The Parkinson's disease genes Fbxo7 and Parkin interact to mediate mitophagy

Victoria S Burchell[1,*], David E Nelson[2,*], Alvaro Sanchez-Martinez[3,4,*], Marta Delgado-Camprubi[1], Rachael M Ivatt[3,4], Joe H Pogson[3,4], Suzanne J Randle[2], Selina Wray[1], Patrick A Lewis[1], Henry Houlden[1], Andrey Y Abramov[1], John Hardy[1], Nicholas W Wood[1], Alexander J Whitworth[3,4,¶], Heike Laman[2,¶] and Helene Plun-Favreau[1,¶].

Author affiliations:

1 Department of Molecular Neuroscience, UCL Institute of Neurology, Queen Square, London WC1N 3BG, UK.

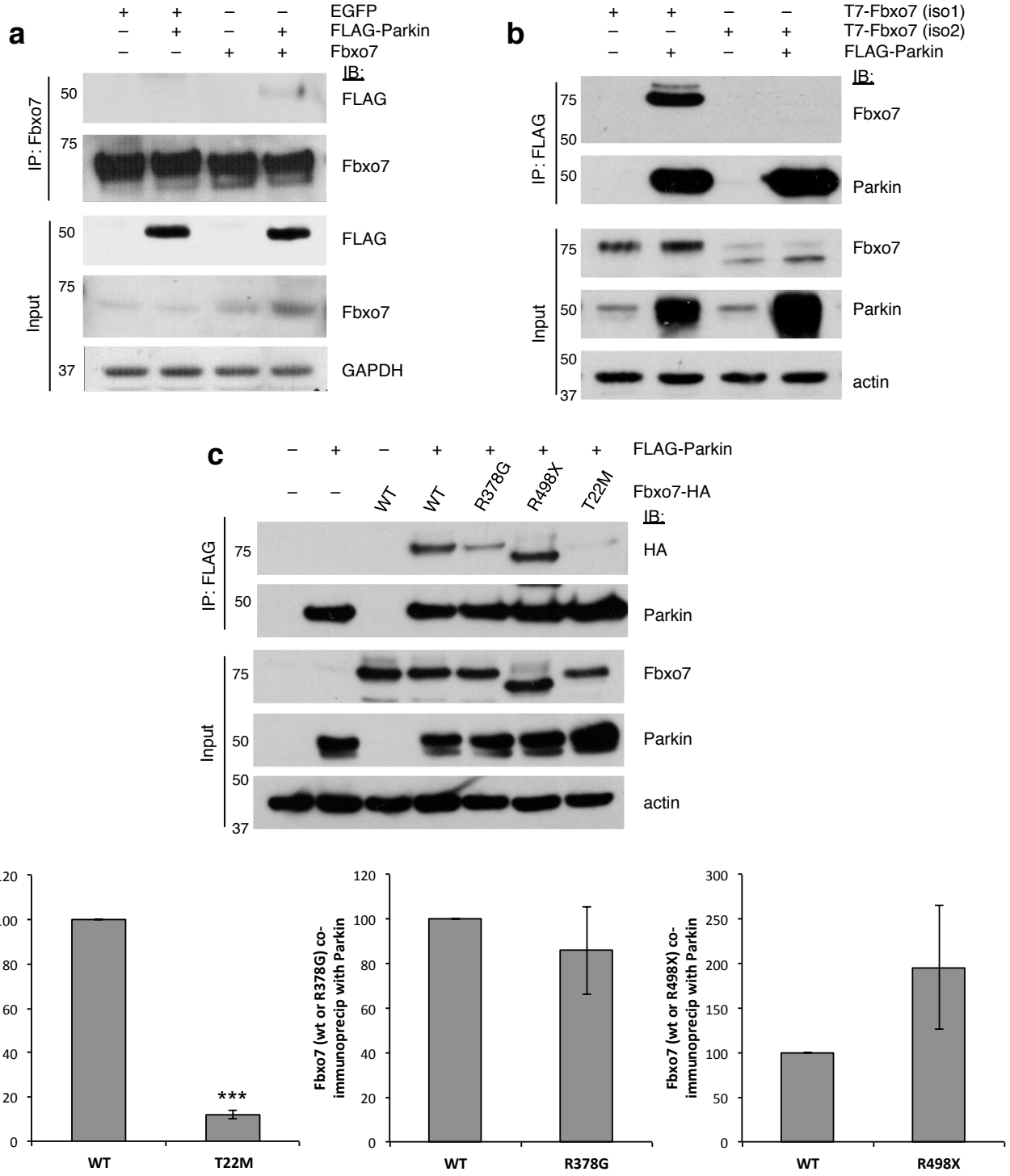
2 Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK.

