Supporting Information

Rera et al. 10.1073/pnas.1215849110

SI Materials and Methods

Fly Stocks. The standard laboratory strains w^{1118}, Canton S, and *w*Dahomey were used to detect intestinal barrier defects during aging, and *w*;*Drs*-GFP was used to examine *drosomycin* (*Drs*) expression during aging. Subunit b of succinate dehydrogenase (*sdhB*) mutants and the genomic rescue control, described previously (1), were used to examine the impact of mitochondrial dysfunction on intestinal barrier function.

Lifespan, Hyperoxia, and Fly Culture. Flies were cultured in a humidified, temperature-controlled incubator with a 12-h on/off light cycle at 25 °C in vials containing standard cornmeal medium (1% agar, 3% brewer's yeast, 1.9% sucrose, 3.8% dextrose, and 9.1% cornmeal; all concentrations given in wt/vol). Adult animals were collected under light nitrogen-induced anesthesia, housed at a density of 27–32 flies per vial, and flipped to fresh vials and scored for death every 2–3 d throughout adult life. Dietary-restricted and rich media composition was 1% agar, 0.5 or 5% yeast extract, 5% sucrose, and 8.6% cornmeal. Survival under hyperoxia was maintained as described previously (1).

Smurf Assay. Unless stated otherwise, flies were aged on standard medium until the day of the Smurf assay. Dyed medium was prepared using standard medium with dyes added at a concentration of 2.5% (wt/vol). The blue no. 1 and red no. 40 dyes were purchased from SPS Alfachem, and fluorescein was purchased from Sigma-Aldrich. Flies were maintained on dyed medium for 9 h. A fly was counted as a Smurf when dye coloration was observed outside the digestive tract. The Smurf increase rate (SIR) was calculated by plotting the average proportion of Smurfs per vial as a function of chronological age, with the SIR defined as the slope of the calculated regression line. When the Smurf assay was performed on *w;Drs*-GFP animals, GFP expression level was scored before exposure to blue dye no. 1.

Identification of FluorSmurf and *Drs*-GFP$^+$ Flies. Animals were sorted on ice and imaged using a Zeiss Discovery v12 stereomicroscope. The *Drs*-GFP expression assay was conducted in a binary fashion. An animal was considered GFP$^+$ when GFP expression was seen outside of the female sperm storage organ, previously described as a site of constitutive expression in these flies (2).

Internal Bacterial Load. Internal bacterial load samples were prepared as described previously (3) and maintained at −80 °C before plating. Serial sample dilutions were plated on mannitol media.

Quantitative Real-Time PCR. RNA extractions were carried out in TRIzol (Invitrogen) following the manufacturer's directions. cDNA synthesis was performed using a First-Strand cDNA Synthesis Kit (Fermentas). PCR was performed with Power SYBR Green Master Mix on an ABI 7300 Real-Time PCR System (Applied Biosystems), under the following cycling conditions: 95 °C for 10 min, 95 °C for 15 s, then 60 °C for 60 s, cycled 40 times.

All calculated gene expression values were normalized to the value of the loading control gene, *Actin5c*. The following primer sequences were used: *Actin5c*_L: TTGTCTGGGCAAGAGGATCAG, *Actin5c*_R: ACCACTCGCACTTGCACTTTC; *Dro*_L: CCATCGAGGATCACCTGACT, *Dro*_R: CTTTAGGCGGGCAGAATG; *Drs*_L: GTACTTGTTCGCCCTCTTCG, *Drs*_R: CTTGCACACACGACGACAG; *Dpt*_L: ACCGCAGTACCCACTCAATC, *Dpt*_R: CCCAAGTGCTGTCCATATCC; *InR*_L: GCACCATTATAACCGGAACC, *InR*_R: TTAATTCATCCATGACGTGAGC; *Thor*_L: TACACGTCCAGCGGAAAGTT, *Thor*_R: CCTCCAGGAGTGGTGGAGTA; and *ImpL2*_L: GCCGATACCTTCGTGTATCC, *ImpL2*_R: TTTCCGTCGTCAATCCAATAG.

