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论文题目： Decoding Parkinson's Disease associated
protein trafficking genes

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Decoding Parkinson's Disease associated protein trafficking genes

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Abstract:

Parkinson's disease (PD) is associated with mitochondrial dysfunction, impaired autophagy-lysosome activity, and aberrant activation of leucine-rich repeat kinase 2 (LRRK2) via the Rab small GTPases. However, the pathogenic mechanism of the recently identified PD-associated Rab32 S71R variant remains unclear. Here, we investigated how Rab32 S71R affects LRRK2 signaling, protein assembly, mitochondrial homeostasis, and mitophagy. Co-immunoprecipitation demonstrated that both wild-type (WT) Rab32 and S71R interacted with LRRK2, whereas the S71R enhanced LRRK2 kinase activity more strongly than WT. Native-PAGE analysis revealed an increased propensity of S71R protein to form higher-order assemblies than WT. Furthermore, Rab32 showed mitochondrial localization, played a role in maintaining MOM integrity, and promoted the initiation of mitophagy after CCCP treatment. In contrast, the S71R variant reduced autophagosome formation and impaired the maturation of autolysosomes. To investigate its effects *in vivo*, the isogenic RAB32 and S71R knock-in induced pluripotent stem cell lines were generated from H9 cells using CRISPR-based genome editing. Both lines successfully formed approximately 5-mm cerebral organoids by day 20. Collectively, Rab32 S71R may contribute to PD through a dual mechanism involving aberrant activation of LRRK2 and disruption of mitochondrial autophagy-lysosome homeostasis.

Keywords: Parkinson's Disease; Protein Trafficking; Rab32 S71R; LRRK2

I. Background Introduction

Parkinson's disease (PD) is a progressive and currently incurable neurodegenerative disorder with the accumulation of misfolded α -synuclein protein in Lewy bodies and the dopaminergic neurons loss in the SN region (Lim et al. Lancet Neurol 2024). In addition to bradykinesia, resting tremor, and rigidity, PD commonly causes cognitive impairment, sleep disturbances, and depression, imposing heavy burden on patients and healthcare systems. According to the Global Burden of Disease 2021 estimates, approximately 11.77 million people worldwide were living with PD in 2021, with about 1.34 million new cases and 388,000 associated deaths.

Although aging is the strongest risk factor, genetic susceptibility and environmental stress converge on several interconnected processes, including mitochondrial dysfunction, impaired proteostasis, vesicular trafficking defects, oxidative stress, and neuroinflammation. Increasing evidence places disruption of the autophagy-lysosome pathway as a key hub. Macroautophagy, chaperone-mediated autophagy, and mitophagy collectively remove damaged organelles and aggregation-prone α -synuclein protein (Cuervo et al. Science. 2004). Defective lysosomal acidification, cargo delivery, or autophagosome-lysosome fusion can therefore promote α -synuclein oligomerization, while aggregated α -synuclein can further compromise the lysosomal and mitochondrial function, establishing a self-amplifying pathogenic cycle. The genetic association of PD with *GBA1*, *ATP13A2*, *VPS35*, *SNCA*, *PINK1*, *PRKN*, and *LRRK2* strongly supports this model. LRRK2 is a multidomain kinase that is recruited to stressed endolysosomal membranes and phosphorylates many Rab small GTPases, including Rab8A, Rab10, Rab29, and Rab43 (Steger et al. eLife. 2016). Rab proteins act as molecular switches that control organelle identity, vesicle formation, trafficking, and membrane fusion. Pathogenic LRRK2 mutations increase kinase activity and Rab phosphorylation, thereby altering Rab-effector interactions and disrupting endolysosomal trafficking, lysosomal repair, autophagic flux, and mitophagy. In particular, LRRK2-dependent phosphorylation of Rab10 at Thr73 has emerged as a widely used indicator of LRRK2 pathway activation (Steger et al. eLife. 2016).

Rab32, one member of the Rab32/Rab38 subfamily, has recently emerged as a

compelling candidate linking mitochondrial homeostasis, endolysosomal trafficking, and LRRK2 signaling to PD. In 2024, genetic analyses revealed the heterozygous Rab32 Ser71Arg (S71R) (213C>G in CDS) mutant in multiple families and unrelated individuals with parkinsonism (Gustavsson et al. Lancet Neurol 2024; Hop et al. Nat Genet 2024). The variant cosegregated with autosomal dominant disease in 3 families and was associated with PD across combined cohorts, although penetrance was incomplete and the confidence interval for the estimated risk was broad. Affected carriers shared a founder haplotype and displayed variable ages at onset, supporting S71R as a rare, incompletely penetrant PD-associated variant rather than a deterministic mutation in every carrier. Nevertheless, how the Rab32 variant affects mitochondrial function, and autophagy-lysosome activity remains unknown. More importantly, it has not been established whether activation of LRRK2-Rab10 signaling by Rab32 S71R is sufficient to impair α -synuclein clearance and promote its aggregation. Whether S71R plays a functional and causal role in PD pathogenesis keeps unknown.