3 MRC Centre for Developmental and Biomedical Genetics, Sheffield S10 2TN, UK.

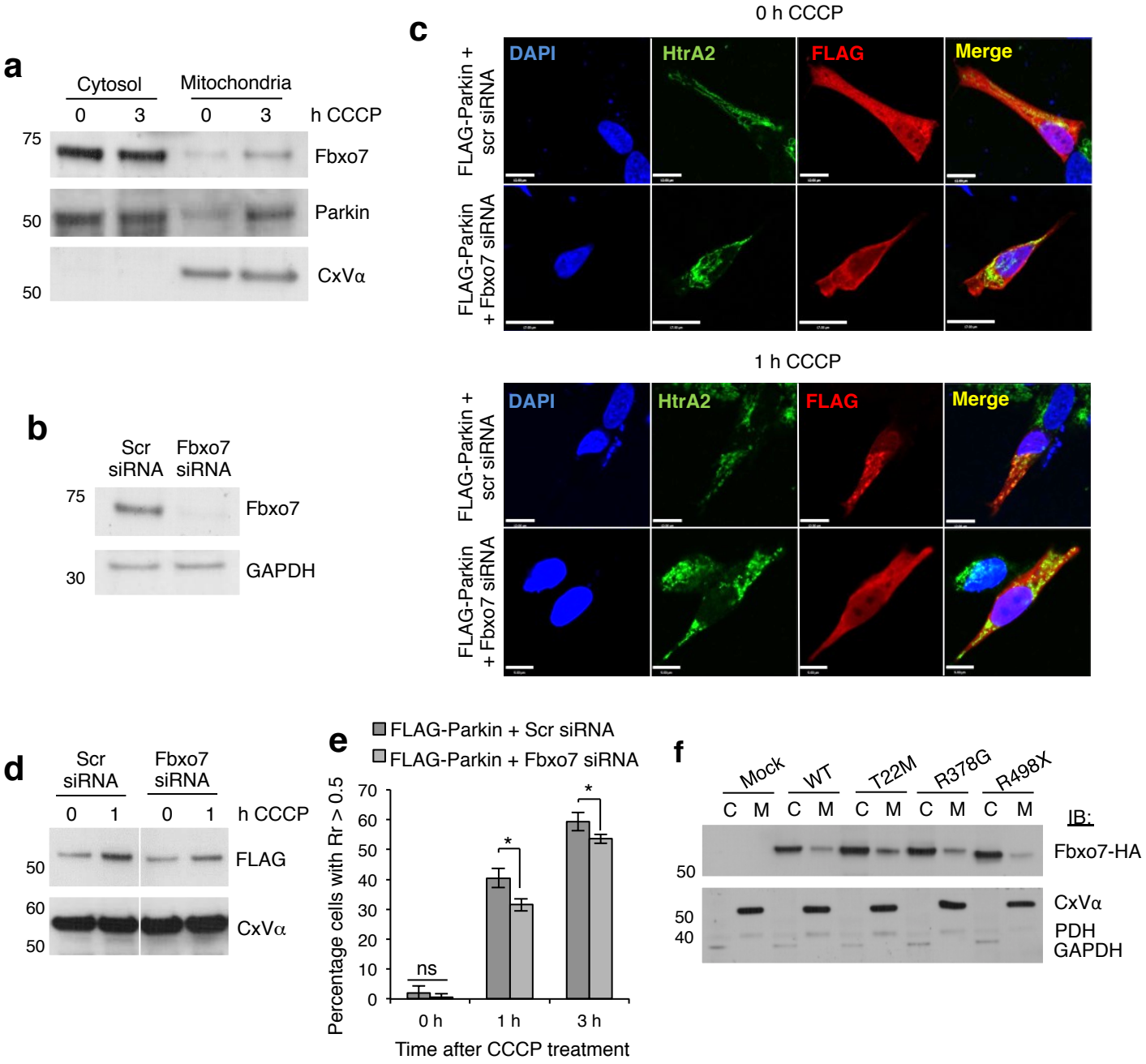
4 Department of Biomedical Sciences, University of Sheffield, Sheffield S10 2TN, UK.

*These authors contributed equally to this work.

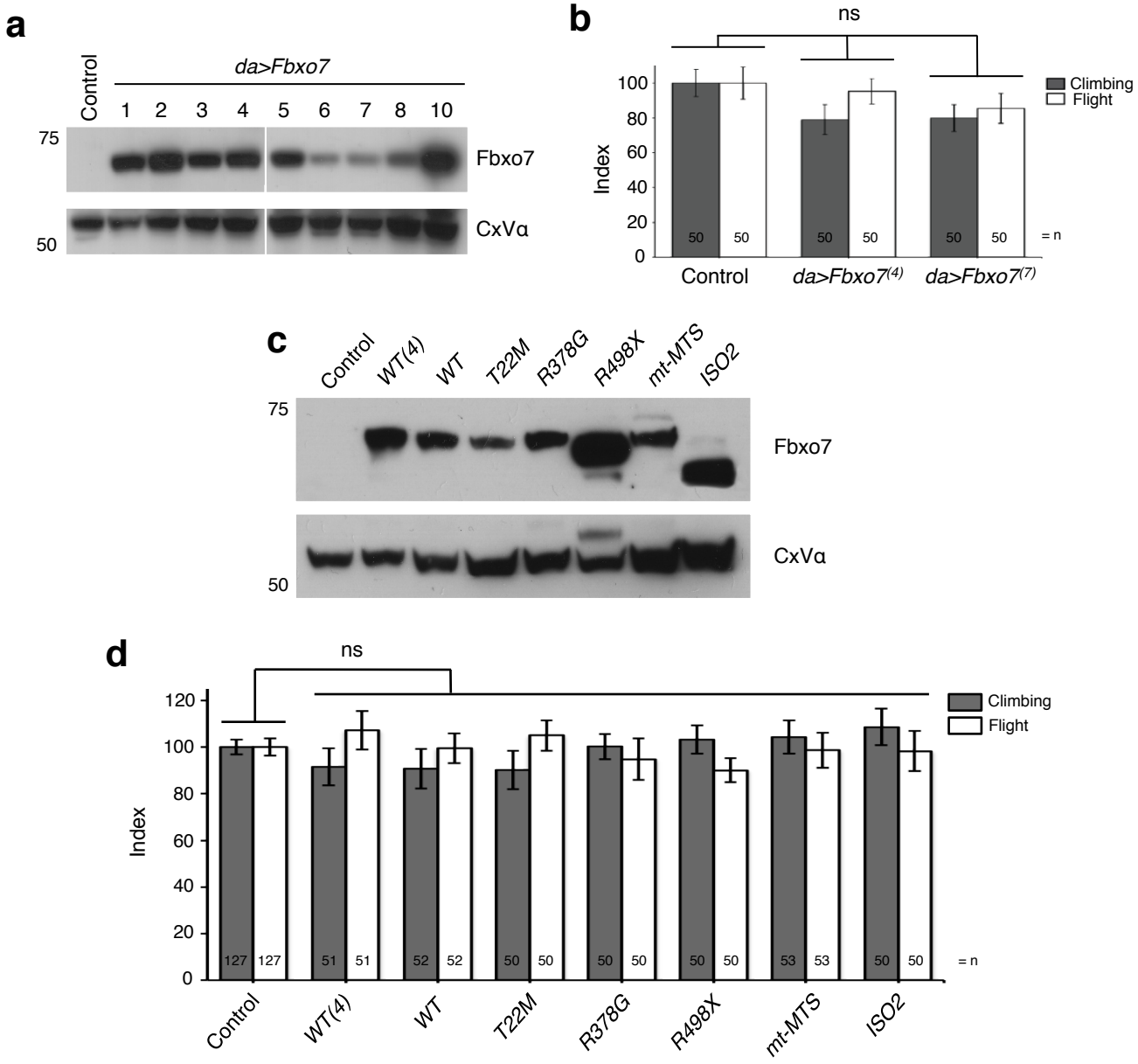
¶ Correspondence should be addressed to H.P-F., H.L. or A.J.W. (e-mail h.plun-favreau@ucl.ac.uk, hl316@cam.ac.uk, or a.whitworth@sheffield.ac.uk).



