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Supplemental Information

Upregulation of the Autophagy Adaptor p62/SQSTM1

Prolongs Health and Lifespan

in Middle-Aged *Drosophila*

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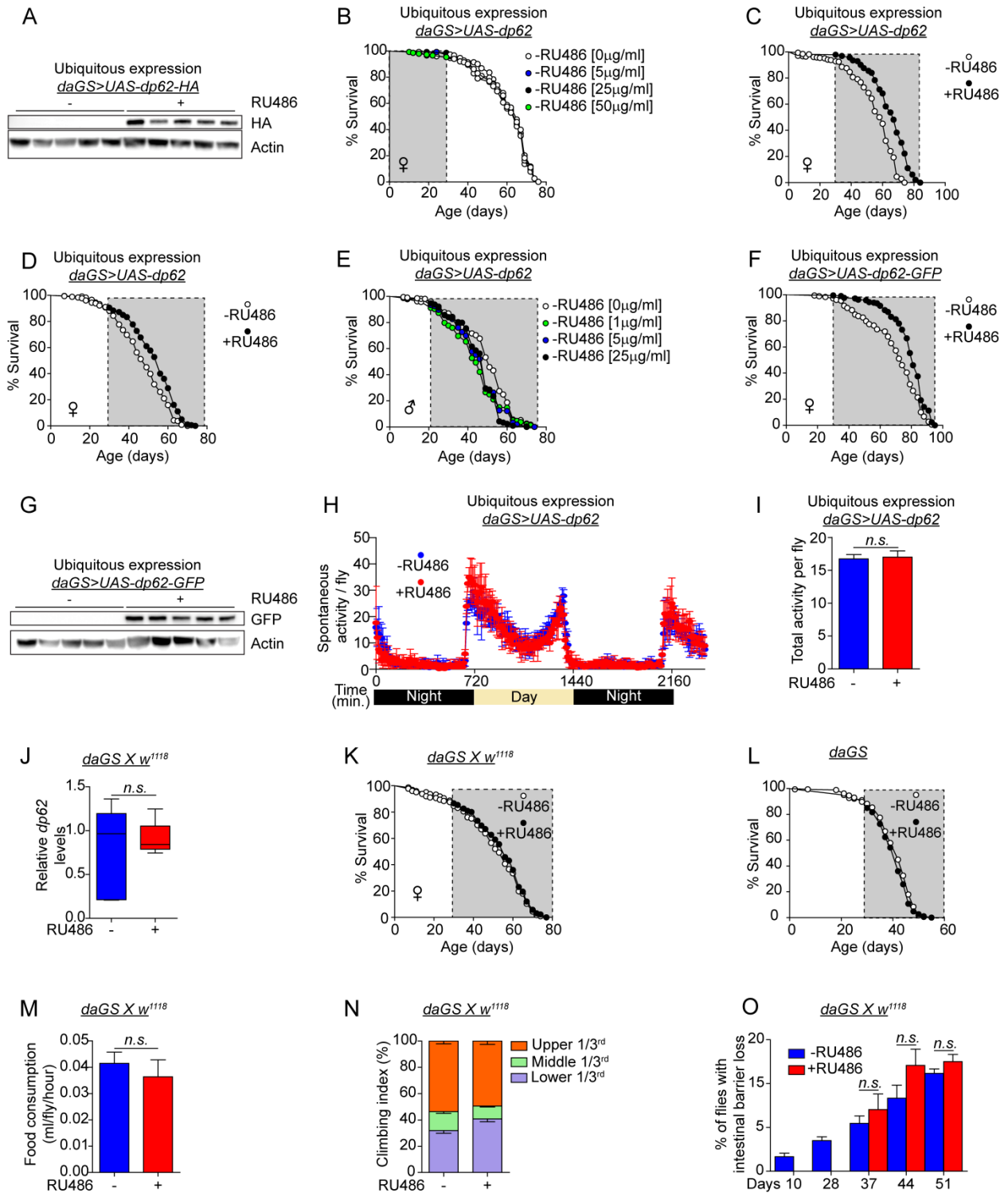


Figure S1, related to Figure 1. Midlife *dp62* induction extends lifespan and RU486 treatment in control flies has no effect on lifespan and healthspan.

- (A) Western blot detection of HA levels in day 37 *daGS>UAS-dp62HA* females with or without RU486-mediated transgene induction from day 30 to day 37.
- (B) Survival curves of *daGS>UAS-dp62* females with or without RU486-mediated transgene induction from day 1 until day 30. The shaded area indicates the duration of *dp62* induction. $p = 0.9980$, log-rank test; $n > 150$ flies.
- (C) Survival curves of *daGS>UAS-dp62* females with or without RU486-mediated transgene induction from day 30 onwards. The shaded area indicates the duration of *dp62* induction. $p < 0.0001$, log-rank test; $n > 300$ flies.
- (D) Survival curves of *daGS>UAS-dp62* females with or without RU486-mediated transgene induction from day 30 onwards. The shaded area indicates the duration of *dp62* induction. $p < 0.0001$, log-rank test; $n > 180$ flies.
- (E) Survival curves of *daGS>UAS-dp62* males with or without RU486-mediated transgene induction from day 30 onwards. The shaded area indicates the duration of *dp62* induction. $p = 0.0002$, log-rank test; $n > 200$ flies.
- (F) Survival curves of *daGS>UAS-dp62GFP* females with or without RU486-mediated transgene induction from day 30 onwards. The shaded area indicates the duration of *dp62* induction. $p < 0.0001$, log-rank test; $n > 175$ flies.