Western Blot Analysis. Three female flies were homogenized in 0.1 mL of 2× NUPAGE LDS sample buffer (Invitrogen), with 10 μL of lysate loaded per lane. Protein was transferred to PVDF membrane. Antibodies used were anti-Akt from rabbit (9272, 1:1,000 dilution; Cell Signaling Technologies) and anti–S505-phosphorylated *Drosophila* Akt (4054, 1:1,000; Cell Signaling Technologies). The secondary antibody was HRP anti-rabbit IgG (7074, 1:2,000; Cell Signaling Technologies). Proteins were detected with Supersignal West Pico (Pierce), and total protein was visualized using Ponceau S staining. Analysis was carried out in ImageJ 1.46j.

Walking Activity. *Drs*-GFP$^+$ and *Drs*-GFP$^-$ flies were separated under light nitrogen anesthesia and transferred into 5-cm Petri dishes containing 5 mL of standard fly food. Flies were then allowed to recover from anesthesia for 2 h before recording. The Petri dishes were then placed on the top of a white light transilluminator and recorded for 1 h (5:00 PM–6:00 PM) using a 1,080-p Logitech HD Pro C920 Web cam at 15 frames/second. The video was then converted to 8-bit TIFF files (15/s) on which the position of each individual fly was determined using ImageJ. For each fly, the average speed of movement for the 1 h video was calculated. The *Drs*-GFP$^{+/-}$ results were then compared using the Mann–Whitney *U* test.

Quantification of Triglycerides. Lipids were extracted from five whole female flies in a chloroform:ethanol solution (2:1 vol/vol), and nonpolar lipids (fatty acid, triacylglycerol) were separated by thin-layer chromatography with a n-hexane/diethylether/glacial acetic acid solution (70:30:1, vol/vol/vol). Plates were air-dried and stained (with 0.2% Amido Black 10B in 1 M NaCl), and lipid bands were quantified by photo densitometry using ImageJ and normalized to body weight.

Quantification of Glycogen. Five decapitated female flies were homogenized in 200 μL of buffer (1× PBS, 1 mM EDTA; pH 7.4). Glucose and glycogen were measured with a Biovision Glycogen Assay Kit using 3 μL of cleared extract in accordance with the manufacturer's instructions. Protein content was quantified with a Thermo Fisher Scientific μBCA Kit and used for normalization.

Statistics. Linear regression lines of Smurf proportion during aging were determined using at least 16 individual points (4 time points and 4 replicates per time point) in GraphPad Prism version 5. Correlation of the datasets was assessed using the Pearson test for linear regressions as implemented in the software. Comparison of slopes and testing for nonnull slope values were done using GraphPad Prism. Comparisons of Smurf proportion per time point were carried out using binomial tests to calculate the probability of having as many Smurfs in population B as in population A. The binomial tests were performed using R version 2.14.2. The comparison of survival curves was done using the log-rank test as implemented in GraphPad Prism. Gene expression levels and bacterial loads were tested for significant differences using the Mann–Whitney *U* test for sample sizes of five or more and the Student *t* test for sample sizes less than five, both implemented in R version 2.14.2. All statistical tests were two-sided.

All binomial regressions were done using R version 2.14.2 and the glm function with family = "binomial" argument. Theoretical Smurf proportions were then calculated using the regression equation for days 10, 20, 30, 40, 50, 60, 70, and 80 to plot the regression lines.

1. Walker DW, et al. (2006) Hypersensitivity to oxygen and shortened lifespan in a *Drosophila* mitochondrial complex II mutant. *Proc Natl Acad Sci USA* 103(44):16382–16387.
2. Ferrandon D, et al. (1998) A *drosomycin*-GFP reporter transgene reveals a local immune response in *Drosophila* that is not dependent on the Toll pathway. *EMBO J* 17(5): 1217–1227.
3. Ren C, Webster P, Finkel SE, Tower J (2007) Increased internal and external bacterial load during *Drosophila* aging without life-span trade-off. *Cell Metab* 6(2):144–152.

