Cell Metabolism, Volume *19*

Supplemental Information

A *Drosophila* In Vivo Screen Identifies Store-Operated Calcium Entry as A Key Regulator of Adiposity

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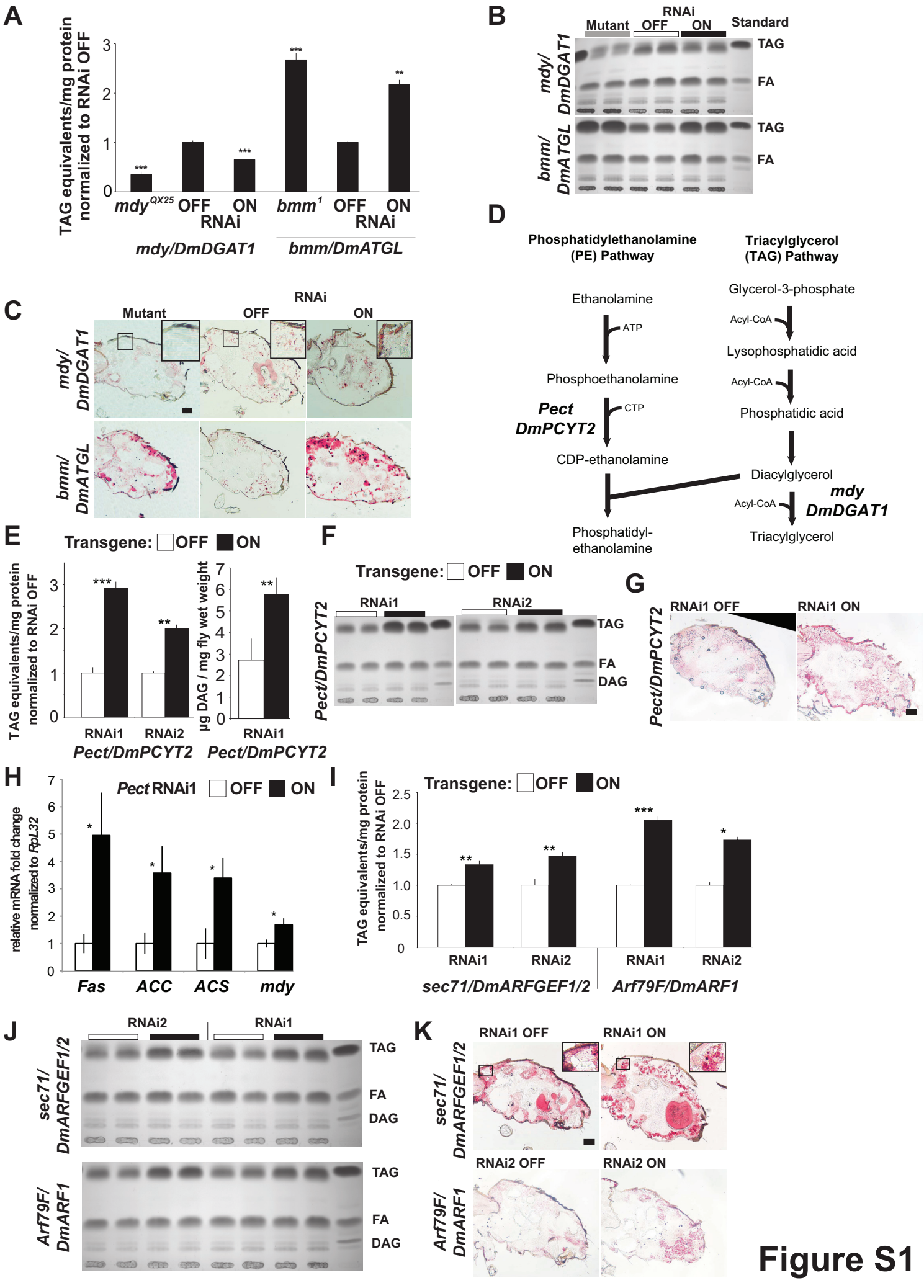

A
TAG equivalents/mg protein normalized to RNAi OFF
mdy^QX25 OFF ON
RNAi
bmm^1 OFF ON
RNAi
mdy/DmDGAT1
bmm/DmATGL
B
RNAi
Mutant OFF ON Standard
mdy/ DmDGAT1
bmm/ DmATGL
TAG
FA
C
RNAi
Mutant OFF ON
mdy/ DmDGAT1
bmm/ DmATGL
D
Phosphatidylethanolamine (PE) Pathway
Triacylglycerol (TAG) Pathway
Ethanolamine
ATP
Phosphoethanolamine
Pect DmPCYT2
CTP
CDP-ethanolamine
Phosphatidyl-ethanolamine
Glycerol-3-phosphate
Acyl-CoA
Lysophosphatidic acid
Acyl-CoA
Phosphatidic acid
Diacylglycerol
Acyl-CoA
mdy DmDGAT1
Triacylglycerol
E
Transgene: OFF ON
TAG equivalents/mg protein normalized to RNAi OFF
µg DAG / mg fly wet weight
RNAi1 RNAi2
Pect/DmPCYT2
RNAi1
Pect/DmPCYT2
F
Transgene: OFF ON
RNAi1 RNAi2
Pect/DmPCYT2
TAG
FA
DAG
G
RNAi1 OFF
RNAi1 ON
Pect/DmPCYT2
H
Pect RNAi1 OFF ON
relative mRNA fold change normalized to RpL32
Fas ACC ACS mdy
I
Transgene: OFF ON
TAG equivalents/mg protein normalized to RNAi OFF
RNAi1 RNAi2 RNAi1 RNAi2
sec71/DmARFGEF1/2
Arf79F/DmARF1
J
RNAi2 RNAi1
sec71/ DmARFGEF1/2
Arf79F/ DmARF1
TAG
FA
DAG
K
RNAi1 OFF
RNAi1 ON
sec71/ DmARFGEF1/2
RNAi2 OFF
RNAi2 ON
Arf79F/ DmARF1

