

**Developmental Cell, Volume 43**

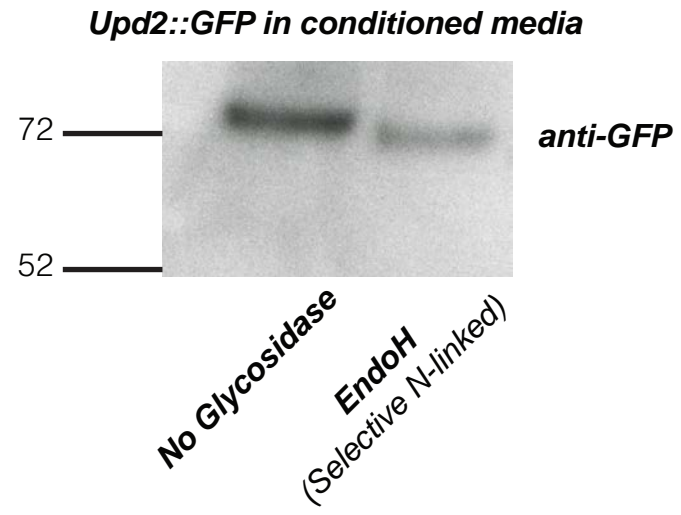
**Supplemental Information**

**A Mechanism Coupling Systemic**

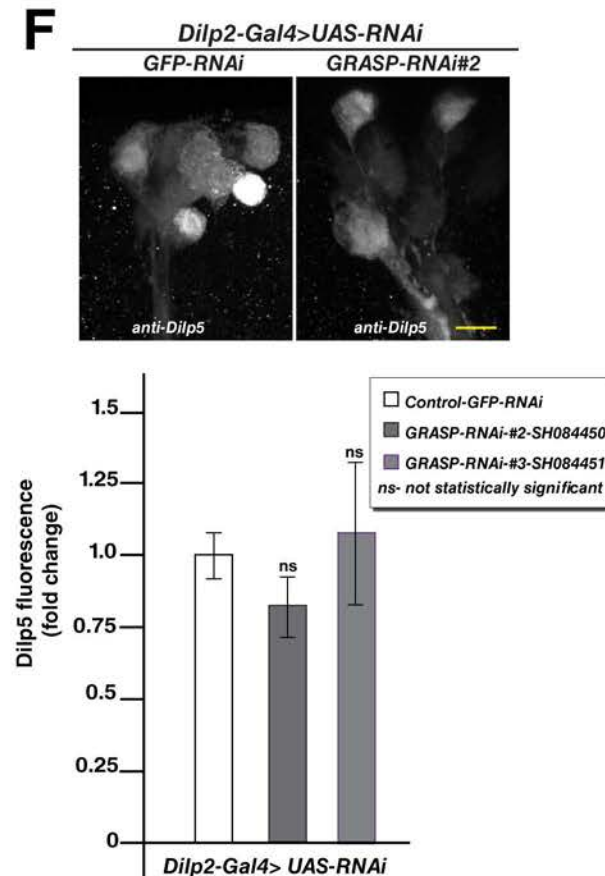
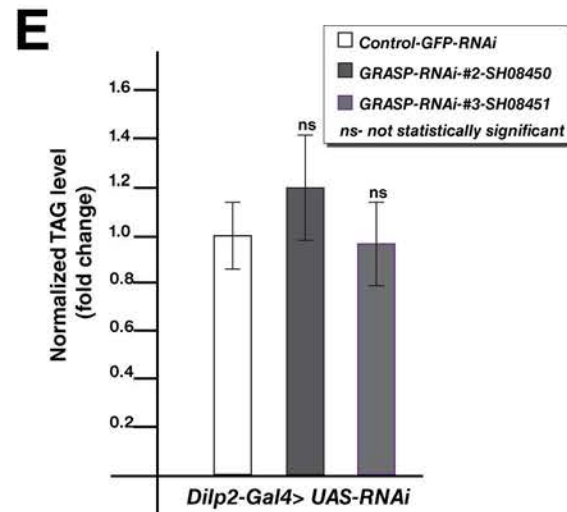