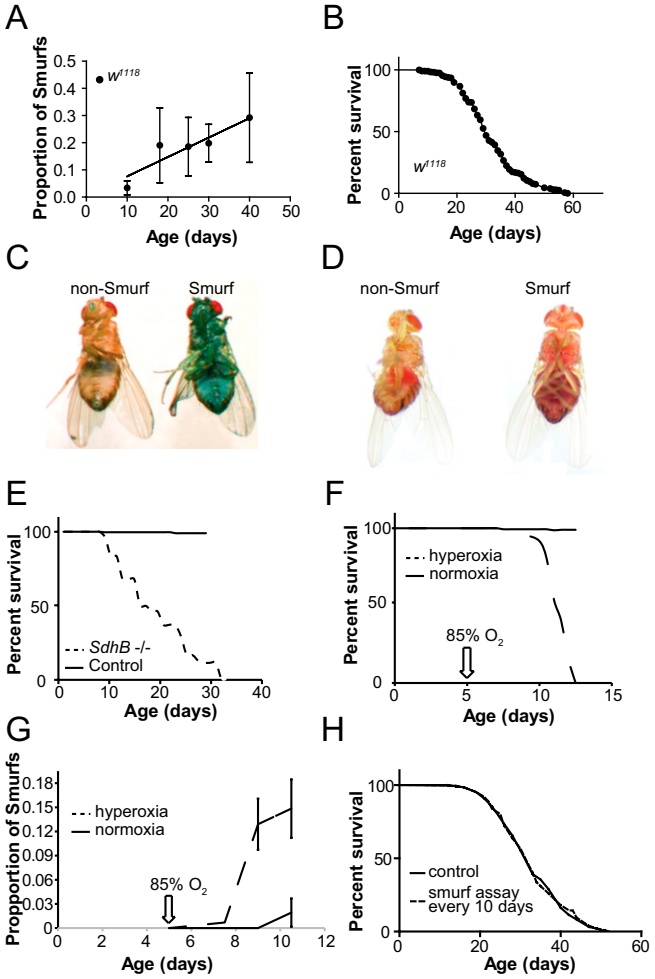


Fig. S1. Loss of intestinal integrity in aging flies and in response to hyperoxia. (*A*) Loss of intestinal integrity in male flies as a function of age using FD&C blue dye no. 1. The slope, or SIR, -0.007914 ± 0.001146 (R^2 = 0.8416; $P_{Pearson}$ = 0.0282), is significantly nonnull ($P_{F\ test}$ = 0.0031). n = 3–7 replicates (vials) per time point. (*B*) Survival of w^{1118} males. n = 976. (*C* and *D*) Smurfs obtained with blue no. 1 (*C*) or red no. 40 (*D*) using Canton S females. (*E*) *sdhB* mutant survival curve. *sdhB* mutants have a lower lifespan compared with control flies carrying a genomic rescue construct ($P < 0.0001$, log-rank test). *sdhB*, n = 78 female flies; control, n = 320 female flies. (*F*) Survival of w^{1118} females under normoxia (solid line) or hyperoxia from day 5 (dashed line). (*G*) Exposure to hyperoxia leads to an increased number of Smurf flies. After 5 d under hyperoxia, 14.7% of the population displayed intestinal barrier defects, compared with 2.3% of control flies under normoxia ($P < 0.0001$, binomial test). $n > 900$ female flies. (*H*) Survival curves of w^{1118} female flies (n = 2,820) assayed every 10 d for the proportion of Smurfs in the population (solid line) and w^{1118} female flies (n = 1,176) not exposed to the blue dye. The two curves overlap completely (P = 0.88, log-rank test). Error bars represent mean ± SEM.

