Figure 3 Effects of 14-3-3η on the E3 activity of parkin. (**A**) *In vitro* autoubiquitylation. The ubiquitylating assay was conducted as described in Materials and methods with or without various amounts of GST-14-3-3η (left panel) or 14-3-3η (right panel). After incubation, the reaction mixtures were subjected to SDS–PAGE, followed by Western blotting with anti-parkin. Arrow on the right indicates the position of His-parkin. (**B**) *In vivo* autoubiquitylation. HA-Ub (3 μg), FL-parkin (3 μg), and Myc-14-3-3η, σ, β, or ζ (6 μg) plasmids were transfected for 48 h into HEK293 cells as indicated. After immunoprecipitation with anti-FL, Western blotting was performed using antibodies against HA and parkin. Western blotting of all lysates was performed to test the expression levels (Lysate). (**C**) Ubiquitylation of synphilin-1 in HEK293 cells. HA-Ub (2 μg), FL-parkin (3 μg), FL-parkin(K161N) (3 μg), FL-parkin(T240R) (3 μg), Myc-14-3-3η (6 μg), and V5-synphilin-1 (4 μg) plasmids were transfected into HEK293 cells as in (B) at the indicated combinations. After immunoprecipitation with anti-V5 antibody, Western blotting was performed using antibodies against HA, FL, V5, and 14-3-3. Asterisk denotes an IgG heavy chain.

V5-synphilin-1. Cotransfection of 14-3-3η resulted in almost complete inhibition of the ubiquitylation of synphilin-1 by parkin and/or self-ubiquitylation of parkin (Figure 3C, top and second panels), as well as inhibition of the interaction between synphilin-1 and parkin (Figure 3C, second panel). 14-3-3η did not interact with synphilin-1 (Figure 3C, bottom panel). Taken together, these results suggest that 14-3-3η does not only inhibit the intrinsic ubiquitylation activity of parkin, but also its binding activity to the substrate and its ubiquitylation.

The ARJP disease-related parkin(K161N) and parkin(T240R) mutants, which cannot bind with 14-3-3η,

could not bind and ubiquitylate synphilin-1 and/or self-ubiquitylation of parkin even in the absence of 14-3-3η (Figure 3C, top panel). Hence, the linker and RING-box domains of parkin are essential not only for the negative regulation by 14-3-3η, but also for the substrate recognition and ubiquitin-ligase activity. These results illustrate the importance of these regions of parkin on its positive and negative regulation.

Since parkin is known to associate with E2 (Shimura et al, 2000), we also examined the effect of 14-3-3η on the ability of parkin to recruit E2. For this purpose, we coexpressed HA-parkin with FL-UbcH7 or FL-Ubc7, both of which are known

to bind to parkin (Shimura *et al* 2000; Imai *et al*, 2001). Almost the same amounts of UbcH7 (Figure 4A) and Ubc7 (Figure 4B) were detected in the anti-parkin immunoprecipitants irrespective of cotransfection with Myc-14-3-3η. These findings indicate that 14-3-3η does not influence the recruitment of E2, that is, UbcH7 or Ubc7, to parkin.

α-Synuclein abrogates 14-3-3η-related parkin inactivation

Based on the above findings, we next examined the mechanism that regulates the 14-3-3η–parkin binding. As α-SN partly has a high homology to 14-3-3 isoforms (Ostrerova *et al*, 1999), we tested the effects of α-SN on the 14-3-3η-induced suppression of parkin. By cotransfection experiments in HEK293 cells, parkin again ubiquitylated synphilin-1, and 14-3-3η inhibited the parkin-mediated ubiquitylation (Figure 5A). Coexpression of α-SN resulted in the recovery of ubiquitylation of synphilin-1 and the association of synphilin-1 with parkin, suggesting that α-SN abrogates the 14-3-3η-induced suppression of parkin (Figure 5A, top panel). Importantly, the familial PD-related mutants of α-SN(A30P) (Kruger *et al*, 1998) and α-SN(A53T) (Polymeropoulos *et al*, 1997) could not abrogate the inhibitory role of 14-3-3η. Similar results were observed by detection of self-ubiquitylation activity of parkin (Figure 5A, second panel).

We then tested whether α-SN can release the binding of Myc-14-3-3η from parkin in cotransfection experiment. As shown in Figure 5B (top panel), FL-parkin was self-ubiquitylated in the absence of 14-3-3η. Coexpression of 14-3-3η inhibited the self-ubiquitylation of parkin, and this was accompanied by the binding of 14-3-3η to FL-parkin. Coexpression of α-SN abrogated the binding of 14-3-3η to

parkin and resulted in the recovery of self-ubiquitylation of parkin. These effects were not seen by coexpression of α-SN(A30P) and α-SN(A53T) (Figure 5B, top panel). In addition, while the 14-3-3η–parkin interaction was considerably reduced by α-SN, it was not reduced by α-SN(A30P) or α-SN(A53T) (Figure 5B, bottom panel). Taken together, α-SN, but not α-SN(A30P) or α-SN(A53T), binds strongly to 14-3-3η and thereby releases parkin from the parkin–14-3-3η complex.

We also tested the interaction of Myc-14-3-3η with FL-α-SN, FL-α-SN(A30P), and FL-α-SN(A53T). Myc-14-3-3η interacted only with FL-α-SN, but not α-SN(A30P) nor α-SN(A53T) (Figure 5C, upper-top panel), suggesting that α-SN relieves parkin activity from binding to 14-3-3η. The 14-3-3η/α-SN interaction was not affected by parkin (Figure 5C, upper-top panel), and parkin was not associated with α-SN (Figure 5C, upper-second panel). Interestingly, FL-α-SN did not interact with Myc-14-3-3σ, β, and ζ in the same experiment (Figure 5C, lower panel). These results further strengthen the notion that α-SN specifically activates parkin through binding 14-3-3η.

