emia occurred simultaneously with normal glucose tolerance. Moreover, in our proband, severe hyperinsulinemia preceded diabetes by many years. Although we cannot exclude an effect of the AKT2 mutation on beta-cell function, it is clear that the major effect of this mutation was on insulin action.

Germline loss-of-function mutations in genes that encode intracellular signaling kinases are being increasingly recognized as causes of human inherited disease. Thus, *JAK3* mutations cause severe combined immunodeficiency disease (*17*), *RPS6KA3* mutations cause Coffin Lowry Syndrome (*18*), and *WNK4* mutations cause an inherited form of hypertension (*19*). The kindred described here demonstrate that AKT2 can be added to this list, the $R^{274}H$ mutation in this enzyme causing a rare form of human diabetes due to a post-receptor defect in insulin signaling. Although AKT2 mutations are unlikely to explain most common forms of diabetes, this mutant uniquely demonstrates the critical role of AKT signaling in maintaining insulin sensitivity in humans.

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20. We thank D. Alessi for AKT2 cDNA and B. Hemmings for helpful discussions. Supported by the Wellcome Trust (S.O.R., J.J.R., M.S., J.C.W., D.B.D., and A.M.U.), the U.K. Medical Research Council (S.G. and P.R.M.), the Canadian Natural Sciences and Engineering Research Council (S.L.G.), the Deutsche Forschungsgemeinschaft (S.S.), the Raymond and Beverly Sackler Foundation (S.G.), Cancer Research–UK (D.B.), and Diabetes UK (R.W.). The GenBank accession number of *AKT2* is M77198.

Supporting Online Material

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11 February 2004; accepted 16 April 2004

S-Nitrosylation of Parkin Regulates Ubiquitination and Compromises Parkin's Protective Function

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Parkin is an E3 ubiquitin ligase involved in the ubiquitination of proteins that are important in the survival of dopamine neurons in Parkinson's disease (PD). We show that parkin is *S*-nitrosylated in vitro, as well as in vivo in a mouse model of PD and in brains of patients with PD and diffuse Lewy body disease. Moreover, *S*-nitrosylation inhibits parkin's ubiquitin E3 ligase activity and its protective function. The inhibition of parkin's ubiquitin E3 ligase activity by *S*-nitrosylation could contribute to the degenerative process in these disorders by impairing the ubiquitination of parkin substrates.

Parkinson's disease (PD) is a common neurodegenerative disorder that leads to the progressive loss of dopamine (DA) neurons (*1*). The majority of PD is sporadic and is thought to be due in part to oxidative stress through derangements in mitochondrial complex–I activity (*1*–*3*). There are also rare familial causes of PD due to mutations in α-synuclein, parkin, and DJ1 (*4*–*6*). Several indices of oxidative stress are also present in PD, including increased nitrotyrosine immunoreactivity (*7*, *8*), reduced glutathione and ferritin levels, increased lipid peroxidation, and increased levels of iron (*1*, *3*, *8*).

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Parkin is a ubiquitin E3 ligase that is responsible for the addition of ubiquitin on specific substrates (*9*–*11*). Mutations in parkin that lead to a loss of parkin's ubiquitin E3 ligase activity are the most common cause of hereditary PD (*12*). Parkin has a number of putative substrates, and the failure to ubiquitinate some of these substrates in the absence of functional parkin protein may play an important role in the demise of DA neurons (*13*–*17*). In addition, parkin may play a more general role in the ubiquitin proteasomal pathway by participating in the removal and/or detoxification of abnormally folded or damaged proteins (*18*). The observation that there are increased markers of nitrosative stress in PD (*7*, *8*) prompted our investigation of whether parkin could be modified by nitric oxide (NO). Here, we show that parkin is *S*-nitrosylated and that this *S*-nitrosylation markedly diminishes parkin's E3 ligase activity and protective function.

