Supplemental Figures:

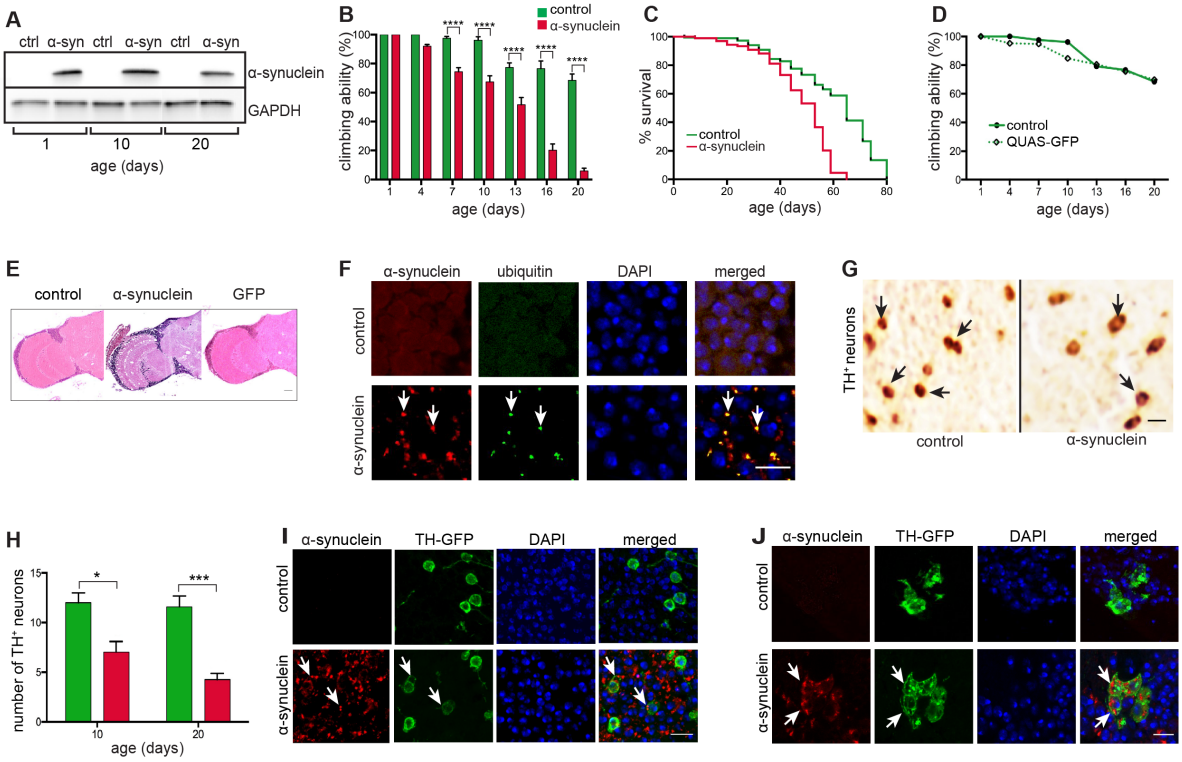

Figure S1. Neurodegeneration and inclusion formation in α-synuclein transgenic flies, Related to Figure 1

(A) Western blot showing α-synuclein levels in 1-, 10- and 20-day-old flies. The blot was reprobed for GAPDH to illustrate equivalent protein loading. Control is *Syb-QF2/+.*

(B) Behavioral analysis of motor deficits using the climbing test indicating changes in motor ability when α-synuclein is expressed using the Q system. n=60 per genotype. Control is *Syb-QF2/+.*

(C) Lifespan analysis showing early death in α-synuclein transgenic flies (red) compared to control flies (green). n=300 per genotype. Kaplan-Meyer curve. $p<0.01$, log rank test. Control is *Syb-QF2/+.*

(D) Behavioral analysis of motor deficits using the climbing test indicating no changes in motor ability when GFP is expressed using the Q system. n=60 per genotype. Control is *Syb-QF2/+.*

(E) Hematoxylin and eosin staining showing no vacuolization in the medulla of 10-day-old GFP flies compared to α-synuclein transgenic flies. Scale bar, 20 μm. Control is *Syb-QF2/+.*

(F) Immunofluorescence staining showing that α-synuclein aggregates are ubiquitin positive in 10-day-old α-synuclein transgenic flies (arrows). Scale bar, 5 μm. Control is *Syb-QF2/+.*

(G) Tyrosine hydroxylase (TH) immunostaining showing loss of TH-immunopositive dopaminergic neurons in the anterior medulla (arrows) in 10-day-old α-synuclein transgenic flies compared to control flies. Scale bar, 5 μm. Control is *Syb-QF2/+.*

(H) Quantification of TH-positive neurons in 10- and 20-day-old α-synuclein transgenic flies compared to control flies. n=6 per genotype. Control is *Syb-QF2/+*.

(I and J) Immunofluorescent staining showing α-synuclein aggregates in TH-positive neurons of the anterior medulla (I) and the central brain (J) in 10-day-old α-synuclein transgenic flies (arrows). Scale bar, 5 μm. Control is *UAS-mCD8-GFP/+; TH-GAL4, Syb-QF2.*

Asterisks indicate *p<0.01, ***p<0.0002 and ****p<0.0001, two-way ANOVA in (B), (D) and (H) with Student's Newman-Keuls test. Data is represented as mean ± SEM.

