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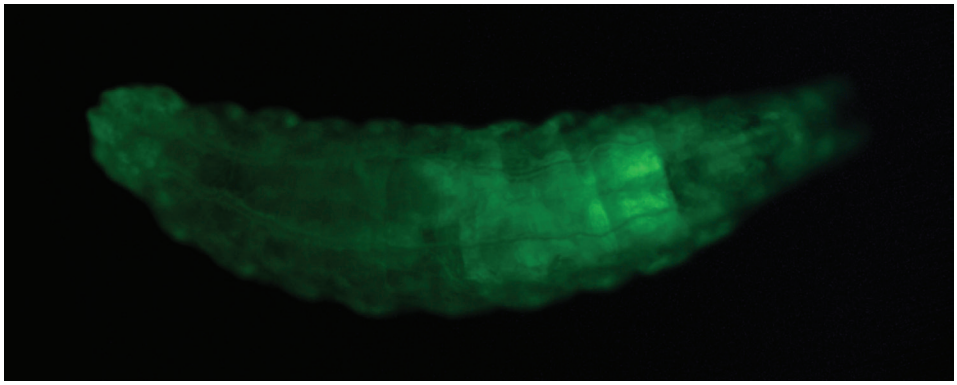
Uncovering a Sugar Tolerance Network: SIK3 and Cabut as Downstream Effectors of Mondo-Mlx



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Uncovering a sugar tolerance network:
SIK3 and Cabut as downstream effectors of Mondo-Mlx

Mari Teesalu

ACADEMIC DISSERTATION

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“The secret of getting ahead is getting started.”
― Agatha Christie

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I Havula, E., **Teesalu, M**., Hyötyläinen, T., Seppälä, H., Hasygar, K., Auvinen, P., Orešič, M., Sandmann, T., and Hietakangas, V. 2013. Mondo/ChREBP-Mlx-regulated transcriptional network is essential for dietary sugar tolerance in Drosophila. PLoS Genet. 9, e1003438.

II Bartok, O#., **Teesalu, M**#., Ashwall-Fluss, R., Pandey, V., Hanan, M., Rovenko, B.M., Poukkula, M., Havula, E., Moussaieff, A., Vodala, S., Nahmias, Y., Kadener, S., and Hietakangas, V. 2015. The transcription factor Cabut coordinates energy metabolism and the circadian clock in response to sugar sensing. EMBO J. 34, 1538–1553.

# - equal contribution

III **Teesalu, M**., Rovenko, B.M., and Hietakangas, V. 2017. Salt-Inducible Kinase 3 Provides Sugar Tolerance by Regulating NADPH/NADP(+) Redox Balance. Curr. Biol. CB 27, 458–464.

The author's contributions:

I
MT performed the experiments for Figures: 2A, 4C, 4F and from supplement: S2, S3D, S5C.
MT optimized and performed the IP and glycogen measurements. In addition, MT carried out a Western blot and a qPCR, while designing and testing the majority of qPCR primers in the study. MT contributed to the writing by drafting up the respective figure legends and materials and methods.

II
MT performed the experiments for Figure 1; Figure 3 C, D, E; Figure 4 and Figure 5.
MT conceived, performed and analyzed the majority of metabolic experiments, including all experiments conducted in cells and larvae. MT and other authors wrote the manuscript.

III
MT performed the experiments for Figure 1 A-C, E,F, I; Figure 2; Figure 3 A-D, H-J and also helped prepare samples for the other experiments.
MT conceived, performed, and analyzed the experiments, and wrote the paper with BMR and VH. MT analyzed sugar tolerance, SIK3 gene regulation, G6PD phosphorylation, and SIK3 genetic interaction with Mondo-Mlx.

Abbreviations

ACC	Acetyl-CoA carboxylase
AcCoA	acetyl-co-enzyme A
AGE	advanced glycation end products
AMPK	adenosine monophosphate-activated kinase
ARRDC4	Arrestin doimain contraining protein 4
ATP	adenosine triphosphate
bHLH	basic-helix-loop helix
bp	base pair
C	carbon
C-terminus	carboxy-terminus
cAMP	cyclic adenosine monophosphate
CCG	clock-controlled gene
ChIP	chromatin immunoprecipitation
ChoRE	carbohydrate response element
ChREBP	Carbohydrate response element binding protein
CNS	central nervous system
CREB	cyclic adenosine monophosphate-responsive element-binding protein
CRTC	cyclic AMP-regulated transcriptional co-activators
CVD	cardiovascular disease
Cyc	Cycle
dClk	*Drosophila* Clock
DCD	dimerization and cytoplasmic localization domain
DEC1	Deleted in esophageal cancer 1
dILP	*Drosophila* insulin-like peptide
E-box	enhancer box
G3P	glycerol-3-phosphate
G6P	glucose-6-phosphate
G6PD	glucose-6-phosphate dehydrogenase
GA3P	glyceraldehyde-3-phosphate
GAL4	Galactose gene transcription factor 4
G6Pase	Glucose-6-phosphatase
Glc	glucose
GlcNAc	N-acetyl-glucosamine
GlcNG	gluconeogenesis
GLUT	glucose transporter
GlyNG	glyceroneogenesis
GRACE	glucose-response activation conserved element
GSM	glucose sensing module
GSSG	glutathione disulfide
F2,6P	fructose-2,6-bisphosphate
$FADH_2$	flavin adenine dinucleotide
FAS	Fatty acid synthase
Fbp	Fru-1,6 bisphosphatase
FFA	free fatty acid
FoxO	Forkhead box O
Fru	fructose
HA	hemagglutinin
HDAC	Histone deacetylase
HK	Hexokinase

HSD	high sugar diet
IRS-1	Insulin receptor substrate 1
LDH-A	Lactate dehydrogenase A
LID	low-glucose inhibitory domain
LKB1	Liver kinase B1
LSD	low sugar diet
LZ	leucine zipper
kDa	kilo-Dalton
Klf	Krüppel-like factor
PK	pyruvate kinase
MCR	Mondo-conserved region
ME2	Malic enzyme 2
Mlx	Max-like protein X
Mlxip	*Mlx-interacting protein*
Mlxipl	*Mlx-interacting protein like*
N-terminus	amino-terminus
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NES	nuclear export signal
NLS	nuclear localization signal
OAA	oxaloacetate
OGT	O-linked N-acetyl-glucosamine transferase enzyme
PDH	Pyruvate dehydrogenase
PDP1ε	PAR domain protein 1ε
PEP	phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
PEPCK-C	cytoplasmic Phosphoenolpyruvate carboxykinase
PEPCK-M	mitochondrial Phosphoenolpyruvate carboxykinase
Per	Period
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase
PI3K	Phosphoinositide 3-kinase
PTG	Protein targeting to glycogen
R5P	ribose-5-phosphate
RNAi	RNA interference
ROS	reactive oxygen species
Sav	Salvador
SIK	Salt-inducible kinase
SREBP1c	Sterol regulatory element binding protein 1c
TAG	triacylglyceride
TCA	tricarboxylic acid cycle, citric acid cycle
TF	transcription factor
Tim	Timeless
TrxR-1	Thioredoxin reductase 1
TXNIP	Thioredoxin interacting protein
UAS	upstream activating sequence
Vri	Vrille
WAT	White adipose tissue
Xu5P	xylulose-5-phosphate
zw	*zwischenferment*

Nucleotides and amino acids are referred to in their standard abbreviation form

Abstract

Simple carbohydrates constitute a big part of our everyday diet, as significant consumption increases have occurred in recent decades. This has coincided with a dramatic rise in people suffering from metabolic disorders, such as diabetes and obesity. However, the genetic factors ensuring a healthy response to sugar intake remain poorly understood. To keep blood glucose in a healthy range even upon overnight fasting or following a rich meal, animals need to be able to adapt quickly. In a healthy organism, high sugar intake leads to rapid conversion of excess sugars into stored glycogen and triacylglycerols, while starvation triggers glucose production through gluconeogenesis and glycogen breakdown. To cope with these fluctuating nutritional conditions organisms possess glucose-sensing mechanisms. Such pathways have a key role in monitoring changes in cellular and organismal nutrient status and readjusting animal physiology accordingly to maintain homeostasis.

Intracellular sugar metabolites are sensed by the conserved Mondo family transcription factors (MondoA and MondoB/ChREBP in mammals, Mondo in *Drosophila*), which act together with transcription factor Mlx. Together, they control the expression of metabolic target genes by binding to the carbohydrate response elements (ChoREs) present in their promoters. The known Mondo-Mlx targets include genes involved in carbohydrate metabolism and biosynthesis of fatty acids and TAGs. Yet, the physiological output of these transcription factors has remained largely elusive. As these transcription factors are very well conserved in *Drosophila*, this model organism has been used in this thesis to understand the physiological role of Mondo-Mlx and their target genes *in vivo*.

We demonstrate that Mondo-Mlx interact in the fly and are crucial for dietary sugar tolerance. Loss of either transcription factor leads to impaired growth and lethality upon high sugar diet. We further characterize the *mlx* mutant phenotype to reveal high circulating glucose, and increased glycogen levels. We uncover transcriptional repressor Cabut as a direct target of Mondo-Mlx. The Cabut promoter contains two conserved ChoREs that are directly bound by Mondo-Mlx in a sugar-dependent manner. Loss of Cabut similarly results in dietary sugar intolerance, pointing to a crucial function in carbohydrate metabolism. We discover that Mondo-Mlx through Cabut are essential for repressing the expression of *pepck* – the rate limiting gene in gluconeogenesis and glyceroneogenesis. A failure to regulate this step results in *pepck* over-expression, leading to several fold higher levels of circulating glycerol, and pupal lethality of *mlx* mutants. Moreover, we reveal that Cabut interconnects nutrient sensing with the circadian clock, and contributes to circadian gene expression oscillation.

Additionally to Cabut, we identify Salt inducible kinase 3 (SIK3) as a direct transcriptional target of Mondo-Mlx required for dietary sugar tolerance. Moreover, we show that the activity of the rate limiting enzyme in the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PD), is increased following sugar feeding. In addition to transcriptional upregulation by Mondo-Mlx, we demonstrate that sugar-augmented increase in G6PD activity is achieved through SIK3-dependent activating G6PD phosphorylation. Collectively, our results demonstrate that Mondo-Mlx-mediated transcriptional upregulation, as well as SIK3-dependent phosphorylation promote G6PD enzyme activity in response to high high sugar diets. The subsequent increase in G6PD activity is required for elevating $NADPH/NADP^+$ ratio in order to reduce glutathione in high sugar conditions. We determine that upregulating G6PD activity through Mondo-Mlx and SIK3 is essential for redox balance maintenance and this mechanism contributes to dietary sugar tolerance.

In sum, the results of this thesis demonstrate that maintaining redox balance, directing the flow of carbon backbones, and regulating the expression of metabolic circadian genes is essential for dietary sugar tolerance. We highlight the central role of Mondo-Mlx in orchestrating the necessary transcriptional response required for safe glucose utilization, and ultimately, for maintaining metabolic homeostasis.

1. Overview of literature

1.1 A brief history of human carbohydrate consumption

For the majority of omnivorous and herbivorous animals, carbohydrates constitute a substantial part of energy intake. However, the diet of a pre-historic man, practicing mainly a hunter's and a forager's lifestyle, was rich in protein, moderately abundant in fats and fairly scarce in carbohydrates. Protein from game meat covered the bulk of daily energetic needs, supplemented with occasional carbohydrates in the form of consumed berries, roots, and leaves (Yudkin, 1967). It is hypothesized that the implementation of cooking has been one of the break-through events in utilizing carbohydrates. Namely, in its raw form dietary starches are ingested only slowly and partially, whereas cooking starchy foods, such as roots and tubers, greatly increases their speed of ingestion (Hardy et al., 2015) and thus opened up a whole plethora of potential energy sources.

The low intake of carbohydrates and especially starches changed dramatically with the adoption of agriculture during the Neolithic revolution 11000 to 3500 years ago (Bellwood et al., 2007). As a striking contrast to the pre-historic man's diet, the cultivation of cereals introduced starches as one of the main macronutrient groups.

Up to the 18th and 19th century, our ancestors had limited access to simple sugars – a rare treat in the form of berries, fruits, and honey. However, by the 20th , simple sugar consumption had exponentially grown and the sweeteners had become a habitual part of our diets (reviewed in (Tappy and Lê, 2010). It is noteworthy, that during more recent decades, the consumption of simple sugars with diet, mainly in the form of fructose (Fru) has increased by 50% since the 1970s in the USA (Vos et al., 2008). According to the modern (2014) Finnish nutrition recommendations (Valtion ravitsemusneuvottelukunta, 2014) 45-60% daily energetic needs are recommended to be covered by carbohydrates, while less than 10% should be consumed in the form of simple sugars. Thus, the majority of carbohydrates currently recommended to be consumed is in the form of starches, such as grains and vegetables.

1.2 The importance of glucose homeostasis

While the quantity of dietary carbohydrates needed for human nutrition is disputed (Westman, 2002), it is noteworthy that certain tissues in the human body depend heavily on glucose (Glc) as a source of energy. These include the brain, erythrocytes, renal medulla, lens and cornea of the eye, and testes (Ferrier, 2013). For example, the brain has only a limited capacity to store glycogen in astrocytes and neurons (Dinuzzo et al., 2010). Moreover, other potential energy sources such as free fatty acids (FFAs) are impermeable to the blood-brain barrier, and alternatives, such as ketone bodies, are generally present in low levels (Gerich, 2000). Although, after prolonged fasting or carbohydrate withdrawal, ketone bodies will be produced to help cover the energetic needs of the human brain (Owen et al., 1967), the plasma Glc is the preferred source of energy for the central nervous system (CNS). Moreover, even a modest drop in plasma Glc concentration can decrease the capacity of the brain cells to take up Glc. Therefore, the body aims to maintain a steady circulating Glc level of 4-9 mM, both in between and following meals (Gerich, 2000). This constitutes about 4 grams of Glc for a person weighing 70 kg (Wasserman, 2009). Depending on the organismal nutritional status, the human brain can consume 20-60% of the whole body's Glc utilization in a resting state (Berg et al., 2011; Erbsloh et al., 1958; Fonseca-Azevedo and Herculano-Houzel, 2012; Gerich, 1993), while most of the remaining body's energetic needs are fulfilled by FFAs (Boden, 1997).

At the systemic level, Glc metabolism is coordinated by the interplay between the core metabolic hormones: insulin and glucagon, while aberrances in their function often result in metabolic disorders. In the anticipation of fluctuating nutrient availability, a subset of consumed dietary carbohydrates are stored in the liver and skeletal muscles in the form of glycogen to be used in between meals. However, following excessive intake of carbohydrates, another pathway is activated to help clear away Glc from the circulation – lipogenesis, where the unneeded Glc is converted and thus trapped into triacylglycerides (TAGs) (Ameer et al., 2014).

There are three main sources for plasma Glc: dietary carbohydrates, hepatic glycogenolysis (glycogen breakdown) and gluconeogenesis (*de novo* synthesis of Glc from other metabolites; GlcNG). In between meals and upon starvation, blood Glc is maintained steady mostly through rapidly functioning glycogenolysis, and the slightly slower pathway of GlcNG (Ferrier, 2013). However, upon dietary carbohydrate withdrawal, glycogen stores are sufficient to supply the body with Glc for only 10 to 18 h (Ferrier, 2013). Moreover, after 40 h of fasting GlcNG will become the sole source of Glc for the body, while all the able tissues will switch to lipid oxidation for energy utilization in order to spare Glc for basal Glc-dependent processes (Soeters et al., 2012).

1.3 Carbohydrate metabolism

The digestion of carbohydrates starts nearly immediately after their consumption in the mouth saliva, and after passing the stomach, is finalized in the small intestine (Dashty, 2013). Polysaccharides composed of 6 carbon (C) units, like starch, are first hydrolyzed by the digestive enzymes, such as amylases, and broken down to smaller fragments of oligosaccharides, and eventually to maltose in the small intestine. Disaccharides, such as maltose, lactose, and sucrose are similarly first hydrolyzed by their corresponding enzymes (maltase, lactase, and sucrase) into Glc and either Glc, Fru, or galactose, respectively. The latter two can subsequently be converted into Glc in the liver (Dashty, 2013). The 5C sugars, including ribose and xylulose, readily diffuse through the absorptive cells of the intestine. The 6C sugars are primarily imported by transporters that function either through an active or facilitative mechanism. The active entry of hexoses requires Na^+/K^+-coupled pumps, while the facilitated transport into the cell can occur through 14 different glucose transporters (GLUT1-14) (Stringer et al., 2015) (**Figure 1**). The GLUTs display different tissue-specificity, affinity, and capacity. Of these, GLUT4 is present in insulin-sensitive tissues and requires insulin-dependent exocytosis to the plasma membrane (Stringer et al., 2015).

Once Glc has entered the cytoplasm of a cell, it is phosphorylated by hexokinases (HK, isoforms I-IV), and results in the formation of Glc-6-phosphate G6P (Garrett and Grisham, 2016; **Figure 1**). This is the first step of the universal Glc breakdown: glycolysis – a non-oxidative pathway. During this process, one Glc molecule is broken down to two 3C molecules that are metabolized into two molecules of pyruvate. Most of the reactions in this pathway are reversible upon sufficient substrate concentrations, with the exception of three: the HK, the phosphofructokinase 1 and the pyruvate kinase (PK) reactions (Berg et al., 2011).

Next, the formed pyruvate enters the mitochondrion via pyruvate translocase, and upon sufficient oxygen conditions is decarboxylated by the pyruvate dehydrogenase (PDH) complex to form acetyl-coenzyme A (AcCoA) (**Figure 1**). The latter enters the tricarboxylic acid (TCA) or the citric acid cycle, by merging with oxaloacetate (OAA) to form citrate (Berg et al., 2011). The subsequent steps in the pathway (as well as several previous reactions) will result in the reduction of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), that subsequently donate their electrons during oxidative phosphorylation to produce most of the adenosine triphosphate (ATP) molecules derived from Glc oxidation. During hypoxic conditions however, the pyruvate from

glycolysis is converted into lactate in the cytoplasm. This reaction is reversible upon re-oxygenation, whereupon the re-acquired pyruvate is metabolized through PDH (Garrett and Grisham, 2016).

Alternatively, the G6P generated by HKs can enter the pentose phosphate pathway (PPP), where the first and the rate-limiting step is catalyzed by Glc-6-phosphate dehydrogenase (G6PD) (**Figure 1**). This cytoplasmic pathway produces nucleotide precursors in the form of ribose-5-phosphate (R5P) and reductive power through NADPH reduction. The latter is important in lipogenesis, and helps to maintain a proper antioxidant profile, including reducing glutathione (Puri, 2014).

