

STEP₆₁ is a substrate of the E3 ligase parkin and is upregulated in Parkinson's disease

Pradeep K. Kurup^a, Jian Xu^a, Rita Alexandra Videira^b, Chimezie Ononenyi^a, Graça Baltazar^b, Paul J. Lombroso^{a,c,d}, and Angus C. Nairn^{c,1}

^aChild Study Center and Departments of ^cPsychiatry and ^dNeurobiology, Yale University School of Medicine, New Haven, CT 06520; and ^bHealth Science Research Center, Faculty of Health Sciences, Universidade da Beira Interior, 6200-506 Covilhã, Portugal

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Parkinson's disease (PD) is characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc). The loss of SNc dopaminergic neurons affects the plasticity of striatal neurons and leads to significant motor and cognitive disabilities during the progression of the disease. *PARK2* encodes for the E3 ubiquitin ligase parkin and is implicated in genetic and sporadic PD. Mutations in *PARK2* are a major contributing factor in the early onset of autosomal-recessive juvenile parkinsonism (AR-JP), although the mechanisms by which a disruption in parkin function contributes to the pathophysiology of PD remain unclear. Here we demonstrate that parkin is an E3 ligase for STEP₆₁ (striatal-enriched protein tyrosine phosphatase), a protein tyrosine phosphatase implicated in several neuropsychiatric disorders. In cellular models, parkin ubiquitinates STEP₆₁ and thereby regulates its level through the proteasome system, whereas clinically relevant parkin mutants fail to do so. STEP₆₁ protein levels are elevated on acute down-regulation of parkin or in *PARK2* KO rat striatum. Relevant to PD, STEP₆₁ accumulates in the striatum of human sporadic PD and in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned mice. The increase in STEP₆₁ is associated with a decrease in the phosphorylation of its substrate ERK1/2 and the downstream target of ERK1/2, pCREB [phospho-CREB (cAMP response element-binding protein)]. These results indicate that STEP₆₁ is a novel substrate of parkin, although further studies are necessary to determine whether elevated STEP₆₁ levels directly contribute to the pathophysiology of PD.

Parkinson's disease | parkin | STEP | ubiquitination | synaptic plasticity

Parkinson's disease (PD) is a common motor disorder with clinical symptoms that include bradykinesia, resting tremor, rigidity, postural instability, and cognitive deficits (1–3). The pathophysiology of PD includes selective loss of dopaminergic neurons in the substantia nigra, with a progressive depletion of striatal dopamine and the presence of intraneuronal cytoplasmic inclusions known as Lewy bodies. Mutations of several genes are implicated in PD and are responsible for ~10% of cases; the remaining cases are classified as sporadic PD. Although specific mutations in genes that include *PARK2*, *PINK-1*, *LRRK2*, and *DJ-1* are known, the effects these mutations have on intracellular signaling and disease progression are not well understood and form an area of intense investigation (2, 4–6).

STEP₆₁ (striatal-enriched protein tyrosine phosphatase) is a brain-specific phosphatase enriched in the striatum and in other regions, including cortex, hippocampus, and substantia nigra (7–9). STEP₆₁ levels are elevated in several disorders, including Alzheimer's disease, schizophrenia, and fragile X syndrome (10–12). STEP₆₁ levels are normally regulated by the ubiquitin proteasome system, and disruption of this pathway leads to an accumulation of STEP₆₁ in both Alzheimer's disease and schizophrenia (10, 11).

Substrates of STEP₆₁ include ERK1/2, Pyk2, Fyn, the GluN2B subunit of the NMDA receptor, and the GluA2 subunit of the AMPA receptor. The current model of STEP₆₁

function is that it opposes the development of synaptic strengthening by dephosphorylating regulatory tyrosines on these substrates. In the case of the kinases, STEP₆₁-mediated dephosphorylation of the regulatory Tyr within the activation loop inactivates these enzymes (13–16). STEP-mediated dephosphorylation of Tyr residues in the glutamate receptor subunits results in internalization of GluN1/GluN2B and GluA1/GluA2 receptor complexes (17–20). As a result, STEP KO mice have an increase in the basal Tyr phosphorylation of its substrates, including ERK1/2 and its downstream target pCREB (21, 22).

Overexpression of STEP disrupts synaptic function, and thereby contributes to cognitive and behavioral deficits (23). Consistent with this hypothesis, genetic or pharmacologic reduction of STEP activity in several disorders in which STEP levels are elevated reverses the biochemical and cognitive deficits that are present (19, 24), and STEP KO mice demonstrate enhanced hippocampal long-term potentiation and enhanced hippocampal- and amygdalar-dependent memory tasks (22, 25).

