

# RUNX3 negatively regulates autophagic degradation of aggregated $\alpha$ -synuclein

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# Abstract

Synucleinopathies are a group of neurodegenerative disorders characterized by the accumulation of  $\alpha$ -synuclein ( $\alpha$ -syn) aggregates in the brain. Elucidating the mechanisms of  $\alpha$ -syn aggregation is important in understanding the pathogenesis of synucleinopathies. In this study, we identified Runt-related transcription factor 3 (RUNX3), a tumor suppressor gene that regulates autophagic activity, as a regulator of the level of intracellular  $\alpha$ -syn aggregates. RUNX3 deficiency reduced the  $\alpha$ -syn aggregates by inducing autophagic activity for the  $\alpha$ -syn degradation, while the overexpression of RUNX3 resulted in an increased level of  $\alpha$ -syn aggregates. Interestingly, treatment of cells with  $\alpha$ -syn aggregates increased the expression of RUNX3, suggesting a feedback loop between  $\alpha$ -syn aggregation and RUNX3 expression. Single nucleus RNA sequencing analysis revealed that RUNX3 expression was significantly increased in brains with multiple system atrophy. This further supports the feedback loop between RUNX3 and  $\alpha$ -syn pathology, identifying RUNX3 as a potential therapeutic target for synucleinopathies.

## Introduction

Parkinson disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA) are a group of neurodegenerative disorders characterized by the accumulation of  $\alpha$ -synuclein ( $\alpha$ -syn) in the brain<sup>1–6</sup>. While monomeric  $\alpha$ -syn is natively unfolded, it can aggregate into cytotoxic filaments with a cross- $\beta$ -sheet structure. Intracellular  $\alpha$ -syn aggregates found in patient brains are phosphorylated and are insoluble to detergents such as N-Lauroylsarcosine (sarkosyl)<sup>7,8</sup>. Aggregated  $\alpha$ -syn can act as “seeds” to induce further aggregation of  $\alpha$ -syn monomers<sup>9–11</sup> and the propagation of  $\alpha$ -syn seeds between cells can induce the prion-like spread of  $\alpha$ -syn pathology in the brain with synucleinopathies<sup>12</sup>. The degradation of  $\alpha$ -syn aggregates is also an important factor in the formation of  $\alpha$ -syn pathology. It involves both the autophagic, lysosomal and proteasomal pathways<sup>13–15</sup>. On the other hand,  $\alpha$ -syn aggregation is also known to impair autophagic activity, which promotes further aggregation and cellular toxicity<sup>16,17</sup>. However, the details of the regulatory mechanisms involved in the formation and the metabolism of  $\alpha$ -syn aggregates are still unknown.

Runt-related transcription factor (RUNX3) is a transcription factor associated with the regulation of inflammation, tumorigenesis and apoptosis by altering the expression of genes related to these cellular processes<sup>18,19</sup>. Originally, the mammalian RUNX family proteins (i.e., RUNX1, 2, 3) were identified as orthologous proteins related to *runt*, a transcription factor critical to the embryonic development of *Drosophila melanogaster*. Knockout mouse studies showed that RUNX3 is important in the differentiation of the lymphocyte and myeloid lineages. A recent study revealed that RUNX3–miR-186-3p axis is critical in the dopaminergic neuron death in PD model induced by MPTP treatment, and loss of miR-186-3p expression significantly decreased the  $\alpha$ -syn expression level<sup>20</sup>. However, the pathophysiological role of RUNX3 in the degradation of  $\alpha$ -syn has not been examined so far.

In this study, we have revealed that RUNX3 can regulate the levels of  $\alpha$ -syn aggregates via autophagic activity. RUNX3 deficiency resulted in a decreased level of  $\alpha$ -syn aggregates, while the overexpression of RUNX3 increased the intracellular  $\alpha$ -syn aggregates. Our results suggest that RUNX3 deficiency increases the degradation of  $\alpha$ -syn aggregates by increasing autophagy. Interestingly, RUNX3 expression was increased specifically by  $\alpha$ -syn aggregates treatment, but not by aggregates composed of tau. Importantly, single nucleus RNA sequencing revealed that RUNX3 expression is increased in the brains of MSA patients, further supporting the involvement of RUNX3 in  $\alpha$ -syn pathology. Our study suggests the role of RUNX3-autophagy axis in the regulation of  $\alpha$ -syn aggregation and its potential as a therapeutic target.

## Materials and methods

### Antibodies

The following antibodies were purchased and used in the experiments: mouse anti- $\alpha$ -syn antibody 4D6 (ab1903, Abcam, Cambridge, UK, 1:1000 dilution), rabbit anti-phosphorylated  $\alpha$ -syn antibody (ab51253, Abcam, Cambridge, UK, 1:1000 dilution), rabbit anti-RUNX3 antibody (#18113, Cell Signaling Technology, MA, USA, 1:1000 dilution), mouse anti-tau antibody (#5A6, Developmental Studies Hybridoma Bank, IA, USA, 1:100 dilution), rabbit anti-HIF-1 $\alpha$  antibody (#36169, Cell Signaling Technology, MA, USA, 1:1000 dilution), mouse anti- $\alpha$ -tubulin antibody (T6199, Merck, Darmstadt, Germany, 1:1000 dilution), rabbit anti-LC3B antibody (#3868, Cell Signaling Technology, MA, USA 1:1000 dilution), rabbit anti-ATG13 antibody (#13273 Cell Signaling Technology, MA, USA 1:1000 dilution), rabbit anti-p62 antibody (ab91526, Abcam, Cambridge, UK, 1:1000 dilution), horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (#515-035-003 Jackson immunoresearch, PA, USA), horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (#111-035-003 Jackson immunoresearch, PA, USA).