II. Materials and Methods

In vitro protein expression and purification

The coding sequences for wild-type (WT) Rab32 and the S71R variant were synthesized by the Tsingke Biotechnology, and subcloned into the pcDNA and pSMT3 vectors, respectively. For recombinant protein production, the pSMT3 constructs encoding WT Rab32 or S71R were introduced into *E. coli* Rosetta (DE3) cells. Bacterial cultures were incubated in 100 mL LB medium containing 40 μ g/mL kanamycin at 37 °C. When the optical density (OD₆₀₀) reached approximately 0.8, 0.1 mM isopropyl IPTG was added to induce protein expression, followed by incubation at 16 °C for 18 hours.

Cells were centrifuged at 5,000 rpm for 10 min at 4 °C. The pellets were resuspended in lysis buffer containing Tris-HCl (50 mM, pH 8.0), NaCl (100 mM), imidazole (10 mM), and dithiothreitol (1 mM) and were then disrupted. Insoluble material was removed by centrifugation at 16,000 rpm at 4 °C for 50 min. The lysate was applied to a His-Trap HP column to get the eluted targeted protein with 50 mM

Tris-HCl (pH 7.4), 500 mM NaCl, 250 mM imidazole, and 1 mM dithiothreitol. Next, the proteins were further separated by gel-filtration using a Superdex200 10/300 column (GE Healthcare) equilibrated with 50 mM Tris-HCl (pH 8.0) and 100 mM NaCl. Fractions corresponding to major elution peaks were collected and examined by the SDS-PAGE method.

Cell culture

The H9 human embryonic stem cell line was obtained from WiCell and maintained on Matrigel-coated culture plates (Corning) in mTeSR medium (STEMCELL Technologies). The culture medium was replaced daily. HEK293T and SH-SY5Y cells were incubated in EMEM (Gibco) supplemented with 10% FBS, 1×GlutaMAX, and 1% P/S (Sigma). All cells were maintained at 37 °C in a humidified incubator containing 5% CO₂.

Co-immunoprecipitation

HEK293T or SY5Y cells were transfected with the indicated epitope-tagged expression constructs 24 h later. At 48 h after transfection, the cells were harvested and lysed in co-immunoprecipitation buffer for 40 min with continuous rotation. The samples were briefly sonicated and then centrifuged at 16K rpm for 15 min to remove insoluble materials. The remaining lysate was divided equally and incubated with anti-FLAG magnetic beads, or IgG control beads for 4-5 h at 4 °C, further subsequently washed with buffer containing 0.1% Triton X100, 50 mM Tris-HCl (pH 7.5), and 150 mM NaCl. Bound proteins and input samples were mixed with the buffer and heated at 95 °C for 8 min before immunoblot analysis.

Plasmid construction and sgRNA design

A CRISPR-Cas9-mediated homology-directed repair strategy was used to introduce the S71R substitution into the human Rab32 locus. Candidate single-guide RNAs were designed using the CRISPR-TEFOR and CCTop online platforms and cloned into the pX458 vector (Addgene). Editing efficiencies were initially evaluated in HEK293T cells, after which two sgRNAs targeting exon 1 of Rab32 were selected for generation of the S71R knock-in cell line. The donor construct was generated using the pBluescript SK(+) vector and contained the intended Rab32 S71R sequence together with an eGFP-

IRES-puromycin selection cassette flanked by loxP sites. The cassette was positioned between homology arms corresponding to sequences surrounding the target region in Rab32 exon 1. Correctly edited clones were identified by DNA sequencing.

Native-PAGE

Purified WT Rab32 and S71R were buffer-exchanged into 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and adjusted to identical molar concentrations. Equal amounts of protein were mixed with native sample buffer without SDS and reducing agents and separated on 12% native polyacrylamide gels at 4 °C. The relative abundance of monomeric and higher-order Rab32 species was quantified by densitometry using ImageJ.

Mitochondrial-cytosolic fractionation

Cells expressing FLAG-tagged WT Rab32 or S71R were harvested, and washed three times with ice-cold PBS. Cell pellets were resuspended in mitochondrial isolation buffer containing 20 mM HEPES-KOH (pH 7.5), 200 mM mannitol, 50 mM sucrose, 1 mM EDTA, and protease inhibitors. Cells were mechanically disrupted and the homogenates were centrifuged at $500 \times g$ for 15 min at 4 °C to remove nuclei and intact cells. The supernatants were collected as the cytosolic fractions, whereas the mitochondrial pellets were washed once and resuspended in mitochondrial isolation buffer containing 1% Triton X-100. Equivalent proportions of each fraction were analyzed by immunoblotting. TOM20, was used as a mitochondrial marker, whereas beta-actin served as a cytosolic marker.

