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Parkin is an E3 ubiquitin-ligase for normal and mutant ataxin-2 and prevents ataxin-2-induced cell death.

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Abstract

Expansion of the polyQ repeat in ataxin-2 results in degeneration of Purkinje neurons and other neuronal groups including the substantia nigra in patients with spinocerebellar ataxia type 2 (SCA2). In animal and cell models, overexpression of mutant ataxin-2 induces cell dysfunction and death, but little is known about steady state levels of normal and mutant ataxin-2 and cellular mechanisms regulating their abundance. Based on preliminary findings that ataxin-2 interacted with parkin, an E3 ubiquitin ligase mutated in an autosomal recessive form of Parkinsonism, we sought to determine whether parkin played a role in regulating the steady state levels of ataxin-2. Parkin interacted with the N-terminal half of normal and mutant ataxin-2, and ubiquitinated the full-length form of both wild type and mutant ataxin-2. Parkin also regulated the steady-state levels of endogenous ataxin-2 in PC12 cells with regulatable parkin expression. Parkin reduced abnormalities in Golgi morphology induced by mutant ataxin-2 and decreased ataxin-2 induced cytotoxicity. In brains of SCA2 patients, parkin labeled cytoplasmic ataxin-2 aggregates in Purkinje neurons. These studies suggest a role for parkin in regulating the intracellular levels of both wild type and mutant ataxin-2, and in rescuing cells from ataxin-2-induced cytotoxicity. The role of parkin variants in modifying the SCA2 phenotype and its use as a therapeutic target should be further investigated.

Keywords

parkin; ataxin-2; SCA2; Parkinson's disease; AR-JP

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Introduction

Ataxin-2 is a polyglutamine (polyQ) protein that causes spinocerebellar ataxia type 2 (SCA2) when its polyQ tract is expanded above 31 glutamine repeats. SCA2 thus belongs to the group of polyQ neurodegenerative diseases (Nakamura et al., 2001; Orr and Zoghbi, 2001; Wanker, 2000). The most common normal ataxin-2 allele contains 22 glutamines. When the polyQ repeat is expanded to 32 or longer, mutant ataxin-2 causes degeneration of Purkinje neurons and other neuronal groups in the brain and spinal cord resulting in progressive ataxia and premature death (Imbert et al., 1996; Pulst et al., 1996; Sanpei et al., 1996; Schols et al., 1997). As in other polyQ diseases, SCA2 becomes more severe and has an earlier age of onset with increasing length of the polyQ repeat (Pulst et al., 2005).

In polyQ diseases as well as in other neurodegenerative diseases such as Parkinson and Alzheimer disease, neuronal death is associated with cytoplasmic or intranuclear aggregation or accumulation of mutant proteins (DiFiglia et al., 1997; Huynh et al., 1999; Huynh et al., 2000a; Klement et al., 1998; Koyano et al., 2002; Koyano et al., 2000; Paulson et al., 1997; Uchihara et al., 2001). Although intranuclear aggregates are seen in a small number of brainstem neurons in SCA2 patients (Koyano et al., 2002; Koyano et al., 2000), and transiently transfected COS1 cells (Huynh et al., 2003b), cytoplasmic aggregates predominate without the formation of large inclusion bodies, and no nuclear inclusions have been observed in Purkinje neurons of SCA2 patients (Huynh et al., 1999; Huynh et al., 2000a; Koyano et al., 2002). In vitro, mutant ataxin-2 activates apoptosis, causes dispersion of the Golgi complex, and cell death in PC12 and COS1 cells (Huynh et al., 2003b).

Parkin is an E3 ubiquitin ligase which ubiquitinates specific proteins targeted for degradation by the ubiquitin-dependent proteasome system (UPS) (Shimura et al., 2000; Zhang et al., 2000). Mutations of the parkin gene or S-nitrosylation of parkin inactivate the E3 ubiquitin ligase function and cause the majority of cases with autosomal recessive juvenile Parkinsonism (AR-JP) (Abbas et al., 1999; Mata et al., 2004; Yao et al., 2004). Several proteins have been found to be interacted with parkin. Among these are proteins associated with the UPS, Pael-R receptor, CDCrel-1, p38 aminoacyl tRNA synthetase, Far Up Stream Element Binding Protein-1 or FBP1, LRRK2, synphilin-1, glycosylated α-synuclein, synaptotagmin XI, and tubulin (Chung et al., 2001; Corti et al., 2003; Huynh et al., 2003a; Huynh et al., 2003b; Imai et al., 2001; Ko et al., 2006; Ren et al., 2003; Shimura et al., 2000; Shimura et al., 2001; Smith et al., 2005; Zhang et al., 2000). Interaction with parkin facilitates the polyubiquitylation and subsequent degradation of Pael-R receptor, CDCrel-1, synphilin-1, and synaptotagmin XI. Proteomic analyses of an exon 3 parkin knockout mouse model showed that the level of CDRel-1/septin, tubulin α-1 chain, synaptotagmin I were increased (Periquet et al., 2005), while analysis of an exon 7 parkin ko mouse line only showed the increase of p38 aminoacyl tRNA synthetase and FBP1 compared with wildtype animals (Ko et al., 2006; Ko et al., 2005).

Several lines of evidence suggest that parkin may play a role in regulating polyQ proteins. *In vitro* expression of parkin with an expanded polyQ repeat fragment derived from ataxin-3 fused to GFP showed that parkin colocalized and interacted with aggregates of GFP-polyQ, and that it ameliorated proteasomal impairment and caspase-12 activation (Tsai et al., 2003).

During our studies with synaptotagmin XI (Huynh et al., 2003a), we serendipitously observed an interaction of parkin with an N-terminal fragment of normal ataxin-2. In this report, we further characterized this interaction and determined that parkin ubiquitinated normal and mutant ataxin-2. Co-expression of wild type, but not mutant, parkin rescued cells from ataxin-2 induced cytotoxicity. In brains from SCA2 patients, parkin labeled ataxin-2 cytoplasmic aggregates in Purkinje cells and parkin subcellular localization was altered. Regulated

expression of parkin reduced steady-state levels of endogenous ataxin-2 indicating a role for parkin in the normal regulation of ataxin-2 levels.

Materials and Methods

In vitro Co-immunoprecipitation

Prior to co-immunoprecipitation (co-ip), we co-transfected equal molar ratios of pCMV-HA-parkin and the respective pEGFP-ataxin-2 expression plasmids (pEGFP-ataxin-2[Q22], pEGFP-ataxin-2[Q104], or pEGFP- truncated ataxin-2) into HEK293 cells at 60–80% confluency in 100mm^2 dishes. After 48 hrs, proteins were extracted with single detergent buffer [100 mM TrisHCl, pH 8.0, 150 mM NaCl, 0.5% NP40, 0.05% NaAzide containing protease inhibitor mixture (Roche)], immunoprecipitated (ip) with rat anti-HA agarose matrix (Roche), and eluted with 1x PAGE-SDS sample buffer. IP products were immunoblotted with anti-GFP antibody (1/1000, Chemicon) and anti-HA conjugated peroxidase (1/500, Roche).

Ubiquitination assays

HEK293 cells were co-transfected with 5 μg of pCMV-Myc-ubiquitin, pEGFP-ataxin-2, and pCMV-HA-parkin^{wt} or pCMV-HA-parkin^{C289G}. pCMV-HA and pEGFP plasmids were used as negative controls. After 36 hrs, the proteasome was inhibited by 20 μM lactacystin for 4 hrs, and proteins were extracted with RIPA buffer containing protease inhibitor pellet (Roche, 1 pellet per 10 ml buffer), and 2 μM N-ethylamimide to inhibit deubiquitination enzymes. Protein extracts were immunoprecipitated with mouse anti-GFP antibody to pull down GFP-tagged ataxin-2, and the ip products were immunoblotted with anti-HA to detect HA-tagged parkin, anti-myc to detect myc-tagged ubiquitin, or rabbit anti-GFP antibody to detect GFP-tagged ataxin-2.

Assessment of Cell Viability: Trypan blue exclusion

HEK293 cells were transfected with 1 μg of pEGFP vector, pEGFP-ataxin-2, pCMV-HA vector, and pCMV-HA parkins (wild-type and missense) using Qiagen polyfect reagent. After 24 hours, cells were stripped from the dishes with 0.05% trypsin/EDTA (Invitrogen/GIBCO) and gently resuspended in equal volume of growth media and trypan blue. Trypan blue positive and negative GFP staining cells were scored separately using a hemacytometer. The percentage of dead (trypan blue positive) cells was determined by dividing the number of trypan blue positive transfected cells with the total number of transfected cells.

Antibodies

Mouse monoclonal antibodies to Golgi58K (Sigma), to β-actin (Sigma), and rabbit antibody to ubiquitin (DAKO) were purchased. The following reagents were purchased from Boehringer Mannheim: mouse anti-HA antibody, anti-HA-conjugated peroxidase, anti-myc-conjugated peroxidase, and anti-HA-conjugated agarose. Rabbit affinity purified anti-HA antibody used for immunofluorescent labeling was purchased from BETHYL Laboratories, INC. The rabbit and chicken antiparkA antibodies were generated against peptide ParkA as described (Huynh et al., 2000a; Huynh et al., 2003a). The mouse anti-parkin monoclonal antibody (MAB5512) was purchased from Chemicon International.

Immunofluorescent stain

After transfection, cells were fixed with 4% paraformaldehyde in DPBS for 20 min on ice, and incubated in solution A [DPBS, 3% goat serum, 0.05% Triton X-100] for 30 minutes. Immunofluorescent staining was performed using established methods in our laboratory (Huynh et al, 2003b). For immunofluorescent staining of paraffin-embedded tissues, sections were deparaffinized and treated with Auto/Zyme buffer (a protease mixture from Fisher/

Biomedia) for 7 minutes at 37°C, followed by treating the tissues with 15 mM ammonium chloride in PBS, pH 7.5 for 20 minutes to decrease background fluorescence, and then immunofluorescent labeling.

