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Supplemental Information

Systematic Discovery of Rab GTPases

with Synaptic Functions in *Drosophila*

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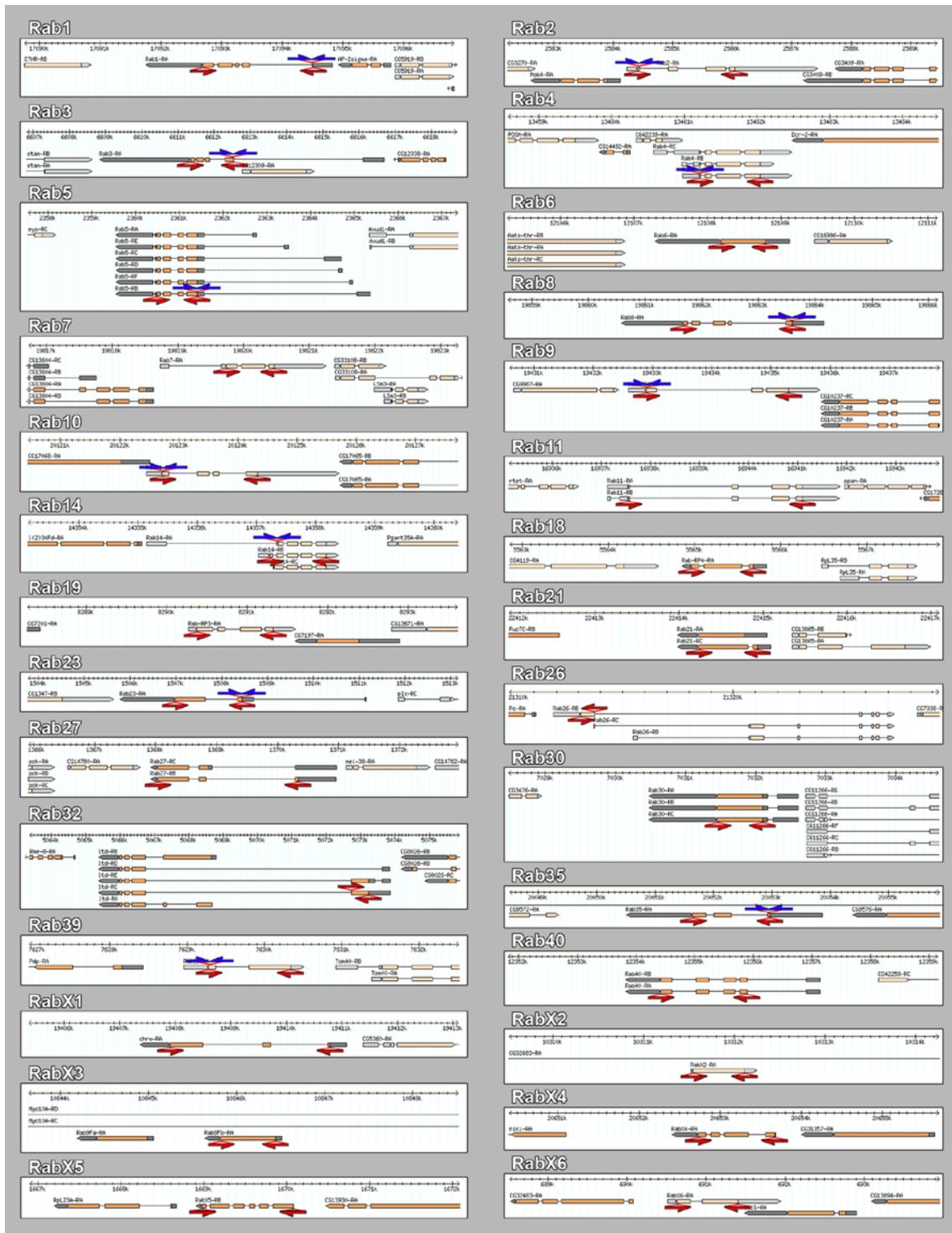


Figure S1. Genomic Loci and Target Vector Design for 29 *rab* Loci

Schematics of genomic loci between 4kb and 11kb are depicted based on FlyBase GBrowse (<http://flybase.org/cgi-bin/gbrowse/dmel/>). the exon/intron structure is shown for every *rab* with coding exons in orange and 5' UTRs and 3' UTRs in grey. If more than one transcript is known (even if the open reading frames are identical) all transcripts are listed. The red half-arrows depict regions replaced for 'ORF knock-ins'; blue half-arrows depicted regions replaced for 'ATG knock-ins'.

