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1 **Sugar sensing by ChREBP/Mondo-Mlx – new insight into downstream**
2 **regulatory networks and integration of nutrient-derived signals**
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13 Short title: ChREBP/Mondo-Mlx in sugar sensing
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15 Body text word count: 3090
16
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Highlights

-ChREBP/Mondo-Mlx is an intracellular sugar sensor of animals with conserved target genes and physiological functions

- ChREBP/Mondo-Mlx integrates multiple inputs through direct sensing of sugar and fatty acid-derived metabolites and cross-talk with other nutrient sensing pathways

-ChREBP/Mondo-Mlx controls several second-tier regulators, including transcription factors and hormones

Abstract

Animals regulate their physiology with respect to nutrient status, which requires nutrient sensing pathways. Simple carbohydrates, sugars, are sensed by the basic-helix-loop-helix leucine zipper transcription factors ChREBP/Mondo, together with their heterodimerization partner Mlx, which are well-established activators of sugar-induced lipogenesis. Loss of ChREBP/Mondo-Mlx in mouse and *Drosophila* leads to sugar intolerance, *i.e.* inability to survive on sugar containing diet. Recent evidence has revealed that ChREBP/Mondo-Mlx responds to sugar and fatty acid-derived metabolites through several mechanisms and cross-connects with other nutrient sensing pathways. ChREBP/Mondo-Mlx controls several downstream transcription factors and hormones, which mediate not only readjustment of metabolic pathways, but also control feeding behavior, intestinal digestion, and circadian rhythm.

Preface

Nutrient intake of animals displays a high degree of variation, which requires fine-tuned control of metabolic pathways. Animals regulate their metabolic homeostasis by continuously reading and converging signals originating from both within the organism and the outside environment. Macronutrients (sugars, amino acids and lipids) are sensed by nutrient sensing pathways that detect different dietary inputs and orchestrate an integrated adaptive response that is appropriate for that particular set of macronutrients. Simple carbohydrates, sugars, are sensed by several mechanisms, of which the so-called intracellular sugar sensing by ChREBP/Mondo-Mlx transcription factors is among the most conserved ones. Studies from mouse and *Drosophila* have shown the importance of this system in providing sugar tolerance; animals lacking ChREBP/Mondo-Mlx die rapidly on a diet containing sugars at a level, which is normally physiologically

52 tolerated [1,2]. We highlight here recent work, which has revealed several novel aspects
53 of the ChREBP/Mondo-Mlx physiological function and regulation by nutrient-derived
54 cues.
55
56 **ChREBP/Mondo-Mlx – the conserved regulator of metabolic homeostasis**
57 Carbohydrate response element binding protein (ChREBP, also known as MondoB and
58 MLXIPL) and its paralog MondoA (MLXIP) are intracellular sugar sensors that are
59 activated by glucose-6-phosphate (G6P) and other phosphorylated hexoses [3].
60 ChREBP and MondoA act together with their common heterodimerization partner Mlx,
61 a necessary step for transcriptional activation. Although both are ubiquitously
62 expressed, ChREBP is more prominently expressed in the liver and adipose tissue
63 whereas MondoA is most highly expressed in the skeletal muscle. Muscle breaks down
64 glucose via the glycolytic pathway to release energy that is used for muscle contraction
65 and in fact many of the MondoA-Mlx target genes are involved in the glycolytic
66 pathway [4,5]. Liver is the main site for *de novo* lipogenesis (DNL), where dietary
67 sugars are converted into triacylglycerols (TAGs) and further transported to adipose
68 tissue for storage. ChREBP target genes include lipogenic genes, such as *fatty acid*
69 *synthase* and *acetyl-CoA-carboxylase*, both of which possess well-established
70 ChREBP-Mlx binding sites, known as the carbohydrate response element (ChoRE), in
71 their promoters [6,7]. ChREBP-Mlx is also a key regulator of glycolytic genes, such as
72 *glucokinase regulatory protein*, *glyceraldehyde-3-phosphate dehydrogenase* and *L-*
73 *type pyruvate kinase*, in the liver and adipose tissue [6-10]. Moreover, ChREBP-Mlx
74 regulates the glucose-induced expression of *glucose-6-phosphate dehydrogenase*
75 (*G6PDH*), the rate-limiting enzyme of the pentose phosphate pathway (PPP) [8].
76
77 The physiological function and key metabolic target genes of ChREBP/Mondo-Mlx are
78 well conserved in animals and studies in *Drosophila* have revealed new insight into the
79 physiological roles and tissue specificity of intracellular sugar sensing. Whereas the
80 vertebrate genome encodes for two Mondo proteins, the fruit fly, *Drosophila*, has only
81 one Mondo protein, displaying high expression in the fat body (corresponding to liver
82 and adipose tissue), midgut (corresponding to small intestine) and Malpighian tubules
83 (corresponding to kidney) [2]. Mondo-Mlx mediates a substantial proportion of
84 *Drosophila* sugar-induced transcription and it activates conserved genes involved in
85 glycolysis, lipogenesis, and the PPP, which is the most strongly over-represented

