The role of the tumor suppressor Lkb1 in energy homeostatis

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Chapter 1

Introduction and outline of this thesis

Metabolism, the process of change

The process of metabolism describes the intricate set of biochemical processes that enable the survival of an organism. The word 'metabolism' originates from the Greek word "μεταβολή," and refers to the action of change. The biochemical processes that 'change' i.e. convert nutrients, facilitate the following three essential organismal processes: (1) generation of energy to run cellular processes, (2) generation of biomolecular substrates such as proteins, lipids, nucleic acids, and carbohydrates, and (3) elimination of harmful and/or waste products. Not only is 'change' referable to the biochemical changes, it also reflects the adaptation of metabolism in order to maintain stable energy levels, a process called homeostasis. Remaining a state of homeostasis by adaptation to changes in nutrient ingestion, is crucial for an organism's survival. However, nutrient availability is nΔot the sole factor that affects energy homeostasis. Deregulation of metabolic processes by itself can disturb homeostasis as well, often accompanied by an increased risk of the development of diseases, for example diabetes or cancer. The metabolic pathways, the role of metabolism in organismal processes, and the role of metabolism in diseases are therefore intensely studied research topics. This thesis contributes to the field of metabolism and disease research with a study of an important tumor suppressor, known as Liver Kinase B1 (LKB1). Using zebrafish larvae as an animal model, the goal of our research was to gain insight into the role of this tumor suppressor in metabolic adaptation and energy homeostasis at a whole organism level.

Glucose metabolism during feeding and fasting

Glucose is the main energetic substrate in cellular metabolism. Under nutrient abundance conditions glucose is absorbed, released into the bloodstream and taken-up by cells. Within the cells, glucose is broken-down to generate energy (ATP) via glycolysis. To maintain glucose homeostasis, signals from various organs - liver, pancreas, skeletal muscle, brain and adipose tissue - interact with each other. After a meal, the release of glucose from the liver is regulated by insulin, produced by β-cells in the pancreas in response to rising blood glucose levels (Figure 1A). Besides the direct use of glucose, insulin also promotes the conversion of glucose to glycogen in the liver, where it can serve as a glucose-reservoir during times of nutrient shortage. During periods of short-term fasting, less glucose is absorbed via the gastrointestinal tract, resulting in lowering of glucose levels in the blood (Figure 1B). This decrease triggers the hydrolyzation of the stored glycogen in the liver, resulting in the generation and release of glucose into the bloodstream. During times of prolonged fasting, after glycogen becomes depleted, further decreased blood glucose levels activate the release of glucagon from α-cells in the pancreas. Glucagon subsequently activates the transcription of gluconeogenesis in liver cells. Gluconeogenesis is the process during which non-carbohydrate sources, such as lactate, glycerol, pyruvate and amino acids, are redirected to the liver to serve as substrate to generate glucose, to restore blood glucose levels. The regulation of glucose metabolism is essential for the survival of an organism; deregulation of one of these processes, resulting in dysregulation of blood glucose levels, contributes to the development of metabolic disorders, such as type 2 diabetes and diseases that are associated with deregulated metabolism including cancer. The current rise in metabolic diseases and the increased understanding of the influence of metabolism in many other life-threatening diseases has focused research on understanding the metabolic regulation in further detail.

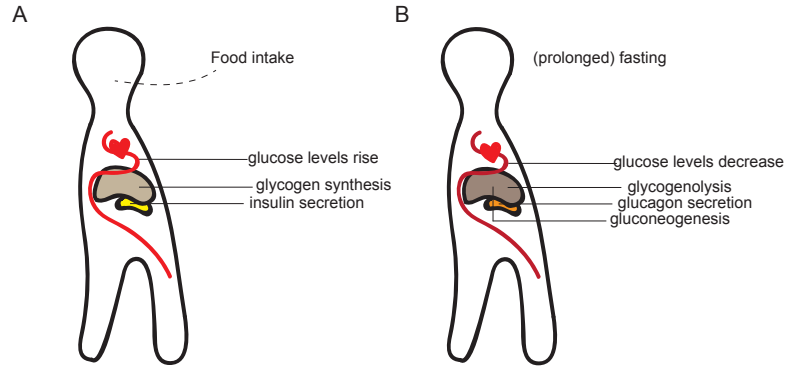

Figure 1. Organismal response to feeding and (prolonged) fasting. (A) After food ingestion, absorption of glucose via the gastrointestinal system results in increases blood glucose levels. In response to rising glucose levels, insulin is produced by β-cells in the pancreas. Insulin subsequently promotes synthesis and storage of glycogen in the liver. **(B)** During periods of short-term fasting, blood-glucose levels decrease, which triggers break-down of stored glycogen (glycogenolysis) in the liver. During times of prolonged fasting, further decreasing blood glucose levels activate the release of glucagon from α-cells in the pancreas. Glucagon subsequently activates the production of 'new' glucose (gluconeogenesis) in liver cells to prevent further lowering blood glucose levels.