Figure S1

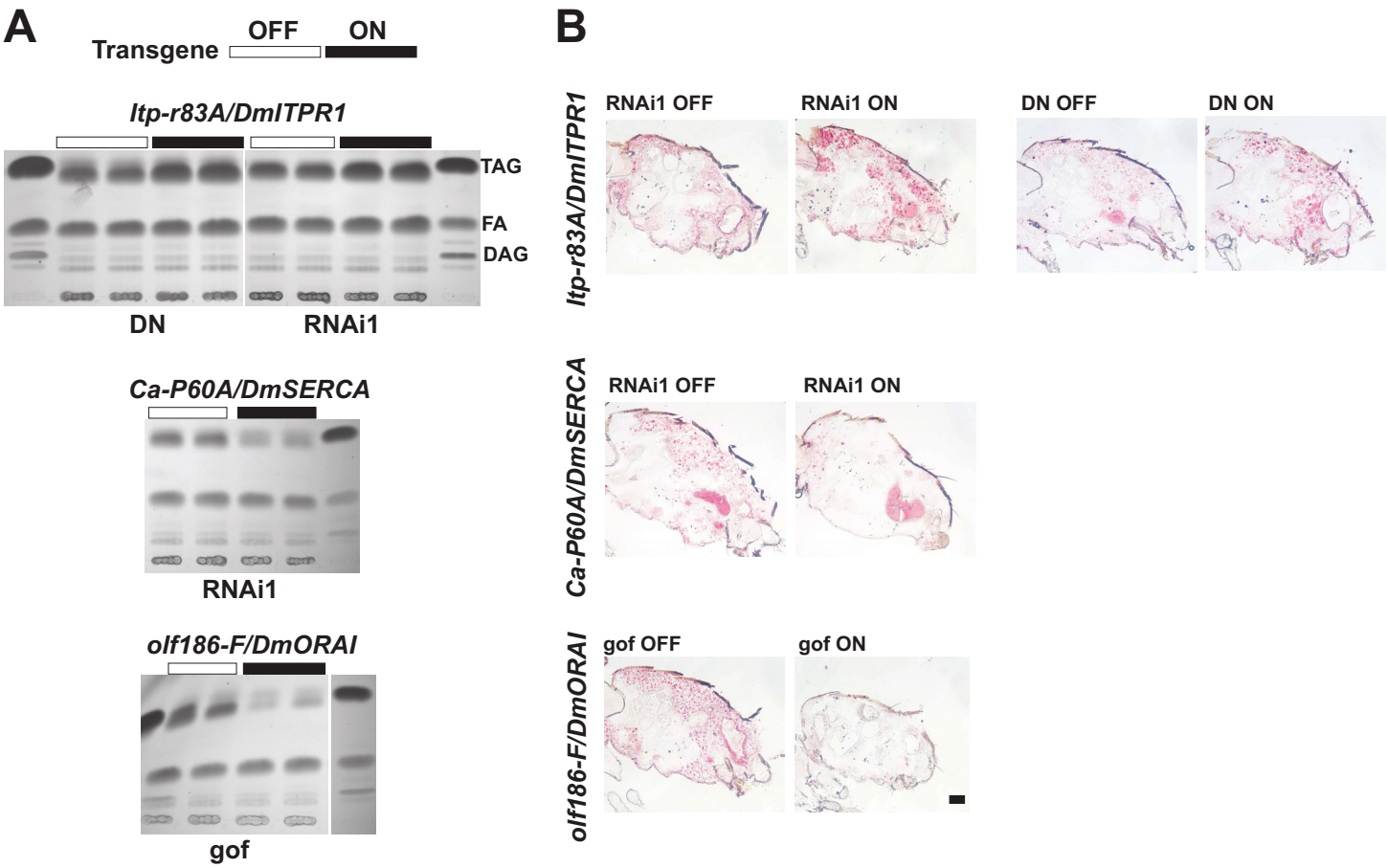

A
Transgene
OFF
ON
Itp-r83A/DmITPR1
TAG
FA
DAG
DN
RNAi1
Ca-P60A/DmSERCA
RNAi1
olf186-F/DmORAI
gof
B
Itp-r83A/DmITPR1
RNAi1 OFF
RNAi1 ON
DN OFF
DN ON
Ca-P60A/DmSERCA
RNAi1 OFF
RNAi1 ON
olf186-F/DmORAI
gof OFF
gof ON