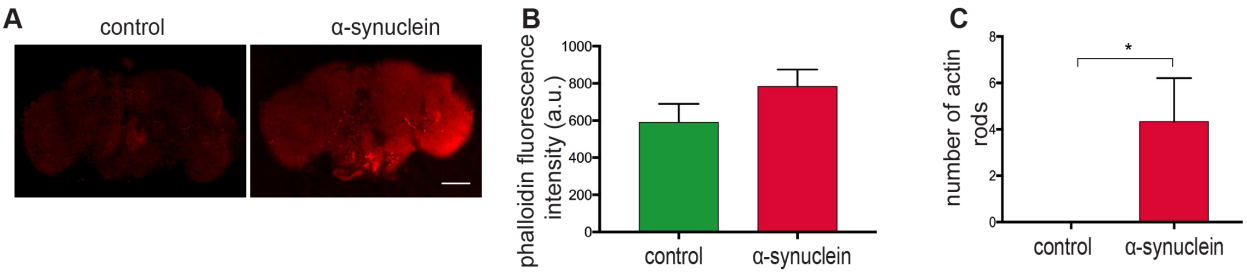

Figure S2. Actin-rich rods are present at 1 day of age in α-synuclein transgenic flies, Related to Figure 2

(A and B) F-actin staining of whole mount brains of 1-day-old α-synuclein transgenic flies compared to control flies. Scale bar, 50 μm. Quantification (B) of fluorescence intensity shows a trend toward increased F-actin in α-synuclein transgenic flies. n=6 per genotype.

(C) Quantification of actin-rich inclusions in α-synuclein transgenic flies reveals the presence of actin-rich rods in 1-day-old α-synuclein transgenic flies. n=6 per genotype.

Asterisks indicate *$p<0.01$, unpaired t-test. Data is represented as mean ± SEM. Flies are 1 day old. Control in (A)-(C) is *Syb-QF2/+*.

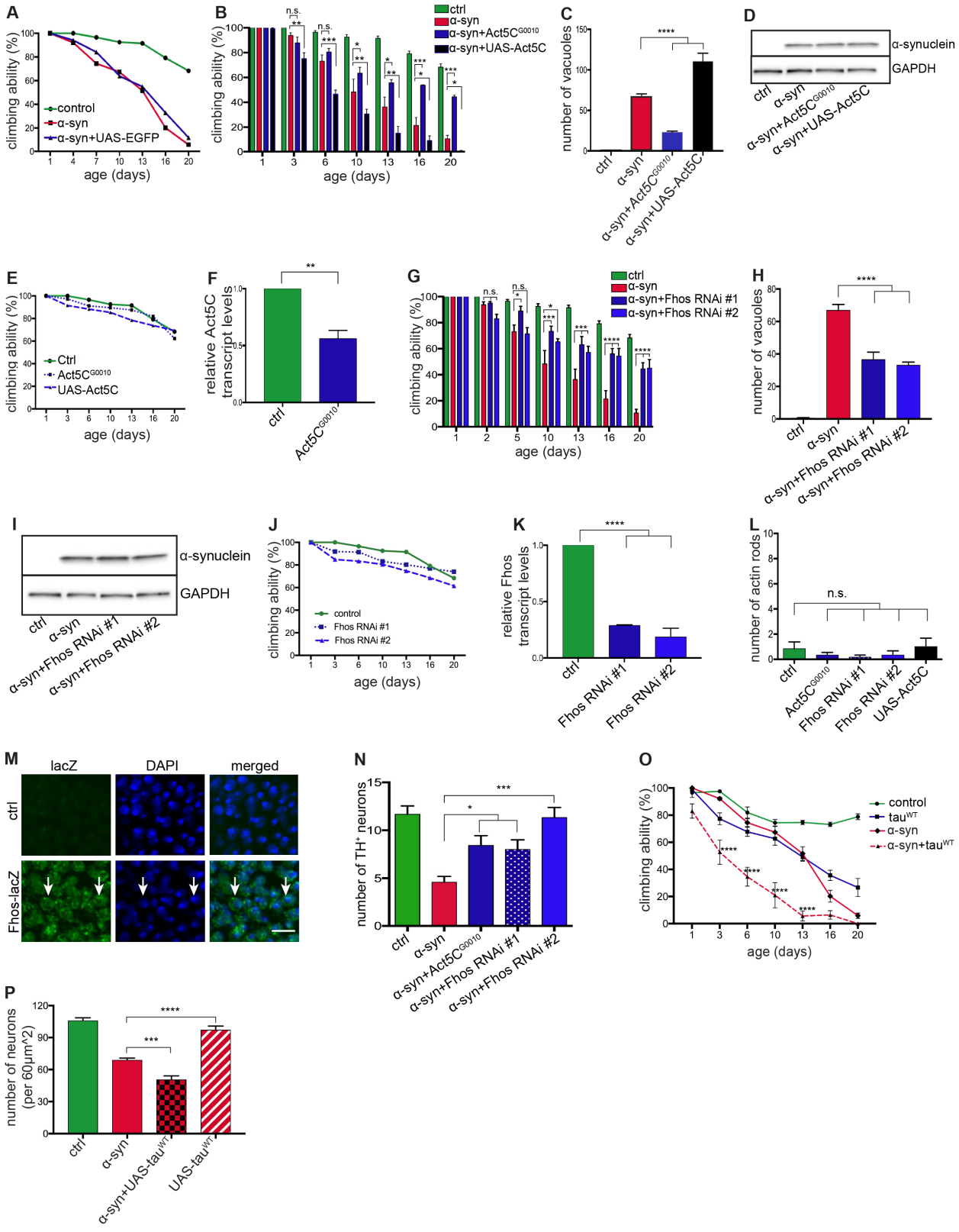
A
climbing ability (%)
age (days)
control
α-syn
α-syn+UAS-EGFP
B
climbing ability (%)
age (days)
ctrl
α-syn
α-syn+Act5C^G0010
α-syn+UAS-Act5C
n.s.
C
number of vacuoles
****
ctrl
α-syn
α-syn+Act5C^G0010
α-syn+UAS-Act5C
D
α-synuclein
GAPDH
ctrl
α-syn
α-syn+Act5C^G0010
α-syn+UAS-Act5C
E
climbing ability (%)
age (days)
Ctrl
Act5C^G0010
UAS-Act5C
F
relative Act5C transcript levels
**
ctrl
Act5C^G0010
G
climbing ability (%)
age (days)
ctrl
α-syn
α-syn+Fhos RNAi #1
α-syn+Fhos RNAi #2
n.s.
H
number of vacuoles
****
ctrl
α-syn
α-syn+Fhos RNAi #1
α-syn+Fhos RNAi #2
I
α-synuclein
GAPDH
ctrl
α-syn
α-syn+Fhos RNAi #1
α-syn+Fhos RNAi #2
J
climbing ability (%)
age (days)
control
Fhos RNAi #1
Fhos RNAi #2
K
relative Fhos transcript levels
****
ctrl
Fhos RNAi #1
Fhos RNAi #2
L
number of actin rods
n.s.
ctrl
Act5C^G0010
Fhos RNAi #1
Fhos RNAi #2
UAS-Act5C
M
lacZ
DAPI
merged
ctrl
Fhos-lacZ
N
number of TH+ neurons
***
*
ctrl
α-syn
α-syn+Act5C^G0010
α-syn+Fhos RNAi #1
α-syn+Fhos RNAi #2
O
climbing ability (%)
age (days)
control
tau^WT
α-syn
α-syn+tau^WT
****
P
number of neurons (per 60μm^2)
****
***
ctrl
α-syn
α-syn+UAS-tau^WT
UAS-tau^WT