When high cellular energy levels result in excess ATP, the AcCoA formed by PDH accumulates, and slows down the TCA cycle. As an alternative output, the carbon backbones can enter *de novo* lipogenesis, mainly taking place in the liver. For this purpose, the abundant AcCoA exits the mitochondrion through interconversion to citrate (**Figure 1**). Next, cytosolic citrate is processed to form AcCoA and OAA. The re-formed cytoplasmic AcCoA is then carboxylated by the rate-limiting enzyme in lipogenesis – Acetyl-CoA carboxylase (ACC). The subsequent elongation steps are catalyzed by the Fatty acid synthase (FAS) requiring NADPH as the reductive power. As a result of this pathway, the excess sugar is converted into FFA that following esterification with glycerol-3-phosphate (G3P), can be stored as TAGs (Berg et al., 2011).

Likewise, following a meal, part of the excess G6P can also be stored in muscle and liver in the form of glycogen via glycogen synthesis (see **Figure 1**). The liver and muscle of an average person generally deposit 100 and 400 g of glycogen, respectively (Beck-Nielsen, 2012; Wasserman, 2009). Glycogen metabolism is heavily regulated, with the synthesis being stimulated by insulin, while breakdown is triggered by glucagon. In healthy subjects, about 80-90% of Glc taken up by the skeletal muscle is converted into glycogen, whereas in people suffering from type 2 diabetes, the percentage is reduced to 50%, thereby greatly contributing to diminished Glc disposal in these individuals (Højlund et al., 2003; Shulman et al., 1990). A noteworthy study analyzed the flux of Glc through different metabolic pathways: 37% was reported to be oxidized and exhaled as CO_2, while 50% of Glc carbon backbones were converted into TAG (including 12% into glycerol and 88% into fatty acids). Of the remainder, 4.5% was converted into glycogen and 6.5% into lactate (Flatt and Ball, 1964).

1.3.1 Gluconeogenesis

Fasting, low carbohydrate consumption, intense exercise, and a diabetic state can result in a shortage of intracellular carbohydrates. Such cues will activate gluconeogenesis (GlcNG) – an evolutionarily old pathway present in nearly all animals, fungi, plants, and bacteria (Berg et al., 2011; Nelson and Cox, 2012). GlcNG is a mainly cytoplasmic pathway producing Glc from other non-carbohydrate precursors, such as lactate, certain amino acids, and pyruvate. Some of the main tissues exhibiting GlcNG include the liver, kidney, brain, erythrocytes, and testes. During an overnight fast, about 85-90% of the GlcNG takes place in the liver (Ekberg et al., 1999). The outline of GlcNG is similar to that of a reversed glycolysis, with the exception of four unique and highly regulated enzymes that substitute the irreversible reactions in glycolysis: pyruvate carboxylase, phosphoenolpyruvate carboxykinase (PEPCK); Fru-1,6 bisphosphatase (Fbp), and Glc-6-phosphatase (G6Pase) (Nuttall et al., 2008) (see **Figure 1**).

Pyruvate carboxylase catalyzes the first step of the pathway: the conversion of pyruvate to OAA. The reaction consumes an ATP and a CO_2 molecule, and takes place in the mitochondrion. Next, the formed OAA is transported to the cytosol in the form of malate via malate-aspartate shuttle, after which it is converted back to OAA (Dashty, 2013). Cytoplasmic OAA is used as a substrate by the cytoplasmic PEPCK (PEPCK-C). This step consumes a GTP molecule and results in the

decarboxylation of OAA, producing the high-energy intermediate phosphoenolpyruvate (PEP). Since PEPCK catalyzes the rate-limiting reaction of GlcNG, its metabolic role and regulation have received the most attention in this pathway (Nuttall et al., 2008). The next unique enzyme in GlcNG is Fru-1,6-bisphosphatase, which results in the conversion of Fru-1,6-bisphosphate to Fru-6-phosphate. The final regulated step in GlcNG is the conversion of G6P to free Glc by G6Pase. In insects, the last step functions only in specific tissues such as testes, while the final product of GlcNG is mostly trehalose (Becker et al., 1996).

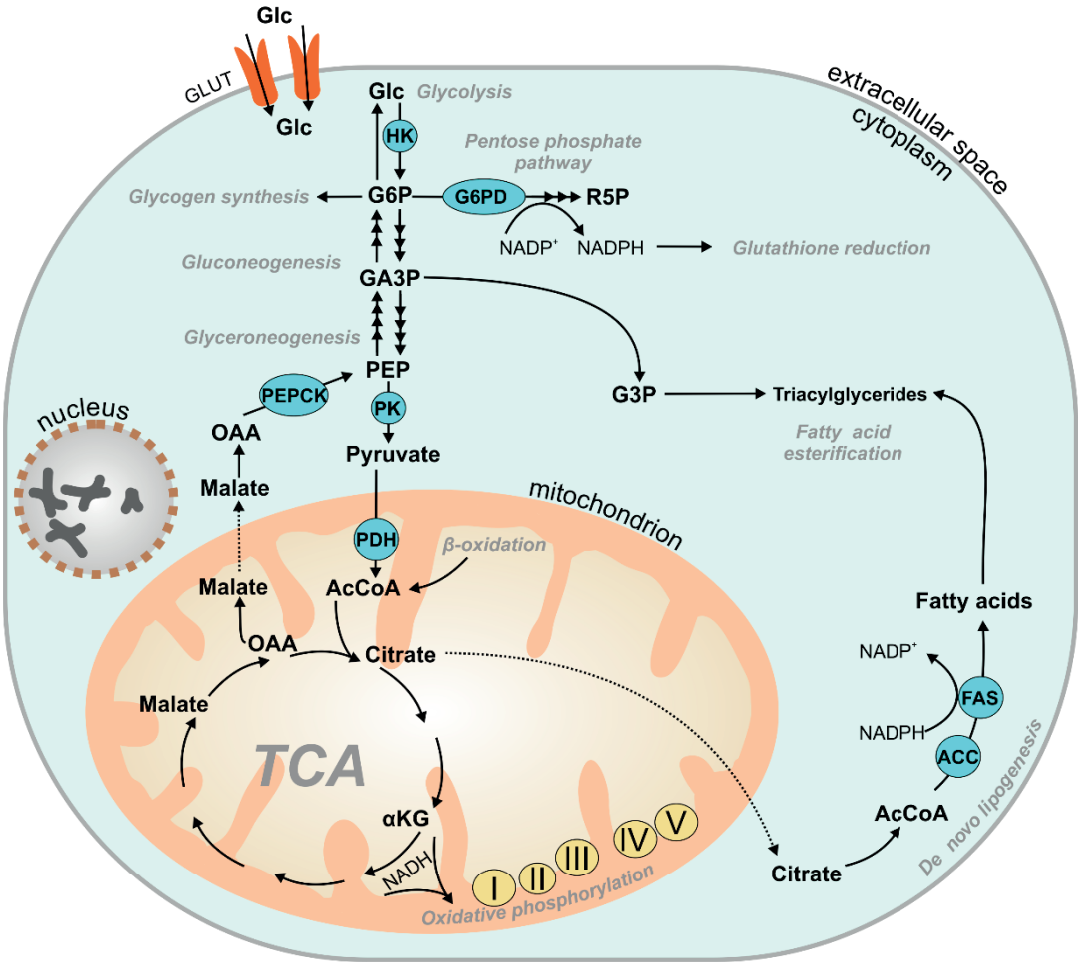

Figure 1: Simplified illustration of main pathways metabolizing Glc. *The Glc taken up into the cell is first phosphorylated by HK (isoforms I-IV), resulting in the formation of G6P. Depending on cellular nutritional status, G6P can either be metabolized further through PPP, glycolysis and TCA, or converted into glycogen. In addition, excess G6P results in the buildup of AcCoA, which can exit the TCA in the form of citrate, and be used as a substrate for lipogenesis. I-V depict the complexes of the respiratory chain. GA3P – glyceraldehyde-3-phosphate. Other abbreviations are explained in the main text.*

1.3.2 Glyceroneogenesis

In 1967, two independent groups described the synthesis of G3P in white adipose tissue (WAT) in what looked to be a shorter version of the GlcNG pathway (Ballard et al., 1967; Reshef et al., 1967). Soon thereafter, this stunted version of GlcNG was named glyceroneogenesis (GlyNG) (Gorin et al., 1969), but its physiological relevance was not appreciated for several decades. GlyNG is known to take place in the liver and also in the adipose tissue, where its function has mostly been studied. Upon fasting, low carbohydrate diet, and prolonged exercise, the body will switch to using FFAs as the main energy source (Wolfe et al., 1990). This requires the hydrolysis of TAGs to FFAs and glycerol by lipases. However, a substantial fraction of the released FFAs will subsequently be re-esterified to re-form TAGs. The re-estrification is more frequent during the post-absorptive state, when up to 70-90% of the released FFAs from lipolysis are esterified back to TAGs (Patel and Kalhan, 1992; Wolfe et al., 1990). While upon starvation, the re-esterification rate can drop down to 10-20% (Frayn and Humphreys, 2012; Wolfe et al., 1990). However, under fasting and low carbohydrate intake conditions, G3P cannot readily be synthesized from Glc. Moreover, the alternative pathway producing G3P through glycerol kinase has very low activity in the WAT (Reshef et al., 2003). Therefore, GlyNG is activated upon such conditions, mostly at the level of *pepck* transcription, to provide G3P for the recycling of excess FFAs that are released from the WAT upon lipolysis (Forest et al., 2003; Reshef et al., 2003). Surprisingly, GlyNG remains as the dominant source of glycerol incorporated into TAGs even with a high sucrose diet (Nye et al., 2008). This indicates that GlyNG is relevant not only during starvation, but also in well-fed conditions.

1.3.3 PEPCK as the rate-limiting enzyme in GlcNG and GlyNG

pepck-c is present as a single copy gene, but the genome of most organisms also contains another *pepck* variant – the mitochondrial PEPCK (*pepck-m*). Although *pepck-m* has been implicated in Glc homeostasis, it has been less studied than its cytoplasmic counterpart, and hence its role remains less understood (Stark and Kibbey, 2014). PEPCK-C functions as the pace-setting enzyme for both GlcNG and GlyNG. In mice, a two-fold increase in *pepck-c* levels leads to elevated hepatic Glc production (Sun et al., 2002), while a 7-fold expression results in hyperglycemia and insulin resistance (Valera et al., 1994). The importance of PEPCK-C in GlyNG, and as a result in lipid homeostasis, is illustrated by studies demonstrating that knock-down of this gene results in altered fat stores in *C. elegans* (Ashrafi et al., 2003). In addition, in mammals, overexpression of PEPCK-C in skeletal muscle increases localized fat content (Ren et al., 2017), while in the adipose tissue it leads to obesity due to increased FFA re-esterification (Franckhauser et al., 2002). As GlcNG contributes to Glc output more in diabetic patients than in control subjects (Wajngot et al., 2001), both mitochondrial and cytoplasmic PEPCK variants have been proposed to contribute to the development of type 2 diabetes (Beale et al., 2007). Moreover, fasting levels of serum glycerol have been found to correlate with type 2 diabetes risk (Mahendran et al., 2013), and could hence be used as a prognostic marker. However, whether this is the consequence of over-active PEPCK and GlyNG, has not yet been studied.

1.4 Consequences of excess Glc intake

There is increasing amount of evidence to demonstrate the direct link between high levels of simple dietary sugars (such as Glc, and especially Fru), and increased incidence of weight gain, type 2 diabetes and metabolic syndrome (reviewed in (Stanhope, 2012; Tappy and Lê, 2010). In addition, more recent results also demonstrate that even the excessive consumption of starches correlates with higher metabolic syndrome risk (Feng et al., 2015). An imbalanced diet high in carbohydrates can lead to hepatic steatosis – the first step in non-alcoholic fatty liver disease (NAFLD). Although it is a relatively benign condition, it can progress to non-alcoholic steatohepatitis (NASH). NASH may

further develop into such life-threatening conditions as cirrhosis or even liver cancer (Yki-Järvinen, 2014). One of the contributors to pathologic sequence is the over-active *de novo* lipogenesis due to chronic excess of simple sugars. Moreover, the aftermaths of excessive sugar consumption, such as chronic hyperglycemia, obesity, and type 2 diabetes, in turn result in a higher risk to develop cancer (Gallagher and LeRoith, 2015). In addition, NAFLD and NASH are serious risk factors for the development of cardiovascular disease (CVD) – one of the main causes of mortality in these patients (Adams et al., 2017; Söderberg et al., 2010).

1.4.1 Hyperglycemia as an inducer of oxidative stress

About 3% of Glc is present in solution in its linear aldehyde form, making it a reducing sugar. Consequently, it can readily react, in a non-enzymatic manner, with amino-groups present in proteins leading to their inadvertent glycation. This process may escalate and result in the following protein alterations: cross-linking, aberrant conformation, and changed flexibility – all due to the formation of advanced glycation end-products (AGEs) (Wautier and Schmidt, 2004). As a result of chronic hyperglycemia, AGEs become more prevalent. AGEs, however, are potent inducers of reactive oxygen species (ROS), and inflammation (Giacco and Brownlee, 2010). In fact, the level of hemoglobin glycation from a blood test is routinely used to assess the extent of hyperglycemic damage.

In addition, hyperglycemia and consequently increased intracellular Glc levels have been demonstrated to lead to elevated ROS generation by mitochondria (Du et al., 2001). Another contributor to increased oxidative stress upon hyperglycemia is the activation of the polyol pathway. Upon high intracellular Glc levels, alternative metabolic pathways, such as the polyol pathway, become activated to aid in Glc clearance. As a result of hyperglycemia, up to 30% of Glc can be channeled into the polyol pathway (Cheng and González, 1986). The first enzyme in the pathway, aldose reductase, converts Glc into an inactive alcohol sorbitol by consuming NAPDH. Upon chronically high Glc levels, this step consumes significant amounts of NADPH. The latter, however, is the central cellular reductive power required for reducing the critical antioxidant glutathione disulfide (GSSG) into glutathione (GSH). As sorbitol is osmotically active, and high levels of it can damage cells, additional steps are important to metabolize it further. The second step in the polyol pathway is catalyzed by sorbitol dehydrogenase, and converts sorbitol into Fru by reducing NADH. Both steps of the pathway have been demonstrated to contribute to hyperglycemia-induced oxidative stress in the lens and nerves (Chung et al., 2003). In sum, Glc stress leads to elevated levels of ROS, and decreased levels of a vital antioxidant. As a consequence, high Glc will expose the body to increased levels of oxidative stress and also hamper its ability to alleviate it.

1.5 Circadian rhythm and metabolic homeostasis

The phrase "*circa diem"* (in Latin) translates into "about a day". Accordingly, the term circadian rhythm is used to characterize the oscillations observed in organismal sleep-wake cycle, behavior, and feeding that occur in a distinctive daily pattern. As the length of a day can differ substantially due to seasonal changes, the intrinsic ability of the circadian clock is to ensure flexibility and adaption to environmental cues, while at the same time preserving inherent cycling upon loss of such stimuli (Edery, 2000).

In vertebrates, the so-called central and the peripheral clocks can be distinguished that are nevertheless heavily interconnected. The central clock located in the suprachiasmatic nuclei in the mammalian brain functions as a pacemaker to relay the information of external light to peripheral clocks, to ensure in-phase activity with the environment (Ray and Reddy, 2016). In the fruit fly, where many of the

core clock components were first characterized, only the peripheral clocks exist, as the information from external light can be passed on not solely through the optic nerve, but virtually by the whole body, hence making the synchronization of the peripheral clocks by the central light-regulated clock unneeded (Hardin, 2011).

Suggestive evidence for a role of circadian rhythm in the regulation of metabolic homeostasis has been present for decades, as a clear correlation between shift workers and increased risk for metabolic disorders has been documented (De Bacquer et al., 2009). Moreover, it has been suggested that in addition to external light, the circadian clocks are sensitive to eating and could thereby be entrained by food (Stokkan et al., 2001). For example, a high calorie diet rich in fats has been shown to disrupt the circadian rhythm in mice (Kohsaka et al., 2007). Curiously, the expression of many metabolic genes is known to oscillate throughout the day, apparently in the anticipation of nutrient consumption or overnight fasting (Vollmers et al., 2009). This may prove to be the underlying explanation of why shift workers experience more metabolic disorders, as their innate circadian rhythms contradict anticipated feeding patterns. Hence, signaling pathways facilitating information about nutritional status and clock cycling are expected to contribute to metabolic homeostasis maintenance.

Although transcription-independent processes, such as phosphorylation and redox changes have been discovered to have a role in circadian rhythm (Kil et al., 2015; Robles et al., 2017), at the heart of the molecular clock lies a transcriptional loop orchestrated by autoregulation and feedback mechanisms. The mammalian and the fruit fly circadian clocks have several homologous components. For the purpose of this thesis, the *Drosophila* molecular clock and its main constituent genes are briefly introduced.

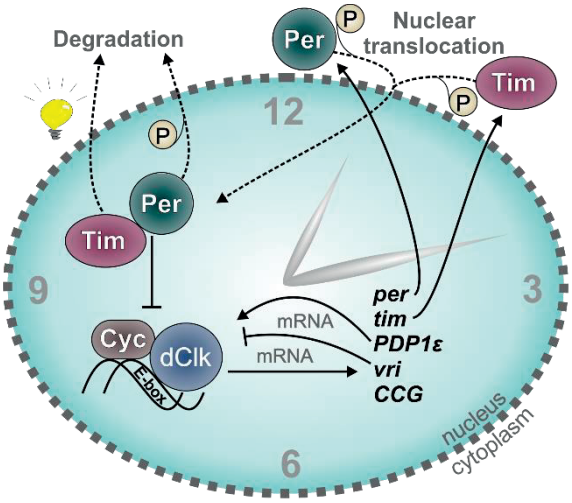

Figure 2: Overview of central circadian regulators and their time-dynamic processes in Drosophila. *Circadian gene oscillations are ensured by fluctuating light conditions, as well as delays in transcription and phosphorylation-triggered processes. The molecular clock in the fly is composed of TFs Cyc and dClk, which form dimers on clock-controlled gene (CCG) promoters. In response to their transcriptional activity, Per and Tim proteins accumulate in the cytoplasm, before they are phosphorylated. They subsequently enter the nucleus, where the Tim-Per dimer will directly inhibit the activity of Cyc-dClk.*

The circadian feedback loop in *Drosophila* is composed of transcription factors (TFs) dClock (dClk; CLOCK in mammals) and Cycle (Cyc; BMAL1 in mammals) that function as a dimer to bind E-box elements present in their target gene promoters. As a result, clock-controlled genes (CCG), including *period* (*per*), *timeless* (*tim*), *PAR domain protein 1 ε* (*PDP1ε*), and *vrille* (*vri*) are expressed (Hardin, 2011; **Figure 2**). Per and Tim proteins accumulate in the cytoplasm, while they undergo phosphorylation to enter the nucleus, where they function as a complex. Once sufficient amount of Per-Tim have accumulated in the nucleus,

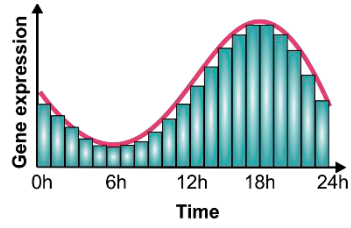

Figure 3: Gene expression oscillation of a CCG, depending on the time of day

they promote dClk phosphorylation, leading to repression of the dClk-Cyc dimer activity, and hence their own expression. Over time, the Per protein accumulates a critical number of phosphorylations in the nucleus, demarking it to be degraded in the proteasome. In parallel, as Tim is sensitive to light-triggered degradation, its protein levels oscillate according to the time of day (Peschel and Helfrich-Förster, 2011). In addition, the CCG PDP1ε promotes the transcription of *dClk,* while Vri represses it (**Figure 2**). This intertwined transcriptional loop with periodic exposure to light, delays in protein translation, (de-) phosphorylation, and degradation is responsible for the cycling of CCGs that oscillate during 24 h (for example, see **Figure 3**).