Baculovirus recombinant parkin protein (BV-parkin) and human embryonic kidney (HEK) 293 cells transfected with myc-parkin were treated with *S*-nitrosoglutathione (GSNO), and both samples were subjected to the *S*-nitrosylation biotin switch assay (Fig. 1A) (*19*, *20*). HEK293-expressed parkin was readily *S*-nitrosylated, whereas *S*-nitrosylation of BV-parkin was not detectable. The absence of *S*-nitrosylated parkin in samples treated with glutathione devoid of NO (GSH) demonstrated the specificity of this modification of parkin. A similar result was observed when we used another NO donor, NOC18, with NOC18 depleted of NO [NOC18(–NO)] as a control (Fig. 1B).

To further control for the specificity of the *S*-nitrosylation of parkin, we examined whether myc–α-synuclein, which contains no cysteines, or myc–ITCH-1, an unrelated ubiquitin E3 ligase, are *S*-nitrosylated. Under conditions that lead to *S*-nitrosylation of parkin, neither α-synuclein (Fig. 1C) nor ITCH-1 (Fig. 1D) were *S*-nitrosylated. Because parkin expressed in HEK293 cells was *S*-nitrosylated but BV-parkin was not, we wondered whether the cellular extract contained a factor that facilitated the *S*-nitrosylation of parkin. Accordingly, we subjected BV-parkin to the *S*-nitrosylation biotin switch assay in the presence or absence of HEK293 cell lysate (Fig. 1E). BV-parkin was *S*-nitrosylated only in the presence of cell lysate. The factor is likely to be proteinaceous, because denaturing the cell lysate by boiling completely blocked the *S*-nitrosylation of BV-parkin (Fig. 1E). To further confirm that parkin is *S*-nitrosylated, we used the 2,3-diaminonaphthalene (DAN) assay on HEK293 cells transfected with myc-parkin (*21*). This assay showed that parkin was *S*-nitrosylated, whereas ITCH-1 and α-synuclein were not (Fig.

1F). We also performed domain mapping to locate the possible site of *S*-nitrosylation and specifically found that three cysteines (within the IBR domain and perhaps the second RING domain) can be modified by NO (fig. S1). These results indicate that parkin is specifically *S*-nitrosylated.

We next determined whether NO modulates the ubiquitin E3 ligase activity of parkin. Parkin ubiquitinates itself; therefore, detection of ubiquitinated parkin serves as a reliable indicator of parkin's ubiquitin E3 ligase activity (*9*–*11*). HEK293 cells transfected with myc-parkin and hemagglutinin (HA)–tagged ubiquitin were treated with the NO donors GSNO and NOR3, as well as with GSH and NOR3 depleted of NO [NOR3(–NO)]. GSNO and NOR3 drastically reduced the autoubiquitination of parkin, whereas GSH and NOR3(–NO) had no effect (Fig. 2A). To further confirm the effect of NO on parkin's E3 ligase activity, we reconstituted the autoubiquitination reaction of parkin in vitro. GSNO markedly reduced the autoubiquitination of parkin in vitro (Fig. 2A), hence NO can directly modulate parkin's E3 ligase activity. Parkin has many putative substrates, including the α-synuclein interacting protein synphilin-1 (*14*). Accordingly, we monitored whether NO could modulate the parkin-mediated ubiquitination of synphilin-1. We transfected cells with FLAG-parkin, myc–synphilin-1, and HA-ubiquitin (Fig. 2B) and treated the transfected cells 1 day later with GSNO and NOR3 as well as GSH and NOR3(–NO). The cell lysates were immunoprecipitated with an antibody to myc and probed with an antibody to HA to monitor ubiquitination. Immunoprecipitated synphilin-1 showed increased anti-HA immunoreactivity consistent with parkin-mediated ubiquitination of synphilin-1, and this ubiquitination was drastically reduced by GSNO and NOR3, whereas GSH and NOR3(–NO) had no effect (Fig. 2B).

To determine whether NO could modulate the function of parkin in situ, we monitored the autoubiquitination of parkin and the parkin-mediated ubiquitination of synphilin-1 in HEK293 cells that stably express neuronal NO synthase (nNOS). nNOS was activated by treating the cells with the calcium ionophore A23187 (8 μM). The activation of nNOS and generation of NO markedly reduced parkin autoubiquitination (Fig. 2C) and parkin-mediated ubiquitination of synphilin-1 (Fig. 2D). A23187 induced reductions in parkin autoubiquitination (Fig. 2C) and in parkin-mediated synphilin-1 ubiquitination (Fig. 2D); this effect was prevented by cotreatment with the NOS inhibitor nitro-L-arginine (N-Arg).

