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Oxidative stress-induced parkin misfolding, aggregation, and toxicity

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Abstract

Background: Excess oxidative stress and protein misfolding are major hallmarks of neurodegenerative disease, including Parkinson's disease (PD). Mutations in the genes encoding the ubiquitin ligase parkin cause autosomal recessive juvenile forms of Parkinsonism by the loss of parkin function in mitochondrial homeostasis and cellular protein quality control, generally. Dysfunction of parkin might also contribute to sporadic forms of PD, yet the underlying mechanisms remain mostly unexplored.

Methods: We obtained key results from studies in human PD brains, a mouse model, yeast, cultured neuronal cells, and in vitro biochemistry. Human postmortem Medial Temporal Gyrus tissue was fixed for immunohistochemistry. We performed biochemical analyses of protein lysates from human brain, mouse brain, yeast and cells to assess parkin modification by oxidative stress under normal growth conditions and more so under oxidative stress.

Results: Our results reveal that oxidative stress damages parkin by inducing the formation of aberrant intra- and inter-molecular disulfide bonds, leading to parkin misfolding and inclusion formation, which is toxic to cells. We furthermore find that parkin is most severely oxidized in its active conformation.

Conclusion: Collectively, our study identifies a mechanism by which protein oxidation can contribute to neurodegeneration in PD by combining loss of function with toxic gain of function mechanisms.

Keywords: Parkinson's disease; Parkin; protein misfolding and aggregation; oxidative stress

Background

Parkinson's disease (PD) is a neurodegenerative disorder characterized by selective loss of dopaminergic neurons in the substantia nigra pars compacta and other brain regions ¹. The majority of PD cases are sporadic and affect mostly older individuals. Yet, PD is also caused by both dominant and recessive mutations in multiple genes ². In particular, mutations in the genes encoding the proteins parkin (PARK2) and PINK1 (PTEN-induced putative kinase 1, PARK6) cause autosomal recessive juvenile Parkinsonism (AR-JP), a severe form of Parkinsonism with an earlier age of onset than sporadic PD ^{3,4}

PINK1 is a protein kinase that localizes to the inner mitochondrial membrane under normal conditions, whereas parkin is an E3 ubiquitin ligase that localizes to the cytoplasm in the absence of cellular stress ⁵. In its inactive state, the N-terminal ubiquitin-like (UBL) domain of parkin folds back on itself, thus blocking the surface of the protein required for E2 recruitment ⁶. Oxidative stress and mitochondrial membrane depolarization transfer PINK1 to the outer mitochondrial membrane ⁷⁻⁹. Here, PINK1 phosphorylates ubiquitin and the UBL domain of parkin, thus inducing an active parkin conformation. Activated parkin ubiquitinates target proteins residing at the mitochondrial outer membrane, which elicits degradation of mitochondria by autophagy (mitophagy) ¹⁰. In addition to its role in mitophagy, parkin has been documented to act in other branches of cellular protein quality control, such as in endoplasmic reticulum associated protein degradation (ERAD) ¹¹. Also, proteomic studies show that hundreds of proteins are ubiquitinated by parkin, indicating that parkin function is not necessarily confined to mitophagy and ERAD ¹²⁻¹⁵. It has been suggested that AR-JP mutations in PARK2 cause neurodegeneration and Parkinsonism solely by the loss of parkin's E3 function ^{16,17}. This loss of parkin function may

cause the accumulation of damaged mitochondria, resulting in neuronal damage and death. By contrast, in sporadic PD, Lewy bodies, large cytoplasmic inclusions in the neurons of PD patients, are the major pathological hallmark. Lewy bodies primarily consist of the protein alpha-synuclein, implying a major role of alpha-synuclein misfolding in the pathogenesis of sporadic PD^{18,19}. Yet, Lewy bodies are typically absent in AR-JP and there is no evidence that alpha-synuclein plays a key role in AR-JP pathogenesis¹⁶. AR-JP and sporadic PD may accordingly only share the specific brain regions affected by neurodegeneration yet no major underlying cellular pathomechanisms. Furthermore, while parkin dysfunction has been debated to contribute to sporadic PD²⁰, supporting experimental evidence is scarce and possible underlying mechanisms remain mostly unexplored.

Analysis of parkin's amino acid sequence reveals an unusually high level of conserved cysteine residues (overall 7.25 %), distributed throughout the entire parkin protein except for the N-terminal UBL domain that contains only one cysteine (Additional file 1: Figure S1A),²¹. This high proportion of cysteines in parkin substantially exceeds the average 2.26 % for all analyzed human proteins²². Many of parkin's cysteines coordinate Zn²⁺ ions, which are required for proper folding, stability, and function of parkin²³. Because cysteine residues are inherently sensitive to oxidative modifications²⁴, this unusually high cysteine content suggests that parkin is highly susceptible to oxidative damage and aberrant disulfide formation following exposure to reactive oxygen species (ROS). Generally, oxidative stress, which plays critical roles in aging and neurodegenerative diseases, including sporadic and familial forms of PD, causes cellular damage by the impairment of proteins²⁵. Accordingly, oxidative stress induces misfolding and aggregation of both wild-type and mutant parkin in cellular and animal models^{26,27}. Moreover, Schlossmacher and coworkers report that Lewy bodies in the brains of sporadic PD patients contain parkin²⁸. It is thus plausible to postulate that oxidatively damaged, misfolded, and aggregated parkin cannot execute its regular function in mitochondrial homeostasis and, more generally, in cellular protein quality control, which may contribute to neurodegeneration in sporadic PD and in AR-JP.

Many neurodegenerative diseases are caused by the toxic gain of function of misfolded, often aggregated proteins, which is arguably the most common and one of the earliest events in these diseases. Here we investigated how oxidative stress induces parkin misfolding, aggregation and

toxicity. We find that in human sporadic PD brains and an MPTP mouse model parkin forms higher molecular weight aggregates due to cysteine oxidation. Using yeast models, cultured mammalian neuronal cells, and purified proteins, we unravel how parkin forms aberrant intra- and inter-molecular disulfide bonds, misfolds, and aggregates and produces cellular toxicity. Our studies suggest a mechanism by which loss of parkin function combined with gain of toxic function of oxidized and misfolded parkin can contribute to neurodegeneration in PD.