We then investigated whether the interaction of 14-3-3η and parkin is direct or indirect by using purified recombinant His-parkin and GST-14-3-3η. GST or GST-14-3-3η was mixed with His-parkin, and pulled down by glutathione beads. His-parkin bound to GST-14-3-3η, but not GST (Figure 5D, left panel), indicating that parkin directly interacts with 14-3-3η. On the other hand, a similar *in vitro* binding assay showed that GST-14-3-3η did not interact with recombinant α-SN (Figure 5D, right panel), suggesting that certain modification(s) of α-SN may be required for the interaction of 14-3-3η.

Subsequently, we measured the binding affinities of parkin and α-SN for 14-3-3η by the surface plasmon resonance (SPR) method. As shown in Figure 5E, parkin bound 14-3-3η with a considerably strong affinity ($K_d = 4.2$ nM, upper), whereas the affinity of α-SN for 14-3-3η was much lower than that of parkin ($K_d = 1.1$ μM, lower). These results are consistent with those of the immunoprecipitation/Western analysis using recombinant proteins (Figure 5D).

Finally, we examined whether 14-3-3η bound to parkin can be released by α-SN. To test this, we first mixed the lysates coexpressing FL-parkin and Myc-14-3-3η of HEK293 cells with those expressing α-SN. Then the mixtures were incubated under three different conditions, as indicated in the upper panel of Figure 5F. Next, the lysates were immunoprecipitated with anti-FL antibody, and followed by Western blotting with anti-Myc and anti-parkin antibodies. As shown in Figure 5F (upper panel), the amount of 14-3-3η bound to parkin was significantly lower in all incubation conditions, when the cell lysates that simultaneously expressed both parkin and 14-3-3η were incubated with α-SN-expressing lysates. Incubation for 1 h at 37°C reduced the amount of 14-3-3η bound to parkin in proportion to the added amount of α-SN-expressing cell lysate (Figure 5F, lower panel). Intriguingly, the α-SN(A30P) and α-SN(A53T) mutants had no effect on the release of 14-3-3η, unlike wild-type (WT) α-SN (Figure 5F, lower panel). These observations strongly indicate that α-SN, but not α-SN(A30P) or α-SN(A53T), can capture and release 14-3-3η from the parkin–14-3-3η complex, which supports our notion that the negative regulation of parkin activity by 14-3-3η is relieved by α-SN (Figure 5A and B).
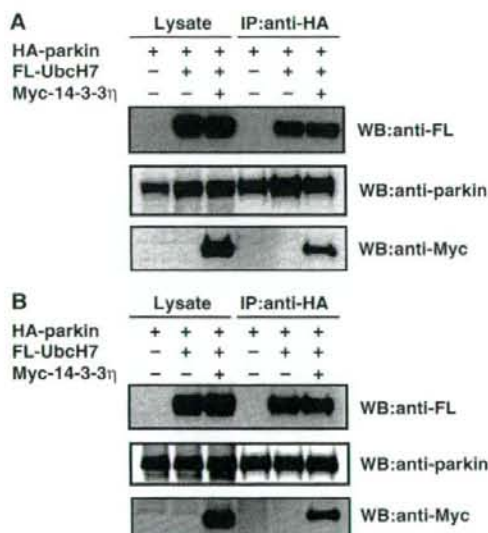

Figure 4 Effect of 14-3-3η on the recruitment of E2 (UbcH7 or Ubc7) to parkin. (**A**) HA-parkin (3 μg), FL-UbcH7 (3 μg), or Myc-14-3-3η (6 μg) plasmids were transfected for 48 h into HEK293 cells at the indicated combinations. After immunoprecipitation with anti-HA antibody, Western blotting was performed using antibodies against FL, Myc, and parkin. (**B**) The experiment was conducted as in (**A**), except that FL-Ubc7 was used instead of FL-UbcH7.

Parkin, 14-3-3η, and α-SN levels in the substantia nigra of PD

Finally, we analyzed the levels of parkin, 14-3-3η, and α-SN in the substantia nigra of the midbrain from patients with sporadic PD. Western blotting revealed no significant differences in parkin, 14-3-3η, and actin in the substantia nigra

