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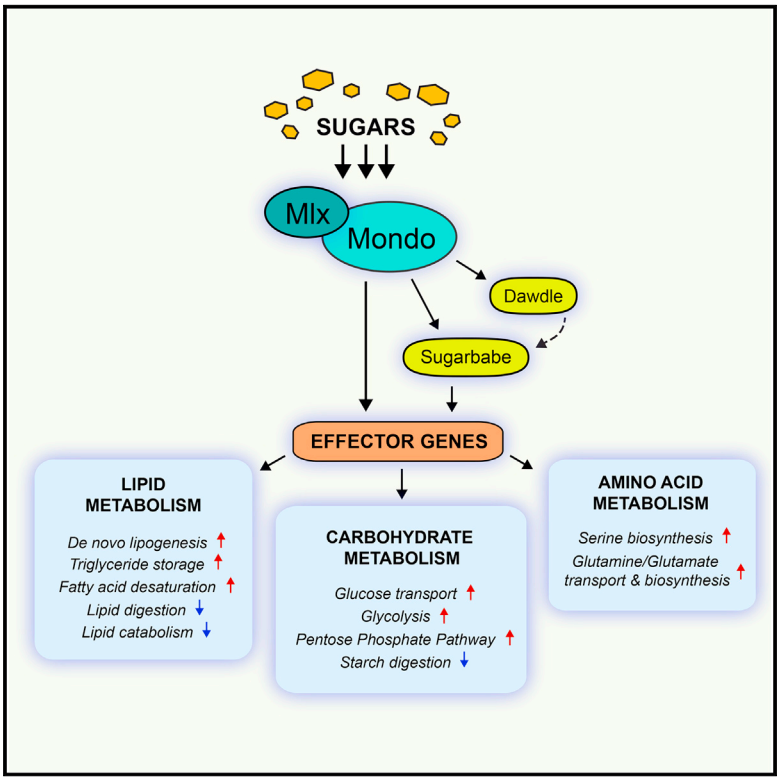
Mondo-Mlx Mediates Organismal Sugar Sensing through the Gli-Similar Transcription Factor Sugarbabe

Graphical Abstract



Highlights

- Mondo-Mlx regulates the majority of *Drosophila* sugar-activated genes in vivo
- Mondo-Mlx controls Activin signaling and the transcription factor Sugarbabe
- Sugarbabe regulates a subset of Mondo-Mlx-dependent processes, including lipogenesis
- Human homologs of Mondo-Mlx targets are enriched among triglyceride-associated genes

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In Brief

Mattila et al. demonstrate that Mondo-Mlx controls the majority of sugar-responsive genes in *Drosophila*, acting both directly and through a downstream regulatory network composed of the Activin pathway and the Gli-similar transcription factor Sugarbabe. Sugarbabe promotes activation of lipid and serine biosynthesis and represses gut amylase expression.

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Mondo-Mlx Mediates Organismal Sugar Sensing through the Gli-Similar Transcription Factor Sugarbabe

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SUMMARY

The ChREBP/Mondo-Mlx transcription factors are activated by sugars and are essential for sugar tolerance. They promote the conversion of sugars to lipids, but beyond this, their physiological roles are insufficiently understood. Here, we demonstrate that in an organism-wide setting in *Drosophila*, Mondo-Mlx controls the majority of sugar-regulated genes involved in nutrient digestion and transport as well as carbohydrate, amino acid, and lipid metabolism. Furthermore, human orthologs of the Mondo-Mlx targets display enrichment among gene variants associated with high circulating triglycerides. In addition to direct regulation of metabolic genes, Mondo-Mlx maintains metabolic homeostasis through downstream effectors, including the Activin ligand Dawdle and the Gli-similar transcription factor Sugarbabe. Sugarbabe controls a subset of Mondo-Mlx-dependent processes, including de novo lipogenesis and fatty acid desaturation. In sum, Mondo-Mlx is a master regulator of other sugar-responsive pathways essential for adaptation to a high-sugar diet.

INTRODUCTION

Adaptation to changes in the diet in order to maintain metabolic homeostasis requires the ability to sense the levels of specific nutrients, such as amino acids, lipids, and sugars. Much is known about both the nutrient-sensing pathways and the physiological changes triggered by nutrients (Efeyan et al., 2015; Havula and Hietakangas, 2012), yet the integration of nutrient-sensing pathway activities with physiological outcome in multicellular animals has remained challenging.

Sugar metabolites are sensed intracellularly by the transcription factor paralogs ChREBP (Carbohydrate-Responsive Element Binding Protein, MLXIPL, or MondoB) and MondoA, both of which heterodimerize with Mlx. The ChREBP/MondoA-Mlx complex is activated by intracellular glucose-6-phosphate and other phosphorylated hexoses (Dentin et al., 2012; Li et al., 2010; Stoltzman et al., 2011), and it regulates genes involved in glycolysis and de novo lipogenesis (Havula and Hietakangas, 2012). *Drosophila melanogaster* encodes single orthologs of ChREBP/MondoA and Mlx, called Mondo and Mlx, respectively. Flies lacking a functional Mondo-Mlx complex display striking intolerance toward dietary sugars (Havula et al., 2013), which resembles the lethality of *ChREBP*$^{-/-}$ mice on a high-carbohydrate diet (Iizuka et al., 2004), suggesting functional conservation. There is emerging evidence linking ChREBP/MondoA-Mlx to human disease-associated phenotypes. Polymorphisms in *ChREBP* (*MLXIPL*) are associated with elevated circulating triacylglycerol (TAG) levels, and deregulated ChREBP in adipose and liver tissues is associated with severe obesity (Herman et al., 2012; Kathiresan et al., 2008; Kooner et al., 2008). Moreover, lipogenic activity of MondoA-Mlx is essential for tumorigenesis by deregulated Myc oncogene (Carroll et al., 2015).

The regulation of key lipogenic ChREBP/MondoA-Mlx target genes, including *Fatty acid synthase* (*FAS*) and *Acetyl-CoA carboxylase* (*ACC*), is conserved in mammals and *Drosophila* (Havula et al., 2013; Ishii et al., 2004; Jeong et al., 2011; Ma et al., 2005, 2006; Musselman et al., 2013). Previous work on ChREBP/MondoA-Mlx-mediated responses has focused on specific cell types, including hepatocytes in mammals (Jeong et al., 2011; Ma et al., 2006) and the *Drosophila* fat body (Havula et al., 2013; Musselman et al., 2013). It has remained unexplored how Mondo-Mlx-mediated sugar sensing contributes to gene

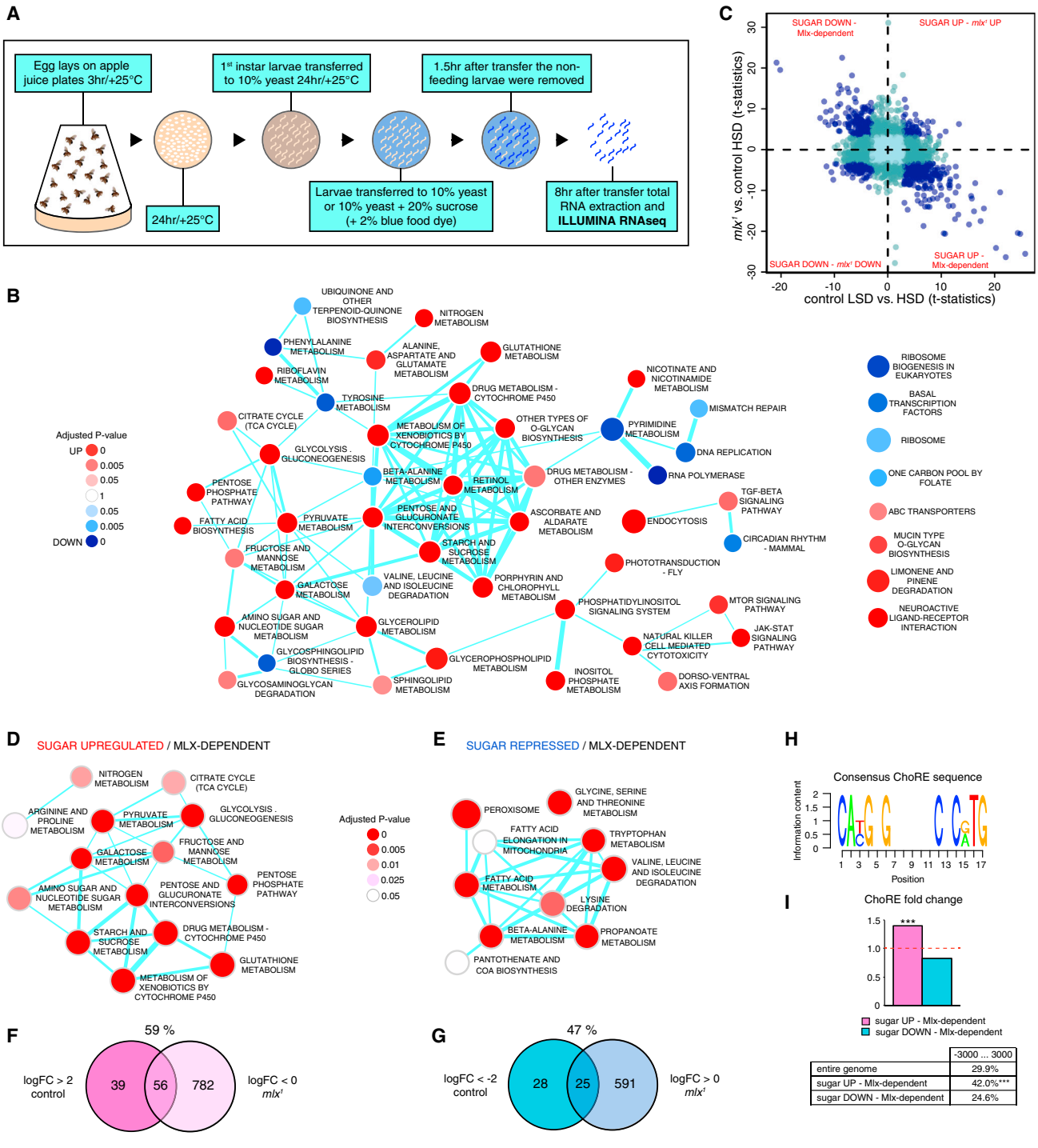

Figure 1. Mondo-Mlx Regulates the Majority of the Sugar-Induced Transcriptome

(A) Schematic presentation of the sugar-induced transcriptome experiment.

