Parkin Somatic Mutations Link Melanoma and Parkinson’s Disease

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ABSTRACT

Epidemiological studies suggest a direct link between melanoma and Parkinson’s disease (PD); however, the underlying molecular basis is unknown. Since mutations in Parkin are the major driver of early-onset PD and Parkin was recently reported to play a role in cancer development, we hypothesized that Parkin links melanoma and PD. By analyzing whole exome/genome sequencing of Parkin from 246 melanoma patients, we identified five non-synonymous mutations, three synonymous mutations, and one splice region variant in Parkin in 3.6% of the samples. In vitro analysis showed that wild-type Parkin plays a tumor suppressive role in melanoma development resulting in cell-cycle arrest, reduction of metabolic activity, and apoptosis. Using a mass spectrometry-based analysis, we identified potential Parkin substrates in melanoma and generated a functional protein association network. The activity of mutated Parkin was assessed by protein structure modeling and examination of Parkin E3 ligase activity. The Parkin-E28K mutation impairs Parkin ubiquitination activity and abolishes its tumor suppressive effect. Taken together, our analysis of genomic sequence and in vitro data indicate that Parkin is a potential link between melanoma and Parkinson’s disease. Our findings suggest new approaches for early diagnosis and treatment against both diseases.

KEYWORDS: Melanoma; Parkinson’s disease; Parkin; Mutation

INTRODUCTION

Melanoma, a melanocytic neoplasm, is responsible for 80% of skin cancer mortality (Flaherty et al., 2012). Parkinson’s disease (PD) is the most common neurodegenerative movement disorder; PD affects 1% of the population above 60 years of age (Veeriah et al., 2010; Pan et al., 2011). Melanoma and PD seem to have very different, even opposite, phenotypes at the cellular level, as melanoma results from an uncontrolled, extensive proliferation of cells, whereas PD is characterized by degeneration and death of dopaminergic neurons. Intriguingly, however, epidemiological studies indicate a potential relationship between melanoma and PD (Herrero Hernandez, 2009; Bertoni et al., 2010; Inzelberg et al., 2011; Liu et al., 2011; Kareus et al., 2012), demonstrating that PD patients and their relatives have a higher frequency of melanoma than...
the general population, and vice versa (Herrero Hernandez, 2009; Gao et al., 2011; Liu et al., 2011). Recently, Parkin was also suggested as a genetic link between melanoma and PD (Hu et al., 2016). However, the molecular mechanism behind this link is still unknown.

Loss-of-function mutations in the Parkin gene (also named PARK2, PRKN, MIM#602544) are responsible for nearly 50% of early onset PD cases (Kahle and Haass, 2004; Guo et al., 2008, 2010). Somatic mutations in the Parkin gene are a known cause of autosomal recessive juvenile PD (MIM#600116), in which a progressive death of neurons in the substantia nigra takes place (Romani-Aumedes et al., 2014). Alterations in the Parkin gene are also associated with other diseases as demonstrated by MalaCards (Table S1), such as lung cancer (MIM#211980), ovarian cancer (MIM#167000) and susceptibility to leprosy (MIM#607572). Parkin is an E3 ubiquitin ligase responsible for substrate recognition in the ubiquitination process (Hershko and Ciechanover, 1998) that leads to substrate degradation in the proteasome (Hampe et al., 2006). The known substrates of Parkin include cell cycle regulators, e.g., Cyclin D1 (Yeo et al., 2012) and Cyclin E (Veeriah et al., 2010); apoptosis mediators, e.g., Bax (Johnson et al., 2012) and Bcl2 (Chen et al., 2010); proteins involved in synaptic transmission, e.g., Synaptotagmin 11 (Kahle and Haass, 2004).

Abnormal Parkin function may underlie certain cancers (Veeriah et al., 2010). The Parkin gene is located on chromosome 6q in the fragile site FRA6E (Wang et al., 2009), which is altered in a variety of human cancers (Letessier et al., 2007). In the absence of Parkin expression, cancer cell lines exhibit extensive proliferation and cell cycle abnormalities (Veeriah et al., 2010; Yeo et al., 2012), while re-expression of wild-type Parkin slows the growth of colorectal carcinoma (Poulogiannis et al., 2010), breast cancer (Letessier et al., 2007; Wang et al., 2009), and lung cancer (Poulogiannis et al., 2010; Veeriah et al., 2010; Yeo et al., 2012) through an unknown mechanism (Liu et al., 2011).

