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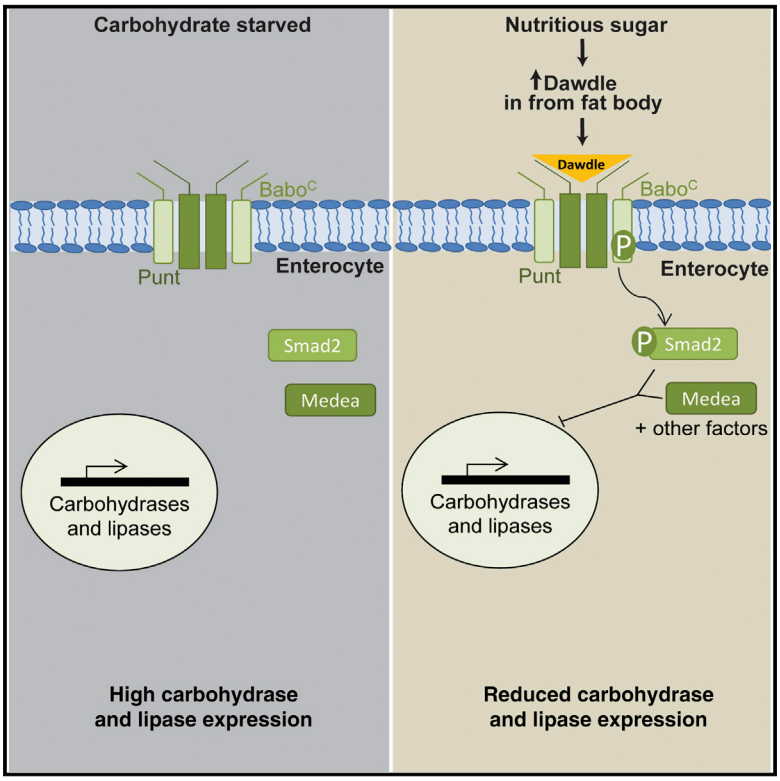
Transforming Growth Factor β/Activin Signaling Functions as a Sugar-Sensing Feedback Loop to Regulate Digestive Enzyme Expression

Graphical Abstract



Highlights

Glucose represses midgut-expressed carbohydrases and lipases

Nutritional value of the sugars and the nutritional state of host affects repression

TGF-β ligand, Dawdle (Daw), derived from the fat body mediates glucose repression

Activation of the TGF-β/Activin signaling in the midgut underlies glucose repression

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In Brief

Organisms modulate their digestive processes to reflect their nutritional state. In this study, Chng et al. demonstrate that the TGF-β/Activin pathway functions as a carbohydrate-sensing mechanism in the adult *Drosophila* midgut to regulate digestive enzyme expression. They show that the TGF-β ligand, Dawdle, and the canonical TGF-β/Activin signaling are essential to couple carbohydrate sensing with digestive enzyme expression. Thus, their study highlights an unexpected function of TGF-β/Activin signaling that is beyond their established roles in development and immunity.

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Transforming Growth Factor β/Activin Signaling Functions as a Sugar-Sensing Feedback Loop to Regulate Digestive Enzyme Expression

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SUMMARY

Organisms need to assess their nutritional state and adapt their digestive capacity to the demands for various nutrients. Modulation of digestive enzyme production represents a rational step to regulate nutriment uptake. However, the role of digestion in nutrient homeostasis has been largely neglected. In this study, we analyzed the mechanism underlying glucose repression of digestive enzymes in the adult *Drosophila* midgut. We demonstrate that glucose represses the expression of many carbohydrases and lipases. Our data reveal that the consumption of nutritious sugars stimulates the secretion of the transforming growth factor β (TGF-β) ligand, Dawdle, from the fat body. Dawdle then acts via circulation to activate TGF-β/Activin signaling in the midgut, culminating in the repression of digestive enzymes that are highly expressed during starvation. Thus, our study not only identifies a mechanism that couples sugar sensing with digestive enzyme expression but points to an important role of TGF-β/Activin signaling in sugar metabolism.

INTRODUCTION

Digestion and absorption are two principal functions of the digestive tract. To efficiently extract nutrients from the ingested food, a repertoire of digestive enzymes is expressed to breakdown the macronutrients in the diet into a form that can be readily absorbed. Given that dietary intake in the environment is inherently variable, organisms need to readily assess their nutritional state and adapt their digestive capacity to the demands for various nutrients. To date, most metabolic studies are focused on the postabsorption events for nutrient homeostasis (Grönke et al., 2007; Havula et al., 2013; Quesada et al., 2008; Saltiel and Kahn, 2001), and our understanding of digestion and its regulation by nutrient-sensing pathways remains limited.

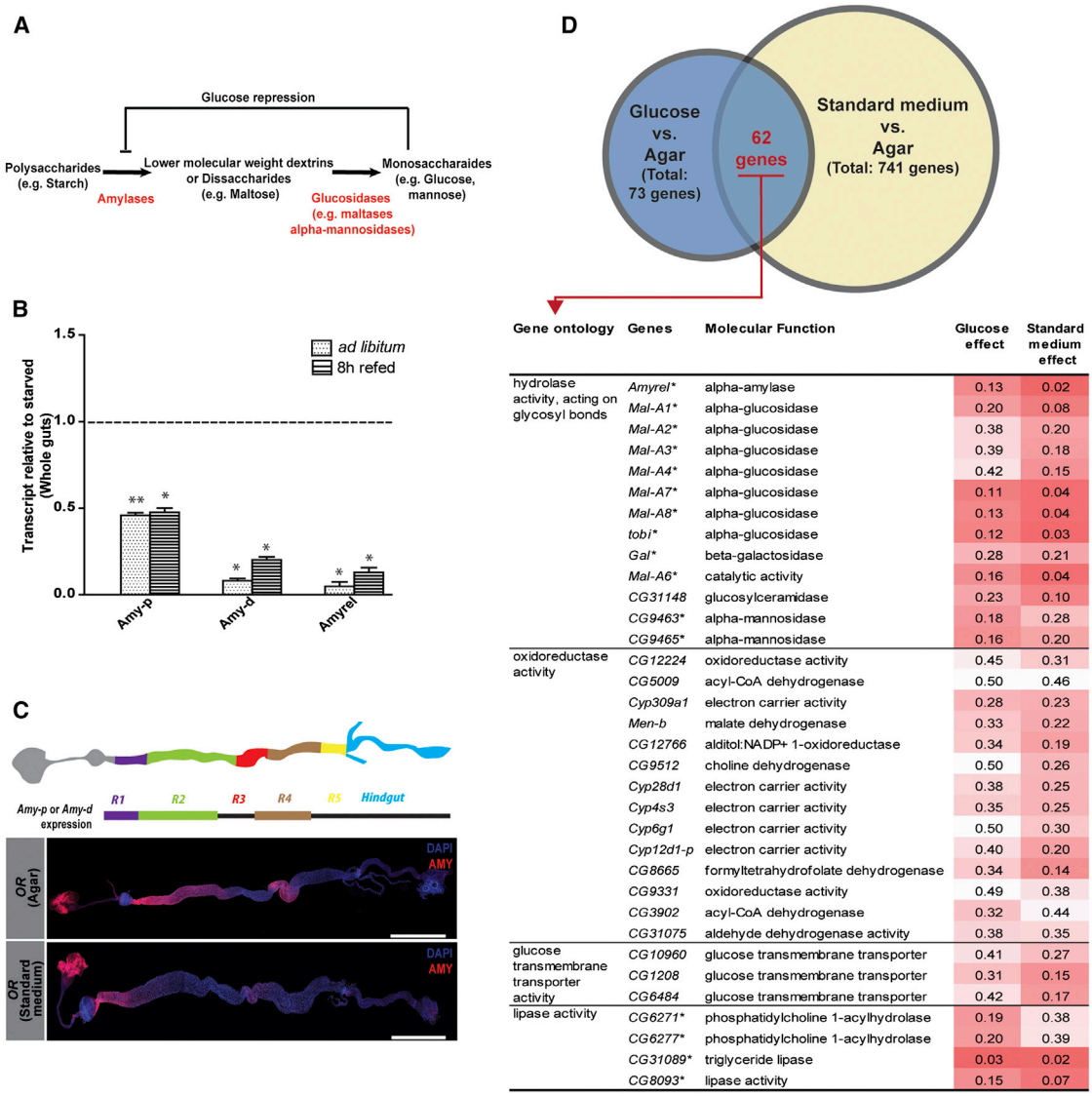
In *Drosophila*, the gut is traditionally divided into three distinct regions: the foregut, the midgut, and the hindgut (Hakim et al., 2010), with digestion and absorption of food being accomplished predominantly in the midgut (Lehane et al., 1995; Terra and Ferreira, 1994). For many dietary components, hydrolysis by endogenous or microbial-derived digestive enzymes precedes absorption through the gut lumen. On the principle of economy, release of digestive enzymes often reflects the quantity of nutrients, with more enzymes generally released in fed than in unfed insects (Karasov et al., 2011). However, several studies in *Drosophila* have reported that the activity of amylase, an enzyme required for the hydrolysis of glucosidic linkages in polysaccharides, is repressed in flies fed with a high-sugar diet. This phenomenon whereby glucose reduces amylase expression was termed glucose repression (Benkel and Hickey, 1986; Hickey and Benkel, 1982; Figure 1A). Whereas the effect of dietary sugar on amylase activity has long been recognized, the mechanistic underpinnings of such regulation and its effects on other digestive enzymes remain to be established.

