Mitochondria-lysosome contacts are recently identified sites for mediating crosstalk between both organelles, but their role in normal and diseased human neurons remains unknown. In this study, we demonstrate that mitochondria-lysosome contacts can dynamically form in the soma, axons, and dendrites of human neurons, allowing for their bidirectional crosstalk. Parkinson’s disease patient derived neurons harboring mutant GBA1 exhibited prolonged mitochondria-lysosome contacts due to defective modulation of the untethering protein TBC1D15, which mediates Rab7 GTP hydrolysis for contact untethering. This dysregulation was due to decreased GBA1 (β-glucocerebrosidase (GCase)) lysosomal enzyme activity in patient derived neurons, and could be rescued by increasing enzyme activity with a GCase modulator. These defects resulted in disrupted mitochondrial distribution and function, and could be further rescued by TBC1D15 in Parkinson’s patient derived GBA1-linked neurons. Together, our work demonstrates a potential role of mitochondria-lysosome contacts as an upstream regulator of mitochondrial function and dynamics in midbrain dopaminergic neurons in GBA1-linked Parkinson’s disease.
Mitochondria-lysosome (M–L) contact sites were recently identified as inter-organelle membrane contacts involving the dynamic tethering of mitochondria with lysosomes. Importantly, M–L contacts allow for the bidirectional regulation of both mitochondrial and lysosomal network dynamics, and mediate their direct interaction in a pathway distinct from mitophagy or lysosomal degradation of mitochondrial-derived vesicles. M–L contact sites are further regulated by TBC1D15, a Rab7 GAP recruited to mitochondria via the outer mitochondrial membrane protein Fis1, which mediates Rab7 GTP hydrolysis to promote M–L contact untethering. However, the formation and role of M–L contacts in human neurons have not been previously investigated.

Parkinson’s disease (PD) is the most common neurodegenerative movement disorder characterized by progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNc), leading to the cardinal motor symptoms of PD. Interestingly, both mitochondrial and lysosomal defects have been genetically and functionally linked to PD. Indeed, the lysosomal enzyme GBA1, which encodes β-glucocerebrosidase (GCase), and catalyzes the hydrolysis of glucosylceramide (GlCer) to glucose and ceramide, represents the greatest genetic risk factor for PD, with GBA1 mutation carriers exhibiting more severe cognitive symptoms. GBA1–PD patient-derived mutant neurons demonstrate both lower GCase protein levels and reduced GCase activity. Moreover, wild-type GCase activity is decreased in both idiopathic and multiple types of familial PD patient neurons, highlighting targeting of GBA1 as a potential therapeutic approach for PD and a key role for GCase activity in PD pathogenesis. However, whether M–L contact dynamics are disrupted in PD patient neurons and further modulated by GCase activity is not known.

Here we investigated the impact of M–L contact sites in neuronal function and GBA1–linked PD pathogenesis. Using super-resolution time-lapse imaging, we found that M–L contact sites dynamically formed in the soma, axon, and dendrites of human iPSC-differentiated dopaminergic neurons. We further reported a role for M–L contacts in PD patient-derived dopaminergic neurons, as contact untethering was disrupted in patient neurons expressing heterozygous mutant GBA1. Finally, we showed that defective M–L contact untethering was caused by decreased GCase lysosomal enzymatic activity leading to GlCer accumulation. This resulted in decreased levels of the untethering protein TBC1D15 which normally mediates Rab7 GTP hydrolysis for contact untethering. Ultimately, these defects resulted in disrupted mitochondrial distribution and function, but could be rescued by TBC1D15 in Parkinson’s patient GBA1–linked neurons. Together, our work identifies an important role for M–L contact sites in human neurons and in GBA1–linked PD pathophysiology.

**Results**

**M–L contact sites dynamically form in human neurons.** To investigate M–L contact sites in human neurons, we utilized induced pluripotent stem cell (iPSC) technology (Supplementary Fig. 1a) to generate human midbrain dopaminergic neurons from healthy controls using previously established protocols. Differentiated neurons expressed the neural specific marker β-III-tubulin (TUJ1) and midbrain dopaminergic neuron-specific markers: tyrosine hydroxylase (TH), forkhead box protein A2 (FOXA2), and LIM Homeobox Transcription Factor 1 Alpha (LMX1A) (Supplementary Fig. 2a, b), and were subsequently imaged for mitochondrial and lysosomal dynamics in live neurons (Supplementary Fig. 3a, b).

Using 3D super-resolution structured illumination microscopy (3D N-SIM), we found that mitochondria and lysosomes (MitoRFP, Lyso-GFP) formed stable inter-organelle contacts in human neurons (Fig. 1a). Neuronal M–L contacts were further confirmed to be <10 nm apart using electron microscopy (EM) (Fig. 1b), consistent with other contact sites. Next, we conducted confocal time-lapse microscopy of live neurons, and found that M–L contacts dynamically formed over time and remained tethered together (yellow arrows) before subsequently untethering from one another (white arrows) (Fig. 1c and Supplemental Movies 1–3). Mitochondria in contact with lysosomes maintained their membrane potential, as visualized by TMRM imaging (Supplementary Fig. 3c–e), and dynamic M–L contact tethering could be further observed in human neurons by imaging outer mitochondrial membrane and lysosomal membrane proteins (TOM20-RFP, LAMP1-GFP) (Supplementary Fig. 3f), as well as by imaging mitochondrial and lysosomal-targeted dyes (MitoTracker, LysoTracker) (Supplementary Fig. 3g). In addition, we could also visualize M–L contacts in human neurons by proximity ligation assay (PLA) imaging of TOM20 and LAMP1 on the outer mitochondrial and lysosomal membrane (Supplementary Fig. 4a–b). M–L contacts in human neurons remained tethered for an average duration of 88.07 ± 5.05 s (Fig. 1d, e), with ~18% of lysosomes in contact with mitochondria at any point in time (Fig. 1f). Thus, M–L contacts can dynamically form to mediate crosstalk between mitochondria and lysosomes in human neurons.

