Molecular chaperone mediated rescue of mitophagy by a Parkin RING1 domain mutant. Rose et al 2010.

Supplementary material

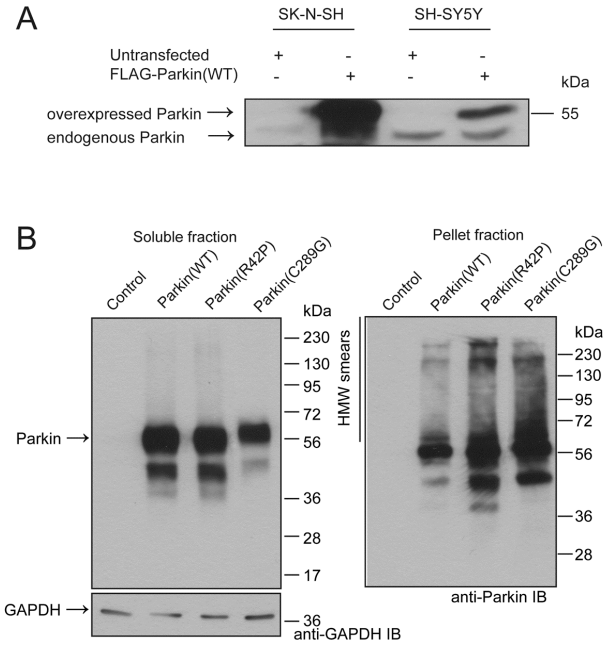

Figure S1. Endogenous and transfected Parkin expression. (**A**) Comparison of endogenous and FLAG-Parkin expression in SK-N-SH and SH-SY-5Y neuroblastoma cells. Untransfected or FLAG-Parkin(WT) transfected SK-N- SH and SH-SY5Y cells were lysed in 1% Triton X-100 in PBS twenty-four hours post- transfection. 20 µL of each total cell lysate were resolved by SDS-PAGE and analyzed by Western blotting with mouse monoclonal anti-Parkin and goat anti-mouse-HRP antibodies. Arrows indicate the position of overexpressed FLAG-Parkin(WT) and endogenous Parkin. Molecular weight markers are shown on the right. (**B**) Comparison of the expression levels and solubility of FLAG-Parkin(WT), FLAG-Parkin(R42P) and FLAG-Parkin(C289G). 24 hours post- transfection cells were lysed in 1% Triton X-100 in PBS and centrifuged to obtain soluble and fractions. Pellets were resuspended in 1% SDS in PBS. 4 µl of soluble fraction and 20 µl of pellet fraction were resolved by SDS-PAGE and analyzed by Western blotting with mouse monoclonal anti-Parkin and goat anti-mouse- HRP antibodies. GAPDH was used as a loading control for the soluble fraction. The position of molecular weight markers is indicated on the right in kDa.

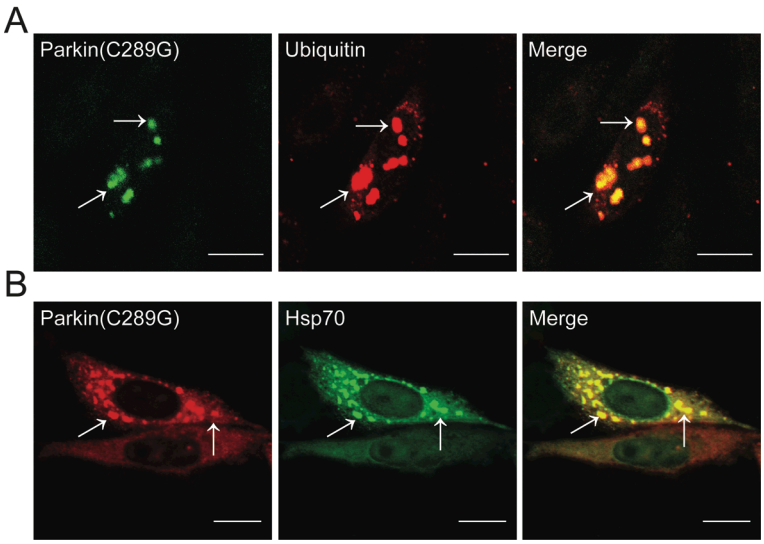

Figure S2. Parkin(C289G) inclusions recruit ubiquitin and Hsp70. SK-N-SH cells transfected with FLAG-Parkin(C289G) and (**A**) myc-ubiquitin or (**B**) GFP-Hsp70. Cells were fixed in methanol and immunostained with (**A**) rabbit monoclonal anti-FLAG and mouse monoclonal anti-myc, followed by donkey anti-rabbit-Cy2 (green) and donkey anti-mouse-Cy3 (red) antibodies; or (**B**) mouse monoclonal anti-FLAG and donkey anti-mouse-Cy3 (red) antibodies. Arrows indicate intracellular inclusions. Scale bar: 10 μm.