Since mutations in Parkin are the major driver of early-onset PD (Lücking et al., 2000; Kahle and Haass, 2004) and there is evidence that Parkin is a key player in cancer progression (Veeriah et al., 2010), we compiled and analyzed sequences of Parkin from 246 melanoma patients. We identified five melanoma-specific non-synonymous mutations in Parkin in exons known to carry mutations in PD patients. We showed that one of these mutations, E28K, abolished the ubiquitination activity of Parkin. We further demonstrated that Parkin is an inhibitor of cell cycle and an apoptotic cell death driver in melanoma cells, whereas Parkin-E28K abolishes Parkin tumor suppressive effects in melanoma. Finally, in our attempt to uncover the role of Parkin in melanoma, we identified potential Parkin substrates in melanoma using immunoprecipitation followed by mass spectrometry, and clustered the identified substrates by functionality analysis. Our data suggest that Parkin links between PD and melanoma and that Parkin may serve as a diagnostic marker.

**RESULTS**

Somatic mutations of the Parkin gene in human melanoma patients

To determine whether mutations in Parkin gene are present in melanoma, we compiled somatic mutation data from whole exome/genome sequencing sources (Berger et al., 2012; Hodis et al., 2012; Krauthammer et al., 2012; Nikolaev et al., 2012). These data and the analysis methods were previously described (Dutton-Regester et al., 2014). Our analysis revealed mutations in 3.6% of the 246 samples analyzed. There were six non-synonymous mutations, three synonymous mutations, and one splice region variant (Table 1). The Parkin protein has an N-terminal ubiquitin-like (UBL) domain, which is responsible for the recognition of proteins destined for ubiquitination. The C-terminal RING box includes two zinc fingers separated by an in-between RING (IBR) domain that is responsible for specific interaction with E2 enzymes (Imai et al., 2000; Shimura et al., 2000). A linker region connects the UBL and RING domains; this linker contains cleavage sites for caspases 1 and 8 (Shimura et al., 2000; Kahle and Haass, 2004) (Fig. 1A). The melanoma-specific mutations are shown in Fig. 1A in relation to the protein domains.

Importantly, our analysis was only performed on point mutations and small indels, and did not include large indels or mutations of exon rearrangements. Therefore, it is possible that Parkin mutational frequency in melanoma may be higher than 3.6%.

The Parkin mutations commonly carried by PD patients (Hedrich et al., 2004; Kay et al., 2010) were indexed by exon and by type of mutation (i.e., point mutations, exon rearrangements, small deletions, and amplifications) and are represented in proportions to their amount in each exon (Fig. 1A, upper panel). Known Parkin mutations span the entire gene. Although mutations in different regions alter different biochemical properties of the Parkin protein, apparently all mutations disrupt the ability of Parkin to degrade substrates, and thus manifest as loss-of-function mutations (Sriram et al., 2005). Interestingly, the most unstable region of the fragile site on chromosome 6q, FRA6E, is found in the region that contains exons 2 through 8 of the Parkin gene (Letessier et al., 2007), and this is the region where most melanoma and PD mutations are observed (Fig. 1A). Exons 2 and 7 of the gene were previously identified as mutational hot spots (Hedrich et al., 2004). Remarkably, melanoma-specific somatic mutations in Parkin appear in the same domains as the most common germline PD mutations (Fig. 1A), supporting the hypothesis of a genetic link between the two diseases.

The structure of Parkin has been determined by crystallography (Protein Data Bank ID 4k95) (Trempe et al., 2013). We used this structure to assess possible effects of the mutations observed in melanoma patients on Parkin activity (Fig. 1B). The E28K mutation is located in the helix of the UBL domain. Based on a corresponding residue in NEDD8 protein, which is conserved and crucial for interaction with its
Table 1

<table>
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<tr>
<th>Chromosome</th>
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<th>Protein position</th>
<th>Codon</th>
<th>Mutation type</th>
<th>Heterozygosity status</th>
<th>Patient sample number</th>
<th>Gene</th>
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<td>Missense</td>
<td>Wild type</td>
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Missense variants are defined as mutations that cause a change in amino acid identity, synonymous variants are defined as mutations that do not alter the amino acid sequence, and a splice region variant is defined as a sequence variant in which a change has occurred within the region of the splice site, either within 1–3 bases of the exon or 3–8 bases of the intron. Reference and variable alleles are given on the plus strand.