**Spatial compartmentalization of neuronal M–L contact dynamics.** Next, we investigated the spatial compartmentalization of neuronal M–L contacts in the soma, dendrites, and axons of human neurons, as both organelles are localized throughout multiple neuronal compartments. Dendrites and axons were identified as being positive for MAP2 or Tau, respectively (Supplementary Fig. 2c). Importantly, we found that mitochondria and lysosomes tethered at contact sites (yellow arrows) within all three neuronal compartments in the soma (Fig. 2a (left) and Supplemental Movie 4), dendrites (Fig. 2a (middle), Supplemental Movie 5), and axons (Fig. 2a (right) and Supplemental Movie 6). M–L contacts in dendrites and axons exhibited decreased mitochondrial and lysosomal motility (yellow arrows) (Fig. 2b). To further examine whether the dynamics of contacts differed by spatial compartmentalization, we quantified the duration of M–L contacts and percentage of lysosomes in contact with mitochondria across different neuronal compartments. Interestingly, live-cell imaging analysis revealed that the average minimum duration of M–L contacts was significantly increased in axons compared to those in the soma (soma: 80.6 ± 6.2 s; dendrites: 82.4 ± 9.7 s; axons: 112.5 ± 13.2 s) (Fig. 2c). In contrast, the percentage of lysosomes contacting mitochondria in the soma, dendrites, and axons did not differ (Fig. 2d). Together, these results demonstrate that M–L contacts are able to form with varying dynamics across multiple neuronal compartments in human neurons.

**Loss of GCase activity disrupts M–L contact untethering in GBA1–PD patient dopaminergic neurons.** PD has been genetically and functionally linked to both mitochondrial and lysosomal defects, but whether M–L contacts are disrupted in PD has not been previously investigated. As mutations in the lysosomal enzyme GCase (GBA1) represent the greatest genetic risk factor for PD, and GCase activity is decreased in both idiopathic and multiple types of familial PD patient neurons, we examined M–L contact dynamics in GBA1–PD (Δhet 84GG) patient neurons. Using PD patient fibroblasts harboring mutant GBA1, we generated human iPSCs and their isogenic controls by CRISPR-
Cas916 (Supplementary Fig. 1a) which were subsequently differentiated into midbrain dopaminergic neurons (mutant GBA1 (ΔGBA); isogenic control (Corr)) (Supplementary Fig. 2a–c). Both mutant GBA1 and its isogenic control did not affect the efficiencies of fibroblast reprogramming to iPSCs or differentiation into midbrain dopaminergic neurons from iPSCs16.

We first confirmed that PD patient-derived mutant GBA1 dopaminergic neurons exhibited decreased GCase protein levels (Fig. 3a, b). We also conducted live-cell GCase activity assays and showed that mutant GBA1 patient neurons had reduced total GCase enzymatic activity compared to isogenic control neurons (Fig. 3c–e). To further compare lysosomal and non-lysosomal GCase activity separately, we used Bafilomycin A1 (BafA1), an inhibitor of lysosomal acidification, as an established protocol for examining lysosomal GCase activity24 and also found a significant decrease in lysosomal GCase activity in patient-derived mutant GBA1 neurons compared to CRISPR-corrected isogenic control neurons (Fig. 3c, d, f), consistent with previous findings16.
Fig. 2 Spatial compartmentalization of neuronal mitochondria-lysosome contact dynamics. a Representative time-lapse confocal images of contacts between mitochondria (red, Mito-RFP) and lysosomes (green, Lyso-GFP) in soma, dendrites, and axons of wild-type human dopaminergic iPSC-derived neurons. Time-lapse recordings were taken at 2 s intervals for 5 min. Yellow arrows mark stable M–L contacts. White arrows mark the site of M–L contacts before contact formation or after contact untethering. Black line shows duration of contacts. Scale bar = 500 nm (see Supplementary Movies 4–6).
b Representative frames of live-cell imaging (left) and dual color kymographs (right) of M–L contacts in dendrites and axons (mitochondria: red, Mito–RFP; lysosomes: green, Lyso–GFP). In confocal image frames (left), contact sites are denoted by yellow arrows. In kymographs: white scale bar = 1 μm, yellow vertical bar = 30 s. Yellow arrows in kymograph point to start and end timepoints of M–L contact tethering. Left black line shows duration of contacts. c, d Quantification of M–L contacts across different neuronal compartments. One-way ANOVA followed by Tukey’s multiple comparisons test. c Comparison of average minimum durations of M–L contacts in soma, dendrites, and axons (n = 86 (soma), n = 35 (dendrite), n = 34 (axon) contacts from 26 neurons, N = 3 independent experiments). *p = 0.0335. d Percentage of lysosomes contacting mitochondria in soma, dendrites, and axons (for >20 s) (n = 14 (soma), n = 13 (dendrite), n = 14 (axon) neurons; N = 3 independent experiments). For all quantifications, data are means ± S.E.M; *p ≤ 0.05, ns: not significant.
We next investigated whether lysosomal contacts with mitochondria were disrupted by loss of lysosomal GCase activity by conducting confocal live-cell microscopy of mitochondria and lysosomes in mutant GBA1 and CRISPR-corrected isogenic control neurons. Interestingly, while M–L contacts dynamically formed in both conditions (yellow arrows) (Fig. 3g and Supplemental Movies 7, 8), the average duration of M–L contact tethering was significantly increased in mutant GBA1 neurons.

