File S1

Supporting Materials and Methods

Molecular Biology. The five 2A peptides selected for investigation in this study were the D2A peptide of *Drosophila C* virus, E2A of *Equine rhinitis* A virus, F2A of foot-and-mouth disease, P2A of *Porcine teschovirus1* and T2A of *Thosea asigna* virus (Donnelly *et al.* 2001). The amino acid sequences of these five 2A peptides are shown in Table S1. Five DNA sequences that encoded the mCD8 transmembrane protein (Lee and Luo 1999) fused to a 2A peptide in a single open reading frame, and incorporating a KpnI restriction between the mCD8 and 2A sequences were synthesized (Epoch Biolabs, Inc. Sugar Land, TX). Following digestion with XbaI and SacI, these constructs were subcloned into the pPacPL vector (a kind gift of Dr. Bruce Paterson; digested with SpeI and SacI) to create the pPacPL-mCD8-2A constructs. The EGFP coding sequence from pEGFP-N2 (BD Biosciences) was subcloned into the pPacPL-mCD8-2A plasmids using the ApaI and NotI restriction sites to generate the five pPacPL-mCD8-2A-EGFP constructs. The pPacPL-mCD8-EGFP control construct was made by digesting both pPacPL-mCD8-2A and pEGFP-N3 (BD Biosciences) with KpnI and NotI and ligating the EGFP fragment into the resulting pPacPL-mCD8-EGFP plasmid.

To generate pPacPL-mCD8-2A-Gal4 constructs, the coding sequence of Gal4 was amplified by PCR using primers that added ApaI (or KpnI) and NotI restriction sites at the 5' and 3' ends. The Gal4 fragment was subcloned into pPacPL-mCD8-2A digested with ApaI or KpnI and NotI, to make the pPacPL-mCD8-2A-Gal4 or pPacPL-mCD8-Gal4 constructs, respectively.

To generate the Bursα-mCD8-EGFP-Gal4 and Bursα-mCD8-EGFP-T2A-Gal4 constructs, a synthetic construct (i.e. Bursα-mCD8-EGFP-T2A-Gal4-Hsp70 pA) including, sequentially, the 252 bp promoter of the bursicon α-subunit gene (Peabody *et al.* 2008), the mCD8-EGFP-T2A-Gal4 coding sequence, and the Hsp70 polyadenylation signal was generated in pBlueScript with KpnI and SpeI restriction sites at 5' and 3' ends, respectively (Epoch Biolabs, Inc. Sugar Land, TX). Using the KpnI and SpeI restriction sites, the bursα-mCD8-EGFP-T2A-Gal4-Hsp70 construct was subcloned into the pC-attB vector (the kind gift of Dr. Chi-Hon Lee) to obtain pC-attB-Bursα-mCD8-EGFP-T2A-Gal4. The pC-attB-bursα-mCD8-EGFP-Gal4 was obtained by digesting pC-attB-bursα-mCD8-EGFP-T2A-Gal4 with ApaI to excise the T2A sequence.

To generate the UAS-rk^{RA} construct, the coding sequence of the rk-RA cDNA (Eriksen *et al.* 2000) was amplified by PCR using primers that introduced an EcoRI restriction site and an optimized translation initiation motif with sequence CAAA immediately before the ATG start codon and a NotI restriction site just after the stop codon. The amplified fragment was then subcloned into the pUAST plasmid for P-element transformation, after EcoRI and NotI restriction digestion.

P[acman] clone CH322-119A8 (Venken *et al.* 2009; Venken *et al.* 2006) (from BAC/PAC Resources Center of Children's Hospital Oakland Research Institute, Oakland, CA) was used to generate the constructs for rk-Gal4 by recombineering using GalK selection (Warming *et al.* 2005). The following synthetic primers (IDT, Coralville, Iowa) were used to amplify the GalK targeting

cassette. Sequences representing homologous arms in the *rk* gene are underlined; T2A-Gal4 sequence introduced for higher efficiency recombineering is in bold face:

For rk^{pan}-GalK, rk^{pan}-Galk F:

<u>ACTTCGAGGAGCACGATGTGAGTGGTCCTGCCACGGGATACGGCTTTGGT</u>**GAGGGCCGCGGCAGCCTGCTGACCTGCGGCGATGTGGAGG**CCTGTTGACAATTAATCATCGGCA

and rk^{pan}-GalK R:

<u>ACCGAACCCGGTTGAAAGTCCTCTGTGGACATACCAGAGAATAGTCCAGT</u>**TCATTACTCCTTCTTGGGGTTGGGGGGGGTATCCTCATCA**TCAGCACTGTCCTGCTCCTT

For rk^{RA}-GalK, rk^{RA}-GalK F:

<u>TGGTCCTGCAGCCGGCAAAGCCTCCTCCCGATCCCAACGATGCACCACTA</u>TCAGCACTGTCCTGCTCCTT

and rk^{RA}-GalK R: <u>GTCTCCTGGTTGGCCCCACCCAATCTTCGCCAGCGCCAGTCCCACCATCA</u>CCTGTTGACAATTAATCATCGGCA.

After obtaining GalK-positive P[acman] clones, the GalK sequence was substituted by the *Drosophilia* codon-optimized T2A-Gal4 sequence. T2A-Gal4 fragments were amplified by PCR with the following primers:

For rk^{pan}-Gal4, rk^{pan}-Gal4 F: <u>TACTTCGAGGAGCACGATGTGAGTGGTCCTGCCACGGGATACGGCTTTGGT</u>GAGGGCCGCGGCAGCCTGCT

and rk^{pan}-Gal4 R: <u>CACCGAACCCGGTTGAAAGTCCTCTGTGGACATACCAGAGAATAGTCCAGT</u>TTACTCCTTCTTGGGGTTGG

For rk ^{RA} -Gal4, rk^{RA}-Gal4 F:

<u>TGGTCCTGCAGCCGGCAAAGCCTCCTCCCGATCCCAACGATGCACCACTA</u>GAGGGCCGCGGCAGCCTGCTGACC

and rk^{RA}-Gal4 R: <u>GTCTCCTGGTTGGCCCCACCCAATCTTCGCCAGCGCCAGTCCCACCATCA</u>TTACTCCTTCTTGGGGTTGGG.

Plasmid DNAs for all constructs were isolated for fly transformation by S.N.A.P. MidiPrep Kit (Invitrogen, Carlsbad, CA).

Fly Genetics. Transgenic flies were generated using standard injection protocols by either Duke University Model System Genomics Group (Durham, NC) or Rainbow Transgenic Flies, Inc. (Camarillo, CA). UAS-rk^{RA} was generated by P-element transformation. All other constructs were generated by ΦC31-mediated transgenesis into the attP2 site on the 3rd chromosome (GROTH *et al.* 2004). All flies were grown on corn meal-molasses medium and maintained at 25°C in a constant 12 h light–dark cycle. UAS-EGFP, UAS-EGFP.nls, UAS-RedStinger and *rickets*^{4} (*rk*^{4}) mutants were from the Bloomington Stock Center (Indiana University). UAS-dnc flies were the kind gift of Dr. Randall Hewes.

SL2 Cell Culture. SL2 cells were grown in serum-free HyQ-CCM3 medium (Hyclone, Logan, UT) to a density of 10^6 cells ml^{-1} in 6-well plates (10^6 cells per well). Cells in each well were transfected with 1.0 µg of each DNA construct purified with S.N.A.P.

MidiPrep kit (Invitrogen, Carlsbad, CA) using the Roche FuGENE6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN). pPacPL-mCD8-2A-Gal4 (or PacPL-mCD8-Gal4) and pUAST-EGFP plasmid DNAs were co-transfected in experiments in which Gal4 activity was monitored by UAS-EGFP expression. Cells were analyzed by confocal microscopy after 2 d incubation at 25 °C.