1.5 Fruit fly as a model to study metabolism

Drosophila melanogaster has been implemented in genetic research for over a century (Morgan, 1910). The advantages of this Dipteran model organism include low cost, compact genome, fast life cycle, and broad selection of available genetic tools. More importantly, a majority of human disease-causing genes have an identified homolog in *Drosophila* (Chien et al., 2002). In addition, many duplicated genes in humans exist as a single copy in the fly, considerably simplifying the aspect of redundancy. Many critical pathway components have first been uncovered in the fly, leading to the subsequent homology-based identification in mammals. The first circadian gene *period* and its mutant were first described in *Drosophila* (Konopka and Benzer, 1971), opening up a whole new field with studies leading to the identification of the underlying circuit of circadian control.

Balancer chromosomes harboring extensive inversions and genetic markers have been generated to prevent recombination, and to aid in lethal stock maintenance (Casso et al., 1999). Moreover, a selection of transposable element insertion mutant stocks are commercially available. These lines may present aberrant expression of a given gene of interest, or alternatively, can be used for imprecise excision-mediated deletion mutant generation (Ryder and Russell, 2003). The genome editing approach based on CRISPR/Cas9 has successfully been implemented in the fly, thereby greatly increasing the number of possible genetic manipulations (Beumer and Carroll, 2014). The adaption of the upstream activating sequence (UAS) – galactose gene transcription factor 4 (GAL4) system in *Drosophila* has offered a genetic advantage to temporally and tissue-specifically modulate gene

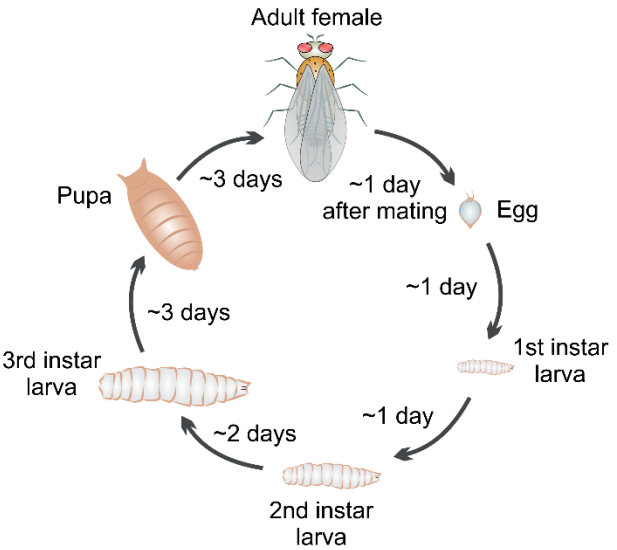

Figure 4: The outline of* Drosophila *life cycle. *At 25 °C, it takes an average of 10 days for a fly to develop from an egg to adult. Females are sexually mature after emerging, while males need about 8 h to be able to mate. The eggs are laid approximately a day after mating. The larval phase is divided into three instars. Molting occurs between the 1st and 2nd, as well as before the 3rd instar stage. The final larval instar stage is followed by a "wandering" phase, and subsequent pupariation. This marks the start of metamorphosis, which takes place in the pupal case. After about three days, the larval tissues have been reorganized into adult counterparts, and the fly emerges from the pupal case in a process termed eclosion.*

expression (McGuire et al., 2003). Currently, several stock centers exist to provide a near complete coverage for RNA interference (RNAi) constructs (Dietzl et al., 2007; Kaya-Çopur and Schnorrer, 2016; Perkins et al., 2015). Due to commercial services, the generation of transgenic flies has become more efficient and faster, with an average wait of about 2 months for balanced lines.

The life cycle of the fly is short, yet can be modified according to experimental needs by adjusting the temperature between 18 and 29° C. At the optimal temperature of 25° C, it takes approximately 10 days for an egg to develop into an adult (**Figure 4**). During the three larval instars, the animal will feed continuously until it leaves the food for a "wandering" stage preceding the pupariation. The metamorphosis lasts several days and relies on pre-existing energy storage. The end of the pupal phase is called the pharate adult stage, where the animal will obtain its final morphology inside the pupal case. This is followed by eclosion – the emergence of adult flies. In contrast to larvae, adult flies are intermittent feeders, and display circadian clock-dictated bimodal activity in their rhythmic behavior (Peschel and Helfrich-Förster, 2011).

Due to a high level of conservation in signaling pathways, *Drosophila* is becoming an increasingly relevant model organism to study growth, and nutritional homeostasis. For example, the mechanism of insulin signaling has remained conserved throughout evolution, and thereby the outline is comparable in flies and mammals. The *Drosophila* genome encodes for 8 insulin-like peptides (dILPs 1-8), and two receptors (*Drosophila* insulin receptor, and Leucine-rich repeat-containing G protein-coupled receptor 3). Notably, only dILPs 2, 3, and 5 are produced and released by the neurosecretory cells (insulin producing cells) situated in the brain (Nässel and Vanden Broeck, 2016).

Drosophila melanogaster, as a food generalist, can be reared on a variety of different diets. This enables manipulation of nutrient levels to investigate their different metabolic aspects. Moreover, numerous well-established methods have been developed to assess metabolic profiles in the fly (Tennessen et al., 2014). The fly intestinal system shows several similarities to mammalian counterparts, both in terms of structure, tissue, and physiology (Apidianakis and Rahme, 2011). Similar to mammals, inter-organ communication is crucial for fly metabolic homeostasis (Ugur et al., 2016). In flies, the equivalents of the adipose tissue and liver are fat body and oenocytes, while Malpighian or renal tubules function as kidneys. The fat body is central for coordinating metabolic sensing, and is largely composed of lipid-containing cells, but also stores glycogen. In the larval stage, the fat body is mainly present as an elongated cluster of cells in the abdomen. During metamorphosis, the fat body is broken down through autophagy and regenerated anew, resulting in the redistribution of the tissue. Consequently, in adults, part of the fat body is located around the brain, while the majority remains in the abdomen (Hwangbo et al., 2004). The uptake of simple sugars from the food occurs passively through the digestive tract into the hemolymph, followed by channeling to the fat body. Once there, Glc is converted into trehalose – a Glc-dimer and the most abundant circulating carbohydrate in insects (Graham and Pick, 2017). For comparison, there is approximately 100-times more trehalose in the fly than Glc.

1.6 Glc-elicited transcriptional response

1.6.1 Carbohydrate response element

Promoter components responding to cellular Glc levels were first investigated more thoroughly for the *L-PK* and *S14* genes (Shih and Towle, 1994, 1992; Shih et al., 1995; Thompson and Towle, 1991). Through such pioneering studies by the Towle laboratory, the term carbohydrate-response element (ChoRE) was coined. Further mapping revealed a consensus sequence in the form of two Enhancer box or E-box elements (CACGTG) spaced by 5 nucleotides: CACGTGnnnnnCACGTG. It is

noteworthy that mutations changing the spacer region length reduce (6 base pair [bp]) or abolish (4 bp) the Glc-responsiveness (Shih et al., 1995). Since then, ChoREs have been identified in the promoters of many lipogenic and glycolytic genes (Ma et al., 2006; Mattila et al., 2015; Rufo et al., 2001; Shih et al., 1995). Further experiments have revealed tolerable aberrances from the perfect ChoRE sequence, while still maintaining carbohydrate responsiveness (Kaytor et al., 1997; Ma et al., 2006; Mattila et al., 2015). In addition, the relative orientation of the two E-boxes in a non-perfect ChoRE may be of critical importance, as their exchanging can result in dramatically different Glc-responsiveness (Kaytor et al., 1997).

E-boxes are well known consensus motives for basic helix-loop-helix (bHLH) leucine zipper (LZ) TFs that bind the DNA element as obligate dimers or tetramers (Prendergast and Ziff, 1991). Given that an ideal E-box element is palindromic, one of the protein monomers attaches to the CAC triplet from one strand, while the other to the CAC from the other strand (Massari and Murre, 2000). In 2001, the transcription factor consequently named carbohydrate response element binding protein (ChREBP) was shown to bind these elements in a sugar-dependent manner (Yamashita et al., 2001). By using affinity chromatography, ChREBP was purified as one of the proteins binding the ChoRE in the *PK* gene promoter. Moreover, in response to feeding rats with a high carbohydrate diet, the DNA-binding of ChREBP increased in liver cells, and correlated with ChoRE-containing promoter reporter activity (Yamashita et al., 2001). For a representation of ChoRE-motif consensus sequence bound by ChREBP, see **Figure 5**.

Figure 5: A representation of a ChoRE motif. *This illustration is based on ChreBP-dependent chromatin immunoprecipitation (ChIP), followed by sequencing experiments. Modified from Poungvarin et al., 2015.*

1.6.2 The sugar sensing TF complexes MondoA Mlx and ChREBP Mlx

Since their discovery almost two decades ago, the Mondo family of bHLH LZ TFs has quickly been established as key-mediators of sugar-dependent transcriptional control (Havula and Hietakangas, 2012). In mammals, two paralogous Mondo proteins exist: MondoA, and MondoB - better known as ChREBP. In order to enter the nucleus and regulate the expression of Glc-responsive genes, both Mondo proteins require dimerization with another bHLH LZ TF: Mad-like protein X (Mlx) (Bellwood et al., 2007; Eilers et al., 2002; Peterson et al., 2010; Stoltzman et al., 2008).

Mlx belongs to the Myc/Max/Mad TF family, and was first discovered from a yeast-two hybrid assay in a search for Mad-family-interacting proteins (Mlx) (Billin et al., 1999). *mlx* is a highly expressed gene displaying a ubiquitous tissue-level distribution pattern (Billin et al., 1999; Meroni et al., 2000). Three alternatively spliced variants of Mlx have been characterized (α, β, γ) that do not display an obvious tissue specificity (Meroni et al., 2000). The Mlx protein has a low molecular weight of 30 kilo-Dalton (kDa), and due to its long half-life (6-8 h), displays relative stability.

MondoA was first identified through a yeast two-hybrid screen for new Mlx-interacting partners (Billin et al., 2000), and therefore, in databases its gene is mainly referred to as *mlx interacting protein* (*mlxip*). The *mlxip* gene expression is detected in most adult tissues, while skeletal muscle displays the highest levels (Billin et al., 2000). Due to the presence of a cytoplasmic localization domain in MondoA, MondoA-Mlx complex displays a mainly cytoplasmic localization pattern (Billin et al., 2000). In fact, in many cell types, MondoA-Mlx dynamically shuttle between the nucleus and the outer mitochondrial membrane (Sans et al., 2006). Such behavior has been suggested to facilitate

communication between the mitochondria and the nucleus, thereby helping to maintain cellular energy homeostasis.

ChREBP/MondoB was discovered based on database comparisons to MondoA, and the gene was subsequently named as *mlx-interacting protein like* (*mlxipl*) (Billin et al., 2000). The ChREBP-Mlx interaction was independently confirmed though a yeast two-hybrid screen (Stoeckman et al., 2004). In contrast to Mlx, the ChREBP protein displays a relatively shorter half-life of 30 min (Cairo et al., 2001). As the ChREBP protein is larger than the cut-off value of 45 kDa for passive transport to the nucleus (Jans and Hübner, 1996), it requires a nuclear localization signal to enter, or an interacting protein possessing one. Motif analysis has revealed the presence of a bipartite nuclear localization signal (NLS) that shows very high level conservation (de Luis et al., 2000). Interestingly, the longer Mlx, rather than the ChREBP protein loop domain, determines the response to Glc by interacting with ChoRE (Ma et al., 2006, 2007).

Through genome-wide association studies in humans numerous polymorphisms in both *mlxip* and *mlxipl* have been characterized. Curiously, several *mlxipl* single nucleotide polymorphisms have been associated with metabolic alterations, such as hypertriglyceridemia, and an increased risk to develop NAFLD and metabolic syndrome (reviewed in Richards et al., 2017). Moreover, the majority of people suffering from Williams-Beuren syndrome, a disease caused by a microdeletion covering 26-28 genes, inlcuding *mlxipl*, display impaired Glc tolerance (Pober, 2010).

1.6.2.1 Structure of Mlx and the Mondo-proteins

The C-terminal domains of Mlx and both Mondo proteins display clear similarities, potentially pointing to evolutionary ancestry (Billin et al., 2000). All three TFs possess a dimerization and cytoplasmic localization domain (DCD) (**Figure 6**). The interaction with DNA is facilitated by the LZ and bHLH domains.

MondoA and ChREBP are big multi-domain proteins with shared structural characteristics. The N-terminal region contains the evolutionarily conserved Glc-sensing module (GSM), composed of low-Glc inhibitory domain (LID), and Glc-response activation conserved element (GRACE) (Havula and Hietakangas, 2012) (**Figure 6**). Strikingly, a 1-196 aa deletion variant of ChREBP results in a constitutively active transcriptional regulator, independent of Glc levels and loss of NLS. This phenomenon has been explained by the intramolecular interaction proposed to take place between LID and GRACE. More specifically, LID is believed to repress GRACE activity under low Glc conditions through a hinge-like conformational mechanism (Davies et al., 2010; Ma et al., 2006). Upon increased Glc conditions, however, an allosteric conformational change is proposed to occur, leading to de-repression of GRACE by LID, resulting in the Glc-dependent activation of the transcription factor. Based on modelling studies, a highly conserved G6P-recognition motif has been predicted to reside in the GRACE domain of Mondo proteins (McFerrin and Atchley, 2012). G6P binding by Mondo-proteins has been suggested to result in an allosteric conformational change that leads to an open complex where LID is unable to hinder GRACE activity. Consequently, the interplay between LID and GRACE functions as a Glc-sensing switch, by being sensitive to the central Glc metabolite – G6P. The extent of GSM conservation is further highlighted by the fact that Glc-responsiveness was retained upon an exchange between the two Mondo proteins, and even by using the same domain from the *Drosophila* orthologue (Ma et al., 2006).

In 2012, a shorter, alternatively spliced variant of ChREBP was identified from adipose tissue, and named ChREBPβ, as opposed to the full length protein - ChREBPα (Herman et al., 2012). The alternative promoter of ChREBPβ contains a ChoRE element in the alternative first exon, and thus its expression depends on ChREBPα. As the first exon of ChREBPα containing the translation start site

is skipped, the ChREBPβ protein coding sequence starts from exon 4, resulting in 177 aa shorter isoform. Consequently, ChREBPβ lack most of the LID, while retaining an intact GRACE (**Figure 6**). As a result, ChREBPβ displays similar transcriptional activity both under low and high Glc conditions, and is approximately 20 times more potent than the full length isoform (Herman et al., 2012) – a behavior more similar to the constitutively active ChREBP mutant lacking the first 196 aa. Therefore, the activation of ChREBPα by high Glc will trigger the expression of a more potent isoform that further amplifies the response to sugar. However, due to the lack of most of the N-terminal domain, and hence loss of stabilizing protein interactions, ChREBPβ may have an even shorter half-life than 30 minutes.

Close analysis of the Mondo protein sequences between different species has revealed the presence of five Mondo-conserved regions (MCRI-V) in the N-terminus (Billin et al., 2000; Davies et al., 2008; Eilers et al., 2002) (**Figure 6**). Specific roles and interaction partners for some of the MCRs have been

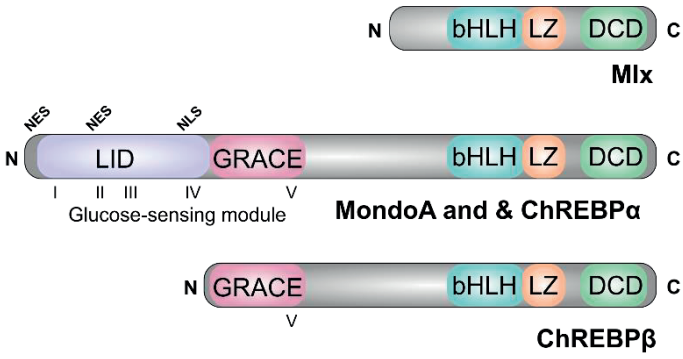

Figure 6: The structural outline of Mlx and Mondo proteins. *All TFs have a similar C-terminal domain. The Mondo-conserved regions are marked with roman numbers. ChREBPβ is an alternatively spliced variant that lacks the LID domain present in MondoA and ChREBPα. NES – nuclear export signal. Proteins not drawn to scale. Modified from Filhoulaud et al., 2013 and Havula and Hietakangas, 2012.*

characterized. For example, MCRII functions as a CRM1 dependent nuclear export signal (NES), while MCRIII contains a 14-3-3 biding site – both interactions are important for cytoplasmic localization (Eilers et al., 2002). In addition, MCR IV had been demonstrated to function as an NLS. Notably, the spacing between MCR II, III, and IV in Mondo proteins is identical between metazoans, potentially suggesting the presence of a functional unit. However, the spacing of neighboring regions differs between MondoA and ChREBP. As a result, mutations of individual MCRs have different outcomes on MondoA and ChREBP activity (Peterson et al., 2010).

1.6.2.2 Evolution and conservation of Mlx and the Mondo proteins

Sequences resembling Mlx and Mondo have been identified from even some of the smallest genomes of some of the simplest known animals, such as *Trichoplax adherens* (McFerrin and Atchley, 2011; Srivastava et al., 2008). This underlines the ancient ancestry of Mlx and Mondo proteins that seemingly predates the origin of animals around 500 million years ago (McFerrin and Atchley, 2011). Moreover, sequences resembling the GSM region of the Mondo proteins seem to be metazoan-specific, possibly indicating that these TFs evolved to coordinate communication between organs for whole body homeostasis maintenance (Richards et al., 2017).

Evolutionary comparison studies have revealed that MondoA and ChREBP are the divergence products of a duplication event that occurred either prior or during early vertebrate evolution (Dehal and Boore, 2005; McFerrin and Atchley, 2011; Singh and Irwin, 2016). This is illustrated by the

presence of *Mlxip* and *Mlxipl* genes in nearly all vertebrates, with an apparent degree of overlapping synteny. In accordance with this, the genome of invertebrate animals, including *C. elegans* and *D. melanogaster*, encodes for a single Mondo protein. Despite multiple genome duplication events at the time of early vertebrate evolution, all examined species among the phylum Chordata harbor only a single gene for *mlx* (McFerrin and Atchley, 2011). This may indicate a high natural selection for the regulation of single functional copies and coordinated interactions (McFerrin and Atchley, 2011; Singh and Irwin, 2016).