Supplementary Figure S1. Fbxo7 interacts with the N-terminus of Parkin.
a, Immunoblot showing detection of FLAG-Parkin following immunoprecipitation with Fbxo7 antibody in HEK293T cells transfected with combinations of FLAG-Parkin, untagged Fbxo7 and a control protein (EGFP). **b**, FLAG-Parkin interacts with isoform 1, but not isoform 2, of Fbxo7 in U2OS cells. **c**, Immunoblot showing interaction of WT, R378G and R498X Fbxo7-HA with FLAG-Parkin in U2OS cells. T22M Fbxo7-HA interaction with FLAG-Parkin is substantially reduced, as shown in Fig. 1g. **d**, Histograms show densitometry analysis of co-immunoprecipitation experiments. Co-immunoprecipitated Fbxo7 proteins were normalised to the input to account for differences in expression levels among Fbxo7 variants and then normalised to immunoprecipitated Parkin. Data is presented as the mean of at least three experiments ± S.E.M. Significance was determined using two-tail student's t-test (*** $p < 0.001$).

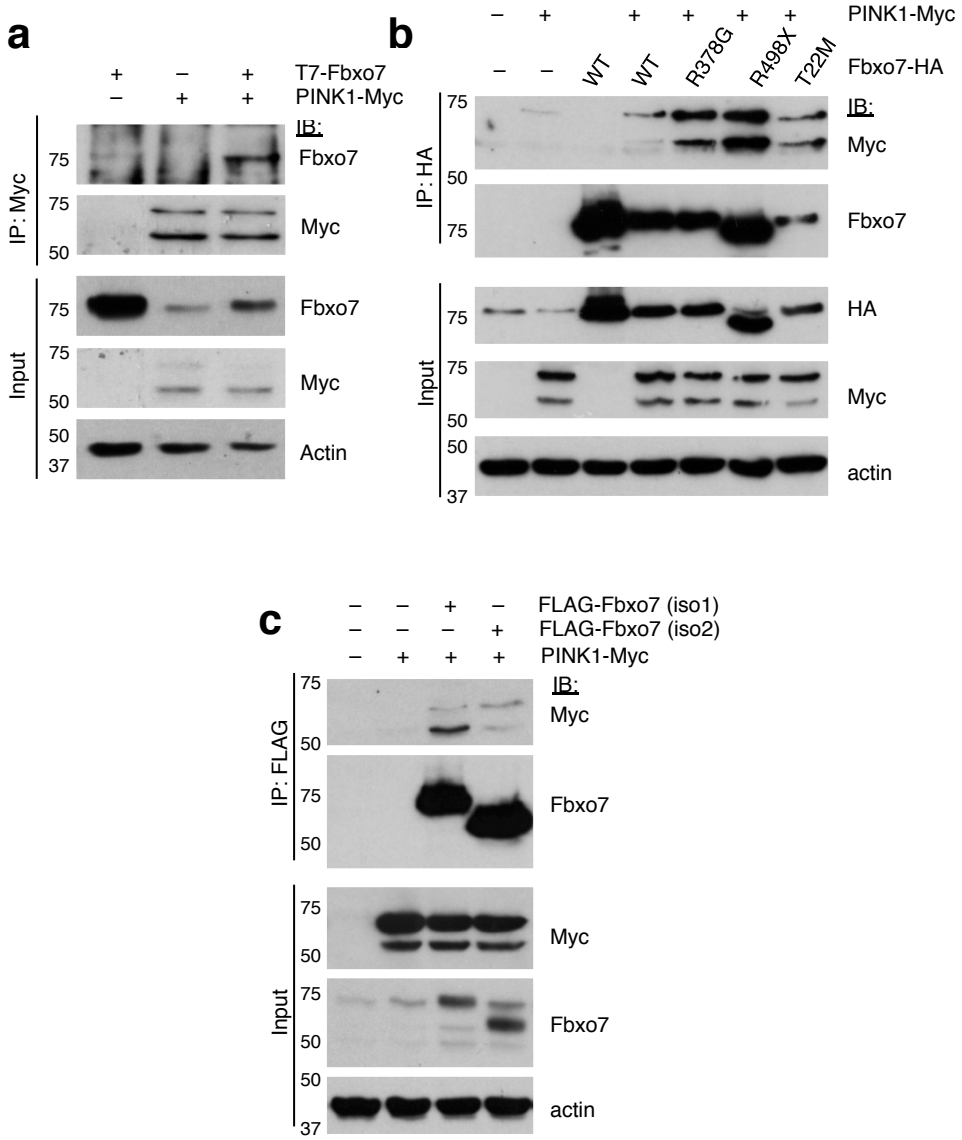


Supplementary Figure S2. Fbxo7 participates in Parkin recruitment to depolarised mitochondria.
a, Immunoblot showing increased Fbxo7 protein levels in the mitochondrial fraction of SH-SY5Y cells following treatment with CCCP (10 μM). **b**, Immunoblot showing Fbxo7 protein levels following transfection of cells with Fbxo7 siRNA compared to scrambled (scr) control. **c**, Representative immunofluorescence images are shown following 0 or 1 h CCCP treatment in SH-SY5Y cells transfected with FLAG-Parkin cDNA plus either scrambled (scr) or Fbxo7 siRNA. Anti-HtrA2 is used as a mitochondrial marker. For corresponding histograms, see Figure 2c. Scale bar, 10 μm. **d**, Immunoblot showing decrease of CCCP-driven FLAG-Parkin relocation to the mitochondria in cells transfected with Fbxo7 siRNA compared to control cells. **e**, The decrease in FLAG-Parkin relocation to depolarised mitochondria by Fbxo7 silencing was verified by an alternative analytical approach in SH-SY5Y cells stably expressing FLAG-Parkin. Images of cells co-stained for FLAG-Parkin and HtrA2 were analysed for Pearson's correlation co-efficient (Rr) on a cell by cell basis. The percentage of cells in which Rr was greater than 0.5 was then calculated to provide a measure of the number of cells in which Parkin relocated to the mitochondria. At least 100 cells were measured per coverslip and the experiment was repeated three times. Data are presented as mean ± S.E.M. * $p < 0.05$. **f**, Immunoblot showing mitochondrial localisation of WT and Parkinson's disease mutant Fbxo7-HA in transfected HEK293T cells. C and M indicate cytosolic and mitochondrial fractions respectively.



