The transcription factor Cabut coordinates energy metabolism and the circadian clock in response to sugar sensing

Osnat Bartok, Mari Teesalu, Reut Ashwall-Fluss, Varun Pandey, Mor Hanan, Bohdana M Rovenko, Minna Poukkula, Essi Havula, Arieh Moussaieff, Sadanand Vodala, Yaakov Nahmias, Sebastian Kadener & Ville Hietakangas

Abstract

Nutrient sensing pathways adjust metabolism and physiological functions in response to food intake. For example, sugar feeding promotes lipogenesis by activating glycolytic and lipogenic genes through the Mondo/ChREBP-Mlx transcription factor complex. Concomitantly, other metabolic routes are inhibited, but the mechanisms of transcriptional repression upon sugar sensing have remained elusive. Here, we characterize cabut, a transcription factor responsible for the repressive branch of the sugar sensing transcriptional network in Drosophila. We demonstrate that cabut is rapidly induced upon sugar feeding through direct regulation by Mondo-Mlx. We found that CBT represses several metabolic targets in response to sugar feeding, including both isoforms of phosphoenolpyruvate carboxykinase (pepck). Deregulation of pepck1 in mlx mutants underlies imbalance of glycerol and glucose metabolism as well as developmental lethality. Furthermore, we demonstrate that cabut provides a regulatory link between nutrient sensing and the circadian clock. Specifically, we show that a subset of genes regulated by the circadian clock are also targets of CBT. Moreover, perturbation of CBT levels leads to deregulation of the circadian transcriptome and circadian behavioral patterns.

Keywords: cabut; circadian; metabolism; nutrient sensing; transcription

Subject Categories: Metabolism; Transcription

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Introduction

Animals respond to changes in food intake through nutrient sensing pathways that readjust metabolism to maintain homeostasis. Various signaling pathways and transcriptional responses are crucial in the adaptation of an organism to changing dietary conditions (Hietakangas & Cohen, 2009; Havula & Hietakangas, 2012). For example, the paralogous basic helix-loop-helix-leucine zipper (bHLHZ) transcription factors (TFs), carbohydrate-responsive element binding protein (ChREBP), and MondoA coordinate energy metabolism with dietary sugar intake (for review see Havula & Hietakangas, 2012; Filhoulaud et al, 2013). These TFs heterodimerize with their common binding partner Mlx to regulate gene expression in response to intracellular glucose-6-phosphate and other phosphorylated hexoses. Drosophila encodes single orthologs for ChREBP/MondoA and for Mlx, called Mondo (Mio, CG18362) and Mlx (Bigmax, CG3350), respectively. We have recently shown that loss of functional Mondo-Mlx in Drosophila leads to dramatic intolerance for dietary sugars and to impaired glucose and lipid metabolism (Havula et al, 2013). In humans, altered ChREBP activity in adipose tissue and liver is associated with severe obesity and polymorphisms of chrebp are linked to elevated circulating triglyceride levels (Kooner et al, 2008; Benhamed et al, 2012; Eissing et al, 2013).

Excess dietary sugars are metabolized into lipids, and there is emerging evidence suggesting that high sugar intake, synergistically with genetic risk factors, contributes to obesity and metabolic syndrome (Qi et al, 2012; Stanhope et al, 2013). Sugar intake activates the ChREBP/Mondo-Mlx-dependent gene expression program to drive fatty acid biosynthesis. This is mediated by binding of ChREBP/Mondo-Mlx to the carbohydrate response elements (ChoREs) in the promoters of lipogenic genes including fatty acid
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synthase (Fas) and acetyl-CoA carboxylase (ACC) (Ishii et al., 2004). Flies lacking functional Mondo-Mlx display elevated circulating glucose, trehalose and reduced adiposity, but the metabolic effector genes underlying these phenotypes are incompletely understood (Sassu et al., 2012; Havula et al., 2013).

In addition to fatty acids, biosynthesis of triglycerides requires glycerol-3-phosphate. Glycerol-3-phosphate is synthesized by three alternative pathways: glycolysis, phosphorylation of diet-derived glycerol by glycerol kinase, and from TCA cycle intermediates via the so-called glyconeogenesis pathway (Ballard et al., 1967; Hanson & Reshef, 2003; Jin et al., 2013). A rate-limiting reaction in the glyconeogenesis pathway is catalyzed by phosphoenolpyruvate carboxykinase (PEPCK) (Hanson & Reshef, 2003). PEPCK controls the cataplerotic flux, which converts the oxaloacetate (OAA) from the TCA cycle into phosphoenolpyruvate (PEP). PEP can be further routed into glucose (trehalose in insects (Becker et al., 1996)) through gluconeogenesis or glycerol-3-phosphate through glyceroneogenesis. PEPCK is present as a cytoplasmic and a mitochondrial isoform, both of which have shown to contribute to gluconeogenesis (Stark et al., 2014). As pepck plays a key role in adjusting the direction of flux of central carbon metabolism, it is under tight nutritional control. It is well established in mammals that the hormonal insulin/glucagon axis controls pepck expression via transcription factors CREB and FoxO1, both of which are active during starvation (high glucagon, low insulin) (Oh et al., 2013). However, whether cell-intrinsic nutrient sensing mechanisms contribute to PEPCK regulation has remained poorly understood.

Circadian clocks time most physiological and behavioral processes to 24-h rhythms (Hardin, 2011; Partch et al., 2014). Among these processes is metabolism, which is strongly controlled by the circadian system (Bass, 2012; Peek et al., 2012; Masri & Sassone-Corsi, 2014; Dibner & Schibler, 2015). In Drosophila, this control manifests mainly at the transcriptional level (Xu et al., 2008, 2011). Metabolism feeds back to the circadian system, as dietary cues (i.e., restriction feeding) and metabolites have profound impact on the circadian system (Mattson et al., 2014). Circadian clocks are spread throughout the body and are present in most cells. Circadian clocks work cell autonomously, although cell-to-cell communication is known to provide robustness to circadian behavior (Weiss et al., 2014). In Drosophila, circadian control of metabolism is achieved mainly in the fat body and it is involved in the timed expression of hundreds of metabolic genes, including the ones involved in sugar and fat metabolism (Xu et al., 2008, 2011). The importance of this regulation is highlighted by the fact that disruption of circadian rhythms either by genetic or environmental cause is strongly associated with metabolic imbalances, diabetes, and obesity (Bass, 2012; Peek et al., 2012).