Methods

Reagents and antibodies

Primary antibodies used in this study include the following: Rabbit phospho-ubiquitin and anti-ubiquitin (abcam); mouse monoclonal anti-parkin and rabbit polyclonal anti-Tom20 (Cell Signaling); rabbit polyclonal and mouse monoclonal anti-Parkin (Abcam); rabbit polyclonal anti-Histone H3 (Proteintech); rabbit polyclonal anti-Pink1 (abcam). The secondary antibodies used for immunofluorescence were Alexa Fluor 488, 594 or 680 goat anti-mouse, rabbit, or goat IgG (Molecular Probe). The HRP- conjugated secondary antibodies used for Western blot analysis were from Bio-Rad. The chemicals used in this study were purchased from Sigma unless otherwise stated.

Human PD and control specimens

Necropsy brain samples from neurologically normal control subjects and individuals diagnosed with PD (formalin-fixed and paraffin-embedded tissues for immunohistochemistry and frozen tissues for protein work) were kindly provided by the PD brain bank at the Banner Health Research Institute. For the western blot analysis, homogenates were prepared from brain tissue (1g) from the median temporal gyrus of three PD patients and three control subjects as described previously ²⁹. SDS-PAGE was performed in the absence and presence of reducing reagents (β -mercapto-ethanol and DTT). Western blots were performed as described below. Immunohistochemistry was performed as described below using the anti-parkin antibody from Abcam (cat#77924) at a concentration of 1:1000.

Immunohistochemistry

Human postmortem Medial Temporal Gyrus tissue was collected in accordance with Institutional Review Board-approved guidelines. Parkinson's disease patients were defined as having two of the three cardinal clinical signs of resting tremor, muscular rigidity and bradykinesia, along with pigmented neuron loss and Lewy bodies in the substantia nigra. Blocks of tissue were fixed for ~2 hrs in 10% neutral buffered formalin. Paraffin-embedded sections were immersed in xylene and decreasing alcohol concentrations and antigen retrieval was done in citrate buffer at pH=6.0. Slides were then blocked for 1 hr in 2.5% horse serum and incubated overnight at 4°C with the

Sigma-Aldrich PARK2 Primary antibody (1:500). Slides were then incubated with the ImmPress kit horse-radish peroxidase micro-polymer solution and Parkin was visualized using Diaminobenzidine (DAB) solution incubation for 10 mins. Slides were stained with hematoxylin, washed and then mounted and covered with cover slips.

Mouse MPTP model

Ten weeks old male C57BL/6 mice weighing 22 to 27g at the beginning of the experiment were used in this study (Charles River Canada, St. Constant, QC). Animals were housed in groups of two or three per cage at a temperature of $22 \pm 1^{\circ}\text{C}$ in a 12 h light/dark cycle with lights on at 7 am and food and water available *ad libitum*. All animal procedures were approved by the University of Western Ontario Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care. All efforts were made to minimize the number of animals used. MPTP treatment was conducted as described before^{30, 31}. Animals were treated once per day with MPTP for 5 consecutive days (25 mg/kg in 0.9 % saline, s.c.). Control animals were treated with 0.9 % saline following the same protocol. All mice were sacrificed 3 days after the last day of treatment for brain harvesting. There was no significant weight loss due to any of the treatments.

Yeast strains, media, and plasmids

To construct pAG423-Parkin-YFP and pAG416-Pink1, human wild-type (wt) Parkin and human wt Pink1 cDNAs were transferred to yeast (*Saccharomyces cerevisiae*) expression vectors using the Gateway cloning system (Invitrogen). Both constructs were confirmed by sequencing. The strains were transformed with the vectors expressing Parkin or Pink1 constructs by the lithium acetate procedure³². To assess parkin protein aggregation and toxicity, cells expressing the different plasmids were grown overnight in synthetic complete media using appropriate selection media to maintain plasmids. Cells were then washed twice with media containing 2% galactose and resuspended to an OD_{600nm} of 0.2 and incubated at 30°C in a rotator drum for the indicated amounts of time to induce protein expression. Spot assays were performed by spotting 5X dilutions of OD_{600nm} 0.2 on agar plates. Plates were photographed using a Gel Doc system (Bio-Rad Laboratories) Liquid growth assays were performed using a Bioscreen C (Thermo Lab Systems, Waltham, MA) plate reader set at 30°C.

Fluorescent microscopy

Parkin was visualized in yeast cells expressing an YFP tagged version of the protein. Fluorescent microscopy was performed on a Leica TCS SP5 II Confocal microscope using a HCX PL APO 63x oil objective. Images were captured using an equipped CCD camera using Leica Application Suite Advance Fluorescence Life V2.6.0 software. Neuronal cells were grown on coverslips, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton-X100, and blocked with 1% BSA. Samples were analyzed using a Leica TCS SP5 II Laser Scanning Confocal Microscope (Carl Zeiss Microimaging, Inc.). Z-sections were acquired (0.7- μ m interval), and final images were obtained after merging planes.

Cell lysis

Yeast - Following 16 h of induction in SGal/Raff media at 30°C, yeast cultures were spun at 3000 g for 5 min to pellet cells. After aspirating supernatants, the pellets were washed with sterile double distilled H₂O. These steps were repeated to wash the cells a second time, after which, the cells were re-centrifuged. Again, supernatants were aspirated, and cell pellets were put through one freeze-thaw cycle at -20°C to assist with lysis. Cells were resuspended in equal volumes of cold lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, SIGMA FAST™ Protease Inhibitor (Sigma), and 50 mM NEM or 2 mM PMSF) and glass beads. Yeast cells were lysed by vortexing in a Disruptor Genie (Scientific Industries) 6 times for 30 sec with 1-min incubations on ice between vortexes. After lysis, samples were spun at 20000×g for 10 min at 4°C. The supernatants were collected and the protein concentration of each sample was determined and normalized using the bicinchoninic acid (BCA) kit (Promega) using bovine serum albumin (BSA) as a standard.

Mammalian cells - Cells were washed with cold PBS which was then removed by aspiration. A cell scraper was used to detach cells from culture plates into cold lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, and SIGMA FAST™ Protease Inhibitor), after which cell lysates were frozen at -20°C. After thawing on ice, lysates were subjected to a Branson Sonifier® ultrasonic cell disruptor for two 3-5 sec pluses. Lysates were then centrifuged at 20000×g for 10 min at 4°C. The supernatants were retained and protein concentrations were determined using the BCA kit (Promega) with BSA as a standard.

