LRRK2 deficiency impairs trans-Golgi to lysosome trafficking and endocytic cargo degradation in human renal proximal tubule epithelial cells

Nathan J. Lanning
Van Andel Research Institute

Calvin Vanopstall
Calvin University

Megan L. Goodall
Van Andel Research Institute

Jeffrey P. Mackeigan
Van Andel Research Institute

Follow this and additional works at: https://digitalcommons.calvin.edu/calvin_facultypubs

Part of the Physiology Commons

Recommended Citation
Lanning, Nathan J.; Vanopstall, Calvin; Goodall, Megan L.; and Mackeigan, Jeffrey P., "LRRK2 deficiency impairs trans-Golgi to lysosome trafficking and endocytic cargo degradation in human renal proximal tubule epithelial cells" (2018). University Faculty Publications. 234.
https://digitalcommons.calvin.edu/calvin_facultypubs/234

This Article is brought to you for free and open access by the University Faculty Scholarship at Calvin Digital Commons. It has been accepted for inclusion in University Faculty Publications by an authorized administrator of Calvin Digital Commons. For more information, please contact dbm9@calvin.edu.
LRRK2 deficiency impairs trans-Golgi to lysosome trafficking and endocytic cargo degradation in human renal proximal tubule epithelial cells

Nathan J. Lanning, Calvin VanOpstall, Megan L. Goodall, Jeffrey P. MacKeigan, and Brendan D. Looyenga

1Van Andel Research Institute, Lab of Systems Biology, Grand Rapids, Michigan; and 2Calvin College, Department of Chemistry and Biochemistry, Grand Rapids, Michigan

Submitted 5 January 2018; accepted in final form 26 July 2018

INTRODUCTION

Activating mutations to human LRRK2 are now well-established drivers of Parkinson’s disease (PD) (30, 39). Because most, if not all, of these mutations increase the kinase activity of LRRK2, pharmacologic inhibition of this enzyme has been an attractive target for PD therapy (16, 34). Two limiting factors for such drugs are their ability to penetrate the brain and the potential for dose-limiting side effects on peripheral tissues. Though the former limitation has largely been overcome, animal studies with brain penetrant LRRK2 inhibitors have demonstrated that chronic inhibition of LRRK2 is associated with toxicity to the pulmonary epithelia (10, 14, 18). This toxicity is phenotypically similar to defects seen in Lrrk2 knockout mice, suggesting a role of LRRK2 in normal Type II pneumocyte function (14, 38). Perhaps surprising, however, is the relative lack of toxicity in the kidneys of drug-treated animals given that both Lrrk2 knockout mice and rats display profound renal dysfunction associated with cellular defects in vesicular trafficking and lysosomal function (4, 38). Whether this points to distinct enzymatic roles for LRRK2 in pulmonary and renal epithelia or a lack of cellular exposure to LRRK2 inhibitors in the kidney is unclear.

The effect of LRRK2 kinase inhibition in the kidney is also of significance based on studies that demonstrate LRRK2 is chromosomally amplified and overexpressed in papillary renal cell carcinoma (pRCC) (2, 23). Perturbation of LRRK2 expression in human pRCC cell lines results in cell cycle arrest and selective inhibition of key cell signaling pathways, most likely via the disruption of signal transduction by growth factor receptors. Other studies have uncovered LRRK2 overexpression or mutation in a variety of solid tumors, as well as epidemiological evidence that PD-associated mutations to LRRK2 (G2019S) increase the risk of several nonskin cancers (1, 20, 33). Together these data suggest that LRRK2 kinase inhibitors may potentially be repurposed for cancer therapy, providing they can be used for a relatively short period of time to avoid peripheral toxicity to the lung. Understanding the molecular role of LRRK2 in cancer and normal tissues is therefore of paramount importance.

Most current literature supports a role for LRRK2 in vesicular trafficking processes downstream of endocytosis, such as autophagy and cargo sorting (3, 24, 26, 35). Precisely where in these processes LRRK2 is involved is less clear, as it appears to interact physically with and/or phosphorylate a number of protein substrates known to be involved in vesicular trafficking. Most prominent among these substrates are Rab family GTPases, particularly those involved in late endosomal sorting (6, 15, 24, 36). Given that the renal and pulmonary phenotypes of Lrk2−/− mice include the epithelial accumulation of intracellular vesicles containing undigested waste, it seems probable that LRRK2 regulates late endosomal compartment homeostasis via its interactions with Rab family GTPases and other vesicular trafficking proteins (19, 38). The central role of this compartment in endocytic cargo sorting may also explain the propensity for amplification or mutation of LRRK2 across several solid tumor types, as it is now well established that alterations to endosomal trafficking machinery play an important role in cancer development (12).

In addition to its interactions with Rab proteins, LRRK2 has also been shown to interact with N-ethylmaleimide-sensitive fusion (NSF) protein, which functions as an ATP-dependent

Address for reprint requests and other correspondence: B. D. Looyenga, Dept. of Chemistry and Biochemistry, Calvin College, 1726 Knollcrest Circle, SE, Grand Rapids, MI 49546 (e-mail: blooye62@calvin.edu).
disassembly factor for cis-SNARE complexes after vesicular fusion (7, 31). Though this activity of NSF is its most prominent function—and the one implicated in its interaction with LRRK2—it has also been shown to mediate restacking of Golgi apparatus fragments into discrete cisternae after the completion of mitosis, which is necessary for proper vesicular trafficking between the Golgi apparatus and other cellular compartments (5, 32). Unlike its SNARE disassembly function, this secondary role for NSF is independent of its ATPase activity though it appears to be conserved in metazoans as simple as Drosophila (28). Whether interactions between LRRK2 and NSF also impact Golgi integrity and spacing between the Golgi and other compartments is unknown. In this study we address this issue in the context of human renal epithelial cells, and present findings that suggest the vesicular trafficking defects previously identified in LRRK2-deficient cells are centrally related to disorganization of the Golgi apparatus.