**Fig. 3** Loss of GCase activity disrupts mitochondria–lysosome contact untethering in GBA1-PD patient dopaminergic neurons. a, b Western blot analysis of PD patient-derived mutant GBA1 dopaminergic neurons (ΔGBA; het 84GG) and its CRISPR-corrected isogenic control (Corr) neurons. GCase level was significantly reduced in ΔGBA neurons (N = 4 independent experiments). Paired two-sided Student’s t-test; **p = 0.0083. c–f ΔGBA and Corr neurons were treated with either DMSO or BafA1 and subjected to live-cell GCase activity analysis. e Quantification of the area under each curve (AUC) demonstrates decreased total GCase activity in ΔGBA neurons. Paired two-sided Student’s t-test; *p = 0.0183. f Lysosomal GCase activity was calculated by subtracting BafA1 values from DMSO. Values are expressed as fold-change compared to isogenic controls (N = 3 independent experiments). Paired two-sided Student’s t-test; *p = 0.0352. g Representative time-lapse confocal images of contacts between mitochondria (red, Mito-RFP) and lysosomes (green, Lyso-GFP) in Corr (left) and ΔGBA (right) human neurons. Yellow arrows mark stable M–L contacts. White arrows mark the site of M–L contacts after contact untethering. Black line shows duration of contacts. Scale bar = 500 nm (see Supplementary Movies 7 and 8). h Quantification of average minimum duration (left) and relative frequency distribution of the duration of stable M–L contacts (right), showing increased duration of stable M–L contacts in ΔGBA neurons (n = 48 contacts from Corr and n = 71 contacts from ΔGBA, N = 3 independent experiments). X-axis of the histogram represents bin centers. Bin width = 60 s. Unpaired two-sided Student’s t-test; *p = 0.0204. i Percentage of lysosomes contacting mitochondria (for >20 s) (n = 12 Corr and n = 36 ΔGBA neurons, N = 3 independent experiments). Unpaired two-sided Student’s t-test. For all quantifications, data are means ± S.E. M.; *p ≤ 0.05, **p ≤ 0.01, ns: not significant.
indicative of inefficient untethering events (Fig. 3h). However, the percentage of lysosomes in contacts was similar between conditions (Fig. 3i), suggesting that the subsequent untethering but not initial formation of M–L contact tethering was disrupted in mutant GBA1 neurons.

To further examine M–L contacts, we conducted PLA imaging of M–L contacts in mutant GBA1 and CRISPR-corrected isogenic control neurons (Supplementary Fig. 4c), and similarly found that M–L contacts could still form in both conditions (Supplementary Fig. 4d). In addition, we conducted EM imaging of M–L contacts in mutant GBA1 and CRISPR-corrected isogenic control neurons (Supplementary Fig. 4e), and also found that M–L contacts tethered together in both conditions, with the length of membrane contact between mitochondria and lysosomes not altered in mutant GBA1 neurons (Supplementary Fig. 4f). Of note, ER–mitochondria contact and ER–lysosome contact formation were also not disrupted in mutant GBA1 neurons (Supplementary Fig. 5a–f). Together, our results suggest that loss of GCase activity does not disrupt M–L contact formation, but preferentially disrupts the untethering of M–L contact sites, resulting in prolonged contact site tethering between mitochondria and lysosomes.

We further examined whether these changes in M–L contact dynamics might be specific to loss of GCase activity rather than general lysosomal enzyme dysfunction. Mutant GBA1 neurons had altered lysosomal morphology, including enlarged lysosomes (Fig. 3g and Supplementary Fig. 3h, i). However, inhibition of other lysosomal enzymes in human neurons which also led to enlarged lysosomal morphology (Supplementary Fig. 6a) including inhibition of lysosomal acid ceramidase (carmofur treatment), cysteine proteases (E64D treatment), or aspartyl proteases (inhibition of lysosomal acid ceramidase (carmofur treatment), enlarged lysosomal morphology (Supplementary Fig. 6a) including inhibition of lysosomal enzymes in human neurons which also led to general lysosomal enzyme dysfunction. Mutant GBA1 neurons was sufficient to restore defective M–L contact dynamics. The modulator S-181 was recently found to disrupt M–L contact dynamics in human neurons (Supplementary Fig. 6c) rescued the prolonged M–L contact tethering in mutant GBA1 neurons (Supplementary Fig. 6d), further highlighting the role of GCase activity in regulating M–L contact dynamics.

Tethering proteins mediating M–L contact are disrupted in GBA1-PD patient dopaminergic neurons. We subsequently examined the potential mechanism through which mutant GBA1 might disrupt M–L contact dynamics. We recently showed that M–L contact untethering is mechanistically regulated by lysosomal Rab7-GTP hydrolysis from GTP to GDP-bound Rab7, and is driven by the GAP (GTPase-activating protein) activity of mitochondrial TBC1D15 (Rab7 GAP)3,4, which is recruited to the outer mitochondrial membrane by Fis14,6. Thus, we investigated whether Rab7-GTP hydrolysis might be disrupted by mutant GBA1 in patient neurons. We first measured the total protein levels of Rab7 together with Fis1 and TBC1D15 in PD patient-derived mutant GBA1 dopaminergic neurons compared to isogenic controls (Fig. 4a–d). We observed no differences in total Rab7 levels (Fig. 4c) or Fis1 levels (Fig. 4d), and Fis1 levels were not altered even after normalizing for mitochondrial levels (Fis1/Tom20; Corr vs GBA1, p = 0.23 (not significant)) (Supplementary Fig. 3j). Of note, TBC1D15 and Fis1 localization to mitochondria (Supplementary Fig. 7a, b), and Rab7 localization to lysosomes (Supplementary Fig. 7c), as well as the levels of the Rab7 GEF (guanine nucleotide exchange factor) complex proteins Mon1 and Ccz1 (Supplementary Fig. 7d) were also not altered in mutant GBA1 neurons. However, we surprisingly found that TBC1D15 levels were significantly decreased in mutant GBA1 patient-derived neurons (\( \ast \alpha p \leq 0.05 \)) (Fig. 4b).