In this study, we have analyzed the mechanism underlying glucose repression in the adult *Drosophila* midgut and show that glucose repression affects many other carbohydrases and lipases. We also show that Dawdle (*Daw*), a transforming growth factor β (TGF-β) ligand, is induced in the fat body by nutritious carbohydrates in the diet and is indispensable for glucose repression. Thus, our study not only identifies a mechanism regulating digestive enzyme expression but points to an important role of the TGF-β/Activin signaling in sugar sensing and metabolism, which is independent of the insulin-signaling pathway.

RESULTS

Amylase Is Repressed in Flies Fed Ad Libitum

Previous studies have shown that intestinal amylase activity is repressed when flies were fed on a glucose-rich diet, an effect described as glucose repression (Benkel and Hickey, 1986; Hickey and Benkel, 1982; Figure 1A). To confirm and further characterize this response, we compared the expression level of all three *Drosophila* amylase genes (*Amy-p, Amy-d*, and *Amyrel*) in the gut of adult flies fed ad libitum on a standard laboratory medium, starved on agar, or refed for 8 hr after starvation on agar. The transcript of all three genes was significantly reduced when flies were fed ad libitum or when flies were refed, as illustrated by the ratio (<1) of amylase expression relative to starved flies (Figure 1B). Consistent with this, we also found



Gene ontology	Genes	Molecular Function	Glucose effect	Standard medium effect
hydrolase activity, acting on glycosyl bonds	*Amyrel**	alpha-amylase	0.13	0.02
	*Mal-A1**	alpha-glucosidase	0.20	0.08
	*Mal-A2**	alpha-glucosidase	0.38	0.20
	*Mal-A3**	alpha-glucosidase	0.39	0.18
	*Mal-A4**	alpha-glucosidase	0.42	0.15
	*Mal-A7**	alpha-glucosidase	0.11	0.04
	*Mal-A8**	alpha-glucosidase	0.13	0.04
	*tobi**	alpha-glucosidase	0.12	0.03
	*Gal**	beta-galactosidase	0.28	0.21
	*Mal-A6**	catalytic activity	0.16	0.04
	CG31148	glucosylceramidase	0.23	0.10
	*CG9463**	alpha-mannosidase	0.18	0.28
	*CG9465**	alpha-mannosidase	0.16	0.20
oxidoreductase activity	*CG12224*	oxidoreductase activity	0.45	0.31
	CG5009	acyl-CoA dehydrogenase	0.50	0.46
	Cyp309a1	electron carrier activity	0.28	0.23
	Men-b	malate dehydrogenase	0.33	0.22
	CG12766	alditol:NADP+ 1-oxidoreductase	0.34	0.19
	CG9512	choline dehydrogenase	0.50	0.26
	Cyp28d1	electron carrier activity	0.38	0.25
	Cyp4s3	electron carrier activity	0.35	0.25
	Cyp6g1	electron carrier activity	0.50	0.30
	Cyp12d1-p	electron carrier activity	0.40	0.20
	CG8665	formyltetrahydrofolate dehydrogenase	0.34	0.14
	CG9331	oxidoreductase activity	0.49	0.38
	CG3902	acyl-CoA dehydrogenase	0.32	0.44
	CG31075	aldehyde dehydrogenase activity	0.38	0.35
glucose transmembrane transporter activity	*CG10960*	glucose transmembrane transporter	0.41	0.27
	CG1208	glucose transmembrane transporter	0.31	0.15
	CG6484	glucose transmembrane transporter	0.42	0.17
lipase activity	*CG6271**	phosphatidylcholine 1-acylhydrolase	0.19	0.38
	*CG6277**	phosphatidylcholine 1-acylhydrolase	0.20	0.39
	*CG31089**	triglyceride lipase	0.03	0.02
	*CG8093**	lipase activity	0.15	0.07

Figure 1. Glucose Repression Affects Carbohydrate and Lipid-Acting Digestive Enzymes

(A) A schematic representation of carbohydrate digestion from polysaccharides to monosaccharaides. Amylases expression is repressed by glucose in the diet (known as glucose repression).

(B) qRT-PCR on whole gut of ad-libitum-fed, starved, or 8 hr refed flies. Transcript levels of all three amylases were reduced (ratio <1) in flies fed ad libitum or after refeeding on a standard medium. Data are expressed relative to starved as mean ± SEM. For western blot analysis of amylase, see also Figures S1A and S1B.

(C) Graphic representation of amylase expression along the anterior/posterior axis of the midgut based on previous microarray data (Buchon et al., 2013; top panel). Amylase expression, as revealed by immunostaining (bottom panel), was mainly observed in region R1, R2, and R4 (red). Amylase expression in the R2 and R4 regions were low when flies are fed. Note that the amylase signal (red) from the crop is due to autofluorescence. The scale bar represents 1,000 μm.

(D) Venn diagram for the RNA-seq results. Sixty-two genes were differentially regulated (at least 2-fold) in both comparison (top panel). Gene Ontology (GO) analysis was done with GOrilla (bottom panel). Thirty-four genes were associated with GO term, including hydrolase activity on glycosyl bonds, oxidoreductases activity, glucose transmembrane transporter activity, and lipase activity. All the 34 genes were repressed. Red shades represent the extent of repression, whereas numeric values represent transcript level relative to starved. The asterisk denotes genes with putative digestive enzyme function (Lemaitre and Miguel-Aliaga, 2013). See also Figures S2 and S3, supplemental text S1, and Table S3.

less amylase protein in ad-libitum-fed flies in western blot analysis using an anti-amylase antibody that we generated (Figures S1A and S1B).

Our laboratory and others (Buchon et al., 2013; Marianes and Spradling, 2013) had previously demonstrated that the adult midgut is compartmentalized into discrete regions, with amylase expression enriched in the anterior (R1 and R2) and posterior (R4) regions of the midgut (Figure 1C, upper panel; Thompson et al., 1992). As such, we immunostained the adult gut with our amylase antibody to visualize amylase expression in the gut of ad-libitum-fed flies and starved flies. Interestingly, amylase repression in the fed state was associated with very low

expression of amylase in the R2 and R4 regions (Figure 1C, lower panel). All these indicate that amylase expression is reduced in the fed state and that the high level of amylase expression during starvation is reversible upon refeeding.

Glucose Repression Extends beyond Amylases

To date, only amylase has been shown to be subjected to glucose repression. In order to extend our results beyond amylases, we performed a RNA sequencing (RNA-seq) study on adult gut samples to identify genes that are differentially regulated by glucose in the diet. To ensure that the gene set derived from our comparison between agar versus glucose-only diet remains relevant for a nutritionally complete diet regime, we identified differentially regulated genes that were common between agar versus glucose and agar versus standard medium conditions. Sixty-two genes were differentially expressed in both comparisons (Figure 1D). A closer examination of these genes revealed that many possess hydrolase activity acting on glycosyl bonds, including several alpha-mannosidases and maltases, enzymes that are involved in the later steps of carbohydrate hydrolysis to produce glucose and/or mannose (Figures 1A and 1D). In addition, our analysis also revealed that several genes with lipase activity, glucose transmembrane transporter activity, as well as genes with oxidoreductase activity were repressed (Figure 1D; see also Figures S2 and S3, Supplemental Text S1, and Table S3). As many of the genes were more repressed by the standard medium, it is possible that other macro- and micronutrients may affect the repression of these genes, either through direct modulation of signaling pathways governing their expression or indirect mechanisms affecting food intake and intestinal transit.

Independent quantitative RT-PCR (qRT-PCR) analysis of carbohydrase and lipase genes identified by our RNA-seq, as well as other members within those gene cluster, showed that seven out of eight maltase genes in the Mal-A cluster (all except *Mal-A5*), all four alpha-mannosidases of the cluster (*CG9463*, *CG9465*, *CG9466*, and *CG9468*), and lipases were downregulated in wild-type (*MyoIA-gal4^{ts} > w^{1118}*) glucose-fed flies relative to starved flies (Figure 5A). Because our qRT-PCR results revealed additional genes subjected to glucose repression (i.e., *CG9466*, *CG9468*, and *CG6283*), the actual pool of affected carbohydrases and lipases could be potentially larger. In agreement with our transcriptome analysis, guts derived from flies fed a glucose-only diet also had reduced amylase activity relative to starved flies (Figure S1C). Collectively, our study demonstrates the ability of the gut to modulate digestive enzymes expression in response to their nutritional states and that the previously characterized glucose repression encompasses a broader digestion response, affecting many carbohydrases and lipases.

