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Parkin is ubiquitinated by Nrdp1 and abrogates Nrdp1-induced oxidative stress

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ABSTRACT

Parkin plays an important role in the pathogenesis of Parkinson's disease. We previously described that Nrdp1, a RING-finger ubiquitin E3 ligase, interacted with Parkin by the yeast two-hybrid assay and by co-immunoprecipitation. Here we further demonstrated that overexpression of Nrdp1 significantly reduced the endogenous Parkin level in an Nrdp1 dosage-dependent and proteasome-dependent manner. More importantly, Nrdp1 ubiquitinated Parkin and catalyzed the poly-ubiquitin chains on Parkin *in vitro* as well as in cells, indicating Parkin is an Nrdp1 substrate. In addition, we demonstrated that overexpression of Nrdp1 increased the production of reactive oxygen species (ROS), which was abrogated by co-expression of Parkin. Conversely, suppression of Nrdp1 by shRNA conferred SH-SY5Y cells a lower ROS level. Together, we provided evidence that interactions between Nrdp1 and Parkin negatively regulated Parkin level and affected ROS production, suggesting that Nrdp1 may play a role in Parkinson's disease.

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by loss of dopaminergic neurons in the substantia nigra pars compacta (SNc). Clinically, PD is defined by four cardinal signs, resting tremor, bradykinesia, rigidity, and postural instability [3]. The etiology of PD remains enigmatic. The genetic involvement in PD has been increasingly recognized during the last 10 years. Identification of mutations in numerous genes in PD patients has yielded crucial insights into the possible pathogenic mechanisms [23].

Mutations in *Parkin*, a gene that encodes an ubiquitin E3 ligase, are the prominent cause of autosomal recessive juvenile Parkinsonism (AR-JP). Loss-of-function mutations in *Parkin* have been found in more than 50% of familial early onset PD patients [24]. Various substrates for Parkin have been identified and characterized, however, the mechanism of how the Parkin protein and its E3 ligase activity are regulated remain poorly understood. By using the yeast two-hybrid screening, we isolated Nrdp1 (neuregulin receptor degradation protein-1, refer to FLRF in mice) to interact with Parkin [25]. Interestingly, Nrdp1 is also a RING finger ubiquitin E3 ligase. The N-terminal half of Nrdp1 possesses a RING finger domain and two zinc finger domains, while its C-terminal half contains a predicted coiled–coiled domain [2]. Nrdp1 has been reported to interact with and to ubiquitinate epidermal growth factor receptor members, ErbB3/ErbB4 and BRUCE, an inhibitor of apoptosis (IAPs) [18,19], indicating that Nrdp1 may play roles in cell proliferation, differentiation and apoptosis.

To further characterize the biological significance of association between Parkin and Nrdp1, we report here that Parkin was a substrate of Nrdp1 for ubiquitination. In addition, we demonstrated that the interaction between Nrdp1 and Parkin influenced the production of reactive oxygen species (ROS), suggesting a potential involvement of Nrdp1 in the pathogenesis of PD.

Chemicals and antibodies: All chemicals, unless otherwise noted, were purchased from Sigma (St. Louis, MO). Antibodies that were used are: anti-Parkin antibody from Cell Signaling Technologies Inc. (Beverly, MA), Parkin antibody (PRK8) from Upstate (Lake Placid, NY), anti-Nrdp1 antibody from Bethyl Laboratories Inc. (Montgomery, TX), anti-flag M2 antibody from Sigma (St. Louis, MO), anti-penta-His antibody from Qiagen (Valencia, CA) and anti-myc (9E10) and anti-tubulin antibodies from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmids: Myc-human *Parkin* (Myc-Parkin) in pcDNA-3.1 (+) vector was described previously [25]. Human *Parkin* mutants were generated by site-directed mutagenesis. Glutathione S-transferase (GST), GST fused Parkin, and GST-Parkin^{C421A} were produced from PGEX-4T-1 vector. *Nrdp1*-flag was released from pcDNA3.1 (+)-*Nrdp1*-flag and subcloned into the pRmHa-3 vector [1] for expression in insect S2 cells (pRmHa-3-*Nrdp1*-flag).