### Preparation of recombinant $\alpha$ -syn

The plasmid of recombinant human wild-type (WT)  $\alpha$ -syn (cloned into pRK127 vector) was gifted by Dr. Masato Hasegawa at the Tokyo Metropolitan Institute of Medical Science<sup>21</sup>.

Recombinant  $\alpha$ -syn was purified according to previous reports<sup>21–24</sup>. Briefly, the WT  $\alpha$ -syn was expressed in BL21 (DE3) (Nippon gene CO., LTD., Tokyo, Japan), and were purified using an anion exchange chromatography column. The eluted  $\alpha$ -syn was precipitated with ammonium sulfate dissolved in 30 mM Tris-HCl (pH 7.5) (2-Amino-2-(hydroxymethyl)-propane-1,3-diol [Tris] (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), Hydrochloric Acid (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan)). Ions were then removed by dialysis and aggregates were removed by ultracentrifugation. Protein concentrations were measured using a BCA protein assay kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Resultant  $\alpha$ -syn monomers were stored at -80°C until use.

# Recombinant $\alpha$ -syn seed preparation

For fibrillization, 5 mg/mL monomeric recombinant  $\alpha$ -syn was shaken at 1,000 rpm, 37°C for 5 days<sup>24,25</sup>. To remove monomers, the fibrillar  $\alpha$ -syn was ultracentrifuged at 45,000 rpm, 25°C for 20 min. The resulting pellet was solubilized as seeds by sonication, and the concentration was measured by BCA assay. The seeds were stored at -80°C until use.

## $\alpha$ -Syn strains derived from patient brains

The MSA brain sample was obtained from the Brain Bank for Aging Research (BBAR) and the Department of Neurology, the University of Tokyo. The DLB brain sample was also obtained from the BBAR at Tokyo Metropolitan Institute for Geriatrics and Gerontology (TMIG) through Japan Brain Bank Net. All cases were diagnosed neuropathologically by the certified neuropathologist.

Experiments using  $\alpha$ -syn seeds derived from human samples were obtained and conducted under the approval of the institutional ethical committee of the National Center of Neurology and Psychiatry (B2022-051), the Graduate School of Medicine (No. G2183-(20)), and the Graduate School of Pharmaceutical Sciences (No. 2-1, 4-5) of the University of Tokyo. They were also approved by the institutional ethical committee of TMIG (No. R20-44).

The  $\alpha$ -syn aggregates were extracted from frozen brain samples of MSA patients as previously described<sup>7,18,19,21,22,26-28</sup>. Briefly, brain samples were homogenized in 20% (w/v) buffer containing 10 mM Tris-HCl (pH 7.5), 0.8 M NaCl, 10% sucrose (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 2% N-Lauroylsarcosine (sarkosyl; Merck, Darmstadt, Germany), cOmplete (Merck, Darmstadt, Germany), and PhosSTOP (Merck, Darmstadt, Germany). After incubation for 30 min at 37°C, the samples were centrifuged at 20,000 x g for 10 min. The supernatant was then centrifuged at 100,000 x g for 20 min. Pellets were washed and resuspended in 0.25% (v/w) 30 mM Tris-HCl (pH 7.5) by sonication for 1 min.

Aggregates from DLB brain samples were extracted using another protocol<sup>33</sup>. In brief, brain samples were homogenized in 10% (w/v) either phosphate-buffered saline (PBS; 15 mM NaCl (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 5 mM Na<sub>2</sub>HPO<sub>4</sub> (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 2.7 mM KCl (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 1.5 mM KH<sub>2</sub>PO<sub>4</sub> (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan)) and samples were centrifuged at 1,000 x g to remove debris. Sarkosyl was added to a final concentration of 2% and incubated at 37°C with shaking for 2 h. A final concentration of 20  $\mu$ g/mL proteinase K (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was added to the samples and incubated for 1 h at 37°C. Phenylmethyl sulfonyl fluoride was added to a final concentration of 1 mM to stop proteinase K reaction. The samples were added 10% (w/v) solution of phosphotungstic acid (pH 7.0) to a final concentration of 2% and incubated at 37°C overnight with shaking. The samples were centrifuged at 16,000 x g for 30 min and the pellet was resuspended in 0.25% (w/v) 30 mM Tris-HCl (pH 7.5) by sonication.

# Preparation of recombinant tau

Recombinant tau repeat domain (RD; 244-332 amino acids of 2N4R tau) with P301S mutation was prepared as previously described<sup>29-31</sup>. In brief, P301S RD of tau was expressed in Rossetta (DE3) (Merck, Darmstadt, Germany), and purified using cation exchange chromatography column. To increase purity, the eluates were applied to reverse-phase chromatography. After evaporating the buffer, the pellet was redissolved in PBS and protein concentration was determined by BCA assay. Tau monomers were stored at -80°C until use.

## Recombinant tau seed preparation

A mixture of 40 µM purified tau RD peptide, 40 µM heparin (Thermo Fisher Scientific, MA, USA), and 2 mM dithiothreitol (Nacalai Tesque, Kyoto, Japan) in PBS was shaken at 1,000 rpm, 37°C, overnight. To remove monomers, the mixture was ultracentrifuged at 113,000 x g, 25°C for 20 min. The pellet was resuspended in PBS by sonication. Protein concentration was determined by BCA assay and tau seeds were stored at -80°C until use.

