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**Supplemental Information**

**Midgut-Derived Activin Regulates  
Glucagon-like Action  
in the Fat Body and Glycemic Control**

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Figure S1

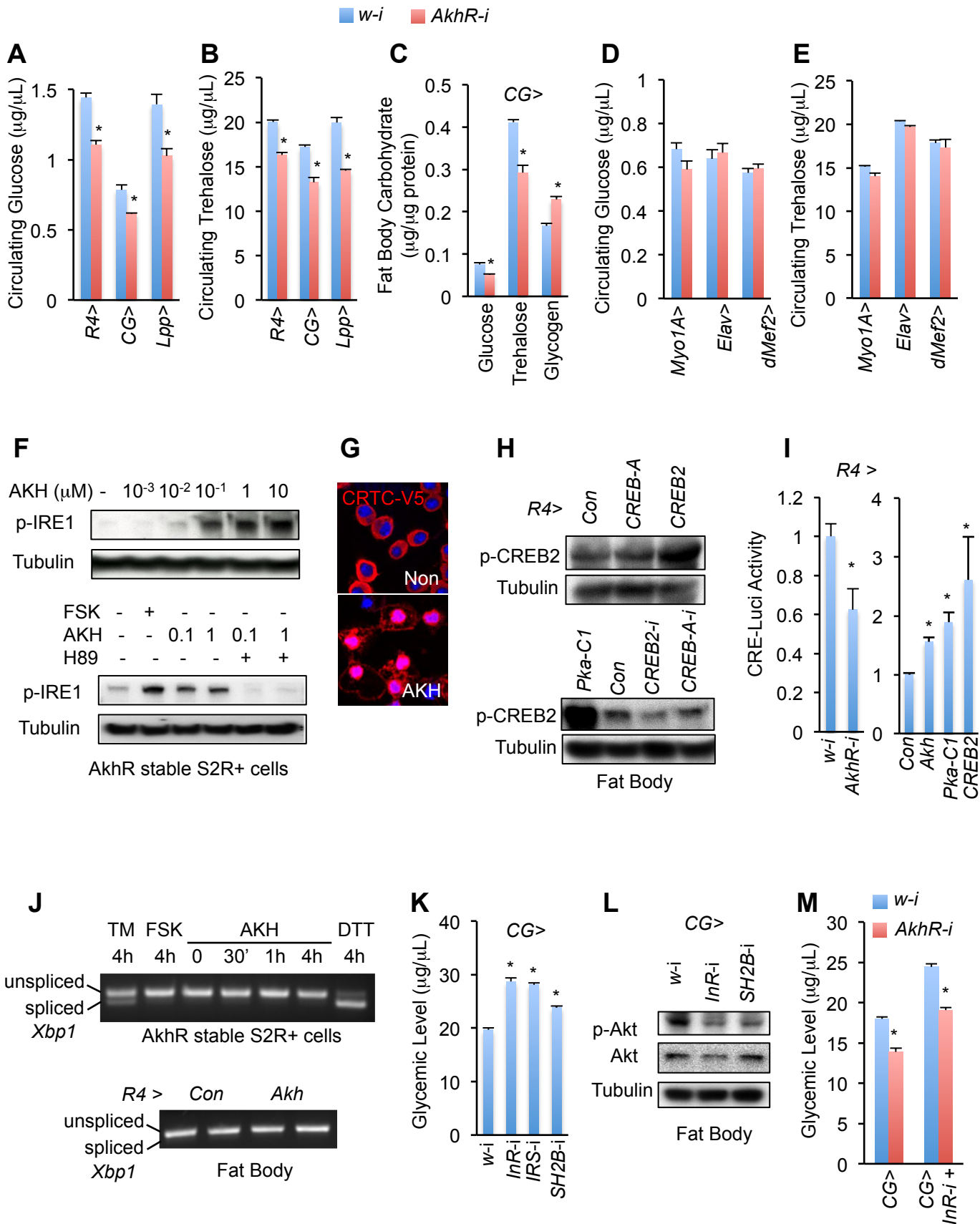
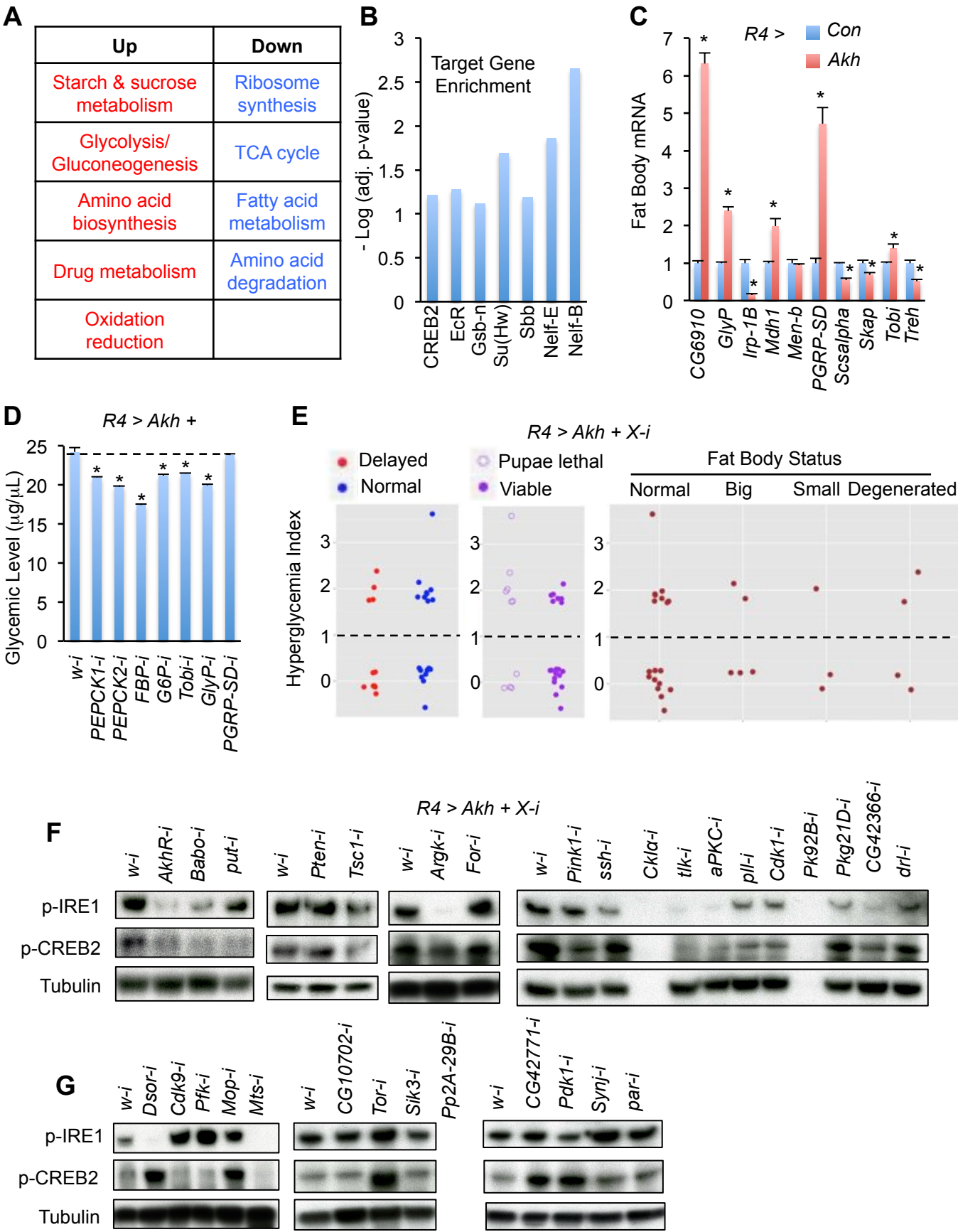