Protein expression, purification and in vitro ubiquitination assay: GST-Parkin and its mutants were purified from *E. coli* BL21 (DE3) [25]. The protein concentrations were determined using Coomassie blue staining with BSA as a standard. The Nrdp1 protein with FLAG tag at C-terminus was purified from S2 cells by immunoprecipitation using anti-flag antibody M2. For *in vitro* ubiquitination assay, GST-tagged Parkin or its mutants were added in the presence or absence of Nrdp1-flag in a 50 μl reaction containing 50 mM

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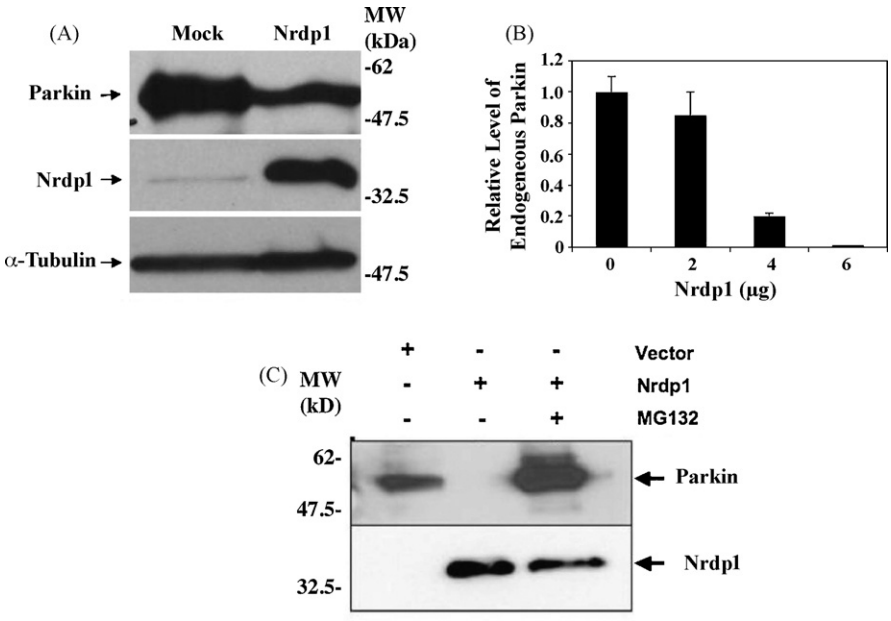

Fig. 1. Overexpression of Nrdp1 reduces endogenous Parkin. (A) The Parkin level in HEK 293 cells transfected with Nrdp1 or control was analyzed by Western blot. (B) The relative levels of the Parkin protein were quantified in HEK 293 cells transfected with different amounts of Nrdp1 by laser densitometry. The means of triplicate analyses with standard deviations were blotted ($P < 0.05$). (C) Parkin was downregulated in cells that overexpress Nrdp1 but stabilized after treatment with 10 μM of MG132 for 8 h.

Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM ATP, 2 mM DTT, 100 ng E1, 1 μg E2 (UbcH5c or UbcH7), and 5 μg His-Ubiquitin (Boston Biochem, Boston, MA). After incubation at 30 °C for 2 h, the reactions were terminated by adding 2X SDS sample buffer and poly-ubiquitin signals were analyzed by Western Blot with antibody against penta-His.

Cell cultures, transfection and biochemical treatment: HEK 293 cells or neuroblastoma SH-SY5Y cells were cultured in Dulbecco's modified eagle's medium (DMEM) or DMEM/F12 supplemented with 10% of FBS and penicillin–streptomycin and maintained in 5% CO_2 at 37 °C. Cells were transfected with pcDNA3-myc-Parkin, pcDNA3-Nrdp1-flag, His-Ubiquitin and/or shRNA using Lipofectamine 2000 (Invitrogen). In some experiments, 36 h after transfection, cells were first treated with proteasome inhibitor MG132 (10 μM) for 6 h and then harvested for further analysis. Insect S2 cells that were grown in Schneider's *Drosophila* medium supplemented with 10% FBS and 50 U/μl Penicillin and Streptomycin (Invitrogen) were transfected with pRmHa-3-Nrdp1-flag. After 48 h transfection, 500 μM $CuSO_4$ was added to induce protein expression for 6–8 h [1].

