Supporting Information

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SI Materials and Methods

Secondary Screening. Unique dsRNAs were prepared for secondary screening. DNA fragments of 400–700 bp were selected using "SnapDragon" (www.flyrnai.org/cgi-bin/RNAi_find_primers.pl) and amplified by PCR. T7 RNA polymerase promoter sequence and the appropriate primers (Dataset S1) were added to each cDNA fragment, and dsRNA was generated using a MEGAscript RNAi kit (Ambion). An irrelevant dsRNA (~500 bp) from the kit was used as a control dsRNA. For knockdown of Mthl10, two unique dsRNAs were constructed, namely, "set-1" and "set-2" (Dataset S2). Data shown in this study were all obtained using set-1, but set-2 yielded similar results. Either S3 cells or $S3^{GCaMP3}$ cells, as indicated, were washed three times with serum-free Schneider's *Drosophila* medium then treated with 20 μg/mL dsRNA for 60 min before readdition of 10% FBS. For cells that were to be used for assaying Ca^{2+} signaling, ~10^5 $S3^{GCaMP3}$ or S3 cells were seeded per well (in 96-well plates). For cells that were to be used for assaying for GDP-TMR binding, ~10^6 $S3^{GCaMP3}$ cells were seeded per well (in six-well plates). In each case, cells were analyzed after 3 d of dsRNA treatment.

Immediately before analysis of Ca^{2+} signaling, each well was washed three times with assay buffer containing 150 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$, 10 mM Hepes, 10 mM D-Glucose, pH 7.2. Then, cells were incubated with 100 μL of the same buffer, plus 1 mM $CaCl_2$ (unless as indicated, Ca^{2+}-free/Ca^{2+}-addback experiments were performed). After no more than 10 min, Ca^{2+} responses were recorded at 25 °C, using $FLIPR^{TETRA}$. Other additions, as indicated in the figures, were made using either 50 or 100 μL of assay buffer as the vehicle. The effect of *Pvr* dsRNA upon Ca^{2+} signaling was studied in wild-type S3 cells using Fluo-4/AM, exactly as described previously (9).

For analysis of GBP-TMR binding, wells were washed three times with Dulbecco's PBS (136.9 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.9 mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.4), then cells were incubated for 2 h with 500 nM of either GBP-TMR or scrambled GBP-TMR (Fig. S1) in Dulbecco's PBS supplemented with 2% BSA, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. Next, cells were washed three times with the Dulbecco's PBS, and TMR binding was determined using a BD LSR II flow cytometer (excitation = 571 nm; emission = 582 nm) at 25 °C in single live cells gated by standard procedures (>10,000 cells for each individual assay condition). For graphical presentation, unsorted data for fluorescence intensity per cell were exported using FlowJo, and then imported into SigmaPlot (v13) for binning (100 bins) and graph plotting.

Analysis of ERK Phosphorylation. S2 cells were lysed immediately after incubation with 50 nM GBP for 3 min at 25 °C. After centrifugation at 17,000 × *g* for 15 min at 4 °C, proteins in the supernatant were resolved by SDS/PAGE and transferred onto an Immobilon-P PVDF membrane (Millipore) and probed in Western blot analysis with anti-phospho-ERK1/2 mouse monoclonal antibodies (M9692; Sigma-Aldrich).

Confocal Immunofluorescence. Brains were dissected from *Drosophila* female adult (day 3), fixed in 4% paraformaldehyde in PBS at 25 °C for 30 min, and extensively washed in PBS containing 0.1% Triton X-100 (PBT). Tissues were then blocked for 1 h in PBT containing 5% normal donkey serum and subsequently incubated with anti-ILP2 antibody (1:800, a gift from Pierre Léopold, Universite Cote d'Azur, Nice, France) at 4 °C for 24 h. For some experiments, anti-Mthl10 antibody (1:200; see *SI Methods and Materials*) was also added. After washing in PBT, tissues were incubated at 25 °C for 3 h with secondary antibody (1:200, goat anti-rat IgG Alexa546, Thermo Fisher Scientific) to record ILP2 and, where appropriate, donkey anti-rabbit IgG Alexa 405 (1:200) (Abcam) to also identify Mthl10. Tissues were imaged using a laser scanning confocal microscope (EZ-Ti system). A He Ne laser, 543-nm excitation, 605/75-nm emission, was used to detect ILP2. A BD laser, 408-nm excitation, 480/25-nm emission, was used to detect Mthl10. ILP2 fluorescence was recorded from a confocal Z series of the ILP-producing cells with 0.75-μm steps, using identical laser power and scan settings. In experiments to determine both ILP2 and Mthl10 immunofluorescence signals, a 0.5-μm confocal step was used. Signal quantification was performed with ImageJ.