Transcription factors (TFs) form hierarchical regulatory networks, which allow the integration of different input signals and the generation of complex and robust biological responses. In the context of sugar sensing, our previous work demonstrated that ChREBP/MondoA-Mlx strongly regulates the TF cabut (cvt) in Drosophila, which is involved in sugar tolerance by a yet unknown mechanism (Havula et al., 2013). The closest mammalian homologs of CBT are Krüppel-like factors Klf10 and Klf11 (Subramaniam et al., 2010; Spittau & Kriegstein, 2012). klf10 expression is promoted by sugar in a ChREBP-dependent manner (Iizuka et al., 2011), whereas klf11 expression is elevated upon starvation (Zhang et al., 2013), suggesting that klf10 is the functional ortholog of cvt in the context of sugar sensing transcriptional network. Interestingly, the circadian clock can regulate klf10 levels, and hence, this transcription factor might act as a link between the circadian and metabolic systems (Guillaumond et al., 2010). Furthermore, a variant of the klf10 gene is proposed to contribute to the risk of type-2 diabetes (Gutierrez-Aguilar et al., 2007).

Here, we found that CBT is a central mediator of a repressive branch of the intracellular sugar sensing transcriptional network in Drosophila. We demonstrate that cvt expression is activated upon sugar feeding through direct regulation by the sugar sensing TF complex Mondo-Mlx. Genome-wide analyses reveal that CBT contributes to rapid and persistent repression of metabolic genes upon sugar feeding. CBT directly represses both pepck isoforms, inhibiting cataplerosis during sugar feeding. Failure in this regulatory axis leads to loss of glucose and glycerol homeostasis and to lethality observed in animals lacking functional Mondo-Mlx. Last but not least, we found that CBT integrates feeding information into a subset of metabolic genes, which are regulated by the circadian clock. In sum, here we identify and characterize a transcriptional circuit that allows animals to readjust their central carbon metabolism as well as circadian clock with respect to sugar feeding.

Results
cvt is regulated by dietary sugar through Mondo-Mlx

Based on our earlier findings on cvt being essential for sugar tolerance (Havula et al., 2013), we decided to explore how cvt is regulated upon sugar feeding. To this end, we measured cvt levels at different times after re-feeding with 5 or 10% sucrose. Indeed, we observed a twofold increase in cvt levels 6 h following sugar re-feeding in adults (Supplementary Fig S1A). In contrast, re-feeding with protein did not lead to increased cvt expression, but rather to a small decrease (Supplementary Fig S1B). Given our previous findings, we hypothesized that the sugar-induced expression of cvt might be mediated by Mondo-Mlx activity. As mlx null mutants (mlx1) are pupal lethal (Havula et al., 2013), we sought to explore cabut expression in the larvae. Indeed, cvt expression was upregulated in larvae that had been transferred to high sugar/high protein diet (10% yeast, 20% sucrose) compared to ones remaining in low sugar/high protein diet (10% yeast), and this upregulation was fully abrogated in mlx1 mutants (Fig 1A). These results suggest that the Mondo-Mlx dimer may directly regulate cvt expression. Supporting this possibility, we found two conserved consensus motifs for putative binding of this heterodimer, the carbohydrate-responsive elements (ChoRE), near the cvt transcription start site (TSS): one was located about one kilobase upstream and the other a few hundred bases downstream the annotated TSS (Fig 1B and C). We performed chromatin immunoprecipitation (ChIP) from Drosophila S2C cells using anti-Mlx antibodies or preimmune serum as a negative control. Indeed, Mlx strongly bound these two putative ChoREs in the cvt promoter region (Fig 1D). Moreover, we observed that Mlx binding to cvt promoter was significantly
Increased in high glucose media (Fig 1D, compare ± Glc). To functionally test the importance of the ChoREs, we constructed a luciferase reporter containing the 1,961 to +641 region of the cbt promoter. We also generated versions of this promoter in which the putative ChoREs were mutated. As the reporter failed to display glucose responsiveness in S2C cells (data not shown), we performed the experiments in human HepG2 hepatocellular carcinoma cells, which are a well-established model system for sugar sensing.

Figure 1. cbt is a direct target of Mondo-Mlx-mediated sugar sensing.

A cbt expression is sugar inducible and Mlx dependent. cbt mRNA levels in control (precise excision of P-element line) and mlx1 mutant 2nd instar larvae after 8 h of 10% yeast (Yeast) or 10% yeast and 20% sucrose feeding (Sugar). Actin was used as a reference gene. Error bars indicate SD, n = 3. *P < 0.05; **P < 0.01.

B Two putative ChoRE sites in the cbt gene region.

C Conservation of the putative ChoRE sites in the cbt gene region in Drosophila species.

D ChIP from S2C cells with anti-Mlx antibody or pre-immune serum in the presence or absence of 50 mM glucose (6 h). The percentage of input pulled down was determined by qPCR. Actin was used as a negative control. Error bars indicate SD, n = 3. *P < 0.05; **P < 0.01, ***P < 0.001.

E ChoRE2 is required for full cbt promoter activity and sugar responsiveness. Luciferase reporter assay with cbt promoter in HepG2 cells grown in low (5.5 mM) or high (25 mM) glucose. Error bars indicate SD, n = 3. **P < 0.01.
ChREBP-mediated gene regulation (Jeong et al., 2011). Indeed, the cbt promoter was activated by glucose stimulation, and the reporter activity was significantly inhibited when ChoRE2 was mutated (Fig 1E). In conclusion, our experiments show that cbt is induced by dietary sugars likely through direct binding of Mondo to the ChoREs in the cbt promoter.