Figure S3. Actin modifiers do not induce toxicity or influence α-synuclein expression, Related to Figure 3.

(A) Behavioral analysis of motor deficits using the climbing test showing no enhancement of motor deficits when the *elav-GAL4* driver is used to express EGFP as a control in α-synuclein transgenic flies. n=60 per genotype.

(B and C) Genetic modification of locomotor ability (B) and vacuole formation (C) in α-synuclein transgenic flies showing rescue by reducing actin in animals heterozygous for $Act5C^{G0010}$ and enhancement with overexpression of actin (*UAS-Act5C*). n=60 per genotype. Flies are 10 days old in (C).

(D and I) Western blot showing normal α-synuclein protein levels following genetic actin manipulation in α-synuclein flies. The blots are reprobed for GAPDH to illustrate equivalent protein loading. Flies are 1 day old.

(E and J) Behavioral analyses using the climbing assay showing no significant locomotor deficits with genetic actin cytoskeleton manipulation in the absence of transgenic α-synuclein expression. n=60 per genotype.

(F and K) Real time quantitative PCR for *Act5C* (F) and *Fhos* (K) transcript levels shows a reduction in *Act5C* and *Fhos* expression in flies heterozygous for $Act5C^{G0010}$ or expressing an RNAi directed to *Fhos*. n=6 per genotype. Flies are 1 day old.

(G and H) Genetic rescue of locomotor dysfunction (G) and vacuole formation (H) in α-synuclein transgenic flies expressing two transgenic RNAi lines directed to *Fhos*. n=60 per genotype. Flies are 10 days old in (H).

(L) Quantification of the number of actin-rich rods in brain sections shows no significant changes with genetic manipulation of the actin cytoskeleton in the absence of transgenic α-synuclein expression. n=6 per genotype. Flies are 10 days old.

(M) Immunofluorescence showing expression of *Fhos* as monitored by the enhancer trap $Fhos^{AA142}$ (arrows). The reporter is nuclear because a nuclear import signal is present on ß-galactosidase. Morphology of positive cells is consistent with neurons. Scale bar, 5 μm. Flies are 1 day old.

(N) Quantitative analysis of the number of TH-immunopositive neurons in the anterior medulla showing rescue of positively stained neurons following genetic manipulation of the actin cytoskeleton. n=6 per genotype. Flies are 20 days old.

(O) Analysis of climbing reflecting enhanced locomotor deficits in α-synuclein transgenic flies expressing wild type human tau. n=60 per genotype.

(P) Quantification of cortical neurons in the anterior medulla showing decreased neuronal density in 10-day-old α-synuclein transgenic flies expressing wild type human tau. n=6 per genotype.

Asterisks indicate *p<0.01, **p<0.001, ***p<0.0002, and ****p<0.0001, two-way ANOVA in (A), (B), (E), (G), (J) and (O), one-way ANOVA in (C), (H), (K), (L), (N) and (P) with Student-Newman-Keuls test, and unpaired t-test in (F). Data is represented as mean ± SEM. Control (ctrl) in (A)-(D), (H), (I), (N) and (P) is *elav-GAL4/+; Syb-QF2/+* and in (E), (F), (J)-(M) and (O) is *elav-GAL4/+*

