Activation of FoxO by LRRK2 induces expression of proapoptotic proteins and alters survival of postmitotic dopaminergic neuron in Drosophila

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Missense mutations in leucine-rich repeat kinase 2 (LRRK2)/Dardarin gene, the product of which encodes a kinase with multiple domains, are known to cause autosomal dominant late onset Parkinson’s disease (PD). In the current study, we report that the gene product LRRK2 directly phosphorylates the forkhead box transcription factor FoxO1 and enhances its transcriptional activity. This pathway was found to be conserved in Drosophila, as the Drosophila LRRK2 homolog (dLRRK) enhanced the neuronal toxicity of FoxO. Importantly, FoxO mutants that were resistant to LRRK2/dLRRK-induced phosphorylation suppressed this neurotoxicity. Moreover, we have determined that FoxO targets hid and bim in Drosophila and human, respectively, are responsible for the LRRK2/dLRRK-mediated cell death. These data suggest that the cell death molecules regulated by FoxO are key factors during the neurodegeneration in LRRK2-linked PD.

INTRODUCTION

Missense mutations in the Leucine-rich repeat kinase 2 (LRRK2) gene cause autosomal dominant late onset familial Parkinson’s disease (PD). This form of PD demonstrates a diverse pathology and includes the progressive loss of nigrostriatal dopaminergic (DA) neurons, α-synucleinopathy and tauopathy (1,2). The clinical symptoms and pathology caused by LRRK2 mutations resemble those of the sporadic form of PD, suggesting that the LRRK2 pathogenic pathway may underlie general PD etiology. This is further supported by a recent finding that several LRRK2 variants increase susceptibility to sporadic PD. The LRRK2 gene encodes a multi-domain protein belonging to the ROCO family proteins. The LRRK2 protein contains a GTPase domain called Ras of complex proteins (Roc) domain, a conserved C-terminal of Roc (COR) domain and a kinase domain. Furthermore, LRRK2 harbors leucine-rich repeats (LRR) at its N-terminus and WD40 repeats at the C-terminus (1,2).

Several amino acid substitutions have been identified throughout the multiple domains, which include R1441G, Y1699C, G2019S and I2020T mutations (3). Numerous pathological substitutions in the kinase domain of full-length LRRK2 such as G2019S and I2020T have been reported to show moderately enhanced kinase activity in vitro (4–6). However, how these mutations cause the progressive loss of DA neurons and the other associated pathologies is currently unknown.

We have previously reported that transgenic expression of the Drosophila LRRK2 orthologue (dLRRK) that contains PD-associated mutations leads to DA neuronal loss in the Drosophila brain (6). LRRK2 has been shown to phosphorylate eukaryotic translation initiation factor 4E (eIF4E)-binding protein (4E-BP), which modulates stress sensitivity and DA...
neuron survival in *Drosophila*. Forkhead transcription factor FoxO, which controls various cellular processes involved in cell cycle, cell death, metabolism and oxidative stress, regulates 4E-BP transcription (7,8). The effects of FoxO are dependent on the cellular context and cell type, as well as the cofactors involved. FoxO controls redox metabolism by the expression of anti-oxidative stress molecules, such as manganese superoxide dismutase and catalase, which may affect the lifespan of animals. On the other hand, cell death-related molecules, such as Hid, Bim, PUMA and Fas ligand, are induced by FoxO in the cells destined to die.

In the current study, we report that FoxO is also phosphorylated by LRRK2, which in turn upregulates cell death regulators, Hid and Bim in *Drosophila* and in human cells, respectively. Moreover, our data suggest that the activation of these molecules contribute to DA neurodegeneration following LRRK2 mutation.