Immunoblotting

For western blot analyses, 30 µg of protein from whole-cell lysates (yeast or/and mammalian cells) were separated on 4–16% Bis-Tris gels and electroblotted. Immunoblots were probed with the indicated primary antibodies, followed by incubation with Alexa-488-conjugated fluorescent secondary antibodies and visualization on a Bio-Rad Chemidoc MP Imaging System.

Semi-denaturing detergent-agarose gel electrophoresis (SDD-AGE).

Samples were resolved by electrophoresis through a 1.8% agarose gel containing 0.1% SDS. For sample preparation, sample buffer (1x TAE, 5% glycerol, and 0.2% SDS) was added to protein extracts, then extracts loaded into lanes of the agarose gel. After electrophoresis, gels were rinsed in TBS (10 mM Tris pH 7.5, and 150 mM NaCl) and proteins transferred to a nitrocellulose membrane (Bio Rad) by capillary force using pre-assembled TurboBlotter™ Rapid Downward Transfer System packs (GE Healthcare). After proteins were transferred to the nitrocellulose membrane, membranes were treated and imaged as described above for standard western blots.

Cell-based models of PD

HEK 293 cells over-expressing FLAG-tagged, parkin (provided by Dr. Rylett), N2a neural cells transiently transfected to express human parkin and SH-SY5Y neuroblastoma cells that express parkin endogenously were cultured in Dulbecco's modification of Eagle's Medium (DMEM) containing 4.5 g/L glucose supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 IU/ml streptomycin and 1X glutamine in a humidified 5% CO₂ atmosphere. Cell lines were passaged using standard 0.25% trypsin with 2.21 mM EDTA incubation followed by washing with fresh DMEM and re-seeding to fresh tissue culture plates.

Transfection

Cells were grown to approximately 90% confluency in DMEM before transfecting cells using Lipofectamine® 2000 (Thermo Fisher) for N2a cells and DNA-In® SY5Y Transfection Reagent (MTI-GlobalStem) for SH-SY5Y cells following the supplier recommended concentrations in Opti-MEM® low serum media. Cells were incubated with transfection reagents and plasmid DNA for 16-20 h at 37°C before washing with PBS. Cells were returned to DMEM media to recover and allow expression of recombinant proteins for 24 h before passaging into 6 well

plates or 8 chamber microscopy slides. Cells were grown for an additional 24 h after passaging before further treatment or analysis.

Chemical stress treatment

Yeast - Spotting assays and Bioscreen growth curve experiments were performed with selective solid or liquid media, respectively, containing either 100 μ M H₂O₂, 500 μ M azetidine-2-carboxylic acid (AZC, Sigma-Aldrich), 10 μ M peroxyntirite (Enzo), 2 mM DTT, 25 μ M radicicol (Sigma-Aldrich) or 50 μ M antimycin A (Sigma Aldrich). Spotting assays were grown for 3-5 days at 30°C under stress conditions, whereas Bioscreen growth experiments were incubated for 2-3 days at 30°C with agitation every 15 min.

Mammalian cells - Mammalian cells were treated with either 75 μ M H₂O₂, 10 μ M MG132, 1 mM AZC, 50 μ M antimycin-A or 10 μ M peroxyntirite for either 8 or 16 h in DMEM at 37°C with 5% CO₂. Following treatment, cells were washed with PBS and used for western blotting or immunofluorescence microscopy (IF).

Cell viability assay

SH-SY5Y cells expressing endogenous parkin and PINK1, HEK 293 cells stably expressing FLAG-parkin but no PINK1, or N2a cells transiently transfected to express parkin, wild-type PINK1, and/or a kinase dead (KD) mutant of PINK1 were used to perform viability assays. Following transfection or standard passaging, cells were given either 1 or 4 days to recover in rich media (DMEM with 10% FBS and 4.5 g/L glucose) or minimal media (DMEM with 1% FBS and 1 g/L glucose) before performing the luminescence based Cell Titer-Glo® Luminescent Cell Viability Assay (Promega). Samples were prepared as recommended by the supplier and loaded into 96 well plates. Plates were then measured using the Victor3V Plate Reader (Perkin Elmer) using the Perkin Elmer 2030 Manager Software.

Parkin expression and purification

Human parkin and 141C (residues 141-465, lacking UBL domain and linker) constructs were expressed as His-Smt3 fusion proteins in BL21 (DE3) *E. coli* cells in LB media as previously described^{33, 34}. Following expression, cells were harvested and suspended in 50 mM Tris, 500 mM NaCl, 0.5 mM TCEP, 25 mM imidazole at pH 8.0 and cells disrupted by homogenization (EmulsiFlex-C5, Avestin). All proteins were purified by Ni²⁺-NTA affinity chromatography on

an AKTA FPLC (GE Healthcare) using a HisTrap FF column. Subsequent cleavage of the His-Smt3 tag used recombinant Ulp1 protease in a 1:50 protease: protein ratio. Cleaved protein solutions were re-applied to the HisTrap FF column and the flow-through containing untagged proteins collected. A final purification stage used a HiLoad Superdex75 gel filtration column in 25 mM HEPES, 100 mM NaCl, 0.5 mM TCEP buffer at pH 7.0. Ubiquitin was expressed from *E. coli* BL21 (DE3) pLysS cells and purified as previously described³⁵.

Parkin phosphorylation

Purified GST-PINK1 was used to phosphorylate parkin and ubiquitin as described⁶. In general, proteins were prepared in 50 mM Tris, 50 mM NaCl, 1 mM DTT buffer at pH 7.5 using 1:2 or 1:30 ratios of parkin:PINK1 or ubiquitin:PINK1 respectively. Each phosphorylation reaction was initiated using 20 mM MgCl₂, 10 mM ATP at 25°C. Reactions were monitored by Phos-tag SDS/Page and mass spectrometry used to confirm phosphorylation. GST-PINK1 was removed from samples using GStrap FF column chromatography as for the PINK1 purification. Phosphorylated proteins were subjected to final purification using a HiLoad Superdex75 gel filtration column.

Oxidation experiments

Oxidation of parkin and 141C proteins was assayed using 60 µL samples containing either 15 µM R0RBR or 15 µM full-length parkin in 50 mM HEPES, 50 mM NaCl buffer at pH 7.2 at 37°C. A concentrated solution of H₂O₂ was added to the protein solutions to a final concentration of 5 mM H₂O₂ at time=0 to initiate the oxidation reaction. At regular time intervals 12 µL aliquots of the oxidation samples were removed and quenched with 6 µL non-reducing SDS sample buffer. Aliquots were visualized by 15% Tris-glycine SDS-PAGE followed by staining with Coomassie Brilliant Blue R-250.