**cbt regulates the expression of genes involved in metabolism**

CBT is known to repress its own expression (Belacortu et al., 2012), but beyond that, the transcriptional targets and function of CBT are still poorly understood. Therefore, we sought to identify CBT-regulated transcriptome in adult *Drosophila*. We utilized the GAL4-UAS system to generate flies in which cbt is down- or upregulated. We tested several GAL4-UAS drivers in combination with UAS-cbt or UAS-cbt RNAi. Most of these combinations resulted in high or total developmental lethality. We were able to obtain adult flies by using a GAL4 driver that expresses the gene of interest only in cells harboring an active circadian clock (tim-gal4 driver). The tim-gal4 driver expresses in approximately half of the cells of the fly head, including the brain, eyes, and the fat body, the fly equivalent of the mammalian liver and adipose tissue (Xu et al., 2008). To overexpress cbt, we utilized the tim-gal4 driver line in combination with a UAS-cbtFLAG transgene (Muñoz-Descalzo et al., 2005); these flies will be referred to as cbtOE flies. To downregulate cbt, we used the same driver in combination with a publicly available cbt RNAi transgene (NIG.4427R from NIG-Fly Stock Center), a UAS-dcr2 transgene, and a mutant cbt allele (cbtE1 (Muñoz-Descalzo et al., 2005)) in heterozygosis. We will call these cbtRNAi flies. After verifying the levels of CBT by Western blot (Supplementary Fig S2A), we assessed the transcriptomes in heads of control, cbtRNAi, and cbtOE flies using oligonucleotide microarrays. More than 1,000 genes were significantly affected by at least one of the manipulations in cbt levels (Fig 2A, Supplementary Dataset S1). Among them, genes related to neuronal function, immunity, and metabolism were specifically enriched (Fig 2B; Supplementary Fig S2B). In particular, we found that genes involved in carbohydrate metabolism were strongly affected by cbt up- or downregulation (Fig 2B and Supplementary Fig S3). Previously, a role of CBT in regulating circadian transcription was suggested by the fact that the circadian transcriptional activator CLK binds CBT promoter in a timely fashion (Abruzzi et al., 2011). Interestingly, we found a significant number of CLK-controlled genes among the genes downregulated by CBT (P < 0.0001), suggesting that in addition to metabolic gene expression cbt might regulate the circadian transcriptome (Abruzzi et al., 2011).

**CBT constitutes a repressive branch of the sugar sensing transcriptional network**

As cbt expression is induced by sugar intake, we decided to determine the role of CBT in the sugar-induced transcriptional response. To do so, we profiled the transcriptomes using 3′ RNA-seq of control and cbtRNAi flies after they had been starved and exposed to different levels of sucrose for 18 h (see scheme in Supplementary Fig S4A). After a very conservative analysis of the data, we found that levels of 72 transcripts were strongly affected by sugar intake at this time point. Most of these mRNAs (51) displayed decreased expression in response to this dietary manipulation (Supplementary Dataset S2). Of these 51 genes, 22 mRNAs were downregulated in a CBT-dependent manner (Fig 2C; Supplementary Dataset S2; Supplementary Fig S4B). In addition, we found eight more CBT-regulated mRNAs by looking at genes that are significantly more affected by sugar intake in cbtRNAi flies compared with control flies (Supplementary Dataset S3). Among the CBT-repressed targets, we found transcripts encoding proteins with function in lipolysis, gluconeogenesis, and glycerol metabolism including *brummer* (*bmm*) lipase, *fructose-1,6-bisphosphatase* (*fbp*), *phosphoenolpyruvate carboxykinase* (*pepck*), and *glycerol kinase* (*Gyk*) (Supplementary Fig S4B). Notably, the *pepck*, *fbp*, and *Gyk* encode proteins with related functions. PEPCK participates with FBP in gluconeogenesis, and *Gyk* and PEPCK control biosynthesis of glycerol-3-phosphate, albeit through two distinct pathways. These results demonstrate that CBT has a central role in repressing the expression of key metabolic genes in response to dietary sugars.

In order to temporally characterize the transcriptional response to dietary sugars with respect to CBT function, we performed a transcriptomic analysis of adult fly heads harvested at different times following sugar intake after starvation. Our analysis identified 520 mRNAs that changed levels significantly at least at one of the time points (Fig 2D; Supplementary Dataset S4). Clustering analysis allowed us to identify ten transcriptional modules with different temporal patterns of expression following sugar intake (Fig 2D and E).
and Supplementary Fig S5; Supplementary Dataset S5; see Materials and Methods for details). Interestingly, the majority of CBT-dependent targets clustered into Group 1 containing genes that are repressed by sugar feeding in a rapid and persistent manner (Fig 2E). This further supports the conclusion that CBT is a sugar-activated transcriptional repressor.
pepck isoforms are negatively regulated by the Mondo-Mix/CBT network

One of the strongest CBT-regulated genes across all our genomic analyses was pepck (CG17725) (Fig 2B and Supplementary Fig S4B). In mammals, PEPCK exists as cytoplasmic (PEPCK-C) and mitochondrial (PEPCK-M) isoforms, displaying partially redundant functions (Stark et al., 2014). Drosophila genome also encodes two genes homologous to mammalian PEPCK isoforms, namely CG17725 (called as Pepck in FlyBase) and the neighboring gene CG10924. CG17725 lacks an identifiable mitochondrial targeting sequence, while MitoProt II predicts that CG10924 is likely targeted to mitochondria (likelihood 0.82). We call the CG17725 and CG10924 as pepck1 and pepck2, respectively. In adult flies, cbt overexpression resulted in strong downregulation of pepck1 expression, whereas cbtRNAi flies displayed elevated pepck1 mRNA levels (Fig 3A). Moreover, sugar feeding rapidly and persistently downregulated the expression of pepck1, and this downregulation was prevented by RNAi-mediated depletion of CBT (Fig 3B and Supplementary Figs S5 and S6A). Feeding with protein caused only a modest downregulation of pepck1 expression (Supplementary Fig S6B). Similar to adults, sugar feeding led to downregulation of pepck1 expression in larvae (Supplementary Fig S6C). In fed larvae, two independent RNAi lines against CBT caused upregulation of pepck1 expression (Fig 3C; Supplementary Fig S6D), providing further evidence for CBT-mediated repression of pepck1. Consistent with the finding that CBT function is dependent on functional Mondo-Mix, pepck1 expression was also strongly elevated in mix mutant larvae (Fig 3C). Moreover, fat body-specific overexpression of CBT in the mix mutant background was sufficient to downregulate pepck1 expression (Fig 3D), consistent with the model that CBT acts downstream of Mondo-Mix to repress pepck1. CBT knockdown and loss of mix led to comparable upregulation of pepck2 (Fig 3E). Moreover, fat body-specific overexpression of CBT in the mix mutant also rescued pepck2 expression levels (Supplementary Fig S6E). In conclusion, our data demonstrate that CBT represses both Drosophila pepck isoforms.

