The role of the tumor suppressor Lkb1 in energy homeostatis

Mans, L.A.

Chapter 3

Deregulated metabolism in *lkb1* mutant zebrafish uncovers susceptibility to metabolic activators for targeted cancer therapy

Laurie A. Mans [1], Laia Querol Cano [1,2], Gaetano Gargiulo [3] and Anna-Pavlina G. Haramis [1#]

1 Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE, Leiden, The Netherlands
2 Present address: Radboud Institute for Molecular Life Sciences - Tumour immunology department, Geert Grooteplein 28, 6525 GA Nijmegen, The Netherlands.
3 Molecular Oncology, Max-Delbrück-Center for Molecular Medicine (MDC), Robert-Rössle-Str. 10, 13092 Berlin, Germany

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Abstract

Non-small cell lung cancer (NSCLC) is one of the most common and deadly cancers. The tumor suppressor LKB1/STK11 is frequently mutated in lung adenocarcinomas (LUAD), often coexisting with oncogenic TP53 and KRAS. *LKB1* tumors show a hypermetabolic phenotype, and enhanced sensitivity to metabolic stressors. In zebrafish, Lkb1 is a critical regulator of metabolic reprogramming during development. Zebrafish *lkb1* mutants show accelerated energy However, whether these metabolic traits can be exploited against *LKB1* mutant tumors has not been fully elucidated. Here we show that *lkb1* mutants show defective glucose homeostasis and a premature and exacerbated fasting response. We leverage the deregulated metabolic features of *lkb1* mutants in a synthetic lethality screen for compounds that could selectively kill the mutants but not wild-type counterparts. We identify that piceatannol and Tyrphostin23, are selectively lethal to *lkb1* mutants, likely by increasing energetic stress. Collectively, our data highlight that Lkb1 has a critical, functionally conserved role in regulating metabolic reprogramming processes and support the combination of genetics and drug screening in zebrafish, as a promising discovery platform for targeted cancer therapy.

Introduction

Tumor cells undergo fundamental changes in their metabolism (metabolic transformation) to meet their increased energetic and biosynthetic demands, often under nutrient-and oxygen-poor environments (Jones and Thompson, 2009; Vander Heiden et al., 2009). However, the molecular mechanisms that underlie this transformation are not well understood. The altered metabolic characteristics of tumors provide vulnerability points exclusive to the cancer cells that can be targeted for efficient treatment. Indeed, it has been suggested that the altered metabolism of cancer cells constitutes the Achilles' heel of cancer (Kroemer and Pouyssegur, 2008).

The tumor suppressor LKB1 (also known as STK11), an important regulator of metabolic reprogramming in cancer cells (Faubert et al., 2014) is a serine threonine kinase that regulates various cellular processes such as cell growth, polarity, autophagy, and metabolism (Shackelford and Shaw, 2009). LKB1 phosphorylates AMP-activated Kinase (AMPK), a central metabolic switch in all eukaryotes that controls metabolism in response to changes in intracellular energy levels (Hardie et al., 2012a; Lin and Hardie, 2018), and 12 other AMPK-related kinases (Li et al., 2014; Lizcano et al., 2004; Sanchez-Cespedes et al., 2002; Shaw et al., 2004b). LKB1 was initially identified as the causal mutation in the rare familial cancer-predisposition syndrome Peutz-Jeghers (Hemminki et al., 1998) and a decade later, inactivating mutations in the *LKB1 locus* were identified in several cancers (Li et al., 2014; Sanchez-Cespedes, 2007) including notably, over 30% of human lung adenocarcinomas (LUADs) (Ji et al., 2007; Sanchez-Cespedes et al., 2002). Indeed, *LKB1* is the third most frequently mutated gene in LUADs (Ding et al., 2008), often coexisting with oncogenic TP53 and KRAS in non-small cell lung cancer (NSCLC) (Sanchez-Cespedes, 2011).

Therapeutically exploiting tumor suppressor mutations is indirectly possible when they confer specific dependencies to the cancer cell (Howard et al., 2016). *LKB1* tumors show a hypermetabolic phenotype (Kottakis et al., 2016), and enhanced sensitivity to metabolic stressors (Momcilovic et al., 2015; Shackelford et al., 2013). In zebrafish, Lkb1 regulates metabolic reprogramming during normal development as *lkb1* larvae show defective response to metabolic stress, premature exhaustion of energy reserves (van der Velden et al., 2011), and fail to activate starvation-induced autophagy (Mans et al., 2017). In this study, we provide a link between metabolic adaptation to energetic demands and Lkb1-deficiency. We expand the characterization of the *lkb1* metabolic phenotype and show that zebrafish *lkb1* larvae have defective glucose homeostasis during development and

exhibit a premature and exacerbated fasting response. We leverage the deregulated metabolic features of *lkb1* larvae in a synthetic lethality screen for compounds that could selectively kill the mutants but not wild-type counterparts and find that piceatannol (a resveratrol analog), and Tyrphostin23, (a mitochondrial uncoupler), are selectively lethal to *lkb1* larvae.

