem cell homeostasis to whole-body metabolic adaptation. Deregulated metabolism can contribute to the development of diseases and is a hallmark of cancer cells. The tumor suppressor LKB1 has an essential and evolutionary conserved role in the regulation of metabolism. Mutations that lead to inactivation of this protein have been discovered in many cancer types. Further knowledge of the effect of inactive LKB1 on metabolic deregulation would contribute to the development of treatments that specifically target cancer cells. The work in this thesis describes the effects of whole-body inactivation of Lkb1 on energy homeostasis in zebrafish larvae in response to the metabolic stress during yolk depletion. We focused on (1) studying the metabolic processes associated with nutrient restriction e.g. regulation of autophagy and glucose metabolism, (2) using the *lkb1* larvae as a screening platform for identifying metabolic drugs that target *LKB1* tumors, (3) composing a transcriptional profile of genes specifically regulated by Lkb1, and (4) studying the effects of inactivation of Lkb1 on hematopoietic stem cells maintenance and hematopoiesis.

Lkb1 is a critical regulator of starvation-induced autophagy

Autophagy is a process during which cellular components are degraded. Under basal conditions it serves a recycling function and during metabolic stress it can be used as a survival mechanism to generate energy (Rabinowitz and White, 2010). In mice, it was shown that inactivation of autophagy is lethal for neonates shortly after birth (Kuma et al., 2004), demonstrating the importance of autophagy in surviving the metabolic stress accompanying the interruption of the maternal nutrient supply. In zebrafish, the role of autophagy during yolk-depletion had not been investigated.

In **Chapter 2**, we show that between 5 and 7 dpf, during yolk depletion, markers associated with autophagy (Beclin, Lc3-II cleavage, atg5) are progressively upregulated in wt larvae. However, in *lkb1* larvae we observed decreased levels of Lc3-II from 6 dpf, and low expression of the other markers at all time-points, which indicates an autophagy defect in *lkb1* larvae. Furthermore, the autophagy receptor and scaffold protein Sqstm1/p62 accumulates in the liver and intestine of *lkb1* larvae from 5 dpf, further confirming impaired autophagy. We successfully experimentally activated autophagy in wt larvae by targeting the mTOR and PI3K pathways (Figure 1A), but this did not rescue the autophagy defects in *lkb1* larvae, since these pathways are affected by the inactivation of Lkb1 (Figure 1B). Interestingly, we show that treatment of larvae with calpeptin, which acts independent of the mTOR and PI3K pathways, induces the expression of autophagy markers, prevents p62 accumulation, and extends the lifespan of *lkb1* larvae. Furthermore, we show that knock-down of p62 by antisense morpholino-injection induces autophagy and increases survival of *lkb1* larvae.

The precise molecular pathways that underlie the lack of autophagy induction in *lkb1* larvae are not understood. Nonetheless, we show that activation of starvation-induced autophagy is an essential survival mechanism in zebrafish, and that Lkb1 is a critical regulator of this process.

Lkb1 is essential for whole-body glucose homeostasis

We addressed glucose metabolism in *lkb1* larvae during development and demonstrate that Lkb1 is essential for glucose homeostasis during embryonic and larval development. We show in **Chapter 3** that *lkb1* larvae display dramatically low glucose levels at 7 dpf. Furthermore, gluconeogenesis, a hallmark of fasting adaptation, is prematurely and excessively induced in *lkb1* larvae, while genes associated with glycolysis are downregulated. However, this adaptive response at the transcriptional level is not sufficient to maintain glucose levels in *lkb1* larvae. Therefore, our data confirm the essential role of Lkb1 as regulator of glucose metabolism.