We next determined whether the reduction in parkin ubiquitination by NOS blockade is reversible with excess substrate. For this experiment, we inhibited NO formation with the competitive NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA), whose effect can be readily reversed by the coadministration of excess substrate L-arginine (L-Arg). Coadministration of L-Arg reversed the effect of L-NMMA and restored the ability of A23187 to reduce parkin autoubiquitination and parkin ubiquitination of synphilin-1 (Fig. 2E). These results indicate that NO inhibits the ubiquitination of parkin both in vitro and in situ through *S*-nitrosylation.

We used 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which induces nigrostriatal dopaminergic pathway damage similar to that observed in PD, to determine whether parkin is *S*-nitrosylated in vivo (Fig. 3). Mice received an injection of MPTP (20 mg/kg body weight) every 2 hours for a total of four doses; such a regimen causes a reduction of more than 90% of striatal dopamine and its metabolites and a loss of DA neurons (*22*). We used the biotin switch assay to monitor *S*-nitrosylation of parkin in the whole brain (Fig. 3A). Parkin was robustly *S*-nitrosylated 2 and 4 hours after the last administration of MPTP, and *S*-nitrosylation of parkin was reduced at 24 hours. Interestingly, another peak of *S*-nitrosylation of parkin occurred between 48 and 72 hours (Fig. 3A). Although we were able to assess *S*-nitrosylation of parkin only in whole brain because of the nature and sensitivity of this assay, enhanced *S*-nitrosylation of parkin after MPTP administration most likely occurs predominantly in the substantia nigra and/or striatum, because MPTP intoxication specifically and selectively increases NO formation in the nigrostriatal pathway (*23*–*25*).

We next determined the source of NO that *S*-nitrosylates parkin by administering MPTP under identical conditions to mice lacking the gene for nNOS or inducible NOS (iNOS). In iNOS knockout mice, parkin was *S*-nitrosylated only at the early 2-hour time point; in nNOS knockout mice, parkin was *S*-nitrosylated only at the 48- and 72-hour time points (Fig. 3A). These results suggest that parkin is *S*-nitrosylated in vivo after MPTP administration, and that both neuronally and microglially derived NO contribute to the *S*-nitrosylation of parkin in a biphasic fashion after MPTP intoxication.

We then assayed *S*-nitrosylation activity in tissue derived from the brains of patients with PD and diffuse Lewy body disease (DLBD) (table S1). These pathologic conditions are associated with a high level of nitrosative stress, as indicated by nitrotyrosine immunoreactivity of α-synuclein (*26*). We first measured the level of *S*-nitrosylation in PD and DLBD brain tissues by the Saville reaction (*27*, *28*). A doubling of *S*-nitrosylated proteins was found in post-mortem brain tissues in PD and DLBD patients (Fig. 3B), which strongly supports the notion that these pathologic conditions have a high level of nitrosative stress. We then determined whether parkin is *S*-nitrosylated in these brain tissues by the biotin switch method. We found that parkin was *S*-nitrosylated in PD and DLBD brains, whereas there was minimal to no *S*-nitrosylation in control brains (Fig. 3C). To further confirm that *S*-nitrosylation of parkin is specific to PD and DLBD patients in the affected brain regions, we also examined brain tissues from Alzheimer's disease patients and from occipital cortex in PD and DLBD patients (which is known to be free of α-synuclein pathology). We found no increase of *S*-

