Parkin protects mitochondrial genome integrity and supports mitochondrial DNA repair

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Mutations in the parkin gene are the most common cause of recessive familial Parkinson disease (PD). Parkin has been initially characterized as an ubiquitin E3 ligase, but the pathological relevance of this activity remains uncertain. Recently, an impressive amount of evidence has accumulated that parkin is involved in the maintenance of mitochondrial function and biogenesis. We used a human neuroblastoma cell line as a model to study the influence of endogenous parkin on mitochondrial genomic integrity. Using an unbiased chromatin immunoprecipitation approach, we found that parkin is associated physically with mitochondrial DNA (mtDNA) in proliferating as well as in differentiated SH-SY5Y cells. In vivo, the association of parkin with mtDNA could be confirmed in brain tissue of mouse and human origin. Replication and transcription of mtDNA were enhanced in SH-SY5Y cells over-expressing the parkin gene. The ability of parkin to support mtDNA-metabolism was impaired by pathogenic parkin point mutations. Most importantly, we show that parkin protects mtDNA from oxidative damage and stimulates mtDNA repair. Moreover, higher susceptibility of mtDNA to reactive oxygen species and reduced mtDNA repair capacity was observed in parkin-deleted fibroblasts of a PD patient. Our data indicate a novel role for parkin in directly supporting mitochondrial function and protecting mitochondrial genomic integrity from oxidative stress.

INTRODUCTION

Parkinson disease (PD) is the second most common neurodegenerative disease after Alzheimer disease. More than 1% of subjects older than 60 years are affected by PD (1). A pathological hallmark of PD is the severe depletion of dopaminergic neurons in the substantia nigra pars compacta. The aetiology of PD is believed to be multi-factorial, including genetic predisposition and environmental influences, with age being one of the most important risk factors. Biochemical abnormalities in the brain of PD patients include oxidative stress and mitochondrial dysfunction. The link between mitochondrial dysfunction and PD has been established by the observation of complex I deficiency in the brains of idiopathic PD patients (2). Another important aspect is that animal models and humans exhibit PD-like phenotypes upon exposure to environmental toxins inhibiting mitochondrial complex I activity (3,4). Recently, an elevated number of mitochondrial DNA (mtDNA) deletions has been detected in ageing brains and PD patients (5,6). Consistent with this observation, variations in the gene encoding polymerase γ (POLG) have been identified as a risk factor in idiopathic PD (7). POLG is the only known mtDNA polymerase, which is responsible for replication as well as for repair.

Reduced mitochondrial activity leads to ATP depletion of the respective cells, while at the same time, oxidative stress increases, which in turn further reduces the activity of essential cellular proteins including the respiratory chain. It is tempting to speculate that genes involved in PD play a neuroprotective role in order to interrupt this vicious cycle, because three of four recessive PD genes are linked to

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mitochondrial function. PINK1 is a mitochondrial protein kinase, which phosphorylates the mitochondrial chaperone TRAP1 (8). DJ-1 is a mitochondrial H$_2$O$_2$ scavenger involved in the response to oxidative stress (9), and parkin mutations lead to mitochondrial deficits in animal models and PD patients (10–12).

Parkin shows E3-ligase activity, mediating the covalent attachment of activated ubiquitin to target proteins. Initially, parkin was thought to function in the ubiquitin proteasomal degradation pathway by formation of poly-ubiquitin chains, and several substrates for parkin-ligase activity have been identified (13). However, for the majority of the putative substrates, no accumulation in knockout mice and Parkinson patients has been observed. Recently, work from several groups demonstrated mono-ubiquitination (14,15) and K63-linked poly-ubiquitination (16,17) mediated by parkin. These activities are proteasome-independent and known to play a role in regulatory processes like signal transduction, transcriptional regulation and protein trafficking rather than in protein degradation.

Current research has linked parkin to mitochondrial function. In patients with parkin mutations, the mitochondrial complex I activity was reduced in leucocytes (10). Mouse parkin knockout models exhibit reduced expression of mitochondrial proteins and some mitochondrial deficits (11,12). In a Caenorhabditis elegans parkin deletion model, a higher vulnerability towards complex I inhibitors was observed (18). In parkin knockout Drosophila melanogaster muscle degeneration with mitochondrial pathology was detected (19). Moreover, parkin rescues the mitochondrial PINK1 knockout phenotype in flies and cellular models, linking the two PD proteins to one pathway (20–22). Together these data suggest that parkin has an important and phylogenetically conserved mitochondrial function. Interestingly, parkin association with mitochondrial D-loop sequences and stimulation of mitochondrial biogenesis have been observed in proliferating cells of a parkin overexpression cell culture system (23). However, it remains cryptic how parkin mediates these effects on mitochondrial integrity and whether or not this function is dependent on parkin E3-ligase activity.

The objective of this study was to gain deeper insight into the role of parkin in mitochondrial function, especially its influence on the integrity of the mitochondrial genome. We used a human dopaminergic neuroblastoma cell line (SH-SY5Y), exploiting the fact that parkin is endogenously expressed in these cells and that they can easily be differentiated. Performing genome-wide chromatin immunoprecipitation (ChIP) experiments, we could identify mtDNA as the major target of parkin, most probably associated through an indirect interaction with the mtDNA binding mitochondrial transcription factor A (TFAM). In an inducible overexpression system, we found parkin to be involved in the regulation of mitochondrial gene expression and in the maintenance and integrity of the mitochondrial genome. We propose that these functions are crucial for the neuroprotective properties of parkin and that impairment of this activity by pathogenic mutations as well as through the administration of environmental insults contributes to the increased vulnerability of dopaminergic cells.

Table 1. Parkin target sequences in the human genome determined by ChIP on chip, sorted by enrichment over input

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
<th>Mitochondrial</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Unknown (protein for IMAGE:3925346); H. sapiens</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>H. sapiens ATP synthase 6</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>H. sapiens ATP synthase 8 (MTATP8)</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>H. sapiens mRNA for OK/SW-CL.16</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>H. sapiens mitochondrial Cox II</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>NADH dehydrogenase subunit 2; [human, brain]</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>NADH dehydrogenase subunit 2; [human, brain]</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>H. sapiens cDNA FLJ45445 fis, clone BRSSN2013696</td>
<td>−</td>
</tr>
<tr>
<td>9</td>
<td>H. sapiens cDNA FLJ45445 fis, clone BRSSN2013696</td>
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</tr>
<tr>
<td>10</td>
<td>H. sapiens sarcoma antigen NY-SAR-71</td>
<td>+</td>
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<tr>
<td>11</td>
<td>H. sapiens Humanin (HN1)</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>H. sapiens clone G4-10-3 mucin 4 (MUC4)</td>
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</tr>
<tr>
<td>13</td>
<td>H. sapiens NADH dehydrogenase 2 (MTND2)</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>H. sapiens cDNA FLJ45445 fis, clone BRSSN2013696</td>
<td>−</td>
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<tr>
<td>15</td>
<td>H. sapiens cytochrome c oxidase I (MTCO1)</td>
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<tr>
<td>16</td>
<td>H. sapiens cDNA FLJ45445 fis, clone BRSSN2013696</td>
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<tr>
<td>17</td>
<td>MTND5; NADH dehydrogenase subunit 5; H. sapiens</td>
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<td>MTND1; NADH dehydrogenase 1; H. sapiens</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>20</td>
<td>H. sapiens FLJ45445 protein</td>
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RESULTS
Parkin is associated with mtDNA and co-associates with TFAM

We first employed ChIP experiments, which are widely used to investigate the binding of regulatory proteins to DNA and the epigenetic modification of nuclear histone proteins in vivo. In a recent study, Kuroda et al. (23) observed in a parkin over-expression system association of parkin with the mitochondrial D-loop region, which contains regulatory sequences. To avoid over-expression artefacts, we used SH-SY5Y human dopaminergic neuroblastoma cells that express parkin endogenously. In order to identify possible parkin target sequences in the human genome, we employed a ‘ChIP on chip’ approach, in which the DNA fragments isolated by ChIP are hybridized on a DNA array. We used the NimbleGen (Madison, USA) promoter tiling array, which represents 24 000 human promoter sequences and additionally includes the mitochondrial genomic sequence. Precipitated DNA of three independent ChIP experiments and the corresponding genomic input DNA was pooled, amplified by ligation-mediated PCR (LM-PCR) and purified. The samples were labelled and hybridized by NimbleGen. In Table 1, sequences corresponding to DNA fragments with the highest enrichment over input are listed. Interestingly, 13 of 20 isolated sequences are coding for mitochondrial genes.