Mondo-Mlx target pathway in *Drosophila* [11]. The PPP has a key role in producing NADPH reductive power, which is needed for the biosynthesis of lipids. Indeed, inhibition of the Mondo-Mlx induced activation of glucose-6-phosphate dehydrogenase leads to a dramatic reduction of TAG stores in *Drosophila* larvae [11]. The PPP is also essential for sugar tolerance. The reductive power of NADPH generated by the PPP prevents oxidative damage upon high sugar feeding through the glutathione system, which contributes to the animal's ability to tolerate a high sugar diet [11,12].

In addition to conserved metabolic pathways, studies in *Drosophila* have revealed novel tissue-specific functions for sugar sensing. Mondo-Mlx is essential for the sugar-induced regulation of digestive enzymes in the midgut. Sugar feeding represses the expression of Amylases and Maltases, which are needed for digestion of complex carbohydrates [13,14]. This mechanism might have a role in regulating glucose intake, in order to prevent glucose overload [15]. Mondo-Mlx also regulates Malpighian tubule-specific glucose transporters, possibly in order to control excretion of excess glucose [11]. Furthermore, Mondo-Mlx is essential for normal flight muscle function and structure. Muscle-specific knockdown leads to accumulation of glycogen stores, possibly reflecting reduced glucose utilization [16]. In conclusion, ChREBP/Mondo-Mlx is a conserved sugar sensor within animals, with essential roles in sugar-induced lipogenesis and coordinating whole body glucose homeostasis by controlling carbohydrate digestion and transport.

ChREBP/Mondo-Mlx controls a gene regulatory network

In addition to direct regulation of key metabolic genes Mondo-Mlx is emerging as a master regulator of other regulatory genes, including transcription factors (TFs) (Figure 1). Mondo-Mlx directly regulates Krüppel-like factor 10 (KLF10, also known as TIEG1) and its *Drosophila* ortholog Cabut. In *Drosophila*, the *cabut* promoter has two high affinity ChoREs. *cabut* is the most strongly downregulated gene in *mlx* mutants and depletion of Cabut by RNAi induces sugar intolerance in *Drosophila* larvae [2]. Similarly, in rat primary hepatocytes KLF10 expression is activated by glucose stimulation and hampered by dominant negative Mlx [17]. Elevated KLF10 expression is also observed *in vivo* in mouse following feeding on a high fat/high sugar diet [18]. Adenoviral overexpression of KLF10 in primary hepatocytes suppressed the glucose-induced activation of ChREBP and several of its target genes, suggesting that KLF10