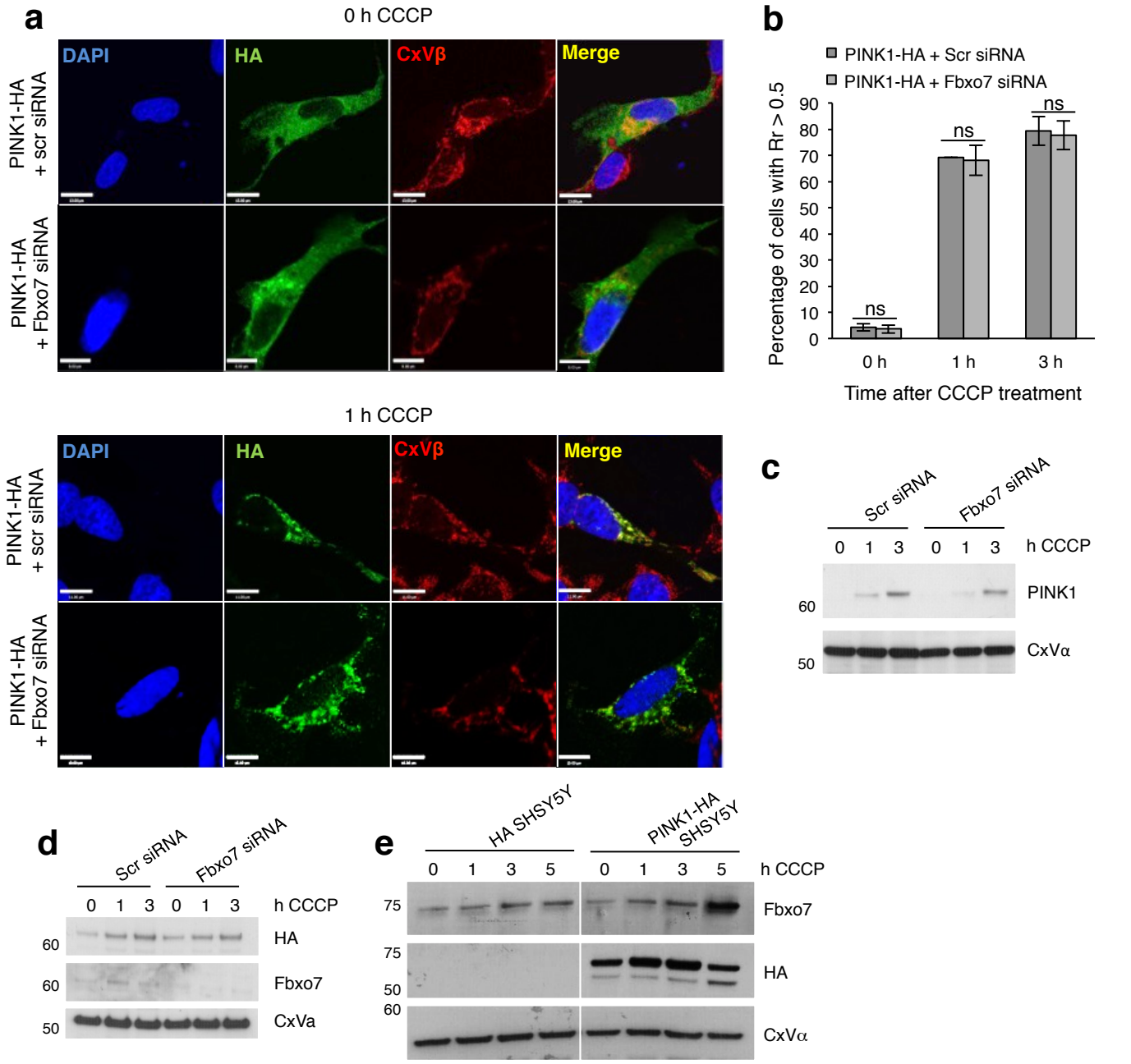
Supplementary Figure S3. Ectopic expression of human Fbxo7 has no significant effect on behaviour in *Drosophila*.

a, Immunoblot of multiple random insertion UAS-Fbxo7 transgenic line expression induced by the ubiquitous *daughterless-GAL4* (*da*) driver reveals varying levels of expression between lines. **b**, Analysis of climbing and flight ability in two transgenic lines overexpressing Fbxo7 (WT) at different levels in a WT background showed no significant difference compared to control. **c**, Immunoblot of site-directed integration (phiC31) transgenic lines expressing Fbxo7 WT, pathogenic mutations and isoform 2, induced by the *da-GAL4* driver. Transgenic line '4' analysed in **a** and **b** is shown for comparison. **d**, Climbing and flight ability of Fbxo7 variant transgenic lines analysed as in **c** in a WT genetic background showed no effect compared to control (daG4/+). Histograms show mean ± S.E.M. Significance was determined by one-way ANOVA with Bonferroni correction. Control genotype is *da-GAL4*/+ genetic background. For climbing and flight assays at least 50 flies were assessed.