Direct mutations of the E3 ligase parkin (*PARK2*) result in autosomal recessive juvenile parkinsonism (AR-JP), with early onset of PD symptoms (26, 27); disruption of parkin activity is also implicated in sporadic PD (28–30). Moreover, PD toxins such as MPTP, rotenone, paraquat, and 6-hydroxydopamine alter parkin levels or its ligase activity and result in the accumulation of parkin substrates (31–35). Identification of new parkin substrates and characterization of their role or roles in synaptic function should result in a better understanding the molecular basis of PD.

Here we identify parkin as an E3 ligase that ubiquitinates STEP₆₁. STEP₆₁ levels are increased in human PD brains and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD models and are associated with a decrease in the phosphorylation of ERK1/2 and CREB. As an increase in STEP₆₁ expression

Significance

In neurons, STEP₆₁ (striatal-enriched protein tyrosine phosphatase) protein levels are tightly regulated, and the protein's up-regulation is implicated in several neuropsychiatric disorders. Here, we demonstrate that parkin is a major E3 ligase regulating STEP₆₁ levels through the ubiquitin proteasome system. In Parkinson's disease, in which parkin function is compromised, STEP₆₁ levels increase, which is associated with down-regulation of synaptic proteins required for neuronal plasticity.

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¹To whom correspondence should be addressed. Email: angus.nairn@yale.edu.

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disrupts synaptic function and contributes to the cognitive deficits in several disorders, these findings suggest that the increase in STEP₆₁ levels in PD may contribute to the pathophysiology of this disorder.

Results

STEP₆₁ Interacts with Parkin. STEP is normally degraded by the ubiquitin proteasome system, and disruption of this pathway leads to increased STEP expression in patients diagnosed with Alzheimer's disease and schizophrenia (10, 11). A previous proteomic study identified STEP (protein tyrosine phosphatase non-receptor type 5, *PTPN5*) as one of several proteins up-regulated in the striatum of 6-hydroxydopamine-treated rats (36), suggestive of altered protein turnover in a PD animal model. However, little is known concerning the regulation of STEP by the ubiquitin proteasome system. We therefore set out to identify the E3 ligase that ubiquitinates STEP.

To determine whether STEP and parkin associate with each other, we performed GST pull-down experiments using lysates from STEP KO mouse brain. GST-STEP₆₁ efficiently pulled down parkin, as well as the known STEP substrate ERK1/2. However, GST-STEP₆₁ did not pull down GluA1, a negative control (Fig. 1A). We next overexpressed V5-tagged STEP₆₁ and Myc-tagged parkin in HEK-293 cells and conducted immunoprecipitation studies with either V5 or Myc antibody. There was a reciprocal immunoprecipitation of STEP₆₁ and parkin (Fig. 1B). STEP₆₁ did not interact with a structural homolog of parkin, HHARI (Fig. 1C), or with two other E3 ligases, Mdm2 (a RING-domain containing ligase) or Nedd4 (a HECT-domain containing ligase), in HEK-293 cells (Fig. S14). We also confirmed the interaction of STEP₆₁ and parkin in primary corticostriatal cultures using coimmunoprecipitation experiments with parkin antibody (Fig. 1D).

Parkin Regulates STEP₆₁ Protein Levels. To determine the functional significance of the interaction of STEP and parkin, we analyzed STEP₆₁ levels in the presence of increasing amounts of parkin in HEK-293 cells. There was a dose-dependent decrease in steady-

