LRRK2 delays degradative receptor trafficking by impeding late endosomal budding through decreasing Rab7 activity

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Abstract

Mutations in the leucine-rich repeat kinase 2 (LRRK2) gene cause late-onset autosomal dominant Parkinson’s disease (PD), and sequence variations at the LRRK2 locus are associated with increased risk for sporadic PD. LRRK2 contains both GTPase and kinase domains flanked by protein interaction motifs, and mutations associated with familial PD have been described for both catalytic domains. LRRK2 has been implicated in diverse cellular processes, and recent evidence pinpoints to an important role for LRRK2 in modulating a variety of intracellular membrane trafficking pathways. However, the underlying mechanisms are poorly understood. Here, by studying the classical, well-understood, degradative trafficking pathway of the epidermal growth factor receptor (EGFR), we show that LRRK2 regulates endocytic membrane trafficking in a Rab7-dependent manner. Mutant LRRK2 expression causes a slight delay in early-to-late endosomal trafficking, and a pronounced delay in trafficking out of late endosomes, which become aberrantly elongated into tubules. This is accompanied by a delay in EGFR degradation. The LRRK2-mediated deficits in EGFR trafficking and degradation can be reverted upon coexpression of active Rab7 and of a series of proteins involved in bridging the EGFR to Rab7 on late endosomes. Effector pull-down assays indicate that pathogenic LRRK2 decreases Rab7 activity both in cells overexpressing LRRK2, as well as in fibroblasts from pathogenic mutant LRRK2 PD patients as compared to healthy controls. Together, these findings provide novel insights into a previously unknown regulation of Rab7 activity by mutant LRRK2 which impairs membrane trafficking at very late stages of the endocytic pathway.
Introduction

Mutations in the Leucine-Rich Repeat Kinase 2 (LRRK2) gene are a common cause of autosomal dominantly-inherited Parkinson’s disease (PD), and risk factor variants contribute to the risk of sporadic PD (1-4). However, despite the importance of LRRK2 for the pathogenesis of both familial and sporadic PD, its biological function, and the mechanism(s) by which pathogenic mutations cause neurodegeneration in PD remain largely unknown. LRRK2 is a large multi-domain containing protein with various protein-interaction domains, a Ras-of-complex (Roc) GTPase domain in tandem with a C-terminal of Roc (COR) domain, and a protein kinase domain. LRRK2 displays GTPase and kinase activities in vitro, and PD-associated mutations either enhance kinase activity (G2019S) or decrease GTPase activity (R1441C/G/H, Y1699C) (5-16). Such altered activities seem to correlate with increased neuronal toxicity in vivo (6,11-13,15,17).

LRRK2 is widely expressed in non-neuronal cell types, with modest expression in the brain (18). Within cells, it is associated with a number of membraneous and vesicular structures suggestive of a role in intracellular membrane trafficking events (19-21). Indeed, LRRK2 has been shown to regulate synaptic vesicle trafficking (22,23), endocytosis (23,24), the autophagy-lysosomal pathway (25-30), retromer-mediated retrograde trafficking from late endosomes to the trans-Golgi network (31) and Golgi complex integrity (15,32,33). How mutant LRRK2 induces such divergent effects on intracellular membrane trafficking pathways remains unclear, and may involve a large range of distinct molecular mechanisms. Alternatively, since membrane trafficking pathways are highly interconnected and interdependent, a small number of LRRK2-mediated cellular alterations may give rise to the large spectrum of observed trafficking alterations.
In the present study, we evaluated effects of wildtype and pathogenic LRRK2 on endocytic trafficking of the epidermal growth factor receptor (EGFR). The trafficking route of the EGFR is purely degradative (34), and distinct from other receptors such as the transferrin receptor, which is recycled back to the cell surface via a recycling compartment, or the mannose 6-phosphate receptor, which undergoes retromer-mediated retrieval back to the Golgi from a late endosomal compartment. Upon binding EGF, the EGFR is internalized by clathrin-mediated endocytosis, and subsequently sorted to the lysosome for degradation. Such trafficking seems dependent on CIN85 (cbl-interacting protein of 85 kDa), which interacts with the late endosomal GTPase Rab7 to recruit dynamin 2 (dyn2), a large GTPase which deforms lipid bilayers causing vesicle scission. Action of the CIN85-dyn2 complex results in post-endosomal budding necessary for EGFR trafficking from a late endosomal to lysosomal compartment for subsequent degradation (35).

Here, we find that LRRK2 causes a delay in EGFR degradation. This is due to a delay in receptor trafficking out of late endosomes which become aberrantly elongated into tubules. Overexpression of CIN85 or dyn2 rescues the LRRK2-induced deficits in late endosomal trafficking, and a similar rescue is observed when overexpressing active Rab7. We find that pathogenic LRRK2 overexpression causes a significant decrease in the levels of active Rab7, and decreased Rab7 activity is also observed in fibroblasts from PD patients carrying a G2019S LRRK2 mutation as compared to healthy controls. Our data implicate pathogenic LRRK2 in the regulation of membrane dynamics important for late endosomal trafficking steps through a mechanism which decreases Rab7 activity, and highlight possible targets for novel therapeutic interventions against LRRK2-induced PD.
Results

Pathogenic LRRK2 delays EGFR degradation

To investigate the role of LRRK2 in EGFR internalization and degradation, we expressed epitope-tagged LRRK2 constructs in HeLa cells and assessed binding and degradation of fluorescently labelled EGF (Alexa555-EGF). Binding of Alexa555-EGF at 4 °C was reduced in the presence of wildtype LRRK2, and this effect was more pronounced in the presence of pathogenic G2019S or R1441C-mutants (Fig. 1A,B). To monitor endocytic movement in cells overexpressing wildtype or mutant LRRK2, cells were incubated with Alexa555-EGF at 4 °C, followed by washing to remove labeled EGF from the medium, and endocytosed Alexa555-EGF was quantified 10 and 30 min upon incubation of cells at 37 °C. Under these conditions, we observed a slight delay in Alexa555-EGF clearance in wildtype LRRK2, and a very pronounced delay in G2019S- and R1441C-mutant LRRK2-expressing cells (Fig. 1C). Such delay was not observed with the kinase-inactive K1906M LRRK2 mutant, which was expressed to similar levels as wildtype or mutant LRRK2 (Fig. 1D), suggesting that the observed effects are LRRK2 kinase activity-dependent. Further support for this notion was obtained when using two distinct and highly selective LRRK2 kinase inhibitors (36,37). Upon prior incubation of cells for 4 h with either 100 nM CZC-25146 (36) or 500 nM GSK2578215A (37), there was no significant change in Alexa555-EGF binding or clearance of Alexa555-EGF in control cells, or in cells expressing wildtype or K1906M mutant LRRK2 (Fig. 2A,B). However, both kinase inhibitors partially rescued the decrease in binding, and fully rescued the delay in clearance of fluorescently labelled EGF in cells expressing either G2019S or R1441C mutant LRRK2 (Fig. 2A,B). Thus, the observed effects seem to be mediated, at least in part, by the kinase activity of pathogenic LRRK2.
As the CIN85-dyn2 complex is important for trafficking of the EGFR out of a late endosomal compartment, we next expressed dominant-negative versions of either CIN85 (CIN85-dn) or dyn2 (dyn2-dn) unable to bind to each other (35). Overexpression of either CIN85-dn or dyn2-dn mimicked the effects of mutant LRRK2, both in terms of Alexa555-EGF surface binding and delayed clearance (Fig. S1). To determine whether the changes in intracellular fluorescent EGF were due to altered EGFR degradation, we performed biochemical EGFR degradation assays in transfected HEK293T cells expressing wildtype or mutant LRRK2. Indeed, cells expressing mutant LRRK2 showed a delay in EGFR degradation compared to mock-transfected cells (Fig. 1E), similarly to what has been previously described when overexpressing CIN85-dn or dyn2-dn binding mutants (35).

