Propagation of α-synuclein aggregates has been suggested as a contributing factor in Parkinson’s disease (PD) progression. However, the molecular mechanisms underlying α-synuclein aggregation are not fully understood. Here, we demonstrate in cell culture, nematode, and rodent models of PD that leucine-rich repeat kinase 2 (LRRK2), a PD-linked kinase, modulates α-synuclein propagation in a kinase activity-dependent manner. The PD-linked G2019S mutation in LRRK2, which increases kinase activity, enhances propagation efficiency. Furthermore, we show that the role of LRRK2 in α-synuclein propagation is mediated by RAB35 phosphorylation. Constitutive activation of RAB35 overrides the reduced α-synuclein propagation phenotype in lrk-1 mutant C. elegans. Finally, in a mouse model of synucleinopathy, administration of an LRRK2 kinase inhibitor reduced α-synuclein aggregation via enhanced interaction of α-synuclein with the lysosomal degradation pathway. These results suggest that LRRK2-mediated RAB35 phosphorylation is a potential therapeutic target for modifying disease progression.
Parkinson’s disease (PD) is a common neurodegenerative disorder characterized by movement symptoms, as well as various non-motor symptoms. PD belongs to a group of diseases known as synucleinopathies because its pathological characteristics include abnormal deposition of α-synuclein aggregates in the forms of Lewy bodies (LBs) and Lewy neurites (LNs) in surviving neurons. The gene for α-synuclein, SNCA, has not only been linked to familial PD but is also strongly associated with sporadic PD. Importantly, not only been linked to familial PD but is also strongly associated with sporadic PD. Importantly, the pattern of aggregate spreading is somewhat correlated with clinical progression, increasing the possibility that the former is the main driver of disease progression.

A growing body of evidence suggests that cell-to-cell propagation of α-synuclein aggregates through interconnected brain regions is the principle mechanism underlying pathological aggregate spreading. Importantly, postmortem analysis of human brains and connectomics studies challenge the prion hypothesis of the aggregate propagation in PD. Nevertheless, aggregate propagation is a real phenomenon that has been confirmed in numerous cell and animal models. Thus, studies of this phenomenon will provide insights into the etiopathology of PD. The aggregate propagation seems to be mediated by exocytosis of aggregates from donor neurons and subsequent aggregate endocytosis by recipient neurons, although a recent study suggested that tunneling nanotubes can enable the neurons to directly transport α-synuclein aggregates. The internalized α-synuclein aggregates are trafficked through the endolysosomal pathway. The current understanding of the propagation details and identification of key regulators of the process remains very primitive.

Mutations in leucine-rich repeat kinase 2 (LRRK2) are considered the most common cause of both familial and sporadic PD. LRRK2 is a member of a multidomain protein family, which also includes leucine-rich repeat kinase 1 (LRRK1) (Supplementary Fig. 1). Domain structures of LRRK proteins are conserved between the isoforms and among different species. *C. elegans* has one LRRK gene, *lrk-1*, which has domain structures homologous to those of mammalian LRRK genes. Overall structural conservation suggests functional similarity between LRK-1 and mammalian LRRKs. It is worth taking into consideration a recent report showing that two LRRK2 kinase inhibitors did not inhibit LRK-1. However, the evidence provided in this study was indirect, and the direct effects of LRRK2 kinase inhibitors on LRK-1 remain to be determined. The most common mutation in LRRK2 is the G2019S substitution in the kinase domain, which leads to an increase in the kinase activity. Interestingly, many PD patients with LRRK2 (G2019S) mutations exhibit α-synuclein-positive LBs, even though nearly half the LRRK2 (G2019S)-PD cases are LB-negative. This suggests that LRRK2 mutations could be implicated in α-synuclein-induced neurodegeneration in PD. Overexpression of LRRK2 has been shown to exacerbate while deletion of the LRRK2 gene suppressed synucleinopathy lesions and α-synuclein-induced neurodegeneration in rodent models of synucleinopathy. Inclusion body formation that was triggered by administration of α-synuclein fibrils in neuronal culture and rat brain was also increased by transgenic expression of LRRK2. RNAi-mediated silencing of *lrk-1* expression led to neuron-to-neuron propagation of α-synuclein in *C. elegans*. However, the mechanism by which LRRK2 exerts its action in synucleinopathy is not fully understood.

Here, we investigate the roles of LRRK2 and LRK-1 in the propagation of α-synuclein in cell culture, nematode, and rodent models. The current study demonstrates that LRRK2 can regulate α-synuclein propagation in a kinase activity-dependent manner via phosphorylation of RAB35. Furthermore, our findings suggest that LRRK2 kinase activity can be targeted for the purpose of reducing synucleinopathy lesions.

**Results**

**lrk-1 and Lrrk2 deficiencies reduced α-synuclein propagation.** We employed two independent in vivo models, *C. elegans* and rat models, which were deficient in *lrk-1* and Lrrk2 genes, respectively. First, we used the *C. elegans* model, in which the N-terminal and C-terminal halves of Venus fluorescent protein were fused to α-synuclein protein (V1S and SV2, respectively) and expressed in pharyngeal muscle and the connected neurons, respectively (Fig. 1a). This model allowed for quantitative analysis of intercellular α-synuclein propagation between pharyngeal muscles and neurons through bimolecular fluorescence complementation (BiFC). We generated three independent BiFC transgenic lines each in wild-type (WT) (V1S and SV2, respectively) and expressed in pharyngeal muscle and the connected neurons, respectively (Fig. 1a). The evidence provided in Supplementary Fig. 2c, d). However, in *lrk-1* background worms, the fluorescence was not increased with aging, showing significantly reduced Venus fluorescence at day 13 compared with the WT worms (Fig. 1b, c and Supplementary Fig. 2c, d). Likewise, the number of Venus-positive inclusion bodies was greatly reduced in *lrk-1(tm18898)* (Fig. 1d and Supplementary Fig. 2e, f). We then examined various degenerative phenotypes in the transgenic lines. Consistent with a previous report, the number of both axonal blebs (Fig. 1e) and fragmentation (Fig. 1f and Supplementary Fig. 2g) was increased in N2; V1S+SV2 lines at old ages. This axonal damage was ameliorated by *lrk-1* gene mutation (Fig. 1e, f). The age-dependent α-synuclein propagation was accompanied by behavioral deficits, in this case, a decline in the pharyngeal pumping rates (Fig. 1g). The *lrk-1* mutation completely eliminated this behavioral phenotype (Fig. 1g). Finally, the decrease in life span in the BiFC transgenic worms was almost completely rescued by *lrk-1* mutation (Fig. 1h and Supplementary Fig. 2h, i). Therefore, α-synuclein propagation and the associated degenerative phenotypes require the function of the *lrk-1* gene in *C. elegans*.

