Kamp et al: **Inhibition of Mitochondrial fusion by αS is rescued by PINK1, Parkin and DJ-1**

Supplementary data

SUPPLEMENTARY MATERIALS AND METHODS

Subcellular fractionation

Cells were harvested and cell pellets were washed twice in PBS and resuspended in RSB (10 mM Tris/HCl pH 7.4, 10 mM NaCl, 1.5 mM $CaCl_2$). After incubation for 10 min on ice and centrifugation for 10 min at 600 x g cell pellets were resuspended in 1:1 ice-cold mixture of RSB and MS (420 mM mannitol, 140 mM saccharose, 10 mM Tris/HCl pH 7.4, 5 mM EDTA pH 8). Cells were homogenized with a glass douncer and 1 volume of MS was added. Mitochondria were harvested from the supernatant after two low spin centrifugations (10 min at 900 x g) by high speed centrifugation for 10 min at 12000 x g and pellets were resuspended in MS. Post-mitochondrial supernatants were subjected to ultracentrifugation for 30 min at 100000 x g to yield microsome-enriched pellets and cytosolic supernatants.

Figure S1:

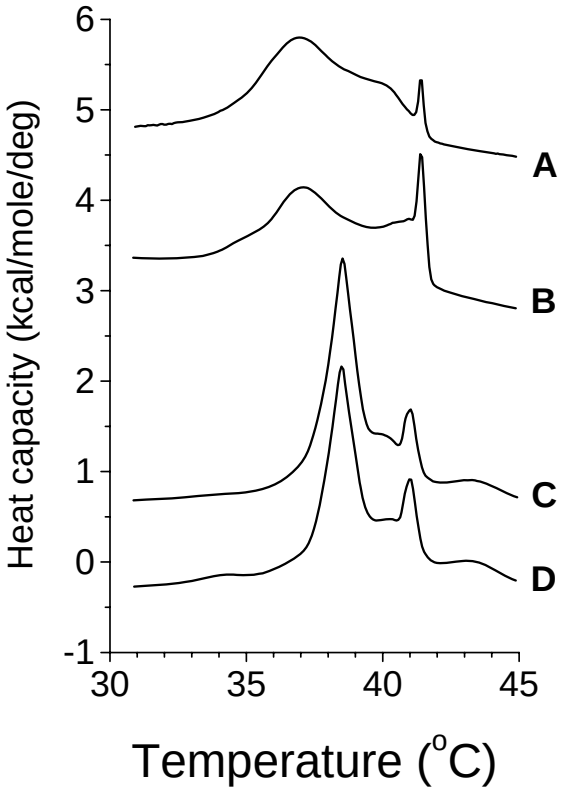

Figure S1: Inhibition of membrane fusion by αS due to removal of lipid-packing stress in highly curved vesicles. Sonicated DPPC-SUV (12.5 mM lipid) were introduced into a calorimeter cell (VP-DSC instrument MicroCal, Northampton, Massachusetts) (A) The heat capacity curve recorded immediately after the sonication displays a broad phase transition around 37 °C. The gel to liquid-crystalline phase transition of DPPC-SUV is severely broadened due to curvature associated lipid-packing stress in the membrane. The second narrow peak at 41 °C is attributed to a small fraction of multilamellar vesicles with low curvature that typically remains in the SUV preparation, displaying the typical T_m (41 °C) of DPPC membranes; (B) DPPC-SUV after 4 days incubation at 30 °C. The broad transition at 37 °C decreased and the narrow peak at 41 C increased, as a result of the spontaneous formation of fusion products with low curvature; (C) and (D) DPPC-SUV with αS (lipid/αS 180 molar ratio) after 10 minutes (C), and after 4 days incubation at 30 °C (D). The phase transition of the SUV was much narrower and the heat capacity maximum was shifted towards higher temperature (T_m = 39 °C) indicating that αS stabilizes defects in the lipid packing of highly curved vesicles. The small peak at 41 °C did not increase within 4 days indicating that αS inhibits spontaneous membrane fusion.