We wanted to test whether the association of parkin with mtDNA was reflected by the subcellular localization of parkin in SH-SY5Y cells. By cell fractionation of SH-SY5Y cells, we observed that even though endogenous parkin is mainly localized in the cytosol of proliferating SH-SY5Y cells, a small proportion resides in the mitochondrial fractions (Fig. 1A). To determine to which mitochondrial compartment parkin localizes, we did NP40 extraction and further fractionation of the mitochondrial lysates (Fig. 1B). Typically,
proteins bound to mitochondrial membranes or to mtDNA are pelleted during centrifugation (P1). We used as marker proteins the outer membrane (OM) protein TOM20, TFAM and POLG. PCR analysis showed that mtDNA was highly enriched in P1 (data not shown). HtrA2/Omi, which is partly associated to mitochondrial membranes and partly resides in the inter-membrane space, served as a soluble mitochondrial protein control. P1 was treated with micrococcal nuclease S7 to interrupt association of DNA-binding proteins with the inner membrane. TFAM was to some extend released into the soluble fraction (S2) upon nuclease digestion and recentrifugation, whereas the OM protein TOM 20 remained in P2. In contrast, POLG and parkin were released from P2, but were degraded by a protease, which was most likely activated by the Ca\textsuperscript{2+}-containing nuclease buffer (Fig. 1B).

In order to confirm the intramitochondrial localization, we first isolated crude mitochondria from HeLa and SH-SY5Y cells and treated the preparation with proteinase K (Fig. 1C and D). Proteins inside mitochondria are protected from protease digestion. TOM 20 was readily digested within the time course of this experiment, whereas the inner mitochondrial membrane protein prohibitin and parkin were resistant to proteinase K cleavage, indicating that a portion of cellular parkin is localized within mitochondria. In HeLa cell mitochondrial preparations parkin was fully protected, whereas in SH-SY5Y cells some OM contamination was still detectable, which was removed by the protease.

We confirmed the ChIP on chip results by performing ChIPs following quantitative real-time PCR (qRT-PCR) with primers specific for mitochondrial sequences from four coding regions of the mitochondrial genome and one mitochondrial D-loop sequence. Precipitation with the anti-parkin antibody led to an up to 8-fold enrichment of mitochondrial sequences over no antibody control (Fig. 2A). DNA that was precipitated with an anti-acetylated histone H3 (AcH3) antibody served as a negative control. MtDNA is not covered by nuclear histone proteins. Thus, no enrichment of mtDNA could be observed with this antiserum. In contrast, active and therefore acetylated, nuclear promoter sequences showed a 9-fold enrichment over input DNA (Fig. 2B). To further test the specificity of the signal, ChIPs have been performed with three polyclonal anti-parkin antisera, raised against different epitopes of the protein, as listed in Materials and Methods. All three antibodies resulted in similar enrichment of mtDNA sequences (data not shown). For all ChIP experiments shown here, the ab15954 anti-parkin antibody was used.

TFAM is a histone-like protein that belongs to the family of high-mobility group (HMG) proteins. A prominent member of this protein family, HMG1, plays important roles in nuclear DNA metabolism. TFAM possesses specific and unspecific DNA-binding properties. Besides its role in transcriptional activation of mitochondrial genes, it is speculated to be involved in replication and repair processes. Moreover, it seems to have histone-like functions as coordinator of the structural unit of mtDNA, the nucleoids (24). Consistently, it has been shown recently to cover the entire mitochondrial genome (25). We wanted to explore whether parkin and TFAM show association with the same mitochondrial sequences. To that end, we performed ChIPs with anti-TFAM antibodies.
In summary, we could show that endogenous parkin is associated with mtDNA in SH-SY5Y cells and that this association was not limited to the regulatory sequences of the D-loop region, but could be confirmed within several coding regions of the mitochondrial genome. Moreover, parkin and TFAM co-associate at all mitochondrial sequences tested in this study.

**Association of parkin with mtDNA is not restricted to proliferating cells**

The cells affected in PD are differentiated dopaminergic neurons. In order to prove that our observation that parkin is physically associated with mtDNA is of potential relevance in PD, we differentiated human dopaminergic SH-SY5Y neuroblastoma cells by sequential treatment with retinoic acid (RA) and brain-derived neuronal growth factor (BDNF) (26). After stimulation, the cells showed a neuron-like differentiation accompanied by a robust network of neuritic processes (Fig. 3A). Induction of the surface BDNF receptor gene (TrkB) expression by RA was monitored by reverse transcriptase–PCR analysis (Supplementary Material, Fig. S2). We performed ChIP experiments with differentiated cells using anti-parkin and anti-TFAM antibodies (Fig. 3B and C). As a control, we precipitated DNA with anti-AcH3 antibody. In contrast to the previous study (23), parkin and TFAM both associated with mtDNA in differentiated SH-SY5Y cells. Comparable to the results obtained with proliferating cells, anti-parkin antibody led to an enrichment of up to 10-fold and anti-TFAM antibody to an enrichment of about 60-fold over control DNA. Thus, endogenous parkin is associated with mtDNA in proliferating as well as in differentiated cells.

**Parkin associates with mtDNA in vivo**

As pointed out earlier, ChIP experiments are an excellent method to study in vivo protein–DNA interaction. We wanted to explore parkin association with mtDNA in brain. Therefore, we homogenized and cross-linked mouse brain tissue and performed ChIPs with anti-parkin and anti-TFAM antibodies. Figure 4A and B represents data from one of two independent experiments with similar results. Using the anti-parkin antibody, we observed an about 2-fold enrichment of specific mitochondrial sequences (Fig. 4A). Enrichment of mtDNA with the anti-TFAM antibody was observed in the same range as in SH-SY5Y cells (Fig. 4B). Taking into account that parkin is not expressed uniformly in all cell types and areas of the mouse brain (27), this result is comparable to the tissue culture experiments. In contrast, TFAM is expressed in all eukaryotic cells as a housekeeper of mitochondrial function. The experiment was repeated with human brain tissue. We used a cerebellum sample from a young individual with no signs of neurodegeneration or mitochondrial disease. In the anti-parkin ChIP, an ~4-fold enrichment of mitochondrial sequences (Fig. 4A). Enrichment of mtDNA with the anti-TFAM antibody was observed in the same range as in SH-SY5Y cells (Fig. 4B). Taking into account that parkin is not expressed uniformly in all cell types and areas of the mouse brain (27), this result is comparable to the tissue culture experiments. In contrast, TFAM is expressed in all eukaryotic cells as a housekeeper of mitochondrial function. The experiment was repeated with human brain tissue. We used a cerebellum sample from a young individual with no signs of neurodegeneration or mitochondrial disease. In the anti-parkin ChIP, an ~4-fold enrichment of mitochondrial sequences could be observed (Fig. 4C). TFAM antibodies resulted in a 40-fold enrichment of the selected sequences (Fig. 4D). As a control, we tested the enrichment of nuclear encoded sequences by the two antibodies. Two selected nuclear regions were precipitated by the anti-AcH3 antibody, but not by the anti-parkin or anti-TFAM antibody, respectively (Fig. 4E). Taken together, these results show that parkin is physically associated to mtDNA in vivo.
Generation of stably transfected inducible parkin-expressing SH-SY5Y cell lines