(B) Enrichment map of sugar-regulated processes ($p < 0.05$).

(C) Four-way plot (t-statistics) presenting the Mlx-dependent sugar-regulated genes ($p < 0.05$, logFC > 0/logFC < 0). Dark blue indicates significant in both comparisons; dark turquoise, significant in one comparison; light turquoise, not significant.

(D) Sugar-upregulated/Mlx-dependent processes (FDR < 0.05).

(E) Sugar-repressed/Mlx-dependent processes (FDR < 0.05).

(F and G) Venn diagram of the sugar-upregulated (logFC > 2)/Mlx-dependent (logFC < 0) transcriptome ($p < 0.05$) (F) and sugar downregulated (logFC < −2)/Mlx-dependent (logFC > 0) transcriptome ($p < 0.05$) (G).

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expression in complex organism-wide settings, enabling diverse tissues to respond to dietary sugars in a concerted manner.

In addition to Mondo-Mlx, other regulators have been implicated in sugar sensing of *Drosophila*. Our recent work has uncovered that Mondo-Mlx directly activates the expression of the Krüppel-like transcription factor Cabut, which represses the expression of *pepck* as well as the circadian cycling of metabolic genes (Havula et al., 2013; Bartok et al., 2015). Moreover, the Gli-similar transcription factor ortholog *sugarbabe* (*sug*) has been shown to be strongly upregulated upon sugar feeding, and it has been proposed to repress genes encoding lipases as well as insulin-like peptides (Varghese et al., 2010; Zinke et al., 2002). However, the mechanism of sugar-dependent regulation of *sug* has not been addressed, and its physiological role remains poorly understood. In addition to transcriptional regulators, activation of endocrine responses upon sugar feeding has been observed. For example, recent studies have shown that Activin signaling plays an essential role in systemic metabolic regulation and the expression of Activin ligand *dawdle* is sugar inducible (Chng et al., 2014; Ghosh and O'Connor, 2014). Whether these sugar-responsive regulators act in parallel or as members of the same regulatory network is, however, unclear.

By using *Drosophila* larvae as a model system, we have addressed the role of Mondo-Mlx in organismal gene regulation in response to sugar feeding. We discovered that the physiological role of Mondo-Mlx is significantly broader than previously appreciated. In addition to the well-established glycolytic and lipogenic gene expression programs, Mondo-Mlx controls nutrient digestion and transport through tissue-specific gene regulation in the gut and renal tubules, respectively. We also identify several previously unknown metabolic targets for Mondo-Mlx, including genes involved in the synthesis of nonessential amino acids serine and glutamine. Interestingly, human homologs of the Mondo-Mlx targets had significantly stronger associations to circulating serum triglyceride levels than would be expected by chance, providing further evidence for the relevance of Mondo-Mlx-regulated transcription in human pathophysiology. In addition to direct regulation of metabolic genes, Mondo-Mlx maintains metabolic homeostasis through downstream effectors. Specifically we show that Mondo-Mlx acts as a master regulator of a hierarchical network composed of the Activin signaling pathway as well as transcription factor Sugarbabe. Analysis of *sug* mutants reveals that Sugarbabe contributes to sugar-responsive metabolic regulation through the control of genes involved in starch digestion as well as biosynthesis of serine and fatty acids.

RESULTS

Mondo-Mlx Regulates the Majority of the Sugar-Induced Transcriptome In Vivo

To characterize the gene expression changes triggered by dietary sugars in vivo, we compared the RNA-sequencing (RNA-seq) gene expression profiles of *Drosophila* larvae grown on a diet rich in protein but low in sugar (LSD) with those exposed to a high-sugar diet (HSD) for 8 hr (Figure 1A). Among the most highly enriched gene sets were the ones related to sugar metabolism, fatty acid biosynthesis, and cytochrome P450 functions (false discovery rate [FDR] < 0.05) (Figure 1B; Table S1). Conversely, genes involved in the metabolism of essential amino acids and ribosome biogenesis were significantly downregulated (FDR < 0.05) (Figure 1B; Table S1). Comparison with published datasets on sugar-regulated genes in *Drosophila* revealed substantial overlap, further validating our data (Figure S1; Table S3) (Chng et al., 2014; Musselman et al., 2013; Zinke et al., 2002).

To determine which part of the sugar-regulated transcriptome is regulated in an Mlx (*bigmax*, CG3350)-dependent manner, we compared the sugar-regulated transcriptomes of control and mlx^1 null mutant animals (Havula et al., 2013) (Figure 1C; Table S2). 325 genes were significantly upregulated (FDR < 0.05) in sugar-fed control animals but displayed significantly lower expression (FDR < 0.05) in mlx^1 mutants ("sugar up, Mlx-dependent") (Figure 1C; Table S4). These included genes involved in glycolysis, pentose phosphate pathway (PPP), pyruvate metabolism, and cytochrome P450-mediated processes (Figure 1D; Table S4). Our analysis also uncovered the genes downregulated on a sugar diet in an Mlx-dependent manner ("sugar down, Mlx-dependent," n = 169, FDR < 0.05) (Figure 1C; Table S4). This group was enriched for genes involved in amino acid and fatty acid metabolism (Figure 1E; Table S4). When focusing on the genes most strongly upregulated by sugar (logFC > 2), the majority of genes (59%) displayed significantly lower expression in the mlx^1 mutants (Figure 1F). On the other hand, 47% of the strongly downregulated genes (logFC < −2) were dependent on Mlx (Figure 1G). Thus, in an organism-wide setting, Mondo-Mlx contributes to the expression of the majority of genes that display strong responsiveness to dietary sugars.

To assess the global distribution of direct Mondo-Mlx targets, we looked for putative carbohydrate response elements (ChoREs) in their promoter regions (Figure 1H; Ma et al., 2006). The genes in the sugar up, Mlx-dependent group displayed a significant enrichment of putative ChoREs in a 6-kB window around the transcriptional start site, suggesting that this group includes direct targets (Figure 1I). In contrast, the sugar down, Mlx-dependent group of genes displayed no significant enrichment of ChoREs, implying these genes being mainly indirect targets (Figure 1I).

Control of Carbohydrate Homeostasis through Tissue-Specific Gene Regulation by Mondo-Mlx

Next, we wanted to systematically explore key groups of metabolic Mondo-Mlx targets and their contribution to organismal homeostasis upon sugar feeding. In the absence of functional Mondo-Mlx, the circulating glucose levels in *Drosophila* larvae are highly elevated on a sugar-containing diet (Havula et al.,

(H) ChoRE sequence used in the ChoRE enrichment analysis.
(I) Presence of putative ChoREs in Mlx-dependent sugar-up- and downregulated groups are displayed as percentage and fold change when compared to the whole genome.
See also Figure S1.