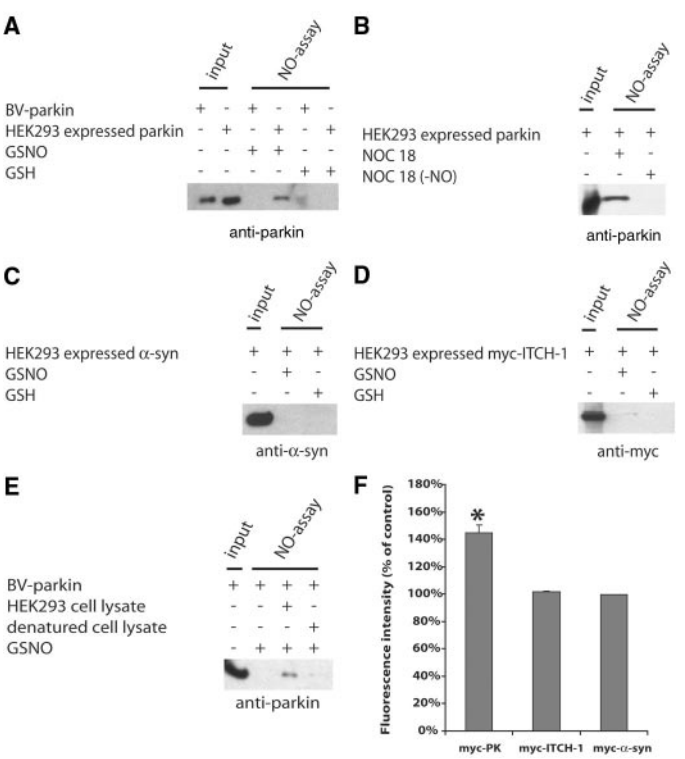

Fig. 1. Parkin can be *S*-nitrosylated by NO in vitro. (**A**) Parkin is selectively *S*-nitrosylated in the presence of the NO donor GSNO (100 μM). (**B**) Parkin is selectively *S*-nitrosylated in the presence of the NO donor NOC18 (100 μM). (**C**) α-Synuclein, which contains no cysteines, is not *S*-nitrosylated under the same conditions. (**D**) ITCH-1, an unrelated ubiquitin E3 ligase, is not *S*-nitrosylated under the same conditions. (**E**) A heat-labile cofactor in the HEK293 cell lysate is required for the efficient *S*-nitrosylation of parkin by NO. (**F**) In vitro *S*-nitrosylation of parkin can be detected by an alternate fluorometric method (*$P < 0.001$ by analysis of variance, Dunnett's test). These results were replicated at least three times.