**LRRK2 regulates late endosomal EGFR trafficking**

Rab5 and Rab7 primarily associate with early and late endosomes, respectively. We next asked whether LRRK2 affects the switch from Rab5 to Rab7 during early-to-late endosomal transition. For this purpose, we coexpressed GFP-tagged versions of Rab5 or Rab7 with LRRK2, and visualized trafficking of fluorescent EGF through these compartments. Previously, we assured that overexpression of GFP-tagged Rab5 or Rab7 did not alter EGFR trafficking in either the absence or presence of mutant LRRK2 (Fig. 3A,B). Most Rab5-positive endosomes were distributed in the cell periphery, whilst Rab7-positive late endosomes were more commonly observed in a perinuclear region (Fig. 3C,D). In mock-transfected cells, at 10 min after a brief pulse of Alexa555-EGF stimulation, most of the fluorescent EGF was found in early endosomes, as evidenced by its colocalization with Rab5 (Fig. 3C,E). After 15 min, most EGF had disappeared out of early endosomes, as evidenced by decreased co-localization with Rab5, and this
decrease was accompanied by an increase in the colocalization with Rab7, indicative of the progression of the EGFR to late endosomal compartments (Fig. 3D, E). In G2019S and R1441C mutant LRRK2-transfected cells, there was a slight decrease in the colocalization of fluorescent EGF with Rab5 after 10 min, and a slight but non-significant increase after 15 min. Importantly, there was a pronounced decrease in the colocalization of fluorescent EGF with Rab7 after 15 min in LRRK2-transfected cells as compared to mock-transfected cells, suggesting impaired entry of the EGFR into the Rab7-positive compartment (Fig. 3E).

The EGFR on endosomes is sorted into intraluminal vesicles (ILVs) within multivesicular bodies (MVBs) prior to delivery to lysosomes for degradation. We examined whether LRRK2 expression affects the sorting of the EGFR into ILVs of GFP-Rab5-Q79L-induced enlarged endosomes upon fluorescent EGF stimulation (Fig. 4 A,B). Overexpression of GFP-Rab5-Q79L did not alter EGFR trafficking in either the absence or presence of mutant LRRK2 (not shown). Quantification of EGF labeling showed that most of the labeling was localized inside individual endosomes and not retained on the limiting membrane in mock-transfected cells. In contrast, both G2019S and R1441C mutant LRRK2 significantly reduced the intraluminal transport of Alexa555-EGF into enlarged endosomes (Fig. 4A,B). Line intensity profiles of individual endosomes showed that most of the EGF was localized within the inner membranes in control cells, but was retained in the limiting membrane in mutant LRRK2-expressing cells (Fig. 4A,B). Thus, intraluminal transport of Alexa555-EGF into enlarged endosomes seems to be delayed/impaired upon LRRK2 expression. Together, these data indicate that transition of EGFR from early to late endosomes, and the sorting of the EGFR into ILVs is affected in mutant LRRK2-expressing cells, both processes which are dependent on the Rab5/7 switch.
Pathogenic LRRK2 impairs vesiculation of late endosomes

We next wondered whether LRRK2 may be recruited onto late endosomes upon EGF stimulation. Whilst overexpressed wildtype and pathogenic mutant LRRK2 were largely cytosolic (not shown), addition of fluorescent EGF allowed occasional detection of pathogenic LRRK2 on GFP-Rab7-positive late endosomal structures (Fig. 5A). Careful analysis showed that at times pathogenic LRRK2 could be detected on the outer membrane of GFP-Rab7-positive structures (Fig. 5B). To gain insight into how mutant LRRK2 may regulate trafficking events from the late endosome, we performed time-lapse microscopy of Rhodamine-EGF (RhEGF)-stimulated HeLa cells co-expressing GFP-Rab7. Expression of wildtype, and to a larger degree G2019S- or R1441C-mutant LRRK2 caused a striking difference in the morphology of GFP-Rab7-positive structures (Fig. 6A,B). Whereas mock-transfected or kinase-inactive K1906M LRRK2-transfected cells showed mainly punctate GFP-Rab7-positive structures and few, short tubules (≤ 1.5 μm), the LRRK2-expressing cells displayed an increased number of tubules, and an increased number of long tubules (> 1.5 μm) which were positive for RhEGF (Fig. 6A,B). Such phenotype was identical to that observed when overexpressing CIN85-dn mutant (Fig. 6B), as previously described (35), indicating that the vesiculation process was attenuated.

We asked whether such phenotype may also be observed in cells expressing endogenous levels of mutant LRRK2. For this purpose, we analyzed Rab7 staining in primary dermal fibroblasts from PD patients carrying the G2019S LRRK2 mutation and age- and sex-matched healthy controls. Rab7 staining was largely punctate in control fibroblasts, and no changes in the amount of Rab7 punctae per area could be observed in the G2019S LRRK2 mutant fibroblasts (Fig. 7A,B). However, most G2019S mutant
fibroblasts displayed at least one Rab7-positive tubule > 5 μm (Fig. 7C). Importantly, both CZC-25146 and GSK2578215A LRRK2 kinase inhibitors significantly decreased the amount of cells displaying rab7-positive tubules (Fig. 7D). These data indicate that pathogenic endogenous mutant LRRK2 interferes with the formation of carriers emanating from the late endosome in a kinase activity-mediated manner, resulting in a delay in EGFR degradation.