Figure S2



**Figure S3**

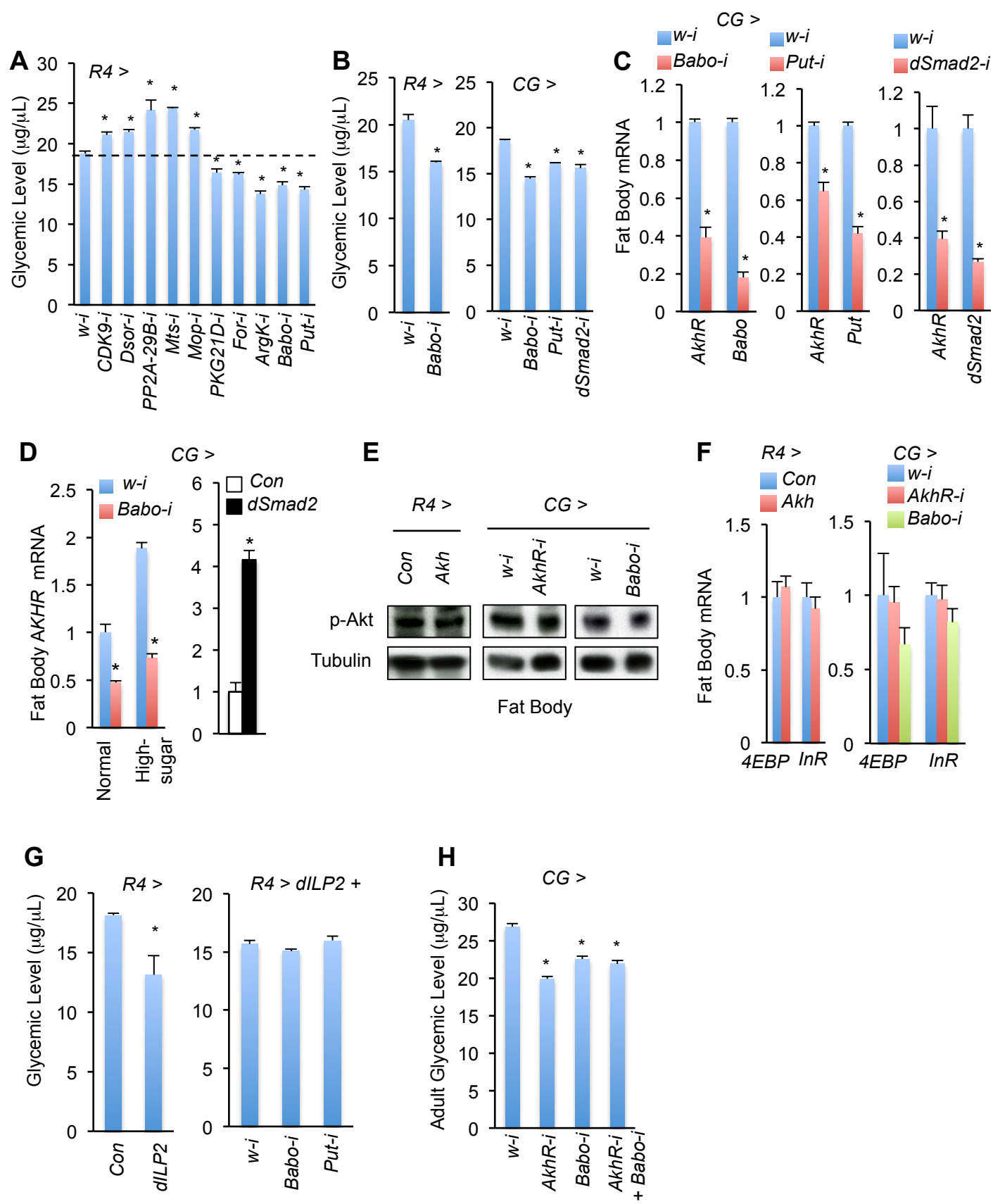


Figure S4