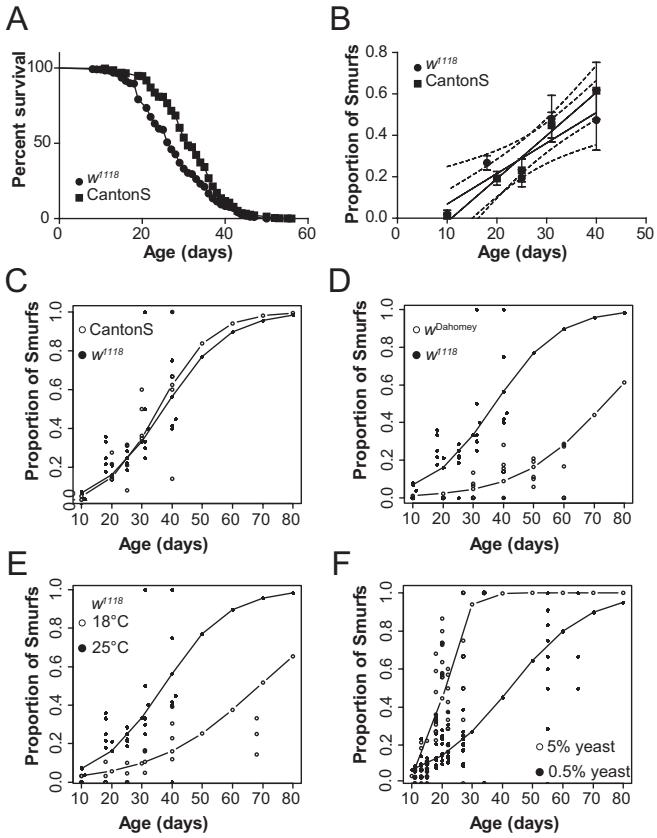


Fig. S2. Loss of intestinal integrity in aging Canton S flies and binomial regression analysis of Smurf proportions. (*A* and *B*) The survivorship of w^{1118} (T_{50} = 27 d; n = 3,786 female flies) is similar to that of Canton S (31 d; n = 739 female flies), and the $SIR_{w^{1118}}$ and $SIR_{CantonS}$ values were not significantly different (0.01472 ± 0.004513 vs. 0.02082 ± 0.003725; P_{Ftest} = 0.3337). Both regression lines show a significantly nonnull slope ($P_{Ftest} < 0.001$) and a significant correlation of the datasets ($R^2 > 0.8$; $P_{Pearson} < 0.05$). n = 3–7 replicates (vials) per time point. Error bars represent mean ± SEM. The dashed lines represent the 95% confidence interval of each dataset. (*C*) Binomial regression lines of Smurf proportions are not significantly different between Canton S and w^{1118} (n = 323 and n = 8,205, respectively; P = 0.37). (*D*) Binomial regression line of the Smurf proportion is significantly lower in $w^{Dahomey}$ (n = 695) flies than in w^{1118} flies ($P < 0.05$). (*E*) Binomial regression line of the Smurf proportion is significantly lower in w^{1118} flies reared at 18 °C (n = 475) than in w^{1118} flies reared at 25 °C ($P < 0.001$). (*F*) Binomial regression line of the Smurf proportion is significantly lower in w^{1118} flies reared on 0.5% yeast extract than in w^{1118} flies reared on 5% yeast extract (n = 1,942 and n = 1,427, respectively; $P < 0.0001$).