RESULTS

LRRK2/dLRRK genetically interacts with FoxO

We have previously reported that LRRK2 phosphorylates 4E-BP, which in turn promotes the translation of numerous proteins (6). While investigating the levels of *Drosophila* (d4E-BP) expressed in dLRRK mutants, we found that the level of d4E-BP transcript was affected by dLRRK protein expression (Supplementary Material, Fig. S1), even though differences in d4E-BP protein level were not apparent (data not shown). It has been reported that 4E-BP transcription is partly regulated by FoxO (7,9). We then tested whether LRRK2 modulates FoxO activity using *Drosophila* genetics (Fig. 1). Overexpression of *Drosophila* FoxO (dFoxO) in the fly eye imaginal disc resulted in mild developmental defects in the eye (Fig. 1B). This result was consistent with that reported previously (7,8). Transgenic expression of either dLRRK or human LRRK2 (hLRRK2), even pathogenic mutants such as dLRRK I1915T and hLRRK2 I2020T, in the eyes resulted in no apparent degeneration (Fig. 1F and G, and Supplementary Material, Fig. S2A and E), a result that has also been reported previously (6). However, the dFoxO-mediated eye defect was significantly worsened by the expression of either a wild-type (WT) dLRRK or a PD-related mutant (I1915T corresponding to I2020T in human) (Fig. 1C and D). Similar results were obtained in dFoxO crosses combined with dLRRK harboring R1069G mutation in the GTPase domain (corresponding to a pathogenic R1441G in human), or with dLRRK harboring Y1383C mutation in the COR domain (Supplementary Material, Fig. S2B, C, F and G). In contrast, the effects of the kinase-dead form of dLRRK (3KD) (6) were not as dramatic (Fig. 1E and Supplementary Material, Fig. S2D). Similar results were also obtained when hLRRK2 WT or PD-related mutant I2020T were co-expressed with dFoxO (Fig. 1H and I). We found that dLRRK exhibited no effect on eye degeneration induced by transgenic expression of another forkhead box protein FoxA (Supplementary Material, Fig. S3) that plays an important role in both the development and maintenance of the midbrain DA neurons in the mammalian central nervous system (10). These results suggest that hLRRK2/dLRRK specifically enhances the toxicity of FoxO through its kinase activity.

Figure 1. LRRK2 enhances FoxO-mediated developmental defects in the *Drosophila* eye. The genotypes are: GMR-Gal4/UAS-EGFP (A), GMR-Gal4, UAS-dFoxO/UAS-EGFP (B), GMR-Gal4, UAS-dFoxO/+,; UAS-dLRRK WT/+ (C), GMR-Gal4, UAS-dFoxO/+,; UAS-dLRRK 11915T/+ (D), GMR-Gal4, UAS-dFoxO/+,; UAS-dLRRK 3KD/+ (E), GMR-Gal4/+; UAS-dLRRK 11915T/+ (F), GMR-Gal4/+; UAS-hLRRK2 I2020T/+ (G), GMR-Gal4, UAS-dFoxO/+,; UAS-hLRRK2 WT/+ (H), GMR-Gal4, UAS-dFoxO, UAS-hLRRK2 I2020T/+ (I), GMR-Gal4, UAS-dFoxO/UAS-AKT1 (J).
**LRRK2/dLRRK activates FoxO through its phosphorylation**

FoxO activity has been shown to be regulated by acetylation, ubiquitination and phosphorylation (11). We did not observe any effects on the development of the eye phenotype caused by dFoxO when overexpressed the FoxO deacetylase SIR2 or removed one copy of the ubiquitin gene (Supplementary Material, Fig. S4). The results may suggest that the acetylation and ubiquitination pathways are not involved in the modulation of dFoxO activity at least in the eye development. Therefore, hLRRK2/dLRRK appeared likely to modulate one of the phosphorylation pathways associated with FoxO activity. We next investigated whether LRRK2 directly phosphorylated mammalian FoxO1. In vitro kinase assays suggested that hLRRK2, but not the kinase-dead form 3KD (6), may act as a FoxO kinase (Fig. 2A). Screening with several mutant FoxO1 proteins revealed that the replacement of the serine 319 residue with alanine (S319A) reduced the phosphorylation signals generated by hLRRK2 (Fig. 2B and Supplementary Material, Fig. S5). The S319 residue was shown to be highly conserved among the mammalian FoxO family and dFoxO (Fig. 2C). As expected, dLRRK also phosphorylated dFoxO in *in vitro* kinase assay, and the introduction of dFoxO S259A mutation, which corresponds to S319A in human FoxO1, reduced the phosphorylation signals generated by dLRRK (Fig. 2D). Overexpression of hLRRK2 (WT or pathogenic G2019S), but not 3KD, was found to specifically stimulate phosphorylation of FoxO1 at S319 (pS319) in cultured HEK 293T cells (Fig. 2E). We generated anti-dFoxO antibody, which specifically recognized endogenous dFoxO (Supplementary Material, Fig. S6A). In *Drosophila*, the level of phosphorylation of endogenous dFoxO (FoxO-P) was increased by dLRRK WT or I1915T overexpression while abolished by 3KD or the loss of the dLRRK gene (Fig. 2F). Reporter assays for FoxO activity suggested that hLRRK2 WT, but not 3KD, stimulates transcriptional activity of FoxO1 (Fig. 3A), whereas the S319A mutation in FoxO1 impairs hLRRK2-mediated activation of FoxO1 (Fig. 3B). The expression of dFoxO S259A has exhibited milder toxicity and has significantly attenuated dFoxO/dLRRK-mediated eye degeneration (Supplementary Material, Fig. S6). In contrast, a phospho-mimic mutant dFoxO S259E enhanced the eye phenotype (Supplementary Material, Fig. S6E). These results indicate that hLRRK2/dLRRK activates FoxO through its phosphorylation. We next examined whether the expression of dLRRK 3KD does not affect the eye phenotype produced by dFoxO S259A. Unexpectedly, the phenotype was dramatically exacerbated, so that the flies came out only under a milder gene expression condition, suggesting the existence of a negative regulation pathway(s) mediated by LRRK2 in FoxO activation (Supplementary Material, Fig. S6H).