MATERIALS AND METHODS

Antibodies and reagents. Rabbit monoclonal or polyclonal antibodies for Rab5, Rab7, NSF, LC3B, and STX6 were used for immunoblotting and immuno-fluorescent staining were purchased from Cell Signaling Technology (Danvers, MA). The anti-LRRK2 (UDD3), anti-LRRK2 (MJJF2) anti-phospho-LRRK2-S935, anti-GBA, and anti-ARSB rabbit monoclonal antibodies were obtained from Epitomics (Epitomics/Abcam, Cambridge, MA). The anti-β-actin and tubulin mouse monoclonal antibodies used for immunoblotting were obtained from Sigma-Aldrich (Sigma, St. Louis, MO). The anti-V5 epitope mouse monoclonal antibody and AlexaFluor-conjugated goat secondary antibodies were obtained from Invitrogen/Life Technologies (Thermo Fisher Scientific, Grand Island, NY). The anti-p62/SQSTM1, EEA1, LAMP1, and gm130 mouse monoclonal antibodies used for immuno-fluorescent staining were obtained from Becton Dickinson (BD Biosciences, San Jose, CA). All antibodies were used at the dilutions recommended by each manufacturer unless otherwise specified.

All chemical reagents were obtained from Sigma-Aldrich unless otherwise indicated. The LRRK2 catalytic inhibitor GNE-7915 was purchased from Selleck Chemicals (Houston, TX) and used at the indicated concentrations. The LRRK2 inhibitor PFE-475 (PFE-06447475) was provided by Dr. Jaclyn Henderson (Pfizer, New York, NY). Vesicular trafficking cargoes AlexaFluor488-transferin, AlexaFluor488-dextran, and BZiPAR [rhodamine 110, bis-(CBZ-L-isoleucyl-L-arginyl-L-arginine amide), and dihydrochloride] were purchased from Invitrogen/Life Technologies and used at the indicated concentrations.

Immunohistochemistry. Murine renal tissues were obtained as a gift from Dr. Ted Dawson (The Johns Hopkins University, Baltimore, MD). The tissues were harvested from necropsied Lkr2−/− animals and wild-type littersmates in compliance with approved animal care guidelines from Johns Hopkins Institutional Animal Care and Use Committee. Tissues were fixed for ~24 h in 4% paraformaldehyde, washed with cold phosphate-buffered saline (PBS), and stored at 4°C until use. All tissues were dehydrated through graded ethanol and methyl salicylate and then embedded in paraffin before sectioning. Kidney sections were cut at 5-μm thickness and floated onto glass slides for drying at 37°C to promote adherence. After drying, sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin using a Symphony Automated H&E stain (Ventana Medical Systems, Tucson, AZ) in the Van Andel Research Institute Core Facility. Images were captured with an ECLIPSE Ci fluorescent microscope (Nikon Instruments, Melville, NY) at ×20 and ×40 resolution.

Cell culture. Normal immortalized human kidney (HK2) cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in RPMI-1640 medium supplemented with 2 mM GlutaMAX (Thermo Fisher Scientific) and 10% fetal bovine serum (FBS). Human embryonic kidney 293FT cells were obtained from Invitrogen/Life Technologies (Thermo Fisher Scientific) and maintained in standard DMEM with high glucose (4.5 g/l) and 10% FBS. Both cell lines were incubated in a humidified and sterile tissue culture incubator at 37°C with 5% CO2 atmosphere. Stable HK2 polyclonal cell lines expressing short hairpin RNAs (shRNAs) were purchased from Dynal BioTech (Thermo Fisher Scientific), 10 mM α-glucose, and 1× RPMI vitamins (Sigma-Aldrich) lacking all amino acids. To block autophagosome processing by lysosomal acidification, cells were treated in parallel with bafilomycin A1 (50 nM) along with amino acid starvation.

Lentiviral vector production. Validated lentiviral shRNA vector plasmids from The RNA Consortium pLKO.1 collection were obtained from Sigma-Aldrich (Sigma). Each lentiviral plasmid was transfected into a 10-cm dish containing 1.5 million 293FT cells along with ViraPower third-generation packaging plasmids (pLP1, pLP2, and pVSVG; Thermo Fisher Scientific) using standard calcium phosphate precipitation. Medium was changed the following day and allowed to incubate on cells for 72 h before harvest. The 10 ml of conditioned medium from each lentiviral vector was removed and filtered through a 0.4-μm syringe filter before freezing at ~80°C in 1-ml aliquots.