The observation that parkin is associated with mtDNA in vitro and in vivo raised the question of functional consequences of this interaction. Specifically, we aimed to elucidate whether parkin is involved in mtDNA metabolism. In order to be able to study the influence of parkin on the integrity of the mitochondrial genome and on mtDNA metabolism, we developed SH-SY5Y cell lines stably expressing wild-type (wt) parkin or one of six parkin derivatives carrying pathogenic point mutations (P36L, K161N, C212Y, R275W, R334C, G430D, respectively) under the transcriptional control of the T-REx™ system (Fig. 5A). Following induction of parkin expression, we isolated DNA, RNA and protein. The parkin-expression level was monitored by western blot analysis (Fig. 5B) and

Figure 3. Association of parkin with mtDNA in differentiated SH-SY5Y cells. (A) SH-SY5Y cells are differentiated by sequential treatment with retinoic acid (RA) and brain-derived neuronal growth factor (BDNF). (B) ChIP of differentiated SH-SY5Y cells with anti-acetyl H3 and anti-parkin antibodies. Primers specific for five mitochondrial genomic sequences and one nuclear promoter region were used. **/*** Significantly different from the anti-acetyl H3 samples. (C) ChIP of differentiated SH-SY5Y cells with anti-acetyl H3 and anti-TFAM antibodies. Primers specific for five mitochondrial genomic sequences and one nuclear promoter region were used. **/*** Significantly different from the anti-acetyl H3 samples.
reverse transcription of parkin mRNA followed by qRT-PCR (Fig. 5C). All the wt and mutant cell lines used in this study showed similar expression of recombinant protein upon induction with doxycycline. In contrast, parkin non-transfected SH-SY5Y cells (TR8) expressing the Tet repressor (TetR) exhibited low endogenous parkin expression.

We also compared parkin wt and mutant over-expression levels and subcellular localization in our system with immuno-cytochemistry (Fig. 5D). Recombinant parkin protein is distributed throughout the cell in all lines tested. There were no obvious differences between the various mutant lines (data shown for R275W and G430D), and no parkin containing inclusion formation was detected in the cells, probably due to the mild expression conditions. Mitochondrial morphology was normal in all cell lines as monitored with mitotracker staining, no fragmentation or prolongation of mitochondria was observed. Partial co-localization of parkin and mitochondria was detected in the wt as well as in the mutant lines (Fig. 5D, merge). Taken together, the results show that our stably transfected cell lines expressed parkin wt or mutant protein, respectively, upon induction, with the approximately same efficiency and cellular distribution. Cellular and mitochondrial morphology remained unchanged as far as detectable by fluorescence microscopy and no inclusion formation caused by over-expression was observed. Our observations are in contrast to other groups reporting mitochondrial morphological changes.

Figure 4. In vivo association of parkin with mtDNA. (A) ChIP of homogenized mouse brain with anti-acetyl H3 and anti-parkin antibodies. Primers specific for four murine mitochondrial genomic regions were used as indicated. (B) ChIP of SH-SY5Y cells with anti-acetyl H3 and anti-TFAM antibodies. Primers specific for four murine mitochondrial genomic regions were used as indicated. (C) ChIP of human cerebellum with anti-acetyl H3 and anti-parkin antibodies. Primers specific for five mitochondrial sequences were used: ATP synthase 6 gene (ATPase 6), cytochrome c oxidase II gene (Cox II), D-Loop region (D-Loop), NADH dehydrogenase subunit I (ND1) and NADH dehydrogenase subunit II (ND2). (D) ChIP of human cerebellum with anti-acetyl H3 and anti-TFAM antibodies. Primers specific for five mitochondrial sequences were used. (E) ChIP of human cerebellum with anti-acetyl H3, anti-parkin and anti-TFAM antibodies. Primers specific for two nuclear encoded sequences (ING1, ATPAF1) were used.
in mutant parkin-expressing cells (28,29). This difference may arise due to the mild over-expression conditions in our inducible cell lines or the manifestation of morphological changes was too subtle to be observed by the methods used.

**Parkin enhances the replication of the mitochondrial genome**

Proper mtDNA synthesis is essentially required for mitochondrial remodelling processes and hereby the number of replicated mtDNA molecules is considered as limiting factor. Very recently, Park and co-workers were able to show in a *Drosophila* model that the PINK1–parkin pathway is involved in the regulation of mitochondrial remodelling processes by promoting mitochondrial fission/fusion events (28,30). The observed phenomenon links mitochondrial biogenesis via mtDNA replication to PD pathology through their PD associated genes but the regulatory mechanism remains uncertain. We were interested in the role of parkin in mitochondrial biogenesis. To this end, we investigated the influence of parkin on mtDNA replication by performing qRT-PCR experiments with our transgenic SH-SY5Y cell lines using primers specific for five different mtDNA sequences. The mtDNA level from different cell lines was normalized to a nuclear encoded housekeeping gene. The ratio of the mtDNA content of wt parkin-expressing cells over non-induced cells is depicted in Figure 5.
Figure 6A. We tested four mitochondrial coding regions as well as the regulatory D-loop region. The mitochondrial genome is encoded on one circular molecule. Consistently, the increase of mtDNA in induced cells compared with non-induced cells was approximately the same for all sequences tested (1.8-fold). A nuclear encoded gene sequence has been used to determine the ratio of nuclear DNA in the cells under the two conditions. In contrast to mtDNA, the nuclear encoded gene of a mitochondrial protein (ATPAF1) did not show any parkin-dependent variation. We also analysed the mtDNA content following induction of mutant parkin. Over-expression of all six mutant parkin derivatives did not result in a change of the overall amount of mtDNA (Fig. 6B, shown for COX II). In summary, induction of wt parkin-expression in SH-SY5Y cells led to an increase of mtDNA in the cells, whereas the amount of nuclear DNA remained unchanged. Thus, we can rule out that mtDNA increase was caused by enhanced general viability of the cells following parkin induction. Parkin expression stimulated the replication of mtDNA and possibly overall mitochondrial biogenesis. This effect was not observed upon induction of the expression of parkin mutants.
Parkin over-expression stimulates transcription of mitochondrial genes

Even though the majority of mitochondrial proteins are encoded in the nucleus, the mitochondrial genome encodes for 13 mitochondrial proteins, two rRNA subunits and 22 tRNAs. It contains three promoters: the heavy strand 1 (H1), the heavy strand 2 (H2) and the light strand (L) promoter. Typical for prokaryotic organisms, mitochondrial genes are transcribed into polycistronic mRNAs. Transcription depends entirely on the action of nuclear encoded proteins: POLG, TFAM and the mitochondrial transcription factors B1 and B2 (TFB1M, TFB2M). We became interested in a potential role of parkin in mitochondrial transcriptional regulation. To test whether parkin over-expression stimulates transcription of mitochondrial genes, we performed reverse transcription qRT-PCR with primers specific for three mitochondrial sequences and with primers for ATPAF1. Induction of wt parkin expression led to a 3.5–4.5-fold increase of mitochondrial transcripts in comparison to the non-induced cells (Fig. 6C). The expression of ATPAF1 remained equal following induction of wt parkin.

Transcriptional stimulation by all six parkin mutants tested was decreased in comparison to stimulation by the wt protein. However, most of the mutants still showed a slight enhancement of transcription in comparison to the non-induced state (1.5–2.5-fold). As only exception, R334C and G430D, which carry point mutations in the IBR- or the second really interesting new gene (RING) domain, respectively, have lost their stimulatory properties completely. We repeated the experiment using cells differentiated with RA and obtained similar results (data not shown). This experiment shows that parkin stimulates the expression of mitochondrial genes on the transcriptional level. Some, but not all pathogenic point mutations significantly decrease this stimulatory effect.

