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# Transcriptional Control of Dietary Sugar Metabolism and Homeostasis by Mondo-Mlx Transcription Factors

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# Transcriptional control of dietary sugar metabolism and homeostasis by Mondo-Mlx transcription factors

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“Just keep swimming”
– Dory

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## LIST OF ORIGINAL PUBLICATIONS

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I **Havula E**, Teesalu M, Hyötyläinen T, Seppälä H, Hasygar K, Auvinen P, Orešič M, Sandmann T, Hietakangas V. 2013. *Mondo-Mlx-regulated transcriptional network is essential for dietary sugar tolerance in Drosophila.* PLoS Genet. Apr;9(4):e1003438.

II Mattila J*, **Havula E***, Suominen E, Teesalu M, Surakka I, Hynynen R, Kilpinen H, Väänänen J, Hovatta I, Käkelä R, Ripatti S, Sandmann T, Hietakangas V. 2015. *Mondo-Mlx mediates organismal sugar sensing through the Gli-similar transcription factor Sugarbabe*. Cell Rep. Oct 13;13(2):350-64

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**Contributions:**

I The author designed all the experiments with VH, performed the majority of the experiment and wrote the manuscript with VH.

II The author designed the experiments with JM and VH, performed the experiments with JM and other authors, and wrote the manuscript with JM and VH.

**ABBREVIATIONS**

| | |
|---|---|
| 2-DG | 2-deoxyglucose |
| 4E-BP | eukaryotic initiation factor 4E binding protein |
| aay | astray |
| ACC | acetyl-CoA carboxylase |
| AcCoAS | acetyl CoA synthase |
| ACS | acetyl-CoA synthetase |
| AgRP | agouti-related peptide |
| AKH | adipokinetic hormone |
| AKT | protein kinase B |
| Ald | aldolase |
| Aldh-III | aldehyde dehydrogenase type III |
| AMP | adenosine monophosphate |
| AMPK | AMPK-activated protein kinase |
| Amy-p | amylase proximal |
| ANGPTL6 | angiopoietin-related protein 6 |
| ApoD | apolipoprotein D |
| ARC | arcuate nucleus |
| ATGL | apidocyte triglyceride lipase |
| ATP | adenosine triphosphate |
| ATPCL | ATP citrate lyase |
| BAT | brown adipose tissue |
| bHLH/LZ | basic helix-loop-helix/leucine zipper |
| CA | corpora allata |
| CC | corpora cardiaca |
| CCHa2 | CCHamide-2 |
| CDC | cytoplasmic and dimerization domain |
| ChoRE | carbohydrate response element |
| ChREBP | carbohydrate-responsive element-binding protein |
| COUP-TF | chicken ovalbumin upstream promoter-transcription factor |
| CREB | cAMP response element binding protein |
| CRH | corticotropin-releasing hormone |
| CRISPR/Cas9 | clustered regularly interspaced short palindromic repeats/caspase 9 |
| DEC1 | deleted in esophageal cancer 1 |
| Desat1 | desaturase 1 |
| Dh44 | diuretic hormone 44 |
| dILP | *Drosophila* insulin-like peptides |
| DNA | deoxyribonucleic acid |
| DNL | *de novo* lipogenesis |
| Dpit47 | DNA polymerase interacting tpr containing protein of 47kD |
| E-box | enhancer box |
| eIF2α | eukaryotic translation initiator factor 2 α |
| ELOVL6 | fatty acid elongase 6 |
| Eno | enolase |
| ER | endoplasmic reticulum |
| FAS | fatty acid synthase |
| FGF21 | fibroblast growth factor 21 |
| FLCN | folliculin |
| FOXO1 | forkhead box O1 |
| FXR | farnesoid X receptor |
| G6P | glucose-6-phosphate |
| G6PDH | glucose-6-phosphate dehydrogenase |

| | |
|---|---|
| Gadd34 | growth arrest and DNA-damage-inducible 34 |
| GAL4 | galactose gene transcription factor 4 |
| Gbp | growth-blocking peptide |
| GCN2 | general control nonderepressible 2 |
| GK | glucokinase |
| GKRP | glucokinase regulatory protein |
| GlcT-1 | ceramide glucosyltransferase |
| GLIS | Gli-similar transcription factor |
| GLUT1-4 | glucose transporter type 1-4 |
| GPAT | glycerol-3-phosphate acyl-transferase |
| GPDH | glycerol 3-phosphate dehydrogenase |
| GRACE | glucose response activation conserved element |
| Gs1 | glutamine synthetase 1 |
| Gs2 | glutamine synthetase 2 |
| GSM | glucose-sensing module |
| GTP | guanosine triphosphate |
| GWAS | genome-wide association studies |
| HAT | histone acetyltransferase |
| HDAC | histone deacetylase |
| HIF1α | hypoxia-inducible factor 1α |
| HK | hexokinase |
| HNF4α | hepatocyte nuclear factor 4α |
| IGF | insulin-like growth factor |
| InR | insulin receptor |
| IPC | insulin-producing cells |
| iPSC | induced pluripotent stem cells |
| IR | insulin receptor |
| KLF10 | krüppel-like factor 10 |
| KLF4 | krüppel-like factor 4 |
| LDH-A | lactate dehydrogenase A |
| LID | low-glucose inhibitory domain |
| LPK | L-type pyruvate kinase |
| Lsd | lipid storage droplet |
| LXR | liver X receptor |
| MAP4K3 | mitogen-activated protein kinase kinase kinase kinase 3 |
| MC4 | melanocortin 4 |
| MCR | Mondo conserved region |
| MFS | major facilitator superfamily |
| MLST8 | mammalian lethal with SEC13 protein 8 |
| MLX | Max-like protein X |
| MML-1 | Myc and Mondo-like protein |
| MODY1 | maturity-onset diabetes of the young 1 |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NAFLD | nonalcoholic fatty liver disease |
| NASH | non-alcoholic steatohepatitis |
| NES | nuclear export signals |
| NLaz | Neural Lazarillo |
| NLS | nuclear localization signal |
| NPY | neuropeptide Y |
| O-GlcNAc | O-linked β-N-acetylglucosamine |
| OCT | octamer-binding transcription factor |
| OMM | outer mitochondrial membrane |
| ORF | open reading frame |
| Pepck | phosphoenolpyruvate carboxykinase |

| | |
|---|---|
| PFK2 | phosphofructokinase 2 |
| PFKFB3 | 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase |
| PG | prothoracic gland |
| PGC1α | PPARγ co-activator 1α |
| Pgd | 6-phosphogluconate dehydrogenase |
| Pgi | phosphoglucose isomerase |
| PI3K | phosphoinositide 3-kinase |
| Pi3K59F | phosphotidylinositol 3 kinase 59F |
| PKA | cAMP dependent protein kinase A |
| PNPLA3 | patatin-like phospholipase domain-containing 3 |
| POMC | pro-opiomelanocortin |
| PPAR | peroxisome proliferator-activated receptor |
| PPP | pentose phosphate pathway |
| PTTH | prothoracicotropic hormone |
| PUFA | polyunsaturated fatty acid |
| PVN | paraventricular nucleus |
| PyK | pyruvate kinase |
| qPCR | quantitative polymerase chain reaction |
| RAPTOR | regulatory-associated protein of TOR |
| RBP4 | retinol binding protein 4 |
| RICTOR | rapamycin-insensitive companion of TOR |
| RNA | ribonucleic acid |
| RNAi | RNA interference |
| Rpi | ribose-5-phosphate isomerase |
| RXR | retinoid X receptor |
| S6K | S6 kinase |
| SCAP | SREBP cleavage-activating protein |
| SCD-1 | stearoyl-CoA desaturase 1 |
| SCN | suprachiasmatic nucleus |
| SIK2 | salt-inducible kinase 2 |
| SLC2 | solute carrier family 2 |
| SOX2 | sex determining region Y-box 2 |
| SRE | sterol regulatory element |
| SREBP | sterol regulatory element-binding protein |
| STAT3 | signal transducer and activator of transcription 3 |
| TAG | triacylglycerol |
| Taldo | transaldolase |
| TFEB | transcription factor EB |
| TGF | transforming growth factor |
| TIEG | TGF inducible early gene-1 |
| TOR | target of rapamycin |
| TORC1 | TOR complex 1 |
| TORC2 | TOR complex 2 |
| TR | thyroid hormone receptor |
| tRNA | transfer RNA |
| TSC2 | tuberous sclerosis complex 2 |
| TXNIP | thioredoxin-interacting protein |
| UAS | upstream activation sequence |
| UCP1 | uncoupling protein 1 |
| Upd2 | unpaired 2 |
| USF | upstream stimulatory factor 1 |
| VLDL | very low density lipoprotein |
| VPS34 | vacuolar protein sorting 34 |
| WAT | white adipose tissue |

| | |
|---|---|
| WBSCR14 | Williams-Beuren syndrome critical region gene 14 |
| Xu-5P | xylulose-5-phosphate |
| Zw | Zwischenferment |

**SUMMARY**

Sugars, amino acids and lipids provide the energy and building blocks for growth and maintenance in all animals. However, animal species display great variation in their dietary preferences. The optimal diet of even closely related species can vary tremendously. Also human populations and individuals vary in their dietary behavior and physiological responses to dietary interventions. Moreover, the high amounts of refined sugars in the modern human diet are suggested to contribute to the development of metabolic diseases such as metabolic syndrome and type 2 diabetes.

Multicellular animals sense and control their energy homeostasis continuously by integrating nutritional, hormonal and neuronal inputs from their internal and external environment. For example, the counteracting hormones Insulin and Glucagon maintain the levels of circulating glucose constant during fluctuating nutritional conditions. At the cellular level, macronutrients are sensed by distinct mechanisms. The nutrient sensors and their downstream signaling pathways are activated in response to specific nutrients, and they ensure the metabolic homeostasis. Sugars are sensed by highly conserved transcription factor paralogs ChREBP (Carbohydrate-responsive element-binding protein) and MondoA, which share the same heterodimerization partner Mlx. ChREBP/MondoA-Mlx heterodimers regulate the majority of sugar-induced transcription in mammals, including genes of the glycolytic and lipogenic pathways. Dysregulation of ChREBP has been associated with the development of type 2 diabetes, and polymorphisms of *ChREBP* with circulating triglyceride levels and increased risk of coronary artery disease.

The *Drosophila* genome encodes single orthologues for ChREBP/MondoA and Mlx called Mondo and Mlx, respectively. The function of the mammalian ChREBP/MondoA-Mlx has been largely studied *in vitro*. The aim of this thesis was to characterize the *in vivo* function of *Drosophila* Mondo-Mlx and to identify its target genes and their roles in regulating sugar metabolism. Due to the lack of genetic redundancy and with the extensive toolkit available, the *Drosophila* provides an optimal *in vivo* model for studying the role of Mondo-Mlx in nutrient sensing and metabolism.

In this thesis, I demonstrate the physiological importance of Mondo-Mlx for organismal sugar tolerance. The *mlx* null mutant animals display severe sugar intolerance and a gene expression profile that confirms the role of Mondo-Mlx as a key regulator of glycolytic and lipogenic genes also in *Drosophila*. Furthermore, we expand the role of Mondo-Mlx as a metabolic regulator by showing that it directly controls the expression of several key enzymes of lipid storage, pentose phosphate pathway and amino acid metabolism in response sugars. We also show that Mondo-Mlx is a master regulator of a gene regulatory network composed of a secondary tier of transcriptional effectors including GLI similar transcription factor Sugarbabe and Krüppel-like factor Cabut. The metabolic profiling of the *mlx* null mutant animals revealed that in addition to being hyperglycaemic, the mutants show signs of amino acid catabolism and elevated ceramide levels that indicate lipotoxicity.

This thesis demonstrates the use of *Drosophila* in studying the genetic basis of dietary sugar tolerance and metabolism. It reveals a number of new metabolic pathways and downstream effectors regulated by Mondo-Mlx, broadening its role as a master regulator of sugar-induced transcription.

## YHTEENVETO

Ravinnosta saatavat sokerit, aminohapot ja rasvat tarjoavat kaikille eliöille paitsi energiaa, myös tarvittavat rakennuspalikat kudosten kasvuun ja ylläpitoon. Eliöt poikkeavat huomattavasti toisistaan niille sopivan ravinnon suhteen ja aivan lähisukuisetkin lajit saattavat suosia hyvin erilaista ravintoa. Myös ihmiset ja ihmispopulaatiot poikkeavat ravintokäyttäytymisessä toisistaan. Nykyisen länsimaisen ravinnon, joka pitää sisällään huomattavan määrän puhdistettuja sokereita, on ehdotettu olevan tyypin 2 diabeteksen sekä metabolisen syndrooman yleistymisen taustalla.

Eläimet aistivat ja kontrolloivat aineenvaihdunnan tasapainoa yhdistämällä hormonaalisia, hermostollisia sekä ravintoperäisiä signaaleja kehosta ja ympäristöstä. Esimerkiksi haiman erittämät vastavaikutteiset hormonit, insuliini ja glukagoni, säätelevät systeemisellä tasolla veren glukoosipitoisuutta. Solutasolla ravinnon aistinnasta vastaavat niin sanotut ”ravintosensorit”, jotka tunnistavat tiettyjä ravintoaineita ja aktivoivat signalointireittejä, jotka puolestaan säätelevät koko kehon aineenvaihdunnan tasapainoa eli homeostaasia. Solutasolla ravinnon sokereiden aistinnasta vastaavat konservoituneet ChREBP ja MondoA transkriptiotekijät, jotka yhdessä saman sitoutumiskumppaninsa, Mlx:n kanssa säätelevät valtaosaa sokeri-indusoituvasta geenien ilmentymisestä nisäkkäillä. ChREBP/MondoA-Mlx kohdegeeneihin lukeutuu muun muassa useita glykolyysin ja rasvasynteesin entsyymejä. ChREBP:n toiminnan häiriöt on yhdistetty ihmisillä tyypin 2 diabeteksen syntyyn. Lisäksi *ChREBP* varianttien (SNP) on havaittu olevan yhteydessä sepelvaltimotaudin syntyyn, sekä plasman triglyseridien määrään.

Toisin kuin nisäkkäillä, banaanikärpäsellä (*Drosophila*) on vain yksi ChREBP/MondoA paralogi, Mondo. ChREBP/MondoA-Mlx:n toimintaa on tutkittu nisäkkäillä lähinnä soluviljelyolosuhteissa ja tämän väitöskirjan tavoitteena oli karakterisoida banaanikärpäsen Mondo-Mlx transkriptiotekijöiden toiminta *in vivo*. Lisäksi selvitimme Mondo-Mlx kohdegeenejä, sekä tutkimme niiden toimintaa sokeriaineenvaihdunnan säätelyssä. Banaanikärpänen on erinomainen malli Mondo-Mlx:n toiminnan tutkimiseen paitsi mahdollisen redundanssin puuttumisen, myös erilaisten geneettisten työkalujen valtavan määrän ansioista.

Osoitan tässä väitöskirjassa Mondo-Mlx transkriptiotekijöiden olevan välttämättömiä banaanikärpäsen sokeritoleranssille. *Mlx* mutanttikärpäset ovat herkkiä ravinnon sokereille ja jo suhteellisen pienet määrät sokeria johtavat $mlx^{1}$ mutanttien kuolemaan kehityksen varhaisessa vaiheessa, sekä selvästi kohonneeseen veren (hemolymfa) glukoosipitoisuuteen. Geeniekspressioanalyysi varmisti Mondo-Mlx:n tärkeän roolin glykolyysin ja lipidisynteesin entsyymien säätelijänä myös banaanikärpäsessä. Nisäkästutkimuksissa aiemmin löydettyjen kohdegeenien lisäksi havaitsin Mondo-Mlx:n säätelevän myös muun muassa rasvojen varastointiin ja aminohappoaineenvaihduntaan liittyvien geenien ilmentymistä. Lisäksi havaitsin Mondo-Mlx:n säätelevän monien transkriptiotekijöiden ilmentymistä, jotka puolestaan säätelevät osaa Mondo-Mlx:n alavirran kohdegeeneistä. *Mlx* mutanttitoukkien metabolinen profilointi paljasti myös monia muita aineenvaihdunnan muutoksia. Alentuneet aminohappotasot sekä kohonneet ureapitoisuudet viittaavat siihen, että $mlx^{1}$ mutantit käyttävät aminohappoja energialähteenään.

Tämä väitöskirja osoittaa banaanikärpäsen olevan geneettisesti sekä fysiologisesti ihanteellinen malli sokeriaineenvaihdunnan tutkimiseen. Löysin työssäni useita uusia Mondo-Mlx säädeltyjä aineenvaihdunnan reittejä ja kohdegeenejä. Nämä tulokset laajentavat käsitystä ChREBP/MondoA-Mlx:n roolista aineenvaihdunnan säätelijänä.

# 1. INTRODUCTION

## 1.1. Nutrient sensing and metabolic homeostasis

Sugars, amino acids and lipids, collectively termed macronutrients, are consumed by all animals to provide energy and building blocks for maintenance and growth. Micronutrients, including vitamins and minerals, are needed in very small quantities, but are nevertheless vital for the function of all cells, where they serve for example as hormones and co-factors for enzymes.

There is tremendous variation between animal species in the composition of optimal diet. For example, some animals prefer high protein diet whereas some animals, such as hummingbirds, feed primarily on high-sugar content flower nectar. In contrast to dietary specialists, the flexible dietary generalists can survive on a wide range of diets. Human populations and individuals for instance show great variation in dietary behavior, also in the course of evolution. The modern human diet rich in carbohydrates and starch differs greatly from the typical diet of our ancestors, where the environment determined the diet of hunter-gatherers and fruits were the main source of dietary sugars (Perry et al. 2007; Luca et al. 2010; Lustig et al. 2012). There is also great variation in the feeding behavior between modern human populations, and between individuals within populations. Despite the differences between diets, most humans still develop a similar body weight and composition. A crosstalk between highly complex nutrient sensing and downstream signaling pathways ensures the proper metabolic homeostasis under fluctuating nutritional conditions.

Animals control their metabolic homeostasis by continuously integrating signals originating both from the body and environment. The endocrine system controls signaling between organs and nutrient partitioning into tissues. These signals can be nutritional, hormonal and neuronal, and they all carry information about the homeostatic status of a given macronutrient or its metabolite. The nutrient sensing mechanisms ensure the energy homeostasis of the body – the balance between catabolic and anabolic processes under fluctuating environmental and nutritional conditions. During fasting, the catabolic processes, such as the β-oxidation of fatty acids, provide an energy source for the cells to maintain essential functions. In turn, when nutrients are available, the anabolic processes that build up tissues and drive growth are activated. The mechanisms regulating the delicate balance of metabolic homeostasis are highly complex, and understanding the interplay between the nutrient sensing pathways is one of the central questions in physiology.

Nutrient sensing is controlled both at the systemic and cellular level. At the systemic level, the organs involved in energy homeostasis integrate multiple inputs from the environment to control appropriate outputs. For example, after feeding, elevated blood glucose levels trigger the release of Insulin from the β-cells of pancreas, which in turn signals mainly to liver, adipose tissue and skeletal muscle to activate glucose uptake in order to maintain stable blood glucose levels (Roder et al. 2016). At the cellular level, glucose is sensed by an intracellular glucose sensor, namely ChREBP/MondoA-Mlx, that initiates a transcriptional response, which ensures that proper metabolic pathways are activated (Havula and Hietakangas 2012).

### 1.1.1. Metabolic organs, tissues and digestion of macronutrients

Key organs in whole-body energy homeostasis include the brain, digestive tract, pancreas, liver, adipose tissue and muscle. The digestive tract is the first organ to receive information from our external environment after feeding. The digestion of macronutrients begins in the mouth by mechanical digestion. Additionally, amylase, an enzyme secreted in the saliva, begins to break down starch. Protein digestion by pepsin starts in the stomach. The main site for digestion is the small intestine, where digestive enzymes secreted from the pancreas and bile from the liver ensure a proper breakdown of all macronutrients. Proteins are degraded into peptides and amino acids by proteases such as trypsin, and lipids are degraded into fatty acids and glycerol by lipases. Pancreatic amylase continues the digestion of starch into simple sugars. The small intestine produces and secretes the disaccharide digesting enzymes sucrase, lactase and maltase. Digested macronutrients are then absorbed through the small intestinal epithelial tissue into the blood vessels lining the intestinal tract. The undigested carbohydrates are also partly broken down by the intestinal bacteria of the colon. In addition to being in charge of the digestion and absorption of nutrients, the digestive tract also secretes hormones that signal to other organs of the nutrient state of the body. For example, Ghrelin secreted by the stomach and small intestine is a

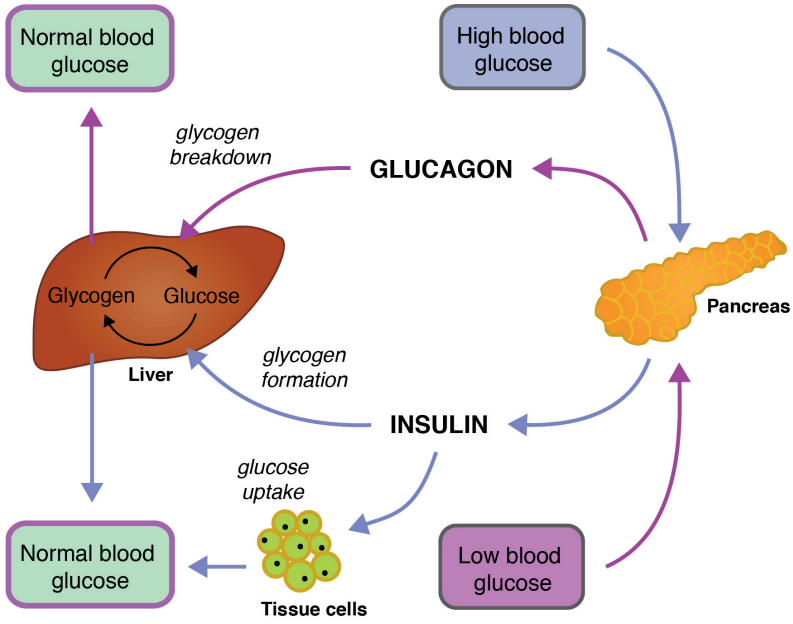


**Figure 1. Systemic regulation of glucose homeostasis by Insulin and Glucagon**
Glucose homeostasis is regulated at the systemic level by the counteracting hormones Insulin and Glucagon. When blood glucose levels rise, Insulin stimulates both the uptake of glucose in tissues and the formation of glycogen in the liver. When blood glucose levels fall, Glucagon stimulates the breakdown of glycogen to glucose. Modified from Benjamin Cummings, 2001.

signal of nutrient shortage, and Glucagon-like peptide 1 secreted by the intestine is a signal of nutrient arrival (Cummings and Overduin 2007).

In addition to secreting most of the key digestive enzymes, the pancreas has a central role in regulating glucose homeostasis via the endocrine system by secreting multiple hormones. The counteracting hormones, Insulin and Glucagon, secreted by the β-cells and α-cells of pancreas, respectively, maintain blood glucose at a constant level (Roder et al. 2016) (Figure 1). During fasting, Glucagon both promotes hepatic glycogenolysis and gluconeogenesis to increase blood glucose levels. Insulin secretion is stimulated by feeding. After binding to its receptors in muscle and adipose tissue, it activates an intracellular signaling cascade that leads to the translocation of GLUT4 glucose transporters on the plasma membrane. In addition, Insulin acts as an anabolic hormone by promoting glycogenesis, lipogenesis and protein synthesis (Biolo et al. 1995; Roder et al. 2016). The pancreas also secretes somatostatin, which inhibits Insulin and Glucagon secretion (Gerich et al. 1974; Hauge-Evans et al. 2009).