Protein extraction and Western blot analysis

Mouse brain tissue was extracted with triple detergent buffer (1% NP40, 0.5% deoxycholic acid, 0.5% SDS, 100 mM Tris-HCl, pH 8.0, .05% NaAzide, and protease inhibitor tablet (Roche, 1 tablet per 10 ml). 500 mg of brain tissue was added to 5 ml of cold triple detergent buffer and homogenized on ice, and the extract was centrifuged for 30 minutes at 4°C. Supernatant was aliquoted and stored at −80°C. Proteins were resolved and blotted using standard Western blot methods used in the laboratory (Huynh et al., 2003a). Western blots were detected with 1 μg/ml of the tested antibody (anti-parkA or anti-GFP (Chemicon)], anti-ataxin-2 (SCA2B) antibody, 1/500 dil of mouse anti-parkin MAB (Chemicon), mouse anti-actin or GAPDH (Sigma) for loading determination, or peroxidase conjugated anti-myc and anti-HA antibodies (Roche). The unconjugated primary antibodies were visualized with a 1/5000 dilution of peroxidase conjugated anti-rabbit, anti-mouse, or anti-chick antibody (Jackson ImmunoResearch) for one hour at room temperature.

Results

Parkin interacts with ataxin-2 in transfected HEK293 cells

To determine whether parkin binds to full-length ataxin-2 containing different polyQ repeats, protein extracts containing HA-parkin and GFP-tagged full-length or truncated ataxin-2 were immunoprecipitated with anti-HA conjugated agarose. The ip products were detected with anti-GFP (Fig. 1A) or anti-HA (Fig. 1B) antibody. The GFP antibody detected proteins at 175, 200, and 230 kDa corresponding to the GFP-tagged full-length ataxin-2[Q22], ataxin-2[Q58], and ataxin-2[Q104], respectively. The anti-HA antibody also precipitated 70 kDa and 90 kDa bands from samples containing the full-length wild type ataxin-2[Q22] and mutant GFP-ataxin-2 [Q104], respectively. The truncated proteins likely represent N-terminal fragments and have been described previously (Huynh et al., 1999;Huynh et al., 2000a;Huynh et al., 2003b). The length of the polyQ repeat did not affect the binding affinity to parkin as the amounts of the precipitated ataxin-2 relative to the expression levels were similar among ataxin-2 proteins with different polyQ repeats. The anti-HA antibody co-precipitated HA-parkin and both the full-length and N-terminal truncated ataxin-2, but did not co-precipitate the GFP or the GFP-tagged C-terminal ataxin-2 fragment (Fig. 1,Fig. 1S) indicating the specificity of the co-ip experiments. A 130 kDa band, which may represent a stable aggregate of the truncated ataxin-2 [Q104], was also detected. To further confirm the interaction between parkin and ataxin-2, HA-tagged parkin was transiently co-transfected with GFP-tagged truncated or full-length ataxin-2 in COS1 cells. Cells were immunolabeled with rabbit anti-HA antibody and detected with Donkey rhodamine conjugated anti-rabbit IgG. HA-parkin colabeled with both the full-length and truncated ataxin-2 with either a wild type (Q22) or mutant (Q58) PolyQ domain. The co-localization of GFP-ataxin-2[Q104] with wild type and mutant parkins is shown in Figure 5. As previously shown (Huynh et al, 2003b), when full-length wild type ataxin-2 is expressed by itself, ataxin-2 is localized with the Golgi apparatus and fine vesicles (Fig. 1F, panel a, white arrow). When parkin was co-expressed with wild type ataxin-2, parkin co-localized with ataxin-2 in perinuclear structures (Fig. 1F, panel c, blue arrow). Parkin did not colocalize with the GFP-ataxin-2 labeled vesicular structures. Although the intensity of the GFP-ataxin-2 labeling in the Golgi apparatus of the parkin/ataxin-2 co-expressing cell was as strong as that in the GFP-ataxin-2 alone, there were fewer GFP-ataxin-2 positive vesicles observed in the parkin/ataxin-2 co-expressing cell; and those vesicles that labeled had less intensity. These observations suggest that, at least in transfected cells, co-expression with parkin reduces the amount of expressed ataxin-2. The N-terminal ataxin-2 fragments

containing 22, 58, and 104 glutamine repeat (Q22N, Q58N, and Q104N) formed cytoplasmic aggregates with different sizes. These aggregates were clustered with HA-parkin and ataxin-2 (Fig. 1F, j-r). Although the C-terminal fragments also formed cytoplasmic aggregates, these aggregates were more uniform and larger in size, and did not label with parkin (Fig. 1F, g–i).

Ubiquitination of ataxin-2 by parkin

To determine whether full-length ataxin-2 was a substrate of parkin, we performed *in vitro* ubiquitination assays as previously described for sytXI (Huynh et al., 2003a). GFP-tagged full-length ataxin-2 (Q22 or Q104) or truncated ataxin-2 (Q22N, Q104N, or C-term) were co-expressed in HEK293 cells with wildtype HA-tagged parkin or mutant parkin^{C289G} and myc-tagged ubiquitin. Twenty-four hours later, cells were treated with 10 μM lactacystin for 4-hours to inhibit degradation of ubiquitinated proteins by the UPS. Protein extracts were then immunoprecipitated with mouse anti-GFP antibody and the ip products were detected with peroxidase conjugated anti-myc (Fig. 2A) or rabbit anti-GFP antibody (Fig. 2B). To control for variation in transfection of the substrate (ataxin-2 fragments), we determined volumes of cell extracts containing approximately equal amounts of ataxin-2 fragments by Western blot analysis. These volumes were then used for western blots with anti-myc and anti-GFP antibodies. A representative western blot is shown in Figure 2. No ubiquitinated products were detected, when either parkin or ubiquitin were omitted from the transfection or when GFP was added as a substrate. Wild type parkin ubiquitinated full-length mutant ataxin-2[Q104] more efficiently than wild type ataxin-2. In contrast, mutant parkin^{C289G} ubiquitinated wild type and mutant ataxin-2 less efficiently than wild type parkin (Fig. 2A). Truncated ataxin-2 was ubiquitinated much less efficiently by parkin than full-length ataxin-2, and when expressed with parkin^{C289G}, no ubiquitination was visible at all. There was evidence of ubiquitination of a C-terminal ataxin-2 fragment. Possible explanations for these observations are given below.

We then examined the possibility that ubiquitinated GFP-ataxin-2 aggregates might not be soluble in our co-ip buffer. Pellets of the protein extraction were resuspended in SDS-sample buffer and then immunoblotted with anti-GFP antibody (Fig. 2C). As suspected, full-length ataxin-2 and higher molecular weight proteins were detected by the anti-GFP antibody in protein extracts of cells expressing full-length GFP-ataxin-2. Although insolubility of proteins prevented co-immunoprecipitation and detection with anti-myc-ubiquitin antibodies, analysis of pellets suggested that increasing amounts of full-length ataxin-2 were found in the pellet in the presence of parkin. Cells transfected with ataxin-2[Q104] showed a greater abundance of a single band migrating at >250 kD protein and a smear of proteins of even higher molecular weight. Significant amounts of the C-terminal fragment were insoluble but only as a monomer since no high molecular weight C-terminal bands were detected. In contrast, all the N-terminal ataxin-2 fragments remained soluble.

Parkin alters the steady state levels of ataxin-2 in parkin tet off PC12 cells and parkin knockout mice. To determine whether variation in parkin levels altered steady-state levels of endogenous ataxin-2, we utilized rat PC12 cells that stably express human parkin (hparkin) under the control of the tet-promoter (Tet-off parkin PC12 cell line). When these PC12 cells are treated with doxycycline, expression of the exogenous human parkin gene is suppressed in a dosage dependent fashion (Darios et al., 2003). To investigate the dose response, tet-off parkin PC12 cells were treated with 0.0, 0.5, and 2.0 μg/ml of doxycycline. After 72 hours of treatment, protein extracts were isolated and subjected to western blot analyses with anti-ataxin-2 (Fig. 3a, top) and anti-parkin (Fig. 3a, bottom) antibodies. Doxycycline at 2.0 μg/ml suppressed expression of hparkin, but as expected did not affect the expression of endogenous rat parkin. Increasing expression of human parkin resulted in decreased abundance of endogenous rat ataxin-2. One of two representative experiments is shown in Fig. 3a. To determine whether parkin deficiency caused an increased level of endogenous ataxin-2 in vivo, we analyzed

ataxin-2 abundance in brains of parkin knockout mice. We performed Western blot analysis of protein extracts from brains of parkin knock-out (ko) and wild type (wt) mice with rabbit anti-ataxin-2 antibody (SCA2B) and a mouse monoclonal antibody to the C-terminal fragment of parkin, or β-actin. Figure 3b shows the western blot of protein extracts from 3 wt and 3 parkin ko mice. In parkin ko mouse brains, parkin was not detected by the antibody to the C-terminal domain of parkin, and the level of mouse endogenous ataxin-2 was significantly increased (Fig. 3b, top panel). The mouse anti-parkin antibody did not detect any parkin band in the parkin ko mice (Fig. 3b, middle). Similar observations were made in a separate analysis of 2 additional wt and 2 parkin ko mice. Densitometric analysis (Fig. 3c) using the Image J program **(http://rsb.info.nih.gov/ij/)** showed that parkin deficiency increased the levels of endogenous ataxin-2 by 22% ($P < 0.007$, $n = 3$, unpaired t-test). Ubiquitinated ataxin-2 in the PC12 cells and parkin ko mice is undetectable in both parkin ko mouse and parkin tet-off PC12 cells as the turn over of ubiquitinated ataxin-2 is likely rapid.

To further access the effect of parkin, cells transfected with GFP-tagged wild type ataxin-2 [Q22] or mutant ataxin-2[Q104] and with pCMV-HA vector or with wild type parkin were treated with puromycin to inhibit protein synthesis. Puromycin is an amino nucleoside antibiotic that inhibits peptidylic transfer on ribosome. At 0, 2, 3, 6, and 24 hrs after puromycin treatment, protein extracts were isolated, and subjected to western blot analyses with mouse anti-GFP antibody (Fig. 3S, supplemental). Figures 3S is a representative experiment of 2 independent experiments. As previously indicated by semi-quantitative experiments using immunofluorescence (Huynh et al, 2003b), mutant ataxin-2[Q104] was degraded much more slowly than wildtype ataxin-2[Q22]. At 6 hrs, 70% of ataxin-2 had been degraded compared with 40% for ataxin-2[Q104]. Parkin facilitated the degradation of both wild-type ataxin-2 [Q22] and mutant ataxin-2[Q104] (Fig. 3S). Compared with expression of HA-vector, expression of parkin reduced levels of ataxin-2[Q22] by 45% and those of ataxin-2[Q104] by 35%.