120 can act as a negative feedback regulator of ChREBP [17]. A different outcome was
121 observed in loss-of-function experiments in *Drosophila*, where Cabut mediated
122 downregulation of a gene set in response to sugar feeding [19]. Among the most
123 strongly repressed Cabut target genes was *phosphoenolpyruvate carboxykinase*
124 (*pepck*), which is a direct Cabut target and also a target of KLF10 in mouse [19,20].
125 PEPCK is the rate-limiting enzyme of cataplerotic flux from the TCA cycle to mediate
126 channeling of carbon towards gluco- and glyceroneogenesis. Consistent with high
127 expression of *pepck*, *Drosophila mlx* mutants display high levels of glycerol and
128 trehalose (a circulating form of glucose in insects), which could be suppressed by
129 simultaneous loss of *pepck* [19]. Interestingly, Cabut also links sugar sensing to
130 regulation of circadian rhythm. Deregulation of Cabut has a dramatic impact on the
131 cycling of circadian metabolic gene output, while having no impact on the cycling of
132 core clock genes [19]. Notably, in rat hepatocytes ChREBP-Mlx also directly regulates
133 BHLHB2/DEC1, which has a well-established role in circadian regulation [21,22]. In
134 conclusion, through direct regulation of downstream transcription factors,
135 ChREBP/Mondo-Mlx-mediated sugar sensing appears to be closely coupled to the
136 circadian clock. Considering the observations on re-setting of the peripheral circadian
137 clock through timed feeding [23], it will be interesting to further explore the
138 physiological importance of the observed interplay between these TFs.
139
140 ChREBP/Mondo-Mlx also controls the expression of TFs involved in lipid
141 homeostasis. The Gli-similar (GLIS) transcription factor Sugarbabe has been long
142 known as the earliest and strongest sugar-responsive gene in *Drosophila* [14]. Recently,
143 *sugarbabe* was found to be a direct target of Mondo-Mlx. Loss- and gain of function
144 analyses revealed that Sugarbabe contributes to the lipid homeostasis *in vivo*. It
145 regulates fatty acid synthesis downstream of Mondo-Mlx by inducing the expression of
146 lipogenic genes *ACC*, *FAS*, *AcCoAS* and *ATPCL* in response to sugar feeding [11]. Gli-
147 similar transcription factor 2, the closest human homologue of Sugarbabe, was
148 originally identified as a bifunctional transcription factor, regulating both the activation
149 and repression of its target genes involved in kidney development [24]. Interestingly,
150 variants of another *sugarbabe* homolog, GLIS3, are now established as one of the
151 strongest known genetic risk factors for both type 1 and type 2 diabetes [25]. In addition
152 to direct regulation of other TFs, ChREBP-Mlx also regulates downstream effectors
153 indirectly. One such example is the fatty acid responsive nuclear receptor PPARγ [26].

154 ChREBP expression is elevated during adipocyte differentiation and ChREBP activity
155 is needed to maintain maximal transcriptional output of adipogenic PPARγ.
156 Consequently, ectopic activation of ChREBP promotes adipocyte differentiation.
157 ChREBP likely affects PPARγ indirectly through lipid biosynthesis as the activating
158 effects of ChREBP depend on functional Fatty acid synthase [26]. In conclusion,
159 ChREBP/Mondo-Mlx contributes to lipid homeostasis through its direct targets as well
160 as through secondary TFs.
161
162 **New hormonal targets of ChREBP/Mondo-Mlx**
163 In addition to downstream transcription factors, ChREBP/Mondo-Mlx contributes to
164 systemic regulation through hormonal signals, including FGF21. In cultured
165 hepatocytes ChREBP-Mlx regulates the expression of *FGF21* in a glucose-dependent
166 manner [8,27]. Levels of circulating FGF21 are rapidly increased in response to
167 fructose in humans as well as mice [28,29]. Interestingly, both baseline and fructose-
168 stimulated levels of FGF21 are significantly elevated in subjects with metabolic
169 syndrome [28]. This is consistent with findings showing elevated activation of hepatic
170 ChREBP in obese individuals [30]. Loss of FGF21 in turn leads to impaired *de novo*
171 lipogenesis in mouse liver, which is accompanied by liver fibrosis following prolonged
172 fructose exposure [29]. In addition to metabolic regulation, FGF21 also regulates sugar
173 feeding. Mice lacking FGF21 consume elevated levels of sucrose, whereas ectopic
174 FGF21 suppresses the intake of sugar in mice as well as cynomolgus monkeys [31,32].
175 The liver-derived FGF21 suppresses sugar feeding in mice by acting through the FGF21
176 receptor complex in the paraventricular nucleus of the hypothalamus [31]. A similar
177 regulatory system likely exists in humans, since variants of *FGF21* are strongly
178 associated with sweet preference, as judged by the consumption of candy [33].
179
180 Research in *Drosophila* has revealed new connections between Mondo-Mlx and
181 hormonal metabolic regulation via TGF-β/Activin signaling. TGF-β/Activin ligand
182 Dawdle is highly expressed in the *Drosophila* fat body, from where it is secreted [15].
183 Sugar feeding strongly elevates *dawdle* expression, while loss of *mlx* leads to impaired
184 *dawdle* activation [11,15]. Moreover, Mondo-Mlx directly binds to a ChoRE at the
185 *dawdle* promoter [11]. Dawdle is essential in metabolic regulation as mutants of *dawdle*
186 show elevated circulating trehalose and glucose as well as high levels of glycogen and