The liver has a central role in whole-body energy homeostasis. Excess dietary carbohydrates are either stored as glycogen or transformed into lipids in the liver (Rui 2014). Glycogenesis, a process where glucose monomers are added into long glycogen polymers, is driven by Glycogenin and Glycogen synthase (Roach et al. 2012). In *de novo* lipogenesis (DNL), dietary carbohydrates are converted into fatty acids, which are further esterified with glycerol-3-phosphate to generate triacylglycerol (TAG). TAGs can be stored in the liver or they can be secreted from the hepatocytes as very low density lipoprotein (VLDL) particles and transported to other tissues, mainly adipose tissue, via the circulation (Rui 2014). DNL takes place mainly in the liver and to a lesser extent in the adipose tissue (Bjorntorp and Sjostrom 1978; Trayhurn 1981). DNL is promoted by feeding via two separate mechanisms. Increased Insulin signaling and increased intracellular glucose activate the Sterol regulatory element-binding protein (SREBP) and Carbohydrate-responsive element-binding protein (ChREBP), respectively, which activate the transcription of key lipogenic genes such as *fatty acid synthase* (*FAS*) and *acetyl-CoA carboxylase* (*ACC*) (Dentin et al. 2004). Liver releases also many cytokines, collectively known as hepatokines. Key hepatokines include Angiopoietin-related protein 6 (ANGPTL6), Fibroblast growth factor 21 (FGF21) and Insulin-

like growth factors (IGFs), which signal to other organs and tissues about the metabolic state of the liver (Stefan and Haring 2013).

Adipose tissue can be functionally divided into white, brown and to a more recently identified subclass, beige adipose tissue (Peirce et al. 2014). White adipose tissue (WAT) stores excess dietary calories in the form of triglycerides and is the main site of storage energy to be used by the body during fasting. WAT also communicates with other tissues and organs by secreting adipokines, such as adiponectin and Leptin (Scherer 2006). White fat cells, adipocytes, are morphologically characterized by a single large lipid vacuole that often fills almost the entire cell, leaving only a thin layer of cytoplasm surrounding it together with a nucleus. The main role of brown adipose tissue (BAT) is to generate heat in response to cold (Cypess et al. 2009; Virtanen et al. 2009). BAT also stores energy in the form of lipids, but unlike in WAT, the lipid droplets are small and spread around the cytoplasm. The main characteristic of BAT is the high abundance of mitochondria and high expression levels of *uncoupling protein 1* (*UCP1* or *thermogenin*) (Cannon and Nedergaard 2004). UCP1 is located at the inner mitochondrial membrane and it uncouples substrate oxidation from ATP production to produce heat (Cannon and Nedergaard 2004). Recently another *UCP1* expressing adipocyte cell type was identified. The beige or brite adipocytes, usually located within WAT, rise from WAT "browning" in response to cold exposure (Ishibashi and Seale 2010; Petrovic et al. 2010). Although both beige and brown adipocytes express high levels of *UCP1*, beige adipocytes have gene expression signature distinct from BAT (Wu et al. 2012).

### 1.1.2. Control of food intake

The hypothalamus controls feeding behavior via integrating neural, metabolic and hormonal signals, such as Leptin, Insulin, FGF21 and Ghrelin, which are secreted from the adipose tissue, pancreas, liver and stomach, respectively (Figure 2). Leptin is sensed by two distinct neuron types in the arcuate nucleus (ARC) of the hypothalamus. The Agouti-related peptide (AgRP) and Neuropeptide Y (NPY) expressing neurons are inhibited by Leptin and stimulated by fasting, whereas the pro-opiomelanocortin (POMC) expressing neurons are stimulated by Leptin and inhibited by fasting (Coll et al. 2007). Both AgRP/NPY and POMC neurons signal via Melanocortin 4 (MC4) receptors to control food intake and energy expenditure. Insulin has also been shown to act via the AgRP/NPY and POMC neurons to regulate peripheral glucose metabolism and food intake (Benoit et al. 2002; Konner et al. 2007). FGF21 was recently described as the first liver-derived hepatokine that acts in the brain to control feeding behavior. Sugar-feeding induces ChREBP-dependent expression of FGF21 in the liver, which signals to the paraventricular nucleus (PVN) in the hypothalamus to suppress carbohydrate intake (von Holstein-Rathlou et al. 2016).

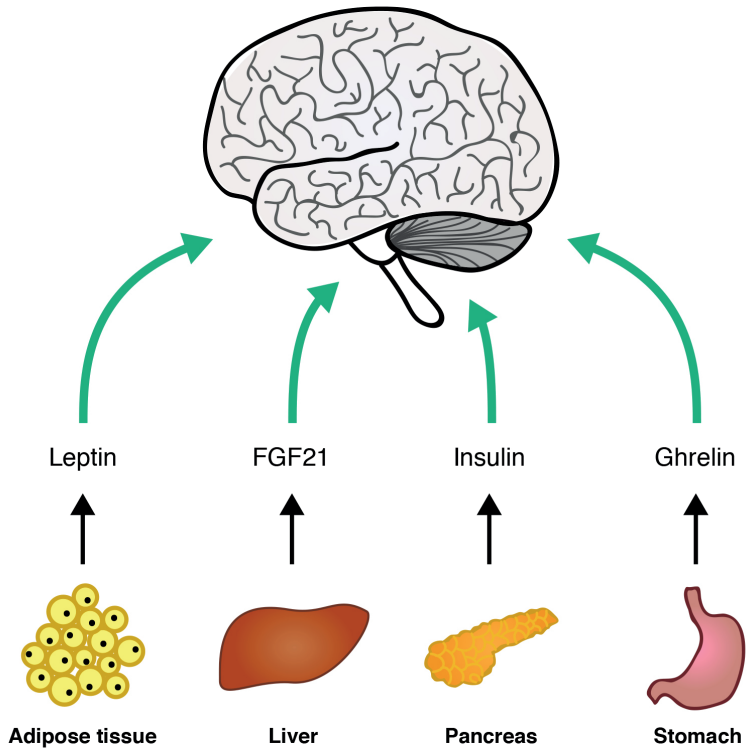


**Figure 2. Control of food intake**
Hypothalamus in the brain controls feeding behavior by integrating hormonal signals from the body. Leptin is a satiety hormone released by adipose tissue. FGF21 is produced in the liver and it controls the intake of sugars. Insulin is secreted by the pancreas in response to elevated blood glucose levels and it reduces food intake. Ghrelin is secreted from the stomach when it is empty and it increases hunger.

### 1.1.3. Nutrient sensing pathways

Ultimately, whole-body metabolism is regulated at the cellular level by specific nutrient sensors. Each nutrient is sensed separately by distinct mechanisms, which further activate downstream signaling pathways that in addition to directly activating metabolic enzymes, often include secondary effectors. Nutrient sensing pathways activate the proper metabolic responses to each nutrient and also communicate with each other to ensure metabolic homeostasis (Figure 3).

Nutrients are metabolized by metabolic enzymes, which are regulated both over short term and long term periods. In the short term, the regulation is mainly allosteric and involves post-translational mechanisms, such as phosphorylation or *O*-GlcNAcylation. In the long term, metabolic enzymes are usually regulated at the transcriptional level. Key transcriptional regulators of metabolic enzymes include SREBP, ChREBP, MondoA, USF, LXR, FXR, RXR, PPAR, HNF4α, STAT3, CREB and FOXO1 (Table 1). Transcription factors can act directly as nutrient sensors or can be activated by upstream sensors. An understanding of the highly complex crosstalk between nutrient sensing pathways is only about to emerge, and new nutrient sensors are being constantly described.

#### 1.1.3.1. Amino acid sensing

Proteins are composed of amino acids that can be further divided into essential and non-essential amino acids. The non-essential amino acids, alanine, asparagine, aspartic acid, arginine, cysteine, glutamine, glycine, glutamic acid, serine, proline and tyrosine can be synthesized by the body, whereas the other nine amino acids, leucine, isoleucine, lysine, histidine, methionine, phenylalanine, threonine, tryptophan and valine, are required in the human diet. During protein synthesis, amino acids are incorporated into polypeptide chains at the ribosome by transfer RNAs (tRNAs). Any uncharged tRNAs are recognized by a protein called General control nonderepressible (GCN2), which upon activation triggers the phosphorylation and inhibition of

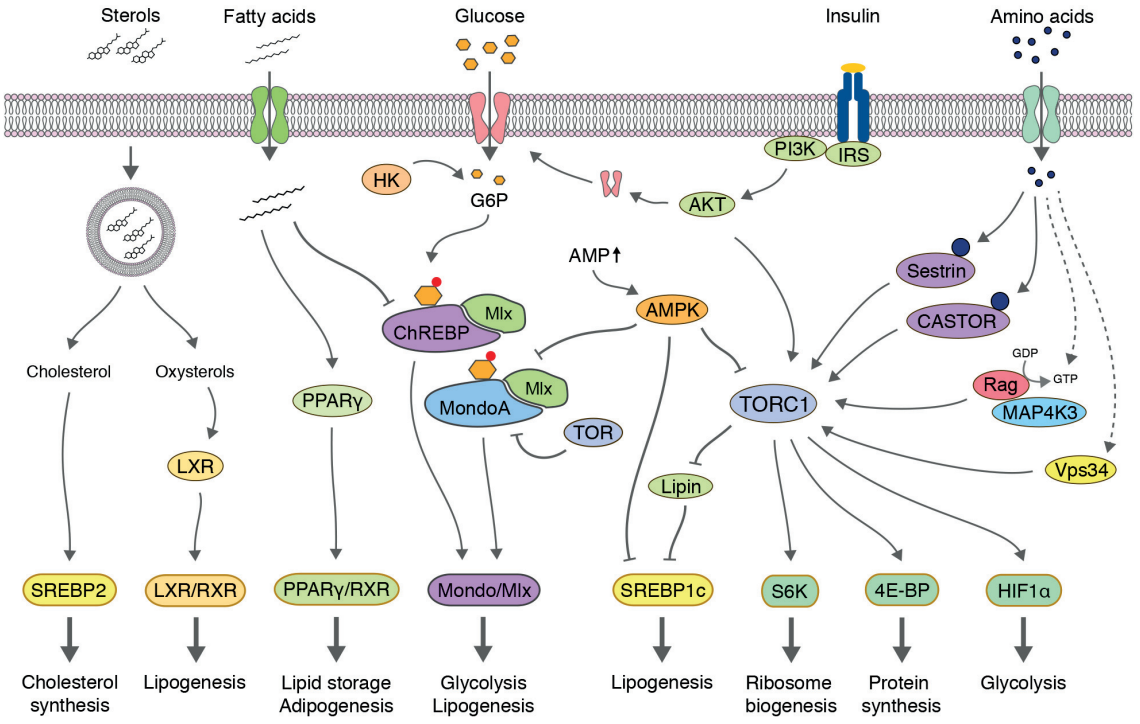


**Figure 3. Intracellular nutrient-sensing pathways**

Abbreviations: AKT: Protein kinase B, AMP: Adenosine monophosphate, AMPK: AMP-activated protein kinase, G6P: Glucose-6-phosphate, HK: Hexokinase, IRS: Insulin receptor substrate, LXR: Liver X receptor, MAP4K3: Mitogen-activated protein kinase kinase kinase kinase 3, PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase, PPAR: Peroxisome proliferator-activated receptor, RXR: Retinoid X receptor, TOR: Target of rapamycin, TORC1: TOR complex 1, SREBP: Sterol regulatory element-binding protein, Vps34: Vacuolar protein sorting 34 (Phosphatidylinositol 3-kinase catalytic subunit type 3, PIK3C3)

**Transcriptional regulators of metabolism**

| Symbol | Gene name | Aliases | Fly homologue | Binding site | Stimuli | Tissue of expression | Pathways regulated | Example target genes | Associated human diseases and/or phenotypes (HGMD and GWAS catalog) |
|---|---|---|---|---|---|---|---|---|---|
| **ChREBP** | Carbohydrate response element binding protein | MondoB, WBSCR14, MLXIPL | Mondo (Mio, CG18362) | ChoRE: CACGTGnnnnnCACGTG | Glucose | Liver, white adipose tissue, small intestine | Lipogenesis, glycolysis, pentose phosphate pathway | ChREBP, L-PK, FAS, ACC, ACLY, SCD-1, GPDH, FGF21, S14, | Metabolic syndrome, Williams-Beuren syndrome, plasma TG levels |
| **MondoA** | | MLXIP | Mondo (Mio, CG18362) | ChoRE: CACGTGnnnnnCACGTG | Glucose | Ubiquitous (Skeletal muscle) | Glycolysis | TXNIP, ARRDC4, LDH-A, HK2, PFKFB3 | N/A |
| **Mlx** | Max like protein X | | Mlx (bigmax, CG3350) | ChoRE: CACGTGnnnnnCACGTG | Glucose | Ubiquitous | Dependent on binding partner (ChREBP/MondoA) | Dependent on binding partner (ChREBP/MondoA) | Dependent on binding partner (ChREBP/MondoA) |
| **Myc** | Myelocytomatosis oncogene | MYC | Myc (Dm, CG10798) | E-box: CANNTG | N/A | Ubiquitous | Glycolysis, glutaminolysis, mitochondrial biogenesis | LDH-A, HK2, GLUT-1, GLUT-2, GLUT-4, GLS1, SREBP-1c | Breast, colorectal and prostate cancer |
| **SREBP-1c** | Sterol regulatory element binding protein | SREBF1 | SREBP (HLH106, CG8522) | SRE: TCACNCCAC | Amino acids | Liver, white adipose tissue | Lipogenesis | SREBP-1c, FAS, ACC, ACLY, SCD-1, mGPAT, PK, GK | Hypolipidaemia, T2D, atherosclerosis, Parkinson's disease, schizophrenia |
| **SREBP-1a** | Sterol regulatory element binding protein | SREBF1 | SREBP (HLH106, CG8522) | SRE: TCACNCCAC | Cholesterol | Liver, white adipose tissue | Cholesterol uptake and synthesis | shares many same targets with SREBP-1c and SREBP2 | see above |
| **SREBP-2** | Sterol regulatory element binding protein | SREBF2 | SREBP (HLH106, CG8522) | SRE: TCACNCCAC | Cholesterol | Liver, white adipose tissue | Cholesterol uptake and synthesis | HMGCS, HMGR, FDPS, FDFT1, | Birth weight, plasma cholesterol levels, schizophrenia |
| **USF1** | Upstream stimulatory factor 1 | FCHL1, HYPLIP1, MLTFI, UEF | Usf (CG17592) | E-box: CANNTG | Insulin | Ubiquitous | Lipogenesis | SREBP-1c, FAS, ACC, ACLY, SCD-1, mGPAT | Familial combined hyperlipidemia (FCHL), inflammatory bowel disease |
| **LXR** | Liver X receptor | NR1H2/NR1H3 | EcR (CG1765) | LXRE: AGGTCAnnnnAGGTCA | Oxysterols | Ubiquitous (Liver and white adipose tissue) | Lipogenesis, bile acid formation, cholesterol synthesis and transport | SREBP-1c, ChREBP, LXR, GLUT4, FAS, ACC, SCD-1, APOE, LPL | Metabolic syndrome, obesity, fasting glucose, HDL cholesterol, coronary heart disease, |
| **FXR** | Farnesoid X receptor | NR1H4 | EcR (CG1765) | FXRE: G/AGGTCAnTGACCT | Bile acid | Intestine | Bile acid efflux | IBABP, BSEP, ApoE | Cholestasis, squamous cell carcinoma |
| **RXR** | Retinoid X receptor | NR2B1/NR2B2 | usp (CG4380) | Dependent on binding partner | 9-cis retinoic acid | Muscle, liver | Dependent on binding partner (PPARalpha/PPARgamma/LXR/FXR) | Dependent on binding partner | Corneal thickness, Crohn's disease, blood pressure |
| **COUP-TFII** | Chicken ovalbumin upstream promoter-transcription factor II | NR2F2 | svp (CG11502) | DR1: GTGTCAnAGGTCA | orphan | Pancreas, liver, gut | Development, insulin secretin, glucose homeostasis | ALDH2, HNF-4α, LPL, FXR, PCK1, PPARα, RBP2 | insulin resistance |
| **PPARγ** | Peroxisome proliferator activated receptor gamma | NR1C31, CIMT1, GLM1 | N/A | PPRE (DR1): AGGTCAnAGGTC | Fatty acids | White and brown adipose tissue | Lipid storage, glucose and fatty acid uptake, insulin sensitivity | FABP4, ADIPOQ, CDF, ACSL1, LPL, C/EBP, PEPCK, PGK1, PKM1/2 | Obesity, T2D, dyslipidaemia, plasma leptin levels |
| **PPARα** | Peroxisome proliferator activated receptor alpha | NR1C1 | N/A | PPRE (DR1): AGGTCAnAGGTC | Fatty acids | Muscle, liver, brown adipose tissue, heart | Fatty acid oxidation and transport | LEPR, ACSL1, FABP1, FGF21, ELOVL6, AGPAT2, G6PC, TXNIP, | Cholesterol levels, very long-chain saturated fatty acid levels |
| **HNF4α** | Hepatocyte nuclear factor 4 alpha | NR2A1, FRTS4, MODY1, TCF | Hnf4 (CG9310) | DR1: AGGTCAnAGGTCA H4-SBM: CAAAGTCCA | Linoleic acid | Liver, gut, kidney, pancreas, intestine | Fatty acid and cholesterol metabolism, glycolysis, insulin secretion | ApoA-C, L-PK, HNF-4α, FABP2, ALDOB, PPARα, G6PC, PEPCK, SLC7A2, | MODY, T2D, hypoglycemia, cholesterol levels |
| **STAT3** | Signal transducer and activator of transcription 3 | ADMIO, APRF, HIES | Stat92E (CG4257) | TTCnnnGAA | Leptin | Ubiquitous | POMC | MYC, HIF-1α, p53, TWIST, FAS, FOXO1, Oct-1, TGF-β | Inflammatory bowel disease, multiple sclerosis, Crohn's disease |
| **CREB** | cAMP response element binding protein | CREB1 | CrebB (CG6103) | CRE: TGACGTCA | Glucagon | Insulin sensitive tissues | Gluconeogenesis, insulin resistance, appetite regulation, mitochondrial biogenesis | C-FOS, SST, PER1, PER2, G6PC, PEPCK | Obesity |
| **FOXO1** | Forkhead box O1 | FKH1 | foxo (CG3143) | FRE: (T/C)(G/A)AAACAA | Low insulin | Insulin sensitive tissues | Gluconeogenesis, lipolysis, proteolysis, food intake | G6PC, PCK1, PGC-1α, IRS2 | Corneal structure and thickness |

**Table 1. Key metabolic transcription factors**

eukaryotic translation initiator factor 2 α (eIF2α) (Berlanga et al. 1999; Dong et al. 2000). GCN2 could be therefore considered an intracellular amino acid sensor.

There is no specific definition of a nutrient sensor. Broad definitions that include a wide variety of nutrient recognizing molecules such as taste receptors have been suggested (Miyamoto et al. 2013). Taste receptors indeed activate signaling cascades leading all the way to the hypothalamus to control our feeding behavior. In the following I will however focus on those key molecules that recognize their specific substrates shortly after they are taken into the cells, and regulate metabolic target genes and signaling cascades to adjust whole-body metabolism to nutritional input.

Target of rapamycin (TOR) is perhaps the most extensively studied nutrient sensor. Although TOR is not a direct amino acid sensor, it plays an indisputable role in amino acid sensing and its activity is regulated by amino acid levels. However, the exact mechanisms of amino acid sensing are only becoming elucidated. TOR was originally identified as a gene that provides resistance to the antifungal drug called rapamycin (Heitman et al. 1991). It is a serine/threonine kinase and a member of the Phosphoinositide 3-kinase (PI3K)-related kinase family and acts as the catalytic subunit of two distinct complexes composed of different sets of proteins, and distinct upstream regulators and downstream functions (Laplante and Sabatini 2012). TOR complex 1 (TORC1) and 2 are both composed of TOR, Mammalian lethal with SEC13 protein 8 (MLST8) and the non-core components, but differ by key scaffold proteins that also define the mechanisms by which the complexes control growth. In TORC1, the binding partner of TOR is the Regulatory-associated protein of TOR (RAPTOR), and in TORC2, the Rapamycin-insensitive companion of TOR (RICTOR) (Laplante and Sabatini 2012). As the name of RICTOR states, TORC2 is insensitive to rapamycin. According to current knowledge, TORC2 is regulated by growth factors but not amino acids. It regulates survival, metabolism and cytoskeletal organization, however, information on TORC2 is still limited (Gaubitz et al. 2016).

TORC1 regulates growth by integrating information about nutritional status and growth signals to control a wide variety of cellular biosynthetic processes such as ribosome biogenesis and protein synthesis via its downstream targets, including S6 kinase (S6K) and Eukaryotic initiation factor 4E binding protein (4E-BP) (Holz et al. 2005; Tee and Blenis 2005; Ma and Blenis 2009). Other functions of TORC1 include control of lipid biosynthesis via S6K and SREBP, and glycolysis via Hypoxia-inducible factor 1α (HIF1α) (Duvel et al. 2010; Li et al. 2011). TORC1 also inhibits autophagy by phosphorylating and thus suppressing the kinase complex required for the initiation of autophagy (Ganley et al. 2009; Hosokawa et al. 2009; Jung et al. 2009). In the absence of amino acids, especially leucine and arginine, TORC1 is not activated (Blommaart et al. 1995; Hara et al. 1998).

The amino acid dependent activation of TORC1 takes place at lysosomes and is mediated by Rag GTPases that bind RAPTOR (Kim et al. 2008a; Sancak et al. 2008). The regulation of TORC1 activity is complex and involves a number kinases, including AMPK-activated protein kinase (AMPK), PI3K and AKT (Protein kinase B), which however regulate TORC1 in response to signals other than amino acids, including Insulin and Glycogen (Hietakangas and Cohen 2009). The Mitogen-activated protein kinase kinase kinase kinase 3 (MAP4K3) and the class III PI3K VPS34 (Vacuolar protein sorting 34) in turn have been linked to the amino acid sensing function of TORC1. MAP4K3 is activated by amino acids and it physically interacts with Rag GTPases (Bryk et al. 2010). VPS34 has been shown to be required for the amino acid dependent activation of TORC1 (Byfield et al. 2005).

In addition, a number of cytosolic proteins, such as Folliculin (FLCN)/FLCN-interacting protein and GATOR-proteins, have been recently suggested to provide a link between amino acid sensing, modulation of Rag GTPases and TORC1 (Tsun et al. 2013; Shimobayashi and Hall 2016). Most recently, leucine was shown to directly bind Sestrin 2, leading to disruption of Sestrin 2-GATOR2 interaction and to TORC1 activation (Chantranupong et al. 2014; Saxton et al. 2016b; Wolfson et al. 2016). Arginine, another potent activator of TORC1, was also recently shown to be directly bound by CASTOR1, another GATOR2 interacting protein (Chantranupong et al. 2016; Saxton et al. 2016a) (Figure 3).

Amino acids enter the cell via amino acid transporters, which have been proposed to serve as

amino acid sensors and regulators of TORC1 activity (Goberdhan et al. 2016). However, TORC1 signaling is also active in the absence of extracellular amino acids, suggesting that intracellular amino acid pools or biosynthesis are sufficient to activate TORC1, and that the amino acid sensing takes place inside the cell (Sancak et al. 2008). Increasing evidence suggests that different amino acids are sensed in different compartments of the cell, and that TORC1 responds to different upstream signals and activates distinct downstream effector pathways (Goberdhan et al. 2016).