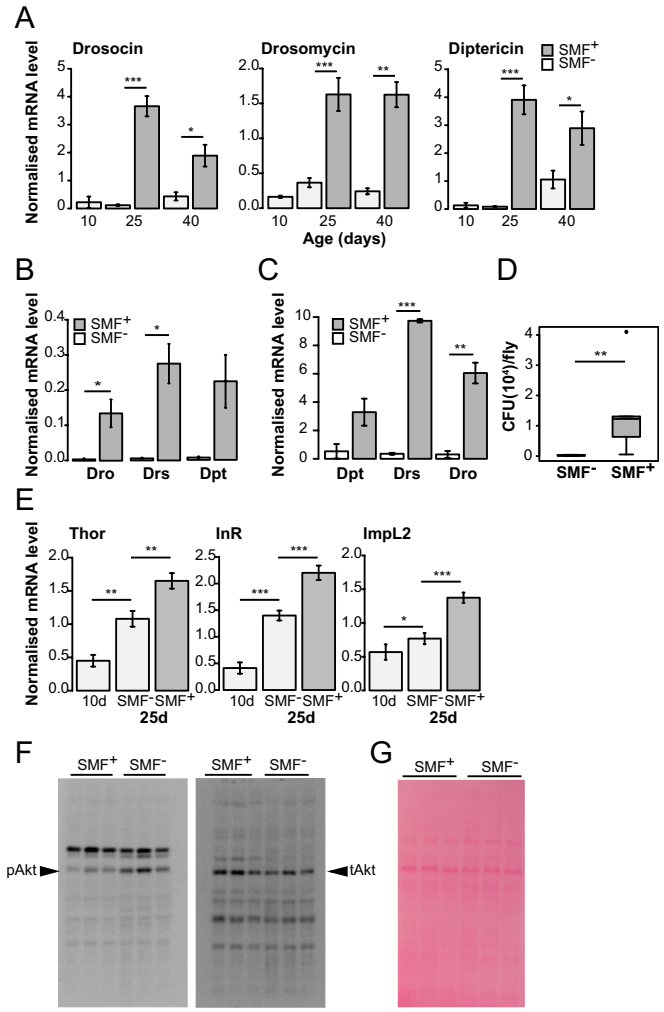


Fig. S3. Loss of intestinal integrity, antimicrobial peptide (AMP) expression, and insulin/insulin-like growth factor signaling in aging flies. (*A*) Systemic expression of drosomycin (*Drs*), *drosocin* (*Dro*), and *diptericin* (*Dpt*) in 10-d-old non-Smurf and in 25- and 40-d-old non-Smurf (SMF^{-}) and Smurf (SMF^{+}) Canton S females. At 10 and 25 d, $n = 3$ flies, 8 replicates; at 40 d, $n = 3$ flies, 5 replicates. Increases in *Dro* and *Dpt* expression between 10 d and 40 d in the SMF^{-} population were also statistically significant (*P* values: *Dro*, < 0.05; *Dpt*, < 0.01), *Drs* expression was significantly increased in the SMF^{-} population only at 25 d ($P < 0.01$). (*B*) Expression of *Drs*, *Dro*, and *Dpt* in 25-d-old non-Smurf and Smurf gut tissue from w^{1118} females. $n = 3$ flies, 3 replicates. (*C*) Systemic expression of *Drs*, *Dro*, and *Dpt* in 25-d-old age-matched non-Smurf and Smurf w^{1118} females, maintained on 0.5% yeast. $n = 3$ flies, 3 replicates. (*D*) Internal bacterial loads in 25-d-old non-Smurf and Smurf Canton S females. $n = 3$ flies, 5 replicates. (*E*) Systemic expression of *Thor*, *Insulin-like receptor* (*InR*), and *ImpL2* in 10-d-old non-Smurf and in 25- and 40-d-old non-Smurf (SMF^{-}) and Smurf (SMF^{+}) Canton S females. At age 10 and 25 d, $n = 3$ flies, 8 replicates; at age 40 d, $n = 3$ flies, 5 replicates. Increases in expression between 10 d and 40 d in the SMF^{-} population were also statistically significant (*P* values: *Thor*, < 0.05; *InR*, < 0.01; *ImpL2*, < 0.05), *Thor* and *InR* expression was also significantly increased at 25 d (*P* values: *Thor*, < 0.01; *InR*, < 0.001). (*F*) Western blots probed for phosphorylated Akt levels (pAkt; *Left*), and total Akt levels (tAkt; *Right*), in 25-d-old w^{1118} non-Smurf and Smurf females. $n = 3$ flies, 3 replicates. (*G*) Western blot of the samples shown in *F*, stained for total protein using Ponceau S. There was no significant difference in total protein levels between non-Smurf and Smurf samples. Error bars represent mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

