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Molecular chaperone-mediated rescue of mitophagy by a Parkin RING1 domain mutant

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Mitochondrial dysfunction is characteristic of many neurodegenerative diseases. The Parkinson's disease-associated ubiquitin–protein ligase, Parkin, is important in the elimination of damaged mitochondria by autophagy (mitophagy) in a multistep process. Here, we show that a Parkin RING domain mutant (C289G) fails to redistribute to damaged mitochondria and cannot induce mitophagy after treatment with the mitochondrial uncoupler carbonyl cyanide m-methylhydrazone, because of protein misfolding and aggregation. Parkin(C289G) aggregation and inclusion formation were suppressed by the neuronal DnaJ/Hsp40 chaperone HSJ1a(DNAJB2a). Importantly, HSJ1a and DNAJB6 also restored mitophagy by promoting the relocation of Parkin(C289G) and the autophagy marker LC3 to depolarized mitochondria. The rescue of Parkin activity and suppression of aggregation were J domain dependent for HSJ1a, suggesting the involvement of Hsp70 in these processes, but were not dependent on the HSJ1a ubiquitin interaction motif. HSJ1a expression did not enhance mitophagy mediated by wild-type Parkin. These data show the potential of molecular chaperones to mediate the functional recovery of Parkin misfolding mutants and to combat deficits associated with Parkin aggregation in Parkinson's disease.

INTRODUCTION

Parkinson's disease is the most common neurodegenerative movement disorder. It is characterized clinically by bradykinesia, resting tremor, rigidity and postural instability. Its pathological features include the progressive degeneration of dopaminergic neurons in the midbrain substantia nigra pars compacta and the formation of intracytoplasmic inclusions called Lewy bodies. Most cases of Parkinson's disease are sporadic, although several genes have been associated with familial Parkinson's disease. Mutations in PARK2, the gene encoding the protein Parkin, were linked to autosomal recessive juvenile onset Parkinsonism (1). Structurally, Parkin contains an N-terminal ubiquitin-like domain (Ubl) and two C-terminal RING domains. It functions as a ubiquitin–protein ligase (2–4) and has been implicated in the formation of Lewy bodies (5).

Recent evidence suggests that Parkin is involved in mitochondrial dynamics. This activity may relate to its neuroprotective functions as it reduces cytochrome *c* release from damaged mitochondria, thereby preventing subsequent induction of the apoptotic cascade (6). Parkin knockout flies have a reduced lifespan, locomotor deficits, male sterility, mitochondrial defects and increased oxidative stress (7). Mutations in PTEN-induced putative kinase 1 (PINK1) cause autosomal recessive familial Parkinsonism (PARK6), and PINK1 is a mitochondria-targeted serine–threonine kinase. PINK1 is also an important regulator of mitochondrial homeostasis. Interestingly, Parkin and PINK1 knockout flies have a similar phenotype (8,9), and overexpression of Parkin can rescue the PINK1 fly knockout phenotype. The phosphorylation of Parkin by PINK1 has been suggested to mediate its relocation to mitochondria (10). A small percentage of Parkin is detected in mitochondrial cristae in proliferating cells (11), but significantly Parkin redistributes to mitochondria when their mitochondrial membrane potential is disrupted by uncoupling agents or reactive oxygen species (12). Moreover, Parkin appears to interact either directly or indirectly with several proteins that are involved in mitochondrial metabolism (13).

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Parkin has recently emerged as an important factor in mitochondrial quality control mechanisms. Indeed, the mitochondrial morphological abnormalities associated with Parkin deficiency highlight a role for Parkin in mitochondrial fusion and fission events (14). Overexpression of dynamin-related protein 1 (Drp1), which promotes mitochondrial fission, rescued the mitochondrial morphological defects in Parkin-deficient flies (15). Moreover, Parkin overexpression can protect against deleterious mtDNA mutations (16). Mitochondrial fission is required for the selective degradation of depolarized mitochondria by autophagy (mitophagy) (17). Interestingly, a function for Parkin in mitophagy has also been described (12). This function appears to be a multistep process that involves PINK1. Initially, PINK1 is stabilized by mitochondrial depolarization, and the kinase activity of PINK1, either directly or indirectly, leads to the recruitment of Parkin to mitochondria. Parkin then stimulates the perinuclear accumulation of mitochondria, via a microtubule-dependent mechanism, before the recruitment of LC3 and the remaining autophagic machinery to stimulate mitochondrial degradation (18–23).

Pathogenic Parkin mutants have been useful in delineating these steps. For example, some mutants are competent for recruitment to depolarized mitochondria, but are unable to stimulate the degradation of damaged mitochondria (19–23). The propensity of some disease-related Parkin mutants to misfold and aggregate into insoluble inclusion bodies, however, has made it difficult to discriminate between residues that are essential for these functions and those that are only important for correct folding. For example, RING1 domain mutants appear to be the most prone to misfolding and aggregation (24,25). Conserved cysteine residues in Parkin's RING domains provide structural stability to the protein (26), explaining the higher misfolding propensity of these mutants. However, mutations located outside of the RING fingers can also reduce the solubility of Parkin and lead to its aggregation into inclusion bodies (27–31).

Molecular chaperones are important for facilitating correct protein folding, the cellular response to protein misfolding and the degradation of misfolded proteins (32). Members of the Hsp70 family of molecular chaperones participate in the folding and assembly of newly synthesized proteins, aggregation prevention, dissolution and refolding of aggregated proteins, translocation of proteins across membranes, clathrin uncoating and protein degradation (33). Hsp70 activity is regulated by co-chaperones of the Hsp40/DnaJ family that stimulate ATP hydrolysis and substrate binding (34). The neuronal DnaJ/Hsp40-like chaperone HSJ1(DnaJB2) combines an N-terminal J domain through which it regulates Hsp70 activity and two C-terminal ubiquitin interaction motifs (UIMs) to promote the sorting of ubiquitylated substrate proteins to the proteasome for degradation (35). The smaller cytoplasmic and nuclear isoform HSJ1a reduced protein aggregation and inclusion body formation in models of polyglutamine expansion diseases (35–37).

The aim of this study was to test the hypothesis that expression of HSJ1a could inhibit the aggregation of mutant Parkin and provide proof of concept that molecular chaperones can functionally rescue a Parkin misfolding mutant for mitophagy.

RESULTS

The Parkin misfolding mutant C289G is defective for mitophagy

The expression of wild-type Parkin [FLAG-Parkin(WT)] and the disease-related Parkin mutants FLAG-Parkin(R42P) and FLAG-Parkin(C289G) were compared in SK-N-SH neuroblastoma cells. SK-N-SH cells express low levels of endogenous Parkin compared with SH-SY-5Y cells, and transient transfection increased the level of Parkin dramatically (Supplementary Material, Fig. S1). Immunocytochemistry revealed that Parkin(WT) localized predominantly to the cytoplasm of cells (Fig. 1). In a minority (5%) of cells, FLAG-Parkin(WT) formed small cytoplasmic inclusions (Fig. 1A and B). The FLAG-Parkin(R42P) mutant, which carries a mutation in Parkin's Ubl domain, was expressed at levels similar to Parkin(WT), but showed a tendency to misfold. Indeed, Parkin(R42P) aggregated into multiple small perinuclear inclusions in approximately 22% of cells ($P < 0.05$ compared with WT) and more was present in the pellet fraction (Supplementary Material, Fig. S1B). In contrast, the RING1 domain mutant FLAG-Parkin(C289G) was expressed at slightly lower levels than Parkin(WT) and was strongly aggregation-prone (Fig. 1 and Supplementary Material, Fig. S1B). A larger proportion was detergent-insoluble and formed multiple large inclusions throughout the cytoplasm and nucleus in approximately 86% of cells ($P < 0.001$ compared with WT). To confirm that the Parkin(C289G) inclusions were aggregates of misfolded protein, myc-ubiquitin or GFP-Hsp70 and FLAG-Parkin(C289G) were transfected into SK-N-SH cells (Supplementary Material, Fig. S2). Cytoplasmic and nuclear FLAG-Parkin(C289G) inclusions were decorated with ubiquitin and Hsp70. Moreover, all Parkin inclusions were positive for ubiquitin or Hsp70. These findings suggest that ubiquitin and Hsp70 associate with the mutant Parkin and that FLAG-Parkin(C289G) is probably misfolded and aggregated.