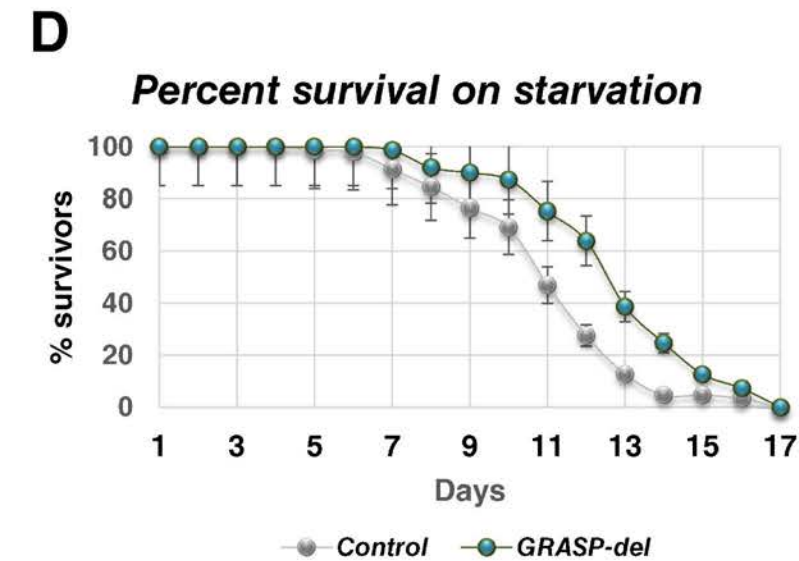
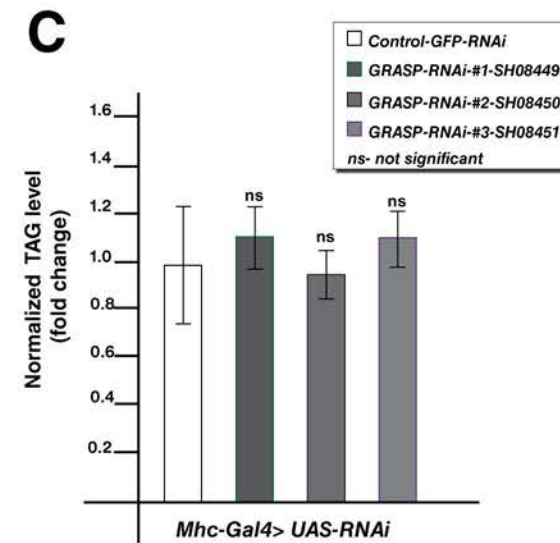
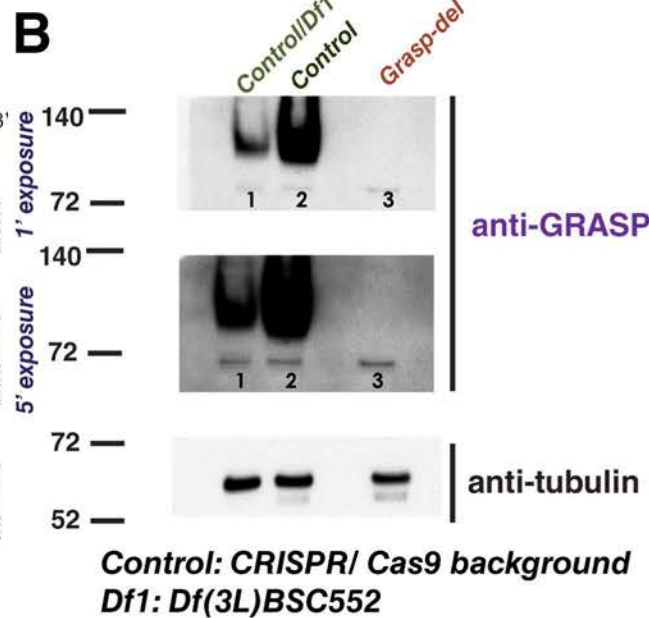
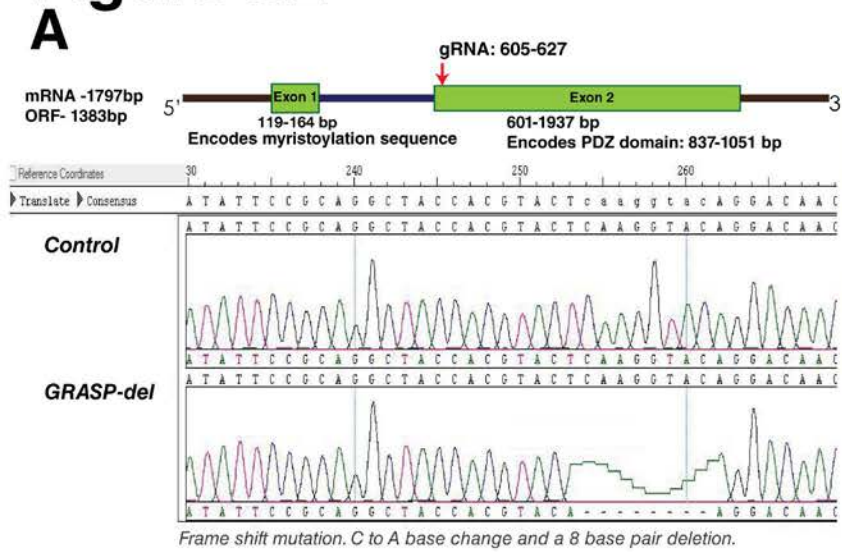
**Energy Sensing to Adipokine Secretion**