Parkin suppresses cell death induced by mutant ataxin-2: As cell death induced by mutant ataxin-2 appears to result from the accumulation of mutant ataxin-2, we investigated whether parkin ameliorated the cytotoxic effect of mutant ataxin-2 in HEK293 cells (Fig. 4). We determined cell death by using trypan blue exclusion of GFP expressing cells 48 hours after transfection. About 200 to 300 transfected cells were scored for each of the experiments described below. In the absence of parkin, mutant ataxin-2[Q104] with an expanded polyQ repeat caused significantly more cell death than wild-type GFP-ataxin-2[Q22] (15.2.0%±1.4 vs. 5.0%±1.013, $n=4$, $P \leq 0.001$, One-way ANOVA analysis). In the presence of parkin, cell death induced by mutant ataxin-2[Q104] was significantly reduced compared to mutant ataxin-2[Q104] alone (8.2%±1.0 vs. 15.2%±0.2, $P \leq 0.01$). Mutant parkin^{C289G} and parkin^{C418R} did not reduce cytotoxicity induced by mutant ataxin-2 ($P \geq 0.05$). When mutant parkin^{C289G} was co-expressed with wild type ataxin-2[Q22], cell death actually increased to 9.8%±1.1 as compared to 5.0%±1.0 and 7.1%±1.8 for wild type GFP-ataxin-2[Q22] with wild type parkin or HA-vector control ($P<0.001$), respectively. Cell death difference between wild ataxin-2 alone and with parkin is not statistically different ($n \geq 0.05$). Coexpression with parkin^{C418R}, a RING2 missense mutation, did not enhance cell death induced by either wild type or mutant ataxin-2.

Parkin reduces Golgi dispersion caused by mutant ataxin-2[Q104]

In cell culture, endogenous and exogenous ataxin-2[Q22] colocalize with the Golgi 58kD marker protein (Huynh et al., 2003b). Expression of mutant ataxin-2 [Q104] (Fig. 5a-d) causes dispersion of the 58K-labelled Golgi compartment (Huynh et al., 2003b). Since parkin reduced cell death caused by the overexpression of mutant ataxin-2, we investigated whether parkin also decreased alterations in Golgi morphology using triple immunofluorescent labeling (Fig.

5). HEK293 cells expressing GFP-ataxin-2[Q104] and parkin or vector control (Fig. 5) were labeled with rabbit anti-HA (Fig. 5c, g, k) and mouse anti-Golgi58K (Fig. 5b, f, j) antibodies and imaged by laser confocal microscopy. Golgi morphology alteration was scored by cells possessing diffuse versus perinuclear Golgi58K labeling. We found that co-expression with wild-type parkin (Fig. 5e-h) greatly reduced the number of transfected cells with an abnormally dispersed distribution of GFP-ataxin-2[Q104] compared to GFP-ataxin-2[Q104] with HA-vector (Fig. 5a-d) or with mutant HA-parkin^{C289G} (Fig. 5i-l).

Cells expressing ataxin-2[Q104] had Golgi alterations in 69%± 7.1 compared with only 32.3% ±11.7 of cells co-expressing parkin and ataxin-2[Q104] (Fig. 5S, n=3, P≤0.001, One-way ANOVA). Cells coexpressing ataxin-2[Q104] and parkin ^{C418R} (not shown) or parkin^{C289G} had Golgi abnormalities in 67.3%±10.3 and 85.6%±14.8, respectively. The increase in Golgi abnormalities upon transfection with parkin^{C289G} was significant compared with HA-vector transfected cells ($p<0.05$).

To examine whether parkin and ataxin-2 interacted in neurons primarily affected by SCA2, we labeled cerebellar sections from 3 normal controls (Fig. 6A) and 2 SCA2 patients (Fig. 6B) with antibodies against parkin (Huynh et al., 2003a) and ataxin-2 (Huynh et al., 2003b). In normal Purkinje neurons, both parkin and ataxin-2 were strongly expressed confirming previous observations (Huynh et al., 1999;Huynh et al., 2000b). Both proteins localized in distinct punctate structures with strong co-localization in the cell plasma membrane (Fig. 6A, blue arrow). The cell bodies of normal Purkinje neurons were strongly immunoreactive for ataxin-2, while staining was almost undetectable in neurites (Fig. 6A, red). In contrast, parkin labeling was more pronounced at the plasma membrane and in neurites (Fig. 6A, green). In SCA2 Purkinje neurons, ataxin-2 localization was confined to cytoplasmic aggregates in the cell body and extended to large neuritic processes (Fig. 6B). Parkin immunoreactivity was now undetectable at the plasma membrane but confined to large bodies or puncta along the dendritic tree (Fig. 6B). In addition, parkin was colocalized with ataxin-2 in selected cytoplasmic (white arrow) and perinuclear aggregates (blue arrow). Overall levels of parkin immunoreactivity were reduced in SCA2 Purkinje neurons compared to normal Purkinje neurons.

Discussion

In human and mouse brains, expression of ataxin-2 with expanded polyQ repeats results in cytoplasmic aggregates, altered Purkinje cell morphology and motor dysfunction in the respective organism (Huynh et al., 1999; Huynh et al., 2000a). Cell death *in vitro* is associated with alterations in Golgi morphology and the activation of apoptosis (Huynh et al., 2003b). Based on our identification of a physical interaction between ataxin-2 and parkin, we investigated the effects of parkin overexpression on abundance and localization of ataxin-2 and ataxin-2 induced cell death.

Parkin mediated ubiquitination and degradation of ataxin-2

Parkin interacted with full-length wildtype ataxin-2[Q22], mutant ataxin-2[Q104], and an N-terminal ataxin-2 fragment, but not with a C-terminal fragment confirming the specificity of the interaction (Fig. 1). Parkin interaction with ataxin-2 was further confirmed when cells co-expressing HA-tagged parkin and GFP-tagged ataxin-2 showed strong colocalization between ataxin-2 and parkin (Fig. 1F). Although parkin bound ataxin-2[Q22] and ataxin-2[Q104] with equal strength, ubiquitination of wild type ataxin-2[Q22] was weaker suggesting that parkin-mediated ubiquitination of polyQ proteins may selectively favor proteins with expanded polyQ repeats. Parkin has been shown to interact with another polyQ protein, ataxin-3, but in those studies, interaction was only observed for a shorter fragment of ataxin-3 containing the polyQ repeat (Tsai et al., 2003).

Parkin with a missense mutation in the RING1 domain (parkin^{C289G}) had reduced ability to ubiquitinate full-length ataxin-2 (Fig. 2). This observation is consistent with recent findings by (Sriram et al., 2005) that parkin missense mutations decrease substrate binding, E3 ubiquitin ligase activity, and substrate degradation. Although parkin bound to the N-terminal truncated ataxin-2 (Fig. 1), the level of ubiquitination of these truncated ataxin-2 fragments was significantly reduced. It is possible that ubiquitination levels of truncated ataxin-2 were below our level of detection as only 7 lysine residues are contained within the truncated fragment, whereas the C-terminus contains 25 clustered lysine residues, which are preferred sites for attachment of ubiquitin (Pickart, 2001). The strong clustering of lysine residues in the C-terminal fragment may explain why this fragment showed some ubiquitination in HEK293 cells despite the lack of visible interaction in co-immunoprecipitation assays (Fig. 1 and 2). The interaction may have been below our level of detection.

Consistent with previous findings with other parkin substrates (Chung et al., 2001; Huynh et al., 2003a; Imai et al., 2001; Zhang et al., 2000), exogenous expression of parkin altered the steady-state levels of ataxin-2 (Fig. 3). In tet-off PC12 cells expressing human parkin, increased parkin expression resulted in a dosage-dependent decrease in the abundance of endogenous rat ataxin-2 (Fig. 3). As rat ataxin-2 does not contain a polyQ repeat (Accession#XP_213779), this observation also suggests that parkin may bind to a site different from the polyQ repeat. Future direct interaction studies with rat ataxin-2 will be required to confirm this hypothesis.

The sensitivity of endogenous ataxin-2 to parkin levels is interesting as mice deficient for parkin failed to show any changes in the abundance of other known parkin interactors such as Pael-R and synphilin-1 (Goldberg et al., 2003; Palacino et al., 2004; Periquet et al., 2005). However, another study using an exon 7 parkin ko model found that only the parkin interactors, p38 tRNA synthetase and FBP1 were increased (Ko et al., 2006; Ko et al., 2005). Unfortunately, ataxin-2 was not assayed in either model. Our analysis of parkin KO mice now confirms that ataxin-2 steady-state levels are increased in KO mice *in vivo*.

Parkin effects on ataxin-2-induced cell death

Overexpression of several parkin substrates including CDCrel-1 and Pael-R causes cell death in mammalian cell lines. In Drosophila and cultured cells, expression of parkin substrates resulted in cell death, which was ameliorated by co-expression of parkin (Chung et al., 2001; Imai et al., 2001; Imai et al., 2003; Ko et al., 2005). These observations are consistent with our observations that expression of wild type parkin reduced Golgi alteration and cell death induced by mutant ataxin-2[Q104] very significantly (Fig. 4). Co-expression of mutant parkins did not rescue cells from ataxin-2-induced cytotoxicity. Quite unexpectedly, parkin^{C289G} increased cell death, especially when co-expressed with wild type ataxin-2[Q22]. It is not clear at this point whether this represents a direct toxic action of parkin^{C289G} or a dominant negative action by interference with the function of endogenous parkin.

Purkinje cells are the site of strong expression of ataxin-2 and are the cell type primarily affected in SCA2. *In vivo*, both ataxin-2 and parkin are expressed in the substantia nigra and in Purkinje cells ((Huynh et al., 1999; Huynh et al., 2001; Huynh et al., 2000b). In brains from SCA2 patients, both proteins were co-localized in discrete cytoplasmic and large perinuclear aggregates in Purkinje neurons (Fig. 6B). The large aggregate depicted in Fig. 6B is likely perinuclear rather than nuclear as we have detected aggregates in transfected cells that originated in the cytoplasm but indented or invaginated into the nucleus. In addition, studies by us and others have not detected nuclear aggregates in Purkinje cells in SCA2 or in any other polyQ disease (Koyano et al, 2002; Huynh et al, 1999, Huynh et al, 2000a).