Results

Defective glucose metabolism in *lkb1* larvae

Depletion or interruption of the maternal nutrient supply induces gluconeogenesis to restore glucose levels in mammals (Gustafsson, 2009; Pilkis and Granner, 1992b) and in zebrafish (Gut et al., 2013). Since zebrafish *lkb1* larvae die prematurely, shortly after yolk depletion, we measured glucose levels during the period of yolk depletion using an enzymatic assay and found that *lkb1* larvae show dramatically lower glucose levels at seven days post fertilization (7 dpf). Since the trend of lower glucose levels was observed already at 5 dpf (Figure 1A), these data indicate that *lkb1* larvae are unable to restore glucose levels following yolk depletion, and prematurely enter a glucose-deprivation state.

Upregulation of gluconeogenesis- and downregulation of glycolysis-related genes in *lkb1* larvae

To dissect how Lkb1 deficiency impacts glucose metabolism, we analysed the glucose metabolism transcriptome in *lkb1* versus wild-type larvae (Figure 1B) and observed overexpression in *lkb1* larvae of *phosphoenolpyruvate carboxykinase 1 (pck1)*, the first rate-limiting enzyme in gluconeogenesis which converts oxaloacetate into phosphoenolpyruvate (Yabaluri and Bashyam, 2010), and *glucose-6 phosphatase catalytic subunit (g6pca.1)*, the last enzyme in the gluconeogenic-cascade that converts glucose-6-phosphate to glucose (Yabaluri and Bashyam, 2010). Furthermore, we also observed an upregulation of genes associated with a fasting state such as *pyruvate dehydrogenase kinase 2b (pdk2b*) (Sugden and Holness, 2006), a kinase that inhibits the pyruvate dehydrogenase complex thereby promoting the conversion of Acetyl-CoA to pyruvate, and *isocitrate dehydrogenase 3a (idh3a)*, an TCA cycle-associated enzyme that is activated when ATP levels are low (Al-Khallaf, 2017). In contrast, genes associated with glycolysis, such as *pyruvate kinase (pklr)*, which converts phosphoenolpyruvate (PEP) to pyruvate, and *enolase 4 (eno4)*, an enzyme that catalyzes the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP) were expressed significantly lower in *lkb1* compared to wt larvae. Overall, these data show that *lkb1* larvae have defects in glucose metabolism and show a transcriptional profile associated with a glucose-deprived (prolonged fasting) state.

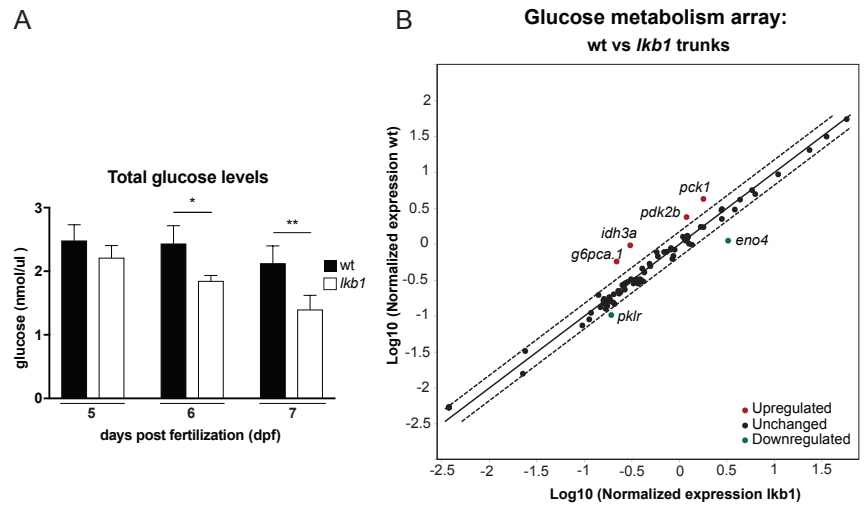


Figure 1. Attenuated glucose metabolism in *lkb1* larvae. (A) Enzymatic assay of total glucose levels in lysates of wt and *lkb1* trunks between 5-7 dpf. Total glucose levels decrease over time in *lkb1* compared to wt larvae and become significantly lower at 6 and 7 dpf. Data represent the means, ± standard errors of the means (SEM) and are pooled from three independent experiments (5 trunks of larvae/sample). * P value < 0.05, ** P-value <0.02, 2way ANOVA with Sidak's multiple comparison test. **(B)** Gene expression analysis of total RNA in wt and *lkb1* trunks at 6 dpf, using a Zebrafish Glucose metabolism PCR array. Genes associated with 'low energy levels' and gluconeogenesis are upregulated (*pck1, g6pca.1, idh3a and pdk2b*). Genes associated with glycolysis are downregulated (*eno4* and *pklr*). Data were analysed with the online software package and represent two independent experiments (10 trunks of larvae/sample). Differential expression was set at a log2 fold change of >1.5 or <-1.5.

Premature upregulation of gluconeogenesis-related genes in *lkb1* larvae

To study the timing of the upregulation of gluconeogenic gene expression in *lkb1* larvae, we analysed the expression of *pck1* and *g6pca.1*, the first and last enzyme in gluconeogenesis respectively, before, during, and after yolk depletion. To enrich for tissues involved in metabolic processes, we isolated mRNA from dissected trunks containing liver, pancreas, intestine and kidney. We compared wt and *lkb1* samples between 5 and 7 dpf and, to address the transcriptional status of these genes in prolonged starvation, we analysed trunks of wt unfed larvae from 8 to 11 dpf.