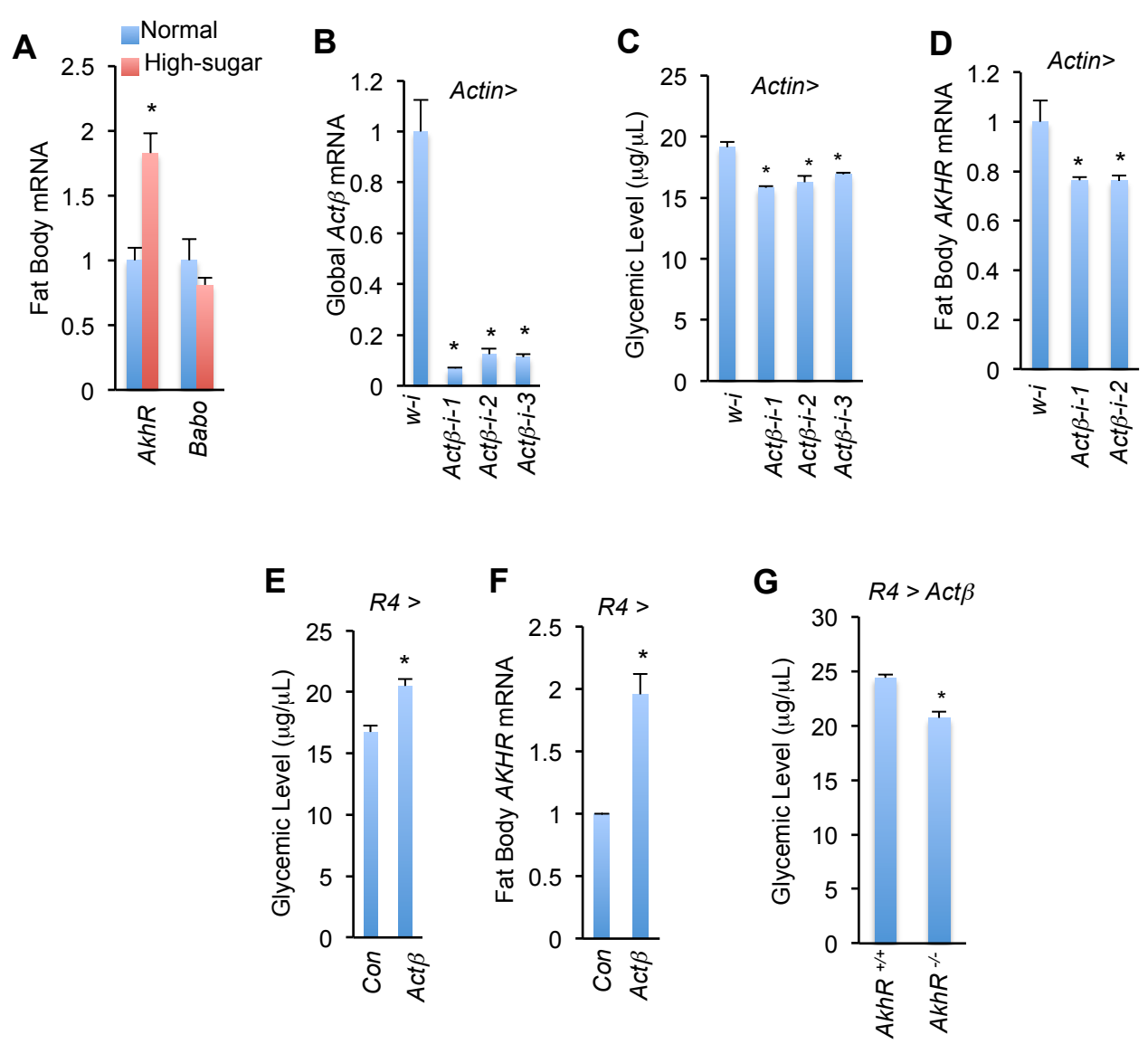


Figure S5

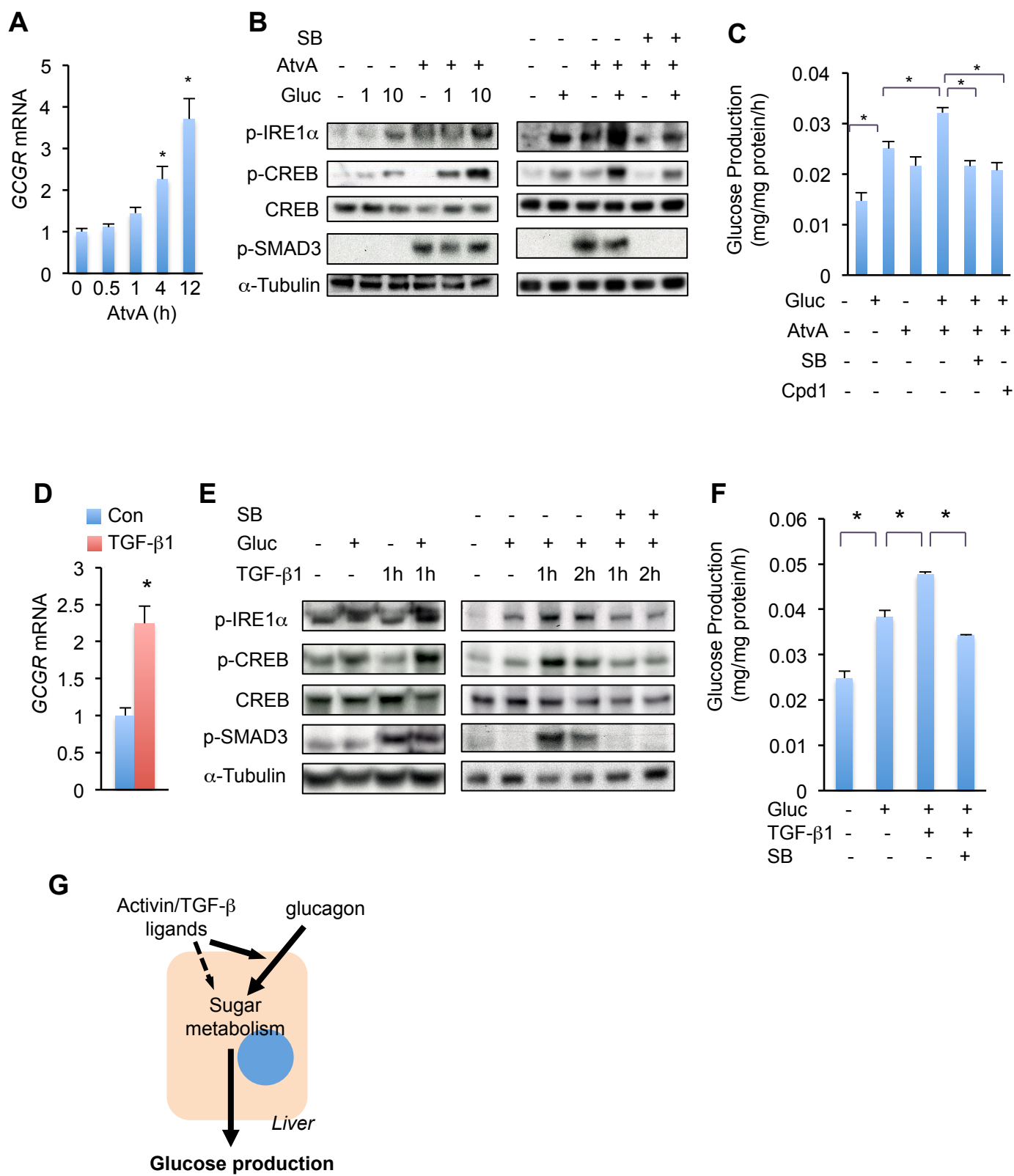