## Cell culture and seeded aggregation of α-syn and tau

HEK293A cells (#CCL-1573, ATCC) were cultured in Dulbecco's Modified Eagle's Medium High Glucose with L-Glutamine (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal bovine serum (Cosmo Bio, Tokyo, Japan) and 50 units/mL penicillin and streptomycin (Thermo Fisher Scientific, MA, USA), and maintained at 37°C in a 5% CO<sub>2</sub>, 95% air atmosphere incubator<sup>24,32</sup>.

Monoclonal HEK293A cells expressing Cas9 and α-syn, or 1N4R P301S tau were generated using lentiviral infection as previously described<sup>24,32</sup>. For cell treatment, 100 mM bafilomycin A1 (AdipoGen Life Sciences, CA, USA) in dimethyl sulfoxide (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was added to the medium at a final concentration of 100 nM for 3 h. Filtered 100 mM CoCl<sub>2</sub> (KANTO CHEMICAL, Tokyo, Japan) dissolved in sterile water was added to the medium to reach the indicated concentrations for 3 h before collection.

α-Syn seeds were transfected using MultiFectam (Promega, WI, USA) as previously described<sup>24,27</sup>. Tau seeds were transfected using Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA) as previously described<sup>29</sup>. Cells were passaged into half at 48 h after seed transfection and incubated overnight before sarkosyl fractionation or flowcytometry.

## siRNA transfection

siRNA transfection was conducted as previously described<sup>32</sup>. siRNA targeting *RUNX3* was designed as below and a non-targeting control siRNA (D-001810-10-20, Thermo Fisher Scientific, MA, USA) were purchased. Briefly, 10 nM of siRNAs were reverse transfected into cells using Lipofectamine RNAiMax Reagent (Thermo Fisher Scientific, MA, USA). Cells were incubated for 24 h and transfected with  $\alpha$ -syn seeds, or incubated for 48 h before  $\text{CoCl}_2$  treatment.

*RUNX3* target sequence: 5'-aaccugaugccauagacucau-3'

## Overexpression of *RUNX3*

For the overexpression of *RUNX3*, cDNA of *RUNX3* was amplified with the indicated primers and cloned into pLV5IN-CMV Neo Vector (Takara Bio, Shiga, Japan) as previously described<sup>29</sup>. Plasmids were transfected using Polyethyleneimine Max (Polysciences, PA, USA) according to the manufacturer's instructions<sup>24,32</sup>.

Forward: 5'-atggcatcgaacagcatcttcgac-3'

Reverse: 5'-tcagtagggccgccacacg-3'

## Knockout of *RUNX3*

3 different gRNA sequences targeting *RUNX3* (shown below) were inserted onto a vector with EGFP (Addgene #48138) and a mixture of the 3 plasmids transfected using PEI. Cells positive for EGFP were sorted using flowcytometry. Monoclonal KO cell lines were created by limiting dilution of EGFP positive cells.

#1 forward: 5'-aagtggcgacctaccaccga-3'

#1 reverse: 5'-tcggtggtaggtcgccactt-3'

#2 forward: 5'-gcggaccacgcaggcgagct-3'

#2 reverse: 5'-agctcgctgcgtggtccgc-3'

#3 forward: 5'-gcaatgcctcggccgtcatg-3'

#3 reverse: 5'-catgacggccgaggcattgc-3'

## Flowcytometry

For flowcytometry analysis of  $\alpha$ -syn aggregation, 10 mM  $\alpha$ -syn filaments specific probe C05-05<sup>33</sup> dissolved in DMSO was added to the medium to the final concentration of 1  $\mu\text{M}$  and incubated for 30

min at 37°C in a CO<sub>2</sub> incubator. Medium was changed and cells were incubated for an additional 1 h before collection.

Cells were washed with PBS and harvested with 0.1% Deoxyribonuclease (Nippongene), 20 units/mL Papain (Worthington) solution in PBS. Cell suspension was filtered through 40 µM EASYstrainer small (Greiner, Kremsmünster, Austria) and stored on ice until analysis. Flowcytometry was conducted using Sony MA 900 cell sorter (Sony Corporation, Tokyo, Japan) with 405 nm laser and 665/30 nm emission filter for C05-05 and 488 nm laser and 525/50 nm emission filter for EGFP detection.

## Biological analysis of sarkosyl-insoluble α-syn

Sarkosyl fractionation was conducted as previously described<sup>29</sup>. In brief, cells were extracted with a sarkosyl solution containing 0.2% sarkosyl, EDTA-complete (Merck, Darmstadt, Germany), and phosSTOP (Merck, Darmstadt, Germany). The extract was sonicated for 10 sec and incubated at 37°C for 30 min. After the ultracentrifugation at 35,000 rpm for 20 min at 4°C, the supernatants were collected as the sarkosyl-soluble fraction and added 5 x sample buffer (10% SDS, 400 mM Tris-HCl (pH6.8), 75% glycerol, 0.0125% Brilliant green (FUJIFILM WAKO chemical Pure Chemical Corporation, Osaka, Japan), 0.03125% Coomassie Brilliant Blue G-250). The resulting pellets were washed with PBS and collected as the sarkosyl-insoluble fraction by adding 1 x sample buffer and sonicated for 10 sec. These samples were used for immunoblotting. Detected bands were quantified using the ImageJ (NIH) software and the band intensities were compared as the ratio of α-syn in the insoluble fraction to the sum of α-syn in the soluble and insoluble fractions (total α-syn).