Glucose Repression Is Induced by Nutritious Carbohydrate

Previous studies have shown that *Drosophila* can also utilize a variety of sugars (Hassett, 1948)—hereafter referred to as nutritious sugars—but not arabinose and L-glucose, two sweet-tasting sugars with no nutritional value (Fujita and Tanimura, 2011; Hassett, 1948). To determine if the nutritional value of sugars is essential for repression, we examined the effect of a glucose, fructose, trehalose, arabinose, or L-glucose-only diet on repression. For this and subsequent analysis, we quantified *Mal-A1* and *Amy-p* transcript in flies, as they provide appropriate readout for glucose repression. Both *Mal-A1* and *Amy-p* are strongly enriched in the midgut (Chintapalli et al., 2007), repressed by glucose in the diet (Figure 1B), and encompass the early and later steps of polysaccharide digestion. *Amy-p* was also demonstrated to be one of the two dietary amylases expressed in adults (Haj-Ahmad and Hickey, 1982; Hickey et al., 1988). Whereas nutritious sugars (glucose, fructose, and trehalose) efficiently repressed *Mal-A1* and *Amy-p*, arabinose and L-glucose did not (Figure 2A). In addition, we also observed a statistically significant repression of *Mal-A1* and a weak repression of *Amy-p* (not statistically significant) by a starch-only diet (Figure S1D). Amylases and maltases are suited for their respective substrates: polysaccharides for amylases and lower-molecular-weight dextrin/disaccharides for maltases. Hence, enzymes involved in different stages of polysaccharide digestion may have different sensitivity toward glucose repression. In contrast, both *Mal-A1* and *Amy-p* transcripts were not repressed by a casein (protein) or palmitic acid (fatty-acid)-only diet (Figure 2A), asserting that the repression of *Mal-A1* and *Amy-p* was not determined solely by the caloric content of the nutrients. Of note, the level of *Mal-A1* and *Amy-p* expression in flies fed with our standard laboratory medium (a nutritionally complete diet) was already low and was not further repressed by supplementing the same medium with 10% glucose (Figure S1E). Together, these results indicate that *Mal-A1* and *Amy-p* repression depends upon the carbohydrate contents of the diet, which activates a negative feedback loop that reduces digestive enzymes expression when flies are in the starved state, but not further when flies are in the fed state.

Glucose Repression Is Independent of Insulin and AKH Signaling

In *Drosophila*, the insulin and adipokinetic hormone (AKH)-signaling pathways are two critical mediators of sugar homeostasis (Brogiolo et al., 2001; Kim and Rulifson, 2004; Lee and Park, 2004; Rulifson et al., 2002). As such, a simple mechanistic explanation for glucose repression would be through the insulin and/or AKH-signaling pathway. To determine if insulin signaling is required for repression of amylase and maltase, we examined glucose repression in *chico^{1}* homozygous mutant flies lacking the insulin receptor substrate (Böhni et al., 1999) and flies whereby *dILP3* producing cells were ablated through the overexpression of a proapoptotic gene, *reaper* (*rpr*). The *dILP3-gal4* driver was used for targeted ablation of the insulin-producing cells in the brain and in the midgut (Veenstra et al., 2008). Interestingly, in *chico^{1}* homozygous flies and *dILP3-gal4 > rpr* cell-ablated flies, glucose repression was unaffected (Figures 2B). In addition, glucose repression was unaffected in *AKHR* mutants or when we knocked down *AKH* using two independent RNAi lines with an *AKH-gal4* driver (Figures 2C and 2D). Thus, our data indicate that glucose repression of *Mal-A1* and *Amy-p* involves a carbohydrate-sensing mechanism independent of the insulin- and AKH-signaling pathway.

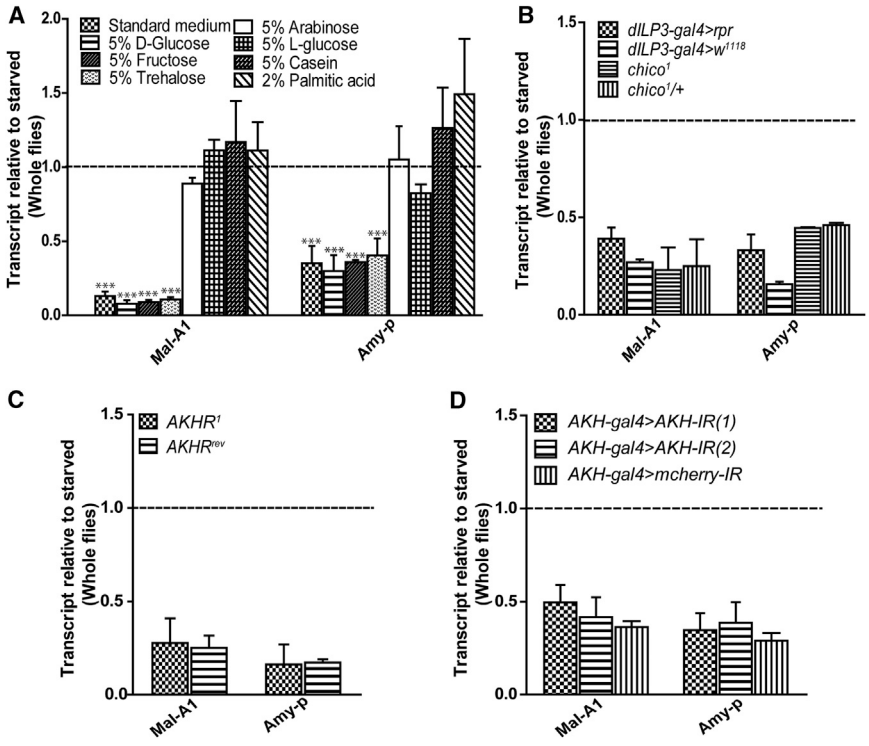


Figure 2. Nutritious Sugars Repress *Mal-A1* and *Amy-p* Independent of the AKH- and Insulin-Signaling Pathway

(A) Repression analysis on flies fed ad libitum with various sugars (glucose, fructose, trehalose arabinose, or L-glucose), protein (casein), fatty acid (palmitic acid), or standard medium for 24 hr. Only nutritious sugars (glucose, trehalose, and fructose) reduced *Mal-A1* and *Amy-p* expression. Data are expressed relative to starved as mean ± SEM. For glucose repression of amylase enzymatic activity, see Figure S1C. For effects of starch and the effects of glucose under non-starving conditions, see also Figures S1D and S1E.

(B) Ablation of *dILP3*-expressing cells by overexpression of reaper (*dILP3-gal4>rpr*) and compromised insulin signaling (*chico*^{1}—an insulin receptor substrate mutant) did not abolish repression in flies fed on a glucose-only diet. Data are expressed relative to starved as mean ± SEM.

(C) Glucose repression in AKH-receptor mutants (*AKHR*^{1}) and a genotype-matched control (*AKHR*^{rev}). Glucose repression was not affected in *AKHR*^{1} in flies fed on a glucose-only diet. Data are expressed relative to starved as mean ± SEM.

(D) Reducing AKH expression with two independent RNAi lines (*UAS-AKH-IR*) using *AKH-gal4* did not affect glucose repression in flies fed on a glucose-only diet. Data are expressed relative to starved as mean ± SEM.

Loss of *Smad2* Abolished Glucose Repression

To obtain insights into how glucose repression is achieved, we performed an in vivo RNAi screen on 60 candidate transcription factors, selected based on their expression profile (see Supplemental Experimental Procedures). For each RNAi line driven by an inducible enterocyte driver in the adult midgut (*MyoIA-gal4*^{ts}), the expression of both *Mal-A1* and *Amy-p* were monitored in glucose-fed and starved flies to assess for glucose repression. Among the transcription factors screened, knocking down *Smad2* abolished glucose repression of both *Mal-A1* and *Amy-p* (Table S1). Three independent RNAi lines against *Smad2* all abrogated glucose repression (Figure 3A). A *Smad2*-null mutant (*Smad2*^{F4}), whereby the entire coding region is deleted, has previously been described (Peterson et al., 2012). Because *Smad2*^{F4} mutants are larval lethal, we used second instar larvae (L2) maintained on a standard medium without glucose or on the same medium but supplemented with 10% glucose to confirm if Smad2 is required for glucose repression. Whereas *Mal-A1* and *Amy-p* were strongly repressed by glucose supplementation in wild-type larvae, this was lost in *Smad2*^{F4} larvae (Figure 3B).