Figure S2

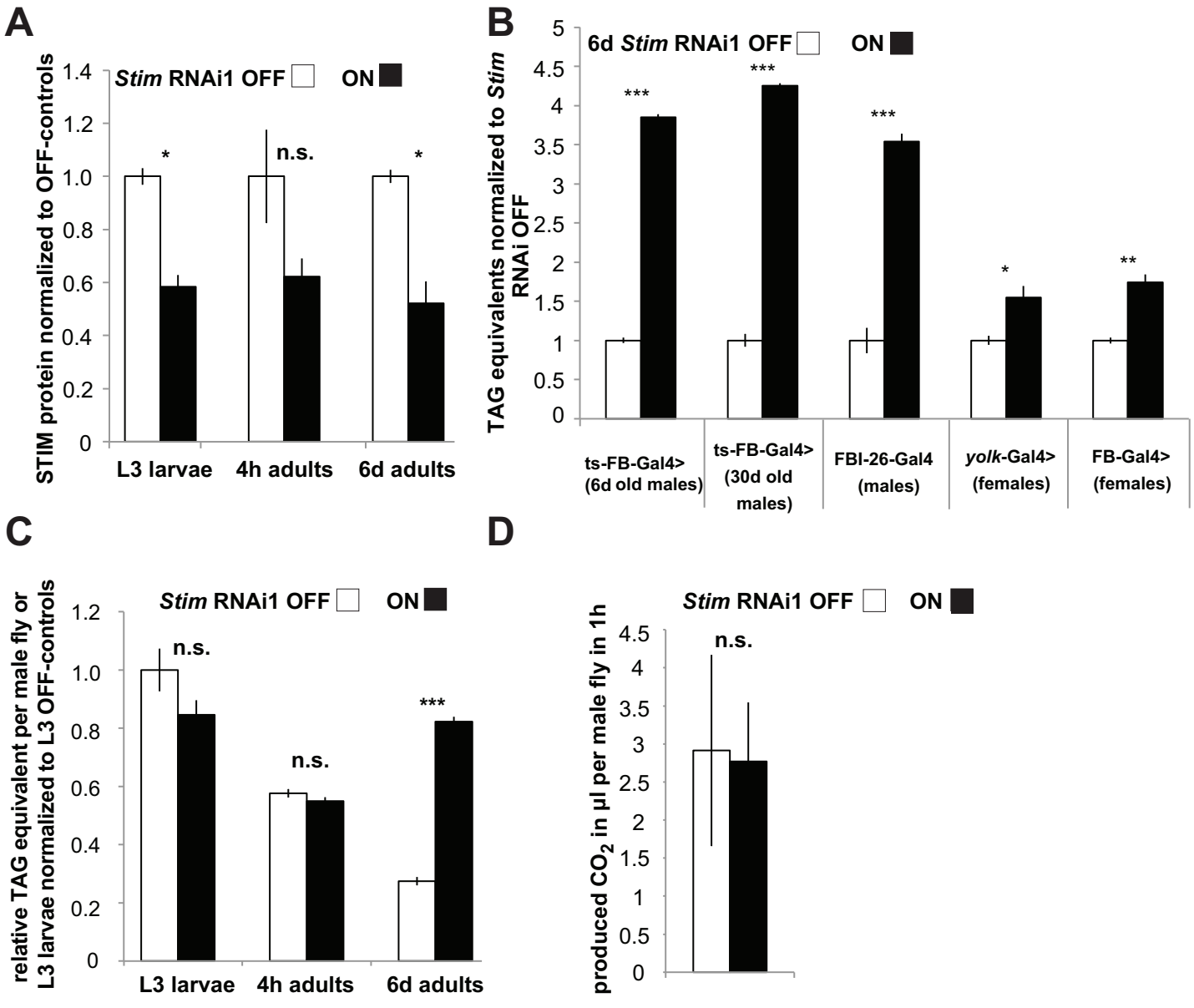
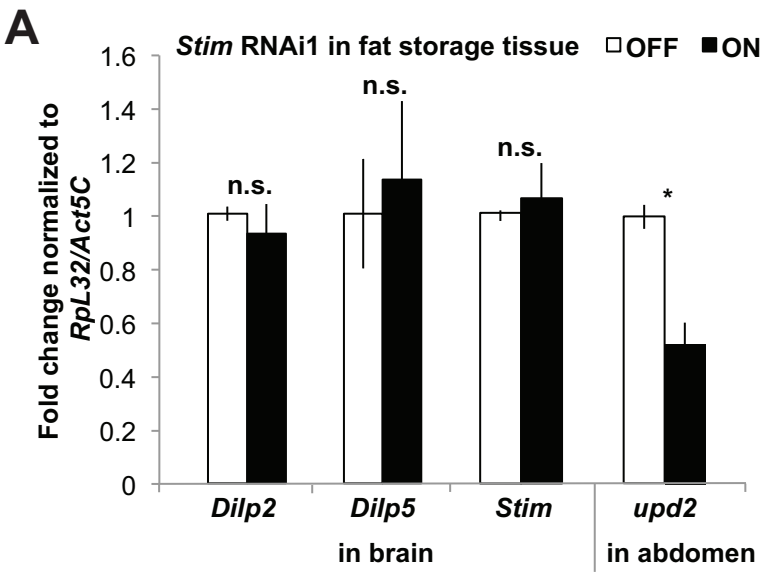
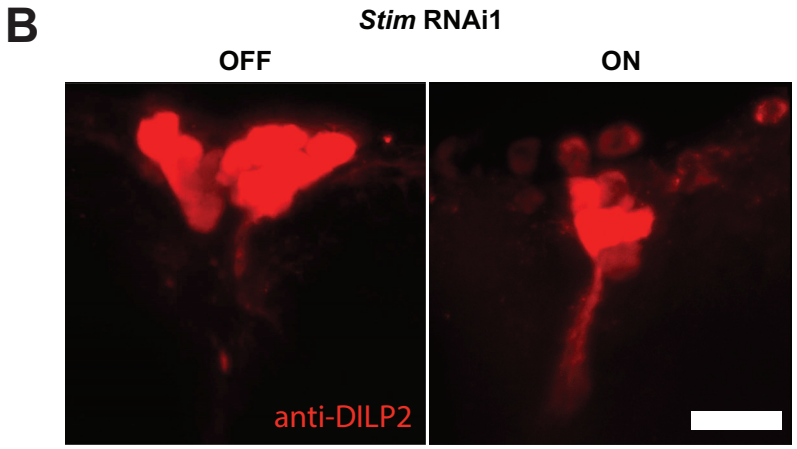
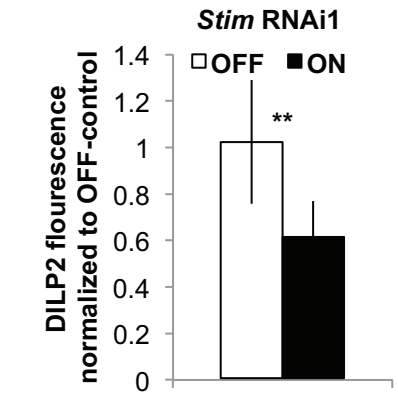

A
Stim RNAi1 OFF
ON
STIM protein normalized to OFF-controls
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
n.s.
*
L3 larvae
4h adults
6d adults
B
6d Stim RNAi1 OFF
ON
TAG equivalents normalized to Stim RNAi OFF
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
***
***
***
*
**
ts-FB-Gal4> (6d old males)
ts-FB-Gal4> (30d old males)
FBI-26-Gal4 (males)
yolk-Gal4> (females)
FB-Gal4> (females)
C
Stim RNAi1 OFF
ON
relative TAG equivalent per male fly or L3 larvae normalized to L3 OFF-controls
1.2
1.0
0.8
0.6
0.4
0.2
0
n.s.
n.s.
***
L3 larvae
4h adults
6d adults
D
Stim RNAi1 OFF
ON
produced CO2 in µl per male fly in 1h
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
n.s.