- (G) Western blot detection of GFP levels in day 37 *daGS>UAS-dp62GFP* females with or without RU486-mediated transgene induction from day 30 to day 37.
- (H) Spontaneous physical activity of 37 days old *daGS>UAS-dp62* females with or without RU486-mediated transgene induction from day 30 to day 37.
- (I) Quantification of total activity per fly per hour from spontaneous activity graph (S1H). n = 3 vials of 10 flies per condition; $p > 0.5$ and is non-significant (n.s.); Unpaired t-test.
- (J) qPCR analyses of *dp62* mRNA levels on day 37 in *daGS X w¹¹¹⁸* females with and without RU486 mediated transgene induction for 7 days (day 30 to day 37). n = 5 biological replicates with 3 flies per replicate; $p > 0.5$ and is non-significant (n.s.); Unpaired t-test.
- (K) Survival curves of *daGS X w¹¹¹⁸* females with or without RU486-mediated transgene induction from day 30 onwards. The shaded area indicates the duration of RU486 feeding. $p = 0.2732$, log-rank test; $n > 220$ flies.
- (L) Survival curves of *daGS* females with or without RU486-mediated transgene induction from day 30 onwards. The shaded area indicates the duration of RU486 feeding. $p = 0.1204$, log-rank test; $n > 178$ flies.

(M) Capillary FEeding assay (CAFE) of 37 day old *daGS X w¹¹¹⁸* females with or without RU486-mediated transgene induction from day 30 to day 37. n = 10 vials of 10 flies per condition; $p > 0.5$ and is non-significant (n.s.); Unpaired t-test.

(N) Climbing index as a measure of endurance of 37 day old *daGS X w¹¹¹⁸* females with or without RU486-mediated transgene induction from day 30 to day 37. n = 3 biological replicates with 100 flies per replicate; $p > 0.5$ and is non-significant (n.s.); Unpaired t-test.

(O) Intestinal integrity during aging of *daGS X w¹¹¹⁸* females with or without RU486-mediated transgene induction since midlife (day 30) onwards. n = 371 flies on day 10; $p > 0.5$ and is non-significant (n.s.); one-way ANOVA/Bonferroni's multiple comparisons test.

RU486 was provided in the media at a concentration of 25 $\mu\text{g/ml}$ for (A, C-D, F-O); 5 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ for (B) and 1 $\mu\text{g/ml}$, 5 $\mu\text{g/ml}$ and 25 $\mu\text{g/ml}$ for (E).

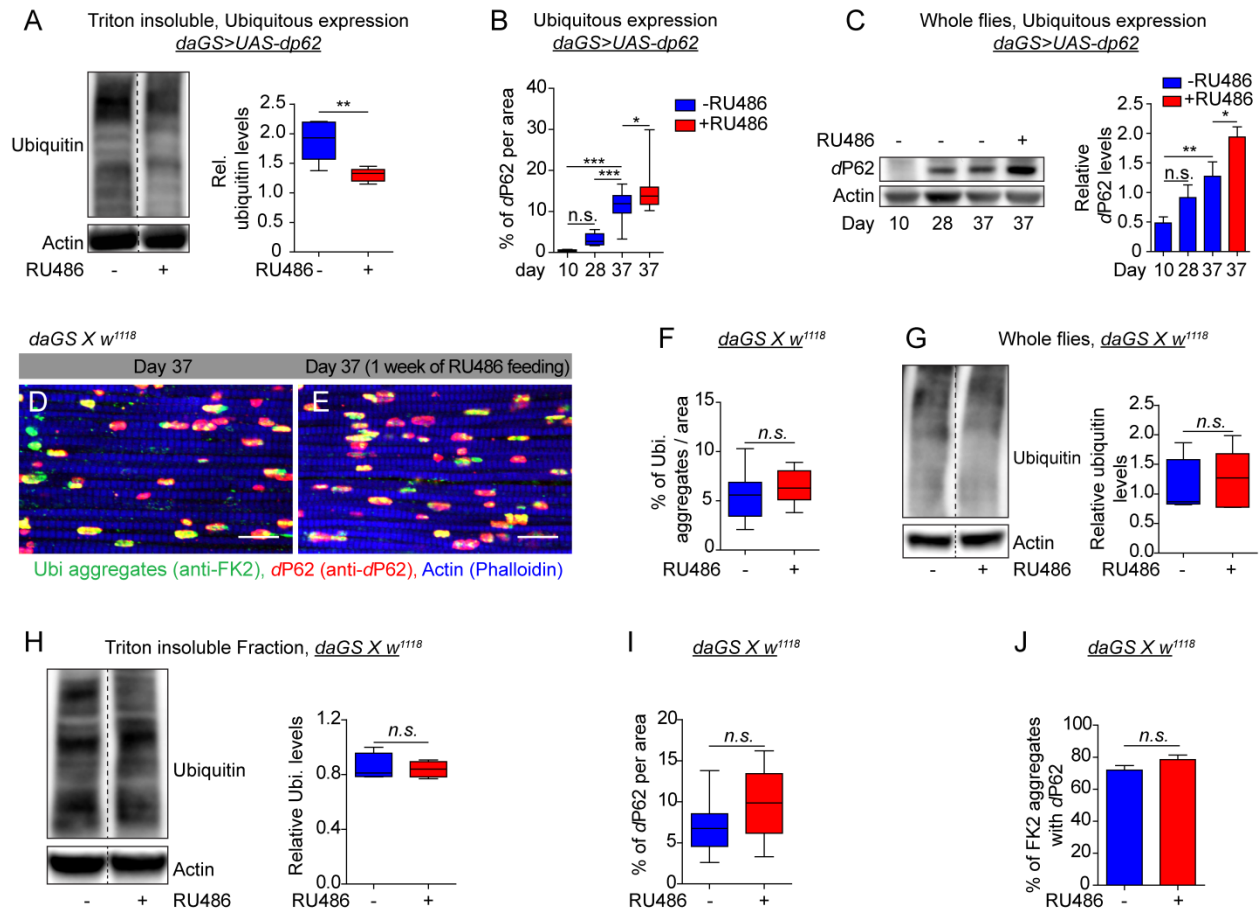


Figure S2, related to Figure 2. Midlife *dp62* induction improves proteostasis and RU486 treatment in control flies has no effect on proteostasis in aged flies.