**1.1.3.2. Lipid sensing**

There are thousands of different types of lipids in mammals, but they can be roughly categorized into six groups: fatty acids, glycerolipids (triglycerides), (glycerol)phospholipids, sphingolipids, sterol lipids and prenol lipids (LIPID MAPS classification). Glycerolipids, or triglycerides, are formed by the esterification of glycerols with different fatty acids, and serve as a main storage form of fat in animals. Phospholipids form lipid bilayers due to their hydrophobic fatty acid tails and hydrophilic phosphate head groups, and are the major component of cell membranes. Sphingolipids, including ceramides, sphingomyelins and glycosphingolipids, are structurally more complex lipids. They are found in the plasma membrane but have also an important role as signaling molecules. Cholesterol, the most well-known sterol, is an abundant component of cell membranes, but acts also as a precursor for the synthesis for steroid hormones.

Lipid sensing nuclear receptors include the Peroxisome proliferator-activated receptors (PPARs), their co-activator PPARγ co-activator 1α (PGC1α), Liver X receptor (LXR) and Hepatocyte nuclear factor 4α (HNF4α). PPARs are transcription factors that are activated by fatty acids (Christodoulides and Vidal-Puig 2010). Different PPAR subtypes (PPARα, -β/δ and -γ) regulate different processes. PPARα is activated during starvation, and it regulates gluconeogenesis, lipid trafficking and inhibition of DNL (Atherton et al. 2009; Vacca et al. 2015). PPARγ in turn promotes adipogenesis, lipid storage, fatty acid and glucose uptake (Lehrke and Lazar 2005). LXRs are activated by oxysterols and act as heterodimers with Retinoid X receptors (RXRs) to control lipogenesis and cholesterol synthesis (Janowski et al. 1996; Calkin and Tontonoz 2012). HNF4α regulates glucose, fatty acid and cholesterol metabolism (Jiang et al. 1995; Odom et al. 2004; Martinez-Jimenez et al. 2010). HNF4α binds linoleic acid, however the functional role for linoleic acid binding is still not clear, since the binding does not have a significant effect on the transcriptional activity of HNF4α (Yuan et al. 2009). HNF4α and PPARs regulate each other's expression and thereby coordinate their functions (Martinez-Jimenez et al. 2010). Fatty acids are also sensed by the G-protein-coupled receptors GPR40 and GPR120, which upon activation, stimulate glucose uptake (Itoh et al. 2003; Oh et al. 2010).

Intracellular cholesterol sensing is a well-characterized process involving SREBP proteins and SREBP cleavage-activating protein (SCAP), which are bound as a complex to the endoplasmic reticulum (ER). When cholesterol is abundant, SCAP binds cholesterol and the complex remains anchored in the endoplasmic reticulum (ER) (Brown et al. 2002; Yang et al. 2002). When cholesterol levels are low, the SREBP-SCAP complex dissociates from the ER and shuttles to the Golgi where SREBP is cleaved and the amino terminal transcription factor domain translocates to the nucleus and activates the transcription of genes involved in cholesterol synthesis (Radhakrishnan et al. 2008; Motamed et al. 2011; Jeon and Osborne 2012). The regulation and functions of SREBPs are discussed in more detail in Chapter 1.3.2.

**1.1.3.3. Carbohydrate sensing**

Dietary carbohydrates include sugars, starches and fibers. Digestive enzymes break down starch and sugars (disaccharides) into simple sugars that can be absorbed through the small intestine into the circulation. Blood glucose levels are maintained in a very narrow range by the counteracting hormones Insulin and Glucagon. The β-cells of pancreas express and secrete Insulin in response to elevated blood glucose levels and increased glucose metabolism (Poitout et al. 2006). The binding of Insulin to Insulin receptors (IR) induces an intracellular signaling cascade, which leads to an enrichment of insulin sensitive Glucose transporters (GLUT4) on the plasma membrane and glucose uptake (James et al. 1988; Furtado et al. 2002). When blood glucose levels fall, the α-cells of pancreas release Glucagon, which stimulates glycogenolysis, the breakdown of glycogen into glucose molecules that are released into the bloodstream (Wasserman et al. 1989).

Sugars are transported into the cells via sugar transporters. The GLUT (Solute carrier family 2, SLC2) family of glucose transporters includes 14 members belonging to the Major facilitator superfamily (MFS) of membrane transporters, from which the GLUTs 1-4 are the best characterized (Thorens and Mueckler 2010; Mueckler and Thorens 2013). GLUT1 and 3 are mainly expressed in fetal tissues and adult brain (Simpson et al. 1994; Shin et al. 1997; Simpson et al. 2008). GLUT1 was the first glucose transporter to be characterized, and it is also the main glucose transporter of red blood cells (Mueckler et al. 1985). The insulin responsive GLUT4 is most highly expressed in adipose tissue, skeletal and cardiac muscle (James et al. 1988; James et al. 1989). The expression of GLUT2 is highest in hepatocytes and pancreatic β-cells, and unlike other GLUTs, it has a low affinity for glucose (Thorens 1992) .

When glucose is taken into the cell, it is readily phosphorylated into glucose-6-phosphate (G6P) by hexokinases. The mammalian hexokinases I-III have a high affinity for glucose and they are all inhibited by G6P, whereas the type IV, commonly referred to as "glucokinase", has a very low affinity to glucose (Wilson 2003). Despite its name, glucokinase can also phosphorylate hexoses other than glucose, similarly to other hexokinases (Cardenas et al. 1998). Glucokinase is only active when glucose levels rise above a certain threshold (half-saturated at 8 mmol/L) and it is expressed at high levels in the liver and pancreas. Glucokinase is thus considered as a systemic glucose sensor that controls circulating glucose levels by promoting Insulin release from the pancreas and glycogenesis in the liver, only when the blood glucose levels reach a threshold level (Postic et al. 2001; Matschinsky 2002; Iynedjian 2009). Glucokinase and GLUT2 share the same expression pattern and low affinity for glucose. Together they form a glucose sensor that promotes glucose import and metabolism in pancreas followed by release of Insulin during hyperglycemia, whereas low blood glucose levels lead to liver GLUT2-mediated export of glucose originating from hepatic glycogenolysis and gluconeogenesis.

In addition to systemic glucose sensing, glucose and energy levels are sensed by intracellular mechanisms. AMPK is activated by increased cellular levels of AMP and ADP and it serves as a regulatory switch between catabolic and anabolic pathways depending on cellular energy status (Hardie et al. 2012). AMPK phosphorylates and inhibits the activity of lipogenic genes *SREBP* and *ACC*. In response to low energy levels, it also inhibits TORC1 and protein synthesis via phosphorylation of RAPTOR and Tuberous sclerosis complex 2 (TSC2) (Inoki et al. 2002; Gwinn et al. 2008).

The true sugar sensor, Carbohydrate response element binding protein (ChREBP), was discovered in 2001 by the laboratory of Kosaku Uyeda. This study followed the work of Howard Towle's lab, which had identified the carbohydrate response element (ChoRE) as a motif mediating the expression of hepatic genes in response to glucose (Thompson and Towle 1991; Shih and Towle 1992; Shih and Towle 1994; Shih et al. 1995). The Uyeda group studied the mechanism by which glycolytic and lipogenic genes were transcriptionally induced in response to elevated carbohydrate levels, and were able to purify the ChREBP transcription factor from the promoter of the rat *L-type pyruvate kinase* (*LPK*) gene (Yamashita et al. 2001). ChREBP had been actually already found some years before as a new binding partner for Mlx, the recently identified novel member in the Myc-network of transcription factors (Billin et al. 2000; Meroni et al. 2000). The same study identified two Mondo paralogues, MondoA and MondoB (MondoB was named ChREBP by Yamashita et al.) (Billin et al. 2000). Since their discovery, the regulation and functions of ChREBP/MondoA-Mlx transcription factors have been studied extensively, which is also the focus of this thesis.

## 1.2. Carbohydrate Response Element Binding Protein ChREBP, MondoA and Mlx

### 1.2.1. ChREBP, MondoA and Mlx belong to the bHLH/LZ family of transcription factors

The basic helix-loop-helix/leucine zipper (bHLH/LZ) class of transcription factors is a large family of transcription factors. They belong to the bHLH superfamily and are characterized by an additional leucine zipper domain that mediates dimerization in addition to the HLH domain. All bHLH proteins recognize and bind specific hexanucleotide sequences (5'-CANNTG-3') known as Enhancer box (E-box) (Massari and Murre 2000). The basic domain mediates binding to the E-box, and the consensus E-box sequence varies between different bHLH families. The E-box motif recognized specifically by ChREBP/MondoA-Mlx is called the carbohydrate response element

(ChoRE) and it is composed of two E-box elements spaced by 5 nucleotides, to which ChREBP/MondoA-Mlx bind as heterotetramers (Figure 4) (Shih et al. 1995; Ma et al. 2006). Other members of the bHLH/LZ transcription factors include SREBPs and the Myc-family. SREBPs recognize a modified type of E-box named sterol regulatory element (SRE) (TCACNCCAC), and they regulate cholesterol and fatty acid biosynthesis (Yokoyama et al. 1993; Kim et al. 1995; Brown and Goldstein 1997).

Myc is one of the most extensively studied transcription factors. The Myc-family is composed of 5 members. The first one, c-Myc, was originally identified in Burkitt lymphoma patients as a cellular homologue to the viral oncogene found in the avian myelocytomatosis retrovirus (v-Myc) (Vennstrom et al. 1982). Myc is also a well-known proto-oncogene mutated in many human cancers (Dang 2012). Myc works together with its binding partner Max to activate the transcription of a great number of target genes that regulate proliferation, apoptosis and metabolism (Blackwell et al. 1990; Blackwood and Eisenman 1991; Amati et al. 1993a; Amati et al. 1993b; Dang 1999; Prendergast 1999). Heterodimerization with Max is required for Myc to activate transcription. The Mad-family members compete with Myc for binding to Max, but in contrast to Myc, the Mad-Max heterodimers act as transcriptional repressors, thus antagonizing the function of Myc-Max (Ayer et al. 1993; Grandori et al. 2000) (Figure 5). Myc-family members also contain the bHLH/LZ domain and they bind single E-boxes together with their dimerization partner Max (Blackwood and Eisenman 1991; Kretzner et al. 1992; Amati et al. 1993a).

Another specialized class of bHLH proteins is the family of bHLH-O TFs that are characterized by an Orange domain. The function of the Orange domain is not completely understood, but it has been suggested to mediate specificity, transcriptional repression and also dimerization (Steidl et al. 2000; Davis and Turner 2001). Based on their structure, the bHLH-O family is further divided into Hes, Hey, Helt and Stra13/Dec subfamilies (Sun et al. 2007). bHLH-O TFs act as transcriptional repressors and regulate a wide variety developmental processes by modulating Notch signaling (Artavanis-Tsakonas et al. 1999; Davis and Turner 2001). The Stra/Dec subfamily members are known regulators of circadian rhythm (Honma et al. 2002), but have also been shown to have roles in peripheral tissues, for example as regulators of lipid metabolism, in response to hypoxia (Yun et al. 2002; Choi et al. 2008). Stra13/Dec proteins bind to tandem E-box elements as homodimers and act as transcriptional repressors in a histone deacetylase (HDAC)-dependent manner (Fujimoto et al. 2007).


**Figure 4. ChREBP/MondoA-Mlx recognize and bind ChoREs**
The consensus sequence of a carbohydrate response element (ChoRE). ChREBP/Mlx and MondoA/Mlx recognize and bind ChoREs, which are composed of two E-box elements spaced by 5 nucleotides. Modified from Poungvarin et al. 2015.

### 1.2.2. Myc-Max-Mad-Mlx-Mondo transcription factor network

Early studies on the Mondo-network involved identification of MondoA and MondoB (ChREBP) as proteins able to bind Mlx (Max-like protein X), which in turn had been previously identified as a new bHLH/LZ protein interacting with Mad1 and 4 *in vitro* (Billin et al. 1999; Billin et al. 2000; Meroni et al. 2000). MondoB was also independently identified in humans as a gene deleted in Williams-Beuren syndrome (called as WBSCR14; Williams-Beuren syndrome critical region gene 14) (de Luis et al. 2000; Cairo et al. 2001).

The most important clue of the molecular function of Mondo-proteins came from a study that identified ChREBP as the glucose-activated transcription factor regulating the *L-type pyruvate kinase* (*L-PK*) gene by binding to its promoter via the ChoRE (Yamashita et al. 2001). The carbohydrate response element in *L-PK* promoter had been identified previously, but its regulation had remained elusive (Thompson and Towle 1991). The identification of ChREBP as the carbohydrate responsive transcription factor was followed by the demonstration of functionally essential heterodimerization with Mlx in the context of glucose sensing, as well as the glucose responsiveness of the ChREBP orthologue

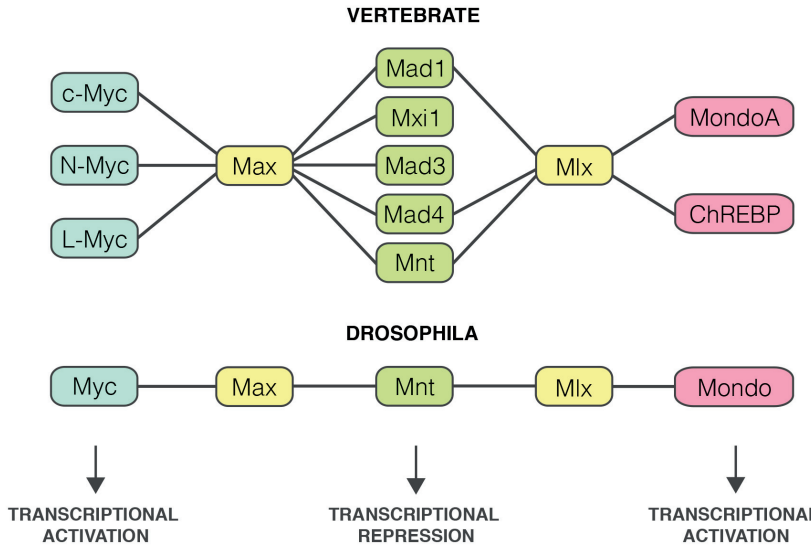


**Figure 5. The Myc-Max-Mad-Mlx-Mondo network**
The vertebrate and *Drosophila* Myc-Max-Mad-Mlx-Mondo networks. Myc, when bound to Max, is a transcriptional activator. Max is also bound by the Mad-family members, which leads to transcriptional repression. Mlx has been identified as an interactor for Mnt, Mad4 and Mad1 *in vitro*. Mlx is the obligate binding partner of ChREBP and MondoA for transcriptional activation. The *Drosophila* genome encodes a minimal Myc-Max-Mad-Mlx-Mondo network with only one paralogue for each member of the network.

MondoA (Stoeckman et al. 2004; Li et al. 2006; Stoltzman et al. 2008). The functional characterization of ChREBP led to the adaptation of the name over MondoB.

Although Mlx has been shown to be able to bind also some of the Mad-family members in an *in vitro* setting, the *in vivo* function of Mad-Mlx heterodimers remains to be established. (Billin et al. 1999; Meroni et al. 2000). However, several studies have indicated that Mlx does not bind to Myc-family members and that Mondo proteins do not bind to Max (Billin et al. 1999; Meroni et al. 2000; Cairo et al. 2001), thus implying that the Mlx-Mondo network forms an independent side branch to the Myc-Max-Mad-Mlx-Mondo network of bHLH/LZ transcription factors (Figure 5).

### 1.2.3. Mondo proteins are highly conserved

MondoA (human gene *MLXIP*) and ChREBP (*MLXIPL*) are located on different chromosomes (12q21.31 and 7q11.23 in human, respectively). They both regulate glucose-induced transcription, but in a tissue specific manner: ChREBP is predominantly expressed in the liver, adipose tissue and pancreas, whereas MondoA function is most important in the skeletal muscle (Billin et al. 2000; Yamashita et al. 2001; Wang and Wollheim 2002; He et al. 2004; Iizuka et al. 2004; Dentin et al. 2006). MondoA and ChREBP share many target genes, suggesting a possible functional redundancy between the paralogues, but there also appears to be major differences, reflecting for example the site of action.

Mondo and Mlx proteins are highly conserved in evolution. The vertebrate genome encodes two Mondo proteins, MondoA and ChREBP. Furthermore, in mammals the ChREBP gene encodes two isoforms, *ChREBP-α* and *ChREBP-β*, which are transcribed from different promoters (Herman et al. 2012). ChREBP-α is activated by glucose and it stimulates the expression of *ChREBP-β*, which has 20-fold higher transcriptional activity when compared to ChREBP-α (Herman et al. 2012). The *ChREBP-β* isoform is found in mammals, birds, reptiles and amphibians but is absent in fish, indicating that the novel isoform developed during the divergence of the tetrapod lineage (Singh and Irwin 2016). The same study also found that MondoA has changed less during evolution compared to ChREBP, suggesting a more ancient and strictly controlled role for MondoA in regulating metabolism, namely glycolysis (Singh and Irwin 2016).

The *Drosophila* genome contains a single *Mondo* (*Mio*, CG18362) and an *Mlx* (*Bigmax*, CG3350) gene along with single orthologues for *Myc*, *Max* and *Mad* (Gallant et al. 1996; Peyrefitte et al. 2001; McFerrin and Atchley 2011) (Figure 5). In *Caenorhabditis elegans,* the network is markedly different and composed of a single Mlx orthologue *Mxl-2*, two Max orthologues *Mxl-1* and *Mxl-3*, and a common orthologue for Myc and Mondo, called *Myc and Mondo like-1* (*Mml-1*) (Billin and Ayer 2006; Pickett et al. 2007; McFerrin and Atchley 2011).

The origin of the Max and Mlx network has been estimated to precede the origin of animals (over 500

Ma), and members of the network have been identified basically in all multicellular animals (McFerrin and Atchley 2011). Genome and gene duplication events during vertebrate evolution have probably resulted in the radiation of Myc, Mxd (Max, Mlx and Mnt) and Mondo proteins. However, whereas there are multiple copies of Myc, Mad and Mondo, only single copy of Max, Mlx and Mnt exists in vertebrates, suggesting that they have been under strong selection during evolution.

### 1.2.4. Glucose-6-phosphate regulates the activity of ChREBP/MondoA

Since their identification, ChREBP/MondoA-Mlx have been established to be nutrient regulated transcription factors. Their activity is induced by sugars and suppressed by polyunsaturated fatty acids (PUFAs) (Dentin et al. 2004; Dentin et al. 2005a; Meng et al. 2016). Although the exact mechanism how sugars activate ChREBP/MondoA-Mlx is still under investigation, several lines of evidence suggest that ChREBP and MondoA sense and bind glucose-derived intermediates directly. Whereas the first studies pointed to the direction of xylulose-5-phosphate (Xu-5P) (Kabashima et al. 2003), the intermediate of the pentose phosphate pathway, a large number of subsequent studies have shown that ChREBP and MondoA are activated by glucose-6-phosphate (G6P) (Stoltzman et al. 2008; Li et al. 2010a; Dentin et al. 2012). Recent studies have also suggested that MondoA-Mlx can sense and be activated by other phosphorylated hexoses as well, including glucosamine (Stoltzman et al. 2011). Furthermore, it has been shown that fructose 2,6-bisphosphate is required for the recruitment of ChREBP-Mlx to the promoters of selected set of target genes, suggesting that the activity of ChREBP-Mlx is also fine-tuned by metabolites other than G6P (Arden et al. 2012).

ChREBP and MondoA are large multidomain proteins (~ 900 amino acids) (Figure 6). The C-terminal bHLH/LZ domain mediates DNA binding and heterodimerization with Mlx. The transcriptional activation domain is supplied by the Mondo proteins, whereas the much shorter Mlx lacks the transactivation capacity and is considered an always abundant passive partner of the complex (Billin et al. 1999; Billin et al. 2000; Meroni et al. 2000; Stoeckman et al. 2004). The C-terminus of MondoA, ChREBP and Mlx also contains an additional cytoplasmic and dimerization domain (CDC), which mediates both the cytoplasmic localization activity and interaction between ChREBP/MondoA and Mlx (Cairo et al. 2001; Eilers et al. 2002).

The N-terminal region of ChREBP/MondoA contains the glucose-sensing module (GSM), which is responsible for the glucose responsiveness of ChREBP and MondoA. The glucose-sensing

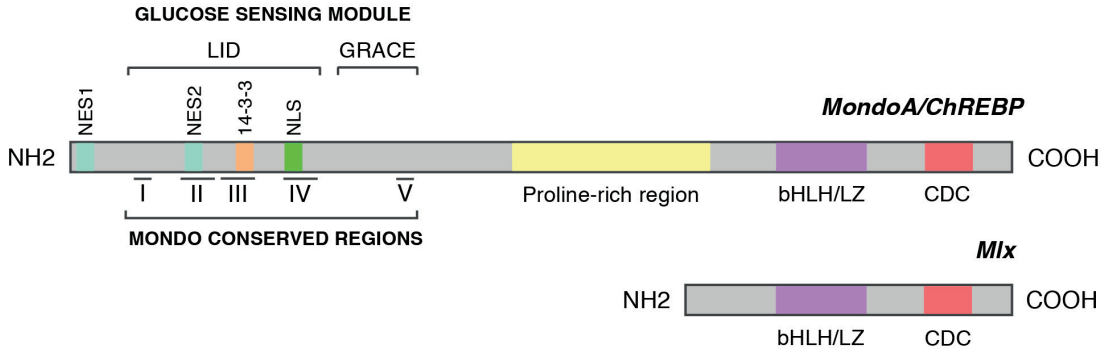


**Figure 6. The domain structure of ChREBP/MondoA and Mlx**

ChREBP, MondoA and Mlx are multidomain proteins. The homologous C-terminal regions contain the dimerization and cytoplasmic localization domain (DCD) and basic helix-loop-helix leucine zipper region (bHLH/LZ), which mediate their heterodimerization and DNA binding. The glucose sensing module in the N-terminus of ChREBP and MondoA, contains the low-glucose inhibitory domain (LID) and the glucose-response activation conserved element (GRACE), which mediate the glucose-responsiveness of ChREBP/MondoA. The N-terminal region has been also divided into five functional subdomains called Mondo conserved regions (MCR). MCRI-IV corresponds to LID and GRACE contains the MCRV. The subcellular localization of ChREBP/MondoA-Mlx is controlled by a number of proteins. CRM1 (Exportin 1) binds the nuclear export sequences NES1 and NES2. 14-3-3 binding site is located in MCRIII. The nuclear localization signal in MCRIV is bound by Importin-α. Modified from Havula & Hietakangas, 2012.

function of GSM is highly conserved – replacing the GSM of ChREBP with MondoA GSM retains the glucose responsiveness of the molecule (Li et al. 2006). The GSM, also known as the Mondo conserved region (MCR), is functionally divided into low-glucose inhibitory domain (LID) and glucose response activation conserved element (GRACE), which roughly respond to MCRI-IV and MCRV regions, respectively (Eilers et al. 2002; Li et al. 2006). Under low glucose conditions, the LID domain inhibits GRACE (Li et al. 2006). When glucose levels rise, the repression of GRACE by LID is relieved. The current models suggest that a direct binding of G6P to LID leads to a conformational change, thus allowing the interaction with Mlx and other coactivators (Davies et al. 2010; McFerrin and Atchley 2011). This model, where LID acts as a glucose signal mediating inhibitory domain, is supported by the notion that a LID-deletion form of ChREBP is highly active also under low glucose conditions (Davies et al. 2010). Moreover, the recently identified novel ChREBP-β isoform lacks the LID domain and its transcriptional activity is not dependent on glucose levels (Herman et al. 2012).