Figure S3


A
Stim RNAi1 in fat storage tissue
OFF
ON
Fold change normalized to RpL32/Act5C
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
n.s.
n.s.
n.s.
*
Dilp2
Dilp5
Stim
upd2
in brain
in abdomen


B
Stim RNAi1
OFF
ON
anti-DILP2


C
Stim RNAi1
OFF
ON
DILP2 flourescence normalized to OFF-control
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**

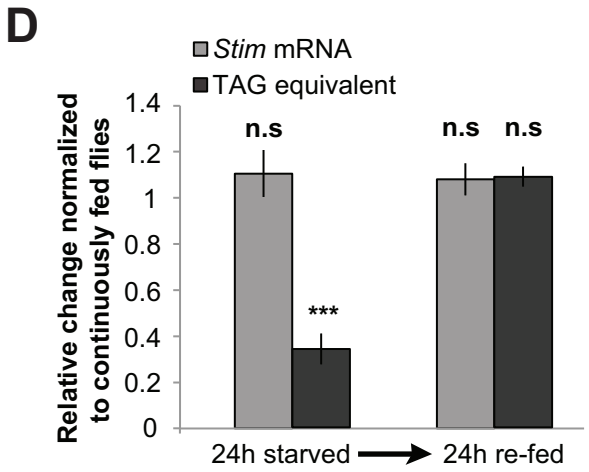

D
Stim mRNA
TAG equivalent
Relative change normalized to continuously fed flies
1.4
1.2
1
0.8
0.6
0.4
0.2
0
n.s
n.s
n.s
***
24h starved
24h re-fed

Figure S4

Supplemental figure and table legends

Table S1 related to Figure 1: Large-scale genetic *in vivo* screen for fly obesity-associated genes. Primary screen results, validation scheme information, gene ontology analyses and functional classification of obesity and anti-obesity genes identified in the *in vivo* screen using fat storage tissue targeted gene knockdown.

Figure S1 related to Figure 2: Fly obesity and anti-obesity genes involved in lipid metabolism and vesicle trafficking. Comparison of adult male glycerolipid metabolism mutants mdy^{QX25} and bmm^{1} to flies subjected to conditional gene knockdown of these genes in the fat storage tissue by total body fat analysis (**A**), thin layer chromatography (**B**), and Oil red O staining on sagittal cryosections of adult male fly abdomen (**C**; Note that homogenous Oil Red O staining in the ejaculatory bulb is unrelated to fat body). **D** Scheme of the catalytic steps and the intersection of the PE and TAG biosynthesis pathways. Increase of total body fat and DAG (**E**), TAG (**F**) and sub-cuticular lipid accumulation (**G**) as well as elevated expression of lipoanabolic genes (**H**) in response to *Pect/DmPCYT2* gene knockdown in the adult male fat storage tissue. Body fat increase in adult male flies upon adipose tissue knockdown of evolutionarily conserved small GTPase genes *Arf79F/DmARF1* and guanine nucleotide exchange factor genes *sec71/DmARFGEF1/2*, involved in the ER-Golgi vesicle transport. Shown are increases in total body fat (**I**), body TAG content (**J**), and sub-cuticular lipid

accumulation (**K**) compared to control flies. Error bars represent SD. *p≤0.05, **p≤0.01 and ***p≤0.001 for all comparisons to RNAi OFF. Scale bar represents 100μm.

Figure S2 related to Figure 3: Body fat control by store-operated calcium entry genes. Increase of body TAG content (**A**) and sub-cuticular lipid accumulation (**B**) upon adipose tissue-targeted knockdown of *Itp-r83A/DmITPR1* or over-expression of a dominant-negative version (DN) of the gene. Decrease of TAG content (**A**) and sub-cuticular lipid depletion (**B**) upon adipose tissue-targeted knockdown of *Ca-P60A/DmSERCA* or over-expression of *olf186-F/DmORAI*. Shown is thin layer chromatography of adult male fly homogenates (**A**) and Oil red O staining on sagittal abdominal cryosections (**B**) compared to control flies. Scale bar represents 100μm.

Figure S3 related to Figure 4: Physiological phenotypes in response to fat storage tissue targeted *Stim* and *Cam* gene knockdown. **A** Depletion of STIM protein in total L3 larvae or abdomen of adult flies of different ages in response to fat tissue targeted gene knockdown shown by quantitative Western blot (normalization based on ß-Tubulin quantification). **B** *Stim*-dependent obesity scored by total body fat content is independent of sex, age and mode of gene knockdown in the adipose tissue. Obesity in adult male flies of different ages subjected to TARGET-system-mediated conditional knockdown (ts-FB-Gal4), adult males flies subjected to SWITCH-system-mediated conditional *Stim*-knockdown

(FBI-26-Gal4), and adult female flies subjected to either conditional TARGET-dependent (ts-FB-Gal4) or –independent (*yolk*-Gal4) *Stim*-knockdown in adult adipose tissue or knockdown in this tissue throughout development (FB-Gal4). **C** No body fat increase upon *Stim*KD in fat storage tissue of L3 larvae or immature adult flies. **D** No change in metabolic rate (estimated by average CO_2 production per hour) during at day six of *Stim*KD-dependent adiposity progression. Error bars represent SD. *p≤0.05, **p≤0.01 and ***p≤0.001, n.s. not significant

Figure S4 related to Figure 5: Proximal and remote regulatory effects of fat storage tissue targeted functional impairment of *Stim*. **A** Down-regulated abdominal *upd2* but unchanged *Dilp2*, *Dilp5* and *Stim* mRNA abundance in the brain of *Stim*KD flies. **B,C** Decreased DILP2 content in brain IPCs from *Stim*KD flies compared to controls. Maximum intensity projections (**B**) and corresponding fluorescence value quantifications (**C**) of anti-DILP2 immunohistochemistry. Scale bar: 20μm. **D** *Stim* is not nutritionally regulated. Unchanged abdominal *Stim* mRNA abundance contrasts the severe body fat depletion / re-accumulation of six day old male w^{1118} flies subject to a 24h starvation -> 24h re-feeding regimen. Error bars represent SD. *p≤0.05, **p≤0.01 and ***p≤0.001, n.s. not significant

Table S2 related to Extended experimental procedures: List of fly stocks used in this study.

Table S3 related to Extended experimental procedures: List of oligonucleotides used in this study.

