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Midgut-derived Activin regulates glucagon-like action in the fat body and glycemic control

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Abstract

While high-caloric diet impairs insulin response to cause hyperglycemia, whether and how counter-regulatory hormones are modulated by high-caloric diet is largely unknown. We find that enhanced response of *Drosophila* adipokinetic hormone (AKH, the glucagon homolog) in the fat body is essential for hyperglycemia associated with a chronic high-sugar diet. We show that the activin type I receptor Baboon (Babo) autonomously increases AKH signaling without affecting insulin signaling in the fat body via, at least, increase of Akh receptor (AkhR) expression. Further, we demonstrate that Activin-β (Actβ), an activin ligand predominantly produced in the enteroendocrine cells (EEs) of the midgut, is up-regulated by chronic high-sugar diet and signals through Babo to promote AKH action in the fat body, leading to hyperglycemia. Importantly, activin signaling in mouse primary hepatocytes also increases glucagon response and glucagon-induced glucose production, indicating a conserved role for activin in enhancing AKH/glucagon signaling and glycemic control.

Graphical Abstract

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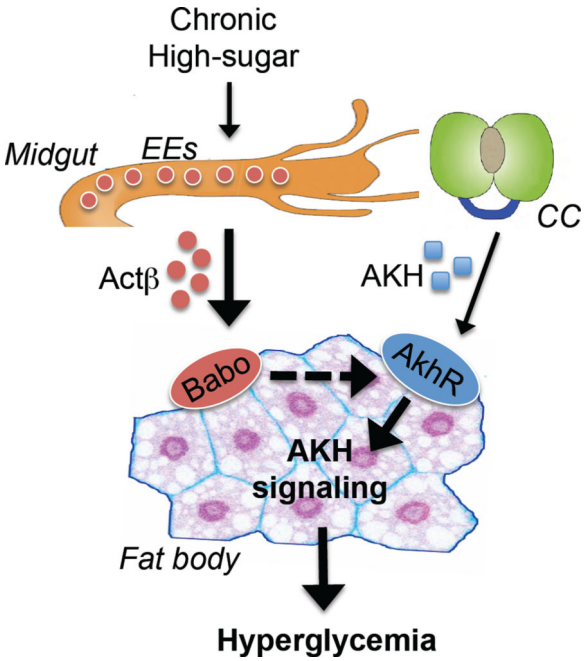
Author Contributions

W. S. conceived the study and designed and performed experiments. D. C. and Y. H. performed RNA-seq and bioinformatics analysis. D. C., B. S. and N. W. performed RNAi screen. S. H. and P. P. performed primary hepatocyte experiments. C. Z. and M. B. O. generated transgenic flies. W. S. and N. P. discussed results and wrote manuscript.



Introduction

In both vertebrates and invertebrates, Insulin and counter-regulatory hormones have evolved to maintain stable circulating carbohydrate levels in response to nutritional and environmental cues (Haselton and Fridell, 2010; Unger, 1971). Insulin promotes circulating carbohydrate clearance, while counter-regulatory hormones increase carbohydrate release into circulation. However, high-caloric diets can cause an imbalance between insulin and counter-regulatory hormone regulations resulting in elevated glycemic levels, a condition known as hyperglycemia (Buettner et al., 2007; Musselman et al., 2011). Much has been learned about how impaired insulin action or insulin resistance contributes to the carbohydrate metabolic dysregulation associated with hyperglycemia (Frojdo et al., 2009; McNelis and Olefsky, 2014). By contrast, very little is known about the role of counter-regulatory hormone regulation of glycemic control in caloric overload conditions.

Glucagon, an important insulin counter-regulatory hormone, is secreted from pancreatic α-cells during fasting or exercise and promotes glucose production in the liver via glucagon receptor signaling (i.e. GCGR/cAMP/PKA signaling) (Jiang and Zhang, 2003; Unger and Cherrington, 2012). Glucagon action in hepatocytes is abnormally increased in response to a high-caloric diet in obese or diabetic subjects, and contributes to glucose intolerance and hyperglycemia (Matsuda et al., 2002; Sheng et al., 2012). Importantly, inhibition of hepatic glucagon signaling using antisense oligonucleotides has been shown to improve glucose tolerance and to restore normal glycemic levels in diet-induced obese or diabetic mice (Liang et al., 2004). A similar effect of glucagon enhancement has also been reported in diabetic patients (Ali and Drucker, 2009). Previous studies have indicated that the inflammatory factor NIK and the ER stress sensor IRE1α are involved in hepatic glucagon

signal transduction in obese mice under high-fat diet feeding (Mao et al., 2011; Sheng et al., 2012).

As a homolog of mammalian glucagon, *Drosophila* adipokinetic hormone (AKH) is a well-established insulin counter-regulatory hormone involved in carbohydrate metabolic homeostasis (Galikova et al., 2015; Kim and Rulifson, 2004; Lee and Park, 2004). Under starvation conditions, AKH is secreted from corpora cardiaca (CC) endocrine cells and promotes nutrient utilization in the fat body, leading to increased glycemic levels in circulation. The prohormone processing and secretion regulations of AKH and glucagon are similar. AKH also activates a G protein-coupled receptor, AkhR, and triggers cAMP/PKA signaling to regulate carbohydrate metabolism in the fat body, which is reminiscent of glucagon signaling (Bharucha et al., 2008; Gronke et al., 2007; Kim and Rulifson, 2004; Lee and Park, 2004; Rhea et al., 2010). Further, similar to the effects of high-fat diet in mammals, subjecting *Drosophila* to a chronic high-sugar diet has been shown to increase adiposity, impair insulin signaling in the fat body, and cause hyperglycemia in larvae (Musselman et al., 2011). Thus, we reasoned that investigating how AKH response is regulated by high-sugar diet would provide novel insights into how carbohydrate imbalance and hyperglycemia develop in response to excess caloric intake.

In this study, we uncover that fat body AKH action is abnormally enhanced in the context of a high-sugar diet, causing hyperglycemia. We further demonstrate that, in response to high-sugar feeding, midgut produces Actβ to increase AKH response in the fat body via activation of Babo/dSmad2 signaling and elevation of AkhR expression, thus leading to a hyperglycemic state.

Results

Regulation of carbohydrate metabolism by AKH

To characterize the role of AKH at the organismal level, we used RNAi to knockdown the *Akh receptor* (*AkhR*) in various metabolic tissues and measured circulating carbohydrate levels. Knockdown of *AkhR* in the larval fat body, but not in neurons, the midgut, or muscle, led to a significant reduction in circulating levels of both glucose and trehalose, a disaccharide form of glucose that is the major carbohydrate in the fly hemolymph (Fig. 1A–B and Fig. S1A–B, S1D–E). Knockdown of *AkhR* in the adult fat body also led to a significant reduction in glycemic levels, i.e. circulating glucose and trehalose (Fig. S3H). *AkhR* knockdown in the larval fat body dramatically decreased glucose/trehalose storage but increased glycogen storage (Fig. S1C), suggesting that AKH signaling in the fly fat body regulates circulating carbohydrate levels via both glycogen breakdown and glucose/trehalose production.

Mammalian studies have shown that glucagon activates PKA, leading to increased phosphorylation of IRE1α and CREB, CRTC2 nuclear translocation, and CREB transcriptional activity (Mao et al., 2011; Wang et al., 2009). To address whether the AKH signal is transduced in a similar manner in *Drosophila*, we generated a *Drosophila* S2R+ cultured cell line stably expressing AkhR. The addition of synthetic AKH to the culture media led to increased phosphorylation, not nuclease activity, of IRE1, the fly ortholog of

IRE1α, in a dose- and PKA-dependent manner, and promoted nuclear translocation of Crtc, the fly ortholog of CRTC2 (Fig.S1F–G and S1J). Consistently, treatment with synthetic AKH of wild type larval fat bodies also led to an increase in phosphorylation of IRE1 and CREB2, the fly ortholog of CREB. Moreover, knockdown of *AkhR* potently blocked this AKH-induced increase in p-IRE1 and p-CREB2 levels (Fig. 1E, antibody that recognizes p-CREB2 in Fig. S1H). We also found that AKH signaling led to an increase in CREB2 transcriptional activity in the fat body (Fig. S1I). Altogether, our results indicate that AKH signaling in the fly fat body is analogous to glucagon signaling in the mammalian liver.

A chronic high-sugar diet promotes glycemic level by enhancing AKH action

An increase in *AkhR* expression in the larval fat body was previously shown to be associated with chronic high-sugar feeding (Musselman et al., 2013); however, whether AKH directly contributes to high-calorie diet-induced hyperglycemia is not known. To address this question, we fed larvae a chronic high-sugar diet to induce hyperglycemia and monitored AKH signaling. Compared to the larvae fed normal food, larvae fed a high-sugar diet showed an increase in both their response to synthetic AKH stimulation and homeostatic AKH signaling output in the fat body, as evidenced by increased p-IRE1 and p-CREB2 levels (Fig. 1F), indicating that a chronic high-sugar diet enhances larval fat body AKH response. Abolishing AKH signaling via disruption of *AkhR*, as achieved using either a null mutation of *AkhR* or fat body-specific RNAi, significantly alleviated both increased AKH signaling and hyperglycemia associated with high-sugar diet (Fig. 1C–D, 1F). These data indicate that high-sugar diet-induced hyperglycemia is attributable to increased AKH signaling in the fat body.