Comparisons of the two Mondo proteins in mammals have revealed a stronger selection pressure on the MondoA protein, in comparison to ChREBP, in all regions except the DCD. This may indicate a more ancient and evolutionarily a more critical function for MondoA (Singh and Irwin, 2016). In addition, in contrast to fish, tetrapods possess an alternative, auto-regulatory isoform of ChREBP (ChREBP-β), demonstrating the development of a more recent control (Singh and Irwin, 2016).

1.6.2.3. Regulation of MondoA and ChREBP

1.6.2.3.1 Glc-sensing through intermediary metabolites

Due to their high level of similarity, the regulation of MondoA and ChREBP activity displays several common features. However, many differences have also been reported. Despite extensive studies, the exact mechanism of how MondoA and ChREBP are activated in response to sugars, remains poorly understood. Three carbohydrate metabolism intermediates have been implicated to facilitate the information about intracellular Glc levels to modulate MondoA and ChREBP activity. These include xylulose-5-phosphate (Xu5P), G6P, and Fru-2,6 bisphosphate (F2,6P).

Based on experimental evidence, Xu5P was demonstrated to activate the necessary PP2A-family phosphatases, catalyzing the removal of the critical phosho-groups from AMP-activated kinase (AMPK) and cyclic adenosine monophosphate (cAMP)-dependent kinase (PKA) target sites (S196, S568 and T666), and thus resulting in ChREBP nuclear translocation and increased DNA-binding (Kabashima et al., 2003) (**Figure 7**). However, nuclear localization has been shown to be insufficient for MondoA-Mlx transcriptional activation, as it does not lead to increased promoter occupancy (Peterson et al., 2010). Therefore, G6P has been proposed as the more dominant metabolite in facilitating ChREBP and MondoA activation (Li et al., 2010; Peterson et al., 2010; Stoltzman et al., 2008) (**Figure 7**). HK - enzymes generating G6P, were demonstrated to be crucial for MondoA activation (Stoltzman et al., 2008). G6P has also been demonstrated to be sufficient for ChREBP-Mlx nuclear translocation and activation (Dentin et al., 2012; Li et al., 2010). Moreover, a putative G6P-recognition motif has been predicted to reside in the GRACE domains of Mondo proteins (McFerrin and Atchley, 2011). However, experimental evidence of direct G6P binding by Mondo proteins is currently lacking. In addition to G6P, another glycolytic intermediate has been linked to activating Mondo proteins: F2,6P (**Figure 7**). This metabolite is an allosteric activator of glycolysis, and inhibitor of GlcNG (Hue and Rider, 1987). Notably, F2,6P has been demonstrated to facilitate ChREBPs sugar-induced translocation to the nucleus in liver cells. Moreover, suppressing F2,6P production downstream of G6P, decreases target gene promoter occupancy by ChREBP-Mlx in hepatocytes (Arden et al., 2012). Similarly, the importance of F2,6P in hepatocytes has also been demonstrated for MondoA-Mlx recruitment to target gene promoters (Petrie et al., 2013). This may indicate the presence of conserved regions between MondoA and ChREBP that facilitate F2,6P sensing. In sum, it is possible that several metabolites delegate the Glc-induced activation of Mondo proteins. Currently, the most convincing data point to G6P being the primary link between increased intracellular Glc levels and MondoA/ChREBP-Mlx activity.

1.6.2.3.2 Inhibitors of MondoA/ChREBP-Mlx activity

Numerous reports exist to point to an intricate regulation of MondoA/ChREBP-Mlx by different pathways. However, successive studies have made certain discrepancies in published results apparent. Thus, further research is needed to help understand the underlying mechanisms of MondoA/ChREBP-Mlx regulation.

To date, several key metabolic kinases have been demonstrated to phosphorylate ChREBP. For example, there are at least three PKA phosphorylation consensus sites in the ChREBP protein, of which Ser196 and Thr666 were confirmed to be critical in regulating nuclear localization and DNA-binding ability (Kawaguchi et al., 2001; Yamashita et al., 2001). *In vitro* experiments demonstrated that PKA-dependent phosphorylation inhibits the ChREBP DNA-binding ability by 90%, which can partially be rescued by the addition of the phosphatase PP2A (Yamashita et al., 2001) (**Figure 7**). However, other research groups have observed only modest effects on nuclear localization upon mutating these phospho-sites (Eilers et al., 2002; Ma et al., 2006). Moreover, as these supposed Glc-sensitive phospho-sites are not conserved in MondoA or in the *Drosophila* Mondo protein (Billin and Ayer, 2006; Ma et al., 2005), additional mechanisms than nuclear localization determinants relaying Glc-responsiveness are believed to exist.

As carbohydrate metabolism lies at the center of cellular metabolic homeostasis, reciprocal regulation through organismal nutritional status would be expected to occur. In agreement with this, metabolites increased upon starvation, such as acetate and certain ketone bodies, have been found to inhibit ChREBP-Mlx activity (Kawaguchi et al., 2002; Nakagawa et al., 2013). In addition, certain saturated and unsaturated fatty acids also potently reduce ChREBP-Mlx nuclear translocation (Dentin et al., 2012; Kawaguchi et al., 2002; Sato et al., 2016). The underlying mechanism of this inhibition depends on increased AMP levels. Due to allosteric binding, AMP promotes the interaction between ChREBP and 14-3-3, and subsequent cytoplasmic sequestration (Sato et al., 2016) (**Figure 7**).

In addition, in response to elevated AMP levels, the AMPK has been proposed to inhibit ChREBP function. Ser568 in ChREBP was mapped as the kinase target site that upon phosphorylation leads to the inactivation of DNA-binding ability (Kawaguchi et al., 2002) (**Figure 7**). However, consecutive studies revealed that the regulatory understanding based on PKA and AMPK regulations is incomplete, as additional ChREBP-Mlx activating steps are required for full high-Glc response (Stoeckman et al., 2004).

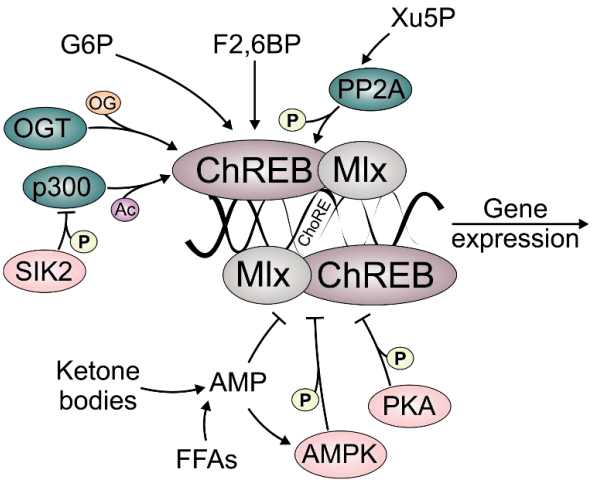

Figure 7: Examples of regulatory mechanisms affecting Mondo proteins, illustrated on ChREBP. *AMP has been demonstrated and G6P is proposed to regulate the TF complex activity through allosteric binding. In addition, several post-translational modifications affecting nuclear localization, stability and DNA-binding have been reported. See main text for details.*

1.6.2.3.3 Post-translational activation of ChREBP

In addition to phosphorylation, ChREBP is subjected to other types of post-translational modifications that affect its activity. For example, ChREBP is reported to be acetylated by the histone acetyltransferase co-activator p300 (Bricambert et al., 2010) (**Figure 7**). The acetylation of Lys672, that is located in the DNA-binding domain, increases ChREBP-Mlx DNA-binding, and thereby promotes target gene expression. Notably, however, p300 is subjected to Salt-inducible kinase (SIK) 2-dependent inhibitory phosphorylation (Bricambert et al., 2010) (**Figure 7**). Therefore, SIK2 silencing in liver retains p300 activity, and promotes increased ChREBP-Mlx target gene expression, resulting in hepatic steatosis. As SIK2 activity has been demonstrated to be influenced by the organismal nutritional status (Du et al., 2008), it likely functions to coordinate metabolic needs by modulating ChREBP-Mlx activity through p300.

Secondly, ChREBP interacts with and is targeted by the O-linked N-acetyl-glucosamine (GlcNAc) transferase enzyme (OGT). Moreover, the GlcNAc-modifed ChREBP displays higher protein stability and increased transcriptional activity towards its target genes (Guinez et al., 2011) (**Figure 7**). As GlcNac synthesis is limited to the availability of its substrates, Glc and Fru, this may add an additional hexose-regulated mechanism layer to ChREBP function. However, it has not yet been investigated whether MondoA is similarly targeted by OGT.

In sum, numerous metabolites, as well as signaling pathways, have been demonstrated to regulate the MondoA/ChREBP-Mlx complex (**Figure 7**). The large number of regulators may indicate a central role at the metabolic nexus for ChREBP/MondoA-Mlx. However, much detailed information about these regulatory mechanisms remains unclear. Moreover, it is highly likely that other, as yet uncharacterized, regulatory processes exist to fine-tune ChREBP/MondoA-Mlx activity.

1.6.2.4. Target genes and physiological output

1.6.2.4.1. Interplay between the two Mondo proteins

ChREBP expression is most abundant in the liver and adipose tissue, while MondoA in its ubiquitous pattern shows the highest levels in the skeletal muscle. Consequently, it has been thought that the paralogs have diverged to regulate sugar-responsive gene expression in their respective tissues. However, experimental evidence suggests that MondoA has specific functions also in the liver (Ahn et al., 2016; Petrie et al., 2013), while ChREBP-dependent gene expression regulation is important in metabolic type determination in muscle fibers (Hanke et al., 2011). Despite their vast structural similarity and responsiveness to Glc, very few shared direct target genes have been identified to date. In addition to tissue-specific differences, this may be partially explained due to recognized promoter elements. ChREBP-Mlx have been demonstrated to bind the ChoRE elements, composed of two E-boxes, spaced with 5 critical nucleotides. The two E-boxes are each recognized and bound by a heterodimer, resulting in the formation of a ChREBP-Mlx tetramer that is sensitive to the spacer region length. Such multimeric ChREBP-Mlx complexes have experimentally been demonstrated to form at certain target gene promoters. Although ChREBP-Mlx dimers are able to bind to single E-box elements, albeit more weakly, such reporters fail to respond to Glc (Ma et al., 2007). Simultaneous binding of two E-boxes instead of one is believed to stabilize the interaction of ChREBP-Mlx with more degenerate and less perfect elements. In contrast, several identified MondoA-Mlx targets have one or several consecutive E-box elements in their promoters, and not necessarily a full ChoRE element (Sans et al., 2006). The exceptions here are the ChoRE elements in the *thioredoxin interacting protein* (*TXNIP*) and *arrestin domain containing protein 4* (*ARRDC4)* promoters (Parmenter et al., 2014; Stoltzman et al., 2008) – two paralogous genes with overlapping function in inhibiting Glc uptake. The former gene has also been identified as a target of ChREBP (Cha-Molstad et al., 2009).

Based on functional conservation, the paralog ARRDC4 may also be a target of ChREBP, but this has not yet been experimentally reported (Peterson and Ayer, 2011). It is thus possible that a single E-box element is sufficient for MondoA-Mlx mediated transcriptional response, while TXNIP (and, possibly ADDRC4) promoter is a hybrid version capable of sustaining regulation by both ChREBP and MondoA.

1.6.2.4.2. MondoA-Mlx regulate glycolysis and Glc uptake

Experiments conducted in myoblast and kidney cells have helped uncover a dual role for the MondoA-Mlx complex in Glc metabolism. Particularly, through its target genes, MondoA has been found to promote the rate of glycolysis (Sans et al., 2006), while simultaneously inhibiting further Glc uptake (Stoltzman et al., 2008). Several genes related to glycolysis, including *HKII* (see **Table 1**), have been identified as direct targets of MondoA-Mlx in myoblasts. However, other reports have contradicted the proposed correlation between increased MondoA activity and higher glycolytic rate. For example, it has been reported that the glycolytic rate in skeletal muscles of mice lacking MondoA was in fact increased, while Mlx-dependent glycolytic gene expression regulation was not apparent in muscle precursor cells (Hunt et al., 2015; Imamura et al., 2014).

The MondoA-dependent inhibition of Glc uptake occurs through direct Glc-dependent upregulation of *TXNIP* and *ARRDC4* (Parmenter et al., 2014; Stoltzman et al., 2008, 2011). TXNIP and ARRDC4 are paralogs belonging to the same protein family (Patwari et al., 2009). The fact that these genes inhibit further Glc uptake, illustrates the presence of a feedback loop through modulation of MondoA-Mlx activity. In addition, inhibiting MondoA activity in liver and skeletal muscle has been linked to lower TAG synthesis due to reduced lipogenic gene expression (Ahn et al., 2016). MondoA has also been demonstrated to induce the expression of glycogen synthesis genes in hepatocytes (Petrie et al., 2013). Therefore, MondoA-Mlx promote the metabolism excess intracellular Glc by directing it through different pathways, while limiting excessive Glc uptake.

Table 1: Examples of direct MondoA-Mlx activated targets:

Gene	Functions:	Cell type/tissue	Reference
ARRDC4	Glc uptake inhibition	Melanoma cells	Parmenter et al., 2014
HKII	Glc phosporylation	Myoblast	Sans et al., 2006
LDH-A[a]	Lactate metabolism	Myoblast	Sans et al., 2006
PFKFB3[b]	Carbohydrate metabolism	Myoblast	Sans et al., 2006
PTG[c]	Glycogen metabolism	Hepatocyte	Petrie et al., 2013
TXNIP	Glc uptake inhibition; redox regulation	HA1ER	Stoltzman et al., 2008

[a] *LDH-A – lactate dehydrogenase A*; [b] *PFKFB3 – phosphofructo-2-kinase/fructose-2,6-bisphosphatase*; [c] *PTG- protein targeting to glycogen*

1.6.2.4.3. ChREBP-Mlx regulate the expression of lipogenic genes

Since its discovery, ChREBP has rapidly been unveiled as a master regulator of several key enzymes in glycolysis and lipogenesis (for direct targets, see **Table 2**). Curiously, ChREBP and the Sterol regulatory element binding protein 1 c (SREBP1c) have been found to act synergistically on several of their lipogenic and glycolytic targets (Dentin et al., 2004). Moreover, experimental evidence exists to indicate direct regulation of the *SREBP1* gene by ChREBP-Mlx in HepG2 cells (Jeong et al., 2011). Based on microarray data, approximately 75% of Glc-elicited transcriptional response in hepatocytes appears to be mediated by Mlx (Ma et al., 2006). As the more prevalent Mondo protein in the liver, majority of Mlx-dependent changes seen in hepatocytes are therefore expected to be facilitated by ChREBP. It is noteworthy that pre-diabetic and type 2 diabetic patients display lower expression of

ChREBP and its targets in WAT, while elevated expression is apparent in liver samples (Kursawe et al., 2013). Moreover, the same study found a clear positive correlation between insulin sensitivity and ChREBP expression in WAT. Thus, the role of ChREBP-Mlx in regulating carbohydrate metabolism and conversion to TAG is critical for metabolic homeostasis maintenance. Yet the significance of tissue-specific expression differences and concurrent physiological effects continue to be discussed (Richards et al., 2017).

Table 2: Examples of direct ChREBP-Mlx activated targets:

Gene	Functions:	Cell type/tissue	Reference
6PGD[a]	PPP	Macrophage	Sarrazy et al., 2015
ACC	De novo lipogenesis	Liver	Ishii et al., 2004
DEC1[b]	Transcriptional repressor	Hepatocyte	Iizuka and Horikawa, 2008
FAS	De novo lipogenesis	Liver, macrophage	Ishii et al., 2004; Sarrazy et al., 2015
G6Pase	GlcNG, glycogenolysis	Liver	Arden et al., 2012
GLUT4	Glc transport	HEK293	Ma et al., 2006
GPDH[c]	Glycerol metabolism	HEK293	Ma et al., 2006
KLF10[d]	Transcriptional repressor	Liver	Iizuka et al., 2011
ME2[e]	Mitochondrial malate and NADPH metabolism	Macrophage	Sarrazy et al., 2015
PK	Glycolysis	HEK293	Ma et al., 2006
TXNIP	Glc uptake inhibition; redox regulation	INS1; β-cells	Cha-Molstad et al., 2009

[a] *6PDG - 6-phosphogluconate dehydrogenase;* [b] *DEC1 – deleted in esophageal cancer 1:* [c] *GPDH – glycerol-3-phosphate dehydrogenase;* [d] *Klf10 – Krüppel-like factor 10;* [e] *ME2 – Mitochondrial malic enzyme 2*

Given the key role that ChREBP plays in lipogenesis and carbohydrate metabolism, ChREBP knock-out mice displayed slightly elevated plasma Glc levels and about 50% reduction in circulating FFA levels. Moreover, with a standard diet, the adipose tissue deposits of these animals were significantly smaller, likely due to reduced fatty acid synthesis (Iizuka et al., 2004). Notably, such mice displayed normal embryonic development and adult life span under standard conditions. However, attempts to feed ChREBP -/- mutant mice diets high in simple sugars resulted in dramatic and rapid deterioration of the animals. In particular, a 70% sucrose diet caused the death of more than half of the tested mutant mice within a week, whereas, a diet high in Fru resulted in a terminal decline after just few days (Iizuka et al., 2004). For ChREBP knock-out mice, a diet high in sugar resulted in a further decline of circulating FFA levels, accompanied by progressive hypothermia (Iizuka et al., 2004). Apparently, these animals suffered from metabolic problems that are aggravated by dietary sugars. However, despite the characterization of these initial findings, the underlying reasons for the dietary sugar intolerance have remained unexplored.

1.6.2.4.4. Common modifiers of the circadian clock and ChREBP-Mlx

Overexpression experiments of a dominant negative variant of Mlx in hepatocytes have indicated a transcriptional regulation of *G6PD*, most likely by ChREBP-Mlx (Ma et al., 2006). G6PD is the rate-limiting enzyme in the PPP and produces NADPH by metabolizing G6P. Moreover, another enzyme from the pathway 6-phosphogluconate dehydrogenase (6PGD), that likewise produces NADPH, has been identified as a direct ChREBP target in macrophages (Sarrazy et al., 2015). Through NADPH metabolism, PPP has recently been demonstrated to regulate circadian gene oscillations and

behavioral rhythmicity, underlining the potential contribution of ChREBP-Mlx to the circadian output (Rey et al., 2016).