Figure S6

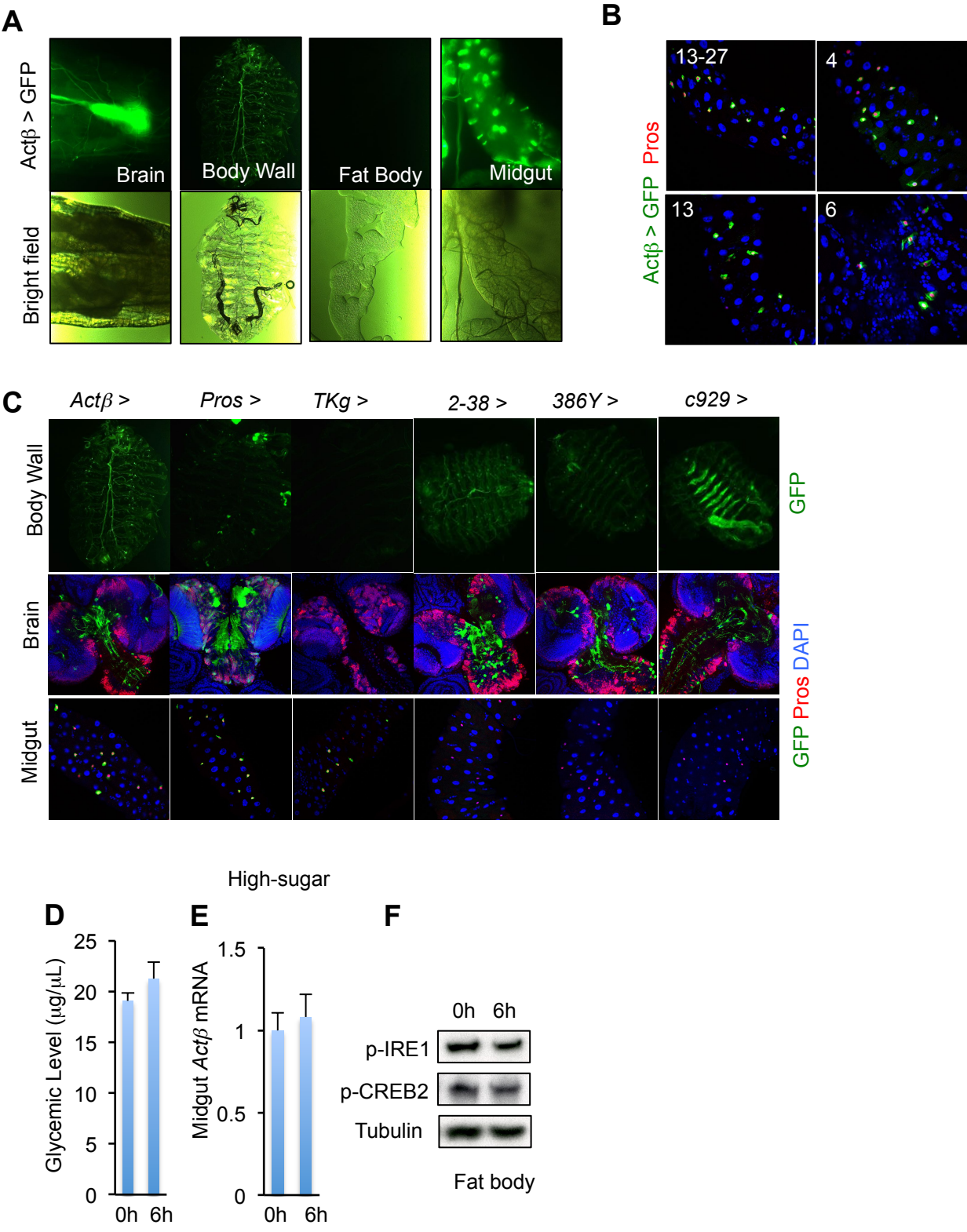
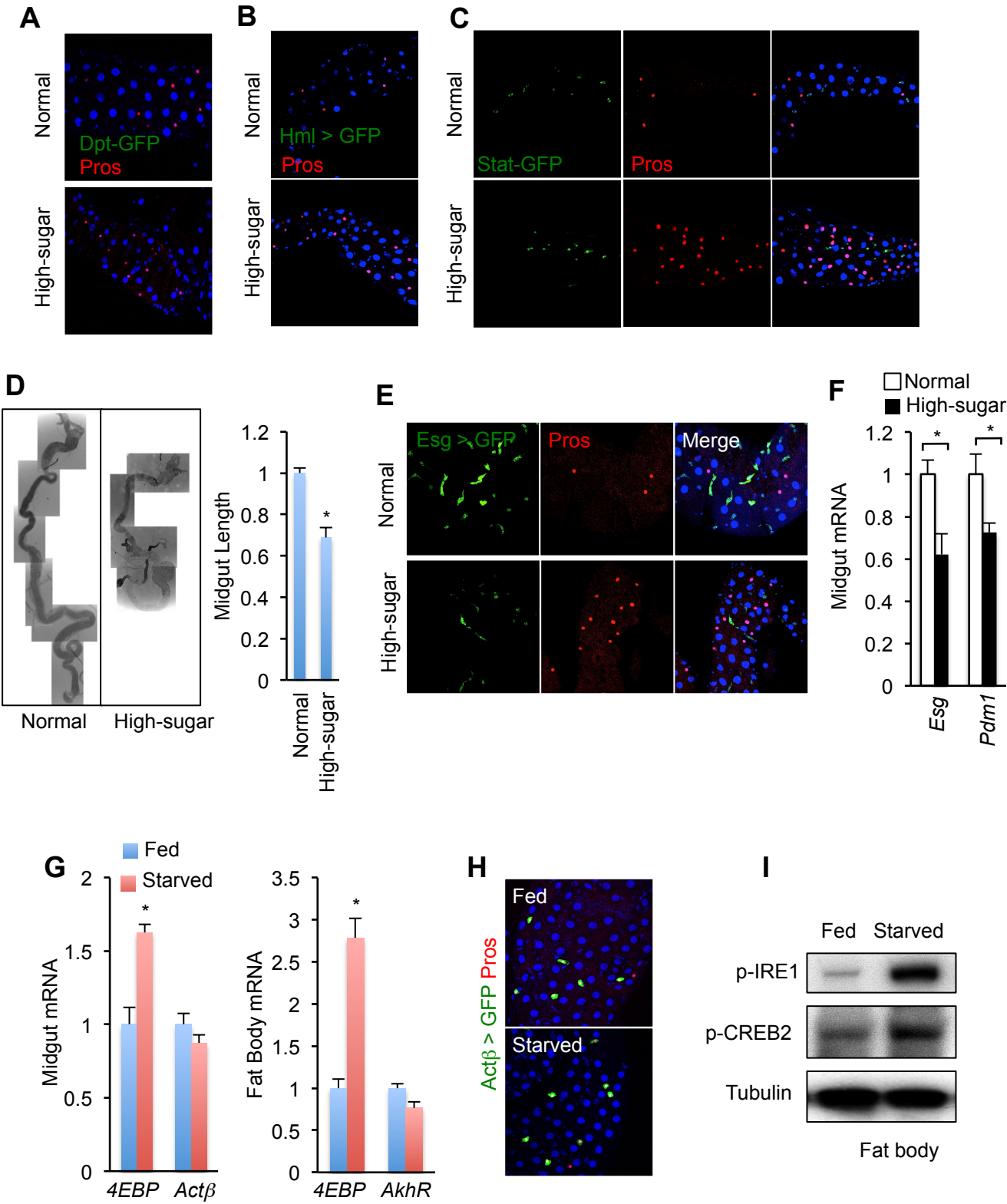