## Immunoblotting

All procedures of immunoblotting were performed as previously described<sup>29,32,34,35</sup>. Total cell lysates were collected with sample buffer and sonicated for 30 sec. Protein concentrations in total lysates or sarkosyl soluble fractions were measured using BCA protein assay according to the manufacturer's instructions. 1% 2-mercaptoethanol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was added to the samples and boiled at 100°C for 10 min. Samples and protein marker, Precision Plus Protein Dual Xtra Standards (BIO-RAD, CA, USA), were applied to 7.5% Tris/Glycine or 15% Tris/Tricine SDS-PAGE gels and transferred onto PVDF membranes (Merck, Darmstadt, Germany). Immunodetection was performed using the ImmunoStar detection kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and Image Quant LAS4000 (GE Healthcare, IL, USA).

## qRT-PCR

qRT-PCR after RNA extraction and cDNA synthesis was performed as previously described<sup>36,37</sup>. In brief, RNA was extracted using Isogen (Nippon gene CO., LTD., Tokyo, Japan) and cDNA was synthesized using

ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Real-time reverse transcription was performed with the indicated primers using THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) and was run on LightCycler 480 Instrument II (Roche).

*RUNX3* forward: ATGGCAGGCAATGACGAGAA

*RUNX3* reverse: AGGGTGAAACTCTTCCCTCG

*BIM* forward: CAAGAGTTGCGGCGTATTGGAG

*BIM* reverse: ACACCAGGCGGACAATGTAACG

*BAX* forward: TCAGGATGCGTCCACCAAGAAG

*BAX* reverse: TGTGTCCACGGCGGCAATCATC

*GAPDH* forward: AGGTCGGTGTGAACGGATTTG

*GAPDH* reverse: TGTAGACCATGTAGTTGAGGTCA

## Single nucleus RNA sequencing

Single nucleus RNA sequencing using human samples were conducted under the approval of the ethical committee of Niigata University (G2018-0034), and the ethical committee of the Graduate School of Pharmaceutical Sciences, The University of Tokyo (5-15). Written informed consent for autopsy including the use of tissue for research purposes was obtained from the next of kin. Single-nucleus RNA sequencing (snRNA-seq) was performed using amygdala tissue samples obtained from six individuals (three patients with pathologically proven MSA and three controls), archived at the Brain Research Institute, Niigata University (Supplementary Table 1). Nuclei were isolated from approximately 20 mg of fresh-frozen tissue using the Minute Single Nucleus Isolation Kit for Neuronal Tissues/Cells (Invent Biotechnologies, Inc., MN, USA). Isolated nuclei were processed using the Chromium Next GEM Single Cell 3' Kit v3.1 (10x Genomics, CA, USA). Approximately 10,000 nuclei per library were partitioned into gel beads-in-emulsion on a Chromium Controller instrument (10x Genomics). Reverse transcription and cDNA amplification were performed, and Illumina-compatible adapter sequences were added to the final libraries via PCR. The libraries were then sequenced on a NextSeq 2000 instrument (Illumina, CA, USA). Raw sequencing data were processed using the Cell Ranger pipeline (v7.0.0, 10x Genomics) for demultiplexing, barcode assignment, read alignment to the human reference genome (GRCh38-2020-A), and gene counting.

Quality control and downstream analysis were conducted using Scanpy (ver. 1.9.3) package in Python (ver. 3.10.13). Low-quality nuclei were filtered out based on the number of detected genes (> 10,000-12,000), the proportion of mitochondrial genes (> 0.1-1%). Doublets were detected and removed using Scrublet (ver. 0.2.3), and 88,731 nuclei finally remained.

After quality control, all samples were integrated into a single dataset, log-normalized and the top 2000 variable genes were selected. Principal component analysis (PCA) was performed to reduce to two dimensions. To account for the batch effects, batch correction was conducted using Harmony (ver. 0.0.10). Uniform Manifold Approximation and Projection (UMAP) embedding was generated based on principal component analysis (PCA) space and clusters were calculated based on the Leiden algorithm. From the algorithm, 21 clusters were generated, and we annotated the clusters based on the previously published marker genes<sup>38,39</sup>

## Statistical analysis

Data analyses were conducted from independent tubes or wells and were not conducted in a blinded fashion. Data are presented as mean values and error bars indicate the standard error of the mean (SEM). Suitable statistical analysis was performed, including Tukey's HSD test and Student's *t*-test with Welch's correction. A *p*-value of less than 0.05 was considered to have a statistically significant difference between groups.

## Data availability

The data that support the findings of this study are available from the corresponding author on reasonable request. Human brain data set used in this study are available from the GEO repository with the accession number of GSE323989.

## Ethics declaration

This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the Brain Bank for Aging Research, the National Center of Neurology and Psychiatry, Tokyo Metropolitan Institute for Geriatrics and Gerontology, Niigata University, and the University of Tokyo.