Impaired insulin signaling is known to elevate glycemic levels and contribute to high-sugar diet-induced hyperglycemia (Musselman et al., 2011). *AkhR* knockdown in the fat body is sufficient to restore normal glycemic levels in the context of *InR* deficiency, which dramatically impairs insulin signaling and leads to elevated glycemic levels (Fig. S1K–M). Thus, we next asked whether AKH signaling suppresses hyperglycemia by promotion of insulin signaling under a normal diet. However, knockdown of *AkhR* failed to affect Akt phosphorylation or FoxO target gene expression (*4E-BP* and *InR*) (Fig. S3E–F), two major readouts of insulin signaling (Owusu-Ansah et al., 2013; Song et al., 2010). Thus, fat body AKH signaling does not impinge on insulin signaling to impact glycemic control.

An AKH-induced hyperglycemia model in *Drosophila*

Ectopic *Akh* overexpression in the fat body using R4-Gal4 (*R4 > Akh*) results in hyperglycemia (Fig. 2A) (Lee and Park, 2004), suggesting that ectopic *Akh* expression activates AKH signaling in the fat body in an autocrine manner. Consistently, *Akh* overexpression leads to a dramatic increase in fat body AKH signaling (Fig. 2B). Further, fat body-specific knockdown of AKH/glucagon signaling components significantly affected AKH-induced hyperglycemia (Fig. 2C).

Little is known about the molecular mechanism by which AKH regulates carbohydrate metabolism. We thus analyzed the fat body transcriptome via RNA-seq and found that ectopic AKH expression led to significant changes in the expression of 702 genes in the fat

body, with 364 down-regulated and 338 up-regulated (Fig. 2D and Table S1). Gene Ontology (GO) enrichment analysis of the differentially-expressed genes revealed a significant enrichment of genes involved in carbohydrate metabolism, including glycogen and sucrose metabolism, glycolysis and/or gluconeogenesis, and the TCA cycle (Fig. S2A). Genes involved in AKH-associated carbohydrate metabolism were further validated by qPCR and genetic analysis (Fig. S2C–D). Consistent with activation of CREB2, GO analysis revealed that CREB2 target genes were significantly enriched (Fig. S2B). Interestingly, the targets of other transcriptional factors than CREB2 were also enriched (Fig. S2B), suggesting potential cross-talk between AKH signaling and other pathways. Note that FoxO target genes were not significantly enriched, consistent with our observation that knockdown of *AkhR* failed to affect insulin/FoxO signaling (Fig. S3E–F).

In vivo RNAi screen for regulators of AKH signaling

The AKH-induced hyperglycemia model described above allowed us to perform an *in vivo* RNAi screen to discover physiological regulators of AKH signaling (Fig. 2E). Specifically, transgenic RNAi fly stocks targeting 305 kinases and phosphatases, many of which were previously validated (Sopko et al., 2014), were crossed to *R4 > Akh* flies and glycemic levels (glucose + trehalose) of their offspring were measured. We used glycemic levels as the major readout in this screen as we found it to be more reliable and inclusive than monitoring signaling (i.e. p-IRE1, p-CREB2, or CREB transcriptional activity; data not shown). The screen assay is robust, as knockdown of glucagon/AKH signaling components consistently affected glycemic levels (Fig. 2C). We normalized glycemic changes to both negative (*w-i*, 1, 100% hyperglycemia) and positive (*AkhR-i*, 0, 0% hyperglycemia) controls using the formula $NG_{RNAi} = (RG_{RNA\text{-}i} - RG_{AkhR\text{-}i}) / (RG_{w\text{-}i} - RG_{AkhR\text{-}i})$ (NG, normalized glycemic levels; RG, raw glycemic levels) (Fig. 2F). A total of 30 candidate genes were identified that significantly affected AKH-induced hyperglycemia (>1.7 or <0.3, hyperglycemic index) (Table S2). These genes have been previously implicated in various biological processes. (Fig. 2G and Table S2). Among the candidates, the mammalian homologs of *Pfk* and *Sik3* have previously been shown to be involved in glucagon signaling (Pilkis et al., 1982; Wang et al., 2011). Consistently, knockdown of *Pfk* and *Sik3* in the *Drosophila* fat body significantly enhanced AKH-induced hyperglycemia (hyperglycemic index values of 3.6 and 2.1, respectively) (Table S2).

In addition to changes in glycemic levels, knockdown of a few genes also resulted in changes in larval fat body morphology, developmental delay, and/or pupae lethality. However, we did not observe a strong correlation between these effects and glycemic changes (Fig. S2E and Table S2). To further assess how these 30 genes regulate AKH action, AKH signaling outputs were measured and potential mechanisms annotated based on their differential effects on p-IRE1 or p-CREB2 levels (Fig. 2H and Fig. S2F–G).

We also performed a small secondary RNAi screen in wild type larvae and found that knockdown of these candidates consistently affect glycemic levels in the absence of AKH overexpression (Fig. S3A).

Babo regulates AKH response and carbohydrate metabolism

RNAi stocks targeting *Babo* and *Punt* (*Put*), which encode a type I and II TGF-β receptors, respectively, were among the reagents exerting the greatest suppression of AKH-induced hyperglycemia in the primary RNAi screen (Table S2 and Fig. 2H). In *Drosophila* there are three different type I TGF-β receptors (Thick-Vein (Tkv), Saxophone (Sax) and Babo) and two shared type II receptors (Put and Wishful-thinking (Wit)), which respond to different ligands and activate distinct downstream transcription factors (Brummel et al., 1999; Gesualdi and Haerry, 2007). Unlike our observation with knockdown of *Babo*, knockdown of the other two type I receptors, *Tkv* or *Sax*, failed to affect AKH-induced hyperglycemia (Fig. 3A). Consistently, only knockdown of *dSmad2* (the transcription factor downstream of Babo), but not *Mad* (the transcription factor downstream of Tkv/Sax), significantly suppressed AKH-induced hyperglycemia (Fig. 3A).

To examine whether Babo signaling directly regulates AKH signaling, we treated larval fat bodies bearing *Babo* RNAi with synthetic AKH peptides. Interestingly, *Babo* RNAi potently blocked the AKH-induced increase in p-CREB2 and p-IRE1 (Fig. 3C). Suppression of Babo signaling using RNAi in the wild-type fat bodies significantly decreased glycemic levels under normal conditions (Fig. S3B). *Babo* RNAi further alleviated hyperglycemia and enhancement of AKH signaling in the fat body associated with high-sugar diet (Fig. 3B–C). Moreover, activation of Babo signaling via overexpression of either active Babo or dSmad2 in the fat body was sufficient to increase AKH signaling and glycemic levels in wild type larvae (Fig. 3D–E), indicating that Babo signaling directly enhances the fat body responses to AKH.

To determine whether the impact of Babo signaling on carbohydrate metabolism is dependent on AKH response, we manipulated Babo signaling in *AkhR*-deficient larval fat bodies. Interestingly, neither *Babo* knockdown nor *dSmad2* overexpression affected glycemic levels when *AkhR* was knocked down in the larval fat body (Fig. 3F). Similar results were observed in adults (Fig. S3H). Taken together, our results suggest that Babo signaling regulates carbohydrate metabolism and glycemic levels via modulation of the fat body response to AKH.

Importantly, knockdown of *Babo* in the fat body failed to affect insulin signaling, as indicated by p-Akt level and expression of FoxO targets (*4E-BP* and *InR*), or dILP2-induced hypoglycemia (Fig. S3E–G). These results indicate that the effects of Babo signaling on glycemic control are independent of insulin/Akt/FoxO signaling in larval fat bodies.

Babo signaling regulates *AkhR* mRNA expression

AkhR is essential for AKH signaling and carbohydrate metabolic regulation, and *AkhR* mRNA levels correlate with chronic high-sugar diet-induced hyperglycemia (Musselman et al., 2013) (Fig. S3D). Therefore, we hypothesized that Babo affects AKH signaling and high-sugar-induced hyperglycemia via, at least, control of *AkhR* mRNA levels. Consistent with our hypothesis, reducing Babo signaling led to a dramatic reduction in fat body *AkhR* mRNA levels under both normal and high-sugar diet conditions (Fig. S3C–D). We further found that overexpression of *dSmad2* in the fat body led to a significant increase in *AkhR*

mRNA levels (Fig. S3D). As Babo/dSmad2 signaling regulates sugar metabolism in an AkhR-dependent manner (Fig. 3F), our results collectively indicate that Babo/dSmad2 signaling mediates fat body AKH response via *AkhR* transcriptional regulation.

Actβ/Babo signaling modulates AKH response in the fat body

Babo expression is not elevated in the fat body in response to high-sugar diet (Fig. S4A). Thus, we examined the role of ligands that activate Babo in modulating AKH action. Two activin ligands, Actβ and Daw, regulate Babo/dSMAD2 signaling in *Drosophila* (Gesualdi and Haerry, 2007). To address whether one or both is involved in Babo-mediated AKH signaling, we first assessed the impact of Actβ on carbohydrate metabolism via a loss-of-function mutation (Actβ ^{ed80}) (Zhu et al., 2008) or ubiquitous knockdown (*Actin > Actβ-i*). In both conditions, we observed a decrease in glycemic levels (Fig. 4A and Fig. S4B–D). Reduction of *Act*β expression led to a decrease in the fat body AKH response and hyperglycemia under high-sugar diet conditions (Fig. 4A, 4D). Conversely, ectopic expression of Actβ in the larval fat body was sufficient to increase fat body AKH signaling and glycemic levels (Fig. 4B, 4D and Fig. S4E–F). The increase in glycemic levels was potently diminished by knockdown of either *Babo* or *AkhR* expression in the fat body (Fig. 4C and Fig. S4G). These data indicate that Actβ is involved in Babo-mediated AKH response and carbohydrate metabolism in the fat body.