Based on these findings, we hypothesized that disrupted M–L contact untethering in mutant GBA1 neurons might be due to defective Rab7-GTP hydrolysis as a consequence of reduced TBC1D15 levels (Rab7 GAP). To test this, we performed GST-RILP (Glutathione Transferase-Rab Interacting Lysosomal Protein) pull-down assays5,7 to measure GTP-bound Rab7 levels, as RILP preferentially binds to GTP-bound Rab726,27 (Fig. 4e). Importantly, we found that PD patient-derived mutant GBA1 dopaminergic neurons demonstrated significantly increased levels of Rab7-GTP/total Rab7 compared to CRISPR-corrected isogenic control neurons (\( \ast \alpha p \leq 0.05 \)) (Fig. 4e, f). Together, these results suggest that decreased TBC1D15 levels in mutant GBA1 neurons disrupted Rab7 GTP hydrolysis, resulting in increased GTP-bound Rab7 and prolonged M–L contact tethering dynamics.

To further investigate the downregulation of TBC1D15 in mutant GBA1 neurons, we analyzed TBC1D15 by qPCR and showed that decreased TBC1D15 protein levels in mutant GBA1 neurons were not due to lower TBC1D15 transcripts levels (Supplementary Fig. 8a). Rather, decreased TBC1D15 protein levels resulted from elevated proteasomal degradation of TBC1D15, which could be inhibited by lactacystin, leading to similar TBC1D15 protein levels between mutant GBA1 and isogenic control neurons (Supplementary Fig. 8b). We next examined whether mitochondrial dysfunction was sufficient to disrupt TBC1D15 expression levels. Treatment of either the mitochondrial uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (CCCP), Complex I inhibitor rotenone, Complex III inhibitor Antimycin, or the ATP synthase inhibitor Oligomycin (Supplementary Fig. 8c–f) did not disrupt TBC1D15 expression levels in HeLa cells. In contrast, increasing GCase activity in mutant GBA1 neurons using the modulator S-181 was sufficient to rescue the decreased levels of TBC1D15 (Supplementary Fig. 6e, f). Thus, these findings suggest that TBC1D15 levels are downregulated at the protein level by proteasomal degradation in mutant GBA1 neurons, due to the loss of GCase activity rather than general mitochondrial dysfunction.

Finally, we examined if loss of TBC1D15 was able to disrupt M–L contact dynamics in human neurons. Knockdown of TBC1D15 in wild-type iPSC-derived neurons (Supplementary Fig. 9a) increased M–L contact tethering duration (Supplementary Fig. 9b), consistent with findings in non-neuronal cells8 and further supporting our results that decreased TBC1D15 levels in PD GBA1-linked patient neurons disrupt M–L contact dynamics.

Inhibition of GCase activity disrupts M–L contact untethering. To further examine whether M–L contact untethering defects were indeed dependent on loss of GCase activity, we treated wild-type human midbrain dopaminergic neurons expressing wild-type GCase with the GCase inhibitor conduritol-b-epoxide (CBE) (50 μM; 7 days) which decreases neuronal GCase activity and promotes GlcCer accumulation14,16,28. Using confocal live-cell time-lapse imaging of mitochondria and lysosomes in both wild-type (Ctrl) and CBE-treated (+CBE) human neurons, we found that M–L contacts dynamically formed in both conditions (yellow arrows) (Fig. 5a). In addition, CBE treatment was effective in increasing lysosomal size as has been previously reported29 (Fig. 5b, c). Importantly, we found that inhibition of GCase activity by CBE treatment resulted in significantly prolonged M–L contact tethering duration (\( \ast \ast \ast \alpha p \leq 0.001 \)) (Fig. 5a, d), consistent with what we observed in mutant GBA1 patient-derived neurons, and further confirming the effect of decreased GCase activity on disrupting M–L contact untethering.
GBA1 mutant total Rab7 (Fig. 5l, m), consistent with what we observed in and CBE-treated neurons support the hypothesis that loss disruption of TBC1D15 levels and Rab7-GTP hydrolysis in of GCase activity, which increases GlcCer levels, leads to decreased TBC1D15 levels (Supplementary Fig. 10f, g). treatment led to similar defects in Rab7 GTP hydrolysis, resulting also showed reduced TBC1D15 levels (Supplementary Fig. 10f, g). treatment led to similar defects in Rab7 GTP hydrolysis, resulting also showed reduced TBC1D15 levels (Supplementary Fig. 10f, g).

We additionally validated these findings in two other wild-type iPSC-derived neuronal lines which we characterized for iPSC and midbrain dopaminergic neuron-specific markers (Supplementary Fig. 1a–c). Inhibition of GCase activity with CBE treatment in both lines also led to significantly prolonged M–L contact tethering duration (Supplementary Fig. 10d, e), further supporting our results that inhibition of GCase activity disrupts M–L contact dynamics in human neurons.