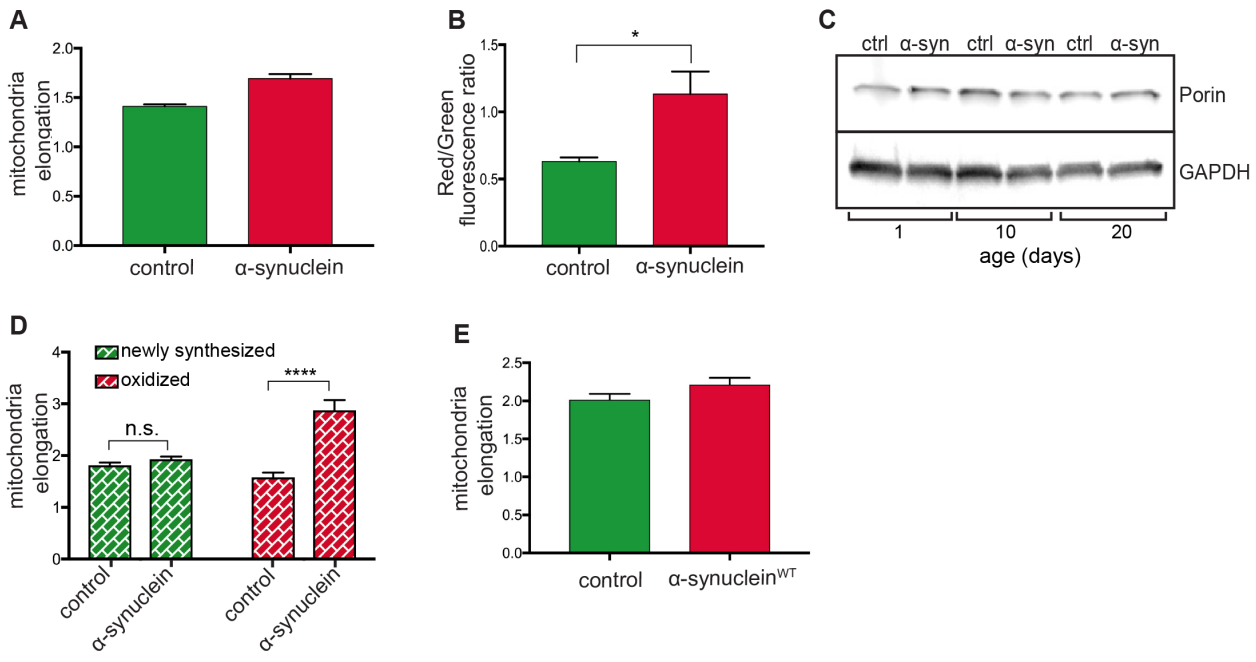

Figure S4. Mitochondrial abnormalities in α-synuclein transgenic flies, Related to Figure 4.

(A) Measurements of mitochondrial elongation showing a trend toward mitochondrial enlargement in 1-day-old α-synuclein transgenic flies. n=6 per genotype. Control is *Syb-QF2/+.*

(B) Mitochondrial protein oxidation as monitored by the transition from green to red fluorescence in MitoTimer protein is consistent with early mitochondrial dysfunction in 1-day-old α-synuclein transgenic flies. n=6 per genotype. Control is *elav-GAL4/+; UAS-MitoTimer/Syb-QF2*.

(C) Western blot probed for with an antibody to porin showing no changes in 1-, 10- and 20-day-old α-synuclein flies compared to controls. The blot is reprobed with an antibody recognizing GAPDH to illustrate equivalent protein loading. Control is *Syb-QF2/+.*

(D) Measurement of elongation in newly synthesized and oxidized mitochondria reveal mitochondrial enlargement in oxidized mitochondria in α-synuclein transgenic flies. n=6 per genotype. Control is *elav-GAL4/+; UAS-MitoTimer/Syb-QF2*.

(E) Measurements of mitochondrial elongation show no changes in mitochondrial morphology in 12-month-old human wild type α-synuclein transgenic mice compared to controls. n=4 per genotype.

Asterisks indicate *$p<0.01$, unpaired t-test. Data is represented as mean ± SEM. Flies are 1 day old in (A and B) and 10 days old in (D).