The CIN85/dyn2/Rab7 pathway is involved in the LRRK2-mediated late endosomal trafficking defects

We sought to determine whether the LRRK2-mediated defects at the late endosome may be rescued by modulating the CIN85/dyn2 complex (35). Overexpressed CIN85 and dyn2 were found to localize to Rab7-positive structures (Fig. S1), as previously described (35). Whilst overexpression of CIN85 or dyn2 had no effect on EGFR downregulation compared to mock-treated cells, they largely rescued the LRRK2-mediated delay in EGFR degradation (Fig. 8A,B). CIN85 was slightly more efficient in rescuing the trafficking deficit as compared to dyn2 (Fig. 8A). As CIN85 is thought to interact with Rab7 on late endosomes, followed by recruitment of dyn2 to vesiculate late endosomal membranes (35), we wondered whether active Rab7 may be implicated in this process. Overexpression of active, GTP-bound GFP-Rab7-Q67L did not affect EGFR trafficking in mock-transfected cells, but suppressed the delay in EGFR degradation induced by LRRK2 (Fig. 9A,B). This was not observed when expressing wildtype Rab7 (Fig. 9A,B), even though both wildtype and active mutant Rab7 were expressed to similar degrees (Fig. 9C).
Pathogenic LRRK2 downregulates the activation state of Rab7

Given the above-mentioned data, we reasoned that LRRK2 may regulate the levels and/or activity of Rab7. No change in Rab7 protein levels were observed in cells overexpressing mutant LRRK2 as compared to mock-transfected cells, fibroblasts from G2019S LRRK2 patients, or postmortem brain extracts from G2019S LRRK2 patients versus healthy controls (Fig. 10A). We thus focused on determining effects of LRRK2 on Rab7 activity. Rab protein activity is regulated by guanine nucleotide exchange factors (GEFs), which activate Rabs by facilitating the exchange of GDP for GTP, and GTPase-activating proteins (GAPs), which inactivate Rabs by accelerating the hydrolysis of the bound GTP to GDP (38,39). Rab proteins only associate with their effector proteins when GTP-bound, and this produces a downstream response associated with GTPase activation of the given Rab protein. Whilst the identity of the mammalian Rab7 GEF remains controversial, TBC1D15 seems to act as a GAP for Rab7 (40,41). Interestingly, TBC1D15 overexpression delayed EGFR degradation (Fig. 10B,C). Such delay could be rescued when expressing catalytically active, GTP-bound Rab7-Q67L but not wildtype Rab7 (Fig. 10B,C), identical to what was observed with mutant LRRK2 expression. These experiments confirm that Rab7-Q67L is functionally active in cells and resistant to the effects of TBC1D15 expression, as previously described (42).

To assess how mutant LRRK2 may affect the membrane cycling activity of Rab7, we performed fluorescence recovery after photobleaching (FRAP) experiments. HeLa cells were transfected with GFP-Rab7, and either empty vector or mutant LRRK2. A small perinuclear region containing GFP-Rab7-positive vesicles was bleached with high laser intensity, and recovery of fluorescence measured (43) (Fig. 11A). Recovery of fluorescence can only occur when the GTP-bound, membrane-bound
Rab7 dissociates from the membrane upon GTP hydrolysis and is replaced by active GFP-Rab7 from the cytosol, thus representing flux through the Rab7 activity cycle. The recovery of fluorescence in the bleached spot was plotted (Fig. 11B), allowing determination of the recovery time ($t_{1/2}$; the time in which 50% of the fluorescence in the bleached spot was recovered) and the mobile fraction $M$ (the percentage of maximally recovered GFP-Rab7). The recovery time in control cells or cells expressing K1906M mutant LRRK2 was similar to what has been previously described, with no full recovery of the initial fluorescence (43,44) (Fig. 11B,C). Cells expressing G2019S or R1441C mutant LRRK2 showed a drastically slower recovery, with similar percentage of GFP-Rab7 in the mobile fraction (Fig. 11C). These data indicate that mutant LRRK2 causes alterations in the rate of membrane exchange of Rab7 in a kinase-dependent manner.

To gain more direct evidence for a LRRK2-induced change in Rab7 activity, we employed an effector pull-down assay using the Rab7 binding domain of RILP to selectively isolate Rab7-GTP from cell lysates (41). Pull-down assays from cells expressing Rab7, or mutants that are predominantly GTP-bound (Rab7-Q67L) confirmed that Rab7-GTP was selectively isolated by GST-RILP (Fig. 12A). The fraction of endogenous Rab7 bound to GTP was drastically reduced in cells expressing TBC1D15, further confirming that TBC1D15 functions as a GAP for Rab7 in vivo (Fig. 12B). When overexpressing G2019S or R1441C mutant LRRK2, a striking decrease in the levels of endogenous Rab7-GTP was observed (Fig. 12 C,D), indicating that pathogenic LRRK2 decreases endogenous active Rab7 levels. Similar results were obtained when analyzing the amount of active Rab7 in extracts from fibroblasts from G2019S mutant LRRK2 PD patients as compared to healthy controls (Fig. 12 E,F).

Alltogether, these results suggest that pathogenic LRRK2 causes a decrease in Rab7
activity, concomitant with a delay in late endocytic trafficking and a decrease in EGFR degradation.

Discussion

In this study, we describe a novel role for pathogenic LRRK2 in decreasing Rab7 activity, with a concomitant deficit in Rab7-mediated membrane trafficking events, including a delay in the Rab5/Rab7 switch and ILV formation, a delay in the trafficking of the EGFR out of late endosomes which become aberrantly elongated, and a delay in degradation of the EGFR. Two prominent pathogenic mutations (G2019S, R1441C) in LRRK2 were found to affect the same late endosomal trafficking pathway, whilst wildtype and kinase-inactive mutant LRRK2 were largely without effect, indicating that this may be a shared pathomechanism of distinct mutant LRRK2 forms. In addition, the significant rescue observed in the presence of two distinct LRRK2 kinase inhibitors suggests that the kinase activity is, at least in part, responsible for the deficits in endosomal trafficking mediated by pathogenic LRRK2.

The effects of pathogenic LRRK2 on late endosomal trafficking could be rescued when overexpressing wildtype CIN85 and dyn2. Previous studies have shown that inhibiting the interaction of CIN85-dyn2 prevents exit of the EGFR out of late endosomes (35), similarly to what is observed when expressing a Rab7 dominant-negative mutant or knocking down Rab7 (45,46). In addition, interfering with the CIN85-dyn2 interaction causes formation of Rab7-positive late endosomal tubules containing EGF, indicating a defect in late endosomal budding (35), similar to what we observed here upon mutant LRRK2 expression. Thus, the CIN85-dyn2 complex seems to form and function at organelles involved in post-endocytic EGFR trafficking in conjunction with Rab7, and may as such recruit active Rab7, and/or modulate Rab7...
activity to bring about efficient EGFR trafficking, which would explain the observed rescue of the LRRK2-mediated late endosomal trafficking deficit with CIN85 and dyn2. CIN85 has also been shown to regulate endocytosis of the dopamine receptor (47) and of LRP6, a Wnt co-receptor (48,49), and alterations in dopamine receptor surface levels or LRP6 via LRRK2 have been observed (50,51), suggesting possible effects of the CIN85-dyn2-Rab7 link beyond the trafficking of the EGFR in signalling pathways directly relevant to PD.