As loss of GCase activity results in significantly increased GlcCer levels (Fig. 5e, f), we then asked whether treatment of exogenous GlcCer in wild-type iPSC-derived neurons could be sufficient to disrupt M–L contact dynamics. Using live-cell microscopy of mitochondria and lysosomes in wild-type iPSC-derived neurons treated with exogenous GlcCer, we found that this also led to significantly prolonged contact site tethering duration (**p < 0.001) (Fig. 5g), highlighting a role for increased GlcCer levels in mutant GBA1 neurons in dysregulating M–L contacts.

Next, we investigated whether inhibition of GCase activity also disrupted M–L contact untethering machinery. We measured the total protein levels of Rab7 together with TBC1D15 and Fis1 in wild-type (Ctrl) and CBE-treated (+CBE) humans neurons (Fig. 5h–k). Indeed, CBE-treated neurons also showed significantly decreased TBC1C15 levels (**p ≤ 0.05) (Fig. 5h, i), without changes in total Rab7 or Fis1 levels (Fig. 5j, k), consistent with mutant GBA1 patient-derived neurons. To further support these findings, wild-type iPSC-derived neurons treated with exogenous GlcCer also showed reduced TBC1D15 levels (Supplementary Fig. 10f, g).

We then examined whether inhibition of GCase activity by CBE treatment led to similar defects in Rab7 GTP hydrolysis, resulting in elevated GTP-bound Rab7 levels. Using the GST-RILP pull-down assay in CBE-treated neurons (Fig. 5l), we further found that GCase inhibition resulted in increased levels of RAB7-GTP/total Rab7 (Fig. 5l, m), consistent with what we observed in mutant GBA1 neurons. In summary, our results from both mutant GBA1 and CBE-treated neurons support the hypothesis that loss of GCase activity, which increases GlcCer levels, leads to disruption of TBC1D15 levels and Rab7-GTP hydrolysis in neurons, resulting in the misregulation of M–L contact untethering dynamics.

Mitochondrial dysfunction due to prolonged M–L contacts is partially rescued by TBC1D15 expression. M–L contacts mediate the bidirectional regulation of both mitochondrial and lysosomal network dynamics, and importantly are able to directly regulate both mitochondrial dynamics and motility. We thus investigated whether mitochondrial dynamics might be disrupted in PD patient-derived mutant GBA1 dopaminergic neurons, consistent with defective M–L contact untethering, by analyzing the distribution of mitochondria in the soma and axons. Healthy mitochondria with intact mitochondrial membrane potential were imaged by live-cell imaging in patient-derived mutant GBA1 (ΔGBA) and CRISPR-corrected isogenic control (Corr) neurons (Supplementary Fig. 3c). Interestingly, while there was no difference in mitochondrial density in the soma (Fig. 6a, b and Supplementary Fig. 3k), we found that axonal mitochondrial density was significantly decreased in mutant GBA1 neurons compared to axons of isogenic control neurons (Fig. 6a, c), suggesting that mutant GBA1 preferentially disrupts axonal mitochondrial distribution in Parkinson’s patient neurons. In addition, we also observed defective mitochondrial respiration as measured by decreased oxygen consumption rate (OCR) in mutant GBA1 neurons (Fig. 6d), as well as decreased AMPK activation (Fig. 6e), compared to isogenic control neurons. We also measured cellular ATP level and found significantly decreased ATP levels in mutant GBA1 neurons compared to isogenic controls (Fig. 6f), even after normalization for mitochondrial mass (**p < 0.001; Corr vs GBA [ATP intensity/TOM20 levels]). Consistent with our findings that TBC1D15 levels were decreased in mutant GBA1 neurons, we also observed mitochondrial dysfunction in TBC1D15 knockdown neurons (Supplementary Fig. 9c).

Based on these findings, we hypothesized that dysregulated M–L contact untethering dynamics in mutant GBA1 neurons...
might contribute to these defects in mitochondrial distribution and function. Thus, to test if these defects could be rescued by promoting M–L contact untethering in mutant GBA1 neurons, we expressed human TBC1D15 in mutant GBA1 neurons by lentiviral transduction (Fig. 6g and Supplementary Fig. 11a). The expression of exogenous TBC1D15 in neurons was confirmed by western blot analysis and fluorescence imaging (Supplementary Fig. 11b, c). Importantly, consistent with our previous findings that TBC1D15 promotes M–L contact untethering2, transduction of wild-type TBC1D15 in mutant GBA1 neurons (ΔGBA+TBC1D15) was able to promote M–L contact untethering, resulting in significantly decreased M–L contact durations compared to lentiviral vehicle-treated neurons (ΔGBA+veh) (***p ≤ 0.001) (Fig. 6h).