In order to determine the biological role of Parkin in melanoma, we first examined the effect of Parkin overexpression on melanoma clonogenic propensity. WM3314 and A375 melanoma cell lines were transiently transfected with a vector for Parkin expression or an empty vector as a control and were subjected to a colony formation assay. In both melanoma cell lines, Parkin expression significantly decreased the number of detected colonies (Fig. 2A). Further, Parkin overexpression significantly attenuated metabolic activity of WM3314 and A375 cells (Fig. 2B) and growth rate of these cells (Fig. 2C). Moreover, overexpression of Parkin decreased cell cycle progression of WM3314 and A375 cells (Fig. 2D). We found that Parkin induces apoptosis, since overexpression in both WM3314 and A375 melanoma cells increased the percentage of apoptotic and TUNEL-positive cells compared to cells transfected with empty vector (Fig. 2E and F). These results clearly demonstrate that Parkin reduces proliferation and promotes apoptosis in melanoma cells, suggesting that loss-of-function mutations in Parkin enable melanoma cells to avoid cell cycle arrest and proliferate.

Parkin reduces melanoma proliferation and promotes apoptosis

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Parkin-E28K melanoma somatic mutation perturbs Parkin biological activity in melanoma

To determine the effects of the identified melanoma-specific mutations on Parkin function, we generated expression vectors of each melanoma-specific Parkin mutant. We first examined the ubiquitin ligase activity of these mutants by testing and comparing the ubiquitination capacity of wild-type (WT) Parkin and Parkin mutants C360S, E300K, L112F, R275W, and E28K in the presence or absence of a proteasome inhibitor (MG132). In the absence of MG132, less ubiquitination activity was detected upon expression of WT Parkin.
This was likely due to higher levels of degradation of the ubiquitinated substrates by the proteasome. Of the Parkin mutants evaluated, only Parkin-E28K had significantly lower ubiquitination capacity than WT Parkin (Fig. 3A). Since only E28K in the UBL domain abolished Parkin ubiquitination activity (Fig. 3A), we proceeded with this mutation for further functional characterization of Parkin. Structural analysis of a homolog of Parkin ubiquitin-like domain, NEDD8, bound to NAE1, shows that this residue is conserved, and that it forms a salt-bridge with NAE1 residue R251. Therefore, we speculate that E28 is important for the interaction with a partner during the ubiquitination process.

To further evaluate the biological activity of Parkin-E28K, WM3314 melanoma cells were transfected with vectors for the expression of WT Parkin or Parkin-E28K, and proliferation rates were measured relative to cells transfected with a control vector. Cells that expressed WT Parkin grew more slowly than control cells, and cells that expressed Parkin-E28K proliferated significantly more rapidly than the control cells (Fig. 3B). The overexpression of Parkin-E28K resulted in an enhanced cell cycle progression of both WM3314 and A375 cells (Fig. 3C and S1). Finally, the E28K mutation impaired Parkin biological activity as an apoptosis inducer in WM3314 melanoma cells (Fig. 3D and S1). These data demonstrate that in melanoma, a glutamic acid located at the consensus position 28 of Parkin is essential for the E3 ligase activity of Parkin and thus for its biological function as an inhibitor of proliferation and an apoptosis inducer.