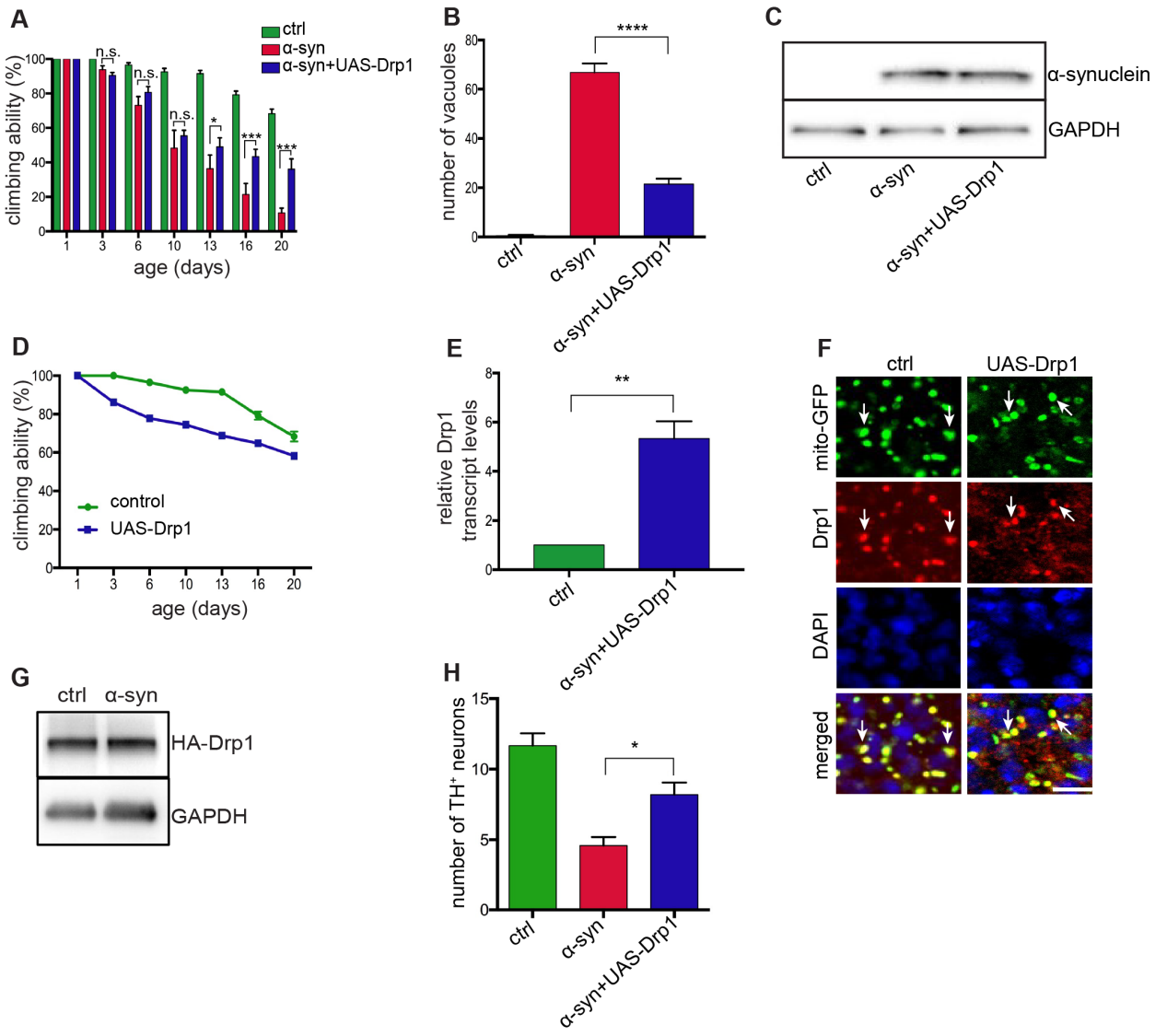

Figure S5. Overexpression of Drp1 does not influence mitochondrial dynamics in the absence of transgenic α-synuclein expression, Related to Figure 5.

(A) Behavioral analysis of motor deficits using the climbing assay showing rescue in locomotor activity following overexpression of a previously characterized (DuBoff et al., 2012), moderately expressing Drp1 line. n=60 per genotype, two-way ANOVA with Student-Newman-Keuls test, *p<0.01 and ***p<0.0002. Control is *elav-GAL4/+; Syb-QF2/+.*

(B) Quantification of vacuoles showing reduced number of vacuoles in the medulla of 10-day-old α-synuclein transgenic flies following overexpression of Drp1 (*UAS-Drp1*). n=6 per genotype, one-way ANOVA with Student-Newman-Keuls test, ****p<0.0001. Flies are 10 days old. Control is *elav-GAL4/+; Syb-QF2/+.*

(C) Western blot showing no change in α-synuclein protein levels when Drp1 is overexpressed. The blot is reprobed for GAPDH to illustrate equivalent protein loading. Flies are 1 day old. Control is *elav-GAL4/+; Syb-QF2/+.*

(D) Behavioral analysis of motor deficits using the climbing assay showing no changes in locomotor activity following Drp1 overexpression. n=60 per genotype, two-way ANOVA with Student-Newman-Keuls test. Control is *elav-GAL4/+; Syb-QF2/+.*

(E) Real time quantitative PCR for *Drp1* transcript levels shows an increase in *Drp1* expression in flies overexpressing the *UAS-Drp1* construct. n=6 per genotype, unpaired t-test, **p<0.001. Flies are 1 day old. Control is *elav-GAL4/+*

(F) Drp1 localization to the mitochondria, as monitored by HA-tagged Drp1 (*HA-Drp1*), is not affected following Drp1 overexpression (arrows). Scale bar, 5 μm. Flies are 10 days old. Control is *elav-GAL4/+; Mito-GFP/+; HA-Drp1/Syb-QF2.*

(G) Western blot showing no changes in Drp1 expression, as monitored by immunoblotting with an antibody directed to HA to detect HA-tagged Drp1 when transgenic α-synuclein is expressed. Flies are 1 day old. Control is *elav-GAL4/+; HA-Drp1/Syb-QF2.*

(H) Quantitative analysis of the number of TH-immunopositive neurons in the anterior medulla showing rescue of positively stained neurons following overexpression of Drp1.

n=6 per genotype, one-way ANOVA with Student-Newman-Keuls test, *p<0.01. Flies are 20 days old. Control is *elav-GAL4/+; Syb-QF2/+.*

Data is represented as mean ± SEM.