## Results

### **RUNX3 expression levels correlated with intracellular $\alpha$ -syn aggregation.**

To investigate the effect of RUNX3 on  $\alpha$ -syn aggregation, we analyzed the intracellular  $\alpha$ -syn aggregation induced by the transfection of recombinant  $\alpha$ -syn filaments (pre-formed filaments; PFFs) in a monoclonal HEK293A cell line stably expressing  $\alpha$ -syn. RUNX3 knockdown (KD) by siRNA treatment was confirmed by immunoblotting and qRT-PCR (Fig. 1A, B). Flow cytometry after staining the cells with C05-05<sup>40</sup>, a fluorescent probe specific for  $\alpha$ -syn aggregates, revealed that RUNX3 KD decreased the

proportion of cells with intracellular  $\alpha$ -syn aggregates (Fig. 1C, D). Biochemical analysis using sarkosyl fractionation also showed that RUNX3 KD decreased the amount of sarkosyl insoluble phosphorylated  $\alpha$ -syn (p- $\alpha$ -syn) (Fig. 1E, F). On the other hand, the overexpression of RUNX3 increased sarkosyl insoluble  $\alpha$ -syn (Fig. 1G, H), indicating that RUNX3 expression levels correlate with  $\alpha$ -syn aggregation.

We then examined the pathological role of RUNX3 on the  $\alpha$ -syn aggregates induced by patient-derived  $\alpha$ -syn filaments. Flow cytometric analysis revealed that RUNX3 KD decreased C05-05 positive cells with aggregates induced by MSA patient-derived filaments, in a similar manner to that by PFFs (Fig. 2A, B). This result was also confirmed by the biochemical analysis, showing a decrease in sarkosyl-insoluble p- $\alpha$ -syn by RUNX3 KD (Fig. 2C, D). Additionally, in the case of use of DLB patient derived filaments, we also found that RUNX3 KD reduced the insoluble p- $\alpha$ -syn (Fig. 2E, F). These data suggest that RUNX3 is a common regulatory factor independent of the type of synucleinopathies. We further analyzed the effect of RUNX3 on the aggregation of tau, another intracellular protein associated with neurodegenerative diseases. Immunoblotting of lysates of tau stably expressing cells transfected with recombinant tau PFF showed that RUNX3 KD did not affect the amount of sarkosyl insoluble tau (Fig. 2G, H). These suggest that RUNX3 is an  $\alpha$ -syn-specific regulatory factor for the levels of intracellular aggregation.

## **RUNX3 regulates $\alpha$ -syn aggregation by altering autophagic flux.**

Since RUNX3 is associated with autophagy, one of the major degradation pathways of  $\alpha$ -syn aggregates, we hypothesized that RUNX3 deficiency decreased the  $\alpha$ -syn aggregates by increasing the autophagic flux. To test this hypothesis, we treated RUNX3 KD cells with an autophagy inhibitor, bafilomycin A1. Immunoblotting of sarkosyl soluble and insoluble fractions showed that bafilomycin A1 treatment attenuated the effect of RUNX3 on the  $\alpha$ -syn aggregation (Fig. 3A, B). To further investigate the role of RUNX3 and autophagy in  $\alpha$ -syn aggregation, we generated RUNX3 knockout (KO) HEK293 cells, which also stably express  $\alpha$ -syn. We confirmed again that the induction of intracellular  $\alpha$ -syn aggregates was decreased in RUNX3 KO cells (Fig. 3C, D). The increase in autophagic flux can be detected by the increased proportion of LC3-II to LC3-I under bafilomycin A1 treatment. We demonstrated an increase in proportion of LC3-II in RUNX3 KO cells compared to controls (Fig. 3E, F). An increase in ATG13, an autophagy-related protein, was also observed by immunoblotting (Fig. 3G, H). In addition, the decrease of the autophagic substrate, p62, was detected in RUNX3 KO cells (Fig. 3I, J), suggesting an increase in autophagic flux. Furthermore, since RUNX3 is known as a transcription factor, we examined the expression of RUNX3-regulated downstream factors that may be involved in autophagic flux. BIM and BAX are apoptosis-related proteins that can bind to Beclin1, potentially interfering with its role in autophagosome formation<sup>35–37</sup>. A decrease in the expression of *BIM* and *BAX* (sFig. 1A, B) was observed in RUNX3 KO cells, suggesting the possibility of the increased autophagic flux. Collectively, these data suggest that the decrease in RUNX3 levels induces autophagic flux in cells, leading to a reduction in  $\alpha$ -syn aggregation.

To further elucidate the mechanism for RUNX3 in regulating autophagic flux, we also focused on the relationship between RUNX3 and HIF-1 $\alpha$ . RUNX3 is known to decrease the stability of HIF-1 $\alpha$  under hypoxic conditions<sup>41</sup>. And HIF-1 $\alpha$  regulates the expression of autophagy-related genes in hypoxic cells<sup>42</sup>. Under physiological conditions, HIF-1 $\alpha$  is rapidly degraded after expression. However, under hypoxic conditions, the degradation of HIF-1 $\alpha$  is inhibited, and HIF-1 $\alpha$  protein levels increase<sup>43,44</sup>. Indeed, the treatment with CoCl<sub>2</sub>, a hypoxia mimic reagent, increased HIF-1 $\alpha$  expression in control cells, and RUNX3 KD further increased HIF-1 $\alpha$  levels (Fig. 4A, B). Moreover, CoCl<sub>2</sub> treatment reduced the induction of  $\alpha$ -syn aggregates, suggesting that  $\alpha$ -syn aggregates are degraded by HIF-1 $\alpha$  mediated autophagy (Fig. 4C, D). Interestingly, the effect of CoCl<sub>2</sub> treatment on  $\alpha$ -syn aggregates was not observed in RUNX3 KO cells (Fig. 4E, F), suggesting that no additional degradation occurred, as  $\alpha$ -syn aggregates were already reduced in RUNX3 KO cells. Collectively, these data suggest that the RUNX3-HIF-1 $\alpha$  axis is involved in the regulation of  $\alpha$ -syn aggregation, via the regulation of autophagic flux.