Because the repression of amylase in the R2b and R2c subregions of the midgut was most prominent in the fed state (Figure 1C), we monitored amylase expression in the R2b subregion in flies whereby midgut *Smad2* was knocked down (*MyoIA-gal4*^{ts} > *Smad2-IR* (1)). Immunostaining for amylase showed similar levels of amylase protein in the R2b region between agar- and glucose-fed flies (Figure 3C). Knockdown of *Smad2* also affected amylase repression in other regions, such that amylase expression level along the midgut between glucose-fed and starved guts were indistinguishable (Figure S4A). To confirm if the high-amylase signal in the R2b region was indeed due to changes in gene expression, we examined the expression of *Amy-p* along the midgut using a newly generated *Amy-p-EGFP* reporter. The nuclear enhanced GFP (EGFP) reporter expression was under the control of a 2 kb region upstream of the *Amy-p* coding sequence. Whereas *Amy-p-EGFP* was repressed in the R2b region in glucose-fed control flies, *Amy-p* expression remained high in the same region when *Smad2* was knocked down in the midgut (Figure 3D). Because many of our manipulations were conducted such that *Smad2* was only compromised in differentiated adult enterocytes, we expect little, if any, effects on enterocyte cell fate and differentiation. Consistent with this, enterocytes expressed the gut-specific brush border myosin IA (Morgan et al., 1994); the marker of differentiated enterocyte, *Pdm1* (Lee et al., 2009; data not shown); and amylases, even when *Smad2* was silenced.

Next, to determine if the effects of *Smad2* knockdown on glucose repression is cell autonomous, we made positively marked clones of *Smad2* knockdown using the *esg*^{ts}*F/O* system (Jiang et al., 2009) and examined the expression of amylase by immunostaining whole guts derived from glucose-fed flies. In both the R1 and R2b regions, amylase expression was consistently higher within the *Smad2* knockdown clones (Figure 3E). Hence, increased amylase expression when *Smad2* was knocked down in the midgut (*MyoIA-gal4*^{ts} > *Smad2-IR* (1)) was not due to changes in feeding behavior. Instead, *Smad2* is required cell autonomously along the midgut for repression of *Mal-A1* and *Amy-p* in response to dietary glucose.

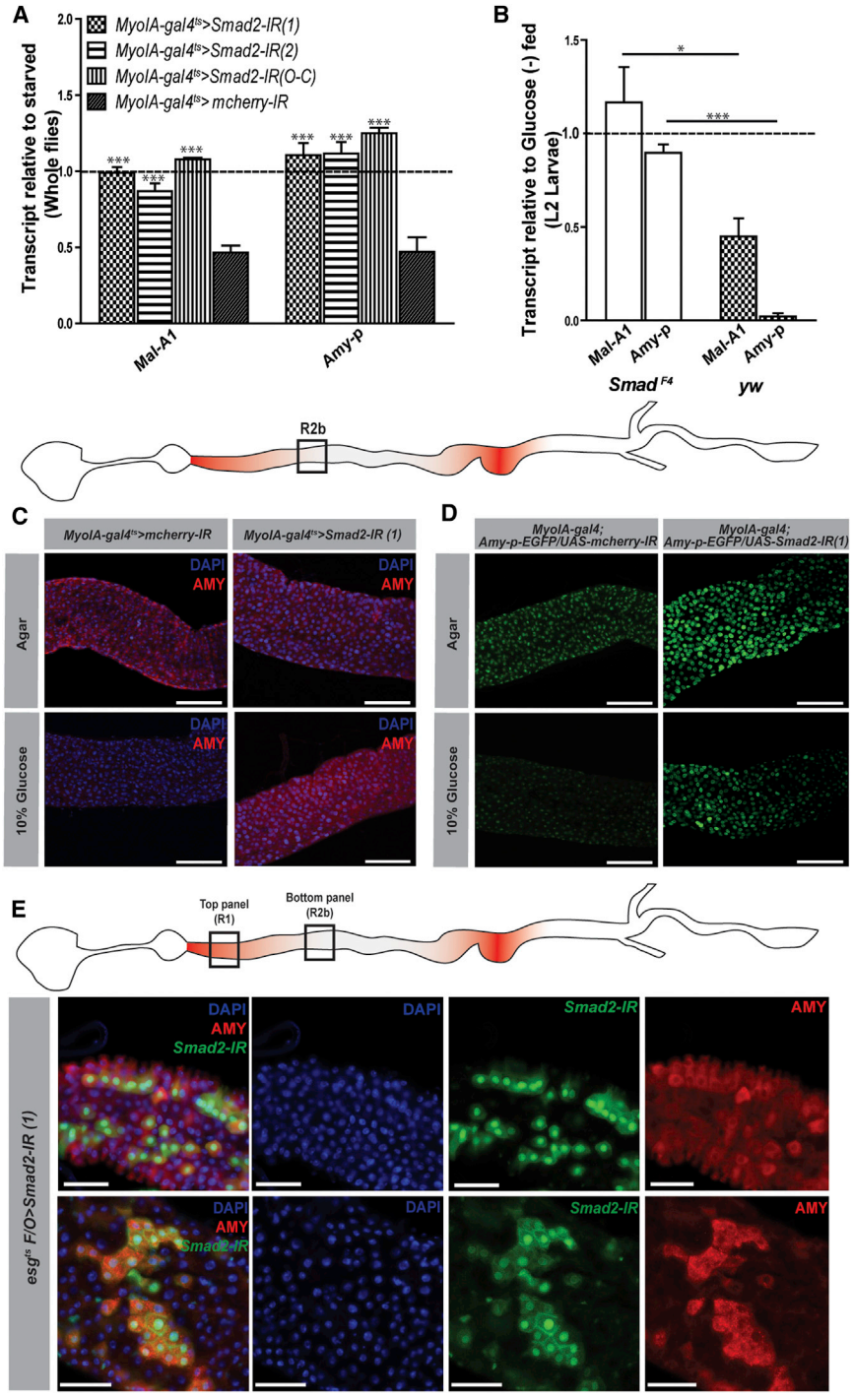


Figure 3. *Smad2* Is Cell Autonomously Required for Glucose Repression

(A) Glucose repression was determined by qRT-PCR on whole flies. Three independent RNAi lines against *Smad2*, all abolished *Mal-A1* and *Amy-p* repression by glucose. Data are expressed relative to starved as mean ± SEM. For RNAi screen, see also Table S1.

(B) *Mal-A1* and *Amy-p* repression in L2 larvae. Feeding L2 larvae were maintained on standard medium without glucose (glucose [−]) or standard medium supplemented with 10% glucose (glucose [+]). Repression of *Mal-A1* and *Amy-p* by glucose was lost in $Smad2^{F4}$ larvae. Data are expressed relative to glucose (−)-fed larvae as mean ± SEM.

(C) Glucose repression of amylase expression in the R2b region as revealed by immunostaining. When *Smad2* was knocked down in the midgut, amylase signal remained high in the R2b region in glucose-fed flies. The scale bar represents 100 μm. For immunostaining images of whole gut, see also Figure S4A.

(D) *Amy-p* expression was monitored using a nuclear localized EGFP reporter under the control of a 2 kb region upstream of the *Amy-p* coding sequence (*Amy-p-EGFP*). In the R2b region of *Smad2* knockdown, *Amy-p* expression remained high in glucose-fed flies. The scale bar represents 100 μm.

(E) Clonal analysis of *Smad2* knockdown using the esg^{ts} *F/O* system. In the midgut, *Smad2-IR*-activated clones (green) expressed higher level of amylase (red) compared to surrounding enterocytes in glucose-fed gut, both in the R1 (top panel) and R2b (bottom panel) region. The scale bar represents 50 μm.

Glucose Repression Is Dependent on the Canonical TGF-β/Activin Pathway

The transcription factor Smad2 is an R-Smad of the TGF-β/Activin pathway. The conserved TGF-β/Activin pathway consists of a tetrameric complex of two type 1 and two type 2 receptor serine/threonine kinases activated by ligand dimers. In *Drosophila*, there is one type 1 receptor, named Baboon (*Babo*), which is specific to the TGF-β/Activin pathway, and two type 2 receptors, Wishful thinking (*Wit*) and Punt (*Put*), common to both the TGF-β/Activin and the BMP/Dpp pathway. To confirm and extend our results with our *Smad2* RNAi, we examined if the TGF-β/Activin pathway is required for glucose repression by targeting *Babo*, *Wit*, and *Put*. Whereas glucose repression was lost by knockdown of *Babo* and *Put*, *Mal-A1* and *Amy-p* expression was still repressed by glucose in *Wit* knockdown flies (Figure 4A).

Previous studies have demonstrated that the activated Babo receptor can also phosphorylate Mad when endogenous Smad2 protein is depleted (Gesualdi and Haerry, 2007; Hevia and de Celis, 2013; Peterson et al., 2012). To exclude ectopic Mad activation as an explanation for the loss of glucose repression, we also knocked down *Dad*, a negative regulator of Mad, to hyperactivate Mad (Ogiso et al., 2011) and showed that *Dad* knockdown did not affect *Mal-A1* and *Amy-p* repression by glucose (Figure 4A). In agreement with this, *Babo* knockdown

(see above), which reduces Smad2 activation without any corresponding Mad phosphorylation, also effectively abolished *Mal-A1* and *Amy-p* repression by glucose. Given that Babo and Smad2 were both required for glucose repression, we reasoned that the hyperactivation of the same pathway would be associated with the repression of *Mal-A1* and *Amy-p* expression, even in absence of glucose. Indeed, when we overexpressed the constitutive active form of Babo (*Babo**) or Smad2 (*Smad2**) in the adult midgut, both *Mal-A1* and *Amy-p* expression was reduced (Figure 4B). Taken together, glucose repression of *Mal-A1* and *Amy-p* in the adult midgut is mediated through the canonical TGF-β/Activin pathway involving Babo, Punt, and Smad2.