CCCP treatment

Mitochondrial depolarization was induced by treating cells with 10 μ M CCCP for 4 h, as specified for each experiment. The same volume of dimethyl sulfoxide was added into control cultures. When lysosomal degradation was examined, bafilomycin A1 (100 nM) was added during the final 2 h of CCCP exposure. Cells were subsequently processed for immunoblotting, confocal microscopy, mitochondrial fractionation, or mitophagy analysis.

Autophagic-flux analysis

Autophagic flux was assessed by measuring LC3B-II turnover in the presence and

absence of lysosomal inhibition. Cells expressing WT Rab32 or S71R were treated with vehicle or CCCP, with parallel cultures receiving bafilomycin A1 during the final 2 h. Cell lysates were analyzed by immunoblotting using antibodies against LC3B, Rab32. LC3B-II accumulation induced by bafilomycin A1 was used to estimate autophagic flux rather than interpreting steady-state LC3B-II abundance alone. Autophagosome formation and maturation were independently examined using the tandem mCherry-EGFP-LC3 reporter. Cells were transfected with the reporter together with WT Rab32, S71R, or the corresponding control vector. Because EGFP fluorescence is quenched following lysosomal acidification whereas mCherry fluorescence is retained, puncta positive for both EGFP and mCherry were classified as autophagosomes, and mCherry-positive/EGFP-negative puncta were classified as autolysosomes. Puncta were quantified on a per-cell basis by an investigator blinded to genotype and treatment.

Statistical analysis

All experiments were repeated independently three times. Quantitative data were analyzed using the GraphPad-Prism (v8.0) and are presented as the mean $\pm$ standard deviation. Comparisons between two groups were performed using Student's t-test. P value less than 0.05 was considered statistically significant.

III. Results

3.1 Rab32 S71R variant interacted and enhanced LRRK2 kinase activity more strongly than WT Rab32.

Whole-genome sequencing has identified the Rab32 S71R mutant as a potential high-risk genetic factor for PD (Gustavsson et al. Lancet Neurol 2024; Hop et al. Nat Genet 2024). To investigate the underlying molecular mechanism, Flag-tagged wild-type (WT) Rab32 or the S71R variant was introduced into SH-SY5Y cells. Co-immunoprecipitation assays revealed that WT Rab32 and S71R interacted with endogenous LRRK2 (Fig. 1A). Next, Rab32-knockout SH-SY5Y cells were reconstituted with WT Rab32, S71R, or an empty vector. Compared with WT Rab32, the S71R variant induced a greater increase in LRRK2 kinase activity, whereas total Rab10 protein levels remained comparable among the groups (Fig. 1B).

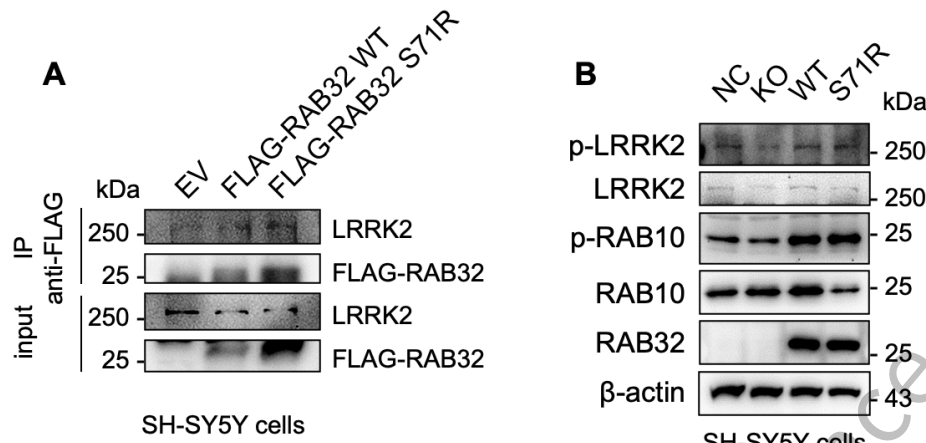


Figure 1. Rab32 and its S71R variant interacted with LRRK2.

To further investigate why WT Rab32 and the S71R variant differentially regulate LRRK2, the two proteins were recombinantly expressed in *Escherichia coli* using the pSMT3 vector. SDS-PAGE analysis showed that both WT and S71R were efficiently expressed and purified, with no evident difference in their electrophoretic profiles (Fig. 2A and 2C). Size-exclusion chromatography on a Superdex-200 column further revealed that both proteins were eluted at approximately 16.4 mL, indicating similar hydrodynamic properties under native solution conditions (Fig. 2B and 2D). In contrast, native-PAGE showed that WT Rab32 was predominantly monomeric, whereas the S71R variant produced abundant slower-migrating species consistent with dimers (Fig. 3). Notably, an additional band at approximately 53 kDa was more readily detectable for S71R than for WT (Fig. 3A). Immunoblotting with an anti-Rab32 antibody further confirmed a predominant Rab32 species at approximately 25 kDa, together with an additional Rab32-immunoreactive species at approximately 53 kDa (Fig. 3B). Glutaraldehyde cross-linking assays further confirmed that Rab32 S71R exhibited a greater propensity to oligomerize than the WT protein. Collectively, these findings suggest that the S71R substitution alters the higher-order assembly of Rab32 without markedly affecting its expression, purification behavior, or overall hydrodynamic size.