187 triacylglycerol [34]. Dawdle acts through multiple target tissues, for example it acts on
188 *Drosophila* insulin producing cells to promote insulin-like peptide secretion [34].
189 Moreover, Dawdle activates its receptor Baboon in intestinal enterocytes and
190 suppresses the expression of genes encoding carbohydrate digestive enzymes,
191 Amylases and Maltases, thus inhibiting carbohydrate digestion in response to excess
192 systemic glucose [15]. Interestingly, Dawdle functionally cooperates with the Mondo-
193 Mlx target TF Sugarbabe, which also contributes to the sugar-induced repression of
194 amylase expression [11]. In conclusion, studies in mammals and *Drosophila* have
195 opened new insight into the role of ChREBP/Mondo-Mlx on the systemic regulation of
196 metabolism via hormones secreted by the liver and fat body, respectively.
197
198 **Direct regulation of ChREBP/Mondo-Mlx by nutrient-derived cues**
199 ChREBP/Mondo TFs respond to sugars via their N-terminal Glucose sensing module
200 (GSM), which can be functionally divided into low-glucose inhibitory domain (LID)
201 and glucose response activation conserved element (GRACE) [35]. Although direct
202 structural evidence about ChREBP/Mondo activation mechanism is still missing, ample
203 indirect evidence suggests that direct binding of G6P, and possibly other
204 phosphorylated hexoses, lead to loss of intramolecular inhibition of GRACE by LID
205 [36,37]. Subsequently activated ChREBP/Mondo-Mlx dissociates from 14-3-3,
206 translocates to the nucleus, and binds to the ChoRE to activate its target genes (Figure
207 2A) [38-40]. Furthermore, glucose-responsive dephosphorylation as well as acetylation
208 have also been shown to contribute to ChREBP activation [41,42]. A detailed
209 representation of ChREBP/Mondo-Mlx activation has been presented elsewhere [3].
210 This core activation mechanism allows ChREBP/Mondo-Mlx to control gene
211 expression in response to sugar availability. However, additional regulatory
212 mechanisms and functional interplay with other nutrient-responsive TFs to integrate
213 other nutrient-derived signals are beginning to emerge. One such signaling mechanism
214 is O-GlcNacylation. The synthesis of the substrate for O-GlcNacylation, UDP-GlcNAc,
215 through the hexosamine biosynthesis pathway (HBP), depends on availability of
216 nutrients, such as glucose and glutamine [43]. Thereby, O-GlcNacylation is considered
217 as a nutrient sensing mechanism. ChREBP is O-GlcNac modified [44-46] and recent
218 mass-spectrometric analysis have revealed multiple target sites [47]. Consistent with
219 the glucose sensitivity of the HBP, the levels of ChREBP O-GlcNac modification are
220 increased upon exposure to high glucose [44,45]. O-GlcNac modification increases