Statistical analyses

Differences among means were analyzed using 1- or 2-way ANOVA, followed, when showing significant differences, by pair-wise comparisons between means using Newman-Keuls post hoc testing. When only 2 groups were compared, Student's *t*-test or Mann-Whitney test was used. In all analyses, the null hypothesis was rejected at the 0.05 level. To quantify changes in yeast cells

growth, growth curves were generated using the BioscreenC and area under the curve was calculated using GraphPad Prism.

Results

Oxidation-based inclusion formation of parkin in PD brains

We first explored whether parkin forms insoluble high molecular weight protein species in post-mortem brains derived from PD patients. The most severely affected brain region in PD, the substantia nigra pars compacta in the midbrain ³⁶, is characterized by massive neuronal cell loss in post-mortem brain tissues derived from PD patients, which would substantially complicate our studies. We therefore decided to focus our experiments on a less affected brain region, the median temporal gyrus (MTG) ³⁷. For our experiments, we used samples derived from sporadic PD patients and age-matched neurologically normal controls and performed western blot experiments in the presence and absence of reducing reagents. We detected an increased amount of oxidized, higher molecular weight parkin protein in PD samples compared to age-matched controls (Figure 1 A, B). Of note, treatment with reducing reagents results in decreased signals for the high molecular protein species, indicating that disulfide bonds are key to the formation of these higher molecular weight species. Our immunohistochemistry experiments also revealed strong accumulation of parkin inclusions in PD brains and, to a much lesser extent, in unaffected, age-matched controls (Figure 1 C, D). We corroborated these results in the well-established MPTP mouse model of PD ³⁸⁻⁴⁰. Immunoblot experiments show increased levels of higher molecular weight, oxidized parkin in MPTP-treated mouse mid-brains as compared to untreated animals (Figure 1 E, F). These data document that parkin is oxidized and forms higher molecular weight aggregates in sporadic PD and the MPTP PD mouse model.

Expression and localization of human parkin in yeast

To study parkin oxidation and aggregation in more detail, we established a yeast model expressing human parkin (Figure 2). Yeast models have been instrumental in deciphering basic mechanisms underlying protein misfolding in neurodegeneration, including PD ^{41,42}. Further, since yeast does not express parkin and PINK1 homologs, interference from endogenous proteins does not confound experimental outcomes. The yeast model thus presents an optimal experimental platform to study parkin oxidation, misfolding, aggregation, and toxicity separated from its cellular functions, such as in mitophagy and ERAD.

We first assessed whether the expression of human parkin affects growth of yeast cultures under normal conditions. Spotting assays on agar plates and growth curves in liquid media show a mild growth defect associated with the expression of human parkin (Fig 2A, B). We next assessed localization of parkin fused at its C-terminus to the yellow fluorescent protein (parkin-YFP). Parkin-YFP was localized diffusely throughout the cytosol as was reported previously for studies in yeast and mammalian cells, including human neurons (Fig 2C) ^{43,44}. Western blots further showed that parkin was stably expressed in yeast (Fig 2D).

Next, we explored if PINK1 modifies parkin folding and stability by determining how the co-expression of PINK1 affects parkin localization, steady-state protein levels, degradation, and toxicity. Human PINK1 does not properly localize to mitochondria in yeast ⁴⁵. We therefore used a modified PINK1 variant that contains a yeast specific N-terminal outer mitochondrial protein (OMP) leader sequence and a C-terminal FLAG tag as reported previously ⁴⁵, which mimics the oxidative stress-induced localization of active PINK1 to the outer mitochondrial membrane as observed for mammalian cells.

In addition, we engineered a kinase dead PINK1 variant bearing two key aspartic acid residues to alanine substitutions (D3682A and D384A, PINK1 kinase dead mutant, KD) in the yeast PINK1 variant ⁴⁶. PINK1 or KD-PINK1 were co-expressed with parkin. We first assessed whether PINK1 and KD-PINK1 expression altered growth in yeast. PINK1 expression in yeast caused a mildly toxic phenotype, which does not occur in cells expressing KD-PINK1 (Fig 2E-H and Additional file 1: Figure S1B, C). Western blots of yeast cells expressing either PINK1 or KD-PINK1 in the absence and presence of parkin showed that PINK1 was stably expressed, but that KD-PINK1 appeared to be expressed at slightly lower levels than PINK1 (Fig 2I and data not shown). Fluorescence microscopy (Fig 2J) showed that when co-expressed with PINK1, parkin-YFP forms cytosolic inclusions and that this almost eliminated diffuse staining of parkin. When co-expressed, PINK1 and parkin caused a severe toxic phenotype, almost completely inhibiting growth (Fig 2E lane 3). Co-expression of parkin with KD-PINK1 was not toxic (Fig 2F-H) and did not produce any parkin aggregates (Fig 2J), suggesting that parkin toxicity and inclusion formation depends, at least in part, on the kinase activity of PINK1. To test whether the toxic effects caused by co-expression of PINK1 and parkin were based on growth inhibition or cell death, a SYTOX® Green-based assay was used (Fig 2H). The SYTOX® assay revealed that separate expression of either parkin or PINK1 caused circa 25% cell death. The co-expression of

the two proteins increased cell death to approximately 55%. Expression of KD-PINK1 and its co-expression with parkin did not cause cell death.

These data establish that parkin co-expressed with PINK1 in yeast results in parkin inclusion formation and cellular toxicity, whereas the separate expression of either parkin or PINK1 showed only mild growth defects and no parkin inclusion formation.

Oxidative stress induces parkin aggregation

To explore the effects of oxidative stress and other cellular stressors on parkin, growth assays and fluorescence microscopy were performed with yeast cells expressing parkin in the presence of various stress-inducing chemicals (Fig 3A and Additional file 1: Figure S2A). No significant changes were observed in the growth rate of parkin expressing yeast cells, but treatment of cells with H₂O₂ and L-Azetidine-2-carboxylic acid (AZC) caused parkin-YFP to accumulate into subcellular inclusions (Fig 3A).

H₂O₂ was chosen for subsequent experiments based on its relevance to disease associated oxidative stress in cells and because the H₂O₂ treatment conditions used in these experiments do not lead to complete collapse of the mitochondrial membrane potential and cell death as has been seen with CCCP (carbonyl cyanide m-chlorophenyl hydrazine, Additional file 1: Figure S2B)⁴⁷. Growth curves with increasing concentrations of H₂O₂ showed that concentrations up to 100 μM had no effect on yeast cell growth in the presence or absence of parkin (Fig 3B).