Lifespan Determination. For lifespan studies, all fly strains were backcrossed for 10 generations to their respective genetic background as recommended previously (35). After establishing the backcrossed strain, test flies were sorted into experimental vials at a density of 10 flies per vial and monitored for their survival in 10 biological replicates (n = 100 per strain). Flies were transferred to fresh food every 2 d, and survival was scored on those days.

Cell Spreading Assay. S2 cells were collected from a stock culture flask 2–3 d after seeding and washed with Schneider's *Drosophila* medium (US Biological). Washed cells were suspended in 500 μL of the same medium (1 × 10^5 cells per mL) and, after leaving on ice for 60 min, cells were used for spreading assays. The degree to which the normally spherical S2 cells adopted a more flattened, spread phenotype was assessed by micrometric analysis. Cells were regarded as spread when they became flattened along their longest axis ≥25 μm.

Fluorescence Polarization. TMR-conjugated GBP peptide (50 nM) was incubated at 25 °C for 10 min with increasing concentrations of Mthl10 ectodomain in 20 mM Hepes pH 7.2, 0.5 mM DTT, and 150 mM NaCl. The assays (50 μL each) were then transferred to a black 96-well half area flat bottomed plate (REF3993; Corning) and fluorescence polarization was read at 25 °C with the PHERAstar microplate reader (BMG LABTECH) with the fluorescence polarization (FP) module (excitation, 590–50 nm; emission, 675–50 nm; focal height, 6.5; flashes per well, 200 per well; cycle time, 200 ms per well). FP values, determined as previously described (36), were expressed in millipolarization units.

Expression and Purification of the Mthl10 Ectodomain. The cDNA of Mthl10 was synthesized by Genscript. The Bac-to-Bac Baculovirus Expression System (Thermo Fisher) was used to express Mthl10 in Sf9 insect cells. The coding sequence for the predicted ectodomain Mthl10 (residues 1–243), C-terminally linked to a TEV cleavage site proximal to a Flag-polyHis sequence (ENLYFQGS-DYKDDDDKHHHHHHHHHH), was transferred into the Gateway compatible pDEST 8 expression vector (Thermo Fisher) via LR Reaction from a pENTR-like plasmid, producing pDEST8-Mthl10/C-TEV-Flag-10xHis. Both the entry clone and the final expression vector were sequenced verified. To generate a recombinant bacmid, pDEST8-TEV-Mthl10/C-Flag-10xHis was transformed into DH10Bac competent cells. The recombinant bacmid DNA was purified and then transfected into Sf9 insect cells using TransIT-Insect Transfection Reagent (Mirus); Sf9 insect cells were maintained in suspension shaking at 150 rpm (Multitron Standard Incubator Shaker model AJ118; INFORS

HT) in 125 mL Thomson Optimum Growth Flasks (Thompson Instrument Company) containing 50 mL HyClone Sfx Media (GE Healthcare) with 3% FBS and 1% Pluronic F-68. The P1 viral stock was harvested via centrifugation after 72 h at 27 °C. Two subsequent rounds of viral amplifications were performed in 50 mL of Sf9 suspension cultures in 125-mL Optimum Growth Flasks (Thomson Instrument Company) with each being harvested after 72 h. The P3 viral stock titer was tested using Sf-9ET cells (provided by Dominic Esposito; Leidos Biomedical Research). The final titer concentration was determined to be 1×10^{-8} IFU/mL.

Expression of the Mthl10 protein was performed in suspension using 9 mL of the P3 viral stock to infect a 900-mL culture of Sf9 cells at concentration of 1×10^6/mL at 27 °C. Due to the N-terminal signal peptide on the ectodomain (residues 1–32; www.predisi.de) the protein enters the secretory pathway. Thus, after 96 h, the culture was harvested and the supernatant was saved and clarified by centrifugation at 1,860 × *g* for 20 min. Recombinant Mthl10 ectodomain was purified by sequential chromatography using Ni-NTA resin, Q Sepharose, and Superdex 200 columns; recombinant protein was verified by TEV protease digestion and Western blotting with anti-flag antibody. The purified Mthl10 was concentrated and stored at −80 °C. Antibodies were raised in rabbits against purified recombinant Mthl10 that was emulsified in TiterMax Gold (TiterMax USA) (37).