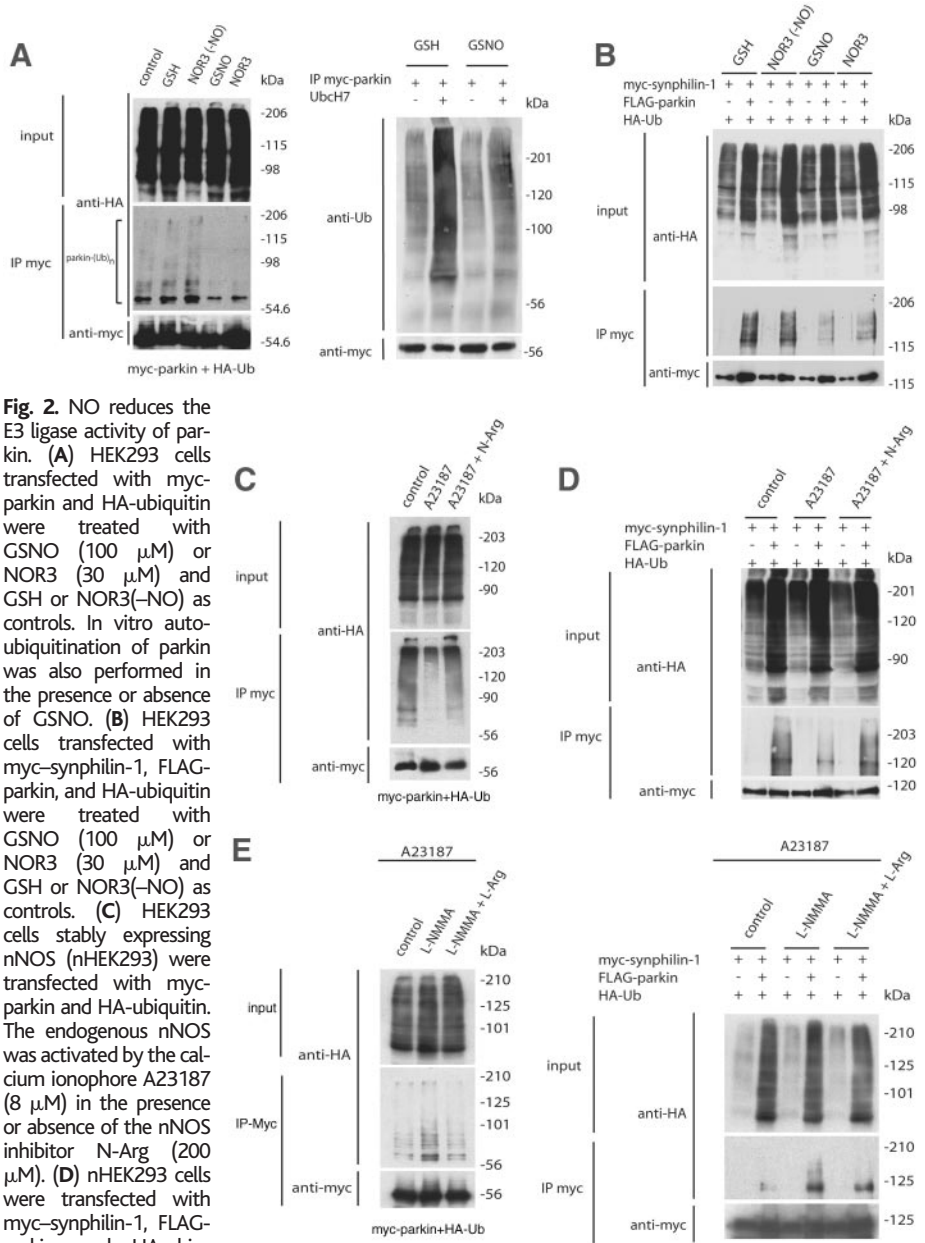

Fig. 2. NO reduces the E3 ligase activity of parkin. (**A**) HEK293 cells transfected with myc-parkin and HA-ubiquitin were treated with GSNO (100 μM) or NOR3 (30 μM) and GSH or NOR3(–NO) as controls. In vitro auto-ubiquitination of parkin was also performed in the presence or absence of GSNO. (**B**) HEK293 cells transfected with myc–synphilin-1, FLAG-parkin, and HA-ubiquitin were treated with GSNO (100 μM) or NOR3 (30 μM) and GSH or NOR3(–NO) as controls. (**C**) HEK293 cells stably expressing nNOS (nHEK293) were transfected with myc-parkin and HA-ubiquitin. The endogenous nNOS was activated by the calcium ionophore A23187 (8 μM) in the presence or absence of the nNOS inhibitor N-Arg (200 μM). (**D**) nHEK293 cells were transfected with myc–synphilin-1, FLAG-parkin, and HA-ubiquitin. The endogenous nNOS was activated by A23187 (8 μM) in the presence or absence of N-Arg (200 μM). (**E**) Similar experiments were performed as in (C) and (D) with the use of another nNOS inhibitor, L-NMMA (500 μM). L-Arg (5 mM) was used to reverse the effect of L-NMMA to show the specificity of the inhibitor. These results were replicated at least three times.