Other research has further supported the role of systemic inactivation of Lkb1 on metabolism regulation. For instance, in 2016, a paper was published in which the authors investigated the effect of Lkb1 inactivation on glucose metabolism in zebrafish during the first five days of development using a gene trap mutant (Kuang et al., 2016). They described aberrant glucose metabolism from 3 dpf, with increased expression of gluconeogenesis-related genes and increased levels of glucose. Furthermore, they showed that *lkb1* larvae are producing high levels of lactate, which is facilitated by the upregulation of *pdk2* expression. Another study described the effect of systemic inactivation of LKB1 in mice (Shan et al., 2016). Since homozygous deletion of *Lkb1* is embryonic lethal in mice, they made use of a tamoxifen-inducible LKB1 KO mouse model. Mice treated with tamoxifen died within 6 weeks, showed a reduction in body weight, had increased levels of glucose, and increased expression of mitochondrial and hepatic gluconeogenesis-related genes. Furthermore, they showed reduced oxygen consumption, carbon dioxide production and energy expenditure. In conclusion, the data from these studies, combined with our work, show that inactivation of LKB1/Lkb1 results in systemic deregulated glucose metabolism, and is lethal under energy restricting conditions.

Metabolic phenotype of *lkb1* larvae before yolk-depletion

The metabolic phenotype as we described in this thesis, focused on the effect of Lkb1 inactivation on the metabolic adaptation in response to yolk depletion. However, during our experiments we found differences between *lkb1* larvae and wt larvae even before yolk depletion. For instance, in **Chapter 2**, we observed reduced expression of autophagy markers and p62 accumulation in *lkb1* larvae is present prior to yolk depletion. Furthermore, the transcriptome data presented in **Chapter 4**, reveal an *lkb1*-specific transcription profile. In this profile, we find *calpain 3, crystallins*, and several other genes to be highly overexpressed in trunks of *lkb1* larvae before yolk-depletion and not in wt during prolonged fasting. These data highlight the complexity of metabolic regulation by Lkb1. Since the upregulation of *crystallins* is striking, and many questions about their functions outside the eye are still unanswered, further investigation of the role of *crystallins* in metabolic regulation is warranted. Finally, it would be interesting to investigate if the upregulation of these genes before yolk depletion is a causative factor in the (in)ability of *lkb1* mutant larvae to adapt to the metabolic challenge of yolk depletion.

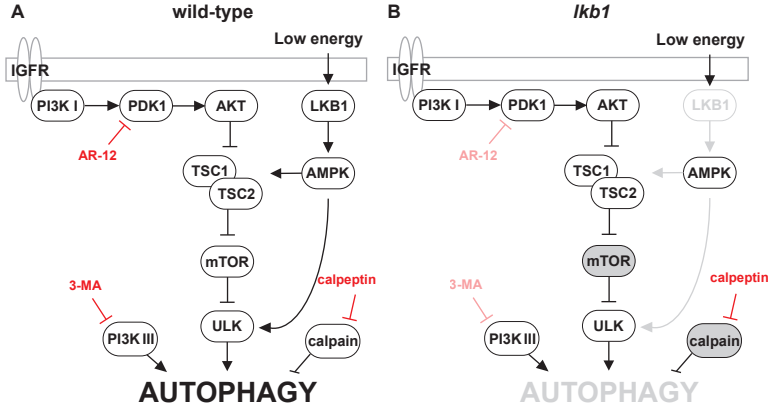

Figure 1. Model illustrating autophagy impairment in *lkb1* larvae. (A) Molecular signalling pathways in wild-type larvae. Under low energy conditions, Lkb1 activates AMPK, which in turn inhibits mTOR signalling. Inhibition of TOR and direct activation of ULK by AMPK lead to induction of autophagy. Signalling from the nutrient-sensing PI3K pathway is dampened under conditions of energetic stress. Input from the class III PI3K pathway acting downstream of ULK contributes to maximal autophagy efficiency to meet energetic demand. In wt larvae, autophagy can be activated is by treatment with AR-12, 3-MA and calpeptin. **(B)** Molecular signalling pathways in *lkb1* larvae. Loss of functional Lkb1 leads to lack of AMPK signalling which results in mTOR upregulation (grey) and lack of ULK1 activation. Impairment of class III PI3K signalling, and possible overexpression of calpains (grey) contributes to impairment of autophagy. Due to insufficient autophagy induction, substrates necessary for survival are not provided leading ultimately to premature death of *lkb1* larvae. Due to deregulated signalling pathways *lkb1* larvae are unsusceptible to AR-12 and 3-MA mediated activation of autophagy. However, activation of autophagy by calpeptin (depicted in red), is successful and prolongs survival of *lkb1* larvae.