(A) Western blot detection and densitometry of ubiquitin conjugated proteins from Triton Insoluble fraction extracted from day 37 *daGS>UAS-dp62* females with or without RU486-mediated transgene induction from day 30 to day 37. n = 5 replicates with 5 flies per replicate; **p < 0.01; Unpaired t test.

(B) Quantification of dP62 in muscle as shown in (Fig 2A'-2D'). n > 8 flies; ***p < 0.001 and *p < 0.1; one-way ANOVA/Bonferroni's multiple comparisons test.

(C) Western blot detection and densitometry of *dP62* in whole flies extracts from day 10, 28 and 37 *daGS>UAS-dp62* females with or without RU486-mediated transgene induction from day 30 to day 37. $n = 3$ replicates with 5 flies per replicate; $**p < 0.01$, $*p < 0.05$ and $p > 0.5$ is non-significant (n.s.); one-way ANOVA/Bonferroni's multiple comparisons test.

(D-E) Immunostaining of indirect flight muscles from day 37 *daGS X w¹¹¹⁸* females with or without RU486 from day 30 to day 37, showing ubiquitinated aggregates (green channel, anti-FK2); *dP62* (red channel, anti-*dP62*); and muscles (blue channel stained with phalloidin/F-Actin). Scale bar is 10 μm .

(F) Quantification of ubiquitin aggregates in muscle as shown in (S2D and S2E). $n > 8$ flies; $p > 0.5$ and is non-significant (n.s.); Unpaired t-test.

(G) Western blot detection and densitometry of total ubiquitin-conjugated proteins from day 37 *daGS X w¹¹¹⁸* females with or without RU486-mediated transgene induction from day 30 to day 37. $n = 5$ replicates with 5 flies per replicate; $p > 0.5$ and is non-significant (n.s.); Unpaired t-test.

(H) Western blot detection and densitometry of total ubiquitin-conjugated proteins from Triton Insoluble fraction extracted from day 37 *daGS X w¹¹¹⁸* females with or without RU486-mediated transgene induction from day 30 to day 37. $n = 5$ replicates with 5 flies per replicate; $p > 0.5$ and is non-significant (n.s.); Unpaired t-test.

(I) Quantification of *dP62* in muscle as shown in (S2D and S2E). $n > 8$ flies; $p > 0.5$ and is non-significant (n.s.); Unpaired t-test.

(J) Quantification of colocalization of ubiquitin aggregates with *dP62* as shown in (S2D and S2E). $n > 8$ flies; $p > 0.5$ and is non-significant (n.s.); Unpaired t-test.

RU486 was provided in the media at a concentration of 25 $\mu\text{g/ml}$.

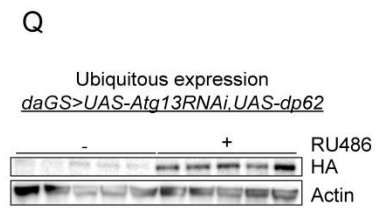
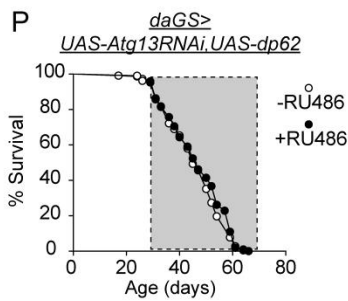
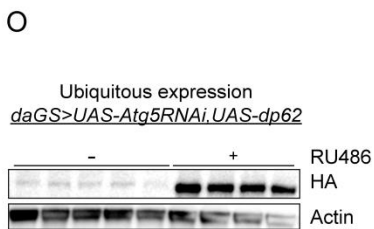
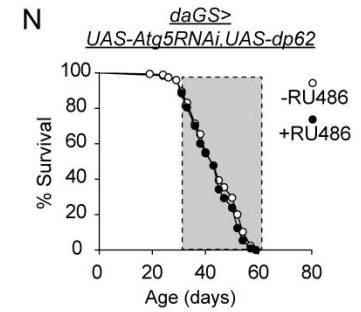
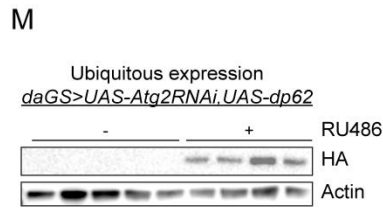
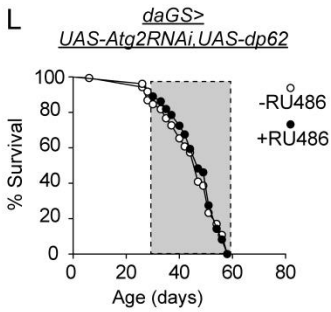
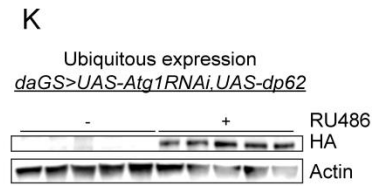
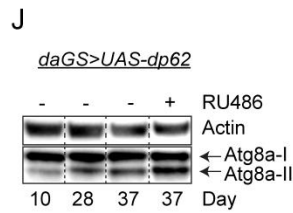
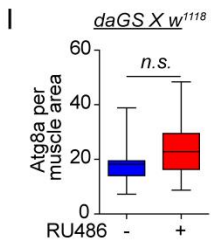
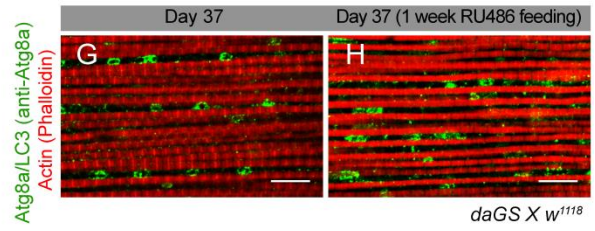
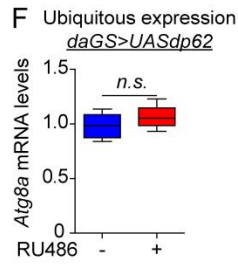
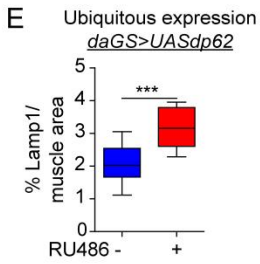
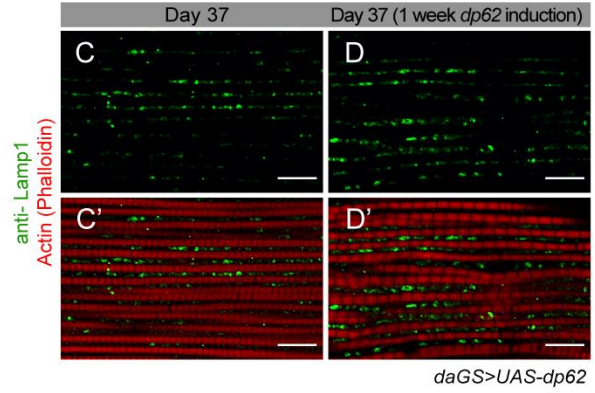
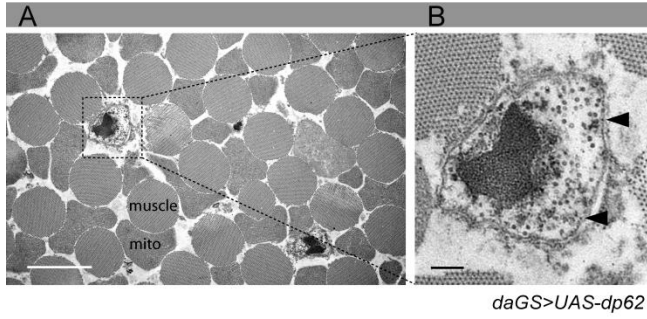