Based on the findings described earlier, we tested whether these disease-associated Parkin mutants would retain their ability to induce mitophagy (12). To investigate this hypothesis, SK-N-SH cells were transfected with the mitochondrial marker MitoDsRed and FLAG-Parkin(WT), FLAG-Parkin(R42P) or FLAG-Parkin(C289G), before treatment with the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP). Mitochondrial dynamics were assessed over time (Fig. 1, Supplementary Material, Fig. S3). The incidence of cells with overlapping FLAG-Parkin(WT) and MitoDsRed staining was quantified over an 8 h time-course (Fig. 1C and D). Mitochondria appeared similar in untransfected cells and in MitoDsRed-only transfected cells (data not shown), suggesting that transfection with a mitochondrial marker did not alter the mitochondrial morphology. SK-N-SH cells express low levels of endogenous Parkin (Supplementary Material, Fig. S1A), which enabled us to investigate the effect of Parkin overexpression on mitochondrial dynamics.

In untreated cells, FLAG-Parkin(WT) staining was cytoplasmic and did not overlap with the MitoDsRed staining (Supplementary Material, Fig. S3A and Table S1). However, after 1 h CCCP treatment, the FLAG-Parkin(WT) staining

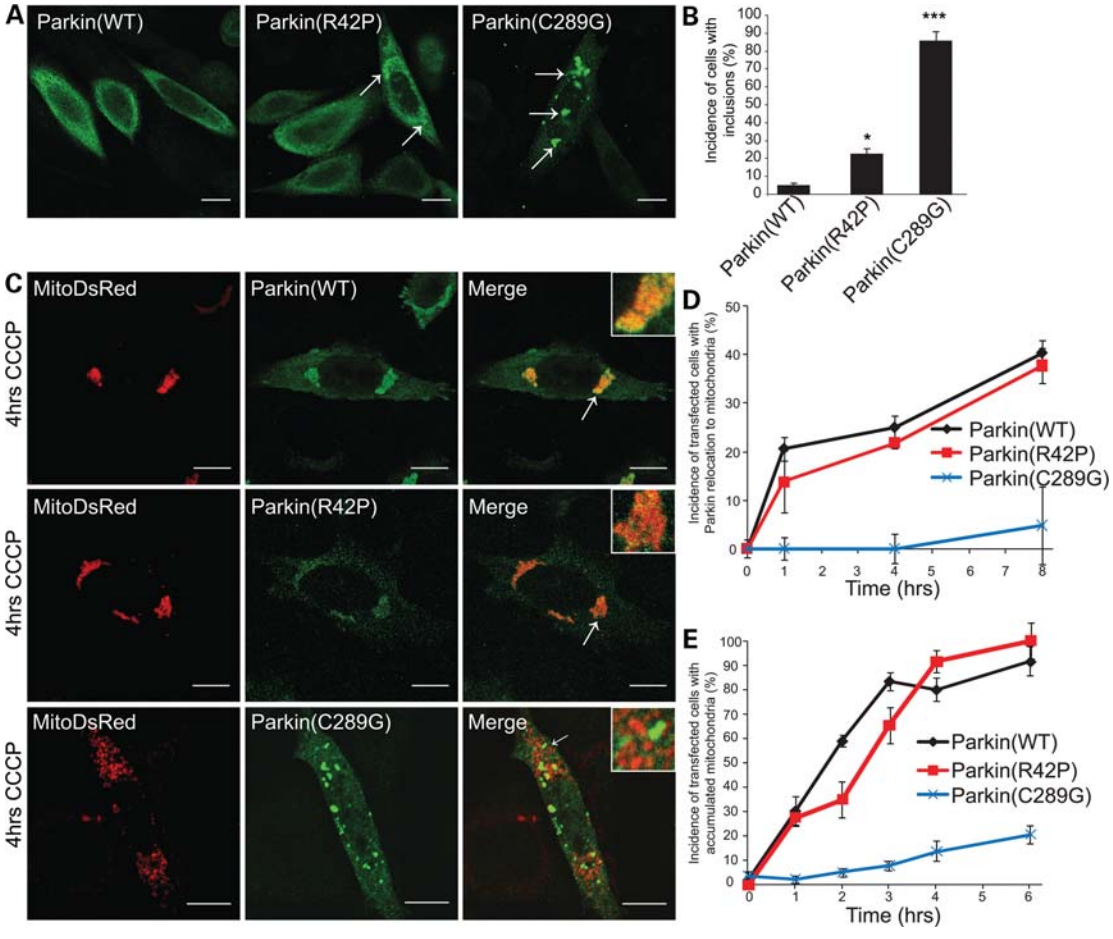

Figure 1. Parkin(C289G) misfolds and does not traffic to depolarized mitochondria. (**A**) SK-N-SH cells were transiently transfected with FLAG-Parkin(WT), FLAG-Parkin(R42P) or FLAG-Parkin(C289G). Twenty-four hours post-transfection, cells were fixed in methanol and immunostained with mouse monoclonal anti-FLAG and donkey anti-mouse-Cy2 antibodies. Arrows indicate intracellular inclusions. Scale bar: 10 μm. (**B**) Quantification of inclusion incidence in cells transfected with wild-type or mutant Parkin. Four groups of more than 100 cells were counted for each condition. Error bars represent standard errors ($\pm$ SE). Asterisks indicate a significant difference between wild-type and mutant Parkin ($^{*}P < 0.05$ and $^{***}P < 0.001$). (**C**) SK-N-SH cells were transfected with MitoDsRed (red) and FLAG-Parkin(WT), FLAG-Parkin(R42P) or FLAG-Parkin(C289G). Twenty hours post-transfection, cells were treated with CCCP (20 μM, 4 h) before fixation in paraformaldehyde and methanol. Cells were immunolabeled with anti-FLAG (green). Arrows indicate areas that are magnified in the inset to show overlap or no overlap between the FLAG-Parkin and mitochondrial staining. Scale bar: 10 μm. (**D**) The incidence of cells with accumulated mitochondria was quantified in cells overexpressing MitoDsRed and FLAG-Parkin(WT) (black), FLAG-Parkin(R42P) (red) or FLAG-Parkin(C289G) (blue) and treated with CCCP (20 μM, 0–6 h). Error bars: $\pm$2 SE, $n = 4$. (**E**) Quantification of the relocation of Parkin mutants to mitochondria. SK-N-SH cells were transfected with MitoDsRed and FLAG-Parkin(WT) (black), FLAG-Parkin(R42P) (red) or FLAG-Parkin(C289G) (blue). Twenty-four hours post-transfection, cells were treated with CCCP (20 μM, 0–8 h). Error bars: $\pm$2 SE, $n = 4$.

became punctuate and overlapped with the MitoDsRed staining (Fig. 1C; Supplementary Material, Fig. S3A and Table S1). FLAG-Parkin(R42P) was also recruited to damaged mitochondria (Fig. 1C) as efficiently as Parkin(WT) (Fig. 1D). In contrast, FLAG-Parkin(C289G) staining did not overlap with the MitoDsRed staining, as it was mainly observed in distinct intracellular inclusions (5% overlap with mitochondria after 8 h CCCP treatment) (Fig. 1C and D; Supplementary Material, Table S2). This suggested that the Parkin RING1 domain mutant could not redistribute to damaged mitochondria because of protein misfolding, whereas Parkin(R42P) was still capable of folding sufficiently to be recruited to mitochondria.