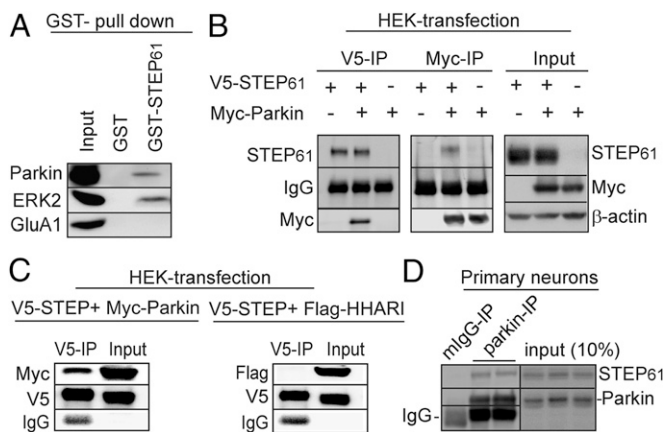


Fig. 1. Parkin interacts with STEP₆₁. (A) GST-STEP₆₁ efficiently pulled down parkin from STEP KO brain lysate. ERK2 served as a positive control, whereas GluA1 or use of GST alone were negative controls ($n = 3$). Samples in this and subsequent figures were analyzed by Western blotting, using the indicated antibodies. (B) Coimmunoprecipitation of STEP and parkin using either V5-tag or Myc-tag antibodies from cotransfected HEK lysate ($n = 4$). (C) STEP₆₁ did not interact with the structural homolog of parkin, HHARI. Immunoprecipitation of STEP₆₁ from Myc-parkin or Flag-HHARI transfected lysate demonstrated that STEP₆₁ immunoprecipitated Myc-parkin, but not Flag-HHARI ($n = 3$). (D) STEP₆₁ was coimmunoprecipitated from primary corticostriatal cultures, using parkin antibody, but not with the control mouse IgG ($n = 3$).

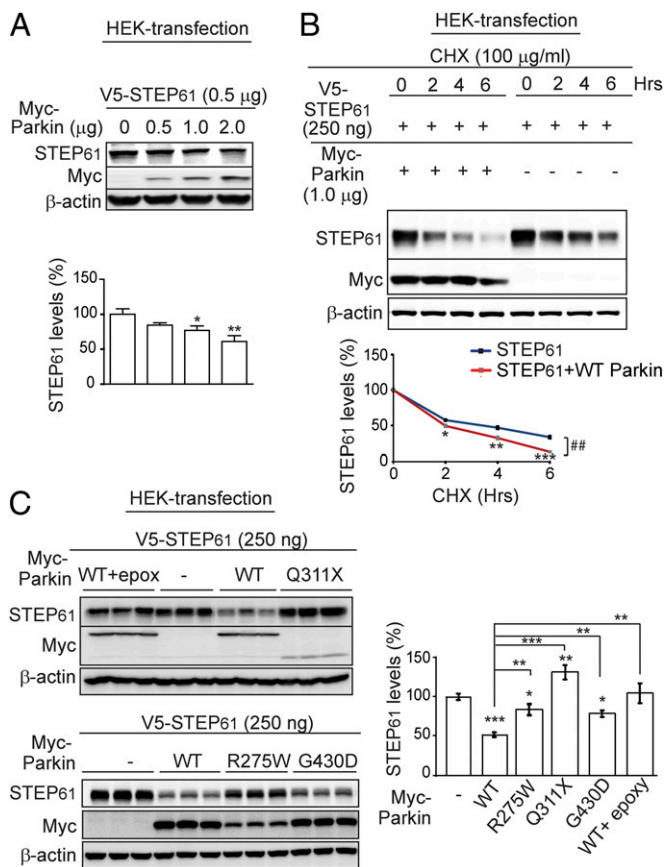


Fig. 2. Parkin and the ubiquitin proteasome system regulate STEP₆₁ levels. (A) Steady-state STEP₆₁ levels were significantly decreased by coexpressing increasing amounts of parkin in HEK cells. STEP₆₁ levels were normalized to β-actin ($n = 3$; mean ± SEM; * $P < 0.05$, ** $P < 0.01$, one-way ANOVA with Tukey test). (B) In the presence of cycloheximide, coexpression of parkin with STEP₆₁ led to an accelerated decrease of STEP₆₁ steady state levels compared with STEP₆₁-alone transfected control. STEP₆₁ levels were normalized to β-actin ($n = 3$; mean ± SEM; * $P < 0.05$, ** $P < 0.01$, Student's t test; ##, results of a two-way ANOVA, with treatment and time as covariants). (C) Expression of WT parkin in HEK cells decreased the steady-state level of STEP₆₁, an effect that was blocked by parkin mutants (R275W, Q311X, and G430D), as well as by the proteasome inhibitor epoxymycin (1 μM). STEP₆₁ levels were normalized to β-actin ($n = 6$; mean ± SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; one-way ANOVA with Tukey test).

state levels of STEP₆₁ as parkin levels increased (Fig. 2A). Further, cycloheximide chase experiments indicated an accelerated decrease in steady-state levels of STEP₆₁ when parkin was coexpressed with STEP₆₁ (Fig. 2B).

We next examined the effects of clinically relevant parkin mutations on STEP₆₁ steady state levels. STEP₆₁ levels were significantly reduced by coexpression of WT parkin, which was blocked by the proteasome inhibitor epoxymycin, suggesting degradation of STEP₆₁ involves the proteasome system (Fig. 2C). The expression of STEP₆₁, along with familial-PD linked parkin mutants Q311X, R275W, or G430D, significantly increased the steady-state STEP₆₁ levels. These results suggest parkin regulates STEP₆₁ expression through proteasomal degradation.