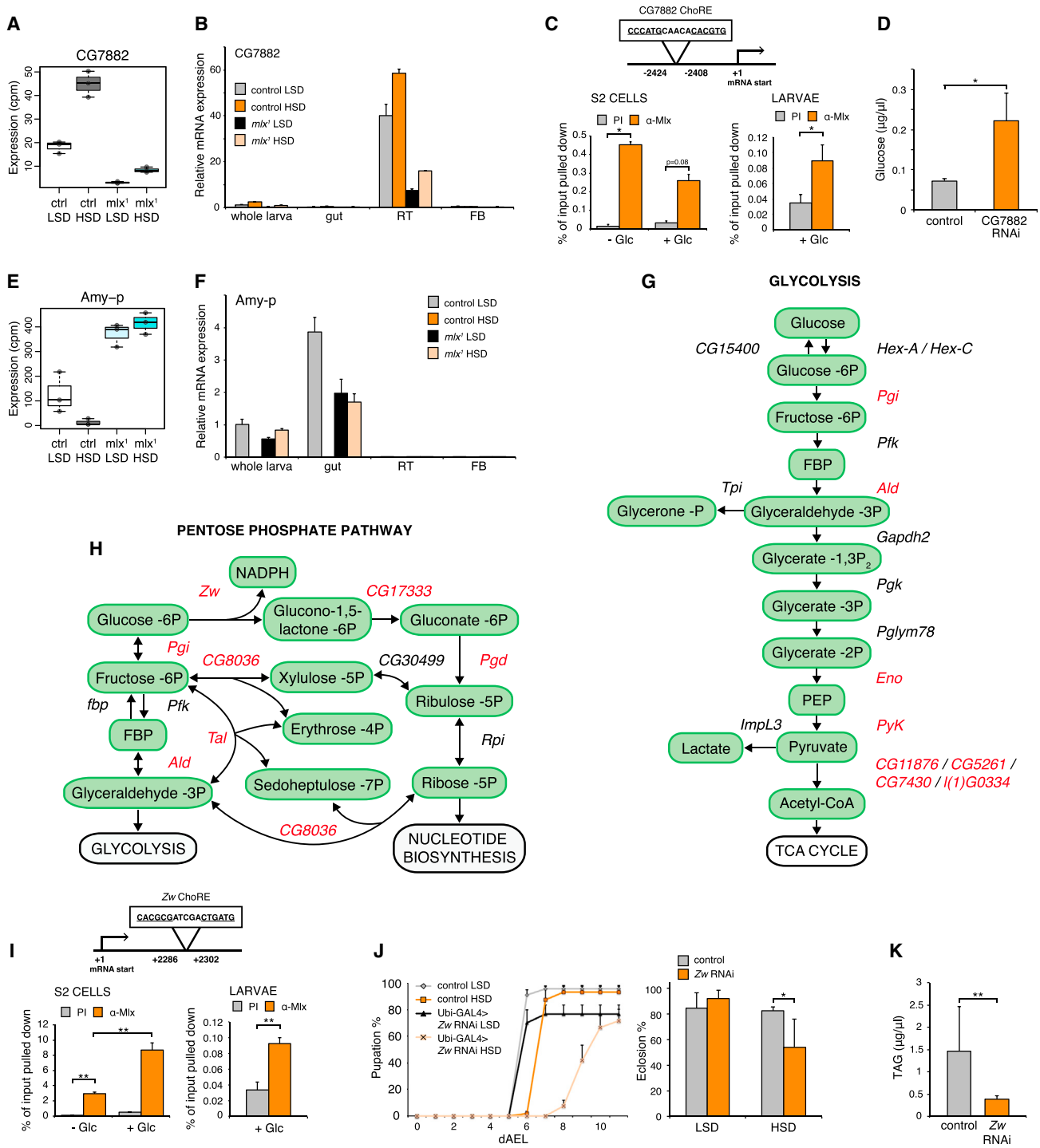

Figure 2. Mondo-Mlx Regulates Sugar Transport and Carbohydrate Digestion

(A) Expression of GLUT CG7882 on LSD versus HSD (*mlx* RNA-seq).

(B) Tissue-specific expression of CG7882 (qPCR). FB, fat body; RT, renal tubules.

(C) CG7882 chromatin immunoprecipitation (ChIP) in S2 cells (low versus high glucose) and larvae in using anti-Mlx antibodies or preimmune (PI) serum.

(D) Hemolymph glucose of control and CG7882 RNAi (tub-GAL4 >) third-instar larvae on a 20% yeast + 10% sucrose diet.

(E) Expression of *Amy-p* on LSD versus HSD (*mlx* RNA-seq).

(F) Tissue-specific expression of *Amy-p* (qPCR) in early third-instar mlx^1 mutant and control larvae following acute transfer to HSD for 24 hr.

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2013), which may reflect deregulation of glucose transport, metabolism, or both. Our RNA-seq data revealed that the expression of five putative sugar transporters, belonging to the GLUT family (Mueckler and Thorens, 2013), was regulated upon sugar feeding in an Mlx-dependent manner (Figures 2A and S2A; Table S4). We performed a tissue-specific expression analysis, focusing on tissues with high Mondo-Mlx expression (Figures 2B and S2B) (Havula et al., 2013). The expression of GLUT CG7882 was highly specific to the renal (Malpighian) tubules and was upregulated by sugar in an Mlx-dependent manner (Figure 2B). Furthermore, chromatin immunoprecipitation (ChIP) assays in S2 cells and larvae provided evidence that CG7882 is a direct target of Mondo-Mlx (Figure 2C). Interestingly, depletion of CG7882 by RNAi led to hyperglycemia (Figure 2D), suggesting that controlled expression of CG7882 may serve as a mechanism to balance circulating glucose levels, possibly by promoting excretion through the renal tubules.

In addition to sugars, *D. melanogaster's* diet contains carbohydrates in the form of polysaccharides, such as starch, which need to be enzymatically digested by α-amylases in the alimentary track. The *Drosophila* genome contains three genes encoding for α-amylases, *Amylase proximal* (*Amy-p*), *Amylase distal* (*Amy-d*), and *Amyrel* (Ruaud et al., 2011), whose expression is repressed by dietary sugar (Benkel and Hickey, 1987). Our RNA-seq data revealed that the repression of all three α-amylases by HSD was Mondo-Mlx dependent (Figures 2E, S2C, and S2D; Table S4). Consistent with their role in digestion, the amylase gene expression is concentrated into the gut (Figure 2F) (Thompson et al., 1992). We failed to identify putative ChoRE elements in the *Amylase* promoters, suggesting that their repression might be mediated through secondary effectors downstream of Mondo-Mlx. Together, these data show that Mondo-Mlx controls tissue-specific expression of genes involved in carbohydrate digestion and glucose transport.

Previous work with mammalian cells has demonstrated that ChREBP/MondoA-Mlx controls glycolytic flux through the transcriptional regulation of glycolytic enzymes (Ishii et al., 2004; Jeong et al., 2011; Ma et al., 2005; Sans et al., 2006). Our RNA-seq data are in line with these observations, showing that a significant fraction of glycolytic enzymes is under the control of Mondo-Mlx (Figures 2G and S3A; Table S4). In addition to the glycolytic enzymes, the gatekeeper enzyme for the PPP, Glucose-6-phosphate dehydrogenase, is a known target of mammalian ChREBP-Mlx (Ma et al., 2006). Our RNA-seq data revealed that along with the *Glucose-6-phosphate dehydrogenase* (*Zwischenferment*, or *Zw*), several other genes encoding for enzymes in the PPP displayed strong Mondo-Mlx-dependent induction upon sugar feeding (Figures 2H, S3B, and S3C; Table S4). At the gene-set level, PPP was among the most strongly enriched processes that were activated by sugar feeding in an Mlx-dependent manner (Figure 1D; Table S4). Furthermore, we demonstrated by ChIP that *Zw* is a direct Mondo-Mlx target (Figure 2I).

mlx^1 mutants are intolerant to dietary sugars and display delayed development and reduced survival on HSD (Havula et al., 2013). Like *mlx^1* mutants, *Zw* RNAi larvae also showed significantly delayed pupation and impaired eclosion on HSD (Figure 2J), demonstrating the physiological importance of PPP activation in the presence of sugar. *Zw* is regulated by Mondo-Mlx in the fat body and gut (Figure S3D). Fat-body-specific knockdown of *Zw* showed impaired development on HSD, while gut-specific knockdown had no detectable effect (Figure S3E). As NADPH produced by the oxidative phase of PPP is needed for de novo lipogenesis, we hypothesized that inhibition of PPP might hamper TAG accumulation in response to sugar feeding. In agreement, the total TAG content of *Zw* knockdown larvae was significantly lower compared to the control (Figure 2K). In conclusion, Mondo-Mlx is essential for the transcriptional upregulation of the PPP, which in turn is necessary for lipid homeostasis and growth upon feeding on a sugar-rich diet.

Activation of Glutamine and Serine Biosynthetic Genes Is Essential for Growth and Survival on HSD

The non-essential amino acids glutamate and glutamine play a central role in amino acid and energy metabolism (DeBerardinis and Cheng, 2010). We observed that several genes involved in glutamate/glutamine metabolism were induced by sugar in an Mlx-dependent manner (Figures 3A and S4A; Table S4) including the glutamate transporter *Eaat1*, a putative glutamate synthase (*GltS*, CG9674), and the Glutamine synthetases 1 and 2 (*Gs1*, *Gs2*). The Glutamine synthetases displayed tissue-specific expression patterns, with *Gs1* being strongly regulated in the fat body and *Gs2* being mostly expressed in the gut (Figure S4B). ChIP analysis revealed that *Gs2* promoter was bound by Mlx (Figure 3B). Consistent with the role of glutamine in anabolic reactions, we observed that knockdown of *Gs2* led to the development of pupae with smaller size than controls on HSD (Figure 3C). This finding implies that glutamine biosynthesis needs to be coordinated with respect to sugar intake to sustain optimal growth and that Mondo-Mlx is a key regulator in this process.