FLAG-Parkin(WT) expression induced the accumulation of impaired mitochondria to perinuclear clusters (91% of transfected cells after 6 h of CCCP treatment) (Fig. 1E; Supplementary Material, Fig. S3). Similarly, the Parkin Ubl domain mutant FLAG-Parkin(R42P) retained its ability to mediate mitochondrial clustering (100% of transfected cells after 6 h of CCCP treatment) (Fig. 1E). However, the RING1 domain mutant FLAG-Parkin(C289G) failed to stimulate mitochondrial clustering (21% after 6 h of CCCP treatment) (Fig. 1E). In the absence of overexpressed FLAG-Parkin, only 10% of the cells had clustered mitochondria after 8 h of CCCP treatment (Supplementary Material, Fig. S3B). These results suggest that Parkin misfolding resulting from C289G amino acid substitution severely compromises the ability of Parkin to modulate mitochondrial dynamics.

Impaired mitochondria are degraded by autophagy (12,17,38). To confirm that Parkin was stimulating autophagy of impaired mitochondria in SK-N-SH cells, we investigated whether the autophagy marker GFP-LC3 would co-localize to damaged mitochondria in FLAG-Parkin(WT)-transfected

cells treated with CCCP for 4 h (Supplementary Material, Fig. S3C). CCCP treatment for 4 h did not alter the cytoplasmic and nuclear localization of GFP-LC3; however, with Parkin overexpression, an overlap of GFP-LC3 staining was observed in the majority of mitochondria [FLAG-Parkin(WT) 58% of cells compared with 1% with no Parkin $P < 0.001$] (Supplementary Material, Fig. S3 and Table S3). To test whether Parkin induces the degradation of damaged mitochondria by autophagy, SK-N-SH cells transfected with FLAG-Parkin(WT) and GFP-LC3 were treated with CCCP and the autophagy inhibitor 3-methyladenine (3-MA) for 24 h (Supplementary Material, Fig. S4). Cells were fixed and immunolabeled with anti-Hsp60 antibody, as a mitochondrial marker. Only 30% of transfected cells that were treated with CCCP for 24 h were immunopositive for Hsp60 staining, suggesting that most cells had lost their mitochondria (Supplementary Material, Fig. S4). In contrast, treatment with the autophagy inhibitor 3-MA significantly increased the number of cells with mitochondrial staining (63% compared with 30%, $P < 0.001$) (Supplementary Material, Fig. S4). In these cells, mitochondria were still clustered around the nucleus. As expected, following inhibition of PI3-kinase by 3-MA, the GFP-LC3 staining did not overlap with the mitochondrial staining. These findings support the hypothesis that Parkin induces the elimination of impaired mitochondria by autophagy.

To verify that Parkin(C289G) was defective for inducing mitophagy, SK-N-SH cells transfected with FLAG-Parkin(C289G) and GFP-LC3 were treated with CCCP and 3-MA for 24 h (Supplementary Material, Fig. S4). Cells were fixed and immunolabeled with an anti-Hsp60 antibody. The majority (90%) of transfected cells treated with CCCP for 24 h were immunopositive for Hsp60, suggesting that FLAG-Parkin(C289G) failed to induce mitophagy. Treatment with the autophagy inhibitor 3-MA resulted in a small, but not statistically significant, increase in the percentage of cells that were immunopositive for Hsp60 staining (97%) (Supplementary Material, Fig. S4). In addition, cells that were subjected to 3-MA treatment displayed LC3 accumulation around the nucleus, which did not overlap with mitochondria. We hypothesized that the accumulation of LC3 around the nucleus may represent LC3 redistribution to inclusions formed by aggregated mutant Parkin and that these inclusions may have been labeled by LC3 prior to the 3-MA treatment. Consequently, inhibition of autophagy by 3-MA treatment would have sustained the accumulation of LC3-decorated mutant Parkin. To test further the hypothesis that Parkin(C289G) might be targeted for autophagic degradation, SK-N-SH cells were transfected with FLAG-Parkin(C289G) and the autophagy marker mCherry-ATG5 (Supplementary Material, Fig. S4F). ATG5 was recruited to the inclusions formed by the mutant Parkin, suggesting that misfolded, aggregated Parkin(C289G) may be subject to autophagic, as well as proteasomal (27,39,40), degradation.

HSJ1a reduces misfolding and aggregation of mutant Parkin

The human neuronal co-chaperone HSJ1a reduces protein aggregation in polyglutamine expansion models of protein misfolding diseases (35–37). To test whether HSJ1a would similarly reduce Parkin inclusion formation, SK-N-SH cells were co-transfected with FLAG-Parkin(C289G) and myc-HSJ1a (Fig. 2). As before, most cells overexpressing FLAG-Parkin(C289G) formed multiple large inclusions throughout the cytoplasm and nucleus, whereas co-expression of HSJ1a significantly reduced FLAG-Parkin(C289G) inclusion incidence (17% compared with 81%, $P < 0.001$) (Fig. 2A and B). Importantly, HSJ1a not only reduced the number of cells with inclusions, but also affected the number of inclusions per cell. Indeed, although most cells did not have inclusions, those that did have an inclusion formed one or two juxtanuclear inclusions (Fig. 2A). In these cells, HSJ1a staining overlapped with the Parkin inclusion, suggesting that HSJ1a recognized misfolded Parkin.

The ability of HSJ1 to reduce inclusion formation in a model of polyglutamine expansion disease is J and UIM domain dependent (35). Therefore, we tested whether the effect of HSJ1a on Parkin inclusions required these domains (Fig. 2). SK-N-SH cells were transfected with FLAG-Parkin(C289G) and myc-HSJ1a(H31Q) or myc-HSJ1a (ΔUIM). The H31Q mutation within the J domain disrupts the HSJ1 interaction with Hsp70 and the HSJ1 stimulation of Hsp70 ATPase activity. The myc-HSJ1a(H31Q) mutant did not significantly reduce the incidence of cells with Parkin inclusions (83% compared with 81%, not significant) (Fig. 2A and B). The co-localization of myc-HSJ1a(H31Q) with mutant Parkin was preserved (Fig. 2A). This may reflect that the HSJ1a J domain mutant can still bind to, but not prevent the aggregation, or facilitate the degradation of mutant Parkin. In contrast, the myc-HSJ1a(ΔUIM) mutant significantly reduced Parkin(C289G) inclusion incidence (27% compared with 81%, $P < 0.001$) and was almost as efficient as myc-HSJ1a (27% compared with 17%, $P < 0.05$). Like wild-type HSJ1a, the HSJ1a(ΔUIM) mutant reduced the number and size of inclusions per cell and decorated the remaining inclusions (Fig. 2A).