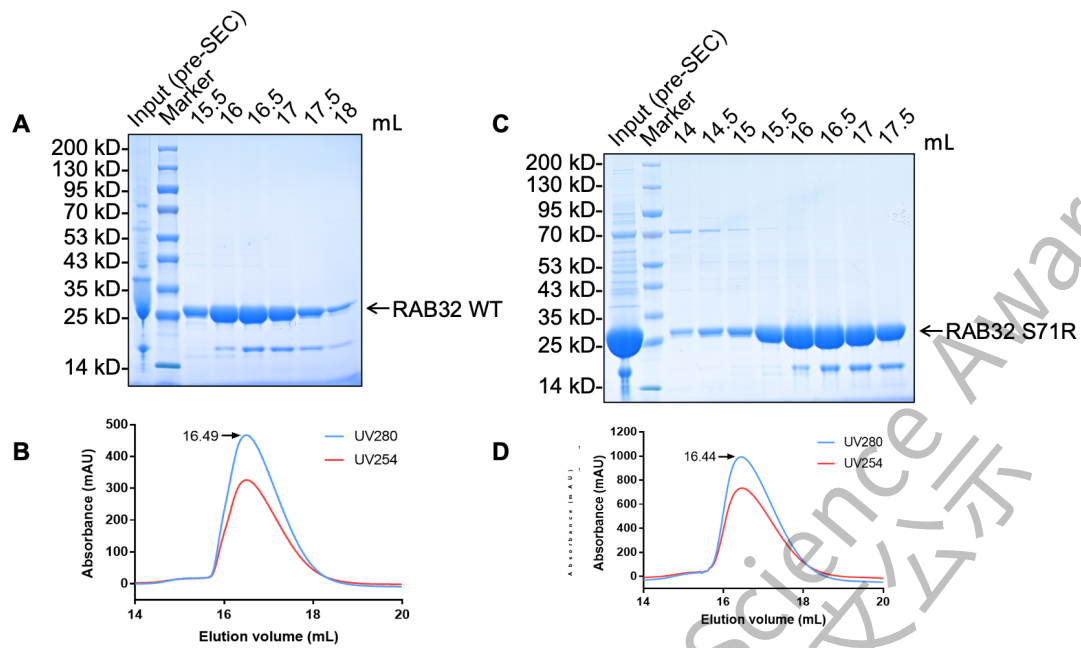


Figure 2. SDS-PAGE and gel-filtration profiles of Rab32 and the S71R proteins.

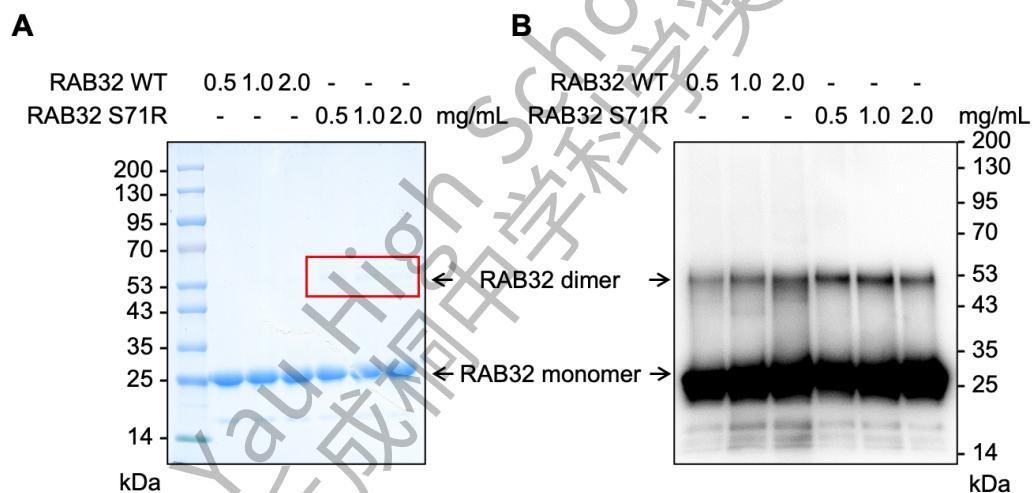


Figure 3. Native-PAGE result of Rab32 and the S71R proteins.