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Short name	Stock number	Genotype	Reference/ source
ts-FB-Gal4	RKF805	*y*w* ; P{w[+mW.hs]=GawB}FB P{w[+m*] UAS-GFP 1010T2}#2; P{w[+mC]=tubP-GAL80[ts]}2*	(Beller et al., 2010)
FB-Gal4	RKF125	*w*; P{w[+mW.hs]=GawB}FB+SNS*	(Grönke et al., 2003)
w-control	RKF1084	*w[1118]*	VDRC60000
to-Gal4	RKF1088	*w*; to-Gal4 (II)*	(Dauwalder et al., 2002)
	RKF1192	*w*; rev[to-Gal4 (II)]#1*	this study
yolk-Gal4	RKF1091	*w*; yolk-Gal4 (II)*	(Georgel et al., 2001)
FBI-26-Gal4	RKF1045	*P{Switch1}FBI-26; UAS-GFP*	(Suh et al., 2007)
mdy -	RKF1004	*w*; mdy[QX25] cn[1] bw[1] / CyO float.*	(Buszczak et al., 2002)
mdy RNAi			VDRC6367
mdy gof	RKF560	*w*; P{w[+mC]UAS-mdy} / TM3 Sb[1] e[1] float.*	this study
bmm -	SGF529	*w*; bmm[1] / TM3, Sb[1] float.*	(Grönke et al., 2005)
bmm RNAi			VDRC37880
bmm gof	SGF533	*w*; + / +; P{w[+mC] bmm[Scer\UAS]=UAS-bmm}#2d*	(Grönke et al., 2007)
nuclear lacZ:eGFP reporter	GÖ1048	*y[1] w[67c23]; P{UAS-GFP::lacZ.nls}2-1; P{GAL4-btl.S}3-1 / TM3 Sb[1] Ser[1]*	(Shiga et al., 1996)
Pect RNAi1			VDRC27459
Pect RNAi2			VDRC109802
Itp-r83A RNAi1			VDRC106982
Itp-r83A RNAi2			BDSC25937
Itp-r83A DN	RKF1139	*UAS-itpr[Ka901] cDNA on II*	(Srikanth et al., 2006)
Ca-P60A RNAi1			VDRC4474
Ca-P60A RNAi2			VDRC107446
Ca-P60A RNAi3			BDSC25928
Stim RNAi1	RKF1112	*w[1118]; UAS-dStim[RNAi]1 / CyO*	(Eid et al., 2008)
Stim RNAi2			VDRC47073
	RKF1113	*w[1118]; UAS-dStim[RNAi]3 / TM3, Sb**	(Eid et al., 2008)
Stim gof	RKF1114	*w[1118]; UAS-dStim[FL]#290 / CyO*	(Eid et al., 2008)
olf186-F gof	RKF1191	*w*; Orai-pUAST#3 / TM3, Sb[1] e[1]*	(Venkiteswaran and Hasan, 2009)
sec71/DmARFGEF1/2 RNAi1			VDRC33634
sec71/DmARFGEF1/2 RNAi2			VDRC100300

Arf79F/DmARF1 RNAi1			VDRC23082
Arf79F/DmARF1 RNAi2			VDRC103572
Cam RNAi			VDRC102004
	JBF1387	*w*; UAS-dStim[RNAi]1 / CyO float.; UAS-mdy RNAi / TM3 Ser[-]*	this study
	JBF1385	*w*; UAS-dStim[RNAi]1 / CyO float.; P{w[mç]UAS-GFP.nls} / TM3 Ser[-]*	this study
	JBF1386	*w*; P{w[mç]UAS-GFP.nls}; UAS-mdy RNAi*	this study
	JBF1404	*w*; UAS-dStim[RNAi]1 / CyO float.; P{w[+mC] bmm[Scer\UAS]=UAS-bmm}#2d/TM3 Sb[-]*	this study
sNPF gof	JBF1393	*w*; UAS-sNPF; UAS-sNPF*	(Hong et al., 2012)
sNPF RNAi	RKF1320	*w*;+/+; UAS-sNPF.RI III*	(Hong et al., 2012)
sNPF-Gal4	RKF1321	*w*; +/+; sNPF.RI-GAL4*	(Hong et al., 2012)
CaLexA reporter	JBF1324	*LexAop-CD8-GFP-2A-CD8-GFP / LexAop-CD8-GFP-2A-CD8-GFP; UAS-mLexA-VP-NFAT C del (4-2), LexAop-CD2-GFP / TM6B*	(Masuyama et al., 2012)
FB-Gal4>*Stim* RNAi3	JBF1397	*w*; P{w[+mW.hs]=GawB}FB+SNS; UAS-UAS-dStim[RNAi]3 / TM3 Ser[-]*	this study
ts-FB-Gal4	RKF805	*y*w* ; P{w[+mW.hs]=GawB}FB P{w[+m*] UAS-GFP 1010T2}#2; P{w[+mC]=tubP-GAL80[ts]}2*	(Beller et al., 2010)
FB-Gal4	RKF125	*w*; P{w[+mW.hs]=GawB}FB+SNS*	(Grönke et al., 2003)
w-control	RKF1084	*w[1118]*	VDRC60000

Table S3 related to Extended experimental procedures: List of oligonucleotides used in this study.

Detected gene	Sequence / Identity	Reference
RpL32	QT00985677	www.qiagen.com
Fas (CG3523)	5` CCCCAGGAGGTGAACTCTATCA 3` 5` GACTTGACCGATCCGATCAAC 3`	(Seegmiller et al., 2002)
mdy	5` AAGACAGGCCTCTACTATTGT 3` 5` CCCATCATGCCCATAAATGCC 3`	this study
Stim	QT00923020	www.qiagen.com
ACS	5` AAATCCCATGGACCGATGAC 3` 5` TGTAGAGCATGAACAATGGATCCT 3`	(Seegmiller et al., 2002)
ACC	5` GTGCAACTGTTGGCAGATCAGT 3` 5` TTTCTGATGACGACGCTGGAT 3`	(Seegmiller et al., 2002)
bmm	QT00964460	www.qiagen.com
Act5c	5` GTGCACCGCAAGTGCTTCTAA 3` 5` TGCTGCACTCCAAACTTCCAC 3`	(Bauer et al., 2009)
upd2	5` CGGAACATCACGATGAGCGAAT 3` 5` TCGGCAGGAACTTGTACTCG 3`	(Rajan and Perrimon, 2012)
sNPF	5` CCCGAAAACTTTTAGACTCA 3` 5` TTTTCAAACATTTCCATCGT 3`	(Hong et al., 2012)
Dilp2	5` ACGAGGTGCTGAGTATGGTGTGCG 3` 5` CACTTCGCAGCGGTTCCGATATCG 3`	(Wang et al., 2008)
Dilp5	5` GAGGCACCTTGGGCCTATTC 3` 5` CATGTGGTGAGATTCGGAGCTA 3`	(Broughton et al., 2005)