KLF10 (Krüppel-like factor 10) and DEC1 (Deleted in esophageal cancer 1) are two transcriptional repressors that have been proposed to modulate the lipogenic gene expression response elicited by ChREBP-Mlx. Both Klf10 and DEC1 promoters possess a Glc-responsive ChoRE and have been confirmed as direct targets for ChREBP-Mlx in primary hepatocytes (Iizuka and Horikawa, 2008; Iizuka et al., 2011). However, the physiological relevance of this Glc-dependent regulation has not been studied *in vivo*. Curiously, DEC1 has been demonstrated to compete with ChREBP-Mlx complexes in binding the E-box elements present in ChoREs, thereby inhibiting Glc-responsive gene expression, including its own (Iizuka and Horikawa, 2008). More strikingly, as they are both proposed to be direct targets of the molecular clock, KLF10 and DEC1 have been linked to the control and output of the circadian rhythm (Guillaumond et al., 2010; Honma et al., 2002). DEC1 also directly inhibits the expression of clock genes by competing with CLOCK-BMAL1 for E-box elements in CCG promoters. (Honma et al., 2002), whereas KLF10 has been found to be important for circadian gene expression oscillations, especially in terms of lipogenic and carbohydrate metabolism (Guillaumond et al., 2010). *DEC1* gene appears to be more highly expressed in diabetic and insulin-resistant humans (Coletta et al., 2008), while loss of *klf10* in male mice leads to PEPCK-dependent hyperglycemia (Guillaumond et al., 2010). Evidently, both of these TFs contribute to metabolic homeostasis. It is noteworthy that the core molecular clock CLOCK-BMAL1, DEC1, and MondoA/ChREBP-Mlx complexes recognize similar elements in promoters – E-boxes and ChoREs composed of two E-boxes. At least for the ChoRE in the promoter of *DEC1*, the same E-box has been demonstrated to be bound by both the molecular clock and ChREBP-Mlx (Honma et al., 2002; Iizuka and Horikawa, 2008). Therefore, it is possible that CLOCK-BMAL1 and MondoA/ChREBP-Mlx share other direct targets, and potentially either compete or synergize to regulate their expression. Thus, by regulating the expression of circadian modulators such as *DEC1* and k*lf10*, ChREBP-Mlx may coordinate the synchronization of nutrient signals to circadian rhythm. Elaborate feedback loops between ChREBP-Mlx, their targets, and the circadian clock seem to exist to help maintain metabolic homeostasis throughout daily fluctuating nutrient levels.

1.6.2.4.5. ChREBP-Mlx as regulators of antioxidant profile

Similarly to MondoA, ChREBP has also been found to regulate *TXNIP* expression (Cha-Molstad et al., 2009). TXNIP protein is known to interact and, as a result, inhibit the antioxidant properties of thioredoxin. As such, TXNIP has been suggested to possess an inhibitory role in cancer progression (Zhou and Chng, 2013). However, reporter comparisons in HeLa cells have revealed a 7-fold more potent response on *TXNIP* promoter by MondoA, when compared to ChREBP (Yu et al., 2009). Nevertheless, the dominance of *TXNIP* regulation may be tissue dependent. As TXNIP is an antioxidant modifier, changes in ChREBP-Mlx are expected to affect the oxidative stress response.

G6PD and 6PGD, two enzymes from the PPP reducing NADPH, have been demonstrated to lie transcriptionally downstream of ChREBP-Mlx in primary hepatocytes and macrophages, respectively (Ma et al., 2006; Sarrazy et al., 2015). PPP is the main source of cytoplasmic NADPH, while fatty acid synthesis is the main consumer (Fan et al., 2014). Thus, it is fully expected that the upregulation of lipogenic genes by ChREBP-Mlx in response to high sugars would be accompanied by increased NADPH production to sustain lipogenesis. In addition, the mitochondrial Malic enzyme 2 (ME2), that also reduces NADPH, has similarly been identified as a direct ChREBP target in macrophages (Sarrazy et al., 2015). NADPH has a central function in cellular antioxidant maintenance, as it is a substrate for glutathione reduction. Consequently, knocking down ChREBP in macrophages resulted in a 30-fold decrease in the $NADPH/NADP^+$ ratio, subsequently reduced lipid synthesis, and reduced glutathione levels (Sarrazy et al., 2015). Moreover, ChREBP-deficient macrophages displayed higher

susceptibility to ROS inducers, resulting in an increased inflammatory response, caspase activation, and subsequent necrosis. The ChREBP-deficiency dependent inflammation and cell death could be, however, rescued by the addition of exogenous reduced glutathione (Sarrazy et al., 2015). Therefore, at least in macrophages, ChREBP-Mlx dependent gene regulation is crucial to ensure sufficient antioxidant production for ROS tolerance. Yet, macrophages as cells are specialized for ROS generation to help battle infection. Moreover, ROS levels in phagocytes, including macrophages, are substantial (about 2μM for O_2^- and between 1-4 μM for H_20_2; (Slauch, 2011)). Thus, whether ChREBP-Mlx regulate NADPH reduction in metabolic tissues, and if such a modulation has consequences on systemic oxidative stress response, has not yet been investigated.

1.7 AMPK-related kinases

Another well-characterized cellular energy sensor is AMPK, which is nearly universal among eukaryotes (Hardie et al., 2012). AMPK belongs to the AMPK-related kinase family, regulated by the master-kinase Liver kinase B1 (LKB1) (reviewed in (Bright et al., 2009). Its principal function lies in monitoring cellular AMP/ATP ratio. Fasting, hypoxia, and exercising diminish the available ATP in the cell, while the AMP levels undergo a consequential surge. In order to help sustain the metabolic needs of the altered energetic conditions, AMPK activation is triggered (Hardie, 2004). As a result, anabolic energy-consuming reactions, such as protein and glycogen synthesis, gluconeogenesis, and lipogenesis are inhibited, while catabolic processes including fatty acid oxidation, autophagy, Glc uptake, and glycolysis are promoted (Hardie et al., 2012). Moreover, by promoting the salvage pathway of NAD+ synthesis, AMPK also affects the redox state of the cell (Cantó et al., 2009; Han et al., 2016). In addition to its critical role at the energy checkpoint, AMPK has been proposed to elicit anti-proliferative effects on cancer cells, most likely due to shutting down the anabolic metabolic reactions required for cell-division (Alessi et al., 2006). This has prompted researchers to investigate the plausibility of using the antidiabetic drugs functioning through AMPK activation, such as metformin, as treatment options for cancer, and CVD (Pryor and Cabreiro, 2015).

1.7.1 Salt-inducible kinases

In mammals, the AMPK-related kinase family is composed of 14 kinases. In addition to AMPK kinases, however, the regulation of metabolic effectors is also facilitated by another group of kinases from the same family – the Salt-inducible kinases (SIKs) (reviewed by Shackelford and Shaw, 2009). The kinases were so named because the first orthologue, SIK1, was initially cloned and identified from rat adrenal glands fed a diet high in salt (Wang et al., 1999). Subsequent similarity searches in mammalian genomes helped to identify SIK2 and SIK3 (Katoh et al., 2004a). Overall, the kinases demonstrate evolutionary conservation in animals, as the *Drosophila*, Xenopus, and zebrafish genomes all encode for two SIK proteins, while *C. elegans* has a single *SIK* gene (Funato et al., 2016; Okamoto et al., 2004). Moreover, due to a high level of conservation, the SIK3 kinase domain sequence displays extensive identity (71%) and similarity (86%) between humans and flies (Choi et al., 2015). In mammals, SIKs show distinct gene expression patterns: *SIK1* is detectable in adrenal glands, testes and skeletal muscle; *SIK2* is highest in adipose tissue and brain, and functions also in the liver; while *SIK3* is expressed ubiquitously at a more moderate rate (Dentin et al., 2007; Horike et al., 2003; Katoh et al., 2004a; Okamoto et al., 2004). In flies, SIK2 functions as an orthologue of both SIK1 and SIK2, and is mostly expressed in the brain (Choi et al., 2011; Okamoto et al., 2004). The fly SIK3 kinase has been found to be highly expressed in the fat body (Choi et al., 2015; Wang et al., 2011).

Contrary to AMPK, SIKs are single subunit enzymes devoid of glycogen and AMP sensitivity domains (Hardie, 2004) (see **Figure 8**). LKB1-dependent phosphorylation of a conserved and critical

Thr residue in the kinase domain T-loop has been proposed to be essential for nearly all AMPK-related kinases, including thee SIKs (Lizcano et al., 2004) (see **Figure 8**). However, at least for SIK2, other regulatory mechanisms independent of LKB1 have also been suggested (Altarejos and Montminy, 2011; Miranda et al., 2016). The C-terminal domain of the SIK kinases harbors a PKA phosphorylation site that is well conserved, and acts as a NLS (Katoh et al., 2004b) (**Figure 8**). Despite this, under *in vitro* conditions the PKA-dependent phosphorylation seem to have no effect the activity of SIK kinases *per se* (Berdeaux et al., 2007; Katoh et al., 2006). Rather, the main PKA-dependent regulatory mechanism on the SIK kinases is likely mediated by phosphorylation-dependent subcellular sequestration, and subsequent separation from substrates. *In vitro*, mammalian SIK2 has been found to be activated by insulin through AKT-dependent phosphorylation (Dentin et al., 2007). This mechanism seems to be conserved in *Drosophila*, as both fly SIK kinases can be activated through the same mechanism (**Figure 8**), while the fly glucagon counterpart adipokinetic hormone inhibits SIK3 activity through LKB1 (Choi et al., 2015; Wang et al., 2008, 2011). Thus, SIK kinases appear to be regulated by several important metabolic kinases.

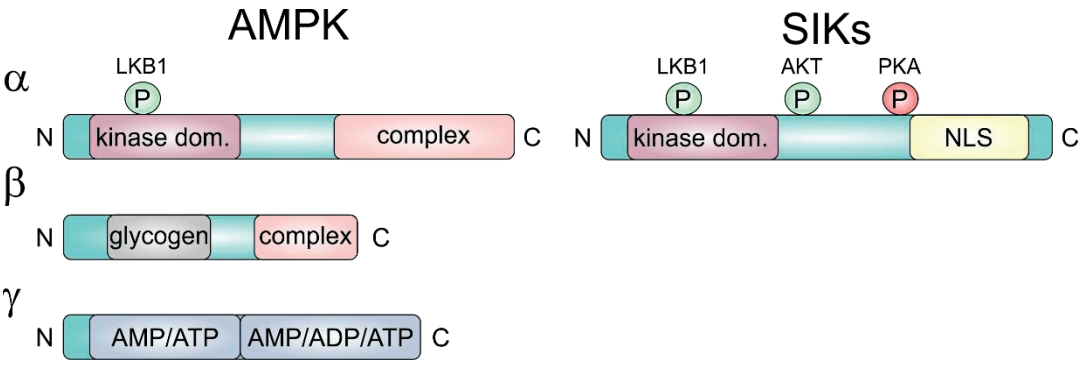

***Figure 8**: A simplified schematic comparison of AMPK and SIK domain structures. AMPK is composed of three subunits: the kinase domain-containing catalytic subunit AMPKα, and the regulatory subunits AMPKβ and AMPKγ. The regions marked as "complex" on the figure dictate the trimer formation. The regulatory subunits contain domains for glycogen and AMP/ADP/ATP binding. An NLS present in certain AMPKα isoforms has not been marked to the figure. Similarly to AMPK, the SIK kinase domain harbors an activating LKB1-phosphorylation site. The SIK C-terminal domain contains an NLS, and the inhibitory PKA-phosphorylation site. Additionally, the activating AKT-phosphorylation site that has yet to be experimentally demonstrated for SIK1 is marked on the figure. Proteins not drawn to scale. Based on Berdeaux, 2011 and Hardie et al., 2012.*

1.7.1.1 SIKs as regulators of growth and metabolism

Studies with different model organisms have helped to uncover the physiological function of SIKs. To date, a number of SIK phosphorylation target proteins have been identified (see **Table 3**), many of which either control or participate in metabolic processes. For example, cAMP-regulated transcriptional co-activators (CRTCs) are well-characterized targets for all SIKs (Patel et al., 2014) (**Table 3)**. CRTC together with its partner cAMP-responsive element-binding protein (CREB) promote the expression of genes involved in GlcNG. As a result, SIKs have been established as crucial inhibitors of GlcNG in the liver, where they seem to function redundantly (Patel et al., 2014). Consequently, due to uninhibited CRTC, SIK2 null flies have been found to be starvation resistant, and possess larger glycogen and lipid deposits (Choi et al., 2011). However, pan-SIK inhibitor treatments induced Glc production even in CRTC-deficient primary hepatocytes (Patel et al., 2014).

Therefore, the SIKs most likely contribute to carbohydrate homeostasis also through additional means.

Another group of well characterized SIK targets is the class IIa histone deacetylases (HDACs) (**Table 3**). All three SIKs have been demonstrated to phosphorylate this subset of HDACs, resulting in their cytoplasmic sequestration, and consequent inactivation (Berdeaux et al., 2007; Walkinshaw et al., 2013). The class IIa HDACs have been linked to Glc homeostasis through activation of Forkhead box O (FoxO), and regulation of GlcNG gene expression (Mihaylova et al., 2011; Wang et al., 2011). Notably, inhibition of class II HDACs may help alleviate hyperglycemia, as witnessed by experiments conducted in type 2 diabetic mouse models (Mihaylova et al., 2011). Curiously, the lack of SIK-dependent HDAC4 inhibition in SIK3 knock-out mice has been demonstrated to result in dwarfism due to impaired chondrocyte growth control (Sasagawa et al., 2012). Moreover, the same mice exhibit diminished lipid stores, hypoglycemia, and impaired cholesterol metabolism (Uebi et al., 2012), but whether these metabolic phenotypes are the result of unregulated HDACs, has not yet been studied. As SIK3 null mutant flies have been published to be early larval (Wang et al., 2011) or pupal lethal (Choi et al., 2015), a hypomorphic allele has been used for experimental purposes in *Drosophila* (Wang et al., 2011). Such flies have reduced TAG levels, are developmentally delayed, and exhibit increased sensitivity to starvation and the oxidative stress-inducer paraquat (Wang et al., 2011). The absence of HDAC4 inhibition through SIK3-dependent cytoplasmic sequestration in response to feeding in these animals leads to deacetylation, and hence, nuclear accumulation and activation of FoxO. As a result, the expression of a FoxO target lipase *brummer* is enhanced in SIK3 hypomorphs, and is consequently proposed to explain the reduced TAG stores, and starvation sensitivity observed in these flies (Wang et al., 2011). Importantly, many of the aforementioned SIK3 hypomorphic fly phenotypes, and also SIK3 null lethality, can be rescued by restoring the kinase expression in the fat body (Wang et al., 2011), indicating that SIK3 function is essential in this metabolic organ. The loss of the single SIK orthologue in *C. elegans* similarly results in a developmental delay. Despite increased food foraging, SIK mutant worms are smaller in size, indicating a potential role in growth control for this kinase. Moreover, these animals are long-lived – a phenotype that can be normalized by simultaneous loss of the *C. elegans* FoxO orthologue (Lanjuin and Sengupta, 2002). Thus, FoxO may also lie downstream of SIK in *C. elegans*, but whether this putative regulation occurs through HDACs, has not been investigated.

Direct evidence of SIK-dependent growth control comes from studies conducted in *Drosophila*. Namely, both SIK2 and SIK3 have been demonstrated to promote growth by inhibiting the Hippo pathway. This regulation occurs through SIK2- and SIK3-dependent phosphorylation of the Hippo pathway scaffolding protein Salvador (Sav), resulting in its inability to bridge the association between Hippo and Warts – the key kinases of the pathway (Wehr et al., 2013). Consequently, the Warts-dependent inhibitory phosphorylation of Yorkie is reduced (Wehr et al., 2013), resulting in its increased nuclear localization. As Yorkie promotes cell proliferation and survival, over-expressing active SIK2 in the fly wing results in significantly increased wing area (Wehr et al., 2013). Moreover, through the involvement of Hippo pathway regulation, both SIK2 and SIK3 have been demonstrated to contribute to insulin-dependent dietary sugar enhancement of tumor growth in an activated Ras/Src fly model (Hirabayashi and Cagan, 2015). Interestingly, in mammals, SIK2 and SIK3 have recently been implicated in promoting tumor progression in ovarian and prostate cancer (Bon et al., 2015; Charoenfuprasert et al., 2011; Miranda et al., 2016). As high levels of SIK2 are often detected in adipocyte-rich ovarian cancer metastases, the kinase is suggested to play a role in tumor metastasizing. Mechanistically, by inhibiting lipogenesis at the level of ACC and activating the pro-survival, pro-proliferation kinase Phosphoinositide 3-kinase (PI3K) by phosphorylating its regulatory subunit, SIK2 has been proposed to link metabolic control and growth in cancer (Miranda et al., 2016).

Table 3: Examples of known SIK-phosphorylation targets:

SIK #	Target protein	Target protein function:	Regulatory consequence	Cell type/tissue	Reference
2	ACC	Lipogenesis	Inhibition	Different ovarian cancer cell lines	Miranda et al., 2016
1, 2, 3	Class IIa HDACs	Deacetylation	Inhibition	HEK293, MEF, Hela, A549 (SIK2 & 3); myocytes (SIK1)	Berdeaux et al., 2007; Walkinshaw et al., 2013
1, 2, 3	CRTC; CRTC2	Transcriptional co-activator	Inhibition	Adrenal cells (SIK1); adipocytes (SIK2); hepatocytes (SIK3)	Itoh et al., 2015; Lee et al., 2015; Park et al., 2014
2	IRS-1[a]	Insulin signal transmission	Inhibition	COS-7	Horike et al., 2003
2	p85α	Regulatory subunit of PI3K	Activation	Different ovarian cancer cell lines	Miranda et al., 2016
2, 3	Sav	Hippo-pathway regulation	Inhibition	S2R+; *Drosophila* wing	Wehr et al., 2013
1	SREBP1c	TF	Inhibition	Liver	Yoon et al., 2009

[a] IRS-1 – Insulin receptor substrate 1

Based on a number of targets, the consensus site for SIK-dependent serine and threonine phosphorylation motifs has been well characterized (Horike et al., 2003; Screaton et al., 2004; Wang et al., 2011; Wehr et al., 2013): ΦX[HKR]XX[S/T]XXXΦ, where Φ stands for a hydrophobic residue, and X for any amino acid. However, whether all the SIK targets contain the aforementioned consensus motif, is currently uncertain. The phosphorylation event in question often forms a 14-3-3 chaperone binding site, resulting in the cytoplasmic sequestration of some substrates, such as CRTC and certain HDACs (Berdeaux et al., 2007; Screaton et al., 2004). However, for SREBP1-c, IRS-1, and p300, the inhibitory effect elicited by SIK phosphorylation occurs independently of 14-3-3 (Berdeaux, 2011; Bricambert et al., 2010; Yoon et al., 2009). For SREBP1-c, the SIK1 dependent phosphorylation of serine residues in the bHLH/LZ domain appears to abolish the DNA-binding ability of the TF, without any effect on its subcellular localization or stability (Yoon et al., 2009).

Evidently, the AMPK and SIK kinases share several common target proteins, including CTRCs, class II HDACs, SREBP1c, ACC, and IRS-1 (Horike et al., 2003; Jakobsen et al., 2001; Koo et al., 2005; Li et al., 2011; McGee et al., 2008; Miranda et al., 2016; Park et al., 2002; Walkinshaw et al., 2013; Yoon et al., 2009). Similar to AMPK, this may indicate a central role for SIK kinases in metabolic homeostasis. Moreover, accumulating experimental evidence points to the possibility that the SIK kinases function to link metabolic regulation to growth control. Such characteristics delineate SIKs as potential targets for metabolic syndrome and cancer treatment. However, much of their function and downstream effector regulation remains uninvestigated, and further experiments are required for more complete understanding, before therapeutic targeting of SIKs can be considered.