Structural Modeling. The model of the ectodomain (residues 43–239, shown in surface presentation) of Mthl10 were predicted by the Swiss-Model algorithm using Mth as a template (https://swissmodel.expasy.org/). The ribbon structure for the transmembrane domain of Mthl10 (residues 244–524) is derived by homology modeling, using the β-2 adrenergic receptor (PDB ID code 3SN6) as the initial template. The GBP peptide (shown in stick and ball model) was docked into the predicted ectodomain model using CABS-dock (biocomp.chem.uw.edu.pl/CABSdock/).

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DmGBP          ILLETTQKCKPGFE-----------LFG-KRCRKPA
HsBD2     GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP
                    *::    *:* *            * * * *:**


GBP:             ILLETTQKCKPGFELFGKRCRKPA
GBP-TMR:         ILLETTQKCKPGFELFGKRCRKPAK-[TMR]
Scrambled-TMR    GCFLKLFRCIPITRQKPEAKGETKK-[TMR]
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Fig. S1. Amino acid sequences of *Drosophila* GBP and other peptides relevant to this study. The *Upper* alignment (generated by Clustal-Omega with slight manual adjustment) compares the biologically active GBP peptide (National Center for Biotechnology Information no. NM_137350.4; after serine protease cleavage) with the human β-defensin, BD2 (Uniprot no. O15263) after cleavage of its N-terminal signal sequence. The *Lower* alignment shows GBP, GBP-TMR (note the addition of the C-terminal Lys), and scrambled-GBP-TMR. These three peptides were purchased from Genemed Synthesis.

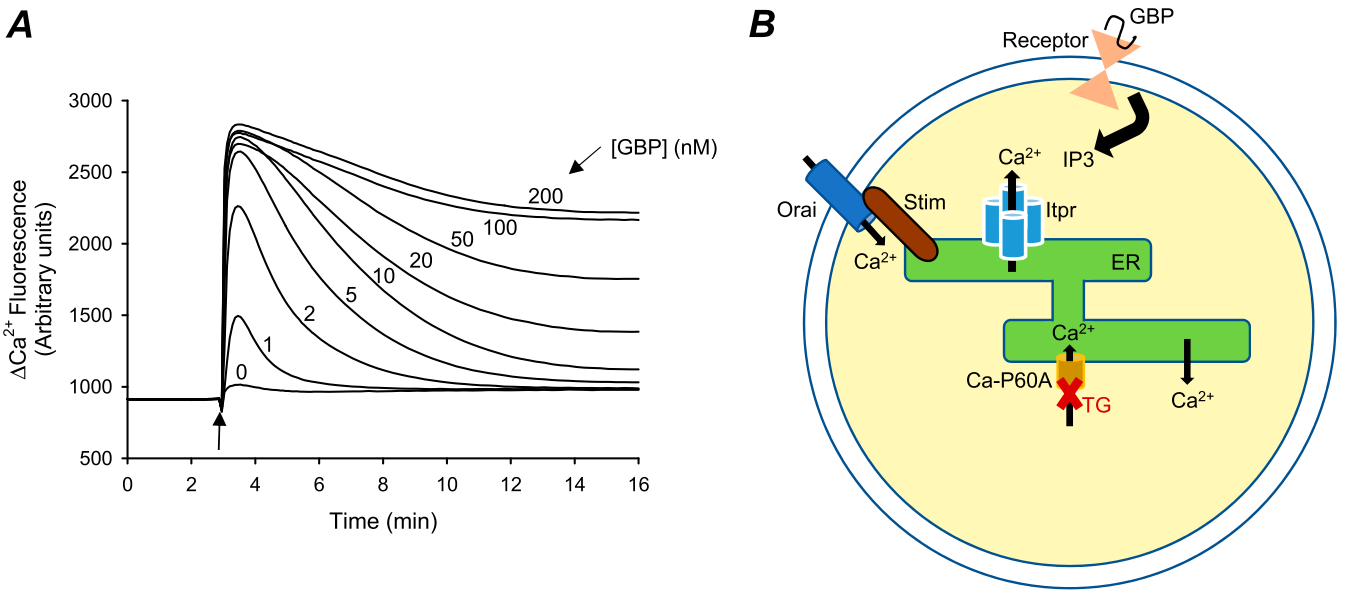

Fig. S2. GBP-mediated Ca^{2+} signaling in $S3^{GCaMP3}$ cells. (*A*) Cytoplasmic [Ca^{2+}], depicted as fluorescence units, in $S3^{GCaMP3}$ cells following the addition (see arrow) of the indicated nanomolar concentration of GBP. (*B*) Graphic illustrating the proposed mechanism by which GBP increases cytoplasmic Ca^{2+} in S3 cells: GBP binds to a cell-surface receptor that stimulates phospholipase C to produce inositol (1,4,5) trisphosphate (IP3), which binds to Itpr (the IP3 receptor), thereby releasing Ca^{2+} from ER stores. Emptying of these Ca^{2+} stores is sensed by Stim, which associates with Orai, through which capacitative Ca^{2+} entry occurs. Also shown is the Ca^{2+}-ATPase (Ca-P60A), which pumps Ca^{2+} into the ER; the activity of Ca-P60A is inhibited by TG, thereby exposing an ongoing leak of Ca^{2+} from ER. TG can therefore be used to study the size of the Ca^{2+} stores, as well the receptor-independent coupling of Ca^{2+} release to Ca^{2+} entry.