**Co-expression of pathogenic dLRRK and FoxO is toxic to post-mitotic DA neurons**

Endogenous dFoxO is widely expressed in the *Drosophila* central nervous system, and is observed in the tyrosine hydroxylase (TH)-positive neurons (Supplementary Material, Fig. S7) while the expression of transgenic dFoxO during the development of tissues is apparently toxic, as shown in Figure 1. To examine the effects of FoxO in post-mitotic neurons, we employed the mifepristone-inducible GAL4 system (GeneSwitch-GAL4) that drives the tissue-specific expression of upstream activating sequence (UAS)-constructs (Fig. 4A). To reduce the probability of non-specific neurotoxicity by conventional overexpression and to modulate the signal pathway more specifically, *Drosophila* crosses was treated with 25 μg/ml of RU486 throughout the lifespan (refer to lanes 2 in Fig. 4A). Pan-neuronal expression of dFoxO in WT (Fig. 4B) or dLRRK null (Fig. 4C) adult *Drosophila* had no effect on survival. The effect of dFoxO on neuronal cells was consistent with that reported previously (12). The combined expression of dLRRK WT and dFoxO had little effect on the lifespan (Fig. 4D), whereas the co-expression of pathogenic dLRRK and dFoxO significantly shortened lifespan (Fig. 4E and F). The number of some clusters of TH-positive neurons observed in adult *Drosophila* co-expressing dFoxO and dLRRK was found to decrease in an age-dependent manner (Fig. 5A and Supplementary Material, Fig. S8). Consistent with the results of the lifespan assay and the viability of TH-positive neurons, the motor activity of the flies pan-neuronally expressing dFoxO/ dLRRK I1915T by the elav-GeneSwitch-GAL4 (elav-GS) driver was more impaired with age than that of flies expressing elav-GS, dFoxO or dLRRK I1915T alone (Fig. 5B). Treatment with 1 mM t,3,4-dihydroxyphenylalanine (t-DOPA) significantly improved the locomotor activity of dFoxO/dLRRK I1915T-expressing flies (Fig. 5B). In this context, the introduction of the S259A mutation in dFoxO attenuated the toxic interaction of pathogenic dLRRK and dFoxO in both lifespan and TH-neuronal loss (Figs 4G and 5C). These results suggest that dLRRK affects DA neuron survival through phosphorylation of dFoxO at the S259 residue.