Zebrafish as a model to study whole-body energy homeostasis

Since regulation of metabolism is complex, for in-depth understanding it is important to study the processes involved at cellular and whole-organismal level. Studying metabolism at the cellular level will provide a more detailed view and might shed light on processes such as cell cycle regulation or intracellular, cell-type specific responses to environmental changes. Studying the metabolic processes at the organismal level, on the other hand, will provide insight into the integrated response to metabolic changes and the interplay between multiple organs and endocrine signalling. Our research has been focused on studying metabolic adaptation and energy homeostasis, at the whole-body level. Therefore, the use of an organism as research model would provide most valuable insights. In this thesis, we have used the zebrafish as our scientific model. The regulation of systemic metabolism is often studied in zebrafish because of its small size and vertebrate physiology (Schlegel and Gut, 2015a; Schlegel and Stainier, 2007; Varga et al., 2015). Importantly, fundamental principles of energy homeostasis are highly conserved between humans and zebrafish (Schlegel and Gut, 2015b; Seth et al., 2013).

Metabolic adaptation and its regulation by LKB1

During zebrafish development, metabolic adaptation occurs during depletion of the yolk. The yolk is the sole nutrient source during the first days of development. However, between 5 and 7 days post fertilization (dpf), it becomes depleted and larvae need to start scavenging for external resources to meet their energetic demands (Figure 2A). The transition from a continuous maternal nutrient supply to an external intermittent nutrient supply is a period of nutrient restriction, and mechanisms of metabolic adaptation need to be activated for the organism to survive. Our group has shown that Lkb1 is a key player is this process, since lkb1 mutant larvae die shortly after yolk depletion (Figure 2B) showing physical and biochemical signs of starvation (Mans et al., 2017; van der Velden et al., 2011).

The tumor suppressor LKB1 (also known as STK11) is a serine/threonine kinase that has been discovered as the causal mutation of Peutz-Jeghers syndrome (PJS). Patients with PJS develop benign polyps in the intestine and have increased risk to develop tumors in various organs. Moreover, somatic mutations and deregulation of LKB1 have been identified in many tumor types, and are amongst the most common mutations in lung adenocarcinoma (Sanchez-Cespedes, 2011; Sanchez-Cespedes et al., 2002). LKB1 regulates essential cellular and organismal processes such as metabolism, cell growth and cell polarization via phosphorylation of AMP-activated kinase (AMPK) and 12 other AMPK-related kinases (Li et al., 2014; Lizcano et al., 2004; Sanchez-Cespedes, 2007; Shaw et al., 2004b). Two important downstream targets in the context of glucose metabolism are AMPK and Salt-Inducible Kinases (SIKs). AMPK, also referred to as the cellular 'gauge', measures the cellular energetic status, characterized by the AMP/ATP and ADP/ATP ratios and glucose levels (Hardie et al., 2012a; Lin and Hardie, 2018). Under low energetic conditions, AMPK is activated by LKB1 resulting in the regulation of downstream pathways, such as the mTOR-pathway, resulting in (1) the activation of catabolic pathways, such as autophagy, to generate energy substrates, and (2) inhibition of anabolic pathways, such as protein synthesis, to restrict energy-consuming processes.

In contrast, activation of SIK by LKB1 occurs under normal energy conditions. Activation of SIK promotes the degradation of the transcriptional activator CRTC2, thereby preventing transcriptional activation of gluconeogenesis and other meta-