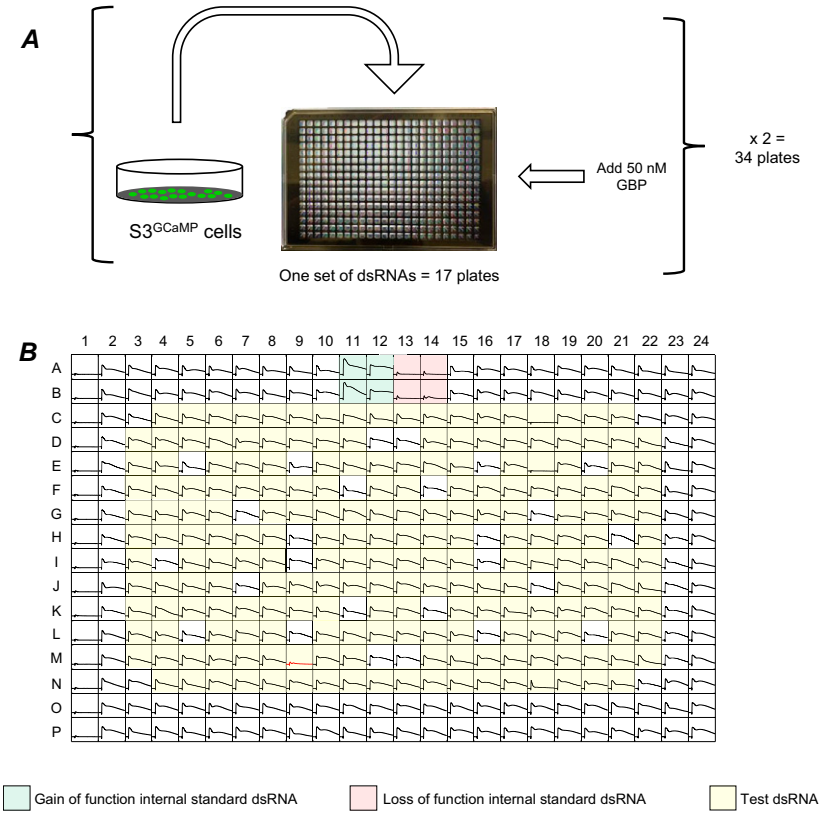

Fig. S3. Description of the screening protocol with data from plate 714B29, which includes the *Mthl10* dsRNA. (*A*) Graphic showing preparation of 384-well plate containing $S3^{GCaMP3}$ cells; 5 d later, 50 nM GBP was added to each well. (*B*) Depiction of the experimental template that was employed for each dsRNA-screening plate. Ca^{2+} signals shown are data obtained from plate 714B29 over a time course of 16 min; after 2 min, GBP (50 nM) was added to each well, except for those in column 1 (vehicle alone). Each square represents a well containing 0.25 μg of one of the following: test dsRNAs, added to the wells that are colored yellow; gain of function positive controls (colored green), using dsRNA against either *Tsr* (wells A11 and B11) or *Atx2* (wells A12 and B12); loss of function controls (colored red), obtained by adding dsRNA against *Itpr* (wells A13, A14, B13, B14). Wells that are not colored contained either vehicle (i.e., water) or dsRNA against an irrelevant gene, *LacZ* (wells D13, D14, F11, F14, H9, H16, I9, I16, J7, J18, K11, K14, L5, L20, M12, M13, N3, N22). The graphic also shows genuine fluorescent signals recording cytoplasmic [Ca^{2+}] in every well. M9 (the trace colored red) contained dsRNA against *Mthl10*.