Immunofluorescence microscopy. Parental HK2 cells or stable polyclonal cell lines expressing shRNAs were seeded to glass coverslips and grown to 90% confluence. Cells were fixed in 4% paraformaldehyde, washed with cold PBS and permeabilized with 0.2% Triton X-100 on ice. After being blocked in 5% normal goat serum (Sigma) in PBS solution, the cells were incubated with the indicated primary antibody diluted in blocking buffer overnight at 4°C. Cells were nuclear counter-stained with DAPI (1 μM) and AlexaFluor-488 coupled goat anti-rabbit and AlexaFluor-546 coupled goat anti-mouse secondary antibodies (Invitrogen/Life Technologies) diluted at 1:1000 in blocking buffer overnight at 4°C. The following day cells were washed with PBS containing 0.05% Tween 20 (PBS-T) and stained with AlexaFluor-488 coupled goat anti-rabbit and AlexaFluor-546 coupled goat anti-mouse secondary antibodies (Invitrogen/Life Technologies) diluted in culture medium and allowed to adhere overnight under standard tissue culture conditions. Treatment of cells before fixation and staining is indicated in each data figure. Cells were fixed with 3.7% formaldehyde in PBS solution and permeabilized with 0.2% Triton X-100 on ice. After being blocked in 5% normal goat serum (Sigma) in PBS solution, the cells were incubated with the indicated primary antibody diluted in blocking buffer overnight at 4°C. The following day cells were washed with PBS containing 0.05% Tween 20 (PBS-T) and stained with AlexaFluor-488 and AlexaFluor-546 coupled goat anti-rabbit and AlexaFluor-488 and AlexaFluor-546 coupled goat anti-mouse secondary antibodies (Invitrogen/Life Technologies) diluted in 1:1000 in blocking buffer for 1 h at room temperature. After a second round of washing in PBS-T, the cells were nuclear counter-stained with DAPI (1 μg/ml) and prepared with gel mounting medium before mounting on glass slides. Epifluorescent images of cells were obtained using a Nikon Ti-E inverted fluorescence microscope equipped with DAPI, FITC, and Texas Red filter sets and processed using the NIS Elements software package (Nikon Instruments). Confocal images were obtained using a Nikon A1Rscanning confocal microscope equipped with 40×, 568-, 640-, and 660-nm solid-state lasers and a 32-detector spectral imager (Nikon Instruments). All images were processed and quantified using the NIS Elements software package (Nikon Instruments).

Immunoblotting. Cells cultured in 6-well dishes were rinsed with cold PBS and harvested into 100 μl of lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM sodium glycerophosphate, 1 mM sodium orthovanadate, 0.5% Nonidet P-40 (NP40), 0.1% Brij35, 0.1% sodium pyrophosphate, 1 mM sodium glycerophosphate, 1 mM sodium orthovanadate, 0.5% Nonidet P-40 (NP40), 0.1% Brij35, 0.1% sodium gluta max, 0.1% SDS, 1 mM EDTA, 150 mM NaCl, and 0.1% Triton X-100). Lysates were precleared with protein G-Sepharose (GE Healthcare) and supernatants were collected. Supernatants were electrophoresed on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes for immunoblotting. Blots were incubated with primary antibodies and AlexaFluor-488 or AlexaFluor-546 conjugates for 1 h at room temperature. Membranes were washed with TBS-T and incubated for 1 h in TBS-T containing 0.1% Brij35 and 5% normal goat serum. After washing, membranes were incubated with primary antibodies diluted in blocking buffer overnight at 4°C. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies. Membranes were washed and incubated with ECL substrate for 1 min. Blots were scanned and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).
deoxycholate] supplemented with mammalian cell protease inhibitor cocktail (Sigma-Alrich). Each lysate was homogenized by brief sonication at 30% power on ice and then cleared by centrifugation at 10,000 relative centrifugal forces (rcf) for 5 min at 4°C. Concentration of each lysate was determined by Bradford assay along with a twofold serial dilution of 10 mg/ml BSA to generate a standard curve. Equal amounts of protein lysate (20–50 μg) were separated by reducing polyacrylamide gel electrophoresis and transferred overnight to nitrocellulose membrane using a traditional wet transfer apparatus (TE62 model; Hoefer, Holliston, MA). The blots were blocked with 3% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) and then probed overnight at 4°C with primary antibodies diluted to the manufacturer’s specification. After unbound primary antibody was washed off, the membranes were incubated for 1 h at room temperature with goat anti-ribbon-IRDyeTM800 and goat anti-mouse-IRDyeTM680 secondary antibodies (Li-Cor, Lincoln, NE) and then imaged with an Odyssey scanner (Li-Cor). Images were processed with the Odyssey Infrared ImagingSystem software (version 3.0.25) to ensure that signal was in the linear range of photon detection before export in TIFF format.

Immuno precipitation. Cells cultured in 10-cm dishes were rinsed with cold PBS and harvested into 0.4 ml of immunoprecipitation buffer [50 mM HEPES (pH 7.0), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 10 mM sodium glycero phosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.1% NP40, 10% glycerol] supplemented with mammalian cell protease inhibitor cocktail and N-ethylmaleimide when indicated (Sigma-Alrich). The lysates were homogenized by shearing through a 25-gauge needle on ice and cleared by centrifugation at 10,000 rcf for 5 min at 4°C. Lysates were quantified by Bradford assay as above, and equal protein amounts (0.5–1 mg) were incubated for 1 h at 4°C with anti-LRRK2-UDD3 antibody (Abcam) diluted 1:100 in a final volume of 1 ml. Affinity complexes were precipitated by addition of 50 μl of equilibrated protein-G agarose beads (Invitrogen/Life Technologies) and incubation at 4°C with rotation for an additional hour. Bead pellets were washed three times with 0.9-m1l volumes of buffer and eluted by boiling in 80 μl of 2X Laemmli Buffer [120 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 0.02% bromophenol blue, 50 mM DTT]. Transmission electron microscopy. HK2 stable cell lines grown in 10-cm dishes were trypsinized, pelleted, washed with PBS, and resuspended in 2% glutaraldehyde for fixation (Sigma). The cell pellets were then embedded in 2% agarose, postfixed in osmium tetroxide, and incubation at 4°C with rotation for an additional hour. Bead pellets were washed three times with 0.9-m1l volumes of buffer and eluted by boiling in 80 μl of 2X Laemmli Buffer [120 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 0.02% bromophenol blue, 50 mM DTT]. Transmission electron microscopy. HK2 stable cell lines grown in 10-cm dishes were trypsinized, pelleted, washed with PBS, and resuspended in 2% glutaraldehyde for fixation (Sigma). The cell pellets were then embedded in 2% agarose, postfixed in osmium tetroxide, and dehydrated with graded acetones. Samples were embedded in PolyBed 812 resin and polymerized at 60°C for 24 h. Ultrathin sections (70 nm) were generated with a Power Tome XL (Boeckeler Instruments, Tucson, AZ) and placed on copper grids. Cells were examined using a JEOL 100C × transmission electron microscope at 100 kV (Tokyo, Japan). Electron microscopy services were performed by the Michigan State University Center for Advanced Microscopy (East Lansing, MI).