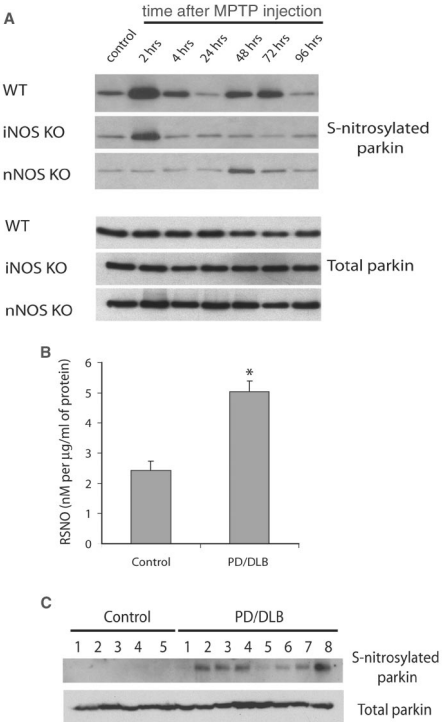

Fig. 3. Parkin is *S*-nitrosylated in vivo. (**A**) The level of *S*-nitrosylated parkin is increased in a biphasic fashion, peaking at 2, 4, 48, and 72 hours in mice after treatment with MPTP. In iNOS knockout mice, parkin is *S*-nitrosylated only at the 2-hour time point; in nNOS knockout mice, parkin is *S*-nitrosylated only at the 48- and 72-hour time points. (**B**) Levels of *S*-nitrosylation in PD and DLBD brain tissues were measured by the Saville reaction. A significant increase of *S*-nitrosylated proteins (RSNO) was found in post-mortem brain tissues (*$P < 0.001$, Student's t test). (**C**) Brain tissues from PD and DLBD patients were subjected to the in vivo *S*-nitrosylation assay. A marked increase of *S*-nitrosylated parkin was observed in these patients. These results were replicated at least three times.

nitrosylation of parkin in these samples (fig. S2). These results suggest that parkin is *S*-nitrosylated in vivo both in the MPTP model of PD and in the post-mortem brains of PD and DLBD patients.

To determine whether the *S*-nitrosylation of parkin has functional consequences, we monitored the ability of parkin to protect cells both in the presence and absence of NO (*29*). We sought to determine whether NO could modulate parkin's rescue of cell death induced by the coexpression of α-synuclein and synphilin-1 in the presence of the proteasome inhibitor MG132. We coexpressed α-synuclein and synphilin-1 in human SHSY5Y cells and observed significant cell death induced by MG132 (10 μM), as reported previously (*30*) (Fig. 4A). Coexpression of parkin significantly attenuated the toxic effect of α-synuclein and synphilin-1 induced by MG132. The protective effect of parkin, however, was completely abolished by NOC18 (Fig. 4A) under conditions that *S*-nitrosylate parkin (see Fig. 1B). Moreover, the protective effect of parkin was dependent on its E3 ligase activity, because the catalytically inactive $Thr^{240} \rightarrow Arg$ (T240R) parkin mutant (*14*) was not protective under the same conditions (Fig. 4A). Coexpression of α-synuclein and synphilin-1 in SHSY5Y cells also resulted in a significant increase of cell death in the presence of the specific proteasome inhibitor lactacystin (10 μM) (Fig. 4B), whereas lactacystin only modestly increased the toxicity of α-synuclein and synphilin-1 expressed alone (fig. S3, A and B). Coexpression of parkin significantly attenuated the toxic effect of α-synuclein and synphilin-1 induced by lactacystin, and the protective effect of parkin was completely abolished by NOC18 (Fig. 4B). Parkin also protected against lactacystin-induced synphilin-1 toxicity, which was reduced by NOC18 (fig. S3B). α-Synuclein/lactacystin toxicity was not influenced by NO donors or parkin (fig. S3A). These results suggest that the protective function of parkin against α-synuclein and synphilin-1 toxicity induced by proteasomal inhibitors can be attenuated by NO modification of parkin.

We find that parkin is *S*-nitrosylated both in vitro and in vivo, and that *S*-nitrosylation inhibits parkin's ubiquitin E3 ligase activity as well as its protective func-