221 ChREBP protein stability and increases the expression of ChREBP target genes [44].
222 Pancreatic β-cells have an inherent glucose sensing capacity and ChREBP activation
223 seems to involve a distinct mechanism in this setting. ChREBP forms a cytoplasmic
224 complex with a Calcium binding protein Sorcin [48]. Calcium influx in response to
225 high glucose releases ChREBP allowing its nuclear localization. In conclusion,
226 ChREBP activity is regulated by sugars through multiple parallel mechanisms.
227
228 In addition to posttranslational modifications, ChREBP/Mondo-Mlx is also regulated
229 at the transcriptional level. In mammals, the *ChREBP* gene encodes two isoforms,
230 *ChREBP-α* and *ChREBP-β*, which are transcribed from different promoters [49].
231 ChREBP-α is contains the N-terminal GSM and is thus directly activated by G6P.
232 ChREBP-α stimulates the expression of the shorter ChREBP-β, which lacks inherent
233 glucose sensing ability, but has a 20-fold higher transcriptional activity than ChREBP-
234 α. The promoter of *ChREBP* contains a binding site for Liver X receptor (LXR), and it
235 has been shown that LXR regulates the expression of *ChREBP* in the liver [50,51].
236 However, the effect of LXR on *ChREBP* expression appears to be independent of
237 nutrient as the expression of *ChREBP* is not altered in high-sugar fed LXR mutant mice
238 [52]. Hepatocyte nuclear factor 4 alpha (HNF-4α) in turn has been shown to activate
239 the expression of both *ChREBP* isoforms in response to glucose [53]. Moreover, HNF-
240 4α has been also shown to physically interact with ChREBP, and this interaction is
241 promoted by glucose [53,54].
242
243 While ChREBP is activated in the liver of animals on a high sugar diet, a high lipid diet
244 has an inhibitory effect. Furthermore, administration of fatty acids inhibits nuclear
245 translocation and activation of ChREBP (Figure 2B) [55,56]. Fatty acids are oxidized
246 into ketone bodies, such as β-hydroxybutyrate and acetoacetate. By using an *in vitro*
247 binding assay with purified ChREBP and 14-3-3 as well as protein-free hepatocyte
248 extracts, Nagakawa and coworkers showed that β-hydroxybutyrate and acetoacetate
249 increase the binding between ChREBP and 14-3-3 [57]. This increased binding anchors
250 ChREBP to the cytoplasm, preventing its target gene activation. Fatty acid
251 administration also significantly elevates the levels of intracellular AMP [55]. AMP
252 activates the AMP-activated protein kinase (AMPK), which phosphorylates ChREBP
253 on Ser568 and inhibits its transcriptional activity, while having no impact on the nuclear

254 localization [55,58]. Interestingly, a recent study suggests that AMP can also inhibit
255 ChREBP directly, through an allosteric mechanism. AMP binds to ChREBP and
256 increases its affinity to 14-3-3, which prevents ChREBP nuclear translocation [58]. In
257 conclusion, recent studies suggest that ChREBP acts as a direct sensor for several fatty
258 acid-derived metabolites, thus integrating multiple nutrient-derived cues.
259
260 **Cross-talk between ChREBP and other nutrient sensing pathways**
261 In addition to nutrient sensing through direct allosteric regulation and posttranslational
262 mechanisms, ChREBP/Mondo acts in close synergy with other nutrient sensing
263 systems, including insulin signaling. A well-established example is SREBP, a target of
264 insulin signaling, which controls synergistically lipogenic gene expression with
265 ChREBP [59]. In *Drosophila*, Mondo was shown to act synergistically with Salt-
266 inducible kinase 3 (SIK3), which is also a target of the insulin signaling pathway
267 [12,60]. Mondo and SIK3 act cooperatively to activate the PPP upon sugar feeding [12].
268 Similarly to Mondo, SIK3 is essential for sugar tolerance and animals with reduced
269 expression of both Mondo and SIK3 display extremely low tolerance towards dietary
270 sugars [12]. Moreover, ChREBP O-GlcNacylation is inhibited by Foxo1, which is an
271 inhibitory target of insulin signaling, thus providing additional cross-talk between
272 insulin-mediated systemic glucose homeostasis and intracellular glucose sensing [46].
273 ChREBP also likely modulates insulin signaling, since genome-wide analysis of
274 ChREBP chromatin binding in mouse liver and adipose tissue revealed significant
275 enrichment in ChREBP binding near genes encoding components of the insulin
276 signaling pathway [61].
277
278 Evidence about cross-talk between sugar sensing and amino acid sensing is also
279 emerging (Figure 2C). Glutamine is a critical metabolic intermediate, acting as a carbon
280 and ammonium carrier. Glutaminolysis is critical in replenishing the TCA cycle, when
281 TCA cycle carbon is channeled to biosynthesis. Interestingly, glutamine modifies
282 glucose sensing by inhibiting the transcriptional output of MondoA [62]. Consequently,
283 glutamine inhibits the glucose-induced activation of *TXNIP*, a negative regulator of
284 glucose transporter GLUT1 [63], thus increasing cellular glucose uptake [62].
285 Glutamine has no impact on MondoA nuclear localization or DNA binding, but it likely
286 impacts MondoA cofactors [62]. MondoA can also control the uptake of glutamine. In
287 endothelial cells infected with Kaposi's Sarcoma-associated Herpesvirus, glutamine