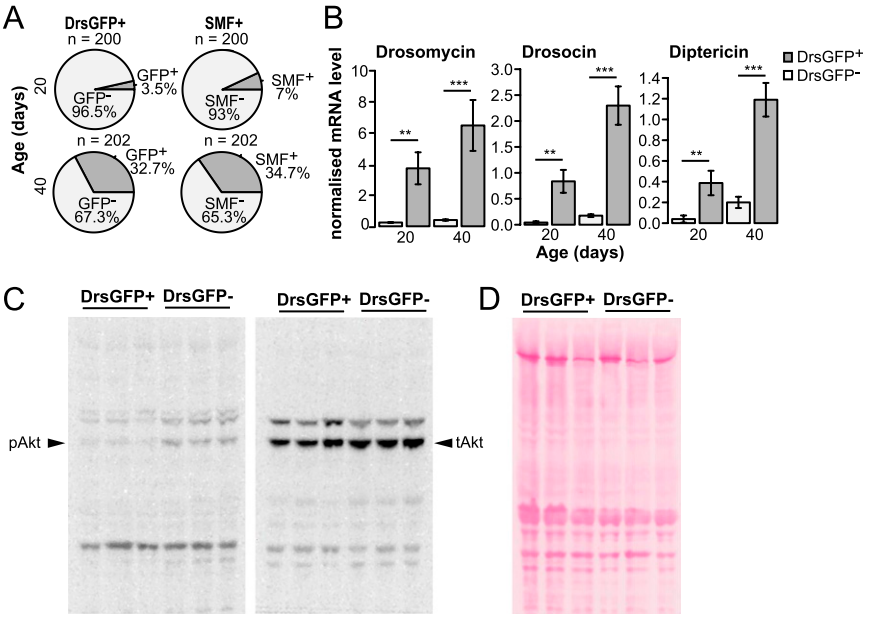


Fig. S4. *Drs*-GFP$^+$ flies show increased expression of other AMPs and reduced levels of activated Akt. (*A*) Proportions of GFP$^+$ and Smurf flies in a *w*;*Drs-GFP* female population at age 20 d and 40 d. Both show a significant increase from 20 d to 40 d (GFP$^+$, $P < 0.0001$; Smurfs, $P < 0.0001$, binomial tests). (*B*) Systemic expression of *Drs*, *Dro*, and *Dpt* at age 20 d and 40 d in GFP$^-$ and GFP$^+$ *w*;*Drs*-GFP females. At 20 d, $n = 3$ female flies, 6 replicates; at 40 d, $n = 3$ female flies, 8 replicates. Increases in AMP expression between 20 d and 40 d in the GFP$^-$ population were also statistically significant (P values: *Drs*, < 0.001; *Dro*, < 0.01; *Dpt*, < 0.05). (*C*) Western blots probed for phosphorylated Akt levels (pAkt, *Left*), and total Akt levels (tAkt, *Right*), in 40-d-old GFP$^-$ and GFP$^+$ *w*;*Drs-GFP* females. $n = 3$ flies, 3 replicates. (*D*) Western blot of the samples shown in *C*, stained for total protein using Ponceau S. Error bars represent mean SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