We further investigated the effect of rescuing M–L contact dynamics on axonal mitochondrial density in mutant GBA1 neurons. Interestingly, while TBC1D15 expression did not alter mitochondrial densities in the soma of mutant GBA1 neurons (Fig. 6g, i), it significantly rescued the decreased mitochondrial density in axons of mutant GBA1 neurons (Fig. 6g, j). Moreover, TBC1D15 expression was also able to partially rescue ATP levels in mutant GBA1 neurons (Fig. 6k). Together, our results suggest that upregulation of TBC1D15 in PD patient mutant GBA1 neurons is sufficient to rescue the misregulation of M–L contact
Inhibition of GCase activity disrupts mitochondria-lysosome contact untethering in dopaminergic neurons. **a** Healthy control WT human iPSC-derived dopaminergic neurons were treated with vehicle (Ctrl) or CBE (50 μM; 7 days) to inhibit GCase activity. Representative time-lapse confocal images of contacts between mitochondria (red, Mito-RFP) and lysosomes (green, Lyso-GFP) in Ctrl (left) and CBE-treated (right) human neurons. Yellow arrows mark stable M–L contacts. White arrows mark the site of M–L contacts after contact untethering. Black line shows duration of contacts. Scale bar = 500 nm. **b** Representative confocal images of lysosomes (LAMP1-GFP) in the Ctrl (left) and CBE-treated (right) human neurons. Scale bar = 1 μm. **c** The effects of CBE treatment on lysosomal morphology were confirmed by quantification of the percentage of lysosomes that were enlarged (diameter >0.5 μm) in M–L contacts (n = 12 neurons per condition, N = 3 independent experiments). Unpaired two-sided Student’s t-test; **p = 0.0001. **d** CBE treatment in control neurons increased the average minimum duration of stable M–L contacts (n = 60 contacts per condition, N = 3 independent experiments). Unpaired two-sided Student’s t-test; ***p = 0.0001. **e** Representative confocal images of immunocytochemistry of GlcCer in the Ctrl (left) and CBE-treated (right) human neurons. Scale bar = 5 μm. **f** Quantification of CBE treatment leading to increased GlcCer levels as measured by immunofluorescence signal of GlcCer (n = 21 neurons per condition, N = 3 independent experiments). **g** Exogenous GlcCer treatment in control neurons increased the average minimum duration of stable M–L contacts (n = 93 contacts from Ctrl, n = 94 contacts from +GlcCer neurons, N = 3 independent experiments). Unpaired two-sided Student’s t-test; **p = 0.0001. **h–k** Western blot analysis of TBC1D15, Rab7, and TIS15 in Ctrl and CBE-treated neurons. Protein levels were normalized to loading control GAPDH. Values are expressed as fold-change compared to Ctrl (N = 5 independent experiments). Paired two-sided Student’s t-test; *p = 0.0104. **l** GST-RILP pull-down to measure GTP-bound Rab7 levels in Ctrl and CBE-treated neurons. **m** Rab7-GTP levels were normalized to total Rab7 normalized to GAPDH. Values are expressed as fold-change compared to Ctrl (N = 3 independent experiments). Paired two-sided Student’s t-test; *p = 0.0242. For all quantifications, data are means ± S.E.M., “p ≤ 0.05,” ***p ≤ 0.001.

**Discussion**

Our study demonstrates that M–L contact sites dynamically form in human neurons, and further investigates their role in neurons from patients with GBA1-linked PD. We found that loss of lysosomal GCase enzymatic activity in PD patient-derived dopaminergic neurons led to prolonged M–L contact tethering dynamics due to defective contact untethering machinery, and resulted in misregulated axonal distribution of mitochondria and decreased ATP levels. Importantly, we showed that rescuing M–L contact site dynamics in PD patient neurons is sufficient to ameliorate defects in mitochondrial distribution and function, thus highlighting a potential role for M–L contact site dysregulation in PD pathogenesis.

Multiple genes linked to mitochondria or lysosomes have been identified as causative or risk genes of PD. Moreover, both mitochondrial and lysosomal dysfunction have been implicated in PD, suggesting a functional crosstalk between these two organelles. We previously found that oxidized dopamine mediates convergence of mitochondrial and lysosomal dysfunction in human but not in mouse dopaminergic neurons, highlighting the importance of patient-derived dopaminergic neurons for studies of PD pathogenesis.

Despite the previously studied role of mitochondria and lysosomes in PD pathogenesis, a direct homeostatic relationship between these two organelles that is independent of eventual lysosomal degradation of mitochondria has not been examined. In this context, our work provides evidence for the role of M–L contacts not only in the homeostasis of dopaminergic neurons but also as a link between mitochondrial and lysosomal dysfunction in PD pathogenesis. We specifically focused on GBA1 mutations which represent the greatest genetic risk factor for PD. Importantly, wild-type GCase enzyme activity is also reduced in patient neurons with genetic or idiopathic PD who do not harbor GBA1 mutations, suggesting that loss of GCase activity is an important contributor to PD pathogenesis. Previously, we reported that the loss of GCase function in patient neurons compromises lysosomal protein degradation which contributes to other key PD pathogenic phenotypes including α-synuclein accumulation. In addition to its primary lysosomal dysfunction, GBA1 mutations and abnormal GCase activity have also been linked to mitochondrial dysfunction.

In this study, we show that GBA1 mutant PD patient neurons have defective M–L contact dynamics, resulting in prolonged contact tethering. While previous studies did not examine the GBP-bound state of Rab7, we found that loss of GCase activity resulted in an increased percentage of active GBP-bound lysosomal Rab7, which directly mediates M–L contact tethering. This is likely due to decreased levels of TBC1D15 (Rab7 GAP) in mutant GBA1 patient neurons, as TBC1D15’s GAP activity promotes Rab7 GTP hydrolysis and subsequent M–L contact untethering. Indeed, we found that increasing TBC1D15 expression was sufficient to rescue the prolonged M–L contact tethering we observed in mutant GBA1 neurons. In addition, we further observed mitochondrial dysfunction in patient neurons, including abnormal mitochondrial distribution in axons, decreased mitochondrial respiration and lower ATP levels. Of note, TBC1D15 expression was also able to rescue mitochondrial dysfunction. Thus, dysregulation of M–L contacts may play an important role in GBA1-linked PD pathogenesis, and targeting contact machinery may help ameliorate downstream mitochondrial dysfunction. Given that mitochondria play key roles as energy suppliers especially in the synapses of active neurons, we hypothesize that such abnormal distribution of mitochondria may further contribute to synaptic dysfunction in PD.