Figure S3, related to Figure 3. Midlife *dp62* induction requires autophagy to promote longevity and RU486 treatment in control flies does not alter *Atg8a* levels.

(A-B) Transmission electron microscopy coupled with immuno staining of indirect flight muscle from day 37 *daGS>UAS-dp62* females, showing *Atg8a* antibody can label autophagosomes/autolysosomes (AP/AL); double membrane structures enclosing cellular materials. mito (mitochondria). Scale bar 2 μm (A) and 400 nm (B).

(C-D and C'-D') Immunostaining of indirect flight muscles from day 37 *daGS>UAS-dp62* females with or without RU486-mediated transgene induction from day 30 to day 37, showing Lamp1 (green channel, anti-Lamp1); and muscles (red channel, stained with phalloidin/F-actin). Scale bar is 10 μm .

(E) Quantification of Lamp1 in muscle as shown in (S3C and S3D). $n > 13$ flies; *** $p < 0.0001$; Unpaired t-test.

(F) qPCR analyses of *Atg8a* mRNA levels on day 37 in *daGS>UAS-dp62* females with and without RU486 mediated transgene induction from day 30 to day 37. $n = 5$ biological replicates with 3 flies per replicate; $p > 0.5$ and is non-significant (n.s.); Unpaired t-test.

(G-H) Immunostaining of indirect flight muscles from day 37 *daGS X w¹¹¹⁸* females with or without RU486-mediated transgene induction from day 30 to day 37, showing *Atg8a*/LC3 foci (green channel, anti-*Atg8a*); and muscles (red channel, stained with phalloidin/F-actin). Scale bar is 10 μm .

- (I) Quantification of Atg8a/LC3 foci in muscle as shown in (S3G and S3H). $n > 10$ flies; $p > 0.5$ and is non-significant (n.s.); Unpaired t-test.
- (J) Western blot detection of Atg8a and Actin levels in thoraces extracts from day 10, 28 and 37 *daGS>UAS-dp62* females with or without RU486-mediated transgene induction from day 30 to day 37.
- (K) Western blot detection of HA levels in day 37 *daGS>UAS-Atg1RNAi, UAS-dp62HA* females with or without RU486-mediated transgene induction from day 30 to day 37.
- (L) Survival curves of *daGS>UAS-Atg2RNAi, UAS-dp62* females with or without RU486-mediated transgene induction from day 30 onwards. The shaded area indicates the duration of *Atg2RNAi* and *dp62* induction. $p = 0.6270$, log-rank test; $n > 176$ flies.
- (M) Western blot detection of HA levels in day 37 *daGS>UAS-Atg2RNAi, UAS-dp62HA* females with or without RU486-mediated transgene induction from day 30 to day 37.
- (N) Survival curves of *daGS>UAS-Atg5RNAi, UAS-dp62* females with or without RU486-mediated transgene induction from day 30 onwards. The shaded area indicates the duration of *Atg5RNAi* and *dp62* induction. $p = 0.1705$, log-rank test; $n > 203$ flies.

(O) Western blot detection of HA levels in day 37 *daGS>UAS-Atg5RNAi, UAS-dp62HA* females with or without RU486-mediated transgene induction from day 30 to day 37.