**Parkin interactors in melanoma**

In order to further explore the molecular mechanism of Parkin activity in melanoma, we identified Parkin substrates using mass spectrometry analysis. WM3314 melanoma cells were transfected with an expression vector of GFP-tagged-Parkin or with a GFP control vector. Following immunoprecipitation of GFP-Parkin with anti-GFP antibody (Fig. 4A, left panel), associated proteins were identified using mass spectrometry analysis. The experiment was repeated twice to increase the significance of the results. Parkin interacting proteins that
were identified in two independent experiments (Table S2), are represented in a functional protein association network constructed using STRING in Fig. S2. As expected, since Parkin is a known E3 ubiquitin ligase (Moore et al., 2008), the majority of identified proteins are associated with the ubiquitination process and are components of the 26S proteasome system, which strengthen the validity of our analysis. Additionally, four known Parkin substrates were identified in our mass spectrometry analysis, HSP70 (Moore et al., 2008), alpha and beta Tubulin (Ren et al., 2003) and VDAC1 (Narendra et al., 2010). Importantly, this is the first time that these substrates are identified as Parkin interactors in melanoma. In line with our experimental results, gene ontology analysis revealed a significant enrichment of cell cycle regulators and mitochondrial proteins (Fig. 4B and Table S3), emphasizing the role of Parkin-E3 ligase in melanoma cell cycle regulation.

Fig. 2. Parkin reduces melanoma proliferation and promotes apoptosis. WM3314 and A375 cells were transiently or stably transfected with a vector for Parkin expression or a vector control, respectively. A: Colony formation assay. Number of colonies is plotted; error bars represent ±SEM, and * indicates P < 0.05 (n = 3). B: XTT assay for mitochondrial activity. Data are relative to color levels on day 1. Error bars represent ±SEM, and * indicates P < 0.05 (n = 3). C: Crystal violet staining to determine cell number. Data are relative to color levels on day 0. Error bars represent ±SEM, and * indicates P < 0.05 (n = 3). D: Cell cycle analysis. Data are presented as fraction of cells in G1 phase relative to G2/M phase. Error bars represent ±SEM, and * indicates P < 0.05 (n = 3). E: Annexin-PI staining to quantify apoptotic cells. Cells were subjected to serum-free medium and stained with Annexin V and PI. Apoptotic cells were measured by FACS Calibur flow cytometer. Error bars represent ±SEM, and * indicates P < 0.05 (n = 3). F: TUNEL labeling analysis using fluorescence microscope. Cells were subjected to serum-free medium and were labeled with TUNEL (red) and DAPI (blue).
DISCUSSION

The co-occurrence of PD and melanoma has been reported in several epidemiological studies (Olsen et al., 2006; Gao et al., 2009), and recently in a genetic study (Hu et al., 2016); however, the molecular link is still unknown. To the best of our knowledge, no study has stratified PD patients by common PD mutations (Parkin, DJ-1, SNCA, etc.) and looked for melanoma appearance in each group. Epidemiologically, only a positive correlation between the two diseases has been reported. For many years, melanoma appearance in PD patients was thought to be related to levodopa treatment (Fiala et al., 2003). It was shown that levodopa treatment does not increase the risk of melanoma (Fiala et al., 2003), and the risk for melanoma is increased before PD is diagnosed (Olsen et al., 2006). Thus, it is reasonable that the joint susceptibility for both diseases is based on genetic components. Here we focus on Parkin as a plausible candidate underlying the PD-melanoma link. Germline mutations in the Parkin gene are major cause of the familial forms of PD (Lücking et al., 2000), and mutations or deletions of Parkin promote various types of cancers development (Veeriah et al., 2010). We discovered five somatic missense mutations in the Parkin gene in human melanoma samples; these mutations map to regions where the majority of Parkin mutations were located in PD patients. In our analysis, one of the mutations, Parkin-E28K, nearly
abolished Parkin ubiquitination activity. This mutation is located in the UBL domain, and thus probably disrupts substrate binding. Another UBL located mutation R42P was reported previously to be involved in PD, and was shown to potentially disrupt binding to the proteasome (Sriram et al., 2005).

In PD, there is massive loss of dopaminergic neurons in the substantia nigra (Lim et al., 2005; Hampe et al., 2006; Oyama et al., 2010), whereas cancer cells are characterized by uncontrolled cell division and growth (Hanahan and Weinberg, 2011). Parkin is expressed in a wide variety of tissues and is well conserved from nematodes to humans (Xu et al., 2014). Parkin is expressed in a wide variety of tissues and is well conserved from nematodes to humans (Xu et al., 2014).