$Babo^C$ Receptor Isoform Mediates Glucose Repression of Carbohydrases and Lipases

The *Drosophila* genome encodes a single *Babo* gene that is alternatively spliced to produce three different receptor isoforms, each with distinct extracellular domains. Specifically, the $Babo^C$ receptor isoform was previously found to be enriched in the larval gut (Jensen et al., 2009). Consistent with those findings, we also found $Babo^C$ receptor to be the major isoform expressed in the adult gut (Figure 4C). Importantly, knocking down $Babo^C$ (but not $Babo^A$ and $Babo^B$) with an isoform-specific RNAi (Awasaki et al., 2011) also abolished glucose repression of *Mal-A1 and Amy-p* (Figure 4D). Similarly, knocking down $Babo^C$ or *Put* in specific subset of clones using the $esg^{ts}F/O$ system (Jiang et al., 2009) also renders those cells insensitive to glucose repression (Figures 4E and S4B).

To verify if the TGF-β/Activin pathway may act to repress other putative digestive enzymes identified in our RNA-seq analysis, we examined the effects of $Babo^C$ knockdown on amylases, maltases, alpha-mannosidases, and lipases identified from our RNA-seq analysis. Strikingly, glucose repression of all amylase, maltase, alpha-mannosidase, and lipase genes tested was abolished when $Babo^C$ was knocked down in the adult midgut (Figure 5A). Consistent with this observation, glucose repression of amylase activity was also abrogated when *Smad2* and *Babo* were silenced in the adult midgut (Figure 5B).

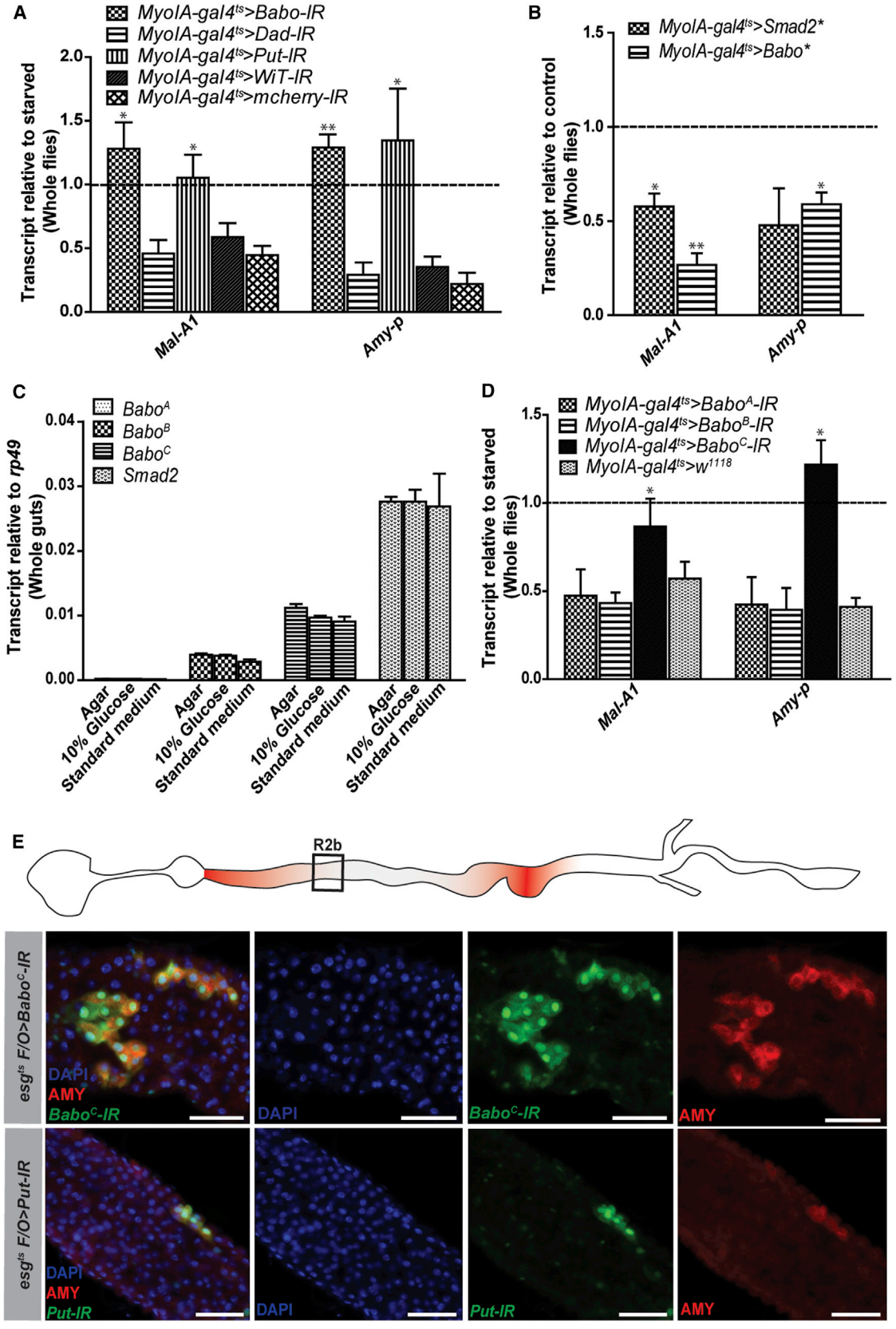
We then analyzed the metabolic consequence of disrupting the TGF-β pathway in the midgut. Glucose is the principal sugar absorbed through the gut lumen into the hemolymph. To determine if increased digestive enzyme expression may affect postprandial level of circulating glucose, we quantified the level of glucose in the hemolymph before refeeding and 1 hr post refeeding. As expected, the increase in postprandial level of glucose was consistently higher in the flies whereby the TGF-β/Activin signaling was compromised in the midgut (Figure 5C). However, there were no consistent changes in the level of trehalose 1 hr postrefeeding (data not shown). Thus, we examined the glucose, trehalose, glycogen, and triglyceride (TAG) levels in flies fed on a high-sugar diet for 2 weeks. Unexpectedly, we did not observe any significant differences between $Babo^C$ or *Smad2* knockdown flies and control (Figures 5D and S5A). Also, *Smad2* knockdown flies fed on a nutrient-poor medium, deprived of sugar, had similar survival kinetics as control (Figure S5B). Hence, there are probably compensating mechanisms to counteract any postprandial increase in circulating sugars when TGF-β/Activin pathway is disrupted in the midgut. Taken together, the TGF-β/Activin signaling through $Babo^C$ is required in the adult midgut to repress both carbohydrate and lipid-acting digestive enzymes in response to glucose in the diet; however, the metabolic significance of this process remains unclear.

Nutritious Sugar Induces *Daw* Expression, which Represses Amylase and Maltase

The transcript levels of all three *Babo* receptor isoforms and *Smad2* were similar in the guts derived from flies fed ad libitum with agar, glucose, or standard medium (Figure 4C). Thus, the modulation of the TGF-β/Activin pathway after feeding does not rely on transcriptional changes involving *Babo* receptor or *Smad2*. In *Drosophila*, the TGF-β/Activin pathway can potentially be activated by four different ligands: Dawdle (*Daw*), Myoglianin (*Myo*), Maverick (*Mav*), and Activin-β (*Actβ*) (Lo and Frasch, 1999; Nguyen et al., 2000; Parker et al., 2006; Zhu et al., 2008). To test which of the ligands are sensitive to glucose, we compared their expression in glucose-fed flies relative to starved flies. Among the four ligands, only *Daw* level was induced by glucose (Figure 6A). More importantly, *Daw* transcript was also increased in flies fed the various nutritious sugars (Figure S6A), which repressed both *Mal-A1* and *Amy-p* (Figure 2A). Casein, palmitic acid, and the nonnutritious sugar arabinose did not stimulate *Daw* expression. These results suggest that *Daw* may act as the nutritional cue to repress digestive enzyme expression in the midgut in response to sugar in the diet. To confirm whether these ligands have a repressive function, we examined the level of *Mal-A1* in flies individually overexpressing the various ligands of the TGF-β/Activin pathway using a ubiquitous driver in adults. Among the four ligands tested, only *Daw* significantly reduced *Mal-A1* level (Figures 6B and S6B). Because *Daw* was also the only TGF-β/Activin ligand that was induced by glucose, we focused our subsequent analysis only on *Daw*. As expected, overexpression of *Daw* also reduced amylase protein expression in western blot and immunostaining analysis on gut samples (Figures 6C and S6C). We then confirmed if *Daw* is essential for glucose repression by using a previously described null mutant of *Daw* (Gesualdi and Haerry, 2007). Given that *Daw* mutants (Daw^{Acct1}) do not survive to adulthood, we studied glucose repression in feeding L2 larvae. Concurring with our results whereby overexpression of *Daw* repressed *Mal-A1*, glucose repression was abolished in Daw^{Acct1} larvae (Figure 6D). Altogether, our data demonstrate that *Daw* expression is induced by nutritious sugar and one of its functions is to reduce digestive enzyme expression in the adult midgut.