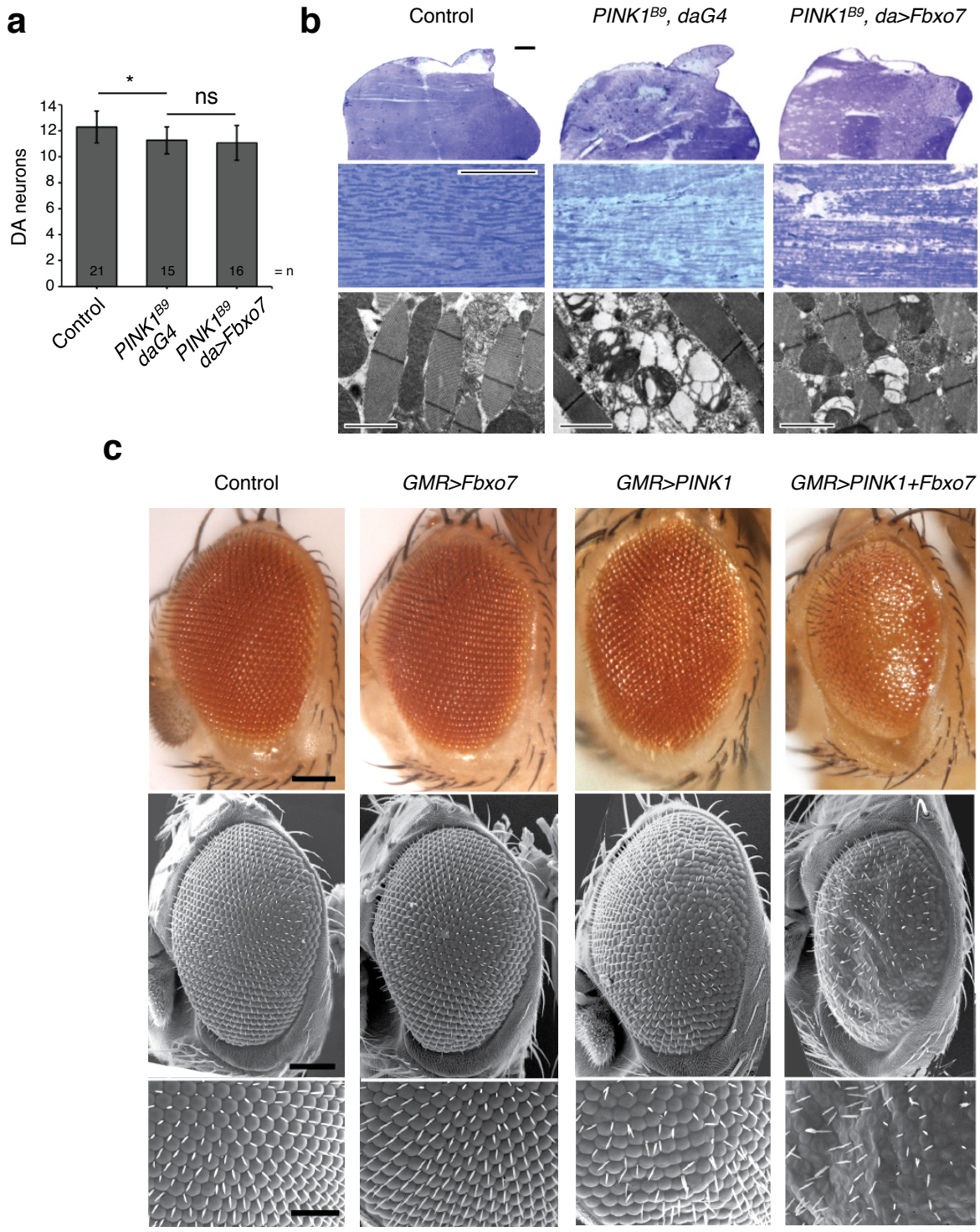


Supplementary Figure S4. Fbxo7 interacts with PINK1.
a, Immunoblot showing detection of Fbxo7 in complex with PINK1-Myc following immunoprecipitation with Myc antibody in U2OS cells. **b**, Immunoblot showing co-immunoprecipitation of WT and PD mutant Fbxo7-HA with PINK1-Myc in U2OS cells. **c**, PINK1-Myc co-immunoprecipitates with both isoforms of Fbxo7.

Supplementary Figure S5



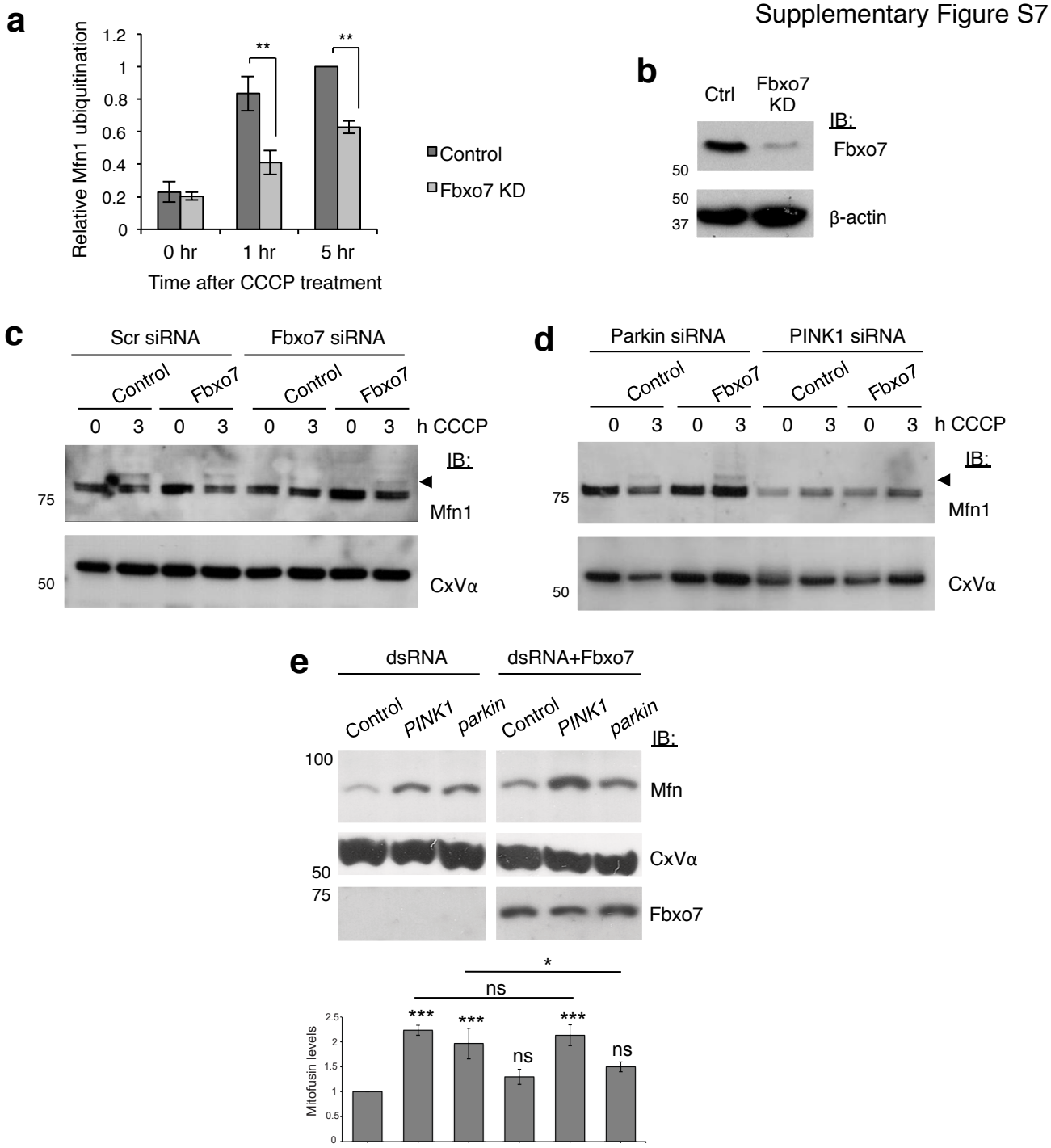
Supplementary Figure S5. Fbxo7 is not required for accumulation of PINK1 on depolarised mitochondria. **a**, Representative images are shown for staining of PINK1-HA following 0 or 1 h treatment with CCCP (10 μM) in SH-SY5Y cells transfected with PINK1-HA cDNA plus either scrambled or Fbxo7 siRNA. For corresponding histograms, see Figure 5a. Scale bar, 10 μm. **b**, The lack of effect of Fbxo7 silencing on mitochondrial accumulation of PINK1 was verified by an alternative analytical approach in HA-PINK1 overexpressing SH-SY5Y cells. Images of cells co-stained for HA-PINK1 and Complex V β subunit (CxVβ) were analysed for Pearson's correlation co-efficient (Rr) on a cell by cell basis. The percentage of cells in which Rr was greater than 0.5 was then calculated to provide a measure of the number of cells in which PINK1 accumulated on the mitochondria. At least 100 cells were measured per coverslip and the experiment was repeated three times. Data are presented as mean ± S.E.M. **c-d**, Immunoblots confirming no effect of Fbxo7 siRNA on accumulation of either endogenous PINK1 in SH-SY5Y cells (**c**) or overexpressed PINK1-HA in SH-SY5Y cells transfected with scrambled (scr) or Fbxo7 siRNA (**d**) in the mitochondrial fraction following CCCP treatment. **e**, Fbxo7 accumulation in the mitochondrial fraction following treatment with CCCP (10 μM) is enhanced in SH-SY5Y cells stably overexpressing PINK1-HA compared to SH-SY5Y cells overexpressing HA.



