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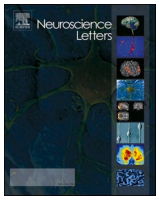

Research article

Absence of motor impairments or pathological changes in TMEM230 knockout rats

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ABSTRACT

Parkinson's disease (PD), which is the second most common neurodegenerative disorder, is characterized by progressive movement impairment and loss of midbrain dopaminergic neurons in the substantia nigra. Although mutations in *TMEM230* are linked to familial PD, the pathogenic mechanism underlying TMEM230-associated PD remains to be elucidated. To explore the effect of TMEM230 depletion *in vivo*, we created TMEM230 knockout rats using CRISPR-Cas9 technology. TMEM230 knockout rats did not exhibit any core features of PD, including impaired motor function, loss of dopaminergic neurons in the substantia nigra, or altered expression of proteins related to autophagy, the Rab family, or vesicular trafficking. In addition, no glial reactions were observed in TMEM230 knockout rats. These results indicate that depletion of TMEM230 may not lead to dopaminergic neuron degeneration in rats, further supporting that PD-associated *TMEM230* mutations lead to dopaminergic neuron death by gain-of-toxic function.

1. Introduction

Parkinson's disease (PD), is the second most common neurodegenerative disorder with no effective treatment, leads to slowness of movement, tremors, rigidity, and cognitive impairment in late stages of the disease [1,2]. The defining pathological features of PD are loss of midbrain dopaminergic (DA) neurons in the substantia nigra (SN) and accumulation of a α-Synuclein-containing Lewy bodies [3]. Several genetic loci with pathogenic mutation is associated with PD have been identified in the last two decades [4–6]. For example, increased LRRK2 kinase activity leads to nigrostriatal degeneration and Lewy body formation [4], and G2019S mutation of *LRRK2* is linked to decreased GTPase, which affects signaling cascades and endosomal trafficking [5]. Mutations in *PINK1* and *Parkin* are the most common causes of recessive early-onset PD and loss of function in either these genes results in selective loss of DA neurons and locomotor impairment [4,6]. Springer *et al.* showed that PINK1 functions in cooperation with Parkin to identify and label damaged mitochondria for selective degradation via autophagy [7]. Mutations in *VPS35* are a rare cause of PD, and *VPS35* is not only a critical player in pathways associated with α-synuclein accumulation and clearance, but also has a key role in ensuring mitochondrial function [8]. Despite the increasing accumulation of clinical and preclinical evidence, a detailed understanding of PD pathogenesis remains elusive.

Deng *et al.* first described that the *TMEM230* gene as causative of PD in an autosomal-dominant manner [2]. TMEM230 is present as both a

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long and short isoform, with the short form being highly conserved and accounting for ~ 95 % of the total TMEM230 protein in humans, suggesting its functional importance [2]. Several research groups also identified PD-linked mutations in *TMEM230*. For example, a German patient with classical PD symptoms was found to have a rare mutation (*p.R68H*) [9] located in the same domain as the pathogenic *p.Y92C* mutation reported by Deng et al. [2]. An Italian woman with a positive family history and typical PD symptoms exhibited a heterozygote variant (*p.I125M*) [10]. In addition, some groups found no pathogenic mutations in the *TMEM230* gene or any missense variants that were significantly associated with PD [11–14].Therefore, more genetic screening and validation studies are required to gain a better understanding of the genetic basis of PD.

TMEM230 is associated with both vesicle trafficking and Rab family proteins in the endosome pathway [2,15]. Deng *et al.* reported disrupted vesicle trafficking after the expression of mutant *TMEM230* in mouse primary neurons [2]. Kim *et al.* [15] demonstrated that CI-M6PR retromer trafficking and autophagic clearance are disrupted by TMEM230 knockdown in the HEK293-FT cell line. Furthermore, Wang *et al.* reported that both mutant *TMEM230* and knockdown of endogenous TMEM230 induce neurodegeneration in a SHSY-5Y cell line [16]. Together, these studies suggest that *TMEM230* mutation or knockdown plays a critical role in PD development, but the molecular mechanisms are still uncertain.

Here, we explored the effect of *TMEM230* depletion in TMEM230 knockout rats. However, we observed no alterations in these KO rats at 18 months of age.