Figure S7





## Supplementary Figure and Table Legends

**Figure S1, related to Figure 1. AKH signaling regulates carbohydrate metabolism.** (A-C) Circulating glucose (A), circulating trehalose (B), and fat body stored carbohydrate levels (C) in 3<sup>rd</sup> instar larvae with fat body knockdown of *AkhR*. (D-E) Circulating glucose (D) and trehalose (E) in 3<sup>rd</sup> instar larvae with *AkhR* knockdown in different tissues (*Myo1A*, enterocytes in midgut; *Elav*, pan-neuron; *dMef2*, muscle cells) (n=4, 40 larvae). (F) Immunoblots of AKHR-stable S2R+ cells treated with synthetic AKH ( $\mu$ M) or 10  $\mu$ M Forskolin (FSK, PKA agonist) for 30 min, with or without prior treatment with 10  $\mu$ M H89 (PKA antagonist) for 1h. (G) Nuclear translocation of V5-tagged CRTC in *AkhR*-stable S2R+ cells treated with or without 1  $\mu$ M AKH for 4h. (H) Immunoblots of p-CREB2 in larval fat bodies. (I) CRE-Luciferase measurements representing CREB2 transcriptional activity in larval fat bodies (n=4, 60 larval fat bodies). (J) IRE1 activity is not affected by AKH/PKA signaling. Spliced and unspliced *Xbp1* transcripts detected by PCR in *AkhR*-overexpressing S2R+ cells that were treated with 10  $\mu$ g/mL tunicamycin (TM), 10  $\mu$ M Forskolin (FSK), 10  $\mu$ M AKH, or 5 mM DTT for the indicated times (up) and in 3<sup>rd</sup> instar larval fat bodies overexpressing *AKH* (down). (K, L) Glycemic levels (K) (n=4, 40 larvae) and insulin signaling in fat body (L, indicated by p-AKT) in 3<sup>rd</sup> larvae with knockdown of insulin signaling components. (M) Glycemic levels in 3<sup>rd</sup> larvae with knockdown of *AkhR*, *InR*, or both in fat bodies (n=4, 40 larvae). Data are presented as means  $\pm$  SEM. \* p < 0.05.

**Figure S2, related to Figure 2. Large-scale RNAi screen in hyperglycemic *R4 > Akh* flies.** (A, B) GO enrichment (A) and transcriptional factor target gene enrichment (B) of differentially expressed genes in AKH-expressing larval fat bodies. (C) Carbohydrate metabolic genes were validated by qPCR (n=4, 40 larvae). (D) Knockdown of certain carbohydrate metabolic genes in fat body alleviated AKH-induced hyperglycemia (n=4, 40 larvae). (E) No strong correlation was observed between glycemic effects and fat body status, developmental

delay, or pupal lethality associated with candidate gene knockdown. *AkhR-i* did not affect developmental delay, pupal lethality, or fat body status as compared to *w-i*. In each knockdown genotype, fat body protein level that was increased or decreased by > 40% was defined as “big” or “small” accordingly, while fat body that was degenerated and hard to collect was defined as “degenerated”. > 70% failed to emerge to adult flies were defined as “pupae lethality”. Larval development into pupa that was delayed by > 24h was defined as “developmental delay”. (F, G) Immunoblots of p-IRE1 and p-CREB2 in the 3<sup>rd</sup> instar larval fat bodies with AKH overexpression and candidate gene knockdown. Gene knockdowns are associated with suppressed (F) or enhanced (G) hyperglycemia. Note that knockdown of *Cklα*, *Pk92B*, *mts*, or *Pp2A-29B* causes fat body degeneration in *R4 > Akh* larvae and makes it difficult to evaluate AKH signaling regulation. Data are presented as means ± SEM. \* p < 0.05.

**Figure S3, related to Figure 3. Babo signaling regulates AKH signaling and glycemic level.** (A) Knockdown of several candidates from the RNAi screen in wild type larval fat body also affects glycemic levels (n=4, 40 larvae). (B) Knockdown of Babo signaling components reduced glycemic levels in wild type 3<sup>rd</sup> instar larvae (n=4, 40 larvae). (C) *AkhR* mRNA levels in 3<sup>rd</sup> instar larval fat bodies with knockdown of indicated Babo signaling components (n=4, 40 larval fat bodies). (D) *AkhR* mRNA levels in *Babo* knockdown (left) or *dSmad2* overexpression (right) larval fat bodies (n=4, 40 larval fat bodies). (E) Immunoblots of p-AKT, indicative of insulin signaling, in 3<sup>rd</sup> instar larval fat bodies. (F) qPCR results of mRNA levels of two FoxO target genes, *InR* and *4EBP*, in 3<sup>rd</sup> instar larval fat bodies (n=4, 40 larval fat bodies). (G) Glycemic levels in 3<sup>rd</sup> instar larvae with fat body overexpression of *dILP2* and knockdown of *Babo* or *Put* (n=4, 40 larvae). (H) Glycemic levels of 5d old adult flies with fat body knockdown of *AkhR*, *Babo*, or both (n=4, 120 adult flies). Data are presented as means ± SEM. \* p < 0.05.

**Figure S4, related to Figure 4. Act $\beta$  regulates glycemic levels in an AkhR-dependent manner.** (A) mRNA expression levels in the fat body (n=4, 40 larval fat bodies). (B-D) Global Act $\beta$  mRNA levels (B, n=4, 40 larvae), glycemic levels (C, n=4, 40 larvae), as well as fat body AkhR mRNA levels (D, n=4, 40 larval fat bodies), in *Actin > Act $\beta$ -i 3<sup>rd</sup>* instar larvae (*Act $\beta$ -i-1*, RNAi line JF03276 from TRiP. *Act $\beta$ -i-2* and -3, RNAi lines 11062R-1 and 11062R-2 from NIG, respectively). (E, F) Glycemic level (E) and fat body AkhR mRNA level (F) in *R4 > Act $\beta$  3<sup>rd</sup>* instar larvae. (G) Glycemic levels in 3<sup>rd</sup> instar larvae with indicated genotypes (*R4-Gal4/UAS-Act $\beta$  V.S. AkhR<sup>-</sup>/AkhR<sup>-</sup>; R4-Gal4/UAS-Act $\beta$* ). Data are presented as means  $\pm$  SEM. \* p < 0.05.

**Figure S5, related to Figure 4. Activin/TGF- $\beta$ 1 signaling promotes glucagon action in primary mouse hepatocytes.** (A) mRNA levels of *glucagon receptor* (*GCGR*) in primary mouse hepatocytes treated with 100 ng/mL Activin A (ActA) (n=4). (B) Immunoblots in hepatocytes treated with 100 ng/mL AtvA and 10nM SB-431542 (SB), activin type I receptor inhibitor, prior to glucagon stimulation (Gluc, 1 or 10 nM (left) or 10 nM (right)). (C) Glucose productions in primary mouse hepatocytes treated with 100 ng/mL AtvA, 10 nM SB, or 10  $\mu$ M Cpd1 for 4h prior to treatment with 10 nM glucagon for 4h (n=4). (D) mRNA levels of *glucagon receptor* (*GCGR*) in primary mouse hepatocytes treated with 10 ng/mL TGF- $\beta$ 1 for 1h (n=4). (E) Immunoblots of p-IRE1 $\alpha$  and p-CREB in hepatocytes treated with 10 ng/mL TGF- $\beta$ 1 and 10nM SB for 1h or 2h prior to glucagon stimulation (Gluc, 10 nM). (F) Glucose production in primary mouse hepatocytes (h) treated with 10 ng/mL TGF- $\beta$ 1 or 10 nM SB for 1h prior treatment with 10 nM glucagon for 4h (n=4). (G) Model of the regulation of AKH/glucagon action and glycemic levels by activin signaling. Data are presented as means  $\pm$  SEM. \* p < 0.05.