Western blot analysis of protein lysates of yeast cells expressing parkin prepared under reducing conditions showed no noticeable difference in parkin's electrophoretic mobility between untreated and hydrogen peroxide, antimycinA or peroxyntirile-treated samples (Fig 3C, lanes 1, 3, 5, 7). In the absence of reducing agents, however, parkin migrated differently than parkin treated with reducing agents (Fig 3C lanes 2, 4, 6, 8). N-Ethylmaleimide (NEM) irreversibly alkylates cysteine residues in proteins. When protein lysates from yeast cells expressing parkin were treated with 50 mM NEM, the odd mobility for parkin observed by Western blot with non-reducing buffer was eliminated (Additional file 1: Figure S2C). A closer analysis of the parkin signal under non-reducing conditions revealed a predominant species that migrated faster than under reducing conditions and a smeared signal of higher molecular weight species. These results suggest that parkin is modified by oxidative damage even under normal growth conditions and that this oxidative damage is exacerbated by H₂O₂ treatment.

To confirm the results from our yeast model in mammalian cells, we employed two human cell lines expressing different levels of parkin: HEK 293 cells stably over-expressing parkin bearing an N-terminal FLAG epitope tag (FLAG-parkin) and dopaminergic neuronal SH- SY5Y cells expressing only endogenous parkin. In both cases, western blots performed under non-reducing conditions revealed a smear of high molecular weight parkin following exposure of cells to H₂O₂ that could be reversed through the addition of reducing agents (Fig 3D). The HEK293 cell lines that constitutively express high levels of FLAG-parkin showed a strong shift in electrophoretic mobility. Lysates from SH-SY5Y cells expressing endogenous parkin levels showed a similar shift albeit with lesser intensity. In addition, we found that under non-reducing conditions the main parkin band migrated faster than under reducing conditions (Fig 3E and Additional file 1: Figure S2D, E). This was observed in cells under both control and H₂O₂-treated conditions.

Next, we determined if oxidative stress treatment caused changes to parkin subcellular localization (e.g. aggregation, association with mitochondria) in mammalian cells. N2a cells transiently transfected to express parkin-YFP and SH-SY5Y cells expressing endogenous parkin were treated with cellular stress-inducing chemicals and parkin localization was assessed using fluorescence- and immunofluorescence microscopy, respectively (Fig 3E and Additional file 1: Figure S3A). Untreated SH-SY5Y cells expressing endogenous parkin and N2a cells transiently transfected to express parkin-YFP presented several small foci and crescent-shaped inclusions of parkin throughout the cytoplasm. However, upon stress treatment, endogenous parkin in SH- SY5Y cells and parkin-YFP in N2a cells accumulate in larger foci that often appear interconnected with one another throughout the cytoplasm. These results document that parkin forms inclusions upon exposure of cells to reactive oxygen species (ROS) in both the yeast model and in two different mammalian neuronal cell models.

Genes regulating oxidative stress modulate parkin toxicity and localization

We next explored genetic interactions between parkin and yeast strains bearing deletions of genes involved in oxidative stress management (Fig 3F and Additional file 1: Figure S3B). To this end, parkin and parkin-YFP expressing plasmids were transformed into the selected yeast deletion strains. Deletion strains expressing untagged parkin were spotted onto inducing media and growth was assessed. These experiments identified two deletion strains that show reduced

growth due to the expression of human parkin: deletion of SOD2, encoding a mitochondrial superoxide dismutase that protects against toxicity caused by superoxide radicals^{48,49}; and the deletion of YAP1, encoding a transcription factor required for oxidative stress tolerance⁵⁰. The SOD2 and SGT2 deletion strains also showed changes in parkin-YFP localization (Fig 3G), i.e. subcellular accumulation of parkin into inclusions; all other strains, including the Yap1 deletion strain, showed diffuse parkin localization as seen in wild-type yeast cells (Fig 3G). SGT2 is a cytoplasmic co-chaperone that is part of a protein complex required to mediate post-translational insertion of tail anchored proteins into the endoplasmic reticulum (ER) membrane⁵¹, suggesting that altered capability of cells to undergo proper protein trafficking and folding affects parkin localization. These results document that in yeast cells with reduced capacity to handle oxidative stress, parkin can cause growth defects and inclusion formation.

Parkin forms inclusions in the cytosol and at mitochondria

A prerequisite for parkin's activation during mitophagy is its translocation from the cytosol to mitochondria, as suggested by data derived from cells upon dissipation of mitochondrial membrane potential by CCCP treatment⁴³. Activated PINK1 phosphorylates parkin's UBL domain and ubiquitin both at serine 65, which stimulates parkin's ligase activity and recruitment to mitochondria⁵². Yet, our immunofluorescence experiments carried out in SH-SY5Y cells probing for phosphorylated parkin (p-parkin) and phosphorylated ubiquitin (pS65- Ub) indicates that with non-lethal oxidative stress treatment parkin inclusions are localized to the cytoplasm, the nucleus, mitochondria, and possibly mitochondria-derived vesicles (MDVs), and not exclusively to mitochondria (Fig 4A-L).

We also showed that cytosolic parkin does not completely translocate to mitochondria in either SH-SY5Y cells expressing endogenous parkin or transiently transfected N2a cells expressing parkin-YFP (Fig 4 and Additional file 1: Figure S4A-F), upon exposure to either 100 μ M of H₂O₂ or 10 μ M proline analogue azetidine-2-carboxylate (AZC), an proline analogue, which can induce protein misfolding. More than 80% of cells analyzed exhibited either one large focus or several smaller discrete parkin foci in both untreated cells and cells exposed to stress treatment, rather than showing a normal diffuse parkin fluorescence. Under these experimental conditions, image analysis showed that only about 50% of parkin inclusions co-localized with the mitochondrial protein TOM20 (Fig 4L). Of note, the co-localization of parkin with TOM20 in our

experiments can indicate parkin's localization to mitochondria or mitochondria-derived vesicles (MDVs) ⁵³. In sum, our data show that oxidative and protein misfolding stress induce the formation of parkin inclusions that are not limited to mitochondria but rather localize to multiple cellular compartments, including the cytosol.