**Akhila Rajan, Benjamin E. Housden, Frederik Wirtz-Peitz, Laura  
Holderbaum, and Norbert Perrimon**

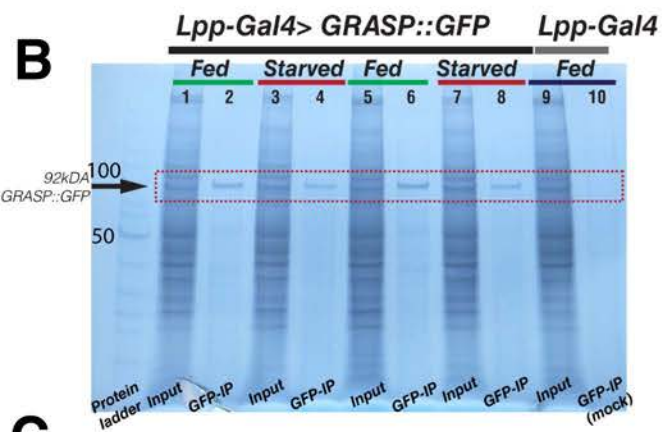
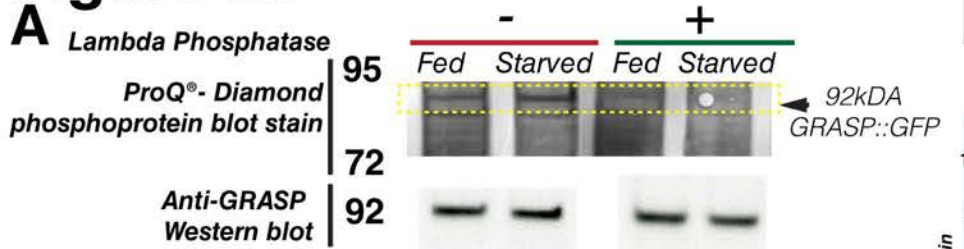
# Figure S1



# Figure S2

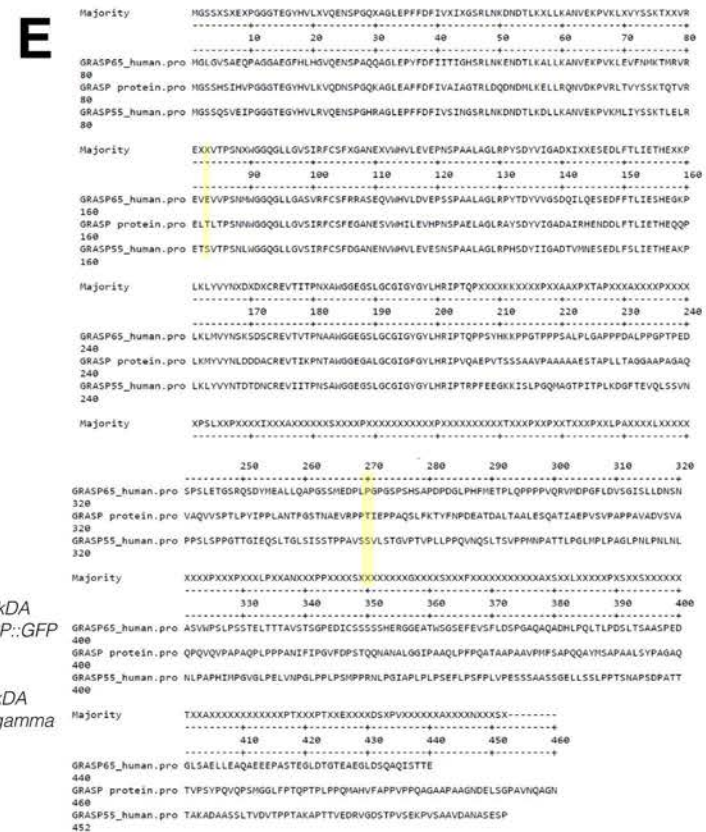
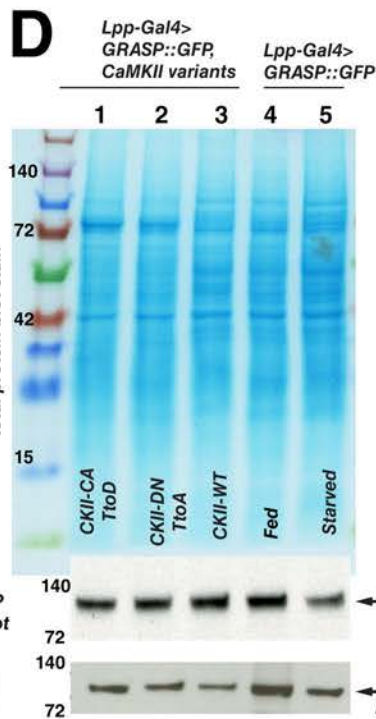