**FoxO targets hid and bim affect viability of post-mitotic DA neurons**

We next examined which target of FoxO contributes to the FoxO/LRRK2-mediated neurotoxicity. A lot of transcriptional targets of FoxO have been characterized, which includes the molecules involved in cell cycle arrest, oxidative stress resistance, programmed cell death and metabolism (11). We tested the reported FoxO targets, which may be involved in the neuronal maintenance by a combined screening of the *Drosophila* eye assay and quantitative RT–PCR (qRT–PCR), and determined the proapoptotic *hid* gene as a responsible target. Overexpression of *hid* caused dramatic eye degeneration as reported (Fig. 6A) (13), whereas the removal of one copy of *hid* genes significantly improved the eye phenotype of dFoxO/dLRRK I1915T co-expression (Fig. 6B compared with Fig. 1D). *Drosophila* inhibitor of apoptosis (DIAP) inhibits cell death program by promoting the degradation of an initiator caspase Dronc (13). Hid inactivates DIAP through binding, which in turn activates the caspase activity and executes cell death (13). Heterozygosity for DIAP promoted loss of the ommatidia, while reduction in the gene dose of *Dronc* partially rescued the eye phenotype of dFoxO/dLRRK I1915T co-expression (Fig. 6C and D). dLRRK stimulated expression of *hid* transcripts in *Drosophila* and a pathogenic
Figure 2. LRRK2/dLRRK phosphorylates FoxO. (A and B) In vitro kinase assay of hLRRK2 using recombinant GST-FoxO1 as a substrate. hLRRK2 WT or 3KD was immunoprecipitated from hLRRK2-transfected HEK293T cells for kinase sources. Mock immunoprecipitate (Mock) served as a control. Autoradiography (P32) and Coomassie brilliant blue (CBB) staining of the gels are shown. (B) FoxO1 S319 represents a major phosphorylation site for hLRRK2 in vitro. (C) Alignment of putative hLRRK2/dLRRK target sequences in the FoxO family. The arrow indicates the potential phosphorylation sites. (D) In vitro kinase assay of dLRRK using recombinant GST-dFoxO and its mutant S259A as substrates. The assay was performed as in (B). (E) Lysate from 293T cells transfected with hLRRK2 WT, PD mutant (G2019S) and 3KD or β-galactosidase (control) was treated with or without dephosphorylation reaction (CIP) and analyzed by western blot. Overexpression of hLRRK2 with kinase activity stimulated phosphorylation of the S319 residue in endogenous FoxO1. The graph shows relative levels of phospho-S319 (pS319) after normalization with total FoxO1 levels. Data are presented as mean ± SE of three experiments (∗∗P < 0.01, Tukey–Kramer test). (F) The levels of dFoxO phosphorylation are decreased in the dLRRK null fly. Fly brain extracts treated with (+) or without (−) dephosphorylation reaction with alkaline phosphatase (CIP) were subjected to western blot analysis using dFoxO antibody (left panel). Endogenous dFoxO expression was analyzed in the flies harboring the UAS genes (EGFP, dLRRK, dLRRK I1915T and dLRRK 3KD) crossed with the ubiquitous daughterless (Da)-Gal4 driver (right), dLRRK(+/-) and dLRRK(−/−) (middle). Bands corresponding to phosphorylated (dFoxO-P) and non-phosphorylated forms (dFoxO) of dFoxO are indicated. Actin signals indicate that equivalent amounts of lysates were loaded. The genotypes of dLRRK(+/-) and dLRRK(−/−) are w− and dLRRKo35680/dLRRKo35680, respectively.
mutant I1915T had a higher activity (Fig. 6E). Clusters of FoxO response elements were reported in the first intron of the hid gene (14). We confirmed that dFoxO binds to the sites in the first hid intron by chromatin-immunoprecipitation (ChIP) for endogenous dFoxO in Drosophila S2 cells (Fig. 6F). Furthermore, a combined expression of dFoxO S259A along with dLRRK I1915T mutant impaired the binding of FoxO to the hid gene in S2 cells and subsequent hid expression in flies, compared with dFoxO WT/dLRRK I1915T co-expression (Fig. 6G and H). We next examined whether Hid and its mammalian homologue are involved in the DA neurodegeneration by FoxO/LRRK2 signalling. DA neurodegeneration caused by the inducible expression of dFoxO/dLRRK I1915T in the adult fly brain tissues was significantly suppressed on the hid heterozygous genetic background (Fig. 6I). In mammals, FoxO family regulates the expression of a proapoptotic Bcl-2 family protein Bim in various contexts of neuronal death, which is a cellular regulation similar to Hid in Drosophila (15–17). Bim, especially a shorter isoform Bim-S, stimulates caspase activation by promoting the release of cytochrome c from mitochondria (18,19). A clear increase of alternative splicing isoforms of Bim (Bim-L and Bim-S) expression was observed in cells transfected with hLRRK2 G2019S mutant as well as a constitutive active form of FoxO (Fig. 7A), suggesting that hLRRK2 G2019S activates endogenous FoxO through phosphorylation. A similar tendency was also seen in hLRRK2 WT-transfected cells although the difference was not statistically significant (Fig. 7A). The FoxO/LRRK2-mediated cell death was also observed in SH-SY5Y cells. Co-transfection of hLRRK2 along with FoxO1 resulted in around 30% of cell death, which was partially suppressed by one of the mammalian IAP family proteins XIAP (Fig. 7B). We next examined to what extent endogenous hLRRK2 and its downstream Bim contribute to FoxO/LRRK2-mediated cell death in SH-SY5Y cells. RNA interference (RNAi) against hLRRK2 or Bim significantly inhibited cell death by FoxO1 (Fig. 7C and D). Furthermore, a FoxO1 mutant that is resistant to phosphorylation by LRRK2 (FoxO1 SA) attenuated the cell death (Fig. 7E). These results suggested that Hid/Bim is required downstream of FoxO/LRRK2 signaling for neurodegeneration.