Extended experimental procedures

Fly stocks and husbandry

Transgenic RNAi fly strains used in this study were received from either the VDRC (GD and KK library; (Dietzl et al., 2007) and http://stockcenter.vdrc.at/) or the BDSC (Harvard TRiP library; www.flyrnai.org) fly stock centers and are summarized in Table S1. All other fly stocks used in this study are listed in Table S2. Young flies 24-48h after hatching were kept for 5-6d on standard food (20l H_2O, 125g agar, 360g dry yeast, 200g soy flour, 440g treacle, 1.6kg cornmeal, 1.6kg malt, 125ml propionic acid and 30g nipagine). Flies containing the ts-FB-Gal4 driver were kept under repressed conditions (18°C). Afterwards these flies were induced for further 6 days at active conditions (30°C). Flies containing the FBI-26-, *yolk*-or the FB-Gal4 drivers were continuously kept at 25°C. For low/medium/high sugar experiments flies were placed for 5d on a 10% yeast, 1.5% agar food (Skorupa et al., 2008) including preservatives (see above) containing low (2.3%), medium (8.7%) or high (30.6%) sucrose concentrations. For conditional transgene induction using the SWITCH system (Roman et al., 2001) (FBI-26-Gal4 driver) the flies were kept for 5d on standard food containing either 200µM RU486 (mifepristone; Sigma M8046; stock solution 10mM in 80% ethanol; ON-condition) or solvent only (OFF-condition).

Preparation and imaging of adult fly guts

Flies carrying the ts-FB-Gal4 were crossed to nuclear ßGal:eGFP reporter flies and male progeny was kept for 6d at 30°C (active condition) or 18°C (repressed condition), respectively. Subsequently, adult fly guts were manually dissected in 1xPBS and reproductive tract structures removed. Afterwards guts were mounted in 1xPBS/30% glycerol with DAPI (1:1000; Invitrogen D1306) for at least 10min and imaged as described below. Alternatively, guts were stained with 0.5% Oil Red O (Sigma O-0526; solved in propylenglycol) for 30min at 65°C, washed three times with propylenglycol and 1xPBS before mounting in 1xPBS/30% glycerol. Specimens were imaged with a Zeiss Axiovert 200M at 5x magnification. Images were assembled using Adobe Photoshop CS3.

Gene ontology and orthology analysis

For obesity and anti-obesity genes all available gene ontology (GO) terms in the categories “biological process” and “molecular function” were retrieved. Statistically significant over- or underrepresented GO terms in the obesity / anti-obesity gene set as compared to the reference gene set were identified using the GOToolBox (GTB) suite (at http://genome.crg.es/GOToolBox/; last update: 2009-07-22) with hypergeometric calculation mode, level cutting 5 and Benjamini & Hochberg correction. Enriched (E) and depleted (D) GO terms are shown in Table S1. Enriched GO terms in the category “biological process” were filtered according to

p<0.012, reference gene set range 4-100 and enrichment factor >3 and ranked according to their E value (Table S1; GO biological process). Human and mouse orthologs (inparalog score 1) of *Drosophila* genes were identified using InParanoid 7 (Ostlund et al., 2009).

Metabolic and physiological analyses

Lipid analysis

Organismal fat content (expressed as glycerides) of migrating L3 larvae, 0-4h old freshly hatched and 6d old male flies was quantified by an coupled colorimetric assay as described (Grönke et al., 2003; Hildebrandt et al., 2011). Depicted are representative experiments with average values of triplicate measurements (on five larvae or flies per replicate) and corresponding standard deviations. Experiments were repeated at least twice.

Fly lipids were extracted according to Bligh and Dyer (Bligh and Dyer, 1959). Briefly, the wet weight of duplicate samples of 3-8 6d old male flies was determined and the flies were homogenized in 150μl methanol, 75μl chloroform and 60μl water in a Bioruptor sonifier (www.diagenode.com). Lipids were extracted for 1 hour at 37^{o}C before adding 75μl chloroform and 75μl 1M KCl. Separation of phases was achieved by centrifugation (3000 rpm for 2min) and the chloroform phase solvent

was evaporated in a SpeedVac concentrator. Lipid pellets were resuspended in 25-50μl chloroform/methanol (1:1).

Lipids extracted from 1-3.5mg fly wet weight were separated on high performance thin layer chromatography (HPTLC) plates (Merck, 105633) using n-hexane/diethylether/acetic acid (70:30:1, v/v/v; Merck) for unpolar lipids TAG, DAG and FA, respectively, as liquid phase along with the following standard lipids: trioleoylglycerol (TAG; Sigma T7140), pentadecanoin (DAG; Sigma D8508) and stearic acid (FA; Fluka 85679). Plates were air dried, dipped into 8% (w/v) H_3PO_4 containing 10% (w/v) copper (II) sulfate pentahydrate and charred for 10 min at 180°C. Fly lipid classes were quantified by photo densitometry (LAS-1000 and Image Gauge V3.45, FujiFilm) scaled to a dilution series of the corresponding lipid standard.