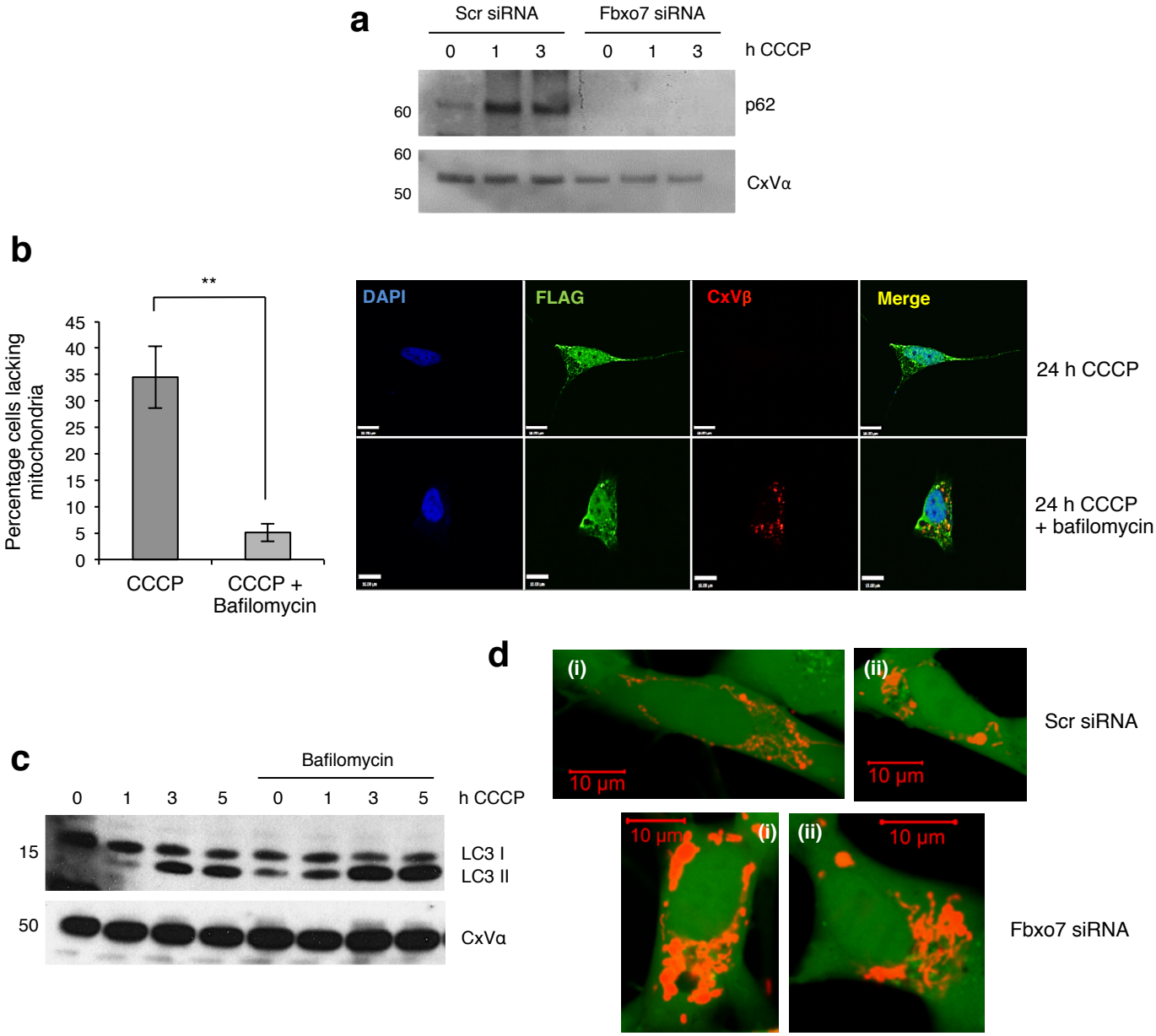
Supplementary Figure S6. *Fbxo7* does not suppress PINK1 phenotypes but genetically interacts with *PINK1* overexpression.

a, Overexpression of Fbxo7 does not suppress dopaminergic neurodegeneration in the PPL1 cluster of *PINK1* mutants. **b**, Toluidine blue stained sections of adult thorax and (top panels) TEM images of muscle show Fbxo7 overexpression does not suppress muscle degeneration nor mitochondrial disruption (n = 3 animals analysed). Toluidine blue scale bars show (top) 200 μm and (middle) 20 μm. TEM scale bars show 2 μm. Control genotype is *PINK1*^{B9}/+; da-GAL4/+.. **c**, Compound eye from a control fly (GMR/+) showing wild type regular arrangement of ommatidia. Overexpressing Fbxo7 causes no phenotype while PINK1 overexpression causes a mild disruption of the ommatidial array. Fbxo7 co-expression synergistically enhances that PINK1-induced rough eye. Light microscopy images; scale bars show 100μm. Scanning electron microcopy images; scale bars show (middle panels) 100 μm and (bottom panels) 50 μm. Charts show mean ± S.E.M. Significance was determined by one-way ANOVA with Bonferroni correction (* P<0.05). Images are representative of more than 20 animals per genotype.