Cytoplasmic and nuclear protein aggregation has been implicated in several polyQ associated diseases (DiFiglia et al., 1997; Huynh et al., 2000a; Klement et al., 1998; Koyano et al.,

2002; Paulson et al., 1997), although the formation of large aggregates may actually be protective (Cummings et al., 2001). Our studies were not designed to examine the direct pathogenicity of aggregates. However, parkin reduced steady state levels of ataxin-2, which may contribute to reduced aggregation and cytotoxicity. Of note, parkin immunoreactivity was significantly reduced in the cytoplasm of SCA2 Purkinje neurons compared with wildtype Purkinje neurons. This may indicate that parkin was sequestered into ataxin-2 aggregates, which in turn could affect steady-state levels of other parkin-interacting proteins as well. In light of the recently recognized importance of the proteasome system in synaptic function (Bingol and Schuman, 2006; Willemeur et al, in press) the greatly reduced parkin immunoreactivity in Purkinje cell dendrites in SCA2 brains may suggest a direct link between parkin sequestration and synaptic dysfunction in SCA2.

Parkin mutations are the most common cause of familial early-onset PD (Abbas et al., 1999; Lohmann et al., 2003; Mata et al., 2004). Some SCA2 patients and even some SCA2 pedigrees have a predominant L-DOPA responsive Parkinsonian phenotype with bradykinesia, tremor, and rigidity (Gwinn-Hardy et al., 2000; Ragothaman et al., 2004; Shan et al., 2001). This is consistent with expression of ataxin-2 in dopaminergic neurons of the substantia nigra (Huynh et al., 2000b). A pair of sisters with SCA2 exhibited neuronal loss in the substantia nigra with evidence of intranuclear ubiquitin and ataxin-2 associated inclusions in neurons (Armstrong et al., 2005) suggesting that mutant ataxin-2 was ubiquitinated. Furthermore, we found ataxin-2 in Lewy bodies in nigral neurons in two of five individuals with diffuse Lewy body Dementia (Huynh and Pulst, pers. Comm.). The physical interactions of parkin and ataxin-2 raise interesting prospects in view of the phenotypic overlap seen in some PD families with SCA2 mutations and should lead to investigation of parkin single nucleotide polymorphisms as modifiers of the SCA2 phenotype and vice versa. Finally, the ability to rescue cells from ataxin-2-induced cytotoxicity by parkin overexpression may provide therapeutic options that could be tested using *in vivo* models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Abbas N, Lucking CB, Ricard S, Durr A, Bonifati V, De Michele G, Bouley S, Vaughan JR, Gasser T, Marconi R, Broussolle E, Brefel-Courbon C, Harhangi BS, Oostra BA, Fabrizio E, Bohme GA, Pradier L, Wood NW, Filla A, Meco G, Denefle P, Agid Y, Brice A. A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe. French Parkinson's Disease Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease. Hum Mol Genet 1999;8:567–574. [PubMed: 10072423]

Armstrong J, Bonaventura I, Rojo A, Gonzalez G, Corral J, Nadal N, Volpini V, Ferrer I. Spinocerebellar ataxia type 2 (SCA2) with white matter involvement. Neurosci Lett 2005;381:247–251. [PubMed: 15896478]

Bingol B, Schuman EM. Activity-dependent dynamics and sequestration of proteasomes in dendritic spines. Nature 2006;441:1144–1148. [PubMed: 16810255]

Chung KK, Zhang Y, Lim KL, Tanaka Y, Huang H, Gao J, Ross CA, Dawson VL, Dawson TM. Parkin ubiquitinates the alpha-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. Nat Med 2001;7:1144–1150. [PubMed: 11590439]

Corti O, Hampe C, Koutnikova H, Darios F, Jacquier S, Prigent A, Robinson JC, Pradier L, Ruberg M, Mirande M, Hirsch E, Rooney T, Fournier A, Brice A. The p38 subunit of the aminoacyl-tRNA synthetase complex is a Parkin substrate: linking protein biosynthesis and neurodegeneration. Hum Mol Genet 2003;12:1427–1437. [PubMed: 12783850]

Cummings CJ, Sun Y, Opal P, Antalffy B, Mestril R, Orr HT, Dillmann WH, Zoghbi HY. Over-expression of inducible HSP70 chaperone suppresses neuropathology and improves motor function in SCA1 mice. Hum Mol Genet 2001;10:1511–1518. [PubMed: 11448943]

Darios F, Corti O, Lucking CB, Hampe C, Muriel MP, Abbas N, Gu WJ, Hirsch EC, Rooney T, Ruberg M, Brice A. Parkin prevents mitochondrial swelling and cytochrome c release in mitochondria-dependent cell death. Hum Mol Genet 2003;12:517–526. [PubMed: 12588799]

DiFiglia M, Sapp E, Chase KO, Davies SW, Bates GP, Vonsattel JP, Aronin N. Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science 1997;277:1990–1993. [PubMed: 9302293]

Goldberg MS, Fleming SM, Palacino JJ, Cepeda C, Lam HA, Bhatnagar A, Meloni EG, Wu N, Ackerson LC, Klapstein GJ, Gajendiran M, Roth BL, Chesselet MF, Maidment NT, Levine MS, Shen J. Parkin-deficient mice exhibit nigrostriatal deficits but not loss of dopaminergic neurons. J Biol Chem 2003;278:43628–43635. [PubMed: 12930822]

Gwinn-Hardy K, Chen JY, Liu HC, Liu TY, Boss M, Seltzer W, Adam A, Singleton A, Koroshetz W, Waters C, Hardy J, Farrer M. Spinocerebellar ataxia type 2 with parkinsonism in ethnic Chinese. Neurology 2000;55:800–805. [PubMed: 10993999]

Huynh DP, Del Bigio MR, Ho DH, Pulst SM. Expression of ataxin-2 in brains from normal individuals and patients with Alzheimer's disease and spinocerebellar ataxia 2. Ann Neurol 1999;45:232–241. [PubMed: 9989626]

Huynh DP, Dy M, Nguyen D, Kiehl TR, Pulst SM. Differential expression and tissue distribution of parkin isoforms during mouse development. Brain Res Dev Brain Res 2001;130:173–181.

Huynh DP, Figueroa K, Hoang N, Pulst SM. Nuclear localization or inclusion body formation of ataxin-2 are not necessary for SCA2 pathogenesis in mouse or human. Nat Genet 2000a;26:44–50. [PubMed: 10973246]

Huynh DP, Scoles DR, Ho TH, Del Bigio MR, Pulst SM. Parkin is associated with actin filaments in neuronal and nonneural cells. Ann Neurol 2000b;48:737–744. [PubMed: 11079537]

Huynh DP, Scoles DR, Nguyen D, Pulst SM. The autosomal recessive juvenile Parkinson disease gene product, parkin, interacts with and ubiquitinates synaptotagmin XI. Hum Mol Genet 2003a;12:2587–2597. [PubMed: 12925569]

Huynh DP, Yang HT, Vakharia H, Nguyen D, Pulst SM. Expansion of the polyQ repeat in ataxin-2 alters its Golgi localization, disrupts the Golgi complex and causes cell death. Hum Mol Genet 2003b; 12:1485–1496. [PubMed: 12812977]

Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, Takahashi R. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. Cell 2001;105:891–902. [PubMed: 11439185]

Imai Y, Soda M, Murakami T, Shoji M, Abe K, Takahashi R. A product of the human gene adjacent to parkin is a component of Lewy bodies and suppresses Pael receptor-induced cell death. J Biol Chem 2003;278:51901–51910. [PubMed: 14532270]

Imbert G, Saudou F, Yvert G, Devys D, Trottier Y, Garnier JM, Weber C, Mandel JL, Cancel G, Abbas N, Durr A, Didierjean O, Stevanin G, Agid Y, Brice A. Cloning of the gene for spinocerebellar ataxia 2 reveals a locus with high sensitivity to expanded CAG/glutamine repeats. Nat Genet 1996;14:285–291. [PubMed: 8896557]

Klement IA, Skinner PJ, Kaytor MD, Yi H, Hersch SM, Clark HB, Zoghbi HY, Orr HT. Ataxin-1 nuclear localization and aggregation: role in polyglutamine-induced disease in SCA1 transgenic mice. Cell 1998;95:41–53. [PubMed: 9778246]

Ko HS, Kim SW, Sriram SR, Dawson VL, Dawson TM. Identification of far up stream element binding protein-1 as an authentic parkin substrate. J Biol Chem. 2006

Ko HS, von Coelln R, Sriram SR, Kim SW, Chung KK, Pletnikova O, Troncoso J, Johnson B, Saffary R, Goh EL, Song H, Park BJ, Kim MJ, Kim S, Dawson VL, Dawson TM. Accumulation of the authentic parkin substrate aminoacyl-tRNA synthetase cofactor, p38/JTV-1, leads to catecholaminergic cell death. J Neurosci 2005;25:7968–7978. [PubMed: 16135753]

Koyano S, Iwabuchi K, Yagishita S, Kuroiwa Y, Uchihara T. Paradoxical absence of nuclear inclusion in cerebellar Purkinje cells of hereditary ataxias linked to CAG expansion. J Neurol Neurosurg Psychiatry 2002;73:450–452. [PubMed: 12235319]

Koyano S, Uchihara T, Fujigasaki H, Nakamura A, Yagishita S, Iwabuchi K. Neuronal intranuclear inclusions in spinocerebellar ataxia type 2. Ann Neurol 2000;47:550. [PubMed: 10762173]

Lohmann E, Periquet M, Bonifati V, Wood NW, De Michele G, Bonnet AM, Fraix V, Broussolle E, Horstink MW, Vidailhet M, Verpillat P, Gasser T, Nicholl D, Teive H, Raskin S, Rascol O, Destee A, Ruberg M, Gasparini F, Meco G, Agid Y, Durr A, Brice A. How much phenotypic variation can be attributed to parkin genotype? Ann Neurol 2003;54:176–185. [PubMed: 12891670]

Mata IF, Lockhart PJ, Farrer MJ. Parkin genetics: one model for Parkinson's disease. Hum Mol Genet 13 Spec No 2004;1:R127–133.