2. Materials and methods

2.1. Animals

TMEM230 knockout (KO) rats were generated using Crispr/Cas9 technology, and we cross-bred these KO rats with wild-type Sprague Dawley rats for six generations to remove potential CRISPR/Cas9 off-target effects. All rats were maintained on the Sprague-Dawley rat background. A pair of primers were used to amplify the targeted region: 5′-GGAATCAAGTCTTCACTCACC-3′ and 5′-ACAGCTACATGACTTTAACACT-3′, then sequenced the PCR product with a primer to detect any alterations in the targeted region: 5′-AATTAGGCAGGAAACATTGGT-3′. Both male and female rats were used in this study.

2.2. Behavioral testing

As described previously [17], the mobility was assessed by both Open-field and accelerating Rotarod analysis (Med Associates, Inc. VT, USA). The total distance that a rat traveled in the Open-field within 10 min was recorded. The latency to fall off the accelerating rod (0–40 rpm) for each testing rat was recorded over 5 trials for 2 min per trial. Each type of rat n = 10, male/ female, 18 months.

2.3. Immunoblotting

Rat midbrains were dissected and homogenized in RIPA lysis buffer and separated via SDS-PAGE for immunoblotting analysis [17]. Nitrocellulose membranes were used for protein transfer and then probed with the specific primary antibodies: anti-GFAP (Millipore, cat#-MAB3402), anti-Iba1 (Wako: 016–20001), anti-TMEM230 (Sigma: HPA009078-100UL), anti-LAMP2a (Abcam, ab18528), and antibodies from proteintech: anti-tyrosine hydroxylase (TH) (66334–1-Ig), anti-VPS35 (10236–1-AP), anti-LC3 (14600–1-AP), anti-p62 (18420–1-AP), anti-Beclin 1 (11306–1-AP), anti-RAB3A (15029–1-AP), anti-RAB5A (11947–1-AP), anti-RAB6A (10187–2-AP), anti-RAB7A (55469–1-AP), anti-RAB30 (16328–1-AP), anti-GAPDH (60004–1-Ig). Densitometric analysis were performed by NIH ImageJ 7.0. Each type of rat n = 5, male, 18 months.

2.4. Immunostaining

Rat brains were fixed in 4 % paraformaldehyde and cryopreserved in 30 % sucrose prior to cryostat sections. As previesly described [18], serial coronal sections of 10 μm were collected for rat brains. Every 10th section were stained with mouse anti-TH, and the number of TH positive neurons in the SNpc was estimated by image J software. And the glial reaction was detected by anti-GFAP (a marker for astrocytes) and anti-IBa1 (a marker for microglia) antibodies. Each type of rat n = 5, male, 18 months.

2.5. RNA isolation and quantitative reverse transcription RT-PCR

As described previously [17], the rat brains were quickly removed and RNA were extracted using Trizol according to manufacturer's instructions (Sigma). cDNA were synthesized using the ProtoScript® First Strand cDNA Synthesis Kit (BioLabs). RT-PCR was performed as previously reported [19]. Primers used for TMEM230: 5′-AAAAGCCCTCCTAAGATCCC-3′ and 5′-CAGGAACACCAAGATGCCAA-3′; Primers used for GAPDH: 5′- GAAGGTGAAGGTCGGAGTC −3′ and 5′- GTAAACCATGTAGTTGAGGTC −3′. Each type of rat n = 6, male/ female, 18 months.

2.6. Statistics

Behavioral tests were analyzed by two-way ANOVA and unpaired *t*-test. RT-PCR were analyzed by one-way ANOVA followed by Tukey's post hoc test. Statistical analysis was performed using Prism 7.0 (Graphpad Software, San Diego, CA, USA).

3. Results

3.1. Creation of TMEM230 KO rats

To examine the effect of TMEM230 deficiency *in vivo*, we created TMEM230 knockout (TMEM230$^{-/-}$, KO) rats using Crispr-Cas9 techonology. The short form of *TMEM230* shares the highest similarity with the corresponding isoform in rats. Sanger sequencing showed that these KO rats have one nucleotide deletion but harbor two nucleotide insertions (Fig. 1A-B). These changes lead to a premature stop codon (TAG) in the second exon of the *TMEM230* gene. Quantitative PCR showed that TMEM230 mRNA decreased by 35 % and 57 % in heterozygote and homozygote KO rats, respectively (Fig. 1C). Consistently, immunoblotting analysis revealed a dramatic reduction of TMEM230 and no detectable TMEM230 expression in homozygote KO rats (Fig. 1D-E). These results suggest that we successfully created TMEM230-depleted rats.