We next tested the role of LRRK2 in a rat model in which neuronal-to-neuron α-synuclein transfer and long-distance protein spreading are triggered by injection of recombinant adeno-associated viral vectors (AAVs) encoding human α-synuclein into the vagus nerve. In this model, targeted overexpression of the exogenous protein in the dorsal medulla oblongata triggers progressive caudo-rostral spreading of the protein towards the pons, midbrain and forebrain, affecting regions that are anatomically connected to the dorsal motor nucleus of the vagus (DMNX) and the nucleus of the tractus solitarius. Experiments were carried out using Charles River Long-Evans wild-type (WT) controls and homozygous LRRK2-deficient (-/-) rats on the Charles River Long-Evans background strain. The absence of LRRK2 in the latter group of animals was confirmed by RT-PCR and immunohistochemistry (Supplementary Fig. 3a, b). Rats were killed at 8 and 12 weeks after intravaginal injection of AAVs. Neurotoxicity was assessed by stereological counting of DMNX neurons, and transduction efficiency was estimated by calculating the percentage of DMNX neurons overexpressing human α-synuclein. Animals in the different experimental groups were matched based on lack of neurodegeneration and similar counts of transduced DMNX neurons (approximately 20% of the total neuronal number; Supplementary Fig. 3c, d). Axons immunoreactive for human α-synuclein were observed in brain regions rostral to the medulla oblongata of either WT or Lrrk2-/- rats,
indicating inter-neuronal spreading of the exogenous protein. Positive fibers were characterized by robust immunoreactivity within irregularly spaced varicosities (Fig. 2a). The number of labeled axons was determined and compared in the left (AAV-injected side) pons of WT vs. Lrrk2−/− animals. In agreement with previously reported findings, a temporal progression of human α-synuclein spreading was indicated by higher counts at 12 weeks compared with the earlier time point (Fig. 2b). When the number of immunoreactive fibers was compared between WT and Lrrk2−/− rats, decreased counts were observed in the latter group at both 8 and 12 weeks (Fig. 2b). To enhance the specificity and sensitivity of this comparison, unbiased measurements of length and density of human α-synuclein-positive fibers were taken in a pontine region encompassing the coeruleus/subcoeruleus complex and the parabrachial nuclei using the Space Balls stereological tool. Data at 8 and 12 weeks were pooled and analyzed together. The results showed that the mean total length and mean density of labeled axons were both significantly decreased in Lrrk2−/− animals (Fig. 2c, d). A final set of analyses involved counting of immunoreactive axons in more rostral brain regions. Similar to the results in the pons, counts were found to be markedly lower in sections from the caudal (−69%) and rostral (−69%) midbrain and from the forebrain (−55%) of Lrrk2−/− rats (Fig. 2e). Taken together, the rat data demonstrated that LRRK2 plays a critical role in cell-to-cell propagation and long-distance spreading of α-synuclein in vivo.

**LRRK2 kinase promotes α-synuclein propagation.** We then generated recombinant adenoviral vectors containing LRRK2 WT
or the G2019S or D1994A mutant. We confirmed the ectopic expression of LRRK2 WT and its mutant variants in differentiated SH-SY5Y neuroblastoma cells (Supplementary Fig. 4). The overall expression levels were similar among the LRRK2 variants. The intracellular distribution of LRRK2 proteins was indistinguishable among the experimental groups. Note that most of the SV2 cells were infected with the virus and represented approximately 50% of the total cells in the co-culture (Supplementary Fig. 4). The cell-derived extracellular α-synuclein proteins that were used in this experiment contained both α-synuclein monomers and aggregates (Supplementary Fig. 5). Upon internalization, the monomers were collected mostly in the Triton X-100 soluble fractions, whereas the aggregates were found in the insoluble fractions (Fig. 3a,c). The cumulative levels of α-synuclein in Triton X-100 soluble fraction were not altered by overexpression of WT LRRK2 (Fig. 3a,b). However, WT LRRK2 overexpression brought an increase in accumulation of α-synuclein aggregates in the Triton-insoluble fraction (Fig. 3c,d). The expression of G2019S, the PD-linked mutant with increased kinase activity, further increased the accumulation of α-synuclein both in the Triton-soluble and -insoluble fractions in the recipient SH-SY5Y cells (Fig. 3a–d). In contrast, the expression of LRRK2 D1994A, a kinase-deficient variant, did not alter the accumulation of internalized α-synuclein (Fig. 3a–d).

Propagation of α-synuclein was quantitatively analyzed using a dual-cell BIFC system consisting of two different stable cell lines, which overexpressed either V1S or SV226. Although both V1S- and SV2-expressing cells can secrete and internalize α-synuclein, our previous study showed that V1S cells secrete substantially more α-synuclein protein than SV2 cells, which makes the latter cells primary recipients in this assay system26. Transduction of SV2 recipient cells with WT LRRK2 resulted in an increase in the propagation of α-synuclein aggregates, while expression of the G2019S variant further increased the propagation to significantly higher levels than observed with WT LRRK2. These effects were not observed when the kinase-dead D1994A variant was expressed (Fig. 3e,f, Supplementary Fig. 6). Next, we investigated whether the increase in LRRK2 kinase activity provokes the secretion of α-synuclein aggregates. Because α-synuclein is constantly secreted, internalized, and then secreted again26, the extracellular α-synuclein in the media is the sum of ‘primary’ secretion and the ‘secondary’ secretion (or internalization and rescertainment). It is very difficult to measure only the ‘primary secretion’. However, we can measure the ‘secondary secretion’ by measuring the Venus fluorescence in the media, which represents co-aggregation between the internalized α-synuclein and the recipient-expressed α-synuclein. This secondary secretion was increased in the cultured cells overexpressing WT LRRK2 and even more increased in the cells expressing G2019S LRRK2 (Fig. 3g). For further validation of the role of LRRK2 kinase, we inhibited the LRRK2 kinase activity using the selective inhibitor HG-10-102-0127. Treatment of the cells with this inhibitor decreased LRRK2 autophosphorylation on several serine residues (Fig. 3h). Inhibition of LRRK2 kinase activity near completely nullified the effects of LRRK2 WT and G2019S on the propagation of α-synuclein (Fig. 3i,j). The inhibitor did not affect α-synuclein propagation in mock-transduced cells, probably due to the low expression levels of endogenous LRRK2 in SH-SY5Y cells. These results demonstrated that LRRK2 regulates α-synuclein propagation through its kinase activity.