As CBT has been shown to possess transcriptional repressor activity (Belacortu et al., 2012), we speculated that CBT might directly repress the transcription of the pepck genes. To test this possibility, we performed ChIP from fly heads using an anti-CBT antibody followed by quantitative PCR. As CBT is known to act as an autoregulator (Belacortu et al., 2012), we used cbt promoter as a positive control. We found that CBT strongly binds to a region between the two pepck genes (Fig 3F and G), providing evidence that the expression of pepck isoforms is directly repressed by CBT. Krüppel-like TFs bind to GC-rich areas (Brown et al., 2005; Belacortu et al., 2012), and indeed, the region enriched for CBT binding possesses three GC-rich segments (Fig 3G).

pepck1 is dispensable for viability and growth but essential for glycerol and glucose homeostasis

To address the physiological role of PEPCK1 in Drosophila, we generated a pepck1 deletion mutant by imprecise P-element (EY10042) excision (pepckΔ84; Fig 4A). The deletion removed 108 amino acids from the conserved PEPCK1 N-terminus (34.5% identical to this region of human PEPCK-C). Quantitative RT–PCR revealed that the deletion strongly reduced pepck1 mRNA levels (Fig 4B). Thus, the deletion mutant is a null or a strong hypomorphic allele of pepck1. To reduce possible compensatory effects by pepck2, we used the pepckA84 allele in trans with a deficiency uncovering both pepck loci (Df(2R)ED3636). Surprisingly, we found that the pepckA84/Df(2R)ED3636 mutants were fully viable (Fig 4C). Careful phenotypic characterization of the pepckΔ84/Df(2R)ED3636 mutants revealed modest, but significant reduction of feeding in the pepck1-deficient animals (Supplementary Fig S7A). However, we did not observe a delay of development or reduced pupal volume, which are typical phenotypes of starved animals (Supplementary Fig S7B and C).

Next, we wanted to analyze whether pepck1-deficient animals displayed alterations of metabolite profile. Levels of circulating glucose and trehalose were moderately, but significantly downregulated in the pepck1-deficient animals (Fig 4D and E). Trehalose is the most abundant circulating sugar in insects and the product of fat body gluconeogenesis and glycogenolysis (Becker et al., 1996). In addition to the moderate effects on glucose metabolites, steady state levels of circulating glycerol were strongly downregulated in pepckA84/Df(2R)ED3636 larvae as well as in pepckA84 homozygotes (Fig 4F). RNAi against either of the pepck isoforms also led to reduced circulating glycerol levels (Supplementary Fig S7D), suggesting that the pepck isoforms have overlapping functions, but are not fully functionally redundant. Moreover, triglyceride levels were significantly reduced in the pepck1 mutant flies (Fig 4G). In contrast to the glucose and glycerol metabolites, levels of glutamate and glutamine remained unchanged (Supplementary Fig S7E), demonstrating that the loss of pepck1 leads to a specific metabolic imbalance. Metabolites, which are downstream of PEPCK-mediated cataplerosis, are present at lower levels in pepck1 mutants. In conclusion, our data reveal a prominent role for pepck1 in glycerol and triglyceride homeostasis and a moderate effect on steady state glucose and trehalose levels. However, the metabolic changes in the pepck1-deficient animals have no impact on growth or survival of the larvae.

The Mondo-Mix/CBT network inhibits PEPCK-mediated cataplerosis

Next, we addressed the physiological consequences of the PEPCK regulation by the Mondo-Mix/CBT network. As PEPCK catalyzes the conversion of oxaloacetate (OAA) to phosphoenolpyruvate (PEP), we started by measuring the ratio of these metabolites. Interestingly, we observed that cbt knockdown as well as loss of mix led to a significant elevation of the PEP/OAA ratio (Fig 5A and B), consistent with the elevated pepck expression in these genotypes. As pepck1 mutants displayed reduced level of circulating glycerol, we analyzed glycerol levels upon inhibition of the Mondo-Mix/CBT network. While cbtRNAi showed no dramatic changes in glycerol homeostasis (data not shown), mix1 mutants displayed strongly elevated circulating glycerol levels. Similar results were obtained by mondonRNAi (Supplementary Fig S8A). Moreover, we could rescue this phenotype by fat body-specific expression of Mix (Fig 5C). To directly test whether this phenotype is due to deregulated PEPCK activity, we analyzed the phenotypes of mix1; pepckΔ84/Df(2R)ED3636 double mutants. Indeed, the high circulating glycerol levels of mix1 mutants were rescued
**Figure 3.** The Mondo-Mlx/CBT network negatively regulates the expression of pepck isoforms.

A. pepck2 mRNA levels in heads of cbtRNAi, control (yw; UAS-cbt), and cbtOE adult flies. pepck2 levels were normalized to rp49. Error bars indicate SD, n = 6. **P < 0.01.