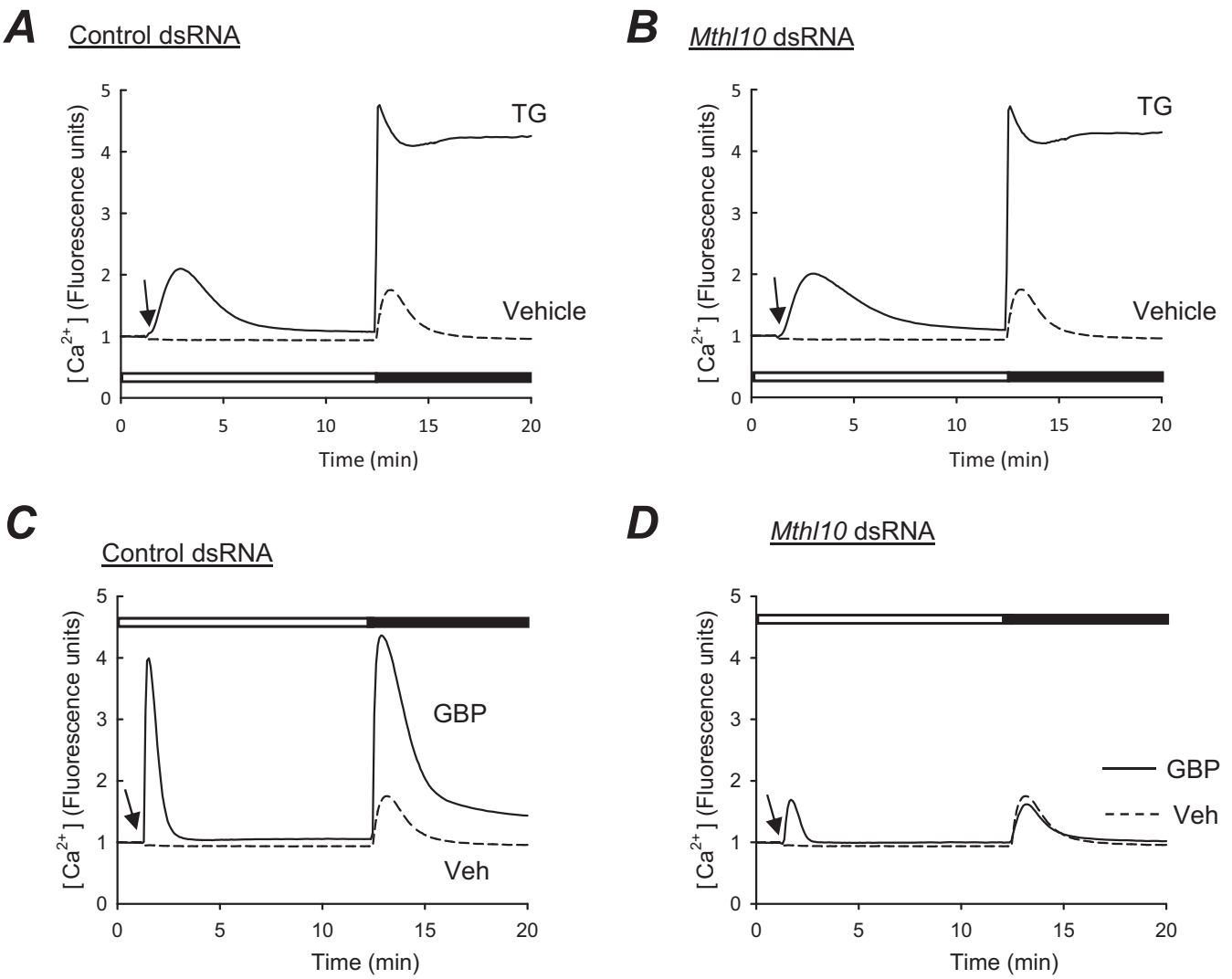

Fig. S4. *Mthl10* knockdown does not affect receptor-independent Ca^{2+} entry in $S3^{GCaMP3}$ cells. (*A* and *B*) Either 2 μM TG or vehicle was added to control (*A*) or Mthl10 dsRNA treated $S3^{GCaMP3}$ (*B*) cells as indicated (see arrows), incubated in Ca^{2+}-free medium (open horizontal bar); the timing of the "Ca^{2+} addback" (final concentration = 1 mM) is indicated by the filled horizontal bar. *C* and *D*, as for *A* and *B*, except 50 nM GBP or vehicle were added as indicated by the arrow.