**DISCUSSION**

Mutations in the LRRK2 gene have been reported to be the most common cause of familial PD (20). In addition, recent genome-wide association studies have identified LRRK2 as well as SNCA/α-synuclein as major risk loci for general PD, strongly suggesting that LRRK2 signal pathway has a central pathogenic role across the spectrum of PD (21,22). However, the physiological and pathogenic functions of LRRK2 are poorly understood. Here we have found that LRRK2 phosphorylates and stimulates transcriptional activity in both human and Drosophila FoxO proteins through phosphorylation. Numerous transcriptional targets of FoxO have been characterized, and include molecules involved in cell cycle arrest, oxidative stress, programmed cell death and metabolism (11). We tested by a combination of fly genetics and qRT–PCR the reported key target molecules that might have roles in neurodegeneration, which included 4E-BP (7,8), Polo (23), Cyclin B (23) and Hid (14) (Fig. 6 and Supplementary Material, Fig. S1 and S9). Abnormal activation of Cyclins and its regulators has been reported to be one of the causes for DA neuron death (24,25). However, there was no evidence that Polo and Cyclin B contribute to the neurodegeneration by FoxO/dLRRK in Drosophila (Supplementary Material, Fig. S9). In contrast, hid expression showed the best correlation with the neuronal loss in this context. Although hid is not conserved in the mammalian system, known programmed cell death target molecules such as Bim, PUMA and Fas ligand are possible candidates for effectors of the LRRK2/FoxO pathway in humans (11). Indeed, Bim expression and subsequent cell death were promoted by a combined expression of FoxO and LRRK2 in the human cultured cells, whereas the knockdown of Bim or overexpression of XIAP suppressed cell death in this context. Thus, Bim seems to be a functional homologue of Hid downstream of FoxO/LRRK2 signaling in mammalian system.

We have previously reported that LRRK2 phosphorylates one of the transcriptional targets of FoxO, 4E-BP, and attenuates 4E-BP function (6). The relationship between LRRK2, FoxO and 4E-BP appears to be complex. Although LRRK2/dLRRK stimulates the expression of 4E-BP in parallel with Bim/Hid through FoxO phosphorylation, our previous study suggested that LRRK2 promotes the inactivation of a neuroprotective function of 4E-BP through its phosphorylation. The idea that LRRK2 has a dual effect on 4E-BP is supported by a result of the genetic interaction analysis, where the removal of d4E-BP gene exacerbated the FoxO/dLRRK2-mediated DA neurodegeneration (Supplementary Material, Fig. S10).

We have demonstrated that the co-expression of FoxO and LRRK2/dLRRK causes synergistic effect of neurotoxicity. However, single expression of PD-related LRRK2/dLRRK mutants by the GMR-GAL4 driver was not toxic in the eyes,
suggesting that the phosphorylation of endogenous FoxO by pathogenic LRRK2/dLRRK is not sufficient for the retinal degeneration in developing eyes. Cell death signaling pathways are known to have neutralization mechanisms, in which the balance between death activators and repressors determines the threshold for cell death. Hid induction by ectopic expression of LRRK2/dLRRK will lower the threshold for cell death, neutralizing the cell protective activity of DIAP, but it might not be sufficient to undergo neurodegeneration in this context. In fact, we observed age-dependent DA neurodegeneration by pathogenic LRRK2/dLRRK alone (6,26), suggesting that ageing and cell type are key factors to modulate the cell death threshold.

The S319 residue in FoxO1 has been reported to constitute one of the major phosphorylation sites for the serine–threonine kinases AKT/protein kinase B and serum- and glucocorticoid-inducible kinase (SGK) (11). Phosphorylation of S319 along with T24 and S256 by AKT and SGK has been shown to stimulate sequential phosphorylation of S322 and S325, which in turn accelerates nuclear export of FoxO1 and suppresses its activity in mammalian cells (27). In addition, the co-expression of AKT with dFoxO has been
reported to partially rescue dFoxO-mediated eye degeneration in *Drosophila* (8). In our study, we also observed inhibitory effects of AKT on the eye phenotype in *Drosophila* (Fig. 1J), and in a FoxO reporter assay in mammalian cells (data not shown). In contrast to AKT, LRRK2 appears to stimulate FoxO activity by selectively targeting S319, and the phosphorylation status at S322 and S325 was relatively unchanged (Supplementary Material, Fig. S11). Thus, selective phosphorylation of FoxO S319 site by LRRK2 may have a novel effect on FoxO function. For example, specific phosphorylation of S319 might stimulate the recruitment of a co-factor(s) of dFoxO, which may activate cell death targets of FoxO preferentially.

In contrast to the results reported here, Tain *et al.* (28) have demonstrated that FoxO protects DA neurons in a parkin-linked PD model fly. We speculate that the inconsistency comes from differences in a primary cause of neurodegeneration. Loss of *parkin* causes mitochondrial degeneration in tissues with high-energy demands in *Drosophila*. Since it has been reported that the genes for mitochondrial biogenesis are regulated by FoxO in *Drosophila* (29), FoxO might compensate for the mitochondrial loss by *parkin* inactivation via the expression of those genes as well as 4E-BP. Thus the effects of FoxO appear to be context-dependent.