B. pepck2 mRNA levels in the heads of control (+/cbtE1-UAS-cbt RNAi) or cbtRNAi adult flies that were starved for 16 h and then exposed to food containing different concentrations of sucrose for 18 h. RNA-seq data. Error bars indicate SD, n = 3. *P < 0.05.

C. pepck1 mRNA levels in 1st instar cbt knock-down and mix1 mutant larvae. Actin was used as a reference gene. Control (Tub-GAL80ts; Tub-GAL4 > ), cbtRNAi (Tub-GAL80ts; Tub-GAL4 > cbtRNAI NIG 4427R-1), control for mix1, and mix12 mutants were grown on 20% yeast. Error bars indicate SD, n = 3. *P < 0.05, **P < 0.01.

D. pepck1 mRNA levels in 1st instar control (precise excision of P-element line) larvae, mix1 mutants, and in mix12 mutants with fat body-specific over-expression of cbt (mix1, r4-GAL4 > UAS-cbt). Actin was used as a reference gene. Error bars indicate SD, n = 3 (control and mix1) and n = 7 (mix1, r4-GAL4 > UAS-cbt). **P < 0.01.

E. pepck2 mRNA levels in 1st instar cbt knock-down and mix12 mutant larvae. Actin was used as a reference gene. Control (Tub-GAL80ts; Tub-GAL4 > ), cbtRNAi (Tub-GAL80ts; Tub-GAL4 > cbtRNAI NIG 4427R-1), control and mix12 mutants were grown on 20% yeast. Error bars indicate SD, n = 3. *P < 0.05, **P < 0.001.

F. Fold enrichment of ChIP-PCR in the promoter regions of cbt and pepck2 performed from adult heads. The fold enrichment was obtained by normalization of signals in the immunoprecipitates to those in input fractions. Normalization was performed using primer pairs that amplify non-specific DNA regions. Error bars indicate SD, n = 3.

G. Schematic representation of the CBT binding sites in between the PEPCK-encoding genes. GC-rich regions are highlighted.
**Figure 4.** pepck1 mutants are viable but display strongly reduced free glycerol and triglyceride levels.

A  Schematic representation of the pepckΔ84 allele generated by imprecise P-element excision. The resulting deletion spans 825 base pairs and removes the transcription start site, start codon, and sequence encoding 108 amino acids of the N-terminal region of the protein.

B  qPCR analysis of pepck1 expression levels in control (precise excision of P-element line crossed with y,w), pepckΔ84, and pepckΔ84/Df(2R)ED3636 larvae. Results indicate that pepckΔ84 allele is likely null or a strong hypomorph. Error bars indicate SD, n = 3. **P < 0.01.

C  PEPCK1 is dispensable for viability, but pepckΔ84 homozygotes display lethality likely due to genetic background. Pupation and adult emergence of pepckΔ84/+, pepckΔ84, and pepckΔ84/Df(2R)ED3636 animals.

D  Loss of PEPCK1 leads to modest downregulation of circulating trehalose. Hemolymph was harvested from 3rd instar larvae grown on 20% yeast. Error bars indicate SD, n = 10. **P < 0.01.

E  Loss of PEPCK1 causes reduction of circulating glucose levels. Hemolymph was harvested from 3rd instar larvae grown on 20% yeast. Error bars indicate SD.

F  Loss of PEPCK1 strongly reduces circulating glycerol levels. Hemolymph was harvested from 3rd instar larvae grown on 20% yeast. Error bars indicate SD. **P < 0.01.

G  Triglyceride levels are reduced in the pepck1 mutant male adult flies. Flies were aged for 5 days after eclosion on regular fly food. Errors bars indicate SD. **P < 0.01.
by simultaneous loss of pepck1 (Fig 5D). We have earlier shown that the mlx\textsuperscript{A} mutants display strongly elevated levels of circulating glucose and trehalose (Havula et al, 2013). Interestingly, we observed that the levels of circulating trehalose were normalized in the mlx\textsuperscript{A}; pepck\textsuperscript{A84/Δ84}(Df(2R)ED3636 double mutants (Fig 5E), while in the case of circulating glucose, there was no significant rescue observed (Supplementary Fig S8B). mlx\textsuperscript{A} mutant larvae display near complete pupal lethality (Havula et al, 2013). Strikingly, also this
phenotype was efficiently suppressed by the simultaneous loss of pepck1 (Fig 5F). In sum, our data demonstrate that deregulation of pepck1 expression leads to impaired metabolism in mlx mutant animals and contributes to the lethality of these animals.

**CBT modulates the circadian transcriptional network**

The data presented above demonstrated a role for CBT in metabolic gene regulation. Interestingly, ctb promoter region is bound by the circadian transcription factor CLK in a circadian fashion (Abruzzi et al., 2011). In addition, our gene expression profiling (see earlier chapter on CBT target genes) revealed that CBT targets display a significant overlap with CLK-regulated genes. Together, these suggest a role for CBT in integrating the circadian clock with metabolism. In order to determine the influence of CBT in the circadian system, we utilized the cbtOE flies, which would mimic a situation of high CBT activity in sugar-fed animals. Circadian rhythms in locomotor activity are the most studied behavioral output of the circadian system in *Drosophila*. Interestingly, CBT overexpression led to severe defects in circadian locomotor activity rhythms. While control flies were strongly rhythmic, most cbtOE flies (85.1%; Fig 6A) displayed no or very weak rhythms. Moreover, the small percentage of flies displaying circadian behavioral rhythms did so with very low power (Fig 6A). To determine whether these defects were associated with impaired oscillation of the core or output circadian-controlled genes, we generated RNA-seq libraries from control and cbtOE fly heads collected at 6 different times of the day. Despite the strong defects on circadian behavior, cbtOE flies displayed normal oscillations of the mRNA of core clock circadian components such as tim, clk, vri, and per (Fig 6B and data not shown). However, cbtOE flies had serious defects in clock-controlled output genes. While a small subset of genes displayed strong and significant oscillations in control and cbtOE strains, a large number of mRNA had altered oscillations in cbtOE flies (Fig 6B). Interestingly, we noticed that genes that oscillate only in control flies, but cease to oscillate in cbtOE flies, are strongly enriched for terms related to metabolism (Fig 6C). We also found a similar number of mRNAs that cycle only in cbtOE flies. This could be attributed to the fact that the overexpression of ctb is done using the *tim-gal4* driver, which displays strong oscillations in activity (our own unpublished observation), hence making a subset of CBT transcriptional targets to artificially oscillate in this strain.