Carbohydrate analysis

For circulating sugar analysis from hemolymph, 15 male flies were subjected to thorax perforation (10 pricks with a fine needle). Perforated flies were placed in a 0.5ml-tube (with a hole in the bottom, covered with a cotton plug) caged in a 1.5ml tube and hemolymph was extracted by centrifugation (2min, 14.000 rpm, 4°C). Hemolymph (0.5µl per assay) was used immediately for sugar determination. Hemolymph glucose levels were determined with Infinity Glucose Hexokinase solution (Thermo Electron TR15421) and by measuring the absorbance at 340nm (T_1). For the determination of hemolymph trehalose levels, trehalase (Sigma

T8778; final: 0.05U/ml) was added and the samples were incubated at 37ºC overnight followed by a second reading of absorbance at 340nm (T_2). The amount of trehalose is equal to the final absorbance reading (T_2) minus that of the first reading (T_1). Final glucose and trehalose concentrations were determined by reference to standard curves (Glucose standard: Sigma G7528, Trehalose standard: Sigma T3663). Glycogen levels of whole flies were determined according to published protocols (Iijima et al., 2009; Palanker et al., 2009) with modifications. In detail, five male flies were quick frozen in liquid nitrogen and homogenized in 1ml 0.5% Tween 20 in 1xPBS. Homogenates were immediately incubated at 70°C for 5min and centrifuged at 12.000rpm for 3 min. Thirty µl aliquots of the supernatant were added to either 100µl 1xPBS, 100µl Glucose Assay Kit solution (Sigma, GAGO20-1KT) and 100µl Glucose Assay Kit solution + 0.3 U Amyloglucosidase (Sigma, 10115-1G-F) in a 96-well microplate format. Microplates were incubated for 30min at 37°C and absorbance at 540nm was determined in a microplate reader (BioRad Microplate Reader Benchmark). Glucose and glucose plus glycogen content were determined using a standard curve (Glycogen standard: Sigma G0885). The amount of glycogen was determined by subtracting the glucose from the glucose plus glycogen values.

For the glycogen mobilization analysis, *Stim* RNAi1 flies were crossed against *w*-control and ts-FB-Gal4, respectively, and F1 males were raised for 6d on OFF-conditions, induced for further 6d on ON-conditions and subsequently subjected to water-only starvation. Triplicates of five male flies were collected and quick frozen

in liquid nitrogen at different time during the starvation paradigm until the median starvation time was reached. Glycogen levels were quantified as described above.

Feeding and food intake

Food intake quantification of adult male flies under *ad libitum* feeding and pair fed conditions was done as described (Beller et al., 2010) using a modified CAFE system (Ja et al., 2007). In detail, freshly hatched F1 male progeny of *Stim* RNAi1 flies crossed against *w*-control and ts-FB-Gal4, respectively, were raised for 6d on *Stim* RNAi repressed conditions (18°C). Afterwards flies (n=22) were transferred into individual chambers of a modified CAFE system (24-well plate) with 100% humidity at 25°C (active condition) and 12:12 LD cycle. Flies were *ad libitum* fed on liquid diet (5% sucrose/5% dry yeast) in 5µl capillaries (ringcaps, Hirschmann, Ref 9600105) and food intake was determined daily when fresh food was provided. Food consumption reads were corrected by an evaporation control (empty fly chamber).

For pair feeding the same genotypes like above were provided with the daily food ration consumed by *ad libitum* fed control flies only. To this aim capillaries were loaded with the respective food volume overlayed by 2µl of mineral oil (Sigma, M-5904). After six days of feeding, body fat content was measured as described above.

Energy expenditure

The metabolic rate of male progeny flies of *Stim* RNAi1 crossed against *w*-control and ts-FB-Gal4 flies raised and analyzed on repressed (18°C)- and active (25°C)-conditions was estimated on the basis of manometric measurement of the CO_2 production as described in (Kucherenko et al., 2011) for four different time periods during the circadian light cycle. Triplicates of three male flies of each genotype were placed in a sealed fly chamber with CO_2 absorber (Soda lime: Wako, 196-10525), connected to an eosin-stained (Sigma-Aldrich, HT110232-1L) H_2O reservoir by a 50µl capillary (Brand, 708733) in a closed TLC running chamber. The change in the meniscus of the capillaries was determined every 30min during a 2h time period. Data sets were normalized against the respective genotypes under OFF-conditions and daily averages were calculated on the basis of the four points in time.

Starvation assay

For the glycogen mobilization analysis male flies were pre-treated and starved as described above.

For determination of the *Stim* expression response to starvation, male *w*-control flies (aged 0-24h) were kept for 6d at 25°C on standard food. Afterwards flies were subjected to water-only starvation for 24h or continuously fed in parallel. Groups of flies were collected for *Stim* mRNA and body fat quantification. The starved flies were re-fed for additional 24h and compared to continuously fed flies (see Fig. S4D).

Locomotor activity

Male progeny flies (32 per genotype, aged 0-24h) of *Stim* RNAi1 crossed against *w*-control and ts-FB-Gal4 flies were analysed in the TriKinetics DAM2 system (http://www.trikinetics.com/) on standard food. The activity was monitored for 6d at 18°C (repressed condition) followed by further 6 days at 25°C (active condition). The cumulative activity was determined and normalized to OFF condition.

DILP2 Immunohistochemistry

Male progeny flies of *Stim* RNAi1 crossed against *w*-control and ts-FB-Gal4 flies were raised for 6d on OFF-conditions and induced for further 6d on ON-conditions. Brains were dissected in 0.3% Triton-X100 /1xPBS and fixed in 4% paraformaldehyde for 25min. Fixed brains were intensively washed in 0.3% TritonX100/1xPBS, blocked for 2h in 5% BSA in 0.3% Triton-X100/1xPBS and incubated with an anti-DILP2 antibody (1:400, (Géminard et al., 2009)) overnight at 4°C. After further washing, brains were incubated with an anti-rat IgG antibody (1:500, Invitrogen A-11077) labelled with Alexa Flour 568 for 2h at room temperature. Brains were washed, embedded in VECTASHIELD mounting medium (Vector laboratories, contains DAPI, H-1500) and imaged by confocal microscopy. To quantify the DILP2-dependent fluorescence, maximum intensity projections were used for the mean gray value and ImageJ for quantification.