### 1.2.5. Regulation of ChREBP activity by posttranslational modifications and protein interactions

ChREBP and MondoA shuttle between the cytosol and nucleus both under low and high glucose concentrations, however the nuclear entry rate is increased by high glucose levels (Kawaguchi et al. 2001; Davies et al. 2008; Peterson et al. 2010) (Figure 7). Heterodimerization between ChREBP/MondoA and Mlx is required for the binding to ChoREs, transcriptional activity and nuclear entry (Eilers et al. 2002; Stoeckman et al. 2004; Ma et al. 2007; Davies et al. 2008; Stoltzman et al. 2008; Peterson et al. 2010). Moreover, MondoA-Mlx has been shown to shuttle between nucleus and mitochondria in the cytoplasm, where it physically interacts with the outer mitochondrial membrane (OMM) (Sans et al. 2006; Stoltzman et al. 2008). This type of retrograde communication between mitochondria and nucleus has been suggested to serve as a sensory mechanism for the cellular energy status (Sans et al. 2006). Although physical interaction between ChREBP and mitochondria has not been demonstrated, its localization in hepatocytes resembles the punctuated cytoplasmic localization typical for mitochondria (Kawaguchi et al. 2001).

Although the primary mode of ChREBP/MondoA-Mlx regulation seems to take place by the cytoplasmic-nuclear localization induced by glucose, nuclear localization alone is not sufficient for the transcriptional activation of ChREBP/MondoA-Mlx (Davies et al. 2008; Peterson et al. 2010), and additional regulatory steps are required for target gene activation. In the cytoplasm, the activity of ChREBP/MondoA-Mlx is regulated by multiple post-translational modifications as well as by interactions with other proteins. The N-terminus of ChREBP/MondoA contains a nuclear localization signal (NLS) and two nuclear export signals (NES1 and NES2) (de Luis et al. 2000; Eilers et al. 2002; Fukasawa et al. 2010; Ge et al. 2011). Also, the interaction between ChREBP and 14-3-3 has been shown to regulate the subcellular localization of ChREBP and MondoA (Eilers et al. 2002; Merla et al. 2004; Li et al. 2008; Sakiyama et al. 2008) (Figure 7).

Regulation of ChREBP activity via cAMP dependent protein kinase A (PKA) and AMPK mediated phosphorylation has been under extensive investigation, however none of the identified phosphorylation sites have been exclusively shown to be required for ChREBP activation or inhibition (Kawaguchi et al. 2001; Kawaguchi et al. 2002; Li et al. 2006; Tsatsos and Towle 2006; Denechaud et al. 2008a; Tsatsos et al. 2008). However, a simultaneous mutation of multiple phosphorylation sites within LID leads to the inhibition of glucose-responsiveness, suggesting that regulation of ChREBP/MondoA activity by phosphorylation involves a complex interplay between multiple phosphorylation sites (Tsatsos et al. 2008).

In addition to phosphorylation, ChREBP is also modified by acetylation and *O*-GlcNAcylation (Bricambert et al. 2010; Sakiyama et al. 2010; Guinez et al. 2011). Histone acetyltransferase (HAT) coactivator recruitment takes place before the transcriptional activation of both ChREBP and MondoA (Cha-Molstad et al. 2009; Peterson et al. 2010). The transcriptional coactivator p300 interacts and acetylates ChREBP, thereby promoting DNA binding of the complex (Bricambert et al. 2010). p300 activity in turn is inhibited by Salt-inducible kinase 2 (SIK2), the activity of which is regulated by nutritional signals such as starvation (Du et al. 2008; Bricambert et al. 2010). *O*-GlcNAcylation both stabilizes ChREBP protein and increases its transcriptional activity (Guinez et al. 2011) (Figure 7). To conclude, the

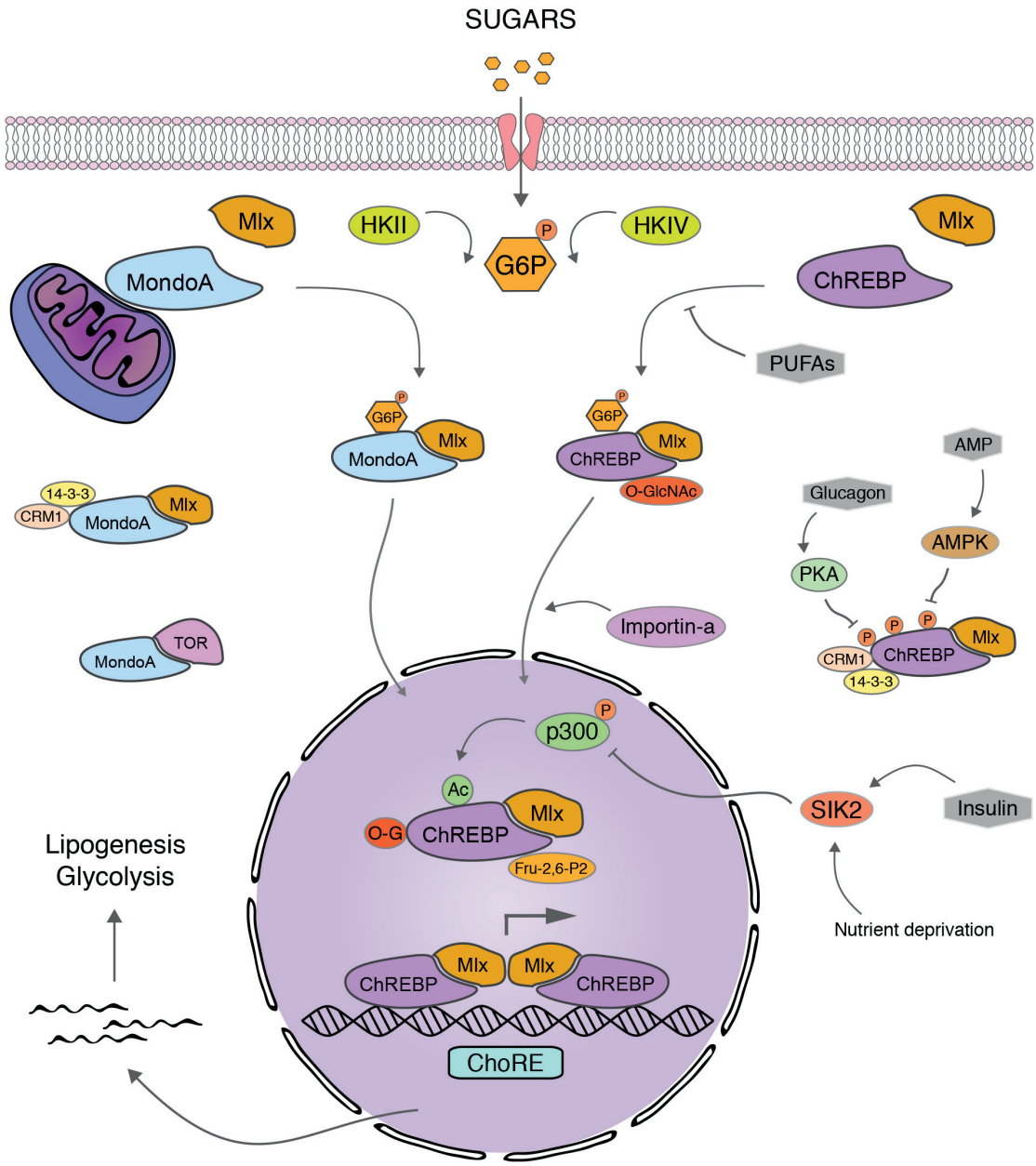


**Figure 7. Regulation of ChREBP/MondoA-Mlx activity**
Abbreviations: Ac: Acetylation, AMP: Adenosine monophosphate, AMPK: AMP-activated protein kinase, ChoRE: Carbohydrate response element, CRM1: Chromosome region maintenance 1 (Exportin 1), Fru-2,6-P2: Fructose 2,6-bisphosphate, G6P: Glucose-6-phosphate, HKII: Hexokinase II, HKIV: Hexokinase IV (Glucokinase), O-GlcNAc/O-G: O-linked β-N-acetylglucosamine, PKA: Protein kinase A, PUFA: Polyunsaturated fatty acids, SIK2: Salt inducible kinase 2

regulation of ChREBP/MondoA-Mlx activity seems to be a complex interplay between posttranslational modifications and a coordinated action of regulatory proteins that control both the nuclear entry and transcriptional activity of the complex in response to nutritional signals.

### 1.2.6. Transcriptional regulation of ChREBP/MondoA-Mlx

In addition to posttranslational modifications, ChREBP, MondoA and Mlx are also regulated at the transcriptional level. Posttranslational

mechanisms are in charge of the rapid, nutrient-triggered regulation of ChREBP/MondoA-Mlx activity. However, it has become evident, especially after identification of the ChREBP-β isoform, that transcriptional regulation plays an important role in the long term regulation of ChREBP/MondoA-Mlx activity. *ChREBP* expression is induced in the liver, especially after high carbohydrate feeding (Dentin et al. 2004; Meng et al. 2016). *ChREBP* transcript levels are also increased by Insulin via the Octamer transcription factor-1 (Oct-1), which has been shown to bind *ChREBP* promoter after Insulin stimulation (He et al. 2004; Sirek et al. 2009).

The interrelationship between nuclear factors and ChREBP is perhaps the most extensively studied field of transcriptional regulation of *ChREBP* (Poupeau and Postic 2011). Nuclear receptors, including PPARs, LXR and HNF-4α, are transcription factors that are activated by ligands, such as hormones and a variety of small molecules (Robinson-Rechavi et al. 2003; Evans and Mangelsdorf 2014). The ligand binding can take place in the cytoplasm, followed by a translocation to the nucleus, or the nuclear receptor can be retained in the nucleus in its inactive state. For some nuclear receptors, the activating ligand is not known, and they are thus called orphan receptors, such as the chicken ovalbumin upstream promoter-transcription factor (COUP) (Li et al. 2009b; Evans and Mangelsdorf 2014). There are approximately 50 nuclear receptors in mammals, whereas in *Drosophila* there are only approximately 20, although these represent a member of each receptor subfamily (King-Jones and Thummel 2005; Thomson et al. 2009).

LXR is an important regulator of glucose, fatty acid and cholesterol homeostasis, especially in the liver, but also in the adipose tissue (Kalaany et al. 2005; Laurencikiene and Ryden 2012; Beaven et al. 2013). It functions as a transcriptional activator together with its heterodimerization partner RXR (Peet et al. 1998). The promoter of ChREBP contains a binding site for LXR, and it has been shown that LXR can regulate the expression of *ChREBP* in the liver (Cha and Repa 2007; Gauthier et al. 2010). However, the expression of *ChREBP* was not altered after high sugar feeding in LXR mutant mice, implying that the effect of LXR on *ChREBP* expression is independent of nutrition (Denechaud et al. 2008a). Next to the LXR/RXR binding site in the *ChREBP* promoter lies another LXR element (LXRE), but instead of LXR, it is bound by TR/RXR heterodimers. Thyroid hormone receptor (TR) can activate *ChREBP* expression in liver and adipose tissue, but activation also seems to be independent of nutritional status (Gauthier et al. 2010).

HNF-4α is a conserved nuclear receptor that is highly expressed in the liver but also in the gut, kidney and pancreas (Duncan et al. 1994; Taraviras et al. 1994). It binds DNA as a homodimer and is a crucial transcriptional regulator of glucose and lipid metabolism in the liver and pancreas (Jiang et al. 1995; Odom et al. 2004). In addition to directly regulating the expression of a number of genes involved in glucose and lipid metabolism, HNF-4α also regulates the expression of many transcription factors involved in metabolic regulation (Kuo et al. 1992; Taraviras et al. 1994; Bolotin et al. 2010). HNF-4α is also known as *MODY1* (*Maturity-onset diabetes of the young 1*), since it has been discovered as a gene often mutated in this subtype of familial type 2 diabetes. Recently, HNF-4α was shown to activate *ChREBP* transcription. More importantly, HNF-4α was shown to activate *ChREBP* expression (both *ChREBP-α* and *ChREBP-β*) in response to glucose in mouse primary hepatocytes and HepG2 cells (Meng et al. 2016), thus providing the long-sought mediator of glucose-induced *ChREBP* expression. Interestingly, Meng and colleagues also found that the expression of *ChREBP-β* was not only promoted by the previously shown ChREBP-α isoform, but also by HNF-4α. HNF-4α is also known to physically interact with ChREBP, and this interaction is promoted by glucose, suggesting that positive feedback loop between ChREBP-α, ChREBP-β and HNF-4α (Adamson et al. 2006; Herman et al. 2012; Meng et al. 2016). The co-operation of HNF-4α and ChREBP in regulating *L-PK* expression in response to glucose has been also previously demonstrated, extending the regulation to their target genes as well (Burke et al. 2009).

### 1.3. ChREBP/MondoA as a key regulator of sugar-induced transcription

Since their discovery, ChREBP and MondoA have proven to be key transcription factors mediating glucose induced expression of glycolytic and lipogenic genes. The first direct target gene of ChREBP to be identified was *L-PK* (also known as liver pyruvate kinase), which catalyzes the final step of glycolysis in hepatocytes (Yamashita et al. 2001). This study also gave the first clue about the

glucose-responsiveness of the transcription factors. The glucose-dependent activation of MondoA was later demonstrated by the use of 2-deoxyglucose (2-DG), a glucose analog that is phosphorylated but not processed further (Stoltzman et al. 2008). In this setting, the expression of more than 75% of the glucose-responsive genes was shown to be dependent on MondoA-Mlx (Stoltzman et al. 2008). The tissue specific expression pattern of the paralogues is reflected in their downstream target genes. However, they also share many targets, suggesting a possible functional redundancy between ChREBP and MondoA.

### 1.3.1. Regulation of glycolysis and lipogenesis by MondoA and ChREBP

The utilization of dietary sugars by different tissues depends on their physiological role. Skeletal muscle is one the most important sites for dietary glucose utilization. Muscles break down glucose via the glycolytic pathway to release energy that is used for muscle contraction. MondoA expression is highest in the skeletal muscle, and most of the MondoA-Mlx target genes are involved in the glycolytic pathway, such as *hexokinase II* (*HKII*), *lactate dehydrogenase A* (*LDH-A*) and *6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase* (*PFKFB3*) (Billin et al. 2000; Sans et al. 2006).

Liver is the main site for *de novo* lipogenesis (DNL), where the dietary sugars are converted into TAGs and further transported to adipose tissue for storage. DNL also takes place in adipose tissue. ChREBP displays its highest expression in liver and adipose tissue, and the best characterized ChREBP target genes include the key lipogenic target genes *acetyl-CoA carboxylase* (*ACC*) and *fatty acid synthase* (*FAS*) (Ishii et al. 2004; Jeong et al. 2011).

In addition to FAS and ACC, ChREBP-Mlx regulates also the expression of a number of other genes involved in the lipogenic pathway, including the *stearoyl-CoA desaturase 1* (*SCD-1*), *fatty acid elongase 6* (*ELOVL6*) and *glycerol 3-phosphate dehydrogenase* (*GPDH*) (Ishii et al. 2004; Ma et al. 2005; Ma et al. 2006; Wang et al. 2006; Iizuka et al. 2009b; Jeong et al. 2011; Poungvarin et al. 2015). Moreover, ChREBP-Mlx regulates the glucose-induced expression of *glucose-6-phosphate dehydrogenase* (*G6PDH*), the rate limiting enzyme of the pentose phosphate pathway (PPP) (Ma et al. 2006). This step in PPP also produces the NADPH required by the DNL pathway. Glycolysis and DNL are tightly linked particularly in the liver, where the main function for the hepatic glycolytic pathway is to transform carbohydrates into fat. ChREBP-Mlx is also a key regulator of glycolytic enzymes in the liver and adipose tissue (Ishii et al. 2004; Ma et al. 2005; Ma et al. 2006; Jeong et al. 2011) (Figure 8).

One of the best characterized target genes regulated both by MondoA and ChREBP is the *thioredoxin-interacting protein* (*TXNIP*, also known as *vitamin D3-upregulated protein 1*) (Ma et al. 2006; Stoltzman et al. 2008; Cha-Molstad et al. 2009). TXNIP belongs to the alpha-arrestin protein family and is an important regulator of redox signaling. Moreover, TXNIP inhibits cellular glucose uptake by binding and inducing the endocytosis of GLUT1 (Wu et al. 2013), thereby providing a potential negative feedback loop for glucose metabolism and uptake.

### 1.3.2. SREBP-1c controls expression of lipogenic genes in response to Insulin

Sterol regulatory element binding proteins (SREBPs) are transcription factors that regulate the expression of genes involved in lipogenesis and cholesterol uptake and synthesis (Briggs et al. 1993; Tontonoz et al. 1993; Wang et al. 1993; Magana and Osborne 1996; Goldstein et al. 2006). The SREBP family consists SREBP-1a, SREBP-1c and SREBP-2, which are encoded by two genes, SREBPF1 and SREBPF2 (Hua et al. 1995; Shimomura et al. 1997).

SREBPs belong to the bHLH/LZ class of transcription factors and are synthesized as inactive precursor proteins that are anchored to the endoplasmic reticulum (ER) membrane (Brown and Goldstein 1997; Osborne and Espenshade 2009). SREBP-2 controls cholesterol metabolism genes and its activity is mainly mediated by the intracellular cholesterol levels. SREBP-1c is the main isoform expressed in the liver and adipose tissue, where it is activated by Insulin (Shimomura et al. 1997; Kim et al. 1998; Foretz et al. 1999; Shimomura et al. 1999; Ferre and Foufelle 2007). In contrast to SREBP-2 and SREBP-1a, which are induced and activated by low cholesterol levels, SREBP-1c levels are regulated by changes in nutritional status (Horton et al. 1998; Kim et al. 1998; Bizeau et al. 2003; Commerford et al. 2004). High-carbohydrate diet induces *SREBP-1c* expression, whereas fasting reduces the levels. Insulin has been shown to induce both transcription and cleavage of SREBP-1c, thus regulating its


**Figure 8. Regulation of hepatic glycolysis and *de novo* lipogenesis by ChREBP-Mlx**
Enzymes regulated by ChREBP-Mlx are highlighted in red.

activity on multiple levels (Kim et al. 1998; Foretz et al. 1999; Hegarty et al. 2005). Glucagon on the other hand has been shown to inhibit *SREBP-1c* transcription (Foretz et al. 1999). Furthermore, Insulin has been shown to mediate its effect on SREBP-1c via the PI3K/AKT/TORC1 pathway (Porstmann et al. 2008; Duvel et al. 2010; Li et al. 2010b). The mechanism by which TORC1 regulates SREBP-1c activity is still largely unresolved. However, inhibitory phosphorylation of Lipin-1 by TORC1 in response to Insulin has been suggested to increase SREBP-1 transcriptional activity (Peterson et al. 2011).

The transcriptional regulation of *SREBP-1c* is known to require the Insulin-TORC1 axis, which ultimately leads to a feed-forward stimulation where SREBP-1c can autoregulate its own expression via SREs present in their promoters (Sato et al. 1996; Amemiya-Kudo et al. 2000; Li et al. 2010b). LXRα has been also shown to induce the transcription of *SREBP-1c* in response to dietary cholesterol (Repa et al. 2000). In addition to direct transcriptional regulation, LXR is required for the Insulin-mediated activation of SREBP-1c, further linking cholesterol and fatty acid metabolism (Chen et al. 2004). *SREBP-1c* promoter also contains a ChoRE element, and ChREBP has been shown to mediate the glucose-induced expression of *SREBP-1c* (Jeong et al. 2011; Poungvarin et al. 2015).

SREBP-1c is a key regulator of DNL in the liver, and its target genes include *acetyl-CoA synthetase* (*ACS*), *ACC*, *FAS*, *SCD-1* and *glycerol-3-phosphate acyl-transferase* (*GPAT*) (Edwards et al. 2000). The regulation of *glucokinase* (*GK*) expression is also dependent on Insulin and SREBP (Van Schaftingen 1994; Foretz et al. 1999; Kim et al. 2004). However, the Glucokinase regulatory protein (GKRP) that controls GK activity, is regulated by glucose and ChREBP (Ma et al. 2006; Jeong et al. 2011), thus providing a synergistic regulatory mode for glucokinase, an important sensor and regulator of whole body glucose-levels and glucose metabolism.

### 1.3.3. Coordinated regulation of *de novo* lipogenesis by ChREBP and SREBP

For a long time, SREBP-1c was thought to be the master regulator of DNL. However, expression of lipogenic genes is reduced only by approximately 50% in the livers of *SREBP-1c* knockout mice (KO) (Liang et al. 2002). Moreover, although *SREBP1-1c* is expressed in adipose tissue, the expression of lipogenic enzymes in the adipose tissue of *SREB*P KO mice is normal, and thus it has been suggested that DNL in adipose tissue is regulated solely by ChREBP (Shimano et al. 1997; Shimomura et al. 1997; Herman et al. 2012).

Although the role of SREBP-1c as a key regulator of DNL is indisputable, the characterization of ChREBP has provided a more complete picture of transcriptional regulation of DNL. Today it is known that SREBP-1c is regulating DNL together with other transcription factors such as ChREBP, HNF-4α and LXR, in a highly concerted manner (Chen et al. 2004; Dentin et al. 2005b; Wong and Sul 2010). Most of the lipogenic genes harbor both SRE and ChoRE elements in their promoters, highlighting the importance of co-regulation of DNL both by Insulin and glucose initiated signals (Shih et al. 1995; Rufo et al. 2001; Griffin et al. 2007; Wong and Sul 2010) (Figure 9). In addition, the Patatin-like phospholipase domain-containing 3 (PNPLA3), better known as Adiponutrin, promotes hepatic lipid synthesis and is regulated both by ChREBP and SREBP in response to glucose and Insulin, respectively (Dubuquoy et al. 2011; Kumari et al. 2012; Perttila et al. 2012).

### 1.3.4. ChREBP-Mlx controls the glucose induced expression of genes related to circadian rhythm

MondoA and ChREBP have shown to act mainly as transcriptional activators, however their role in glucose induced transcriptional repression has been also suggested by a number of studies (Ma et al. 2006; Noordeen et al. 2010; Arden et al. 2011; Boergesen et al. 2011; Jeong et al. 2011; Noriega et al. 2011; Poungvarin et al. 2015). Interestingly, MondoA/ChREBP-Mlx target genes also include many transcriptional regulators, suggesting that MondoA/ChREBP-Mlx mediates some of its effects via a secondary tier of transcription factors, including transcriptional repressors.