(P) Survival curves of *daGS>UAS-Atg13RNAi, UAS-dp62* females with or without RU486-mediated transgene induction from day 30 onwards. The shaded area indicates the duration of *Atg13RNAi* and *dp62* induction. $p = 0.2952$, log-rank test; $n > 210$ flies.

(Q) Western blot detection of HA levels in day 37 *daGS>UAS-Atg13RNAi, UAS-dp62HA* females with or without RU486-mediated transgene induction from day 30 to day 37.

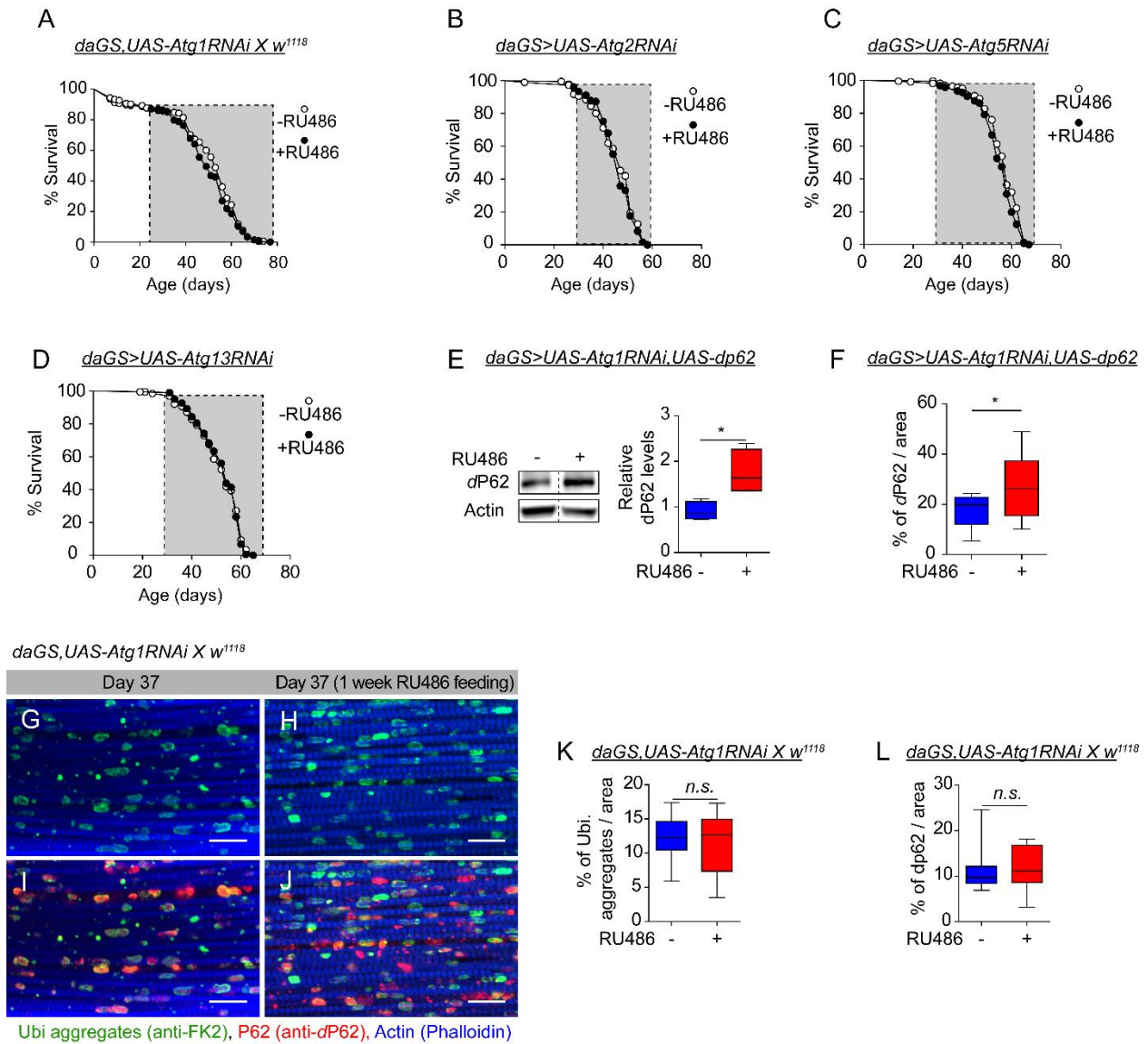


Figure S4, related to Figure 3. Midlife RNAi of Atg genes does not alter fly lifespan and RU486 treatment in Atg1 RNAi flies does not alter proteostasis or dP62 levels.

(A) Survival curves of *daGS, UAS-Atg1RNAi X w¹¹¹⁸* females with or without RU486-mediated transgene induction from day 30 onwards. The shaded area indicates the duration of *Atg1RNAi* induction. $p = 0.1092$, log-rank test; $n > 205$ flies.

(B) Survival curves of *daGS>UAS-Atg2RNAi* females with or without RU486-mediated transgene induction from day 30 onwards. The shaded area indicates the duration of *Atg2RNAi* induction. $p = 0.3511$, log-rank test; $n > 181$ flies.

(C) Survival curves of *daGS>UAS-Atg5RNAi* females with or without RU486-mediated transgene induction from day 30 onwards. The shaded area indicates the duration of *Atg5RNAi* induction. $p = 0.0079$, log-rank test; $n > 180$ flies.

(D) Survival curves of *daGS>UAS-Atg13RNAi* females with or without RU486-mediated transgene induction from day 30 onwards. The shaded area indicates the duration of *Atg5RNAi* induction. $p = 0.5924$, log-rank test; $n > 203$ flies.