At 5 dpf, when there is still yolk present, both *pck1* and *g6pca.1* were already upregulated in *lkb1* compared to wt larvae (Figure 2A, B). The expression of both genes sharply increased at 6 dpf in *lkb1* larvae and subsequently decreased at 7 dpf. In comparison, in wt larvae expression of both genes increased significantly only after 7 dpf, peaking at 8 dpf followed by a gradual decrease until 11 dpf. These data show that gluconeogenesis is aberrantly and prematurely activated in *lkb1* larvae.

To study the mechanism underlying the premature upregulation of gluconeogenesis in *lkb1* larvae, we studied the expression of the transcription factors that regulate transcription of gluconeogenic enzymes (Altarejos and Montminy, 2011; Short et al., 1986). CREM is activated by PKA in response to increased cAMP, resulting in the transcriptional activation of PEPCK, G6Pase and peroxisome proliferator γ-activated receptor coactivator 1-α (PGC-1α)(Altarejos and Montminy, 2011). PGC-1α in turn, acts as a co-activator to further increase gluconeogenic gene expression during prolonged fasting (Herzig et al., 2001). We assessed the expression of *cremb* and *pgc1a* by qPCR analysis over time in wt and *lkb1* trunks from 5 to 7 dpf and wt trunks until 11 dpf. In wt trunks, *pgc1a* and *cremb* levels increased between 5 and 7 dpf (Figure 2C, D), and remained constant until 11 dpf. This is consistent with *pck1* and *g6pca* levels being significantly increased around 7-8 dpf. In *lkb1* trunks, however, levels of *pgc1a* and *cremb* were elevated at 7 dpf, at which time-point *pck1* and *g6pca.1* levels are already decreasing, suggesting that upregulation of *pck1* and *g6pca.1* expression at 5 dpf precedes *pgc1a* or *cremb*. Interestingly however, both genes are highly overexpressed in *lkb1* larvae compared to wt suggesting that the dramatic increase in expression of these genes is Lkb1-regulated. These results indicate that since the premature upregulation of gluconeogenesis in *lkb1* larvae precedes *cremb* and *pgc1a* expression, it is most likely not mediated by *cremb* and *pgc1a*, but through other Lkb1-regulated mechanisms.

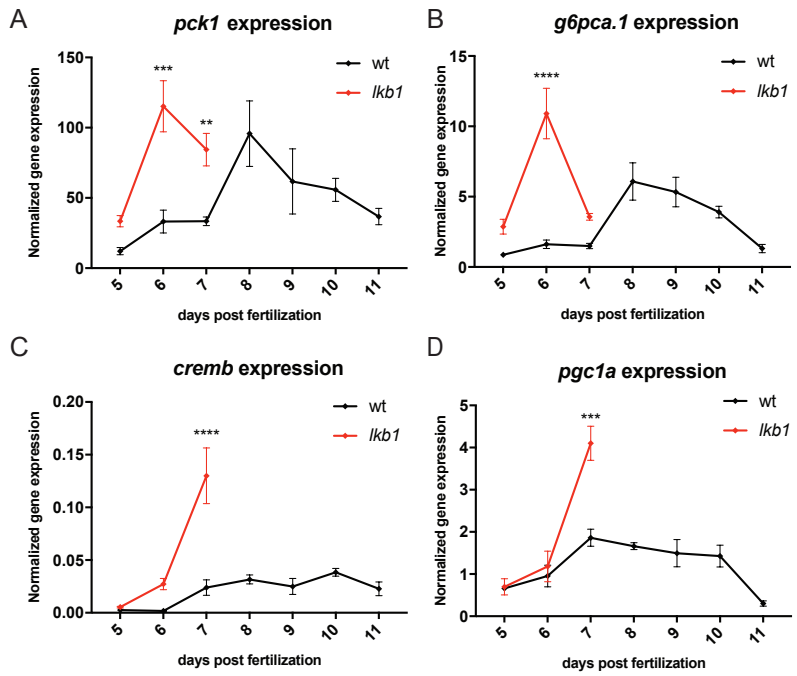
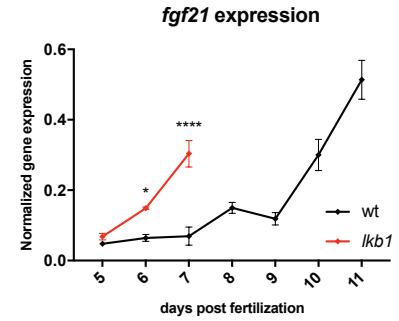