Immunostaining and Image Acquisition. Excised nervous system whole mounts from wandering third-instar larvae or pharate adults were dissected in PBS, and fixed in 4% paraformaldehyde in PBS for 20–30 min, followed by post fixation in 4% paraformaldehyde/PBS plus 0.5% Triton X-100 for 15 min. Primary antibodies were used at the following concentrations: rabbit anti-bursicon α-subunit (PEABODY *et al.* 2008), 1:5000 dilution; mouse anti-mCD8, 1:100 dilution (Invitrogen, Carlsbad, CA); rabbit anti-Gal4, 1:100 dilution (Santa Cruz Biotech, Inc., Santa Cruz, CA). Secondary antibodies used were: AlexaFluor 488 goat anti-mouse, AlexaFluor 568 goat anti-rabbit, and AlexaFluor 680 goat anti-rabbit (all from Invitrogen) were used at 1:500 dilution. Immunolabeled samples were mounted in Vectashield (Vector Laboratories) prior to confocal imaging on a Nikon C-1 confocal microscope. *Z*-series were acquired in 1 µm increments using a 20× objective and 488 nm, and 543 nm, and 633nm laser emission lines for fluorophore excitation. The images shown are maximal projections of volume rendered *z*-stacks of confocal sections collected by incrementally stepping through the entire nervous system.

References

DONNELLY, M. L., L. E. HUGHES, G. LUKE, H. MENDOZA, E. TEN DAM *et al.*, 2001 The 'cleavage' activities of foot-and-mouth disease virus 2A site-directed mutants and naturally occurring '2A-like' sequences. J Gen Virol **82:** 1027-1041.

ERIKSEN, K. K., F. HAUSER, M. SCHIOTT, K. M. PEDERSEN, L. SONDERGAARD *et al.*, 2000 Molecular cloning, genomic organization, developmental regulation, and a knock-out mutant of a novel Leu-rich repeats-containing G protein-coupled receptor (DLGR-2) from Drosophila melanogaster. Genome Research **10:** 924-938.

GROTH, A. C., M. FISH, R. NUSSE and M. P. CALOS, 2004 Construction of transgenic Drosophila by using the site-specific integrase from phage phiC31. Genetics **166:** 1775-1782.

LEE, T., and L. LUO, 1999 Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron **22:** 451-461.

PEABODY, N. C., F. DIAO, H. LUAN, H. WANG, E. M. DEWEY *et al.*, 2008 Bursicon functions within the Drosophila CNS to modulate wing expansion behavior, hormone secretion, and cell death. J Neurosci **28:** 14379-14391.

VENKEN, K. J., J. W. CARLSON, K. L. SCHULZE, H. PAN, Y. HE *et al.*, 2009 Versatile P[acman] BAC libraries for transgenesis studies in Drosophila melanogaster. Nat Methods **6:** 431-434.

VENKEN, K. J. T., Y. C. HE, R. A. HOSKINS and H. J. BELLEN, 2006 P[acman]: A BAC transgenic platform for targeted insertion of large DNA fragments in D-melanogaster. Science **314:** 1747-1751.

WARMING, S., N. COSTANTINO, D. L. COURT, N. A. JENKINS and N. G. COPELAND, 2005 Simple and highly efficient BAC recombineering using galK selection. Nucleic Acids Res **33:** e36.