Expression of GTP-locked active Rab7, but not wildtype Rab7, was found to rescue the endosomal trafficking deficits in the presence of mutant LRRK2 or upon expression of TBC1D15, a Rab7 GAP protein. The GTP-locked Rab7 mutant has been reported to be insensitive to TBC1D15 action (42), which may explain such differential rescue, and is consistent with the observed partial rescue of the neurite outgrowth phenotype in primary neurons expressing mutant LRRK2 with GTP-locked active Rab7, but not wildtype Rab7 (31).

Rab7 acts as a major regulator of a variety of intracellular trafficking steps, including endosomal maturation, transport from the late endosome to the lysosome, endo-lysosomal positioning via regulation of trafficking of cargo along microtubules, retromer-mediated trafficking, lysosome reformation and autophagosome maturation (43,45,52-55). Thus, decreased Rab7 activity would be expected to affect all above-mentioned events, albeit possibly to distinct degrees. Indeed, evidence for a role of LRRK2 in endocytosis (23,24), endo-lysosomal positioning (29), retromer-mediated trafficking (31), lysosomal abnormalities (30,56) and autophagic events (25-30,56-59) have been reported.

The given Rab7-dependent cellular process affected by mutant LRRK2 may be dependent on the precise cellular state. For example, the LRRK2-mediated effects on
lysosomal positioning have been only observed under conditions which favour high flux through the endocytic system (29), and translocation of LRRK2 to autophagosomal membranes, with concomitant effects on autophagic flux, are most prominently observed upon stimulus-dependent autophagy induction (27,58,59). Thus, distinct cellular triggers may recruit LRRK2 to distinct subcellular compartments, where it may locally alter Rab7 activity in a compartment-specific manner. Alternatively, distinct stimuli may increase the need for Rab7 activity only in select compartments, and thus preferentially uncover LRRK2-mediated effects on Rab7 activity on the vesicular trafficking steps upregulated by those stimuli, and further work will be necessary to distinguish between these possibilities.

The manner by which mutant LRRK2 decreases the amount of GTP-bound, active Rab7 requires further clarification. Conceptually, mutant LRRK2 may preferentially bind to inactive Rab7, which may alter the pool of activatable Rab7 able to be recruited to membranes. However, inconsistent with this model, our FRAP studies indicate no change in the mobile, recruitable pool of Rab7 upon mutant LRRK2 expression. Alternative scenarios include LRRK2-mediated effects on Rab7 GAP and/or GEFs, or local regulation of Rab7 activity on membranes, for example through alterations in the intraluminal ion content of vesicular Rab7 target organelles (30), and further work is warranted to dissect the precise links between luminal ion content, Rab7 activity and mutant LRRK2 action.

Apart from Rab7, LRRK2 has been shown to interact with other Rab proteins including rab5 (24) and Rab29/Rab7L1, a Golgi-resident Rab (31,33). Whilst GAPs, GEFs and effector proteins for Rab29/Rab7L1 remain to be discovered, it will be interesting to determine whether the activity of this Rab protein is altered by mutant LRRK2 as well. In sum, our data highlight a mechanism by which mutant LRRK2 may
alter various intracellular vesicular trafficking events dependent on Rab7 activity, and hopefully provide a basis for further studies aimed at elucidating pathomechanism(s) underlying LRRK2-related PD.

**Materials and Methods**

**DNA constructs and site-directed mutagenesis**

Double myc-tagged wildtype and pathogenic mutant LRRK2 constructs were obtained from Addgene, or generously provided by E. Greggio (University of Padova, Italy) and M. Cookson (NIH, Bethesda, USA). The myc-tagged kinase-dead K1906M LRRK2 construct was generated by site-directed mutagenesis (QuickChange, Stratagene), and identity of the construct verified by sequencing of the entire coding region. DNA was prepared from bacterial cultures grown at 28 °C using a midiprep kit (Promega) according to manufacturer’s instructions. GFP-Rab7, GFP-Rab7-Q67L and GFP-Rab7-T22N constructs were provided by Dr. S. Ponnambalam (University of Leeds, Leeds, UK), GFP-Rab5 by Dr. P. Woodman (Manchester University, Manchester, UK) and GFP-rab5Q79L was from Addgene. FLAG-CIN85 wild-type and mutant (ACW-Y) constructs, and FLAG-Dyn2 and FLAG-Dyn2-CBM mutant constructs were kindly provided by M. McNiven (Mayo Clinic, Rochester, USA). The Rab7 binding domain of RILP (murine, amino acids 220-299) and myc-tagged TBC1D15 constructs were generously provided by A. Edinger (University of California, California, USA).

**Cell culture and transfections**

HEK293T cells were cultured as described (30) and transfected at 80 % confluence with 2 µg of LRRK2 constructs and 6 µl of LipoD293 (SignaGen Laboratories) per well of a 6 well plate overnight in full medium. HeLa cells were
cultured in 100 mm dishes and grown at 37 °C in 5 % CO₂ in full medium (DMEM medium containing 10 % fetal bovine serum, non-essential amino acids and high glucose). Confluent cells were harvested using 0.05 % trypsin and 0.02 mM EDTA in PBS, and subcultured at a ratio of 1:4 to 1:6. Cells were plated onto 6-well plates and the following day, at 70–80 % confluency, transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer’s specifications for 4 h in DMEM, followed by replacement with fresh full medium. Single transfections were performed using 5 μg of plasmid of interest and 10 μl of Lipofectamine 2000, and double-transfections using 4 μg of LRRK2 and 1 μg of plasmid of interest. Transfected cells were re-plated the next day at a 1:3 ratio onto coverslips in 6-well plates. LRRK2 was expressed for 48 h. Cells were incubated with 500 nM GSK2578215A (Tocris) and CZC-25146 (obtained through the Michael J. Fox Foundation) for 4 h at 37 °C as indicated before analysis.