Figure 2. Premature upregulation of gluconeogenesis in lkb1 **larvae.** Gene expression analysis from total RNA extracted from *lkb1* trunks at 5-7 dpf, and wt trunks at 5-11 dpf. **(A)** In wt samples, *pck1* expression gradually increases until 8 dpf, followed by a decrease until 11 dpf. In *lkb1* samples, *pck1* expression is significantly upregulated compared to wt, and peaks at 6 dpf. **(B)** *g6pca.1* expression, similar to *pck1*, gradually increases until 8 dpf, followed by a decrease in wt samples. In *lkb1* samples, *g6pca.1* is significantly upregulated at 6 dpf followed by a drop at 7 dpf. (C-D) Gene expression analysis of gluconeogenesis-regulators *cremb* **(C)** and *pgc1a* **(D)** in total RNA extracted from *lkb1* trunks at 5-7 dpf, and wt trunks at 5-11 dpf. In wt samples, expression of *cremb* and *pgc1a* rises at 7 dpf. In *lkb1* samples, both genes are upregulated at 6 dpf and are expressed at much higher levels compared to wt at 7 dpf. Data represent the means, ± standard errors of the means (SEM) and are pooled from three independent experiments. * P value < 0.05, ** P-value <0.02, *** P-value < 0.002, **** P-value <0.0001; 2way ANOVA with Sidak's multiple comparisons test

Metabolic stress markers *fgf21*, *ucp2* and *lpin1* are upregulated in *lkb1* larvae

The lower glucose levels and premature upregulation of gluconeogenesis in *lkb1* larvae suggested that they are in a state of severe metabolic stress. During the different phases of fasting, prolonged fasting and starvation, distinct metabolic programs are activated to enable organisms to adapt to the varying degrees of nutrient limitation. The hormone Fibroblast growth factor 21 (FGF21) plays an important role in the response to starvation (Inagaki et al., 2007; Zhang et al., 2015) as it acts locally and in an endocrine manner, to orchestrate adaptation of glucose and lipid metabolism during starvation in mammals (Zhang et al., 2015). We found that in wt trunks, *fgf21* expression is gradually upregulated after yolk depletion, reaching very high levels at 11 dpf (Figure 3A). Interestingly, *lkb1* larvae showed premature upregulation of *fgf21* already from 6 dpf onwards, suggesting that the larvae are under metabolic stress shortly after yolk depletion. Uncoupling protein 2 (Ucp2) is also involved in the response to starvation and metabolic stress (Gremlich et al., 2005; Nakatani et al., 2002) and we observed that although in wt trunks *ucp2* expression is dynamic over time and does not appear to correlate with prolonged fasting (Figure 3B), in *lkb1* trunks *ucp2* is highly upregulated at 6 and 7 dpf, indicating that *lkb1* larvae are indeed under metabolic stress as early as 6 dpf. Finally, we examined the expression of *lipin1* (*lpin1*), which is directly involved in lipid metabolism, and indirectly in the transcriptional activation of PGC1a and PPARα in the liver, thereby regulating β-oxidation (Finck et al., 2006). In accordance with our previous observations, we found that in wt larvae *lpin1* is upregulated from 8 dpf, while in *lkb1* larvae *lpin1* is already upregulated at 6 dpf (Figure 3C). These data collectively show that the *lkb1* larvae are prematurely in a state of severe metabolic stress.

A



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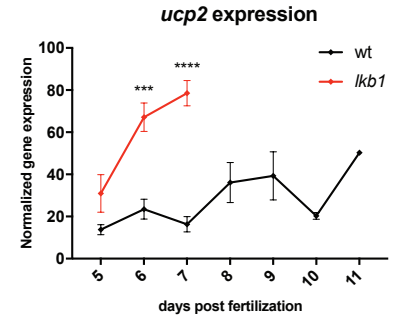

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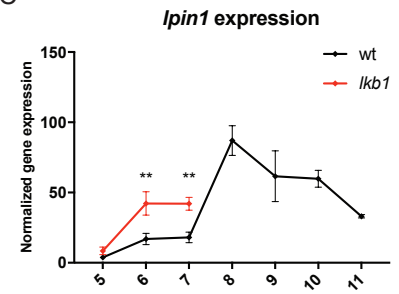

Figure 3. Markers of metabolic stress are upregulated in *lkb1* larvae. Gene expression analysis of metabolic stress markers *fgf21* **(A)**, *ucp2* **(B)** and *lpin1* **(C)** in total RNA extracted from wt and *lkb1* trunks at 5-7 dpf. **(A)** In wt samples, *fgf21* expression increases from 9 dpf onwards. In *lkb1* samples, *fgf21* expression is significantly upregulated compared to wt at 6 and 7 dpf. **(B)** In wt samples *ucp2* levels are dynamic between 5 and 11 dpf (n.s.). In *lkb1* samples *ucp2* expression is significantly upregulated compared to wt at 6 and 7 dpf. **(C)** In wt samples *lpin1* expression peaks at 8 dpf, followed by a gradual decrease until 11 dpf. In *lkb1* samples, *lpin1* expression is significantly higher at 6 and 7 dpf. Data represent the means, ± standard errors of the means (SEM) and are pooled from three independent experiments. * P value < 0.05, ** P-value <0.02, *** P-value < 0.002, **** P-value <0.0001; 2way ANOVA with Sidak's multiple comparisons test.

Lkb1 larvae are susceptible to activators of metabolism

We next sought to exploit the altered metabolic characteristics of *lkb1* larvae to uncover vulnerabilities that could be used for targeting an Lkb1 deficient setting, such as that seen in many cancers. To that end, we performed a synthetic lethality screen aiming to identify compounds that target specifically *lkb1* larvae (Figure 4). We tested 80 kinase and 33 phosphatase inhibitors for their ability to selectively kill the *lkb1* larvae and not wt counterparts. We distributed wt, heterozygous and *lkb1* larvae at 4 dpf in a 96-well plate (3 larvae/well) containing 10 μM of compounds. We monitored morphology and survival of the larvae for three days, since the majority of *lkb1* larvae die at 8 dpf. At 8 dpf, all surviving larvae were collected and genotyped to distinguish the *lkb1* larvae.