## RUNX3 expression is elevated in MSA patient brains

To determine the role of RUNX3 in synucleinopathy pathogenesis, we observed RUNX3 expression under  $\alpha$ -syn aggregation. Treatment of  $\alpha$ -syn stable cells with  $\alpha$ -syn PFF increased the mRNA levels of *RUNX3* (Fig. 5A). On the other hand, treatment of tau stable cells with tau PFFs did not increase RUNX3 expression (Fig. 5B). Therefore,  $\alpha$ -syn aggregation and RUNX3 expression may create a vicious cycle: increased  $\alpha$ -syn aggregation causes increase in RUNX3 expression, further enhancing the aggregation of  $\alpha$ -syn. This cycle may also be the cause that underlies the selectivity of RUNX3 for  $\alpha$ -syn versus tau.

To reveal if this cycle actually occurs in synucleinopathy brains, we performed single-nucleus RNA sequencing to compare gene expression in the brain of MSA patients and non-patient controls (Supplementary Table 1). Each cluster was annotated to astrocytes, endothelial/ peripheral cells, excitatory neurons, inhibitory neurons, microglia/ macrophages, neurons, oligodendrocyte progenitor cells, oligodendrocytes and T cells by the expression of cell type markers (Fig. 6A). We found that RUNX3 expression was mainly high in microglia and T-cells, but was also seen in other cell types as well (Fig. 6B). Importantly, the expression levels of RUNX3 were significantly increased in the microglia (Fig. 6C) and T cells (Fig. 6D) of MSA patients. Although not significant because of less expression, the increase of RUNX3 expression was also seen in excitatory neurons (Fig. 6E) and oligodendrocytes (Fig. 6F) of MSA patients as well. These data suggest that RUNX3 is a negative regulator for the degradation of  $\alpha$ -syn aggregates via autophagy in the pathological process of synucleinopathies.

## Discussion

In this study, we identified RUNX3 as a novel negative regulator of  $\alpha$ -syn aggregation. RUNX3 deficiency decreased  $\alpha$ -syn aggregates, whereas RUNX3 overexpression increased them (Fig. 1). Furthermore, we demonstrated that RUNX3 deficiency reduces  $\alpha$ -syn aggregates by increasing autophagic flux (Fig. 3), which may be partially due to the increased HIF-1 $\alpha$  levels under reduced RUNX3 expression (Fig. 4). We

also confirmed that induced expression of HIF-1 $\alpha$  by CoCl<sub>2</sub> treatment leads to a decrease in  $\alpha$ -syn aggregation, further suggesting the role of HIF-1 $\alpha$  mediated autophagy in the regulation of  $\alpha$ -syn aggregation (Fig. 4). It is known that RUNX3 directly binds to HIF-1 $\alpha$ , leading to its hydroxylation and destabilization<sup>41</sup>. Furthermore, HIF-1 $\alpha$  is known to regulate autophagy by increasing BNIP3 and BNIP3L<sup>45</sup>. These molecules bind to Bcl2, thereby freeing Beclin1 from Bcl2-Beclin1 complexes. Beclin1 then binds to other autophagy-related proteins, inducing autophagy. Although the role of BNIP3 and BNIP3L in  $\alpha$ -syn degradation remains unclear, Bcl2 has been reported to be involved in  $\alpha$ -syn pathogenesis<sup>41</sup>, supporting the involvement of autophagy in  $\alpha$ -syn degradation. Moreover, RUNX3 can also physically interact with the promoter region and increase the expression of BIM<sup>45,46–48</sup> and BAX<sup>40</sup>, which may potentially inhibit autophagy by interacting with Beclin1<sup>45</sup>. As the expression of *BIM* and *BAX* was decreased in RUNX3 KO cells (sFig. 1), this may also contribute to increased autophagy in RUNX3 deficient cells. Since RUNX3 is involved in various pathways<sup>49–52</sup>, we do not exclude the possibility that other pathways may also contribute to autophagy-mediated degradation of  $\alpha$ -syn aggregates. However, our results indicate that autophagy flux induced by the RUNX3 may play an important role in the metabolism of  $\alpha$ -syn aggregates.

Our results also demonstrated that RUNX3 may be involved in the pathogenesis of synucleinopathies, supported by the increased RUNX3 expression in  $\alpha$ -syn treated cells (Fig. 5A) and in MSA patient brains (Fig. 6). These findings are in line with the previous report showing increased RUNX3 expression in PD model mice<sup>20</sup>. In this model, MPTP treatment increases RUNX3 expression, which leads to miR-186-dependent apoptosis of dopaminergic neurons. Interestingly, other studies have shown that miR-186 can bind to the 3'-UTR regions of HIF-1 $\alpha$  and inhibit its expression<sup>53,54</sup>. Together with our findings, miR-186 may also be involved in the RUNX3-HIF-1 $\alpha$  axis in regulating  $\alpha$ -syn aggregation and contribute to the pathogenesis of synucleinopathies.