Although Parkin inactivation has mostly been shown to cause neuronal dysfunction, it is reasonable that it will also affect other tissues. Indeed, Parkin alterations are involved in various types of cancer (Veeriah et al., 2010), supporting a central role for Parkin in various tissues. It appears, therefore, that mutations in Parkin cause different phenotypes in different cell lineages. This contradiction might be due to various Parkin regulators and substrates in different cell types, in accordance with the different cellular roles, function, differentiation state, proliferative capacity and other unique features of each cell type. One prominent difference between cancer cells and neurons is their ability to progress through cell cycle. Neurons are fully differentiated post mitotic cells that do not proliferate, whereas cancer cells partly lose their differentiation state and retain high proliferative capacity (Hanahan and Weinberg, 2011). This may indicate that cell proliferative state dictates the opposite outcomes of Parkin function. In accordance with previous works demonstrating that Parkin functions as a tumor suppressor (Veeriah et al., 2010; Yeo et al., 2012), we showed here that Parkin expression decreased the ability of melanoma cells to progress through cell cycle and inhibited proliferation. In addition, the mutant Parkin-E28K could not suppress, and possibly promote melanoma cell cycle progression. As differentiated neurons that receive signals for proliferation die (Sumrejkanchanakij et al., 2006), it will be interesting to examine whether Parkin functions as a kind of a cell cycle guardian to prevent neuronal cells from entering cell cycle, and its absence causes re-entrance to cell cycle leading to apoptotic cell death.

In melanoma, Parkin expression results in increased apoptotic cell death; this was not observed when the Parkin-E28K mutant was expressed. These results are contradictory to numerous previous reports that intact Parkin is required for neuronal survival and health, and that dysfunctional Parkin leads to neuronal death (Shen and Cookson, 2004), like in PD. Taken together, however, these results strongly support the fact that mutated Parkin leads to opposite phenotypes in different lineages.

In our study, we focus on the effect of the E28K variant in melanoma cells, since it is the only mutation that perturbed Parkin E3-ligase ubiquitination activity in our prior biochemical assays. However, other mutations that were identified here, including the R275W variant, may also impair Parkin activity and induce changes of cellular phenotypes. For example, Sriram et al. (2005) have previously demonstrated that the R275W variant does not impair Parkin E3-ligase ubiquitination activity. Additionally, Xiong et al. (2015) reported on the presence of germline R275W mutation in familial lung cancer patients. It will be very interesting to further investigate the effect of each mutation on intracellular localization, substrate binding and degradation, solubility, and enzymatic activity.

Interestingly, the majority of Parkin mutations we identified did not decrease, and even elevated, the ubiquitination activity of Parkin (Fig. 3A and 3A). Additionally, differences in extracting expressed mutants from lysed cells were observed. Specifically, Parkin mutants do not appear equally in the Triton X-100 soluble fraction, as the insoluble fraction (including cell membranes) are excluded from the final analysis. Specifically Parkin-R275W, E300K, and C360S were not soluble in Triton X-100 when compared with WT Parkin (Fig. S3B, lower panel). Importantly, all Parkin mutant mRNAs were expressed at similar levels (Fig. S3C). These observations suggest that some mutations of Parkin may change the protein cell localization (Sriram et al., 2005); alternatively, mutation might lead to a conformational change, thus affecting protein solubility, and possibly protein activity.
Here we identify a possible genetic association between PD and melanoma by using somatic mutations data of melanoma patients. Of note, Parkin mutations in PD patients are germline (Kahle and Haass, 2004; Guo et al., 2008, 2010). Recently, Hu et al. (2016) published a work in which they identified Parkin germline mutations in melanoma patients; the results are in line with what we propose here, and further demonstrate the genetic connection between melanoma and PD. It is highly likely that germline PD mutations in Parkin play a role in melanoma predisposition, either in the initial development of the tumor or its progression. Interestingly, most of the Parkin somatic mutations found in variety of cancers (Veeriah et al., 2010) occur at the same domains and residues as the germ-line Parkin mutations implicated in PD. In addition, although Parkin KO mice do not recapitulate signs central to Parkinsonism, these mice are prone to cancer development (Fujitawara et al., 2008). Mice with homozygote Parkin exon 3 deletions spontaneously develop hepatic tumors with characteristics of hepatocellular carcinoma (HCC). Moreover, Parkin heterozygous deletion accompanied by APC heterozygous deletion promotes intestinal adenoma development in mice. As HCC appears in the mice at advanced age, and there is a dependency between Parkin and APC mutations in adenoma development, it is possible that a mutation in Parkin alone is not sufficient to initiate development of some types of cancer, and it may require additional risk factors. Moreover, we identified Parkin interacting proteins in melanoma cells by immunoprecipitation followed by mass spectrometry. The proteins detected are mainly connected to cell cycle, mitochondrial function, and proteasome function; this result is consistent with the study of Sarraf et al. (2013). In conclusion, our data indicate that Parkin has a tumor suppressive role in melanoma. Our findings open new possible methods for early diagnosis and treatments against both diseases.