EdU cell proliferation assay. Identification of proliferating cells found in the S-phase of the cell cycle was performed using the Click-iT EdU AlexaFluor488 imaging kit from Invitrogen/Life Technologies. In this assay the thymidine analog 5-ethynyl-deoxyuridine (EdU) was pulsed to cells at 10 μM for 1 h, and then incubated with 4-methylumbelliferyl-β-d-glucosaminide (MNDG) as a substrate. Cleaveage of the glycosidic bond in this substrate by lysosomal hexosaminidases releases 4-methylumbelliferyl, which can be quantified by fluorescence plate reader. Assays were performed on 0.50 μg of total protein from each fraction, which was diluted in a final volume of 50 μl in 250 mM citrate buffer (pH 4.6) containing a saturating concentration of 1 mM MNDG. Fluorescence values at Ex/Em:355/460 nm were measured on a Synergy H1 plate reader (BioTek) at 1-min intervals over a 15-min assay and then converted to product concentrations using a standard curve of known 4-methylumbelliferone concentration. The slope of the linear plot from each assay was used to determine NAG activity values in mmol·min⁻¹·mg⁻¹ of lysate. Each assay was performed three times in triplicate to determine average activity values and the standard deviation of activity in each fraction.

Quantitative RT-PCR. Total RNA was isolated from cells using an RNeasy kit (Qiagen) and then reverse transcribed to produce cDNA libraries with an iScript Select kit (Bio-Rad), both of which were utilized according to the manufacturers’ suggested protocols. Three separate biological replicates of cells in each condition were analyzed by quantitative PCR (qRT-PCR) using intron-spanning primers tar-
LRRK2 DEFICIENCY IMPAIRS VESICLE TRAFFICKING IN TUBULE CELLS

Depletion of LRRK2 in human renal epithelial cells promotes vesicular accumulation. Consistent with several prior studies, we found that histological staining of renal tissue from Lrrk2/− mice at 3 mo of age reveals the accumulation of optically clear vesicular inclusions within the cytoplasm of cortical tubule epithelia (Fig. 1A). These inclusions are most prominent in the proximal tubule cells, which can be identified by their intraluminal brush border that is absent in distal tubule cells. To determine whether human cells derived from renal proximal tubule epithelia display similar defects in the absence of LRRK2, we stably infected an immortalized human cell line derived from this tissue with a lentiviral vector (pLKO.1) that expresses a previously validated shRNA targeted to the LRRK2 mRNA (23). As a control, HK2 cells were also transduced with a nontargeting shRNA that lacks homology to the human coding genome. Simple phase-contrast microscopy of these cells demonstrates a notable increase in the percent of cells bearing large vesicular inclusions, many of which have prominent vacuole-like structures (Fig. 1B).

Given the wide range of vesicular trafficking phenomena that have been associated with LRRK2 function in various cellular and whole animal models, we sought to determine the identity of the large vacuole-like structures that were frequently observed in the LRRK2-knockdown line of HK2 cells. We used immunofluorescent staining with a variety of specific antibodies that mark specific vesicular populations or organelles in the mammalian cell as indicated in Fig. 1C. We particularly focused on markers that elucidate the autophagic, endo-lysosomal, and recycling pathways for vesicular trafficking, all of which have been related to defects in LRRK2 activity.

Immunofluorescent imaging of LRRK2-deficient and control cells showed no obvious defects in the early endosome (Fig. 1D) or autophagic pathways (Fig. 1E), though significant differences in localization of late endosome/lysosomal (Fig. 1F) and Golgi markers (Fig. 1G) were apparent. Specifically, we found that roughly 70% of cells contained large, perinuclear vacuole-like inclusions uniformly stained positive for the late endosome marker Rab7 and that these inclusions were typically situated in the perinuclear region of cells next to LAMP1-positive lysosomes (Fig. 1, F and H). These observations are consistent with a role for LRRK2 in vesicular sorting in renal epithelia, most likely in a postendocytic compartment associated with cargo trafficking from the Rab7-positive late endosome to the lysosome.

Depletion of LRRK2 in human renal epithelial cells causes Golgi fragmentation. In addition to the vesicular accumulation phenotype noted above, immunofluorescent staining of LRRK2-deficient cells also revealed widespread Golgi fragmentation (Fig. 1G). Markers for distinct compartments of the Golgi (STX6 and GM130) were distributed over a larger area of the cell with an average size of nearly 2.5 times that found in control cells (Fig. 1I). Although this expansion and fragmentation of the Golgi apparatus was apparent in all LRRK2-deficient cells, it was especially prominent in those that also displayed an enlarged Rab7-positive endosomal compartment (Fig. 1J). In contrast, the relatively small percentage of control cells that contained an enlarged Rab7-positive endosomal compartment showed no difference in average Golgi area compared to those with typical Rab7 staining (Fig. 1J).