Different Babo isoforms respond to individual ligands and result in diverse physiological outputs (Gesualdi and Haerry, 2007; Jensen et al., 2009). $Babo_a$ and $Babo_c$ are highly expressed in the larval fat body as revealed by RNA-seq analysis (Fig. 5A). We determined which Babo isoform(s) is required for Actβ regulation of AKH action using RNAi targeting distinct isoforms (Awasaki et al., 2011). Strikingly, only knockdown of $Babo_a$, but not $Babo_b$ or $Babo_c$, in the larval fat body significantly decreased Actβ-induced AKH signaling and hyperglycemia (Fig. 5B–C). Based on the similarity between Actβ and $Babo_a$ in neuronal development and growth regulation (Gesualdi and Haerry, 2007; Zhu et al., 2008), our results suggest that Actβ regulate carbohydrate metabolism in the fat body via $Babo_a$. Moreover, knockdown of $Babo_a$ also significantly alleviated AKH signaling and hyperglycemia in the AKH-overexpression flies (Fig. 5D–E). Our results thus demonstrate that Actβ/$Babo_a$ signaling modulates AKH activity and glycemic control in *Drosophila*.

Daw does not enhance AKH signaling in the fat body

The activin ligand Daw was previously shown to activate $Babo_c$ signaling and control glycemic levels (Ghosh and O'Connor, 2014; Jensen et al., 2009). Consistently, we observed that systemic knockdown of *Daw* decreased, whereas overexpression of *Daw* in the fat body increased, glycemic levels (Fig. 5F). However, unlike *Actβ, Daw* overexpression failed to enhance AKH signaling and *AkhR*- or *Babo*-loss in the fat body had little effect on Daw-associated glycemic changes (Fig. 5G–H). Thus, Daw is not likely to regulate glycemic levels by increasing AKH action in the fat body.

We further tested the role of Myo, another activin ligand, which is produced by muscles and functions via Babo/MAPK, but not Babo/dSmad2, signaling in the fat body (Demontis et al.,

2014). Ectopic expression of Myo in the larval muscle or fat body failed to enhance AKH signaling (data not shown).

Activin signaling enhances glucagon action in mouse hepatocytes

As activin signaling pathways are highly conserved between *Drosophila* and mammals, we hypothesized that, as in *Drosophila*, mammalian activin signaling might also regulate liver glucose metabolism via modulation of glucagon response. We test this using primary cultured mouse hepatocytes. Similar to the increase in *AkhR* mRNA levels by Actβ in *Drosophila*, stimulation by Activin A led to a significant increase in expression of the *glucagon receptor* (*GCGR*) in primary hepatocytes (Fig. S5A). Activin A further significantly enhanced glucagon signaling, as indicated by increased p-CREB and p-IRE1α levels, as well as glucagon-induced glucose production in primary hepatocytes (Fig. S5B–C). Treatment with the glucagon receptor antagonist, Cpd1 (Qureshi et al., 2004), potently blocked the effects of Activin A on glucagon-induced glucose production (Fig. S5C), demonstrating a glucagon-dependent mechanism. We also noticed an increase in glucose production under Activin A treatment alone (Fig. S5C), suggesting potential mechanism(s) independent of glucagon as well.

Next, we examined whether inhibition of activin signaling attenuates hepatic glucagon response by treating with the inhibitor SB 431542 (SB) (Inman et al., 2002). SB led to a significant suppression of activin signaling, as indicated by p-SMAD3, and alleviated activin A-enhanced glucagon signaling and hepatic glucose production (Fig. S5B–C). Interestingly, similar results were observed for TGF-β1. Specifically, we observed that TGF-β1, but not Myostatin (data not shown), significantly increased *GCGR* expression, glucagon signaling, and glucagon-induced glucose production in primary mouse hepatocytes (Fig. S5D–F). Taken together, our results indicate that, in mammals, activin signaling regulates hepatic carbohydrate metabolism, at least, via modulation of the glucagon response (Fig. S5G).

Midgut-derived Actβ regulates fat body AKH action and glycemic levels

We next examined the source(s) of Actβ that regulates AKH action and carbohydrate metabolism in the fat body. Consistent with previous results (Gibbens et al., 2011; Kim and O'Connor, 2014; Ting et al., 2007), we observed Actβ expression in the larval central neuron system (CNS), neuroendocrine cells, and motor neurons using Actβ-Gal4-driving GFP expression (Fig. S6A, C). Strikingly, we also observed Actβ expression in most enteroendocrine cells (EEs), as indicated with the EE marker Prospero (Pros), in the larval midgut (Fig. 6A and Fig. S6B). Actβ-expressing EEs also produce Tachykinin (TK), an abundant gut hormone (Amcheslavsky et al., 2014; Song et al., 2014), in the posterior midgut (Fig. 6B–C). Actβ expression in the midgut was confirmed by qPCR and further was found to be upregulated in response to a chronic high-sugar diet (Fig. 6D). High-sugar diet also increased Actβ induction in the motor neurons in the body wall, but not in the brain (Fig. 6D). Importantly, overexpression of Actβ using *Actβ-Gal4* potently enhanced AKH signaling in the fat body and led to hyperglycemia (Fig. 6E–F), mimicking the impact of a high-sugar diet on Actβ elevation and carbohydrate metabolic regulation.

To determine which cell types or tissues produce Actβ to enhance AKH response in the fat body, we used different Gal4 drivers to specifically overexpress Actβ in EEs (i.e. using *Pros-Gal4* or *TKg-Gal4*) or in motor neurons (*2–38-Gal4*) (Fig. S6C). Surprisingly, Actβ overexpression in all EEs (*Pros-Gal4*) or only in TK-expressing EEs (*TKg-Gal4*) was sufficient to increase AKH signaling in the fat body, as well as glycemic levels (Fig. 6E–F). Notably, the elevation in glycemia associated with Actβ overexpression in EEs was significantly diminished by *AkhR* deficiency (Fig. 6G), indicating that the hyperglycemia associated with EE-derived Actβ is dependent on AKH signaling. By contrast, Actβ overexpression in motor neurons (*2–38-Gal4*) failed to enhance fat body AKH signaling activity (Fig. 6H–I). We also excluded the effects of neuroendocrine cell-derived Actβ, as neither fat body AKH signaling nor glycemic levels were affected following Actβ overexpression in neuroendocrine cells (*386Y–Gal4* and *c929-Gal4*) (Fig. 6H–I and Fig. S6C). Therefore, our results indicate that Actβ production in EEs remotely regulates fat body AKH response and glycemic homeostasis.

A high-sugar diet leads to an increase in the number of Actβ-producing EEs

The *Drosophila* midgut is an important endocrine organ that senses environmental stresses and nutrient availability and produces multiple peptides or hormones that regulate systemic lipid and carbohydrate metabolism (Chng et al., 2014; Li et al., 2016; Song et al., 2014). We next investigated how chronic high-sugar feeding regulates Actβ expression in the EEs. To determine whether the effects of Actβ reflect an acute or a chronic condition, 3rd instar larvae were fed a high-sugar diet for 6h, a time sufficient to evoke an acute response. In contrast to chronic feeding, acute feeding of a high-sugar diet failed to elevate midgut *Act*β expression, AKH signaling or glycemic levels (Fig. S6D–F). We next asked whether hemocyte infiltration-associated immune and/or inflammatory responses, as suggested by induction of Jak/Stat or NF-κB signaling (Woodcock et al., 2015), contribute to high-caloric diet-induced Actβ induction in EEs. However, we failed to observe any changes in hemocyte infiltration (Hml > GFP) in the midgut, and did not observe changes in Jak/Stat signaling (Stat-GFP reporter) or NF-κB signaling (Dpt-GFP reporter) in EEs (Fig. S7A–C), suggesting that immune and/or inflammatory responses do not directly regulate *Act*β expression in EEs.

During this analysis we did note a striking observation that the number of EEs and the expression of EE marker *Pros* in the midgut were potently increased by chronic high-sugar feeding (Fig. 7A–C and Fig. S7A–C). These results indicate that chronic high-sugar feeding perturbs midgut homeostasis and leads to an increase in EE production. Consistently, the number of Actβ-producing EEs was also significantly increased under chronic high-sugar feeding (Fig. 7A–B). In contrast, the overall midgut size, as well as the mass of intestinal stem cells (ISCs) and enterocytes (ECs), were significantly decreased (Fig. S7D–E). The qPCR results also revealed that mRNA levels of the ISC marker *esg* and EC marker *Pdm1* were significantly decreased (Fig. S7F).

As starvation causes effects that are similar to what we observe with chronic high-sugar feeding, including suppressed insulin signaling and increased AKH signaling (Fig. S7G, I), we further examined whether starvation affects EE number and Actβ expression in the

midgut. 3rd instar larvae were cultured for 6h on food containing only agar. However, we failed to observe an induction in the number of EEs, *Act*β expression in the midgut, or *AkhR* expression in the fat body (Fig. S7G–H). These results indicate that Actβ levels are not induced by acute nutrient deprivation.