MATERIAL AND METHODS

Cell culture and transfection

WM3314 and A375 melanoma cells were generously provided by Dr. Levi A. Garraway (Department of Medical Oncology and Center for Cancer Genome Discovery, Dana-Farber Cancer Institute, Boston, MA, USA). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, USA) and 1% penicillin/streptomycin/glutamine (Invitrogen, USA). For establishment of stable cell lines, A375 cells were transfected with a vector for expression of WT Parkin-FLAG or control vector, and were selected by culture with 100 mg/mL G418 (Sigma—Aldrich). Constructs were transfected using jetPEI™ according to the manufacturer’s instructions.

Plasmids and cloning

Site-directed mutagenesis was performed using the Stratagene Quick Change method (Stratagene, USA) according to manufacturer’s protocol. pEGFP-N1 and pEGFP-C2 vectors were purchased from Clontech (USA). The pRK5-Parkin-myc expression vector is a gift from Dr. Simone Engleender (The Rapport Institute for Research, Faculty of Medicine, Technion – Israel Institute of Technology, Haifa, Israel). The pDNA3.1-Parkin-FLAG expression vector was kindly provided by Dr. Luc Morris (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). To tag Parkin with GFP, a fragment spanning the WT or mutant Parkin coding region was amplified from RSK-Parkin constructs, digested with EcoR I-SAL I, and cloned into pEGFP-C2.

Colony-forming assay

Cells were selected using G418 until visible separated colonies were formed. Cells were then fixed with 10% acetic acid and 10% ethanol for 40 min. Colonies were stained with 0.2% (w/v) crystal violet (Sigma—Aldrich) in 10% acetic acid and 10% ethanol for 1 h, washed, and counted.

Crystal violet staining

Cells were washed with PBS and fixed with 10% acetic acid and 10% ethanol solution. Cells were then stained using 0.2% (w/v) crystal violet in 10% acetic acid and 10% ethanol for 1 h. For quantification of the crystal violet staining, the dye was re-dissolved in 10% acetic acid solution. Color intensity was measured using a UV—vis plate reader at 595 nm.

Mitochondrial activity assay

Cells were treated with activated XTT solution (Biotium, USA). The signal was measured according to the manufacturer’s protocol at 490 nm and normalized to signal at 690 nm.

Cell cycle analysis

Cells were synchronized in G1 phase by treatment with 2 mMol/L thymidine (Sigma—Aldrich) for 22 h. Cells were then washed twice with PBS and incubated for 18 h in DMEM with 10% serum. Cells pellets were fixed with 70% ice cold ethanol and resuspended in propidium iodide (PI) solution containing 0.05% Triton X-100, 0.05 mg/mL PI (Sigma—Aldrich), and 0.1 mg/mL of RNase A. Cell cycle phases were determined using a BD FACSCalibur flow cytometer and analyzed by Kaluza analysis software.

Annexin-PI staining

Stress was induced by growing cells in serum-free medium for 48 h. The cell pellets were suspended in binding buffer containing 0.1 mol/L HEPES, pH 7.4, 1.4 mol/L NaCl, and 25 mmol/L CaCl2. To 10^5 cells, 1 mg/mL of Annexin V (Biolegend, USA) and 0.05 mg/mL PI were added. Samples were incubated in the dark at room temperature for 15 min then analyzed on a BD FACSCalibur flow cytometer with Flowjo analysis software. Samples treated with Annexin V only and PI only were used as controls.
TUNEL assay

Stress was induced by growing cells in serum free medium for 48 h. Cells were fixed in 4% PFA for 1 h at room temperature and then incubated on ice for 2 min in permeabilization solution containing 0.1% Triton X-100 in 0.1% sodium citrate. Cells were then incubated with TUNEL reaction mixture (in situ cell death detection kit, Roche, USA) for 1 h and then stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich). Cells were analyzed by fluorescence microscopy.