To determine whether the expansion of the Golgi compartment was more specifically related to a loss of LRRK2 kinase activity, we treated cells with two selective LRRK2 kinase inhibitors (GNE-7915 and PFE-475) before imaging of the Golgi apparatus by immunofluorescent microscopy. Immunoblot analysis of total protein levels and the autophosphorylation site at S935 demonstrate that both drugs confer kinase inhibition up to 24 h with little impact on protein stability (Fig. 2, A and B). Cells treated for 24 h with each drug were subsequently imaged after staining cells with the Golgi markers STX6 and GM130 (Fig. 2C). In contrast to the effect of complete LRRK2 knockdown with shRNA, we observed few cells with complete Golgi fragmentation when LRRK2 kinase activity was pharmacologically blocked. We did, however, observe a milder expansion of the Golgi apparatus, which was still significantly enlarged compared with vehicle (DMSO)-treated cells (Fig. 2D). These data suggest that the Golgi expansion phenotype observed after stable genetic depletion of LRRK2 is at least in part a result of its kinase activity being absent in HK2 cells but that absence of protein, or a longer timeframe, may be required to elicit complete Golgi fragmentation.

Genetic depletion of NSF phenotypically mimics lrrk2 knockdown. Prior studies of LRRK2 function in neuronal vesicular trafficking identified the protein NSF as an interaction partner and target of LRRK2 kinase activity (7, 31). This finding is intriguing because NSF has been previously shown to play a key role in the disassembly and reassembly of Golgi stacks during and after mitosis, respectively (28, 32). The former role depends upon its ATPase activity, whereas the latter occurs independent of its known enzymatic function. Cellular depletion of NSF in epithelial cells promotes Golgi fragmentation and defects in receptor recycling, though it has little effect on cell viability or endocytosis (11). Given the defects seen in LRRK2-deficient HK2 cells, these data suggested a functional link between NSF and LRRK2 in renal epithelia.

To test this hypothesis, we stably infected HK2 cells with an shRNA that targets NSF and compared the phenotype of these cells with LRRK2 knockdown (Fig. 3). Immunofluorescent staining for the early and late endosomal compartments in
these cells demonstrates the presence of Rab7-positive vesicular inclusions that are phenotypically similar to those seen after stable LRRK2 knockdown (Fig. 3A). Further characterization of the two knockdown lines by transmission electron microscopy revealed an increase in the number and size of electron-dense vesicular structures, which are characteristic of late endosomes and lysosomes, compared with control cells (Fig. 3B). The contents of these vesicles include whole organelles, membrane whorls, and electron-dense aggregates, suggesting that these represent endocytosed or autophagic material that is destined for lysosomal degradation but has failed to be properly digested (Fig. 3, C and D).
LRRK2 and NSF physically and functionally interact in renal epithelial cells. The similarity in phenotypes between cells lacking either LRRK2 or NSF prompted us to investigate whether the two proteins colocalize to the same compartment in HK2 cells using confocal microscopy. Because endogenous LRRK2 is found at very low levels in cultured HK2 cells, we expressed exogenous FLAG-LRRK2 under control of the human EF1a promoter to facilitate immunofluorescent detection (Fig. 4A). As a positive control, we first stained cells for total LRRK2 versus the FLAG epitope to show that signals overlapped in the expected pattern (Fig. 4B). After confirming a strong overlap correlation between these signals, we then stained and imaged cells for the FLAG epitope versus endogenous NSF, which also demonstrated statistically significant overlap by confocal microscopy (Fig. 4, A and B). It is notable that NSF was detected on all vesicles that stain positive for LRRK2, though a sizable portion of NSF-positive vesicles do not appear to contain LRRK2 (Fig. 4A, inset). This observation suggests that NSF is likely to play a broader role in vesicular trafficking events than LRRK2, which appears to be more restricted in its subcellular localization.

The relevance of LRRK2-NSF colocalization in cells is reinforced by results of coimmunoprecipitation experiments that demonstrate that NSF can be precipitated with endogenous LRRK2 in HK2 cells (Fig. 4C). The interaction between these two proteins is enhanced by addition of the NSF inhibitor N-ethylmaleimide (NEM) to cell lysis buffer, which effectively locks NSF into its homohexameric, ATP-bound state by inhibiting ATPase activity (8, 27). These findings demonstrate that LRRK2 and NSF physically interact in renal epithelia and suggest that these two proteins are functionally related to each other in the process of vesicular trafficking in the endolysosomal system.

A potential insight into how LRRK2 and NSF interact became apparent from monitoring levels of endogenous NSF in HK2 cells that stably expressed an shRNA targeted to LRRK2. We observed that stable knockdown of LRRK2 leads to a roughly 40% decrease in NSF at both the mRNA and protein levels (Fig. 4, D and E). Precisely why endogenous NSF expression at the mRNA level would be decreased by LRRK2 depletion is unclear, though we suggest it is an indirect relationship because overexpressing LRRK2 at a variety of levels using a pseudotyped baculovirus system (BacMam) fails to increase NSF levels (Fig. 4F). Furthermore, exogenous reexpression of V5-tagged human NSF in cells with LRRK2 deficiency fails to rescue the Golgi expansion defect seen when LRRK2 is depleted (Fig. 4E, data not shown). Together, these data support the possibility of a functional link between LRRK2 and NSF but exclude a mechanism in which LRRK2 simply regulates NSF protein levels in the renal epithelia.