An increase in RUNX3 expression can lead to insufficient degradation of  $\alpha$ -syn aggregation, resulting in further enhancement of RUNX3 expression, exacerbating  $\alpha$ -syn pathology (Fig. 7). Importantly, the increase in RUNX3 expression was observed in various types of cells in the brains of patients; the increasing trend of RUNX3 expression seen in neurons and oligodendrocytes is possibly related to cell types with  $\alpha$ -syn inclusions in MSA patients<sup>55</sup>. In addition, a significant increase in RUNX3 expression was observed in microglia and T cells, which are not only major cell types expressing RUNX3 in the brain, but are also activated by  $\alpha$ -syn<sup>56,57</sup>. Furthermore, while microglia are capable of degrading  $\alpha$ -syn via autophagy<sup>58</sup>,  $\alpha$ -syn can suppress microglial autophagy<sup>59</sup>. Thus, our study suggests the role of RUNX3 in the pathogenesis of synucleinopathies by modulating T cell response and microglial  $\alpha$ -syn degradation in the brains. However, while the role of microglia is well studied in PD patients, the involvement of microglia in MSA pathogenesis has not been well studied. The further studies clarifying the contribution of microglia and its expression of RUNX3 in other synucleinopathies will be needed.

Further analysis is required to elucidate the signaling pathways involved in the RUNX3 upregulation by  $\alpha$ -syn. The expression of RUNX3 is known to be regulated by TGF- $\beta$ <sup>60,61</sup>. Interestingly, aggregated  $\alpha$ -syn is

known to induce TGF- $\beta$  signaling in astrocytes<sup>62</sup>, and elevated TGF- $\beta$  levels have been observed in PD and MSA<sup>63,64</sup>, in line with our results showing upregulation of RUNX3 by  $\alpha$ -syn treatment (Fig. 5A) and in MSA patient brains (Fig. 6). On the other hand, TGF- $\beta$  signaling is decreased in patients with Alzheimer disease (AD)<sup>65–67</sup>. Decreased expression of TGF- $\beta$  receptor has been reported in the brains of AD patients, but not PD or DLB patients<sup>67</sup>. Additionally, tau aggregates in the AD patient brains are known to interact with SMADs, transducers of TGF- $\beta$  signals, and interfere with their nuclear translocation<sup>68,69</sup>, suggesting the reduced TGF- $\beta$  signaling by tau. Therefore, the difference in the regulation of TGF- $\beta$  signaling by  $\alpha$ -syn and tau aggregates may explain the difference in RUNX3 expression under  $\alpha$ -syn and tau treatment (Fig. 5A, B).

Overall, our study demonstrates the significance of RUNX3 as a novel regulator of  $\alpha$ -syn aggregation that may shed light on the pathogenesis of synucleinopathies. As RUNX inhibitors have been utilized in cancer research<sup>70–73</sup>, they may be applicable to synucleinopathies as well. While further research is needed, the inhibition of RUNX3 may be a potential therapeutic strategy in the treatment of synucleinopathies.

## Abbreviations

**AD:** Alzheimer disease

**ATG13:** autophagy-related 13

**BCA:** bicinchoninic acid

**CoCl<sub>2</sub>:** cobalt(II) chloride

**DLB:** dementia with Lewy bodies

**DMSO:** dimethyl sulfoxide

**EGFP:** enhanced green fluorescent protein

**GAPDH:** glyceraldehyde-3-phosphate dehydrogenase

**gRNA:** guide RNA

**HEK293A:** human embryonic kidney 293A cells

**HIF-1 $\alpha$ :** hypoxia-inducible factor 1 $\alpha$

**KD:** knockdown

**KO:** knockout

**LC3:** microtubule-associated protein 1A/1B-light chain 3

**MPTP:** 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

**MSA:** multiple system atrophy

**p- $\alpha$ -syn:** phosphorylated  $\alpha$ -synuclein

**PBS:** phosphate-buffered saline

**PCA:** principal component analysis

**PD:** Parkinson disease

**PFF:** pre-formed filament

**RUNX3:** Runt-related transcription factor 3

**SEM:** standard error of the mean

**siRNA:** small interfering RNA

**SMAD:** Sma- and Mad-related protein

**snRNA-seq:** single-nucleus RNA sequencing

**TGF- $\beta$ :** transforming growth factor  $\beta$

**Tris:** 2-amino-2-(hydroxymethyl)-propane-1,3-diol

**UMAP:** Uniform Manifold Approximation and Projection

**WT:** wild-type

**$\alpha$ -syn:**  $\alpha$ -synuclein

## Declarations

## Ethics approval and consent to participate

All experiments involving human-derived materials were conducted in accordance with the Declaration of Helsinki. Experiments using  $\alpha$ -syn seeds derived from human brain tissue were approved by the institutional ethical committees of the National Center of Neurology and Psychiatry (B2022-051), the Graduate School of Medicine (G2183-(20)) and the Graduate School of Pharmaceutical Sciences (2-1, 4-5) of the University of Tokyo, and the Tokyo Metropolitan Institute for Geriatrics and Gerontology (R20-44). Single-nucleus RNA sequencing using human samples was approved by the ethical committees of

Niigata University (G2018-0034) and the Graduate School of Pharmaceutical Sciences, the University of Tokyo (5-15). Written informed consent for autopsy, including the use of tissue for research purposes, was obtained from the next of kin of all donors.

## Consent for publication

Not applicable.

## Availability of data and material

The data that support the findings of this study are available from the corresponding author on reasonable request. The human brain single-nucleus RNA sequencing dataset generated in this study is available from the Gene Expression Omnibus (GEO) repository under the accession number GSE323989.

## Competing interests

The authors declare no competing financial interests associated with this study.