The results described above demonstrate that CBT is sufficient for sculpting the transcriptional profile of circadian-regulated mRNAs related to metabolism. To explore the importance of endogenous CBT in circadian regulation, we performed a similar experiment in cbtRNAi flies. As in the cbtOE flies, downregulation of CBT did not significantly affect the oscillations of the circadian clock components (Fig 6D). A subset of mRNAs ceased to oscillate in cbtRNAi flies, suggesting that their oscillation is CBT dependent. Strikingly, we found a large subset of mRNAs, which cycles only in cbtRNAi flies (Fig 6D). This subset is highly enriched for metabolic genes (Fig 6E). In order to investigate this finding further, we compared the amplitude of oscillation of genes in both cbtRNAi and control flies. We found a small but statistically significant increase in the average amplitude of oscillating genes in cbtRNAi flies (Supplementary Fig S8C). In addition, we found that there are more cycling genes in the cbtRNAi flies and that this is true for all amplitude cutoffs (Fig 6F). These results demonstrate that CBT represses the oscillation of an important subset of metabolic genes regulated by the circadian clock.

**Discussion**

In this study, we establish the *Drosophila klf10* ortholog, ctb, as a repressive effector of the sugar sensing transcriptional network. Specifically, we showed that: (i) ctb expression is activated by dietary sugars in *mlx*-dependent manner, (ii) ctb is a direct target of Mlx, (iii) many key metabolic genes are rapidly repressed by CBT upon sugar feeding, (iv) CBT binds to the proximity of pepck genes, (v) pepck1 is dispensable for viability, but essential for glucose and glycerol homeostasis, (vi) deregulation of pepck1 underlies lethality of *mlx* mutants, and (vii) CBT modulates the circadian system by controlling the cycling of a subset of circadian output genes. Based on these findings, we propose a model in which CBT serves as a repressive downstream effector of the Mondo-Mlx-mediated sugar sensing, which contributes to diet-induced physiological readjustment, including flux of central carbon metabolism and cycling of metabolic circadian clock targets.

By uncovering the CBT-mediated repression of pepck isoforms downstream of Mondo-Mlx, our study provides a mechanistic explanation to the regulation of cataplerosis in response to intracellular sugar sensing. *Drosophila* Mondo-Mlx is known to drive activation of glycolysis, for example, by promoting the expression of *phosphofructokinase 2* (Havula et al., 2013). Placing the rate-limiting enzymes of gluconeogenesis downstream, the same sensor mechanism that activates glycolysis provides an elegant mechanism to adjust the direction of flux of glucose metabolism in response...
to sugar input. Such simple network topology provides a robust safeguard against loss of energy in futile cycles caused by simultaneous high activity of glycolysis and gluconeogenesis.

Mondo-Mlx also activates the expression of lipogenic gene expression (e.g., FAS and ACC) in order to promote conversion of excess sugars into triglycerides (Sassu et al., 2012; Havula et al., 2012).
2013). In addition to fatty acid moieties, which are built by FAS and ACC, triglyceride biosynthesis requires glycerol-3-phosphate. Substrate-labeling studies in mammals have shown that a significant portion of glycerol in triglycerides is in fact derived from the PEPCK-dependent glyceroneogenesis pathway (Kalhan et al., 2001; Chen et al., 2005; Nye et al., 2008; Jin et al., 2013). This is supported by our findings showing significantly lower circulating glycerol levels in well-fed pepck1-mutant larvae. The impact of glyceroneogenesis on triglyceride homeostasis is also likely reflected in the reduced triglyceride levels in pepck1 mutant flies. Similarly, mammalian studies have shown that elevated expression of PEPC-K-C in adipose tissue increases fat mass, whereas reduced PEPC-K-C expression leads to lower fat content (Franckhauser et al., 2002; Olswang et al., 2002). Moreover, in humans, adipose tissue expression of PEPC-K-C positively correlates with adiposity and plasma triacylglycerol levels (Chang et al., 2008). Control of pepck through CBT places both branches of triglyceride biosynthesis under Mondo-Mlx. Inhibition of PEPC-K-mediated cataplerosis upon high sugar intake allows maximal conversion of excess glucose-6-phosphate into the glycerol moieties of triglycerides through the glycolytic route of glycerol-3-phosphate synthesis. Simultaneous impairment of de novo lipogenesis and failure to suppress glyceroneogenesis likely leads to the breakage of glycerol homeostasis and massive accumulation of circulating glycerol, as observed in mls mutants. Interestingly, a recent study showed that fasting serum levels of glycerol predicted development of hyperglycemia and type 2 diabetes (Mahendran et al., 2013). It will be interesting to learn whether this diagnostic marker is associated with deregulation of pepck isoforms and the activity of ChREBP/MondoA-Mlx and Klf10-dependent transcriptional network.

According to the current view, Mondo/ChREBP and Mix act mainly in nutrient sensing and metabolic regulation. In contrast, CBT and its human ortholog Klf10 are multifunctional transcription factors. In Drosophila, cbt was originally identified as a development regulator with an essential function in dorsal closure early in development (Muñoz-Descalzo et al., 2005). Moreover, cbt is a direct transcriptional target of the circadian transcription factor CLK (Kadener et al., 2007; Abruzzi et al., 2011), and we have established here that it is deeply involved in the control of the circadian transcriptional network. While CBT overexpression leads to strong behavioral abnormalities, they are not accompanied by noticeable changes in the oscillation of the core clock components in the fly heads. This suggests that it reflects a specific effect on circadian output. If the behavioral patterns were due to an effect in the general health of the animal, we would expect deregulation of core clock components. Despite the null effect of cbt overexpression in the expression of core clock genes, we observed that cbt modulates the expression of an important subset of CLK and circadian-controlled genes, most of which are involved in metabolic functions. We observed also strong effects of cbt downregulation in circadian oscillation of metabolic genes, establishing CBT as a new regulator of the circadian transcriptome. Interestingly, downregulation of CBT in circadian cells decreases the amplitude of oscillation of a large number of circadian-controlled genes, providing a direct link between food intake, circadian gene expression, and behavior. Given the established link between feeding time, metabolism, and the circadian system in Drosophila (Xu et al., 2008, 2011; Mattson et al., 2014), it will be interesting to further analyze the importance of CBT in this coordination.