Parkin does not influence the concentration of reactive oxygen species in SH-SY5Y cells

Parkin has been speculated to act neuroprotectively by directly influencing the concentration of intracellular reactive oxygen species (ROS). Consistent with this idea, a decrease of cellular ROS in neuroblastoma cells over-expressing parkin has been reported (31). In contrast, using a tyrosinase expressing cell model, we did not observe changes of the cellular ROS concentration dependent on the parkin-expression level (32). Because we were interested in mtDNA integrity under oxidative stress conditions, we wanted to rule out that parkin decreased the ROS levels in our system, which in consequence could reduce oxidative DNA damage. To that end, we measured the cellular ROS concentration following H2O2 treatment in our stably transfected SH-SY5Y cells with and without induction of wt parkin over-expression. Cells were stressed within a range of 100–500 μM H2O2 for 30 min. Intracellular ROS levels in relative fluorescence units/mg protein were determined and the ratio of non-treated cells over H2O2-treated cells is depicted in Figure 7A. Parkin did not show any influence on the intracellular ROS levels at all H2O2 concentrations tested. More specifically, we wanted to test the degree of mitochondrial oxidative stress. Following treatment with 500 μM H2O2, we stained the cells with MitoSOX and measured the amount of positive cells by FACS analysis (Fig. 7B). Approximately 60% of the stressed cells showed positive staining regardless of parkin over-expression. Together, these results show that parkin did not influence the intracellular or mitochondrial ROS level upon oxidative stress infliction in our experimental setting.

Parkin protects mtDNA from damage caused by oxidative stress

Damage of mtDNA is known to be a cause of mitochondrial diseases but plays also a role in ageing and neurodegeneration. Recently, the accumulation of mtDNA deletions in ageing individuals as well as in Parkinson patients has been reported (5,6,33). We asked whether parkin expression could influence mtDNA damage caused by oxidative stress. In order to quantify mtDNA damage in relation to oxidative stress, we developed a method which was based on the qRT-PCR amplification of mtDNA fragments of different lengths. The smaller, 87 bp fragment, served as an internal control to monitor the total mtDNA concentration. Stress conditions were adjusted to a level that did not result in measurable amounts of nicks, breaks, base modifications or bulky DNA adducts (designated lesions) in the small fragment. Amplification of a bigger, 1037 bp fragment, served as experimental probe to observe the level of lesions introduced by oxidative stress. It is important to note that by comparing the amplification of the two fragments our data provide a measure of lesions irrespective of the mtDNA copy number or the purification efficiency of mtDNA. The PCR conditions for the different fragments were optimized to achieve similar amplification efficiencies, a prerequisite for the comparison of the two reactions. We treated the inducible wt parkin cell line with H2O2 and measured mtDNA damage with and without wt parkin induction. Non-H2O2-treated cells served as a baseline control. Figure 7C depicts the lesions detected per 10 kb mtDNA. Upon incubation with 500 μM H2O2, non-induced cells showed ~3.3 lesions/10 kb mtDNA, whereas in cells over-expressing wt parkin only 1.6 lesions/10 kb mtDNA were detected. We repeated the experiment with another SH-SY5Y cell line, used before in a different study (32), stably expressing FLAG-tagged parkin. These cells were compared with a vector transfected control and showed similar results (data not shown). These data suggest that parkin protects mtDNA from oxidative damage inflicted by chemical insults.

Parkin supports mtDNA recovery under conditions of oxidative stress

Cellular models resistant towards chronic oxidative stress show a lower initial mtDNA damage and an elevated activity of mtDNA repair enzymes (34). Higher concentrations of ROS generating compounds were required to cause equivalent initial damage in resistant variants when compared with wt cells. This report made us curious whether the resistance from H2O2-inflicted damage in our system was related to stimulation of DNA repair. To answer this question, we stressed the cells with H2O2 and allowed for DNA repair, measuring the relative damage at different time points following treatment. As before, we compared the non-induced cells with wt parkin...
over-expressing cells. To rule out that the higher level of initial damage was overwhelming the repair machinery in the non-induced cells, we adjusted the initial damage to comparable levels by applying different H₂O₂ concentrations. Thus, non-induced cells were treated with 250 mM H₂O₂, whereas parkin-expressing cells were treated with 500 mM H₂O₂. Mitochondrial ROS levels were determined by FACS analysis of MitoSOX positive cells. (C) Parkin protects mtDNA from oxidative damage. SH-SY5Y cells with and without doxycycline induction were treated with 500 μM H₂O₂. Lesions in mtDNA were determined by qRT-PCR. (D) Parkin supports mtDNA recovery. SH-SY5Y cells were treated with 500 μM (+ parkin) or 250 μM (− parkin) H₂O₂, respectively. Lesions in the mtDNA were detected by qRT-PCR directly after the oxidative insult and following recovery.

**Figure 7.** Parkin protects mtDNA from oxidative damage and supports mtDNA recovery. (A) ROS levels are not changed in cells over-expressing parkin. SH-SY5Y cells with and without doxycycline induction were treated with 500 μM H₂O₂. The ROS levels are indicated as ratio of relative fluorescence units (RFU)/mg protein. (B) Mitochondrial ROS levels are not changed in cells over-expressing parkin. SH-SY5Y cells were treated with 500 μM H₂O₂. Mitochondrial ROS levels were determined by FACS analysis of MitoSOX positive cells. (C) Parkin protects mtDNA from oxidative damage. SH-SY5Y cells with and without doxycycline induction were treated with 500 μM H₂O₂. Lesions in mtDNA were determined by qRT-PCR. (D) Parkin supports mtDNA recovery. SH-SY5Y cells were treated with 500 μM (+ parkin) or 250 μM (− parkin) H₂O₂, respectively. Lesions in the mtDNA were detected by qRT-PCR directly after the oxidative insult and following recovery.

**mtDNA is more susceptible to ROS in parkin mutant fibroblasts**

Impaired mitochondrial function and mitochondrial morphology abnormalities have been recently described in human parkin-mutant fibroblasts (29). To determine whether endogenous parkin protects against ROS-derived mtDNA insults, we studied human parkin-mutant and control fibroblasts. Western blot analysis showed a moderate parkin level in control fibroblasts, but no parkin expression was detectable in patient fibroblasts (Fig. 8A). qRT-PCR analysis revealed a 22% reduction of the mtDNA copy number in parkin-mutant fibroblasts (Fig. 8B; *p < 0.01).

We first investigated whether the protective effect of parkin on mtDNA observed in our parkin over-expressing cellular system was abolished in parkin-mutant fibroblasts. Therefore, fibroblasts were exposed to H₂O₂ followed by monitoring the mtDNA damage level and the mtDNA copy number course. Relative to control fibroblasts, initial mtDNA damage was slightly increased in parkin-mutant fibroblasts upon treatment with 50 μM H₂O₂ (Fig. 8C). In addition, following incubation with 50 μM H₂O₂ mtDNA damage was reversed by 70% in control and 25% in parkin −/− fibroblasts after 1 h (Fig. 8C). Complete mtDNA recovery was observed in control and in 93% of the parkin −/− fibroblasts after 48 h (Fig. 8C). Elevated mtDNA damage induced by exposure with 200 μM H₂O₂ was reduced by 28% in control and by 15% in parkin-mutant samples after 1 h recovery; and 48 h recovery time resulted in
a 50% reduction of DNA damage in healthy control fibroblasts, whereas parkin-mutant fibroblasts exhibited 23% reduced DNA damage (Fig. 8D). These data indicate that parkin-mutant fibroblasts are more susceptible to oxidative stress and show a decelerated DNA repair capacity compared with control fibroblasts.

Recently, ROS have been observed to act as a key modulator of mtDNA copy number (35), suggesting that mtDNA recovery being a concerted mechanism of mtDNA repair, mtDNA synthesis and degradation. To test the influence of ROS on the mtDNA copy number in our system, we quantified the mtDNA copy number throughout the experiment. The mtDNA copy number was not changed in control and parkin−/− fibroblasts treated with 50 μM H2O2 (Fig. 8E), respectively. Incubation of fibroblasts with a higher H2O2 concentration (200 μM) resulted in a 1.6-fold increase of mtDNA copy number in both control and parkin−/− fibroblasts (F). After 48 h recovery, the mtDNA copy number was significantly reduced to 17% in control and 11% in parkin−/− fibroblasts. All values are expressed as mean ± SEM of at least three independent experiments.