Finally, we examined whether the increase in EE-derived Actβ is essential for high-sugar diet-induced hyperglycemic levels by knocking down *Act*β specifically in EEs. Knockdown of *Act*β using the Pros-Gal4 driver caused a dramatic decrease in *Act*β mRNA levels in the midgut under chronic high-sugar diet (Fig. 7D). Importantly, *Act*β knockdown in EEs significantly lowered the increases in both AKH signaling in the fat body and glycemic levels associated with chronic high-sugar feeding (Fig. 7E–F). Taken together, our results indicate that chronic high-sugar feeding perturbs homeostasis of EEs and increases Actβ production in the midgut, leading to a non-cell autonomous enhancement of AKH signaling in the fat body and hyperglycemia (Fig. 7G).

Discussion

Chronic high-caloric diets have been reported to impair insulin action and elevate glycemic levels in both vertebrates and invertebrates (Musselman et al., 2011; Pasco and Leopold, 2012; Susini et al., 1979). Using *Drosophila* as a conserved model of metabolic regulation, we demonstrate that enhanced AKH action, which mobilizes energy storage and increases circulating carbohydrate levels as an insulin counter-regulatory hormone, is also essential for high-sugar diet-induced hyperglycemia. We show that chronic high-sugar feeding abnormally augments AKH sensitivity in the fat body, consistent with previous observation of increased *AkhR* expression in this tissue (Musselman et al., 2013). Importantly, impaired AKH signaling in the fat body due to *AkhR* deficiency significantly alleviates the hyperglycemia induced by chronic high-sugar feeding or insulin resistance. However, acute high-sugar feeding fails to increase AKH action or glycemic level, indicating a pathogenic, but not physiological, enhancement of AKH response in the fat body under chronic high-sugar diet. Altogether, our study identifies an insulin-independent mechanism for diet-associated hyperglycemia.

Identification of regulators of AKH signaling

To study how intracellular AKH signaling is enhanced, we performed an *in vivo* RNAi screen targeting kinases and phosphatases and identified 30 potential novel candidates that modulate AKH signaling and glycemic control. Future studies that aim to elucidate how these candidates regulate AKH action in the fat body in response to a chronic high-sugar diet will offer a more comprehensive understanding of diet-associated hyperglycemic pathogenesis beyond insulin regulation.

In addition to AKH signaling in the fat body as presented here, AKH production or secretion from CC cells may also contribute to diet-associated hyperglycemia. Interestingly, several metabolic regulators, including AMPK and water sensor ppk28, have previously been shown to affect AKH production and/or secretion (Braco et al., 2012; Kim and Neufeld, 2015; Kim and Rulifson, 2004; Waterson et al., 2014). Thus, regulation of AKH production and/or

secretion in the context of high-caloric feeding will be another interesting question to address.

Actβ/Babo$_a$ regulate AKH response in the fat body

Our results demonstrate that Actβ-induced Babo action in the fat body potentiates AKH signaling. Specifically, we found that Babo signaling triggers an increase in dSmad2 transcriptional activity and *AkhR* expression. Interestingly, in addition to Actβ, Babo also responds to other activin ligands, including Daw and Myo. Among these activin ligands, only Actβ and Daw signal through dSmad2 (Gesualdi and Haerry, 2007). However, several lines of evidence suggest that Actβ and Daw exert different effects on Babo signaling and trigger distinct downstream outputs. First, Daw deficiency results in reduced insulin signaling and hyperglycemia, but fat body overexpression of an active form of Babo fails to restore normal glycemic levels in *Daw* mutants (Ghosh and O'Connor, 2014). Consistently, our results revealed that Babo deficiency in the fat body fails to affect Daw-induced hypoglycemia (Fig. 5H), indicating that Daw regulation of carbohydrate metabolism is independent of fat body Babo signaling. A plausible explanation is that Daw controls dILP2 secretion to modulate systemic insulin effects in a non-cell autonomous manner (Bai et al., 2013; Ghosh and O'Connor, 2014). In contrast, Actβ promotion of AKH signaling is dependent on Babo function in the fat body. Second, Daw preferentially targets Babo$_c$ (Jensen et al., 2009), but the Babo isoform used for Actβ signaling has not previously been reported. In this study we demonstrate that Actβ modulates AKH signaling and hyperglycemia via Babo$_a$ in the fat body, but not via Babo$_b$ or Babo$_c$. Collectively, we conclude that Actβ/Babo$_a$ regulates AKH signaling in the fat body, whereas Daw/Babo$_c$ regulates dILP2 secretion in CNS, to differentially control carbohydrate homeostasis. Note that we exclude the possibility that Mav, another activin ligand, is relevant to AKH signaling as it has only a slight effect on Babo signaling *in vivo* (M. O'Connor, unpublished data). We also exclude another activin ligand, Myo, which signals through Babo/MAPK in the fat body (Demontis et al., 2014), as Myo shows no effects on AKH signaling (unpublished data).

Actβ produced in midgut EEs affects fat body AKH signaling

One of our striking findings is that Actβ produced by midgut EEs affects the fat body AKH signaling, implicating a midgut-to-fat-body axis in glycemic control. Actβ has been previously characterized as a neuronal factor and is abundantly expressed in the central and peripheral nervous systems (PNS) to modulate neuronal development and activity (Kim and O'Connor, 2014; Zhu et al., 2008). We demonstrate for the first time that Actβ is abundantly expressed in EEs of the larval midgut. Midgut EEs are key endocrine cells that sense environmental cues, including stresses and dietary nutrients, and secrete gut hormones that modulate the functions of other tissues (Chng et al., 2014; Li et al., 2016; Song et al., 2014). Strikingly, Actβ overexpression in EEs (Pros-Gal4) leads to a dramatic increase in hyperglycemia in a manner dependent on *Akh* response in the fat body, suggesting inter-organ communication between the midgut and the fat body. Further, using a midgut-specific TKg-Gal4 that targets TK EEs (i.e. about ~23% of Actβ-positive EEs), we show that overexpression of Actβ only in a few EEs is sufficient to lead to a significant increase in fat body AKH signaling activity and glycemic levels. These results are consistent with a model in which Actβ production in midgut EEs affects fat body physiology. Finally, knockdown of

Actβ in the midgut using RNAi potently alleviates the increased fat body AKH action and hyperglycemia associated with a chronic high-sugar diet, indicating an essential role for midgut Actβ in diet-associated fat body AKH action and carbohydrate metabolism.

We also noticed that Actβ overexpression in neuroendocrine cells (386Y–or c929-Gal4) or in motor neurons (2–38-Gal4) failed to enhance AKH response in the fat body. Possibly, Actβ may function as a regional hormone, as the larval midgut is surrounded by the fat body, which is relatively far from neuroendocrine cells in the brain and motor neurons in the body wall. Alternatively, only mature Actβ hormone that is produced from the midgut through specific post-translational modifications is able to enhance AKH response in the fat body. In support of this model, different processing regulations of the Ast B prohormone in EEs and neurons have been observed in *Drosophila* (Baggerman et al., 2002; Reiher et al., 2011). The release of active mammalian TGF-β is also context-dependent of distinct activation/localization of TGF-β complexes (Annes et al., 2003).

Intriguingly, we found that a chronic high-sugar diet is associated with an increase in EE number. Several signaling pathways have been implicated in ISC proliferation and EE differentiation in the adult midgut (Zeng et al., 2015), in particular JAK/STAT signaling (Lin et al., 2010). Interestingly, we have observed an increase in JAK/STAT signaling in ISCs under chronic high-sugar feeding (data not shown). Further, Upd3, a Jak/Stat ligand, is elevated in both hemocytes (Woodcock et al., 2015) and enterocytes (data not shown) under high-caloric diet. Further studies that elucidate in detail the signaling crosstalk that controls EE generation in the late larval midgut will be required to understand how a chronic high-sugar diet increases EE number and Actβ production.

Relevance to Mammalian Physiology

Maintenance of stable circulating carbohydrate levels via a balance between insulin and counter-regulator hormones, particularly glucagon, is conserved in mammals and *Drosophila*. Whereas insulin regulation is well studied, less is known about AKH/glucagon. Here, we demonstrate that AKH action in the *Drosophila* fat body is highly similar to glucagon action in the mammalian liver with regards to diet-induced hyperglycemia. Interestingly, and reminiscent of AKH regulation in *Drosophila*, liver glucagon action is aberrantly enhanced in high-fat diet conditions and a reduction of glucagon signaling specifically in the liver improves glucose tolerance in type II diabetic or insulin resistant subjects (Ali and Drucker, 2009; Liang et al., 2004; Matsuda et al., 2002). Moreover, newly-characterized physiological regulators of mouse glucagon signaling, including the unfolded protein response (UPR) sensor IRE1α and the inflammatory NF-κB signaling (Mao et al., 2011; Sheng et al., 2012), have been shown to play similar roles in AKH regulation in *Drosophila* (Fig. 2). Thus, our study provides a conserved model of glucagon regulation and will help to comprehensively characterize regulatory networks involved in liver glucagon signaling.