Systemic Release of Daw Can Mediate Glucose Repression in the Gut

In adults, *Daw* is highly expressed in the fat body and muscle; whereas in the brain, gut, and ovary tissues, *Daw* expression is low (Bai et al., 2013; Chintapalli et al., 2007). To determine the source of adult *Daw* induction in glucose-fed flies, we examined in which of those tissues *Daw* might be induced. After 24 hr on a glucose diet, transcript levels of *Daw* were elevated only in the abdomen carcass (without gut and ovaries; containing mainly fat body) samples. Expression of *Daw* in the head (brain), thorax



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(consisting mainly muscle), and whole-gut samples was not significantly altered (Figure 6E). All these results suggest that glucose repression is unlikely mediated by a local release of Daw from the gut. Next, to confirm that Daw can function in an endocrine manner, we overexpressed *Daw* in the fat body (using *yolk-gal4* and *ppl-gal4*), visceral muscle (using *how-gal4^{ts}*), or gut (using *MyoIA-gal4^{ts}*). Overexpression with the different *gal4* drivers all repressed *Mal-A1* and *Amy-p* expression (Figures 6F and S6D), showing that Daw can function in an endocrine manner via the circulation to regulate midgut expression of *Mal-A1* and *Amy-p*.

To better define the source of Daw for glucose repression, we used various *gal4* drivers to knock down *Daw* in a tissue-specific manner (Figures 6G and S6E). Whereas glucose repression remained when *Daw* was knocked down in the adult midgut (*MyoIA-gal4^{ts}*), the muscle (*MHC-gal4*), and the brain (*elav-gal4*), glucose repression was abolished when *Daw* expression was compromised in the fat body (*ppl-gal4* and *C564-gal4^{ts}*). Fat body knocked down of *Daw* also increased the expression of *Mal-A1* and *Amy-p* (Figure S6F). In conclusion, our results are consistent with a model whereby *Daw* expression is induced and secreted by the fat body in response to nutritious carbohydrate in the diet and, via the circulation, represses digestive enzyme expression in the midgut through the TGF-β/Activin-signaling pathway (Figure 7).

DISCUSSION

Digestive enzymes expression is subjected to complex regulation. However, apart from the regulation of *magro* (lipase) by the nutrient-sensitive DHR96 and dFOXO (Karpac et al., 2013; Sieber and Thummel, 2012), regulatory mechanisms controlling digestive enzymes expression have not been carefully studied in *Drosophila*. A rare but poorly characterized example of digestive enzyme regulation concerns amylase repression by the end product of carbohydrate digestion, glucose (Benkel and Hickey, 1986; Hickey and Benkel, 1982). Although glucose repression was initially described in the context of amylase, our transcriptome analyses have showed that glucose repression encompasses a broader spectrum response. Glucose also repressed genes with predicted carbohydrase, glucose transport, and lipase function. Many of the genes affected by glucose repression are expressed almost exclusively in the midgut (Chintapalli et al., 2007) and are organized in clusters in the genome (e.g., lipase cluster: *CG6283*, *CG6271*, and *CG6277*), a feature pertaining to many digestive-enzyme-encoding genes (Lemaitre and Miguel-Aliaga, 2013). It is noteworthy that our arbitrary threshold for RNA-seq analysis has rejected several genes whose repression was more subtle (Figure S2). For this, we have independently verified *Amy-p*, *Amy-d*, *CG9466*, *CG9468*, and *CG6283* to be repressed by glucose through qRT-PCR. Thus, the actual repertoire of carbohydrases and lipases affected by glucose could be potentially larger.

To date, little is known about the contribution of digestion on sugar homeostasis. Although a detailed profiling for the metabolic effects of digestion is beyond the scope of this paper, it seemed likely that glucose repression of carbohydrases and lipases is aimed at reducing the amount of sugars and lipids that are available for absorption. Consistent with this view, we also found glucose transmembrane transporters among genes that were downregulated by dietary glucose. A high-sugar diet in *Drosophila* is associated with dire consequences such as hyperglycemia, insulin resistance, and increased fat accumulation (Havula et al., 2013; Musselman et al., 2011). Thus, reducing both carbohydrases and lipases expression may restrict the nutritional load available for absorption into the circulation when carbohydrate stores in the organism are sufficient and fats are accumulating. In accordance with this, early postprandial glucose level was elevated in the hemolymph when TGF-β/Activin pathway function was compromised in the midgut, a condition associated with elevated digestive enzymes expression. However, when we monitored the levels of TAG, glycogen, glucose, and trehalose after 2 weeks on a high-sugar diet, we did not observe any significant differences between flies whereby *Smad2* or *Babo* were knocked down in the midgut and control. Sugar homeostasis is a tightly regulated process involving multiple tissues. One possibility would be that the postprandial increase in glucose was counteracted by early acting satiety response when hemolymph glucose level passed a certain threshold, thus limiting the net amount of glucose entering the circulation. Clearly, the role of glucose repression in sugar homeostasis and metabolism warrants additional research. An understanding of how the repertoire of digestive enzymes respond to other nutriments in the diet will provide insights into how an organism may rebalance its diet after ingestion and improve our understanding of nutrients homeostasis.

In this study, we also showed that digestive enzyme repression is induced only by nutritious carbohydrates in the diet. Arabinose, a sweet-tasting sugar with no nutritional value, and L-glucose, another nonutilizable sugar (Fujita and Tanimura,

Figure 4. The Canonical TGF-β/Activin Pathway Is Required for Glucose Repression in the Adult Midgut

(A) *Mal-A1* and *Amy-p* repression was determined by qRT-PCR on midgut-activated *Babo-IR* (does not distinguish between isoforms), *Dad-IR*, *Put-IR*, and *Wit-IR* flies. Glucose repression of *Mal-A1* and *Amy-p* was lost in *Babo-IR* and *Put-IR*. Data are expressed relative to starved as mean ± SEM.

(B) Overexpression of the constitutive active form of Babo (*Babo**) and Smad2 (*Smad2**) in the adult midgut reduced *Mal-A1* and *Amy-p* expression in starved flies. Data are relative to control flies (*MyoIA-gal4^{ts} > yw* for *Smad2**; *MyoIA-gal4^{ts} > w^{1118}* for *Babo**) as mean ± SEM.

(C) The transcript level of *Smad2* and the three *Babo* isoforms were monitored by qRT-PCR on adult gut from flies fed ad libitum on various medium for 24 hr. *Babo^{C}* and to a lesser extent *Babo^{B}* receptor isoform were expressed in the adult gut. Expression of *Babo* receptors and *Smad2* was not sensitive to the different diet. Data are expressed relative to *rp49* as mean ± SEM.

(D) Effects of knocking down specific Babo receptor isoform on glucose repression. Loss of *Babo^{C}* receptor isoform, but not *Babo^{A}* and *Babo^{B}*, abolished glucose repression. Data are expressed relative to starved as mean ± SEM.

(E) Clonal analysis of *Babo^{C}* or *Put* knockdown using the *esg^{ts} F/O* system. In the R2b region of the gut, knockdown of *Babo^{C}* or *Put* (green) increased amylase (red) expression relative to surrounding enterocytes in glucose-fed flies. This effect was also observed in the R1 region of the midgut (Figure S4B). The scale bar represents 50 μm.