Immunoprecipitation and Western blot: Cells were collected and whole cell lysates were prepared with radioimmunoprecipitation (RIPA) buffer. After pre-cleared with protein-A/G agarose, cell lysates were incubated with 2 μg of antibody against Parkin or rabbit IgG at 4 °C. After 3 h incubation, cell lysates were added with 50 μl of protein A or G slurry to pull down the immunocomplex. Immunoprecipitates that were washed twice in TBS supplemented with various strengths of detergent were examined by Western blot to detect ubiquitination with antibodies against His, Parkin or myc.

ROS measurement: Approximately 1×10^7 neuroblastoma SH-SY5Y cells were pre-incubated with DCFH-DA (10 μM) for 30 min at 37 °C and then washed three times with ice-cold PBS [9]. The cells were resuspended in PBS buffer supplemented with 2% FBS and analyzed immediately on BD LSR II (CA, USA) interfaced to BD Diva software (Beckton Dickinson, Mountain View, CA). The fluorescence of cells was recorded under the 488 nm excitation. Green fluorescence from DCFH was measured in the 530 nm emission filter bandpassed for DCF.

Overexpression of Nrdp1 reduced the endogenous level of Parkin: To characterize the functional consequences of the interaction between Nrdp1 and Parkin, HEK 293 cells were transfected with flag-tagged Nrdp1. Western blot analysis with an anti-Parkin antibody showed that Nrdp1 overexpression significantly reduced the endogenous Parkin level (Fig. 1A). The down-regulation of Parkin protein was in an Nrdp1-dosage dependent manner (Fig. 1B). For instance, transfection of 2 μg of Nrdp1 reduced Parkin to 80% of the control while Parkin was hardly detectable in cells with 6 μg of Nrdp1 (Fig. 1B). To examine how Nrdp1 down-regulated Parkin, the proteasome inhibitor MG132 was applied to Nrdp1 transfected cells. We found that Nrdp1-induced dramatic decrease in Parkin level (Fig. 1C, lane 2). In contrast, a significant recovery of Parkin was observed in cells treated with MG132 (Fig. 1C, lane 3), suggesting that Nrdp1 prompted the degradation of endogenous Parkin in a proteasome-dependent manner.

Mutant Parkin^{C421A} was ubiquitinated by Nrdp1 in vitro: Parkin is an ubiquitin E3 ligase and self-ubiquitinates. To limit the potential complication, we generated several Parkin mutants. We demonstrated that GST-Parkin^{C421A} lost its self-ubiquitination activity (Supplementary Fig. 1). With GST-tagged Parkin^{C421A} as a substrate and Nrdp1-flag protein produced from S2 cells (Fig. 2A) as an E3 ubiquitin ligase, effect of Nrdp1 on Parkin ubiquitination was assessed in *in vitro* assays. Poly-ubiquitin chains linked to Parkin were detected by Western blot using anti-His antibody. Our results demonstrated that mutant Parkin^{C421A} was poly-ubiquitinated in the presence of Nrdp1 (Fig. 2B and C, lanes 2–5) while Nrdp1 or mutant Parkin^{C421A} alone showed no poly-ubiquitination signals (Fig. 2B and C, lanes 1–3 and 6).

Parkin was ubiquitinated by Nrdp1 in cells: To further validate the effects of Nrdp1 E3 ligase activity on Parkin ubiquitination, experiments in cultured cells were performed. Wild type or *Nrdp1* mutants, His-ubiquitin and *Parkin* were co-transfected into HEK 293 cells. At 48 h post-transfection, cells were harvested and whole cell lysates were subjected to immuno-precipitation with anti-Myc antibody 9E10. The immunoprecipitates were analyzed by immunoblotting with antibodies against His to detect signals of the ubiquitinated Parkin. Ubiquitination of wild type Parkin was clearly