between control (patients without PD) and PD patients, whereas α-SN was significantly increased in the substantia nigra of PD patients (Figure 6A, upper panel). As parkin did not interact physically with α-SN in our immunoprecipitation analysis (Figure 5C; data not shown), we then examined the interactions of 14-3-3η with parkin or α-SN by measuring
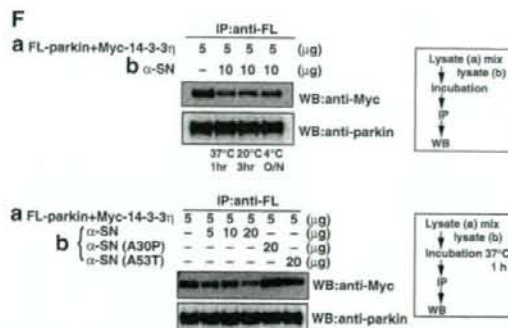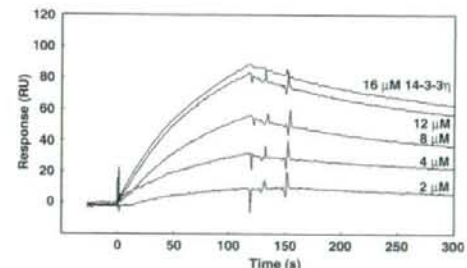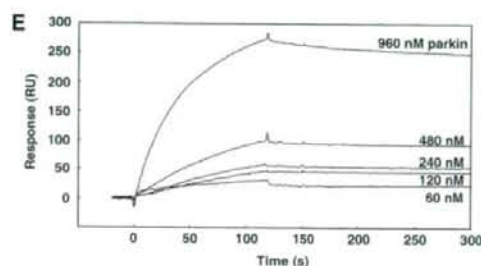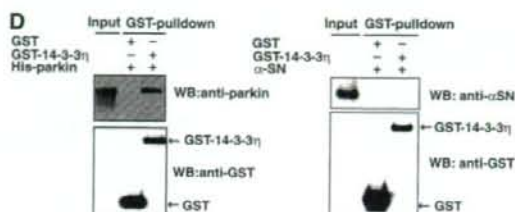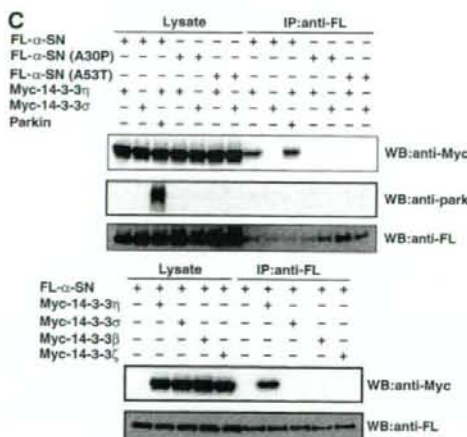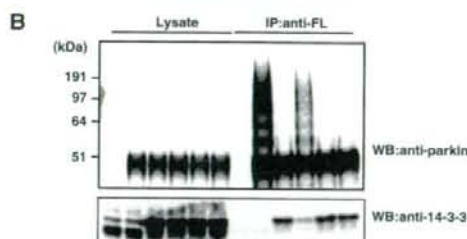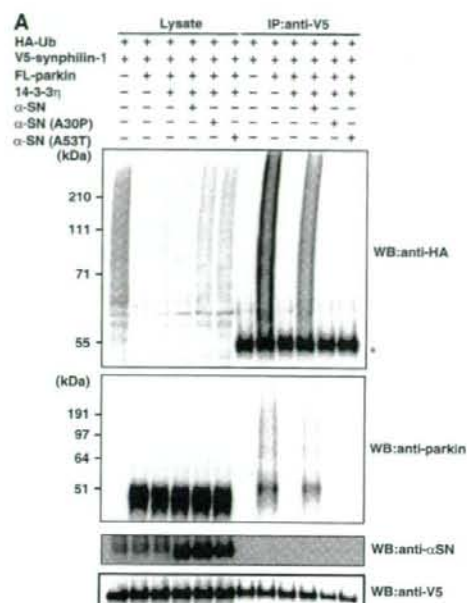
these proteins in the anti-14-3-3η immunoprecipitant. Whereas the levels of parkin associated with 14-3-3η from PD appeared to be decreased relative to the control, the levels of α-SN that interacted with 14-3-3η were clearly increased in patients with PD (Figure 6A, lower panel). Thus, it is suggested that the elevated levels of α-SN are associated with its interaction with 14-3-3η and the activity of parkin may be aberrantly regulated in the substantia nigra of sporadic PD.

Discussion

The major finding of the present study was the identification of 14-3-3η as a novel regulator of parkin. First, parkin was in a complex with 14-3-3η, but not β, γ, ε, or τ isoforms, in the mouse brain (Figure 1). 14-3-3η could bind primarily to the linker region of parkin, but not with the ARJP-causing missense mutant parkin (K161N), which has a mutation in the
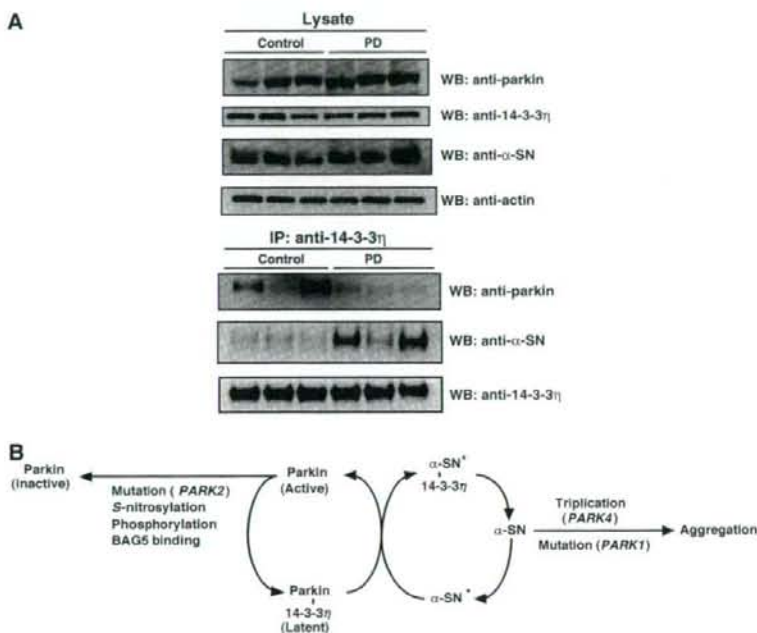
Figure 6 (A) Levels of parkin, 14-3-3η, and α-SN in the substantia nigra of PD. Brain of a representative patient with PD (upper panel). Samples (30 μg) of the crude extract of the brains (substantia nigra) of control (patients without PD) and PD patients were subjected to SDS–PAGE, following Western blotting against antibodies against parkin, 14-3-3η, α-SN, and actin. Physical interaction between 14-3-3η and parkin or α-SN (lower panel). After the same samples used in the upper panel were immunoprecipitated with anti-14-3-3η, Western blotting was carried out using antibodies against parkin, α-SN, and 14-3-3η. (B) A schematic diagram showing the pathways involved in the regulation of parkin activity by 14-3-3η and α-SN. α-SN, α-synuclein; α-SN*, modified form of α-SN. Note that whether parkin is phosphorylated to bind to 14-3-3η remains unknown at present. See text for details.