**Fibroblasts**

Age- and sex-matched human skin fibroblasts established from skin biopsies taken from five healthy control and five PD patients with the G2019S mutation, with informed consent and ethical approval, were grown in IMDM and 10 % fetal bovine serum, and media changed every two days. Cells were subcultured at a ratio of 1:2, and seeded at equal densities on coverslips. Cells were fixed with 2 % paraformaldehyde (PFA) in PBS for 10 min at room temperature, followed by permeabilization in 0.05 % saponin in PBS for 3 min, and incubated in blocking buffer (2 % goat serum (Vector Laboratories) in 0.05 % saponin in PBS) for 1 h at room temperature. Exposure to primary antibody (1:50, rabbit polyclonal, Sigma) was performed in blocking buffer for 1 h at room temperature. Cells were washed 3 times 5 min in PBS, and incubated with goat anti-rabbit AlexaFluor-488-conjugated secondary antibody (1:1000, Invitrogen)
diluted in PBS for 1 h at room temperature, followed by washes in PBS. Fixed cells were mounted using mounting medium containing DAPI (Vector Laboratories). All analyses were carried out on passages 4-12, and no passage-dependent differences were observed.

**Immunofluorescence and laser confocal imaging**

HeLa cells were fixed using 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature, and permeabilized in 0.5 % Triton-X100/PBS (3 x 5 min) followed by preincubation in blocking buffer (10% goat serum, 0.5 % Triton-X100/PBS) for 1 h at room temperature. Cells were incubated with primary antibody (mouse monoclonal anti-c-Myc, Sigma, clone 9E10, 1:100), in blocking buffer for 1 h at room temperature to identify cells overexpressing myc-tagged LRRK2. Cells were washed in 0.5 % Triton-X100/PBS and incubated with secondary antibodies (Alexa488- or Alexa594-conjugated goat anti-mouse antibodies, 1:1000; Invitrogen) for 1 h, followed by washes and mounting in mounting medium with or without DAPI (Vector Laboratories).

Images were acquired on a Leica TCS-SP5 confocal microscope using a 63X 1.4 NA oil UV objective (HCX PLAPO CS). Images were collected using single excitation for each wavelength separately (488 nm Argon Laser line and a 500-545 nm emission band pass; 543 HeNe Laser line and a 556-673 nm emission band pass; 405 nm UV diode and a 422-466 nm emission band pass (12.5 % intensity). Ten to fifteen image sections of selected areas were acquired with a step size of 0.4 μm, and z-stack images analyzed and processed using Leica Applied Systems (LAS AF6000) image acquisition software. The same laser intensity settings and exposure times were used for image acquisition of individual experiments to be quantified.
The JACoP pluggin of ImageJ was used for the quantification of colocalization of GFP-Rab5- or GFP-Rab7-positive compartments with Alexa555-EGF. After image thresholding, the percentage of colocalization was obtained by calculating the Mander’s coefficients (M1 for red channel (Alexa555-EGF)), and percentage of colocalization was obtained by multiplying M1 by 100 (54).

To measure the total number of Alexa555-EGF structures per cell, cells were circled, and a modified NIH ImageJ macro (GFP-LC3 macro) was employed. For each condition per experiment, 20 independent cells were analyzed. All analysis was done by an observer blind to conditions.

For determination of sorting of fluorescent EGF into multivesicular bodies, cells were co-transfected with empty control vector or mutant LRRK2 construct and GFP-Rab5Q79L. Cells were serum-starved and incubated with Alexa555-EGF as previously described, and fixed 20 min later. Individual enlarged endosomes (diameter > 1 µm) were imaged from distinct cells (total 50-70 endosomes scored per experiment per condition), and data are presented as percentage of endosomes where Alexa555-EGF is localized to the lumen.

**Alexa555-EGF binding and uptake assays**

Co-transfected HeLa cells were re-seeded onto coverslips the day after transfection and serum-starved for 16 h. The following day, medium was replaced with fresh, serum-free medium containing 100 ng/ml fluorescent Alexa555-EGF (Invitrogen) at 4 ºC, and cells incubated for 30 min at 4 ºC. This treatment allows ligand binding to the receptor, but prevents internalization. To confirm that labelled EGF was only surface-bound, control cells were washed twice with PBS, followed by acid stripping (0.5 M NaCl, 0.2 M acetic acid, pH 2.5) for 3 min at 4 ºC. Transfected cells were
washed twice in ice-cold PBS, and transferred to pre-warmed serum-free medium for
the indicated periods of time to allow uptake of bound Alexa555-EGF. At the assay
endpoint, cells were fixed (4% PBS in PBS, 15 min at room temperature) and softly
permeabilized (0.5% Triton-X100/PBS for 3 min). To identify myc-LRRK2-expressing
cells under those conditions, immunofluorescence was performed as described, but
detergent was omitted from all incubations.

**Rhodamine-EGF uptake and in vivo imaging**

For live-cell fluorescence microscopy, co-transfected cells were reseeded onto
35-mm glass-bottom dishes (IBIDI Biosciences) the following day, and serum-starved
for 16 h. Medium was replaced by phenol-free, serum-free DMEM (GIBCO), and cells
were incubated with Rhoadmine-EGF (Rh-EGF, 200 ng/ml, Invitrogen) for 90 min at
37 ºC before imaging as previously described (35). Time-lapse images were acquired on
a Leica TCS-SP5 confocal microscope using a 100X 1.4 NA oil UV objective (HCX
PLAPO CS). Images were taken every 1.4 sec over a total time of 86 sec for each movie
(total 60 frames) with pinhole 1.22 airy. During imaging, cells were maintained at 5 %
CO₂ and at 37ºC. The length GFP-Rab7-positive tubules were quantified using Leica
Applied Systems (LAS AF6000) image acquisition software. GFP-Rab7-positive
structures were scored over the entire imaging period as late endosomal tubules if they
were RhEGF-positive and > 900 nm. 7-15 cells were quantified per condition per each
experiment.

**Fluorescence after photobleaching (FRAP) experiments**

FRAP experiments were performed on a Leica TCS-SP5 confocal microscope at
37 ºC under 5 % CO₂ using a 63X 1.4 NA oil UV objective (HCX PLAPO CS). Cells
were co-transfected with GFP-Rab7 and either empty vector or LRRK2 constructs, and re-seeded onto 35-mm glass-bottom dishes the following day. Forty-eight hours after transfection, defined GFP-Rab7-positive endolysosomal structures which did not display long-range motility in the perinuclear region were photobleached for 10 sec at 488 nm with 100% of argon laser power. Fluorescence recovery was imaged every 20 s for a total of up to 500 sec using the Argon laser at the same power as used for pre-bleach fluorescence levels. Regions of interest (ROIs) were drawn around bleached GFP-Rab7 vesicles and the fluorescence intensity at each time point quantified using Leica Applied Systems (LAS AF6000) image acquisition software. Data were exported to Microsoft Excel and fluorescence recovery curves for each condition averaged and plotted. Pre-bleach was set to 1 and the first post-bleach time point was set to 0 for each ROI. The recovery curves were corrected for loss of total fluorescence due to bleaching and imaging.