Nakamura K, Jeong SY, Uchihara T, Anno M, Nagashima K, Nagashima T, Ikeda S, Tsuji S, Kanazawa I. SCA17, a novel autosomal dominant cerebellar ataxia caused by an expanded polyglutamine in TATA-binding protein. Hum Mol Genet 2001;10:1441–1448. [PubMed: 11448935]

Orr HT, Zoghbi HY. SCA1 molecular genetics: a history of a 13 year collaboration against glutamines. Hum Mol Genet 2001;10:2307–2311. [PubMed: 11673415]

Palacino JJ, Sagi D, Goldberg MS, Krauss S, Motz C, Wacker M, Klose J, Shen J. Mitochondrial dysfunction and oxidative damage in parkin-deficient mice. J Biol Chem 2004;279:18614–18622. [PubMed: 14985362]

Paulson HL, Perez MK, Trottier Y, Trojanowski JQ, Subramony SH, Das SS, Vig P, Mandel JL, Fischbeck KH, Pittman RN. Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. Neuron 1997;19:333–344. [PubMed: 9292723]

Periquet M, Corti O, Jacquier S, Brice A. Proteomic analysis of parkin knockout mice: alterations in energy metabolism, protein handling and synaptic function. J Neurochem 2005;95:1259–1276. [PubMed: 16150055]

Pickart CM. Mechanisms underlying ubiquitination. Annu Rev Biochem 2001;70:503–533. [PubMed: 11395416]

Pulst SM, Nechiporuk A, Nechiporuk T, Gispert S, Chen XN, Lopes-Cendes I, Pearlman S, Starkman S, Orozco-Diaz G, Lunkes A, DeJong P, Rouleau GA, Auburger G, Korenberg JR, Figueroa C, Sahba S. Moderate expansion of a normally biallelic trinucleotide repeat in spinocerebellar ataxia type 2. Nat Genet 1996;14:269–276. [PubMed: 8896555]

Pulst SM, Santos N, Wang D, Yang H, Huynh D, Velazquez L, Figueroa KP. Spinocerebellar ataxia type 2: polyQ repeat variation in the CACNA1A calcium channel modifies age of onset. Brain 2005;128:2297–2303. [PubMed: 16000334]

Ragothaman M, Sarangmath N, Chaudhary S, Khare V, Mittal U, Sharma S, Komatireddy S, Chakrabarti S, Mukerji M, Juyal RC, Thelma BK, Muthane UB. Complex phenotypes in an Indian family with homozygous SCA2 mutations. Ann Neurol 2004;55:130–133. [PubMed: 14705123]

Ren Y, Zhao J, Feng J. Parkin binds to alpha/beta tubulin and increases their ubiquitination and degradation. J Neurosci 2003;23:3316–3324. [PubMed: 12716939]

Sanpei K, Takano H, Igarashi S, Sato T, Oyake M, Sasaki H, Wakisaka A, Tashiro K, Ishida Y, Ikeuchi T, Koide R, Saito M, Sato A, Tanaka T, Hanyu S, Takiyama Y, Nishizawa M, Shimizu N, Nomura Y, Segawa M, Iwabuchi K, Eguchi I, Tanaka H, Takahashi H, Tsuji S. Identification of the spinocerebellar ataxia type 2 gene using a direct identification of repeat expansion and cloning technique, DIRECT. Nat Genet 1996;14:277–284. [PubMed: 8896556]

Schols L, Gispert S, Vorgerd M, Menezes Vieira-Saecker AM, Blanke P, Auburger G, Amoiridis G, Meves S, Epplen JT, Przuntek H, Pulst SM, Riess O. Spinocerebellar ataxia type 2. Genotype and phenotype in German kindreds. Arch Neurol 1997;54:1073–1080. [PubMed: 9311350]

Shan DE, Soong BW, Sun CM, Lee SJ, Liao KK, Liu RS. Spinocerebellar ataxia type 2 presenting as familial levodopa-responsive parkinsonism. Ann Neurol 2001;50:812–815. [PubMed: 11761482]

Shimura H, Hattori N, Kubo S, Mizuno Y, Asakawa S, Minoshima S, Shimizu N, Iwai K, Chiba T, Tanaka K, Suzuki T. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nat Genet 2000;25:302–305. [PubMed: 10888878]

Shimura H, Schlossmacher MG, Hattori N, Frosch MP, Trockenbacher A, Schneider R, Mizuno Y, Kosik KS, Selkoe DJ. Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. Science 2001;293:263–269. [PubMed: 11431533]

Smith WW, Pei Z, Jiang H, Moore DJ, Liang Y, West AB, Dawson VL, Dawson TM, Ross CA. Leucine-rich repeat kinase 2 (LRRK2) interacts with parkin, and mutant LRRK2 induces neuronal degeneration. Proc Natl Acad Sci U S A 2005;102:18676–18681. [PubMed: 16352719]

Sriram SR, Li X, Ko HS, Chung KK, Wong E, Lim KL, Dawson VL, Dawson TM. Familial-associated mutations differentially disrupt the solubility, localization, binding and ubiquitination properties of parkin. Hum Mol Genet 2005;14:2571–2586. [PubMed: 16049031]

Tsai YC, Fishman PS, Thakor NV, Oyler GA. Parkin facilitates the elimination of expanded polyglutamine proteins and leads to preservation of proteasome function. J Biol Chem 2003;278:22044–22055. [PubMed: 12676955]

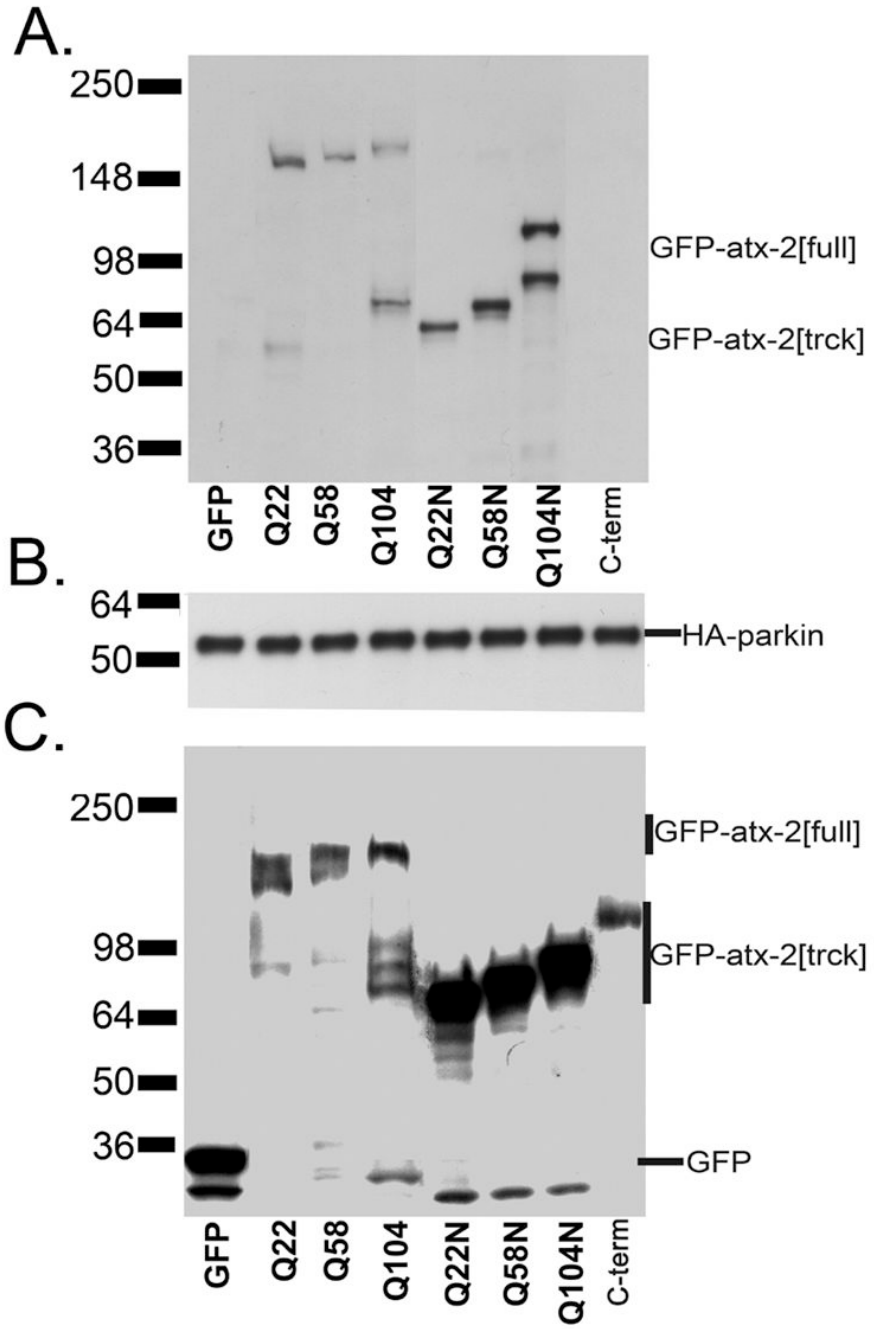
Uchihara T, Fujigasaki H, Koyano S, Nakamura A, Yagishita S, Iwabuchi K. Non-expanded polyglutamine proteins in intranuclear inclusions of hereditary ataxias--triple-labeling immunofluorescence study. Acta Neuropathol (Berl) 2001;102:149–152. [PubMed: 11563629]

Wanker EE. Protein aggregation and pathogenesis of Huntington's disease: mechanisms and correlations. Biol Chem 2000;381:937–942. [PubMed: 11076024]

Willeumier K, Pulst SM, Schweizer FE. Proteasome Inhibition Triggers Activity-Dependent Increase in the Size of the Recycling Vesicle Pool in Hippocampal Neurons. J Neurosci in press. 2006