Like the response of *Drosophila* Babo to Actβ, the mammalian receptors ALK4 and ALK5 respond to the ligands activin A, TGF-β1, and Myostatin to activate Smad2/3 transcriptional activity (Pauklin and Vallier, 2015; Rebbapragada et al., 2003). We found that long-term treatment with activin A or TGF-β1, but not Myostatin, potently enhances glucagon

signaling to increase glucagon-induced glucose production in mouse primary hepatocytes. Activin A and TGF-β1 are produced in multiple organs in mammals, including EEs, and regulate liver growth and inflammation (Bogunovic et al., 2007; Kreidl et al., 2009; La Rosa et al., 2004). Enhanced glucagon signaling and increased activin A/TGF-β1 levels have been coincidently observed in obese or diabetic animals (Mao et al., 2011; Sheng et al., 2012; Yadav et al., 2011; Zaragosi et al., 2010). Importantly, mice with a mutation in *Inhba*, which encodes the inhibin βA subunit of Activin A, are lean and show improved glucose tolerance and hyperglycemia (Li et al., 2009). TGF-β1 or Smad3 deficiency also protects mice from diet-induced diabetes (Yadav et al., 2011). These observations are consistent with our proposal that activin A/TGF-β1 regulates hepatic glucagon action and glucose production to impact glycemic homeostasis. However, consistent with an *in vitro* study indicating that acute treatment of activin A alone promotes liver glycogen breakdown and glucose production (Kojima et al., 1995), we also observed a basal induction of glucose production by activin A alone, suggesting the presence of a glucagon-independent mechanism. Thus, it will be of interest to characterize the nature and role of glucagon-dependent and -independent mechanisms in regulating liver glucose production under different conditions, such as long- or short-term activin ligand treatment.

Methods

Drosophila studies

Strains and culture methods used in this study are listed in the Sup Methods.

High-sugar diet and carbohydrate measurement

25% extra sucrose was added into the standard diet to generate the high-sugar diet food. Determination of carbohydrate levels has previously been described (Kwon et al., 2015; Song et al., 2010). Trehalase (E-TREH) and glucose reagent (K-GLUC) were obtained from Megazyme.

RNA-seq transcriptome analysis

10 fat bodies from late 3rd instar larvae of each genotype were collected for RNA-seq. Details on samples preparation and data analyses are in the Sup. Methods. RNA-seq data were deposited in the Gene Expression Omnibus (Accession number GSE92350).

Generation of stable AkhR-expressing S2R+ cells

A full-length *AkhR* cDNA was generated by RT-PCR using primers 5'-CCGGAATTCGAGGCAAATCCTTGATGCAG and 5'-AGAATGCGGCCGCACTTCTGGCGGATCGGGGAT, verified by DNA sequencing, and inserted into vector pAC5-Stable2-Puro (Gonzalez et al., 2011). Stable cell selection is described in the Sup. Methods.

Primary hepatocyte culture, HGP assays, and immunoblots

Mouse primary hepatocytes preparation and HGP assays have been previously described (Hong et al., 2015; Sheng et al., 2012). Immunoblots were performed as previously described (Song et al., 2010).

qPCR

10 fat bodies, 10 midguts, 10 body walls, or 10 brains from late 3rd instar larvae of each genotype were collected for RNA extraction and qPCR analysis as previously described (Song et al., 2014). Primers are listed in the Sup. Methods.

Immunostaining

Immunostainings of S2R+ cells and larval midgut and brain have been previously described (Song et al., 2010; Song et al., 2014).

Statistical Analyses

Data are presented as the mean ± SEM. Student's t tests were used to compare two groups. $p < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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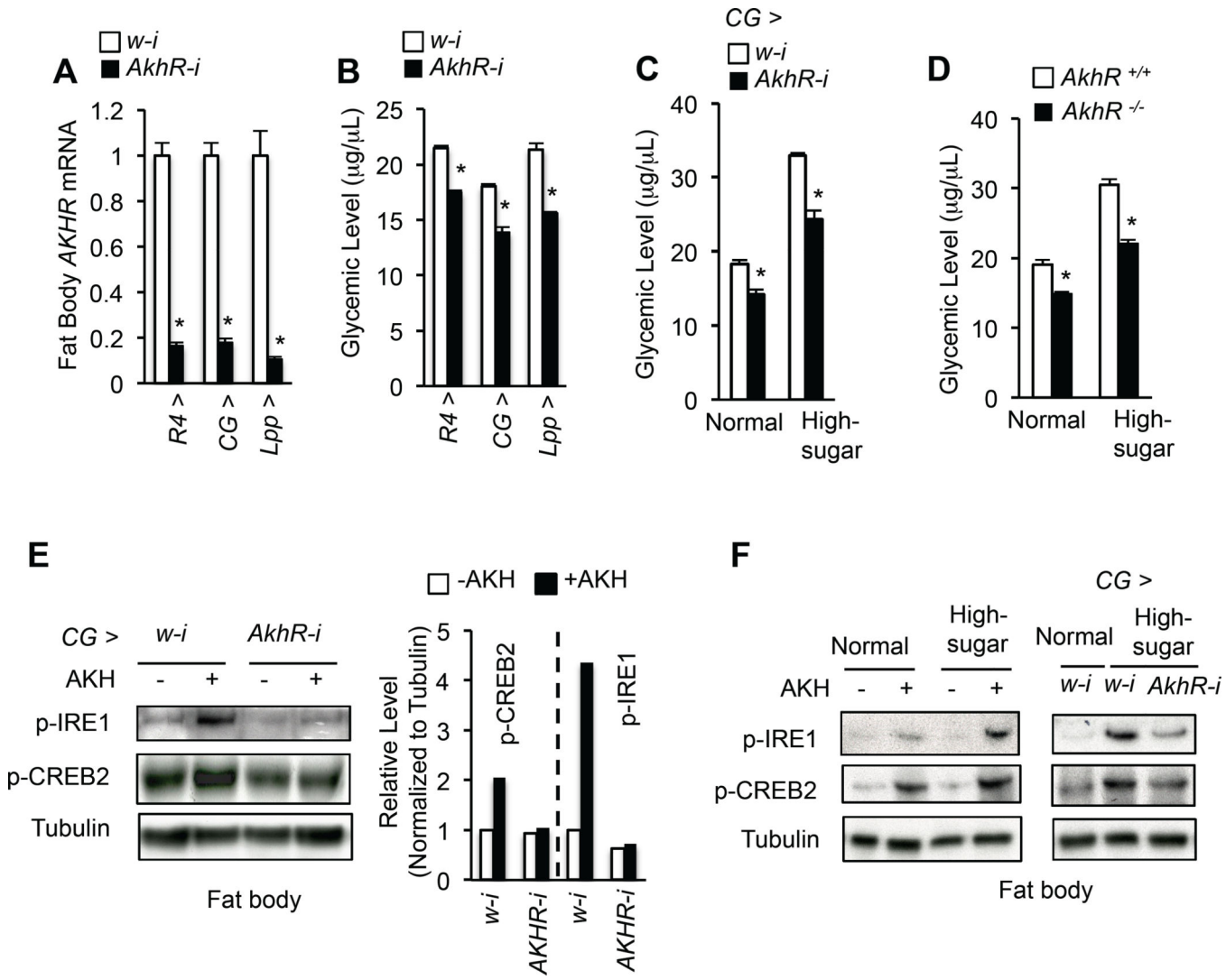

Figure 1. Enhanced AKH signaling contributes to hyperglycemia in response to a high-sugar diet

(**A, B**) Fat body *AkhR* mRNA levels (**A**, n=4, 40 larval fat bodies) and glycemic levels (**B**, circulating glucose + trehalose, n=4, 40 larvae) in 3rd instar larvae expressing RNAi against *w* (*white*, control) or *AkhR* with different fat body Gal4 drivers. (**C, D**) Glycemic levels in *CG > AkhR-i* (*CG-Gal4/+; UAS-AkhR-RNAi/+*) (**C**) or *AkhR* $^{-/-}$ (**D**) 3rd instar larvae were fed with normal or high-sugar food (n=4, 40 larvae). (**E**) Immunoblots (left) and quantification (right) of p-IRE1 and p-CREB2 in freshly isolated 3rd instar larval fat bodies that were treated with or without 1 μM synthetic AKH peptide for 30 min. (**F**) Immunoblots of p-IRE1 and p-CREB2 in fresh isolated wild type 3rd instar larval fat bodies treated with or without 1 μM synthetic AKH peptide for 30 min (left) and in fresh isolated fat bodies of 3rd instar larvae fed with or without high-sugar diet (right). Data are presented as means ± SEM. * $p < 0.05$.