After validation, we selected two compounds, piceatannol and tyrphostin23 for further characterization. Piceatannol (3,3′, 4,5′-trans-trihydroxystilbene) is a naturally occurring, metabolically more stable analogue of resveratrol (Piotrowska et al., 2012; Setoguchi et al., 2014), implicated in various cellular processes, such as induction of apoptosis, promoting stem cell maintenance and activation of various cellular pathways (Piotrowska et al., 2012). While studies have addressed the effectiveness of piceatannol in treatment of cancer (Seyed et al., 2016), cardiovascular diseases (Tang and Chan, 2014) and metabolic diseases (Kershaw and Kim, 2017), there is no sufficient evidence to recommend its clinical use as yet. For validation experiments, we tested a range of concentrations to identify the maximum tolerated dose. We found that *lkb1* larvae were susceptible to piceatannol treatment in a dose-dependent manner (Figure 5). Specifically, treatment with 50 μM of piceatannol led to death of 75% of *lkb1* larvae, 48 hours post-treatment (hpt) (at 6 dpf), while, 100% of the untreated *lkb1* larvae were still alive and only minor lethality was observed in treated wt or heterozygous (het) larvae. At 72 hpt, all piceatannol-treated *lkb1* larvae were dead and only 5% of wt/het larvae had died. These results indicate that *lkb1* larvae are exclusively susceptible to piceatannol at a concentration in which the vast majority of wt/het larvae are not affected.

Tyrphostin23 (Ty23) is a member of the tyrphostins, which were developed as synthetic inhibitors of protein tyrosine kinases. The action of Ty23 has been linked to many cellular processes including blocking EGFR (Ligeza et al., 2011), increasing glycolytic rate and lactate production (Blumrich et al., 2016), accelerating the mitochondrial TCA cycle (Hohnholt et al., 2017), and lowering ATP levels via mitochondrial uncoupling properties (Soltoff, 2004). Treatment with 25 μM Tyr23 led to 50% of *lkb1* larvae dying at 60 hpt, while untreated *lkb1* larvae remained alive (Figure 6) and at this concentration, Tyr23 was toxic to 20% of wt larvae at 96 hpt. These results show that, similarly to a response to piceatannol, *lkb1* larvae are more susceptible to Tyr23 than wt counterparts.

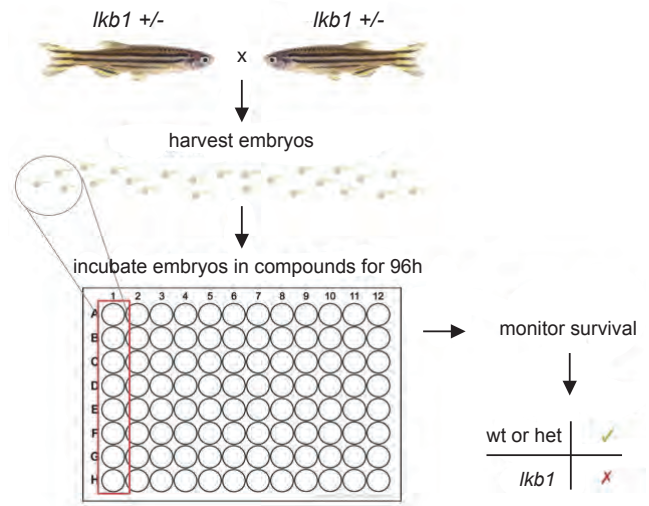

Figure 4. Schematic overview of experimental set-up of the synthetic lethality screen. Heterozygous *lkb1* adults are set-up in single matings for timed fertilization. Embryos are harvested and grown in incubators until 4 dpf. At 4 dpf, larvae are distributed (3 larvae/well) and incubated in library compounds. Survival is monitored and larvae are stored individually for genotyping. Compounds that are synthetic lethal in combination with Lkb1-inactivation will kill *lkb1* larvae but leave wt larvae unharmed.

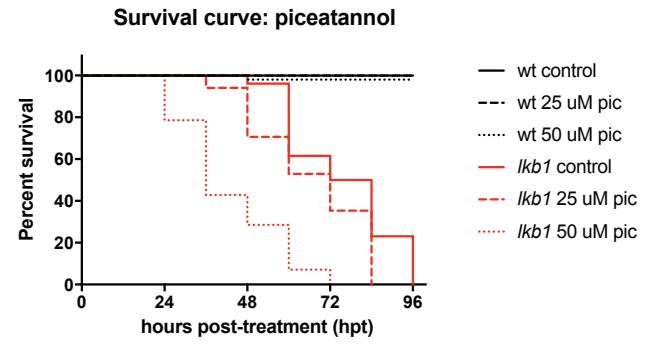

Figure 5. *lkb1* larve are sensitive to piceatannol treatment in a dose-dependent manner. Survival analysis of wt and *lkb1* larvae after treatment with 25 μM, 50 μM piceatannol (pic) or vehicle. Larvae were treated at 4 dpf and monitored until 8 dpf. Treatment of *lkb1* larvae with piceatannol results in premature death of *lkb1* larvae from 24 hpt onwards in a dose-dependent manner. (*lkb1* 25 μM *, *lkb1* 50 μM ****, wt 25/50 μM n.s.). * P-value <0.05, **** P-value <0.001, n.s. P-value not significant; All P-values were calculated in comparison to control treatments and calculated with Log-rank (Mantel-Cox) test.