**Cell extracts and Western blotting**

Cells were collected, washed in PBS and resuspended in cell lysis buffer (1% SDS in PBS containing 1 mM PMSF, 1 mM Na₃VO₄, 5 mM NaF). Extracts were sonicated, boiled, and centrifuged at 13’500 rpm for 10 min at 4 °C. Protein concentration of supernatants was estimated using the BCA assay (Pierce), and extracts immediately resolved by SDS-PAGE and analyzed by Western blot. Antibodies used for immunoblotting included a mouse monoclonal anti-myc antibody (1:1000, Sigma), a rabbit polyclonal anti-GFP antibody (1:2000, Abcam), a mouse monoclonal anti-flag antibody (1:1000, Sigma), a mouse monoclonal anti-tubulin antibody (clone DM1A, 1:10’000, Sigma), a rabbit polyclonal anti-Rab7 antibody (1:1000, Sigma) and a rabbit
polyclonal anti-EGFR antibody (1:500, Cell Signaling), and a mouse monoclonal anti-
LRRK2 antibody (1:1000, NeuroMab, 75-253).

For EGFR degradation assays, we employed HEK293T cells, as overexpression
and transfection efficiencies were much higher than in HeLa cells. Cells were
transfected as described above in 100 mm plates using LipoD293, and replated the
following day at 80 % confluence. The following day, cells were serum-starved for 1 h
in the presence of cycloheximide (1 µg/ml, Calbiochem) to block novel protein
synthesis, followed by stimulation with non-labelled EGF (100 ng/ml, Invitrogen) for
the indicated time points. Cell extracts were collected and processed as indicated above.
Westerns were developed with ECL reagents (Roche), and a series of timed exposures
undertaken to ensure that densitometric analyses were performed at exposures within
the linear range. Films were scanned and densitometric analysis performed using
QuantityOne (Biorad).

**Human tissues and sample preparation**

Freshly frozen brain samples from deceased human subjects were collected at
autopsy following informed consent from the next of kin under a protocol approved by
the local ethics committee. Caudate brain regions from healthy control and genotyped
LRRK2 G2019S mutant PD patients were analyzed. For all samples, patient age,
gender, time to postmortem tissue collection and postmortem pathological analysis was
known. Sample preparation and analysis was performed as previously described (60).

**GST-RILP pull-downs**

GST-RILP pull-downs were essentially performed as described (41). GST-RILP
or empty GST vectors were transformed into E. coli strain BL21. 250 ml of LB was
inoculated with 1 ml of an overnight culture and grown at 37 °C to an OD of 0.6-0.8. Isopropyl-1-thio-β-D-galactopyranoside (EMD Biosciences) (0.5 mM) was added and bacteria induced for protein production for 3-4 h at 30 °C. Bacterial cells were pelleted, washed with cold PBS and cell pellets frozen at -20 °C. The pellet was resuspended in 5 ml ice-cold purification buffer (25 mM Tris-HCl, pH 7.4, 1 M NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 0.1 % Triton-X100 and protease inhibitor cocktail (Roche)), lysates were sonicated, cleared by centrifugation, and another 5 ml of cold purification buffer was added to the supernatant. GST-RILP was purified by adding 300 μl of a pre-equilibrated 50 % slurry of glutathione-Sepharose 4B beads (GE Healthcare) and incubation for 1 h at 4 °C. Beads were washed with purification buffer, resuspended to a 50 % slurry, and kept at 4 °C. A sample (5 μl) was separated by SDS-PAGE and analyzed by Coomassie brilliant blue staining to determine protein purity, and protein concentration estimated by the BCA assay (Pierce). For best results, beads were used with cell lysates within 2 days of preparation. Transfected HEK293T cells were collected by centrifugation, washed in PBS, resuspended in pulldown buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 5 mM MgCl2, 1 % Triton-X100 and protease inhibitor cocktail (Roche)), and lysates cleared by centrifugation at 13’500 rpm for 10 min at 4 °C. GST-RILP pull-downs were performed in 1 ml pulldown buffer containing 300 μg (HEK293T) or 200 μg (human dermal fibroblasts) of cell lysate and 60 μl of 50 % slurry beads pre-equilibrated in pulldown buffer. Beads were rocked overnight at 4 °C, washed twice with ice-cold pulldown buffer, bound proteins eluted by adding 2x sample buffer/β-mercaptoethanol and incubated at 72 °C for 10 min prior to separation by SDS-PAGE.
Statistical analysis

All data are expressed as means ± s.e.m. Data were analyzed by one-way ANOVA with Tukey’s post-hoc test, and p < 0.05 was considered significant.

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Conflict of Interest statement. None declared.

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References


Figure Legends

Figure 1. LRRK2 delays EGFR degradation. (A) HeLa cells were transfected with empty pCMV vector (ctrl) or the indicated myc-tagged LRRK2 constructs, and processed for immunocytochemistry with an anti-myc antibody (blue) after binding of Alexa555-EGF for 30 min at 4 °C. Scale bar, 15 μm. (B) The amount of surface-bound fluorescent EGF was quantified using ImageJ from 20 transfected cells per condition per experiment. N=8 experiments (ctrl versus LRRK2), 7 (ctrl versus R1441C), 4 (ctrl versus G2019S) and 2 (ctrl versus K1906M) (1-way ANOVA; Tukey’s post-hoc, *p < 0.05; **p<0.005). (C) Upon binding of fluorescent EGF at 4 °C, cells were washed to remove unbound EGF, and shifted to 37 °C to allow internalization of bound EGF. Quantification of internalized Alexa555-EGF was performed at 0 min, and after 10 min and 30 min of internalization. Values are normalized to the amount of Alexa555-EGF binding at t=0. N=8 experiments (ctrl versus LRRK2), 7 (ctrl versus R1441C), 4 (ctrl versus G2019S) and 2 (ctrl versus K1906M). G2019S versus control, 1-way ANOVA, Tukey’s post-hoc, **p<0.005; R1441C versus control, 1-way ANOVA, Tukey’s post-hoc, **p<0.005. (D) Hela cells were transfected with either empty pCMV (ctrl) or the indicated LRRK2 constructs, and cell extracts (50 μg) analyzed by Western blot for overexpression levels using an anti-LRRK2 antibody, and tubulin as loading control. Arrow points to endogenous LRRK2. (E) To determine effects on EGFR degradation, we employed HEK293T cells, which are transfected at much higher efficiency with LRRK2 constructs as compared to Hela cells. Cells were transfected with empty pCMV (ctrl) or mutant LRRK2, serum-starved for 1 h in the presence of cycloheximide to block novel protein synthesis, and EGFR internalization stimulated with non-labelled EGF for the indicated time points. Cell extracts (40 μg) were analyzed by Western blot
for EGFR levels and tubulin as loading control, as well as for levels of LRRK2 protein using an anti-myc antibody. The experiment was repeated twice with similar results.