2. Aims of the study

MondoA and ChREBP, along with their transcriptional partner Mlx, have been demonstrated to mediate the majority of Glc-responsive gene expression. While efforts have been made to investigate the biological significance of MondoA/ChREBP-Mlx-dependent transcriptional control, most physiological consequences of their target gene expression regulation remain unknown.

The *Drosophila* genome encodes for single proteins for Mondo and Mlx (McFerrin and Atchley, 2011). More importantly, the key Glc-sensitive element GSM of *Drosophila* Mondo protein can readily substitute for its mammalian counterpart in ChREBP, without decreasing sugar-responsiveness (Ma et al., 2006). This demonstrates high level of functional conservation. The aim of this work was to investigate the physiological role of the Mondo-Mlx complex, by using *Drosophila* as a model organism. Another major goal was to dissect the relevance of Mondo-Mlx dependent gene expression in response to sugar diet.

In sum, the specific goals for this thesis were:

1) To broaden the understanding of Mondo-Mlx function *in vivo*
2) To identify direct Mondo-Mlx targets, and investigate the physiological role of their transcriptional regulation downstream of these TFs
3) To characterize novel signaling pathways involved in sugar sensing

3. Materials and methods

Detailed descriptions of the materials and methods used throughout this study can be found in the respective articles and their corresponding supplementary information. A summarizing table outlining the applied methods is listed below (**Table 4**). The fly lines that were used, and their origin, can be found in **Table 5**. The plasmids and primary antibodies implemented throughout the thesis are shown in **Table 6** and **Table 7**, respectively.

Table 4: Summary table of the methods used

Method	Publication
ChIP	II, III
Circulating Glc and trehalose measurement	I, II, III
Circulating glycerol measurement	II
Co-immunoprecipitation and HA-pulldown	I, III
Colorimetric feeding assay	II
Fly husbandry	I, II, III
Glycogen measurement	I
Hemolymph pH	III
In vitro kinase assay	III
Luciferase reporter assay	II
Molecular cloning	II, III
Pupal volume measurement	II
RNA extraction and qPCR	I, II, III
S2 cell culture and transfection	I, III
TAG measurement	II, III
Western Blotting and Phos-Tag	I, III

***Drosophila* fly husbandry**

Flies were kept at 25 °C in 12 h light: 12 h dark cycles. For defined nutrient experiments, 1st instar larvae were collected from apple juice plates onto food at a fixed density. The low sugar diet (LSD) contained 20% (w/v) dry baker's yeast, 0.5% (w/v) agarose, 2.5% (v/v) Nipagin (methylparaben), and 0.7 % propionic acid in PBS. For sugar feeding, the LSD was supplemented with 5-20% sucrose. A diet containing 20% sucrose in this setting was referred to as high sugar diet (HSD) For starvation, 2nd instar larvae were transferred to 0.5% agarose in PBS media for 16 h, followed by 4 h of refeeding with 0.5% agarose and 10% Glc in PBS media. For sugar only feeding, 1st instar larvae were collected to 0.5% agarose in PBS supplemented with 20% sucrose. For acute sugar feeding experiments, late 2nd instar or early third instar larvae were transferred onto high sugar (10 or 20%) diet for 16 h before samples were harvested. The used fly and RNAi lines are listed in Table 5.

Table 5: Used fly lines

Fly line	Stock nr. and source or reference	Publication
Cabut RNAi	4427R-1, NIG[a]	I, II
Canton-S	64349, BDSC[b]	II
cbt^{E1}	Muñoz-Descalzo et al., 2005	II
Control for *mlx[1]*	Generated by V. Hietakangas for this study	I, II, III
Control for PEPCKΔ84	This study	II
Df(2R)ED3636	150416, BDSC	II
Mlx RNAi	v110630 (KK), VDRC[c]	I
mlx[1]	Generated by V. Hietakangas for this study	I, II, III
Mondo RNAi	v109821 (KK), VDRC	I, III
P{EPgy2}EY1004	19965, BDSC	II

Table 5, continued

Fly line	Stock nr. and source or reference	Publication
pepck1 (CG17725) RNAi	v50253 (GD), VDRC	II
pepck2 (CG10924) RNAi	36915, BDSC	II
PEPCKΔ84	Generated by M. Poukkula	II
PHGPx RNAi	33939, BDSC	III
r4-GAL4	(Lee and Park, 2004)	I, II
SIK3 RNAi	28366, BDSC	III
SIK3Δ72	(Wang et al., 2011)	III
Tim-GAL4	(Zhao et al., 2003)	II
TrxR-1	110648 (KK), VDRC	III
Tub-GAL4	(Lee and Luo, 1999)	I, II, III
Tub-GAL80^{ts}	(McGuire et al., 2003)	I, II
UAS-cbt-Flag	(Muñoz-Descalzo et al., 2005)	II
UAS-Dicer 2	(Dietzl et al., 2007)	II
UAS-mlx-Flag	Generated by V. Hietakangas for this study	I, II
UAS-strep-Zw-6xP-mut-PB-3HA	Generated for this study	III
UAS-strep-Zw-PB-3HA	Generated for this study	III
Ubi-GAL4	32551, BDSC	III
y^{1} w^{1118}	6598, BDSC	III
Zw RNAi	v108898 (KK), VDRC	III

[a] BSC – Bloomington Drosophila Stock Center
[b] NIG - National Institute of Genetics, Japan
[c] VDRC - Vienna Drosophila Resource Center

HepG2 and S2 cell culture and transfection

HepG2 (human hepatocellular carcinoma) cells were grown in complete MEM media (5.5 mM Glc), supplemented with or without high Glc (25 mM, final concentration). Transfections were carried out with Lipofectamine 2000 based on manufacturer's guidelines.

Drosophila S2 (circulatory macrophage-like) cells were grown at 25°C in standard or Glc-free Shield's and Sang M3 media (Sigma and US Biological), supplemented with 2% of fetal bovine serum (Gibco), 1× insect medium supplement (Sigma) and penicillin/streptomycin (Gibco). The transfections with plasmids listed in Table 6 were performed using Effectene (Qiagen), according to manufacturer's protocol. Expression from pMT and pUAST vectors was induced with 1.2 µM CuSO4 24 h post-transfection, cells were harvested 48 h post-induction.

Table 6: Plasmids used in this thesis

Plasmid	Reference or source	Publication
pUAST-Mlx-Flag	Generated by V. Hietakangas for this study	I
pMT-strep-Mondo-3xV5	Generated by J Mattila	I
pMT-GAL4	(Klueg et al., 2002)	I
pRL-SV40	Promega, E2231	II
pGL3 basic	Promega, E1751	II
pGL3 Cabut ChoRE 1 & 2 wt	Generated for this study	II
pGL3 Cabut ChoRE 1 mut, ChoRE2 wt	Generated for this study	II
pGL3 Cabut ChoRE 1 wt, ChoRE2 mut	Generated for this study	II
pGL3 Cabut ChoRE 1 & 2 mut	Generated for this study	II

Table 6, continued

Plasmid	Reference or source	Publication
pMT-strep-G6PD-PA-3HA	Generated for this study	III
pMT-strep-G6PD-PB-3HA	Generated for this study	III
pMT-strep-SIK3-PA-3V5	Generated for this study	III
pMT-strep-SIK3-PB-3V5	Generated for this study	III
pUAS-strep-G6PD-PB-3HA	Generated for this study	III
pUAS-strep-G6PD-6xP-mut-PB-3HA	Generated for this study	III

Table 7: Used primary antibodies

Primary antibody	Source or reference	Publication
Guinea pig anti-Mlx	Generated by E. Havula for this study	I
Mouse anti-V5	Life Technologies, R96025	I, III
Mouse anti-HA	Life Technologies, 326700	III
Rabbit anti-Kinesin	Cytoskeleton, AKIN01	I, III

Statistical analysis

All experiments were performed in minimum three biological replicates. The data are represented as the average ± standard error of the mean (SEM) (**I**) or the average ± standard deviation (**II**, **III**). All quantitative data were analyzed by Student's t-test (two-tailed, unequal variance), where $p < 0.05$ was considered significant. The significance of pupariation and survival curves for publication III was evaluated by using the Log-Rank test (JMP Pro 12 software).

4. Summary of results and discussion

4.1 The Drosophila Mondo and Mlx physically interact and show functional conservation

In mammals, two Mondo proteins exist that after dimerizing with Mlx, promote the expression of Glc-responsive genes. Due to common ancestry, MondoA and ChREBP share a visibly similar structure, and are also proposed to be regulated by the same metabolites (Havula and Hietakangas, 2012; McFerrin and Atchley, 2011; Petrie et al., 2013). In addition, both TFs recognize similar elements in their target gene promoters. Despite their different predominant tissue expression pattern, both ChREBP and MondoA have been described to function in the liver and skeletal muscle (Ahn et al., 2016; Hanke et al., 2011; Ma et al., 2006; Petrie et al., 2013). Moreover, at least one common direct target gene is shared between the paralogous TFs, while similar pathways have been demonstrated to be regulated by MondoA and ChREBP. Therefore, these TFs may be partially redundant in mammals.

In contrast, the *Drosophila* genome harbors a single copy of both Mondo and Mlx: Mondo (CG18362, also known as *Mio*), and Mlx (CG3350; official name *bigmax*). This eliminates the possibility of redundancy. The level of functional conservation and sugar-responsiveness between the GSM of the fly and mammalian Mondo proteins has been experimentally demonstrated (Ma et al., 2006). Moreover, when we compared the Mlx protein sequences in flies and humans, we discovered a high level of identity (32.2%) and similarity (45.3%) (**Figure 9**).

```
MTEPGASPEDPWVKASPVGAHAGEGRAGRARARRGAGRRGASLLSPKSPTLSVPRGCRED
------------------------------------------------------------MSD

SSHPACA-KVEYAYS-DNSLDPGLFVESTRKGSVVSRANSIGST-SA-SSVPNT-DDEDS
NNN-ALATK-EDVFGMEH--DQ----DHTSK-HY-SRCSSAGSTHTPNSSAHNSDDDDDS

-D-YHQELYKE--SYKDRRRRAHTQAEQKRRDAIKRGYDDLQTIVPTCQQQDFSIGSQKL
GDARHSAAANSTLSYKERRREAHTQAEQKRRDAIKKGYDSLQELVPRCQPNDSS-G-YKL

SKAIVLQKTIDYIQFLHKEKKKQEEEVSTLRKDVTALKIMKVN-YEQIVKAHQD-NPHEG
SKALILQKSIEYIGYLNQQKLKQEDEGSALQKEVTALRIIK-NGYENMLQ-HQQANP--G

-ED-QVSDQVKFNVFQGIMDSLFQSFNASISVASFQELSACVFSWIEEHCKPQTLREIVI
PEEARLTDEAKFQVFQAIMEEMFETFQ-HIPMENFKQLTTGIIPWLEEHCKPHILRNI-L

G-VLHQLKNQ-LY----------------- 298
SRTLQQMAQEAM-EKQELQAMEQESSEGFS 254
```

Figure 9: Pairwise alignment of human (γ-isoform; upper row) and Drosophila Mlx. *We analyzed the protein sequences with Emboss Needle. Identical residues are marked in dark blue, and similar residues with lighter blue color.*

In mammals, the paralogous TFs MondoA and ChREBP interact with Mlx to form heteromers (Ma et al., 2007; Peterson et al., 2010) – a pre-requisite for nuclear entry and transcriptional regulation. However, whether the fly counterparts also directly interact, has not yet been shown. Thus, by carrying out a co-immunoprecipitation experiment with over-expression constructs in *Drosophila* S2 cells, we confirmed the interaction of the fly Mondo and Mlx proteins (**I**, Fig. 2A).

A loss of one of the protein partners would be expected to abolish the function of the Mondo-Mlx complex. Thus, to investigate the physiological role of these TFs, we generated an mlx^1 mutant fly through imprecise P-element excision and, in parallel, a precise P-element excision line to be used as a genetic control. Upon an Mlx-specific Western blot, three protein bands were visible in control larval sample that were absent in the mlx^1 homogenate. However, in an S2-cell lysate sample, two prominent protein bands were seen (**I**, Fig. S2). In both cases, despite different intensity ratios, the upper-most band appeared to be the most prevalent one. However, Mlx is reported to possess only a single splice-variant in the fly. Moreover, proteolytic processing cannot explain the detection of several bands, as N- and C-terminal tag-mediated affinity-purification resulted in comparable band intensities (data not shown). In addition, by using alkaline phosphatase treatment, we ruled out phosphorylation. In addition, treatment with an OGT inhibitor failed to change the Mlx protein band pattern (data not shown). Therefore, the nature of the multi-band Mlx protein

remains unresolved. Sumoylation, ubiquitinylation, or other post-translational modifications could be plausible candidates.

4.2 Mondo-Mlx are required for dietary sugar tolerance

While analyzing the *mlx*^{1} mutants on a standard lab diet, we noticed a moderate developmental delay, culminating in pupal lethality during the pharate stage. In mammals, MondoA/ChREBP-Mlx have been implicated in carbohydrate utilization through sugar-responsive gene expression control. Moreover, mice lacking ChREBP have been demonstrated to deteriorate after being fed a diet high in simple sugars. By adding increasing concentrations of sucrose to the fly food, and monitoring the developmental kinetics of *mlx*^{1} mutants or Mondo RNAi animals, we demonstrated that the functional Mondo-Mlx complex is required for dietary sugar tolerance in *Drosophila* (**I**, Fig. 1D, E).

By qPCR, we determined the tissue-specific expression of *mlx* and *mondo*. In accordance with the mammalian data, the fly orthologues displayed highest expression in peripheral metabolic tissues, especially in the fat body, gut, and Malpighian tubules (**I**, Fig. 5A). Moreover, expressing *mlx* in the fat bodies of *mlx*^{1} animals rescued pupal lethality on LSD and intolerance to HSD (**I**, Fig. 5B). Thus, Mondo-Mlx function is essential in the fat body for dietary sugar tolerance.

4.3 Loss of Mondo-Mlx function results in imbalanced carbohydrate profile

To gain further insight into this striking phenotype, we analyzed the metabolic profile of *mlx*^{1} mutants. We uncovered elevated levels of sorbitol (**III**, SFig 2A), and circulating levels of Glc and trehalose (**I**, Fig. 4A, B). These aberrations are a clear indication of impaired carbohydrate metabolism. Notably, however, a sugar-dependent increase was apparent only in sorbitol and Glc levels, while trehalose was also elevated in response to a LSD. Compared to circulating Glc levels, trehalose levels have been proposed to be less responsive to environmental cues (Graham and Pick, 2017; Ugrankar et al., 2015). This may explain the lack of further increase in trehalose levels following sugar feeding. Importantly, loss of either Mondo or Mlx also lead to high body glycogen levels (**I**, Fig. 4C, F). This result is in agreement with observations from ChREBP knock-out mice, where loss of this TF similarly resulted in elevated glycogen levels (Iizuka et al., 2004). Therefore, Glc uptake to cells likely remains functional in these animals, but further Glc metabolism through glycolysis or lipogenesis may be hampered. Curiously, knock-down of lipogenic genes *ACC* and *FAS* has been demonstrated to result in increased fat body glycogen levels (Garrido et al., 2015). As both of these genes are expressed at a lower rate in the *mlx*^{1} mutants (**I**, Fig. 6D), it is plausible that the observed elevated glycogen levels are a direct consequence of impaired lipogenesis. Lipogenesis and glycogen synthesis predominantly take place in the fat body, where Glc from hemolymph is directed. Upon the impairment of one of these Glc-storage pathways, it is conceivable that the other one is upregulated for compensation purposes. Accordingly, the high glycogen phenotype was rescued in glycogen synthase and ACC or FAS double knock-down animals (Garrido et al., 2015). However, whether impaired conversion of Glc to glycogen results in increased lipogenesis has not yet been addressed in flies.

4.4 Mondo-Mlx directly regulate the expression of TF *Cabut*

As Mondo-Mlx act as TFs, the phenotypes observed upon their loss are likely to be the consequence of deregulated gene expression. Thus, in order to discover candidate genes downstream of Mondo-Mlx contributing to sugar intolerance, we performed a microarray analysis from *mlx*^{1} mutants fed a moderate (5%) sugar diet. Because the sugar tolerance depends on Mondo-Mlx function in fat bodies, we chose this tissue to investigate their regulatory output. Nearly 200 genes were differentially

expressed (> 2-fold change; adjusted p-value <0.05) in *mlx*^{1} mutant fat bodies. Based on the KEGG and gene-set enrichment analysis, many of the affected genes are categorized as functioning in fatty acid and nitrogen metabolism (**I**, Figure 6B, C).

Interestingly, one of the most strongly downregulated genes in *mlx*^{1} mutants was *Cabut*. Moreover, our results demonstrated that *Cabut* expression is induced by sugar feeding and this induction depends on Mondo-Mlx (**II**, Fig 1A). Cabut belongs to the family of Krüppel-like (Klf) transcription factors, with Klf10 and Klf11 being its closest mammalian homologs. These TFs have been implicated in a broad range of cellular processes, including proliferation, differentiation, and apoptosis (Spittau and Krieglstein, 2012). In addition, Klf11 has been demonstrated to protect from fatty liver disease (Zhang et al., 2013), while genetic variants of *klf10* have been linked to susceptibility to type 2 diabetes (Gutierrez-Aguilar et al., 2007).

It is noteworthy that the Klf10 promoter possesses a sugar-responsive ChoRE that has been demonstrated to be directly bound by ChREBP-Mlx (Iizuka et al., 2011). However, the metabolic consequence of this supposed regulation has not been studied. Thus, to investigate whether Cabut is a direct target of Mondo-Mlx, we first examined the promoter region of the gene. We found two putative ChoRE sequences from the *Cabut* gene region: one upstream of the transcription start site, the second in an intron (**II**, Fig. 1B). Comparisons among Drosophilids revealed that these elements are conserved, and may hence be functionally relevant. To test this, we constructed luciferase-reporter vectors containing combinations of wild type and mutated ChoREs. Next, we measured the activity of these constructs in *Drosophila* S2 cells. However, we failed to detect a Glc-dependent response in these cells. Given that S2 cells have been proposed display characteristics of a macrophage-like lineage (Luce-Fedrow et al., 2008), it is possible that this cellular context lacks certain co-regulators necessary for Mondo-Mlx-dependent gene expression regulation. Thus, the human hepatocarcinoma cell line HepG2, which has been established as a relevant experimental setting for ChREBP (Jeong et al., 2011), we analyzed the Glc-responsiveness of the candidate *Cabut* ChoRE elements in this setting. The *Cabut* promoter reporter containing two ChoREs, or only ChoRE2, demonstrated increased activity in response to high Glc in the media, while loss of ChoRE1 reduced this significantly (**II**, Fig. 1E). This suggests that ChoRE1 may be relevant for sugar-responsiveness. To confirm direct regulation, we performed an Mlx-dependent chromatin immunoprecipitation (ChIP) assay from *Drosophila* S2 cells grown with or without Glc for 6h. This revealed that both ChoRE elements were significantly bound by Mondo-Mlx, and the enrichment was further increased by the addition of Glc (**II**, Fig. 1D). While looking into the physiological relevance of this regulation, we discovered that similar to *mlx*^{1}, ubiquitous knock-down of Cabut in flies resulted in dietary sugar intolerance (**I**, Fig 7A). In sum, our data demonstrates that Mondo-Mlx dependent regulation of Cabut is direct, sugar-dependent, and required for dietary sugar tolerance.