ChREBP regulates the expression *krüppel-like factor 10* (*KLF10*) and *DEC1* (*BHLHB2/BHLHE40/STRA13/STRA14*) in response to glucose (Iizuka and Horikawa 2008; Iizuka et al. 2011). KLF10, also known as TGFβ inducible early gene-1 (TIEG), is an ubiquitously expressed protein originally identified as a cell cycle regulator and potential tumor suppressor (Subramaniam et al. 2010; Song et al. 2012). *KLF10* expression levels are regulated by the circadian clock, a molecular timing system composed of multiple transcriptional regulators that coordinate physiology, metabolism and behavior in response to external light-dark cycle input (Guillaumond et al. 2010; Gooley 2016; McGinnis and Young 2016). The core clock is composed of the four clock proteins Clock, Bmal1, Per and Cry. Clock/Bmal1 heterodimers activate the expression of *PER* and *CRY*, which in turn heterodimerize and inhibit the function of Clock/Bmal1 via direct interaction (Partch et al. 2014). Furthermore, KLF10 has been shown to be an important regulator of lipid and carbohydrate metabolism (Guillaumond et al. 2010). DEC1 (Deleted in Esophageal cancer 1) and its paralogue

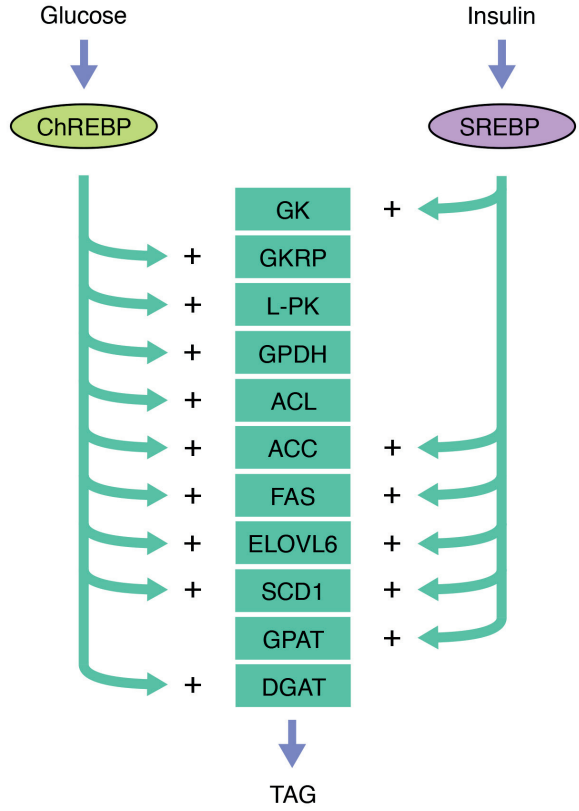


**Figure 9. Coordinated regulation of DNL by ChREBP and SREBP**

ChREBP and SREBP regulate the expression of glycolytic and lipogenic genes in response to glucose and Insulin, respectively. Modified from Postic & Girard, 2008.

DEC2 regulate the clock by inhibiting the expression of *PER1* via competing with Clock/Bmal1 for binding to an E-box in the *PER1* promoter (Honma et al. 2002). Clock/Bmal1 in turn directly regulates expression of *DEC1* and *DEC2*, thus forming a negative feedback loop in the clock (Kato et al. 2014).

Interestingly, it was shown already in 1986 by Newman & Hospod that the uptake of glucose in the suprachiasmatic nucleus (SCN) of the brain differs between day and night (Newman and Hospod 1986; Hamada et al. 1993), suggesting that circadian rhythm controls metabolism and feeding behavior. SCN is the area of the hypothalamus that contains the master clock, however the peripheral tissues also express and have functional, self-sustained molecular clocks (Yoo et al. 2004). Today, the dysfunction of circadian rhythm has been linked to many metabolic diseases, for example metabolic syndrome, obesity and diabetes (Spiegel et al. 2005; Turek et al. 2005; Knutson et al. 2007; Arble et al. 2009; Marcheva et al. 2010; Maury et al. 2010; Shimba et al. 2011). For example, loss of Bmal1 specifically in the pancreas leads to impaired Insulin production and severe glucose intolerance (Sadacca et al. 2011), whereas loss of Bmal1 in the adipose tissue causes a shift in the diurnal food intake rhythm, which leads to obesity in mice (Paschos et al. 2012).

The circadian rhythm regulated glucose uptake and the finding that ChREBP-Mlx regulates some of the key regulators and mediators of the clock suggest that nutrient sensing, metabolism and circadian rhythm are tightly connected processes. The details are only beginning to emerge, along with the role of MondoA and ChREBP in these processes.

### 1.3.5. ChREBP and MondoA in human disease

ChREBP and MondoA, together with their binding partner Mlx, are key transcriptional regulators of carbohydrate and lipid metabolism. Studies on ChREBP and MondoA mutant mice have confirmed their critical role in metabolic regulation. *ChREBP-/-* mice display various metabolic defects, including reduced liver fatty acid levels and adiposity, hyperglycemia, and increased Insulin and glycogen levels (Iizuka et al. 2004). *ChREBP* knockout mice are also highly sugar intolerant and are unable to survive on a high-carbohydrate diet. However, the underlying reason for the sugar intolerance has not been explored (Iizuka et al. 2004). *MondoA* knockout mice are viable, have a normal body weight, fasting glucose and plasma lipid profiles (Imamura et al. 2014). However, due to defective glycolysis, the animals use fatty acid oxidation to provide energy, and the pyruvate is shunted to lactate which is reflected by the high plasma lactate levels of *MondoA-/-* mice (Imamura et al. 2014). Perhaps not surprisingly, dysregulation of both ChREBP and MondoA has been linked to many human diseases, including type 2 diabetes, metabolic syndrome and cancer.

*ChREBP* was originally identified as a gene deleted in William-Beuren syndrome, a neurodevelopmental disorder characterized by multiple physiological abnormalities, including cardiovascular complications and diabetes, which is observed in 75% of adult patients (de Luis et al. 2000; Cairo et al. 2001; Pober 2010). Variants of the human *ChREBP* (also known as *MLXIPL*) have been associated with high plasma triglyceride levels as well as with elevated concentrations of plasma liver enzymes and increased risk of coronary artery disease (Kathiresan et al. 2008; Kooner et al. 2008; Wang et al. 2008; Willer et al. 2008; Pan et al. 2009). Most recently, the expression levels of the novel *ChREBP-β* isoform in adipose tissue have been found to correlate positively with the insulin sensitivity in humans (Herman et al. 2012; Kursawe et al. 2013). On the other hand, increased ChREBP-β activity has been linked to elevated DNL in obesity (Eissing et al. 2013).

Hepatic ChREBP activity has been suggested to play a role in the pathobiology of nonalcoholic fatty liver disease (NAFLD), the most common cause of liver dysfunction in Western countries (Denechaud et al. 2008b). NAFLD is a presenting symptom of metabolic syndrome. It is characterized by steatosis and abnormal fat accumulation in the hepatocytes, and it is tightly linked to type 2 diabetes and obesity (Marchesini et al. 2001; Charlton 2004; Cheung and Sanyal 2010). NAFLD can develop into non-alcoholic steatohepatitis (NASH), which in turn can lead to cirrhosis and liver cancer (Hassan et al. 2014). Although overexpression of *ChREBP* in mice leads to induction of lipogenesis and hepatic steatosis, increased ChREBP activity also has beneficial effects, for example improved glucose tolerance (Benhamed et al. 2012). In fact, *ChREBP* expression has been found to correlate positively with the degree of hepatic steatosis and negatively with insulin resistance in NASH patients, suggesting that the two metabolic conditions can occur independently, and that modulation of

ChREBP activity can be used to protect NAFLD patients from developing diabetes (Benhamed et al. 2012).

Modulation of ChREBP activity has been also studied in the commonly used *ob/ob* obesity mouse model (Ingalls et al. 1950; Dentin et al. 2006; Iizuka et al. 2006). The *ob/ob* mice also develop insulin resistance and are used as a model for type 2 diabetes (Genuth 1969; Beloff-Chain et al. 1975). Identification of the mutation leading to the various metabolic defects of *ob/ob* mice led to the discovery of Leptin hormone, a satiety signal the deficiency of leads to excessive feeding and obesity in *ob/ob* mice (Friedman et al. 1991; Zhang et al. 1994; Campfield et al. 1995). Interestingly, *ob/ob* mice with liver-specific inhibition of *ChREBP*, or *ob/ob –ChREBP-/-* double mutant mice, display markedly improved metabolic profiles, including reduced lipogenic gene expression, hepatic DNL and enhanced glucose tolerance and insulin sensitivity (Dentin et al. 2006; Iizuka et al. 2006).

Finally, both MondoA and ChREBP have been suggested to play a role in the metabolic reprogramming of cancer cells. Tumor cells often rely on glycolysis to provide the high amount of energy and carbons needed for anabolic reactions such as nucleotide biosynthesis that drive the fast growth and proliferation. This phenomenon is also known as the Warburg effect. Increased ChREBP activity in colorectal and hepatic cancer cell models has been shown to promote anabolic pathways including glycolysis, DNL and nucleotide biosynthesis (Tong et al. 2009). Loss of MondoA in tumors that rely on deregulated Myc in growth leads to synthetic lethality, suggesting that MondoA is required by the Myc-dependent metabolic reprogramming of cancer cells (Carroll et al. 2015).

### 1.4. *Drosophila melanogaster* as a model organism

The basis for modern genetics was established in the early 1900s in the lab of Thomas Hunt Morgan who was using the fruit fly, *Drosophila melanogaster*, in his studies. He was able to show that chromosomes, and later genes within them, formed the basis of heredity (Morgan 1910; Morgan et al. 1920). These findings gave Morgan the Nobel Prize in Physiology in 1933. Morgan's work also made *Drosophila* very popular among scientists around the world, becoming one of the most favored model organisms, which it still is today. Altogether, the work done with *Drosophila* has resulted in 7 Nobel prizes in Physiology and Medicine, among them Morgan's former student Herman Muller, who discovered that X-ray irradiation causes mutations (Muller 1927). Other famous discoveries include the conserved homeotic HOX-genes that control embryonic development and patterning, and most recently work that elucidated some fundamental aspects of innate immunity (Lewis 1978; Nusslein-Volhard and Wieschaus 1980; Lemaitre et al. 1996).

*Drosophila*'s popularity as a model organism can be attributed to many factors, including its low cost, small size, short generation time and ease of maintenance (Figure 10). Its small genome (only 4 pairs of chromosomes) was fully sequenced in 2000 and although it is only about 5% of the size of the human genome (0.18 Gb vs. 3.3Gb), the difference in the number of genes is surprisingly small (Adams et al. 2000). *Drosophila* genome encodes approximately 15 000 genes, whereas in humans the number has been estimated at 20 000 (Adams et al. 2000; Kornberg and Krasnow 2000; Lander et al. 2001; Ezkurdia et al. 2014). This difference arises mainly from the presence of several paralogous genes in mammals, whereas there is less redundancy in *Drosophila* - often an advantage in genetic studies. Male flies also lack meiotic recombination, further simplifying the genetics in fly experiments.

It has been estimated that more than 75% of all human disease genes have functional orthologues in flies (Reiter et al. 2001). Moreover, the extensive genetic toolkit available, including *in vivo* RNAi and UAS-ORF libraries, tissue specific GAL4-driver lines, and the recessive lethal balancer chromosomes that carry visible genetic markers and do not undergo crossing over, allowing lethal alleles to be maintained as stable heterozygous stocks, have greatly contributed to *Drosophila*'s success as a model organism. The GAL4/UAS (galactose gene transcription factor 4/upstream activation sequence) system allows spatiotemporally controlled transgene expression *in vivo* (Brand and Perrimon 1993), and the fly research community has made the majority of stock lines available in public stock centers such as Vienna *Drosophila* RNAi Center (VDRC) and Bloomington *Drosophila* Stock Center (BDSC). Recent developments in targeted genome editing technologies, especially the CRISPR/Cas9 system,

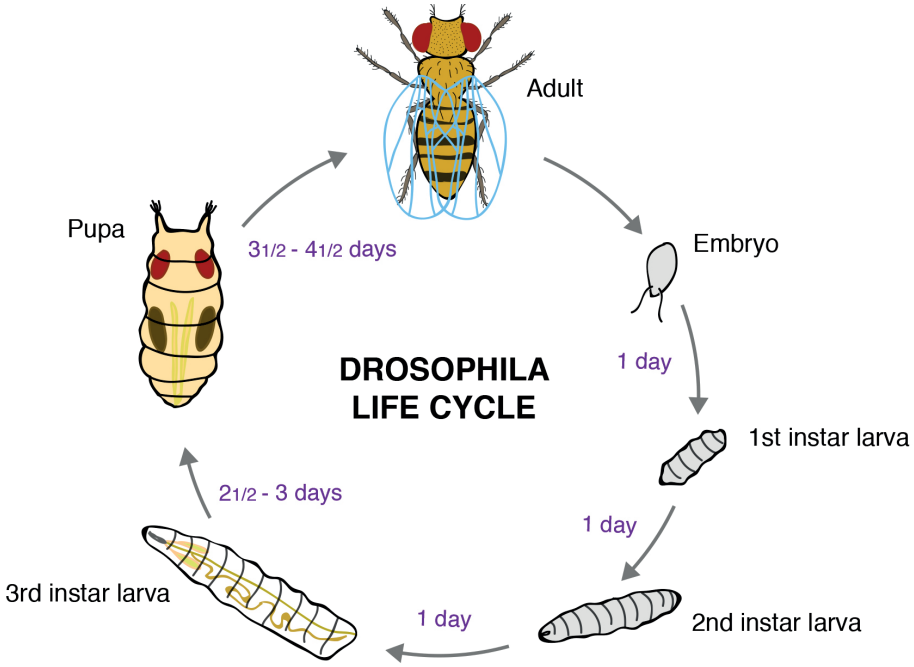


**Figure 10. *Drosophila* lifecycle**
The development of an adult fly from an egg takes optimally ~10 days. Flies undergo a complete metamorphosis from an embryo to adult via three larval stages and a pupal stage.

has also enabled rapid generation of mutant and transgenic flies (Bassett et al. 2013).

*Drosophila* has a long history as a model organism to study development and neurobiology. However, the use of fly as a model to study mammalian physiology and metabolism has only started to reach its full potential. Flies were long thought to differ from mammals to the extent that they do not share the same principles of physiology, a view that has proved to be largely incorrect. Although fruit flies differ greatly from vertebrates in their morphology, they have basically the same organ systems, performing the same functions as their mammalian counterparts (Figure 11). In addition, the rapid development of a vast array of basic and more sophisticated metabolic assays, has made *Drosophila* a popular model for human metabolic diseases such as obesity and type 2 diabetes (Baker and Thummel 2007; Leopold and Perrimon 2007; Rajan and Perrimon 2013; Owusu-Ansah and Perrimon 2014; Padmanabha and Baker 2014; Tennessen et al. 2014).

### 1.4.1. Physiology and tissue organization of *Drosophila melanogaster*

*Drosophila* maintains its metabolic homeostasis with the action of the same analogous metabolic organs and signaling pathways as mammals. Although there are clear physiological and anatomical differences between the fly and mammals, functional analogy can be found from many aspects in their physiology. The fly undergoes major morphological and physiological rearrangements during its development from an embryo into an adult, via 3 larval stages (instars) interrupted by molts, and a pupal stage that is finalized by a complete metamorphosis (Figure 10). Metamorphosis and the timing of larval molting are controlled by steroid hormones, which are secreted in response to growth signals by the ring gland located in the brain (Gilbert et al. 2002; Yamanaka et al. 2013). The metabolism of larval stages is markedly different from the adult in that the larvae are feeding continuously and accumulate a substantial amount of fat and glycogen to carry through the pupal stage. Adult flies do not grow, and their size is determined by the nutritional status and growth rate during the larval stages (Edgar 2006; Mirth and Shingleton 2012). The change in regulation and downstream effects of many signaling pathways have been shown to reflect this transition from the active growth period of larvae into maintenance of metabolic homeostasis in the adult.

*Drosophila*, like most other invertebrates, have an "open" circulation system where the blood-like

fluid called hemolymph circulates throughout the body aided by the pumping action of a tubular heart (Choma et al. 2011). Although *Drosophila* has an open circulation, its tubular, beating heart has many characteristics similar to vertebrate heart (Bier and Bodmer 2004). Another special characteristic is the use of trehalose, a disaccharide formed of two glucose molecules, as a form of circulating storage sugar (Reyes-DelaTorre 2012). Trehalose is considered to be an adaptation for flight, which requires high levels of circulating sugars. One of the major differences between mammals and fly is the regulation of glucose transport in response to Insulin. In mammals, GLUT4 transporter translocates to the plasma membrane in response to Insulin, however in the fly, Insulin does not seem to control the rate of glucose uptake (Ceddia et al. 2003). Another key difference is that flies are not able to synthesize cholesterol (Clark and Block 1959; Clayton 1964).

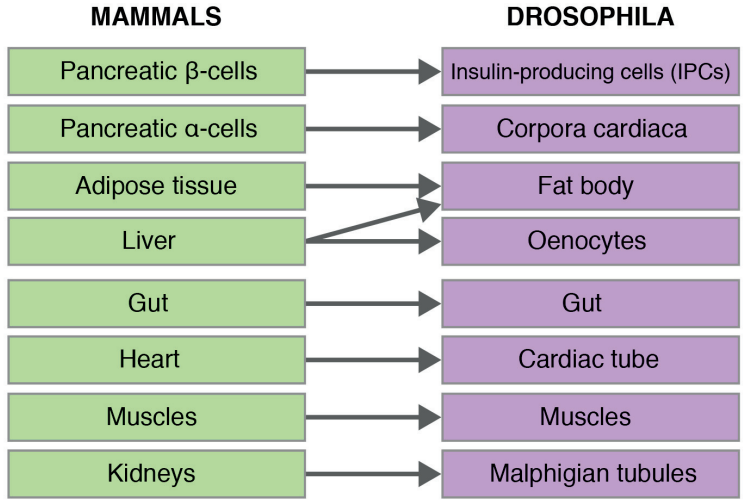


**Figure 11. The functional conservation of *Drosophila* organ systems**
Comparison of the mammalian and *Drosophila* organ systems. Modified from Diop & Bodmer 2015.

Most *Drosophila* organs are morphologically different from their mammalian counterparts but still carry out the same functions. The fly gut, although structurally much more simple, is a highly compartmentalized organ divided into foregut, midgut and hindgut, which display functional similarities to the human gastrointestinal tract (Buchon et al. 2013a). The foregut is in charge of food mixing, detoxification and storage, and the midgut is the main site for digestion and food absorption. Water reabsorption and waste excretion takes place in the hindgut. Furthermore, the midgut has been shown to be regionally organized into 14 subregions displaying distinct gene expression patterns and morphology, and also into functionally diverse intestinal cell types similar to the cells in the mammalian intestinal epithelium (Buchon et al. 2013b; Dutta et al. 2015). Malpighian tubules, or renal tubules, are the major excretory and osmoregulatory organs of the fly and thus functionally analogous to vertebrate kidneys (Jung et al. 2005).

The fat body is a specialized organ, functionally orthologous to mammalian adipose tissue and liver, composed of adipocytes that are organized into loose sheet-like structures. The fat body is distributed throughout the body and it serves both as a nutrient sensor as well as a site for triglyceride and glycogen storage (Colombani et al. 2003). It is the major metabolic organ, synthetizing and secreting the majority of circulating proteins and metabolites that in turn control growth and metabolism in other sites of the body (Arrese and Soulages 2010). In fact, the fat body is not only in charge of storing nutrients, but acts as an endocrine organ that regulates organismal growth and development by integrating nutritional and hormonal signals (Colombani et al. 2003; Arrese and Soulages 2010).

The Insulin producing β-cells of pancreas have functional counterparts in the fly brain, where Insulin-producing cells (IPCs) secrete *Drosophila* Insulin-like peptides (dILPs) into the circulation (Brogiolo et al. 2001; Rulifson et al. 2002; Broughton et al. 2005). dILPs are also secreted from other organs and display developmental stage-dependent expression patterns. For example, from the eight dILPS encoded by the *Drosophila* genome, only dILP2, 3 and 5 are secreted from IPCs, dILP4 is secreted by the embryonic and larval gut and dILP6 by the fat body (Gronke et al. 2010; Padmanabha and Baker 2014).

The ring gland is located next to the IPCs and is a key neuroendocrine organ composed of the corpora cardiaca (CC), corpora allata (CA) and prothoracic gland (PG). CC is analogous to mammalian pancreatic α-cells and secretes the Glucagon-like

peptide adipokinetic hormone (AKH) (Kim and Rulifson 2004). AKH, like its mammalian counterpart Glucagon, regulates circulating sugar levels. It binds to its receptors in the fat body and activates Glycogen phosphorylase, which induces the breakdown of glycogen and secretion of trehalose into the circulation (Staubli et al. 2002; Van der Horst 2003; Kim and Rulifson 2004; Lee and Park 2004). Juvenile hormone secreted by CA, and Ecdysone and Prothoracicotropic hormone (PTTH) secreted by PG, control the molting and metamorphosis in insects (Doane 1973; McBrayer et al. 2007).

**1.4.2. Nutrient sensing pathways in *Drosophila***

The metabolic homeostasis of flies and mammals is controlled by the same basic endocrine hormones. For example, the counteracting hormones AKH and dILPs regulate circulating glucose levels similarly to Glucagon and Insulin. The brain dILPs are secreted into the circulation in response to feeding, promoting the uptake of sugars from the hemolymph and their storage as glycogen and TAGs, whereas AKH stimulates the breakdown of glycogen and TAGs during starvation (Kim and Rulifson 2004; Lee and Park 2004; Geminard et al. 2009). Loss of dILPs leads to hyperglycemia, similar to mammals (Rulifson et al. 2002).

The major signaling pathways involved in lipid biosynthesis, storage and mobilization are highly conserved in *Drosophila*. Like other metabolic pathways, lipid metabolism is more simple in flies when compared to mammals that often have multiple paralogues for many key metabolic regulators and enzymes. A special characteristic of *Drosophila* lipid metabolism is the inability to synthesize cholesterol, which flies require from their diet (Clark and Block 1959; Clayton 1964). In line with this, *Drosophila* SREBP activity is not regulated by sterols but by phosphatidylethanolamine, and SREBP is thought to regulate only the synthesis of fatty acids (Dobrosotskaya et al. 2002; Seegmiller et al. 2002). *Drosophila SREBP* mutants die as 2$^{nd}$ instar larvae and display markedly reduced fatty acid synthesis. The role of *Drosophila* SREBP as a key regulator of fatty acid synthesis is supported by the finding that the lethality of *SREBP* mutants can be rescued by supplementing fatty acids, namely oleate, in their diet (Kunte et al. 2006).

The fat body serves as the main site of fat storage in the fly. Flies have also a specialized group of cells, termed oenocytes, which have the ability to mobilize stored lipids from the fat body, similarly to the mammalian liver (Gutierrez et al. 2007). During nutrient deprivation, AKH stimulates the breakdown of glycogen and lipolysis (Kim and Rulifson 2004; Lee and Park 2004). The regulation and the key enzymes of lipolysis are conserved in the fly. The adipocytes in the fat body store lipids in droplets, which are coated by lipase-protecting proteins, including perilipins (Teixeira et al. 2003; Gronke et al. 2005). The *Drosophila* genome encodes two perilipins that are thought to display opposing functions, with Lsd-2 (Lipid storage droplet-2) protecting lipid droplets from lipolysis, and Lsd-1 promoting lipid mobilization (Teixeira et al. 2003; Gronke et al. 2005; Beller et al. 2010; Bi et al. 2012). The expression of *brummer*, the *Drosophila* homologue of the *apidocyte triglyceride lipase* (*ATGL*), is upregulated during starvation and is essential for mobilization of lipids (Gronke et al. 2005).

Metabolic signaling pathways are also highly conserved in the fly, and the groundwork for resolving many key aspects of nutrient sensing and signaling in mammals has been done in fly studies. One the best and most studied nutrient sensing and signaling systems is the Insulin/Insulin-like growth factor (IGF) and TOR signaling pathway, which controls growth and metabolism both in humans and the fly. In fact, *Drosophila* has become one the most important model systems to study Insulin signaling (Teleman 2010). The core pathway and its functions are highly conserved but compared to mammals, the fly displays a reduced pathway with less complexity. For example, the fly genome encodes only single homologues for the *insulin receptor* (*InR*), *insulin receptor substrate* (*chico*) and *FOXO* (*foxo*) (Fernandez et al. 1995; Bohni et al. 1999; Puig et al. 2003; Teleman 2010). The single InR is activated by all dILPs, however different dILPs seem to respond to different nutritional cues (Ikeya et al. 2002). dILP2 secretion is promoted by amino acids, whereas the release of dILP3 from IPCs is dependent on sugars (Geminard et al. 2009; Kim and Neufeld 2015). The fat body specific *dILP6* is expressed in response to starvation and regulates the expression and release of dILP2 from the IPCs (Okamoto et al. 2009; Slaidina et al. 2009; Bai et al. 2012).