Golgi fragmentation after NSF or LRRK2 depletion occurs independent of cell division. Because prior studies from our laboratory showed a decrease in cellular proliferation rate when LRRK2 is stably depleted from renal cancer cells, we asked the question of whether the effect of LRRK2 knockdown on Golgi structure in normal HK2 cells is simply an artifact of mitotic arrest (23). To answer this question, we performed pulse-chase labeling of cells with the thymidine analog EdU and subsequently fixed and stained them for incorporation of this marker using fluorescent click chemistry along with immunofluorescent staining for the Golgi marker GM130 (Fig. 5A). EdU incorporation provides a good proxy for S-phase entry in renal epithelia.
cells, which showed little difference when either LRRK2 or NSF was knocked down in HK2 cells (Fig. 5B). Importantly, EdU incorporation had no impact on Golgi size in control cells, though depletion of either LRRK2 or NSF again caused an expansion in the gm130-positive Golgi compartment (Fig. 5C).

To evaluate whether the effect of LRRK2 and NSF on Golgi fragmentation occurred concomitant with cell cycle progression, we performed two experiments. In the first experiment, we pulse-labeled cells with EdU for 2 h and then immediately fixed and stained them for GM130. In this context, control cells with EdU incorporation showed a small but insignificant increase in Golgi size, whereas LRRK2- and NSF-depleted cells showed no difference, though their overall Golgi area was still significantly larger than that of control cells (Fig. 5D). These data show that while in S-phase before mitotic Golgi fragmentation has occurred cells lacking LRRK2 or NSF already have expanded Golgi compartments.

In the second experiment, we performed EdU pulse labeling as before but followed that with a 6-h medium chase to allow for cells to progress through S-phase and into mitosis. Here we observed a significant expansion of the Golgi area in EdU-positive cells, consistent with a mitosis-associated fragmentation of the Golgi (Fig. 5E). Importantly, however, we also found that EdU-negative cells from the LRRK2- and NSF-depleted lines still displayed significantly expanded Golgi compartments, indicating that loss of these two proteins leads to a loss of Golgi compaction independent of progression through S-phase and into mitosis.

Fig. 3. Depletion of NSF phenocopies LRRK2 deficiency and results in the accumulation of vesicular waste cargo. A: confocal immunofluorescent images of HK2 cells transduced with nontargeting shRNAs or shRNAs targeted to LRRK2 or NSF. Cells were stained with antibodies for endogenous EEA1 (red) and Rab7 (green) to indicate the early and late endosomal compartments, respectively. DAPI (blue) costain of nuclei is also shown in the merged image. B–D: transmission electron microscopy was performed on dissociated cell pellets of HK2 cells that were stably depleted of LRRK2 or NSF using lentiviral shRNAs. B: whole cell images of stable lines showing the accumulation of electron-dense vesicles in HK2 cells after depletion of LRRK2 or NSF. Cells also displayed large vacuolar inclusions in some instances (red arrows), which suggest that some of these vesicles may be autophagic in origin. C: vesicles in HK2 cells lacking LRRK2 display whole organelles encased in vesicles (black arrows), which suggest that some of these vesicles may be autophagic in origin. D: higher magnification images of LRRK2-deficient HK2 cells demonstrate the variety of waste cargo in vesicles, which includes: 1) membrane whorls, 2) lipid droplets, and 3–4) electron dense aggregates of undetermined identity. LRRK2, leucine-rich repeat kinase 2; HK2, normal human kidney cells; NSF, N-ethylmaleimide-sensitive fusion protein; shRNA, short hairpin RNA.
Depletion of LRRK2 and NSF impairs trafficking of endocytic cargo. A previous study of HeLa cells after knockdown of NSF suggested that although endocytosis itself was unimpaired, the ability of cells to recycle endocytosed receptors back to the cell surface was blocked by the absence of NSF (11). We recapitulated these findings in HK2 cells using fluorescently labeled dextran as a marker for fluid phase bulk endocytosis and labeled transferrin as a marker for receptor-mediated endocytosis. After knockdown of LRRK2 or NSF, we observed no change in the rate of dextran uptake relative to control cells, which is consistent with the absence of a general endocytic defect (Fig. 6, A and B). In contrast, the rate of transferrin uptake was significantly decreased when either of these proteins was depleted from HK2 cells (Fig. 6C). Because the continuous uptake of transferrin by its receptor (TfR) requires postendocytic recycling, this finding suggests that both LRRK2 and NSF are required to maintain the recycling pathway after internalization of membrane cargo from the cell surface.

We next asked whether the late endosomal defect we first observed in HK2 cells lacking LRRK2 could be a result of improper sorting of endocytic cargo to the lysosomal compartment. In this context we used syntaxin-7 (STX7) as a positive control because this protein is required for proper transport of late endosomal proteins to the lysosome (29). As expected, knockdown of STX7 had no impact on uptake of dextran or...
transferrin, though it significantly decreased trafficking of a fluorogenic peptide substrate (BZiPAR) to the lysosomal compartment (Fig. 6, A–D). A similar decrease in trafficking of this peptide was elicited by pretreatment of cells with the inhibitor dynasore (30 μM), which prevents dynamin-mediated scission of endocytic vesicles (Fig. 6D). Interestingly, we found that LRRK2 depletion resulted in a roughly 25% increase in peptide trafficking rate to the lysosome, whereas depletion of NSF had no effect. This finding suggests that the waste accumulation defect seen in Lrrk2/H11002 mouse kidneys and in HK2 cells after LRRK2 knockdown may not be a result of decreased lysosomal trafficking per se but rather a loss of lytic activity toward specific lysosomal substrates.