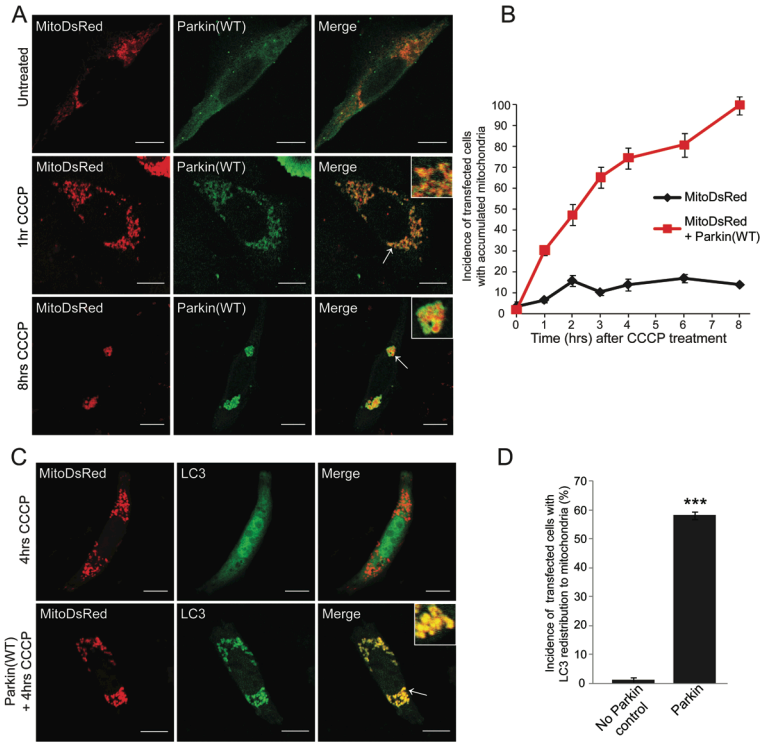

Figure S3. Parkin is recruited to depolarized mitochondria and stimulates mitochondrial clustering. (**A**) SK-N-SH cells transfected with MitoDsRed and FLAG-Parkin(WT). After 16 hours, cells were treated with CCCP (20 µM, 0 to 8 hours, as indicated) before fixation in paraformaldehyde and methanol. Cells were immmunolabeled with anti-FLAG (green). Arrows indicate areas where FLAG-Parkin(WT) staining overlaps with mitochondria. Insets are magnifications of the areas highlighted by the arrows. Scale bar: 10 µm. (**B**) Quantification of FLAG-Parkin(WT) expression on the percentage of cells with clustered mitochondria after CCCP treatment over time. 4 groups of more than 100 cells were counted for each condition. Error bars: ± 2 S.E., *n*=4 (**C**) SK-N-SH cells transfected with the mitochondrial marker MitoDsRed (red), FLAG-Parkin(WT) and the autophagy marker GFP-LC3 (green). 20 hours post-transfection, cells were treated with CCCP (20 µM, 4 hours) before fixation in paraformaldehyde and methanol. The arrow indicates LC3 staining on mitochondria. The inset is a magnification of the area highlighted by the arrow. Scale bar: 10 µm. (**D**) The percentage of cells with overlapping GFP-LC3 and MitoDsRed staining was quantified in control cells, or in cells co-transfected with FLAG-Parkin(WT), treated with CCCP (20 µM, 4 hours). Error bars:± 2 S.E.; *n*=4 (***$p<0.001$).