Figure 5 Effects of α-SN on 14-3-3η-induced suppression of parkin E3 activity and interaction between 14-3-3η and α-SN in HEK293 cells. (A) Ubiquitylation of synphilin-1. Transfection was conducted at various combinations, as in Figure 3C, except for cotransfection of 4 μg of α-SN, α-SN(A30P), and α-SN(A53T). After immunoprecipitation with anti-V5 antibody, Western blotting was carried out with antibodies against HA, parkin and α-SN, and V5. Asterisk denotes an IgG heavy chain. (B) Autoubiquitylation of parkin. Transfection was performed as in (A). After immunoprecipitation with anti-FL antibody, Western blotting was carried out with antibodies against parkin and 14-3-3. (C) Interaction of 14-3-3η and α-SN with or without parkin. Various expression vectors at the indicated combinations were transfected. Immunoprecipitation was conducted by anti-FL antibody and the resulting immunoprecipitates were used for Western blotting with antibodies against Myc, parkin, and FL. (D) Physical interaction between 14-3-3η and parkin (left panel) or α-SN (right panel) in recombinant proteins. After recombinant His-tagged parkin produced from baculovirus-infected HiFive insect cells (3 μg) or GST-α-SN expressed in *E. coli* whose GST moiety was removed by PreScission Protease digestion prior to use (3 μg) was incubated for 1 h at 32°C with 3 μg of GST or GST-tagged 14-3-3η expressed in *E. coli*, glutathione-Sepharose was added and the incubation vessels were slowly rotated for 3 h at 4°C. The washed Sepharose resin was eluted with 50 μl of 50 mM Tris–HCl (pH 8.0) buffer containing 10 mM reduced glutathione, and aliquots (15 μl) were analyzed by Western blotting with antibodies against parkin (left-top panel), α-SN (right-top panel), and GST (bottom panel). Input: 500 ng of parkin or α-SN. (E) SPR analyses of parkin and α-SN binding to 14-3-3η. Upper: subtracted sensorgrams of interaction between a subset of parkin concentrations and immobilized 14-3-3η. Lower: subtracted sensorgrams of interaction between a subset of 14-3-3η concentrations and immobilized α-SN. (F) Sequestration of 14-3-3η by α-SN from the parkin–14-3-3η complex. Various expression vectors were transfected as indicated. Upper panel: 5 μg of the lysate-(a) from cells co-expressing FL-parkin and Myc-14-3-3η were mixed with 10 μg of cellular lysate-(b) expressing α-SN. The mixtures were incubated under various conditions; that is, 37°C for 1 h, 20°C for 3 h, or overnight at 4°C (O/N), then immunoprecipitation by anti-FL antibody was conducted, followed by Western blotting with antibodies against Myc (14-3-3η) and parkin. Lower panel: the experiments were conducted as for the top panel, except that incubation was carried out at 37°C for 1 h using α-SN-, α-SN(A30P)-, or α-SN (A53T)-expressing lysates as indicated. The experimental protocol is shown in the flow charts on the right.

linker region (Figure 2). Second, the binding of 14-3-3η to parkin was associated with suppression of the ubiquitin-ligase activity, suggesting that certain parkin bound to 14-3-3η is present at a latent status in the brain (Figure 3). Third, overexpression of α-SN abrogated the 14-3-3η-induced suppression of parkin activity, indicating that α-SN relieves the negative regulation of parkin by 14-3-3η (Figure 5A and B). Intriguingly, PD-causing A30P and A53T mutations of α-SN could not bind 14-3-3η and failed to activate parkin. These results indicate that 14-3-3η is a regulator that functionally links parkin and α-SN, as illustrated in Figure 6B.

It is of particular note that we report unusual isoform specificity for 14-3-3η to interact with parkin among all 14-3-3 species examined. However, the possibility that the other species are also involved in the interaction by forming a heterodimer with 14-3-3η cannot be excluded *in vivo*, because 14-3-3 bands immunoprecipitated by anti-parkin antibody from the brain extracts showed doublet with one weak signal for Western blotting (Figure 1A). Nevertheless, herein we address that recombinant parkin could directly bind to 14-3-3η (Figure 5D, left panel), with considerably high affinity (K_d = approximately 4 nM) (Figure 5E) and that the 14-3-3η homodimer is a negative factor for autoubiquitylating activity of parkin *in vitro* (Figure 3A).

It is known that the 14-3-3 family proteins interact with the majority, but not all, proteins after their phosphorylation (Aitken *et al*, 2002; Bridges and Moorhead, 2004; Mackintosh, 2004). Indeed, parkin contains the RKDSPP sequence in the linker region that resembles the typical binding motifs with a potential phosphorylation residue for 14-3-3 proteins (Yaffe *et al*, 1997; Mackintosh, 2004). It is also known that parkin has several possible phosphorylation sites, and recent studies showed that parkin is phosphorylated *in vitro* (Yamamoto *et al*, 2004), although there is no direct evidence demonstrating phosphorylation of parkin *in vivo* to date. However, it remains elusive whether or not phosphorylation of parkin is responsible for its specific binding to 14-3-3η, because known potential phosphorylation motifs are capable of associating with many 14-3-3 species in general. The specificities of 14-3-3: client–protein interactions do not result from different specificities for the phosphopeptide-binding motifs, but probably arises from contacts made on the variable surface of 14-3-3 outside the binding cleft, as discussed previously by Yaffe *et al* (1997). In this regard, some reports showed functional specificities of 14-3-3 isoforms (Aitken, 2002; Aitken *et al*, 2002; Roberts and de Bruxelles, 2002), and indeed several enzymes retain several nonphosphorylated binding motifs for 14-3-3s (Hallberg, 2002; Sribar *et al*, 2003), though parkin lacks such 14-3-3-interacting sequences. Thus, parkin, in particular its linker region, may have a new binding motif(s) for 14-3-3η, but the interacting motif(s) remains to be identified. If 14-3-3η binds to parkin through two sites as a dimmer, it is plausible that the phosphorylation of parkin is involved in their interactions at least in part.

With regard to the mechanistic action of 14-3-3η, it may suppress parkin activity by preventing access of the substrate, because the binding of synphilin-1 (used here as a model substrate to parkin) was inhibited by 14-3-3η (Figure 3C). Accumulating evidence suggests that parkin can bind various targets by the UBL domain or the RING box, in particular the RING 1 domain (Dawson and Dawson, 2003). Accordingly,

14-3-3η may have function(s) other than suppressing the access of the substrate to parkin. Indeed, 14-3-3η strongly inhibits substrate-independent self-ubiquitylation of parkin, indicating blockage of the intrinsic E3 activity. It was also anticipated that 14-3-3η hinders the recruitment of E2 to parkin. However, this was not the case, because 14-3-3η had no effect on the binding of UbcH7 and Ubc7 to parkin (Figure 4). Thus, while the mechanism of 14-3-3η-induced suppression of parkin activity remains to be identified, it is possible that it involves preventing the positioning of the ubiquitin-charged E2 toward the target Lys residue by steric hindrance due to the association of 14-3-3η to parkin.