Depletion of LRRK2 and NSF impairs trafficking of lysosomal hydrolases. The finding that postendocytic lysosomal sorting was normal, if not accelerated, in LRRK2 deficient cells prompted us to examine whether the two central vesicular trafficking defects seen upon LRRK2 or NSF depletion (expansion of the late endosome and fragmentation of the Golgi apparatus) are functionally related by a defect in trans-Golgi to late endosome transport. Among the various cargoes of interest in this pathway are a variety of lysosomal hydrolases, which are initially produced in the secretory pathway but then sorted to the endosome rather than being secreted outside the cell. Pharmacologic or genetic collapse of the trans-Golgi is known to impair this process and to result in defective lysosomal function (17).

Defects in trans-Golgi to late endosome transport can be evaluated by measuring the steady-state ratio of lysosomal hydrolases in the lysosome versus the secretory pathway; defective sorting results in decreased lysosomal enzyme content and increased secretory pathway content. We used density...
**A** LRRK2 DEFICIENCY IMPAIRS VESICLE TRAFFICKING IN TUBULE CELLS

**B**

![Graph showing AF488-Dextran Fluorescence vs. Time](image)

**C**

![Graph showing AF488-Transferrin Fluorescence vs. Time](image)

**D**

![Bar chart showing BZP2AR Conversion Rate](image)

**E**

![Western Blots for LAMP1, GBA, ARSB](image)

**F**

![Bar chart showing P2 and P3 activity](image)

**G**

<table>
<thead>
<tr>
<th>Non-target shRNA</th>
<th>(-) amino acids</th>
<th>(+) BafA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT-sh</td>
<td>P2, P3</td>
<td>P2, P3</td>
</tr>
<tr>
<td>LRRK2-sh</td>
<td>P2, P3</td>
<td>P2, P3</td>
</tr>
<tr>
<td>NSF-sh</td>
<td>P2, P3</td>
<td>P2, P3</td>
</tr>
<tr>
<td>STX7-sh</td>
<td>P2, P3</td>
<td>P2, P3</td>
</tr>
</tbody>
</table>

**H**

<table>
<thead>
<tr>
<th>LRRK2-sh</th>
<th>(-) amino acids</th>
<th>(+) BafA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>P2, P3</td>
<td>P2, P3</td>
</tr>
<tr>
<td>LRRK2</td>
<td>P2, P3</td>
<td>P2, P3</td>
</tr>
<tr>
<td>NSF</td>
<td>P2, P3</td>
<td>P2, P3</td>
</tr>
<tr>
<td>STX7</td>
<td>P2, P3</td>
<td>P2, P3</td>
</tr>
</tbody>
</table>

**I**

<table>
<thead>
<tr>
<th>NSF-sh</th>
<th>(-) amino acids</th>
<th>(+) BafA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>P2, P3</td>
<td>P2, P3</td>
</tr>
<tr>
<td>LRRK2</td>
<td>P2, P3</td>
<td>P2, P3</td>
</tr>
<tr>
<td>NSF</td>
<td>P2, P3</td>
<td>P2, P3</td>
</tr>
<tr>
<td>STX7</td>
<td>P2, P3</td>
<td>P2, P3</td>
</tr>
</tbody>
</table>

**J**

<table>
<thead>
<tr>
<th>STX7-sh</th>
<th>(-) amino acids</th>
<th>(+) BafA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>P2, P3</td>
<td>P2, P3</td>
</tr>
<tr>
<td>LRRK2</td>
<td>P2, P3</td>
<td>P2, P3</td>
</tr>
<tr>
<td>NSF</td>
<td>P2, P3</td>
<td>P2, P3</td>
</tr>
<tr>
<td>STX7</td>
<td>P2, P3</td>
<td>P2, P3</td>
</tr>
</tbody>
</table>
dependent organelle fractionation of hypotonically lysed cells to isolate the lysosomal (P2) and microsomal fractions (P3) and then performed immunoblotting for various proteins known to traffic to the lysosome. Three such proteins (LAMP1, GBA, and ARSB) showed decreased abundance in the lysosomal fraction and/or increased abundance in the microsomal fraction of cells deficient in LRRK2 and NSF compared with control cells (Fig. 6E). These data were reinforced with a quantitative enzymatic assay for N-acetylglucosaminidase (NAG) activity, which is mediated by the lysosomal enzyme hexosaminidase-B. Data from this assay also showed increased NAG activity in the microsomal fraction and decreased activity in the lysosomal fraction of cells deficient in LRRK2 and NSF compared with control cells (Fig. 6F). Together, these data imply that the undigested waste material seen in cellular vesicles by electron microscopy (Fig. 3, B–D) accumulates because of insufficient trafficking of digestive hydrolases to the lysosome due to trans–Golgi fragmentation.

In contrast to the defects in endocytic recycling, we did not find any evidence of acute defects in autophagic flux in HK2 cells after knockdown of LRRK2 or NSF. Cells starved of amino acids to induce autophagy demonstrated proper accumulation and subsequent turnover of LC3B and p62/SQSTM1 in the lysosomal fraction of cells deficient in LRRK2 and NSF compared with control cells (Fig. 6G). Together, these data imply that the undigested waste material seen in cellular vesicles by electron microscopy (Fig. 3, B–D) accumulates because of insufficient trafficking of digestive hydrolases to the lysosome due to trans–Golgi fragmentation.