Our data also uncovered an Mlx-dependent regulation of metabolism of another non-essential amino acid, serine. A key route for serine biosynthesis is the three-step reaction through 3-phosphoglycerate, an intermediate of glycolysis (Figure 3D). Interestingly, enzymes of this route, including 3-phosphoglycerate dehydrogenase (*3-PGDH*, CG6287), Phosphoserine

(G) The glycolytic pathway and the predicted *Drosophila* genes encoding the enzymes. Genes in red are significantly (FDR < 0.05) upregulated on HSD in an Mlx-dependent manner (*mlx* RNA-seq) (Table S4). Genes in blue are significantly (FDR < 0.05) downregulated on HSD in an Mlx-dependent manner (*mlx* RNA-seq) (Table S4).
(H) The pentose phosphate pathway and the predicted *Drosophila* genes encoding the enzymes.
(I) *Zw* ChIP in larvae and S2 cells.
(J) Pupation kinetics and eclosion of control and *Zw* RNAi larvae (Ubi-GAL4 >).
(K) TAG levels of control and *Zw* RNAi (Ubi-GAL4 >) on HSD.
Error bars show SD. *p < 0.05, **p < 0.01, ***p < 0.001. For tissue-specific data, the error bars show the SD of three technical replicates from pools of three replicate cDNA samples. See also Figures S2 and S3.

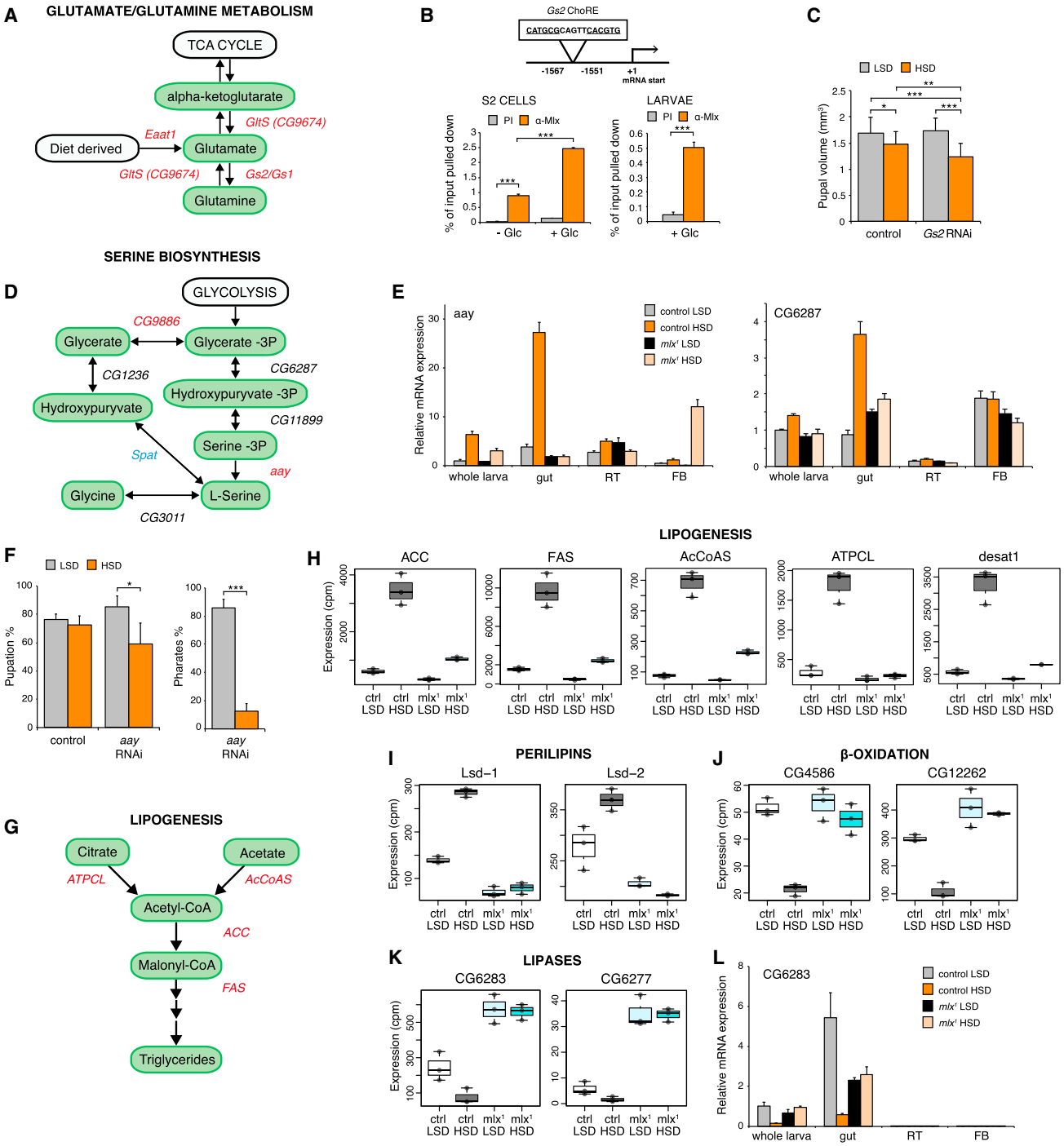

Figure 3. Mondo-Mlx Regulates Amino Acid and Lipid Metabolism

(A) Predicted *Drosophila* genes encoding enzymes of glutamine/glutamate metabolism. Genes in red are significantly (FDR < 0.05) upregulated on HSD in an Mlx-dependent manner (*mlx* RNA-seq) (Table S4). Genes in blue are significantly (FDR < 0.05) downregulated on HSD in an Mlx-dependent manner (*mlx* RNA-seq) (Table S4).

(B) *Gs2* ChIP in larvae and S2 cells.

(C) Pupal volume of *Gs2* RNAi (tub-GAL4 >) and control animals.

(D) Predicted *Drosophila* genes encoding enzymes of de novo serine synthesis pathway.

(E) Tissue-specific expression of *aay* and CG6287 (qPCR).

(F) Left: pupation of *aay* RNAi (tub-GAL4 >) on HSD. Right: *aay* RNAi animal development to the pharate pupal stage.

(G) Predicted *Drosophila* genes encoding enzymes of the de novo lipogenesis pathway.

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aminotransferase (*PSAT*, CG11899) and Phosphoserine phosphatase (*astray*, *aay*) were upregulated by sugar (Figure S4C; Table S2). Tissue-specific analysis of the serine biosynthetic genes showed that especially the sugar-induced expression in the gut was strongly dependent on Mlx (Figure 3E).

To test the functional importance of the serine biosynthetic pathway, we used RNAi-mediated depletion of *aay*, which led to late (pharate) pupal lethality on LSD. However, this phenotype was strongly enhanced on HSD, leading to lethality already during early pupal stages (Figure 3F). In conclusion, our data uncovered a previously unknown role for Mondo-Mlx in the control of glutamine and serine biosynthesis. Failures in these regulatory axes lead to compromised growth and survival on HSD.

Regulation of Lipid Homeostasis by Mondo-Mlx

Loss of functional ChREBP-Mlx in mammals and *Drosophila* leads to reduced expression of the lipogenic genes *FAS* (CG3523), *ACC*, *Acetyl-CoA synthetase* (*ACS*/*AcCoAS*), *ATP citrate lyase* (*ACL*/*ATPCL*), and *stearoyl-CoA desaturase* (*SCD*/*desat1*) (Havula et al., 2013; Jeong et al., 2011; Ma et al., 2005, 2006; Musselman et al., 2013). Our data confirmed these earlier findings (Figures 3G and 3H; Table S4) but also revealed additional, previously unknown roles for Mondo-Mlx in lipid homeostasis. For example, we observed that sugar feeding upregulated *Lsd-1* and *Lsd-2,* two members of the Perilipin family, in an Mlx-dependent manner (Figure 3I; Table S4). Perilipins surround lipid droplets and protect lipids from lipase activity (Beller et al., 2010). In addition, several genes encoding enzymes necessary for the β-oxidation pathway were downregulated by dietary sugar in an Mlx-dependent manner, including two genes encoding for Acyl-CoA dehydrogenases (CG4586 and CG12262) (Figure 3J; Table S4). Also, the sugar-induced repression of several lipases is under the control of Mlx, including the tandem duplicated genes CG6283 and CG6277, homologs of mammalian pancreatic lipases (Figure 3K; Table S4). These lipases were exclusively expressed in the gut (Figure 3L), pointing toward a role in dietary lipid hydrolysis. CG6283 and CG6277 contain predicted N-terminal signal sequences (Figure S4D), suggesting that these enzymes are indeed secreted. In sum, Mondo-Mlx has a broader role in lipid homeostasis than previously anticipated.

Mondo-Mlx Is the Master Regulator of the Activin-Sugarbabe Network

As many of the Mlx-dependent target genes lacked any evident ChoREs (see Figure 1I), they are likely to include indirect targets. Therefore, we sought for possible downstream regulatory genes controlled by Mondo-Mlx. Interestingly, among the genes that were induced by sugar in a Mondo-Mlx-dependent fashion was the Activin ligand *dawdle* (*daw*) (Figures 4A and S5A; Table S4). The sugar-dependent regulation of *daw* had been described previously, but the underlying regulatory mechanisms have remained unknown (Chng et al., 2014). In agreement with earlier observations, induction of *daw* was most prominent in the fat body (Figure 4B), a tissue with high Mondo and Mlx expression (Havula et al., 2013). ChIP from *Drosophila* S2 cells and larval lysates revealed that the *daw* promoter, which contains a putative ChoRE, was occupied by Mlx (Figure 4C). RNAi-mediated depletion of *daw* in animals sustained on HSD throughout the larval stages caused pupal lethality, while animals fed an LSD survived into adulthood (Figure 4D). This confirmed the earlier findings by Ghosh and O'Connor (2014) on the physiological importance of Activin signaling on a sugar-rich diet.