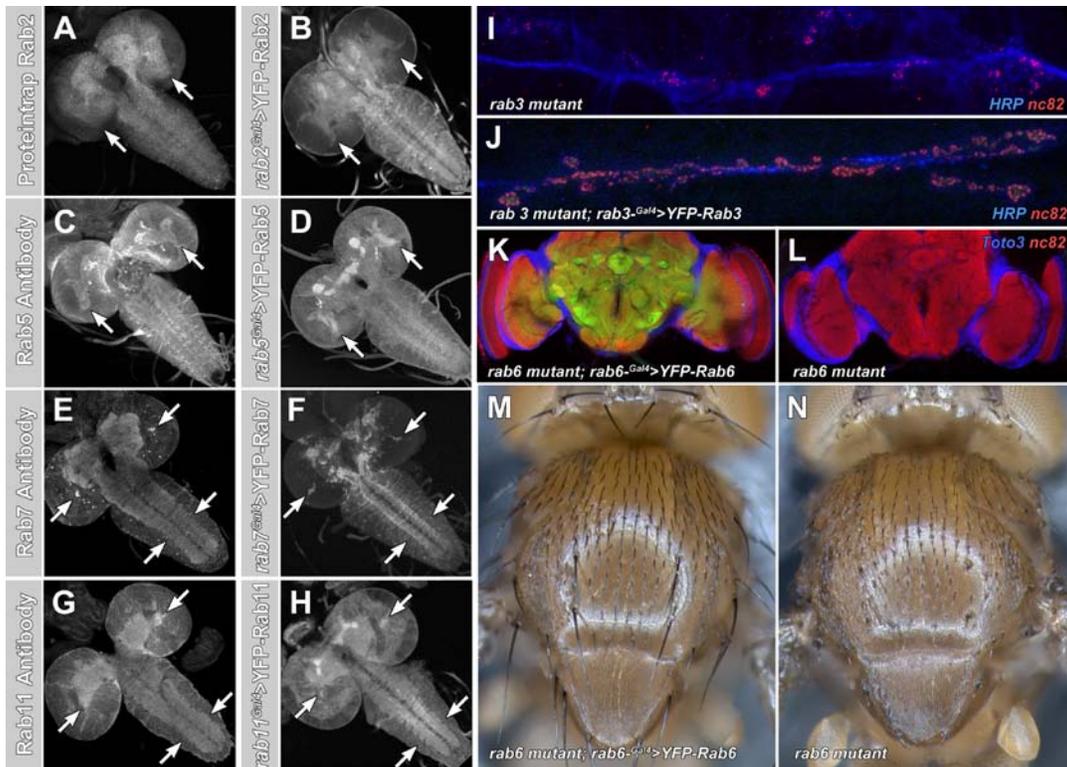


Figure S2. Verification of rab-Gal4 Lines with Proteintrap, Antibodies, and Rescue Experiments

(A, B) A protein trap has been identified for the *rab2* locus where a GFP-containing exon is randomly spliced into the *rab2* open reading frame (Buszczak et al., 2007). The resulting GFP fusion protein is at the detection limit of standard confocal microscopy and is not functional (homozygous lethal) and may thus exhibit altered subcellular localization. Despite these drawbacks, the protein trap expression pattern (A) exhibits remarkable similarity to YFP-Rab2 driven by *rab2*-Gal4, as shown in (B).

(C, D) A commercially available polyclonal anti-Rab5 antibody (abcam ab31261) shows immunolabeling (C) similar to YFP-Rab5 driven by *rab5*-Gal4 (D). The antibody specificity has been tested for YFP-Rab5 expressing neurons compared to all other YFP-tagged Rabs (compare Fig. 4 and Fig. S5). The antibody has not been specificity-tested in a *rab5* mutant and is likely to have some background labeling.

(E, F) A polyclonal anti-Rab7 antibody (gift from P. Dolph) shows immunolabeling (E) similar to YFP-Rab7 driven by *rab7*-Gal4 (F). Again, the antibody has been tested for labeling of YFP-Rab7 compared to all other YFP-tagged Rabs, but not specificity-tested in a *rab7* mutant.

(G, H) A mouse monoclonal antibody against Rab11 (BD Bioscience clone 47) exhibits immunolabeling (G) similar to YFP-Rab11 driven by *rab11*-Gal4. The antibody has been tested for recognizing YFP-Rab11 (Suppl. Fig. 5). *rab11* mutants are cell lethal. All arrows point at comparable structures between the complementary labeling.

(I, J) L3 larval neuromuscular junctions labeled for HRP (blue) and the active zone marker *nc82*/Bruchpilot (red). *rab3* null mutants (*rab3^{rup}/Df(2R)BSC639*) are viable and exhibit a partial loss of active zones (I). Expression of YFP-Rab3 by *rab3*-Gal4, as used in this study for profiling, rescues this phenotype.

(K, L) Confocal cross-sections of adult brains labeled for neuropil/active zones with *nc82* (red) and nuclei with *Toto-3* (blue). *rab6* hypomorphic mutants (*rab6^{AS1}*) are viable and exhibit no abnormal brain morphology at the level of light microscopy (L). Widespread and predominantly synaptic localization of YFP-Rab6 is apparent for *rab6*-Gal4 driven YFP-Rab6 in an otherwise *rab6* mutant brain (K).

(M, N) Adult nota are shown for the same genotypes as in (C, D). Hypomorphic *rab6* causes a bristle phenotype that is rescued by *rab6*-Gal4 driven expression of YFP-Rab6. *rab6* null mutants (*rab6^{D23D}/Df(2L)ED775*) are lethal, and the lethality can be rescued by expressing YFP-Rab6 using *rab6*-Gal4 (data not shown).

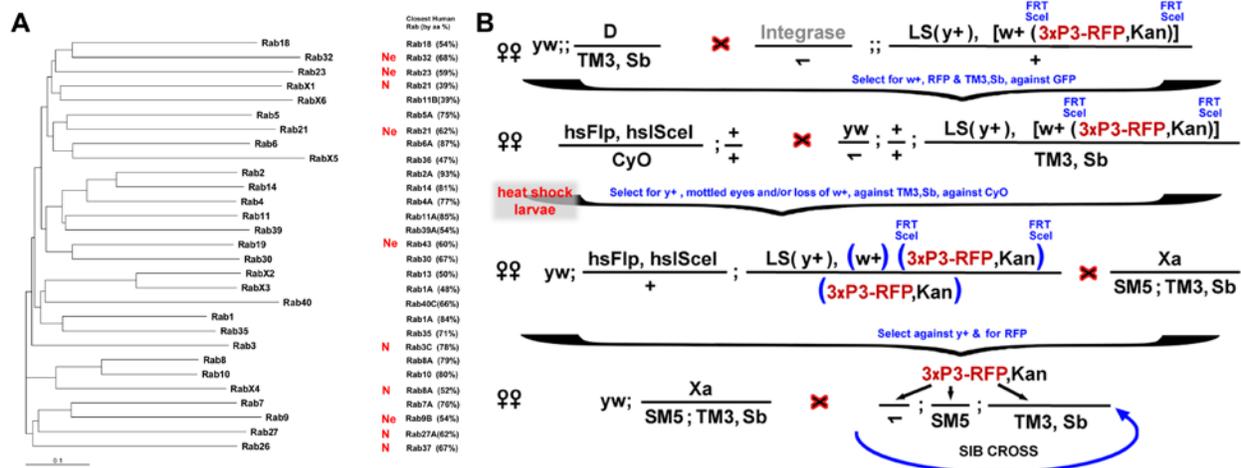


Figure S3. Phylogenetic Tree of *Drosophila* Rab Proteins and Crossing Scheme for the Mobilization of the Targeting Cassette from the Landing Site into the Endogenous Locus