3.2. No motor impairment in TMEM230 KO rats

To investigate whether complete loss of *TMEM230* results in behavioral alterations in rats, we performed systematic and detailed characterization at 18 months of age. Groups of rats were tested via the open-field and accelerating rotarod. There was no significant difference in the total distance travelled between TMEM230 KO rats and their widetype littermates at the age of 18 months (Fig. 2A). Similarly, there was no significant difference in rotarod performance (Fig. 2B). These results show that inactivation of the *TMEM230* gene does not cause motor deficits in rats.

3.3. No dopaminergic neuron loss in TMEM230 KO rats

The major neuropathological hallmark of PD is DA degeneration in the SN pars compacta, which causes striatal DA deficiency through the DA nigrostriatal pathway. Therefore, rat brains were dissected at 18 months of age, and immunohistochemistry was performed to examine

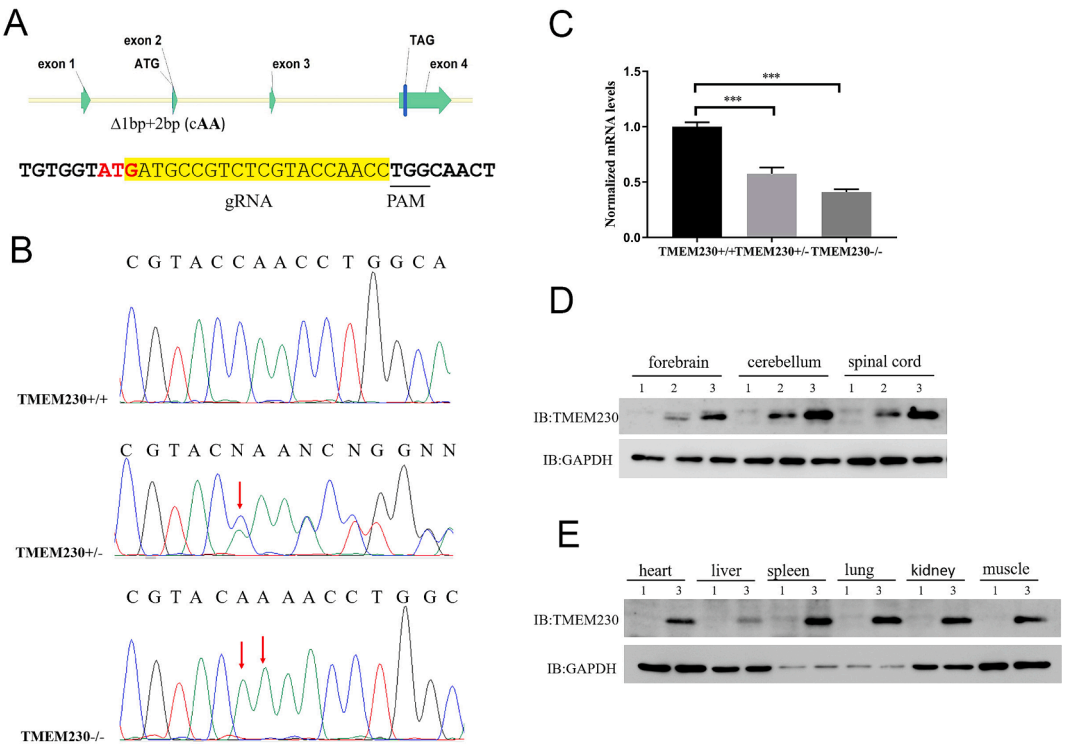

Fig. 1. Characterized TMEM230 knockout rats. (A) Strategy to create TMEM230 knockout rats using CRISPR/Cas9 technology. (B) Sanger sequencing revealed that a deletion of one nucleotide and an insertion of two nucleotides at the targeted region in *TMEM230* gene. (C) qPCR revealed the relative mRNA level in TMEM230 KO rats. Data are expressed as the mean ± SD (n = 6), ***$p < 0.05$. (D) Immunoblot analysis revealed TMEM230 exprssion in the central nerval system. (E) Immunoblot analysis revealed TMEM230 exprssion in multiple organs of rats. GAPDH was used as the loading control. Note: 1, homozygote; 2, heterozygote; 3, wild-type rats.