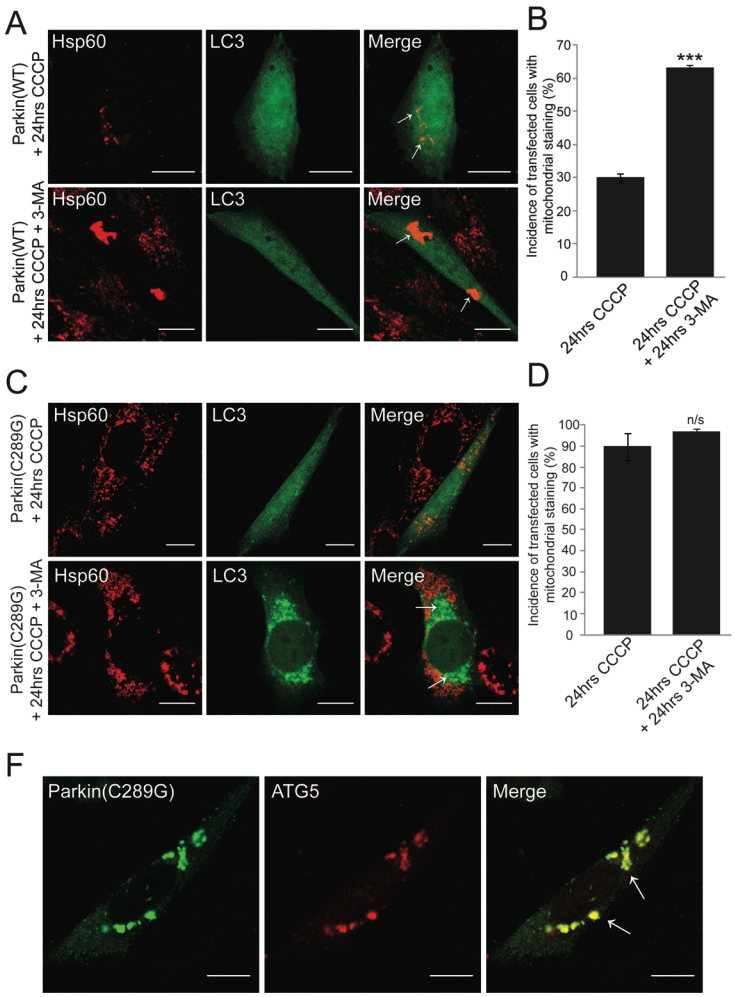

Figure S4. Parkin stimulates mitophagy of damaged mitochondria. (**A-D**) SK-N-SH cells transfected with (**A**) FLAG-Parkin(WT), or (**C**) FLAG-Parkin(C289G), and GFP-LC3 (green). 20 hours post-transfection, cells were treated with CCCP (20 µM, 24 hours) and 3-MA (10 mM, 24 hours) before fixation in paraformaldehyde and methanol. Cells were immunolabeled with the mitochondrial marker, anti-Hsp60 (red). Arrows indicate areas of mitophagy. Scale bar: 10 µm. Quantification of the number of transfected cells with mitochondrial staining in cells treated with CCCP (20 µM, 24 hours) and 3-MA (10 mM, 24 hours) for (**B**) FLAG-Parkin(WT) or (**D**) FLAG-Parkin(C289G). Error bars: ± 2 S.E., n=4, ***p<0.001, n/s: not significant. (**F**) SK-N-SH cells transfected with FLAG-Parkin(C289G) and mCherry-ATG5 (red). 24 hours post transfection, cells were fixed in paraformaldehyde and methanol, before immunolabeling with anti-FLAG (green). Arrows indicate overlapping staining pattern between Parkin(C289G) and ATG5. Scale bar: 10 µm.