The role of fat body as an important nutrient sensor regulating whole-body metabolism has been shown by several studies, which have also given clues

about nutrient-dependent regulation of conserved signaling pathways. For example, the amino acid transporter Slimfast mediates the effects of whole-body amino acid levels to TOR signaling in the fat body (Colombani et al. 2003). Fat body specific knockdown of *slimfast* phenocopies the growth suppressive phenotype of TOR signaling inhibition or amino acid starvation. The fat body also secretes many peptides, cytokines and other endocrine molecules, which signal other sites of the body about the nutritional status. Unpaired 2 (Upd2), the functional homologue of mammalian Leptin, is secreted by the fat body in response to feeding (Rajan and Perrimon 2012). Similar to the mammalian Leptin, Upd2 stimulates the JAK/STAT pathway, which ultimately leads to the secretion of dILPs (Rajan and Perrimon 2012). Whereas Slimfast responds to amino acids, Upd2 secretion is promoted only by fats and sugars (Colombani et al. 2003; Rajan and Perrimon 2012). Recently, an amino acid sensitive adipokine and TNF-α homologue Eiger was shown to be secreted from the fat body in response to amino acid starvation, and to mediate the reduced expression of dILPs by binding to its receptor Grindelwald in the IPCs (Agrawal et al. 2016). The epidermal growth-like factors Growth-blocking peptide 1 and 2 (Gbp1 and Gbp2) in turn are produced and secreted from the fat body in response to increased amino acid levels and TOR signaling (Koyama and Mirth 2016). Gbp1 and Gbp2 act directly in the brain and stimulate dILP secretion (Koyama and Mirth 2016).

In addition to Leptin, multiple other hormonal factors have been shown to be activated and secreted in response to carbohydrates in *Drosophila*. CCHamide-2 (CCHa2) is expressed and secreted by the gut and fat body in response to sugars. It signals directly to the IPCs and triggers the expression and release of dILP5 and also the release of dILP2 by binding to its receptor CCHa2-R (Sano et al. 2015). The TGF-β/Activin-like ligand Dawdle is also secreted from the fat body in response to sugars, regulating the release of dILP2 and dILP5 from the IPCs (Ghosh and O'Connor 2014). Moreover, Dawdle-activated Activin signaling in the gut has been shown to mediate the sugar-induced repression of amylases (Hickey and Benkel 1982; Benkel and Hickey 1987; Chng et al. 2014). High-sugar feeding also triggers the production and release of Neural Lazarillo (NLaz), a lipocalin peptide highly homologous to mammalian Retinol binding protein 4 (RBP4) and Apolipoprotein D (ApoD) (Pasco and Leopold 2012). High serum levels of RBP4 and ApoD have been associated with development of insulin resistance (Yang et al. 2005; Graham et al. 2006; Do Carmo et al. 2009), which is also true in *Drosophila*, where partial *NLaz* deficiency protects against high-sugar diet induced insulin resistance (Pasco and Leopold 2012).

The *Drosophila* genome encodes a minimal Myc-Max-Mnt-Mlx-Mondo(ChREBP) network composed of a single copy of each gene (Figure 5). At the time when this thesis was started, there were no published studies on *Drosophila* Mondo-Mlx. However, the conserved glucose-sensing function of Mondo had been suggested by a mammalian *in vitro* study, where the replacement of the mouse ChREBP N-terminal region including the glucose sensing module (GSM) with the corresponding *Drosophila* Mondo region retained the glucose-inducible activation of the reporter (Li et al. 2006). In addition, the expression of *mlx* has been shown to be upregulated in response to high sugar diet in *Drosophila* larvae (Zinke et al. 2002), suggesting a conserved role in sugar sensing. An observation described in a review article from the lab of Donald Ayer also suggests that *Drosophila* Mondo is necessary for viability, since partial loss of *mondo* led to reduced viability (Billin and Ayer 2006). They also proposed a synthetic lethal genetic interaction between *Drosophila* Myc and Mondo: whereas the *myc* null mutants were viable, loss of both *myc* and *mondo* led to a complete lethality with no adult survivors.

### 1.4.3. Studies on nutrient-regulated transcription in *Drosophila*

Flies develop obesity and insulin resistance in response to high carbohydrate and high fat diets in a manner similar to mammalian models (Surwit et al. 1988; Musselman et al. 2011; Pasco and Leopold 2012). Additionally, the analysis and phenotyping of several different mutant flies have indicated that the pathobiology of several metabolic diseases is conserved in the fly (Baker and Thummel 2007; Teleman 2010). This makes *Drosophila* an appealing model organism to study the molecular mechanism underlying various human complex diseases. A major advantage compared to human studies is the possibility to manipulate and strictly control the environmental conditions, especially the diet. Moreover, unlike for example mice, *Drosophila* larvae eat continuously, which allows a reliable assessment of metabolic effects in response to dynamic dietary treatments.

Metabolic enzymes and regulators are often regulated at the transcriptional level. Thus much of research has focused on identifying genes that are differentially expressed in response to different dietary interventions. The major groundwork for identifying genes that are regulated transcriptionally in response to nutritional signals came from the study by Zinke and collegues in 2002. In this study, they investigated the gene expression (Affymetrix microarray) profiles of larvae after 1, 4 and 12 hours on high-sugar diet feeding or starvation, and as expected, found that the number of genes that were differentially expressed increased as a function of time (Zinke et al. 2002). The gene that displayed the highest and earliest expression response to sugar feeding was found to be a zinc finger transcription factor CG3850, which they named *sugarbabe* (*sug*). The sugar-induced expression of *sugarbabe* was found to be most prominent in the fat body, gut and Malpighian tubules, and the study suggested that it has a role in transcriptional repression of downstream target genes in response to dietary sugars (Zinke et al. 2002). In addition to *sugarbabe*, among the genes that responded most strongly to dietary sugars were *acetyl-CoA carboxylase* (*ACC*, CG11198), *acetyl-CoA synthase* (*AcCoAS*, CG9390), *ATP citrate lyase* (*ATPCL*, CG8322), glucose-6-phosphate dehydrogenase *Zwischenferment* (*Zw*, CG12529) and the phosphoserine phosphatase *astray* (*aay*, CG3705).

## 2. AIMS OF THE STUDY

Metabolic diseases, such as type 2 diabetes, are the leading cause of death in developed countries (WHO 2016). Excess intake of calories, especially in the form of added sugars, has been suggested to underlie this epidemic (Lustig et al. 2012). However, individuals display remarkable differences in their physiological and metabolic responses to nutrients. Also considerable natural variation in diet exists between animal species. Although the systemic control of glucose homeostasis by Insulin and Glucagon is well-understood, the regulation and control of sugar-induced metabolic processes at the cellular level are still largely unresolved. ChREBP/MondoA-Mlx transcription factors are activated by sugars, and their target genes include mainly glycolytic and lipogenic genes. Interestingly, mice lacking *ChREBP* are intolerant to dietary carbohydrates (Iizuka et al. 2004), suggesting a role for ChREBP-Mlx in regulating organismal sugar tolerance. Variants of human *ChREBP* have also been associated with increased triglyceride levels, a predisposing factor for the development of type 2 diabetes (Kathiresan et al. 2008; Kooner et al. 2008; Wang et al. 2008; Willer et al. 2008; Pan et al. 2009). The role of mammalian ChREBP/MondoA-Mlx in sugar-induced transcription has been studied mainly in an *in vitro* setting. Mondo and Mlx proteins are well conserved throughout metazoan evolution. The *Drosophila* genome encodes single orthologues of ChREBP/MondoA and Mlx, which we call Mondo and Mlx, respectively.

The specific aims of this thesis were:

I Characterization of the *in vivo* role of the *Drosophila* Mondo-Mlx

II To study the role of Mondo-Mlx in sugar-induced transcription and to identify the targets of Mondo-Mlx

III To further characterize the role of the secondary tier of transcriptional effectors downstream of Mondo-Mlx in sugar metabolism

## 3. MATERIALS AND METHODS

### Fly strains

| Strain | Source |
|---|---|
| P[XP]*bigmax*$^{d07258}$ | BDSC |
| mlx$^{1}$ | Havula et al. 2013 |
| UAS-Mlx FLAG | Havula et al. 2013 |
| UAS-Aldh-III | Havula et al. 2013 |
| UAS-Mlx RNAi 110630 KK | VDRC |
| UAS-Mondo RNAi 109821 KK | VDRC |
| UAS-Cabut RNAi 4427R-1 | NIG |
| UAS-Aldh-III RNAi 107110 KK | VDRC |
| UAS-FAS RNAi 108339 KK | VDRC |
| UAS-PFK2 RNAi 25959 GD | VDRC |
| UAS-CG7882 RNAi 109918 KK | VDRC |
| UAS-Zw RNAi 101507 KK | VDRC |
| UAS-Gs2 RNAi 32929 GD | VDRC |
| UAS-aay RNAi 110661 KK | VDRC |
| UAS-daw RNAi 105309 KK | VDRC |
| UAS-Smox RNAi 105687 KK | VDRC |
| Sug$^{17\Delta}$ | Mattila et al. 2015 |
| UAS-sug | Zinke et al. 2002 |
| UAS-sug RNAi$^{1}$ 3850R-1 | NIG |
| UAS-sug RNAi$^{2}$ 3850R-3 | NIG |
| Df(2R)Exel7123 | BDSC |
| tub-GAL4 | BDSC |
| ppl-GAL4 | BDSC |
| r4-GAL4 | BDSC |
| Elav-GAL4 | BDSC |
| Mef2-GAL4 | BDSC |
| Ubi-GAL4 | BDSC |
| Hs-GAL4 | BDSC |
| Cg-GAL4 | BDSC |
| Fb-GAL4 | BDSC |
| NP1-GAL4 | BDSC |

Stock centers:
BDSC (Bloomington *Drosophila* Stock Center)
NIG (Fly Stocks of National Institute of Genetics, Japan)
VDRC (Vienna *Drosophila* Resource Center)

### Fly husbandry

Fly stocks were maintained at +25°C on standard food containing 0.6% (w/v) agar, 3.2% (w/v) semolina, 6.5% (w/v) malt, 1.8% (w/v) dry baker's yeast, 2.4% (v/v) nipagin and 0.7% (v/v) propionic acid.

The defined nutrient studies were conducted in food containing 0.5% (w/v) agar, 2.4% (v/v) nipagin and 0.7% (v/v) propionic acid in PBS supplemented with varying concentrations of dry baker's yeast (w/v), sucrose (w/v), glucose (w/v), fructose (w/v) or potato starch (w/v). Larvae were grown at controlled density (30 larvae per vial).

| Method | Reference | Used in publication |
|---|---|---|
| Generation of transgenic fly strains | I, II | I, II |
| Generation of mutant fly by imprecise P-element excision | Venken and Bellen 2005 Nature | I |
| Generation of mutant fly by CRISPR/CAS9 | Kondo and Ueda 2013 | II |
| *in vivo* UAS-GAL4 methods | I, II | I, II |
| Recombinant protein expression and purification | I | I |
| SDS-PAGE | I | I |
| Western blotting | I | I |
| Co-immunoprecipitation (Co-IP) | I | I |
| Standard RNA and DNA techniques | I, II | I, II |
| RNA extraction | I, II | I, II |
| Quantitative real-time PCR (qRT-PCR) | I, II | I, II |
| Chromatin immunoprecipitation (ChIP) | II | II |
| Triglyceride assay | Palanker et al. 2009 | II |
| Glucose and trehalose assay | Zhang et al. 2011 | I, II |
| Glycogen assay | Parrou et al. 1997 | I |
| Cell culture | I, II | I, II |
| Transfection of cells | I, II | I, II |
| Metabolomics | I | I |
| Lipid analysis by mass spectrometry | II | II |
| Agilent Drosophila gene expression microarray | I | I |
| Illumina RNA sequencing | II | II |
| Gene enrichment analysis | II | II |

## 4. RESULTS

### 4.1. Mondo-Mlx is essential for dietary sugar tolerance in *Drosophila* (I)

#### 4.1.1. Loss of *mlx* leads to late pupal lethality in *Drosophila*

To study the function of *Drosophila* Mondo-Mlx, we first created an *mlx* null mutant fly via imprecise excision of a P-element (P[XP]*bigmax*$^{d07258}$). The *mlx*$^{1}$ mutant allele lacks the entire coding region of the *mlx* gene and 17 C-terminal amino acids of the neighbouring gene CG3368. Lines where the P-element was precisely excised were recovered to serve as controls. We generated a polyclonal antibody against Mlx and confirmed the complete absence of Mlx protein by Western blot from 3$^{rd}$ instar larvae. Also the mRNA expression, measured by qRT-PCR, was lost in the *mlx* null mutant animals. The loss of *mlx* led to pupal lethality, with only very few animals able to emerge as adult flies.

In mammals, Mlx acts as a heterodimeric pair of two Mondo paralogs, ChREBP and MondoA, whereas *Drosophila* genome encodes a single *mondo* orthologue. We demonstrated with co-immunoprecipitation in *Drosophila* S2 cells that Mlx biochemically interacts with Mondo, suggesting that the heterodimeric function is conserved in the fly.

#### 4.1.2. Mondo-Mlx deficient animals show intolerance to dietary sugars

On our standard laboratory food, *mlx*$^{1}$ mutant animals displayed a one-day delay in pupation and late pupal (pharate) lethality. Interestingly, we found that the delay in pupation displayed significant variation between the so-called "standard laboratory diets". Since the mammalian ChREBP/MondoA-Mlx had been identified as a sugar responsive transcription factor, we hypothesized that the dietary component responsible for the observed differences in developmental delay would be the amount of carbohydrates.

To test this, we prepared a minimal diet rich in protein (20% baker's yeast (w/v) in PBS + 0.5% agarose and 2.5% Nipagin as a preservative) with increasing amounts of sucrose (5-20% w/v). Whereas the *mlx*$^{1}$ mutants developed with similar kinetics as control animals on 20% yeast only food, adding sucrose into the diet gradually slowed down their development. With 20% yeast + 15% sucrose diet that still sustained normal development of control animals, virtually all of the *mlx*$^{1}$ mutant larvae failed to pupate. Furthermore, the mutants were unable to survive on a 20% sucrose only diet.

To confirm that the sugar sensitive phenotype was due to loss of Mondo-Mlx function, we used RNAi-mediated knockdown of *mlx* and *mondo*. Ubiquitous knockdown of both *mondo* and *mlx* led to decreased adult emergence and increased pupal lethality on high sugar diet, respectively. The sugar sensitivity of *mondo* knockdown was enhanced by removing one copy of *mlx*, providing further genetic support for joint function and evolutionary conservation of the system. Moreover, sugar intolerance and pupal lethality were rescued by ubiquitous expression of transgenic *mlx*.

#### 4.1.3. Loss of Mondo-Mlx leads to severe metabolic defects *in vivo*

The severe intolerance toward dietary sugars prompted us to study in detail the metabolic phenotypes of *mlx*$^{1}$ mutants. We measured the circulating glucose levels from the haemolymph and found that already on the yeast only diet, the *mlx*$^{1}$ mutants displayed significantly higher circulating glucose levels. The hyperglycaemic phenotype became more severe by adding only 5% of sucrose into the diet. We also measured the levels of the circulating trehalose. Trehalose is composed of two glucose molecules and it is the main form of circulating sugar in insects (Becker et al. 1996). Circulating trehalose levels were also significantly higher in the *mlx*$^{1}$ mutants, both on yeast only and 20% yeast +5% sucrose diets. To rule out the possibility that the neighbouring gene CG3368 would play a role in the phenotypes observed in the *mlx*$^{1}$ mutants, we performed a series of *in vivo* knockdown studies with CG3368. Knockdown of CG3368 had no influence on sugar tolerance, circulating glucose or trehalose levels.

To get a comprehensive view of the metabolic profile of the *mlx*$^{1}$ mutants, we performed a lipidomics analysis with mass spectrometry, in collaboration with Dr. Matej Oresic and Dr. Tuulia Hyötyläinen in VTT Otaniemi. The analysis revealed significant changes especially in lipid and phospholipid metabolism. Phospholipids such as phosphatidylethanolamine and lysophosphatidylcholine were significantly downregulated in the *mlx*$^{1}$ mutants. The total triglyceride levels also showed a lower trend in the

*mlx*$^{1}$ mutants, although the difference to control was not statistically significant. Interestingly, the profile of triglyceride species was remarkably altered. The *mlx*$^{1}$ mutants showed strong enrichment in triglyceride species with long fatty acid tails. In addition, the ceramide levels were strongly elevated, suggesting that the *mlx*$^{1}$ mutants might suffer from lipotoxicity. The *mlx*$^{1}$ mutants also showed a dramatic increase in urea levels, whereas the total amino acid levels were significantly downregulated, suggesting that the mutants are unable to utilize sugars and therefore catabolize amino acids as their energy.

#### 4.1.4. *Mondo* and *mlx* are expressed in the metabolic tissues of *Drosophila*

In mammals, the two Mondo paralogs *MondoA* and *ChREBP* are both ubiquitously expressed, however *MondoA* is predominantly expressed in skeletal muscles whereas the highest expression of *ChREBP* is found in the liver and adipose tissue (Billin et al. 2000; Yamashita et al. 2001; Iizuka et al. 2004). The gene duplication event that took place before or during vertebrate evolution most likely contributed to the existence of two Mondo proteins in mammals (McFerrin and Atchley 2011). It is possible that during the course of evolution, the functions of the ancient Mondo protein were divided between MondoA and ChREBP leading to two distinct glucose-responsive pathways operating in different tissues in a similar fashion.

To identify the most critical tissues for *Drosophila* Mondo-Mlx function, we first analysed their gene expression using qPCR. Expression of *mondo* and *mlx* mRNA showed spatial correlation, being highest in the fat body, gut and renal tubules (Malpighian tubules). Next we utilized the UAS-GAL4 system to rescue *mlx* expression tissue specifically in the *mlx*$^{1}$ mutant background. As expected, restoring *mlx* expression ubiquitously with tub-Gal4 rescued both the viability and sugar-sensitivity of *mlx*$^{1}$ mutants. While restoring *mlx* expression in neurons (Elav-GAL4) or muscle (Mef2-GAL4) did not improve the mutant phenotype, ectopic expression of *mlx* in the fat body (Ppl-GAL4, Cg-GAL4, r4-GAL4) rescued both viability and sugar-sensitivity. This implies that fat body, which is functionally equivalent to mammalian liver and adipose tissue, is the most critical tissue for Mondo-Mlx function.

### 4.2. Identification of putative Mondo-Mlx target genes by Agilent gene expression analysis (I)

In order to identify the downstream targets of Mondo-Mlx responsible for the mutant phenotypes, we performed Agilent gene expression microarrays specifically from the fat bodies of 3rd instar control and *mlx*$^{1}$ mutant larvae kept on 20% yeast vs. 20% yeast + 5% sucrose diets. Comparison of gene expression profiles between the different diets did not show any significant changes in control or *mlx*$^{1}$ mutants, suggesting that addition of 5% sucrose to the diet does not have any long-term physiological effects, at least at the transcriptional level. However, the comparison between control and *mlx*$^{1}$ mutants on the 20% yeast + 5% sucrose diet revealed significant changes in a number of genes (>100 with >2-fold change and adjusted p-value<0.05), most of them having a role in different metabolic processes. As expected, *mlx* was the most downregulated gene in the microarray. We did a Gene Set Enrichment Analysis (GSEA) to identify the pathways and processes affected by loss of *mlx*, and found that glycolysis, fatty acid metabolism and nitrogen metabolism (KEGG categories) were strongly downregulated in the *mlx*$^{1}$ mutants.

Among the most downregulated genes in the *mlx*$^{1}$ mutants were the 3-hydroxybutyrate dehydrogenase *shroud* (sro, CG12068), phosphoserine phosphatase *astray* (aay, CG3705), *glycerol-3-phosphate dehydrogenase* (*Gpdh*, CG9042), stearoyl-CoA desaturase *desaturase 1* (*Desat1*, CG5887), *aldehyde dehydrogenase type III* (*Aldh-III*, CG11140), *glutamine synthetase 1* (*Gs1*, CG2718) and *amylase proximal* (*Amy-p*, CG18730). Interestingly, one of the most strongly downregulated genes in *mlx*$^{1}$ mutant fat body was the Krüppel-like transcription factor *cabut* (*cbt*, CG4427), suggesting that Mondo-Mlx regulates its downstream targets at least in part through secondary transcriptional effectors.

We also looked at the homologues of known mammalian ChREBP/MondoA-Mlx targets in our microarray data and found that the expression levels of *fatty acid synthase* (*FAS*, CG3523), *acetyl-CoA carboxylase* (*ACC*, CG11198) and *phosphofructokinase 2* (*PFK2*, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, CG3400) were all downregulated in *mlx*$^{1}$ mutant fat bodies, however they did not pass our significance cut-offs.

**4.3. Sugar intolerance and hyperglycaemia are phenotypes that can be genetically uncoupled (I)**

To test whether any of the downregulated genes would explain the *mlx*$^{1}$ mutant phenotypes, we carried out a systematic *in vivo* sugar sensitivity RNAi screen for >100 genes downregulated in the *mlx*$^{1}$ mutants. We also included in the screen a selected set of genes that are known targets of the mammalian ChREBP/MondoA-Mlx, but did not pass our strict cut-offs.

We used the Vienna *Drosophila* Research Centre RNAi line collection for the screen, in which we crossed the lines with a ubiquitous tub-GAL4 driver, collected the 1st instar larvae into 20% yeast (low sugar diet) and 20% yeast + 15% sucrose (high sugar diet) diets and scored for pupation and hatching kinetics. Out of the 105 lines tested, 6 showed embryonic lethality, 21 died during the larval stage and 16 resulted in pupal lethal phenotype, while 59 had no apparent diet-dependent phenotype. Four lines showed a diet-dependent developmental phenotype. Knockdown of *cabut*, *Aldh-III* and *PFK2* phenocopied the sugar-sensitive phenotype of *mlx*$^{1}$ mutant animals, whereas knockdown of *FAS* led to a reverse phenotype where the larvae displayed significantly lower survival into the pupal stage on the 20% yeast diet compared to the 20% yeast + 15% sucrose diet.

Knockdown of the transcription factor *cabut* and *PFK2* led to a dramatic sugar-sensitive phenotype, with only few animals able to reach the pupal stage on high sugar diet. The knockdown of *Aldh-III* also led to late pupal (pharate) lethality on low sugar diet, to a lower pupation rate on high sugar diet, earlier pupal lethality, and also to a lower larval survival rate on the 20% sucrose only diet. Importantly, restoring the *Aldh-III* expression in the *mlx*$^{1}$ mutant background improved the larval survival on the 20 % sucrose only diet. However, ectopic expression of *Aldh-III* did not rescue the *mlx*$^{1}$ mutant survival on the high sugar diet, suggesting that additional downstream targets contribute to the complex metabolic phenotype of *mlx*$^{1}$ mutants.

In addition to the dietary sugar-intolerance, the *mlx*$^{1}$ mutants display high circulating glucose levels. Surprisingly, only the knockdown of *PFK2* phenocopied the hyperglycaemic phenotype, while loss of *Aldh-III* or *cabut* did not have any significant effect on the circulating glucose levels. The dietary sugar intolerance and circulating glucose levels are phenotypes that can thus be genetically uncoupled.