Yao D, Gu Z, Nakamura T, Shi ZQ, Ma Y, Gaston B, Palmer LA, Rockenstein EM, Zhang Z, Masliah E, Uehara T, Lipton SA. Nitrosative stress linked to sporadic Parkinson's disease: S-nitrosylation of parkin regulates its E3 ubiquitin ligase activity. Proc Natl Acad Sci U S A 2004;101:10810–10814. [PubMed: 15252205]

Zhang Y, Gao J, Chung KK, Huang H, Dawson VL, Dawson TM. Parkin functions as an E2-dependent ubiquitin- protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. Proc Natl Acad Sci U S A 2000;97:13354–13359. [PubMed: 11078524]



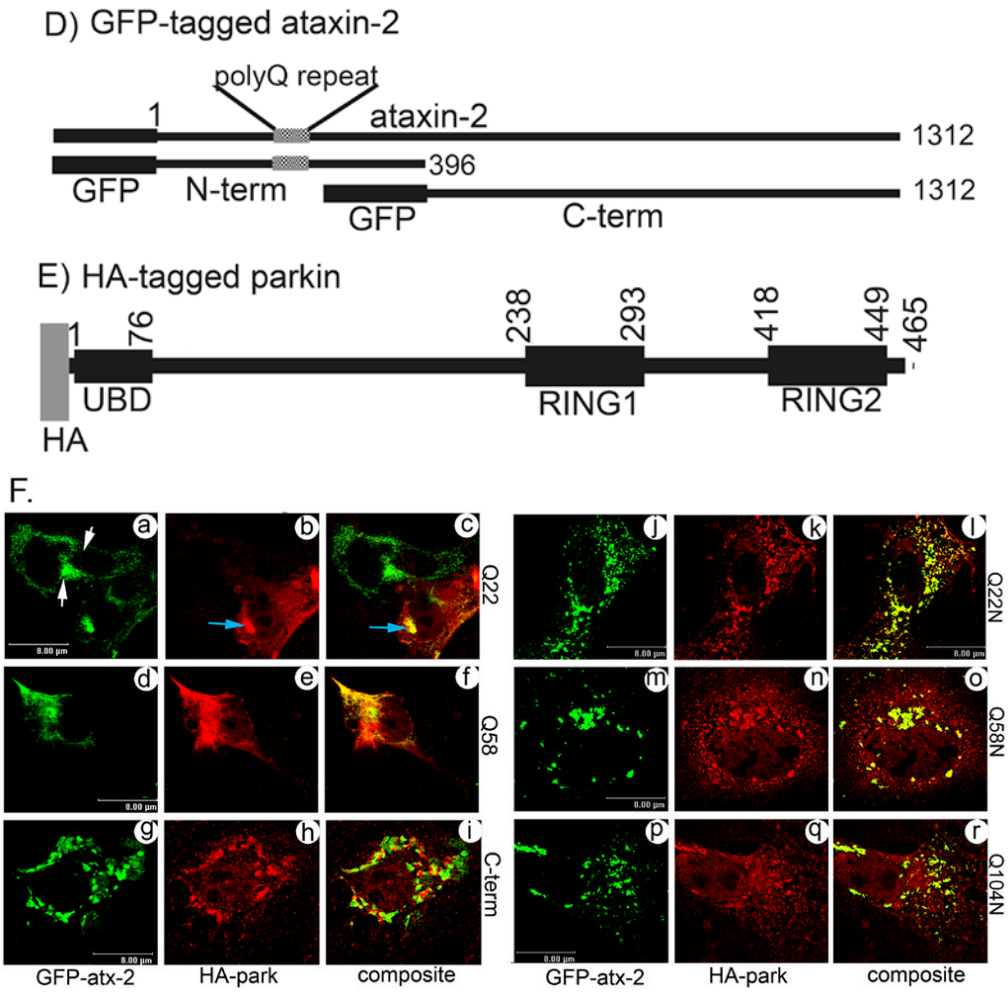


Figure 1.

Both full-length and truncated ataxin-2 interact with parkin (panel A). HEK293 cells were co-transfected with wildtype parkin and GFP-tagged full-length (atx-2[full], N-terminal (N-term), or C-terminal (C-term) fragments of ataxin-2. For the full-length and N-terminal fragments constructs with different polyQ repeats (Q22, Q58, or Q104) were tested. The GFP vector and the GFP-tagged C-terminal domain of ataxin-2 were used as negative controls. Protein extracts were ip with anti-HA antibody conjugated to agarose, and detected with either anti-GFP (A) or anti-HA-peroxidase (B) antibodies. To determine the levels of transfected proteins, western blot of transfected HEK293 cells lysates were detected with anti-GFP (C). Panels D and E show the schematic structure of GFP tagged ataxin-2 (D) and HA-tagged parkin (E) proteins. Panel F demonstrates the colocalization of HA-tagged parkin to GFP tagged full-length and truncated ataxin-2 in COS1 cells. Cells were co-transfected with expression plasmids expressing HA-parkin and GFP-tagged full-length or truncated ataxin-2. Cells were stained with rabbit anti-HA antibody and detected with donkey Rhodamine-RED conjugated anti-rabbit IgG. Images were visualized and acquired by Leica Confocal Laser Microscopy. Bar represents 8 μm.

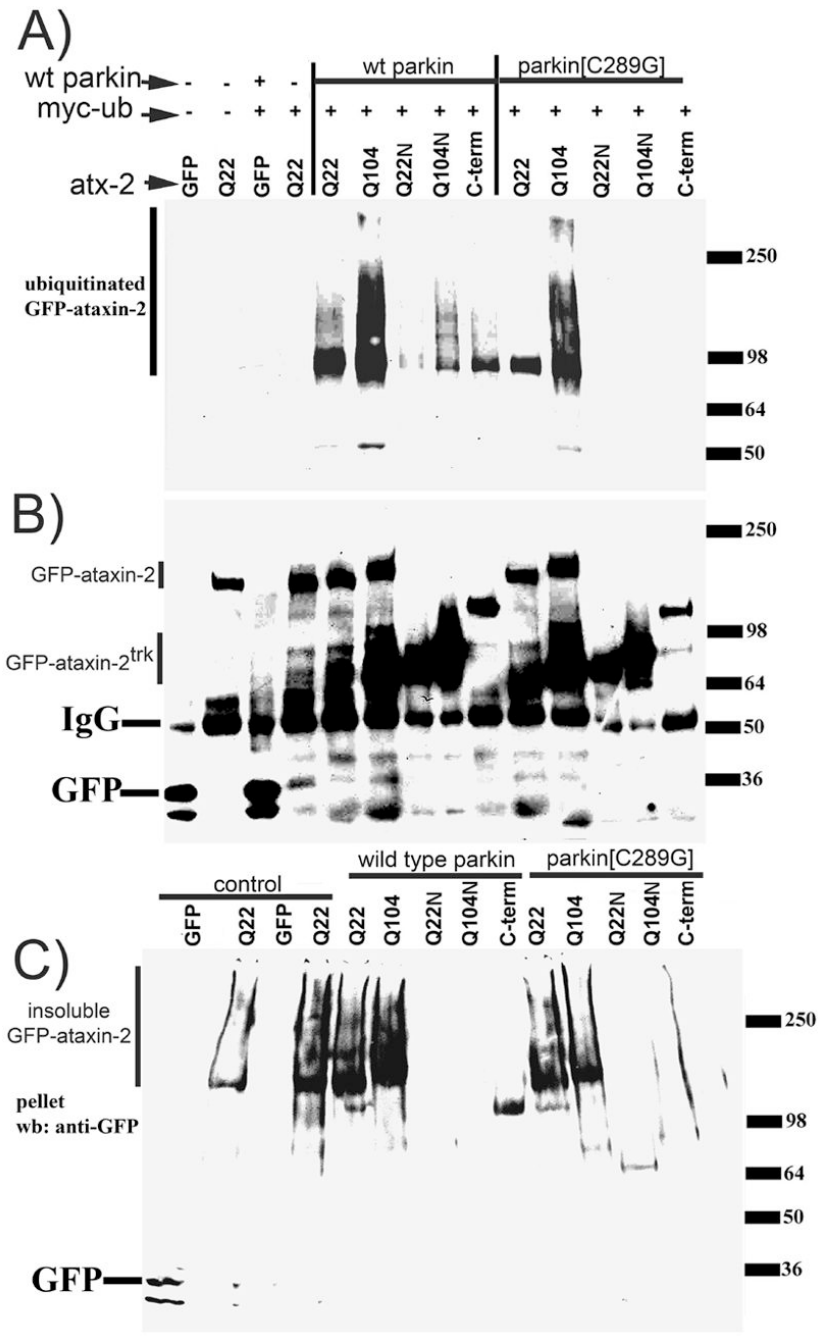


Figure 2.

Parkin ubiquitinates full-length ataxin-2. Ubiquitylation of ataxin-$2^{N\text{-term}}$ (Q22N and Q104N) and ataxin-$2^{C\text{-term}}$(C-term) is much less pronounced. HEK293 cells overexpressing HA-parkins or controls with the corresponding myc-ubiquitin and GFP-tagged ataxin-2 were treated with lactacystin for 4 hours, and protein extracts were immunoprecipitated with anti-GFP antibody. IP products of the ubiquitination assays were detected with an antibody to the myc tag (A) and anti-GFP antibody (B). Note the lack of ubiquitinated products in cells expressing HA-parkin and GFP, GFP tagged truncated ataxin-2. Cells expressing parkin mutants and GFP- ataxin-2 produce a lower amount of ubiquitin-conjugated ataxin-2 compared to the wild-type (wt) parkin. The anti-GFP antibody detects near equal amounts of GFP-

ataxin-2 monomer (B) in all samples containing GFP-ataxin-2. Controls using GFP and GFP-ataxin-2[Q22] with and without parkin did not show any ubiquitination species. Bottom panel (C) shows western blot analysis of pellets of the protein extracts detected with anti-GFP antibody. Large amounts of high molecular weight species were detected in protein extracts from cells coexpressing both wild parkin and full-length ataxin-2 compared with full-length ataxin-2 alone or full-length ataxin-2 and missense mutated parkin. Although significant amounts of C-terminal truncated ataxin-2 remain in the pellet, no high molecular weight species were detectable.