(E) Western blot detection and densitometry of *dP62* in day 37 old *daGS>UAS-Atg1RNAi, UAS-dp62* females with or without RU486-mediated transgene induction from day 30 to day 37. $n = 5$ replicates with 5 flies per replicate; $*p < 0.05$; unpaired t test.

(F) Quantification of *dP62* in muscle as shown in (Fig 3M' and 3N'). $n = 14$ flies; $*p < 0.1$; Unpaired t-test.

(G-H and I-J) Immunostaining of indirect flight muscles from day 37 *daGS, UAS-Atg1RNAi X w¹¹¹⁸* females with or without RU486-mediated transgene induction from day 30 to day 37, showing ubiquitinated proteins (green channel, anti-FK2); *dP62* (red channel, anti-*dP62*) and muscles (blue channel, stained with phalloidin/F-actin). Scale bar is 10 μm .

(K) Quantification of ubiquitin aggregates in muscle as shown in S4G and S4H. $n = 10$ flies; $p > 0.5$ and is non-significant (n.s.); Unpaired t-test.

(L) Quantification of *dP62* in muscle as shown in S4I and S4J. $n = 10$ flies; $p > 0.5$ and is non-significant (n.s.); Unpaired t-test.

RU486 was provided in the media at a concentration of 25 $\mu\text{g/ml}$.

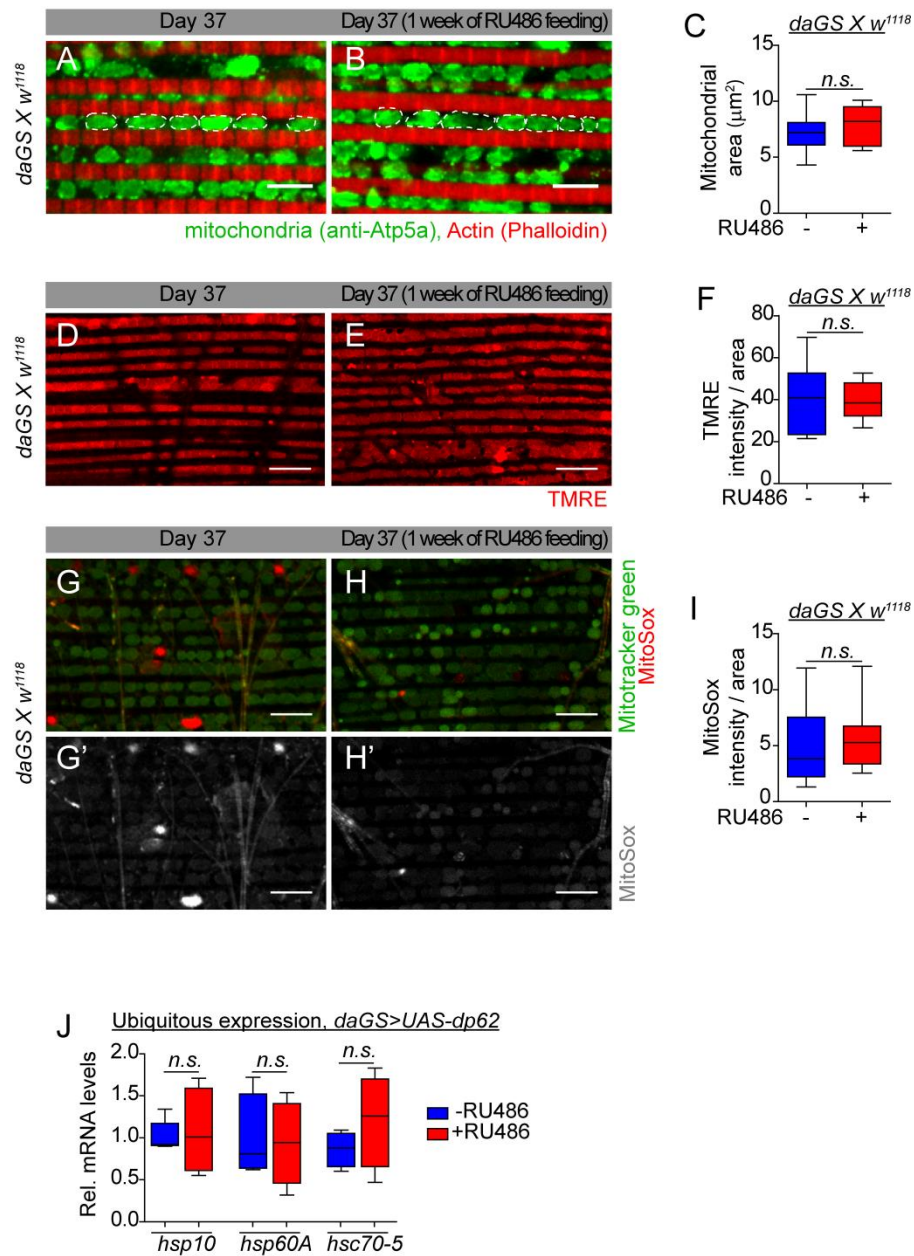


Figure S5, related to Figure 4. RU486 treatment in control flies has no effect on mitochondria function.

(A-B) Immunostaining of indirect flight muscles from day 37 *daGS X w¹¹¹⁸* females with or without RU486-mediated transgene induction from day 30 to day 37, showing mitochondrial morphology (green channel, anti-ATP5a); muscles (red channel, stained with phalloidin/F-actin). Scale bar is 5 μm .

(C) Quantification of mitochondrial area in muscle as shown in (S4A and S4B). $n = 9$ flies; $p > 0.5$ and is non-significant (n.s.); Unpaired t-test.

(D-E) Staining of indirect flight muscles from day *daGS X w¹¹¹⁸* females with or without RU486-mediated transgene induction from day 30 to day 37 showing TMRE fluorescence. Scale bar is 10 μm .