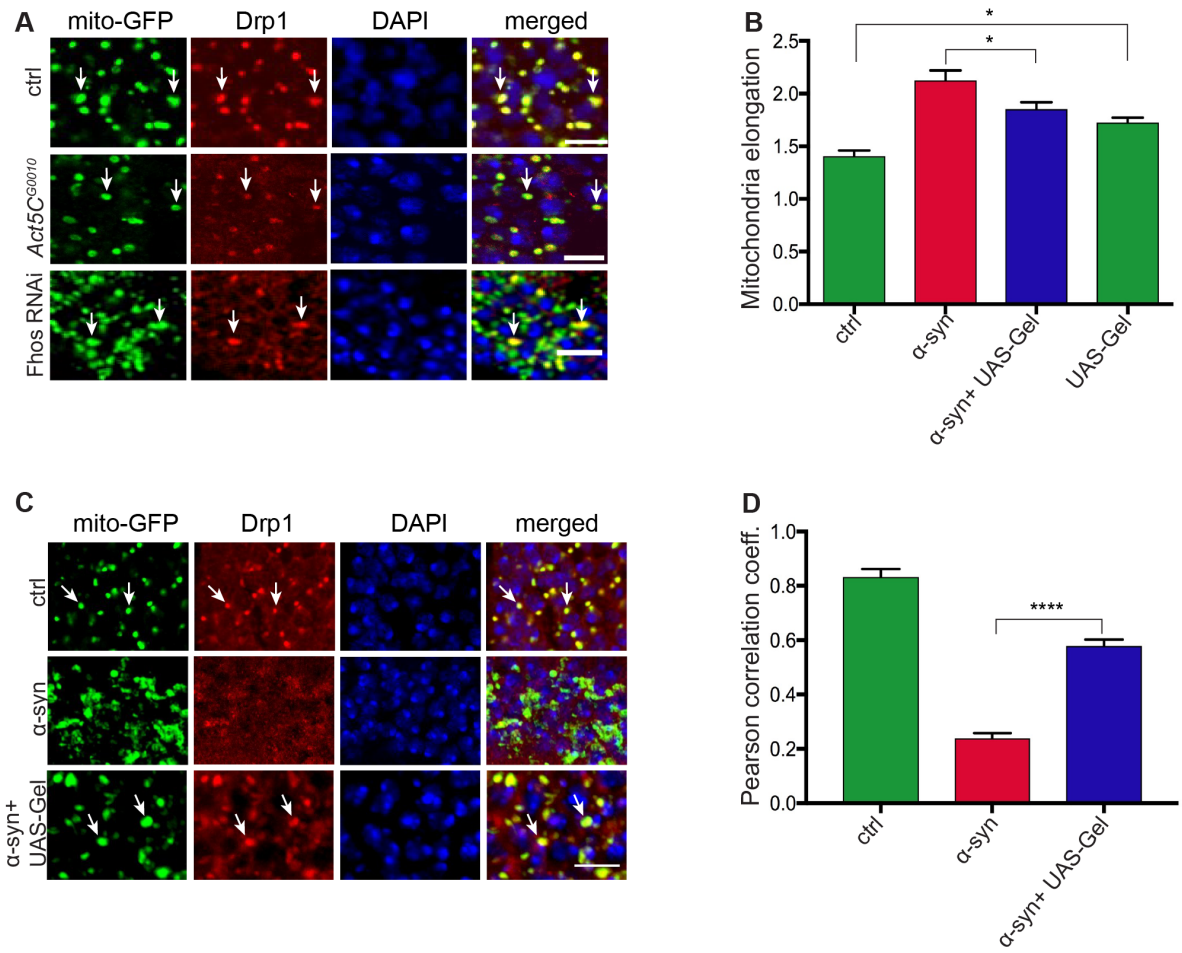

Figure S6. Drp1 translocation to the mitochondria outer membrane is dependent on actin dynamics, Related to Figure 6.

(A) Drp1 localization to the mitochondria, as monitored by HA-tagged Drp1 (*HA-Drp1*), shows no mislocalization in animals with one copy of *Act5C*^{G0010} or following expression of an RNAi line directed to *Fhos* (arrows) in the absence of α-synuclein overexpression. Scale bar, 5 μm. Control is *elav-GAL4/+; Mito-GFP/+; HA-Drp1/Syb-QF2.*

(B) Quantification of mitochondrial morphology showing reduced enlargement in α-synuclein flies overexpressing Gelsolin. n=6 per genotype. Control is *elav-GAL4/+; Syb-QF2/+.*

(C and D) Drp1 localization to the mitochondria, as monitored by HA-tagged Drp1 (*HA-Drp1*) and quantification (D) shows rescued Drp1 colocalization following overexpression of Gelsolin (arrows). Scale bar, 5 μm. n=6 per genotype. Control is *elav-GAL4/+; HA-Drp1/Syb-QF2.*

Asterisks indicates *p<0.01, **p<0.002, one-way ANOVA with Student-Newman-Keuls test. Data is represented as mean ± SEM. Flies are 10 days old.