Parkin Ubiquitinates STEP₆₁ in Vivo and in Vitro. To determine whether parkin directly ubiquitinates STEP₆₁, we coexpressed STEP₆₁ with hemagglutinin (HA)-ubiquitin and WT parkin or mutant parkin constructs (R275W, G430D) and conducted ubiquitin affinity pull-down experiments. Overexpression of WT parkin resulted in an increase in ubiquitinated high-molecular-weight

STEP bands. In contrast, coexpression of either parkin mutant resulted in a decrease in the levels of high-molecular-weight STEP species relative to the V5-STEP₆₁ alone control (Fig. 3A). HEK-293 cells express endogenous parkin (37), and the R275W and G430D parkin mutants had a dominant-negative effect on STEP₆₁ ubiquitination. We then conducted *in vitro* ubiquitination reactions, demonstrating that STEP₆₁ is directly ubiquitinated by parkin in the presence of the E1 ligase ubiquitin-activating enzyme E1 (UBE-1) and the E2 ligase ubiquitin-conjugating enzyme H7 (UBCH7) (Fig. 3B).

Down-Regulation of Parkin Increases STEP₆₁ Levels. We used several complimentary approaches to examine STEP₆₁ levels in rodent models with lower levels of parkin. We first measured STEP₆₁ levels in rat corticostriatal cultures after shRNA down-regulation of parkin. Decreasing parkin levels ($43.0 \pm 1.4\%$) resulted in a significant increase in STEP₆₁ expression ($142.0 \pm 9.0\%$) compared with control shRNA-treated neurons (Fig. 4A). There was no effect of reduced parkin expression on the level of STEP₆₁ mRNA in striatal neurons (Fig. S1B) [although note that a previous study in PC12 cells exposed to ceramide suggested that parkin overexpression could influence STEP mRNA expression (38)]. We also examined STEP₆₁ levels in a rat Park2 KO model. There was a significant increase in STEP₆₁ in striatum of 12-month-old Park2 KO rats ($122.0 \pm 2.0\%$) compared with WT controls (Fig. 4B). In contrast, there was no significant change in STEP₆₁ expression in cortex (Fig. 4C).

STEP₆₁ Is Up-Regulated in MPTP-Lesioned Mice Striatum and Human PD Striatum. Administration of MPTP in mice is a well-accepted PD model that recapitulates many of the pathologic and behavioral symptoms of human PD (39, 40). To examine the effects of MPTP on STEP₆₁ expression, we measured STEP₆₁ levels, as well as the phosphorylation of STEP₆₁ substrates in striatum. There was a significant increase in STEP₆₁ and a concomitant decrease in pERK1/2 and pCREB phosphorylation compared with vehicle-treated controls (Fig. 5A). MPTP treatment also led to an increase in Tyr-phosphorylation of parkin (Fig. 5B), as previously shown (32). As the ability of STEP₆₁ to bind to and dephosphorylate its substrates requires the dephosphorylation of STEP₆₁ at a regulatory serine (Ser²²¹) within the substrate-binding domain [kinase interaction motif (KIM) domain], we

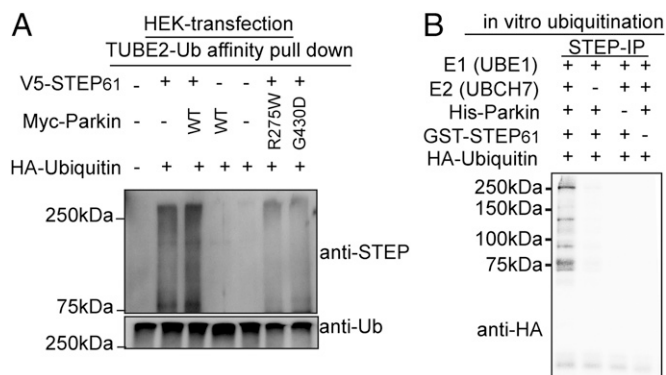


Fig. 3. Parkin ubiquitinates STEP₆₁ *in vivo* and *in vitro*. (A) HEK cells were transfected with STEP₆₁, along with HA-ubiquitin or WT parkin, or with parkin mutants. Ubiquitinated proteins were enriched using tandem ubiquitin binding entity 2 (TUBE2) affinity pull-down and probed with anti-STEP antibody or anti-ubiquitin antibody. STEP₆₁ ubiquitination was increased by WT parkin but decreased by the parkin mutants R275W or G430D ($n = 3$). (B) GST-STEP₆₁ was ubiquitinated by recombinant parkin in the presence of the E1 ligase UBE1, the E2 ligase UbCh7, and HA-ubiquitin. STEP was immunoprecipitated and probed with anti-HA antibody, which shows HA immunoreactivity for higher-molecular-weight STEP-ubiquitin conjugates ($n = 4$).