transporter SLC1A5 is activated in a MondoA-Mlx-dependent manner to facilitate glutaminolysis [64]. In conclusion, MondoA coordinates the intracellular balance of glucose and glutamine catabolism.

The mechanistic target of rapamycin (mTOR) is a conserved intracellular amino acid sensing system, which also has been shown to modulate the output of ChREBP/Mondo-Mlx. mTOR inhibitors increase the expression of MondoA-Mlx target gene *TXNIP* [65]. mTOR physically interacts with MondoA and sequesters it from forming an active complex with Mlx [65]. Similarly to MondoA, ChREBP is inhibited by mTOR through protein-protein interaction [66]. mTOR inhibition leads to increased expression of *TXNIP*, which causes death of pancreatic β-cells [66]. Interestingly, the levels of TXNIP are strongly elevated in diabetic islets, further suggesting that retaining the interaction between cellular amino acid and glucose sensing is essential for normal β-cell homeostasis. Recent evidence from *C. elegans* implies that Mondo-Mlx has a capacity to modulate mTOR signaling, since loss of *C. elegans* homolog of Mondo and Myc, MLL-1, leads to mTOR activation [67]. MLL-1 represses the expression of leucyl-tRNA synthetase *lars-1*, whose activity promotes mTOR activation via RAG-GTPases [67,68]. Through the regulation of mTOR signaling, MLL-1 is essential for promoting longevity [67]. In sum, mTOR and ChREBP/Mondo pathways appear to be closely interconnected, which potentially provides cross-coordination between phenotypic outputs of dietary sugars and amino acids.

Concluding remarks

To conclude, recent work in the field has revealed that ChREBP/Mondo-Mlx regulates a large regulatory network, including transcription factors and hormones, which controls not only metabolic pathways, but also feeding activity and intestinal digestion. In addition to intracellular sugar sensing, ChREBP/Mondo-Mlx integrates multiple nutrient-responsive inputs, placing it at the very heart of an interlinked regulatory system mediating physiological re-adjustment in response to nutrient intake. Such integrated control allows animals to flourish in highly variable nutrient landscapes.

However, several questions remain to be addressed. These include elucidating the structural basis of the function of the Glucose sensing module. This would allow better

understanding of the "nutrient sensor" mechanism of ChREBP/Mondo and possibly to enable design of new pharmaceuticals to modulate its activity. Given the availability of animal models, understanding of the physiological roles of ChREBP/Mondo in different tissues and cell types will certainly arise. Moreover, a wider perspective is needed to understand the natural variation in dietary sugar intake, and the variation between species and individuals with respect to their healthy nutrient landscape. As ChREBP/Mondo-Mlx is a key determinant in sugar tolerance in mice and *Drosophila*, it will be interesting to analyze its function in animals with differential sugar intake - and perhaps differential inherent sugar tolerance. Understanding the genetic factors predisposing humans to disease on a high sugar diet would allow disease prevention through personalized nutrition.