The mammalian Gli-similar transcription factor homolog *sug* is among the most strongly sugar-induced genes in *Drosophila* (Zinke et al., 2002). Interestingly, our analysis revealed that induction of *sug* is strongly dependent on Mondo-Mlx (Figures 4E and S5B; Table S4). *sug* expression was highest in the fat body, gut and renal tubules, similarly to Mondo-Mlx expression (Figure S5C) (Havula et al., 2013) and *sug* expression was Mlx-dependent in all of these tissues (Figure 4F). ChIP assay with S2 cells and larval lysates showed Mlx enrichment on a putative ChoRE positioned 415bp upstream of the *sug* transcriptional start site, consistent with direct regulation of *sug* by Mlx (Figure 4G).

Having identified regulatory genes, the Activin ligand *daw* and the transcription factor *sug,* as direct targets of Mondo-Mlx, we next explored the interdependence of their gene expression. While *daw* expression was independent of *sug* (Figure S5D), *sug* expression was strongly dependent on *dawdle* (Figure 4H). Dawdle is an activator of Activin signaling, which acts through the transcription factor Smox (SMAD2, CG2262) (Parker et al., 2006; Serpe and O'Connor, 2006). If *sug* was a downstream target of the Activin signaling pathway, we would predict that depletion of *Smox* by RNAi suppresses the sugar-induced expression of *sug*. This was indeed the case, confirming the involvement of Activin signaling in *sug* regulation (Figure 4I). In conclusion, these results are consistent with a model that Mondo-Mlx acts as a master regulator of the hormonal regulatory axis controlled through the Activin ligand Dawdle. Sugarbabe is a downstream effector of the Activin signaling pathway, but it is also directly regulated by Mondo-Mlx through the ChoRE located in its promoter.

Sugarbabe Regulates a Subset of Mondo-Mlx-Dependent Target Genes

To functionally explore the role of *sug*, we established a deletion mutant ($sug^{17\Delta}$) lacking parts of the coding region as well as the promoter of the *sug*-RA isoform, including the identified Mlx binding site (Figure 5A). When placed in *trans* with a deficiency, $sug^{17\Delta}$ displayed >10-fold reduction in *sug* expression, rendering it a strong hypomorph (Figure S5E). The *sug* mutants were viable but displayed delayed larval growth and pupation kinetics on HSD, while displaying no delay on LSD (Figure 5B)

(H–K) Expression of *FAS*, *ACC*, *AcCoAS*, *ATPCL*, and *desat1* (H), *Lsd-1* and *Lsd-2* (I), CG4586 and CG12262 (J), and CG6287 and CG6277 (K) on LSD versus HSD (*mlx* RNA-seq).

(L) Tissue-specific expression of CG6283 (qPCR).

Error bars show SD. *p < 0.05, **p < 0.01, ***p < 0.001. For tissue-specific data, the error bars show the SD of three technical replicates from pools of three replicate cDNA samples. See also Figure S4.

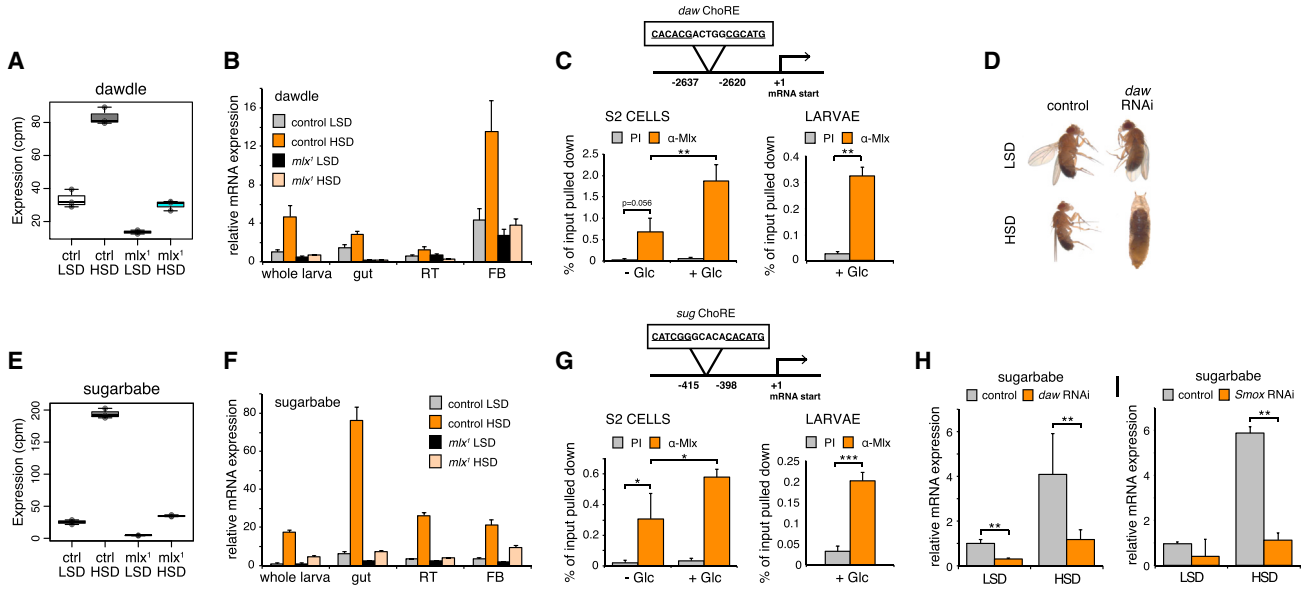

Figure 4. Mondo-Mlx Is a Direct Activator of *dawdle* and *sugarbabe*

(A) Expression of *daw* on LSD versus HSD (*mlx* RNA-seq).

(B) Tissue-specific expression of *daw* (qPCR).

(C) *daw* ChIP in S2 cells and larvae.

(D) *daw* RNAi (tub-GAL4 >) causes pupal lethality on HSD.

(E) Expression of *sug* on LSD versus HSD (*mlx* RNA-seq).

(F) Tissue-specific expression of *sug* (qPCR).

(G) *sug* ChIP in S2 cells and larvae.

(H and I) *sug* induction on HSD (qPCR) upon *daw* (H) *and Smox* (I) knockdown (tub-GAL4 >).

Error bars show SD. *p < 0.05, **p < 0.01, ***p < 0.001. For tissue-specific data, the error bars show the SD of three technical replicates from pools of three replicate cDNA samples. See also Figure S5.

and this phenotype was confirmed by two independent RNAi lines (Figure S5F). Furthermore, survival of first instar larvae on 20% sucrose-only diet was significantly impaired upon *sug* knockdown (Figure S5G). Thus, Sugarbabe is essential for sugar tolerance, but it displays a more moderate phenotype than *mlx* mutant animals.

To characterize the contribution of Sugarbabe in genome-wide sugar-dependent transcription, we performed RNA-seq of the *sug* mutants using similar experimental conditions as with mlx^1 mutants (Figures 1A and 5C; Table S5). The sugar-regulated gene sets dependent on Sugarbabe displayed highly significant overlap with the Mlx-dependent genes (Figure 5D; Table S6). Consistent with the idea that Sugarbabe is a downstream effector of Mondo-Mlx, the total number of Sugarbabe-dependent targets was lower than that dependent on Mlx (Table S7).

Analysis of the specific Sugarbabe targets uncovered that a subset of the Mondo-Mlx-dependent processes detailed above are, in fact, placed downstream of Sugarbabe. The Sugarbabe-dependent targets included genes mediating serine biosynthesis (Figure 5E and S5H; Table S7). In line with the finding that Sugarbabe activation depends on Activin signaling, depletion of Dawdle also hampered the sugar-induced activation of *aay* (Figure 5F). This is consistent with the model that the Activin-Sugarbabe axis acts downstream of Mondo-Mlx to control serine biosynthesis during high-sugar feeding. Our RNA-seq analysis also revealed that the sugar-dependent repression of gut-specific amylase-encoding genes was strongly prevented in the absence of Sugarbabe (Figure 5G; Table S7). Moreover, overexpression of *sug* caused downregulation of the *Amy-p* transcript (Figure 5H). Larvae overexpressing *sug* survived poorly on a diet with high starch content (10% yeast + 10% potato starch) compared to the control animals (Figure 5I), providing evidence for the functional importance of Sugarbabe-mediated regulation of gut amylases. Together, these results indicate that Mondo-Mlx suppresses starch breakdown on HSD through its downstream effector Sugarbabe.