**LRRK2-mediated RAB35 phosphorylation.** Next, we turned our attention to substrates of LRRK2 kinase. We were particularly interested in RAB proteins, small GTPases that regulate vesicle trafficking and sorting28. A recently published study showed that several RABs, including RAB3, 8, 10, 12, 35, and 43, were phosphorylated by LRRK229,30. Our recent in vitro screen using protein chips identified three RAB proteins, RAB1, RAB8, and RAB35, as substrates of LRRK2 kinase31. All these RAB proteins contain a Thr residue in the Switch II domain. It has been shown that the Thr residue in this domain is the preferred substrate of LRRK2 kinase29. Substitution of Thr 72 with Ala abolished the LRRK2-mediated RAB35 phosphorylation31. These earlier studies encouraged us to identify the specific RAB proteins that are involved in LRRK2-mediated α-synuclein propagation. First, we determined which of these three RAB proteins co-localize with internalized α-synuclein in the recipient cells. RAB proteins were expressed using lentiviral vectors in the recipient cells. RAB1 and RAB35 exhibited a more diffuse pattern than RAB8 (Supplementary Fig. 7a), However,
vesicular patterns were present for all the RABs tested. We found that approximately 35% of the internalized α-synuclein colocalized with RAB35 (Fig. 4a, b and Supplementary Fig. 7b), whereas colocalization with RAB1 and RAB8 was less than 10% (Fig. 4a, b). Pearson’s coefficient also showed a correlation between RAB35 and transmitted α-synuclein (Fig. 4c). Co-immunoprecipitation experiments confirmed that LRRK2 interacted with RAB35 in neuronal cells (Fig. 4d–g). We then examined the effects of ectopic expression of a dominant-negative mutant (S22N) form of RAB35. Inhibition of RAB35 via cell transduction with RAB35 S22N completely nullified the LRRK2-stimulated α-synuclein propagation (Fig. 4h, i).

**RAB35 phosphorylation on α-synuclein propagation.** To further validate the role of RAB35 in α-synuclein propagation in vivo, BiFC transgenes (V1S and SV2) were introduced into WT Venus BiFC.
(N2) and rab-35 mutant (tm2058) C. elegans, and three lines with similar α-synuclein expression levels were selected for analysis (Supplementary Fig. 8a, b). The rab-35 mutant worms showed effects similar to those in lrk-1 worms, exhibiting reduced levels of Venus fluorescence and Venus-positive inclusion numbers compared with the N2 control worms (Fig. 5a–c and Supplementary Fig. 8c–f). Consistent with the reduced aggregate propagation, the rab-35 mutant worms showed reduced nerve degeneration, improved pharyngeal pumping, and increased life span (Fig. 5d–g and Supplementary Fig. 8g, h).

Next, we tried to reverse the phenotypes of rab-35 mutant worms with ectopic expression of either the wild-type or T72A mutant form of human RAB35 protein; the latter is deficient in phosphorylation by LRRK2. We expressed either WT or T72A RAB35 transgenes in BicF C. elegans, and the expression levels of human RAB35 were comparable (Supplementary Fig. 9a, b and d, e). Expression of the wild-type human RAB35 was able to recover the propagation of a-synuclein, whereas that of T72A RAB35 was not (Fig. 6a–c and Supplementary Fig. 9f, g and j, k). Likewise, nerve degeneration, pumping rate, and life span were significantly exacerbated in the wild-type RAB35-expressing worms, whereas the T72A RAB35-expressing worms did not show significant differences in these phenotypes compared with the rab-35 mutant worm itself (Fig. 6d–g and Supplementary Fig. 9n, o).

We then examined whether expression of constitutively active RAB35 (Rab35 Q67L) can reverse the phenotypes of lrk-1 mutant worms. We generated three independent RAB35 Q67L transgenic lines in the lrk-1 mutant BicF worms (Supplementary Fig. 9c–e). RAB35 Q67L worms reversed the lrk-1 phenotypes, increasing the propagation of a-synuclein and inclusion formation (Fig. 7a–c and Supplementary Fig. 9h, i and l, m). These worms exacerbated the degenerative phenotypes, such as nerve degeneration, pharyngeal pumping behavior, and life span, compared with the lrk-1 worms (Fig. 7d–g and Supplementary Fig. 9p, q). Collectively, these results suggest that phosphorylation of RAB35 on Thr72 and subsequent activation of this protein is the downstream effector of lrk-1 in the regulation of a-synuclein propagation.

**Effect of an LRRK2 inhibitor on a-synuclein pathology.** To investigate the role of LRRK2 kinase activity in a-synuclein deposition, we administered an LRRK2 inhibitor (HG-10-102-01, 10 mg kg⁻¹) via intraperitoneal (IP) injection to either non-transgenic (non-tg) or a-synuclein tg mice for 4 weeks. Inhibition of LRRK2 kinase activity was confirmed by measuring LRRK2 autophosphorylation in the brain homogenates, which showed a significant 70% reduction in non-tg and a-synuclein tg mice treated with HG-10-102-01 compared with vehicle (Supplementary Fig. 10a, e). Levels of total LRRK2 were not different among the four groups (Supplementary Fig. 10a, d). Immunohistochemical analysis demonstrated that in the vehicle-treated a-synuclein tg mice, there was abnormal accumulation of a-synuclein aggregates in the neocortex (Fig. 8a–c), striatum (Fig. 8a, d), and corpus callosum (Fig. 8a, e). Moreover, a-synuclein was highly accumulated in neuronal cell bodies (Fig. 8a–c), neuropils (Fig. 8a, b–e), and axons (Fig. 8a–d) of a-synuclein tg mice. The a-synuclein aggregates were highly phosphorylated (pSer129) (Fig. 8f–h) and showed proteinase-K (PK) resistance (Fig. 8f, i, j). Administration of the LRRK2 inhibitor significantly decreased the levels of a-synuclein aggregates in a-synuclein tg mice in the neocortex, striatum and corpus callosum (Fig. 8a–e). The levels of phosphorylated α-synuclein in the corpus callosum (Fig. 8f, h) and PK-resistant aggregates in the neocortex and corpus callosum (Fig. 8f, i, j) were also decreased by LRRK2 inhibitor treatment. In particular, administration of the LRRK2 inhibitor significantly reduced trans-axonal α-synuclein aggregates, a possible mechanism for a-synuclein propagation. Immunoblot analysis also showed that in the vehicle-treated a-synuclein tg mice there was an accumulation of both α-synuclein monomers and high molecular weight aggregates, which were clearly decreased by treatment with the LRRK2 inhibitor (Supplementary Fig. 10a, b). Taken together, these results support the notion that functional inhibition of LRRK2 kinase ameliorates a-synuclein pathology in a mouse model of PD.