(A) A total of 29 Rab amino acid sequences were aligned with CLUSTAL W and the tree generated with CLUSTAL X (Neighborhood Joining method) (Thompson et al., 1994). The right columns shows the closest human Rab proteins based on amino acid sequence identity (in brackets). The central columns in red demarcates neuron-specific (N) and neuron-enriched (Ne) Rabs in *Drosophila* based on the study presented here.

(B) All rab-Gal4 lines presented here were generated by integrating the rab-Gal4 constructs in a landing site on the third chromosome with the original PhiC31 integrase (marked with 3xP3 GFP and 3xP3 RFP on the X). If the integrase has not yet been cleaned, this first balancing step removes it. Second, hsFlp and hslSce are crossed into the same genetic background. Early larvae are heat shocked to induce FLP and ISCE1 expression according to published protocols outlined in (Gong and Golic, 2003). Third, non-balanced females are selected that contain the landing site (yellow+) and the knock-in cassette (3xP3-RFP). Imprecise excision of the targeting cassette may cause mottled eyes for $w+$ from the P[acman] backbone. These females are crossed to a double balancer stock. Fourth, all flies that lose the landing site (yellow) and retain the knock-in cassette (3xP3-RFP) represent mobilization events and potential knock-outs. For details see protocols and text.

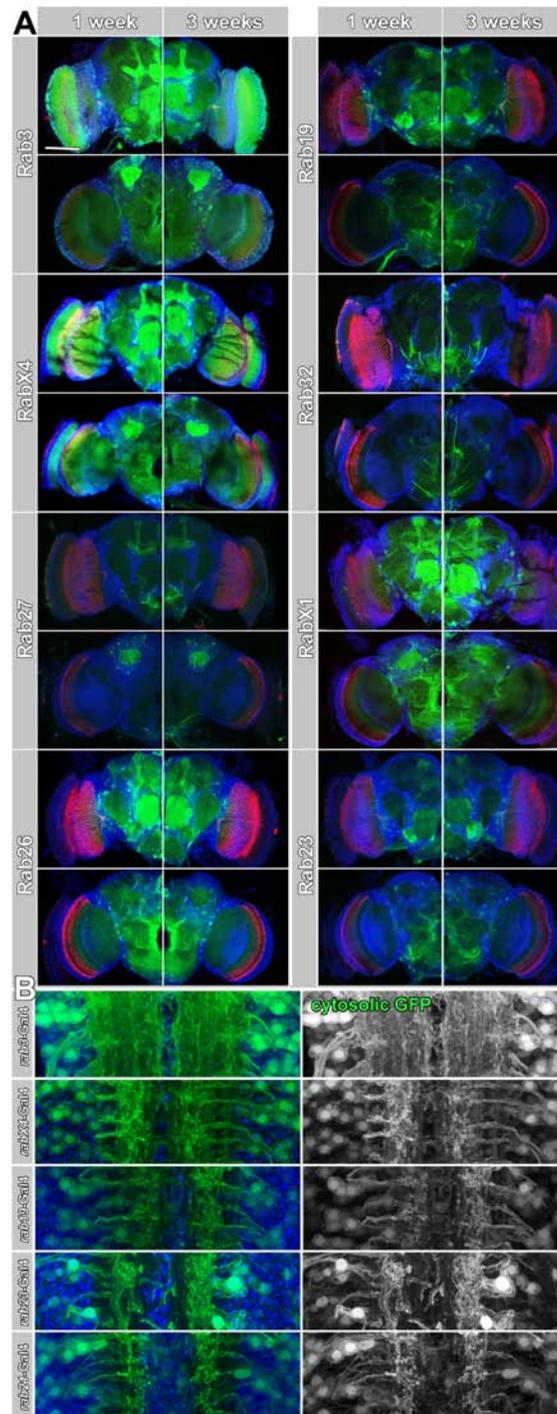


Figure S4. Neuronal *rabs* Exhibit Stable Expression in Adult Brain Neurons and Cytosolic GFP Evenly Localizes to Cell Bodies and Synapses

(A) Rab-Gal4 driven CD8-GFP expression for eight neuronal *rabs* for one week and three week old brains. Anterior and posterior part of the brains are shown as in Figure 3B. Scale bar for all panels: 50 μ m.

(B) Cytosolic GFP exhibits both cell body and synaptic localization when expressed with neuronal *rab*-Gal4 lines. Note that expression of YFP-tagged *Rabs* using the same Gal4 lines reveals synaptic localization specific to the *Rab* proteins.