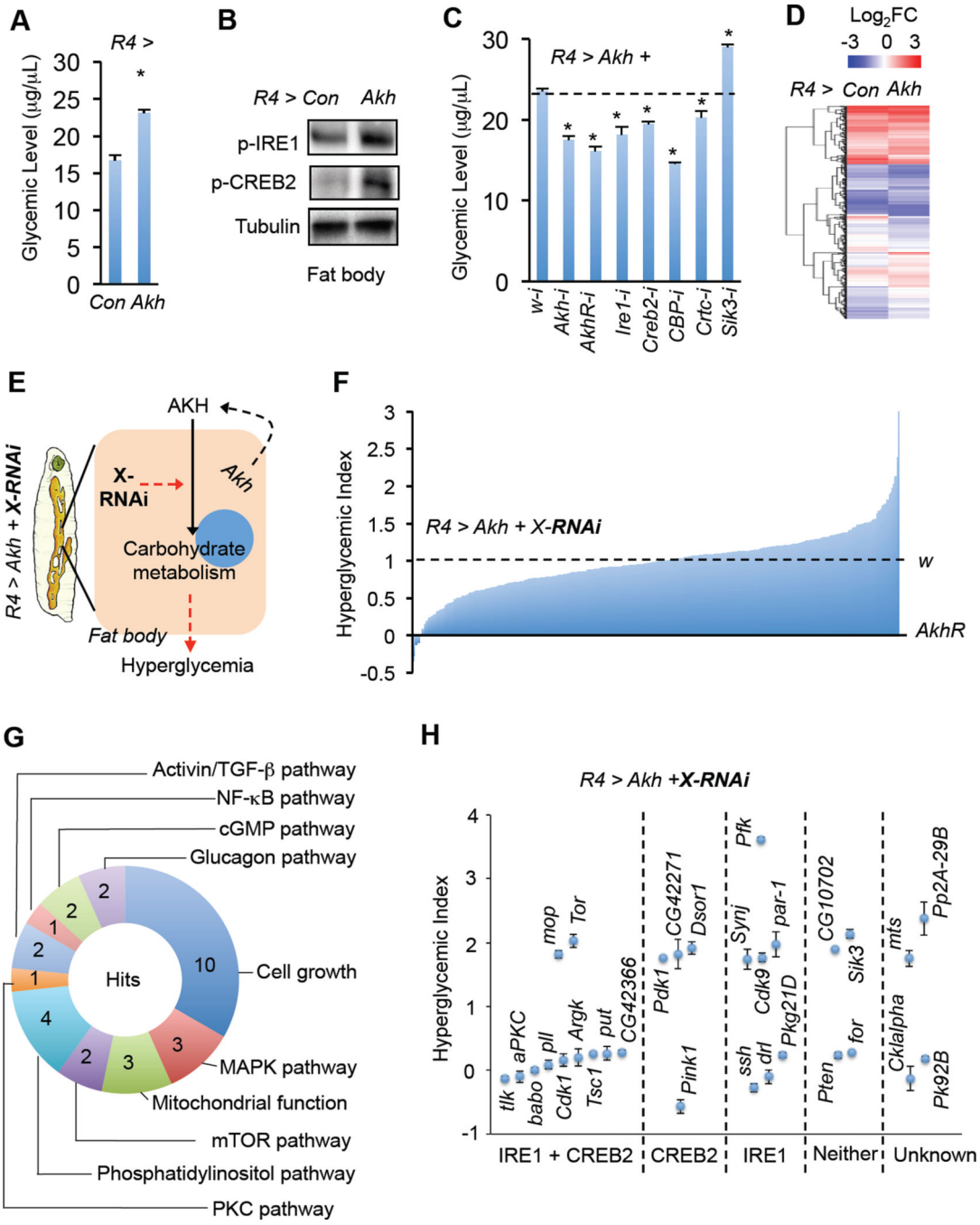

Figure 2. An AKH-induced hyperglycemia model and *in vivo* RNAi screen

(**A–D**) Glycemic levels (glucose + trehalose) (**A**, n=4, 40 larvae), immunoblots (**B**), and gene expression (**D**) in fat bodies from *R4 > AKH* (*UAS-Akh/+; R4-Gal4/+*) 3rd instar larvae. (**C**) Glycemic levels of *R4 > AKH* larvae with knockdown of AKH/glucagon signaling components (n=4, 40 larvae). Schematic (**E**) and results (**F**) of the *in vivo* RNAi screen of kinases and phosphatases in AKH-induced hyperglycemic flies. Glycemic levels in *R4 > Akh + w-i* (*UAS-Akh/+; R4-Gal4/UAS-w-RNAi*) and *R4 > Akh + AkhR-i* (*UAS-Akh/+; R4-Gal4/UAS-AkhR-RNAi*) were normalized to 1 (100%) and 0, respectively (n=3,

30 larvae). (**G**) Annotation of novel positive gene 'hits' identified in the *in vivo* RNAi screen (> 1.7 or < 0.3 as cutoffs) for signaling pathways or biological processes. (**H**) 30 hits modulate AKH-induced hyperglycemia via regulation of p-IRE1 or p-CREB2. Data are presented as means ± SEM. * $p < 0.05$.

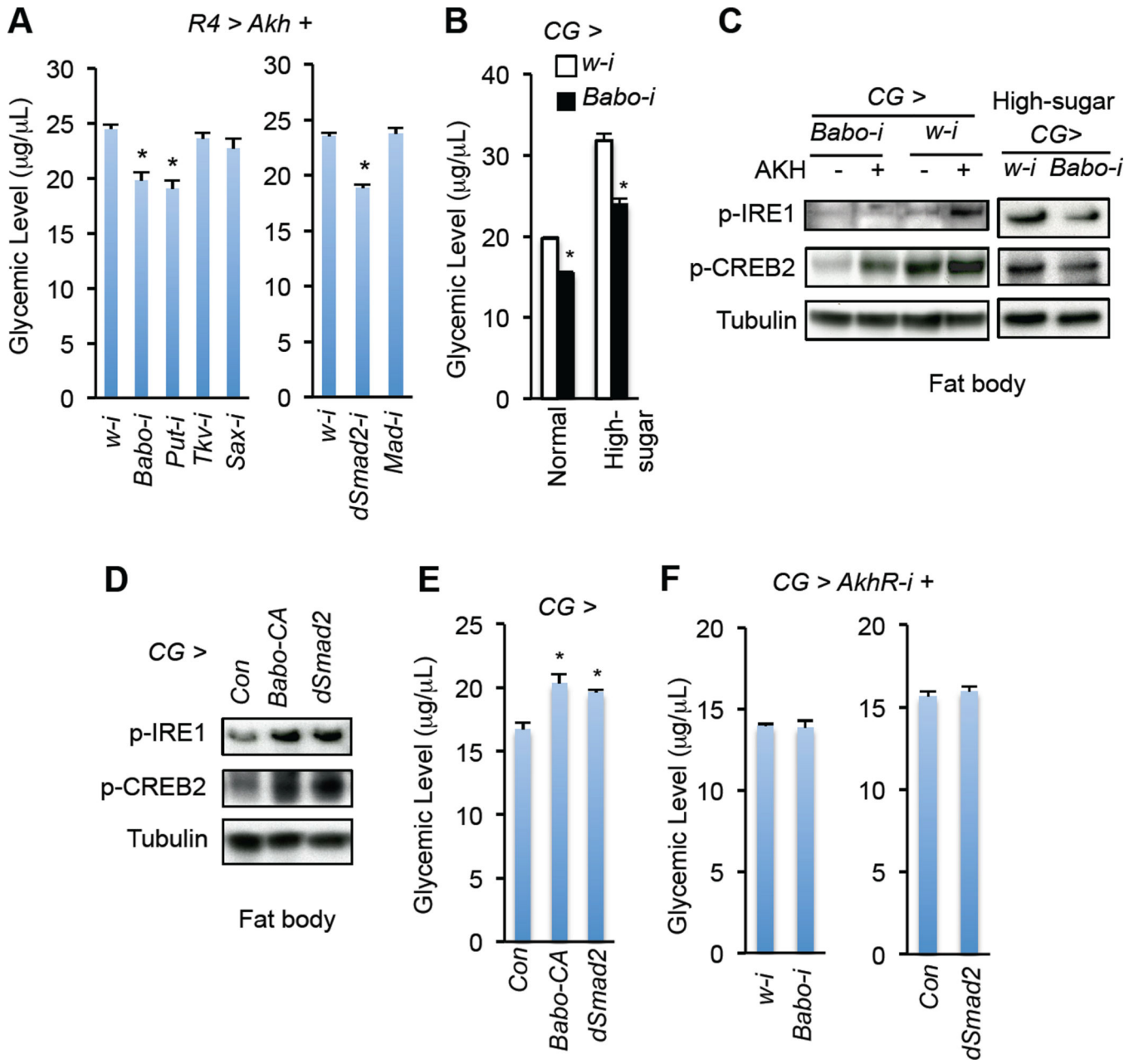

Figure 3. Babo signaling regulates AKH action and glycemic control

(**A**) Effects of knockdown of individual TGF-β-like receptors (left) or downstream transcription factors (right) in the fat body on glycemic levels (glucose + trehalose) in *R4 > Akh* 3rd instar larvae (*UAS-Akh/+; R4-Gal4/UAS-RNAi*) (n=4, 40 larvae). (**B**) Glycemic levels in *CG > Babo-i* larvae (*CG-Gal4/+; UAS-babo-RNAi/+*) fed with normal or high-sugar food (n=4, 40 larvae). (**C–D**) Immunoblots of protein from freshly isolated larval fat bodies that were treated with or without 1 μM synthetic AKH peptide for 30 min (**C**, left) or freshly isolated fat bodies of 3rd instar larvae fed a high-sugar diet (**C**, right) or a normal diet (**D**). (**E**) Glycemic levels in *Babo* or *dSmad2* overexpressing 3rd instar larvae (n=4, 40 larvae). (**F**) Glycemic levels in 3rd instar larvae bearing *Babo* knockdown (left, *CG-Gal4/+;*

UAS-AkhR-RNAi/UAS-Babo-RNAi) or *dSmad2* overexpression (right, *CG-Gal4/+; UAS-AkhR-RNAi/UAS-dSmad2*) in the context of *AkhR* deficiency in fat body (n=4, 40 larvae). Data are presented as means ± SEM. * $p < 0.05$.