3.2 The S71R variant reduced autophagosome formation and impaired the maturation of autolysosomes.

To determine whether Rab32 is associated with mitophagy, the mitochondrial fractionation analysis following CCCP treatment was performed. Immunoblot analysis revealed that Rab32 was present in both fractions and remained detectable in the mitochondrial fraction following CCCP treatment, indicating that a pool of Rab32 is

associated with mitochondria (Fig. 4A). Next, the mitochondrial homeostasis was assessed with the abundance of TOM20, an outer mitochondrial membrane protein. Under basal conditions, overexpression of Rab32 WT did not significantly alter TOM20 protein levels. In contrast, following CCCP-induced mitochondrial depolarization, TOM20 levels were significantly higher in cells overexpressing Rab32 WT than in control cells (Fig. 4B-C). Notably, inhibition of lysosomal acidification and autophagic degradation with bafilomycin A1 (BafA1) abolished the difference in TOM20 levels between Rab32 WT-overexpressing and control cells. Together, these findings indicate that Rab32 associates with mitochondria and modulates TOM20 abundance in response to mitochondrial stress, potentially through a mechanism involving the autophagy-lysosome pathway.

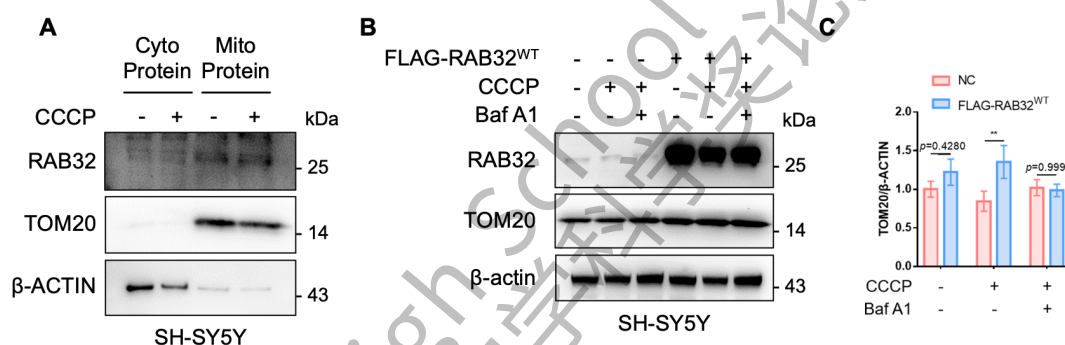


Figure 4. Rab32 localizes to mitochondria and maintains mitochondrial outer membrane integrity.

Following Rab32 overexpression, the level of LC3B-I decreased (Fig. 5), whereas LC3B-II accumulated further in the presence of Baf A1. Because Baf A1 inhibits vacuolar H⁺-ATPase, thereby suppressing lysosomal acidification and late-stage autophagic degradation, the enhanced accumulation of LC3B-II after Baf A1 treatment is consistent with increased autophagic flux and autophagosome formation. These results suggest that Rab32 facilitates autophagosome biogenesis and participates in mitophagy, potentially by promoting the progression of autophagosomes toward autolysosomal degradation (Fig. 5).

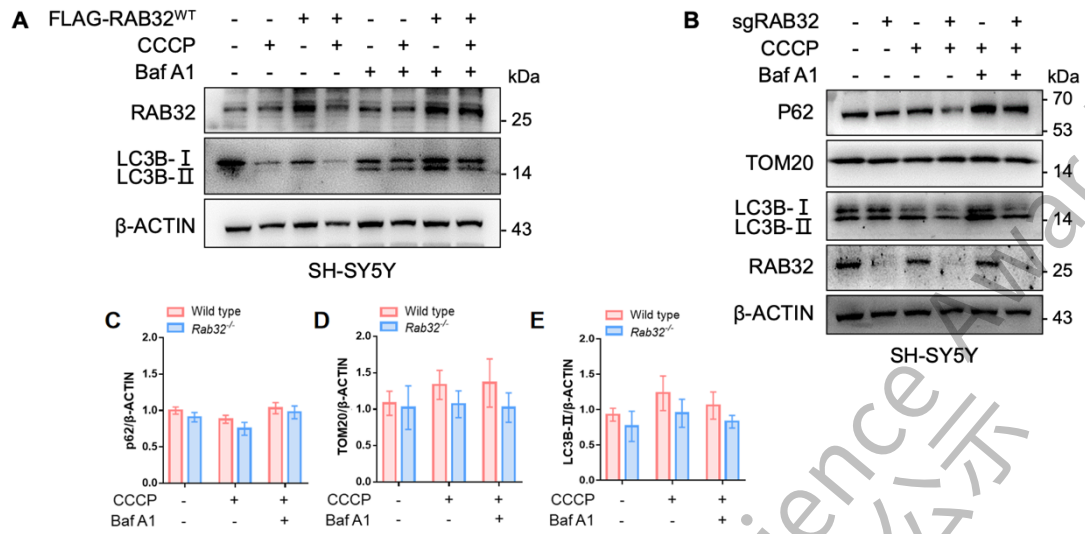


Figure 5. Rab32 promoted the initiation of mitophagy.