Inactivation of Lkb1 leads to a cachexia-like phenotype

Cachexia, or 'wasting syndrome' is associated with various diseases including infectious diseases and cancer. Patients suffer from weight loss, muscle atrophy, deregulated metabolism, excessive inflammation and anemia (Engineer and Garcia, 2012). Despite its devastating effects, the molecular mechanisms underlying cachexia are poorly understood. In **Chapter 4**, we show that a panel of genes that are associated with a cachexia-pathophysiology are highly upregulated only in *lkb1* larvae. For instance, the metabolism and immune-related *leptin b, il1 and il6*, as well as *ucp2* and *pgc1a*, markers that are associated with cancer cachexia (Argiles et al., 2014) are highly expressed in *lkb1* larvae, but not in starved wt. In addition, *lipin 1* expression, which has been associated with conditions involving muscle atrophy such as prolonged fasting, uremia and cachexia (Lecker et al., 2004; Reue and Zhang, 2008), is also increased in *lkb1* larvae. Finally, we show that *calpain 3*, also associated with cachexia (Costelli et al., 2005), is highly overexpressed in *lkb1* larvae even before yolk-depletion.

The underlying cause and consequences of the induction of these cachexia-associated genes in *lkb1* larvae, remains unclear. It is interesting that, while most of these genes are significantly upregulated in *lkb1* larvae at 7 dpf, the upregulation of *calpain 3* occurs prior to yolk-depletion. This could indicate a more causal role of Calpain in the development of the cachexia-like phenotype in *lkb1* larvae. Interestingly, in **Chapter 2** we show that treatment of *lkb1* larvae with the calpains inhibitor Calpeptin prolonged their survival.

Finally, cachexia syndrome is typically associated and initiated by malignant diseases, or infection. However, the *lkb1* larvae are exhibiting a cachexia-like pathophysiology without the presence of a tumor or infection. Therefore, the *lkb1* model may be a valid model to study the molecular interplay of systemic metabolism and inflammation that underlie cachexia pathophysiology.

Lkb1 affects hematopoiesis

The effects of (systemic) metabolism on stem cell maintenance is an intensively studied field, as reviewed in **Chapter 5**. Furthermore, it has become evident that LKB1 has an important cell-autonomous role in the maintenance of hematopoietic stem cells (HSC) (Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010a). However, a putative role for systemic LKB1 inactivation on hematopoietic stem cell maintenance has not been investigated.

In **Chapter 6**, we uncover that whole-body inactivation of Lkb1 had little effect on HSC maintenance in zebrafish. However, in a subset of *lkb1* larvae (~ 20%) we observed no HSCs in the thymus throughout development, which is puzzling since

it suggests that Lkb1 affects seeding of HSCs specifically to the thymus, albeit with a low penetrance.

Furthermore, we show that the neutrophil cell population is not maintained in *lkb1* larvae only at 7 dpf, suggesting that this phenotype results from lack of Lkb1 signalling and is exacerbated by metabolic stress.

Finally, staining for *gata1*$^{+}$ erythrocytes revealed a dramatic absence in all *lkb1* larvae throughout development. *Gata1* is an essential the regulator of erythrocyte development, and inactivation of *gata1* results in premature lethality in mice (embryonic)(McDevitt et al., 1997), and zebrafish larvae (8 - 15 dpf) (Belele et al., 2009). The absence of *gata1*$^{+}$ cells in *lkb1* larvae during embryonic development suggests a direct role for Lkb1 in the development of this cell type. How Lkb1 regulates the expression of *gata1*, or the differentiation of *gata1*$^{+}$ cells requires further investigation.