The UBL domain reduces parkin aggregation

To provide a rationale for the mechanisms driving parkin oxidation and aggregation, we generated truncation variants of parkin and mapped changes in inclusion formation and cellular toxicity. We focused on the UBL domain with its established autoinhibitory function and the catalytic RING2/Rcat domains and constructed four different truncation variants that allow us to test oxidative effects in cells and in vitro (Fig 5A).

We first established yeast models of all the parkin truncation variants, testing toxicity, subcellular localization and expression. Spotting assays and growth curves revealed that all truncation variants caused only mild growth defects similar to those observed with full-length parkin (Fig 5B, C and Additional file 1: Figure S 5A, B). We also tested expression levels of the parkin truncation variants in yeast. Because it was not possible to compare all variants using the same anti-parkin antibody, three different antibodies were used to quantify the expression of the different truncation variants (Additional file 1: Figure S5C). The variant lacking the UBL domain and adjoining linker (141C) was present at higher steady-state protein levels than wild-type parkin, whereas the three remaining truncation variants were expressed at similar levels as wild-type full-length parkin.

We next explored co-expression of parkin truncations and PINK1 (Fig 5C, D, F and Additional file 1: Figure S4B). PINK1 co-expressed with truncation variant 141C and additionally lacking the RING2/Rcat domain (141-409) showed a similar reduced growth phenotype as full-length parkin. In all cases, the addition of the C-terminal YFP tag reduced the growth defect slightly when compared to their untagged counterparts (Additional file 1: Figure S5B, C). Co-expression of parkin and the parkin truncation variants with KD-PINK1 were all non-toxic (Fig 5E, F), suggesting that this toxic effect is, as observed for full-length parkin, at least partially dependent on the kinase activity of PINK1.

We then performed localization studies with C-terminally YFP tagged parkin truncation variants (Additional file 1: Figure S 5A, D). Fluorescence microscopy showed that, like parkin, the UBL

domain and the 321C (IBR+RING2/Rcat) fragment were diffusely localized throughout the cytosol, but 141C and 141-409 had altered subcellular localization (Fig 5G) 141C formed small puncta, similar in size and distribution to those caused by AZC or H₂O₂ treatment of cells on full-length parkin-YFP with some additional diffuse staining. Truncation variant 141-409 resulted in more severe localization change, with the protein product forming either a few large or several small puncta with no diffuse staining remaining. These findings suggest that both the UBL and RING2/Rcat domains of parkin play central roles in maintaining its proper folding and localization. We performed fluorescence microscopy to test if PINK1 co-expression caused changes to parkin localization (Additional file 1: Figure S5D). Truncation variants 141C-YFP, 141-409-YFP, and 321C-YFP form cytosolic inclusions when expressed alone and co-expressed with PINK1. Co-expression of PINK1 causes a subtle change to Ubl-YFP localization, leading to the formation of very small fluorescent foci in some cells.

To validate these results in mammalian cells, we expressed 141C and 141-409 parkin truncation variants in N2a and SH-Y5Y to assess their aggregation and toxicity (Fig 5H). Western blots of transfected cell lysates showed that, in contrast to results in yeast, 141C protein levels are lower than that of full-length parkin (Additional file 1: Figure S5E). Immunofluorescence microscopy revealed that 141C transfected cells show increased inclusion formation compared to full-length parkin (Fig 5H). Viability assays of cells transfected with 141C showed that it is toxic (Fig 5I) as it significantly reduced the cell viability over time, which might contribute to its lower protein levels (Additional file 1: Figure S5F). These data document that truncated versions of parkin, particularly those lacking the amino-terminal UBL domains, are prone to form inclusions and can be toxic.

Parkin misfolds and aggregates upon oxidative damage in vitro

We next asked whether oxidative damage directly modifies parkin via cysteine oxidization using purified proteins. First, we tested exposure of full length parkin and the parkin variant 141C to 50 μ M H₂O₂ for increasing periods of time. As shown in Figure 6A, prolonged exposure to H₂O₂ alters the electrophoretic mobility of parkin. Starting at early time points, exposure to H₂O₂ produces a faster migrating protein species and after longer H₂O₂ exposure parkin form high molecular weight protein species. This pattern of H₂O₂-mediated modification is even more pronounced for 141C as it readily forms the faster migrating band and after longer

H₂O₂ exposure it is converted to high molecular weight species (Fig 6A, left panel).

We also assessed whether parkin phosphorylation and its interaction with phosphorylated ubiquitin, both of which activate parkin, modulate parkin oxidation. To this end we analyzed full length recombinant parkin and phosphorylated parkin (p-parkin) proteins in the presence of ubiquitin and phosphorylated ubiquitin (p-ubiquitin) after treatment with H₂O₂ and analyzed the parkin protein under reducing and non-reducing conditions. A profound accumulation of high molecular weight parkin bands was observed following H₂O₂ treatment under non-reducing conditions (Fig 6B). Activated parkin, either by phosphorylation or due to the presence of p- ubiquitin, shows increased levels of high molecular weight parkin species compared to unphosphorylated parkin alone. Notably, in addition to these high molecular weight parkin species, H₂O₂ treatment led to a significant mobility shift in monomeric parkin, consistent with intra-molecular disulfide formation (Fig 6B) as we observed for parkin and 141C alone upon H₂O₂ treatment. To further confirm the formation of intra- and inter-molecular disulfides, oxidized parkin was treated with the reducing agent β -mercaptoethanol after H₂O₂ treatment, which resulted in the complete collapse of all parkin species into one distinct protein band (Fig 6B).

Many protein aggregates are resistant to the ionic detergent sodium dodecyl sulfate (SDS), whereas amorphous, non-amyloid inclusions are solubilized by SDS ^{54,55}. We analyzed parkin by semi-denaturing agarose gel electrophoresis (SDD-AGE), which separates monomers from large multimers following their exposure to low concentrations of SDS. We again found that those parkin samples incubated with p-ubiquitin are more prone to aggregation compared to parkin alone (Additional file 1: Figure S 6).

We thus demonstrate that upon exposure to reactive oxygen species, purified parkin forms a faster migrating protein species, indicative of a more compact parkin conformer with intra-molecular disulfide bonds, and high molecular weight aggregates, indicative of parkin adducts formed by inter-molecular disulfide bonds. The presence of phospho-ubiquitin exacerbates oxidation-based parkin aggregation. These results reveal that parkin is highly susceptible to cysteine oxidation, resulting in conformational changes caused by both intra- and intermolecular disulfide bonds and that the enzymatically active parkin conformation is more susceptible to parkin oxidation.