Next, to investigate the effects of WT Rab32 and the S71R variant on autophagic flux and autophagosome maturation, SH-SY5Y cells were co-transfected with a tandem mCherry-GFP-LC3 reporter and plasmids encoding Flag-tagged Rab32 or S71R. Following treatment with CCCP for 4 h, the cells were exposed to BafA1 (100 nM) for an additional 2 h (Fig. 6). Confocal fluorescence microscopy revealed that, following CCCP treatment, overexpression of Rab32 increased the accumulation of both autophagosomes (GFP⁺/mCherry⁺ puncta, appearing yellow in merged images) and autolysosomes (GFP⁻/mCherry⁺ puncta, appearing red) compared with the vector control. In contrast, cells expressing S71R exhibited fewer autophagic structures than those expressing WT, suggesting that the S71R variant compromises the ability of Rab32 to support CCCP-induced autophagy (Fig. 6). Upon inhibition of lysosomal acidification and autophagic degradation with BafA1, a further accumulation of autophagosomes was observed in Rab32 WT-expressing cells, whereas this accumulation was less pronounced in cells expressing S71R.

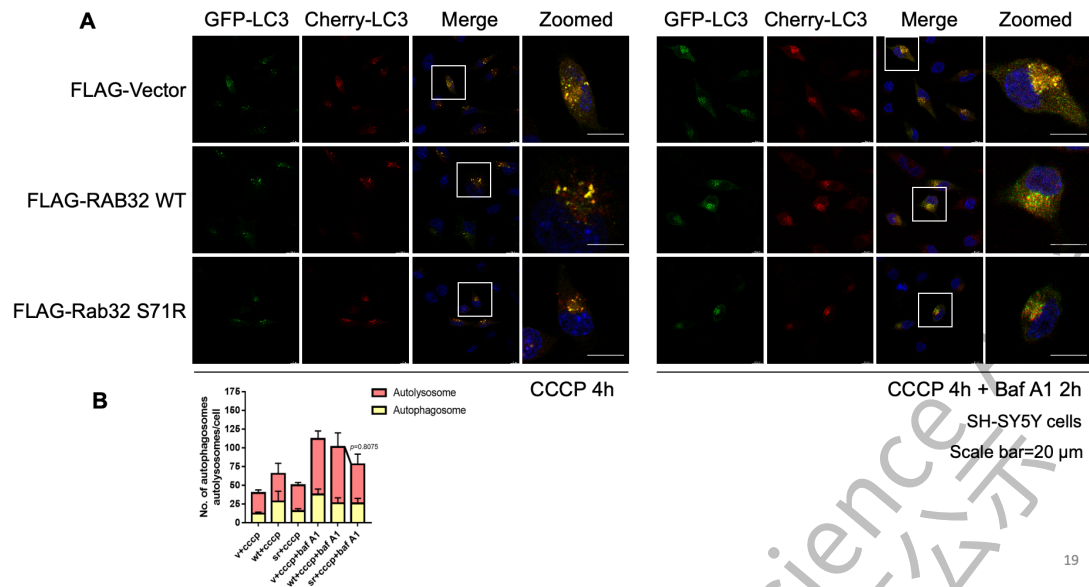


Figure 6. S71R mutation impaired the maturation of autolysosomes.

3.3 Construction fo RAB32 WT and S71R knock-in iPSC and cerebral organoids.

Organoids have become valuable experimental models for investigating tissue and organ development and disease pathogenesis, particularly in studies of brain tumors and neurodegenerative disorders (Bagley et al. Nat Methods. 2017). To further define the role of the Rab32 S71R variant in Parkinson's disease, we used CRISPR-Cas9-mediated genome editing to introduce a heterozygous c.213C>G substitution, corresponding to the p.Ser71Arg mutation, into the endogenous Rab32 locus of H9 human embryonic stem cells (hESCs). The donor construct contained a dTomato fluorescent reporter and a puromycin-resistance cassette for selection. Cre-loxP-mediated recombination was subsequently performed to excise the floxed selection cassette and generate the heterozygous Rab32 S71R knock-in hESC line (Fig. 7).