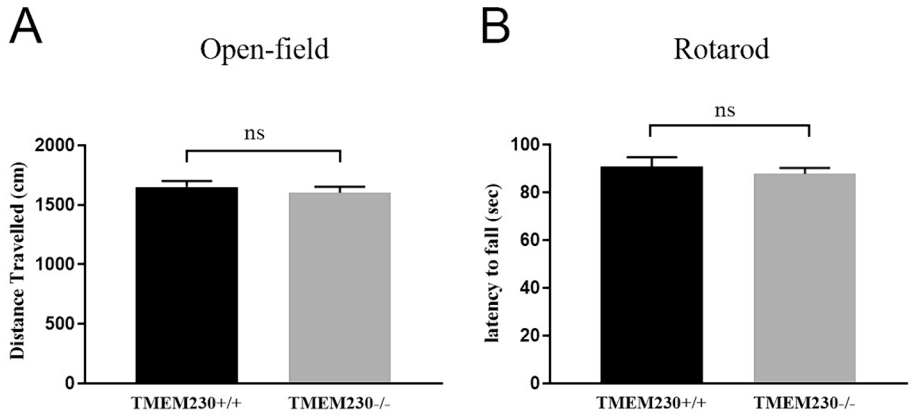

Fig. 2. No alterations in behavioral tests. (A) Analysis of the total distance travelled during the open-field test. (B) Analysis of the latency to fall during the rotarod test. Data are expressed as the mean ± SD (n = 10), $p > 0.05$, ns indicate "not significant".

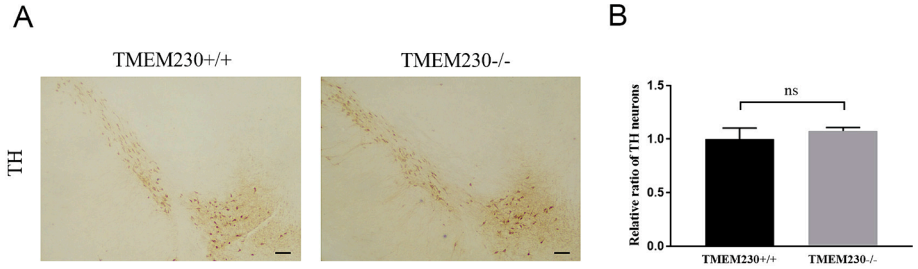

Fig. 3. No dopaminergic neuron loss in the SNpc of TMEM230 KO rats. (A) TH staining revealed normal immunoreactivity in the SNpc of TMEM230 KO rats. (B) TH-positive neurons were estimated using image J software. The data are shown as the mean ± SD. Student's *t* test, n = 5, $p > 0.05$, ns indicate "not significant". Scale bar = 150 μm.

tyrosine hydroxylase (TH)-positive neurons. TH-immunoreactivity was similar between TMEM230 KO rats and widetype rats (Fig. 3A), which was further confirmed by image J software estimating for TH positive neurons (Fig. 3B). These results suggest that complete loss of *TMEM230* does not cause DA neuron degeneration in rats.

3.4. No changes in PD-associated protein expression in TMEM230 KO rats

Increasing evidence suggests that impaired autophagy-lysosomal function plays a key role in the pathogenesis of PD. Importantly, TMEM230 knockdown results in accumulation of p62 and LC3-II in HEK-293 T cells [15]. Unlike these previous in vitro data, our immunoblotting analysis showed similar levels of p62 and LC3-II expression between TMEM230 KO and wild-type rats (Fig. 4A). Two other autophagy-related proteins, Beclin 1 and LAMP2a, the latter of which is a receptor in the lysosomal membrane that serves as a substrate protein or chaperone-mediated autophagy, were also expressed at similar levels.

A previous study reports that TMEM230 is required for RAB8A mediated vesicle trafficking and retromer trafficking [15]. When we measured levels of the endosome markers RAB5A and RAB7A, no significant difference was obtained between TMEM230 KO and wild-type rats (Fig. 4B). We also tested the levels of other RAB proteins, such as RAB3A, RAB6A, RAB30, but there were no clear differences (Fig. 4B). Mutations of *VPS35* were recently shown to cause PD and to play a key role in the neurodegenerative process [20]. We found similar VPS35 protein expression between TMEM230 KO and wild-type rats, as well as similar expression of two other PD-causing genes, PARK8 and LRRK2 (Fig. 4C).