To distinguish the direct effect of Lkb1 inactivation from the effects of the premature starvation or prolonged fasting on hematopoiesis, it would be interesting to examine the presence of HSCs, neutrophil cells and erythrocytes in non-fed 11 dpf wild-type 11. Furthermore, analysis of more blood cell types with WISH and qPCR, and possibly lineage tracing would provide a more complete picture of both primitive and definitive hematopoiesis and its regulation by Lkb1.

lkb1 zebrafish as a model to study regulation of energy homeostasis

Regulation of metabolism involves inter-organ communication as well as responses of the whole organism to external signals (nutrients, oxygen) as well as systemic signals (hormones, glucose levels, amino acids and lipids). Therefore cell-based assays cannot recapitulate the complexity of interactions involved in the communication of organs and integration of external signals to organism physiology. We have chosen the zebrafish as an alternative model for the study of metabolism since regulation of metabolism in fish is highly conserved with humans (Seth *et al*. 2013). By using the zebrafish larvae to study the effect of Lkb1-inactivation on metabolism-related processes we show the deregulation of glucose metabolism-related processes and autophagy. Furthermore, in **Chapter 4** we find transcriptional profiles affected by Lkb1 inactivation, highlighting the importance of Lkb1 in the control of metabolism at the whole-organism level. However, the complexity of an organism also accompanies the difficulty of distinguishing direct from indirect effects. This prevented us in some cases defining cause from consequence. For instance, it is difficult to prove whether the deregulation of metabolic pathways in *lkb1* larvae is caused by the direct and specific regulation by Lkb1, or possibly by indirect, secondary effects resulting from Lkb1 inactivation. Nonetheless, the

data presented is this thesis provide many new insights into the effects of systemic inactivation of Lkb1 on energy homeostasis, and reveal mechanisms previously unknown to be affected by Lkb1.

The potential value of *lkb1* larvae for metabolism-related research lies not only in the description and discovery of deregulated pathways. The synthetic lethality screen described in **Chapter 3**, demonstrates that the *lkb1* zebrafish is a valid discovery platform to identify compounds that specifically target *lkb1* mutations.

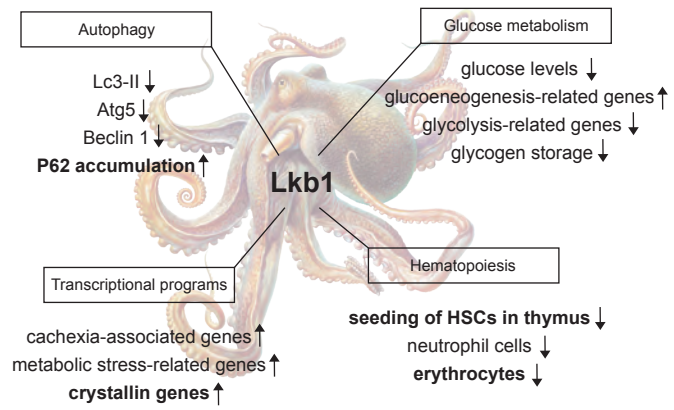

Figure 2. Lkb1 as regulator of metabolic processes. Lkb1 has an essential role in the regulation of cellular and organismal processes. The data in this thesis shows that Lkb1 regulates glucose metabolism, autophagy, transcriptional programs and hematopoiesis. Under each process, the molecular characteristics of *lkb1* larvae at 7 dpf, after yolk-depletion, are depicted. The arrows represent up and downregulation, in comparison to wt larvae at 7 dpf. The characteristics in bold are also deregulated in *lkb1* larvae at 5 dpf, prior to yolk-depletion.

Concluding remarks

The work in this thesis describes the fundamental role of Lkb1 as a conductor of metabolism-related processes in zebrafish larvae. We show that Lkb1 is essential for the regulation of glucose metabolism, the activation of autophagy, and hematopoiesis under conditions of metabolic stress. Furthermore, we also uncovered gene transcription profiles and hematological characteristics that are specific to *lkb1* larvae, and independent of metabolic stress. Finally, we illustrate and highlight the potential of *lkb1* larvae as screening platform in research related to metabolism, hematopoiesis, and tumors bearing *LKB1* mutations. Overall, we have strengthened the value of *lkb1* zebrafish larvae as a model to study the effects of Lkb1-inactivation on various metabolism-related processes.

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