Figure 8. Mitochondrial DNA (mtDNA) integrity in control and parkin-mutant fibroblasts. (A) Western blot analysis of control and parkin-mutant fibroblasts using anti-parkin and anti-actin antibodies. In control fibroblasts, a strong parkin band was detected, whereas in patient fibroblasts no parkin protein was observed. (B) Quantitative analysis of the mtDNA copy number. Total DNA from control and parkin-mutant fibroblasts was isolated, amplified by RT-PCR using primers specific for mitochondrial sequences and normalized using primers specific for nuclear sequences. In parkin-mutant fibroblasts, the mtDNA copy number was significantly reduced by 22% compared with control fibroblasts. *P < 0.01. (C and D) Parkin-mutant fibroblasts exhibit a higher mtDNA damage rate and an impaired mtDNA recovery after oxidative insult. Fibroblasts were exposed to 50 μM (C) or 200 μM (D) H2O2 for 30 min, respectively. Cells were harvested immediately or allowed to recover for 1 or 48 h. Total DNA was isolated and mtDNA lesions were detected by qRT-PCR. Relative to control fibroblasts, initial mtDNA damage was significantly increased in parkin−/− fibroblasts by 40% incubated with 50 μM H2O2 (C) and 5% with 200 μM H2O2 (D), respectively. Lesioned mtDNA was recovered by 70% in control and 25% in parkin−/− fibroblasts after 1 h and complete mtDNA recovery was observed in control and 93% in parkin−/− fibroblasts after 48 h treated with 50 μM H2O2 (C), respectively. Elevated mtDNA damage induced by high ROS level exposure (200 μM H2O2) was reduced by 28% in control and 5% in parkin−/− fibroblasts after 1 h recovery; and 48 h recovery time resulted in a 50% reduction of DNA damage in healthy control fibroblasts, whereas parkin-mutant fibroblasts exhibited 23% reduced DNA damage, respectively (D). (E and F) Determination of the mtDNA copy number. Throughout the experiment, the mtDNA copy number did not vary in control and parkin−/− fibroblasts treated with 50 μM H2O2 (E), respectively. Incubation of fibroblasts with higher H2O2 concentration (200 μM) resulted in a 1.6-fold increase of mtDNA copy number in both control and parkin−/− fibroblasts (F). After 48 h recovery, the mtDNA copy number was significantly reduced to 17% in control and 11% in parkin−/− fibroblasts. All values are expressed as mean ± SEM of at least three independent experiments.
parkin−/− fibroblasts (Fig. 8F). Following a 48 h recovery period, the mtDNA copy number was reduced to 17% in control and 11% in parkin−/− fibroblasts, respectively, indicating the promotion of mtDNA degradation by high levels of mtDNA damage.

DISCUSSION

The neuroprotective function of parkin is well established. However, little is known about the pathways involved. Parkin is an ubiquitin E3 ligase, and for many years, its influence on protein degradation was the focus of scientific interest. Recently, a growing number of studies revealed that parkin shows beside its poly-ubiquitination activity, which targets substrate proteins to the proteasome, involvement in the lysosomal pathway (16) and mono-ubiquitination activity (14,15,17,36), which support regulatory pathways independent of protein degradation. Furthermore, the influence of parkin on mitochondrial function and integrity became increasingly apparent. In this study, we provide evidence for a novel mitochondrial pathway, which enables parkin to protect from oxidative stress by supporting the mitochondrial genome integrity.

Depending on the antibodies used and on the method applied, conflicting data have been published concerning the subcellular localization of parkin. Mainly cytoplasmic and cellular vesicle (37,38), but also nuclear and mitochondrial staining has been reported (23,39,40). Even though the influence of parkin on mitochondrial function is widely accepted, the spatial association of parkin with mitochondria is still poorly established. The main reason for this gap of information, disregarding the reliability of the various antisera, is the fact that the majority of parkin molecules are located in the cytoplasm. This makes fractionation experiments challenging and prevents parkin from being detected with all those methods that are based on enrichment of the protein in the mitochondrial fraction over the cytoplasm, e.g. the analysis applied for the recently published mitochondrial proteome (41). Moreover, mitochondrial fractionation is frequently hampered by over-expression of parkin, which leads to a high cellular parkin concentration, which is mainly cytoplasmic, and therefore promotes a massive contamination of the mitochondrial preparation. This material is mainly bound to the OM and easily removed by protease digestion and leads to the assumption that parkin is an OM protein (42). To avoid the over-expression problem, we chose to perform cell fractionation experiments using cell lines endogenously expressing parkin. In contrast to the previous study (23), parkin was not restricted to mitochondria in proliferating cells, but the majority of the protein was located in the cytoplasm as reported by other groups (37,38,42). Furthermore, we could show, consistent with the observations from Kuroda et al. (23), that parkin antibodies immunoprecipitated mtDNA in a ChIP approach. In summary, we conclude that a fraction of endogenous cellular parkin is physically associated with mtDNA, most likely through an indirect interaction with TFAM, and thus is localized within mitochondria.

Transcription and replication of mtDNA are prerequisites for proper mitochondrial biogenesis and function. When compared with non-induced cells, we found an increase of total mtDNA to ~1.8-fold and of mRNA to ~4-fold. This is in accordance with the former study (23) and indicates that parkin supports mitochondrial replication and transcription. At this point, an increase of mitochondrial biogenesis could be interpreted either as the direct protective mechanism or as the consequence of improved cellular viability due to a different protective effect of the parkin protein. However, the idea of parkin directly influencing mitochondrial function was supported in a recent study, in which impaired mitochondrial morphology in combination with reduced complex I activity and ATP production was shown in fibroblasts from PD patients with homozygous and compound heterozygous parkin mutations (29). Parkin knockout mice showed mitochondrial deficits including reduced electron transport chain activity. Proteomic analysis revealed decreased abundance of proteins involved in mitochondrial oxidative phosphorylation, particularly subunits of complex I and IV (11). Mitochondrial dynamics, also named mitochondrial remodelling, is assumed to play a prominent role in proper mitochondrial function. In each cell, dozens of mitochondria undergo continuous cycles of mitochondrial fusion and fission, which can be observed as variations of the mitochondrial morphology (43). To maintain mitochondrial function, the balance of mitochondrial fusion and fission seems to be of vital importance (44). In neuronal cultures, extensive and rapid mitochondrial fragmentation (fission) leading to apoptotic cell death can be induced by several Parkinson causing neurotoxins such as 6-hydroxydopamine, rotenone and MPP⁺ (45–47). In another report, PINK1 was shown to control mitochondrial translocation of parkin via direct phosphorylation (72). Interestingly, in PINK1 and parkin Drosophila mutants, the mitochondrial phenotype was reduced by over-expression of Drp1, a regulator for mitochondrial fission, suggesting a substantial role of the PINK1–parkin pathway in mitochondrial remodelling processes. Our results reinforce a functional role of parkin in the vitality of mitochondria by influencing the mitochondrial transcription and translation processes.

One underlying cause of mitochondrial dysfunction is impairment of the mitochondrial genome integrity by accumulation of mtDNA mutations, frequently in the form of common deletions. In mitochondrial ‘mutator’ mice, harbouring a proof reading deficient POLG enzyme, DNA deletions and clonal mutations drive premature ageing (48). Recent studies suggest that mtDNA deletions accumulate in PD patients (5,6). ROS is one major source of mtDNA damage. Accordingly, ROS scavenging is an attractive neuroprotective activity, which has been attributed to parkin. However, our results suggest that mitochondrial ROS level remain constant following parkin over-expression. On the other hand, mtDNA repair is being recognized as an important long-term defence mechanism against oxidative stress, especially in neurons (49–51). Consequently, the role of the mitochondrial repair machinery attracts more and more attention. mtDNA repair depends on the nuclear encoded enzymes of the base excision repair (BER) pathway. Following oxidative stress, the mitochondrial uracil-DNA glycosylase is upregulated (52). Furthermore, over-expression of mitochondria-targeted truncated apurinic/apyrimidinic endonuclease leads to increased mtDNA repair capacity and cell survival upon...
induction of hydrogen peroxide-induced stress (53). We found a lower level of oxidative stress-induced mtDNA damage in parkin-expressing SH-SY5Y cells and furthermore observed increased mtDNA recovery following hydrogen peroxide treatment. It is important to note that we apply sub-apoptotic oxidative stress by using 250 μM H2O2 for 30 min, a concentration leading to 1–2 lesions/10 kb, which represents a physiological damage level for repair. After application of stronger insults, apoptosis will be induced due to overwhelming of the cellular defense mechanisms. Under these conditions, the sensitivity for differences ± parkin induction is lost, which explains the discrepancy between our results and other studies, e.g. (42,54), who did not detect a neuroprotective parkin effect upon application of H2O2. Interestingly, parkin has been suggested recently to promote nuclear DNA repair and to interact with the proliferating cell nuclear antigen, a protein that coordinates DNA excision repair (55). Taken together, these observations hint to a possible involvement of parkin in the BER mechanism.