It is worth noting that parkin does not interact with α-SN directly, because we could not demonstrate the physical binding of parkin to α-SN *in vivo* and *in vitro* (data not shown; see also Dawson and Dawson, 2003). Nevertheless, we found that the negative regulation of parkin by 14-3-3η was relieved by α-SN, which could bind tightly to 14-3-3η *in vivo* (Figure 5A–C). In this regard, it is of note that the amounts of 14-3-3η bound to parkin were decreased when the lysates of cells coexpressing parkin and 14-3-3η were incubated with those expressing WT αSN, but not PD-related α-SN(A30P) or α-SN(A53T) mutants *in vitro* (Figure 5F). These results clearly indicate that 14-3-3η bound to parkin is sequestered by α-SN, but not competition by α-SN toward the binding of 14-3-3η to parkin. Unlike the association between parkin and 14-3-3η, there is little or no interaction between α-SN and 14-3-3η *in vitro*, as recombinant α-SN did not bind to 14-3-3η (Figure 5D, right panel, and E). Thus, it is plausible that certain modification(s) of α-SN is required for its association to 14-3-3η in mammalian cells (see our model in Figure 6B). Judging from the characteristic properties of 14-3-3 family proteins capable of binding many phosphorylated proteins (Yaffe *et al*, 1997), certain phosphorylation(s) of α-SN seems quite possible for the interaction with 14-3-3η. Indeed, there are several reports regarding phosphorylation of α-SN (Fujiwara *et al*, 2002; Hirai *et al*, 2004). Although previous studies clearly demonstrated that α-SN deposited in synucleinopathy brains is extensively phosphorylated at Ser-129 (Fujiwara *et al*, 2002; Hirai *et al*, 2004), this is probably not the case in our study, because the chemically synthesized peptide phosphorylated at Ser-129 of α-SN did not bind to the 14-3-3η (our unpublished results). However, the possibility that α-SN is phosphorylated at other site(s) cannot be exclusively ruled out. Alternatively, one cannot exclude a possible, though yet unknown, modification(s) of α-SN other than phosphorylation as a mechanism responsible for the increased affinity toward 14-3-3η. In this regard, α-SN is structurally related to 14-3-3 family proteins (Ostrerova *et al*, 1999), but it is unknown whether the homologous region is involved in the physical interaction with α-SN. Further studies are required to clarify the mode of α-SN modification.

In the present study, we found reciprocal regulation of parkin activity by α-SN and 14-3-3η, whose tripartite control could enhance our understanding of the pathogenesis of PD. As illustrated in Figure 6B, to date there are several reports on the post-translational modification of parkin. Recent findings indicate that the ubiquitin E3 ligase activity of parkin is modified by nitric oxide (NO). Namely, parkin is S-nitrosylated in PD patients and an *in vivo* mouse model of PD, and S-nitrosylation shows inhibition of the E3 activity of

parkin (Chung *et al*, 2004; Kahle and Haass, 2004; Yao *et al*, 2004), which could contribute to the degenerative process in PD by impairing the ubiquitylation of parkin substrates. Moreover, Kalia *et al* (2004) showed that the bcl-2-associated athanogene 5 (BAG5) enhanced the death of dopaminergic neurons in an *in vivo* model of PD by inhibiting the E3 ligase activity of parkin. In addition, recent studies reported that phosphorylation of parkin causes a small but significant reduction of parkin auto-ubiquitylating activity (Yamamoto *et al*, 2004). More recently, it was reported that Nrdp1/FLRF RING-finger E3 ligase binds and ubiquitylates parkin, resulting in reduction of parkin activity, implying its involvement in the pathogenesis of PD (Zhong *et al*, 2005). Considered together, these results indicate that the apparent loss of parkin E3 ubiquitin ligase activity associated with the pathogenesis of PD (see the model displayed in Figure 6B) is in agreement with the ARJP-linked mutations that lead to loss of function of parkin, and that the functional loss of parkin activity is linked to the death of dopaminergic neurons. In addition, we reported herein the imbalance of tripartite interactions among parkin, 14-3-3η, and α-SN levels in the substantia nigra of sporadic PD, but it is still not clear how these alterations influence parkin activity in neural cells. Thus, parkin is an E3 ubiquitin ligase involved in the ubiquitylation of proteins, irrespective of its involvement of K48- or K63-linked ubiquitylation (Doss-Pepe *et al*, 2005; Lim *et al*, 2005), that are important in the survival of dopaminergic neurons in PD.

In the present study, we found that parkin E3 activity is regulated positively and negatively by α-SN and 14-3-3η, respectively, suggesting that derangements of this regulation may be responsible for ARJP. For instance, the activated parkin free from 14-3-3η may be labile, and thus sensitive to other stresses, such as S-nitrosylation, and inactivated secondarily in PD. This situation resembles the effect of S-nitrosylation, in which nitrosative stress leads to S-nitrosylation of WT parkin, which leads initially to a marked increase followed by a decrease in the E3 ligase–ubiquitin–proteasome degradative pathway (Yao *et al*, 2004). The initial increase in the activity of parkin's E3 ubiquitin ligase leads to autoubiquitylation of parkin and subsequent inhibition of its activity, which would impair ubiquitylation and clearance of parkin substrates. In turn, 14-3-3η may protect against impairment of parkin induced by various environmental stresses, including S-nitrosylation. It is also noteworthy that, although 14-3-3η acts as a negative regulator of parkin, it may play a positive role in maintaining a large pool of parkin by preventing its self-ubiquitylation in the brain. Finally, we assume that gradual reduction of parkin activity may be associated with the development of ARJP as well as sporadic PD.