We demonstrated that pan-neuronal expression of dFoxO and PD-associated mutant dLRRK leads to motor dysfunction and degeneration of DA neurons in *Drosophila*, suggesting that our findings might be relevant to clinical and pathological features of PD. However, we also observed similar degeneration phenotypes in retinal neurons of developing eye tissues by higher expression of dFoxO and LRRK2/dLRRK (Fig. 1). Considering relatively ubiquitous distribution of LRRK2/dLRRK and FoxO in human and *Drosophila* brain tissues (Supplementary Material, Fig. S7) (6,30,31), we speculate
that a sensitivity threshold for cell death could determine selective degeneration of DA neurons as well as other types of neurons. It is widely accepted that early non-motor signs, which include neuropsychiatric complications, autonomic disorders, sleep disturbances and sensory symptoms, precede the onset of motor symptoms in PD. Although the pathophysiology of non-motor symptoms is still poorly understood, the degeneration of both DA and non-DA systems is thought to contribute to their pathogenesis. Thus the FoxO-LRRK2 pathway could be a common cell death pathway in various

Figure 6. A FoxO target Hid is responsible for dLRRK-mediated DA neurodegeneration in Drosophila. (A–D) SEM images of the fly eyes. (A) Ectopic expression of hid resulted in eye degeneration. Reduction of the gene dose of hid (B) and Dronc (D) improved the eye phenotype of dFoxO/dLRRK I1915T shown in Figure 1D, whereas the phenotype was exacerbated on a DIAP heterozygous mutant background (C). (E) dLRRK regulates the level of hid transcript. The amounts of hid and rp49 transcripts were measured by real-time RT–PCR using total RNA from the transgenic flies indicated genotypes crossed with the Da-Gal4 driver. The values are presented as the mean ± SE from three independent experiments. EGFP served as a control. *P < 0.05; **P < 0.01. (F) FoxO binds to the hid locus. ChIP assay was performed using S2 cells incubated in serum-free medium for 16 h. FoxO/DNA complex was immuno precipitated using anti-FoxO antibody or control antibody. The region of the first hid intron containing the FoxO-binding sites was amplified by PCR. (G) FoxO SA mutation impairs the binding of FoxO to the hid gene. S2 cells transfected with dLRRK I1915T along with dFoxO WT or SA were subjected to ChIP assay as in (F). (H) FoxO SA mutation impairs FoxO/dLRRK-mediated hid expression. Pan-neuronal expression of the indicated genes was induced by elav-GS crosses after 100 μg/ml RU486 administration for 4 days. Flies of the indicated genotypes were subjected to real-time RT–PCR for hid as in (E). The values are presented as the mean ± SE from three independent experiments. **P < 0.01. (I) Hid is required for dLRRK/dFoxO-mediated DA neurodegeneration in Drosophila. Graph shows the number of PPM1/2 and PPL1 TH-positive neurons in 24-day-old adult flies (n = 10) as treated in Figure 4E. Data are presented as the mean ± SE from three repeated experiments. *P < 0.05; **P < 0.01; N.S., not significant. Fly genotypes in Figure 6 are: GMR-Gal4, UAS-hid/CyO (A), GMR-Gal4, UAS-dFoxO/+; UAS-dLRRK I1915T/hid1 (B), GMR-Gal4, UAS-dFoxO/+; UAS-dLRRK I1915T/DIAP1 (C), GMR-Gal4, UAS-dFoxO/+; UAS-dLRRK I1915T/DroncG02994 (D), UAS-dFoxO/+; elav-GS/+ [dFoxO], UAS-dFoxO/+; elav-GS/hid1 [dFoxO, hid1/+]; UAS-dFoxO/UAS-dLRRK I1915T, elav-GS/+ [dFoxO, I1915T], UAS-dFoxO/UAS-dLRRK I1915T, elav-GS/hid1 [dFoxO, I1915T, hid1/+] (I).
cell types including DA neuron, but the sensitivity to this signal might be cell-type dependent.

Although the neurotoxic effect of LRRK2/dLRRK appears to require kinase activity, the expression of a kinase-dead form of dLRRK 3KD in the eye also had a mild toxic effect (Fig. 1E compared with B). Previous biochemical studies indicated that a dimeric form of LRRK2 possesses kinase activity in vitro and that kinase-dead forms of LRRK2 lack the dimerization activity (32,33). Therefore it is unlikely that hLRRK2/dLRRK 3KD recruits and activates the endogenous hLRRK2/dLRRK. We recently found that a LRRK2-associated kinase also targets the same residue of FoxO (manuscript in preparation). Such associated kinases might be recruited and activated even by 3KD. Indeed the expression of hLRRK2 3KD mildly increased the pS319 signal of FoxO1 in cultured cells (Fig. 2E), and slightly activated the FoxO reporter construct (Fig. 3A) though the difference is statically not significant.