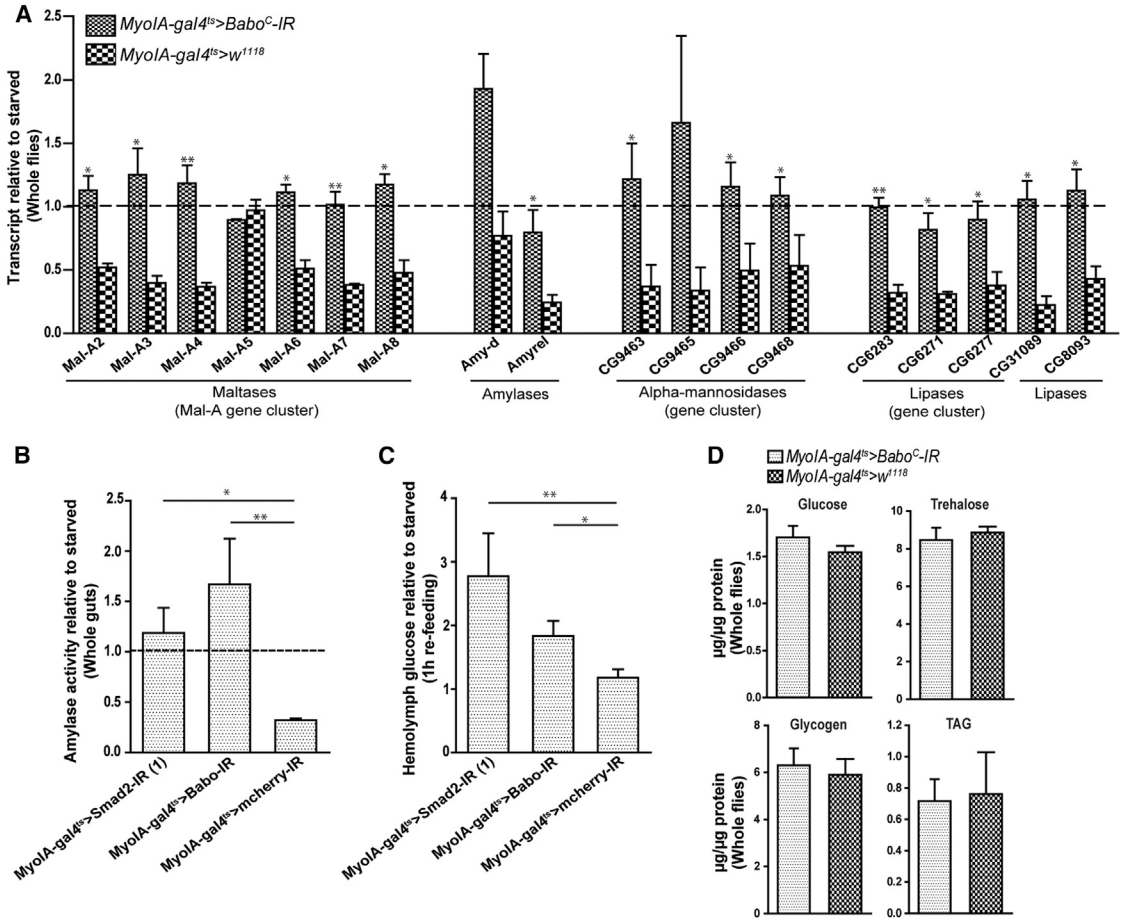

Figure 5. TGF-β/Activin Pathway Affects Repression of Many Digestive Enzymes and Postprandial Glucose Level

(A) Glucose repression analysis for all other maltases, amylases, alpha-mannosidases, and lipases identified by the RNA-seq analysis as well as other members within the same cluster, but not identified in analysis. Knockdown of *Babo*^{C} by RNAi abolished repression of many of the genes in response to glucose in the diet. Data are expressed relative to starved as mean ± SEM.

(B) Glucose repression of amylase activity. Midgut knockdown of *Smad2 or Babo-IR*, but not control, abolished amylase repression. Amylase activities were normalized to protein level (mU activity/µg protein) and expressed relative to starved as mean ± SEM.

(C) Circulating glucose after 1 hr refeeding. Flies were refed with standard medium supplemented with 5% maltose, 5% sucrose, and 2.5% starch. The increase in hemolymph glucose postfeeding was higher in *MyoIA-gal4*^{ts} > *Smad2-IR* (*1*) and *MyoIA-gal4*^{ts} > *Babo-IR* compared to control. Values are hemolymph glucose level relative to level before refeeding.

(D) Amounts of glucose, trehalose, glycogen, and TAG derived from whole flies fed on a high-sugar diet (standard medium supplemented with 20% sucrose, 5% maltose, and 5% starch) for 2 weeks. There were no significant differences between *Babo* knockdown and control. Amounts were normalized to the level of protein and expressed as µg/µg protein. Values are mean ± SEM. For metabolic measurements and sensitivity to sugar deprivation of *Smad2* knockdown, see Figures S5A and S5B.

2011; Hassett, 1948), did not suppress amylase and maltase expression. Hence, we consider postprandial activation of gustatory receptors (Park and Kwon, 2011) in the gut to be an unlikely mechanism for glucose repression of digestive enzymes. Instead, all these are suggestive of an underlying sugar-sensing mechanism to ensure that carbohydrate digestive capacity toward utilizable carbohydrate sources are not comprised until nutritional sufficiency is attained.

In *Drosophila*, sugar homeostasis is often associated with the AKH and insulin signaling, whereas insulin signaling is also modulated by proteins and amino acids in the diet (Brogiolo et al., 2001; Buch et al., 2008; Kim and Rulifson, 2004; Lee and Park, 2004; Rulifson et al., 2002). Recently, Bai and colleagues have showed that *Daw* expression is modulated by insulin signaling and identified *Daw* as a target of dFOXO (Bai et al., 2013), raising the possibility that glucose repression may be similarly affected by insulin signaling. Surprisingly, disrupting both AKH and insulin signaling did not compromise glucose repression. Instead, we identified a key role for TGF-β/Activin signaling in this process. Whereas *Daw* expression may be modulated by insulin signaling, our results clearly showed that glucose repression is mediated through an insulin-independent mechanism. More recently, Ghosh and O'Connor have demonstrated that Daw is required for insulin secretion, suggesting

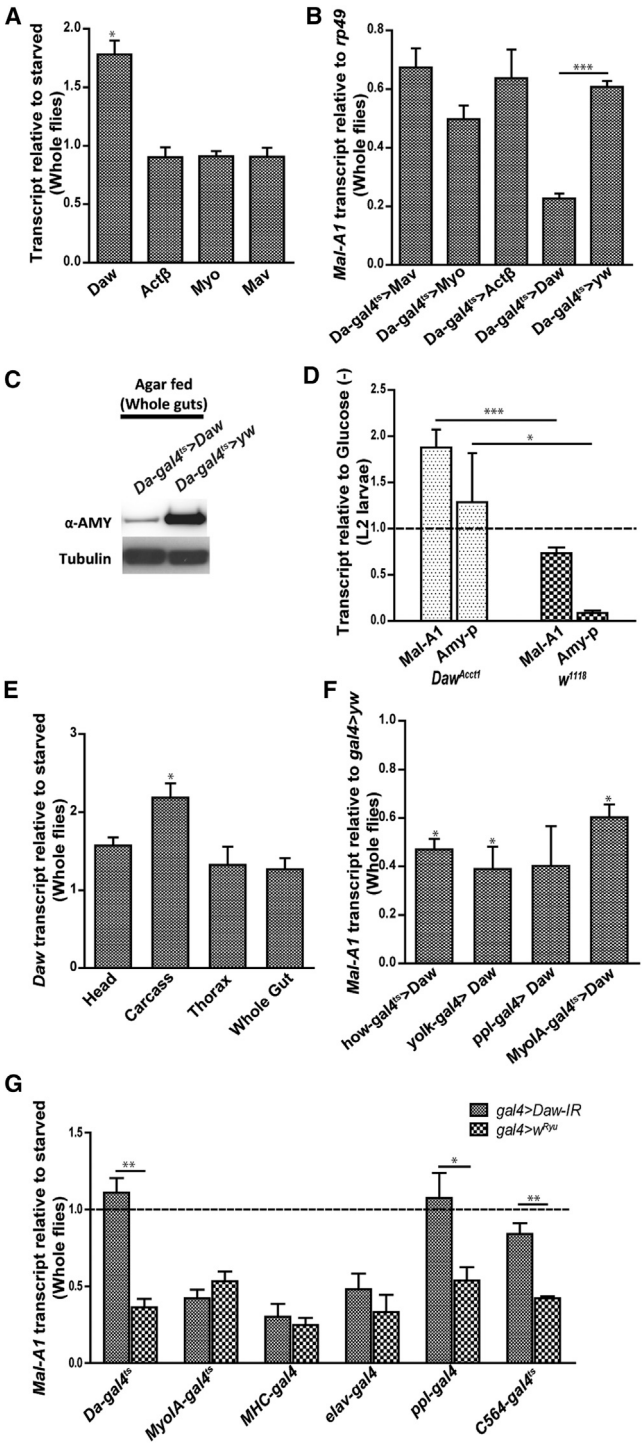

Figure 6. *Daw* Is Induced in the Fat Body by Glucose in the Diet and Is Necessary for Repression of *Mal-A1* and *Amy-p*

(A) qRT-PCR of the four possible TGF-β/Activin pathway ligand genes on flies fed with glucose. Only *Daw* expression was induced upon glucose feeding. Data are expressed relative to starved as mean ± SEM. For the effects of other sugars on *Daw*, see also Figure S6A.

(B) *Mal-A1* level in starved flies ubiquitously overexpressing each of the four TGF-β/Activin ligand genes. Overexpression of *Daw* (*Da-gal4*^{ts} > *Daw*) reduced *Mal-A1* expression relative to control flies (*Da-gal4*^{ts} > *yw*). Data are expressed relative to *rp49* as mean ± SEM. See also Figure S6B for quantification with gut lysate.