**LRRK2 modulates α-synuclein trafficking.** To investigate the mechanisms through which inhibition of LRRK2 might reduce α-synuclein pathology, we next analyzed the levels of RAB35 and their relationship with α-synuclein and lysosomal markers. By western blot, the levels of RAB35 were increased in vehicle-treated α-synuclein tg mouse brains, whereas treatment with the LRRK2 kinase inhibitor reduced RAB35 in tg mice to levels comparable to those in non-tg controls (Supplementary Fig. 10a, c). Our attempts to measure the levels of phospho-RAB35 were unsuccessful, which needs to be addressed in the future. Similar to the western blot results, immunocytochemical analysis demonstrated that the levels of RAB35 were increased in the neocortex.
of vehicle-treated α-synuclein tg mice (Fig. 9a–c). The confocal imaging analysis showed that RAB35 was localized to punctate intraneuronal structures consistent with endosomes; in the vehicle- and LRRK2 inhibitor-treated non-tg mice, these granular structures measured on average 0.942±0.08 and 0.903±0.05 μm, respectively, while in the vehicle-treated α-synuclein tg mice, they measured 1.707±0.19 μm. Treatment with the LRRK2 inhibitor normalized the size of the RAB35-positive endosomes (0.935 ±0.07 μm) in the α-synuclein tg mice. Next, we analyzed co-localization of α-synuclein to the RAB35-positive endosomes. This analysis showed minimal co-localization of α-synuclein with the RAB35 structures in the neuronal cell bodies in the neocortex.
Fig. 4 RAB35 mediates LRRK2-induced propagation of α-synuclein. a-c Localization of transferred α-synuclein in the co-culture of α-synuclein overexpressing SH-SY5Y cells (donor cells) and naïve SH-SY5Y cells (recipient cells labeled with Q tracker). a The localization of transmitted α-synuclein was analyzed by co-immunostaining with RAB proteins (RA81, RA88, and RAB35). Red: RAB proteins, Blue: Qdot 585, recipient cell marker, arrowhead: transmitted α-synuclein, colocalized with RAB proteins, scale bar: 20 μm. The percentage of RAB (+) transmitted α-synuclein was quantified in b. Three independent experiments were performed. Three hundred cells were analyzed per each experiment. F(2,6)=16.09, *p < 0.05 by one-way ANOVA with Dunnett’s post hoc test. The pearson’s coefficient was calculated in (c). Three independent experiments were performed. Fifty cells were analyzed per each experiment. F(2,6)=68.1, ***p < 0.005 determined by one-way ANOVA with Dunnett’s post hoc test. d-g Interaction between LRRK2 and RAB35. WT: LRRK2 WT, GS: LRRK2 G2019S. Pull-down assay of 6X histidine conjugated LRRK2 with nickel beads. The relative levels of eluted RAB35 were calculated in e. N = 3, F(3,8)=39.08, ns: not significant, ****p < 0.0001 by one-way ANOVA with Tukey’s post hoc test. f Immunoprecipitation of RAB35 with 3F10, monoclonal antibody against HA tag. Mouse IgG was used as a control. The relative levels of eluted LRRK2 were quantified in g. N = 3, F(3,8)=66.84, ns: not significant, ****p < 0.0001 by one-way ANOVA with Tukey’s post hoc test. h, i Dominant-negative mutant RAB35 S22N (RAB35 DN) ameliorated LRRK2-induced propagation of α-synuclein. Propagation of α-synuclein aggregates were calculated by measuring the Venus BiFC (+) cell (%). Arrowhead: Venus BiFC (+) puncta, red: RAB35, blue: nuclei, scale: 20 μm. The percentage of Venus BiFC (+) cells was calculated in (i). Five independent experiments were performed. Three hundred cells were analyzed per each experiment. F(4,20)=15.79, ns: non-significant, **p < 0.01 by one-way ANOVA with Dunnett’s post hoc test. Data are represented as mean ± SEM.

Fig. 5 Mutation in rab-35 resulted in the similar phenotypes as lrr-1. a, b Venus BiFC fluorescence in wild-type and rab-35 transgenic models at day 13. The red arrowheads point to Venus BiFC-positive inclusions in pharynx. Twenty worms for each line were used (N = 3 in each group), *p < 0.05, Scale bars: 200 μm. c Worms with Venus BiFC-positive inclusions were quantified at the different ages. The color bars in graph (c) indicate percentages of Venus BiFC-positive inclusions. Thirty worms for each line were used (N = 3 in each group), *P < 0.05. d Quantification of worms that have axonal blebs. e Nerve fragmentation of transgenic line at day 13. f Relative pharyngeal pumping rates at day 13. Thirty worms for each line were used (N = 3 in each group), *p < 0.05. All error bars represent SEM, P value, *p < 0.05, was measured by unpaired, two-tailed Student’s t test. g Mean life span. One hundred worms for each line were used (N = 3 in each group). F(3, 9)=33.730, P value,”p < 0.05, was calculated by one-way ANOVA with Tukey’s post hoc test. All values are represented as mean ± SEM of the non-tg mice (vehicle and LRRK2 inhibitor). In contrast, the vehicle-treated α-synuclein tg mice displayed a significant increase in co-localization between these two markers, and treatment with the LRRK2 inhibitor reduced the % co-localization in the α-synuclein tg mice (Fig. 9a, b). Finally, we evaluated whether the effects of the LRRK2 inhibitor on RAB35 resulted in α-synuclein clearance via lysosomes. For this purpose, confocal analysis was performed in sections double labeled with α-synuclein and cathepsin D. This analysis showed a low % of co-localization between α-synuclein and cathepsin D structures in neuronal cell bodies in the neocortex of the non-tg mice (vehicle and LRRK2 inhibitor). In contrast, the vehicle-treated α-synuclein tg mice showed a moderate increase in co-localization between these two markers, and treatment with the LRRK2 inhibitor significantly increased the % co-localization in the α-synuclein tg mice (Fig. 9d, e). These results suggest that while under pathological conditions α-synuclein localized to RAB35-positive compartments when LRRK2 was activated, inhibition of LRRK2 kinase resulted in reduced localization of α-synuclein in RAB35-positive compartments and increased trafficking to the lysosome (cathepsin D), which might facilitate α-synuclein clearance.

Discussion

Here, we show that LRRK2 plays a critical role in propagation of α-synuclein. The effects of LRRK2 on α-synuclein propagation require the LRRK2 kinase activity. LRRK2 promotes α-synuclein propagation by phosphorylating RAB35. Administration of a selective LRRK2 kinase inhibitor slowed the propagation of α-synuclein in culture and reduced synucleinopathy lesions in a transgenic mouse model.

Propagation of α-synuclein pathology was markedly reduced as a result of lrr-1 deletion in C. elegans and Lrrk2 ablation in rats. The similarity of the findings in these two in vivo models strengthens the likelihood of a relationship between LRRK2 and LRR-1 functions. However, interpretation of the in vivo work assessing α-synuclein propagation requires a few notes of caution. The WT and Lrrk2<sup>−/−</sup> rats used for these studies were of the
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N-terminal region that is present in LRRK2. However, the

functional overlap between these genes. The fact that both

synuclein propagation observed in the present study suggests a

important during embryogenesis and development in mammals,

might have contributed to the reduction in

RAB35 Phosphorylation is necessary for regulation of α-synuclein inclusions in

C. elegans, the lrk-1 gene has an ancient origin distinct from that of LRRK2, while perhaps representing a

converged homologue of mammalian LRRK1. LRRK1 is

important during embryogenesis and development in mammals,

but absent very soon after birth. The relationships between lrk-1 and

LRRK2, which is expressed exclusively in adult mammalian tissues, are not clear. For example, both LRK-1 and LRRK1 lack the N-terminal region that is present in LRRK2. However, the consistency in the effects of lrk-1 and mammalian Lrrk2 on α-synuclein propagation observed in the present study suggests a functional overlap between these genes. Hence, it is possible that the N-terminal region may have functions other than α-synuclein propagation, e.g., regional and cell type vulnerability. In this regard, it is interesting to note that most of the PD-linked mutations are located outside the N-terminal region in LRRK2. Another aspect of lrk-1 to consider when interpreting the worm data is that the worm lrk-1 mutants have several phenotypes, such as abnormal axonal arborizations, synaptic
dysfunction, and mislocalization of axonal proteins in dendrites.