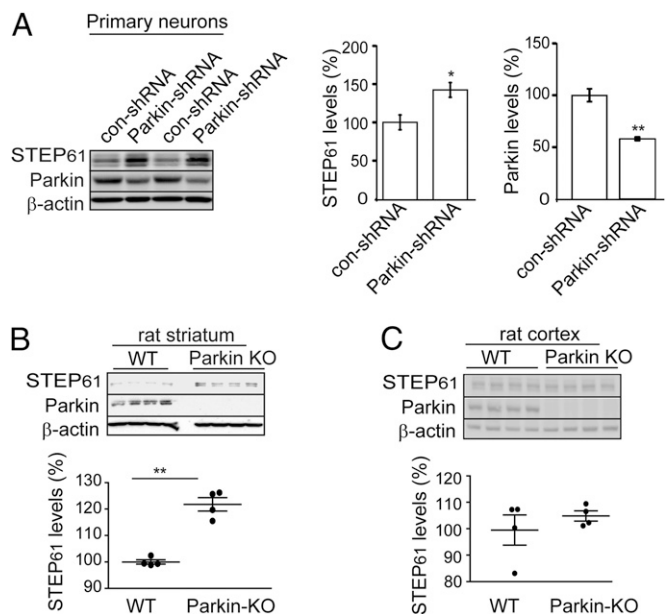


Fig. 4. STEP₆₁ levels are elevated on down-regulation of parkin in primary neuronal cultures and in parkin KO rats. (A) Primary corticostriatal cultures were infected with parkin-specific shRNA, resulting in a significant increase in STEP₆₁ levels compared with control shRNA treatment. Quantification for STEP₆₁ and parkin is shown after normalization with β-actin ($n = 6$; mean \pm SEM; * $P < 0.05$, ** $P < 0.01$, Student's *t* test). (B) STEP₆₁ levels are increased in parkin KO rat striatum at 12 mo of age compared with WT control ($n = 4$; ** $P < 0.01$, Student's *t* test). β-actin served as a loading control. (C) STEP₆₁ levels were not significantly altered in Park2 KO rat cortex ($n = 4$). STEP₆₁ level was normalized to β-actin levels for quantification.

measured the phosphorylation of STEP within the KIM domain, using an antibody that detects only the nonphosphorylated form of STEP Ser²²¹. There was a significant increase in nonphosphorylated STEP₆₁ (more active form) in the MPTP-treated samples compared with control mice (Fig. 5C), suggesting increased STEP levels are associated with increased activity. Further, we also showed that 1-methyl-4-phenylpyridinium (MPP⁺) treatment of a dopaminergic cell line (SH-SY5Y) led to a significant increase in STEP₆₁ and a decrease in the phosphorylation of pERK1/2 and pCREB (Fig. S1C).

Finally, we analyzed STEP₆₁ levels in human sporadic PD cases. There was a significant increase of STEP₆₁ levels in striatum and a decrease in the phosphorylation of pERK1/2 and pCREB levels compared with controls (Fig. 6). As a control, these samples showed a significant increase in aminoacyl-tRNA synthetase interacting multifunctional protein type 2 (JTV), a known parkin substrate. In addition, there was a single case of AR-JP PD among these cases that had a similar increase in STEP₆₁ and decrease in pERK and pCREB (Fig. 6 and Fig. S1D). Of note, there were no significant changes in STEP₆₁, pERK, or pCREB in the cortex of the human sporadic PD cases, in a single case of AR-JP PD samples tested, and in MPTP-treated mice (Fig. S2A–C).

Discussion

Our results indicate that STEP₆₁ is a novel substrate of parkin. Parkin associates with and ubiquitinates STEP₆₁, thereby regulating STEP protein levels through the ubiquitin proteasome system. Several complementary techniques support this finding, including the acute shRNA-down-regulation of parkin, the data from parkin KO rats, and the findings that clinically relevant human parkin mutations all lead to an accumulation of STEP₆₁. Parkin appears likely to be a major E3 ligase for STEP₆₁. Partial