# Figure S3

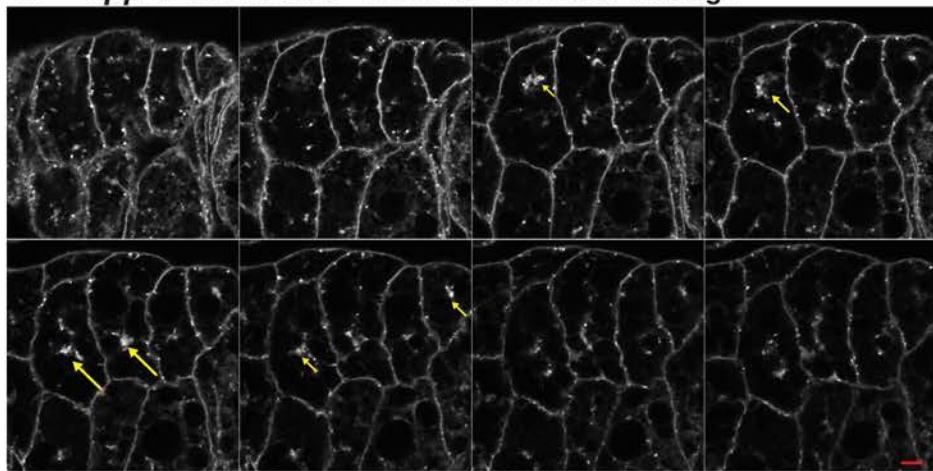


**C**

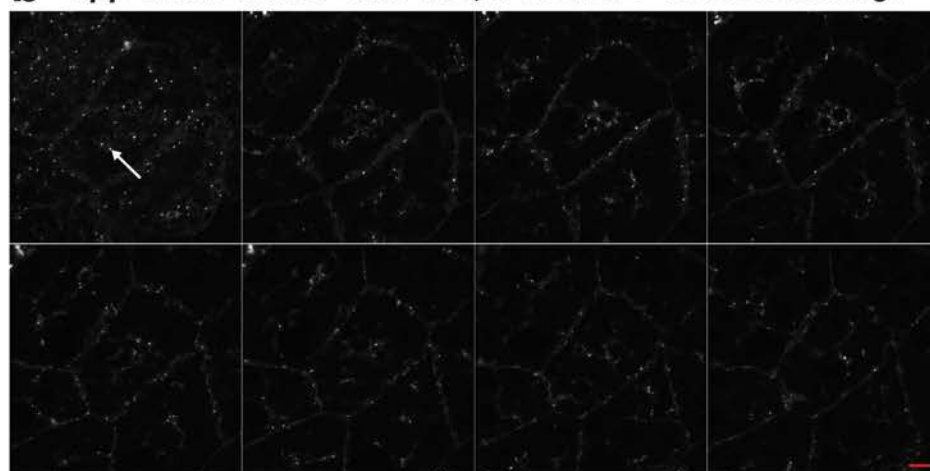
Identified Protein	Fed	Starved
GRASP65	35.8	32.25
Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type (Ca-P60A)	10	8
Calnexin/ Calreticulin	3.3	1.7
Calmodulin (Cam)	5.3	3.0
* Calmodulin dependent kinase II (CaMKII)	7	0.5
Calcium-binding protein 1 (CaBP1)	2	0
Calbindin-32 (Cbp53E)	0	4
Plasma membrane Calcium Channel (PMCA)	0	1