To gain insight into the premature lethality of *lkb1* larvae upon piceatannol treatment, we analysed expression of a panel of genes involved in fasting metabolism on treated wt and *lkb1* larvae at 6 dpf. The most dramatically upregulated gene in both wt and *lkb1* larvae upon treatment was *ucp2* (Figure 7). Together, our data demonstrate that piceatannol treatment likely acts by increasing the energetic burden over already metabolically challenged *lkb1* mutant zebrafish, thus resulting in selective lethality of *lkb1* larvae and no evident toxicity for their wt counterparts.

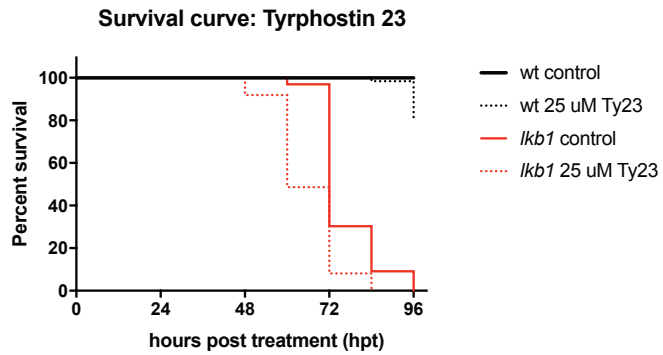

Figure 6. *lkb1* larvae are selectively sensitive to Tyrphostin23 treatment. Survival analysis of wt and *lkb1* larvae following treatment with 25 µM of Tyrphostin (Ty23), or DMSO. Larvae were treated at 4 dpf and monitored until 8 dpf. Ty23- treated *lkb1* larvae die from 48 hpt onwards (****). wt larvae are affected from 96 hpt onwards (***). Data are pooled from three independent experiments (± 48 larvae/experiment). **** P value < 0.0001, *** P-value < 0.0005; all P-values are compared to control treatments and calculated with Log-rank (Mantel-Cox) test.

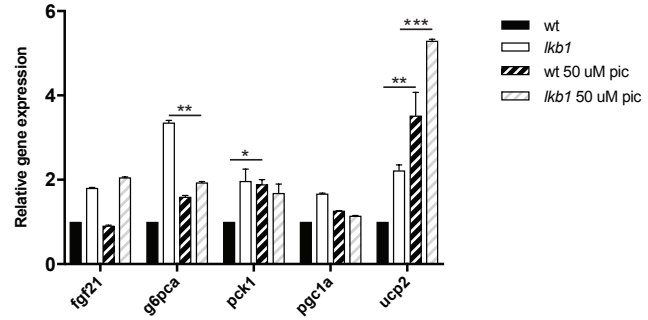

Figure 7. Piceatannol treatment leads to upregulation of *ucp2* expression. Gene expression analysis of *fgf21*, *g6pca*, *pck1*, *pgc1a*, and *ucp2* in total RNA extracted from wt and *lkb1* trunks at 6 dpf. *fgf21* and *pgc1a* do not show differential expression upon treatment. *pck1* is overexpressed in wt larvae upon treatment, while *g6pca* is downregulated in treated *lkb1* larvae. *ucp2* is highly upregulated in both wt and *lkb1* samples upon treatment. Data represent the means, ± standard errors of the means (SEM) and are pooled from three independent experiments. * P value < 0.05, ** P-value <0.02, *** P-value <0.005; two-tailed student's t-test.

Discussion

By using the zebrafish *lkb1* model of deregulated metabolism we show that the tumor suppressor Lkb1 orchestrates metabolic adaptation during development. *Lkb1* larvae exhibit an exacerbated fasting state that ultimately leads to premature starvation and death. We characterize the molecular mechanisms of the overactive fasting metabolism, exploit the deregulated metabolism of *lkb1* larvae and identify compounds that specifically target Lkb1-deficient zebrafish larvae.

Lkb1 larvae display dramatically low glucose levels at 7 dpf in line with a recent study reporting glucose homeostasis defects in a zebrafish gene-trap *lkb1* mutant (Kuang et al., 2016). Glucose levels of *lkb1* larvae are comparable and even higher than those of wt for the first three days of development and begin to decrease at 4 dpf, before yolk depletion (Kuang et al., 2016). We extend this analysis and show that glucose levels further decrease over time in *lkb1* larvae and are dramatically low at 7 dpf. Our results showcase that Lkb1 is essential for glucose homeostasis during embryonic and larval development.