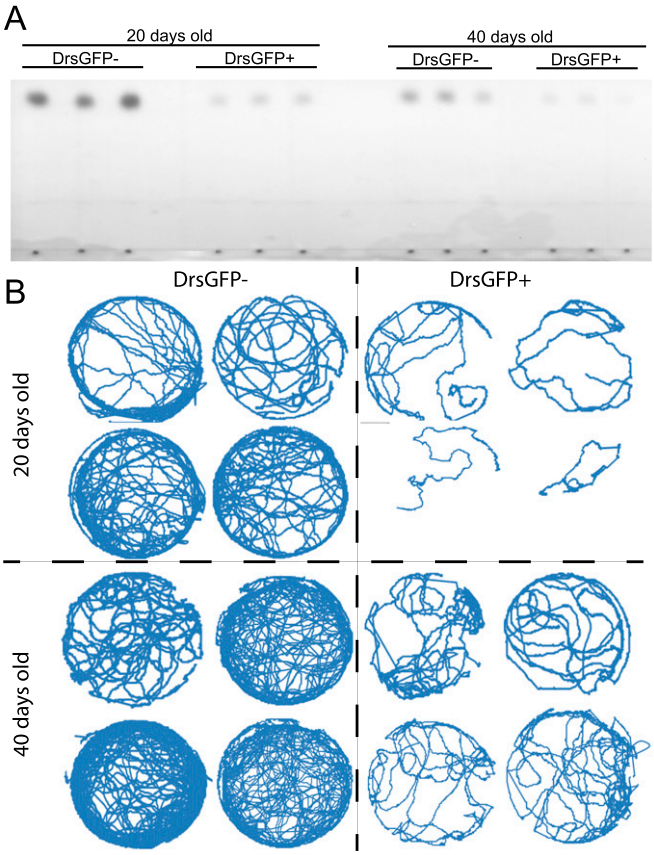
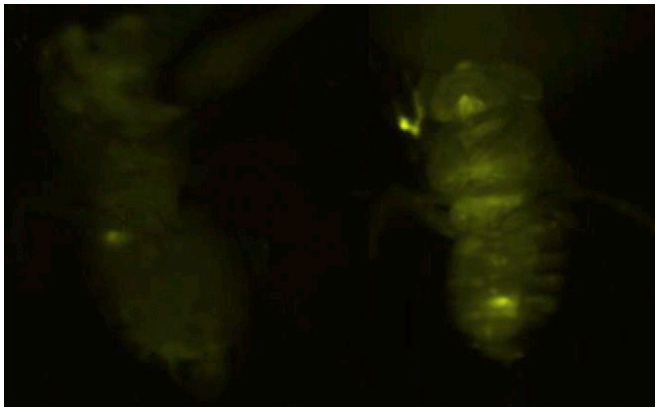


Fig. S5. *Drs*-GFP$^+$ flies show a significantly lower triglyceride content and spontaneous activity compared with age-matched *Drs*-GFP$^-$ flies. (*A*) Scan of the thin-layer chromatography plate developed using a hexane:diethyl ether:glacial acetic acid (70:30:1) solvent phase. (*B*) Representative activity traces of *Drs*-GFP$^-$ and *Drs*-GFP$^+$ flies at age 20 d and age 40 d. The flies were monitored for 1 h ± 5 min.



Movie S1. Live imaging of intestinal barrier dysfunction in aged *Drosophila*. A population of *Drs*-GFP flies was aged on standard medium, and at age 40 d, a GFP$^-$ fly (*Left*) and a GFP$^+$ fly (*Right*) were glued side by side on a microscope slide using UV glue, ventral part up. Flies were then filmed using an epi-fluorescence microscope (Zeiss) at a rate of 5 frames/second. Both flies were fed a 5% sucrose (wt/vol) and 2.5% fluorescein (wt/vol) solution contained in capillaries (Sutter Instruments) until fluorescence was observed in the crop (time, 8 s). The two flies were not fed simultaneously, and the videos of each fly were subsequently realigned to have the signal in the crop at the same time; no intensity adjustment was made on the video. At t = 20 s, the crop is contracting in both flies, and fluorescence can be seen in other parts of the intestine in the GFP$^-$ fly. In contrast, at this time point, the GFP$^+$ fly shows a strong fluorescent signal in the thorax that slowly diffuses throughout the rest of the animal.

Movie S1