DISCUSSION

The discovery of PD-associated mutations in the gene encoding LRRK2 in 2004 produced a surge of interest in how this protein works at the cellular level (30, 39). In the years since that discovery, a wealth of research has demonstrated that LRRK2 primarily functions as a regulator of vesicle trafficking in a variety of cell types, including neurons, immune cells, and in specific epithelial cell populations. Of these latter cell types, Type II pneumocytes of the lung and proximal renal tubule cells have received the most attention due to their especially high expression of LRRK2 and their pathophysiological deficits upon Lrrk2 deletion in rodent models (4, 19). Prior studies of these animal models have demonstrated the progressive accumulation of undigested cellular contents within a poorly defined vesicular compartment that bears features of the late endosome, lysosome, and autophagosome (37, 38).

Precisely how and why this population of vesicles accumulates in the renal epithelium has been of significant interest for three reasons. In the first place, identification of the molecular defects in these cells could potentially provide insights into the cellular pathophysiology of neurons in Parkinson’s disease, thereby providing new therapeutic targets for treatment. Second, identification of peripheral disease markers in patients bearing LRRK2 mutations could potentially provide a means for noninvasive monitoring of disease progression and response to therapy via urine sampling, which is far more tractable than cerebrospinal fluid or tissue sampling (13). Finally, the observation that genetic deletion of Lrrk2 in mice leads to significant pathology in the lung and kidney suggested that prolonged systemic treatment of PD patients with pharmacologic inhibitors of LRRK2 enzymatic activity could be toxic to these organs, thus obviating this approach as a therapy in PD. Although the realization of this concern has varied among the various LRRK2 inhibitors developed to date, it remains a significant issue given that patients treated in such fashion could conceivably be dosed for decades because of the chronic and progressive nature of PD (10, 18).

In this study we developed a cellular model of LRRK2 deficiency in normal immortalized human kidney cells derived from the proximal tubule, which is primarily where LRRK2 is expressed in the kidney (23). These cells phenocopy the early renal defects seen in Lrrk2–/– mice, including the presence of an enlarged late endosomal compartment and accumulation of vesicles with undigested lysosomal cargo. Most importantly, we demonstrate that the LRRK2-NSF interaction is conserved in human kidney cells and that loss of LRRK2 leads to a compensatory destabilization of NSF and Golgi fragmentation.

Trafﬁcking of cargo to and from the Golgi is consequently disrupted by loss of either LRRK2 or NSF, suggesting that the molecular interaction between these two proteins is critical for the maintenance of vesicular trafﬁcking homeostasis in the kidney.

These findings provide important insights into the etiology of endo-lysosomal dysfunction in cells with deficiency or inhibition of LRRK2 by proﬁling the various vesicle trafﬁcking defects in these cells. Though prior studies both in vitro and in vivo have noted the defects in Golgi organization associated with LRRK2 deﬁcits, they did not functionally connect these defects to the accumulation of undigested waste vesicles that are also observed in these cells (21, 22). Here we show that the fragmentation of the entire Golgi apparatus in LRRK2-deﬁcient cells leads to deﬁcits in trans-Golgi to lysosome trafﬁcking, including the trafﬁcking of important lysosomal hydro-
lases. Collectively, these data implicate defects in Golgi apparatus organization and structure as the primary cause for lysosomal dysfunction in renal cells lacking LRRK2.

In this context, it is worth noting that genes whose absence or mutation cause similar defects in protein trafficking have previously been associated with PD, including the genes encoding glucocerebrosidase (GBA) and the retromer complex component VPS35 (9, 25). Whether this implies a general mechanism for the onset of cellular toxicity in PD is unclear at this time, particularly because the relationship of lysosomal dysfunction to the other cardinal hallmarks of PD (mitochondrial dysfunction and alpha-synuclein aggregation) remains somewhat obscure (9). Given the growing number of vesicle trafficking proteins that have been connected to this disease, however, the ongoing search for a unifying mechanism is of considerable importance.

One final point of interest regarding LRRK2 and human disease should be noted in the context of renal cancer. Both we and others have implicated LRRK2 amplification and hyperactivity in the type I subset of papillary renal cell carcinomas that account for ~10% of all human kidney cancer (2, 23). We speculate that these tumors, which are driven by aberrant receptor tyrosine kinase signaling through the hepatocyte growth factor receptor MET, may select for LRRK2 amplification (chromosome 12q12) to promote mistrafficking of MET away from lysosomes and toward the endosome-to-Golgi recycling pathway. Given that LRRK2 knockdown seems to enhance the rate of trafficking from endosomes to the lysosome, it is possible that the converse event (hyperactivation of LRRK2 activity) would slow trafficking of endocytic cargo to the lysosome, leading to aberrant stabilization of MET. We intend to address this question in future studies as a means of providing insights into the molecular events leading to cellular transformation in the human kidney.

ACKNOWLEDGMENTS

We thank Dr. Ted Dawson (Johns Hopkins) for provision of fixed embedded renal tissue from Lrrk2 knockout mice and Dr. Jaclyn Henderson (Pfizer, New York, NY) for provision of the LRRK2 inhibitor PFE-064/47475. Present addresses: N. J. Lanning, California State University, Dept. of Biology, 5151 State University Dr., Los Angeles, CA 90032; C. VanOpstall, University of Chicago, Ben May Dept. for Cancer Research, 5841 South Maryland Ave., Chicago, IL 60637; M. L. Goodall, University of Colorado Denver, Dept. of Pharmacology, 12801 East 17th Ave., Aurora, CO 80045; J. P. MacKeigan, Michigan State University College of Human Medicine, Dept. of Obstetrics, Gynecology and Reproductive Biology, 15 Michigan St., NE, Grand Rapids, MI 49503.

GRANTS

This work has been supported by the National Institutes of Health with funding from National Cancer Institute Grant 1-R15-CA192094.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