Recently, mitochondrial deficits have been detected in non-neuronal tissues including fibroblasts from PD patients with homozygous or compound heterozygous parkin mutations (29). We employed parkin-mutant fibroblasts in order to investigate a possible parkin effect on ROS-derived mtDNA damage in an ex vivo model. Exposure to H2O2 led to elevated mtDNA damage and a reduced repair capacity of parkin −/− fibroblasts compared with healthy control cells.

ROS has been proposed to act as a modulator of the mtDNA copy number which was supported by the observation of an increase of the in vivo mtDNA copy number in isolated yeast mitochondria after treatment with low levels of H2O2 (35). During the DNA recovery process, the mtDNA copy number decreases over time probably due to mtDNA degradation. In our hands, monitoring the mtDNA level throughout the damage assay did not reveal any change of the mtDNA level in fibroblasts treated with 50 μM H2O2. However, a 1.5-fold increase of mtDNA synthesis was observed in both fibroblast lines in response to the exposure to 200 μM H2O2. Following a 48 h recovery period, the mtDNA copy number in parkin-deleted fibroblasts was reduced to 11% and control fibroblasts exhibited 17% of mtDNA of compared with non-treated cells. In reference to the mtDNA damage data analysis, these data point towards the possibility that moderate damaged mtDNA is repaired, whereas more severely damaged mtDNA is degraded. Lower efficiency of mtDNA metabolism and weakened mitochondrial genome integrity in parkin patients might lead to impaired mitochondrial function and to neuronal cell loss.

Cellular energy production is tightly influenced by the amount of mtDNA and the full complement of mtDNA molecules (56). mtDNA disturbances resulting in mitochondrial dysfunction have been associated with many human diseases including cancer, neurodegeneration and ageing (57). The total amount of mtDNA varies widely from tissue to tissue and is related to the energy requirement with brain, retina and muscles possessing the highest energy demands (56). It became evident that the mtDNA copy number plays an important role in mitochondrial vitality, while accelerated mtDNA depletion and resulting mitochondrial disturbances are implicated in cancer, diabetes and neurodegeneration (57). An increase of mtDNA levels has been associated with survival in critically ill patients (58), whereas a reduced mtDNA copy number was observed in renal cell carcinoma (59) and breast cancers (60), among others. In our model, parkin-deleted fibroblasts isolated from a human PD patient showed a 22% reduction of the mtDNA copy number compared with healthy control cells. This result is well in line with the observation that in our parkin-expressing SH-SY5Y cell line mtDNA replication was increased by wt parkin over-expression. Taken together, the regulation of the mtDNA copy number is suggested to correlate with normal cellular function and our data support the hypothesis of a stimulatory parkin effect.

We observed an influence of parkin over-expression on the mtDNA copy number and the mitochondrial mRNA level as well as on mtDNA repair. In addition, ex vivo parkin-deleted fibroblasts exhibited an elevated vulnerability of mtDNA to ROS exposure and a disturbed recovery process. Lower efficiency of mtDNA metabolism and weakened mitochondrial genome integrity in parkin patients might lead to impaired mitochondrial function and to neuronal cell loss. The mechanism by which parkin protects the mitochondrial genome integrity needs to be addressed in future studies. As a RING protein, it might bind directly to DNA and be part of the repair machinery. Alternatively, it might act as E3-ligase activating BER enzymes by mono-ubiquitination or target the BER proteins to the mtDNA. In vivo experiments could clarify, whether parkin is able to protect mtDNA in animals exposed to chemical insults. On the basis of our data, we hypothesise that one aspect of the neuroprotective effect of parkin bases on its ability to conserve mtDNA integrity and thus stalling the progression of the apoptotic pathway. In addition to this protective ability under conditions of sub-lethal stress levels, parkin is suggested to support neuronal survival by promoting the clearance of damaged mitochondria by autophagy (61,62). In conclusion, we speculate that the neuroprotective response of parkin depends on the status of mitochondrial damage, prompting mtDNA conservation and initiating the degradation of excessive damaged mitochondria in order to prevent cell death.

Interestingly, mice with a conditional TFAM knockout in dopaminergic neurons developed a parkinsonian phenotype. The knockout mice showed reduced mtDNA expression and respiratory chain deficiency, which led to motor function impairment, intra-neuronal inclusions and dopaminergic cell loss (63). As another important factor involved in mtDNA metabolism, genetic variations in the POLG gene have been identified as a risk factor in idiopathic PD (7). Taken together, these results strengthen the link between mtDNA integrity, mitochondrial function and neuronal health, implicating premature ageing and cell loss as a consequence of impaired mtDNA maintenance, an idea which is supported by the higher abundance of mtDNA deletions in PD patients. Our finding of a mtDNA protecting pathway of parkin provides novel insights into the pathogenesis of PD and strikes a new path to scientific and therapeutic approaches.

**MATERIALS AND METHODS**

**Antibodies**

For the ChIP experiments, the following antibodies were used: polyclonal anti-parkin #2132 and #4230, and monoclonal...
mouse anti-parkin #4211 (Park8) (CellSignaling, USA), polyclonal anti-parkin ab15954 and ab22652 (Abcam, Cambridge, UK), anti-AchH3 #06-599 (Upstate, USA) and anti-TFAM sc-28200 (Santa Cruz, USA). For immunoblotting experiments, the following antibodies were used: polyclonal anti-parkin ab15954 (Abcam, Cambridge, UK), monoclonal anti-Tom20 sc-17764 (Santa Cruz, USA), monoclonal anti-GAPDH 4699–9555 (Biotrend), monoclonal anti-prohibitin MS-261-P1 (NeoMarker, USA), polyclonal anti-POLG #RB-053-P1ABX (Lab Vision, Fremont, CA, USA), monoclonal anti-alpha-tubulin T 5293 (Sigma), monoclonal anti-beta-actin A5441 (Sigma), polyclonal anti-Omi/HtrA2 Ax-210-906 (Alexis Biochemicals, USA). The anti-hTFAM polyclonal serum was a kind gift from Daniel Bogenhagen (Stony Brook, NY, USA).

For immunocytochemical experiments, the following primary antibodies were used: polyclonal anti-parkin ab15954 (1:500) and polyclonal anti-parkin #2132 (1:1000).

Western blot analysis
Polyacrylamide gel electrophoresis (PAGE) and immunoblotting were performed using standard protocols. For the analysis of the isolation and fractionation of mitochondria, two different PAGE gels were prepared per experiment. Due to the different size and abundance of the proteins of interest and the variable sensitivity of the antisera used, we blotted one 10% gel loaded with 20 μg protein and one 15% gel loaded with 10 μg protein per lane, respectively.

Cell culture and neuronal differentiation
The SH-SY5Y neuroblastoma cell line originated from ATCC (#CRL-2266) and was grown in Dulbecco’s modified Eagle’s medium (DMEM) (Biochrom, Germany) and 10% heat-inactivated fetal bovine serum (PAA Laboratories, Austria). The HeLa cell line originated from ATCC (#CCL-2) and was grown in DMEM (Biochrom) and 10% heat-inactivated fetal bovine serum (PAA Laboratories, Austria). All cells were maintained at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO2. For neuronal differentiation of SH-SY5Y, cells were seeded at an initial density of 5 x 10⁴ cells/cm² in culture dishes (Greiner Bio One, Germany), according to Encinas et al. (26). Briefly, alltrans-RA (Sigma, USA) was added on day 1 after plating to a final concentration of 10 μM. After 5 days in the presence of RA, cells were washed three times with standard medium and incubated with 50 ng/ml BDNF (Tebu-Bio, Germany) in serum free DMEM/HAM F-12 medium for additional 5 days.