Sugarbabe Is a Feed-Forward Regulator of De Novo Lipogenesis and Fatty Acid Desaturation

In a genome-wide comparison, fatty acid biosynthesis was among the most strongly enriched Sugarbabe-dependent processes (Figure 6A; Table S7). Sugarbabe-dependent targets included key drivers of lipogenesis, such as *ACC* and *FAS* as well as *AcCoAS* and *ATPCL* (Figure 6B; Table S7). Moreover, transient overexpression of *sug* under a heat shock inducible GAL4 driver, led to elevated *ACC* and *FAS* levels providing further confirmation on the role of *sug* in lipogenic gene expression (Figure 6C). *ACC* and *FAS* expression were also reduced upon *daw* knockdown (Figure S6A), in agreement with a model placing *sug* downstream of *daw*. Kinetic analysis of *sug* and *FAS* activation upon sugar feeding revealed that *sug* activation

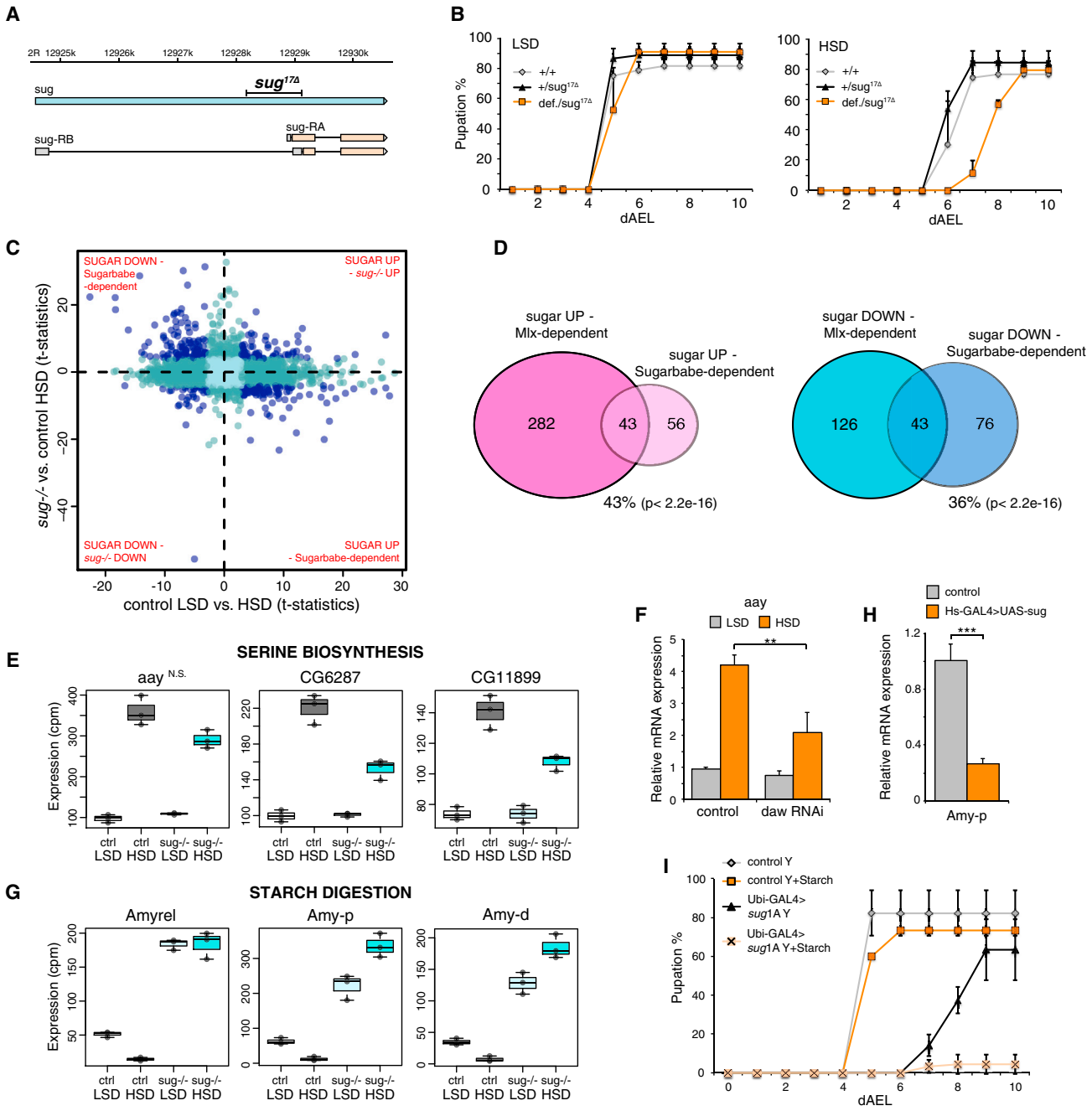

Figure 5. Sugarbabe Regulates a Subset of Mondo-Mlx-Dependent Target Genes

(A) The *sug* locus showing the $sug^{17\Delta}$ deletion.

(B) *sug*-deficient animals (Df(2R)Exel7123/$sug^{17\Delta}$) display delayed pupation on HSD, but not on LSD.

(C) Four-way plot (t-statistics) presenting the Sugarbabe-dependent sugar-regulated genes, $p < 0.05$, logFC > 0/logFC < 0. Dark blue indicates significant in both comparisons; dark turquoise, significant in one comparison; and light turquoise, not significant.

(D) Comparison of Mlx- and Sugarbabe-dependent, sugar up- and downregulated gene sets.

(E) Expression of *aay*, CG6287 and CG11899 on LSD versus HSD (*sug* RNA-seq).

(F) *aay* expression (qPCR) upon *daw* RNAi (tub-GAL4 >).

(G) Expression of *Amyrel*, *Amy-p* and *Amy-d* on LSD versus HSD (*sug* RNA-seq).

(H) *Amy-p* expression (qPCR) upon *sug* overexpression (Hs-GAL4 >).

(I) Pupation kinetics of control and *sug* overexpressing (Ubi-GAL4 >) animals on 10% yeast diet with or without 10% potato starch.

Error bars show SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. N.S., not significant in control LSD versus HSD versus control HSD versus mlx^{1} HSD comparison. See also Figure S5.

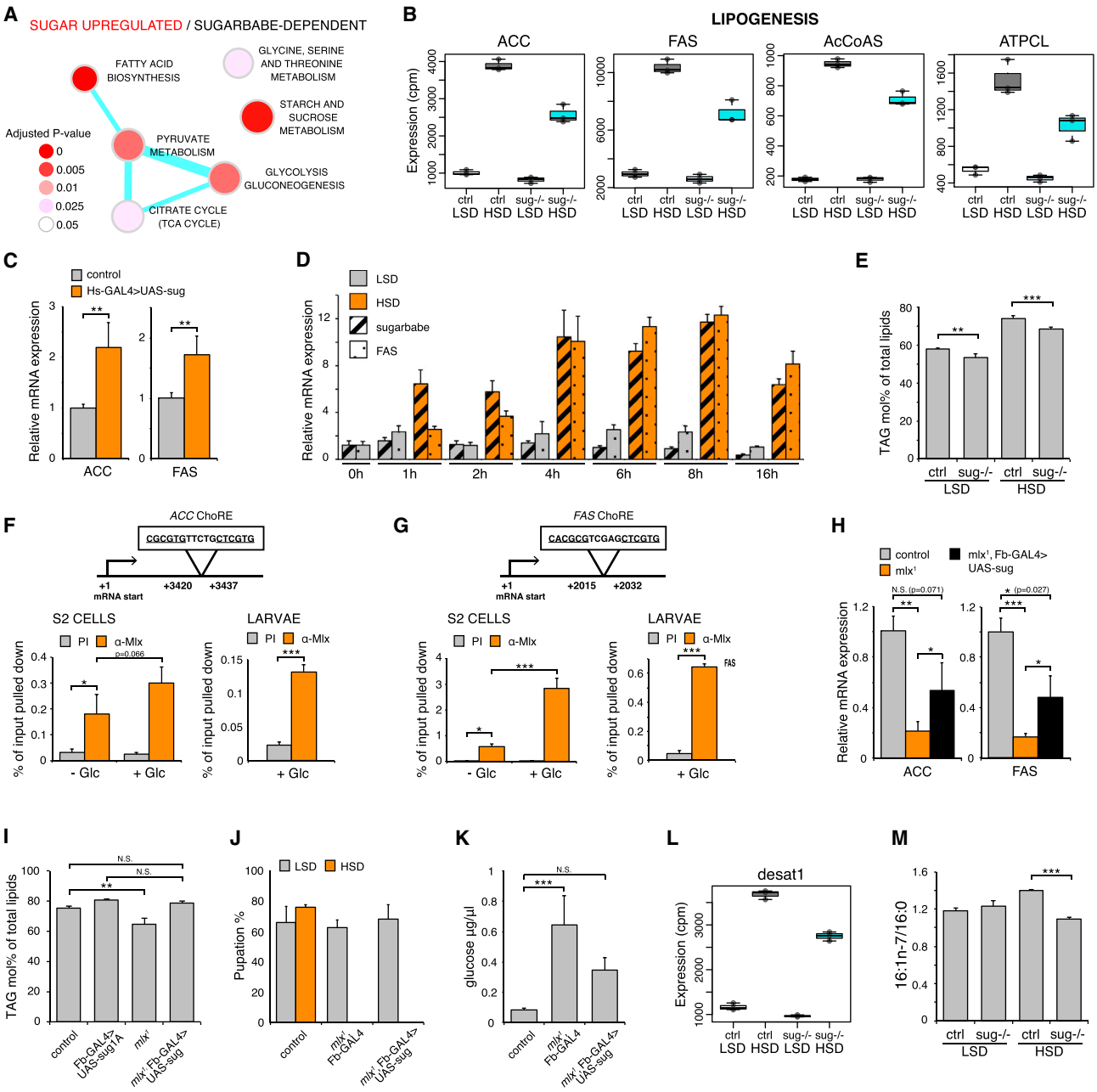

Figure 6. Sugarbabe Is a Feed-Forward Regulator of De Novo Lipogenesis and Fatty Acid Desaturation

(A) Sugar upregulated/Sugarbabe-dependent processes (FDR < 0.05).