Supplementary Figure S7



Supplementary Figure S7. Fbxo7 expression restores Mfn levels in parkin but not PINK1 deficient cells.
a, Densitometry analysis shows a significant reduction in Mfn1 ubuiquitination in Fbxo7 knockdown (KD) cells compared to controls. Mfn1 ubiqitination bands were normalised to Mfn1 levels, then for each experiment results were normalised to the 5 h CCCP response in control cells. Data are represented as mean ±S.E.M. Significance was determined by Student *t*-test (** $p < 0.01$). **b**, Immunoblot showing Fbxo7 protein levels in stable knockdown (KD) SH-SY5Y cells compared to cells transduced with the empty vector (Ctrl). **c**, Higher molecular weight ubiquitinated species of Mfn1 (indicated with arrows) appear following treatment with CCCP (10 μM) in SH-SY5Ycells transfected with scrambled (scr) but not Fbxo7 siRNA, and this can be rescued by exogenous Fbxo7. **d**, Ubiquitination of Mfn1 is prevented in SH-SY5Y cells transfected with siRNA targeting *Parkin*, and this can be rescued by exogenous Fbxo7. Mfn1 ubiquitination is similarly inhibited by PINK1 siRNA, but this cannot be rescued by exogenous Fbxo7. **e**, Mfn steady state levels are increased in PINK1/parkin deficient *Drosophila* S2R+ cells. Fbxo7 expression restores Mfn steady state levels in parkin but not PINK1 deficient cells. Histograms show mean ± S.E.M. of densitometry analysis of Mfn immunoblots above, normalised to Complex V α (CxVα). Significance was determined by one-way ANOVA with Bonferroni correction (*** $p < 0.001$; * $p < 0.05$). Immunoblots; $n = 3$.



Supplementary Figure S8. Fbxo7 participates in CCCP driven mitophagy.
a, p62 accumulates in the mitochondrial fraction of SH-SY5Y cells transfected with scrambled (scr) siRNA but not Fbxo7 siRNA following treatment with CCCP (10 μM). **b**, Mitochondrial disappearance was inhibited by bafilomycin, an inhibitor of autophagy. FLAG-Parkin overexpressing SH-SY5Y cells were treated for 24 h with either DMSO, CCCP (10 μM) or CCCP + bafilomycin (40 nM), then fixed and stained for FLAG-Parkin and complex V β (CxVβ), a mitochondrial marker. Cells were scored visually for the absence of mitochondrial staining. Data are represented as mean ± S.E.M. ** $p < 0.005$. **c**, Pre-treatment of cells with bafilomycin for 3 h prior to the addition of CCCP (10 μM) inhibits clearance of proteins through the lysosome and therefore results in an accumulation of LC3-II at the mitochondrial fraction. **d** Representative images are shown for mitochondria in FLAG-Parkin overexpressing SH-SY5Y cells (i) before and (ii) after treatment with CCCP (10 μM) for 24 h. Cells were either transfected with scrambled (Scr) or Fbxo7 siRNA as indicated. Cell volume was stained using calcein-AM (green) and mitochondria were visualised by transfection with DsRed-Mito (red). Scale bar, 10 μm.

Supplementary figure S9. Full-length images of the blots presented in the main figures.