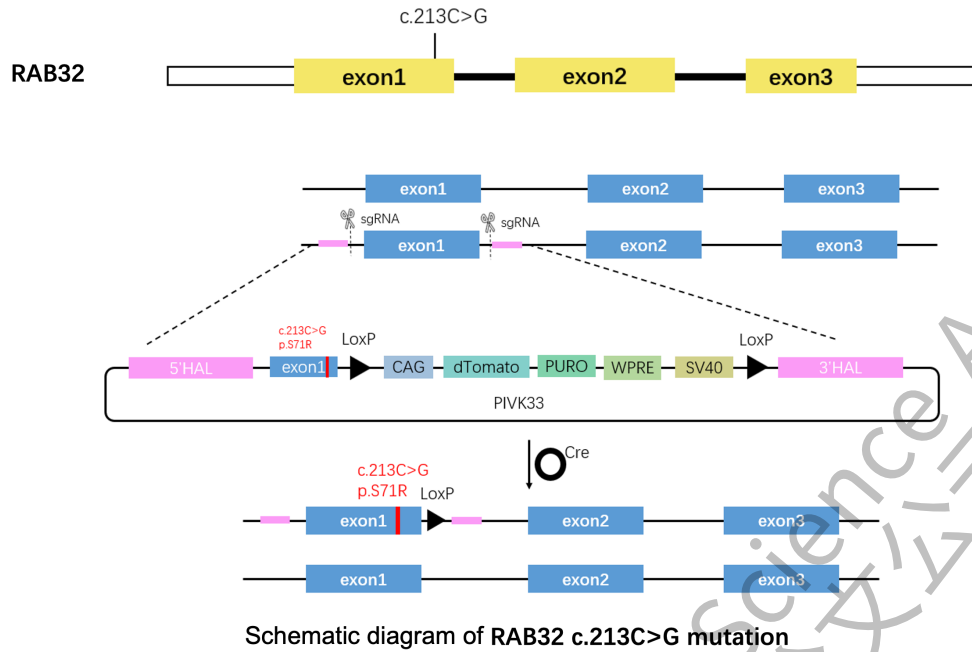


Figure 7. Schematic diagram of Rab32 S71R mutation by gene-editing method.

DNA sequencing of the parental WT and gene-edited H9 cells confirmed successful introduction of the Rab32 S71R mutation (Fig. 8). The edited cells were then differentiated into cerebral organoids using a staged protocol involving matrigel embedding and sequential exposure to defined differentiation factors and culture media. Embryoid bodies began to form at approximately day 6, followed by the appearance of neural buds around day 11 and the development of cerebral organoid-like structures by day 21. As shown in Fig. 9, cerebral organoids derived from WT and Rab32 S71R cells were examined at days 3, 10, 15, and 20. Both groups exhibited apparently normal morphology throughout early development, with no obvious differences in their gross appearance or growth (Fig. 9). By approximately day 20, organoids in both groups reached a diameter of nearly 5 mm, and no significant difference in size was observed between WT and S71R organoids.

Because cellular and molecular phenotypes relevant to neurodegenerative disorders such as PD often emerge only after prolonged organoid maturation, frequently after 60 days or longer, these experiments are ongoing. Mature cerebral organoids will subsequently be analyzed by IHC, WB, and single-cell RNA sequencing to determine whether the Rab32 S71R variant alters dopaminergic-neuron survival, LRRK2

signaling, mitochondrial homeostasis, autophagy-lysosome function, or other PD-associated pathological processes.

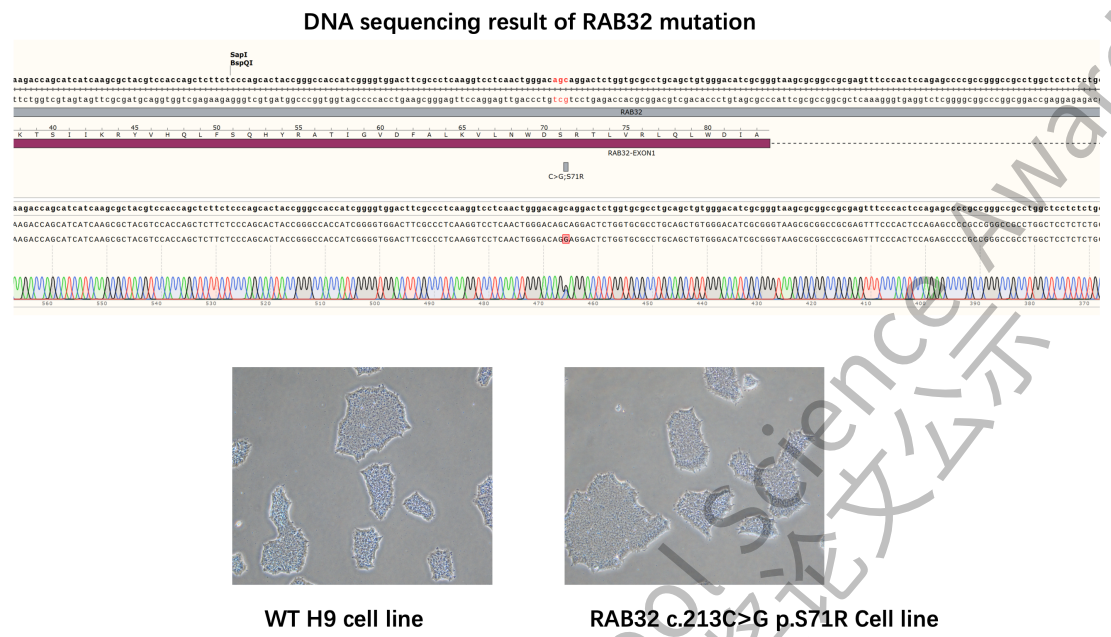


Figure 8. DNA sequencing of the Rab32 mutation in H9 cells.