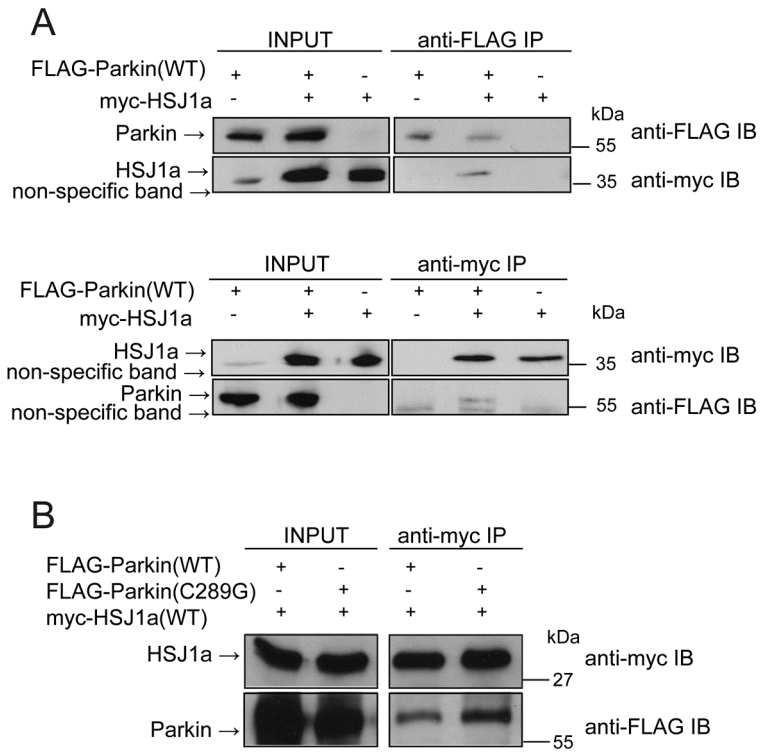

Figure S5. Wild-type and mutant Parkin co-immunoprecipitate with HSJ1a. (**A**) SK-N-SH cells were transfected with FLAG-Parkin(WT) and myc-HSJ1a(WT) as indicated. Twenty hours post-transfection, cells were treated with the proteasome inhibitor MG132 (50 μM, 4hrs). Cells were lysed in 1% Triton X-100, and immunoprecipitated overnight with mouse monoclonal anti-FLAG or mouse monoclonal anti-myc antibodies, as indicated. 10 μl of total cell lysate were loaded as input fraction, while the IP loading corresponded to approximately 40 μl of the input fraction. Samples were resolved by SDS-PAGE and immunoblotted with anti-FLAG and anti-myc primary antibodies, followed by goat anti-mouse-HRP secondary antibody. (B) Preferential interaction of HSJ1a with Parkin(C289G). SK-N- SH cells were transiently transfected with FLAG-Parkin(WT), FLAG-Parkin(C289G) and myc-HSJ1a(WT) as indicated. Twenty hours post-transfection, cells were lysed in 1% Triton X-100 and immunoprecipitated overnight with mouse monoclonal anti-myc antibody. 10 μl of total cell lysate were loaded for the input fraction. The IP loading was equivalent to approximately 40 μl of the input fraction. Samples were resolved by SDS-PAGE and Western blotted with mouse monoclonal anti-myc and mouse monoclonal anti-FLAG primary antibodies, followed by goat anti-mouse-HRP secondary antibody. The position of HSJ1a and Parkin is indicated by the arrows. The position of the molecular weight markers (in kDa) is shown on the right.