Antioxidants reduce parkin aggregation and toxicity

To test if antioxidants affect parkin aggregate formation and toxicity, we treated 141C-YFP transfected N2a and SH-SY5Y cells with resveratrol and epigallocatechin gallate (EGCG), two effective antioxidants, and monitored 141C inclusion formation by fluorescence microscopy and toxicity. As shown in Fig 7A-C, resveratrol and EGCG reduce aggregation formation. Cell viability assays demonstrated that resveratrol and EGCG treatment effectively reduced 141C toxicity (Fig 7D). These results indicate that anti-oxidant treatment can reduce both parkin inclusion formation and toxicity.

Discussion

Protein misfolding and aggregation are major hallmarks of many neurodegenerative diseases ^{56,57}. In PD, oxidative stress plays a key role in dopaminergic neurotoxicity ⁵¹. Environmental factors, including heavy metals, neurotoxins, pesticides, insecticides, and endogenous sources, including dopamine metabolism, mitochondrial dysfunction, neuroinflammation, and aging contribute to increased production of ROS and oxidative stress ^{57,58,59}. Dopaminergic neurons in the substantia nigra pars compacta of the mid brain are particularly vulnerable to oxidative stress and their degeneration causes PD symptoms ⁶⁰. Also, PD-causing mutations in genes encoding DJ-1, PINK1, parkin, alpha-synuclein, and LRRK2 directly or indirectly compromise mitochondrial homeostasis and function, leading to excessive ROS generation and thus oxidative stress ⁶¹. Here, we document how exposure of the PD protein parkin to non-lethal levels of reactive oxygen species (ROS) causes its oxidation, misfolding, and inclusion formation, which directly contributes to cellular toxicity.

The most vulnerable (i.e., reactive) targets to oxidative damage in proteins are the sulfur-containing side chains of methionine and, even more so, cysteine ⁶². Parkin has an unusually high proportion of cysteine residues which makes it highly susceptible to oxidative damage, possibly even at relatively mild levels of oxidative stress. Upon exposure to ROS, cysteine residues on the surface of parkin may be chemically modified, leading to aberrant intra- and then intermolecular disulfide bonds and parkin misfolding and aggregation. Although most of the cysteines in parkin coordinate zinc ions, three-dimensional structures ^{63,64} show that at least three residues C182 in the RING0 domain, C323 in the RING1 domain, and C451 in the RING2/Rcat domain are available on the surface of the autoinhibited form that would be most susceptible to oxidation.

Furthermore, we show that parkin activated by the interaction with phospho-ubiquitin and the phosphorylation of its UBL domain is particularly vulnerable to oxidative damage, more so than its autoinhibited form ⁶⁵. This suggests that the conformational change in parkin not only activates its E3 ubiquitin ligase activity, but also leads to greater susceptibility to oxidation and the ensuing misfolding. Since activation appears to result in movement of the UBL and IBR domains in parkin, cysteines in these regions are likely the most susceptible. Of note, some AR- JP mutations recapitulate partial parkin activation ^{65,66} and may thus also be more prone to oxidation and misfolding. Oxidative stress results from the exposure to neurotoxic chemicals, such as MPTP or rotenone, which cause Parkinsonism in both animal models and humans ^{67,68}. Prolonged exposure

to oxidative stress can result from the exposure to increasing levels of ROS during aging and advanced age is a common risk factor of sporadic PD ⁶⁹. We speculate that the oxidation of parkin contributes to PD during exposure to neurotoxic chemicals and in aged neurons. Our data further document that oxidized parkin forms cellular inclusions and insoluble protein aggregates. Of note, these parkin inclusions do not exclusively locate to mitochondria, but are also found in the cytosol. These results confirm previous studies showing that upon treatment of cells with CCCP, parkin is located primarily at mitochondria where it elicits the degradation of damaged mitochondria by mitophagy and also document cytoplasmic parkin aggregation ^{43,70}. Under our experimental conditions with relatively low and not acutely toxic levels of oxidative stress parkin inclusions form both at mitochondria and apart from them.

Our experiments document that aggregated parkin can be toxic in both yeast and mammalian cells. Several observations support the concept that this toxicity is at least partially due to a toxic gain of function associated with aggregated parkin. First, yeast cells do not express either parkin or PINK1 homologues, thus toxicity cannot arise from loss of function in this system. Further, a truncated version of parkin in which its RING2/Rcat domain is deleted (141-409) and thus lacks its E3 ubiquitin ligase activity and its UBL domain can readily form aggregates and produce cytotoxicity.

Parkin aggregates have been observed in both cell and animal models, and in PD patients, yet ^{35,71} their precise role in PD pathogenesis has not been fully elucidated. Our results offer a novel perspective on the role of parkin in sporadic PD, whereby its oxidation, misfolding, and aggregation directly contributes to neurodegeneration. Our results also show that treatment with antioxidants can reduce parkin aggregation and the associated toxicity. Most antioxidant drugs show general success in PD animal models ^{72,73} while clinical trials for PD prevention and treatment by antioxidants are still in their infancy ^{74, 75}. Our study encourages further trials to determine if antioxidants that are effective in the brain slow or prevent PD progression by inhibiting aberrant parkin oxidation, misfolding, and aggregation. We find it enticing to speculate that a similar oxidative damage and aggregation can also affect other PD proteins that contain vulnerable cysteine residues, such as PINK1, DJ-1, and VPS35 by a similar pathological mechanism. Oxidative damage- and aberrant thiol chemistry-based mechanism might indeed contribute to many different neurodegenerative diseases associated with protein misfolding.

Conclusions

In conclusion, our findings using in vivo and in vitro experimental systems demonstrate that oxidative stress induces the formation of intra- and intermolecular disulfide bonds, which elicit parkin misfolding, formation of inclusion, and cytotoxicity, which may contribute to PD pathogenesis.