Another possibility is that the overexpression of LRRK2/dLRRK proteins in itself is neurotoxic unlike EGFP since we did not see a clear difference of the eye phenotype between WT and pathogenic mutants (Fig. 1C and D, and Supplementary Material, Fig. S1F and G). However, milder expressions of dLRRK WT and mutants using the GeneSwitch system revealed a pathogenic mutant-specific effect in the lifespan assay (Fig. 4). Thus, these findings warrant further study in the neurodegeneration of LRRK2-linked PD.

It is particularly worth noting that the co-expression of dLRRK 3KD and dFoxO S259A dramatically worsened the eye phenotype (Supplementary Material, Fig. S6H). The unexpected effect suggests that there remains a negative regulation mechanism to be clarified, and might explain the aforementioned effect of LRRK2/dLRRK 3KD. One of plausible explanations for this observation is that dLRRK stimulates AKT or its activators, and that dLRRK 3KD acts as a
dominant-negative mutant. However, there is no evidence that LRRK2/dLRRK modulates AKT activity (data not shown). Thus, the elucidation of the entire LRRK2-FoxO pathway must await further studies.

In conclusion, our results identify FoxO proteins as novel substrates in the LRRK2 pathogenic pathway and suggest a role for dFoxO in the neurodegeneration caused by mutant LRRK2/dLRRK in Drosophila. That is, LRRK2/dLRRK confers a neurotoxic activity to FoxO through a novel phosphorylation mechanism in Drosophila. Since the LRRK2-FoxO pathway appears to be partially conserved between human and Drosophila, downstream molecules of Bim/Hid such as IAP family proteins and caspases could be therapeutic targets for LRRK2-linked PD. Further studies in the mammalian system may also uncover other roles for LRRK2 and FoxO in the underlying pathogenic mechanisms mediating sporadic as well as familial PD.

MATERIALS AND METHODS

Drosophila genetics

Drosophila culture and crosses were performed on standard fly food containing yeast, cornmeal and molasses, and flies were raised at 25°C unless otherwise stated. We used UAS-dLRRK WT and mutant lines that exhibit similar levels of protein expression (6). For the construction of UAS-dFoxO S259A and UAS-dFoxO S259E transgenic lines, dFoxO cDNA obtained by RT–PCR from adult Drosophila total RNA was cloned into the pBluescript vector. The mutant forms of dFoxO generated by site-directed mutagenesis were sub-cloned into the µAST vector. The introduction of transgenes into Drosophila germ lines and the establishment of transgenic lines were performed in the w background using the embryonic transformation service of BestGene, Inc. (Chino Hills, CA, USA). All additional general fly stocks and GAL4 lines were obtained from the Bloomington Drosophila stock center. These flies have been described previously: UAS-dLRRK WT, R10969, Y1383C, 19155T, 3KD and e93680 (dLRRK null) (6); UAS-hLRRK2 WT and I2020T (26); elav-GeneSwitch (34); dFoxO21 and dFoxO25 (7); dSir2p2360 (35); UAS-dFoxA (36).

Antibodies

Rabbit anti-dFoxO and anti-hLRRK2 polyclonal antibodies were raised against recombinant GST-N-terminal dFoxO (1–214 amino acids) and GST-hLRRK2 (823–1004 amino acids), respectively, and were affinity-purified with the antigens. Anti-α-tubulin (DM1A), anti-β-tubulin (Tub2.1) and anti-FLAG (M2) antibodies were purchased from Sigma. Anti-FoxO1 (no. 9454), anti-phospho-FoxO1 (Ser319, no. 2487; Thr24, no. 9464; Ser256, no. 9461) and anti-Bim (no. 2819) were obtained from Cell Signaling Technology. Anti-Actin (MAB1501) and anti-phospho-FoxO1 (Ser319, 511361-1) antibodies were purchased from Chemicon and Signalway, respectively. Mouse anti-TH monoclonal antibody was purchased from ImmunoStar. The rabbit anti-Drosophila TH polyclonal antibody has been described previously (37). Anti-phospho-FoxO1 (Ser322 and Ser325) antibodies were kindly provided by Drs G. Rena and C.J. Hastie (38).