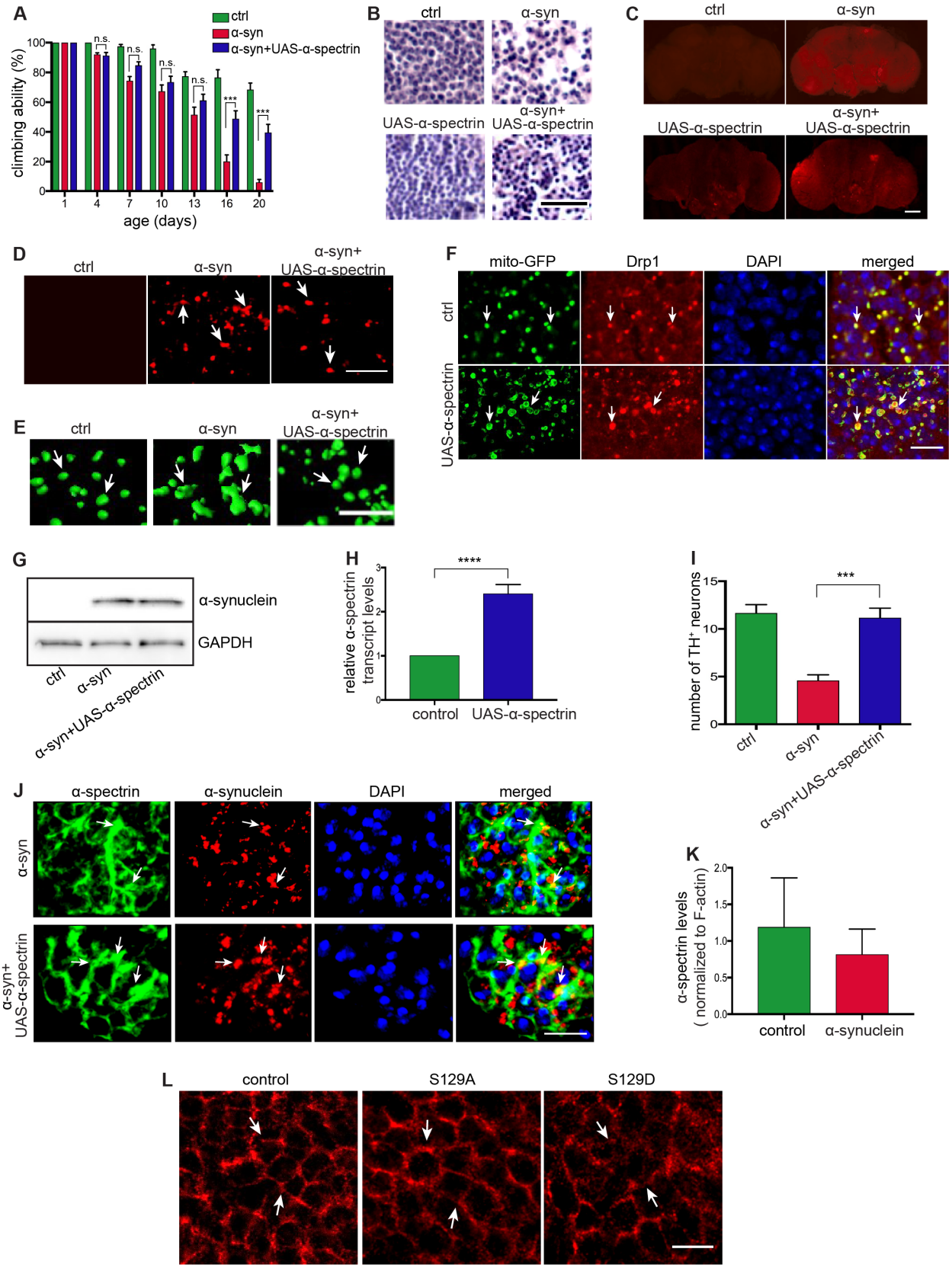
A
ctrl
α-syn
α-syn+UAS-α-spectrin
climbing ability (%)
age (days)
n.s.
***
B
ctrl
α-syn
UAS-α-spectrin
α-syn+ UAS-α-spectrin
C
ctrl
α-syn
UAS-α-spectrin
α-syn+ UAS-α-spectrin
D
ctrl
α-syn
α-syn+ UAS-α-spectrin
E
ctrl
α-syn
α-syn+ UAS-α-spectrin
F
mito-GFP
Drp1
DAPI
merged
ctrl
UAS-α-spectrin
G
α-synuclein
GAPDH
ctrl
α-syn
α-syn+UAS-α-spectrin
H
****
relative α-spectrin transcript levels
control
UAS-α-spectrin
I
***
number of TH+ neurons
ctrl
α-syn
α-syn+UAS-α-spectrin
J
α-spectrin
α-synuclein
DAPI
merged
α-syn
α-syn+ UAS-α-spectrin
K
α-spectrin levels (normalized to F-actin)
control
α-synuclein
L
control
S129A
S129D

Figure S7. Overexpression of α-spectrin does not induce neurotoxicity in the absence of transgenic α-synuclein expression, Related to Figure 7.

(A) Rescue of locomotor deficits by overexpression of α-spectrin (*UAS-α-spectrin*) in α-synuclein transgenic flies. n=60 per genotype, two-way ANOVA with Student-Newman-Keuls test, ***p<0.0002. Control (ctrl) is *elav-GAL4/+; Syb-QF2/+*.

(B) Hematoxylin staining showing rescued neuronal density in 20-day-old α-synuclein flies following overexpression of α-spectrin. Scale bar, 20 μm. Control (ctrl) is *elav-GAL4/+; Syb-QF2/+*.

(C) Phalloidin stained whole brain mounts of α-synuclein transgenic flies overexpressing α-spectrin. Scale bar, 50 μm. Flies are 10 days old. Control is *elav-GAL4/+; Syb-QF2/+*.

(D) Presence of actin rods in the brains of α-synuclein transgenic flies overexpressing α-spectrin as determined by immunostained sections for actin. Scale bar, 3 μm. Flies are 10 days old. Control (ctrl) is *elav-GAL4/+; Syb-QF2/+*.

(E) 3D reconstruction of immunofluorescence-stained mitochondria showing changes in mitochondrial morphology in central brain neurons of α-synuclein transgenic flies overexpressing α-spectrin. Scale bar, 5 μm. Flies are 10 days old. Control is *elav-GAL4/+; Syb-QF2.*

(F) Drp1 localization to the mitochondria is not affected following α-spectrin overexpression (*UAS-α-spectrin*) in the absence of α-synuclein expression (arrows). Scale bar, 5 μm. Flies are 10 days old. Control is *elav-GAL4/+; UAS-Drp1, Mito-GFP/+; HA-Drp1/Syb-QF2.*

(G) Western blot showing no changes in α-synuclein protein levels when α-spectrin is overexpressed in α-synuclein transgenic flies. The blot is reprobed for GAPDH to

illustrate equivalent protein loading. Flies are 1 day old. Control is *elav-GAL4/+; Syb-QF2/+.*

(H) Real time quantitative PCR for *α-spectrin* transcript levels shows an increase in *α-spectrin* expression in flies overexpressing α-spectrin. n=6 per genotype, unpaired t-test, ****p<0.0001. Flies are 1 day old. Control is *elav-GAL4/+.*

(I) Quantitative analysis of the number of TH-immunopositive neurons in the anterior medulla showing rescue of positively stained neurons following overexpression of α-spectrin. n=6 per genotype, one-way ANOVA with Student-Newman-Keuls test, ***p<0.0002. Flies are 20 days old. Control is *elav-GAL4/+; Syb-QF2/+.*

(J) 3D projection of immunofluorescence-stained *Drosophila* Kenyon cells showing colocalization of α-spectrin and α-synuclein signals in α-synuclein transgenics and in α-synuclein flies overexpressing α-spectrin. Scale bar, 5 μm. Flies are 10 days old.