**Fa** *Lpp-Gal4* > GRASP-WT::GFP-XY slices-Montage

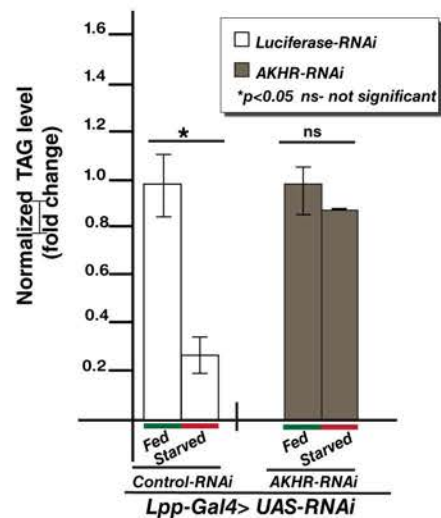


**Fb** *Lpp-Gal4* > GRASP-CKII-T83,270-D::GFP-XY slices-Montage

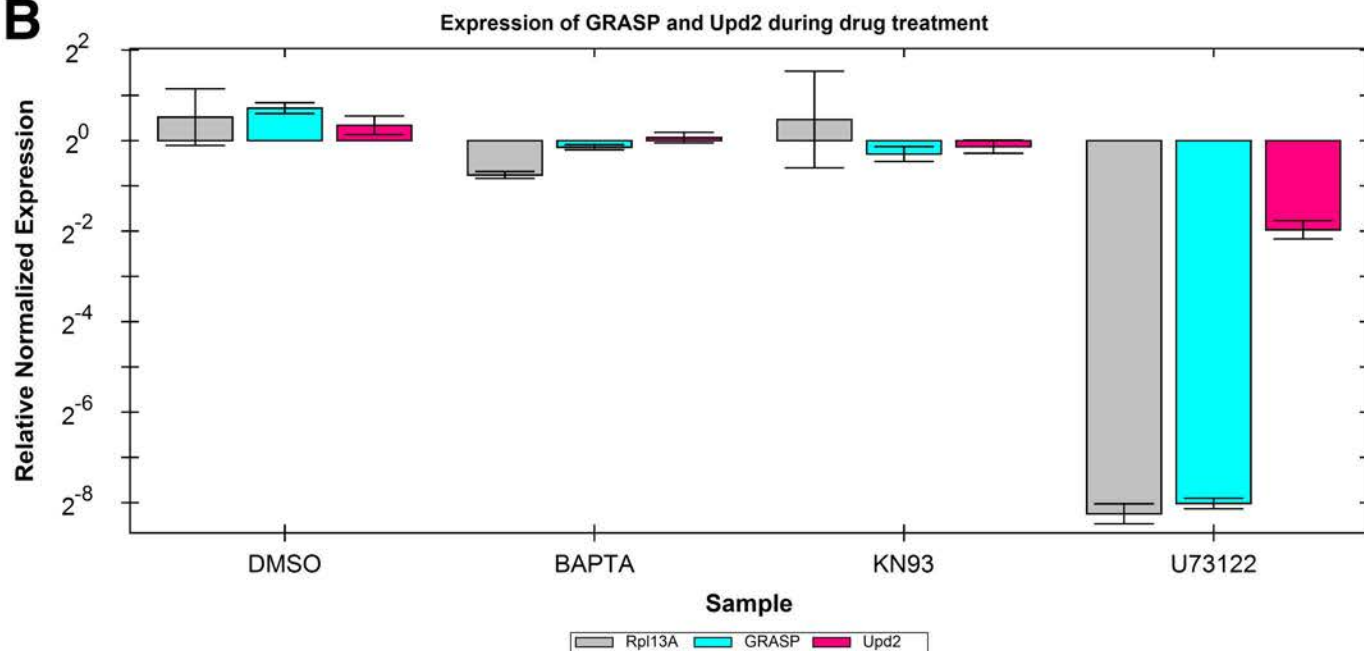


# Figure S4

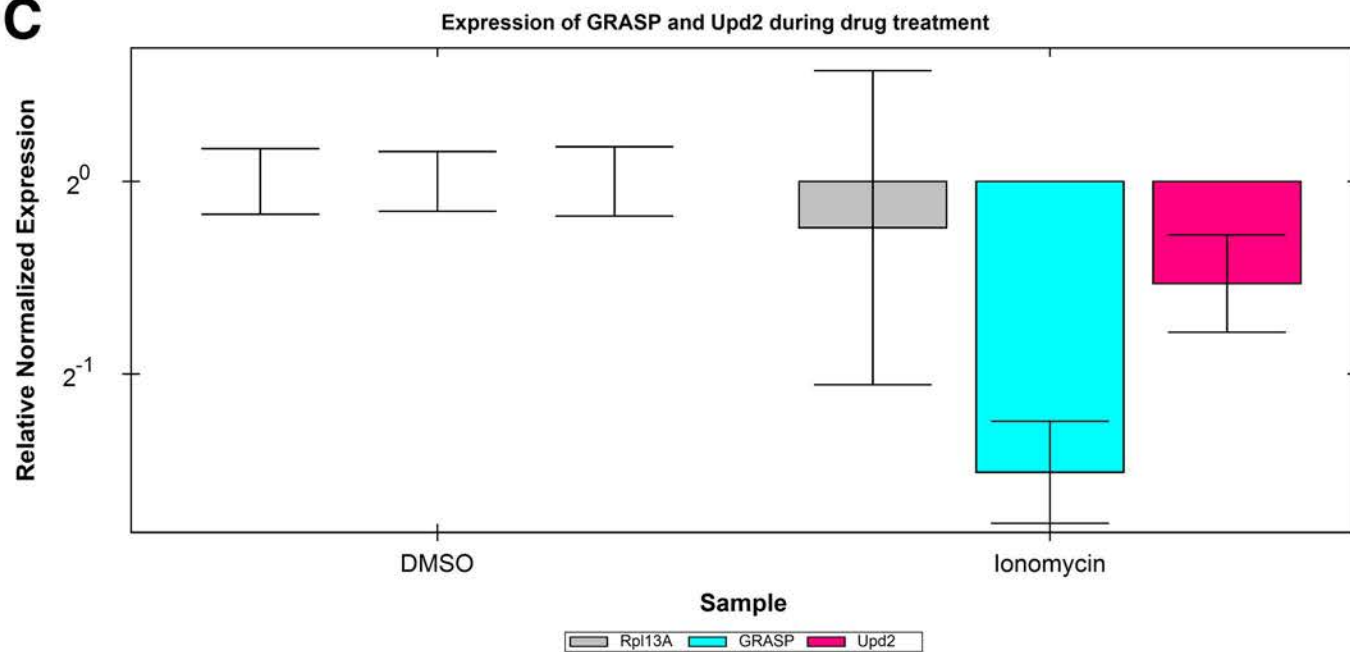
## A



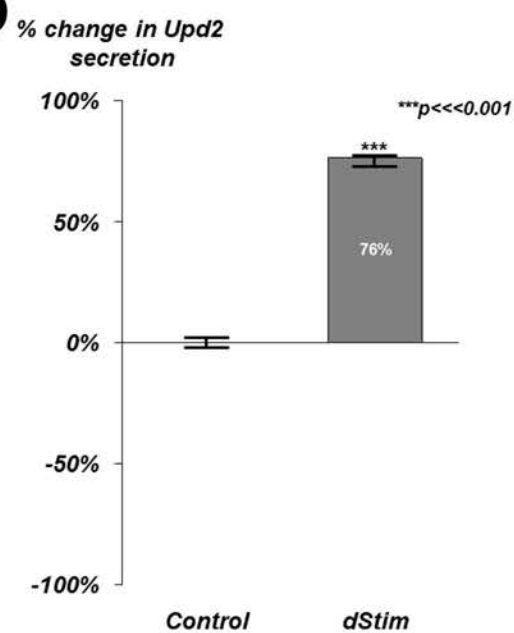
## B



## C



## D



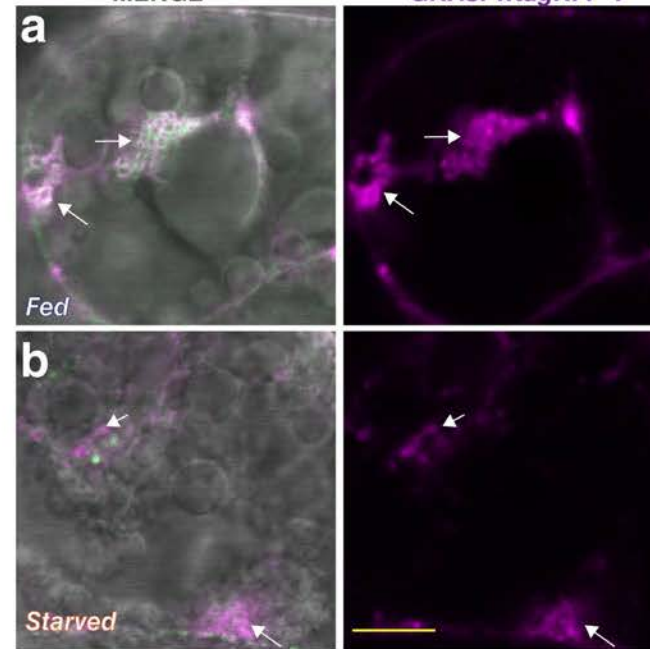


# Figure S6

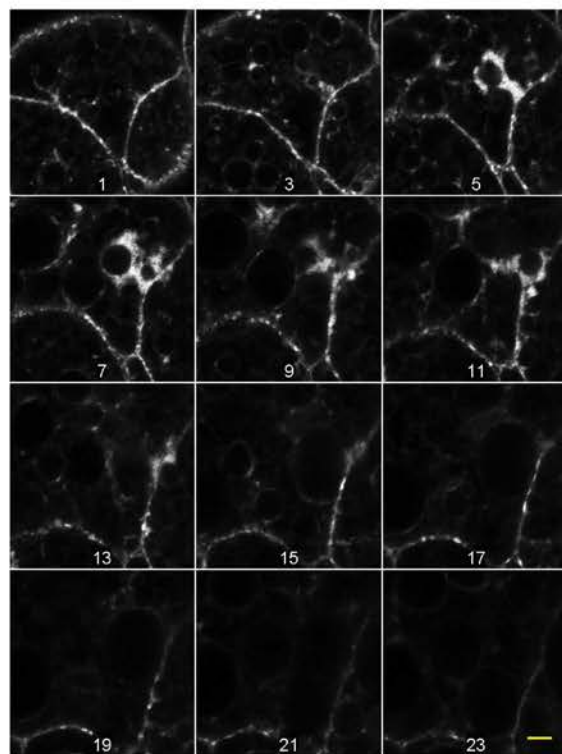
## A

*Lpp-Gal4>GRASP::GFP; GRASP::tagRFP-T*

MERGE *GRASP::tagRFP-T*

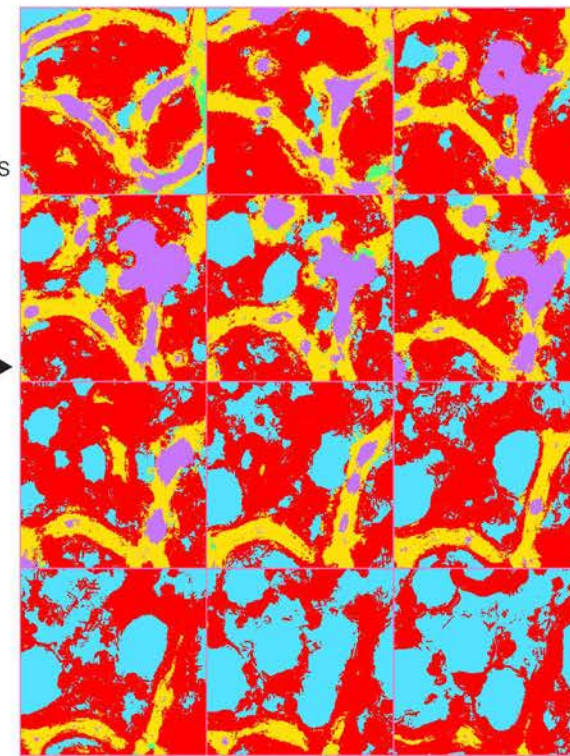
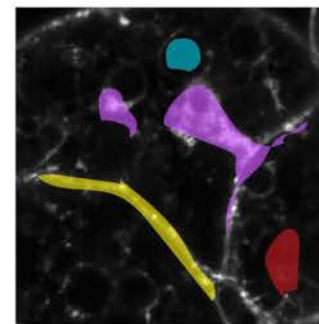


## B



- — Punctae
- — Membrane
- — Hubs/GRASP compartments
- — Background

Segmentation using Weka training classifier



Custom ImageJ Macro obtain % Area of every color /slice

**Figure S1: Related to Figure 1- *Drosophila* Upd2 adopts an unconventional secretion route mediated by GRASP.**

Anti-GFP Western blot of GFP IP from conditioned media of S2R+ cells transfected with Upd2::GFP. GFP immunoprecipitate (IP) digested with EndoH that selectively removes N-linked glycans. Control is Upd2::GFP IP not treated with glycosidases. Note the mobility shift in the EndoH lane.