Gel electrophoresis, immunoblotting, and immunoprecipitation

Cells were lysed 48 h after transfection in buffer containing 50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.5% Triton X-100, 1 mmol/L EDTA, and protease inhibitor cocktail (Sigma–Aldrich). For Input, cells were resuspended in 4% SDS, 0.1 mol/L Tris pH 7.5. Once protein concentration was determined, 0.1 mol/L DTT was added to the lysate. Samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and exposed to the appropriate antibodies: mouse anti-hParkin (MAB14381; R&D Systems, USA), mouse anti-β-actin (AC-74; Sigma–Aldrich), and rat anti-HA (3F10; Roche, Switzerland). Proteins were visualized with SuperSignal Chemiluminescent Substrates (Pierce, USA) using horseradish peroxidase-conjugated anti-mouse secondary antibody (7076; Cell Signaling, USA) or goat anti-rat (sc-2006; Santa Cruz Biotechnology, USA).

Ubiquitination assay

For ubiquitination assay, HEK293 cells were co-transfected with constructs for expression of WT or mutant myc-tagged Parkin or control vector and with HA-tagged ubiquitin, followed by immunoprecipitation of Parkin with anti-myc. Ubiquitination was detected by anti-HA. MG132 (Sigma–Aldrich) treatment was done at 10 μmol/L for 4 h before harvest.

Sample preparation for mass spectrometry analysis

WM3314 cells were transfected with GFP-tagged-Parkin or with vector control. After 48 h, cells were incubated for 4 h with 20 μmol/L MG132 and then washed twice with PBS. Cells were lysed in lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris pH 7.5, 1% NP-40, 1 mmol/L Na3VO4, 1 mmol/L NaF, 0.1% β-mercaptoethanol, and protease inhibitor. Proteins were incubated with mouse anti-GFP antibody (SC-9996; Santa Cruz Biotechnology, USA) for 18 h at 4°C and then incubated with protein A/G agarose beads for 2 h at 4°C. Proteins were eluted in 50 mmol/L Tris, pH 8.5, 8 mol/L urea, and 1 mmol/L DTT for 2 h and in 50 mmol/L Tris, pH 8.5, 8 mol/L urea, and 5 mmol/L IAA for 5 min, followed by overnight incubation at room temperature in the dark.

Mass spectrometry analysis

The tryptic peptides were desalted over C18 resin, dried, and resuspended in 0.1% formic acid. The peptides were separated by reversed-phase chromatography on 0.075 × 180-mm fused silica capillaries (Agilent J&W, USA) packed with Reprosil reversed-phase material (Dr. Maisch GmbH, Germany). The peptides were eluted using a linear 60-min gradient of 5%—28% solvent A, 15-min gradient of 28%—95% solvent A, and 15 min solvent B. Solvent A was 95% acetonitrile with 0.1% formic acid in water and solvent B was water. The flow rate was 0.15 μL/min. Mass spectrometry was performed by Q Exactive plus mass spectrometer (Thermo Scientific, USA) in a positive mode using repetitively full MS scan followed by collision induced dissociation of the 10 most dominant ions selected from the first MS scan. The mass spectrometry data from two biological repeats was analyzed using Proteome Discoverer 1.4 software with Sequest (Thermo Scientific, USA) and Mascot (Matrix Science, UK) algorithms against Human Uniprot database with 1% FDR. Semi-quantitative analysis was done by calculating the peak area of each peptide based its extracted ion currents, and the area of the protein was calculated as the average of the three most intense peptides from each protein.

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SUPPLEMENTARY DATA

Fig. S1. Parkin reduces melanoma proliferation and promotes apoptosis.
Fig. S2. Parkin interactors in melanoma.
Fig. S3. Mutations in Parkin results in altered solubility.
Table S1. Parkin-related diseases.
Table S2. Mass spectrometry analysis of PARK2 potential substrates in melanoma.
Table S3. Significant functional annotations of PARK2 potential substrates in melanoma.
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgg.2016.05.005.

REFERENCES