Furthermore, we show that gluconeogenesis, a hallmark of fasting adaptation, is prematurely and excessively induced in *lkb1* larvae. The glucose-metabolism transcriptome showed that Lkb1 deficiency increases transcription of rate-limiting gluconeogenesis genes *pck1* and *g6pca.1*, already before yolk depletion. This finding may be attributed to the reported role of LKB1 in directly suppressing gluconeogenesis, likely through inhibition of SIK kinases (Patel et al., 2014). The enhanced gluconeogenic response is further exacerbated by the onset of metabolic stress following yolk depletion. Analysis of the gene-trap *lkb1* larvae reported increased gluconeogenesis and increased glycolysis in *lkb1* larvae up to 5 dpf (Kuang et al., 2016). Our analysis focuses on larvae shortly after yolk depletion (5-7 dpf) to gain insight into how Lkb1 orchestrates the response to metabolic stress. Interestingly, we show that genes involved in glycolysis are downregulated in *lkb1* larvae at 7 dpf. Additionally, the observed increase in gluconeogenesis is not accompanied by restoration of glucose levels, and it does not appear to be regulated by the most common regulator Pgc1a.

Whole-body knockout of *Lkb1* in mice leads to early embryonic lethality (Ylikorkala et al., 2001) precluding the study of Lkb1 in energy homeostasis during development. We previously showed that in zebrafish, Lkb1 is critical to maintain whole-body energy homeostasis (Mans et al., 2017; van der Velden et al., 2011) and conditional Lkb1 deletion in adult mice results in body weight reduction, increased mortality, and increased gluconeogenesis (Shan et al., 2016). These findings highlight that Lkb1 has an essential, conserved role in regulating metabolism in vertebrates.

We show that *lkb1* larvae exhibit a premature and exacerbated fasting response and several markers associated with (severe) metabolic stress are upregulated in *lkb1* larvae. In particular, *fgf21* was markedly upregulated in *lkb1* larvae already at 6 dpf. Fgf21 has a pivotal role in metabolism and the response to fasting (Badman et al., 2007; Inagaki et al., 2007). In mice, *FGF21* is upregulated as soon as 6 hours after fasting, whereas in humans FGF21 levels only rise after 7 to 10 days of fasting (Fazeli et al., 2015). We found that in wt zebrafish larvae, *fgf21* is upregulated only at 10 dpf. Thus, this 'late' *fgf21* upregulation in fish more closely resembles the starvation response in humans than in mice, confirming the zebrafish as a suitable model to study human metabolism.

Importantly, we exploited the hyperactivated metabolism of *lkb1* larvae to uncover vulnerabilities specific to cells/tissues harboring *LKB1* mutations. In an unbiased synthetic lethality screen we identified two compounds that selectively kill the *lkb1* larvae, leaving their wt counterparts largely unharmed. Interestingly, the identified compounds challenge metabolism and thus, we reason that the *lkb1* larvae are more sensitive to the additional energetic stress. We propose that piceatannol induces an activated metabolic state, and that *lkb1* larvae, which are already under severe metabolic stress cannot cope with the additional challenge. Tyr23, a small molecule that inhibits tyrosine kinases, also acts as a mitochondrial uncoupler, inhibiting ATP production (Soltoff, 2004). As *lkb1* larvae have profoundly low ATP levels (van der Velden et al., 2011), it is conceivable that further decrease in ATP levels upon Tyr23 treatment may be deleterious.

In the NSCLC setting, LKB1 has been identified as the third most frequently mutated gene in LUAD in humans (Ding et al., 2008) and an important regulator of metabolism, however, the precise molecular mechanisms underlying LKB1 functions are not fully understood. It has been additionally suggested that selective killing of *LKB1*-deficient tumor cells may be achieved by mimicking energetic stress (Momcilovic and Shackelford, 2015). Indeed, *LKB1*-deficient NSCLC cells and engineered mouse models are sensitive to the anti-diabetic drug phenformin (Momcilovic et al., 2015; Shackelford et al., 2013). Therefore, it would be interesting to validate the selective LKB1-targeting effects of piceatannol and tyr23 compounds in mammalian NSCLC models bearing LKB1 mutations.

Our data collectively demonstrate that the zebrafish *lkb1* mutant is a valid discovery platform to expand the repertoire of metabolic drugs that would selectively target *LKB1* tumors, thereby supporting the combination of genetics and drug screening in zebrafish as a promising setting for drug discovery for targeted cancer therapy.

Materials and Methods

Zebrafish strains and Screening Methods

Zebrafish were handled in compliance with the local animal welfare regulations and were maintained according to standard protocols (zfin.org). Their culture was approved by the local animal welfare committee (DEC) of the University of Leiden and all protocols adhered to the international guidelines specified by the EU Animal Protection Directive 2010/63/EU. Genotype analysis for *lkb1*-mutant embryos was performed with SNP-specific PCR analysis.

Glucose assay

Zebrafish larvae at 5, 6 or 7 dpf derived from single-matings were sedated and dissected under a stereomicroscope. For each individual embryo, the tail was used for genotyping and the trunk was snap-frozen in 25 ul assay buffer. After genotyping, 4 embryos, either wt, heterozygous or *lkb1*, were pooled, homogenized, and centrifuged for 10' at 12.000 rpm. The lysate was used for further analysis. Total glucose in each sample was determined using the Glucose Colorimetric Assay Kit (Biovision, K606) with a read-out at 570 nm. Total glucose in each sample was calculated using the glucose assay standard supplied with the kit.