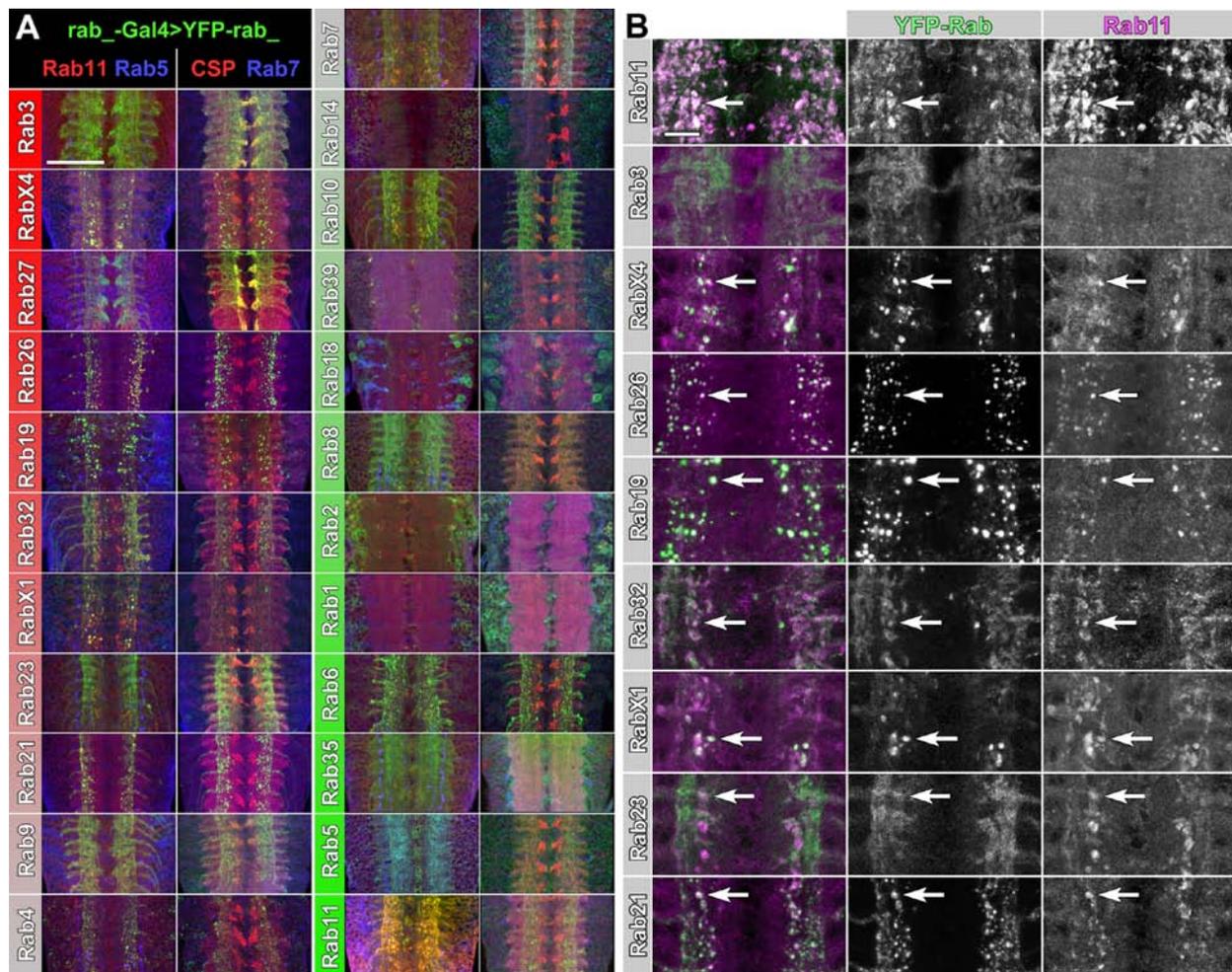


Figure S5. Compartment Characterization of Neuronal and Control YFP-Rab Proteins

(A) Restricted projection views of two double immune-labelings of the posterior larval brain ventral ganglion at high resolution are shown for each YFP-Rab (green). Anti-Rab11 (red, recycling endosomes) and anti-Rab5 (blue, early endosomes) labeling are shown in the left column and anti-CSP (red, synaptic vesicles) and anti-Rab7 (blue, late endosomes) in the right column. In these ventral ganglion sections cell bodies are on the outside with synaptic neuropils proximally and midline glia centrally. All Rabs are sorted from most neuron-specific marked on the left in red, via mixed expression in grey to the most ubiquitously expressed Rabs in green (similar as in Figure 2). Note that each YFP-Rab protein is expressed by the corresponding *rab-Gal4* line, i.e. *rab3-Gal4*>UAS-YFP-Rab3, *rabX4-Gal4*>UAS-YFP-RabX4, etc. A full resolution version is available online. Magnified sections of the subcellular colocalization for the neuron-specific rabs as well as the control co-labelings of Rab5, Rab7 and Rab11 are shown in Figure S5A. Scale bar for all panels: 50 μ m.

(B) Seven neuronal Rabs cause synaptic Rab11 accumulations. High-resolution sections of the synaptic neuropils of ventral ganglions are shown. YFP-Rabs expressed using their corresponding *rab-Gal4* lines are shown in green and in single channel in the middle column; Rab11 antibody labeling is shown in magenta and as a single channel in the right column. Arrows indicate enlarged Rab11 accumulations. Scale bar for all panels: 10 μ m.