**Figure S6, related to Figure 6. Act $\beta$  expression pattern in 3<sup>rd</sup> instar larva. (A)** *Act $\beta$  > GFP* expression (green) in 3<sup>rd</sup> instar larval brain, body wall, and midgut, but not in fat body. **(B)** GFP (green) indicates that independent *Act $\beta$ -Gal4* lines express in EEs, labeled with Pros (red). **(C)** Expression patterns of different Gal4 line-driving GFP in body wall, brain, or midgut. **(D-F)** Glycemic level **(D)** (n=4, 40 larvae), midgut *Act $\beta$*  mRNA expression indicated by qPCR **(E)** (n=4, 40 larval midguts), and fat body immunoblots **(F)** in 3<sup>rd</sup> instar larvae fed with high-sugar diet for 6h. Data are presented as means  $\pm$  SEM. \*  $p < 0.05$ .

**Figure S7, related to Figure 7. Chronic high-sugar feeding increases Act $\beta$  EE cell number. (A-C)** *Dpt-GFP* reporter **(A)**, *Hml > GFP* expression **(B)**, and *10XSTAT-GFP* reporter **(C)** in 3<sup>rd</sup> instar larval midgut fed with normal or high-sugar diet through development. *Dpt-GFP* represents Relish signaling activity, and *Hml > GFP* labels hemocytes. These reporters are not expressed in the larval midgut under either normal or high-sugar diet. The *10XSTAT-GFP* reporter, indicating JAK/STAT signaling, is only expressed in small cells other than EEs in the midgut. **(D)** Image (left) and length (right) of 3<sup>rd</sup> instar larval midgut under a chronic high-sugar diet. **(E-F)** Cell morphology **(E)** and gene expression **(F)** in the larval midgut under a chronic high-sugar diet (n=4, 40 midguts). **(G-I)** Gene expression in the midgut (left) and fat body (right) **(G)**, Act $\beta$ -positive cells (green) and EEs (red) in the midgut **(H)**, and phosphorylation of IRE1 and CREB2 in the fat body **(I)** of 3<sup>rd</sup> instar larvae with or without 6h starvation. Data are presented as means  $\pm$  SEM. \*  $p < 0.05$ .

**Table S1, related to Figure 2. RNA-seq analysis of differentially expressed genes in 3<sup>rd</sup> instar larval fat body.**

**Table S2, related to Figure 2. Novel hits in AKH action regulation from the *in vivo* RNAi screen.** Glycemic levels were normalized to both *AkhR-i* and *w-i*

that were set as “0” and “1” (100%) hyperglycemia, respectively. RNAi hits that significantly affect hyperglycemia ( $< 0.3$  or  $> 1.7$ ) were considered as significant regulators.

## Supplementary Methods

### ***Drosophila* strains.**

Flies were maintained at 25°C on a 12h light, 12h dark light cycle. Flies were raised on a standard cornmeal/agar diet (10 g/L agar, 230 g/L soy flour, 30 g/L yeast, 100 g/L cornmeal, 74 g/L molasses, 4.5 g/L propionate, and 6 g/L Nipogen). All experiments were performed using 3<sup>rd</sup> instar stage larvae (12~24h before wandering). *CG-Gal4*, *R4-Gal4*, and *Lpp-Gal4* were used to target the larval fat body. *CG-* and *R4-Gal4* are stronger than *Lpp-Gal4* and therefore were used for most assays in this study. Fat body genetic manipulation using *CG-* or *R4-Gal4* results in the same changes in both physiology and signaling, except that *CG-Gal4/UAS-Akh* causes lethality. The genotype of *AKH* overexpressing flies is *UAS-Akh; R4-Gal4 / T(2;3)B3, CyO: TM6B, Tb*. Fat body overexpression of *Actβ* or *Daw* (*CG-* or *R4-Gal4*) causes sterility or adult lethality, we therefore performed genetic manipulations in fat body-expressing *Actβ* or *Daw* flies by crossing UAS lines to *Cg-Gal4, tub-Gal80<sup>ts</sup>; UAS-Actβ* or *Cg-Gal4, tub-Gal80<sup>ts</sup>; UAS-Daw* flies at 18°C and culturing offspring larvae at 29°C until they reached the 3<sup>rd</sup> instar larval stage.

The following Gal4 driver lines were used to target various cells/tissues: *CG-Gal4* (BLM 7011), *R4-Gal4* (BLM 33832) and *Lpp-Gal4* (fat body) (Palm et al., 2012), *dMef2-Gal4* (muscle) (Owusu-Ansah et al., 2013), *Myo1A-Gal4* (midgut) (Amcheslavsky et al., 2014; Song et al., 2014), *Elav-Gal4* (pan-neuron) (Song et al., 2014), *TKg-Gal4* (TK EE) (Amcheslavsky et al., 2014; Song et al., 2014), *386Y-Gal4* and *c929-Gal4* (neuroendocrine cell) (Marques et al., 2003), *Hml-Gal4* (BLM 30140) (hemocytes). *Pros-Gal4* (peripheral neuron, a kind gift from Allison Bardin) and *2-38-Gal4* (EE, a kind gift from Katja Brückner) were previously described (Hang et al., 2014; Makhijani et al., 2011). *Actβ-Gal4-4* was from the HHMI Janelia Research Campus (BLM 49078). Other *Actβ-Gal4* lines, including -6, -13, and -13+27, were created by using sense primer 5'-GGCAATGGACGACGGAAGCAGTTTAC-3' and antisense primer 5'-GGCAAGCGTGTAGTACAGGCACTGT-3' to amplify a 5.6 kb *Drosophila* Actβ promoter/enhancer. The region was initially cloned in a PCR II TOPO vector (Invitrogen) before it was transferred to the pPelican-Gal4 vector that bears insulation DNA sequences (Barolo et al., 2000). These four *Actβ-Gal4* lines exhibited very similar expression patterns in 3<sup>rd</sup> instar larvae.

Fly stocks with RNAi transgenes targeting *white* (JF01545), *Akh* (HMS00477), *AkhR* (JF03256), *InR* (JF01482), *Ire1* (HMS03003), *Creb2* (JF02494), *Crtc* (HM05069), *Sik3* (JF03002), *Babo* (JF01953), *Put* (HMS01944), *Sax* (JF03431), *Tkv* (JF01485), *dSmad2* (JF02320), *Mad* (JF01263), *Daw* (HMS01110), *Actβ* (JF03276), *Chico/IRS* (JF02964), *CerbA* (JF02189), *Pten* (HMS00044), *Tsc1* (JF01484), *Cdk9* (HMS01391), *Dsor1* (JF03100), *Pp2A-29B* (JF03316), *Mts*

(JF02805), *Mop* (HMS00706), *Pkg21D* (JF02766), *For* (JF01181), and *ArgK* (JF02699) were obtained from the TRiP at Harvard Medical School (<http://www.flyrnai.org/TRiP-HOME.html>). RNAi fly stocks targeting *Actβ* (11062R-1 and 11062R-2) were obtained from the NIG-FLY stock center ([www.shigen.nig.ac.jp/fly/nigfly](http://www.shigen.nig.ac.jp/fly/nigfly)), and *SH2B* (32892 and 103646) were obtained from the VDRC ([stockcenter.vdrc.at](http://stockcenter.vdrc.at)). RNAi fly stocks targeting *CBP* (BLM 32576) and specific *Babo* isoforms (a, BLM 44400; b, BLM 44401; c, BLM 44402) (Awasaki et al., 2011) were obtained from the Bloomington *Drosophila* Stock Center ([flystocks.bio.indiana.edu](http://flystocks.bio.indiana.edu)). Multiple RNAi fly stocks were used to confirm the knockdown effect and the most significant ones are listed here.