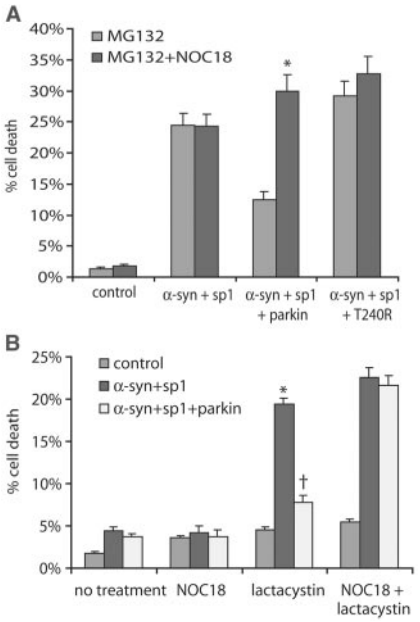

Fig. 4. NO impairs the protective function of parkin. (**A**) Increased cell death is observed in SHSY5Y cells transfected with α-synuclein and synphilin-1 (sp1) in the presence of 10 μM MG132. Coexpression of parkin selectively attenuates the toxic effect of α-synuclein and synphilin-1 induced by MG132, which is completely abolished by NOC18 (100 μM) (*$P <$ 0.001, Student's *t* test). (**B**) Increased cell death is observed in SHSY5Y cells transfected with α-synuclein and synphilin-1 in the presence of 10 μM lactacystin. Coexpression of parkin selectively attenuates the toxic effect of α-synuclein and synphilin-1 induced by lactacystin, which is abolished by NOC18 (100 μM) (*$P <$ 0.01, control vs. sp1; †$P <$ 0.01, sp1 vs. parkin; analysis of variance, Dunnett's test). NOC18 (100 μM) has minimal toxicity alone.

tion. NO modulation of parkin function is likely to play an important role in the physiologic and pathophysiologic regulation of parkin's E3 ligase activity. Because there are a number of E3-ubiquitin ligases with similar RING structures (*31*), it is likely that NO could regulate other E3 ubiquitin ligases through *S*-nitrosylation.

Our results link parkin function with the more common sporadic form of Parkinson's disease and the related α-synucleinopathy, DLBD, through nitrosative and oxidative stress. The *S*-nitrosylation of parkin may be relevant to a number of other pathophysiological conditions with oxidative and nitrosative stress. A number of heterozygous point mutations in parkin have been observed in PD patients without mutations in parkin on the adjacent allele, which has led to the suggestion that these mutations might be risk factors for disease (*32*, *33*). A single parkin heterozygous mutation coupled with nitrosative stress could lead to haploinsufficiency and might account for these mutations in parkin being associated with sporadic PD. In addition, the impairment of RING finger E3 ligases by NO could contribute to the proteasomal dysfunction observed in PD. The elucidation of the pathways by which NO *S*-nitrosylates and inhibits parkin may contribute to the development of new therapies for PD and other disorders associated with nitrosative and oxidative stress.

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34. Supported by U.S. Public Health Service grant NS38377, the Edward D. and Anna Mitchell Family Foundation, and the Mary Lou McIlhaney Scholar Award. We thank H. Ischiropoulos, R. Cole, and C. Lowenstein for helpful comments and advice on detection of *S*-nitrosylation.

Supporting Online Material
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21 November 2003; accepted 13 April 2004
Published online 22 April 2004;
10.1126/science.1093891
Include this information when citing this paper.

Regeneration of Male Germline Stem Cells by Spermatogonial Dedifferentiation in Vivo

Crista Brawley and Erika Matunis*

Although the ability of engrafted stem cells to regenerate tissue has received much attention, the molecular mechanisms controlling regeneration are poorly understood. In the *Drosophila* male germline, local activation of the Janus kinase–signal transducer and activator of transcription (Jak-STAT) pathway maintains stem cells; germline stem cells lacking Jak-STAT signaling differentiate into spermatogonia without self-renewal. By conditionally manipulating Jak-STAT signaling, we find that spermatogonia that have initiated differentiation and are undergoing limited mitotic (transit-amplifying) divisions can repopulate the niche and revert to stem cell identity. Thus, in the appropriate microenvironment, transit-amplifying cells dedifferentiate, becoming functional stem cells during tissue regeneration.

In the *Drosophila* testis, germline stem cells (GSCs) attach to a cluster of quiescent somatic cells called the hub (Fig. 1A), which creates a special localized microenvironment, or niche, by producing the ligand Unpaired (Upd). Upd locally activates the Jak-STAT pathway within GSCs to maintain stem cell fate (*1*, *2*). GSC divisions are stereotypically oriented (*3*), which ensures that one daughter remains at the hub, while the other (displaced from the niche) differentiates (Fig. 1A).

Because GSCs null for the *Drosophila* STAT homolog *stat92E* differentiate (*1*, *2*), we hypothesized that a temperature-sensitive allele of *stat92E* (*stat92E^{F}*) (*4*) would allow us to reversibly control GSC differentiation. *stat92E^{F}/stat92E^{06346}* fruit flies die during embryogenesis at 29°C but are viable and fertile at 18°C (*4*), with testes indistinguishable from wild type (Fig. 1C). Therefore, to follow the effects

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