**Figure 2.** Kinase inhibitors significantly rescue decreased binding and degradation of EGF. (A) Cells were transfected with the indicated constructs, treated for 4 h with 100 nM CZC-25146 or GSK2578215A as indicated, followed by quantification of the amount of surface-bound fluorescent EGF. N=3 independent experiments. Both kinase inhibitors significantly rescued the decrease in EGF binding in the presence of pathogenic LRRK2 (G2019S versus G2019S plus CZC-25146 or GSK2578215A; 1-way ANOVA; Tukey’s post-hoc, *p < 0.05; R1441C versus R1441C plus CZC-25146 or GSK2578215A; 1-way ANOVA; Tukey’s post-hoc, *p < 0.05). (B) Quantification of internalized Alexa555-EGF was performed at 0 min, and after 10 min and 30 min of internalization with the indicated constructs in the absence or presence of kinase inhibitors. Values are normalized to the amount of Alexa555-EGF binding at t=0. N=3 independent experiments. G2019S versus control, 1-way ANOVA, Tukey’s post-hoc, **p<0.005; R1441C versus control, 1-way ANOVA, Tukey’s post-hoc, **p<0.005.

**Figure 3.** Mutant LRRK2 causes a delay in the Rab5/Rab7 switch. (A,B) HeLa cells were transfected either with empty pCMV vector (ctrl), or co-transfected with mutant LRRK2 and the indicated constructs, and Alexa555-EGF binding and uptake experiments performed as described in legend to Figure 1. N=3 experiments (1-way ANOVA; Tukey’s post-hoc, *p < 0.05; **p<0.005). (C,D) Hela cells were co-transfected with empty vector (pCMV) or mutant LRRK2, and either GFP-Rab5 or GFP-Rab7, and Alexa555-EGF uptake performed for either 10 min or 15 min. Colocalization of Alexa555-EGF with either GFP-Rab5 or GFP-Rab7 was quantified as
described in Materials and Methods. Scale bar, 20 μm. (E) The average percentage of colocalization of Alexa555-EGF with GFP-Rab5 (left) or GFP-Rab7 (right) 10 min and 15 min after shifting cells to 37 °C was quantified from 10-15 cells per experiment. N=3 experiments (G2019S versus pCMV, 1-way ANOVA; Tukey’s post-hoc, *p < 0.05; R1441C versus pCMV; 1-way ANOVA; Tukey’s post-hoc, *p < 0.05).

Figure 4. Decreased ILV formation in the presence of mutant LRRK2. (A) Left: HeLa cells were cotransfected with GFP-Rab5-Q79L and either empty vector (pCMV) or R1441C mutant LRRK2, and stimulated with Alexa555-EGF for 20 min. Scale bar, 6 μm. Middle: Images from boxed regions in control (top) or mutant LRRK2 (bottom) transfected cells showing individual enlarged endosomes. Scale bar, 1.5 μm. Right: Areas of colocalization are shown by line intensity profiles. (B) Quantification of Alexa555-EGF localization in the lumen of GFP-Rab5-Q79L-induced enlarged endosomes. Data are presented as the percentage of enlarged endosomes which containing fluorescent EGF inside their lumen. N=3 independent experiments, average of 50-70 endosomes scored per experiment. G2019S versus pCMV, 1-way ANOVA; Tukey’s post-hoc, *p < 0.05; R1441C versus pCMV; 1-way ANOVA; Tukey’s post-hoc, *p < 0.05.

Figure 5. Mutant LRRK2 can localize to late endosomes upon starvation and EGF stimulation. (A) HeLa cells were co-transfected with GFP-Rab7 and the indicated constructs, starved overnight, and Alexa555-EGF uptake performed for 60 min, followed by staining for LRRK2 with an anti-myc antibody. Scale bar, 15 μm. (B) For higher resolution images of mutant LRRK2 localization to individual GFP-Rab7-labelled late endosomes, cells were co-transfected as indicated, EGF uptake
experiments performed with non-fluorescent ligand, and staining for LRRK2 performed using an Alexa594-coupled secondary antibody. Scale bar, 4 μm.

Figure 6. Mutant LRRK2 prevents late endosomal budding and increases GFP-Rab7 tubule length. (A) Movie stills of HeLa cells co-expressing GFP-Rab7 and indicated constructs and stimulated with RhEGF. Arrows point to long Rab7-positive tubules containing RhEGF. Scale bar, 8 μm. (B) Quantification of the total number of GFP-Rab7 tubules (left), and the number of long tubules (> 1.5 μm) (right). GFP-Rab7 tubules containing RhEGF were quantified over the entire imaging period. 7-15 cells were quantified per condition in each experiment. N=4 experiments (except 3 experiments for R1441C, 2 for K1906M). 1-way ANOVA; Tukey’s post-hoc, *p < 0.05*.

Figure 7. Fibroblasts from G2019S LRRK2 PD patients display increase in Rab7-positive tubular endosomes. (A) Fibroblasts from healthy control or G2019S LRRK2 PD patients were stained for endogenous Rab7. As Rab7-positive late endosomal tubules are very sensitive to fixation and detergent conditions, a specific protocol was necessary as described in Materials and Methods. Scale bar, 30 μm. (B) Individual cells were scored for the amount of Rab7-positive structures, and fluorescent signal normalized to cell size. (C) The percentage of cells containing at least one Rab7-positive tubular structure > 5 μm was determined by analyzing between 30-50 cells per fibroblast line (n=5 control lines, n=5 G2019S LRRK2 PD lines). 1-way ANOVA; Tukey’s post-hoc, **p < 0.005. (D) Cells were treated for 4 h with 100 nM CZC-25146 or GSK2578215A as indicated, and the percentage of cells containing at least one Rab7-positive tubular structure > 5 μm was determined by analyzing between 30-50 cells per
fibroblast line (n=5 control lines, n=5 G2019S LRRK2 PD lines). Kinase inhibitors significantly decreased the amount of cells with Rab7-positive tubular structures (G2019S versus G2019S plus CZC-25146 or GSK2578215A, 1-way ANOVA; Tukey’s post-hoc, **p < 0.005).