Figure S10

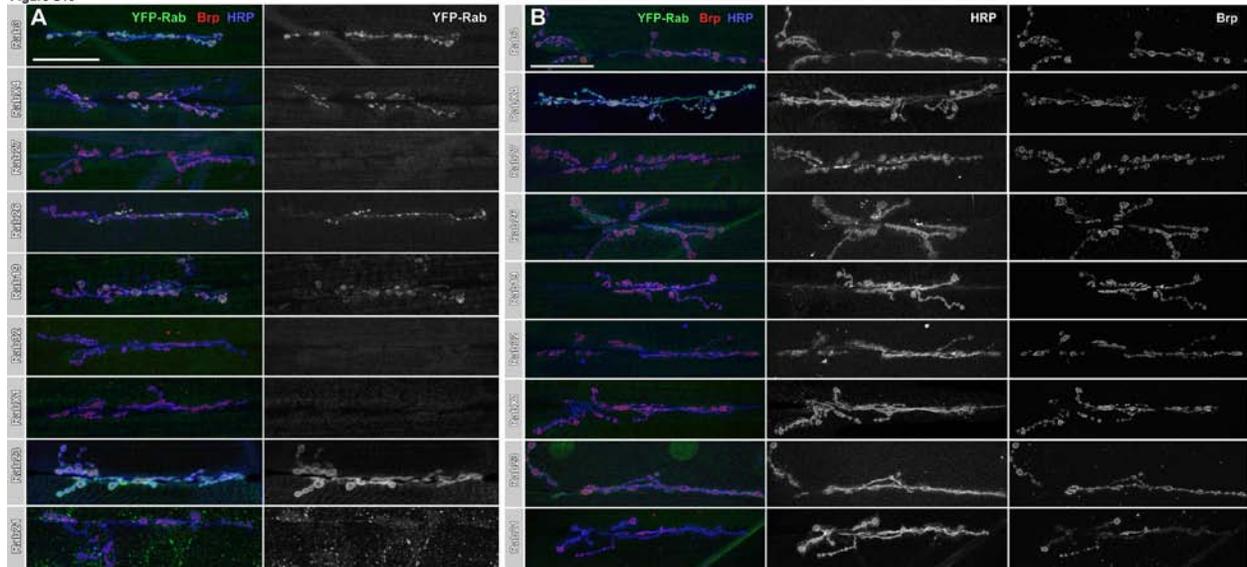


Figure S6. Analysis of Neuronal Rabs at the Larval Neuromuscular Junction

(A) 3rd instar larval NMJs at muscle 6/7, third or fourth segment are shown. YFP-Rabs are expressed with their corresponding *rab*-Gal4 lines. Red: Brp (nc82) labeling of active zones; Blue: HRP labeling of neuronal membranes. Rab3, RabX4, Rab26 and Rab19 exhibit presynaptic localization inside boutons; RabX4, Rab19 and Rab26 mark larger compartments that are not present in all boutons. Rab23 is localizes to the outside of the presynaptic boutons. Rab21 mostly labels compartments in the postsynaptic muscle. No YFP-Rab signal was detected for Rab27, Rab32 and RabX1, all of which have more restricted expression patterns. Scale bar for all panels: 50µm.

(B) Analysis of dominant negative Rab expression of larval neuromuscular junctions. 3rd instar larval NMJs at muscle 6/7, third or fourth segment are shown as in (A). YFP-Rab dominant negatives are expressed with their corresponding *rab*-Gal4 lines. Red: Brp (nc82) labeling of active zones; Blue: HRP labeling of neuronal membranes. None of the dominant negative Rabs cause dramatic alterations of NMJ morphology. Scale bar for all panels: 50µm.

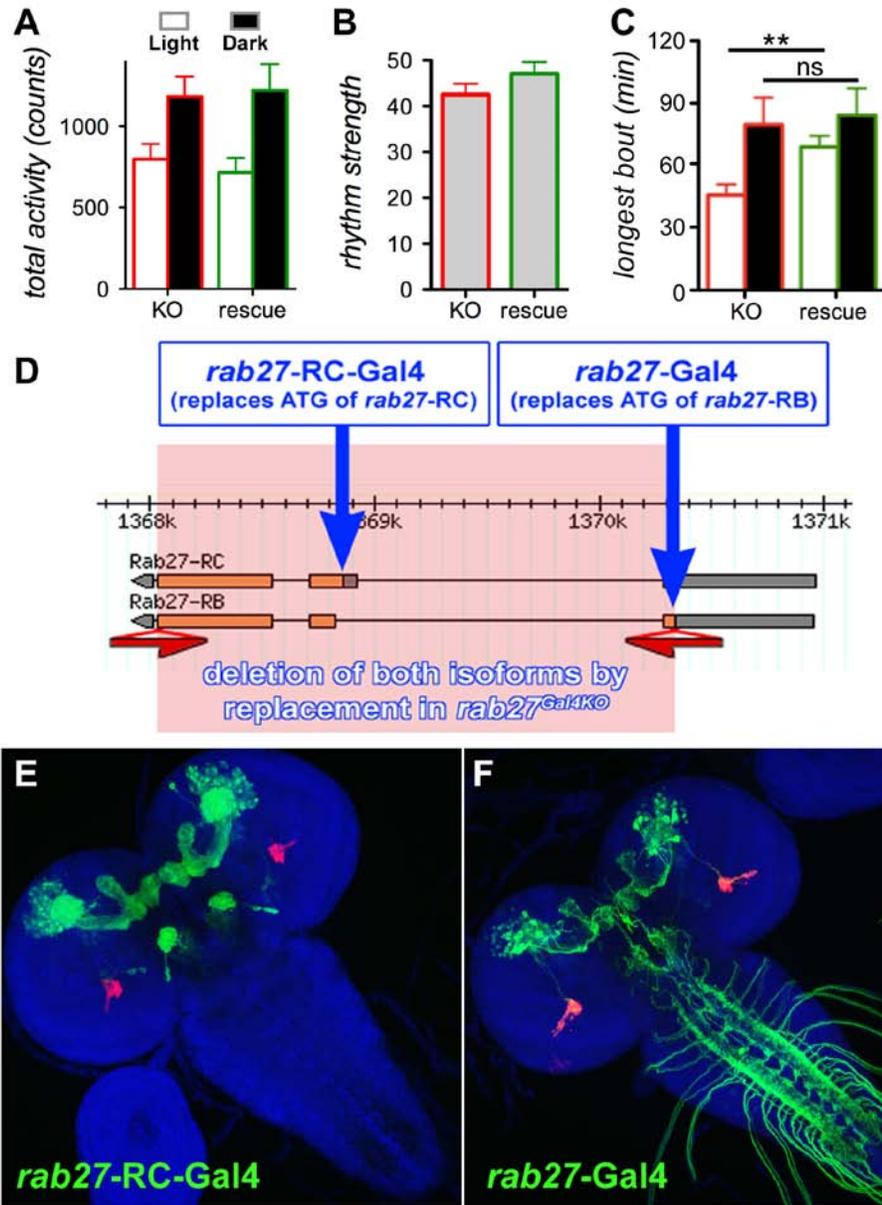


Figure S7. Characterization of *rab27^{Gal4-KO}* Homozygous Mutants and Isoform-Specific Knockins

(A-C) Phenotypic characterization of *rab27^{Gal4-KO}* homozygous mutant flies. The *rab27* mutants exhibit normal total activity (A), normal rhythm strength (B), but altered sleep bout length (C).