Many of these alterations might be due to disturbed neuronal development or general worm health in the absence of a LRRK1/2 homologue and might impact α-synuclein propagation indirectly. In the present study, we did not analyze the previously described phenotypes of lrk-1 mutant worms nor did we analyze these phenotypes in rab-35 mutant worms. Therefore, it is not clear whether rab-35 is involved in the lrk-1 phenotypes other than its role in α-synuclein propagation.

In a recent study by Tyson et al., the role of LRK-1 in α-synuclein propagation was investigated in a C. elegans model. This study reported that silencing of lrk-1 expression by approximately 20% increased α-synuclein propagation, whereas in our current study, where the lrk-1 null mutant worm was used, the opposite result was observed, specifically, deletion of the lrk-1 gene enhanced the propagation. The source of this discrepancy is not known. There are several differences in experimental design between these studies, which may account for the different results. The former study employed EGFP-BiFC, whereas our study used Venus BiFC. In addition, Tyson et al. designed their worm model in such a way that they could monitor neuron-to-neuron propagation, while our model allows monitoring of propagation between neurons and muscle cells. Perhaps the most

same genetic background, albeit not littermates; this raises the possibility that other genetic variations besides the lack of Lrrk2 might have contributed to the reduction in α-synuclein spreading in deficient animals. In C. elegans, the lrk-1 gene has an ancient origin distinct from that of LRRK2, while perhaps representing a conserved homologue of mammalian LRRK1. LRRK1 is important during embryogenesis and development in mammals, but absent very soon after birth. The relationships between lrk-1 and LRRK2, which is expressed exclusively in adult mammalian tissues, are not clear. For example, both LRK-1 and LRRK1 lack the N-terminal region that is present in LRRK2. However, the consistency in the effects of lrk-1 and mammalian Lrrk2 on α-synuclein propagation observed in the present study suggests a functional overlap between these genes. Hence, it is possible that the N-terminal region may have functions other than α-synuclein propagation, e.g., regional and cell type vulnerability. In this regard, it is interesting to note that most of the PD-linked mutations are located outside the N-terminal region in LRRK2. Another aspect of lrk-1 to consider when interpreting the worm data is that the worm lrk-1 mutants have several phenotypes, such as abnormal axonal arborizations, synaptic
dysfunction, and mislocalization of axonal proteins in dendrites. Many of these alterations might be due to disturbed neuronal development or general worm health in the absence of a LRRK1/2 homologue and might impact α-synuclein propagation indirectly. In the present study, we did not analyze the previously described phenotypes of lrk-1 mutant worms nor did we analyze these phenotypes in rab-35 mutant worms. Therefore, it is not clear whether rab-35 is involved in the lrk-1 phenotypes other than its role in α-synuclein propagation.

In a recent study by Tyson et al., the role of LRK-1 in α-synuclein propagation was investigated in a C. elegans model. This study reported that silencing of lrk-1 expression by approximately 20% increased α-synuclein propagation, whereas in our current study, where the lrk-1 null mutant worm was used, the opposite result was observed, specifically, deletion of the lrk-1 gene enhanced the propagation. The source of this discrepancy is not known. There are several differences in experimental design between these studies, which may account for the different results. The former study employed EGFP-BiFC, whereas our study used Venus BiFC. In addition, Tyson et al. designed their worm model in such a way that they could monitor neuron-to-neuron propagation, while our model allows monitoring of propagation between neurons and muscle cells. Perhaps the most
important difference is the way and the extent of the manipulation of lrk-1 expression. To reduce lrk-1 expression, Tyson et al. used RNAi, resulting in a reduction of only 20–30% according to mRNA measurement. In contrast, we used mutant worms with lrk-1 deletion that completely eliminated gene expression. Therefore, it is possible that due to a dose effect and/or differences in compensatory responses, a small decrease in lrk-1 expression yields a different phenotype than that produced by complete lrk-1 depletion. It is also noteworthy that total elimination of the Lrrk2 gene in rats resulted in an effect similar to that observed in lrk-1 mutant worms, suggesting that the propagation phenotype of lrk-1 deletion is conserved in mammals and underscores a direct relationship between the absence of lrk-1 or Lrrk2 and inhibition of α-synuclein spreading.

In worm models, inclusion bodies and other degenerative phenotypes were analyzed on day 13, at which time these phenotypes are well expressed. It needs to be noted that about half the worms were already dead by day 13. Therefore, some of the quantitation values, such as 20% worms with inclusion bodies, may be underestimated because a number of the dead worms may have had inclusions before death. In the present study, the relationships among inclusion bodies, axonopathies, and mortality were not clearly addressed. The inclusion bodies may be an independent phenotype from axonal pathology and increased mortality because the worms with inclusions were less frequent than those with the axonal phenotype.

In the worm and SH-SY5Y BiFC models, it is not clear which species are transmitted from one cell to another. The data can be explained by V1S monomers being secreted by one group of cells in the co-culture and then being taken up by cells expressing SV2 monomers, or vice versa. Additionally, one cell type could release monomers, or vice versa. Additionally, one cell type could release monomers forming dimers in the medium being ruled out in an experiment in which the culture media from V1S and SV2 cells were separately collected and mixed. The latter possibility of V1S monomers being secreted by one group of cells explained by V1S monomers being secreted by one group of cells.

In cells and animal models, LRRK2 has been shown to function in vesicle trafficking through the endolysosomal and autophagic pathways. Defects in this function might lead to a reduced number of neuritic processes in developing neurons. α-Synuclein aggregates secreted from neuronal cells have been shown to be internalized into other neurons through endocytosis. These internalized aggregates were transported through the endosomal pathway and eventually wound up in lysosomes.

Therefore, it has been postulated that perturbation of this pathway interferes with the degradation of internalized α-
Synuclein, thereby inducing aggregation of endogenous α-synuclein protein. Defects in the endolysosomal pathway indeed caused an increase in α-synuclein propagation. Mutations in lysosomal hydrolases, such as GBA1 and cathepsins, augmented the propagation. Expression of mutant LRRK2 caused endolysosomal defects, leading to accumulation of abnormal lysosomal organelles. These results suggest that diversion from endosomal trafficking and subsequent lysosomal degradation are the pivotal events in the propagation of α-synuclein. Interestingly, expression of mutant LRRK2 also disturbed RAB7-dependent internalized α-synuclein and increased propagation of α-synuclein. These results suggest that diversion from endosomal trafficking and subsequent lysosomal degradation are the pivotal events in the propagation of α-synuclein. Interestingly, expression of mutant LRRK2 caused endolysosomal defects, leading to accumulation of abnormal lysosomal organelles. Expression of mutant LRRK2 also disturbed RAB7-dependent internalized α-synuclein and increased propagation of α-synuclein. These results suggest that diversion from endosomal trafficking and subsequent lysosomal degradation are the pivotal events in the propagation of α-synuclein. Interestingly, expression of mutant LRRK2 caused endolysosomal defects, leading to accumulation of abnormal lysosomal organelles.
late endosome maturation, resulting in impaired lysosomal degradation of endocytosed receptors. Therefore, LRRK2 might regulate α-synuclein propagation by perturbing endolysosomal functions, shunting the internalized α-synuclein aggregates from lysosomal degradation in recipient cells.