3.5. Absence of glial reactions in TMEM230 KO rats

Glial reactions are observed in post-mortem PD brains and some animal models of PD [21]. Therefore, we performed immunofluorescence staining with the microglial marker Iba1 and astroglial marker GFAP. However, there were no distinct differences in Iba1 or GFAP immunoreactivity between TMEM230 KO and wild-type rats (Fig. 5).

4. Discussion

Recently, Deng et al. identified a mutation in the *TMEM230* gene on the short arm of chromosome 20 as causative of PD in an autosomal-dominant manner. [2]. Subsequently, additional novel variants were detected in PD patients [22,23]. These studies suggest that TMEM230 is a novel genetic cause of PD and provide new insights into the pathogenesis of PD-related neurodegeneration. However, evidence from more recent studies failed to find PD-related pathogenic variants in *TMEM230, suggest*ing that TMEM230 is a rare cause of PD. In the present study, we created TMEM230 KO rats to explore whether TMEM230 depletion plays a role in neurodegeneration.

PD is characterized by progressive movement disorder and is associated with loss of midbrain DA neurons in the SN. One recent study reports that PINK1 KO rats exhibit age-dependent loss of DA neurons beginning at 6–8 months of age [24,25]. Locomotor behavior deficits appear as early as 4 months of age and include reduced rearing frequency and less distance traveled in the open-field test. Similar phenotypes were observed in DJ-1 KO rats [25]. Also, LRRK2 KO mice display abnormal exploratory activity in the open field test and remain longer on an accelerating rotarod than wild-type rats [26]. However, Parkin KO rats show no significant behavioral deficits or age-dependent nigral DA loss [25].These reports indicate that the inactivation of some genes produces behavioral deficits and progressive neurodegeneration, suggesting that these genes are essential for the survival of DA neurons, but some do not. In our study, we used both the open field and accelerating rotarod tests to examine rat mobility, and immunohistochemistry was performed to examine TH levels. Neither locomotor impairment nor DA neuron loss was observed in TMEM230 KO rats. Therefore, we speculate that TMEM230 depletion is not causative of rat behavioral dysfunction or DA loss.

The autophagy-lysosome pathway plays a key role in the clearance of aggregated proteins and neurodegenerative conditions [27]. Alterations in autophagy processes, including macroautophagy and chaperone-mediated autophagy are reported in PD. For example, Issa *et al.* showed that LAMP2a expression stimulates selective autophagy in the adult Drosophila brain [27]. In addition, accumulation of LAMP2a and HSPA8 is oberved in the midbrain and striatum in aged LRRK2^{R1441G} knock-in mice. In our study, however, there was no difference in the