(D) Knock-out and isoform-specific knock-ins for the *rab27* open reading frame. The pink underlined box shows the complete open reading frame removed in the *rab27^{Gal4-KO}* mutant flies. Note that the *rab27* locus encode two different isoforms with different ATG start codons, both of which are removed in *rab27^{Gal4-KO}* homozygous mutants. *rab27-Gal4* and the *rab27^{Gal4-KO}* knock-in replaces the ATG of isoform Rab27-RB with the ATG of Gal4; the *rab27-RC-Gal4* line replaces the ATG of the *rab27-RC* isoform with the ATG of Gal4.

(E, F) Comparison of expression driven by *rab27-RC-Gal4* (E) and *rab27-Gal4* (F). Note that in the *rab27-RC-Gal4* line expression in the ventral ganglion is lost, whereas highly specific expression in the mushroom bodies and developing antennal lobes is increased. Hence, both *rab27* isoform that are removed in the *rab27^{Gal4-KO}* are specifically expressed in the mushroom bodies.

Error bars are standard error of the mean.

Supplemental Experimental Procedures

Recombineering Protocols

Overview

1. Transform DY380 cells with BAC DNA containing a *rab* locus
2. Soeing PCR to generate 1 kb homologous arms fragment
3. BP reaction to transfer the 1kb fragment into the destination vector P[acman]-KO
4. First round recombineering to clone the genomic fragment into P[acman]-KO-1kb
5. Second round recombineering to knock in the Gal4-3xP3-RFP-Kanamycin cassette into P[acman]-KO-genomic
6. PhiC31-mediated transformation of the final targeting vector P[acman]-KO-Gal4

1. Transform DY380 cells with BAC DNA containing a *rab* locus

1. Order BAC DNAs containing the genomic region of interest from <http://bacpac.chori.org/>
2. Transform the BAC into DY380 cells. DY380 cells grow slowly, and are Tetracyclin-resistant. The BACs carry Chloramphenicol-resistance.
3. Plate the transformants onto LB- Chloramphenicol(+)(12.5ug/ml) Tetracyclin (+)(10ug/ml);
4. Place the plate in **30C** incubator for **24** hours;
5. Select for Chloramphenicol(+) and Tetracyclin (+) clones; set up overnight liquid culture.
6. The above liquid culture or purified plasmid can be used as template for the Soing PCR to generate 500 bp homology arms for the first round recombineering.

2.1 Generation of 500bp DNA fragments

Goal: Amplification of 500 bp homology arms on both sides of target DNA, about 5-20 kb away from each end of the gene of interest by PCR. The homology arms define the size of the genomic DNA to be cloned into P[acman]-KO. The outer primers include attB sites for Gateway BP cloning; the inner primers are overlapping and introduce a BamH1 restriction site between the two 500bp arms.

1. Primer design: Two primer sets are required to amplify two 500bp DNA fragments that are e.g. 40kb apart from each other (LA and RA). The *rab* locus is in the center of this region. Make sure that there is no other BamH1 site in LA and RA. Include Gateway attB1 and Isce-I sites at 5' end of LA; include attB2 and Isce-I sites at 3' end of RA. Note that the Gateway attB and attP sites are unrelated to the PhiC31 attB and attP sequences.
2. Use Invitrogen Platinum Taq High Fidelity DNA polymerase (or any DNA polymerase that has proof reading activity).

3. Pipetting instructions:

- a. DNA (liquid culture) 2 ul
- b. Primer1 (10uM) 1 ul
- c. Primer2 (10uM) 1 ul
- d. MgSO₄ 0.5 ul
- e. Buffer (10mM) 2.5 ul
- f. dNTP 0.5 ul
- g. Platinum Tag 0.25 ul
- h. H₂O to 25 ul

Run PCR samples on a 1% agarose gel; then do gel extraction to recover the 500bp bands. Save DNA in -20C for subsequent soeing PCR (1kb).

2.2 Soeing PCR to generate 1kb DNA fragments

Goal: PCR to ligate Left Arm (500bp) and Right Arm (500bp) into a 1kb fragment for subsequent BP recombination into P[acman]-KO.

1. Use Invitrogen Platinum Taq High Fidelity DNA polymerase (or any DNA polymerase that has proof reading activity)
2. Pipetting instructions:

- a. Left Arm 2 ul
- b. Right Arm 2 ul
- c. Primer1 (10uM) 2 ul
- d. Primer2 (10uM) 2 ul
- e. MgSO₄ 2 ul
- f. Buffer (10mM) 5 ul
- g. dNTP 1 ul
- h. Platinum taq 0.5 ul
- i. H₂O to 50 ul

3. Run PCR samples on a DNA gel; then do gel extraction to recover the 1kb band. Save DNA in -20C for subsequent BP recombination.

3. BP reaction

Goal: Clone 1 kb soing PCR products into P[acman]-KO vector using gateway BP clonase II enzyme mix from Invitrogen (Cat # 11789-020).

1. Add the following components to a PCR tube at room temperature and mix:
 - attB-PCR product (≥ 10 ng/ μ l; final amount ~15-150 ng) 1-7 μ l
 - Donor vector (150 ng/ μ l) 1 μ l
 - TE buffer, pH 8.0 to 8 μ l

2. Thaw on ice the BP Clonase™ II enzyme mix for about 2 minutes. Vortex the BP Clonase™ II enzyme mix briefly twice (2 seconds each time).
3. To each sample (Step 2, above), add 2 µl of BP Clonase™ II enzyme mix to the reaction and mix well by vortexing briefly twice. Microcentrifuge briefly.
4. Incubate reactions at 25°C for 4 hours. Use PCR machine for this step.
5. Add 1 µl of the Proteinase K solution (2 µg/µl) to each sample to terminate the reaction. Vortex briefly. Incubate samples at 37°C for 10 minutes.