Acknowledgements

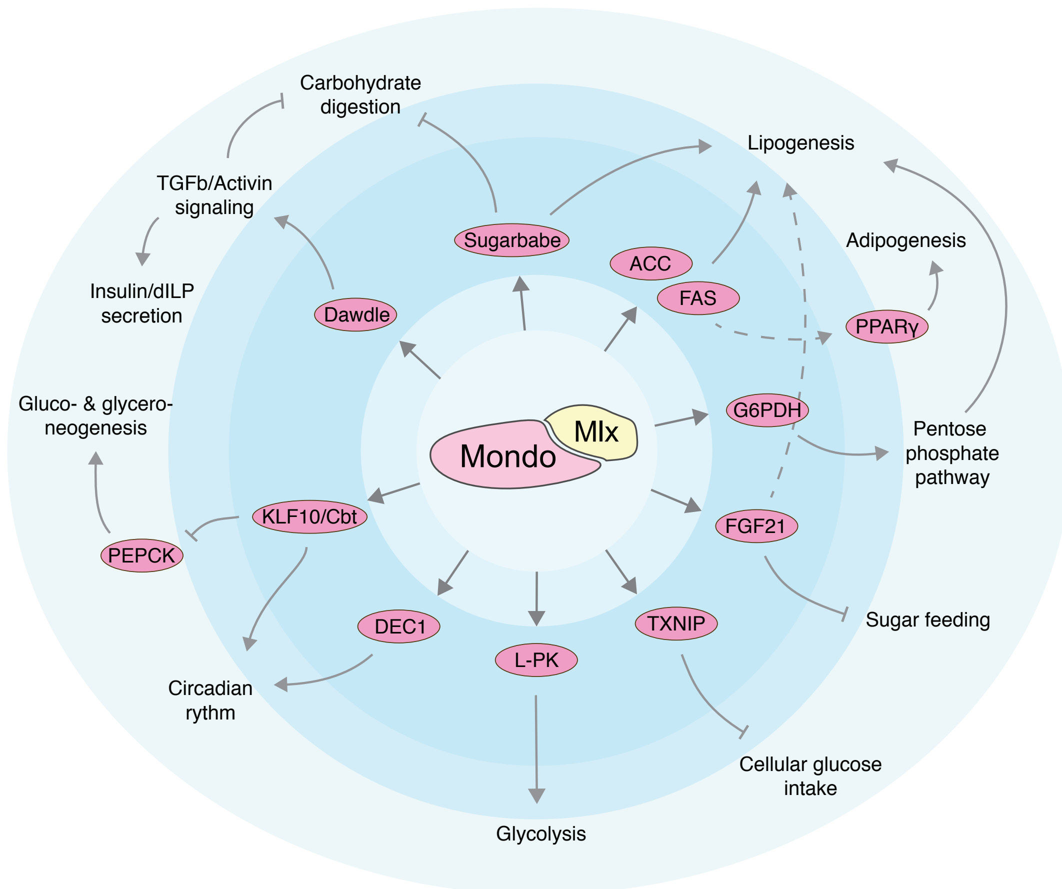
We thank Ryan Giblin and Mari Teesalu for feedback. Our research is supported by Academy of Finland (grant no. 286767 to V.H.), Sigrid Juselius Foundation (to VH), Novo Nordisk Foundation (NNF16OC0021460 to V.H.), Helsinki Institute of Life Science (to VH), and the Finnish Diabetes Research Foundation (to V.H.).

355

356 **Figures**

357

358



359

360 **Figure 1. ChREBP/Mondo-Mlx is a master regulator of a sugar-induced** gene
361 **regulatory network.** In response to sugars, ChREBP/Mondo-Mlx activates the
362 expression of a number of metabolic genes, but also other regulatory genes involved in
363 the control of carbohydrate and lipid metabolism, sugar feeding and circadian rhythm.
364 The figure summarizes key targets of mammalian ChREBP and MondoA as well as
365 *Drosophila* Mondo. Abbreviations: ACC: acetyl-CoA carboxylase, Cbt: Cabut, DEC1:
366 deleted in esophageal cancer 1, FAS: fatty acid synthase, FGF21: fibroblast growth
367 factor 21, G6PDH: glucose-6-phosphate dehydrogenase, KLF10: krüppel-like factor
368 10, L-PK: L-type pyruvate kinase, PEPCK: phosphoenolpyruvate carboxykinase,
369 PPARγ: peroxisome proliferator-activated receptor gamma, TXNIP: thioredoxin-
370 interacting protein.