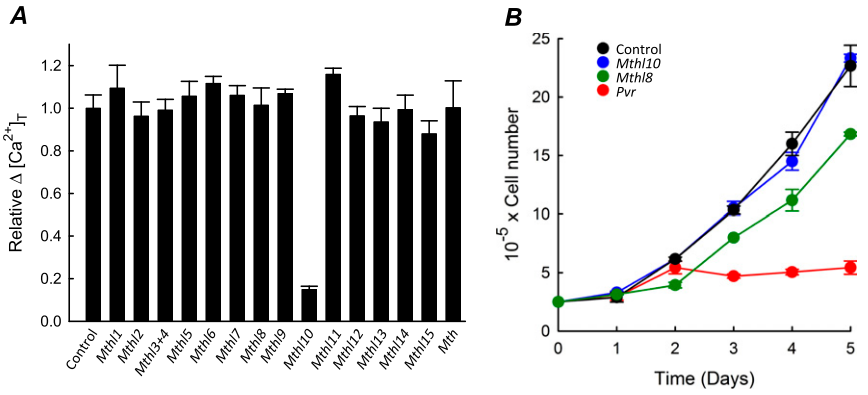

Fig. S5. The effects of gene knockdowns upon Ca^{2+} mobilization and cell growth. (*A*) Bar graphs show total Ca^{2+} release (i.e., $[Ca^{2+}]_T$) relative to controls (set to unity), calculated by integrating the areas under the Ca^{2+}-mobilization curves (means ± SEM; $n = 3$), following dsRNA-mediated knockdown of the indicated genes. Data for *mthl8* are corrected for the 30% decrease in proliferation rate observed after knockdown of this gene; none of the other knockdowns affected proliferation. (*B*) Cell growth data. At time 0, 2.5×10^5 $S3^{GCaMP3}$ cells were seeded per well (in 24-well plates) in the presence of either control dsRNA or dsRNA against either *Mthl10*, *Mthl8*, or *Pvr*. The number of live cells was recorded every 24 h using an automated cell counter, Countess (Life Technologies).