A filter trap assay was performed to test the effect of HSJ1a on Parkin aggregation (Fig. 2C). FLAG-Parkin(C289G) and myc-HSJ1a were transfected into SK-N-SH cells. Twenty-four hours post-transfection, cells were lysed in sodium dodecyl sulfate (SDS) lysis buffer. Cell lysates were applied onto a cellulose acetate membrane in a dot blot apparatus and analyzed by immunoblotting with mouse monoclonal anti-Parkin antibody. Whereas myc-HSJ1a and myc-HSJ1a(ΔUIM) reduced by 75% the amount of insoluble Parkin that was retained on the cellulose acetate membrane, no reduction was observed when myc-HSJ1a(H31Q) was co-expressed (Fig. 2C). These data suggest that a functional J domain, but not the UIM domain, is required for HSJ1a to reduce Parkin aggregation.

The molecular chaperone HSJ1 functionally rescues Parkin(C892G) for mitophagy

The lack of a requirement for the HSJ1a UIM domain, which is important for recognizing and protecting ubiquitin chains as part of proteasomal targeting (35), suggested that HSJ1a could be stimulating some refolding of Parkin and not just enhancing the degradation of the misfolded mutant protein. To test this hypothesis, we investigated whether HSJ1a could rescue the relocation of Parkin(C289G) to damaged mitochondria

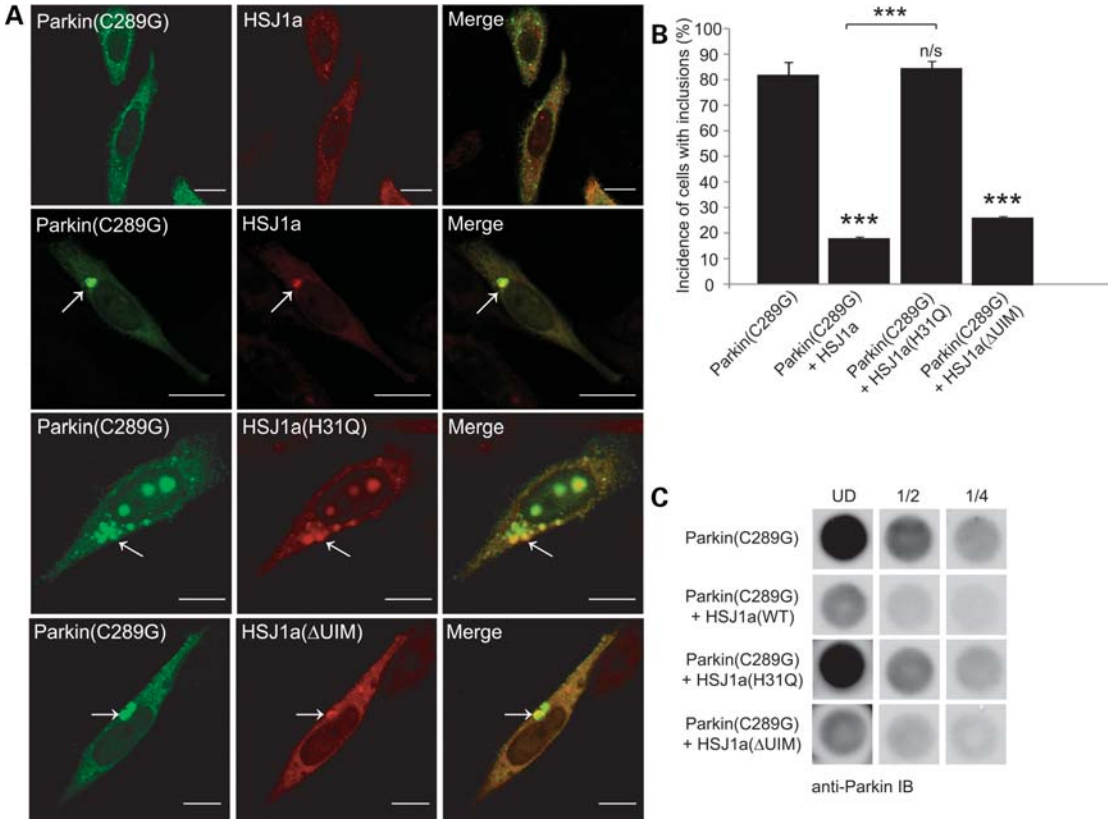

Figure 2. HSJ1a reduces the aggregation of Parkin(C289G). (**A**) SK-N-SH cells were transfected with FLAG-Parkin(C289G) and myc-HSJ1a(WT), myc-HSJ1a(H31Q) or myc-HSJ1a(ΔUIM), as indicated. Twenty-four hours post-transfection, cells were fixed in methanol and immunostained with anti-FLAG (green) and sheep anti-HSJ1 antibodies (red). The upper panel shows a representative image, whereas the middle and lower panels show co-localization of HSJ1a in a rare inclusion-bearing cells. Arrows indicate inclusions. Scale bar: 10 μm. (**B**) Quantification of the incidence of cells with inclusions in cells overexpressing FLAG-Parkin(C289G) and myc-HSJ1a, myc-HSJ1a(H31Q) or myc-HSJ1a(ΔUIM). Error bars: ± 1 SE, $n = 4$, $^{***}P < 0.001$, n/s: not significant. (**C**) Filter trap showing HSJ1a reduction of mutant Parkin aggregation. SK-N-SH cells were transfected with FLAG-Parkin(C289G) and myc-HSJ1a(WT), myc-HSJ1a(H31Q) or myc-HSJ1a(ΔUIM), as indicated. Cells were lysed in 2% SDS 24 h post-transfection. Total cell lysates were diluted in 2% SDS lysis buffer. An aliquot of 100 μl of each cell lysate was loaded onto a cellulose acetate membrane on a dot blot apparatus. Samples were analyzed by immunoblotting with mouse monoclonal anti-Parkin and goat anti-mouse-HRP (UD: undiluted; 1/2 and 1/4: dilution factors).

(Fig. 3). SK-N-SH cells were transfected with MitoDsRed, FLAG-Parkin(C289G) and myc-HSJ1a(WT). Twenty hours post-transfection, cells were treated with CCCP for 4 h before fixation and immunolabeling with mouse monoclonal anti-FLAG antibody. As described earlier, the RING1 domain mutant FLAG-Parkin(C289G) failed to relocate to impaired mitochondria and to induce their mitophagy. In addition, FLAG-Parkin(C289G) formed multiple large cytoplasmic and nuclear inclusions. However, co-expression with myc-HSJ1a(WT) appeared partially to rescue the relocation of FLAG-Parkin(C289G) to damaged mitochondria. Indeed, the FLAG-Parkin(C289G) staining overlapped with the MitoDsRed staining in many cells (37% after 8 h) (Fig. 3B; Supplementary Material, Table S2), and the staining overlapped entire mitochondria.

To investigate further whether the HSJ1a-mediated rescue of mutant Parkin's relocation to damaged mitochondria was dependent on the HSJ1a anti-aggregation activity, SK-N-SH cells were transiently transfected with MitoDsRed, FLAG-Parkin(C289G) and myc-HSJ1a(H31Q) or myc-HSJ1a (ΔUIM), followed by CCCP treatment (Fig. 3A and B). The myc-HSJ1a(H31Q) failed to rescue FLAG-Parkin(C289G) redistribution to damaged mitochondria. Indeed, after 8 h of CCCP treatment, only 11% of myc-HSJ1a(H31Q)-positive cells showed relocation of FLAG-Parkin(C289G) to mitochondria, compared with 37% in cells overexpressing myc-HSJ1a(WT) (Fig. 3B). In contrast, myc-HSJ1a(ΔUIM) partially rescued the relocation of mutant Parkin to mitochondria in 19% of transfected cells (Fig. 3B, Supplementary Material, Table S2).