The overexpression and other fly stocks, including *UAS-Akh* (BLM 27343), *UAS-Pka-C1* (BLM 35555), *UAS-Creb2* (BLM 9232), *UAS-CrebA* (BLM 32572), *UAS-Babo*, *UAS-dSmad2*, *UAS-Actβ*, *UAS-Daw*, *actβ<sup>ed80</sup>* mutant, *UAS-dILP2*, *CRE-Luci*, and *10XSTAT-GFP* were previously described (Bach et al., 2007; Ghosh and O'Connor, 2014; Gibbens et al., 2011; Rajan and Perrimon, 2012; Song et al., 2014; Zhu et al., 2008). *AkhR* null mutant (a kind gift from Ronald Kühnlein) and *Dpt-GFP* (a kind gift from Paula I. Watnick) were previously described (Gronke et al., 2007; Siudeja et al., 2015).

### **High-sugar diet and carbohydrate measurement.**

25% extra sucrose was added into the standard diet to generate the high-sugar diet food. For chronic high-sugar feeding, the parental females were allowed to lay eggs on high-sugar food media, such that larvae were cultured on high-sugar



food throughout development. For acute high-sugar feeding, late 3<sup>rd</sup> instar larvae were transferred to a high-sugar diet for 6h. The method we used to determine circulating trehalose levels has previously been described (Song et al., 2010). Briefly, 2  $\mu$ L hemolymph from 3<sup>rd</sup> instar larvae or 5d old adult flies was diluted in 38  $\mu$ L sugar buffer (5 mM Tris [pH 6.6], 137 mM NaCl, 2.7 mM KCl) and heated at 70°C to inactivate endogenous trehalase activity. After a brief centrifugation, 10  $\mu$ L supernatant was treated with 0.2  $\mu$ L water or trehalase (Megazyme, E-TREH) at 37°C for 20 min to digest trehalose into glucose, then glucose levels were measured by incubation with 150  $\mu$ L glucose assay reagent (Megazyme) at 37°C for 5 min. The absorbance at 510 nm was measured on a Molecular Devices SpectraMax Paradigm plate reader. Circulating trehalose levels were determined by subtracting circulating free glucose levels from total glucose levels after enzyme digestion. We defined circulating trehalose + glucose as the 'glycemic level' in this study.

Measurement of carbohydrates stored in the larval fat body has been previously described (Song et al., 2010). Briefly, 10 3<sup>rd</sup> larval fat bodies were homogenized in 300  $\mu$ L PBS containing 0.1% Triton-X and protease inhibitor and heated at 70°C for 5 min to inactivate endogenous enzymes. After centrifugation at 13,000 rpm for 10 min at 4°C, 10  $\mu$ L supernatant of lysate was treated with 0.2  $\mu$ L trehalase (Megazyme, for trehalose measurement) or 1  $\mu$ L amyloglucosidase (Sigma, for glycogen measurement) at 37°C for 20 min, and glucose levels were measured using a glucose assay reagent (Megazyme). 10  $\mu$ L supernatant was used for protein level measurement using the Bradford reagent (Sigma). A

relative level of trehalose or glycogen was determined by subtracting free glucose levels from total glucose levels after enzyme digestion and then normalized to protein levels.

**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 the plasmid vector pAC5-Stable2-Puro, which allows for multicistronic expression of the target protein and GFP as well as a protein conferring puromycin resistance (Gonzalez et al., 2011). S2R+ cell transfection was performed using the non-liposomal reagent Effectene (Qiagen) following the manufacturer's instructions. 72 hours after transfection, cells were incubated for two weeks in selective medium, i.e. Schneider's medium (Invitrogen) containing 10% FBS, standard cell culture antibiotics, and 10 µg/mL puromycin. Cells were split as necessary and again incubated in selective medium. Stable cells were maintained with selective medium and AKH treatment performed in medium without puromycin.

### **qPCR**

10 fat bodies, 10 midguts, 10 body walls, or 10 brains from late 3<sup>rd</sup> instar larvae of each genotype were collected on ice and stored at -80°C. Total RNA from fat

bodies or mouse primary hepatocytes were extracted using Trizol (Invitrogen) and cDNA transcribed using the iScript cDNA Synthesis Kit (Biorad). qPCR was then performed using iQ SYBR Green Supermix on a CFX96 Real-Time System/C1000 Thermal Cycler (Biorad). *Drosophila* and mouse gene expression were normalized to *RpL32* and  *$\beta$ -actin*, respectively. Primers are listed as below:

*Drosophila*

<i>RpL32-F:</i>	GCTAAGCTGTGCGCACAAATG
<i>RpL32-R:</i>	GTTGATCCGTAACCGATGT
<i>AkhR-F:</i>	GCTATCCACGGACCTGATGTG
<i>AkhR-R:</i>	CTGTCGAGCGATATGCAGACC
<i>Act<math>\beta</math>-F:</i>	ACGGCAAATTTTGACAAAGC
<i>Act<math>\beta</math>-R:</i>	TTGGTATCATTCGTCCACCA
<i>Babo-F:</i>	GCTGGCCGCTGATAACAGAA
<i>Babo-R:</i>	AGGCTGAGCAATGTAGATGGG
<i>Put-F:</i>	TTTTGCCCGGAAGTCATGGG
<i>Put-R:</i>	TGCTCTATCCGTGTTTCACATTG
<i>dSmad2-F:</i>	CCGACTCCATTGTGGACTATCC
<i>dSmad2-R:</i>	CCCAATTCCATGTACTGCGG
<i>Pros-F:</i>	TTTGACCGGAGATGGTGACG
<i>Pros-R:</i>	GGTCGTTCTGCCCAGTTTC
<i>Esg-F:</i>	ATGAGCCGCAGGATTTGTG
<i>Esg-R:</i>	CCTCCTCGATGTGTTTCATCATCT
<i>Pdm1:</i>	AGCTGTCCTAACGAGTTCCG