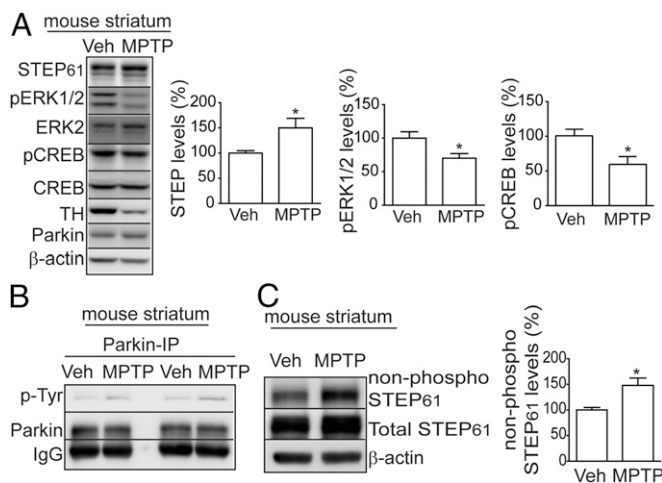


Fig. 5. Striatal STEP₆₁ levels are elevated after MPTP lesion. (A) Immunoblot and quantification indicate that STEP₆₁ expression was increased and pERK1/2 phosphorylation and pCREB levels decreased in MPTP-treated mouse striatum compared with vehicle-treated controls. STEP₆₁ levels were normalized to β-actin for quantification ($n = 8$; mean \pm SEM; * $P < 0.05$; Student's t test). (B) Representative immunoblot indicates that parkin tyrosine phosphorylation was increased in MPTP-treated striatal lysates, after immunoprecipitation of parkin and detection with a pan phospho-tyrosine antibody ($n = 2$). (C) Active, non-phospho-STEP₆₁ and total STEP₆₁ levels were increased in MPTP-lesioned mouse striatum compared with vehicle-treated controls. STEP₆₁ levels were normalized to β-actin ($n = 8$; * $P < 0.05$; Student's t test).

knock-down of parkin in primary neurons had a significant effect on STEP₆₁ protein levels independent of its mRNA levels, and other closely related or distinct E3 ligases did not interact with STEP₆₁.

Increased expression of STEP₆₁ was observed in human postmortem PD samples and parkin KO rats. The increase in STEP was only noted in the striatum, not in cortex. This finding parallels results from previous studies that showed that the parkin substrates JTV and parkin interacting substrate (PARIS) were elevated only in the striatum and not in the cortex of human PD cases (31, 32, 41). The increase in STEP₆₁ levels in the human sporadic PD cases tested is of interest. There was only one case of a *PARK2* mutation in the postmortem samples, suggesting that STEP₆₁ levels are also disrupted in non-*PARK2* cases, a hypothesis that is currently being tested. Taken together, the results suggest that genetic mutations or environmental toxins that disrupt parkin activity result in the accumulation of STEP₆₁.

In striatum, STEP₆₁ is expressed in medium spiny neurons (MSNs), which make up 90–95% of all striatal neurons (42, 43). STEP₆₁ is also likely to be present in axonal terminals that project to striatum, including dopaminergic neurons from substantia nigra (8, 44). STEP₆₁ expression in the human PD samples and the PD rodent KO model is presumably increased in both pre- and postsynaptic compartments in which parkin is expressed (45, 46). Little is known about its presynaptic function, but STEP may play an important role in the regulation of neurotransmitter release. STEP KO mice have elevated pERK1/2 and increased phosphorylation of synapsin I at ERK1/2-specific sites within presynaptic terminals (22), and phosphorylation of synapsin I at these sites is known to increase the probability of vesicle release (47). In this context, amperometric recordings in the striatum of *PARK2* KO mice showed reduction in evoked dopamine release and impaired long-term potentiation and long-term depression in striatal MSNs (48), whereas similar analyses in KO mice for *PINK-1* (an upstream regulator parkin) revealed decreases in evoked dopamine release in striatal slices (49).

However, it is currently not known whether the increase in STEP expression in presynaptic terminals disrupts dopamine release, with concomitant disruption of striatal synaptic transmission.

STEP₆₁ is highly expressed in MSNs, where it is regulated by glutamatergic and dopaminergic signaling through the protein kinase A/dopamine- and cAMP-regulated phosphoprotein MWT 32 (DARPP-32)/protein phosphatase 1 signaling pathway (42, 50). With respect to PD, the increase in STEP₆₁ might influence dopamine signaling in MSNs. Dopamine D1 receptor stimulation in direct pathway MSNs activates protein kinase A, which phosphorylates STEP₆₁ at ser²²¹ within its substrate-binding domain (42). Phosphorylation at this site decreases the binding of STEP₆₁ to substrates and prevents dephosphorylation of these targets. As a result, the Tyr phosphorylation of STEP substrates increases, including pERK1/2, and its downstream signaling pathways (14, 50). The current findings show increased STEP₆₁ levels in human PD and animal models that are associated with a decrease in pERK and its downstream target CREB, which are involved in synaptic plasticity mechanisms by regulating gene expression (51, 52). This suggests that increased STEP₆₁ levels in PD will negatively affect ERK- and CREB-mediated pathways in MSNs.