LRRK2 is a large multidomain protein containing a kinase domain, a GTPase domain, and several protein-interaction domains. Several researchers have searched for LRRK2-interacting proteins and the substrates of LRRK2 kinase under the assumption that these proteins might act as effectors of LRRK2 functions. In agreement with its functions in vesicle trafficking, LRRK2 has been shown to interact with RAB7L1 (RAB29), a member of the RAB protein family that is involved in selective sorting of vesicles in the transport process. The search for LRRK2 kinase substrates identified several RAB proteins, including RAB3A, RAB8A, RAB10, and RAB12. Our in vitro substrate screen identified RAB1 and RAB35 in addition to RAB3 and RAB8. Among these RAB proteins, we demonstrated that RAB35 mediates the function of LRRK2 in α-synuclein propagation.

How RAB35 activation regulates α-synuclein propagation is unclear. RAB35 plays important roles in endosomal recycling and actin filament remodeling. Through these functions, RAB35 has been recognized as the regulator of neurite outgrowth, cytokinesis, phagocytosis, cell adhesion, cell migration, cell polarity, and exosome secretion. Considering its role in endosomal recycling, it is tempting to speculate that activation of the LRRK2-RAB35 pathway would hijack the internalized α-

Fig. 9 Blockade of LRRK2 kinase regulates trafficking of α-synuclein in mice. a Non-tg and α-syn tg mice were treated with either vehicle or LRRK2 inhibitor for 4 weeks. Brain sections were double-immunolabelled with antibodies against α-synuclein and RAB35. Scale bars, 25 μm. b Co-localization analysis of α-synuclein and RAB35 in neocortex of non-tg and α-syn-tg mice. Treatment F(1,20)=79.8, Genotype F(1,20)=156, Interaction F(1,20)=79.15, *** p < 0.001, ### p < 0.001. c Fluorescence intensity analysis for RAB35 in neocortex of non-tg and α-syn tg mice. Treatment F(1,20)=18.58, Genotype F(1,20)=208.1, Interaction F(1,20)=46.07, **** p < 0.001, #### p < 0.001. d Non-tg and α-syn tg mice were administrated with either vehicle or LRRK2 inhibitor for 4 weeks. Brain sections were double-immunolabelled with antibodies against α-synuclein and Cathepsin-D. Scale bars, 25 μm. e Co-localization analysis of α-synuclein and Cathepsin D in neocortex of non-tg and α-syn-tg mice. Treatment F(1,20)=21.55, Genotype F(1,20)=65.25, Interaction F(1,20)=22.01, #### p < 0.001. All data were analyzed by using two-way ANOVA with Tukey’s post hoc test. All analysis was done with N = 6 mice per group. Data are represented as mean ± SEM.

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synuclein aggregates from the lysosomal degradation pathway, leading to amplification of aggregates and continuous propagation (Supplementary Fig. 12). Therefore, excessive activation of RAB35 might be pathogenic by causing impaired homeostasis in endosomal trafficking of α-synuclein aggregates, particularly at the crossroads between lysosomal degradation and endosomal recycling. It is interesting to note that RAB35 expression is elevated in the serum of PD patients and in brain tissues of mouse models of PD, including LRRK2 G2019S transgenic mice67. In a neuroblastoma cell model, overexpression of RAB35 led to increased aggregation and secretion of α-synuclein A53T68.

Our results showed that the LRRK2 kinase inhibitor reduced LRRK2 autophosphorylation but also the RAB35 and α-synuclein levels in mouse brain. This may be the result of the rescue effects of the LRRK2 inhibitor. Given our proposal that RAB35 phosphorylation is a downstream effect of LRRK2, direct assessment of the role of RAB35 phosphorylation in α-synuclein propagation in mice becomes important. The reduction in RAB35 levels could be due to either increased degradation or reduced synthesis of RAB35 protein upon LRRK2 inhibitor treatment. The role of LRRK2-mediated RAB35 phosphorylation in the reduction of RAB35 levels would be another interesting topic for investigation. To address these problems, one has to overcome the technical difficulties of measuring RAB35 phosphorylation in vivo.

In conclusion, LRRK2 kinase phosphorylates RAB35 and causes dyshomeostasis of α-synuclein aggregate trafficking through the endosomal pathway (Supplementary Fig. 12). Consequently, propagation of α-synuclein is augmented by LRRK2-mediated RAB35 phosphorylation. Thus, LRRK2 kinase activity and RAB35 function might be therapeutic targets for slowing or halting disease progression.

Methods

Materials

The following antibodies were used in this study: α-synuclein monoclonal antibody (1:500 dilution, #610787, BD Biosciences, San Diego, CA), α-synuclein monoclonal antibody #274 (1:500 dilution), mouse anti-human α-synuclein clone syn211 (1:0,000,000 dilution, #36-008, Merck Millipore, Darmstadt, Germany), β-actin monoclonal antibody AC-15 (1:0,000,000 dilution, A5441, Sigma-Aldrich, St. Louis, MO), Anti-MVC polyconal antibody (1:0,000,000 dilution, ab9106, Abcam, Cambridge, MA), Anti-LRRK2 (phospho S910) antibodies, UDD1 15(3) (1:0,000,000, ab133449, Abcam), Anti-LRRK2 (phospho S955) antibodies, UDD2 10(12) (1:0,000,000 dilution, ab133450, Abcam), Anti-LRRK2 (phospho S955) antibodies, MJF-R11 (75-1) (1:0,000,000 dilution, ab169521, Abcam), Rab1 monoclonal antibody (1:0,000,000 dilution, sc-515308, Santa Cruz Biotechnology, Santa Cruz, CA), Rab8 monoclonal antibody (1:500,000 dilution, #610844, BD Biosciences), RAB35 monoclonal antibody (1:0,000,000 dilution, #113229-2-AP, ProteinTech Group Inc., Chicago, IL), 3F10 rat monoclonal antibody (#1251867001, Sigma-Aldrich), HRP-conjugated goat anti-rabbit IgG (H+L+) (1:1,000,000 dilution, #78766, Bio-Rad Laboratories, Hercules, CA), and HRP-conjugated goat anti-rabbit IgG (H+L+) (1:1,000,000 dilution, #707651, Bio-Rad Laboratories). Fluorescence dye-conjugated goat anti-rabbit IgG was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). TO-PRO-3 iodide (T3605) and Qtracker 585 cell labeling kit were purchased from Invitrogen (Carlsbad, CA).