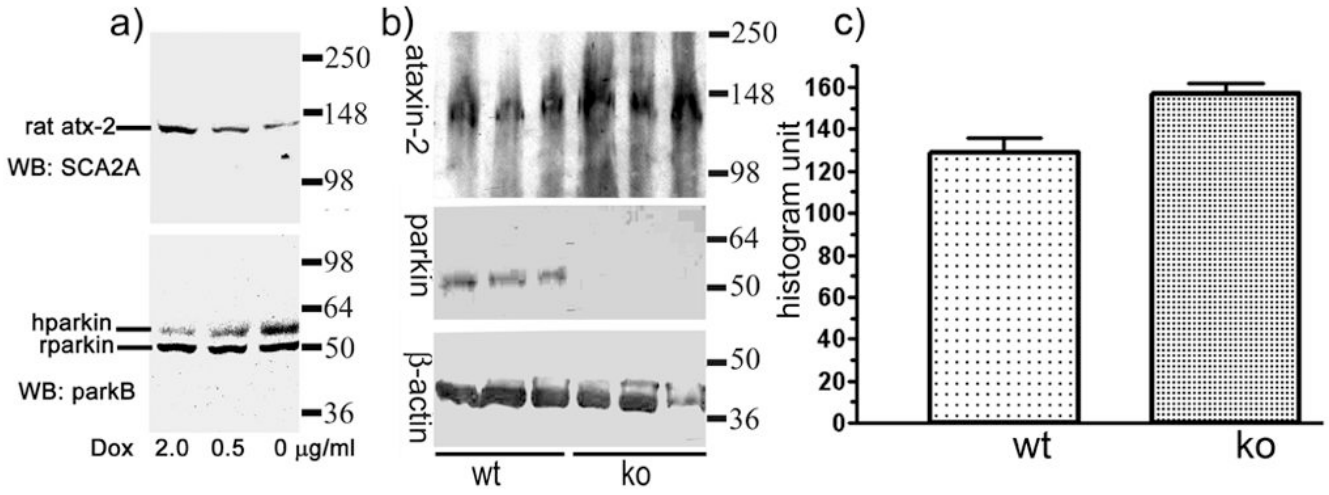


Figure 3.

a) Exogenously expressed wildtype human parkin (hparkin) facilitates the turn over of endogenous wildtype rat ataxin-2. Tet-off hparkin PC12 cells were grown in the presence of different concentrations of Dox for 72 hours. Protein extracts were obtained and proteins were detected with the rabbit anti-ataxin-2 antibody (panel a, top) or the anti-parkin antibody (panel a, bottom). Increased expression of human parkin resulted in decreased abundance of endogenous wildtype ataxin-2. b) Parkin deficiency causes an increase in the level of endogenous ataxin-2 in exon parkin ko mice. Protein extracts from wt and parkin ko mice were detected with anti-ataxin-2 (2 µg/ml SCA2B antibody), anti-parkin (mouse antibody to the C-terminal domain of parkin, 1/500 dil), and antibody to β-actin (1/5000 mouse monoclonal antibody to β-actin). The parkin antibody detects parkin in the wt but not in the ko mice, while ataxin-2 antibody detects a higher level of endogenous ataxin-2 in the ko than the wild type mice. c) Histogram analyses of 3 wt and 3 parkin ko mice using the Image J program. Parkin gene ko causes a 22% increase in endogenous ataxin-2 level ($P < 0.007$, $n = 3$, unpaired t-test).

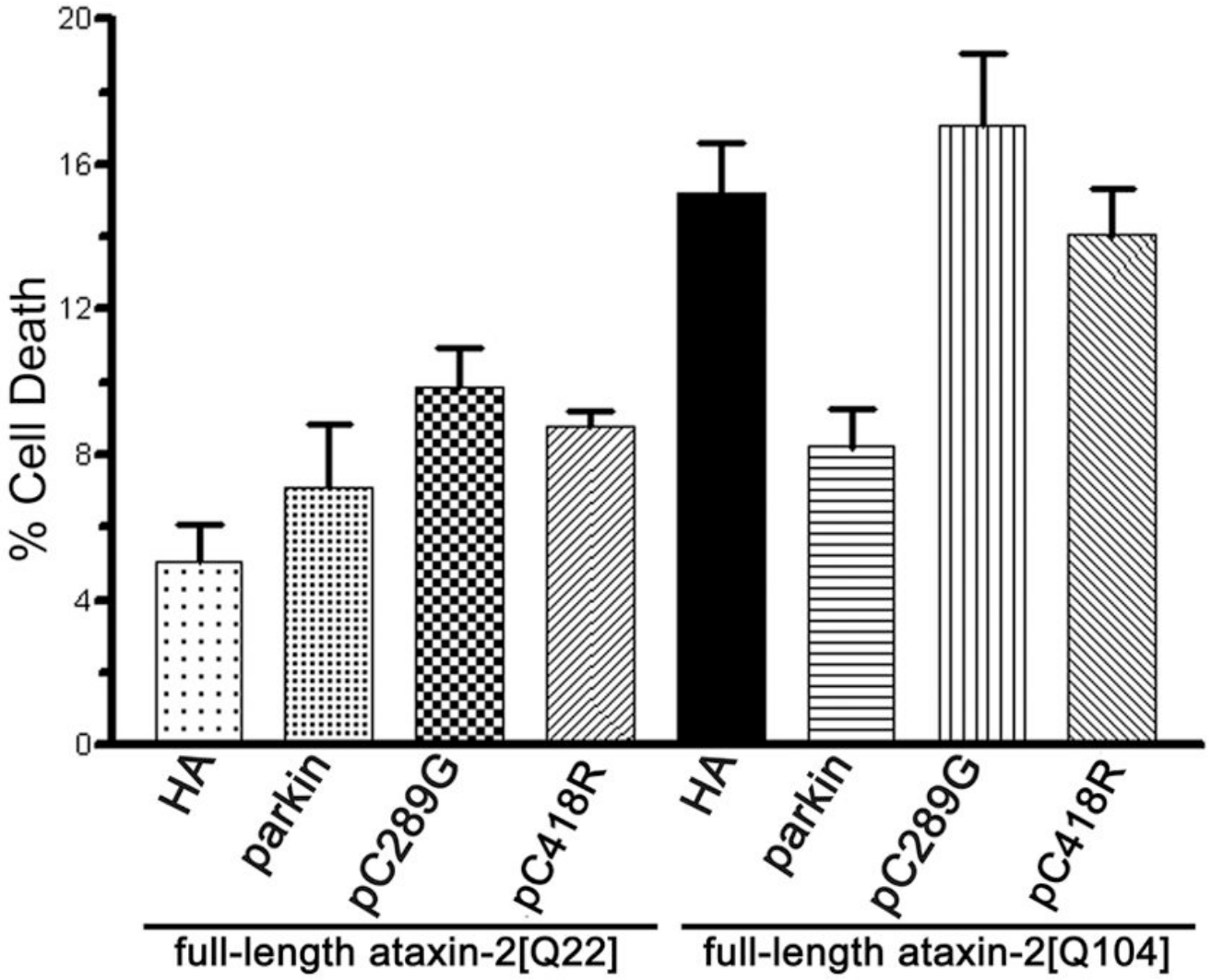


Figure 4.

Wildtype but not missense mutated parkin inhibits cell death induced by mutant GFP-ataxin-2 [Q104]. Cells were co-transfected with wildtype GFP-ataxin-2[Q22] or ataxin-2[Q104] and HA-vector control (vector), HA-parkin (parkin), mutant parkin^{C289G} (pC289G) or parkin^{C418R}(pC418R). Expression of full-length mutant ataxin-2 in HEK293 cells at 48 hours increased the number of cells that were unable to exclude trypan blue. Co-expression with wildtype parkin suppressed toxicity of mutant ataxin-2, while co-expression with mutant parkin^{C289G} and parkin^{C418R} showed no cytoprotective effect. Slight enhancement of cell death was observed for parkin^{C289G}, n=4.