Transformation

1. Transform 2 µl of each BP reaction into 50 µl of One Shot® Top 10 chemically competent *E. coli* from Invitrogen (Catalog no. C4040-10). Incubate on ice for 10 minutes.
2. Heat-shock cells by incubating at 42°C for 45 seconds. Transfer on ice for 2 minutes.
3. Add 300 µl of S.O.C. Medium and incubate at 37°C for 1 hour with shaking. Plate cells of each transformation on LB-Agar plates onto selective plates (Ampicillin 100 µg/ml).
4. Pick colonies and set up O/N liquid cultures @ 37°C.
5. Purify DNA and transform 1 µL into 50 µL TransforMax™ EPI300™ electrocompetent *E. coli* (Cat # EC300110, Epicenter Biotechnologies, Madison, WI) for higher plasmid yield.

4. First round recombineering to clone the genomic fragment into P[acman]-KO-1kb

Overview: Cut P[acman]-KO-1kb with BamH1 for 6 hours, transform into heat-shocked DY380/BAC-rab for gap repair.

Note: make sure that 1) P[acman]-KO-1kb is at high concentration (> 3ug/ul); and 2) P[acman]-KO-1kb is 100% cut.

Day 1

1. Restriction digestion of P[acman]-KO-1kb w/ BamH1-HF
 - a. Pipetting instruction

i. 2-4 µl	DNA (~ 10 µg)
ii. 5 µl	Buffer 4 (10X)
iii. 2 µl	BamH1-HF enzyme (NEB, Cat # R3136S)
iv. To 50 µl	H2O
 - b. Incubate the reaction @ 37C for 6 hours.
2. Set up liquid culture of DY380/BAC-Rab
 - a. Dig some of the frozen glycerol stock; inoculate into 5ml LB-Chl(+)-Tet(+) media (Chroramphenicol (12.5ug/ml); Tetracyclin (10ug/ml)
 - b. Grow in **30C** shaker for O/N

Day 2

3. Set up two 15 ml cultures; 1 for heat-shock induction, 1 for non-HS ctrl.
 - a. Inoculate 600 μ l of the saturated O/N DY380/BAC-Rab culture into each 15 ml LB-Chl(+)-Tet(+) media.
 - b. Grow on 30C shaker until OD600 reaches 0.4 (usually about 2.5-3.5 hours).
4. Purify the BamH1-cut pAcman-1kb
 - a. Run the sample on 1% agarose gel for 25 min @ 120V
 - i. Include an uncut ctrl
 - b. Take a gel photo
 - c. Cut the band of the right size (~14 kb)
 - d. Extract the DNA using kit following manufacturer's manual. We used Zymoclean Gel DNA Recovery Kit (Zymoresearch, Cat # D4008). Final elution in 12 μ l dH₂O.
 - e. Spec.
5. Heat-shock the culture at **42C** for exactly 14 minutes in the water bath (to induce the gap-repair capability). Let ctrl tubes stay at 30C.
6. Swirl the culture on ice for 5 min to chill.
7. Make fresh competent cells. Perform all of the following steps @4C
 - a. Centrifuge at 1500g for 5 min @ 4C
 - b. Discard the supernatant. Re-suspend the pellet with 10ml cold sterile H₂O.
 - c. Centrifuge at 1800g for 5 min @ 4C
 - d. Discard the supernatant. Re-suspend the pellet with 1ml cold sterile H₂O. Transfer to 1.5ml tubes
 - e. Centrifuge at 2200g for 2 min @ 4C in tabletop microcentrifuge
 - f. Discard the supernatant until the 100 μ l mark on the tube. Re-suspend the pellet with 1ml cold sterile H₂O.
 - g. Centrifuge at 2200g for 2 min @ 4C in tabletop microcentrifuge
 - h. Suck up ~900ul of H₂O. Resuspend the pellet in the remaining 100ul water.
8. Transformation
 - a. Add 5ul BamH1-cut DNA to the cells and transfer into a cuvette. Electroporate at 1800V.
 - b. Add 300ul SOC medium. Let recover @ 30C shaker for 2 hours.
 - c. Plate the cells onto LB-Amp(+)-Tet(+) plate for antibiotic selection. Grow @ 30C for 24 hours.

Day 3

9. Pick colonies. Set up liquid culture. Grow in 30C shaker O/N.

Note: A successful recombineering event should result in tens of colonies. If you get no colonies, try to improve on DNA quantity and/or the competency of the cells. On the other hand, if you see hundreds of colonies, that means P[acman]-KO-1kb was most likely NOT cut completely.

Day 4

- 10. PCR using boiled liquid culture as DNA template to screen for inserts.
- 11. Save frozen stock of the PCR-verified clones for the subsequent recombineering from HS plates.

5. Second round recombineering to knock in the Gal4-3xP3-RFP-Kanamycin cassette into P[acman]-KO-genomic

Goal: to replace the rab locus (or any base pair-precise region) in the genomic region with Gal4 cassette. Our Gal4 cassette is 6.7kb. By PCR, two 120bp fragments flanking the knock-in site are added to both ends of the cassette. At least 100bp arms are required for recombineering the 6.7kb Gal4 cassette into the genomic DNA.

Note 1: after recombineering, the transformants are spread onto LB-Amp(+)/Kan(+)/Tet(+) plate. PCR to verify the colonies.

Note 2: sequence, sequence, and sequence. You are using PCR to amplify a 6.7kb fragment. It is likely that some errors have been introduced by PCR.