**4.4. Sugar-induced transcription in *Drosophila melanogaster* (II)**

**4.4.1. Mondo-Mlx regulates the majority of sugar-induced transcription**

Our Agilent gene expression analysis provided us with a number of putative Mondo-Mlx targets, however we were surprised to find no effect between the yeast and yeast + 5% sucrose diets at the level of gene expression. Mondo-Mlx is activated by intracellular glucose, and thus we hypothesized that in our experimental setting, the long exposure to moderate sugar levels led to metabolic and transcriptional adaptations. The mammalian *in vitro* data shows that ChREBP/MondoA-Mlx is very rapidly translocated to the nucleus after glucose intake (Kawaguchi et al. 2001; Stoltzman et al. 2008). Also, the transcriptional responses to sugar feeding in flies are known to occur extremely fast (Zinke et al. 2002), suggesting that a more dynamic experimental approach is needed to define the sugar-induced transcriptome and the role of ChREBP/Mondo-Mlx in the process. In addition, the mammalian studies on identifying target genes of the mammalian ChREBP/MondoA-Mlx have largely focused on specific cell types (Ma et al. 2006; Jeong et al. 2011), and whereas our microarray was done from the fat bodies, the tissue-specific expression profile of Mondo-Mlx suggests a broader role for its function.

These observations, together with the improved feasibility of next generation sequencing techniques, prompted us to perform an Illumina RNAseq gene expression profiling of whole larvae. The animals were first kept on a low-sugar diet and subsequently transferred either to low (10% yeast) or high-sugar diets (10% yeast + 20% sucrose) for 8 hours, followed by total RNA extraction. We chose the 8h time point based on the expression kinetics of *FAS* and *sugarbabe*, two genes known to be regulated by dietary sugars (Zinke et al. 2002). Both the expression of *FAS* and *sugarbabe* peaked at 8h after sugar-feeding, and therefore we chose this time point for our genome-wide analysis. Furthermore, we used food coloured with blue dye to confirm that the animals to be analysed had indeed eaten.

First we looked at the sugar-regulated transcriptome in control animals, and found that dietary sugar is regulating multiple metabolic processes at the transcriptional level. As expected, among the most highly upregulated processes in response to high-sugar diet were glycolysis, gluconeogenesis, pentose phosphate pathway, pyruvate metabolism and fatty acid biosynthesis. In contrast, valine, leucine and isoleucine degradation and ribosome biogenesis were significantly downregulated in response to sugar feeding.

Previous studies on cultured mammalian cells have shown that Mlx together with ChREBP or MondoA plays a key role in sugar-regulated transcription (Ma et al. 2006; Sans et al. 2006; Stoltzman et al. 2008; Jeong et al. 2011). Therefore, we wanted to determine which part of sugar-induced transcription is regulated in an Mlx-dependent manner in the context of the whole organism. To do this, we compared the sugar-induced transcriptomes of control and $mlx^1$ mutant animals. In agreement with the mammalian data, we found that of the most strongly (logFC>2) sugar upregulated genes ~60% were significantly downregulated in $mlx^1$ mutants, confirming a key role for Mondo-Mlx as the main transcriptional activator in response to dietary sugars also in flies. However, the sugar downregulated-$mlx^1$ upregulated group (47% dependent on Mlx with logFC<-2) also contained a number of important metabolic regulators such as amylases, lipases and trehalase, suggesting that Mondo-Mlx plays a crucial role in both sugar induced and sugar attenuated gene expression.

We further looked at the sugar-regulated/Mlx-dependent processes, and found that of the upregulated processes, those most highly Mlx-dependent were glycolysis, gluconeogenesis, pentose phosphate pathway, pyruvate metabolism, glutathione metabolism and the cytochrome p450 genes. Of the downregulated processes, valine, leucine and isoleucine degradation and fatty acid metabolism were most highly dependent on Mlx.

The mammalian ChREBP/MondoA-Mlx recognizes ChoREs in the promoter regions of their target genes to which they bind as heterotetramers to activate transcription. In order to assess which part of the sugar-regulated genes are direct targets of Mondo-Mlx, we looked at putative ChoREs in the promoters of sugar regulated/Mlx-dependent set of genes. Putative ChoREs were significantly enriched in the sugar-upregulated but not in the sugar-downregulated group, further confirming the role of Mondo-Mlx as a transcriptional activator, and suggesting that the down-regulated genes are indirect targets of Mondo-Mlx.

#### 4.4.2. Mondo-Mlx is a key regulator of glycolysis and lipogenesis

The mammalian ChREBP/MondoA has been characterized as a key regulator of glycolysis and lipogenesis (Ishii et al. 2004; Ma et al. 2005; Ma et al. 2006; Sans et al. 2006; Jeong et al. 2011), which was confirmed by our RNAseq data. The glycolytic target genes of the mammalian ChREBP/MondoA-Mlx include *HKII*, *PFKFB3* and *L-PK* (Sans et al. 2006; Ma et. al. 2005; Ishii et al. 2004; Jeong et al. 2011). Our data however suggest an even broader role for Mondo-Mlx in regulating the glycolytic flux. We found that the genes encoding *phosphoglucose isomerase* (*Pgi*, CG8251), *aldolase* (*Ald*, CG6058), *enolase* (*Eno*, CG17654), *pyruvate kinase* (*PyK*, CG7070), several members of the pyruvate dehydrogenase complex (CG11876, CG5261, CG7430, l(1)G0334) and the *acetyl Coenzyme A synthase* (*AcCoAS*, CG9390) were strongly induced upon sugar feeding in control animals, but not in the $mlx^1$ mutants.

Perhaps the best characterized role of ChREBP-Mlx is the regulation of *de novo* lipogenic enzymes *FAS* and *ACC* in response to glucose stimulus. In addition to the previously known lipogenic targets *FAS*, *ACC*, *acetyl-CoA synthetase* (*ACS*/*AcCoAS*), *ATP citrate lyase* (*ACL*/*ATPCL*) and *Desat1*, we found that Mondo-Mlx also regulates many other aspects of lipid metabolism. For instance, we found that Mlx regulates the sugar-induced lipid storage and breakdown through the transcriptional control of perilipins, lipases and several genes of the beta-oxidation pathway.

#### 4.4.3. Novel roles for Mondo-Mlx in the regulation of carbohydrate transport and digestion in response to sugar-feeding

Intriguingly, our RNAseq data revealed a number of new putative targets for Mondo-Mlx, many of them presenting completely new metabolic pathways potentially regulated by sugar feeding and Mondo-Mlx. One of the first metabolic phenotypes we identified in the $mlx^1$ mutants were the elevated circulating glucose levels. Whereas our microarray analysis did not reveal any obvious candidate target genes responsible of the hyperglycaemic phenotype, the RNAseq data

revealed that the expression of several putative sugar transporters was regulated by sugar-feeding in an Mlx-dependent manner. Hemolymph glucose can be regulated both at the level of glucose uptake in the gut as well as by excretion through the renal (Malpighian) tubules. Furthermore, our tissue-specific gene expression analyses by qPCR showed that many of these transporters were expressed tissue-specifically in response to sugar feeding and that this induction was dependent on Mondo-Mlx. We found that the sugar-induced expression of the putative sugar transporters CG7882 and CG15406 were highly specific to renal tubules, whereas CG4797 and CG4607 showed specificity to the gut.

The *Drosophila* genome encodes three α-amylases, enzymes that are needed to breakdown dietary starch. The expression of *α-amylases* is known to be repressed by dietary sugars (Benkel and Hickey 1987), and our data showed that this sugar-induced repression was highly dependent on Mondo-Mlx. The absence of ChoREs in the promoters of *α-amylases*, and the growing evidence of Mondo-Mlx acting only as a transcriptional activator, suggest that the transcriptional control of α-amylases is regulated by a secondary effector downstream of Mondo-Mlx.

**4.4.4. Transcriptional activation of the pentose phosphate pathway is essential for growth on high sugar diet**

One of the most strongly sugar upregulated/Mlx-dependent processes was the pentose phosphate pathway (PPP). *Glucose-6-phosphate dehydrogenase* (*G6PDH*), the gatekeeper enzyme for PPP, is a known target of mammalian ChREBP-Mlx (Ma et al. 2006). Its *Drosophila* orthologue *Zwischenferment* (*Zw*), is upregulated after sugar feeding of *Drosophila* larvae through an unknown mechanism (Zinke et al. 2002). Surprisingly we found that in addition to *Zw*, Mondo-Mlx regulates virtually every step of the PPP in response to sugar. The expression of 6-phosphogluconolactonase (CG17333), *6-phosphogluconate dehydrogenase* (*Pgd*, CG3724), *transaldolase* (*Tal*, CG2827), *ribose-5-phosphate isomerase* (*Rpi*, CG30410) and transketolase (CG8036), all displayed strong Mlx-dependent upregulation upon sugar feeding.

Moreover, the knockdown of *Zw in vivo* led to severely impaired growth on high-sugar diet, demonstrating for the first time the physiological importance of the PPP activation in the presence of sugar. Since the NADPH produced by the oxidative phase of PPP is needed for DNL, we hypothesized that inhibition of PPP might hamper triglyceride accumulation in response to sugar feeding. This was exactly the case, and the total TAG content of *Zw* knockdown larvae was significantly lower compared to the control.

**4.4.5 Regulation of the biosynthesis of non-essential amino acids glutamine and serine by Mondo-Mlx**

Interestingly, our analysis uncovered previously unknown roles for Mondo-Mlx in the control of amino acid metabolism. Several genes involved in glutamine and serine biosynthesis were induced by dietary sugars in an Mlx-dependent manner. The non-essential amino acids glutamate and glutamine play a central role in amino acid and energy metabolism (DeBerardinis and Cheng 2010). Functional analysis of the putative Mondo-Mlx-targets revealed that knockdown of *Glutamine synthetase 2* (*Gs2*, CG1743) led to impaired growth on high-sugar diet in vivo.

Regulation of serine metabolism is important for the biosynthetic capacity of cells through so-called one carbon metabolism pathway. The main routes for serine biosynthesis are through 3-phosphoglycerate, an intermediate of glycolysis, or through glycine. The rate-limiting enzyme in the glycolytic route of serine biosynthesis, the phosphoserine phosphatase *astray*, was upregulated by sugar in an Mlx-dependent manner. Also the knockdown of *astray* led to a growth defect on high sugar diet, indicating that the biosynthesis of the non-essential amino acids glutamine and serine must be coordinated with respect to sugar intake to sustain optimal growth, and that Mondo-Mlx is a key regulator of these process.

**4.5. Mondo-Mlx governs a secondary tier of transcriptional effectors (II)**

In our microarray study, we found the transcription factor *cabut* to be among the most highly Mlx-regulated genes. This finding was confirmed by the sugar-induced transcriptome data where cabut was found to be the most highly Mlx-dependent sugar regulated gene. *Krüppel-like factor Klf10*, the closest mammalian homologue of *cabut* along with *Klf11*, has been shown to be a target of mammalian ChREBP-Mlx (Iizuka et al. 2011), and it has been associated with the development of type 2 diabetes (Gutierrez-Aguilar et al. 2007). A parallel study in our group examined further the role of Cabut in sugar sensing, and found that it is a direct target of

Mondo-Mlx, acting as a transcriptional repressor, inhibiting the expression of *phosphoenolpyruvate carboxykinase* (*Pepck*, CG17725) (Bartok et al. 2015). This study also showed that the deregulation of Pepck in the *mlx*$^{1}$ mutants is the underlying reason for the imbalance between glycerol and glucose metabolism, and also causes developmental lethality (Bartok et al. 2015).

In addition to *cabut*, the RNAseq data revealed a number of other transcriptional effectors that were regulated by Mlx. *Sugarbabe* (*sug*, CG3850, Glis family), *clockwork orange* (*cwo*, CG17100, *BHLHE40/41*), *kahuli* (*Kah*, CG17181, Snail/Scratch family), *grain* (*grn*, CG9656, GATA-type), *hormone receptor-like in 96* (*Hr96*, CG11783, nuclear receptor subfamily, *LXR*) and *seven up* (*svp*, CG11502, nuclear receptor subfamily, *NR2F2/COUP-TFII*), all displayed Mlx-dependent expression after sugar feeding.

Previous studies in flies have shown that *sugarbabe* is one of the earliest genes induced by sugar and that its expression is one of the highest among sugar-regulated genes, however its function has remained largely elusive (Zinke et al. 2002). The closest mammalian homologue of Sugarbabe is the Gli-similar transcription factor 2 (GLIS2). The GLI-similar family (GLIS1-3) of transcription factors belong to the Krüppel-like family of zinc finger proteins, and they have been shown to have a critical role in kidney development (Kim et al. 2008b). Dysfunction of GLIS proteins has been associated various human diseases, including the development of diabetes, cystic kidney disease and cancer (Lichti-Kaiser et al. 2012). Furthermore, similar to Myc, GLIS1 has been shown to greatly enhance the reprogramming of somatic cells into induced pluripotent stem cells (iPSCs), forming the famous cocktail of four transcription factors including Oct3/4, Sox2 and Klf4, required for the generation of iPSCs (Maekawa et al. 2011).

#### 4.5.1. Sugarbabe and Dawdle are direct targets of Mondo-Mlx and required for organismal sugar tolerance

We studied further the role of Sugarbabe *in vivo* in *Drosophila* and found that knockdown of *sugarbabe* leads to impaired growth on high-sugar diet. We also created a deletion mutant of *sugarbabe*, which displayed a similar sugar-sensitive phenotype, further confirming its role in dietary sugar tolerance.

In addition to *sugarbabe*, the Activin ligand *dawdle* was among the sugar up-regulated/Mlx-dependent group of genes. *Dawdle* had been recently identified as a sugar induced gene in *Drosophila,* but the underlying upstream regulatory mechanism had remained unknown (Chng et al. 2014). Loss of Activin signalling has been also associated with a nutrient-dependent phenotype in *Drosophila*, where the *dawdle* null larvae grown on a high protein diet showed a striking growth advantage compared to larvae grown on standard laboratory diet (Ghosh and O'Connor 2014). We found that the knockdown of *dawdle* leads to severe diet-dependent phenotype – on high sugar diet the *dawdle* RNAi animals die at the late pupal stage, whereas on low sugar diet they reach adulthood.

This led us to ask how Mondo-Mlx, Sugarbabe and Dawdle are connected at the regulatory level. We first showed that both *sugarbabe* and *dawdle* are direct targets of Mondo-Mlx both by *in vitro* and *in vivo* ChIP analysis. Furthermore, we found that while *dawdle* expression was independent of Sugarbabe, the expression of *sugarbabe* was strongly dependent on Dawdle, suggesting that Sugarbabe acts downstream of Activin signalling.

#### 4.5.2. Sugarbabe regulates a subset of Mondo-Mlx targets

Next we wanted to study in more detail the role of Sugarbabe in sugar-induced transcription. To do this, we performed a similar sugar-induction experiment with *sugarbabe* mutants as described for *mlx*$^{1}$ mutants, followed by RNA sequencing. The sugar-regulated gene sets dependent on Sugarbabe displayed significant overlap with Mlx-dependent genes, including *astray*, the enzyme that catalyses the last step in the biosynthesis of serine from carbohydrates. The knockdown of *dawdle* also suppressed the sugar-induced expression of *astray*, further confirming the finding that the Activin-Sugarbabe regulatory axis functions downstream of Mondo-Mlx.

The sugar-induced/Sugarbabe-dependent group of genes included also *α-amylases*. Furthermore, we found that larvae overexpressing *sugarbabe* survive poorly on a diet with high starch content compared to control animals, providing evidence for functional importance of Sugarbabe-mediated regulation of *amylase proximal*. Moreover, it has been previously demonstrated that Activin signaling is required for the sugar-mediated repression of *α-amylases* (Chng et al. 2014).

Together, these results indicate that Mondo-Mlx suppresses starch breakdown on high sugar diet through its downstream effectors Dawdle and Sugarbabe.

Of the sugar-induced/Sugarbabe-dependent processes, fatty acid biosynthesis was among the most strongly enriched. To test whether Sugarbabe activity on lipogenic genes *FAS* and *ACC* is dependent on Mondo-Mlx, we rescued *sugarbabe* expression in the fat bodies of $mlx^{1}$ mutants. Indeed, the ectopic expression of *sugarbabe* was sufficient to rescue both the expression of *FAS* and *ACC*, and the triglyceride levels of $mlx^{1}$ mutant animals. Thus, Sugarbabe is acting downstream of Mondo-Mlx also as a feed-forward regulator of lipogenesis. We also found that *FAS* and *ACC* levels were reduced in *dawdle* RNAi animals, providing further support for our model in which Mondo-Mlx is acting as a master regulator of transcriptional network composed of several secondary targets, including Sugarbabe, which is also acting downstream of TGF-β/Activin signalling.

**4.6. Human homologs of Mondo-Mlx targets are enriched among triglyceride-associated variants (II)**

Finally, we compared our RNAseq data on existing human GWAS studies on circulating triglycerides, a phenotype associated with human *ChREBP* (*MLXIPL*) polymorphism (15). We found a striking correlation with putative Mondo-Mlx targets and human genomic variants associated with circulating triglyceride levels (ENGAGE Consortium study, (Surakka et al. 2015)). Moreover, we found that for some of the loci, the actual causal genes underlying the metabolic phenotypes could be predicted based on our data. In conclusion, our data suggest that the data on *Drosophila* Mondo-Mlx targets can be used to predict putative causal genes in the vicinity of the triglyceride-associated genomic variants in humans.

## 5. CONCLUSIONS AND FUTURE PERSPECTIVES

### 5.1. Mondo-Mlx is a master regulator of a sugar-induced gene regulatory network

In this thesis I have characterized the *in vivo* roles of *Drosophila* Mondo-Mlx in sugar-induced transcription. One of the first and major findings was that the loss of functional Mondo-Mlx leads to dietary sugar intolerance *in vivo*. To our knowledge, this is a phenotype that has never been reported in the fly before. Most importantly, this phenotype resembles the one of *ChREBP-/-* mice that are also intolerant to dietary carbohydrates (Iizuka et al. 2004), demonstrating the functional conservation of ChREBP/Mondo-Mlx across phyla. The RNAseq analysis of *Drosophila* Mondo-Mlx target genes further confirmed the conservation at the level of target gene regulation, including for example the well-established mammalian ChREBP-Mlx targets *FAS*, *ACC* and *G6PDH*. Moreover, our study that utilized a transient sugar exposure *in vivo*, was the most comprehensive sugar-induced transcriptome profiling performed in a physiological setting so far. The mammalian ChREBP/MondoA-Mlx are best known for their roles in regulating glycolysis and lipogenesis. However, other metabolic pathways and downstream effectors have also been indicated as their targets, including *Krüppel-like factor 10* and *DEC1*. This thesis further broadened the view of ChREBP/MondoA-Mlx as a metabolic regulator, extending its role in *Drosophila* also to for example amino acid metabolism.

One of the genes most prominently regulated by sugar and Mondo-Mlx in our study was the Gli-similar transcription factor *sugarbabe*. Although *sugarbabe* had been identified as the earliest and strongest sugar-responsive gene in *Drosophila* more than 10 years ago, data on its role in regulating glucose metabolism had remained limited. A suppressive role for Sugarbabe in regulating the repression of lipolytic genes before the activation of lipogenic genes was suggested by Zinke and colleagues, who proposed in 2002 that: "*Based on these observations, we propose a model in which an as yet unknown transcription factor(s) is activated by a sugar signal. This factor(s) then activates sug, as well as a set of genes involved in fatty acid synthesis, such as acetyl CoA carboxylase and ATP citrate lyase*" (Zinke et al. 2002). This prediction turned out the be accurate, and we and others have now identified many of the missing links in the proposed model (Figure 13).

We found that *sugarbabe* is a direct target of Mondo-Mlx, and that it regulates fatty acid synthesis downstream of Mondo-Mlx by inducing the expression of lipogenic genes *ACC*, *FAS*,

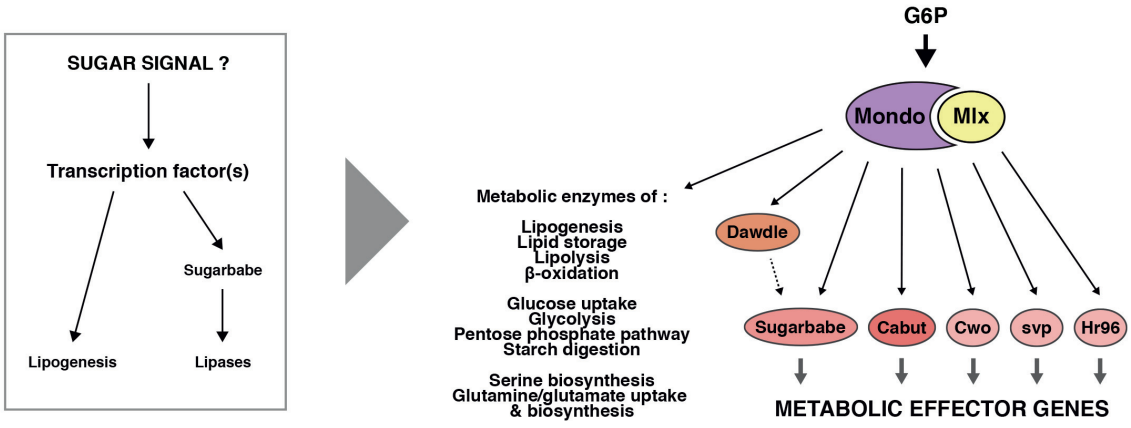


**Figure 13. The sugar-induced gene regulatory network**
Left: In the model proposed by Zinke et al. 2002, a sugar signal activates a transcription factor(s) that activates lipogenesis and the transcription factor Sugarbabe, which represses the expression of lipases in response to sugars. Right: The model proposed in this thesis: The sugar-activated Mondo-Mlx activates directly the expression of many enzymes involved in variety of metabolic processes, including lipogenesis, lipid storage, lipolysis, β-oxidation, glucose uptake, glycolysis, pentose phosphate pathway, starch digestion, serine biosynthesis, and glutamine and glutamate uptake and biosynthesis. In addition, Mondo-Mlx induces the expression of secondary effectors that mediate part of its functions in response to sugars. These effectors include Sugarbabe, Cabut, Clockwork orange, Seven up and Hormone receptor-like in 96.

*AcCoAS* and *ATPCL* in response to sugar feeding. Furthermore, our RNAseq analysis of the *sugarbabe* mutants revealed that the expression of CG6271, a lipase that is repressed in response to sugar, was also strongly upregulated in mutants, supporting the findings and model proposed by Zinke et al. In addition, we found that Sugarbabe is regulating the repression of *α-amylases*. Amylase repression is also inhibited in the $mlx^{1}$ mutants, and thus we propose a model in which Sugarbabe is mediating the sugar/Mondo-Mlx-dependent repression of *α-amylases*. Gli-similar transcription factor 2, the human homologue of Sugarbabe, was originally identified as a bifunctional transcription factor, regulating both the activation and repression of its target genes involved in kidney development (Zhang et al. 2002). Interestingly, variants of another member of the GLIS-family, GLIS3, are now established as one of the strongest known genetic risk factors for both type 1 and type 2 diabetes (Yang and Chan 2016). GLIS3 is a key regulator of both the expression of *insulin* and proliferation of β-cells (Kang et al. 2009; Yang et al. 2009; Yang et al. 2013; ZeRuth et al. 2013). According to FlyBase, the closest homologue of GLIS3 in *Drosophila* is a gene called *lame duck* (CG4677). However, Lame duck is a regulator of myogenesis, and we found the expression of *lame duck* to be only moderately induced by dietary sugars. In fact, *sugarbabe* is expressed in the IPCs where it regulates the expression of *dILP*s (Varghese and Cohen 2007), suggesting that it might be the functional homologue of GLIS3.

However, as with any model, answers give rise to new questions, and the major future challenges include resolving the roles of other transcriptional effectors downstream of sugar signals and Mondo-Mlx. In addition to *sugarbabe*, our RNAseq profiling revealed a number of other transcriptional regulators regulated by sugar in a Mondo-Mlx dependent manner, including *cabut*, *clockwork orange*, *seven up* and *hormone receptor-like in 96*.