Generation of stable inducible SH-SY5Y cells
Human neuroblastoma SH-SY5Y cell clone TR8 (64) stably expressing the hybrid Tet repressor encoded by the pcDNA6/TR regulatory vector of the T-Rex-system (Invitrogen) was cultured in low glucose DMEM (Biochrom) containing 5.5 mM glucose, 1 mM sodium pyruvate, 2 mM glutamine supplemented with 10% tetracycline-negative fetal bovine serum (PAA Laboratories, Austria) and 7 μg/ml blastidin (InvivoGen, San Diego, CA, USA) for pcDNA6/TR selection at 37°C under humidified 5% CO2/air (64). TR8 cells were transfected using FuGene 6 reagent (Roche, Switzerland), and positive parkin clones were selected with 300 μg/ml zeocin (InvivoGen) and single stable clones were separated. Parkin expression was induced by the addition of 1 μg/ml doxycycline (MP Biomedicals, Solon, OH, USA) to the culture medium.

Subcellular fractionation
Cell pellets pooled from up to 10⁹ SH-SY5Y cells were used for the subcellular fractionation according to the previously described method including minor modifications (65). By carrying out differential centrifugation in density gradients, nuclear, cytosolic, mitochondrial and microsomal fractions can be isolated. The procedure was completely conducted at 4°C or on ice. Briefly, pooled cell pellets were rinsed once in five packed cellular volume (pcv) of cold 250-STMDPS buffer containing 250 mM sucrose, 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂ and freshly added 1 mM DTT (Roth, Germany), 25 mg/ml Spermine, 25 mg/ml Spermidine and 1 mM PMSF (Sigma). The pellet was homogenized in a precooled douncer in four pcv of 250-STMDPS buffer. After centrifugation at 1200g for 10 min, the supernatant (C1) containing mitochondria, cytosol and microsomes was separated from the nuclear pellet (N1) which was rinsed once with 250-STMDPS and further homogenized using an ultrasonic generator Sonoplus (Bandelin, Germany). After centrifugation at 1200g for 10 min, pellet (N2) was resolubilized in 2 mM STMDPS buffer containing 2 mM sucrose, 50 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, 1 mM DTT, 1 mM PMSF, freshly added 25 mg/ml Spermine and 25 mg/ml Spermidine, and homogenized with an additional ultrasound treatment. The suspended N2 pellet was layered on a cushion of 2 mM STMDPS buffer and centrifuged at 10 000g for 1 h in a Sorvall ultracentrifuge (ThermoFisher, USA). The nuclear proteins were extracted from the resulting pellet (N3) by incubating for 30 min at 4°C in four pcv of NE buffer containing 20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.5 mM NaCl, 0.2 mM EDTA and 20% glycerol. The nuclei were harshly lysed with Ten passages through a 18G needle and centrifuged at 9000g for 30 min. The resulting supernatant contained the soluble nuclear proteins and non-directly DNA binding proteins. The pellet was resuspended in NET buffer (NE Buffer including 1% Triton X-100, 1 mM DTT and 1 mM PMSF), followed by an additional needle lysis procedure, and the lysate was centrifuged at 9000g for 30 min. The remaining supernatant was enriched with nuclear membrane proteins including DNA binding proteins.

Supernatant (C1) was centrifuged at 6000g for 15 min to separate supernatant (C2) containing cytosol and microsomes, whereas the pellet (M1) contained mitochondria. Microsomal fraction (C3) was pelleted and isolated from pure cytosol (supernatant) by centrifugation of C2 fraction at 100 000g for 1 h. Microsomal proteins were extracted by incubating the resuspended C3 pellet in ME Buffer [20 mM Tris–HCl (pH 7.8), 0.4 mM NaCl, 15% glycerol, 1 mM DTT, 1 mM PMSF and 1.5% Triton X-100] for 1 h. After centrifugation at 9000g for 30 min, the supernatant contained microsomal
proteins. The mitochondrial pellet (M1) was resuspended in hypotonic HDP Buffer [10 mM HEPES (pH 7.9), 1 mM DTT and 1 mM PMSF] and incubated for 30 min. The mitochondria were lysed by sonication with high power settings. After centrifugation at 9000g for 30 min, the supernatant was saved, which contained soluble mitochondrial matrix proteins. The pellet was extracted in ME Buffer, incubated for 1 h, centrifuged at 9000g for 30 min resulting in the supernatant containing mitochondrial membrane proteins.

Isolation of mitochondria

Mitochondria have been isolated from tissue culture cells as described in (66). The procedure was scaled down for the preparation of mitochondria from twenty 15 cm tissue culture dishes. Shortly, cells (HeLa or SH-SY5Y as indicated) from dishes with 80–90% confluence were scrapped into the culture medium, centrifuged at 1000g for 5 min and washed once with PBS (Gibco, Germany). The following steps have been performed on ice. DTT and protease inhibitors [PMSF (Fluka, Swiss), complete mini (Roche, Germany)] have been added when appropriate. The pellet was resuspended in five points 5, 10, 20 and 40 min, 20 μl of the reaction was stop solution. The samples were centrifuged at 20000g for 30 min. The supernatant (S1) was saved for immunoblot analysis, the pellet (P1) was resuspended in 100 μl nuclease buffer [20 mM HEPES (pH 8.0), 0.25 mM sucrose, 0.5% NP40, 1 mM DTT, 2.5 mM CaCl₂]. To 20 μl of the suspension of S7 micrococcal endonuclease (N3755, Sigma) solution (2.5 U in H₂O) was added, 20 μl of the suspension served as mock control (H₂O). After 30 min incubation on ice, the samples were centrifuged at 20000g for 30 min. The supernatant was designated S2, the pellet P2. All fractions were diluted in PAGE sample buffer for immunoblot analysis.

Proteinase K treatment of mitochondria

Crude mitochondria were resuspended in MSH to ~5 mg/ml. For the proteinase K treatment, 100 μl of the suspension was used; 20 μl of the crude mitochondria was removed and mixed with 2 μl stop solution (4 μl 0.5 mM EDTA, 8 μl 0.1 mM PMSF, 8 μl H₂O₂) at t = 0 min 4 μl proteinase K (Merck, Germany) (1 mg/ml) was added to the remaining suspension. At time points 5, 10, 20 and 40 min, 20 μl of the reaction was removed and mixed with 2 μl stop solution. The samples were pelleted at 16 000g for 10 min and resuspended in the appropriate buffer depending on the following procedure.

Micrococcal nuclease treatment of mitochondrial pellets

Crude mitochondria were resuspended in 0.5% NP40 buffer [20 mM HEPES (pH 8.0), 1 mM EDTA, 0.25 mM sucrose, 0.5% NP40, 1 mM DTT] to ~0.5 mg/ml. Lysis was allowed for 30 min on ice. The lysate was centrifuged at 20000g for 30 min. The supernatant (S1) was saved for immunoblot analysis, the pellet (P1) was resuspended in 100 μl nuclease buffer [20 mM HEPES (pH 8.0), 0.25 mM sucrose, 0.5% NP40, 1 mM DTT, 2.5 mM CaCl₂]. To 20 μl of the suspension of S7 micrococcal endonuclease (N3755, Sigma) solution (2.5 U in H₂O) was added, 20 μl of the suspension served as mock control (H₂O). After 30 min incubation on ice, the samples were centrifuged at 20000g for 30 min. The supernatant was designated S2, the pellet P2. All fractions were diluted in PAGE sample buffer for immunoblot analysis.