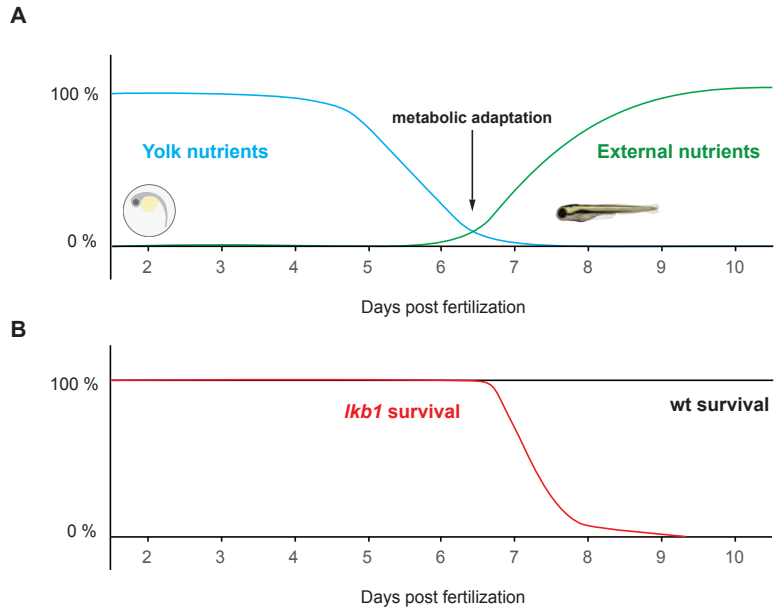

Figure 2. Lkb1 is essential for metabolic adaptation during zebrafish development. (A) Schematic overview of nutrient sources during larval development. During the first days of development, zebrafish larvae are supplied by nutrients from the yolk. Between 5 and 7 dpf, the yolk becomes depleted and larvae need to scavenge for external nutrient sources. This transition from endo- to ecto-nutrition is accompanied by nutrient restriction, and mechanisms of metabolic adaptation need to be activated for the organism to survive. **(B)** Schematic representation of survival of *lkb1* and wt larvae. Lkb1 is essential for metabolic adaptation since *lkb1* larvae die shortly after yolk depletion showing signs of starvation, whereas fasted wt larvae can survive for over a week longer.

bolic programs. Under conditions of low blood glucose levels, glucagon is released in the bloodstream and binds to receptors in hepatocytes resulting in the activation of PKA. PKA subsequently blocks SIK activation, overruling the activation by LKB1, resulting in the translocation of CRTC2 to the nucleus and initiation of transcription of genes involved in glucose and lipid metabolism. These diverse effects of LKB1 on downstream targets highlight the complexity of metabolic regulation by LKB1 under varying energetic conditions; activation of AMPK occurs under low energetic conditions, resulting in direct changes in programs involved in metabolic adaptation, whereas activation of SIK occurs under normal energetic conditions, resulting in the repression of transcriptional programs involved in metabolic adaptation (Figure 3).

The discovery of LKB1 as conductor of these essential processes has resulted in many studies, which have led to an increase in knowledge over the past years. The effects of cell-specific inactivation of LKB1 have provided us with fascinating insights into the different effects of LKB1 deficiency in specific organs (Granot et al., 2009; Koh et al., 2006; Sun et al., 2010). The effects of whole-body inactivation of LKB1, however, have been proven difficult to study in vertebrate organisms, since whole-body deficiency of Lkb1 in mice is embryonic lethal (Ylikorkala et al., 2001). The zebrafish *lkb1* mutant however, survives embryonic development and therefore provides a model organism to study the effects of whole-body inactivation of Lkb1.

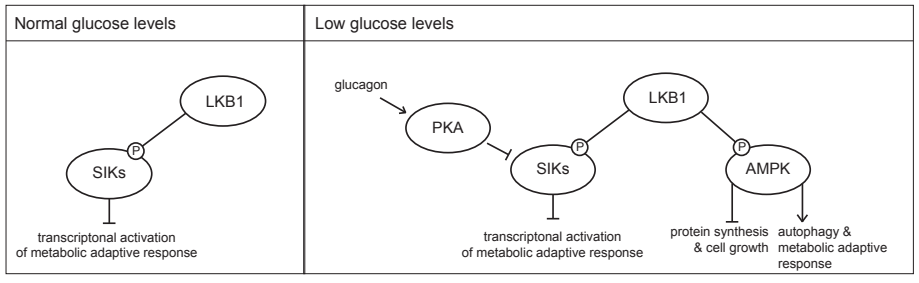

Figure 3. Lkb1 regulation of metabolism via AMPK and SIK. Under normal glucose level conditions, LKB1 activates SIK, resulting in inhibition of transcriptional activation of gluconeogenesis and other metabolic programs. Under low energy conditions, LKB1 activates AMPK resulting in the activation of catabolic pathways and inhibition of anabolic pathways. Furthermore, glucagon is released in the bloodstream resulting in the activation of PKA. PKA subsequently blocks SIK activation, overruling the activation by LKB1, resulting in initiation of transcription of genes involved in glucose and lipid metabolism.

Metabolic adaptation of tumor cells

Besides the role of metabolic adaptation in maintaining whole-body energy homeostasis, adaptation of certain metabolic programs is also a hallmark of cancer cells. Enhanced glycolysis and suppressed oxidative phosphorylation, also known as the 'Warburg' effect', are key-features of cancer cells, which facilitate survival and proliferation under conditions of nutrient and oxygen limitation (Vander Heiden et al., 2009). Moreover, other metabolic processes, including fatty acid and amino acid metabolism, and mitochondrial function are often deregulated in cancer as well. These altered metabolic characteristics of cancer cells distinguish them from normal cells and could be exploited for selective targeting. For instance, *LKB1* tumors are characterized by a deregulated metabolism, or hypermetabolic phenotype (Kottakis et al., 2016). This hypermetabolic phenotype was shown to correlate with enhanced sensitivity to metabolic stressors (Momcilovic et al., 2015; Shackelford et al., 2013). Thus, a better understanding of the role of LKB1 in metabolic adaptation will contribute to the development of treatments that specifically target the altered metabolic state of cancer cells (Howard et al., 2016).