The ability of HSJ1a to restore Parkin(C289G) relocalization to mitochondria was confirmed by a mitochondrial fractionation assay (Fig. 3C). SK-N-SH cells were transiently transfected with FLAG-Parkin(C289G) and myc-HSJ1a(WT). At 20 h post-transfection, cells were treated with CCCP for 4 h before cell lysis and differential fractionation into cytosolic and mitochondrial fractions. Cell lysates from each condition were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and subjected to western blotting with mouse monoclonal anti-FLAG and mouse monoclonal anti-Hsp60 antibodies. FLAG-Parkin(C289G) was exclusively detected in the cytosolic fraction. However, upon myc-HSJ1a(WT) co-expression, some of the FLAG-Parkin(C289G) shifted to the mitochondrial fraction following CCCP treatment. These data support the hypothesis

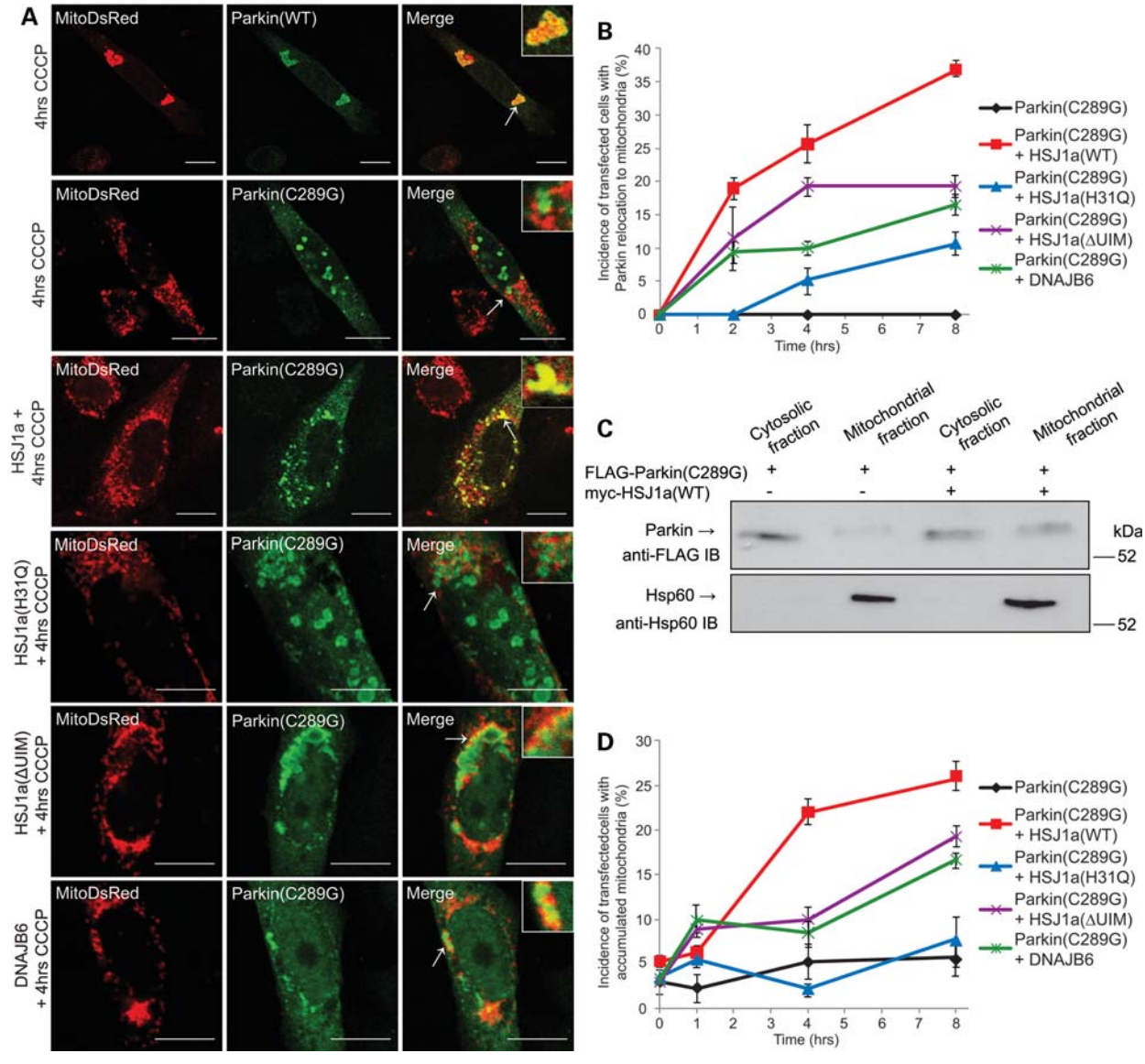

Figure 3. HSJ1a rescues the relocation of Parkin(C289G) to damaged mitochondria. (**A**) SK-N-SH cells transfected with the mitochondrial marker MitoDsRed (red), FLAG-Parkin(WT) (green), FLAG-Parkin(C289G) (green) and myc-HSJ1a(WT), myc-HSJ1a(H31Q), myc-HSJ1a(ΔUIM) or myc-DNAJB6, as indicated. Twenty hours post-transfection, cells were treated with CCCP (20 μM, 4 h) before fixation in paraformaldehyde and methanol. Cells were immunolabeled with anti-FLAG (green). Arrows indicate areas that are magnified in the inset to show overlap or no overlap between FLAG-Parkin and mitochondrial staining. Scale bar: 10 μm. (**B**) Quantification of the effect of myc-HSJ1a(WT) (red) or myc-HSJ1a(H31Q) (blue), myc-HSJ1a(ΔUIM) (purple), myc-DNAJB6 (green) or no chaperone (black) on the relocation of FLAG-Parkin(C289G) to mitochondria. Error bars: ± 2 SE, $n = 4$. (**C**) Cell fractionation assay. SK-N-SH cells were transiently transfected with FLAG-Parkin(C289G) $\pm$ myc-HSJ1a(WT), as indicated. Twenty hours post-transfection, cells were treated with CCCP (20 μM, 4 h) before cell lysis. Cell lysates were fractionated into cytosolic and mitochondrial fractions. An aliquot of 20 μl of cell lysates or equivalent fraction was resolved by SDS–PAGE and western blot analysis was performed with anti-FLAG and anti-Hsp60 antibodies. The position of the molecular size markers (in kDa) is shown on the right. (**D**) Quantification of the effect of myc-HSJ1a(WT) (red), myc-HSJ1a(H31Q) (blue), myc-HSJ1a(ΔUIM) (purple), myc-DNAJB6 (green) or no chaperone (black) on the ability to rescue mitochondrial accumulation induced by FLAG-Parkin(C289G). Error bars: ± 2 SE, $n = 4$.

that HSJ1a can partially rescue the redistribution of a Parkin RING1 domain mutant to impaired mitochondria.

HSJ1a(DNAJB2a) is a member of a subfamily of type II DnaJ proteins that share C-terminal homology beyond the J domain (41). Other proteins within this subfamily, DNAJB6 and DNAJB8, do not share the UIM domain with HSJ1, but are also potent anti-aggregation chaperones (42). The levels of *DNAJB6* and *HSJ1* mRNA transcripts are increased in Parkinson's disease brains (43), and DNAJB6 is a component of Lewy bodies (44). As the UIM domain of HSJ1 did not appear to be critical, we assessed the ability of DNAJB6 to restore Parkin(C289G) relocation to damaged mitochondria. We found that myc-DNAJB6 behaved similar to the HSJ1a(ΔUIM) mutant and rescued the redistribution of Parkin(C289G) in 16% of cells (Fig. 3B).