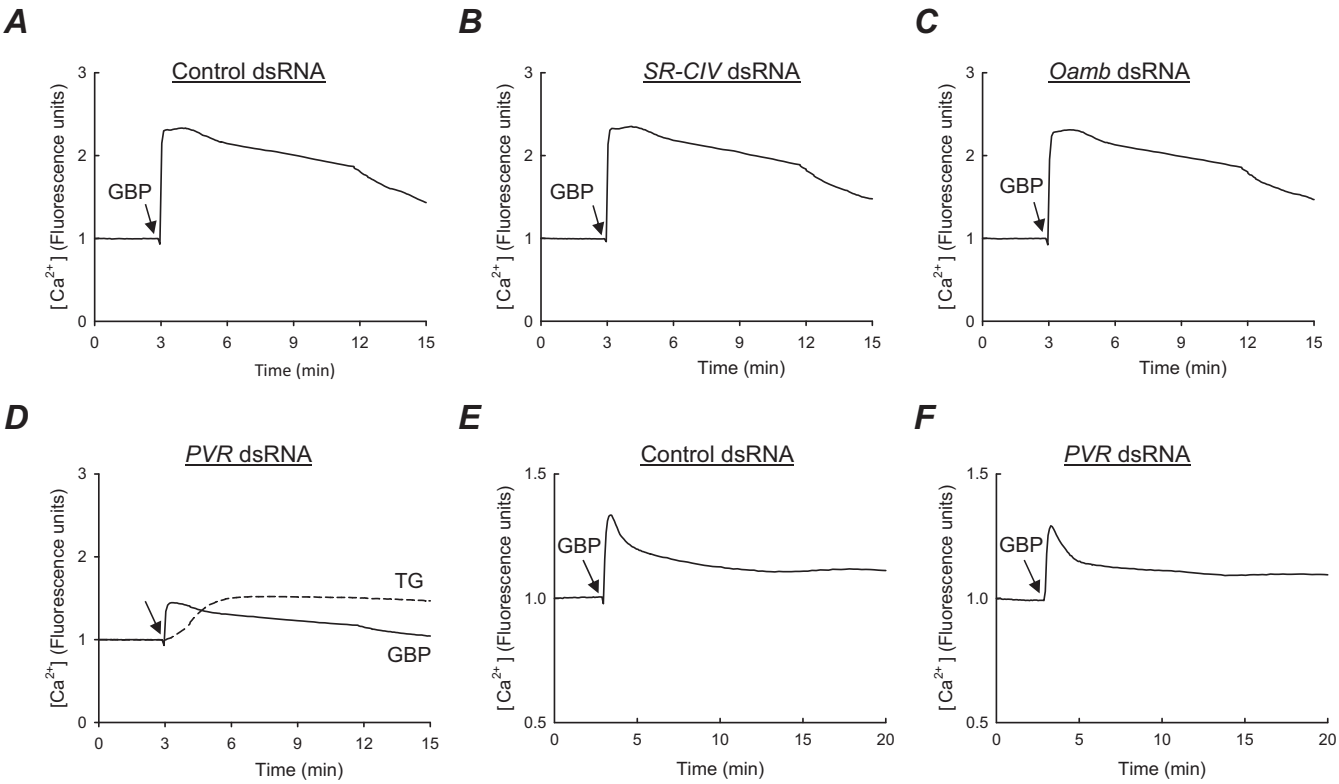

Fig. S6. Knockdown of either *SR-CIV, Oamb,* or *Pvr* does not affect GBP-mediated Ca^{2+} mobilization. Cytoplasmic [Ca^{2+}] (depicted as fluorescence units for GCaMP) is shown following the addition (as indicated by the arrow) of either 50 nM GBP (solid lines) or 2 μM TG (broken line) to $S3^{GCaMP3}$ cells that were pretreated with either control dsRNA (*A*), *SR-CIV* dsRNA (*B*), *Oamb* dsRNA (*C*), or *PVR* dsRNA (*D*). Cytoplasmic [Ca^{2+}] (depicted as fluorescence units for Fluo-4) is shown following the addition of 50 nM GBP (as indicated by the arrow) to S3 cells pretreated with either control dsRNA (*E*) or *PVR* dsRNA (*F*). Each experiment was performed three times.