In PD models, the plasticity of MSNs is significantly altered. This includes loss of long-term potentiation, long-term depression, changes in glutamate receptor subunits, and dystrophic changes of dendritic terminals of MSNs (53–57). STEP dephosphorylates a number of substrates that can directly and indirectly influence synaptic plasticity. These include Fyn, Pyk2, and ERK1/2, where STEP dephosphorylation inactivates these enzymes (13–16). Other STEP substrates include the GluN2B subunit of the NMDA receptor and the GluA2 subunit of the AMPA receptor, and STEP dephosphorylation results in internalization of both GluN1/GluN2B and GluA1/GluA2 receptors (18, 58). The increase in STEP₆₁ levels that results from loss of parkin function would therefore be expected to result in impairment in the regulation of synaptic plasticity.

An increase in STEP levels in mouse striatum after MPTP exposure is also likely to converge with other aspects of signaling in MSNs. Studies in mice suggest that MPTP treatment leads to Cdk5 activation and phosphorylation of DARPP-32 at Thr⁷⁵

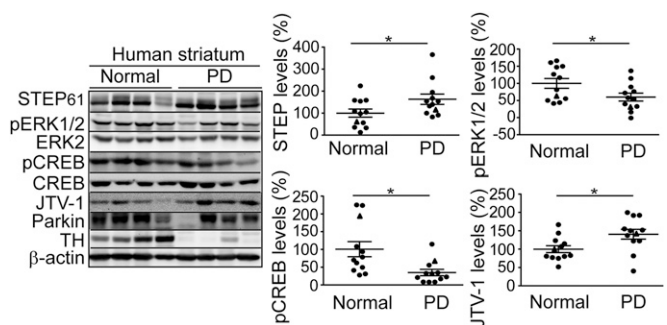


Fig. 6. STEP₆₁ is elevated in human sporadic PD and AR-JP PD striatum. Immunoblot (Left) and quantification (Right) of STEP₆₁ expression in striatum from normal and sporadic PD. There was an increase in STEP₆₁ levels in PD samples that was accompanied by a decrease in phosphorylation of pERK1/2, as well as the downstream target of pERK1/2, pCREB, compared with normal controls. STEP₆₁ levels were also increased and pERK and pCREB phosphorylation were decreased in a single case of human AR-JP PD (▲) striatum compared with an age- and sex-matched control. JTV-1, another parkin substrate, was increased in the human PD striatum compared with controls. β-actin served as a loading control for quantification ($n = 12$; mean \pm SEM; * $P < 0.05$ for STEP₆₁, pERK1/2, and pCREB, as determined by ANCOVA, using PMI, age, and sex as covariates).

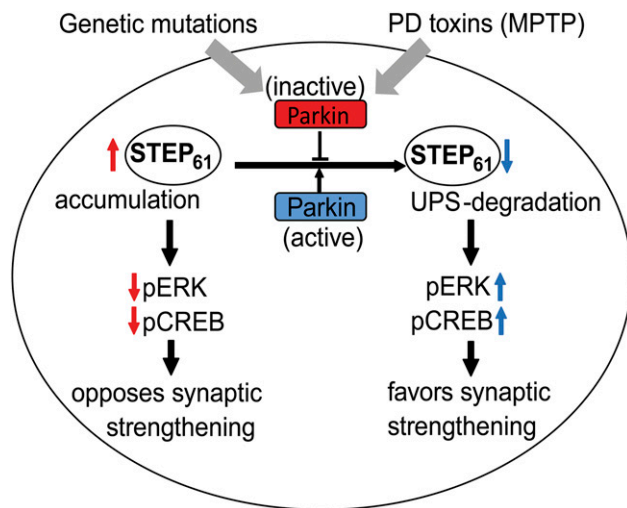


Fig. 7. Schematic model of regulation of STEP₆₁ by parkin in PD. The E3 ligase parkin regulates STEP₆₁ levels through the ubiquitin proteasome pathway. STEP₆₁ normally opposes the development of synaptic strengthening by Tyr dephosphorylation of substrates that include ERK. Genetic mutations and PD toxins result in impaired parkin function that leads to the accumulation of STEP₆₁ and a disruption of ERK and CREB-mediated synaptic plasticity events.