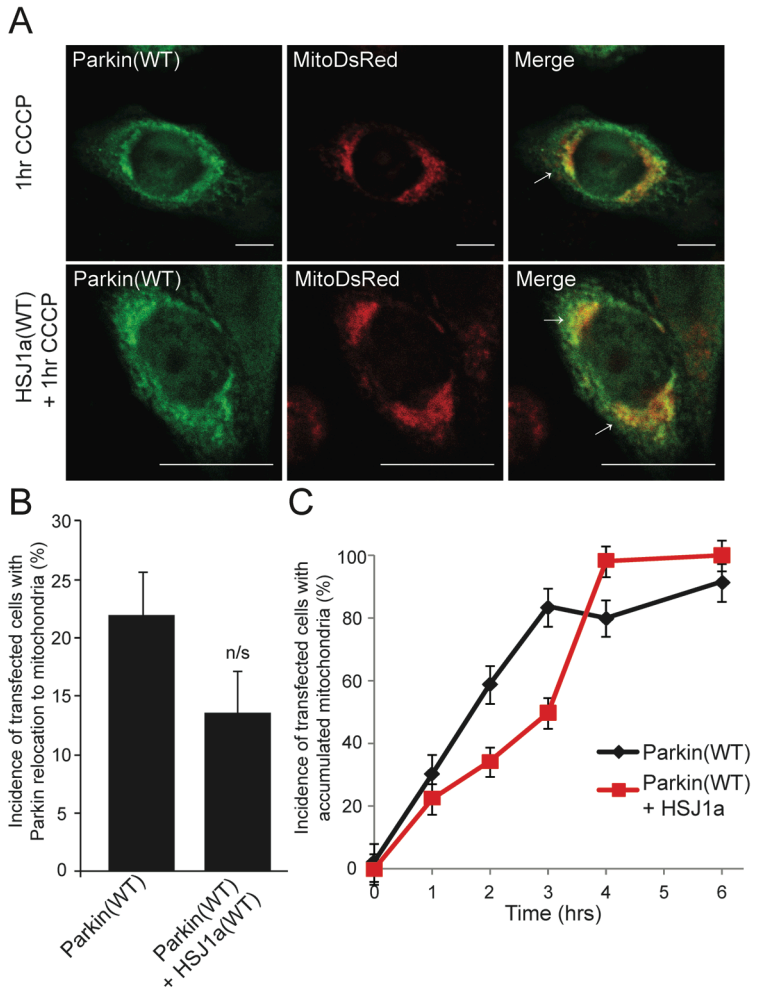

Figure S6. HSJ1a does not affect the relocation of wild-type Parkin to impaired mitochondria. (**A**) SK-N-SH cells were transfected with MitoDsRed, FLAG-Parkin(WT) and myc-HSJ1a(WT). 24 hours after transfection, cells were treated with CCCP (20 μM, 1hr), before fixation in paraformaldehyde and methanol. Cells were immunolabelled with mouse monoclonal anti-FLAG and donkey anti-mouse-Cy2 (green) antibodies. Arrows indicate areas of overlapping Parkin and mitochondrial staining. Scale bar: 10 μM. (**B**) Quantification of the incidence of cells with Parkin relocation to mitochondria in cells co-expressing HSJ1a and treated with CCCP (20 μM, 1hr). 4 groups of more than 100 cells were counted for each condition. Error bars represent ± 2 S.E.; *n*=4, n/s: not significantly different. (**C**) Quantification of the effect of HSJ1a (red) expression on the ability of FLAG-Parkin(WT), compared to FLAG-Parkin(WT) alone (black), to induce the accumulation of damaged mitochondria after CCCP treatment (20 μM, 0 to 6 hrs). 4 groups of more than 100 cells were counted for each condition. Error bars: ± 2 S.E., *n*=4.

Table S1. Co-localization coefficients for Parkin overlap with MitoDsRed in response to CCCP

Conditions	Pearson's coefficient	Mander's coefficient
untreated	0.711	0.225
1 hour CCCP	0.769	0.627
8 hours CCCP	0.798	0.843

Table S2. Co-localization coefficients of Parkin overlap with MitoDsRed after 4 hours CCCP treatment: affect of parkin mutations and chaperones

Conditions	Pearson's coefficient	Mander's coefficient
Parkin(WT)	0.860	0.860
Parkin(C289G)	0.429	0.174
Parkin(C289G) +HSJ1a(WT)	0.740	0.577
Parkin(C289G) +HSJ1a(H31Q)	0.357	0.254
Parkin(C289G) +HSJ1a(ΔUIM)	0.531	0.589
Parkin(C289G) +DNAJB6	0.498	0.367

Table S3. Co-localization coefficients for LC3-GFP overlap with MitoDsRed after 4hrs CCCP treatment in the presence or absence of Parkin(WT)

Conditions	Pearson's coefficient	Mander's coefficient
No Parkin(WT)	0.471	0.031
Parkin(WT)	0.947	0.871

Table S4. Co-localization coefficients of LC3-GFP overlap with Hsp60 after 4 hours CCCP treatment in the presence of Parkin(C289G): the effect of HSJ1a

Conditions	Pearson's coefficient	Mander's coefficient
No HSJ1a	0.227	0.260
HSJ1a	0.298	0.437

Image co-localization analysis.

Images were analysed using the ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) (Abramoff et al., 2004) with JACoP plugin to obtain Pearson's and Mander's thresholded coefficients. Pearson's coefficient values vary between "1" for complete colocalisation and "-1" for total exclusion while readings close to "0" correspond to random localisation of the fluorescent signals. Pearson's coefficient is not dependent on a constant background and image brightness (Menager et al., 2007) but is affected by addition of non-co-localizing signals (e.g. cells with positive staining in one channel only). This problem is addressed by the use of Mander's single-channel specific coefficients measuring the proportion of overlap of each channel with the other. Values vary from "1" for total overlap to "0" for no overlap. Although Mander's coefficients are not affected by the intensity of overlapping pixels (Costes et al., 2004) they are sensitive to a background, making it necessary to set a threshold for each channel. Briefly, RGB.tiff files of confocal images of representative cells (either showing potential overlap or not as determined by the quantitative analyses) were split into 8-bit grayscale channel projections, cleared from background noise outside of cells when necessary and analysed in pairs. Threshold settings were calculated via the modified IsoData method executed by ImageJ.

References

Abramoff M.D., Magelhaes P.J., Ram S.J. (2004) Image Processing with ImageJ. *Biophotonics International*, **11**, 36-42.

Menager M.M., Menasche G., Romao M., Knapnougel P., Ho C.H., Garfa M., Raposo G., Feldmann J., Fischer A., de Saint Basile G. (2007). Secretory cytotoxic granule maturation and exocytosis require the effector protein hMunc13-4. *Nat. Immunol*. **8**, 257–267.

Costes S.V., Daelemans D., Cho E.H., Dobbin Z., Pavlakis G., Lockett S. (2004) Automatic and quantitative measurement of protein-protein colocalization in live cells. *Biophys J*., **86**, 3993-4003.