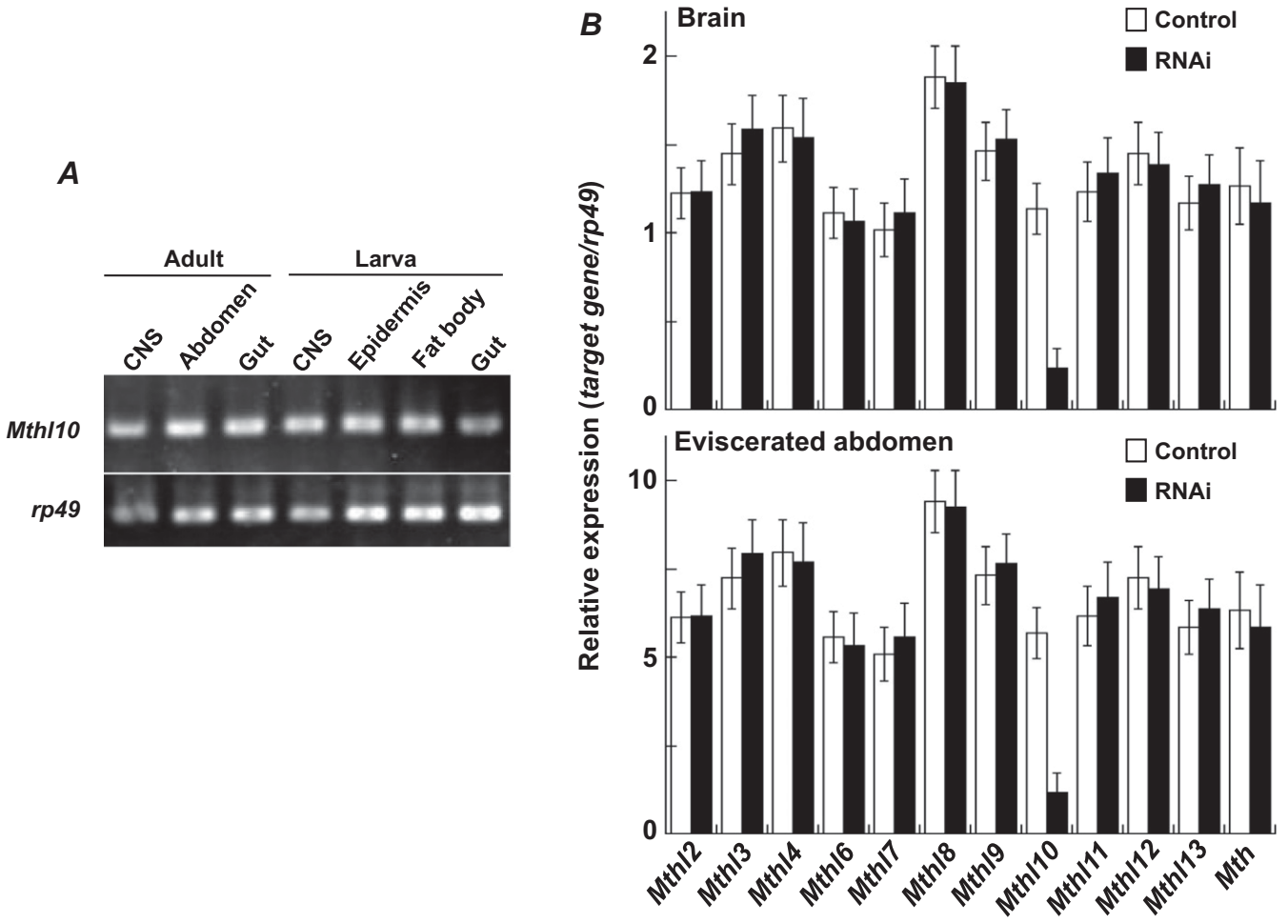

Fig. S7. Expression of *Mth* superclade genes in specific *Drosophila* tissues. (*A*) Analysis of *Mthl10* expression by RT-PCR in the indicated tissues. (*B*) Analysis by qPCR of the expression of *Mth* superclade genes in brain and eviscerated abdomen (including the fat body) from the adult female *hs-Gal4>UAS-dsMthl10* strain, following induction (filled bars) or no induction (open bars) of *Mthl10* RNAi. Data are means ± SEM; $n = 3$.

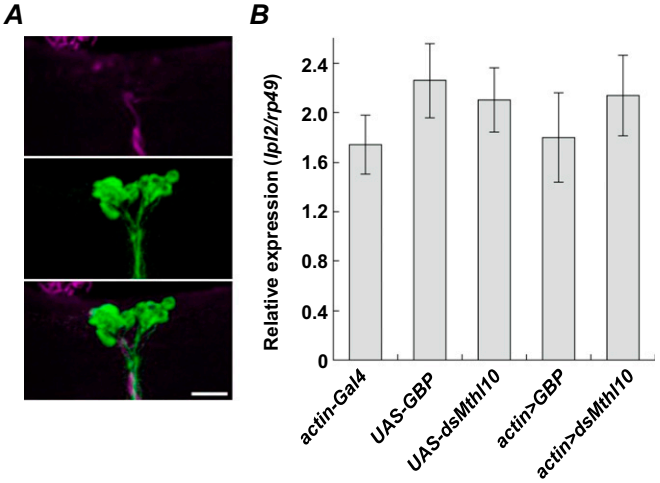

Fig. S8. Control experiments for studying changes in expression of *Ilp2* and *Mthl10* in the brains of *Drosophila* adult females. (*A*) Validation of the specificity of our anti-Mthl10 antibody in brains from female adult *Drosophila* (*hs-Gal4>UAS-dsMthl10*) after induction of *Mthl10* RNAi. Shown are representative images of double immunostaining with anti-Mthl10 (*Top*) and anti-ILP2 antibodies (*Middle*) in the ILP-producing cells. *Bottom* shows the merged image. Compare with Fig. 3*E* in which the antibody is used to stain wild-type female brains. (Scale bar: 15 μm.) (*B*) Neither *GBP* overexpression nor *Mthl10* knockdown caused any significant change in *Ilp2* expression levels in the brains of *Drosophila* adult females. Values shown are mean ± SEM ($n = 4$).

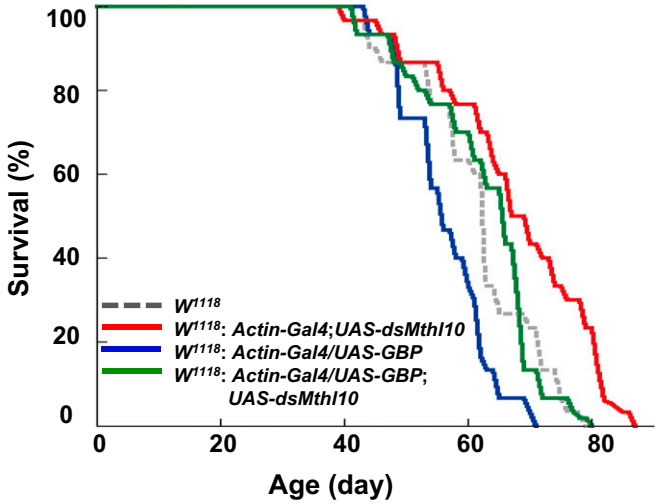

Fig. S9. Effects of *GBP* overexpression and *Mthl10* knockdown on lifespan of *Drosophila* male flies. Lifespan curves of W^{1118};*Actin-Gal4*;*UAS-dsMthl10* ($P = 1.28 \times 10^{-2}$) and W^{1118};*Actin-Gal4/UAS-GBP* ($P = 1.29 \times 10^{-2}$) were significantly different from control (W^{1118}) (log rank test).

Other Supporting Information Files

Dataset S1 (XLSX)
Dataset S2 (XLSX)