Chromatin immunoprecipitation

ChIP assays were performed according to a previously published study with slight modifications (67). Briefly, 10⁸ SH-SY5Y neuroblastoma cells, or ~0.5 cm² of fresh mouse brain (C57 B16, Charles River, Germany) or human cerebellar tissue (IS171), respectively, were cross-linked with formaldehyde for 10 min, harvested and washed twice with PBS. The cell pellets were lysed and the lysate was sonicated to shear the DNA into fragments of 300–800 bp. The resulting extract was precleared with Salmon sperm blocked protein A agarose (Upstate, USA). An aliquot was retained as input control. DNA fragments were enriched by immunoprecipitation with specific antibodies (5 μg per 10 ml sample volume). After thermal cross-link reversion and subsequent proteinase K digestion, DNA was purified performing phenol/chloroform extraction followed by ethanol precipitation. The DNA concentration was determined by real-time Fluorimeter assay using SYBR Green I dye (Amresco, USA).

Genome-wide ChIP analysis

ChIP based parkin-specific enriched DNA and input DNA samples from proliferating SH-SY5Y cells were amplified by LM-PCR. Further sample labelling and hybridization as well as detection and raw data analysis of the human HG17 Promoter 1.5K ChIP array were performed by NimbleGen Systems (Iceland).

Detection of ROS

Intracellular ROS production was measured with the fluorescence dye 2,7-dichlorodihydrofluorescein diacetate (DCFH₂-DA, Molecular Probes). Briefly, 1.0 × 10⁶ wt parkin cells cultured in the presence or absence of doxycycline (1 μg/ml) were washed twice with PBS (Gibco) and then incubated in serum-free DMEM containing 20 μM of DCFH₂-DA at 37°C for 45 min. After two PBS washes, cells were stressed with H₂O₂ (Sigma-Aldrich, Germany) as indicated in serum-free DMEM at 37°C for 30 min and lysed in 200 μl of 1 N NaOH for 5 min on ice. Lysates were centrifuged at 14 000g for 10 min at 4°C and the fluorescence of dichlorofluorescein in the supernatant was measured at excitation wavelength 480 nm and emission wavelength 520 nm (Wallac 1420 Victor fluorescence spectrophotometer). To normalize for variations of the cell number in each well, the protein concentration was measured in parallel (bca assay kit, Pierce).

Intra-mitochondrial ROS level in living cells was measured using MitoSOX Red (Invitrogen) according to the manufac-
turer’s protocol using CyAn Cytometer (DakoCytomation). Briefly, wt parkin ± doxycycline treated 5 × 10^5 cells were washed twice with PBS and stressed with 500 μM H_2O_2 in serum-free DMEM at 37°C for 30 min. After one washing step with PBS, two with PBS, 2 mM EDTA and one with PBS, cells were incubated in PBS containing 5 μM MitoSOX Red at 37°C for 15 min. Following PBS washing step, cells were resuspended in 1 ml PBS and the fluorescence of MitoSOX Red was measured at 580 nm excitation wavelength and emission wavelength of 510 nm. A minimum of 50,000 events was acquired for each sample.

Oligonucleotides

All primers for the real-time applications (RT) were designed using Primer 3 software, synthesized and HPLC purified by Metabion international, Germany. Cloning primers were synthesized Metabion international. The sequence of all primers used in this study can be found in supporting information, Table 1.

Mutagenesis and generation of expression constructs

Wt, P37L, K161N, C212Y, R275W (68), R334C and G430D (68) mutant parkin cDNAs were generated by fusion PCR using the mutagenesis and parkin subcloning primers (supporting information, Table 1). Parkin inserts were subcloned into the Bam HI/Not I sites of a pcDNA4/TO vector (Invitrogen, USA) by standard molecular techniques introducing one glycine residue following the start codon.

Quantitative qRT-PCR

ChIP with parkin, TFAM and acetyl H3 K9/14 antibodies was performed with SH-SY5Y cells in triplicate, human cerebellum and whole mouse brain, respectively. PCR primer was designed to amplify 150–250 bp fragments from selected genomic regions. RT-PCR was carried out in duplicate on each sample and input DNA using Fast Start DNA Master plus SYBR Green I mix (Roche) according to manufacturer’s instructions in a Light cycler 2.0 RT-PCR system (Roche). The product specificity was monitored by melting curve analysis, followed by photographic recording of the gel stained with ethidium bromide.

PCR of the TrkB receptor gene

To verify the differentiation of SH-SY5Y cells, the relative expression level of the TrkB receptor gene was analysed by PCR as followed (70): 1 μl of freshly synthesized cDNA was amplified using cDNA primer for TrkB and GAPDH. PCR amplification was conducted under the following conditions: for TrkB, initial denaturation at 95°C for 10 min, 30 s at 94°C, 30 s at 56°C and 30 s at 72°C for 35 cycles, followed by 10 min at 72°C. For GAPDH, initial denaturation at 95°C for 10 min, 30 s at 94°C, 30 s at 59°C and 30 s at 72°C for 20 cycles, followed by 10 min at 72°C. After amplification, 10 μl aliquots were run on a 2% agarose gel by electrophoresis, followed by photographic recording of the gel stained with ethidium bromide.

Immunofluorescence microscopy

Stable inducible Neuroblastoma cells were seeded onto poly-d-lysine (Sigma) coated coverclips and induced with 1 μg/ml doxycycline for 48 h. Immuncytochemistry was performed according to standard protocols. Mitochondria were stained with 50 nM Mitotracker CMXRos (Invitrogen) for 30 min prior to fixation with Akku stain (Sigma) for 30 s. Cells were permeabilized with 0.2% Triton X-100 in PBS for 30 min, and blocked in 3% normal goat serum for 1 h. Primary antibodies were applied overnight followed by anti-rabbit IgG Alexa 488 conjugates (Molecular Probes) for 1 h. Cell nuclei were counter stained with Hoechst 33342 (Molecular Probes, H1399). Fluorescent images were analysed using Axio imager Z1 laser microscope with Apotome (Zeiss, Germany) equipped with HeNe-Green (543 nm), HeNe-Red (633 nm) and Ar (488 nm) laser units.

DNA damage and repair assays

Stable inducible Neuroblastoma cells (± 1 μg/ml doxycycline for 48 h) were exposed to hydrogen peroxide (H_2O_2) in
serum-free media for 0.5 h as indicated. H₂O₂ was removed by two times washing with standard culture medium. Cells were harvested immediately or allowed to repair for indicated time points. Total DNA was isolated using DNA Blood and Tissue kit (Qiagen). DNA quantity and purity was determined by spectrometric analysis. mtDNA lesion rates were determined by sequence specific qRT-PCR amplification of mtDNA amplicons of 1037 and 87 bp in size relative to DNA isolated from untreated cells. The small amplification product serves as a mitochondrial copy number normalization and as undamaged mtDNA reference (71). The cycling conditions include a pre-incubation phase of 10 min at 95 °C followed by 40 cycles of 10 s 95 °C, 10 s 60 °C and 10 s 72 °C (small fragment) or 50 s 72 °C (large fragment), respectively. Each sample was assayed in quadruplicate.

Fibroblast study

Punch skin biopsies were taken from a healthy control individual and a patient with compound heterozygous mutations in the parkin gene (c.101delAG + het del ex3–4). Genotyping was performed using direct DNA sequencing and the Multiplex ligation-dependent probe amplification was performed using direct DNA sequencing and the Multi-allelic and a patient with compound heterozygous mutations in the parkin gene, as well as other known Mendelian PD genes. Control fibroblasts were obtained from healthy age-matched control subject. Primary fibroblast cells were cultured continuously in RPMI medium with 10% fetal healthy age-matched control subject. Primary fibroblast cells were cultured continuously in RPMI medium with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate. For the determination of the mtDNA copy number and for western blot analysis, control and parkin −/− fibroblasts were harvested and total DNA and proteins were isolated as previously described. DNA damage and repair assays were conducted as follows: fibroblasts were exposed to hydrogen peroxide (H₂O₂) in sodium pyruvate-free media for 0.5 h as indicated. H₂O₂ was removed by two times washing with standard culture medium. Cells were harvested immediately or allowed to repair for indicated time points.

Statistical analysis

The data are presented as the mean ± SE of three independent experiments unless stated otherwise. The data were compared using a standard t-test, and statistical significance was determined at the 0.05 (*), 0.01 (**) and 0.001 (***) level.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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