Fig. 1

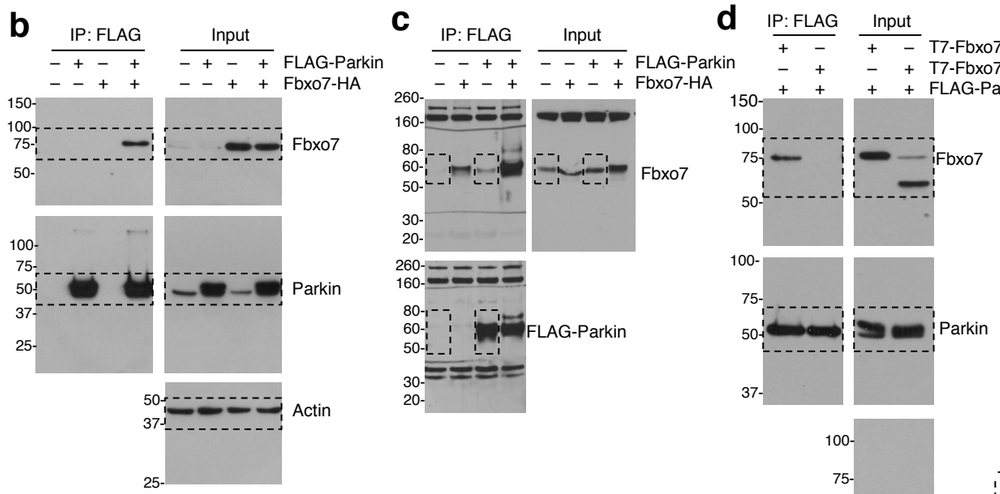


Fig. 2

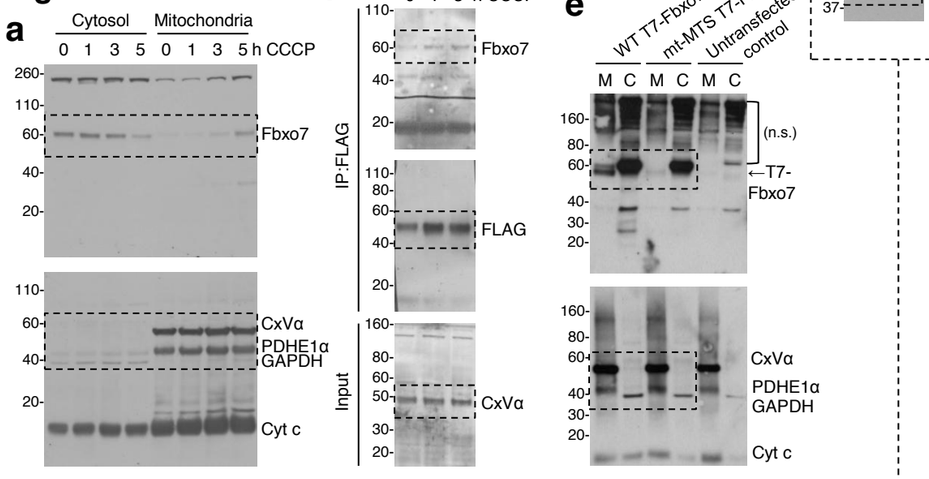


Fig. 4

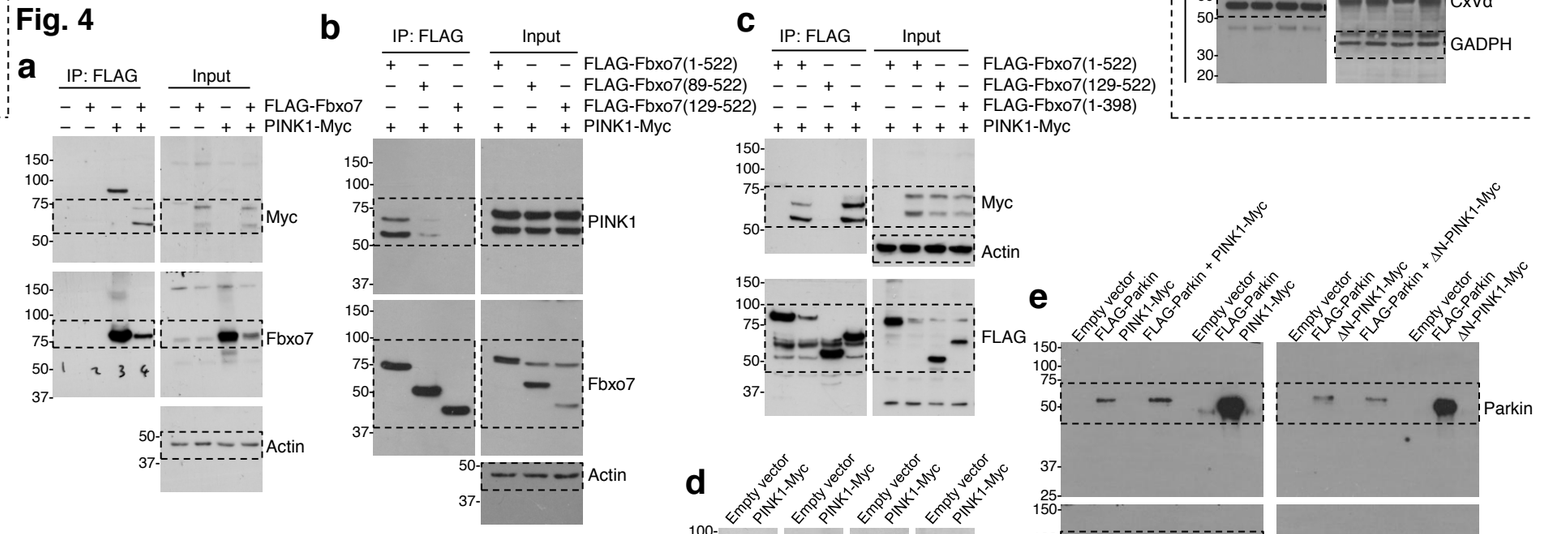


Fig. 5

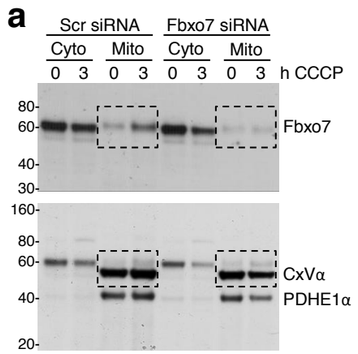


Fig. 6

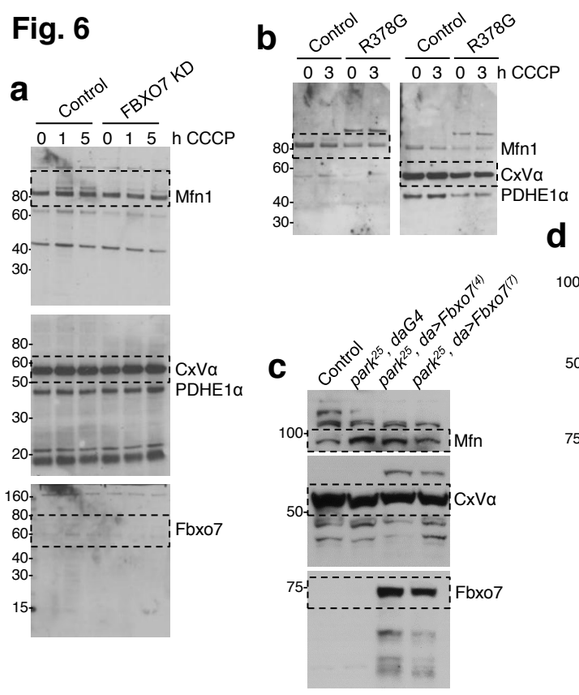


Fig. 7

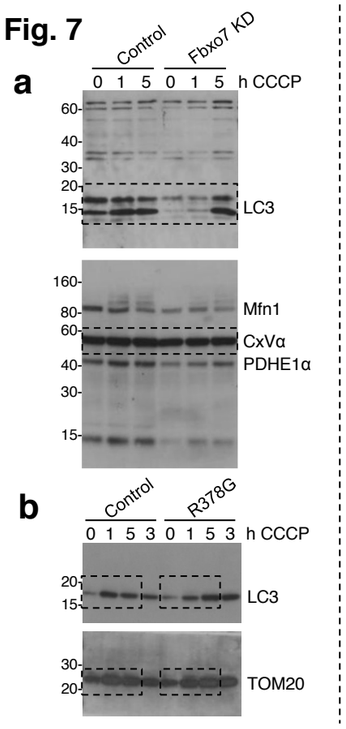


Fig. 8