RT–PCR and plasmids

For real-time RT–PCR analysis, RT and PCR reactions with total RNA extracted from adult flies were performed using Superscript VILO cDNA Synthesis Kit (Invitrogen) and SYBR GreenER qPCR SuperMix (Invitrogen), respectively. To generate GST-FoxO1 for bacterial expression, we amplified the corresponding coding sequences from pcDNA3-mouse FoxO1-Myc-His (a kind gift from Dr T. Furuyama) and cloned it into the pGEX6P-1 vector. The introduction of mutations was performed using the QuikChange II XL Site-directed Mutagenesis Kit (Stratagene). Although the S316 residue in mouse FoxO1 corresponds to S319 in human FoxO1, we describe both residues as S319 to avoid confusion. Plasmids for FLAG-hLRRK2, FLAG-dLRRK, FLAG-XIAP, FLAG-XIAPΔRING and myr-AKT have been reported elsewhere (6,39,40). The luciferase reporter plasmid for FoxO (TK.IRS3) were generated by the insertion of three copies of an insulin response sequence derived from the IGFBP-1 promoter to 81 bp upstream of the thymidine kinase promoter and firefly luciferase cDNA. The characterization of TK.IRS3 has been reported elsewhere (41).

ChIP assay

ChIP assay was performed using ChIP-IT Express Kit (Active Motif). S2 cells treated with 1% formaldehyde were subjected to homogenization, sonication and subsequent fractionation. Immunoprecipitation for dFoxO was carried out using anti-dFoxO in the sheared chromatin fractions. PCR was performed with ExTaq (TAKARA bio) to estimate bound genomic DNA in the precipitates. The hid first intron region was amplified with the same primer pairs as reported (14).

In vitro phosphorylation assay

FLAG-hLRRK2, FLAG-dLRRK or mock fractions immunopurified from transfected and mock-transfected 293T cells were used as kinase sources. Five micrograms of GST-FoxO1 was incubated with FLAG-hLRRK2 or FLAG-dLRRK in kinase reaction buffer containing 20 mM HEPES (pH 7.4), 15 mM MgCl2, 5 mM EGTA, 0.1% Triton X-100, 0.5 mM DTT, 1 mM β-glycerophosphate and 2.5 μCi [γ32P]-ATP for 30 min at 30°C. The reaction mixture was then suspended in SDS sample buffer and subjected to SDS–PAGE and autoradiography.

Cell culture, immunopurification and western blot analysis

Transfection of 293T and SH-SY5Y cells, immunopurification of FLAG protein from the transfected cell lysate and western blot analysis were performed as described previously (42,43). For the preparation of fly samples for western blot analysis, fly heads were directly homogenized in 20 μl/head of SDS sample buffer using a motor-driven pestle. After centrifugation at 16 000g for 10 min, the supernatant was subjected to SDS–PAGE and subsequent western blot analysis. Densitometric analysis was performed using Image J software from the US National Institute of Health (http://rsb.info.nih.gov/ij/).
Scanning electron microscopy analysis
Adult flies directly soaked in 70% ethanol were subjected to stepwise dehydration, and the samples were processed as described previously (6). Scanning electron microscopy (SEM) images were obtained at The Biomedical Research Core of Tohoku University Graduate School of Medicine.

Lifespan and climbing assays
Twenty female adult flies per vial were maintained at 29°C, transferred to fresh fly food vials containing 250 μl of yeast paste and 25 μg/ml of RU486 and scored for survival every 4 days. To control for isogeny, the dLRRK(−/−) mutant and the UAS-dFoxO WT flies were backcrossed to w− wild-type background for six generations. The dLRRK and dFoxO S259A transgenics were generated on w− background and thus have matched genetic backgrounds. Climbing assay was performed as described previously using 24-day-old female adult flies as treated in the lifespan assay (6).

Whole-mount immunostaining
Total TH-positive neuron numbers were calculated following whole-mount immunostaining of brain samples as described previously (37). Brain tissues were isolated and stained by T.K. and TH-positive neurons were counted by Y.I. under blinded conditions. All immunohistochemical analyses were performed using a Carl Zeiss laser scanning microscope system.

Cell death assay
SH-SY5Y cells transiently co-transfected with Venus (an improved version of YFP) and various combinations of plasmids were fixed and stained with DAPI 48 h after transfection. At least 260 Venus-positive cells with healthy or apoptotic nuclei from randomly chosen fields were counted. For a combination of RNAi and plasmid transfection experiments in Figure 7C, Stealth RNAi duplexes (Invitrogen) were introduced with LipofectAMine RNAiMAX reagent (Invitrogen) 24 h before plasmid transfection for maximum gene knockdown efficiencies. Total amounts of DNA for transfection were adjusted to equal amounts with a plasmid for β-galactosidase upon combined transfection.

Statistical analysis
One-way repeated measures ANOVA was performed to determine significant differences between multiple groups unless otherwise indicated. If a significant result was achieved (P < 0.05), the mean of the control and the specific test groups was analyzed using the Tukey–Kramer test. For lifespan assays, the Kaplan–Meier analysis with log-rank test was performed.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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