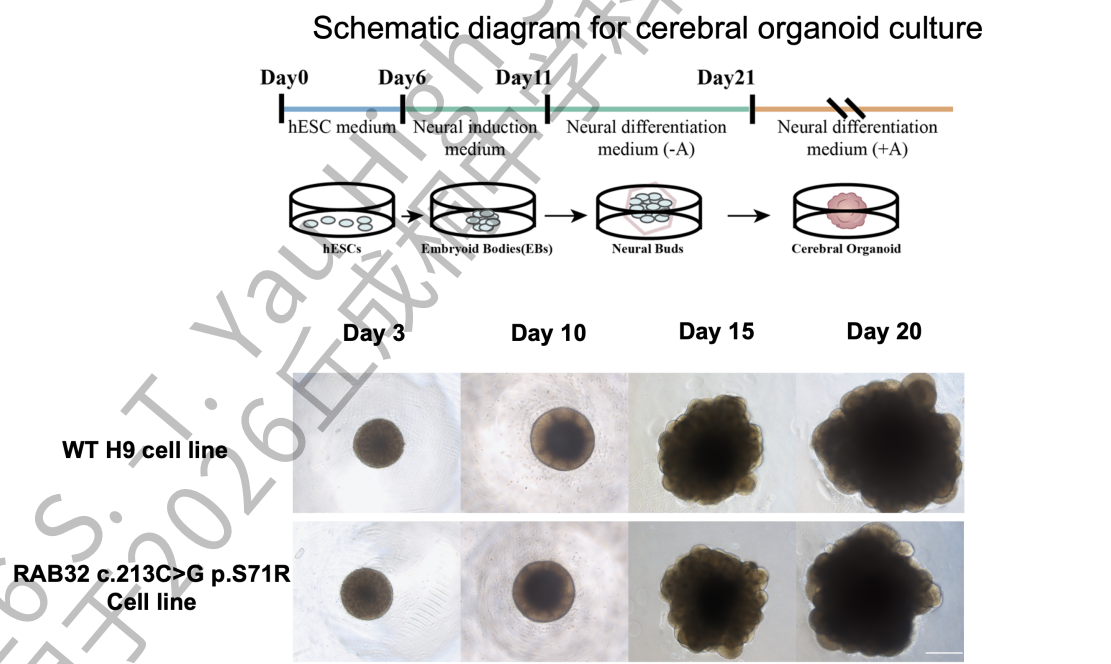


Figure 9. Schematic diagram and images for WT and S71R Rab32 cerebral organoids. (top) Schematic diagram of cerebral organoid culture. (bottom) Images of WT and S71R Rab32 cerebral organoids at different time points (day 3, 10, 15, and 20). Scale bar, 1 mm.

IV. Conclusion and Discussion

Rab-family small GTPases regulate vesicular trafficking, organelle identity, membrane fusion, autophagy, and mitochondrial quality control, all of which are frequently disrupted in Parkinson's disease (PD) (Steger et al. eLife. 2016). The PD-associated kinase LRRK2 phosphorylates many Rab proteins, including Rab8A, Rab10, Rab12, Rab29, and Rab43, thereby influencing endolysosomal trafficking, autophagosome maturation, and mitophagy (Wauters et al. Autophagy. 2020). Rab32 is associated with mitochondria and lysosomes, and the recently identified S71R variant further connects dysregulated Rab signaling to familial PD (Gustavsson et al. Lancet Neurol 2024; Hop et al. Nat Genet 2024).

The current study demonstrates that both WT Rab32 and S71R interact with endogenous LRRK2, whereas S71R induces substantially greater LRRK2 kinase activity (Fig. 1). The two recombinant proteins exhibited comparable expression, SDS-PAGE migration, and size-exclusion chromatography profiles. However, native PAGE and glutaraldehyde cross-linking revealed that S71R has a greater propensity to form dimers or higher-order oligomers, which may facilitate LRRK2 activation. Rab32 also partially localized to mitochondria, contributed to mitochondrial outer-membrane integrity, and regulated lysosome-dependent TOM20 turnover following CCCP-induced damage (Fig. 2-4). LC3B analysis indicated that WT Rab32 promoted autophagosome formation, whereas tandem mCherry-GFP-LC3 imaging showed that S71R reduced autolysosome formation and impaired autophagosome maturation (Fig. 5-6). These findings support a model in which Rab32 S71R contributes to PD through both aberrant activation of LRRK2 signaling and disruption of autophagy-lysosome-dependent mitochondrial quality control.

Importantly, a heterozygous Rab32 S71R knock-in H9 hESC line was successfully generated and differentiated it into cerebral organoids (Fig. 7-9). WT and mutant organoids reached approximately 5 mm by day 20 and showed no evident differences in early growth or gross morphology. Because PD-related phenotypes may emerge only after prolonged neuronal maturation, future studies will examine mature organoids

using immunohistochemistry, WB, and scRNA-seq. This isogenic human organoid model will enable investigation of whether Rab32 S71R promotes LRRK2-Rab10 signaling, defective mitophagy, α -synuclein aggregation, and selective dopaminergic-neuron loss, thereby unveiling its mechanistic contribution to PD.

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