(59), which has an inhibitory influence on protein kinase A activity. This would be predicted to lead to a decrease in STEP₆₁ phosphorylation and an increase in its activity (42). Consistent with this, our results show that phosphorylation of STEP₆₁ at the regulator protein kinase A site (ser²²¹) is decreased in MPTP-treated striatum. Protein kinase A-mediated phosphorylation of STEP was also recently found to be correlated with motor learning (60). Chagniel and colleagues reported that attenuation of striatal STEP₆₁ activity through protein kinase A phosphorylation may be linked to a molecular pathway in the dorsal striatum that leads to the consolidation of complex motor skills during motor learning (60). PD neurotoxins such as MPTP directly inhibit parkin activity through tyrosine phosphorylation by c-Abl. Phosphorylation of parkin at a critical Tyr¹⁴³ decreases its ubiquitin ligase activity, resulting in the accumulation of parkin substrates (31, 32), whereas inhibitors of c-Abl reverse this process and ameliorate motor deficits in PD mouse models (61). The increase in tyrosine phosphorylation of parkin in the MPTP-treated mouse model, coupled with the increase in STEP₆₁ expression, suggests that STEP is not the phosphatase that normally dephosphorylates parkin.

Protein tyrosine phosphatases (PTPs) have previously been implicated in PD (62). Inhibition of PTPs protects dopaminergic neurons from PD toxins by increasing neurotrophin (BDNF) signaling and activation of ERK1/2 (63). We currently do not know whether STEP₆₁ is the PTP that regulates this process. However, these results are intriguing, as they are consistent with our recent observation that STEP and BDNF levels are regulated by a reciprocal negative feedback mechanism. BDNF leads to a rapid ubiquitin-mediated degradation of STEP₆₁, whereas increased STEP levels oppose pERK1/2 and pCREB-mediated expression of BDNF in psychotomimetic models.

In summary, the data identify STEP₆₁ as a novel substrate of parkin and indicate that STEP₆₁ is elevated in human sporadic PD samples and MPTP-treated mice. Increased STEP₆₁ levels are known to disrupt synaptic function and contribute to the cognitive deficits in several neuropsychiatric and neurodegenerative disorders. With an already established role of STEP₆₁ in opposing the development of synaptic strengthening, our findings suggest that the increase in STEP₆₁ expression may contribute to the disruption of synaptic function and the motor impairments that are associated with PD (Fig. 7).

Materials and Methods

Antibodies. The antibodies used are listed in Table S1.

Animals. The Yale Institutional Animal Care and Use Committee approved all experiments at Yale, and the Direção Geral de Alimentação e Veterinária (Portugal) approved the MPTP experiments. Long-Evans WT and *PARK2* KO rats were purchased from SAGE Labs and maintained in the animal facility for 12 mo before being killed for biochemical experiments.

Human Tissue. Postmortem brain tissue (striatum and cortex) from patients with PD and matched control patients were obtained from the National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders (University of Maryland, Baltimore, MD) and stored at -80°C until processed by Western blot analyses. Detailed information of human samples (age, sex, and postmortem intervals) is summarized in Table S2. For immunoblotting, frozen striatum or cortex were homogenized in Tris buffer (in mM): 10 Tris base at pH 7.6, 320 sucrose, 150 NaCl, 5 EDTA, 5 EGTA, 1 NaF, 1 Na₃VO₄, 1 DTT, and protease inhibitor mixture (Roche). Homogenates were centrifuged at $800 \times g$ to remove nuclei and large debris (P1), and synaptosomal membrane (P2) fractions were prepared by centrifugation of S1 at $9200 \times g$ for 15 min.

MPTP-Induced Lesion in Mice. For MPTP lesion, C57BL/6 male mice (3 mo old) were used. Treatment consisted of a single intraperitoneal injection per day (30 mg/kg) for 5 consecutive days (64). Injections were performed at the same time of the day, and animals were killed 7 d after the last injection. Control mice were injected with saline (0.9% NaCl; 100 μL). Brains were quickly removed, and striatum were dissected, frozen on dry ice, and stored at -80°C .

Western Blot Analyses. Proteins (30–50 μg) were loaded onto 10% (wt/vol) SDS/PAGE gels and transferred to nitrocellulose membranes, held overnight at 4°C , followed by incubation with secondary antibody. Bands were visualized by chemiluminescence, using a G:BOX system with GeneSnap image program and quantified using Image J 1.33 (NIH).

Statistical Analysis. All experiments were repeated at least three times before statistical analyses. Data were expressed as means \pm SEM. Statistical significance was determined by Student's *t* test or one-way or two-way ANOVA, as appropriate, with post hoc Tukey test. *P* values <0.05 were considered significant. ANCOVA (IBM SPSS Statistics v19) was used for the human postmortem data analyses to determine the differences between groups (controls and PD), using PMI (postmortem interval), age, and sex as covariates.

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