**Figure 8.** The CIN85/dyn2 pathway regulates the LRRK2-mediated delay in EGFR degradation. (A) HeLa cells were transfected either with empty pCMV vector (ctrl), or co-transfected with mutant LRRK2 and the indicated constructs, followed by quantification of the amount of surface-bound fluorescent EGF. N=3 independent experiments. Co-expression of CIN85 fully rescued binding, whilst coexpression of dyn2 partially rescued binding. Control versus LRRK2; 1-way ANOVA; Tukey’s post-hoc, *p < 0.05; control versus G2019S, 1-way ANOVA; Tukey’s post-hoc, **p < 0.005; control versus R1441C, 1-way ANOVA; Tukey’s post-hoc, **p < 0.005; control plus dyn2 versus G2019S plus dyn2, 1-way ANOVA; Tukey’s post-hoc, *p < 0.05. (B) Quantification of internalized Alexa555-EGF was performed in cells transfected with the indicated constructs after 10 min and 30 min of internalization. Values are normalized to the amount of Alexa555-EGF binding at t=0. N=3 independent experiments. G2019S versus control, 1-way ANOVA, Tukey’s post-hoc, *p<0.05; R1441C versus control, 1-way ANOVA, Tukey’s post-hoc, **p<0.005.

**Figure 9.** Rab7-Q67L rescues the LRRK2-mediated delay in EGFR degradation. (A) HeLa cells were transfected either with empty pCMV vector (ctrl), or co-transfected with mutant LRRK2 and the indicated constructs, followed by quantification of the amount of surface-bound fluorescent EGF. N=3 independent experiments. Co-expression of Rab7-Q67L fully rescued binding, whilst coexpression of Rab7 was
without effect. Control versus LRRK2; 1-way ANOVA; Tukey’s post-hoc, *p < 0.05; control versus G2019S, 1-way ANOVA; Tukey’s post-hoc, **p < 0.005; control versus R1441C, 1-way ANOVA; Tukey’s post-hoc, **p < 0.005; control plus Rab7 versus LRRK2 plus Rab7, 1-way ANOVA; Tukey’s post-hoc, *p < 0.05; control plus Rab7 versus G2019S plus Rab7, 1-way ANOVA; Tukey’s post-hoc, **p < 0.005; control plus Rab7 versus R1441C plus Rab7, 1-way ANOVA; Tukey’s post-hoc, **p < 0.005. (B) Quantification of internalized Alexa555-EGF was performed in cells transfected with the indicated constructs after 10 min and 30 min of internalization. Values are normalized to the amount of Alexa555-EGF binding at t=0. N=3 independent experiments. LRRK2 versus control, 1-way ANOVA, Tukey’s post-hoc, *p<0.05; G2019S versus control, 1-way ANOVA, Tukey’s post-hoc, **p<0.005; R1441C versus control, 1-way ANOVA, Tukey’s post-hoc, **p<0.005. (C) Transfected cell extracts were analyzed by Western blotting to assure similar levels of overexpression of the various GFP-tagged Rab constructs.

**Figure 10.** Mutant LRRK2 and the Rab7 cycle. (A) Western blots of HEK293T extracts (30 µg per lane) transfected with the indicated constructs (left), dermal fibroblast extracts (30 µg per lane) from five healthy controls and five G2019S LRRK2 PD patients (middle), or extracts from postmortem caudate brain (50 µg per lane) from three healthy controls and two G2019S LRRK2 PD patients (right) display no significant changes in Rab7 protein levels. (B,C) HeLa cells were transfected either with empty pCMV vector (ctrl), or co-transfected with the Rab7 GAP TBC1D15 and the indicated constructs, and Alexa555-EGF binding and uptake experiments performed as described in legend to Figure 1. N=3 experiments. 1-way ANOVA; Tukey’s post-hoc, *p < 0.005.
**Figure 11.** Mutant LRRK2 modifies the functional cycle of Rab7. (A) HeLa cells were co-transfected with GFP-Rab7 and either empty vector (pCMV) (top) or mutant LRRK2 (bottom). Scale bar, 10 μm. Right: Representative time-lapse images of individual GFP-Rab7 vesicles in the presence of empty vector (pCMV) (top) or mutant LRRK2 (bottom). The GFP fluorescence of late endosomal structures was bleached (bleach), and fluorescence recovery (relative to the starting value, pre) as a function of time measured as detailed in Material and Methods. Scale bar, 1.9 μm (top); 1.5 μm (bottom). (B) FRAP of GFP-Rab7-positive vesicles indicates that mutant LRRK2 shows decreased rate of fluorescence recovery as compared to control. Average from four control cells and four cells expressing R1441C mutant LRRK2, where fluorescence (F) was related to the initial fluorescence before bleaching. *, p < 0.05. (C) Quantification of the recovery time (t½) and mobile fraction (M) deduced from the recovery curves from cells co-transfected with GFP-Rab7 and either empty vector (pCMV) or the indicated LRRK2 constructs. N=3 experiments. Control versus LRRK2, 1-way ANOVA; Tukey´s post-hoc, *p < 0.05; control versus G2019S, 1-way ANOVA; Tukey´s post-hoc, **p < 0.005; control versus R1441C, 1-way ANOVA; Tukey´s post-hoc, *p < 0.05.

**Figure 12.** Mutant LRRK2 causes a decrease in Rab7 activity. (A) HEK293T cells were transfected with the indicated constructs, and the Rab7 binding domain of RILP coupled to GST was used to pulldown the GTP-bound form of Rab7 from cell lysates. No signal was detected when eluates from beads coupled to GST alone were evaluated (not shown). (B) HEK293T cells were transfected with either empty pCMV vector (ctrl), or with TBC1D15, and the amount of endogenous active Rab7 isolated by GST-RILP.
pulldown. (C) HEK293T cells were transfected with either empty pCMV vector (ctrl) or indicated constructs, and endogenous active Rab7 isolated by GST-RILP pulldown. Input (10%) was run alongside pulldowns to demonstrate equal levels of total Rab7 protein in control and LRRK2-expressing cells, and overexpression levels of various LRRK2 constructs analyzed on separate gels. (D) Experiments of the type described in (C) were quantified, and the amount of Rab7 isolated by GST-RILP expressed relative to input. N=3 independent experiments (N=2 experiments for K1906M). Control versus R1441C, 1-way ANOVA; Tukey’s post-hoc, **p < 0.005; control versus G2019S, 1-way ANOVA; Tukey’s post-hoc, **p < 0.005. (E) Fibroblast extracts from five healthy controls and five G2019S LRRK2 PD patients were subjected to GST-RILP pulldown assays, and 20% of input was blotted separately. The amount of Rab7 isolated by GST-RILP expressed relative to input is indicated below each fibroblast line. (F) Experiments of the type described in (E) were quantified, and amount of Rab7 relative to input plotted. Control versus G2019S, 1-way ANOVA; Tukey’s post-hoc, **p < 0.005. Experiments were repeated twice with similar results.