**Figure S2: Related to Figure 2- *Drosophila* GRASP, via its role in Upd2 secretion, affects systemic lipid homeostasis.**

**(A)** Schematic of gRNA design used for generating the *GRASP* deletion (*GRASP-del*) mutant using Cas9/CRISPR. Snapshot of the sequencing read that identified the frame shift mutation in *GRASP-del* allele.

**(B)** Anti-GRASP Western blot of total protein lysates from adult fat bodies. Control/*Df(3L)BSC552* (lane 1), Control (lane 2), *GRASP-del* (lane 3). The antibody detects a band that runs at M.W. close to 72 kDa in wildtype (lane 2), which is reduced in heterozygotes (lane 1) and absent in homozygous animals for *GRASP-del* (lane 3). Anti-tubulin Western blot of the same membrane is shown as loading control. Control (Cas9/CRISPR background strain); *Df(3L)BSC552* (is a large deletion line that removes *GRASP* and neighboring genes).

**(C)** Normalized triacylglycerol (TAG) levels in adult *Drosophila* males relative to controls. Muscle specific *GRASP* knockdown, using three independent transgenic strains (*GRASP-RNAi*), compared to control (*GFP-RNAi*).

**(D)** Quantification of percent survival on starvation. Comparison between Control (Cas9/CRISPR background strain) and *GRASP-del*. 10 biological replicates, with N=15 adult male flies per replicate was used for this assay.

**(E)** Quantification of normalized TAG levels in flies that have insulin neuron specific knockdown of *GRASP* (*GRASP-RNAi*) relative to control (*GFP-RNAi*).

**(F)** Projection of optical XY sections of IPCs in *Drosophila* adult brain, stained with antibody against *Drosophila* insulin (*Dilp5*), in control (*Dilp2-Gal4 > UAS-GFP-RNAi* background) and IPC specific *GRASP*-knockdown (*Dilp2-Gal4 > UAS-GRASP-RNAi*). Quantification of the relative change in total *Dilp5* fluorescence in IPC cell bodies from images acquired under the same conditions from control and two independent transgenic strains (*GRASP-RNAi*). 7-10 brains per genotype were used for quantification. Scale bar- yellow line- represents 10µM.

**Figure S3: Related to Figure 3- GRASP phosphorylation in *Drosophila* adult fat cells is nutrient sensitive and regulated by CaMKII.**

**(A)** Protein lysates from flies expressing GFP-tagged GRASP specifically in fat cells. 25µg of protein was loaded per lane from flies subjected to starvation versus those fed *ad libitum*. Lysates were either untreated (-) or treated with lambda phosphatase, an enzyme that removes phosphorylation from Ser and Thr residues. The blots were stained with ProQ®-Diamond phosphoprotein stain and were laser scanned (see



Experimental Procedures) to detect phosphorylation signature. Note the lack of band at 92 kDa when lysates were treated with lambda phosphatase. The same blots were probed with anti-GRASP antibody to check for levels of GRASP protein.

**(B)** To identify GRASP interactors, specific to a particular systemic nutrient state (Fed or starved), GFP tagged GRASP expressed in fat cells was immunoprecipitated (IP). The zinc stained gel shows the representative GRASP::GFP samples that were subject to mass-spec analyses. Lanes 1, 3, 5, 7, 9 are loaded with 20% input; lanes 2, 4, 8, 10 contain 33% of GRASP::GFP IP. Note that lane 10 is a mock, and lacks the 92kDa GRASP band. Also, note that equal amount of inputs and IP products from two different physiological states (Fed vs. Starved) were used for mass-spec.

**(C)** Table summarizing the average spectral counts of Calcium (Ca<sup>2+</sup>) related proteins that were recovered from 4 independent GRASP complex identification mass-spec experiments. Similar spectral counts were recovered for the bait GRASP under fed and starved states (Grey highlight row). Proteins highlighted in blue were recovered as GRASP interactors with approximately equal spectral counts under fed and starved states. Those highlighted in green interacted predominantly in the fed state. Red rows include Ca<sup>2+</sup> related proteins that were identified as GRASP interactors only under the starved state.

**(D)** Total protein stain on the blotted membrane as performed lysates from flies expressing GFP-tagged GRASP specifically in fat cells (companion to Figure 3B) shows comparable loading of total protein by staining with *MemCode*<sup>®</sup> total protein stain. 25µg of protein was loaded per lane. See band at 42kDa is comparable in all 5 lanes. Blot was also probed with anti-Lsp1-gamma, a protein that is enriched in fat tissue and is at a molecular weight comparable to GRASP::GFP. Note that similar to GRASP::GFP, comparable levels of Lsp1-gamma is expressed in lysates despite opposing manipulations of CaMKII (compare lane 1 and 2), whereas similar to GRASP::GFP levels, despite equal protein loading there is a reduction in Lsp1-gamma levels between fed and starved states (lane 4 and 5) indicating a global translational change.

**(E)** Clustal W alignment of *Drosophila* GRASP with human GRASP55 and GRASP65, used for prediction of conserved CaMKII phosphorylation sites. CaMKII has potentially two conserved phosphorylation motifs (RXXS/T) on *Drosophila* GRASP (T83 and T270 - see highlighted residues). Note the conservation of both CaMKII motifs with human GRASP55 protein. GRASP::GFP transgenic flies were generated in which these two putative phosphorylation sites are mutated [Threonine (T) to Aspartate (D)] to mimic a constitutively phosphorylated state, i.e., phosphomimetic versions. See Figure 3G for effect on GRASP localization.