(F) Quantification of mitochondrial membrane potential measured by TMRE staining as shown in (S4D and S4E). $n = 13$ flies; $p > 0.5$ and is non-significant (n.s.); Unpaired t-test.

(G-H and G'-H') Staining of indirect flight muscles from 37 day old *daGS X w¹¹¹⁸* females with or without RU486-mediated transgene induction from day 30 to day 37, showing mitochondria (green channel, Mitotracker green staining) and levels of superoxide radicals (red channel, staining with MitoSOX reagent). Scale bar is 10 μm .

(I) Quantification of free superoxide radicals as shown in (S4G' and S4H'); $n > 14$ biological replicates; $p > 0.5$ and is non-significant (n.s.); Unpaired t-test.

(J) qPCR analyses of *Hsp10*, *Hsp60A* and *Hsc70-5* mRNA levels on day 37 in *daGS > UAS-dp62* females with and without RU486 mediated transgene induction for 7 days (day 30 to day 37). $n = 5$ biological replicates with 3 flies per replicate; $p > 0.5$ and is non-significant (n.s.); one-way ANOVA/Bonferroni's multiple comparisons test.

RU486 was provided in the media at a concentration of 25 $\mu\text{g/ml}$.

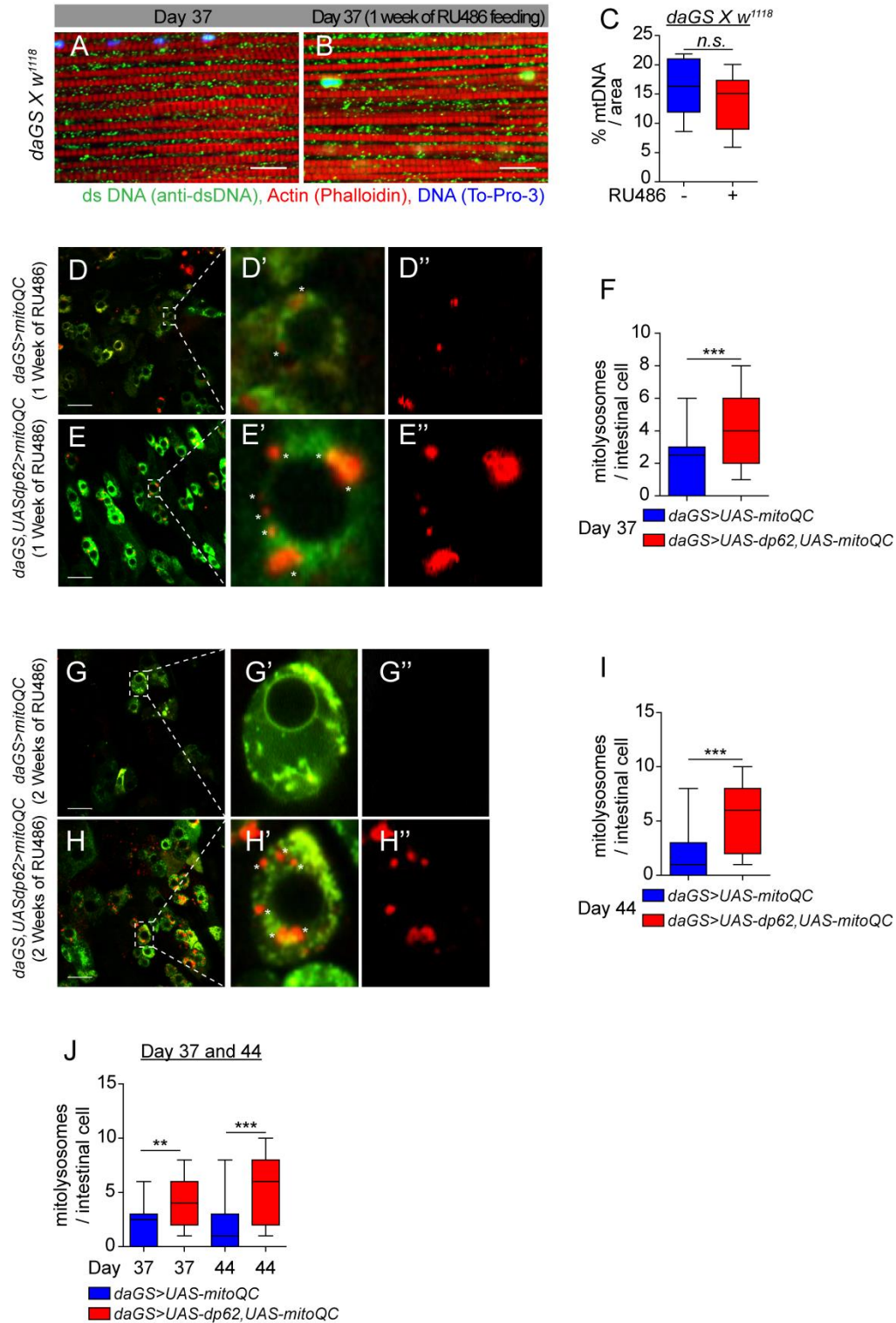


Figure S6, related to Figure 5. Midlife p62 induction stimulates mitophagy in intestinal cells and RU486 treatment in control flies has no effect on mtDNA levels.

(A-B) Immunostaining of indirect flight muscles from day 37 *daGS X w¹¹¹⁸* females with or without RU486-mediated transgene induction from day 30 to day 37, showing dsDNA (green channel, anti dsDNA); muscles (red channel, stained with phalloidin/F-actin); and DNA (blue channel, To-Pro 3 stain). Scale bar is 5 μm .

(C) Quantification of mitochondrial DNA in muscle as shown in (S5A and S5B). $n = 10$ flies; $p > 0.5$ and is non-significant (n.s.); Unpaired t-test.