RNA extraction for RT-qPCR and glucose metabolism array

Trunks of embryos from single-matings were individually stored in RNAlater (Invitrogen, AM7021) until genotyped. Total RNA was isolated from 10 trunks of either *lkb1*, het or wt trunks, with the RNaqueous micro Total RNA isolation kit (Thermo Fisher Scientific, AM-1931) following manual. Briefly, 10 trunks were lysed in 200 ul Lysis solution, supplemented with 100 ul 100% EtOH and applied to the column. Total mRNA was eluted in 2x10 ul Elution solution concentrations were measured using the Nanodrop (ND-1000).

Gene expression analysis

500-1000 ng of total RNA was reverse transcribed using iScript cDNA synthesis kit (BioRad, 1708890). 1:10 Diluted cDNA was analysed with real-time quantitative RT-PCR performed in a Bio-Rad CFX96 system. For the glucose metabolism array (RT² Profiler™ PCR Array Zebrafish Glucose Metabolism (SABiosciences, PAZF-006Z), plates with aliquoted primers for the genes shown in Table 1 were used. Primer sequences for other reactions are shown in Table 2. Data analysis of qPCR results represents data from three independent experiments. Error bars represent the means ± standard errors of the means (SEM). Statistical significance was calculated using 2way ANOVA using a Tukey's multiple comparisons test.

Table 1. Genes analysed in glucose metabolism array

Glucose Metabolism

Glycolysis: *aldoaa, aldoab, aldob, aldoca, bpgm, eno1a, eno2, eno3, eno4, gapdh, galm, gck, gpia, gpib, hk1, hk2, LOC570106, pgam2, pgk1, pgm1, pgm2, pgm3, pklr, tpi1b.*
Gluconeogenesis: *fbp1a, fbp1b, fbp2, g6pca.1, g6pc3, pc, pck1, pck2.*
Regulation of Glucose Metabolism: *pdk1, pdk2, pdk3a, pdk4, pdp2, pdpr.*
Tricarboxylic Acid Cycle (TCA) Cycle: *aclya, aco1, aco2, dlat, dldh, dlst, fh, idh1, idh2, idh3a, idh3b, mdh1aa, mdh2, ogdha, ogdhb, pc, pck1, pck2, pdha1a, pdhb, sdha, sdhb, sdhc, sdhda, sucla2, suclg1, suclg2, zgc:113076.*
Pentose Phosphate Pathway: *g6pd, h6pd, pgls, prps1b, rbks, rpe, rpia, taldo1, tktb.*

Glycogen Metabolism

Glycogen Synthesis: *gys1, gys2, si:ch211-213e17.1.*
Glycogen Degradation: *agla, pgm1, pgm2, pgm3, pygma.*
Regulation of Glycogen Metabolism: *gsk3ab, gsk3b, phkb, phka1, phkg1a, phkg1b, phkg2.*

Table 2. Primers used for qPCR

Gene	Forward	Reverse
pck1	CATCACGCATCGCTAAAGAG	GTCGCTCTCAGATTCCCTTCTTT
g6pca.1	GAGACTGGCTGAACCTCGTC	GATTGAAAGCAACGCTGTGA
pgc1a	TGAGGAAAATGAGGCCAACT	AGCTTCTTCAGCAGGGAAGG
cremb	TGTGACCGGAGATGAGACAG	CCAGATGCAGGAGAGGACAT
fgf21	CTCCGTCAAAGGCTCTCCTG	CCGTGCAGAGTAATGATGCTGT
ucp2	CTCTGATGACTGATGATCTTCC	CTGACGAACATAACCACATTC
ppial	ACCATCCACAACCTTCCCGAAC	GCACTGAAACACGGAGGCAAAA

Synthetic lethality screen

Larvae were treated with compounds of the ENZO Kinase inhibitors (ENZO life sciences, BML-2832, 80 inhibitors) and phosphatase inhibitors (ENZO, BML-2834, 33 inhibitors) between 4 and 7 dpf. Stocks were diluted in embryo medium for treatment with a final concentration of 10µM, 0,2% DMSO. Screening was performed in 96-wells plates with 3 larvae per well, using 8 wells per compound. Larvae that died during treatment and all surviving larvae at 8 dpf were genotyped for the *lkb1* gene. Compounds that selectively killed *lkb1* larvae were validated in a secondary screen in 6-well plates using 24 larvae per treatment. The secondary screen and validations were performed in triplicate.

Compound treatments

Wild type or *lkb1* mutant zebrafish embryos were treated from 4 to 9 dpf in embryo-medium at 28 °C with either of the following treatments: 25-50 µM Piceatannol (ENZO life sciences, ALX-270-202), 25 µM Tyrphostin23 (ENZO life sciences, BML-EI191), or 0,2% DMSO. Stock solutions were prepared in DMSO and diluted in embryo medium for treatment with a final concentration of 0,2% DMSO. Larvae were collected at the specified time points and genotyped for the *lkb1* gene. Each treatment was performed in triplicate with 48 embryos per experiment. Kaplan-Meier curves were made with Graphpad Prism 7c, combining all three experiments. Treatments curves were compared to control curves were using the Log-rank (Mantel-Cox) test, with P-value <0.05 as statistically significant.

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