Fluorescence microscopy of live worms.

Worms were collected, washed with M9 buffer (22 mM KH2PO4, 22 mM Na2HPO4, 85 mM NaCl, 1 mM MgSO4), and then immobilized with M9 buffer containing sodium azide (S2002, Sigma-Aldrich), and subsequently PCRF reaction for target genes was performed in Bio-Rad MyCycler PCR Thermal Cycler system (Bio-Rad Laboratories Inc., Hercules, CA).

Western blotting of C. elegans.

Protein samples derived from each BiFC transgenic line were loaded onto 12% SDS-PAGE gels, and then transferred to a nitrocellulose membrane. The following antibodies were used for immunoblotting in this study: monoclonal anti-α-synuclein antibody, Syn-1 (1:1,500 dilution; #610787, BD BioScience), polyclonal anti-RAB35 antibody (1:200 dilution; #113229-2-AP, ProteinTech Group Inc.), HRP-conjugated goat anti-mouse IgG (H+L+) (1:1,000,000 dilution, #707651, Bio-Rad Laboratories), HRP-conjugated goat anti-rabbit IgG (H+L+) (1:1,000,000 dilution, #707651, Bio-Rad Laboratories). Chemiluminescence detection was performed with ECL prime solution (GE Healthcare Life Sciences Amersham, RP22232) using the Amersham imager 600 (GE Healthcare Life Sciences, Marlborough, MA, USA). Images were quantified with Multi Gauge (v3.0) software (Fujifilm, Tokyo, Japan).

Plasmid construction for C. elegans.

Plasmids for C. elegans, including Pmyo-2::V1S and Pflp-21::SV2-ICR-DsRed vector, were generated as described below. The hrk-1 promoter (Phrk-1) was amplified from genomic DNA obtained from wild-type N2 worms. Phrk-1 was generated by replacing the PCR product with the Pmyo-2::V1S vector69. To make vectors containing human RAB35 (hRAB35) wild-type (WT) and mutants, a sense primer containing a SalI site, 5′- ATC GTC GAC ATG GTC GGC GGC GAC TAC GAC CA-3′ and an antisense primer containing BgfII site, 5′- TAG AAG TCT CTA GCA GCA GCG TTT CTT TCG-3′ were used to amplify the hRAB35 WT obtained from pcDNA3.1 Myc-His RAB35 vector, generous gifts from BD Lee (Kyoung Hee University, Seoul, Korea). The V15 fragment of Phrk-1::V1S was replaced by the PCR-amplified hRAB35 WT fragment to construct Phrk-1::hRAB35 WT. Based on Phrk-1::hRAB35 WT, Phrk-1::hRAB35 Q67L, which was created by substitution of a leucine for a glutamine acid at the 67th codon site, and Phrk-1::hRAB35 T72A, which was produced by replacing a threonine with an alanine acid at the 72th codon position, plasmids were generated using a QuickChange Site-Directed Mutagenesis Kit (#200521, Stratagene, La Jolla, CA).

Life span assay

Life span of N2, mutants and transgenic lines, was counted for 1 min at room temperature (RT) using Axio Observer A1 inverted microscope (Carl Zeiss MicroImaging Inc., Gottingen, Germany). The values were expressed as Pumps Per Minute (PPM).

Vagal AAV injections in rats.

Recombinant AAV (serotype 2 genome and serotype 6 capsid) was used for transgene expression of human wild-type α-synuclein under the control of the human Synapsin1 promoter (Sirion Biotech, Martinsried, Germany). The AAV genome contained a woodchuck hepatitis virus post-
transcriptional regulatory element and a polyadenylation signal sequence downstream to the human α-synuclein sequence. All experiments were approved by the ethical committees of the State Agency for Nature, Environment and Consumer Protection in North Rhine Westphalia. Surgical procedures were performed as described below. Young adult (200-250 g) female WT controls and Lrrk2−/−/ rats on the Charles River Long-Evans background strain (LEH-Lrrk2 m1sage, Horizon Discovery, Saint Louis, MO, USA) were anesthetized with 2% isoflurane mixed with O2 and N2O. An incision was made at the same line of the rat neck, the left vagus nerve was isolated, and the vector solution (2 µl) was injected at a titer of 7.5 × 10^12 genome copies ml−1. WT and Lrrk2−/− rats were treated in parallel. At 8 and 12 weeks after AAV injections, animals were killed with pentobarbitol and perfused through the ascending aorta with saline and 4% (w/v) paraformaldehyde. Brains were immersion-fixed in 4% paraformaldehyde and cryopreserved in 25% (w/v) sucrose solution.

### Molecular biology analyses in Lrrk2−/− rats

To assess expression of LRRK2 in WT and Lrrk2−/− rats, total RNA was extracted from paraformaldehyde-fixed cortical tissue using the RecoverAllTM Total Nucleic Acid Isolation kit (Ambion, TX, USA), and cDNA was synthesized using SuperScriptTM VILOSTM Master Mix (Thermo Fisher, Carlsbad, CA, USA). The following primers were used for RT-PCR analysis: 5′-CAG TTC ATG ACC CAG AGA GCC CTG-3′ forward and 5′-TGT GCC CAC CAG AAT CGC CG-3′ reverse (Lrrk2); 5′-GAC CGG TTC TGTC AATG CTG G-3′ forward and 5′-ACC TGG TTC ATC ATC AAC-3′ reverse (Hprt). PCR products were separated on agarose gel.

### Histological analyses in the AAV rat model

Coronal brain sections (40 µm) were cut using a freezing microtome, and free-floating sections were processed for immunohistochemistry.

### Preparation of conditioned medium

Preparation of conditioned medium. SH-SY5Y cells were transduced by lentiviral vectors for 6 h. After washing out the viruses, the cell lysates were incubated with serum-free DMEM and incubated with serum-free DMEM for 18 h. Medium was collected described above. After 2 days of infection, cells were washed three times with fresh medium, and the culture media was collected. Following the centrifugation to remove the cell debris, the secondary secretion of BfC(+) α-synuclein aggregates was analyzed by measuring the Venus BiFC fluorescence in the culture media obtained from co-culture.

### Inhibition of LRRK2 kinase activity in cell culture

The secretion of Venus BiFC-α-synuclein aggregates was performed as described below. After 2 days co-culture of V1S cells and SV2 cells, the culture media was collected. Following centrifugation to remove the cell debris, the secondary secretion of BfC(+) α-synuclein aggregates was confirmed by the reduction in the autofluoresphorylation of LRRK2.

SH-SYSY co-culture assay. The recipient cells were labeled with a Qtracker 585 cell labeling kit. Q dot labeled recipient cells were co-cultured for 3 days with donor SH-SYSY cells infected with a α-synuclein adenoviral vector. After immunofluorescence staining, the transmitted α-synuclein was observed by using an Olympus FV1000 confocal laser scanning microscope (Olympus, Tokyo).