4.5 Cabut modulates molecular clock output

Klf10, Klf11, and Cabut have mostly been described as transcriptional repressors (Belacortu et al., 2012). However, both Klf10 and Klf11 have been demonstrated to regulate gene expression though interacting with transcriptional co-repressors and co-activators (Spittau and Krieglstein, 2012). For example, a recent report demonstrated, that by interacting on the chromatin with the Hippo-pathway effector Yorkie, Cabut functions as a transcriptional activator (Ruiz-Romero et al., 2015). Thus, the nature of the transcriptional regulation by Klf10, Klf11, and most likely for Cabut may depend on cellular context and the activity of other signaling pathways.

As Cabut is a direct sugar-inducible target of Mondo-Mlx, it likely controls a second tier of sugar-regulated gene expression. Moreover, since the loss of Cabut results in dietary sugar intolerance, it is likely that its function as a transcriptional regulator is needed for carbohydrate homeostasis. To study

the Cabut-dependent transcriptome, we collaborated with S. Kadener's lab in Israel. Microarray and RNA-sequencing experiments were performed from flies, where Cabut expression had been manipulated. Cabut is a vital developmental gene, and is crucial for such processes as dorsal closure (Belacortu et al., 2011). Moreover, due to its auto-regulatory function (Belacortu et al., 2012), forced Cabut expression may lead to unexpected outcomes. Therefore, it was not surprising that ubiquitous and even fat body-specific manipulations in Cabut expression resulted in embryonic or early lethality (data not shown). However, as Cabut expression has been suggested to be regulated by the core circadian clock (Abruzzi et al., 2011), a Tim-GAL4 driver was tested. Overexpression or knock-down of Cabut using this driver resulted in viable flies. Since the nature of the circadian rhythm is much less understood in larvae than adults, the latter developmental stage was chosen for our experiment setup. Tim is known to be heavily expressed in the brain. Moreover, in adults, a subset of the fat body surrounds the brain (Hwangbo et al., 2004). Consequently, the transcriptomic experiments were performed from fly heads, where Cabut was either knocked down or overexpressed by Tim-GAL4. For this, the animals were first starved and fed either increasing concentrations of a sucrose or exposed to sucrose diet for different periods of time (0-18 h).

The data from the microarray experiment demonstrated that many Cabut-affected genes fall into such categories as metabolism, immune response, and CCGs (**II**, Fig. 2A). Hyperglycemia is known to cause an increase in ROS levels (Giacco and Brownlee, 2010). ROS, on the other hand, are both mediators and inducers of defense responses such as inflammation and immune signaling (Mittal et al., 2014). Thereby, Cabut may function to modulate the inflammatory response elicited by dietary sugar-generated ROS. In addition, the evidence of both CCGs and metabolic genes downstream of Cabut suggests that this TF may coordinate nutritional and time-of-day cues. In support of this hypothesis, lost behavioral rhythmicity in flies was detected upon Cabut over-expression (**II**, Fig. 6A). Such a phenotype is a clear indicator of aberrant circadian rhythm in flies. Experimental evidence from ChIP tiling array assays indicates that similar to its mammalian counterpart *klf10*, *cabut* promoter is directly bound by the components of the molecular clock (Abruzzi et al., 2011). Thus, Cabut may be regulated by the circadian clock and dietary sugars to coordinate downstream circadian gene expression control with nutritional cues. To this end, an RNA-sequencing experiment from fly heads harvested during different times throughout the day was performed, and the effect of Cabut manipulation on circadian gene oscillations was analyzed. The cycling of core circadian genes (*Tim*, *Clk*, *vri*) was unaffected by Cabut (**II**, Fig 6B, D). Strikingly, however, a number of genes ceased to oscillate in both Cabut RNAi and over-expression samples. More peculiarly, a subset of genes not cycling in control background, could be seen oscillating upon Cabut manipulation (**II**, Fig 6B, D). When the affected genes were analyzed according to their gene ontology classification, the Cabut RNAi group was significantly enriched in metabolic processes (**II**, Fig 6E). In, sum, these data indicate that sugar-induced Cabut feeds nutritional information into circadian rhythm by executing oscillatory transcriptional control.

4.6 Cabut represses metabolic genes, including both PEPCK isoforms

The sugar-induced temporal RNA-sequencing experiment demonstrated that Cabut functions to repress a number of metabolic genes in response to sugar feeding (**II,** Fig. 2D, E). Cabut-mediated repression appears to be rapid, as the full effect is visible already after 6 h of sugar-feeding. The evidence of such targets as the lipase *brummer* and gluconeogenic genes (*pepck*, *fbp*) suggests that Cabut likely functions to inhibit energy generation from lipolysis and Glc-production from other metabolites, when sugars are present in the food.

The strongest affected gene in the Cabut-dependent transcriptome experiments encodes PEPCK-C PEPCK (CG17725; *pepck1*). PEPCK-C is the rate-limiting enzyme of both GlcNG and GlyNG and is thus subjected to strict regulation. The main level of PEPCK-C regulatory control has been

suggested to occur at the level of transcription (Hanson and Reshef, 1997). Many TFs sensitive to the insulin-glucagon balance are potent inducers of GlcNG gene expression in response to starvation. These include the CREB-CRTC complex upon short term starvation (Altarejos and Montminy, 2011), as well as the FoxO, HNF4α, and PGC1-α mediated transcriptional regulation upon prolonged fasting (Puigserver et al., 2003; Rhee et al., 2003). FoxO, especially, has received attention in GlcNG gene expression control, as increased hepatic Glc output upon insulin insensitivity can be attenuated by loss of FoxO (Samuel et al., 2006). Thus, it is unsurprising that PEPCK-C has been implicated in the development of type 2 diabetes (Beale et al., 2007).

Notably, in the *Drosophila* genome, the gene encoding the mitochondrial variant of PEPCK (PEPCK-M encoded by *pepck2*; CG10924) is located immediately next to the cytoplasmic variant. Moreover, both isoforms appear to be regulated by Cabut, as their levels are several-fold higher in knock down animals (**II**, Fig 3, A, C, E). Through ChIP, we confirmed this regulation to be direct. *pepck1* expression was induced by fasting and rapidly inhibited by sugar re-feeding (**II**: Fig 3B; SFig. 6A, C). However, depleting Cabut by RNAi largely abolished the sugar-dependent downregulation of *pepck1* expression (**II**, Fig. 3B). These results suggest that in response to dietary sugars Cabut inhibits the expression of *pepck* genes.

In accordance with Cabut being an important effector of Mondo-Mlx, we showed that mlx^1 mutants demonstrated similar elevated gene expression levels of both *pepck* isoforms (**II**, Fig. 3C, E). More importantly, by expressing *Cabut* in the fat bodies mlx^1 mutants, we were able to alleviate the high expression of both *pepck* variants (**II**, Fig. 3D; SFig 6E). We conclude that fat body Mondo-Mlx mediated control of *Cabut* expression is crucial for *pepck* inhibition.

4.7 Cytoplasmic PEPCK is not essential for viability, but controls glycerol levels

To investigate the physiological function of PEPCK-C downstream of Mondo-Mlx and Cabut, an imprecise P-element excision mutant of *pepck1* was generated (*pepckΔ84*). To avoid genetic background-dependent effects, we used the mutant line *in trans* with a genomic deficiency line that lacks both *pepck* gene coding regions (*PEPCKΔ84/Df(2R)ED3636*). Accordingly, as a control, we used a precise excision P-element line in *trans* with *yw* background. qPCR experiments revealed that the *pepck1* expression in mutant/deficiency combination larvae was approximately 1000-times lower, when compared to controls (**II**, Fig. 4B), indicating that the *pepckΔ84* allele is likely null or at least strongly hypomorphic for *pepck1*. Mice lacking cytoplasmic PEPCK-C have been reported to succumb to neonatal lethality due to extreme hypoglycemia (Hakimi et al., 2005). In contrast, we observed comparable pupariation and eclosion rates between the *PEPCKΔ84/Df(2R)ED3636* and heterozygous *pepck1* flies (**II**, Fig. 4C). Moreover, their developmental speed and pupal volume were unaffected (**II**, SFig. 7B, C). This demonstrates that *pepck1* is non-essential for viability in flies. Notably, the expression of *pepck2* was unaltered in *PEPCKΔ84/Df(2R)ED3636* animals (data not shown). Thus, it can be speculated, that the mitochondrial variant may compensate the lack of *pepck1* to avoid lethality.

PEPCK-C is the rate limiting enzyme in GlcNG, with trehalose as the final product in insects. Therefore, we assayed both circulating Glc and trehalose levels from *PEPCKΔ84/Df(2R)ED3636* larvae. Despite being well fed, these animals displayed moderately reduced levels of both metabolites (**II**, Fig.4 D, E). In addition to limiting GlcNG, PEPCK-C is also crucial for the functioning of GlyNG – a stunted version of GlcNG, where the final product is G3P. To this end, we measured circulating glycerol levels in larvae lacking *pepck1*. Notably, *PEPCKΔ84/Df(2R)ED3636* larvae had nearly 60% less glycerol in their hemolymph, despite being well fed (**II**, Fig.4F). Similar results were obtained with pepck1 and pepck2 RNAi constructs (**II**, SFig. 7D), indicating that both PEPCK isoforms affect systemic glycerol levels. As G3P is a substrate for FFA esterification in the formation of TAG, we

measured whole body TAGs from animals lacking pepck1. In accordance with the circulating glycerol data, the TAG stores in *PEPCKΔ84/Df(2R)ED3636* adult flies were decreased by 57%, but this difference was only apparent in males (**II**, Fig. 4G). Thus, PEPCK function may have contrasting metabolic consequences in males and females. In agreement with this, through Klf10, PEPCK-dependent effects have been reported to result in sex-dependent alterations in metabolite levels in mice (Guillaumond et al., 2010). We conclude that loss of cytoplasmic PEPCK leads to reduced glycerol output, and consequently, lower TAG levels.

4.8 Mondo-Mlx and Cabut orchestrate glycerol homeostasis through *pepck* regulation

Due to very low *cabut* expression (**II**; Fig. 1A), mlx^1 mutant larvae display 5-7-times higher *pepck1* expression (**II**, Fig. 3C,D). As *pepck* directly reflects on circulating glycerol (**II**, Fig. 4B, F), we assayed the levels of this metabolite in mlx^1 mutant larvae. Importantly, we observed a more than 5-fold increase in the level of circulating glycerol (**II**; Fig. 5C, D) that correlated well with *pepck1* expression in mlx^1 mutants. To directly test whether excessive *pepck1* expression resulted in hemolymph glycerol accumulation in these animals, we combined the mlx^1 mutants with *PEPCKΔ84/Df(2R)ED3636*. Indeed, this combination resulted in a full rescue of high circulating glycerol levels, while elevated trehalose levels underwent a moderate decrease (**II**, Fig. 5D, E). We conclude that the elevated circulating glycerol, and to a certain extent, high trehalose levels in mlx^1 mutants are the consequence of high *pepck* expression.

Moreover, we noticed that the pupal lethality observed in mlx^1 mutants on standard lab diet was overcome by simultaneous loss of *pepck1* in these animals (**II**, Fig. 5F). However, the mlx^1 dietary sugar intolerant phenotype was unaffected in this combination (data not shown). It is likely, that control of GlyNG at the level of *pepck* expression by Mondo-Mlx and Cabut functions to direct the flow of carbon backbones obtained from Glc. Namely, upon high Glc conditions, efficient Glc-clearance is achieved by promoting Glc-utilizing pathways, while competing processes, such as GlcNG and GlyNG, are inhibited. However, upon loss of functional Mondo-Mlx, glycolysis and lipogenesis are impaired. Moreover, due to low *cabut* expression, excess glycerol and trehalose are produced through PEPCK-dependent GlcNG and GlyNG. Combined, these aberrations tip the metabolic imbalance, leading to pupal lethality in mlx^1 mutants. In accordance with high the *pepck* expression in these animals, the PEP to OAA ratio was significantly increased in both mlx^1 mutant and Cabut RNAi larvae (**II**, Fig. 5A, B). However, Cabut RNAi animals displayed no significant differences in circulating glycerol levels (data not shown). This may be explained by functional lipogenic gene regulation in these animals **(II**, SFig. 8D). Therefore, upon loss of Cabut, excess carbon backbones from Glc, and glycerol produced through GlyNG are likely converted into TAGs. In agreement with this, female knockout mice of the mammalian orthologue Klf10 display increased plasma TAG content (Guillaumond et al., 2010). In sum, we conclude that Mondo-Mlx dependent regulation of Cabut is essential for proper *pepck* expression, and consequently, for glycerol and trehalose homeostasis.

4.9 Mondo-Mlx regulate SIK3 expression to promote sugar tolerance

While looking for other Mondo-Mlx downstream effectors in addition to Cabut, we identified SIK3 as their direct target. The *SIK3* promoter contains a putative ChoRE that shows conservation among *Drosophilae* (**III**, Fig. 2D). However, compared to the Cabut ChoRE elements, the SIK3 ChoRE sequence appears to be more degenerate, and as a result, displayed lower, yet still significant levels of enrichment in an Mlx-dependent ChIP (compare **II**, Fig. 1D and **III**, Fig. 2E). In agreement with this, mlx^1 mutants clearly displayed lower levels of *SIK3*, both in terms of developmental stage and tissue-specific expression (**III**, Fig. 2A, B). Moreover, *SIK3* expression was elevated in response to

sugar feeding in a Mondo-Mlx-dependent manner (**III**, Fig 2C). However, *cabut* expression appears to depend more on Mondo-Mlx than *SIK3*, as observed by lower basal-level expression in mlx^1 mutants (**II**, Fig 1A). This may indicate that other transcriptional regulators than Mondo-Mlx are potentially involved in controlling *SIK3* expression. We conclude that *SIK3* is transcriptionally downstream of Mondo-Mlx.

To study the physiological role of SIK3 downstream of Mondo-Mlx, we took use of published mutant flies. Two mutant SIK3 alleles have been generated through imprecise P-element excision by the Montminy lab: a null variant *SIK3Δ72* and a hypomorph *SIK3Δ48* (Wang et al., 2011). Complete loss of SIK3 in *SIK3Δ72* mutants has been reported to result in early larval lethality, while the hypomorphs are viable, yet display reduced starvation sensitivity due to lower TAG stores (Wang et al., 2011). However, when we tested the null mutation on a LSD, we noticed that close to half of the animals managed to develop to the pupal stage. Although, adding 15 % sucrose to the diet, abolished pupariation (**III**, Fig. 1A). Therefore, SIK3 is required for dietary sugar tolerance. This phenotype suggests that the "standard" lab diet used by Wang et al. (2011) likely had relatively high sugar concentration, and thus led to early larval lethality in *SIK3Δ72*.

4.10 Loss of SIK3 results in high sorbitol and trehalose levels

To obtain further insight to the sugar-intolerant phenotype observed upon loss of SIK3, we analyzed the levels of several relevant metabolites. Due to the lack of a precise P-element excision line, we used a *yw* line, which corresponds to the original genetic background of *SIK3Δ72* mutant animals, as a control genotype. In contrast to mlx^1, but similarly to loss of Cabut (**I**, Fig. 4A, 7E), *SIK3Δ72* mutants displayed comparable circulating Glc levels with controls (**III**, Fig. 1E). This suggests that dietary sugar intolerance cannot solely be explained by hyperglycemia, and is likely a complex phenotype. Similar to mlx^1 mutants, however, the *SIK3Δ72* mutants showed elevated sorbitol and circulating trehalose levels (**III**, Fig. 1F, H). These results indicate, that even though circulating Glc levels are maintained in a normal range upon loss of SIK3, it may come at a cost of increased polyol pathway activity, resulting in sorbitol build-up. We demonstrated that the high circulating trehalose in mlx^1, however, is at least partly the result of high *pepck* expression in these mutants (**II**, Fig. 3C; 5E). Importantly, the SIK kinases are well-established repressors of the co-activator CRTC (Choi et al., 2011; Itoh et al., 2015; Lee et al., 2015). CRTC together with CREB function to promote the expression of GlcNG genes, including *pepck.* In addition, over-expression of HDAC4 in flies has been demonstated to result in elevated *pepck* expression (Wang et al., 2011). Importantly, due to the lack of inhibitory phosphorylation, HDAC4 has been found to be over-active in SIK3 mutant flies (Wang et al., 2011). In agreement with this, we observed high *pepck1* expression in *SIK3Δ72* mutants (**Figure 10**). Thus, similar to mlx^1, overactive GlcNG may contribute to increased circulating trehalose levels in *SIK3Δ72* mutants.

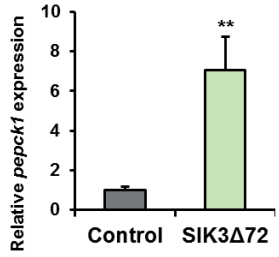

Figure 10: The expression of* pepck1 *is elevated in* SIK3Δ72 *mutants. *qPCR experiment from 1st instar larvae grown on LSD. Gene expression was normalized to Actin.*

We also confirmed the previously published observations that loss of SIK3 leads to reduced TAG stores (Wang et al., 2011; **III**, SFig. 1B). Moreover, we determined that lower TAG levels were not a consequence of reduced lipogenic gene expression profile in these animals (**Figure 11**). On the contrary, lipogenic gene expression seems to be enhanced in SIK3 mutants in a manner that is independent of Mondo-Mlx (data not shown). In mammals, SIK1 is known to inhibit the function the lipogenic gene regulator SREBP1-c (Yoon et al., 2009). Flies, however, lack this SIK variant, and

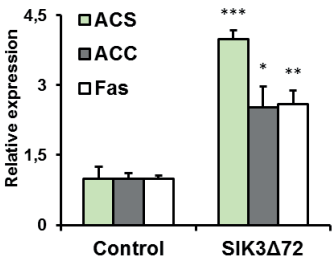

Figure 11: The expression of lipogenic genes is increased upon loss of SIK3. *qPCR data from 1st instar larval samples grown on LSD. Gene expression was normalized to Actin.*

instead, SIK2 has been proposed to cover the function of both SIK1 and SIK2. Yet, SIK2 displays a predominant expression in the brain (Choi et al., 2011; Wang et al., 2008). Thus it is possible that in more metabolic tissues, such as the fat body, where SIK3 is the predominant variant in the fly, SREBP function may also be modulated by SIK3.