(K) Quantitative western blot analysis of α-spectrin levels from biotinylated phalloidin precipitated from α-synuclein transgenic fly heads and controls shows a trend toward lower concentrations of α-spectrin in α-synuclein transgenics. α-spectrin levels were normalized to F-actin. n=10 per genotype. Flies are 10 days old. Control is *elav-GAL4/+; Syb-QF2/+.*

(L) Immunofluorescence staining showing the normal subplasmalemmal staining pattern in controls (*elav-GAL4/+*) and in flies expressing the nontoxic phosphorylation incompetent (S129A) α-synuclein (arrows), and disruption of the spectrin cytoskeleton in flies expressing the toxic phosphomimic (S129D) version of α-synuclein (arrows). Scale bar, 5 μm. Flies are 10 days old.

Data is represented as mean ± SEM.

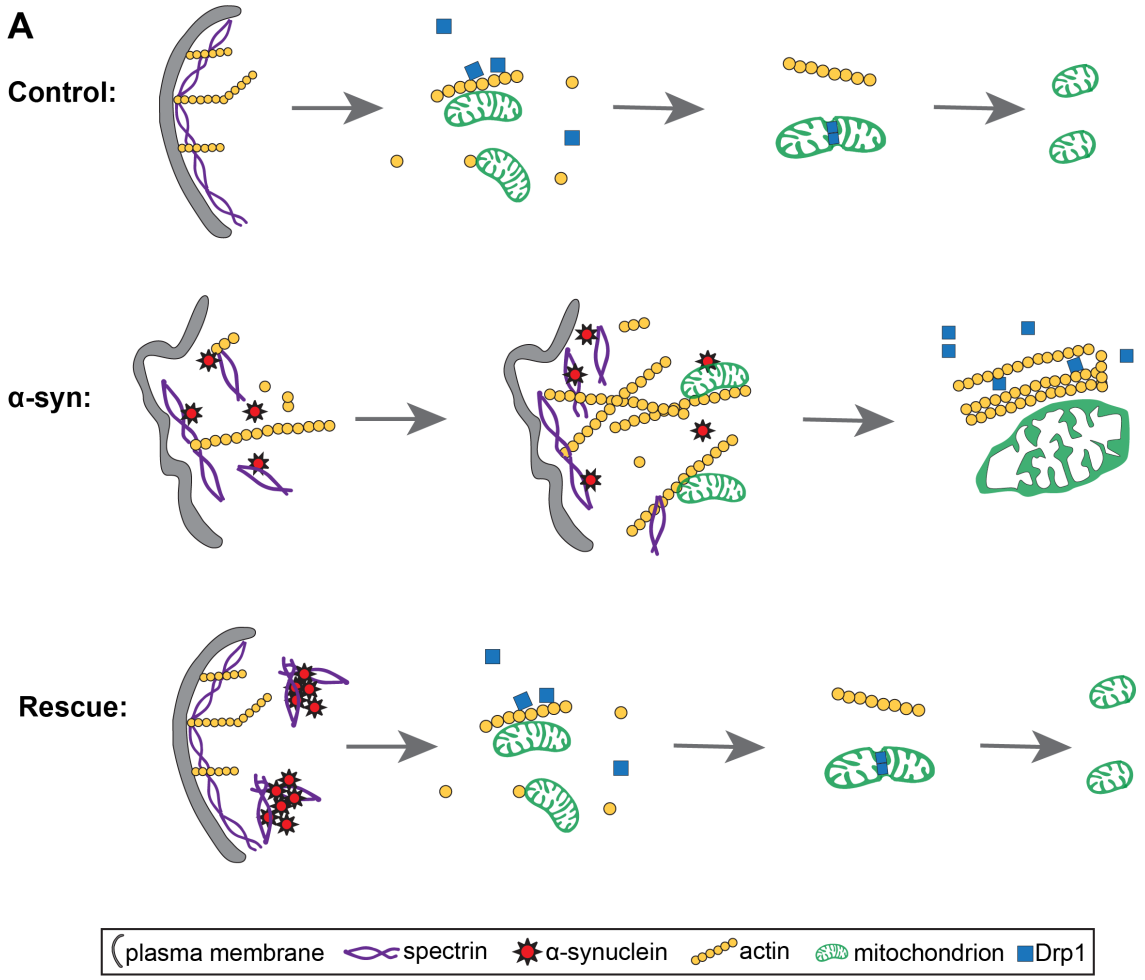

Figure S8: Mechanism of α-synuclein induced neurodegeneration, Related to Figure 7

(A) Schematic of proposed mechanism of α-synuclein induced mitochondrial abnormalities in α-synucleinopathy. α-synuclein binds to α-spectrin, disrupting normal actin cytoskeletal organization, including promoting excess F-actin stabilization at mitochondria. Loss of actin dynamics and increased levels of F-actin at mitochondria inhibits productive Drp1 localization to mitochondria and mitochondrial fission, leading to mitochondrial dysfunction. Increasing the levels of α-spectrin restores normal actin

cytoskeletal organization, Drp1 localization and mitochondrial fission by promoting sequestration of toxic α-synuclein species into large inclusions.