It remained to be determined whether HSJ1a's ability to rescue mutant Parkin relocation to impaired mitochondria could also restore later steps in the mitophagy pathway. To test this hypothesis, the incidence of cells with clustered perinuclear mitochondria was quantified (Fig. 3D). Cells

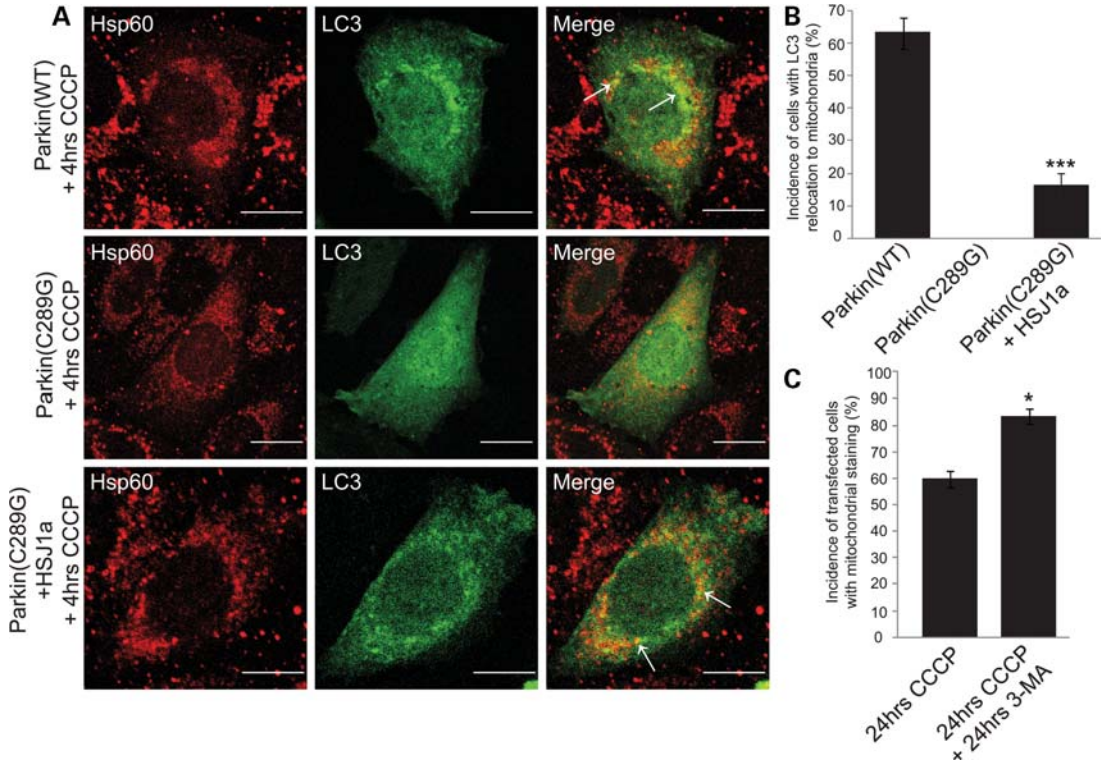

Figure 4. HSJ1a rescues Parkin(C289G) for mitophagy. (**A**) SK-N-SH cells co-transfected with FLAG-Parkin(WT) or FLAG-Parkin(C289G), myc-HSJ1a(WT) and GFP-LC3 (green) were treated with CCCP (20 μM, 4 h) and immunolabeled with anti-Hsp60 (red). (**B**) The percentage of cells with overlapping GFP-LC3 and MitoDsRed staining was quantified in cells co-transfected with FLAG-Parkin(WT), FLAG-Parkin(C289G) ± myc-HSJ1a(WT) and treated with CCCP (20 μM, 4 h). Error bars: ±2 SE, $n = 4$, $^{***}P < 0.001$. (**C**) Quantification of the incidence of transfected cells with mitochondrial staining in cells treated with CCCP (20 μM, 24 h, left bar), CCCP + 3-MA (10 mM, 24 h, right bar). Error bars: ±2 SE, $n = 4$, $^{*}P < 0.5$.

transfected with FLAG-Parkin(C289G) failed to undergo mitochondrial clustering. In contrast, co-expression of myc-HSJ1a(WT) with FLAG-Parkin(C289G) rescued the accumulation of damaged mitochondria in up to 26% of transfected cells [compared with 6% in cells overexpressing FLAG-Parkin(C289G)] (Fig. 3D). The J domain mutant myc-HSJ1a(H31Q) failed to have an effect, as only 8% of the cells displayed signs of mitochondrial accumulation (Fig. 3D). myc-HSJ1a(ΔUIM) and myc-DNAJB6 had an intermediate effect between HSJ1a and HSJ1a(H31Q), as they restored mitochondrial clustering in 19 and 17% of cells, respectively (Fig. 3D). This supports the hypothesis that HSJ1a's ability to rescue mutant Parkin relocation to impaired mitochondria also restores mitochondrial accumulation and that this effect requires a functional J domain.

To test whether HSJ1a can rescue the later stages of Parkin-mediated mitophagy for Parkin(C289G), the relocation of GFP-LC3 to mitochondria was quantified in cells expressing FLAG-Parkin(WT), FLAG-Parkin(C289G) and myc-HSJ1a (WT), which were treated with CCCP for 4 h (Fig. 4A; Supplementary Material, Table S4). In cells expressing FLAG-Parkin(WT), the LC3 staining overlapped with the mitochondrial staining (63%). In contrast, cells expressing FLAG-Parkin(C289G) did not demonstrate any overlap between the LC3 and mitochondrial staining. However, co-expression of myc-HSJ1a(WT) with FLAG-Parkin(C289G) significantly increased the incidence of cells with LC3 staining on mitochondria [16% compared with 0% in FLAG-Parkin(C289G)-expressing cells, $P < 0.001$]. These findings suggest that HSJ1a partially recovers the ability of a Parkin RING1 domain mutant to induce the redistribution of LC3 to depolarized mitochondria.

To verify whether the rescued Parkin RING1 domain mutant could fully induce the clearance of damaged mitochondria by mitophagy, SK-N-SH cells transfected with FLAG-Parkin(C289G), myc-HSJ1a(WT) and GFP-LC3 were treated with CCCP and the autophagy inhibitor 3-MA for 24 h (Fig. 4C). Cells were fixed and immunolabeled with an anti-Hsp60 antibody. About 60% of FLAG-Parkin(C289G)- and myc-HSJ1a(WT)-expressing cells were immunopositive for Hsp60 staining, suggesting that 40% of cells had lost their mitochondria [with Parkin(C289G) alone only 10% lost their mitochondria, Supplementary Material, Fig. S4D]. In contrast, treatment with the autophagy inhibitor 3-MA significantly increased the number of cells with mitochondrial staining (83% compared with 60%, $P < 0.5$). These findings support the hypothesis that the chaperone-mediated rescued Parkin(C289G) is fully competent for enhancing mitophagy of damaged mitochondria.