Recently, a high carbohydrate diet was demonstrated to cause high carbohydrate-dependent lethality in *Drosophila* mutants of the TGFβ ligand *Dawdle*. Curiously, this phenotype was accompanied by hemolymph acidification (Ghosh and O'Connor, 2014). Intriguingly, we found the same phenotype manifested by both mlx^1 and *SIK3Δ72* mutant larvae (**III**, Fig. 1I; SFig. 2B), indicating that loss of SIK3 downstream of Mondo-Mlx may lead to lowering of hemolymph pH levels. Next, we investigated whether elevated lactate levels could help explain the acidification observed in *SIK3Δ72* mutants. We discovered, however, that the low hemolymph pH occurs independently of dietary sugar feeding, while lactate elevation is only apparent on a HSD (**III**, Fig. 1G, I). Ghosh and O'Connor reported that the systemic acidosis due to low hemolymph pH is the consequence of accumulated TCA intermediates in *Dawdle* mutants. Whether such a metabolic imbalance also occurs in *SIK3Δ72* and mlx^1 mutants, has yet to be determined.

4.11 SIK3 directly interacts with Glucose-6-phosphate dehydrogenase to induce its phosphorylation

Our group recently demonstrated that Mondo-Mlx regulate the PPP by increasing the expression of several genes in the pathway in a sugar-inducible manner (Mattila et al., 2015). Moreover, Mondo-Mlx directly bind to the promoter of the *zwischenferment* (*zw*) gene that encodes the rate-limiting enzyme of the pathway: Glucose-6-phosphate dehydrogenase (G6PD) (Mattila et al., 2015). Consequently, the mlx^1 mutants display strongly reduced *zw* expression. Moreover, knock-down of G6PD in the fly renders the animals sugar intolerant and results in decreased TAG levels, likely due to a decreased level of $NADP^+$ reduction (Mattila et al., 2015). As HSD causes similar aberrations in *SIK3Δ72* mutants, we looked for evidence of potential cross-talk between these two Mondo-Mlx targets.

To this end, we performed a co-immunoprecipitation between different SIK3 and G6PD isoforms over-expressed in S2 cells. The results demonstrate that SIK3 and G6PD directly interact in an isoform-independent manner (**III**, Fig. 3A). In addition, by performing a Phos-Tag Western blot (Kinoshita et al., 2006), we noted the presence of several phosphorylated bands of G6PD upon SIK3 co-over-expression (**Figure 12**, upper panels). Through *in vitro* kinase assay, we further confirmed

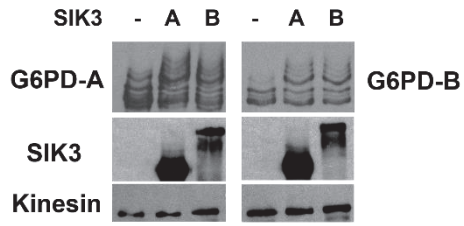

Figure 12: Co-expression of G6PD and SIK3 in S2 cells results in the appearance of phosphorylated bands. *Phosphorylation is isoform (A and B, for both SIK3 and G6PD) independent. Tagged constructs were expressed in Drosophila S2 cells. Samples were lysed and resolved on a Phos-Tag Western blot. Kinesin was used as a loading control.*

the SIK3-dependent phosphorylation of G6PD (**III**, Fig. 3C). Although a SIK3-dependent phosphorylation consensus sequence has been characterized based on several targets (Wang et al., 2011; Wehr et al., 2013), we did not find any visible matches in the G6PD protein. This may indicate either that other, as yet uncharacterized, SIK3 consensus sequences exist, or the witnessed SIK3-dependent G6DP phosphorylation occurs indirectly through an unknown SIK3-activated kinase. Thus, to identify the SIK3-dependent phosphorylation sites in G6PD, a mass-spectrometric analysis from purified G6PD protein co-expressed with or without SIK3 was performed. This resulted in the identification of 8 phosphorylation sites, of which 6 were detected only upon SIK3 co-expression (**III**, Fig., 3D; SFig. 3B). Notably, as SIK3 is a Ser/Thr kinase, the presence of Tyr384 among the SIK3-dependent phosphorylation sites points to the presence of secondary phosphorylation event.

4.12 SIK3 promotes G6PD activation in a sugar-dependent manner

To dissect the physiological relevance of SIK3-dependent G6PD phosphorylation, we created transgenic flies with: a wild type G6PD, and a 6-fold mutant G6PD, where the SIK3-dependent phosphorylation sites were mutated into either Ala (Ser and Thr), or Phe (Tyr). Both protein variants were strep- and hemagglutinin (HA)-tagged. By using a ubiquitous GAL4 driver, we expressed the G6PD transgenes in larvae grown on LSD, and exposed to 16 h of HSD. Next, the larvae were lysed and subjected to affinity purification by using Strep-Tactin microplate wells. This was followed by direct measurement of the purified G6PD enzymatic activity. To normalize the obtained values to the amount of input G6PD protein, we performed an HA-dependent Western blot from the total lysate, followed by digital signal intensity quantification with the Odyssey infrared imaging system. When we compared the normalized enzymatic activity of the two affinity purified proteins, we observed that the activity of the wild type G6PD was increased by HSD, while no change occurred in the 6-fold mutant (**III**, Fig. 3E). This result suggested that SIK3-dependent G6PD phosphorylation functions to increase the enzyme activity in response to sugar feeding.

To further confirm this, we measured G6PD enzyme activity from *SIK3Δ72* larvae exposed to HSD for 16h. In agreement with the previous experiment, the enzyme activity in control animals was increased after sugar feeding. However, this response was absent in stage-matched *SIK3Δ72* mutants (**III**, Fig. 3F). Furthermore, *zw* expression upon loss of SIK3 was unchanged (**III**, Fig 3H). Thus, we conclude that the SIK3-dependent activation of G6PD in response to dietary sugar clearly occurs at a post-translational level, and depends on some or all of the identified phosphorylation sites.

4.13 Mondo-Mlx and SIK3 synergize to regulate G6PD and sugar tolerance

Both *SIK3* and *zw* lie transcriptionally downstream of Mondo-Mlx, and their expression is promoted in response to sugar feeding (Mattila et al., 2015). Loss of the sugar-induced gene expression regulation would be expected to reflect also in G6PD enzyme activity. In agreement with this, G6PD RNAi reduced G6PD enzyme activity (**III**, SFig. 3C). To this end, we sought out to measure G6PD activity in mlx^{1} mutants. Similar to *SIK3Δ72*, we observed the lack of sugar-induced upregulation of G6PD activity in mlx^{1} mutants (**III**, Fig. 3G). Thus, by increasing *zw* expression and promoting the transcription of its upstream activator, Mondo-Mlx appear to promote G6PD activity on two levels. In terms of physiological consequence, such a bifunctional control may allow fine-tuning the G6PD enzymatic activity over broader ranges of dietary sugar intake. Therefore, a loss of both activating regulators would be expected to exacerbate the dietary sugar intolerance in these animals. To test this, we performed a double knock-down experiment by targeting both Mondo and SIK3, and feeding these animals a moderate (5 %) sugar diet. Single RNAi against either targets did not impair pupariation, while simultaneous knock-down of Mondo and SIK3 significantly slowed down development and abolished pupariation in the presence of 5 % sugar (**III**, Fig 3I). In order to find further support for

the synergistic effect on sugar tolerance by SIK3 and Mondo-Mlx, we combined the *SIK3Δ72* and *mlx*^{1} mutants. The resulting animals appeared to do unwell even on LSD (data not shown), as none of them developed past the 2nd instar stage. To escape the developmental problem while investigating dietary sugar intolerance, we reared the double mutant larvae on a diet containing 20 % sucrose as the sole nutritional source. Our results demonstrated that knock down of either SIK3 or Mondo-Mlx decreases larval survival in these conditions (**I**, Fig. 1C; **III**, Fig. 1C). A loss of both SIK3 and Mondo-Mlx, however, further sensitized the animals to dietary sucrose (**III**, Fig. 3J). Combined, our results demonstrate the synergistic control of dietary sugar tolerance by Mondo-Mlx and SIK3.

4.14 SIK3-dependent G6PD activation is crucial for diet-dependent redox balance maintenance

The PPP and G6PD are key mediators of reducing cytoplasmic $NADP^+$ to NADPH. By being a substrate for reducing glutathione disulfide into glutathione, NADPH is necessary for the maintenance of proper cellular antioxidant profile. The cellular pool of reduced glutathione is vital for fighting oxidative damage (Pompella et al., 2003). As the G6PD activity appears to be decreased upon HSD in *SIK3Δ72* and *mlx*^{1}, we measured the balance of NADPH to $NADP^+$ in these animals. In accordance with our previous data, the NADPH/$NADP^+$ ratio in control animals underwent an increase in response to HSD, while this response was absent in *SIK3Δ72* and *mlx*^{1} mutants (**III**, Fig 4A; SFig. 4A). Because NADPH is essential for reducing glutathione disulfide, we also measured the reduced to oxidized ratio of glutathione in *SIK3Δ72* mutants. As a direct reflection of the NADPH/$NADP^+$ ratio, loss of SIK3 led to relatively lower levels of reduced glutathione (**III**, Fig 4C).

Hyperglycemia is a known inducer of oxidative stress (Giacco and Brownlee, 2010). Remarkably, experiments using SIK3 hypomorphs have demonstrated a slightly increased susceptibility to oxidative stress-inducer paraquat in adult flies (Wang et al., 2011). Thus, we hypothesized that a failure to upregulate G6PD activity in response to HSD, and consequently, the inability to maintain a sufficient reduced glutathione may render the *SIK3Δ72* mutants more susceptible to oxidative stress. Thus, as a readout of oxidative damage, we measured the levels of lipid peroxides in *SIK3Δ72* larvae. LSD had no effect on lipid peroxidation in control or mutant animals, while HSD significantly increased the level of lipid peroxides in *SIK3Δ72* larvae (**III**, Fig. 4F). Thereby, to test whether an impaired antioxidant profile and subsequently increased susceptibility to oxidative stress could contribute to sugar intolerance, we knocked down an enzyme involved in glutathione metabolism – Thioredoxin reductase 1 (TrxR-1). This enzyme is responsible for reducing thioredoxin in a NADPH-dependent manner (**III**, Fig 4B). In *Drosophila,* reduced thioredoxin is used to reduce glutathione (Kanzok et al., 2001). In support of the concept that glutathione reduction contributes to dietary sugar tolerance, the TrxR-1 RNAi had a lower pupariation rate on HSD, when compared to LSD (**III**, Fig. 4E).

The described results so far support the notion that HSD induces an increased requirement of antioxidants, and a failure to comply leads to dietary sugar intolerance. Thereby, the supplementation of exogenous antioxidants through feeding may relieve the level of oxidative stress, and consequent sugar intolerance. To test this, we compared the pupariation kinetics of *SIK3Δ72* mutant animals grown on LSD or moderate (5 %) sugar diet, with or without the addition of reduced glutathione. The supplementation of glutathione had no effect on control animals, nor on *SIK3Δ72* mutants reared on LSD. However, on a moderate sugar diet, the addition of glutathione significantly increased the number of pupariated animals (**III**, Fig. 4 D). This result further supports the notion that the dietary sugar intolerance witnessed in *SIK3Δ72* mutants is at least partly the consequence of impaired redox balance.

5. Final remarks and future perspectives

The work carried out in this thesis takes use of conserved pathways in *Drosophila*, to aid in the characterization of the physiological role of Mondo-Mlx TFs upon sugar feeding. Our results demonstrate that Mondo-Mlx activity is essential for dietary sugar tolerance. Furthermore, we provide additional evidence, that by directing the flow of carbon backbones, Mondo-Mlx promote Glc-clearance. Furthermore, our data implies that metabolic gene regulation and circadian rhythm control converge at the level of Mondo-Mlx target Cabut. We uncover a double regulatory function of Mondo-Mlx, whereby antioxidant profile in the form of glutathione is upregulated in the anticipation of higher oxidative stress due to increased sugar consumption.

Specifically, we characterize the phenotype of flies lacking *mlx*. As a striking observation, we note the developmental impairment of these animals in high dietary sugar conditions. Despite increased channeling of Glc into glycogen, they are hyperglycemic and also display high circulating trehalose levels. By performing a microarray and ChIP experiments, we identify TF Cabut as a direct, sugar-inducible target, and secondary effector of Mondo-Mlx. Similar to its upstream regulators, we show that Cabut is required for dietary sugar tolerance. Moreover, we characterize Cabut as sugar-dependent transcriptional inhibitor of many metabolic genes, including both *pepck* variants. We dissect the importance of *pepck* gene expression regulation, and subsequent glycerol homeostasis, mediated by Mondo-Mlx through Cabut. The inability to inhibit *pepck* results in the accumulation of circulating glycerol that contributes to pupal lethality upon loss of Mondo-Mlx activity. We propose, that in order to help utilize the Glc consumed with food, this regulatory mechanism functions to limit GlyNG and GlcNG following feeding. Notably, we report that in *Drosophila*, the cytoplasmic PEPCK is not essential for viability, but whether this is due to functional redundancy of the mitochondrial PEPCK, has to be addressed by future studies. In addition, we demonstrate that Cabut interconnects nutrient sensing with circadian rhythm output. Altered Cabut activity results in aberrant locomotor rhythmicity. This reveals that Cabut integrates nutrient sensing and circadian rhythm output. However, whether Mondo-Mlx contribute to the cycling of circadian genes independently of Cabut, has yet to be determined.

We describe the importance of increasing reductive power in response to high sugar diet. Specifically, we demonstrate that G6PD activity directly correlates with organismal antioxidant profile in the form of reduced glutathione. Furthermore, we provide mechanistic evidence, that in addition to transcriptional upregulation of *G6PD*, Mondo-Mlx also promote the activity of this enzyme through their direct target gene encoding kinase SIK3. With the use of mass-spectrometric analysis, we identify a number of amino acid residues in G6PD that are phosphorylated in a SIK3 dependent-manner. In addition, we provide evidence, that these phosphorylation sites are important for augmenting the G6PD enzyme activity post-translationally following high sugar consumption. However, whether G6PD is directly phosphorylated by SIK3, or if secondary kinases downstream of SIK3 are involved, will be the topic of future studies. As a result, loss of either SIK3 or Mondo-Mlx function abolishes the sugar-dependent increase in G6PD activity and subsequent elevation in the $NADPH/NADP^+$ ratio. As a direct repercussion, SIK3 mutant animals have lower levels of reduced glutathione, and consequently, display hallmarks of oxidative damage. These defects in SIK3 mutants directly contribute to dietary sugar tolerance, as observed by a moderate rescue of this phenotype following reduced glutathione supplementation with the diet. Interestingly, both SIK3 function and reduced NADPH levels have recently been implicated in circadian control (Funato et al., 2016; Rey et al., 2016). Therefore, the regulation of *G6PD* and *SIK3* expression by Mondo-Mlx suggests that, in addition to Cabut-mediated cycling of circadian genes, these Mondo-Mlx may have a more extensive involvement in circadian rhythm.

Thus, the results of this thesis describe the relevance of directing the flow of carbon backbones, maintaining a redox balance, and coordinating circadian rhythm function in response to sugar sensing

(see summarizing **Figure 13** on the next page). In sum, our results emphasize that dietary sugar tolerance is genetically multi-factorial, as loss of Mondo-Mlx and several of their targets (Cabut, SIK3, G6PD and others) results in impaired survival upon high sugar diet. Thus, we characterize Mondo-Mlx as master regulators of pathways required for healthful Glc-utilization. As the components of the identified networks are all well conserved between *Drosophila* and mammals, the results of this thesis help understand the steps required for healthful sugar intake also in the context of human health. Moreover, such knowledge can be valuable in designing new treatment approaches or drugs to help fight metabolic disorders.

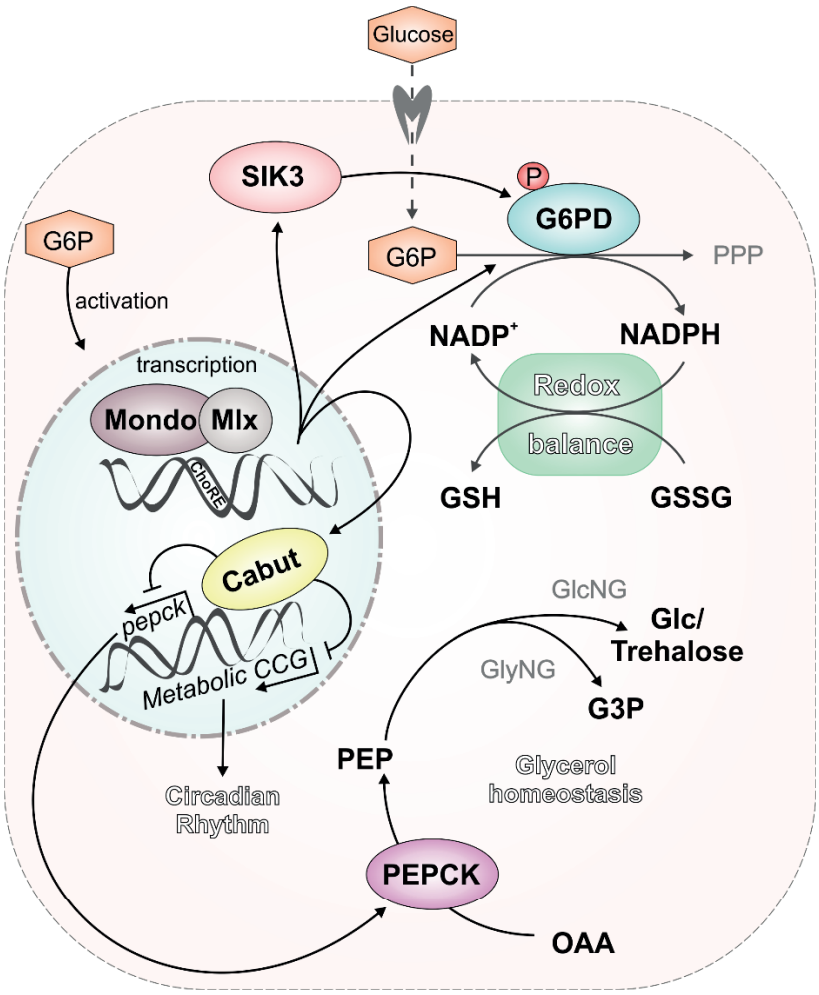

Figure 13: Summary of the uncovered Mondo-Mlx-regulated pathways in this thesis. *In response to increased Glc levels, Mondo-Mlx complex promotes the expression of target genes* G6PD *(Mattila et al., 2015),* SIK3 *and* Cabut *by binding to ChoRE elements present in their promoters. Cabut functions as a secondary effector downstream of Mondo-Mlx, and inhibits the expression of many metabolic genes, including both* pepck *variants and CCGs. This regulation is required to maintain glycerol homeostasis and proper circadian rhythm in response to sugar feeding. Secondly, we demonstrate that the Mondo-Mlx transcriptional targets SIK3 and G6PD interact at the protein level, resulting in the phosphorylation and subsequent activation of G6PD. As a result, the cellular reductive power in the form of increased NADPH/NADP$^+$ ratio is elevated, leading to increased levels of reduced glutathione. Combined, the regulation of G6PD through SIK3 and Mondo-Mlx is necessary for redox balance maintenance and dietary sugar tolerance.*

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