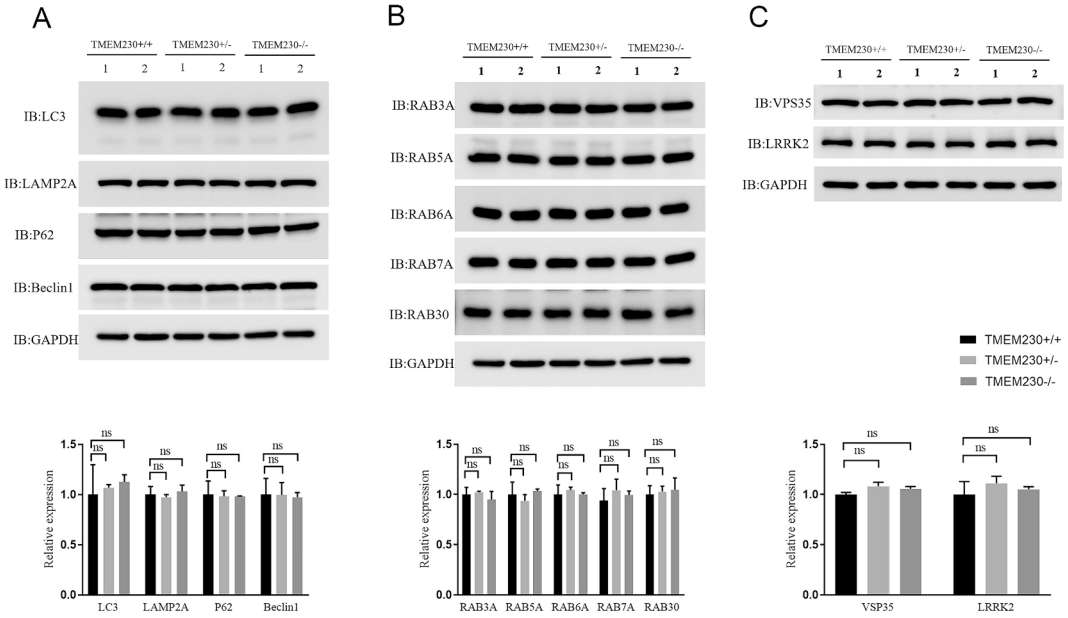

Fig. 4. Relative protein expression levels were not altered in TMEM230 KO rats. (A) Similar expression levels of autophagy related proteins between TMEM230 KO and wild-type rats. (B) Similar expression levels of RAB proteins between TMEM230 KO and wild-type rats. (C) Similar expression levels of VPS35, PARK8, and LRRK2 between TMEM230 KO and wild-type rats. Data are expressed as the mean ± SD (n = 5), $p > 0.05$, ns indicate "not significant".

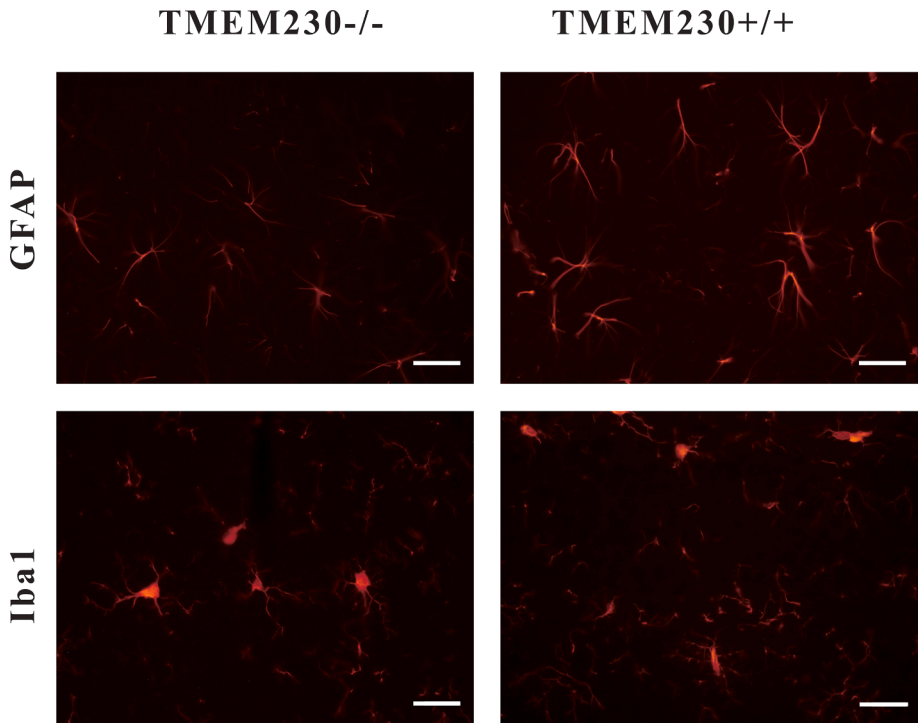

Fig. 5. Glial reactions were not oberserved in TMEM230 KO rats. Brain sections were immunostained with anti-GFAP, an astrocytic marker, and anti-Iba1, a microglial marker. Scale bar = 50 μm.