Current evidence suggests that α-SN increases in response to various stresses (Sherer *et al*, 2002; Gomez-Santos *et al*, 2003). This finding is compatible with the results of recent studies that dopamine-dependent neurotoxicity (Tabrizi *et al*, 2000; Zhou *et al*, 2000; Junn and Mouradian, 2002) is mediated by the formation of protein complexes that contain α-SN and 14-3-3, which are selectively increased in the substantia nigra in PD (Xu *et al*, 2002). Further studies are needed to determine the levels of parkin, 14-3-3η, α-SN, and parkin and α-SN-14-3-3η complexes in the substantia nigra of the midbrain of patients with sporadic PD.

Here we suggest that α-SN and parkin function through the same pathway. Indeed, both proteins, if not all, are associated

with presynaptic vesicles (Dawson and Dawson, 2003). So far, however, the physiological role of α-SN is largely unknown, though various roles including its involvement in synaptic plasticity have been suggested (Liu *et al*, 2004). We here provided the first evidence that α-SN acts as a positive regulator of parkin E3 activity. It is worth noting that disease-causing mutations of α-SN(A30P) and α-SN(A53T) could not activate the latent parkin–14-3-3η complex, and thus, these mutations may accelerate the development of PD by failing to activate parkin. Our results identified a functional link between these two familial PD-gene products, thus highlighting the existence of a novel regulatory mechanism that could help us further understand the pathogenesis of ARJP as well as sporadic PD. However, it must be stressed here that α-SN is the causative gene product of familial PD. It is noteworthy that α-SN is an aggregation-prone protein due to its natively unfolded protein nature. It is of note that the locus of *PARK4* is triplication of the α-*SN* gene (*PARK1*) (Singleton *et al*, 2003), indicating that overexpression of α-SN itself is toxic and induces dopaminergic neuronal death. Indeed, α-SN tends to self-aggregate, and this tendency, which is augmented in the α-SN(A30P) and α-SN(A53T) mutants (Conway *et al*, 2000) (see our model in Figure 6B), causes autosomal dominant PD (Narhi *et al*, 1999). Both WT and mutant α-SN form amyloid fibrils akin to those seen in LBs, as well as nonfibrillary oligomers termed protofibrils (Dawson and Dawson, 2003; Bossy-Wetzel *et al*, 2004). However, whether aggregation and fibrillary formation of α-SN- and PD-linked mutants play a role in neuronal dysfunction and death of neurons in PD are a matter of fierce debate. At this point of view, we emphasize that the feature of α-SN as an aggregation-prone protein is probably not linked directly to its role as a potent activator of parkin E3 in the pathogenesis of PD. Even if these two unique properties of α-SN account for the development of PD independently or synergistically, however, it is clear that their mechanistic actions differ as illustrated in Figure 6B.

Materials and methods

Immunological analysis

For immunoprecipitation analysis of endogenous proteins in the brains of adult mouse and human, these brains were homogenized in three volumes of ice-cold lysis buffer (20 mM HEPES (pH 7.9) buffer containing 0.2% NP-40, 1 mM dithiothreitol (DTT) and protease inhibitor cocktail (Sigma, Chemical Co., St Louis, MO)). The tissue homogenate was centrifuged at $20\,000\,g$ at 4°C for 20 min. The supernatant (2 mg protein) was used for immunoprecipitation with one of the following antibodies: anti-polyclonal parkin (Cell Signaling Technology, Beverly, MA) and anti-14-3-3η antibodies (Immuno-Biological Lab. Co., Gunma) or control IgG (700 ng). The resulting immunoprecipitates were resolved in 30 µl of the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer, and one-third of the samples (10 µl) were subjected to SDS–PAGE, followed by Western blotting with anti-14-3-3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-14-3-3β, 14-3-3γ, 14-3-3ε, 14-3-3η, and 14-3-3τ (Immuno-Biological Lab. Co., Ltd, Japan) and anti-monoclonal parkin (1A1) antibodies (Shimura *et al*, 1999). In all, 10 µg of the supernatant (lysate) was used as input (1.5%).

For immunoprecipitation analysis of the cell culture system, HEK293 cells were transfected with the respective plasmids. After 48 h, the cells were washed with ice-cold PBS (in mM, 10 Na_2PO_4, 2 KH_2PO_4, 137 NaCl, and 2.7 KCl), pH 7.4, and harvested in the lysis buffer (600 µl). The lysate was then rotated at 4°C for 1 h, followed by centrifugation at $20\,000\,g$ for 10 min. The supernatant (200 µl) was then combined with 50 µl protein G-Sepharose (Amersham Life

Science, Buckinghamshire, UK), pre-incubated with anti-Myc (Santa Cruz Biotechnology, Santa Cruz), V5 (Invitrogen), and HA (Santa Cruz Biotechnology) antibodies or anti-FL antibody beads (Sigma) for 3 h. The protein G-Sepharose or FL-beads were precipitated and the pellets were extensively washed using the lysis buffer containing 500 mM NaCl. The precipitates were used for Western blot analysis using anti-parkin, Myc, FL, HA, V5, 14-3-3, and α-SN (BD Transduction Lab.) antibodies, as mentioned above. A volume of 5 μl of the supernatant was used as input (7.5%).

In vitro autoubiquitylation assay
Recombinant GST-14-3-3η was produced in *Escherichia coli*. Untagged 14-3-3η was produced from GST-14-3-3η by digestion with PreScission Protease (Amersham Bioscience). Recombinant His-parkin and E1 were produced from baculovirus-infected HiFive insect cells. Reactions were performed for 3 h at 37°C in 50 μl of assay mixture containing 40 mM Tris–HCl buffer (pH 7.5), 5 mM MgCl$_2$, 2 mM ATP, 2 mM DTT, 15 μg ubiquitin (Sigma), 200 ng of E1, and 600 ng of E2 (UbcH7) (Affiniti-Research, Exeter, Devon, UK) in the presence or absence of GST-14-3-3η or 14-3-3η. After incubation, the reaction was terminated by the addition of the sample buffer for SDS–PAGE (17 μl), and aliquots (15 μl) were subjected to SDS–PAGE followed by Western blotting with anti-parkin antibody.