Figure S2:

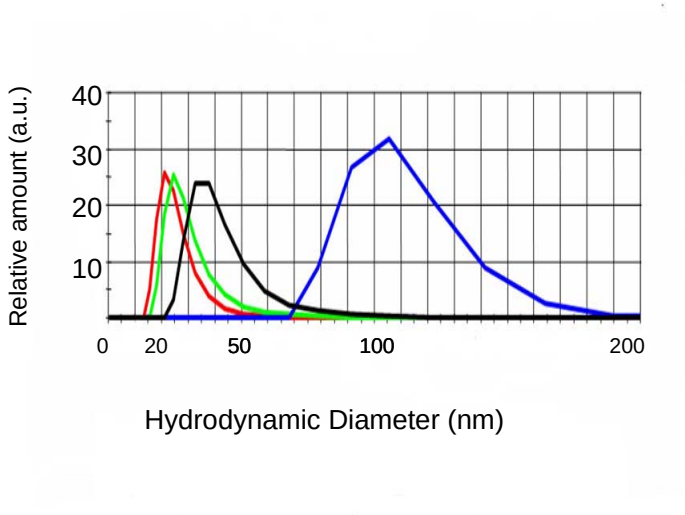

Figure S2: Fusion of DPPC-SUV monitored with Dynamic Light Scattering. 50 µl DPPC-SUV) were analyzed with a Malvern Instruments High Performance Particle Sizer (Herrenberg, Germany). Displayed are the size distributions of the hydrodynamic diameter (D_h) of the vesicles derived from the measured correlograms. Red trace: SUV at t = 0, D_h was 24.4 ± 6.8 nm; Blue trace: Fusion was induced by $C_{12}E_8$ (lipid/detergent = 20 molar ratio). After t = 1 hour the maximum had increased to about 108 ± 10 nm; Green trace: SUV + αS at t = 0 (lipid/αS = 200 mole/mole), D_h was 28.6 ± 3 nm, i.e. slightly higher than in the absence of αS. This may be due to the increase of the effective vesicle diameter when αS binds to the membrane surface; Black trace: SUV + αS, $C_{12}E_8$ induced fusion. After 1 hour the D_h had only increased to 35 ± 7 nm. In all traces: lipid concentration 10 mM, T = 25 C.

Figure S3, related to Figure3:

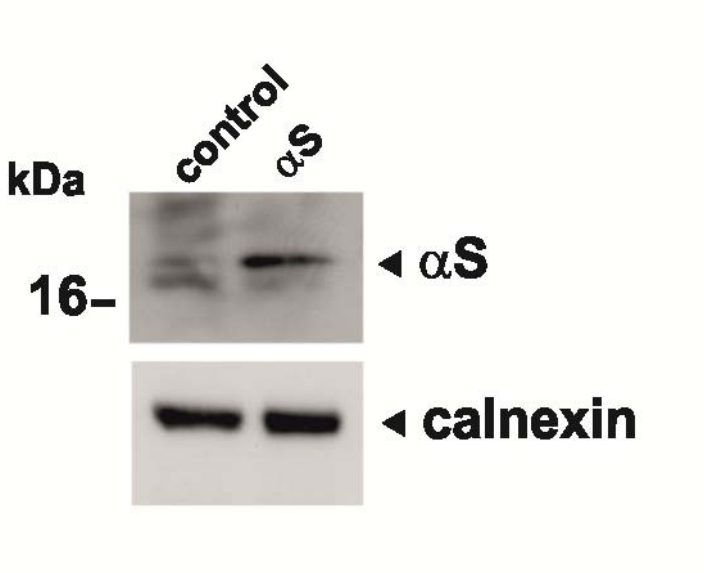

Figure S3: Expression of αS in the experiments shown in Figure 3. Protein levels of SH-SY5Y cells transiently transfected with empty vector (control) or with αS were analyzed by Western blotting using calnexin as loading control.