**(F)** Montage view of XY-slices from *Drosophila* fat cells expressing GFP tagged GRASP wildtype (a) and GRASP putative phosphomimetic for CaMKII (b). The slices represent a series of apical XY slices from a depth of 2 µM to 6µM. Yellow arrows point to LD-associated GRASP clusters; white arrows to punctate localization. Note absence of GRASP membrane and cluster localization in the GRASP putative phosphomimetic versions (b) compared to wildtype GRASP GFP (a) imaged under same conditions. See Figure 3Ga', b' for XY-Projection. Yellow scale bar represents 5µM.

**Figure S4: Related to Figure 4- Intracellular Ca<sup>2+</sup> levels affect GRASP localization in fat cells and Upd2 secretion**

**(A)** Quantification of normalized TAG levels in flies with fat cell specific knockdown of AKH-Receptor (*AKHR-RNAi*) relative to control (*GFP-RNAi*). Note that the starvation control has significantly reduced stored fat compared to *AKHR-RNAi* flies that exhibit defects in fat breakdown on starvation.

**(B)** Relative normalized steady-state mRNA levels of *GRASP* (blue), *Upd2* (pink), and the housekeeping gene *Rpl13A* (grey), in S2R+ cells treated with DMSO, BAPTA-AM (Ca<sup>2+</sup> chelator), KN93 (CaMKII inhibitor) and U73122 (PLC inhibitor). KN93 and BAPTA-AM do not cause significant alterations of *Upd2* and *GRASP* transcription, however, U73122 reduces overall transcription including the housekeeping gene. Despite repression of transcription, increased *Upd2* secretion is observed (4E), suggesting that the effect of U73122 on *Upd2* secretion is post-transcriptional.

**(C)** Steady-state mRNA levels of *Rpl13A* (grey), *GRASP* (blue) and *Upd2* (pink) in S2R+ cells treated with DMSO or Ionomycin (Ionophore- increases intracellular Ca<sup>2+</sup>). Ionomycin does not cause significant alterations of *Upd2* and *Rpl13A* transcription, however, it reduces transcription of the *GRASP*, suggesting that the effect of Ionomycin on *Upd2* secretion may partly depend on *GRASP* transcriptional repression.

**(D)** Quantification of normalized fold change in secreted GFP signal detected using the GFP sandwich ELISA assay performed on conditioned media of S2R+ cells transfected with *Upd2::GFP* and dsRNAs targeting *LacZ* (control) or the *Drosophila* store-operated Ca<sup>2+</sup> regulator *dStim*. Statistical significance quantified by t-test on 6 biological replicates per condition.

**Figure S5: Related to Figure 6- Distribution of tagged GRASP and Upd2 in adult *Drosophila* fat cells.**

**(A)** Schematic of the assay used to image *Drosophila* adult fat body explants. For imaging, the fat tissue was excised from the abdominal region of 5-10 day old adult flies. It is mounted with the cuticle facing down. The side of the fat cell facing the cuticle is referred to as basal.

**(B)** Confocal image of a single optical section along the XY axis of at 3μM depth of adult *Drosophila* fat body expressing GFP tagged GRASP. The dashed boxes are areas whose higher magnification insets are shown in (Ba-Bc). GRASP localization at the surface of LD (Ba); collection of circular GRASP localization (Bb); and punctate and membrane localization of GRASP (Bc). White scale bar represents 10μM and in the Insets (Ba-Bc), yellow scale bar represents 5μM.

**(C)** Confocal image of adult *Drosophila* fat cells expressing membrane targeted myristoylated GFP (*myr::GFP*). Note that there is no LD specific enrichment of *myr::GFP* (red arrows) and that it localizes to the plasma membrane only.

**(D)** Image showing a single optical XY section of *Drosophila* adult fat cells expressing *GRASP::GFP* (green) and *Golgi::RFP* (magenta). Red arrow points to GRASP punctae,

yellow arrow points to GRASP 'hubs'. Note that Golgi RFP does not co-localize with GRASP 'hubs' (yellow), whereas it overlaps with GRASP punctae (red arrow).

In micrographs S5C, D yellow scale bar represents 5 $\mu$ M.

**Figure S6: Related to Figure 7- GRASP localization in *Drosophila* adult fat cells is dependent on systemic nutritional status and regulated by cytosolic calcium.**

**(A)** Confocal images of single optical sections, imaged under the same acquisition conditions, documenting the expression of GRASP::tagRFP-T (magenta) in *Drosophila* adult fat tissue under normal food (a) and starvation (b). Note the presence of apical GRASP (arrow) in fed state (top panel), compared to intensity of apical GRASP in starved state (bottom panel). Note the similar distribution of GFP-tagged GRASP observed in Figure 7A.

**(B)** Image analysis using the WEKA segmentation classifier. The raw image data is processed through the classifier that subdivides the image into 4 colors (see key) based on whether GRASP localization is in 'hubs'- circular structures, membrane, punctae or background. The color coded images are then processed through custom ImageJ macros that quantifies the %area occupied for each color per slice as a percentage of pixels.

In all micrographs yellow scale bar represents 5 $\mu$ M.