### Immunofluorescence staining

Cells grown on poly-L-lysine-coated coverslips were fixed with 4% paraformaldehyde in Phosphate-buffered saline (PBS). After permeabilization with 0.1% Triton X-100 in PBS, cells were incubated with blocking solution (5% bovine serum albumin/3% goat serum in PBS). After blocking, cells were incubated with primary antibodies diluted in blocking solution and washed three times with ice-cold PBS. After incubation with fluorescent dye-conjugated secondary antibodies diluted in blocking solution, nuclei were stained with TOPRO-3 iodide (T3650, Invitrogen). Cells were mounted onto slide glasses in the presence of Prolong Gold Antifade Reagent (P10693, Invitrogen). Images were obtained by using Olympus FV1000 confocal laser scanning microscope.

### Colonization assay

Colony density in cell culture models, 300 cells which contain transmitted α-synuclein were analyzed from each treatment per experiment. Colonization was analyzed by using NIH Image J, Fiji software. Pearson’s coefficients analysis was performed using the Coloc 2 plugin in ImageJ. The values were obtained from three independent experiments.

### Preparation of cell extracts

Cells were washed with ice-cold PBS and lysed in extraction buffer (1% Triton X-100, 1% (w/v) protease inhibitor cocktail (P8340, Sigma-Aldrich) in PBS). Cell lysates were incubated on ice for 10 min and centrifuged at 16000 × g for 10 min. After collecting supernatant, Triton X-100 soluble fraction, Triton X-100 insoluble fraction was resuspended with 1X Laemmli sample buffer and sonicated briefly.

### Immuno-precipitation of HA-tagged human RAB35

In the case of immuno-precipitation of HA-tagged human RAB35, the cell lysates were loaded to equilibrated Ni-NTA agarose (Qiagen, Valencia, CA). After incubation for overnight at 4 °C, beads were washed with washing buffer A (1% Triton X-100 and 20 mM imidazole in PBS), followed by washing twice with buffer B (1% Triton X-100 and 30 mM imidazole in PBS) and buffer C (1% Triton X-100 and 50 mM imidazole in PBS). The protein conjugates were eluted with 2x Laemmli and boiled at 95 °C for 10 min. Eluates were analyzed by western blotting. The relative levels of eluted RAB35 were calculated the ratio versus eluted RAB35 level in SH-SYSY cells overexpressing human α-synuclein. After incubation at 37 °C in a CO2 incubator for the indicated times, the levels of cumulative α-synuclein were measured by western blotting. The levels of internalized α-synuclein were normalized with those of β-actin. The relative levels of α-synuclein in steady states represent the ratio to the maximum level of the internalized total α-synuclein in mock-transduced SH-SYSY cells.

### Western blotting

Protein samples derived from cells were loaded onto 8% or 12% SDS-PAGE gels, and then transferred to a nitrocellulose membrane. After blocking with skim milk, membranes were incubated with indicated primary antibodies. The membranes were incubated with HRP-conjugated secondary antibodies linked. Each was reacted with Amersham ECL prime western blotting substrate (GE healthcare). Images were obtained using the Amersham imager 600 (GE Healthcare Life Sciences, Institute).
Animal treatment: Transgenic mice expressing wild-type human α-synuclein under the mHty1 promoter were used. Briefly, age-matched (9-month-old) 12 non-tg and 12 α-syn-tg mice were injected intraperitoneally with either vehicle (6 mice per group, 10% DMSO, 5% PEG400, 45% Water) or LRRK2 inhibitor (6 mice per group, 10 mg kg⁻¹ HG-10-102-01) for 4 weeks (injected 5 days per week). The right hemi-brains were snap-frozen and stored at −70 °C for biochemistry and pathology analysis, while the other hemi-brains were fixed with 4% paraformaldehyde for neuro-pathological analysis. All animal procedures were approved by the UCSD Institutional Animal Care and Use Committee under protocol #S02221.

Immunohistochemical analysis: Blinded-codged salittal brain sections were treated with primary antibodies against α-synuclein (Syn-1, BD Biosciences) or phospho-α-synuclein (phospho-Ser129, Wako) overnight. To detect proteinase K (PK) resistant α-synuclein, brain sections were pre-incubated with PK (10 μg ml⁻¹) for 8 min, and immunostained against antibody to total α-synuclein (BD Biosciences). Next day, the sections were incubated with biotinylated-secondary antibodies and detected with avidin D-HRP (ABC elite, Vector Laboratories, Burlingame, CA). To determine α-synuclein neuropathology, the brain sections were imaged by Olympus BX41 microscope. The levels of immunoreactivity were determined by optical density analysis using Image Quant 1.43 program (NIH). The numbers of inclusion positive cells were determined per field (100 square μm) of each animal based on cell body recognition using Image Quant 1.43 program (NIH).

Brain tissue extract and western blot analysis: Brains of mice were homogenized with lysis buffer (1 mM HEPES, 5 mM benazamidine, 2 mM 2-mercaptoethanol, 3 mM EDTA, 0.5 mM MOPS, 0.05% NaN₃, protease inhibitor cocktail III, phosphatase inhibitor cocktail II), and centrifuged at 100,000 g for 1 h to obtain cytosolic and particulate (membrane bound) fractions. The fractions were loaded onto 4–12% Bis-Tris SDS-PAGE gels (Invitrogen), transferred onto PVDF membranes, and blocked with bovine serum albumin. After an incubation with indicated primary antibodies, the membranes were incubated with HRP-conjugated secondary antibodies (American Qualex, San Clemente, CA), and reacted with ECL western blotting substrate (PerkinElmer, Waltham, MA). Chemiluminescence detection and densitometry analysis were performed using Versadoc XL imaging apparatus and Quantity One (Bio-rad, Hercules, CA).

Statistical analysis: Values shown in the figures were presented as mean ± S.E.M. To determine the statistical significance, P values were calculated by means of unpaired, two-tailed Student’s t-tests, one-way ANOVA with Tukey’s post hoc tests, one-way ANOVA with Dunnets post hoc test, two-way ANOVA with Dunnets post hoc test, or two-way ANOVA with Tukey’s post hoc test using GraphPad Prism version 7.00 for Mac, Graphpad Software, La Jolla California USA (www.graphpad.com).
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Author contributions
E.J.B., C.L., and H.J.L. designed and performed cell biological experiments and analyzed the data. D.K. designed and performed C. elegans experiments and analyzed the data. C. K., M.M., A.A., and E.R. designed and performed mouse experiments and analyzed the data. E.M. designed the mouse experiments, analyzed the data, and wrote sections of the paper. A.U. performed surgeries and carried out histological analyses and neuronal counts in AAV-injected rats; M.K. performed molecular biology analyses and axonal quantification in rats; D.A.D.M. designed experiments in rats, analyzed data and wrote sections of the paper. G.R.I., J.R.B., and B.D.L. provided the plasmids for RAB35 and essential information on RAB35 phosphorylation. S.J.L conceived and oversaw the study, designed experiments, analyzed the data, and wrote most of the paper with E.J.B.

Additional information
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