Figure S4, related to Figure 7:

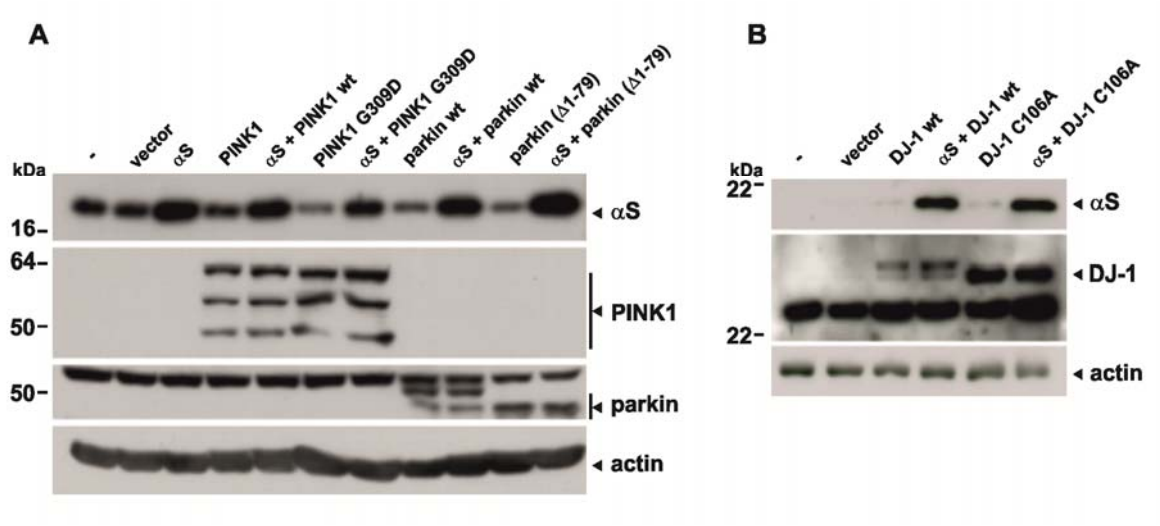

Figure S4: Expression of αS, PINK1, parkin (A) and DJ-1 (B) in the experiments shown in Figure 7. Protein levels of SH-SY5Y cells transiently transfected with the indicated cDNA constructs were analyzed by Western blotting using β-actin as loading control.

Figure S5, related to Figure 8:

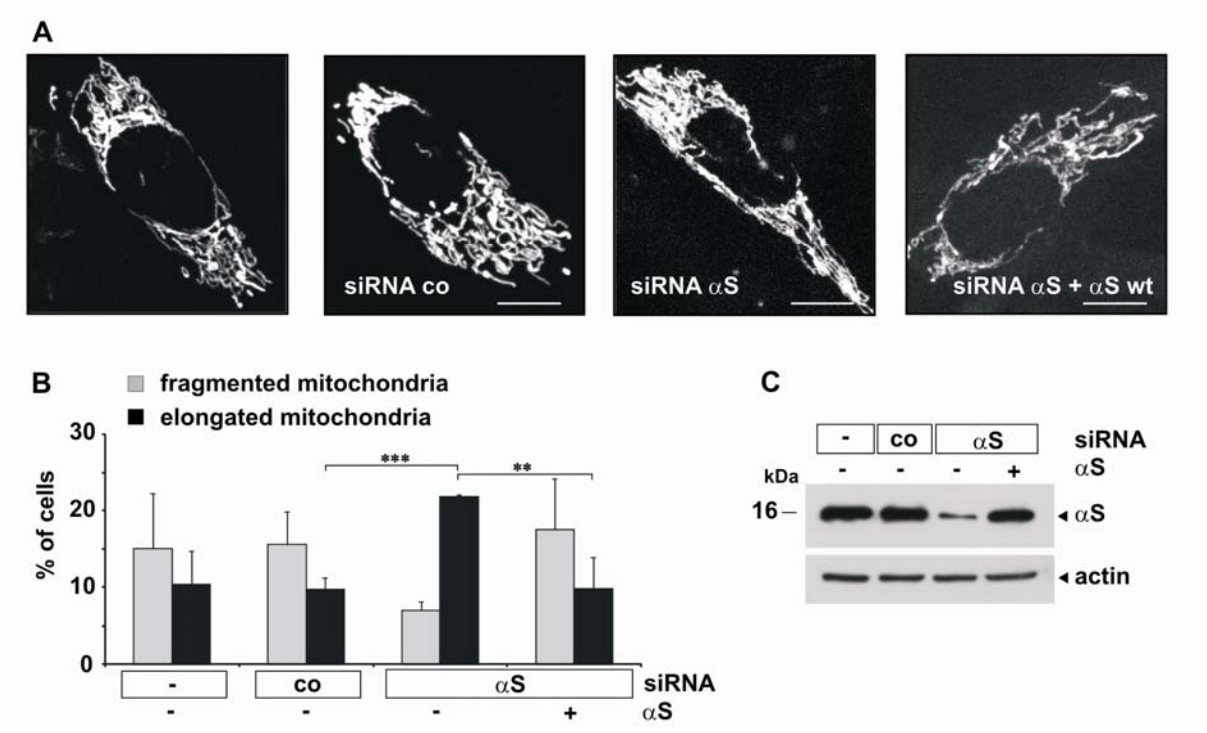

Figure S5: Loss of αS induces elongation of mitochondria. (A) Images of mitochondria fluorescently labeled with mito-GFP. The panels display representative individual cells either untransfected (co) or transfected with control-siRNA, αS-siRNA, and αS-siRNA + αS cDNA. Scale bar 10 µm. (B) Statistical analyses of mitochondrial morphology of cells from the experiments shown in A. Error bars indicate standard deviations. (C) Downregulation of αS in SH-SY5Y cells and retransfection with wild type αS was monitored by Western blotting.

Figure S6, related to Figure 9:

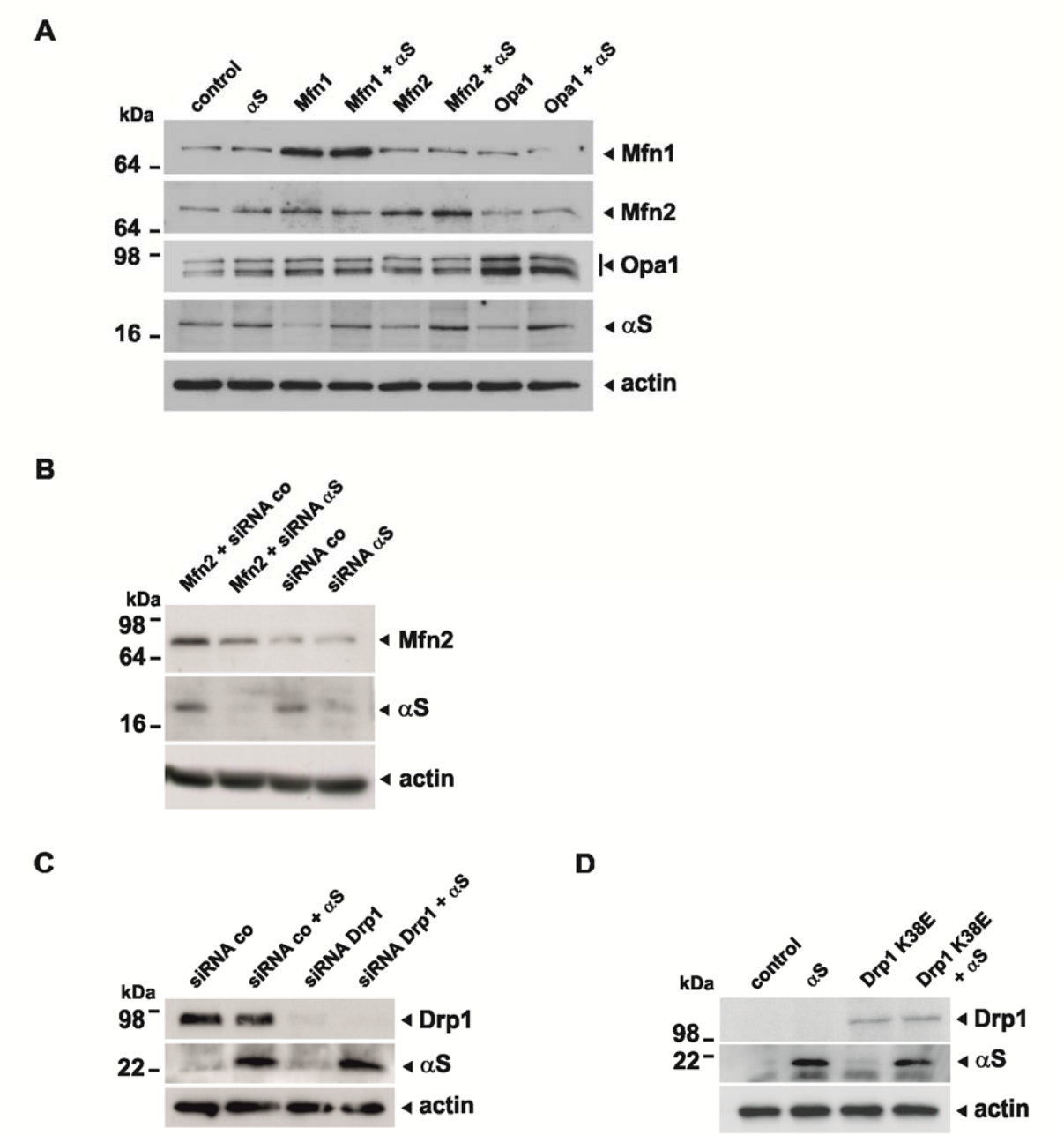

Figure S6: Expression of Mfn1, Mfn2, Opa1, Drp1 and αS in the experiments shown in Figure 9. Protein levels of SH-SY5Y cells transiently transfected with the indicated cDNA constructs were analyzed by Western blotting using β-actin as loading control. (A) Transfection of cDNA constructs as in Figure 9A, (B) Transfection of siRNA and Mfn2 as in Figure 9B, (C) Transfection of siRNA and αS as in Figure 9C, (D) Transfection of cDNA constructs as in Figure 9D.

Figure S7:

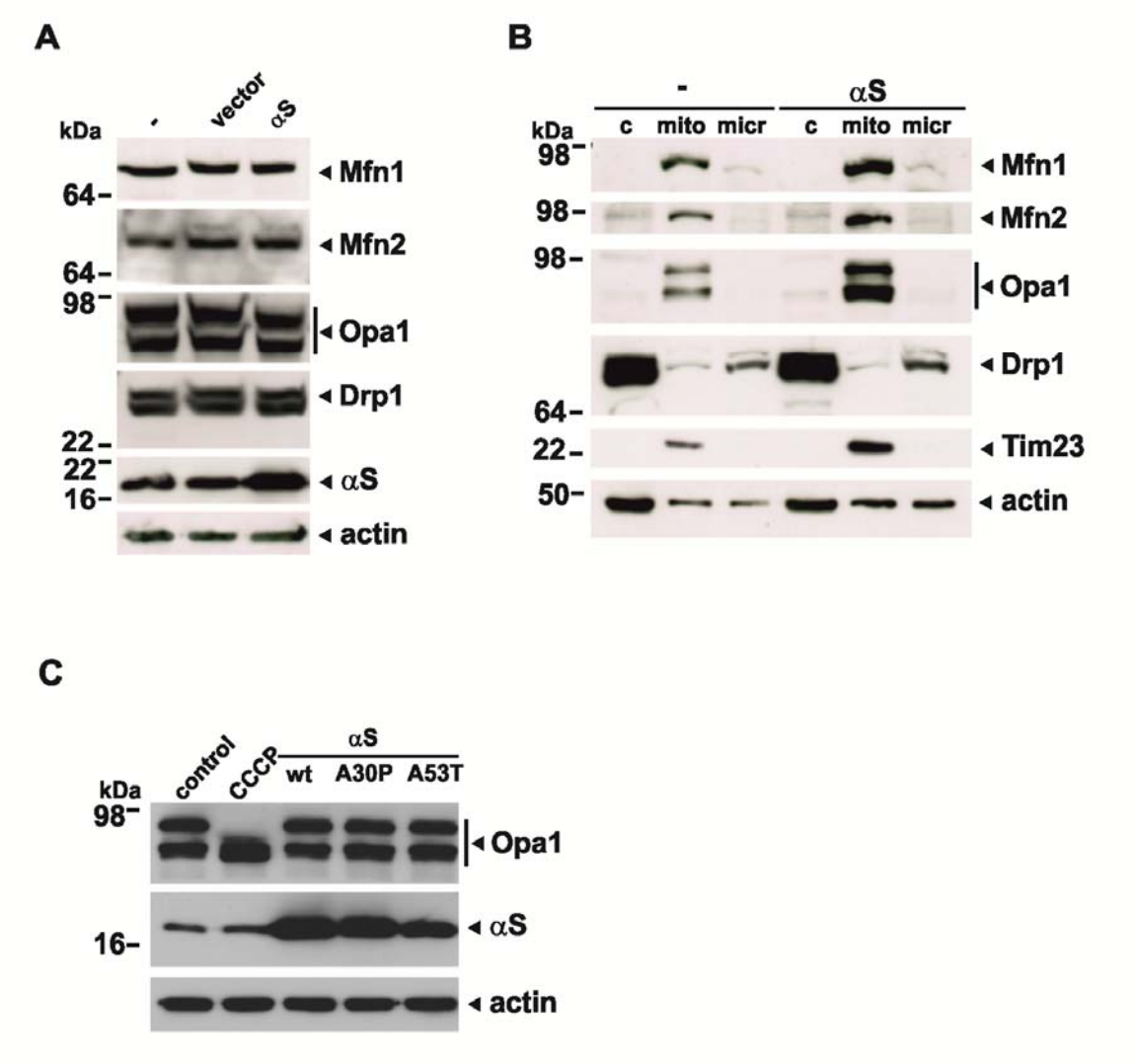

Figure S7: Expression, subcellular distribution and post-transcriptional modifications of proteins involved in mitochondrial fusion or fission are unchanged after expression of αS. (A) SH-SY5Y cells were untreated (-) or transiently transfected with empty vector (vector) or αS. Cell lysates were analyzed by Western blotting for changes in protein levels of endogenous Mfn1, Mfn2, Opa1 and Drp1 using β-actin as loading control. Protein levels were unchanged. (B) Cells were untreated or transiently transfected with αS. Cells were divided into a cytosolic, a mitochondrial and a microsomal fraction by differential centrifugation. Fractions were analyzed for changes in distribution of endogenous Mfn1, Mfn2, Opa1 and Drp1 by Western blotting using Tim23 as marker for mitochondria, calreticulin as marker for

ER, and actin as marker for the cytosol. Subcellular distributions of Mfn1, Mfn2, Opa1 and Drp1 were unchanged. (C) Cells were transfected with vector (control) or with αS wt, αS A30P or αS A53T or incubated in medium containing 20 µM CCCP for 30 min prior to lysis (CCCP). Cell lysates were analyzed by Western blotting for changes in the amounts of Opa1 isoforms using β-actin as loading control. The Opa1 isoform pattern was shifted when cells were treated with CCCP, but unchanged after expression of αS.