(B) Expression of *ACC*, *FAS*, *AcCoAS* and *ATPCL* on LSD versus HSD (*sug* RNA-seq).

(C) *ACC* and *FAS* expression (qPCR) upon overexpression of *sug* (Hs-GAL4 >).

(D) Time course of *sug* and *FAS* expression (qPCR) after acute HSD exposure for indicated times.

(E) TAG levels determined by mass spectrometry from control and *sug* mutant (Df(2R)Exel7123/$sug^{17\Delta}$) third-instar larvae fed on LSD or HSD (ESI-MS/MS).

(F and G) *ACC* (F) and *FAS* (G) ChIP in S2 cells and larvae.

(H–K) *ACC* and *FAS* expression (H), TAG levels (I), total pupation (J), and free glucose levels (K) in control, mlx^1 and mlx^1; Fb-GAL4 > UAS-*sug* animals.

(L) Expression of *desat1* on LSD versus HSD (*sug* RNA-seq).

(M) The ratio of palmitoleic acid (16:1n-7, the product of Δ9-desaturase) and palmitic acid (16:0, the substrate of the desaturase) in control and *sug* mutant third-instar larvae fed on LSD or HSD (gas chromatography).

Error bars show SD. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S6.

Table 1. Top 10 Human Homolog Genes With Association to Triglyceride Levels

Control versus *mlx*^{1} HSD		Control LSD versus HSD		Homolog Mapping		Association with Triglyceride Levels in Humans			
Symbol	logFC	Symbol	logFC	Flybase or BLAST	Human Homolog	Genome Window Examined, Gene Start-End ±100 kb	Effect Allele Frequency	Effect (SE)	p Value (N)
CG32751	−3.521	CG32751	3.760	B	BTD	chr3: 15,743,254–15,787,325	0.081	0.044 (0.011)	7.98E-05 (57,623)
Mdr50	−2.086	Mdr50	1.731	F	TAP1	chr6: 32,912,985–32,921,748	0.035	0.114 (0.018)	2.63E-10 (50,994)
Cyp28d2	−2.050	Cyp28d2	1.443	B	CYP3A7	chr7: 99,402,659–99,432,819	0.060	−0.049 (0.012)	7.68E-05 (60,000)
CG12490	−1.959	CG12490	3.654	B	SLC17A7	chr19: 50,032,654–50,044,808	0.178	0.042 (0.009)	2.61E-06 (54,803)
CG11911	−1.938	CG11911	1.779	F	LPA	chr6: 161,052,514–161,187,407	0.131	−0.051 (0.009)	8.63E-09 (59,949)
Eaat1	−1.021	Eaat1	1.200	F	SLC1A4	chr2: 65,316,494–65,351,000	0.291	−0.034 (0.006)	1.58E-07 (59,949)
CG16771	−0.803	CG16771	0.808	F	LPL	chr8: 19,896,581–19,924,770	0.107	−0.186 (0.010)	9.26E-84 (59,869)
Mio	−0.595	Mio	0.694	F	MLXIPL	chr7: 73,107,523–73,138,870	0.397	0.038 (0.006)	7.63E-10 (59,970)
Best1	−0.568	Best1	0.236	F	BEST1	chr11: 61,817,355–61,831,066	0.381	0.027 (0.006)	2.27E-05 (59924)
grn	−0.403	grn	0.719	F	GATA4	chr8: 11661716–11,717,509	0.392	0.028 (0.006)	3.02E-06 (59,368)

Summary statistics for the most strongly associated variant per genetic locus (gene start-end +100 kb) have been presented here together with the summary statistics of the original *Drosophila* gene. N, number of samples. See also Figure S6.

preceded maximal *FAS* expression, supporting the idea that *sug* activation is needed for maximal *FAS* expression (Figure 6D). Consistent with the reduced lipogenic gene activation the *sug* mutants displayed reduced triacylglycerol levels (Figure 6E).

As *ACC* and *FAS* are well-established direct targets of ChREBP-Mlx in mammals (Jeong et al., 2011; Ma et al., 2005), we tested whether the same is true in *Drosophila*. Indeed, we found putative ChoREs in the proximity of their transcriptional starting sites and used ChIP in S2 cells and larval lysates to confirm that they are bound by Mlx (Figures 6F and 6G). To test, whether *sug* activity on *ACC* and *FAS* depends on functional Mondo-Mlx, we expressed *sug* under the control of a fat body specific GAL4 driver (Fb-GAL4) in the *mlx*^{1} mutant background. Ectopic expression of *sug* was sufficient to activate the expression of *ACC* and *FAS* independent of Mlx (Figure 6H). Similarly, *sug* expression in the fat body of *mlx*^{1} mutants was sufficient to rescue the TAG levels in these animals (Figure 6I), restoring white fat deposits in the rescued larvae (Figure S6B). Restoring the expression of *sug* in the fat body did not rescue the growth or survival phenotypes of *mlx*^{1} mutants on 20% yeast + 10% sucrose (Figure 6J), but the levels of circulating glucose were partially rescued (Figure 6K). These results place Sugarbabe downstream of Mondo-Mlx as a feed-forward regulator of lipogenesis and critical contributor to glucose and TAG homeostasis. In addition to lipogenic gene expression, Sugarbabe regulated the expression of the stearoyl-CoA desaturase *desat1*, which catalyzes fatty acid desaturation (Figure 6L). Consistent with the compromised expression of *desat1*, the *sug* mutant larvae displayed lower level of palmitate desaturation on high sugar diet (Figure 6M). In conclusion, Sugarbabe is an activator of lipid biosynthesis and desaturation in response to sugar feeding.

Human Homologs of Mondo-Mlx Targets Are Enriched among Triglyceride-Associated Genes

Variants of human *ChREBP* (*MLXIPL*) are known to be associated with circulating triglyceride levels, but the underlying target genes are not known. Therefore, we wanted to test, whether human homologs of the Mondo-Mlx targets display association with circulating triglyceride levels in a genome-wide association study by ENGAGE Corsortium (Surakka et al., 2015). We identified the closest human homologs of the *Drosophila* genes upregulated by sugar in Mlx-dependent manner and looked for possible triglyceride-associated variants inside a ±100 kB window around the genes. Strikingly, these genes displayed a clear enrichment among the variants associated with circulating triglycerides ($p = 0.0077$) (Figure S6C; Table S8). Human homologs of the ten most significantly associated *Drosophila* Mondo-Mlx target genes are listed in Table 1. The identified genes include *Lipoprotein lipase* (*LPL*), which is among the best-established triglyceride-associated genes (Johansen et al., 2011). Moreover, lipoprotein(a) (LPA) is strongly associated with increased risk of coronary artery disease (Clarke et al., 2009), which is causally related to high circulating triglycerides (Do et al., 2013). In conclusion, our data suggest that the identified Mondo-Mlx targets can be used to predict putative causal genes in the vicinity of the triglyceride-associated genomic variants in human.

DISCUSSION

Previous studies on mouse and *Drosophila* mutants have established the conservation and physiological importance of ChREBP/Mondo-Mlx (Havula et al., 2013; Iizuka et al., 2004). It is also evident that the regulation of lipogenic genes in the liver and adipose tissue (mouse) or fat body (*Drosophila*) contribute to the physiological function of ChREBP/Mondo-Mlx (Benhamed et al., 2012; Dentin et al., 2006; Havula et al., 2013; Herman et al., 2012; Musselman et al., 2013). Our study now demonstrates a substantially broader in vivo role for the Mondo-Mlx-dependent intracellular sugar-sensing pathway than previously appreciated. We uncover that (1) Mondo-Mlx controls tissue-specific gene expression in gut and renal tubules contributing to nutrient digestion and transport; (2) Mondo-Mlx controls the sugar-induced biosynthesis of glutamine and serine, which are essential for growth and survival of sugar-feeding animals, (3) activated Mondo-Mlx mediates pervasive upregulation of the pentose

phosphate pathway, which is essential for sugar tolerance and sugar-induced lipogenesis, (4) human homologs of *Drosophila* Mondo-Mlx targets display significant enrichment among genomic variants associated with circulating triglyceride levels, providing potential new insight into the functional connection between the genes maintaining triglyceride homeostasis, (5) Mondo-Mlx is a master regulator of a sugar-responsive regulatory network, which functions through hormonal (Activin/Dawdle) and transcriptional (Sugarbabe) axes, Sugarbabe being a downstream effector of Activin signaling, (6) Sugarbabe regulates a subset of Mondo-Mlx-dependent genes, including ones involved in starch digestion, as well as serine and fatty acid biosynthesis, (7) Sugarbabe is essential for triglyceride homeostasis and fatty acid desaturation in sugar feeding animals.