In vivo ubiquitylation assay
HEK293 cells were transfected for 48 h with pcDNA3.1 expression plasmids, in which FL-tagged parkin or FL-parkin mutants, α-SN or

α-SN mutants, 14-3-3η, V5-synphilin-1, and HA-ubiquitin cDNAs were ligated. MG132 (50 μM) was added for 20 min, prior to harvesting of the cells. Then, the cells were washed with cold PBS and lysed by 50 mM Tris–HCl buffer (pH 8.0), containing 150 mM NaCl, 1% Nonidet-P40, 1% deoxycolate, 0.1% SDS, 5 mM ethylenediaminetetraacetic acid, and protease inhibitor cocktail. Preparation of the cell lysate, immunoprecipitation, and Western blot analyses were essentially the same for the immunological analysis as described above. In all experiments, the cell lysates (10 μg, 7.5% input) were used for Western blotting as controls to check the expression levels.

For the method sections of 'Cell culture and transfection', 'Plasmids', and 'Surface plasmon resonance (SPR) analysis', see Supplementary data.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

References
Aitken A (2002) Functional specificity in 14-3-3 isoform interactions through dimer formation and phosphorylation. *Chromosome location of mammalian isoforms and variants. Plant Mol Biol* **50:** 993–1010
Aitken A, Baxter H, Dubois T, Clokie S, Mackie S, Mitchell K, Peden A, Zemlickova E (2002) Specificity of 14-3-3 isoform dimer interactions and phosphorylation. *Biochem Soc Trans* **30:** 351–360
Baxter HC, Liu WG, Forster JL, Aitken A, Fraser JR (2002) Immunolocalisation of 14-3-3 isoforms in normal and scrapie-infected murine brain. *Neuroscience* **109:** 5–14
Berg D, Holzmann C, Riess O (2003) 14-3-3 proteins in the nervous system. *Nat Rev Neurosci* **4:** 752–762
Bossy-Wetzel E, Schwarzenbacher R, Lipton SA (2004) Molecular pathways to neurodegeneration. *Nat Med* **10** (Suppl S2): S2–S9
Bridges D, Moorhead GB (2004) 14-3-3 proteins: a number of functions for a numbered protein. *Sci STKE* **2004:** re10
Chung KK, Thomas B, Li X, Pletnikova O, Troncoso JC, Marsh L, Dawson VL, Dawson TM (2004) S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. *Science* **304:** 1328–1331
Chung KK, Zhang Y, Lim KL, Tanaka Y, Huang H, Gao J, Ross CA, Dawson VL, Dawson TM (2001) Parkin ubiquitinates the α-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson's disease. *Nat Med* **7:** 1144–1150
Conway KA, Lee SJ, Rochet JC, Ding TT, Williamson RE, Lansbury Jr PT (2000) Acceleration of oligomerization, not fibrillization, is a shared property of both α-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy. *Proc Natl Acad Sci USA* **97:** 571–576
Dawson TM, Dawson VL (2003) Molecular pathways of neurodegeneration in Parkinson's disease. *Science* **302:** 819–822
Doss-Pepe EW, Chen L, Madura K (2005) α-Synuclein and parkin contribute to the assembly of ubiquitin lysine 63-linked multiubiquitin chains. *J Biol Chem* **280:** 16619–16624
Forno LS (1996) Neuropathology of Parkinson's disease. *J Neuropathol Exp Neurol* **55:** 259–272
Fujiwara H, Hasegawa M, Dohmae N, Kawashima A, Masliah E, Goldberg MS, Shen J, Takio K, Iwatsubo T (2002) α-Synuclein is phosphorylated in synucleinopathy lesions. *Nat Cell Biol* **4:** 160–164
Gomez-Santos C, Ferrer I, Santidrian AF, Barrachina M, Gil J, Ambrosio S (2003) Dopamine induces autophagic cell death and α-synuclein increase in human neuroblastoma SH-SY5Y cells. *J Neurosci Res* **73:** 341–350

Hallberg B (2002) Exoenzyme S binds its cofactor 14-3-3 through a non-phosphorylated motif. *Biochem Soc Trans* **30:** 401–405
Hirai Y, Fujita SC, Iwatsubo T, Hasegawa M (2004) Phosphorylated α-synuclein in normal mouse brain. *FEBS Lett* **572:** 227–232
Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, Takahashi R (2001) An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell* **105:** 891–902
Junn E, Mouradian MM (2002) Human α-synuclein over-expression increases intracellular reactive oxygen species levels and susceptibility to dopamine. *Neurosci Lett* **320:** 146–150
Kahle PJ, Haass C (2004) How does parkin ligate ubiquitin to Parkinson's disease? *EMBO Rep* **5:** 681–685
Kalia SK, Lee S, Smith PD, Liu L, Crocker SJ, Thorarinsdottir TE, Glover JR, Fon EA, Park DS, Lozano AM (2004) BAG5 inhibits parkin and enhances dopaminergic neuron degeneration. *Neuron* **44:** 931–945
Kawakami T, Chiba T, Suzuki T, Iwai K, Yamanaka K, Minato N, Suzuki H, Shimbara N, Hidaka Y, Osaka F, Omata M, Tanaka K (2001) NEDD8 recruits E2-ubiquitin to SCF E3 ligase. *EMBO J* **20:** 4003–4012
Kawamoto Y, Akiguchi I, Nakamura S, Honjyo Y, Shibasaki H, Budka H (2002) 14-3-3 proteins in Lewy bodies in Parkinson disease and diffuse Lewy body disease brains. *J Neuropathol Exp Neurol* **61:** 245–253
Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, Przuntek H, Epplen JT, Schols L, Riess O (1998) A30P mutation in the gene encoding α-synuclein in Parkinson's disease. *Nat Genet* **18:** 106–108
Lim KL, Chew KC, Tan JM, Wang C, Chung KK, Zhang Y, Tanaka Y, Smith W, Engelender S, Ross CA, Dawson VL, Dawson TM (2005) Parkin mediates nonclassical, proteasomal-independent ubiquitination of synphilin-1: implications for Lewy body formation. *J Neurosci* **25:** 2002–2009
Liu S, Ninan I, Antonova I, Battaglia F, Trinchese F, Narasanna A, Kolodilov N, Dauer W, Hawkins RD, Arancio O (2004) Synuclein produces a long-lasting increase in neurotransmitter release. *EMBO J* **23:** 4506–4516
Mackintosh C (2004) Dynamic interactions between 14-3-3 proteins and phosphoproteins regulate diverse cellular processes. *Biochem J* **381:** 329–342
Martin H, Rostas J, Patel Y, Aitken A (1994) Subcellular localisation of 14-3-3 isoforms in rat brain using specific antibodies. *J Neurochem* **63:** 2259–2265