(C) Western blot analysis of amylase protein on gut lysate derived from flies overexpressing *Daw*. Overexpression of *Daw* reduced amylase protein expression in the gut. Tubulin is shown as loading control. For immunostaining of the gut when *Daw* is overexpressed, see Figure S6C.

(D) *Mal-A1* and *Amy-p* gene repression in *Daw*^{Acct1} was monitored in feeding L2 larvae maintained on standard medium without glucose or standard medium supplemented with 10% glucose. Repression of *Mal-A1* and *Amy-p* by glucose was compromised in *Daw*^{Acct1} larvae. Data are expressed relative to glucose (−)-fed larvae as mean ± SEM.

(E) Expression of *Daw* in different tissues after 24 hr on glucose. *Daw* was induced in the abdomen carcass (mainly fat body tissue) of glucose-fed flies. Data are expressed relative to starved as mean ± SEM.

(F) *Mal-A1* transcript level in starved flies overexpressing *Daw* through different tissue-specific *gal4* drivers. Overexpression of *Daw* by different drivers all reduced *Mal-A1* expression. Data are expressed relative to control (*gal4>yw*) as mean ± SEM. For *Amy-p* expression, see also Figure S6D.

(G) *Mal-A1* repression when *Daw* was knocked down in specific tissue. Fat body knockdown (*ppl-gal4* and *C564-gal4*^{ts}) of *Daw* abolished glucose repression of *Mal-A1*. Data are expressed relative to starved as mean ± SEM. For *Amy-p* repression, see also Figure S6E.

that the TGF-β/Activin pathway may function upstream of the insulin signaling (Ghosh and O'Connor, 2014). It is also noteworthy that, whereas compromising insulin signaling is known to raise circulating sugar levels, this did not affect the ability of flies to repress digestive enzymes in response to dietary glucose. One possible explanation is that *Daw* expression in response to glucose is dependent on the nutritional state perceived cell autonomously by the fat body cells. Thus, if nutrient sensing in these cells is not compromised, *Daw* induction and glucose repression can be achieved. Future research should clarify the mechanism underlying *Daw* induction by nutritious sugar and define the possible interactions between TGF-β/Activin and other sugar-sensing mechanisms.

The TGF-β/Activin pathway in *Drosophila* has been previously studied in the context of larval brain development, neuronal remodeling, wing disc development, and, more recently, aging and pH homeostasis (Bai et al., 2013; Ghosh and O'Connor, 2014; Gibbens et al., 2011; Hevia and de Celis, 2013; Peterson et al., 2012; Zheng et al., 2003; Zhu et al., 2008). This study addresses the physiological function of the TGF-β/Activin pathway in the adult midgut. When we disrupted the TGF-β/Activin signaling in the adult midgut, glucose repression was abolished. Conversely, increasing TGF-β/Activin signaling in the midgut, through the overexpression of the constitutive active form of Babo or Smad2, was sufficient to repress both amylase and maltase expression. Furthermore, we showed that glucose repression is mediated by the TGF-β ligand Daw, produced and secreted from the fat body, a metabolic tissue functionally analogous to the mammalian liver and adipose tissue. Thus, our study uncovers a physiological role for the TGF-β/Activin pathway in adapting carbohydrate and lipase digestion in response to the nutritional state of the organism. Because many features of digestion and absorption are conserved between flies and mammals, it will be of interest to investigate the role of TGF-β/Activin pathway in mammalian digestion.

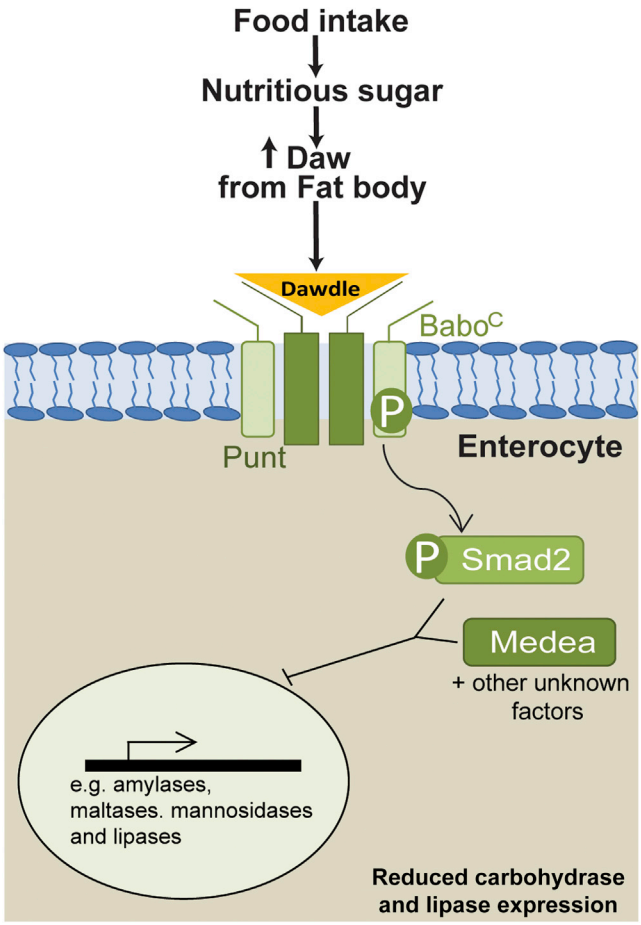

Figure 7. Model for Sugar Sensing and Repression of Carbohydrase and Lipase

Nutritious sugars, when consumed sufficiently, induce *Daw* expression in the fat body. Secreted Daw then activates the canonical TGF-β/Activin signaling in the midgut through Babo^{C} and Punt receptors, leading to the activation of Smad2 and reduction of carbohydrase and lipase expression.

Recent studies have attributed a role for *Daw* in aging (Bai et al., 2013) and pH homeostasis (Ghosh and O'Connor, 2014), two processes tightly linked to metabolism. Thus, it is likely that *Daw* induced from the fat body in response to carbohydrate in the diet will induce a more global response instead of a local response, affecting only digestive enzyme expression. As such, *Daw* may act as a central mediator for glucose homeostasis by regulating sugar level in the circulation. When there are sufficient carbohydrates in the diet, *Daw* expression restricts the expression of carbohydrase and glucose transporters. Concurrently, at the postabsorption level, *Daw* in the circulation may act directly or indirectly (via insulin signaling) to maintain circulating sugar level. A broader role for *Daw* in sugar homeostasis is reinforced by the findings that Daw mutant larvae were more sensitive to a high-sugar diet (Ghosh and O'Connor 2014). Similarly, we found overexpression of *Daw*, but not *Myo*, *Mav*, or *Act*β, renders flies sensitive to sugar starvation (W.A.C., unpublished data). Along this line, in *C. elegans*, the TGF-β signaling is reported to be elevated and required in neurons for satiety (You et al., 2008). There were also several observations that hyperglycemia is linked to increased TGF-β activity in mammals (Iglesias-de la Cruz et al., 2002; Kolm-Litty et al., 1998). Hence, the role of TGF-β/Activin signaling in sugar homeostasis requires further investigation in *Drosophila* and other organisms.

In conclusion, our study revealed a remarkable resilience in the regulation of carbohydrate and lipid-acting enzymes expression to ensure that digestive capacity in the midgut is not compromised before certain metabolic criteria in the fat body is attained. The study also unraveled a role of the TGF-β/Activin-signaling pathway in the adult *Drosophila* midgut, which has not been appreciated. It reinforced the notion that the gut is not a passive tube for nutriment flow. Rather, it dynamically modulates digestive enzyme expression in response to the organism's nutritional state through endocrine signals derived from other metabolic tissues.

EXPERIMENTAL PROCEDURES

Fly Stocks and Maintenance

For stocks and diets, see Supplemental Experimental Procedures. For inducible GAL4 activation, F1 flies were raised at 18°C or 22°C for larval and pupal development and switched to 29°C 3 days after eclosion for at least 5 days. Validation for the various RNAi, overexpression, and cell ablation is provided in Table S2. All crosses and stocks were kept at 25°C on a 12 hr light dark cycle unless otherwise stated.

Statistical Analysis

All analyses were performed in R. For qRT-PCR data, relative ratios of target gene to *rp49* were Log2 transformed. Before performing any parametric test, we used Levene's test to test for equality of variances and the Shapiro-Wilk test to test for normality. For pairwise comparisons, we used Student's t test on the transformed data. ANOVA followed by Dunnett's test was used when comparing more than two groups. Pooled data ($n \geq 3$) were expressed as means ± SEM. ***$p < 0.0005$; **$p < 0.005$; *$p < 0.05$.

ACCESSION NUMBERS

The National Center for Biotechnology Information Gene Expression Omnibus accession number for the RNA-seq raw data reported in this paper is GSE54755.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental Text, six figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.08.064.

AUTHOR CONTRIBUTION

W.A.C. designed the project, assembled the data, analyzed and interpreted the results, and wrote the manuscript. M.S.B.S. analyzed the RNA-seq data and statistics. F.S. collected data for metabolic analysis. B.L. designed the project and wrote the manuscript.

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