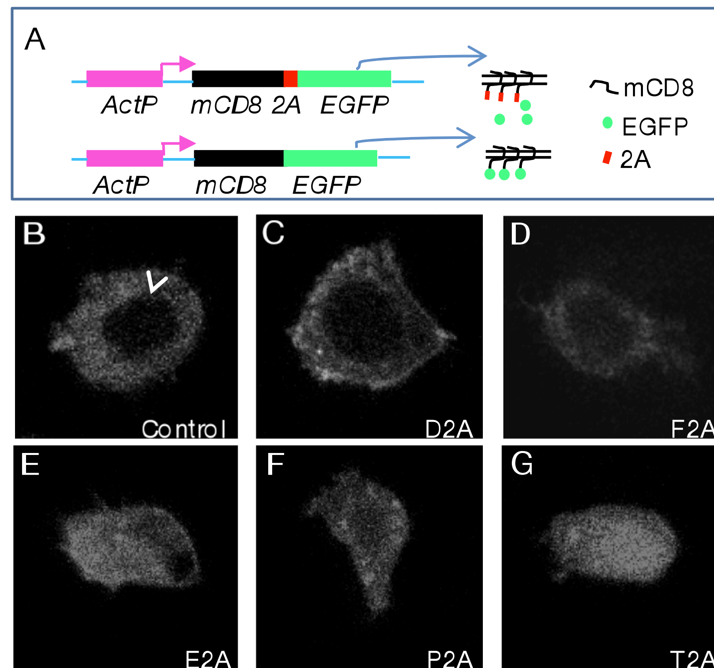

Figure S1 T2A and other 2A peptides facilitate ribosomal skipping in *Drosophila* SL2 cells. (A) Schematic of the mCD8-2A-EGFP construct used to test the faciliation of ribosomal skipping by five 2A peptides (top) and the control mCD8-EGFP construct (bottom). Both constructs are expressed under the control of the *actin* promoter. (B-G) The expression and localization of EGFP in cells transfected with the constructs shown in (A). The images are confocal micrographs of the EGFP distribution in representative individual cells. (B) Tethering EGFP to the membrane by fusing it to mCD8 restricts access of the fluorophore to the nucleus (arrowhead), as is evident in cells made with the control construct lacking a 2A peptide. Constructs made with the D2A (C) or F2A (D) peptides had non-nuclear labeling, similar to controls indicating poor facilitation of ribosomal skipping. In contrast, transfection with the E2A (E), P2A (F), and T2A (G) constructs showed nuclear EGFP localization, with the T2A construct yielding the most uniform labeling patterns suggesting high levels of ribosomal skipping, and the production of soluble, rather than membrane-bound, EGFP. The 2A peptides tested are as indicated in the Materials and Methods.

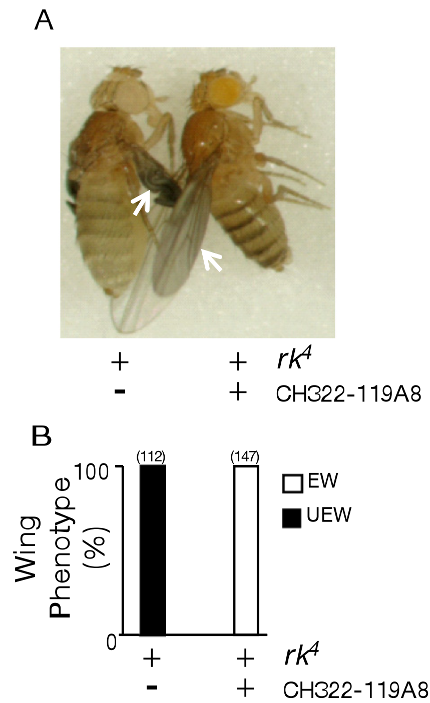

Figure S2 P[acman] clone CH322-119A8, which includes the entire *rk* gene locus as well as 5' and 3' flanking regions, rescues the wing expansion deficits of rk^4 mutants. (A) Unlike flies carrying only the rk^4 mutation (left), flies also bearing a genomic copy of CH322-119A8 (right) expand their wings normally (arrows). (B) Bargraph summarizing the success of rescue with CH322-119A8. Numbers in parentheses indicate the number of flies scored of each genotype.

Table S1 Sequences of 2A peptides tested in this study

2A peptide	Peptide sequence
D2A	AARQMLLLLSG**D**V**E**TN**PG** **P**
E2A	QCTNVALLKLAG**D**V**E**SN**PG** **P**
F2A	VKQTLNFDLLKLAG**D**V**E**SN**PG** **P**
P2A	ATNFSLLKQAG**D**V**E**EN**PG** **P**
T2A	EGRGSLLTCG**D**V**E**EN**PG** **P**

The one-letter amino acid code is used and the conserved –DxExNPG P– motif, which promotes ribosomal skipping, is shown in bold. Sequences are from Donnelly et al. 2001a, as cited in the main text.