expression of LAMP2a between TMEM230 KO and wild-type rats. Many studies report that beclin-1 level is closely associated with neurodegeneration. Spencer *et al.* demonstrated that overexpression of Beclin1 reduces the accumulation of α-synuclein and ameliorates the synaptic and dendrtic pathology in mice [28]. Beclin1 is essential for the initial step of the autophagy pathway, but we found no alteration in the expression of Beclin 1 in TMEM230 KO rats. The loss of p62 in dopaminergic neurons causes Lewy pathology and motor dysfunction in aged mice, likely due to changes in several biological processes [29]. These processes can be assessed through the expression of correlated biomarkers, such as LC3 and ULK1 [30]. Suppressed expression of LC3-II reflects impaired autophagy, and the amount of LC3-II is correlated with the extent of autophagosome formation [17]. We detected no differences in p62 and LC3 between TMEM230 KO and wide-type rats, suggesting that depletion of the *TMEM230* gene does not affect the autophagic-lysosome pathway in rats. Our findings are inconsistent with the previously published work by Dr. Han-Xiang Deng and Dr. Dimitri Krainc, which demonstrated that knockdown of the TMEM230 gene impaired trafficking and autophagy [2,15].The discrepancy in results may stem from the complex interactions and compensatory mechanisms present in a whole organism, which are not fully recapitulated in vitro. Additionally, the shorter lifespan of rats compared to humans could limit the observation of long-term effects, potentially contributing to the absence of consistent phenotypes in our study.

Rab proteins constitute a family of GTP-binding proteins and act as essential regulators of vesicular trafficking. An increasing number of studies reveal that impairment of these proteins contributes to inherited early-onset PD [31]. For example, Deng et al. reported that TMEM230 colocalizes with RAB5A and RAB7A [2]. Membrane-associated GTP-bound RAB3A not only interacts with α-synuclein but also increases α-synuclein sequestration on intracellular membranes by inhibiting RAB3A recycling [32]. Moreover, both Shin et al. [33] and Gomez-Suaga et al. [34] report that both RAB5 and RAB7 interact with LRRK2. Co-expression of RAB5B protein rescues impairments of synaptic vesicle endocytosis induced by altered LRRK2 expression [33]. Another study reports that co-expression of RAB7 alleviates impairment in trafficking from early to late endosomes caused by mutant *LRRK2* [34]. In the present study, we found no differences in RAB3A, RAB5A or RAB7A expression. RAB6 is the most abundant Golgi Rab protein and down-regulation of RAB6A was observed using an RNAi approach to delay Golgi-resident protein recycling to the endoplasmic reticulum (ER) [35]. Moreover, overexpression of GTP-bound forms of RAB6 induces redistribution of Golgi resident proteins into the ER, which stimulates Golgi-ER recycling of Golgi resident glycosylation enzymes [35]. RAB30 localizes to the Golgi and interacts with a diverse set of Golgi proteins [36]. In our study, we still found no alterations in RAB6A or RAB30 expression. Thus, depletion of TMEM230 may not be essential for vesicular trafficking.

VPS35 is essential for endosome-to-Golgi retrieval of membrane proteins, and the LRRK2 protein encodes a large multidomain protein with GTPase and kinase activities and plays a role in autophagy [3]. Mutations in *VPS35* or *LRRK2* have been identified in patients with autosomal dominant PD [37]. The depletion of LRRK2 augments the accumulation of lipofuscin granules and altered levels of LC3-II and p62 [38]. In our study, neither VPS35 nor PARK8 expression was altered in TMEM230 KO rats. In addition, there were no differences in the expression of GFAP or Iba1 between TMEM230 KO and wild-type rats, indicating that TMEM230 loss does not cause glial activation.

In summary, our findings suggest that the complete loss of TMEM230 does not lead to motor impairment or other hallmarks of PD in rats.

5. Ethics approval statement

The study was conducted according to the NIH Guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Thomas Jefferson University.

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Author contributions

C.H. and B.H. conceived and designed the experiments. W.Z., H.P., D. Y., B.H., and C.H. performed the experiments. D.Y., G.S., Y.Z., and J.H. reviewed the manuscript. W.Z., B.H., and C.H. analyzed the data and wrote the paper. All authors have read and agreed to the published version of the manuscript.

CRediT authorship contribution statement

Wenjuan Zhang: Validation, Supervision, Software, Resources, Project administration, Formal analysis, Data curation, Conceptualization. **Hao Peng:** Supervision, Software, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **Daihe Yang:** Validation, Supervision, Software, Resources. **Guohua Song:** Validation, Supervision. **Juan He:** Visualization, Validation, Supervision. **Yun Zhou:** Validation, Supervision, Resources, Project administration, Funding acquisition. **Cao Huang:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Funding acquisition, Formal analysis. **Bo Huang:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Funding acquisition, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Not applicable.

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