While inter-organelle contact sites have been found to be essential subdomains for modulating cellular function and homeostasis, only recently have studies reported the function and molecular architecture of inter-organelle contacts in neurons, such as those between the mitochondria and endoplasmic reticulum. In addition, dysfunction of inter-organelle
Fig. 6 Mitochondrial dysfunction due to prolonged mitochondria-lysosome contacts is partially rescued by expression of TBC1D15 in GBA1-PD patient dopaminergic neurons. a Live-cell distribution of mitochondria (TMRM, red) in the soma and axons from PD patient-derived mutant GBA1 dopaminergic neurons (ΔGBA) and its CRISPR-corrected isogenic control (Corr) neurons. White arrows mark mitochondria in a single axon. Scale bar, 10 μm. b Quantified mitochondrial density in the soma (TMRM-positive pixels/pixels of soma). Corr (n = 47 neurons), ΔGBA (n = 47 neurons) (N = 3 independent experiments). c Quantified mitochondrial density in axons (mitochondria count/length of axon (μm)). Corr (n = 122 axons), ΔGBA (n = 114 axons) (N = 3 independent experiments). d Oxygen consumption rate (OCR) was measured and normalized to total protein content. Corr (n = 9 samples), ΔGBA (n = 9 samples) (N = 3 independent experiments). e Western blot analysis of phospho-AMPKα and AMPKα levels in Corr and ΔGBA neurons. Ratio of p-AMPKα/AMPKα are expressed as fold-change compared to Corr (N = 3 independent experiments). Paired two-sided Student’s t-test; ***p < 0.0001. f Total cellular ATP content was measured and normalized to total protein content (ng/μl). Corr (n = 40 samples), ΔGBA (n = 40 samples) (N = 3 independent experiments). Unpaired two-sided Student’s t-test; ***p < 0.0001. g Live-cell distribution of mitochondria (TMRM, red) in the soma and axons from Corr and ΔGBA human neurons with or without human TBC1D15 lentiviral expression. White arrows mark mitochondria in a single axon. Scale bar, 10 μm. h Quantification of average minimum duration of M-L contacts in Corr-vehicle (n = 62 contacts from 18 neurons), ΔGBA-vehicle (n = 60 contacts from 15 neurons), ΔGBA-TBC1D15 (n = 63 contacts from 19 neurons) (N = 3 independent experiments); ***p < 0.0001. i Quantified mitochondrial density in the soma (TMRM-positive pixels/pixels of soma). Corr-vehicle (n = 32 neurons), ΔGBA-vehicle (n = 31 neurons), ΔGBA-TBC1D15 (n = 38 neurons) (N = 3 independent experiments). j Quantified mitochondrial density in axons (mitochondria count/length of axon (μm)). Corr-vehicle (n = 124 axons), ΔGBA-vehicle (n = 127 axons), ΔGBA-TBC1D15 (n = 127 axons) (N = 3 independent experiments), ***p < 0.0001. k Total cellular ATP content was measured and normalized to total protein content (ng/μl). Corr-vehicle (n = 19 samples), ΔGBA-vehicle (n = 19 samples), ΔGBA-TBC1D15 (n = 19 samples) (N = 3 independent experiments), ***p = 0.0002, *p = 0.0493. h-k One-way ANOVA followed by Tukey’s multiple comparisons test. For all quantifications, data are the means ± S.E.M.; *p ≤ 0.05, ***p ≤ 0.001, ns: not significant.
contacts in disease have been shown to be key contributors in the development of various diseases. Moreover, the recent identification of M–L contact sites has shed new light on the direct relationship between mitochondria and lysosomes in a pathway independent of lysosomal degradation of mitochondria, allowing for direct crosstalk and regulation of both organelles in a dynamic manner. In our previous study, we showed the formation of M–L contacts in non-neuronal cells and identified protein mediators responsible for contact un tethering. Here, we demonstrate that M–L contacts are also key contributors in human neurons and that contacts dynamically form in multiple neuronal compartments, suggesting that they act as important sites for the neuronal regulation of mitochondrial and lysosomal dynamics. Together, our findings not only provide insights into inter-organelle contacts in maintaining the cellular homeostasis of human neurons, but also suggest the importance of M–L contacts as a potential target for therapeutic development in PD.

Methods

Human iPSC culture and characterization. Detailed procedures for iPSC culture and neuronal differentiation have been described previously. Healthy control and PD patient skin fibroblasts were subjected to the Northwestern University Stem Cell Core Facility, using Sendai virus (SeV)-based delivery. All iPSC lines have been routinely characterized by flow cytometry for the expression of Mscs (Stemcell Technologies, #5711). Neurons at day 50–60 were used for immunofluorescence staining for NANOG, OCT4, βIII-tubulin and Tom20, and subjected to computational analysis of mitochondrial and lysosomal contacts. Samples were imaged on a Nikon Eclipse Ti (E400) operated at 40× magnification with a x10 NA oil immersion objective. Images were captured on a Nikon A1R laser scanning confocal microscope with GaAsP detectors.

Electron microscopy. For EM, neurons were grown on PDL/laminin-coated glass coverslips. Neurons were fixed at the beginning of the experiment and which lasted for greater than 20 s. The duration, number of contacts, and the density of mitochondria were analyzed manually in NIS-Elements (Nikon). Two independent experiments were performed for each experimental condition. Samples were imaged on a Nikon FE-SEM operated at 70 kV. Images were captured using a Gatan 750/1200 CCD camera and analyzed using ImageJ (NIH).