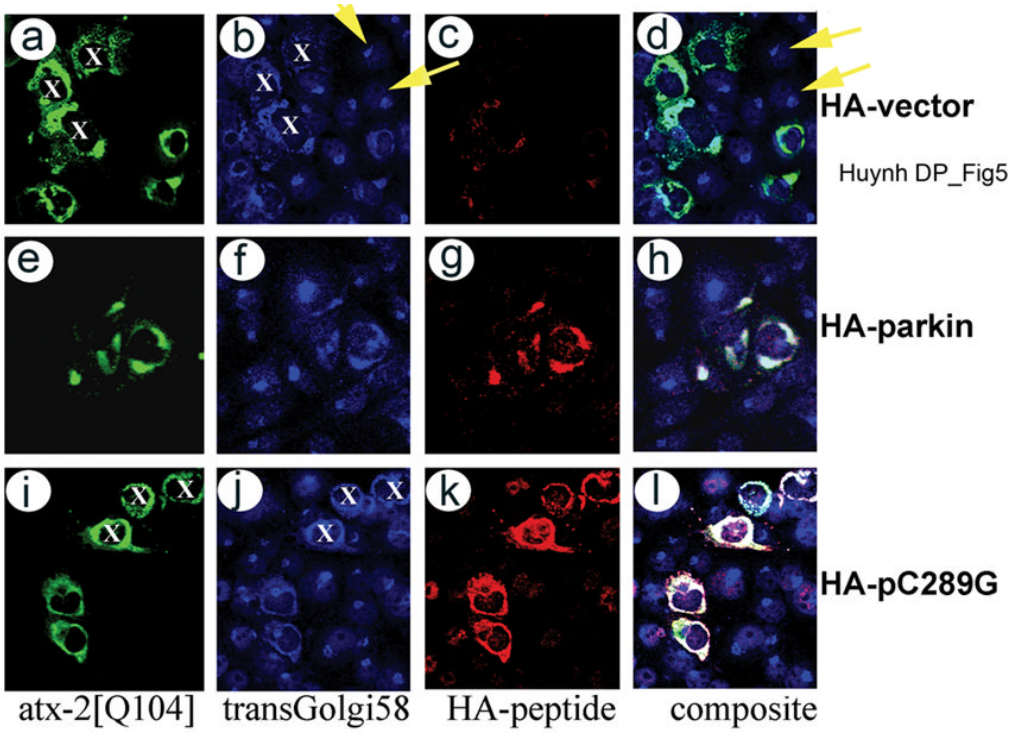
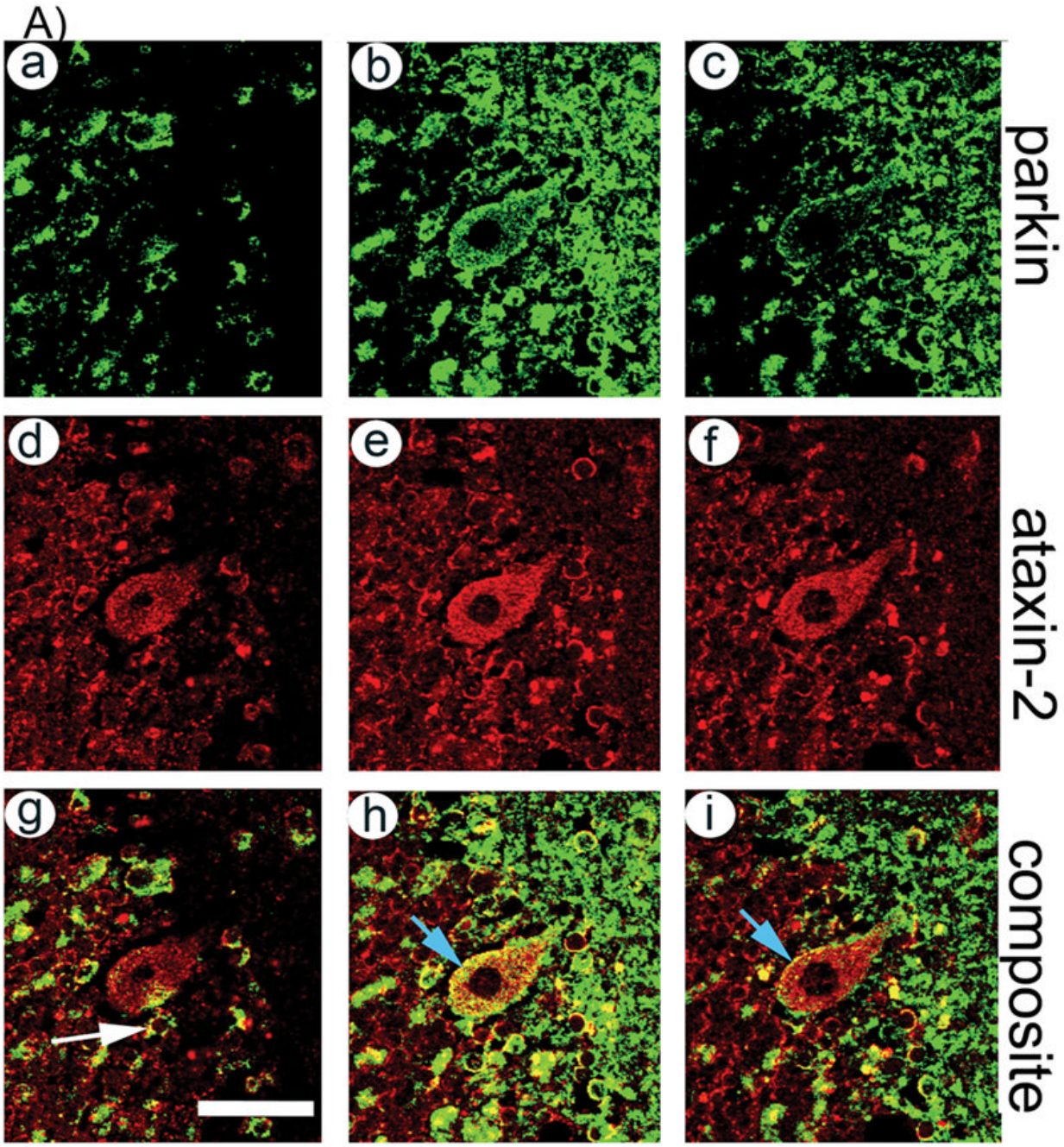


Figure 5.

Parkin reduces Golgi dispersion induced by expanded polyQ repeat ataxin-2[Q104]. HEK293 cells were transiently transfected with pGFP-SCA2[Q104] (a, e, i) and labeled with a monoclonal antibody to transGolgi58K (b, f, j), or rabbit anti-HA antibody (c, g, k). Primary antibodies were detected with anti-rabbit Alexa568 (c, g, k) or anti-mouse Alexa660 (b, f, j). As previously described (Huynh et al, 2003b), GFP-ataxin-2[Q104] disrupts the morphology of the Golgi apparatus (panels a–d); co-expression with wildtype parkin (panels e–h) reduces Golgi abnormalities, while missense parkin ^{C289G} (panels i–l) shows no effect. Note that co-expression of parkin with mutant ataxin-2 restores the co-localization of ataxin-2 with Golgi markers. Parkin largely colocalizes with the Golgi markers resulting in white color in the composite. Images were acquired using the 40X oil immersion objective of the Leica TCSSP microscope. Yellow arrows indicate untransfected cells with normal Golgi58K labeling. White X labels show transfected cells with Golgi dispersion.



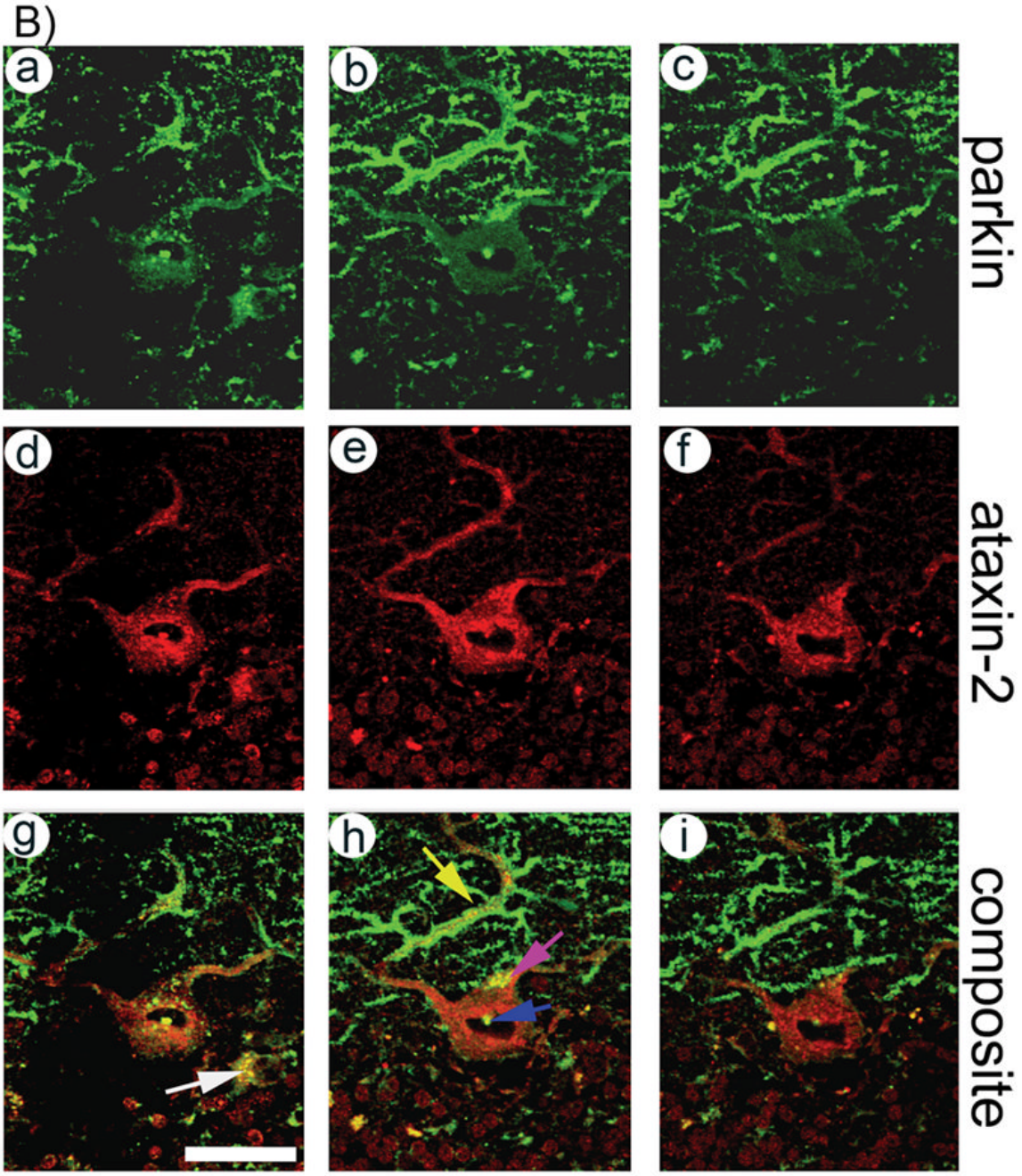


Figure 6.

Expression of ataxin-2 and parkin in Purkinje neurons. Cerebella from normal controls (A) and SCA2 patients (B) were labeled with chicken anti-parkin (green, rows a–c of panels A & B) and rabbit anti-ataxin-2 (red) antibodies. Primary antibodies were visualized with FITC-conjugated anti-chick IgY and Rhodamine-RED conjugated anti-rabbit IgG and imaged by confocal laser microscopy. Each image represents an individual 0.5 μm thick section. The composite represents the overlay of individual images corresponding to parkin and ataxin-2 labeling. Light blue arrow in panel A shows parkin and ataxin-2 colocalization in normal Purkinje neurons. Pink and Blue arrows in panel B show cytoplasmic aggregates and a perinuclear aggregate which invaginates into the nucleus. Most ataxin-2 aggregates also label

with parkin antibodies. Note the lack of parkin staining in Purkinje cell cytoplasm and Purkinje cell processes, but strong staining in fibers likely representing climbing fibers winding around the Purkinje cell arbor (Yellow arrow). Note also the colocalization of parkin and ataxin-2 in small cells near the Purkinje neuron likely representing Golgi cells (White arrow in panels A and B). Bars represent 40 μm.