Although the functional analysis in this study largely focused on the metabolic role of pepck regulation by Mondo-Mlx-CBT network, our microarray and RNA-seq analyses revealed other interesting CBT transcriptional targets including bmm. This gene is an ortholog of the human adipocyte triglyceride lipase gene, and it is an essential regulator of triglyceride stores in Drosophila (Grönke et al., 2005). bmm expression is positively regulated by the Forkhead transcription factor FoxO, which is activated during starvation through inhibition of insulin-like signaling (Wang et al., 2011). The sugar-dependent repression of bmm expression by CBT is in perfect agreement with the lipogenic role of Mondo-Mlx.

It has been proposed that CBT mammalian ortholog Klf10 acts as a negative feedback regulator for ChREBP-activated genes, including lipogenic genes FAS and ACC (Iizuka et al., 2011). This conclusion was based on suppression of ChREBP-activated transcription upon Klf10 overexpression in primary liver cells. We tested this model by analyzing the expression of Mondo-Mlx targets FAS and ACC, but observed no effect by cbtRNAi (Supplementary Fig S8D). In contrast, our genome-wide expression analysis of CBT loss-of-function flies revealed that the CBT-dependent branch of the sugar sensing transcriptional network mediates rapid repression of gene expression. It is interesting to note that while most metabolic targets of CBT are rapidly and persistently downregulated, cbt expression is rapidly attenuated during prolonged sugar feeding (see Supplementary Fig S1A). This is likely due to the negative autoregulation demonstrated earlier (Belacortu et al., 2012) and supported by our ChIP data. The finding that most of the identified CBT-dependent mRNAs are stably repressed for many hours after cbt levels have significantly attenuated suggests that CBT-mediated repression might involve regulation at the chromatin level. This is in agreement with the possible involvement of Sin3A in CBT-mediated repression (Spittau et al., 2007; Belacortu et al., 2012). Through such persistent regulatory marks, sugar feeding may have a long-lasting influence on metabolic homeostasis, which is a topic that certainly deserves to be more thoroughly analyzed in the future.

In sum, our work provides a mechanistic explanation for the transcriptional repression upon Mondo-Mlx-mediated intracellular sugar sensing through the transcription factor CBT. The CBT-mediated repressive branch of the sugar sensing network is involved in securing the mutually exclusive activity of glycolysis and gluconeogenesis and coordination of fatty acid and glycerol biosynthesis with respect to dietary sugar intake. Our study also establishes a mechanism for nutrient input into the circadian gene expression. As intracellular sugar sensing and circadian regulation are highly conserved processes, the insight achieved here in the genetically tractable Drosophila model should provide a new conceptual framework for forthcoming studies in human subjects and mammalian model systems.

Materials and Methods

Fly stocks and maintenance

Flies were reared at 25°C in 12 h light:12 h dark cycles on standard diets (detailed in Supplementary Methods). For defined nutrient studies, 1st instar larvae were collected from apple juice plates onto...
food containing 20% (w/v) dry baker’s yeast, 0.5% (w/v) agarose, 2.5% (v/v) Napagin (methylparaben), and 0.7% propionic acid in PBS supplemented with or without 20% sucrose. For starvation, 2nd instar larvae were transferred to 0.5% agarose in PBS media for 16 h, followed by 4 h of refeeding with 0.5% agarose and 10% glucose in PBS media.

Tum-gal4, ctb2, UAS-Dicer 2, and UAS-ctb-Flag flies were previously described (Zhao et al, 2003; Muñoz-DescaIzo et al, 2005; Dietzl et al, 2007). The P-element line P[EPgy2]EY10042 used to generate the pepekΔ48 allele was from Bloomington Stock Center (19965). As a control for pepekΔ48/Df(2R)ED3636, we used a precise excision line in trans with yw. The RNAi lines used in this study were acquired from the following collections: RNAi lines targeting ctb from NIG-Fl y Stock Center (4427R-1) and Vienna Drosophila RNAi Center (VDRC) (v15555); RNAi-line for pepck1 (CG17725) v50253 from VDRC; and line 36915 for pepck2 (CG10924) from Bloomington Stock Center. Control for mixA, mixB, UAS-mix, and mondoRNAi (v109821) is detailed in Havula et al (2013).

Metabolite assays

Metabolite assays were performed using pre-wandering 3rd instar larvae grown on 20% yeast. Hemolymph was collected, and circulating glucose and trehalose were measured as described previously (Zhang et al, 2011). The circulating glycerol levels were determined by using the Free Glycerol Reagent (Sigma F6428) according to manufacturer’s protocol, scaled down to 100 µl. Triglycerides from pepck1 mutants were measured from 5-day-old adult male flies as described previously (Tennessen et al, 2014); values were normalized to protein. All measurements were done in minimum of three biological replicates. Student’s t-test (two-tailed, unequal variance) was used for statistical analysis.

Sugar and protein feeding

For experimental manipulation, three-day-old Canton-S flies were starved for 16 h and then re-fed with agar-containing sucrose (5% sucrose) or protein (10% casein protein, Sigma). Flies were collected at time zero and at the indicated times after re-feeding.