(D-E and G-H) *mito-QC* of gut cells from 37 (D-E) and 44 (G-H) day old females. Genotypes analyzed were *daGS>UAS-dp62*, *UAS-mito-QC* and *daGS>UAS-mito-QC* females with RU486-mediated transgene induction from day 30 to day 37 and from day 30 to day 44, showing merged image of GFP and mCherry (D-E; D'-E'; G-H and G'-H') and punctate mCherry-only foci (resulting from merged image where GFP has been quenched; mitolysosomes) (D''-E'' and G''-H''). * indicates mCherry-only foci in D'-E' and G'-H'. Scale bar is 20 μm .

(F, I and J) Quantification of mitolysosomes in gut cells as shown in (D''-E'' and G''-H''), day 37 (F), day 44 (I), and day 37 and 44 (J). $n > 10$ cells; *** $p < 0.001$; unpaired t test (F). *** $p < 0.001$; unpaired t test (I). *** $p < 0.001$ and ** $p < 0.01$; one-way ANOVA/Bonferroni's multiple comparisons test (J).

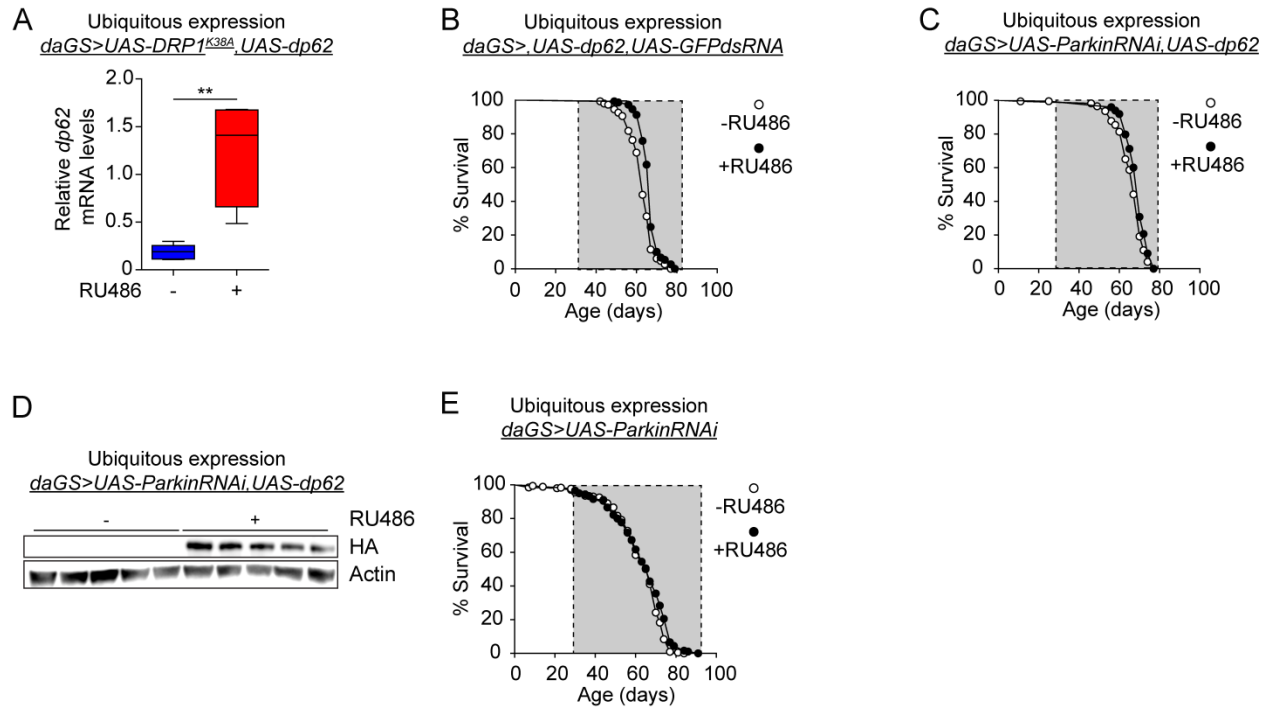


Figure S7, related to Figure 6. qPCR analyses of *dp62* mRNA levels and RU486 treatment-control experiments for lifespan analyses.

(A) qPCR analyses of *dp62* mRNA levels on day 37 in *daGS>UAS-dp62, UAS-DRP1^{K38A}* females with and without RU486 mediated transgene induction for 7 days (day 30 to day 37). $n=5$ biological replicates with 3 flies per replicate; $**p < 0.01$; Unpaired t-test.

(B) Survival curves of *daGS>UAS-dp62, UAS-GFPdsRNA* females with or without RU486-mediated transgene induction from day 30 onwards. The shaded area indicates the duration of *dp62* and *GFP-dsRNA* induction. $p < 0.0001$, log-rank test; $n > 145$ flies.

(C) Survival curves of *daGS>UAS-dp62, UAS-ParkinRNAi* females with or without RU486-mediated transgene induction from day 30 onwards. The shaded area indicates the duration of *dp62* and *ParkinRNAi* induction. $p = 0.0003$, log-rank test; $n > 170$ flies.

(D) Western blot detection of HA levels in day 37 *daGS>UAS-ParkinRNAi, UAS-dp62HA* females with or without RU486-mediated transgene induction for 7 days (day 30 to day 37).

(E) Survival curves of *daGS>UAS-ParkinRNAi* females with or without RU486-mediated transgene induction from day 30 onwards. The shaded area indicates the duration of *ParkinRNAi* induction. $p = 0.0147$, log-rank test; $n > 180$ flies.

RU486 was provided in the media at a concentration of 25 $\mu\text{g/ml}$.