Live-cell time-lapse confocal microscopy. Live-cell time-lapse confocal imaging was conducted of mitochondria and lysosomes labeled with the following reagents: Lentiexpressing Tom20-RFP or LAMP- GFP (MOI = 3, 3 days); Cellight* BacMam 2.0 baculovirus (PPC (particles per cell) = 40, 2 days) (Thermo Fisher: Lysosomes-GFP (#C10596), Lysosomes-RFP (#C10597), Mitochondria-RFP (#C10601), ER-GFP (#C10390)); LysoTracker® Red DND-99 (2 μM, 45 min; LysoTracker® Green FM (0.125 μM, 45 min; LysoTracker® Red #L7528); Mitotracker® Green FM (0.125 μM, 45 min; Fisher Scientific). Live-cell time-lapse imaging was conducted on a Nikon A1R laser scanning confocal microscope with GaAsP detectors using a Plan Apo 100× 1.4 NA oil immersion objective (Nikon) and NIS-Elements software. Images were captured and reconstructed using Nikon NIS-Elements.
The percentage of lysosomes in contacts was quantified as the percentage of lysosomes in contact with mitochondria for greater than 20 s divided by the total number of lysosome-GFP-positive vesicles in the region of interest. The length of neurites and the area of cell bodies were measured using a built-in function of ImageJ (NIH).

**Generation and transduction of lentiviral constructs.** Human MAP2 Lentiviral cDNA vector was purchased from Sino Biological Inc. (#HG13690-ACGLN). Human TBC1D15 ORF in the plasmid generated by Vector Builder was subjected to Site-Directed Mutagenesis (New England Biolabs, #E0554S) to insert Nhel enzyme cutting site (5'-GCTAGC-3') and then cloned into lentiviral vector including BFP tag (Addgene, #117136), mApple-Tom20 (Addgene, #54955) and LAMI-mGFP (Addgene, #34831) were obtained from Addgene plasmids using the Nhel/NotI cut sites and subcloned into pER4 Lentiviral expression vector against TBC1D15 were obtained from Horizon Discovery (RHS4533-EG46786) together with TRC Lentiviral Non-targeting shRNA Control (#RHS6848). To generate the lentivirus control, the nuclear localization signal (NLS) of SV40 large T antigen was introduced after BFP coding sequence in the same lentiviral plasmid (Addgene #117136) used for kTBC1 in lysosomes. Lentiviral vectors were packaged and transduced into HEK 293FT cells using X-treme Gene HP DNA transfection (Roche, #0636636001) together with helper plasmids pSpuAX2 (Addgene, #112260) and pLP3 (Intronvac). Quantitation of retroviral antigens were determined using ZepToMetrix Corporation RETROKit HIV-1 p24 Antibody ELISA kits (Fishier Scientific, #22-156-700). Concentrated retroviruses were aliquoted and kept at −80 °C for future use. For fluorescent expression, neurons were transduced with Map2 Lentivirus and incubated 4 days before live-cell confocal imaging or fixation. For TBC1D15 expression, neurons at day 35 were transduced with hTBC1D15 Lentivirus, incubated 15 days (MOI 5000) until day 50 and were subjected to further analysis. Expression of constructs was monitored by confocal microscopy and quantified using ImageJ (NIH).

**Seahorse XF Cell Mito Stress Test.** OCR of neurons was analyzed using an XF 24 Extracellular Flux Analyzer (Seahorse Biosciences) according to the manufacturer’s protocol; 1.25 × 10^5 neurons were plated on one well of XF24 cell culture microplates. Four empty wells without neurons were used as background control to correct for temperature-sensitive fluctuations in OCR analysis. Before the assay, culture medium was replaced with Seahorse XF medium (Seahorse Bioscience, #103577-100) supplemented with 1 mM sodium pyruvate (Corning®, #25-000-CI), 10 mM d-glucose (Sigma Aldrich, #G6876), and 2 mM glutamine ( Gibco, #25303-081) and incubated for 1 h in a CO2-free incubator. OCR was measured, after sequential injection of 1 µM oligomycin, CCCP, and Antimycin A. After the assay, neurons were lysed and subjected to BCA protein assay (Thermo Fisher, #23225) for normalization.

**Statistical analysis.** For all statistical tests, cells from 3 independent experiments (biological replicates) per condition were used (see text and figure legends). Data were analyzed using unpaired two-tailed Student’s t-test (for two datasets) or one-way ANOVA with Tukey’s post-hoc test (for multiple datasets). Bar graphs presented are in the form of means ± SEM. Statistics and graphing were performed using GraphPad Prism 7 (GraphPad software). Videos and images were processed using NIS-Elements (Nikon) and assembled using ImageJ. All figures were assembled in Adobe Illustrator.

**Data availability** All data that support the findings of this study are included in the manuscript or are available from the authors upon reasonable request. Source data are provided with this paper.
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Author contributions

S.K., Y.C.W., and D.K. provided the conceptual framework and designed the experiments. S.K. performed the cell culture, imaging, and analyzed the data. F.G. performed the cell culture and collected the data. S.K., Y.C.W., and D.K. wrote the manuscript. Figures were created by S.K.

Competing interests

S.K., Y.C.W., and F.G. declare no competing interests. D.K. is a scientific advisor for Prevail Therapeutics and Intellia Therapeutics, as well as a venture partner at Orbimed.

Additional information

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