Analysis of gene expression by real-time PCR

For analyses of fly heads, total RNA was prepared (30 heads per sample) using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. cDNA derived from this RNA (using iScript, Bio-Rad) was utilized as a template for quantitative real-time PCR performed with the C1000 Thermal Cycler Bio-Rad. The PCR mixture contained Taq polymerase (SYBR green, Bio-Rad). Cycling parameters were 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 30 s. Fluorescence intensities were plotted versus the number of cycles by using an algorithm by the manufacturer. mRNA levels were quantified using a calibration curve based upon dilution of concentrated cDNA. mRNA values from heads were normalized to mRNAs encoding ribosomal proteins 49 (rp49) and RpS18.

RNA from larvae was extracted using Nucleospin RNA XS or II kits (Macherey-Nagel). For quantitative RT–PCR, the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) with random hexamer primers was used for first-strand cDNA synthesis. qPCR was performed using Maxima SYBR Green qPCR Master Mix (2×) (Thermo Fisher) and analyzed on Bio-Rad CFX384 Real-Time System with Bio-Rad CFX Manager. mRNA values from larvae were normalized to actin. Primer details are in Supplementary Methods.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed from S2C cells (Mlx binding to ctb promoter) and from fly heads (Ctab targets). Experimental details are in Supplementary Methods.

Microarrays

RNA was used to prepare a probe that was hybridized to Drosophila 2.0 gene expression arrays (Affymetrix). CEL files were imported to Affymetrix Expression Console program, and data were normalized using Robust Multichips Analysis. Statistical analysis was performed using the R statistical environment (http://cran.r-project.org/). Differential expression was tested for all genes that had expression levels above 4.0 (log2 scale) for all measurements in at least one group (mutants or WT). To test for differential expression, we used analysis of variance (ANOVA), controlling for the effect of time of day. The P-values from the ANOVA were adjusted for multiple testing using the false discovery rate (FDR) correction implemented in the R-function p.adjust. We used two linear models with the genotype as the predictor. One model used genotype as the factor, and the second assumed gradual change between RNAi, control and overexpression. The P-values of both models were FDR corrected, and genes with P < 0.2 and at least 1.5 fold change between cbtRNAi and ctbOE were selected.

For enrichment analysis of cycling genes, we used previously published data of ~500 Clk target genes (Abruzzesi et al, 2011) and crossed this list with the list of ctb upregulated or downregulated genes. Statistical significance was determined by comparing the results to multiple crossing of the Clk target genes with random set of genes (100,000 iterations).

RNA extraction, library preparation, and high-throughput sequencing

Fly heads of the indicated genotypes were collected, and RNA was extracted using TRI reagent (Sigma-Aldrich). RNA-seq libraries were prepared as previously described (Engreitz et al, 2013) with the modification of performing polyA selection after fragmentation. RNA-seq reads were aligned and counted per annotated gene.

Analysis of control and cbtRNAi RNA-seq data

We first filtered for expressed genes in each dataset separately (control or cbtRNAi). For each transcript, we determined whether it was differentially expressed in each sucrose concentration in comparison with the time zero control. To determine P-values, we utilized Dunett’s method and performed an FDR correction. We chose those transcripts for which fold change > 1.5 and FDR < 0.4 in at least two different concentrations of sucrose. We determined
CBT dependence in the following cases: (i) genes that fit the significance criteria in control but not in the cbtRNAi dataset; (ii) those genes significantly upregulated in cbtRNAi flies but not in control flies; and (iii) genes that are more than 1.5-fold less affected in cbtRNAi while comparing to control.

Time course RNA-seq profile
We performed ANOVA and FDR correction in order to identify transcripts differentially expressed at any time point following sugar intake. From this group, we selected genes with more than 1.5-fold change. To avoid genes that showed significant changes due to circadian control, we eliminated those genes for which the difference between the first (0 h) and last (18 h) time points was less than 1.5-fold. We followed by clustering the differentially expressed into ten modules using hclust clustering, R bioconductor package. The clustering was based on Euclidean distance measured and clustered using the complete linkage method.

Analysis of cycling genes
The R package JTK cycle was used to detect cycling transcripts as described before (Hughes et al, 2010). We considered cycling transcript as transcript with P-value < 0.01 in the JTK analysis and an amplitude (minimum/maximum value) higher than 2 in all replicas. Chi-square tests were used to determine statistical significance of the differences in the number of cycling genes in the control versus RNAi files. Mann–Whitney U-test was used to determine statistical significance of difference in amplitude size between cycling genes in cbtRNAi and control flies.

Heatmaps were generated using the heatmap.2 function of the gplots package in R. For each heatmap, we used k-means clustering for initial clustering of 6 groups, and a second hierarchical clustering for clustering genes within each group. DAVID functional annotation tool (http://david.abcc.ncifcrf.gov/home.jsp) (Huang et al, 2009) was used to examine gene ontology (GO) enrichment in the different gene groups. P-value < 0.05 after Bonferroni correction was used to determine statistical significance.

Circadian locomotor behavior
Adult male and female flies (3–7 days old) were used for circadian locomotor behavior; these flies were placed separately in behavioral glass tubes containing sucrose–agar food (2% agar, BD; 5% sucrose, BioLab). First, flies were entrained in LD (light:dark) condition for the first 3–4 days, followed by 7–11 days in DD (constant dark); activity was monitored by using Trikinetics Drosophila Activity Monitors (DAM; Trikinetics, Waltham, MA) system. Data were collected by DAM System 3 Data collection software and analyzed using MatLab, as described before (Levine et al, 2002). Rhythm index of > 0.2 in DD was used as threshold for defining activity as rhythmic. Constant humidity and temperature (25 ± 1°C) were maintained during the experiment.

Phosphoenolpyruvate/oxaloacetate ratio measurement
Phosphoenolpyruvate (PEP), pyruvate, and oxaloacetate (OAA) concentrations were measured fluorometrically according to the kits’ manufacturer’s guidelines (MAK102, MAK070, Sigma-Aldrich); the assay is detailed in Supplementary Methods.


regulating lipid synthesis and feeding behavior in Drosophila. Biochem Biophys Res Commun 426: 43–48