<i>Pdm1:</i>	ACATCGCGCATATTTGTGTCAA
<i>CG6910-F:</i>	CCGGGACTACAGTATGGACAC
<i>CG6910-R:</i>	CGAAATCCACAGTCTGGTTCAG
<i>GlyP-F:</i>	GCTCACTGACCAACACCATGA
<i>GlyP-R:</i>	AACGGATGCCATAGCCATAGG
<i>Irp-1B-F:</i>	TCGCCCAGTTCGAGAAAACCTT
<i>Irp-1B-R:</i>	GGATCGAGTAGGGCAGTTGA
<i>Mdh1-F:</i>	CTCGGCTATGACTCGCCTG
<i>Mdh1-R:</i>	CTTAACAGCAGAAATTGGCACAC
<i>Men-b-F:</i>	GCGCTTCAAGACGCAAGAG
<i>Meb-b-R:</i>	CGTACAGATCGCTCAGATAGAGA
<i>PGRP-SD-F:</i>	GACAGCATGGAAACTCCCTTG
<i>PGRP-SD-R:</i>	GTTTTGCAGATTTTGCATGTGC
<i>Scsalpha-F:</i>	CGGAACCGACTTCATCGACTG
<i>Scsalpha-R:</i>	CCTTGATGCCCCGAATTGTACTC
<i>Skap-F:</i>	CCCCTGATCGAAACCGTTAGG
<i>Skap-R:</i>	GGAAACGTGTTCTGACATT
<i>Tobi-F:</i>	GTCATGCATCCTGTGTGGTC
<i>Tobi-R:</i>	GATTTCAGCTGGCTGTTGT
<i>Treh-F:</i>	GGCACCGAGCTTGAGAAATG
<i>Treh-R:</i>	ATTTTGCGTCCCAAGTCCTTC
Mouse	
<i>β-actin-F:</i>	AGTGTGACGTTGACATCCGTA

*β-actin-R*: GCCAGAGCAGTAATCTCCTTCT

*GCGR-F*: TTGCTGGTTGCTGGTAGAGG

*GCGR-R*: AGGGGATGACAAACAGCAGG

**CREB transcriptional activity and IRE1 nuclease activity.** 15 larval fat bodies that carry *CRE-Luci* were isolated and homogenized in 150  $\mu$ L PBS containing 0.1% Triton-X and protease inhibitor. After centrifugation at 13,000 rpm for 10 min at 4°C, 50  $\mu$ L lysate was collected for luciferase activity measurement using a Luciferase Assay Kit (Promega). 10  $\mu$ L lysate was incubated with 200  $\mu$ L Bradford reagent (Sigma) for protein quantification. CREB transcriptional activity was then determined by normalizing CRE-Luciferase activity to protein levels. IRE1 nuclease activity was measured by monitoring *Xbp1* mRNA splicing as previously described (Plongthongkum et al., 2007). Briefly, total RNA from S2R+ cells or from fat bodies was extracted and transcribed into cDNA. Spliced and unspliced *Xbp1* mRNAs were detected by PCR using primer pairs that flank the intron. *Xbp1-F*: CCGAATTCAAGCAGCAACAGCA and *Xbp1-R*: TAGTCTAGACAGAGGGCCACAATTTCCAG.

**RNA-seq transcriptome analysis.** 10 fat bodies from late 3<sup>rd</sup> instar larvae of each genotype were collected on ice and stored at -80°C. Total RNA was extracted using Trizol (Invitrogen). To prepare RNA samples for RNA-seq, RNA integrity (> 6.5) was assessed using an Agilent Bioanalyzer. Sequencing libraries

were constructed using Illumina Hi-seq kits following standard protocol, and 100 bp pair end reads were generated at Novogene Bioinformatics Institute (Beijing, China). Sequence reads were mapped back to the *Drosophila* genome (FlyBase genome annotation version r5.51) using TopHat. Using uniquely mapped reads, gene expression levels were quantified using Cufflinks (FPKM values). Next, we performed data normalization on the read counts and applied a negative binomial statistical framework using the Bioconductor package DESeq to quantify differential expression between experimental and control data. Differentially expressed genes were selected if 2 or more fold changes were consistently observed among replicates. Genes with less than 2 but greater than 1.5 fold changes and with an adjusted p-value of 0.05 or better were also selected. For gene list enrichment analysis, we assembled gene lists, including pathways, complexes and transcription factor target gene sets, from various public resources including Gene Ontology (GO), KEGG, DroID, and other published datasets. CREB2 target genes were predicted based on the presence of a cAMP-responsive element (CRE) “TGG/ACGTCA” in the promoter region. Enrichment p-values were calculated using hyper-geometric distribution, and gene lists with an enrichment p-value of less than 0.05 were presented.

**Primary hepatocyte culture, HGP assays and immunoblots.** Mouse primary hepatocytes were prepared by liver perfusion with type II collagenase (Worthington Biochemical, Lakewood, NJ) and grown at 37°C on collagen-coated

plates to be treated with the desired reagents. HGP assays were previously described (Mao et al., 2011; Sheng et al., 2012). Briefly, after treatment with different reagents (see below), hepatocytes were incubated for 4h in glucose-free DMEM (Sigma) supplemented with 20 mM sodium lactate, 1 mM sodium pyruvate, 2 mM L-glutamine, and 15 mM HEPES, without phenol red. Glucose levels in the culture medium were measured using a glucose assay reagent (Megazyme, K-GLUC) and normalized to hepatocyte protein levels measured with the Bradford reagent (Sigma). Immunoblotting was done as previously described (Song et al., 2010; Song et al., 2014). Briefly, fat bodies dissected from late 3<sup>rd</sup> instar larvae or AKHR-expressing S2R+ cells were incubated in serum-free Schneider's *Drosophila* media (Invitrogen) with or without synthetic AKH or other reagents at room temperature to modulate AKH signaling. Fat bodies, S2R+cells, and primary hepatocytes were lysed in buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1% Nonidet P-40). Rabbit anti-phospho-CREB for mouse p-CREB (1:1000, Cell Signaling), rabbit anti-phospho-IRE1 $\alpha$  for mouse p-IRE1 $\alpha$  and *Drosophila* p-IRE1 (1:1000, Abcam), rabbit anti-phospho-PKA-substrate for *Drosophila* p-CREB2 (1:1000, Cell Signaling), rabbit anti-phospho-SMAD3 for mouse p-SMAD3 (1:1000, Abcam), rabbit anti-CREB for mouse CREB (1:1000, Cell Signaling) and mouse anti- $\alpha$ -Tubulin for mouse  $\alpha$ -Tubulin and *Drosophila* Tubulin (1:5000, Sigma). The following chemicals were used for *in vitro* treatment in this study: synthetic *Drosophila* AKH (pQLTFSPDWa, NeoBioLab) (Park et al., 2002), glucagon



(Sigma), Activin A (R&D), TGF- $\beta$ 1 (Novoprotein), Myostatin (R&D), Forskolin (Abcam), H89 (Sigma), SB 431542 (Abcam), and Cpd1 (Santa Cruz).

**Immunostaining.** Immunostainings of S2R<sup>+</sup> cells and larval midgut and brain were described previously (Song et al., 2010; Song et al., 2014). Samples were fixed for 15 min in 4% formaldehyde/PBS. After fixation samples were washed with PBS containing 0.2% Triton-X and incubated with primary antibodies overnight at 4°C. Next, the samples were incubated with secondary antibodies (1:500) and DAPI (1:1000) for 1h at room temperature, washed and mounted in Vectashield (Vector). Primary antibodies used in this study were: rabbit anti-Tachykinin (1:1000) (Song et al., 2014), mouse anti-Prospero (DSHB, 1:100), and mouse V5 (1:1000, ThermoFisher). Confocal images were obtained using a Leica TCS SP2 AOBS system.

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