5.1 Making a knock-in cassette

- 1) Set up PCRs using the following recipe, making sure to add reagents in the order listed. Use Phusion® High-Fidelity DNA Polymerase (Finnzymes, Cat # F-530S) kit:

Sterile Water -----	71 µL
5X Phusion HF Buffer -----	20 µL
MgCl ₂ (50 mM) -----	2 µL
dNTPs (10 mM TOTAL) -----	3 µL
Forward Primer (10 uM) -----	1 µL
Reverse Primer (10 uM) -----	1 µL
p-ENTR-Gal4 (dilute ≈ 45 ng / µL) -----	1 µL
Phusion DNA polymerase -----	1 µL
Total	100 µL

- 2) Separate into two, 50 µL reactions, and PCR as follows:

98C	30 sec	
98 C	10 sec	2 cycles
55 C	30 sec	
72 C	7 min	

98 C	10 sec		27 cycles
62 C	30 sec		
72 C	7 min		
72 C	10 min		
4 C	<i>Forever</i>		

- 3) Run products on 1% agarose gel (120 V, 27 min).
- 4) Carefully excise the desired bands (6.7 kb) and extract DNA with Zymoclean kit, following the instructions in the manual. Pool together each set of dissolved gel slices by passing them sequentially through single.
- 5) Elute DNA in 20 μ L of DI Water (52 C). Let sit at RT for 1-2 min before centrifugation.
- 6) Determine the concentration of the cassette. Store at - 20 C, aliquot if necessary to avoid freeze-thaw cycles.

5.2 Second Round Recombineering:

- 1) *The Night Before*: Set up 5 mL cultures of verified, first-round clones with appropriate antibiotics.
- 2) Inoculate 600 μ l of the saturated O/N culture into each 15 ml LB-Amp(+)-Tet(+) media. You will need two tubes per *rab* (HS and ctrl). Grow on 30C shaker until OD600 reaches 0.4 (usually about 2.5-3 hours).
- 3) *At T + 2 hrs*: Place all of the materials you will need for recombineering on ice. This includes autoclaved water, electroporation cuvettes, 1.5 mL microcentrifuge tubes and the appropriate cassettes.
- 4) After the cultures reach 0.4-0.6 OD, place HS tubes in water bath for EXACTLY 14 mins. Let ctrl tubes stay at 30C.
- 5) Quickly place all tubes on slushy ice and swirl HS tubes by hand for 2 mins to drop temperature as quickly as possible. Allow tubes to sit on ice for an additional 3 mins (5 mins total).
- 6) Centrifuge at 1500g for 5 mins.
- 7) Aspirate away the supernatant solutions, and add 10 mL ice-cold water to the side of each tube (NOT directly to the pellets). Resuspend pellets as gently as possible, on ice, by hand (swirl and invert tubes).
- 8) Centrifuge at 1800g for 5 mins.

- 9) Aspirate supernatants, resuspend pellets as before in 1 mL ice-cold water. Transfer bacterial solutions to 1.5 mL microcentrifuge tubes.
- 10) Centrifuge at 2200 x g for 2 mins and slowly remove as much supernatant as is needed to reach the 100 μ L mark on each tube.
- 11) Resuspend again in 1 mL water, centrifuge as before, and again remove the supernatant to 100 μ L.
- 12) Add 100 ng of cassette DNA to the appropriate tubes, and then resuspend pellets by flicking and inversion. You will probably need to dilute your cassettes beforehand.
- 13) Transfer 100 μ L of each bacterial solution to a corresponding electroporation cuvette (a little less is OK).
- 14) Electroporate by first tapping the cuvette to remove bubbles, then drying with a Kimwipe. Shock at 1800V with a time constant between 4.0-6.0 ms.
- 15) IMMEDIATELY add 300 μ L SOC medium and let the cuvette sit at RT while your electroporate the other cells.
- 16) Gently transfer the contents of each cuvette to a corresponding culture tube, and allow cells to recover at 30 C with shaking for 2 hrs (being careful not to let them recover for more than 2 hrs).
- 17) Plate the recovered cells on the appropriate antibiotic selection plate, and place at 30 C to grow.
- 18) Colonies should be very small by the next morning (if there at all), and by 4 PM, you should have between 20-200 small-medium colonies if the technique worked effectively. Sometimes you might need to wait until the next morning for the colonies to be sizable.
- 19) Proceed with minipreps and PCR 5' Gal4 junction to verify your recombineering has been successful.

6.1 Increasing DNA copy number using EPI300 cells

1. Transform w/ 1 μ L DNA
 - Mix 50 μ L TransforMax™ EPI300™ electrocompetent *E. coli* with 1 μ L DNA
 - Electroporate the mixture in pre-chilled cuvette at 1800 volts
 - Add 300 μ L SOC medium to the mixture. Transfer to 1.5ml tubes
 - Let recover @ 37C shaker for 1 hour

- Spread the transformants onto LB plates with appropriate antibiotics for selection.
 - Grow @ 37C incubator for overnight.
2. Pick 3 colonies (early morning) and set up 5 ml liquid culture (LB with appropriate antibiotics). Let grow at 37C shaker. After 6-8 hours of shaking, the culture should be semi- saturated.
 3. Verifying the colonies by PCR the 5' Gal4 junction, followed by gel electrophoresis.
 4. Set up liquid LB culture for DNA copy number induction.
 - 5ml of O/N saturated culture + 145 ml of fresh LB + antibiotics + 150 ul of "1000X copy control soln"

Grow @ 37C shaker for 6 hours.

Maxi-prep (Run a small aliquot on get to check quality)

5. Verification of the Gal4 insertion by sequencing.
6. Inject maxi-prep'd DNA into Drosophila embryos (Bloomington 24871), followed by screening for germline transmission.

pENTR with Gal4 cassette

CTTCTCGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCAGCTTTGAGTGAGTGATACCCTCGCCGAGCCGAAACACCGAGCGCAGCGAGTCACTGAGCGAGGA
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ATAACTTGG	loxP	reverse
AAGGTTGGG	pENTR-D/TOPO	forward

Supplemental References

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