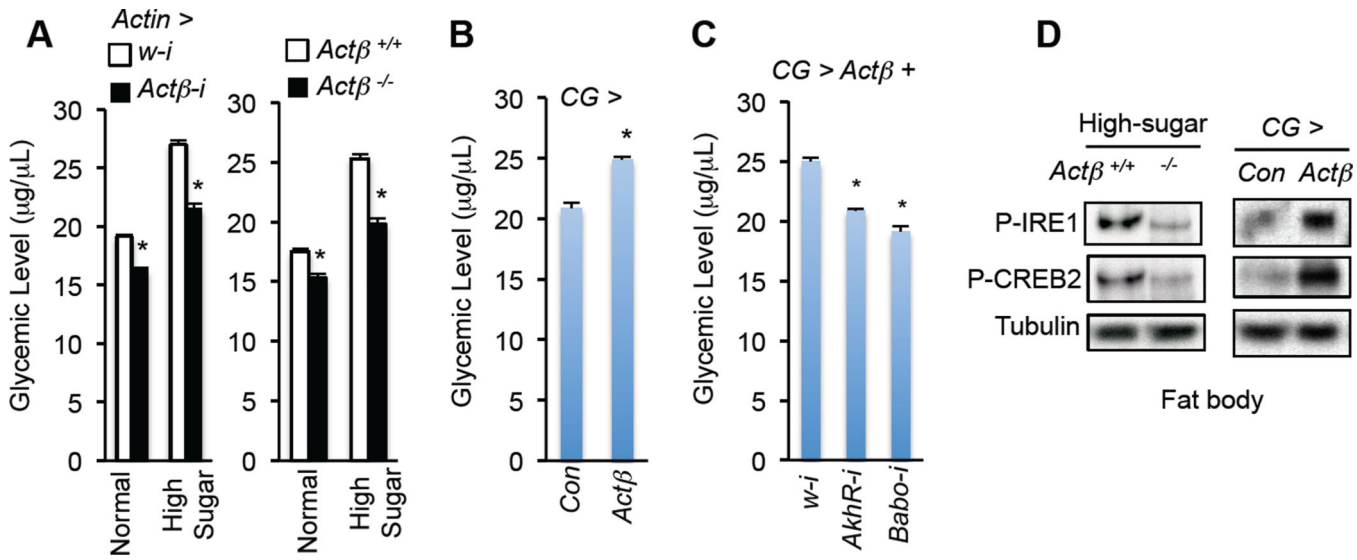

Figure 4. Actβ signals through Babo to affect AKH signaling in the fat body

(**A**) Glycemic levels (glucose + trehalose) in 3rd instar larvae with systemic *Act*β knockdown (left, *Actin-Gal4/UAS-Actβ-RNAi*) or knockout (right, *actβ* ^{ed80} or Actβ $^{-/-}$) fed a standard or high-sugar diet. (**B, C**) Glycemic levels in *CG > Act*β (*CG-Gal4/+; UAS-Actβ/+*) larvae (**B**) or *CG > Act*β larvae with gene knockdown (*CG-Gal4, tub-Gal80*^{ts}*/+; UAS-Actβ/UAS-RNAi*) (**C**) (n=4, 40 larvae). (**D**) Immunoblots in freshly isolated fat bodies of *Actβ*$^{-/-}$ larvae fed a high-sugar diet (left) or *CG > Act*β larvae fed normal food (right). Data are presented as means ± SEM. * $p < 0.05$.

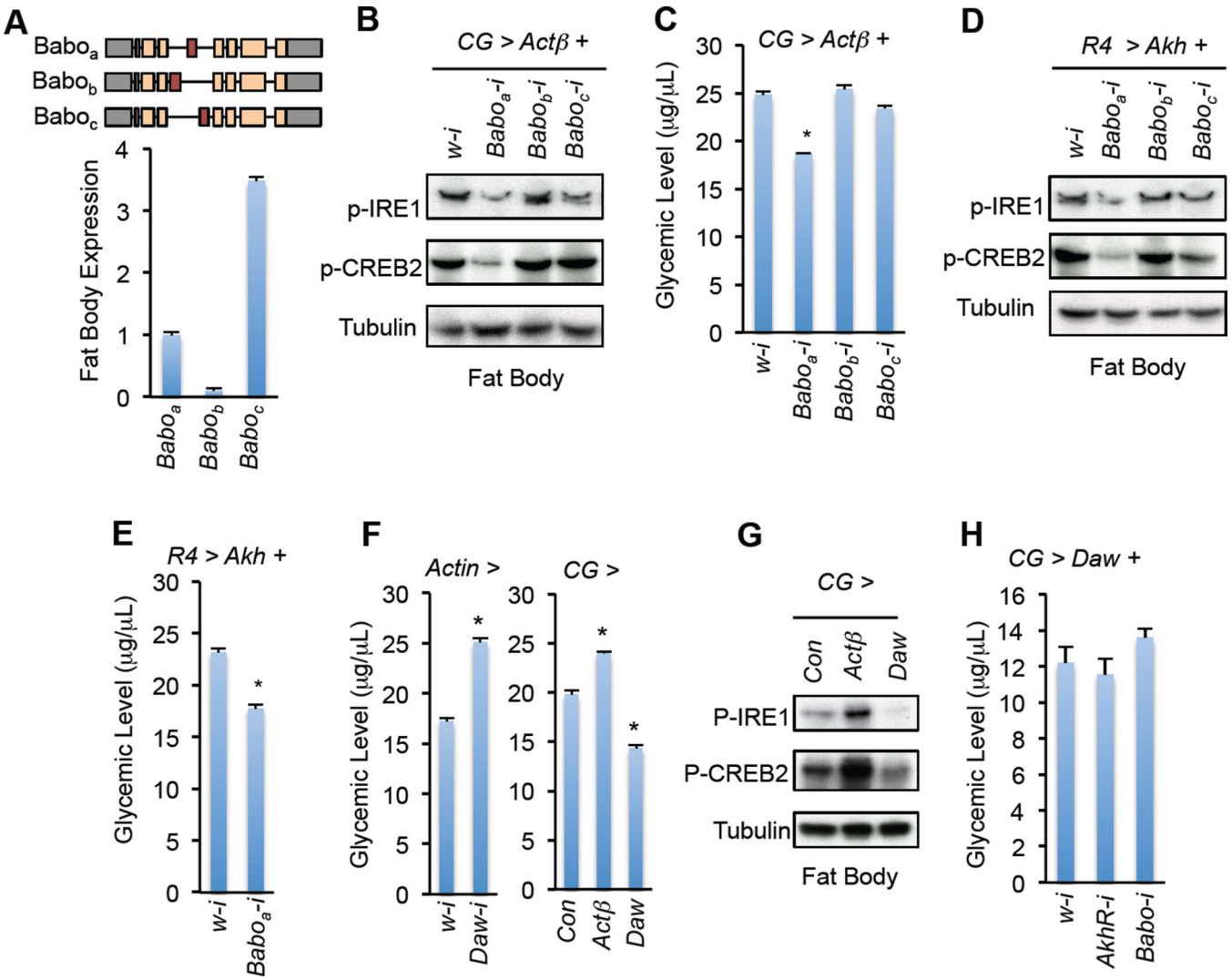

Figure 5. Babo$_a$ responds to Actβ regarding modulation of AKH action and glycemia

(**A**) Schematic and relative FPKM value of different *Babo* isoforms indicated by RNA-seq of 3rd instar larval fat body (n=3, 30 larval fat bodies). (**B, C**) Fat body immunoblots (**B**) and glycemic levels (**C**) (n=4, 40 larvae) of *CG > Actβ* larvae with knockdown of Babo isoforms. Genotype is *CG-Gal4, tub-Gal80^{ts} /+; UAS-Actβ/UAS-RNAi.* (**D, E**) Fat body immunoblots (**D**) and glycemic levels (**E**) (n=4, 40 larvae) of *R4 > Akh* larvae with knockdown of Babo isoforms. Genotype is *UAS-Akh/+; R4-Gal4/UAS-RNAi.* (**F**) Glycemic levels in *Actin > Daw-i* larvae (left, n=4, 40 larvae) and in larvae with fat body overexpression of *Actβ* or *Daw* (right, n=4, 40 larvae). (**G**) Immunoblots of fat body in larvae with fat body overexpression of *Actβ* or *Daw*. (**H**) Glycemic levels in 3rd instar larvae with fat body overexpression of *Daw* plus knockdown of *AkhR* or *Babo* (n=4, 40 larvae). The genotype is *CG-Gal4, tub-Gal80^{ts}/+; UAS-Daw/UAS-RNAi.* Data are presented as means ± SEM. * $p < 0.05$.