Metabolic regulation and stem cell homeostasis

Regulation of systemic metabolism also plays an important role in stem cell homeostasis. Not only are the metabolic characteristics of stem cells distinct from their differentiated progeny, but environmental cues have also been shown to affect the balance between stem cell maintenance and differentiation into downstream lineages (Rafalski et al., 2012). Disruption of stem cell maintenance has detrimental effects on an organism's physiology, resulting in diseases including cancer. The role of LKB1 in hematopoietic stem cell maintenance became evident when three papers were published in 2010, revealing the effects of cell-specific deletion of LKB1 on hematopoietic stem cell (HSC) maintenance in mice (Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010a). Interestingly, the observed effects were at least partially, AMPK- and mTOR-independent. Since hematopoiesis is highly conserved between mammals and zebrafish, *lkb1* zebrafish larvae could provide a new model for studying how Lkb1 and deregulated metabolism affect hematopoiesis. Furthermore, such model could potentially be developed into a screening platform for drugs that ameliorate Lkb1- or metabolism-associated hematopoietic defects.

Outline of this thesis

In this thesis, we further characterize the metabolic phenotype of *lkb1* zebrafish larvae and address the role of Lkb1 in metabolic adaptation in response to the metabolic stress during yolk depletion.

In **Chapter 2**, we show that Lkb1 is essential for the induction of autophagy, a cellular degradation process, after yolk depletion. We show that induction of starvation-induced autophagy occurs in wild-type larvae upon yolk depletion, but not in *lkb1* larvae. The absence of autophagy was accompanied by accumulation of Sqstm1/p62, a protein that under basal conditions is degraded by autophagy. Finally, we demonstrate that the autophagy defect in *lkb1* larvae could be rescued by inhibition of calpains, a class of cysteine proteases.

In **Chapter 3**, we show that *lkb1* larvae exhibit decreased glucose levels, and premature induction of gluconeogenesis- and of metabolic stress-related genes. We leverage the hypermetabolic phenotype of *lkb1* larvae in a synthetic lethality screen aiming to identify compounds that would selectively target the *lkb1* larvae and leave wt larvae unaffected. Indeed, we identified two compounds that selectively target *lkb1* larvae.

In **Chapter 4**, we use RNA sequencing as an unbiased approach to identify the transcriptional changes that occur upon whole-body inactivation of Lkb1. We compared the transcriptome of wt and *lkb1* larvae, before and after yolk depletion, and during prolonged fasting. Our data confirm that *lkb1* larvae are in a premature starvation state shortly after termination of the yolk supply. Interestingly, we discovered gene sets that were deregulated only in *lkb1* larvae after termination of the yolk supply, but not in prolonged fasted wt larvae, indicating that the deregulation of these genes is the result of the interplay of metabolic challenge and inactivation of Lkb1. Finally, we also identified a subset of genes that are deregulated in *lkb1* larvae both before and after yolk-depletion, which is intriguing, since expression of these genes appears to be independent of the metabolic challenge of the organism, and regulated in an Lkb1-specific manner.

In **Chapter 5**, we review the effects of cellular and systemic metabolic state on stem-cell fate and cell cycle regulation, and discuss the most important signalling pathways involved in this process, including LKB1-dependent signalling.

In **Chapter 6**, we investigate the effects of whole-body inactivation of Lkb1 on the maintenance of HSCs and development of downstream lineages. At 7 dpf, when *lkb1* larvae enter a state of starvation, we observed reduced numbers of neutrophil cells in *lkb1* larvae compared to wild type siblings, suggesting that the metabolic state of the larvae influences the maintenance of these cells. Interestingly, *lkb1* larvae showed dramatically low numbers of *gata1*$^{+}$ erythrocytes throughout development, suggesting a cell-specific role of Lkb1 in the terminal differentiation of this lineage. We conclude that the zebrafish *lkb1* model confirms that the metabolic state of an organism has an important role on HSC maintenance and differentiation into downstream lineages, and suggest that Lkb1 has a (cell-) specific role in the development of erythrocytes.

Finally, in **Chapter 7** we summarize and discuss the preceding chapters, and highlight the overarching conclusions of the role of Lkb1 in regulating whole-body energy homeostasis during zebrafish development.

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