List of abbreviations:

AZC - azetidine-2-carboxylic acid

AR-JP – autosomal recessive juvenile Parkinsonism

DAB - diaminobenzidine

DTT – 1,4-dithiothreitol

GAL - galactose

GFP – green fluorescent protein

HRP – horseradish peroxidase

MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MTG – medial temporal gyurs

OD – optical density

PBS – phosphate buffered saline

PD – Parkinson's disease

PINK1 - PTEN-induced putative kinase

Raff - Raffinose

ROS – reactive oxygen species

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDD-AGE – sodium dodecyl sulfate agarose gel electrophoresis

Tris - tris(hydroxymethyl)aminomethane

UBL – ubiquitin-like domain

YFP – fellow fluorescent protein

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Figures Legends

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Fig 3. Cellular stress alters parkin localization in yeast and mammalian cells. (A) Spotting growth assays and fluorescence microscopy of yeast cells expressing C-terminally YFP tagged parkin in the presence of stress reagents (H_2O_2 and AZC). Quantification of three independent experiments are shown in the bottom part of the panel (* $p=0.06$). (B) Growth curves of yeast cells expressing parkin in media containing 1mM, 0.1 mM and 0.05 mM H_2O_2 were performed by growing in inducing selective media at 30°C. (C) Western blot analysis of cell extracts from yeast cells expressing parkin grown in the presence of 100 μM H_2O_2 , 10 μM peroxyntirite, or 50 μM antimycin A were prepared in loading buffer with (1% βME and 100mM DTT) or without reducing agents. (D) Formation of high molecular weight (HMW) parkin species following oxidative stress in stably a stable cell line (HEK293) expressing FLAG-parkin and dopaminergic neuronal cells (SH-SY5Y) expressing only endogenous parkin. The cells were exposed to phosphate buffered saline (control) or 100 μM H_2O_2 for 16 h. Total proteins extract were then probed by western blot against parkin antibody under non-reducing and reducing SDS–PAGE conditions. Quantification of three independent experiments is shown in the bottom part of the panel (* $p=0.001$, and ** $p=0.002$). (E) Fluorescence microscopy images of SH-SY5Y cells treated with 100 μM H_2O_2 , and 10 mM AZC. (F) Spotting assays and fluorescence microscopy were performed with yeast cells expressing C- terminally YFP tagged parkin in a selected set of yeast deletion strains with defects managing oxidative stress mitigation. The arrows indicate subcellular accumulation of parkin into bright puncta. The scale bar represents 5 μm .

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Fig 7. EGCG and resveratrol inhibit parkin inclusion formation in SH-SY5Y cells. (A) Fluorescence images of SH-SY5Y cells transiently transfected with parkin truncation variant (141C) and (B) treated with EGCG and (C) with Resveratrol analyzed by immunofluorescence microscopy. (D) Viability of SH-SY5Y cells transiently transfected to express 141C parkin treated with resveratrol and EGCG was assessed. Quantification of three independent experiments (* $p = 0.003$, ** $p = 0.004$).

Additional file Figure legends

Additional file 1: Figure S1. Yeast parkin model. (A) Schematic representation of parkin domains with all cysteine residues indicated. (B) Spotting assays were performed to assess the effects of parkin C-terminal YFP tags compared to wild type parkin in the absence and presence of PINK1. Quantification of three independent experiments is shown in the right part of the panel ($p = 0.05$). (C) Growth curve of parkin-YFP co-expressed with PINK1 was performed by growing in inducing selective media at 30°C. At mid-log growth all samples were statistically different from the control with a p value ≤ 0.01 as determined from a One-way ANOVA.

Additional file 1: Figure S2. Stress treatment alters parkin localization in yeast and mammalian cells. (A) Spotting assays and fluorescence microscopy of yeast cells expressing C-terminally YFP tagged parkin in the presence of various stress reagents; peroxynitrite, antimycin A, DTT and radicicol. (B) Apoptosis percentage in SH-SY5Y cells treated with H₂O₂ and CCCP was determined by the TUNEL assay. Staurosporine as a positive control induced apoptosis in N2a cells and SH-SY5Y cells to 47.7 and 53.4 %, respectively. A one-way ANOVA followed by a Tukey's multiple comparisons test was used for the analysis with a p value ≤ 0.001 . (C) Western blot analysis of protein extracts from yeast expressing parkin grown in the presence of NEM. (D) Western blot analysis of protein extracts from yeast expressing parkin under non-reducing conditions reveals a predominant 50 kDa with a smear signal above and below the predominant band. (E) Western blot analysis of protein lysates from stably transfected FLAG-parkin (HEK 293) in the presence of 100 μ M H₂O₂, 10mM AZC and 10 mM MG132 A were probed against parkin under non-reducing and reducing SDS-PAGE conditions. Tubulin was used to assess equal loading for extracts.

Additional file 1: Figure S3. (A) N2a cells transiently transfected with parkin-YFP were analyzed by fluorescence microscopy. (B) Spotting assays were performed with yeast cells expressing C-terminally YFP tagged parkin in a selected set of yeast deletion strains of genes involved in oxidative stress control.

Additional file 1: Figure S4. Parkin aggregation by stress treatment. N2a Cells and SH-SY5Y cells transiently transfected with parkin-YFP treated with the indicated reagents were analyzed by microscopy. Parkin and TOM20 were visualized using an AlexaFluor-488 and 568 conjugated secondary antibody, respectively. The nuclei were stained by DAPI.

Additional file 1: Figure S5. Truncations cause subcellular accumulation of parkin in yeast and mammalian cells. (A, B) Spotting assays and growth curves performed of yeast transformed with C-terminally YFP tagged parkin truncation variants (A) and co expression of C-terminally YFP tagged parkin truncation variants and PINK1 (B). At mid-log growth all samples were statistically different from the control with a p value ≤ 0.01 as determined from a One-way ANOVA. (C) Western blot analysis was performed for detecting parkin. Primary antibodies with epitopes specifically recognizing various domains were used to detect parkin and the various truncation variants and compare relative expression. A RING2/Rcat specific antibody was used to detect parkin, 141C, and 321C, a RING1 specific antibody was used to detect parkin, 141C, and 141-409, and a Ubl specific antibody was used to detect parkin and Ubl. (D) Fluorescent images of PINK1 co-transformed with C-terminally YFP tagged parkin truncation variants grown in inducing liquid media for 16 hours were captured using a Leica TCS SP5 II confocal microscope at 63x magnification. (E) Western blot analysis of N2a cells transiently transfected with parkin truncation variant (141C), probed against parkin. Tubulin was used to assess equal loading for extracts. (F). Time course toxicity of N2a cells transiently transfected with parkin truncation variant (141C) and parkin were used to assess the effects of parkin alterations in mammalian cells. P value ≤ 0.01 .

Additional file 1: Figure S6. Aggregation of purified parkin. SDD-AGE of pure parkin exposed to H₂O₂ under different condition detected by anti-parkin antibody.

Figures

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Supplementary Files

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