The human homologues of *cabut* (*KLF10*) and *clockwork orange* (*BHLHE40/41, DEC1/2*) are also known targets of mammalian ChREBP-Mlx. KLF10 and DEC1/DEC2 are both involved in the regulation of the circadian clock, a molecular system stimulated by periodic environmental stimuli that regulates the sleep-wake cycle, feeding behavior and metabolism. Although the master clock is located in the brain, the clock proteins are expressed ubiquitously in all tissues. In fact, a large number of metabolic genes involved in carbohydrate and lipid metabolism are regulated by clock proteins (McGinnis and Young 2016). There is increasing evidence of a link between the dysregulation of the clock and the development of metabolic diseases. Mice with mutations in the core clock components develop obesity, metabolic syndrome and type 2 diabetes (Turek et al. 2005; Marcheva et al. 2010). High fat diet in turn disrupts the function of the clock, which leads to the dysregulation of nuclear receptors and their downstream metabolic target genes (Kohsaka et al. 2007; Barnea et al. 2009). The importance of coordinating feeding behavior with the signals from the clock is also shown by studies in which consumption of food during the biologically inactive period results in metabolic dysregulation including higher postprandial glucose and Insulin levels (Hampton et al. 1996; Mukherji et al. 2015). In fact, it is now known that shift workers are at higher risk for developing metabolic syndrome, type 2 diabetes and coronary heart disease (Kawachi et al. 1995; De Bacquer et al. 2009; Pan et al. 2011).

KLF10 has been proposed to be the transcription factor mediating the information from the clock to the level of metabolic gene expression in the liver (Guillaumond et al. 2010). Our group has also shown that Cabut provides a link between the clock and metabolic regulation in response to sugar feeding (Bartok et al. 2015). Moreover, the mammalian data together with our findings suggest that this process is dependent on ChREBP/Mondo-Mlx (Iizuka et al. 2011; Bartok et al. 2015). Interestingly, humans that carry a specific variant of the core clock gene *CRY1* develop insulin resistance, but only if exposed to a high carbohydrate diet (Dashti et al. 2014). Taken together, the nutritional signals alter the function of the clock, but at the same time the dysfunctional clock causes metabolic dysregulation, suggesting that any alteration in this system can cause a feedforward effect, resulting in metabolic disease. ChREBP/Mondo-Mlx is likely to play a critical role in this process, but the exact mechanisms remain to discovered.

The expression of the human homologue of *seven up*, called *chicken ovalbumin upstream promoter transcription factor II* (*COUP-TFII*), is suppressed by sugar in an ChREBP-Mlx dependent manner (Perilhou et al. 2008). COUP-TFII has been also shown to bind same promoters as ChREBP,

including the *L-PK* promoter, suggesting that COUP-TFII and ChREBP might coordinate their target gene regulation in response to different nutritional signals (Lou et al. 1999). *COUP-TFII* was originally characterized as a gene involved in developmental processes, and the null mutant mice are embryonic lethal (Pereira et al. 1999; Bardoux et al. 2005). COUP-TFII was later shown to play an important role in metabolism; heterozygous mice are insulin resistant and display abnormal Insulin secretion (Bardoux et al. 2005). We found *seven up* expression to be strongly induced in response to dietary sugars, and this induction was dependent on Mondo-Mlx. Interestingly, although the expression of *seven up* was induced after 8 hours of sugar feeding, expression was actually repressed at a shorter time point (1.5h), but not in the $mlx^{1}$ mutant animals (unpublished data). This, together with our unpublished observation that ubiquitous knockdown of *seven up* leads to early larval lethality, suggests a highly conserved role for Seven up/COUP-TFII in regulating development and metabolism in response to sugar feeding.

The Hormone receptor-like in 96 (Hr96, DHR96) is the functional homologue of mammalian LXR. Like its mammalian counterpart, it binds cholesterol and regulates cholesterol homeostasis (Horner et al. 2009). In addition, is has been shown to control the breakdown of dietary fats via its downstream target lipase called *magro*, which is also required for proper cholesterol metabolism in the gut (Sieber and Thummel 2009; Sieber and Thummel 2012). Our data show that *Hr96* is transcriptionally upregulated in response to dietary sugars, and that this induction is dependent on Mondo-Mlx. Moreover, the knockdown of both *Hr96* and *seven up* was recently shown to lead to hyperglycemia (Ugrankar et al. 2015), suggesting an important role for both of these nuclear receptors in regulating glucose homeostasis.

Taken together, in addition to regulating the expression of metabolic enzymes directly, Mondo-Mlx controls a large gene regulatory network composed of multiple downstream effectors. The downstream transcriptional effectors play diverse roles in various biological processes in addition to carbohydrate metabolism, including development, circadian rhythm and cholesterol metabolism. Many of these effectors also appear to be conserved targets of the mammalian ChREBP-Mlx, suggesting that sugar sensing is an evolutionarily conserved process in multicellular animals. It will be of great interest to determine the exact roles of the other Mondo-Mlx transcriptional effectors in regulating metabolism downstream of Mondo-Mlx. *Drosophila,* with its less complex genome and lower redundancy, provides an optimal model to study further the roles of the Mondo-Mlx regulated transcriptional network.

### 5.2. Novel ChREBP isoform, control of feeding behavior and communication with other nutrient sensors

After their discovery, ChREBP and MondoA have been established as key regulators of sugar-induced transcription. The first direct target genes to be identified were enzymes of the glycolytic and lipogenic pathway. Thus, much attention has been directed towards clarifying the role of ChREBP/MondoA-Mlx in regulating these pathways. However, the classical view of ChREBP and MondoA as regulators of only glycolysis and lipogenesis greatly underestimates the role of these transcription factors in regulating a wide range of cellular processes both directly and indirectly via their secondary effectors.

Although much effort has been put into the identification of the upstream signals regulating ChREBP and MondoA activity, many questions still remain. For instance, while the activating role of G6P is now widely accepted, there is still controversy about whether other sugar metabolites participate in the activation process. Similarly, there is no question about the regulatory role of G6P-induced nuclear translocation for ChREBP and MondoA activity, but now it is also known that additional regulatory steps are needed for the final transcriptional activity of the heterodimers. There is no clear consensus yet on these events, and it seems likely that the posttranslational modifications together with co-factors and other regulatory events required for the final activation are cell and tissue type specific. Many of the current studies are conducted *in vitro* with transformed or immortalized cell lines that are known to exhibit altered metabolism. In fact, the role of MondoA in regulating the metabolism of cancer cells has been already established (Wernicke et al. 2012; Carroll et al. 2015), and thus studying ChREBP and MondoA in non-cancerous cell lines or *in vivo* is likely crucial for understanding their roles and regulation in a normal physiological context.

It is also surprising how little is known about the communication and interaction between different

nutrient sensing pathways. The activation of ChREBP and MondoA appears to take place independently of Insulin, however a large number of their target genes are also regulated by the Insulin-activated transcription factor SREBP. Thus it is likely that these pathways communicate with each other to coordinate the expression of their target genes in response to various stimuli. In fact, it has been recently shown that SREBP expression is induced by glucose via ChREBP-Mlx (Jeong et al. 2011, Poungvarin et al. 2015). A number of nuclear receptors also regulate the expression of the same target genes as ChREBP and SREBP. ChREBP has been shown to interact physically and regulate the expression of its target genes together with for example HNF-4α and PGC-1β (Adamson et al. 2006; Chambers et al. 2013). Furthermore, the ChREBP promoter harbors known binding sites for LXRα/RXRα, TRβ1/RXRα, SREBP and for ChREBP itself, suggesting a complex regulatory system including feedback loops and signaling between several nutrient sensing pathways.

One of the most interesting recent studies demonstrated that TOR interacts physically with both MondoA and ChREBP (Kaadige et al. 2015). The authors show that TOR is competing with Mlx for binding of MondoA in the cytoplasm, and thus controlling its transcriptional activity. This interaction is promoted by increased intracellular reactive oxygen species (ROS). The MondoA-Mlx target TXNIP increases intracellular ROS levels and thus the inhibition of MondoA activity by TOR is proposed to serve as a protective feedback loop to reduce the expression of TXNIP. Interestingly, there seems to be a reciprocal regulation between MondoA and TOR, since MondoA-Mlx was found to suppresses TORC1 activity in response to 2-DG treatment (Kaadige et al. 2015). It has been also recently suggested that the *C. elegans* Myc and Mondo-like protein MML-1 is regulating longevity by stimulating autophagy via inhibition of TOR activity (Nakamura et al. 2016). The authors also propose a conserved function for Mondo proteins in the inhibition of TOR activity. They found that both MondoA and ChREBP stimulated the nuclear localization of the TOR signaling inhibitor TFEB during amino acid starvation in HeLa cells. Together, these recent findings indicate a tight communication between Mondo and TOR signaling pathways.

The identification of the novel ChREBP-β isoform has brought a new player to the complex feedback regulatory system of ChREBP. The ChREBP-β isoform is activated by the α–isoform and is a significantly more potent activator of sugar-induced transcription (Herman et al. 2012). Importantly, it was shown that only the expression level of *ChREBP-β* predicts insulin sensitivity in humans (Herman et al. 2012), suggesting that the isoforms display distinct functions in the pathobiology of human metabolic diseases. The newly identified ChREBP-β isoform seems to have developed only later in the evolution of tetrapods, since fish for example lack this isoform (Singh and Irwin 2016). To date, fly studies have not distinguished separate functional isoforms of Mondo or Mlx. However, according to Flybase (R6.12), the *D. melanogaster* Mondo encodes 11 different isoforms, which differ mainly by their 5' untranslated region. One isoform (Mondo-RE) also lacks the first two exons, and it will therefore be interesting to learn whether there are functional differences between the *Drosophil*a Mondo isoforms. Whether the mammalian MondoA encodes differently regulated isoforms also remains to be discovered.

### 5.3. Systemic regulation of whole-body metabolism by Mondo-Mlx

Whereas much of the focus has been on elucidating the mechanisms by which ChREBP/MondoA-Mlx activity is regulated, and what their direct targets are, less is known about the systemic effects of ChREBP/MondoA-Mlx on whole-body metabolism. The Activin ligand Dawdle expressed and secreted from the fat body was recently found to be a hormonal regulator acting in the gut to suppress the expression of *α-amylases* (Chng et al. 2014). Here we show that sugar-induced expression is dependent on Mondo-Mlx, providing evidence on Mondo-Mlx as a regulator of hormonal signals in response to dietary sugars.

*Fibroblast growth factor 21* (*FGF21*) is a hepatokine known to be a direct target of ChREBP-Mlx (Iizuka et al. 2009a). However, only recently has the physiological role of ChREBP- induced expression of *FGF21* been discovered. Liver-secreted FGF21 was shown to act directly on the hypothalamus to suppress food intake and specifically sugar feeding (von Holstein-Rathlou et al. 2016). Although the fly does not have a homologue for FGF21, *Drosophila* Mondo has been suggested to control feeding behavior. Knockdown of *mondo* specifically in the fat body or in the IPCs results in decreased feeding behavior

of adult flies (Sassu et al. 2012; Docherty et al. 2015). It has been also suggested that whereas IPCs seem to respond to amino acid signals, circulating sugar levels are sensed by the corpora cardiaca that secretes AKH, which in turn signals to IPCs to release dILP3 (Kim and Neufeld 2015). Intriguingly, it was recently shown that loss of Mondo in the IPCs leads to an increased expression of *dILP3*, suggesting an autonomous role for Mondo-Mlx in regulating feeding behavior in the brain (Docherty et al. 2015). It will be also interesting to learn whether Mondo-Mlx functions in the recently identified group of neurosecretory cells that were shown to express *diuretic hormone 44* (*Dh44*), a human homologue of *corticotropin-releasing hormone* (*CRH*) and to be activated specifically by dietary sugars (Dus et al. 2015).

In addition to fat body, we found that *mondo* and *mlx* are also highly expressed in the gut. The expression of Mondo proteins in the gut is conserved. In addition to its very prominent expression in skeletal muscle, heart, kidney and liver, *MondoA* is also expressed in the colon and small intestine (Billin et al. 2000). This is also true for *ChREBP,* which although mainly studied in the liver and adipose tissue, shows also strong expression in the gut (Yamashita et al. 2001; Iizuka et al. 2004). Moreover, although Mlx is a ubiquitously expressed protein, its expression in mice has been found to be highest in the villi of the gut (Meroni et al. 2000). The *MML-1/MXL-2* are also highly expressed in the *C. elegans* intestine (Cairo et al. 2001; Pickett et al. 2007). Despite these early findings, the function of ChREBP/MondoA-Mlx in the gut is still largely unstudied. We show here that Mondo-Mlx regulates the expression of gut *amylases* via the secondary effectors Dawdle and Sugarbabe. Furthermore, we found *sugarbabe* to display its highest relative expression in the larval gut, suggesting an important role for gut in the regulation of metabolic responses to sugar feeding.

This is not surprising, since the initial decisions of nutrient absorption are made in the intestine, which is the major site for nutrient intake. The classical view of the gut as a metabolically passive organ, only responsible for the expression of digestive enzymes and subsequent absorption of nutrients, has turned out to be false. The gut is a highly complex and dynamic organ that not only serves as a site for digestion and absorption, but also participates in the regulation of metabolism by various means. For example, gut-secreted hormones regulate metabolism in other tissues, and gut-expressed enzymes dictate the fate of some nutrients before they even are absorbed. The gut has been shown to participate actively in nutrient sensing and metabolism in *Drosophila* as well (Miguel-Aliaga 2012). Recent studies have highlighted the importance of gut microbiota in human health and disease (Cho and Blaser 2012; Boulange et al. 2016), and also support the notion of the gut as a key organ regulating whole-body metabolic homeostasis. The role of ChREBP and MondoA in these processes, and whether there is interaction between the signaling events downstream of nutrient induced switches in microbiota function and ChREBP/MondoA-Mlx activity, are of great interest for the future studies. In fact, the gut microbiota has already been shown to regulate ChREBP activity (Backhed et al. 2004).

Tissue-specific knockout/knockdown models are likely to uncover some of the gut-specific roles of ChREBP/MondoA-Mlx, and *Drosophila* provides an optimal model to study them. Overall, *Drosophila* serves as an excellent cost-efficient model also for screening new regulators of glucose metabolism. A recent *in vivo* study identified more than 160 new genes regulating circulating glucose levels in *Drosophila* in a tissue-specific manner (Ugrankar et al. 2015). Of the characterized hits, more than 70 were novel candidates, and a conserved function for Ck1alpha in regulating glucose metabolism was also shown in mammals. This "glucome screen" study highlights the power of *Drosophila* as a model to pinpoint not only new metabolic regulators, but also their sites of function.

### 5.4. The emerging roles of ChREBP/MondoA-Mlx in human metabolic diseases

Since their discovery, the dysregulation of ChREBP and MondoA has been linked to various human diseases and metabolic phenotypes. However, the data are still limited, and it seems that the dysregulation of ChREBP leads to distinct phenotypes in different tissues. Whereas increased ChREBP activity in the liver has been linked to elevated DNL, obesity and insulin resistance, in adipose tissue ChREBP appears to have a protective function against the development of insulin resistance (Herman et al. 2012; Eissing et al. 2013; Kursawe et al. 2013). It has been also shown that both liver specific and whole-body inhibition of *ChREBP* not only alleviates the hepatic steatosis of *ob/ob* mice, but also improves the whole-body

glucose tolerance and insulin sensitivity (Dentin et al. 2006; Iizuka et al. 2006). On the other hand, the whole-body deficiency of *ChREBP* leads to reduced hepatic DNL and insulin resistance in mice (Iizuka et al. 2004). Although elevated hepatic DNL has been shown to contribute to the development of NAFLD and insulin resistance (Donnelly et al. 2005; Ameer et al. 2014), increased triglyceride accumulation and hepatic steatosis do not always lead to insulin resistance. In fact, it has been suggested that ChREBP activity is the factor that dissociates benign hepatic steatosis from insulin resistance, and that increased ChREBP activity in the liver also protects against insulin resistance (Benhamed et al. 2012).

The classical view of obesity as the cause of insulin resistance has also been challenged by a number of studies that have shown a protective role for certain lipid species and their appropriate storage in the white adipose tissue against the development of insulin resistance (Stefan et al. 2008; Lodhi et al. 2011). The protective role for fat body lipogenesis against the harmful consequences of high-sugar diet feeding has been also shown in *Drosophila* (Musselman et al. 2013). Lipotoxicity, a condition of lipid overload in adipose tissue leading to increased lipid intermediates in blood and non-adipose tissue, in turn has been suggested to contribute to the development of metabolic and cardiovascular diseases (Unger et al. 2010). Lipotoxicity is characterized especially by the accumulation of ceramides, which are thought to be among the most harmful lipid metabolites in the development of metabolic disorders (Chaurasia and Summers 2015). Increased plasma and skeletal muscle ceramide levels are observed in type 2 diabetes (Adams et al. 2004; Haus et al. 2009). Interestingly, our metabolic profiling of $mlx^{1}$ mutant animals revealed an increase in ceramide levels, suggesting that Mondo-Mlx plays an important role in the development of lipotoxicity and in sphingolipid biosynthesis. Our RNAseq data also showed that Mondo-Mlx is regulating the expression of serine palmitoyl transferase subunits *Spt-I* and *lace*, which are the rate limiting enzymes of the sphingolipid biosynthesis pathway. Moreover, we found that the sugar-induced expression of *perilipins* (*Lsd-1* and *Lsd-2*) was dependent on Mondo-Mlx. Lipid droplet formation, and more specifically perilipins, are suggested to play a protective role against lipotoxicity (Borg et al. 2009). Our data suggest that Mondo-Mlx is regulating the delicate balance between healthy lipid storage and toxic overspill of lipid intermediates into other tissues at multiple levels, including *de novo* lipogenesis, lipid storage and biosynthesis of sphingolipids. The beneficial role for Mondo-regulated lipogenesis is supported also by another study, which demonstrated that Mondo-dependent lipogenesis in the fat body protects from high sugar diet induced hyperglycemia (Musselman et al. 2013).

The distinct outcomes of increased ChREBP activity in different tissues, and also the contradictory studies on modulation of ChREBP activity in the liver, have taught us a great deal about the pathogenesis of metabolic diseases such as hepatic steatosis and type 2 diabetes. The complex interplay between different metabolic organs and environmental and genetic factors is likely to dictate the ultimate metabolic outcomes. It seems that ChREBP plays a major role in the development of many metabolic diseases, and we are only beginning to understand its role in these processes. Altogether, the existing studies suggest that modulation of ChREBP activity holds great promise as a therapeutic strategy to treat human metabolic diseases.

### 5.5. Evolutionary view on sugar sensing

Human diet has changed dramatically from traditional hunter-gatherers to agricultural societies and further to modern Western diet. Although the diet of hunter-gatherers showed great variation depending on the environment and culture, compared to modern human diet, the relatively low amount of carbohydrates was a universal feature (Cordain et al. 2000).

The changes in the dietary behavior are also reflected at the genetic level. A common example is the lactase enzyme that breaks down milk-sugar lactose. The production of lactase enzyme declines after weaning in all mammals including humans (Sebastio et al. 1989). However, some humans are lactate persistent, a trait that is particularly common in some parts of the world, for example Northern Europe (Ingram et al. 2009; Itan et al. 2010). Lactose persistence has been suggested to have evolved in cultures where dairy products are consumed in high quantities, and current models trace the mutation associated with the lactase persistence trait back to the time of animal domestication (Enattah et al. 2002; Bersaglieri et al. 2004; Tishkoff et al. 2007; Evershed et al. 2008). Another interesting example of the evolution of

metabolic enzymes in humans is the positive selection on salivary amylase (*AMY1*) gene copy number, which correlates positively with human populations that have favored a high-starch diet (Perry et al. 2007). Whereas the Neanderthals had only one copy of *AMY1*, over 98% of modern humans carry multiple copies of the gene (Perry et al. 2007; Prufer et al. 2014).

The modern Western diet is characterized by a high amount of carbohydrates, estimated to contribute as much as 50% of the total calories in a typical American diet (Freedman et al. 2001; Wright and Wang 2010). The increasing consumption of sugar has been proposed to underlie the increase in prevalence of metabolic diseases world-wide, and according to the newest guideline of the World Health Organization (WHO), the daily intake of added sugars should not exceed 10% of total energy intake. The increasing consumption of fructose, especially from sugar-sweetened beverages, has been suggested to be the major contributing factor to the obesity epidemic and to obesity-related diseases (Lustig et al. 2012). Fructose is metabolized by the liver, and high fructose consumption has been shown to increase hepatic DNL, compromise hepatic insulin sensitivity and to contribute to the development of lipotoxicity (Mayes 1993; Le et al. 2009; Stanhope et al. 2009; Tappy and Le 2010; Lustig et al. 2012; Stanhope 2012). We found that the *mlx*$^{1}$ mutants are intolerant to both glucose and fructose, suggesting that Mondo-Mlx activity is required for proper metabolism of both. Liver ChREBP activity in rats has been shown to respond more strongly to fructose than glucose (Koo et al. 2009). It has been also recently suggested that fructose is a much more potent activator of the newly identified ChREBP-β isoform (Stamatikos et al. 2016). However, the role of Mondo proteins in mediating the specific metabolic effects of different sugars are still largely unknown.

The cactus breeder *D. mojavensis*, has been also shown to
display intolerance to high sugar diet (Matzkin et al. 2011). It will be interesting to learn what are the genetic mechanisms underlying the sugar intolerance of *D. mojavensis*. The genetic analysis of closely related species that differ in their dietary preferences provides an interesting way to discover the mechanisms defining the macronutrient spaces of different animals. Similarly, the analysis of human populations or individuals that show different responses to dietary interventions is invaluable for understanding the variation observed between individuals in their response to different dietary interventions. The example of individuals carrying a *CRY* variant and displaying a high carbohydrate diet-dependent risk of developing insulin resistance (Dashti et al. 2014) suggests that in order to manage metabolic diseases such as type 2 diabetes, we must understand how genetic background interacts with the diet. Certain variants of *ChREBP* have been shown to correlate with increased circulating triglyceride levels, a known risk factor for developing insulin resistance and type 2 diabetes (Kathiresan et al. 2008; Kooner et al. 2008; Wang et al. 2008; Willer et al. 2008; Pan et al. 2009). However, the underlying target genes of ChREBP causing the altered lipid profiles have remained largely undiscovered. We showed that comparing the *Drosophila* Mondo-Mlx regulated transcriptome data to genome-wide association studies (GWAS) can reveal putative causal genes. Comparisons of existing GWAS data sets to functional studies holds a great promise on revealing new potential therapeutic target genes.

Another layer of complexity comes from the discovery that acquired traits can be inherited. It has been shown in several studies that diet induces metabolic reprogramming via chromatin remodeling, and that these epigenetic changes can be also transmitted to the progeny (Ng et al. 2010; Fullston et al. 2013; Leung et al. 2014; Ost et al. 2014). For example, excessive weight gain in early childhood increases the likelihood of developing type 2 diabetes and obesity in the next generation, independent of parental adult obesity (Li et al. 2009a). Moreover, these epigenetic modifications seem to be dependent on genetic background (Leung et al. 2014). Whereas personalized medicine as a concept is widely acknowledged to be critical in treating certain diseases, personalized nutrition as a practice to manage metabolic diseases is only now emerging.

There is no evolution without variation (Darwin 1859). It is clear that species, populations and individuals within them, differ in their metabolism and responses to different diets. This is crucial for adaptation, but also displays a challenge for treating metabolic diseases. Understanding the complex interplay between genetics, epigenetics and diet is the key for developing personalized therapies for metabolic disorders.

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