Preferential co-immunoprecipitation of HSJ1a and Parkin(C289G)

To confirm that HSJ1a could be acting directly on Parkin, we investigated whether the proteins could be co-precipitated in a complex. Reciprocal co-immunoprecipitation showed that a

small proportion of myc-HSJ1a or FLAG-Parkin(WT) precipitated specifically in the presence of the other binding partner (Supplementary Material, Fig. S5A). In contrast, there was greater binding of Parkin(C289G) to HSJ1a compared with Parkin(WT) despite lower levels of protein in the input (Supplementary Material, Fig. S5B). Therefore, HSJ1a can bind both Parkin(WT) and Parkin(C289G), but binds the mutant protein preferentially.

HSJ1a does not enhance mitophagy of wild-type Parkin

Based on the ability of HSJ1a to bind Parkin and partially restore the relocation of a Parkin RING1 domain mutant to mitochondria, thereby rescuing the enhancement of mitophagy, it was hypothesized that HSJ1a may also modulate the activity of wild-type Parkin in mitophagy. To test this hypothesis, SK-N-SH cells were transfected with MitoDsRed, FLAG-Parkin(WT) and myc-HSJ1a(WT) (Supplementary Material, Fig. S6). Twenty-four hours after transfection, cells were treated with CCCP for 1 h and immunolabeled with an anti-FLAG antibody. Co-expression of myc-HSJ1a(WT) with FLAG-Parkin(WT) did not appear to modulate the relocation of wild-type Parkin to damaged mitochondria, which clustered around the nucleus (Supplementary Material, Fig. S6A). Quantification of the effect of HSJ1a co-expression on wild-type Parkin relocation to damaged mitochondria revealed that myc-HSJ1a(WT) expression reduced the incidence of cells with FLAG-Parkin(WT) redistribution to mitochondria (13% compared with 22% after 1 h), although the effect was not significantly different (Supplementary Material, Fig. S6B). Furthermore, myc-HSJ1a(WT) expression appeared initially to delay FLAG-Parkin(WT)-induced mitochondrial clustering, although no difference was observed after prolonged CCCP treatment [100% with myc-HSJ1a(WT) compared with 91% in control cells after 6 h CCCP] (Supplementary Material, Fig. S6C). These findings suggest that HSJ1a overexpression does not affect the function of wild-type Parkin in mitophagy.

DISCUSSION

The elimination of damaged mitochondria by mitophagy is an important quality control mechanism. We have confirmed that the ubiquitin–protein ligase Parkin induces mitophagy by redistributing to mitochondria after membrane depolarization with the mitochondrial uncoupler CCCP. Parkin stimulated the accumulation of impaired mitochondria around the nucleus, induced the relocation of the autophagy marker LC3 to damaged mitochondria and thus facilitated their elimination by mitophagy. These findings are reminiscent of the findings of Narendra *et al.* (12), demonstrating that Parkin selectively eliminates depolarized mitochondria by mitophagy. This role for Parkin in mitochondrial quality control has also been confirmed by others (18–23). The exact mechanism whereby Parkin senses and relocates to damaged mitochondria is currently not clear, but appears to be dependent on PINK1 relocation to, or accumulation in, mitochondria (22,45). However, ubiquitylation of PINK1 by Parkin, or phosphorylation of Parkin by PINK1, *per se* does not appear to be required (46). A role for PINK1 in mitochondrial fission has also been demonstrated (47). Recently, a study has shown that Parkin, PINK1 and DJ-1 form a ubiquitin–protein ligase complex that promotes the catalytic activity of Parkin (48). Whether this complex is implicated in Parkin-mediated mitophagy remains to be determined.

The redistribution of Parkin to mitochondria was inhibited by the disease-related Parkin(C289G) mutation, which could not induce mitophagy, in agreement with other studies (19,22). In contrast, the disease-related Parkin Ubl domain mutant Parkin(R42P) retained its ability to relocate to, and induce mitophagy of, damaged mitochondria. These findings are supported by previous studies demonstrating that R42P and other mutants in the Ubl domain are competent for enhancing mitophagy (19,21–23); however, Lee *et al.* (20) suggested that the R42P mutant was inactive. The reasons for this difference are not clear, but may reflect an increased tendency for R42P to misfold and aggregate in some cell systems. In our assay, the R42P mutant was prone to misfold and aggregate, but not enough to affect significantly its ability to recruit to mitochondria. Therefore, Parkin translocation to mitochondria is RING1 dependent, but does not require the Ubl domain (10). We propose that some RING1 domain residues are not required for the relocation of Parkin to mitochondria *per se*, but rather that they are necessary for the correct folding of Parkin into its final functional structure. Indeed, several studies have shown that Parkin RING domain mutants are prone to misfold (24,25), possibly reflecting the importance of conserved cysteine residues in Parkin's RING domain to bind Zn^{2+} and provide structural stability. In contrast, other mutations in the RING1 domain can result in fully mitophagy-competent proteins (R256C), partially functional (R275W) or soluble, but non-functional proteins (T204R) (19), such that the importance of this domain for Parkin's role in mitophagy still needs to be determined. Similarly, the Parkin(R42P) mutant may cause disease through a distinct, misfolding-independent mechanism such as disruption of its association with interacting partners or the proteasome. As we discover the distinct biochemical and cellular phenotypes associated with Parkin mutations, it will be interesting to determine whether these correspond to differences in clinical presentation and/or disease progression.

Importantly, we have demonstrated that the molecular chaperone HSJ1a rescued Parkin(C289G) relocation to mitochondria in a J domain-dependent manner, thereby restoring its function in mitophagy. In addition, HSJ1a restored the Parkin(C289G)-induced relocation of LC3 to impaired mitochondria. Our results suggest that HSJ1a cooperates with Hsp70 to promote the folding of mutant Parkin. Previous studies have shown that HSJ1a can reduce inclusion formation by enhancing the proteasomal degradation of misfolded substrates, but the chaperone did not appear to promote client protein refolding (35,37). Therefore, the fate of the misfolded client protein appears to vary depending on the client and not on the chaperone network exclusively. The lack of requirement for the HSJ1a UIM function in the suppression of Parkin aggregation would seem to reflect a change in HSJ1a action from a pro-degradation chaperone toward a pro-folding chaperone. We also tested another Hsp70 co-chaperone, DNAJB6, which is present in Lewy bodies

(44). DNAJB6 could suppress Parkin(C289G) aggregation (data not shown) and promote Parkin relocalization to damaged mitochondria. DNAJB6 has a potent anti-aggregation activity for polyglutamine-expanded proteins, but like HSJ1a does not promote the folding of heat-denatured firefly luciferase in cells (42). Whether a pro-folding chaperone, like DNAJB1 and Hsp70 (49), would be more efficient in restoring mutant Parkin activity or whether the anti-aggregation activity is a prerequisite for this recovery remains to be tested.

HSJ1a was highly efficient at reducing Parkin(C289G) aggregation, and this was reflected by the recovery of its ability to localize to uncoupled mitochondria to almost wild-type levels. However, the later processes in Parkin-mediated mitophagy, such as mitochondrial accumulation, LC3 recruitment and mitochondrial elimination, were less well restored. This could be a consequence of the unstable nature of the mutant RING1 domain even in the refolded mutant protein or a compromise in the chaperone machinery leading to suboptimal refolding activity. CCCP treatment depletes cellular ATP levels, and the HSJ1a-mediated Parkin(C289G) refolding activity was dependent on the ATPase Hsp70. Therefore, the reduced rescue observed for later stages in the mitophagy process could reflect a loss of chaperone activity associated with reduced ATP levels within the cell and suggests that if ATP-dependent chaperones were not compromised, then the functional recovery of this Parkin mutant might be even higher. Interestingly, the optimal restoration of mitochondrial clustering also appeared to require the HSJ1a UIM domain. HSJ1 proteins interact with other proteins in ubiquitin-associated proteasomal targeting via the UIM, and whether the ubiquitin–protein ligase activity of Parkin has influenced its relationship with HSJ1a remains to be determined. HSJ1a did not affect wild-type Parkin-mediated enhancement of the elimination of damaged mitochondria. In fact, if anything, HSJ1a caused a slight delay in mitochondrial clustering by Parkin, possibly reflecting a competition with other pathways stimulated by HSJ1a. The requirement for the HSJ1a UIM domain could also reflect the need for continued maximal anti-aggregation chaperone activity to prevent seed formation inhibiting Parkin function.