McNaught KS, Olanow CW (2003) Proteolytic stress: a unifying concept for the etiopathogenesis of Parkinson's disease. *Ann Neurol* **53** (Suppl 3): S73–S84

Narhi L, Wood SJ, Steavenson S, Jiang Y, Wu GM, Anafi D, Kaufman SA, Martin F, Sitney K, Denis P, Louis JC, Wypych J, Biere AL, Citron M. (1999) Both familial Parkinson's disease mutations accelerate α-synuclein aggregation. *J Biol Chem* **274**: 9843–9846

Ostrerova N, Petrucelli L, Farrer M, Mehta N, Choi P, Hardy J, Wolozin B (1999) α-Synuclein shares physical and functional homology with 14-3-3 proteins. *J Neurosci* **19**: 5782–5791

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL (1997) Mutation in the α-synuclein gene identified in families with Parkinson's disease. *Science* **276**: 2045–2047

Roberts MR, de Bruxelles GL (2002) Plant 14-3-3 protein families: evidence for isoform-specific functions? *Biochem Soc Trans* **30**: 373–378

Sherer TB, Betarbet R, Stout AK, Lund S, Baptista M, Panov AV, Cookson MR, Greenamyre JT (2002) An *in vitro* model of Parkinson's disease: linking mitochondrial impairment to altered α-synuclein metabolism and oxidative damage. *J Neurosci* **22**: 7006–7015

Shimura H, Hattori N, Kubo S, Mizuno Y, Asakawa S, Minoshima S, Shimizu N, Iwai K, Chiba T, Tanaka K, Suzuki T (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin–protein ligase. *Nat Genet* **25**: 302–305

Shimura H, Hattori N, Kubo S, Yoshikawa M, Kitada T, Matsumine H, Asakawa S, Minoshima S, Yamamura Y, Shimizu N, Mizuno Y (1999) Immunohistochemical and subcellular localization of Parkin protein: absence of protein in autosomal recessive juvenile parkinsonism patients. *Ann Neurol* **45**: 668–672

Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, Hulihan M, Peuralinna T, Dutra A, Nussbaum R, Lincoln S, Crawley A, Hanson M, Maraganore D, Adler C, Cookson MR, Muenter M, Baptista M, Miller D, Blancato J, Hardy J, Gwinn-Hardy K (2003) α-Synuclein locus triplication causes Parkinson's disease. *Science* **302**: 841

Sribar J, Sherman NE, Prijatelj P, Faure G, Gubensek F, Fox JW, Aitken A, Pungercar J, Krizaj I (2003) The neurotoxic phospholipase A2 associates, through a non-phosphorylated binding motif, with 14-3-3 protein γ and ε isoforms. *Biochem Biophys Res Commun* **302**: 691–696

Tabrizi SJ, Orth M, Wilkinson JM, Taanman JW, Warner TT, Cooper JM, Schapira AH (2000) Expression of mutant α-synuclein causes increased susceptibility to dopamine toxicity. *Hum Mol Genet* **9**: 2683–2689

Ubl A, Berg D, Holzmann C, Kruger R, Berger K, Arzberger T, Bornemann A, Riess O (2002) 14-3-3 protein is a component of Lewy bodies in Parkinson's disease-mutation analysis and association studies of 14-3-3η. *Brain Res Mol Brain Res* **108**: 33–39

Vila M, Przedborski S (2004) Genetic clues to the pathogenesis of Parkinson's disease. *Nat Med* **10** (Suppl): S58–S62

Xu J, Kao SY, Lee FJ, Song W, Jin LW, Yankner BA (2002) Dopamine-dependent neurotoxicity of α-synuclein: a mechanism for selective neurodegeneration in Parkinson disease. *Nat Med* **8**: 600–606

Yaffe MB, Rittinger K, Volinia S, Caron PR, Aitken A, Leffers H, Gamblin SJ, Smerdon SJ, Cantley LC (1997) The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell* **91**: 961–971

Yamamoto A, Friedlein A, Imai Y, Takahashi R, Kahle PJ, Haass C (2004) Parkin phosphorylation and modulation of its E3 ubiquitin ligase activity. *J Biol Chem* **280**: 3390–3399

Yao D, Gu Z, Nakamura T, Shi ZQ, Ma Y, Gaston B, Palmer LA, Rockenstein EM, Zhang Z, Masliah E, Uehara T, Lipton SA (2004) Nitrosative stress linked to sporadic Parkinson's disease: S-nitrosylation of parkin regulates its E3 ubiquitin ligase activity. *Proc Natl Acad Sci USA* **101**: 10810–10814

Zhong L, Tan Y, Zhou A, Yu Q, Zhou J (2005) RING finger ubiquitin-protein isopeptide ligase Nrdp1/FLRF regulates parkin stability and activity. *J Biol Chem* **280**: 9425–9430

Zhou W, Hurlbert MS, Schaack J, Prasad KN, Freed CR (2000) Overexpression of human α-synuclein causes dopamine neuron death in rat primary culture and immortalized mesencephalon-derived cells. *Brain Res* **866**: 33–43