Molecular biology

Quantitative RT-PCR analysis was performed as described (Beller et al., 2010, Grönke et al., 2009). Larvae or adult flies were snap frozen and RNA of whole larvae or abdomen of adults was extracted using the peqGOLD TriFast reagent (www.peqlab.de). For RNA extraction of adult brains, 50 brains were dissected from adult male flies and RNA was extracted as described above. Total RNA (0.8-1µg) was used for cDNA synthesis using the QuantiTect Reverse Transcription Kit (www.qiagen.com). Real-time PCR analysis was performed on an Applied Biosystems StepOnePlus or a Qiagen Rotor-Gene Q using Applied Biosystems Fast SYBR Green Master Mix (www.appliedbiosystems.com) with *Ribosomal protein L32* and/or *Act5C* as internal control. Quantifications of mRNA abundance are shown with standard deviations. The oligonucleotide primers used in this study are listed in Table S3.

Histology

Cryosections were done on adult male fly progeny of RNAi transgenic flies crossed against ts-FB-Gal4 and the *w*-control, raised under OFF-conditions and fed for 6d under OFF- or ON-conditions. Wings were removed and whole flies were incubated in OCT medium (www.leica-microsystems.com) for 6h prior to freezing on dry ice. 18µm sections at -16^{o}C/-13^{o}C chamber/object temperature were done on a Leica CM 3050 S cryostat (www.leica-microsystems.com) and transferred on

coated slides (Superfrost Plus; www.thermoscientific.com). Sections were dried for 5min at 30^{o}C prior to staining.

For ß-galactosidase activity staining, cryosections were thawed and specimens were fixed for 10min in 0.5% glutaraldehyde/1xPBS. Fixation was stopped by washing specimens three times in 1xPBS and slides were exposed to staining solution (5mM $K_3[Fe^{III}(CN)_6]$, 5mM $K_4[Fe^{II}(CN)_6]$, 2mg/ml X-Gal in 1xPBS) at 37^{o}C. After completion of color development, specimens were washed three times in 1xPBS, post-fixed for 30min in 5% paraformaldehyde/1xPBS, washed as above and mounted in 30% glycerol/1xPBS. Microscopic analysis and image acquisition were done using a Zeiss Axiophot (www.zeiss.com) microscope in bright field mode equipped with a ProgRes 3012 camera (www.progres-camera.com).

For Oil Red O staining, cryosections were thawed and fixed for 30min in 5% paraformaldehyde/1xPBS. Specimens were washed three times in 1xPBS and covered with propylenglycol (SAFC W294004-K) for 10min. Sections were stained in 0.5% Oil Red O (Sigma O0625) in propylenglycol for three hours and staining solution was replaced by propylenglycol for 10min followed by three washing steps in 1xPBS. Sections were mounted and imaged as described above.

Electron microscopy

The abdomen of adult male *Drosophila* was placed on a 150µm flat embedding specimen holder using 1-hexadecen (Engineering Office M. Wohlwend GmbH, Sennwald, Switzerland) and frozen in a Leica HBM 100 high-pressure freezer (Leica Microsystems, Wetzlar, D-35578 Germany). The embedding of the

vitrified samples was performed using an Automatic Freeze Substitution Unit (AFS) (Leica).

Substitution was done at -90°C in a solution containing anhydrous acetone, 0.1% tannic acid and 0.5% glutaraldehyde over 72h and in anhydrous acetone, 2% OsO4, 0.5% glutaraldehyde for additional 8h. After a further incubation over 18h at -20°C samples were warmed up to +4°C and washed with anhydrous acetone subsequently. The samples were embedded at room temperature in Agar 100 (Epon 812 equivalent) and polymerised at 60°C for 24h.

Images were taken in a Philips CM120 electron microscope (Philips Inc.) using a TemCam 224A slow scan CCD camera (TVIPS, Gauting, Germany).

Confocal microscopy

For the analysis of the adult fat body by confocal microscopy, female abdomens were opened to remove the gut and the reproductive organs. The fat body enriched carcass was embedded in 1xPBS containing LD540 (Spandl et al., 2009) or BODIPY 493/503 (Invitrogen D3922) (for lipid droplets), DAPI (Invitrogen D1306; for nuclei) and CellMask (Invitrogen C10046; for membranes) dyes. Pictures were taken with a Zeiss LSM-780 at 25x magnification in fixed gain mode. Single optical sections of 15 cells from four independent fat body preparations were analyzed for the lipid area and the cell area quantification in Fig. 3G,H by using ImageJ.

For the imaging with the CaLexA system (Masuyama et al., 2012) fat body enriched carcasses of 10d old females progeny flies from CaLexA reporter flies

crossed against FB-Gal4 flies or FB-Gal4>Stim RNAi3 flies were prepared and images were captured as described above.

Western blot analysis

For Western Blot analysis, protein homogenates from total L3 larvae or adult fly abdomen corresponding to one fly/lane were separated on a 12% SDS-PAGE gel and blotted on nitrocellulose membrane (Thermo scientific #88018). Blots were washed in 1xPBT (Tween-20 0,1%) and blocked over night at 4°C in 1xPBT/5% BSA and incubated with primary antibodies for 1h. After washing in 1xPBT, blots were incubated with secondary antibodies for 30min, washed again in 1xPBT and developed in Super Signal West Pico Chemiluminescent solution (Thermo scientific #34080) for 2min. Signals were visualized on X-ray films (Kodak BioMax XAR Film, CAT 1651454), and scanned images were processed with Adobe Photoshop CS3. For signal intensity normalization developed blots were washed in PBT, restored in Restore Solution (Thermo scientific #21059) and then processed as described above.

Antibodies were used at the following final concentrations: guinea pig anti-STIM and mouse anti-(ß)Tubulin (1:2000 and 1:1.000; this study and Developmental Studies Hybridoma Bank #E7); goat anti-mouse IgG-HRP (1:40.000; Pierce #31430) and donkey anti-guinea pig IgG-HRP (IR Europe 706-055-148; 1:20.000). Guinea pig anti-STIM antibodies were generated against the HRQLDDDDNGNIDLSESDDFLRC peptide and affinity-purified (for the generation

of a corresponding sheep antibody see (Eid et al., 2008). To reduce unspecific binding, anti-STIM antibody (dil. 1:2.000) was pre-incubated to a blot with proteins from *Stim* RNAi knockdown flies prior to use. Signal intensities were quantified in a Fujifilm Intelligent Dark Box II using Fujifilm LAS-1000 Pro 2.11 and Fujifilm Sciencelab99 L Process 1.95.

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