By identifying *daw* and *sug* as targets of Mondo-Mlx our study provides a mechanistic explanation for the activation of these regulatory genes in sugar feeding animals. Although *sug* is a direct target of Mondo-Mlx, its expression depends on *daw* as well. It is conceivable that such a network motif topology has evolved to increase the dynamic range of *sug* regulation contributing to tolerance of a wide range of dietary sugar concentrations. A recent study by Ghosh and O'Connor (2014) revealed metabolic changes in *daw* mutants, including elevated glucose, trehalose, and glycogen levels, that resemble phenotypes observed upon loss of Mlx. On the other hand, *daw* mutants displayed elevated TAG levels, the opposite of what was observed in *mlx* mutants earlier or in *sug* mutants in this study. Future studies are therefore warranted on comparing lipid metabolism of *mlx, sug*, and *daw* mutants.

To adjust organismal nutrient balance, animals control both nutrient intake and excretion. We uncovered that Mondo-Mlx regulates both the expression of digestive enzymes in the gut and putative glucose transporters in renal tubules, potentially to balance nutrient levels in circulation and to prevent metabolic overload. Interfering with the expression of renal tubule expressed GLUT CG7882 led to elevated glucose levels, supporting this hypothesis. Our data confirmed the earlier findings on sugar-dependent repression of digestive α-amylase and lipase genes (Benkel and Hickey, 1987; Zinke et al., 2002) and uncovered that this regulation depends on Mondo-Mlx. A recent study by Chng et al. (2014) demonstrated the role of Dawdle-mediated Activin signaling in sugar-induced repression of amylases, maltases, and lipases in the adult gut. Our data uncovered the underlying molecular mechanism of *daw* regulation by dietary sugars and demonstrated the role of the downstream effector *sug* in the repression of gut amylases. By controlling the digestion of nutrients, Mondo-Mlx also likely modulates the intestinal microenvironment of the gut microbiome. The numbers of *Lactobacillus plantarum* in the *Drosophila* gut have been correlated with the availability of dietary starch and shown to influence growth and mating behavior (Sharon et al., 2010; Storelli et al., 2011). It will thus be interesting to study how the Mondo-Mlx-Sugarbabe network influences the gut microbiome.

Our results revealed a role for Mondo-Mlx in sugar-dependent regulation of glutamine/glutamate and serine metabolism. In growing cells, glutamine is converted to the TCA cycle intermediate α-ketoglutarate through the anaplerotic glutamate dehydrogenase pathway. Glutamine is also an essential nitrogen donor in the synthesis of other non-essential amino acids and nucleotides (DeBerardinis and Cheng, 2010). We noticed that activation of glutamine biosynthesis is essential for growth in sugar-feeding animals, suggesting that sugar and glutamine metabolisms are coupled to ensure proper growth rate. Such a coupling is also observed in rapidly growing mammalian cells, where glucose withdrawal leads to reduced glutamine consumption (Wellen et al., 2010). Notably, glutamine is also consumed during de novo serine biosynthesis (Kalhan and Hanson, 2012), which is also activated upon sugar feeding.

By placing the serine biosynthetic pathway downstream of the Mondo-Mlx/Dawdle-Sugarbabe network, we provide a mechanistic explanation for the sugar-dependent control of serine biosynthesis from 3-phosphoglycerate. Serine metabolism has an essential role in growth of cancer cells (Labuschagne et al., 2014) through so-called one-carbon metabolism, which is essential for biosynthetic processes (Locasale, 2013). In addition, serine acts as an allosteric activator of Pyruvate kinase M2 (PKM2), increasing the glycolytic flux (Chaneton et al., 2012). While we do not yet know the underlying role of serine biosynthetic gene activation, the early lethality of *astray* RNAi animals on HSD highlights the physiological importance of de novo serine biosynthesis upon sugar feeding.

Our study confirmed the previous findings on ChREBP/Mondo-Mlx-mediated regulation of sugar-induced lipogenesis (Havula et al., 2013; Ma et al., 2006; Sassu et al., 2012). Moreover, we demonstrated that while *ACC* and *FAS* are direct Mondo-Mlx targets, activation of Sugarbabe constitutes a feed-forward loop needed for full lipogenic activity. This might produce a sign-sensitive delay (Yosef and Regev, 2011) to ensure maximal activation of lipogenic gene expression only in response to sustained sugar feeding. Mondo-Mlx also increases the expression of perilipins, which surround lipid droplets, and reduces the expression of genes involved in β-oxidation. The PPP, a major source of the NADPH needed for fatty acid synthesis, was induced upon sugar feeding in an Mlx-dependent manner. Unlike lipogenic gene expression, PPP genes are regulated independently of Sugarbabe. Together, our findings show that Mondo-Mlx controls simultaneous activation and repression of multiple metabolic programs that need to be synergistically regulated to shift the lipid homeostasis toward increased TAG storage upon sugar feeding.

Excessive sugar (especially fructose) intake is linked with liver pathologies (e.g., nonalcoholic fatty liver disease [NAFLD]) in humans (Neuschwander-Tetri, 2013). While genetic variants are known to interact with diet in this setting (Sevastianova et al., 2012), our understanding of the underlying mechanisms remains limited. Notably, many of the genes interconnected in our study have been implicated in liver pathologies. First, ChREBP expression is elevated in human patients with severe nonalcoholic steatohepatitis, and high ChREBP activity in the liver leads to hepatic steatosis in mice (Benhamed et al., 2012). In humans, circulating Activin A levels are associated with NAFLD (Yndestad et al., 2009) and mouse models with increased Activin bioavailability in the liver display elevated hepatic TAG content and increased steatosis (Ungerleider et al., 2013). Finally, serine deficiency and altered expression of the Phosphoserine phosphatase are associated with non-alcoholic steatohepatitis

(Mardinoglu et al., 2014). Thus, it will be important to study the contribution and regulatory connections between ChREBP, Activin signaling and serine metabolism in sugar-induced liver pathologies.

In conclusion, by characterizing the regulatory role of Mondo-Mlx upon sugar feeding in vivo, we obtained a first glimpse of the synergistic tissue-specific regulation of nutrient intake, transport, and metabolism and the physiological relevance of the genes involved. Moreover, our study places several sugar-responsive metabolic regulators into the same network, providing a starting point for systems-level understanding of the physiology of sugar sensing.

EXPERIMENTAL PROCEDURES

Fly Strains and Dietary Conditions

The *mlx*^{1} mutant has been described previously (Havula et al., 2013). The following RNAi lines were used: VDRC: Mondo 109174 kk, CG7882 109827 kk, Zw 108898 kk, Gs2 9378 GD, aay 100557 kk, daw 110248 kk, Smox 111163 kk; NIG: sugarbabe 3850R-1 (sug RNAi^{1}) and 3850R-3 (sug RNAi^{2}). The UAS-*sug* line has been described previously (Zinke et al., 2002). *GAL4* driver lines (tub-GAL4, Ubi-GAL4, Hs-GAL4, Cg-GAL4, Fb-GAL4, and NP1-GAL4) and Df(2R)Exel7123 were obtained from the Bloomington Stock Center.

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

RNA-Seq

Total RNA from control and *mlx*^{1} animals kept on LSD or HSD was extracted with the Nucleospin RNA II kit (Macherey-Nagel). Mlx transcriptome sequencing (RNA-seq) was performed with Illumina HiseqTM2000 technology to an average depth of 20 M clean reads per sample. Reads were mapped with BWA v.0.5.9 to the standard *D. melanogaster* reference genome (FlyBase R5.53). Transcription was quantified on the level of annotated exons (FlyBase), and differential expression among the different samples and conditions identified with limma package v.3.18.13 implemented in R/Bioconductor with Benjamini-Hochberg correction (Benjamini and Hochberg, 1995). The Sugarbabe RNA-seq experiment was conducted as above, with the exception that the transcriptome sequencing was performed with Illumina NextSeq500 technology. See Supplemental Experimental Procedures for detailed protocols and bioinformatic analysis.

ChIP

For immunoprecipitation, anti-Mlx-specific antibodies were used (Havula et al., 2013), and coimmunoprecipitated DNA was quantified by qPCR. See additional details in Supplemental Experimental Procedures.

Metabolic Assays

Lipid analysis by mass spectrometry is described in Supplemental Experimental Procedures. Hemolymph glucose was measured from third-instar prewandering larvae using the GAGO-20 kit (Sigma) as described previously (Zhang et al., 2011). The enzymatic TAG assay was conducted as described elsewhere (Palanker et al., 2009).

Statistical Analysis

Statistical analyses were performed by two-way ANOVA in conjunction with Tukey's HSD test or by two-tailed t-test.

ACCESSION NUMBERS

The accession number for the complete expression datasets reported in this paper is GEO: GSE70980.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures, six figures, and eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.081.

AUTHOR CONTRIBUTIONS

J.M., E.H., and V.H. designed experiments, analyzed data, and wrote the manuscript. J.M., E.H., M.T., and R.H. performed the experiments. T.S., E.H., E.S., I.S., H.K., and J.V. performed bioinformatic data analysis. R.K., S.R., and I.H. provided advice and analytical tools.

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