371

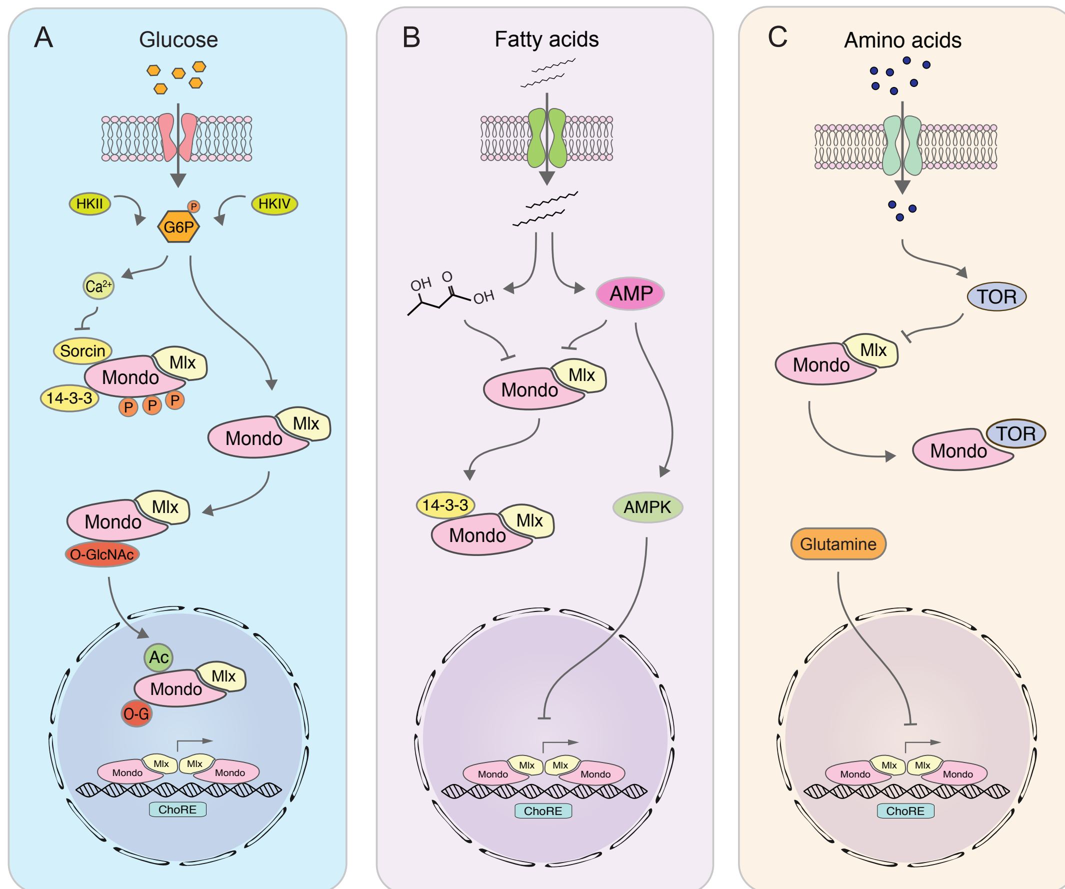


Figure 2. ChREBP/Mondo-Mlx activity is regulated by many nutrient-derived cues. ChREBP/Mondo-Mlx is activated by glucose-6-phosphate (G6P) **(A)** and inhibited both by ketone bodies and AMP, whose levels depend on fatty acids **(B)**. Direct binding of TOR to MondoA and ChREBP inhibits their function and glutamine inhibits the transcriptional activity of ChREBP-Mlx **(C)**.

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