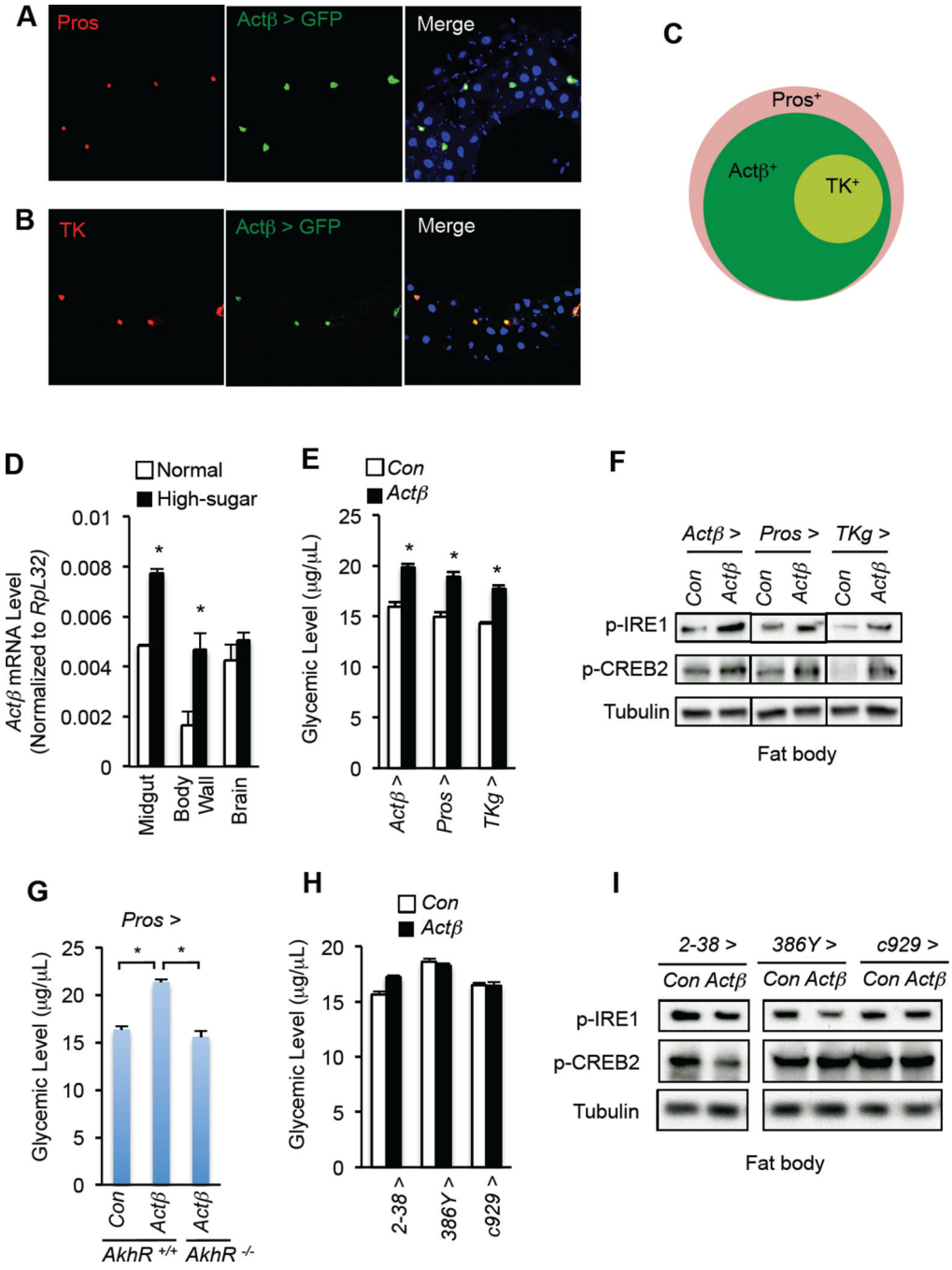

Figure 6. Enteroendocrine cell-derived Actβ modulates AKH action in the fat body

(**A, B**) Expression of *Actβ > GFP* (green, *UAS-srcGFP/+; Actβ-Gal4/+*) in EEs, indicated by Pros (**A**, red) or TK (**B**, red), in 3rd larval posterior midgut. (**C**) Schematic *Actβ > GFP* expression pattern in larval EEs. (**D**) *Act*β mRNA levels in different larval tissues under high sugar diet. (**E–I**) Glycemic levels (glucose + trehalose) (**E, H**) (n=4, 40 larvae) and fat body immunoblots (**F, I**) in Actβ-overexpressing larvae. (**G**) Glycemic levels in *AkhR $^{+/+}$; Pros > Con* (*AkhR $^{+}$/AkhR $^{+}$; Pros-Gal4/+*), *AkhR $^{+/+}$; Pros > Actβ (AkhR $^{+}$/AkhR $^{+}$; Pros-Gal4/*

UAS-Actβ), and *AkhR* $^{-/-}$*; Pros > Actβ (AkhR /AkhR; Pros-Gal4/UAS- Actβ*) larvae (n=4, 40 larvae per group). Data are presented as means ± SEM. * $p < 0.05$.

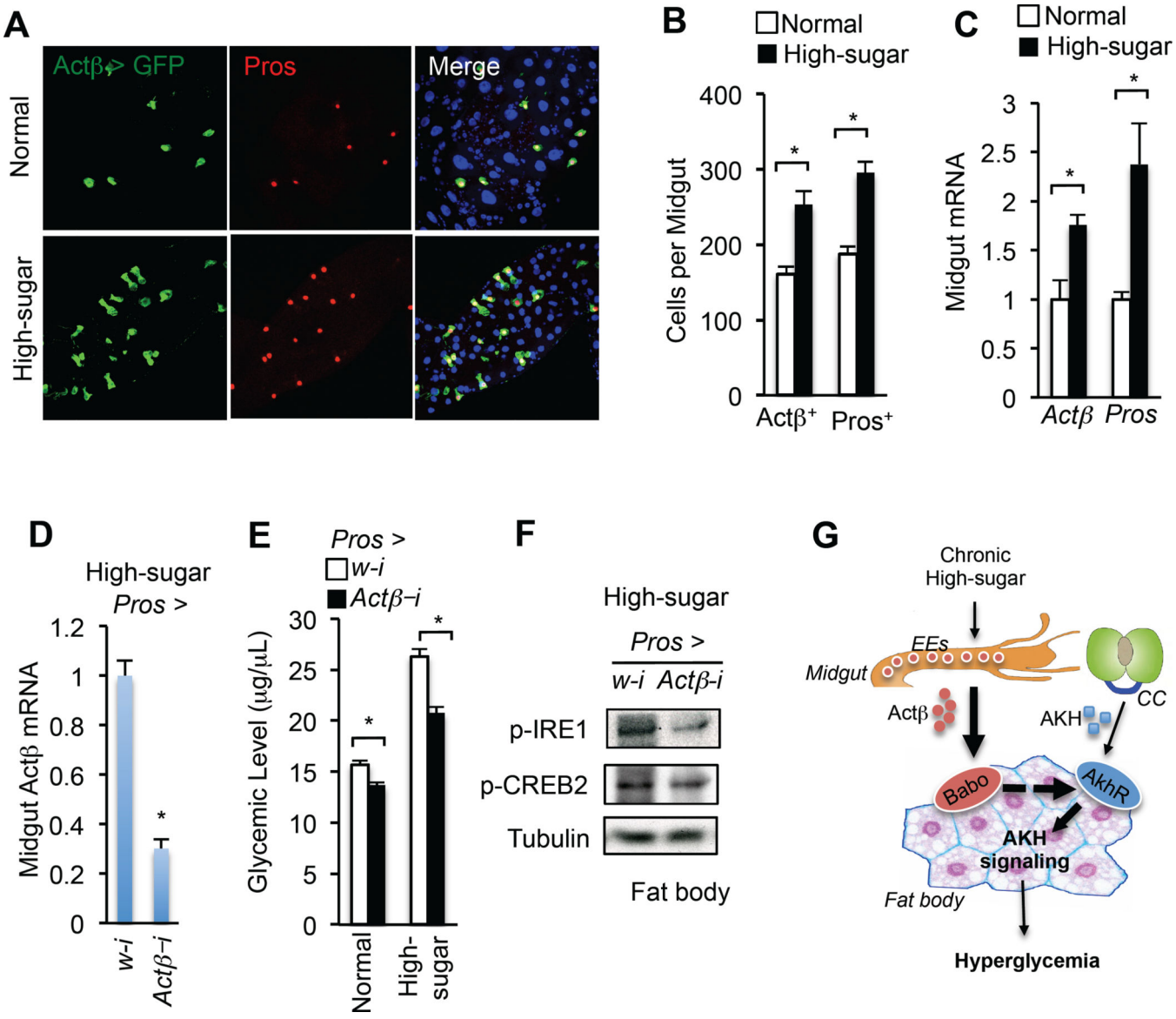

Figure 7. Chronic high-sugar diet increases EE number and Actβ production in the larval midgut

(**A–C**) Confocal image (**A**), number of *Actβ > GFP* or Pros$^+$ cells (**B**) (n=15, 15 larval guts), and gene expression (**C**) (n=4, 40 larval guts), in the larval midgut under high-sugar diet. (**D–F**) Midgut *Act*β mRNA (**D**) (n=4, 40 larval guts), glycemic levels (glucose + trehalose) (**E**) (n=4, 40 larvae), and (**F**) immunoblots of fat body from *Pros > Actβ-i* larvae (*Pros-Gal4/UAS-Actβ-RNAi*) fed a chronic high-sugar diet. (**G**) The midgut-to-fat-body axis in glycemic control. Chronic high-sugar diet leads to an increase in Actβ production in the EEs of larval midgut and enhances the fat body AKH signaling, resulting in hyperglycemia, via Actβ/Babo signaling. Data are presented as means ± SEM. * $p < 0.05$.