A link between mitochondrial dysfunction and protein aggregation has been demonstrated in the past. Indeed, oxidative stress reduces Parkin's solubility, precipitating its inactivation (28,50,51). Furthermore, a transgenic mouse that expresses a truncated form of Parkin (Q311X) is characterized by the presence of Parkin aggregates and develops some of the pathological hallmarks of Parkinson's disease (52), suggesting that Parkin aggregation, in addition to loss of Parkin function, could be a component of Parkinson's disease. Interestingly, depletion of the 26S proteasomal activity in the mouse brain results in the formation of Lewy-body-like inclusions that contain mitochondria (53). Thus, mitochondrial dysfunction and protein aggregation may form a positive feedback loop, in which mitochondrial damage promotes protein misfolding and vice versa.

In summary, to our knowledge, this is the first report demonstrating the functional rescue of a Parkin RING domain mutant in mitophagy. These data could have important implications for the treatment of Parkinson's disease and possibly other neurodegenerative diseases, based on reducing protein aggregation and restoring protein homeostasis.

MATERIALS AND METHODS

Plasmids

The generation of pCMV-myc-HSJ1 wild-type and mutant constructs was described previously (35,54). The pcDNA3.1-FLAG-Parkin construct was as described (27). The MitoDsRed plasmid was purchased from Clontech (Oxford, UK) and the GFP-LC3 and mCherry-ATG5 constructs from Addgene (Cambridge, MA, USA). The pCMV-myc-DNAJB6 construct was generated by subcloning an Mrj(DNAJB6) cDNA insert from the original pTCR-His/Mrj construct (55), a gift from Dr J. Besharse, into an myc-pCMVtag3b vector. The myc-ubiquitin construct was a gift from Dr R. Kopito, and the GFP-Hsp70 construct was obtained from Dr H. Kampinga.

Cell culture and transfections

SK-N-SH cells were cultured in Dulbecco's modified Eagle's medium H-12 medium (1:1) with GlutaMAX^{TM} I (Invitrogen, Paisley, UK), supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Cells were grown at 37°C in 5% (v/v) CO_2. Transfections using a constant amount of plasmid DNA were performed using Lipofectamine^{TM} and PLUS^{TM} reagents (Invitrogen), according to the manufacturer's protocol.

Antibodies and reagents

Mouse monoclonal anti-FLAG (M2), mouse monoclonal anti-Hsp60 (LK1) and mouse monoclonal anti-c-myc (9E10) antibodies were obtained from Sigma-Aldrich (Gillingham, UK). Mouse monoclonal anti-Parkin antibody (Park8) was purchased from Cell Signalling (Hitchin, UK). Generation of the sheep polyclonal anti-HSJ1$^{1-277}$ antibody has been described previously (54). Secondary antibodies coupled to Cy2 or Cy3 were purchased from Jackson Immunoresearch (Newmarket, UK) and goat anti-mouse antibody coupled to horseradish peroxidase (HRP) from Pierce (Cramlington, UK). The drugs CCCP and 3-MA were obtained from Sigma-Aldrich.

Immunocytochemistry and confocal microscopy

Transfected SK-N-SH cells were fixed after 24 h with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 5 min at 22°C and/or in 100% methanol for 5 min at −20°C and washed with PBS. Cells were incubated in blocking solution [3% (w/v) bovine serum albumin and 10% (v/v) donkey serum (Jackson Immunoresearch) in PBS] for 30 min at 22°C. Cells were immunolabeled with primary antibody diluted in blocking solution for at least 1 h. Cells were washed twice in PBS and incubated with secondary antibody diluted in blocking solution for at least 45 min. Cells were washed twice with PBS and mounted with fluorescence

mounting medium (Dako, Ely, UK). Confocal images were obtained using a Zeiss LSM510 confocal microscope at excitation/emission wavelengths of 488/510 nm for Cy2-coupled secondary antibodies and GFP, and 543/570 nm for Cy3-coupled secondary antibodies, DsRed and mCherry. Images were processed in Adobe Photoshop7.0 and Adobe Illustrator CS2.

Quantification and statistical analysis

Cells were analyzed using a Nikon Eclipse 80i fluorescence microscope quantified in four randomly chosen groups comprising at least 100 cells. Inclusion incidence was quantified by counting the number of transfected cells with inclusions. The incidence of transfected cells showing Parkin relocation to mitochondria was defined as cells with distinct re-localization of Parkin that overlapped with mitochondrial staining. Mitochondrial accumulation was scored for cells that had a considerable accumulation of mitochondria around the nucleus, and mitophagy was assessed by the absence of detectable mitochondrial marker staining. The mean, standard deviation and standard error were calculated and statistical significance analyzed using an unpaired two-sample *t*-test. All experiments were carried out at least three times, and the data shown are representative of these, but generally derive from one experiment. To confirm that subjective scoring of overlapping stains corresponded with co-localization of markers, co-localization coefficients were determined using the JACoP plugin for ImageJ (Supplementary Material, Tables S1–S4 and Method).

Western blot analysis

Transfected SK-N-SH cells were lysed after 24 h in 1% (v/v) Triton X-100 in PBS containing 1% (v/v) proteinase inhibitor cocktail (PIC) (Sigma) for 15 min on ice. Cell lysates were scraped and sonicated, before centrifugation at 13 000*g* for 15 min at 4°C. The samples were resolved by SDS–PAGE and detected by western blot analysis with the ECL Plus reagent kit (GE Healthcare, Bucks, UK).

Fractionation assay

Protein extraction for the isolation of mitochondria was performed using the Qproteaome™ mitochondrial isolation kit (Cat. No. 37612, Qiagen, Crawley, UK), according to the manufacturer's protocol.

Filter trap assay

Transfected SK-N-SH cells were washed twice in ice-cold PBS and treated with trypsin-EDTA for 5 min at 22°C. Cells were centrifuged at 1000*g* for 10 min at 4°C. Pellets were washed in ice-cold PBS and resuspended in SDS lysis buffer [10 mM Tris–HCl buffer, pH 8.0, containing 150 mM NaCl and 2% (w/v) SDS] supplemented with 1% (v/v) PIC. Cell disruption was completed by sonication. A serial dilution of the samples was performed in SDS lysis buffer. Undiluted and diluted samples were loaded onto a 0.2 μm cellulose acetate membrane and pre-equilibrated with SDS wash buffer [10 mM Tris–HCl buffer, pH 8.0, containing 150 mM NaCl and 0.1% (w/v) SDS]. After loading, 0.1% (w/v) SDS wash buffer was added to each well. Samples were allowed to flow through the cellulose acetate membrane by gravity for 20 min at 22°C. Thereafter, membranes were washed three times in SDS wash buffer, subjected to a vacuum. Membranes were immunoblotted as described earlier.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None of the authors has a conflict of interest.

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