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## Authors' contributions

I.T., Y.W.C., Y.H. and T.Tomita designed the study. I.T. and Y.W.C. carried out the biological experiments. Y.T., N.H., M.H., M.K., A.M. and T.I. performed the single-nucleus RNA sequencing experiments. T.M., A.I., T.Toda, M.T., A.K., M.M., S.M. and Y.S. provided the human materials. M.O. and M.H. provided the fluorescent probe. I.T., Y.W.C., Y.T., N.H., A.M., T.I., Y.H. and T.Tomita analyzed the data. I.T., Y.H. and T.Tomita wrote the manuscript. All authors discussed the results and approved the final manuscript.

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## Figures

Figure 1

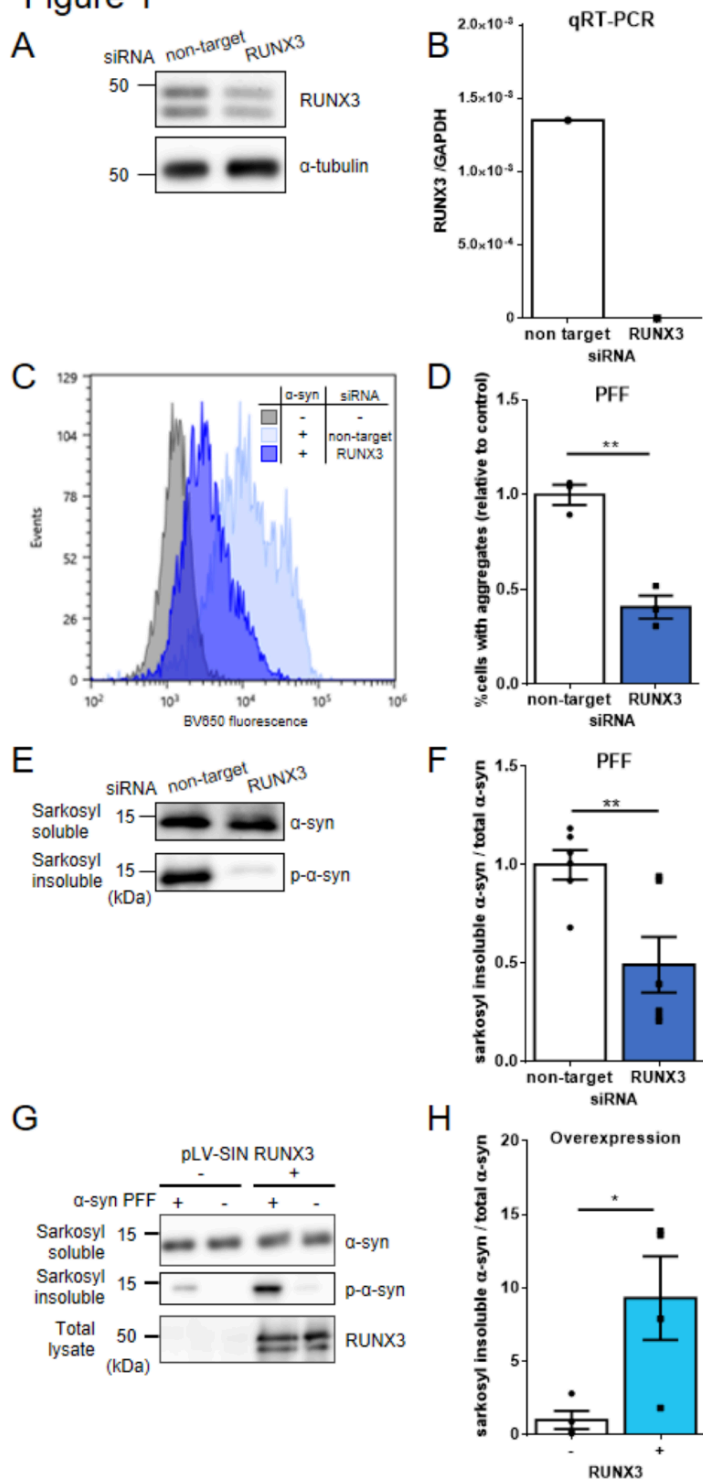


Figure 1

RUNX3 expression levels correlated with intracellular α-syn aggregation.

(A) RUNX3 expression in RUNX3 KD cells were confirmed by immunoblotting.

(B) *RUNX3* mRNA levels in cells treated with siRNAs were measured by qRT-PCR.

(C) Representative histogram of cells treated with non-target siRNA or *RUNX3* siRNA and  $\alpha$ -syn PFFs stained by C05-05.

(D) Quantification of the percentage of cells with aggregates in (C) relative to control (n=3, mean  $\pm$  SEM, Student's t-test, \*\*: p<0.01).

(E) Representative immunoblotting of sarkosyl-soluble  $\alpha$ -syn and sarkosyl-insoluble p- $\alpha$ -syn within cells transfected with PFFs after siRNA treatment.

(F) Quantitative analysis of sarkosyl insoluble p- $\alpha$ -syn in (E) normalized by total  $\alpha$ -syn (n=6, mean  $\pm$  SEM, Student's t-test, \*\*: p<0.01).

(G) Representative immunoblotting of sarkosyl-soluble  $\alpha$ -syn, sarkosyl-insoluble p- $\alpha$ -syn and *RUNX3* in total lysate within cells transfected with PFFs with or without *RUNX3* overexpression.

(H) Quantitative analysis of sarkosyl insoluble p- $\alpha$ -syn in (G) normalized by total  $\alpha$ -syn (n=4, mean  $\pm$  SEM, Student's t-test, \*: p<0.05).

Figure 2

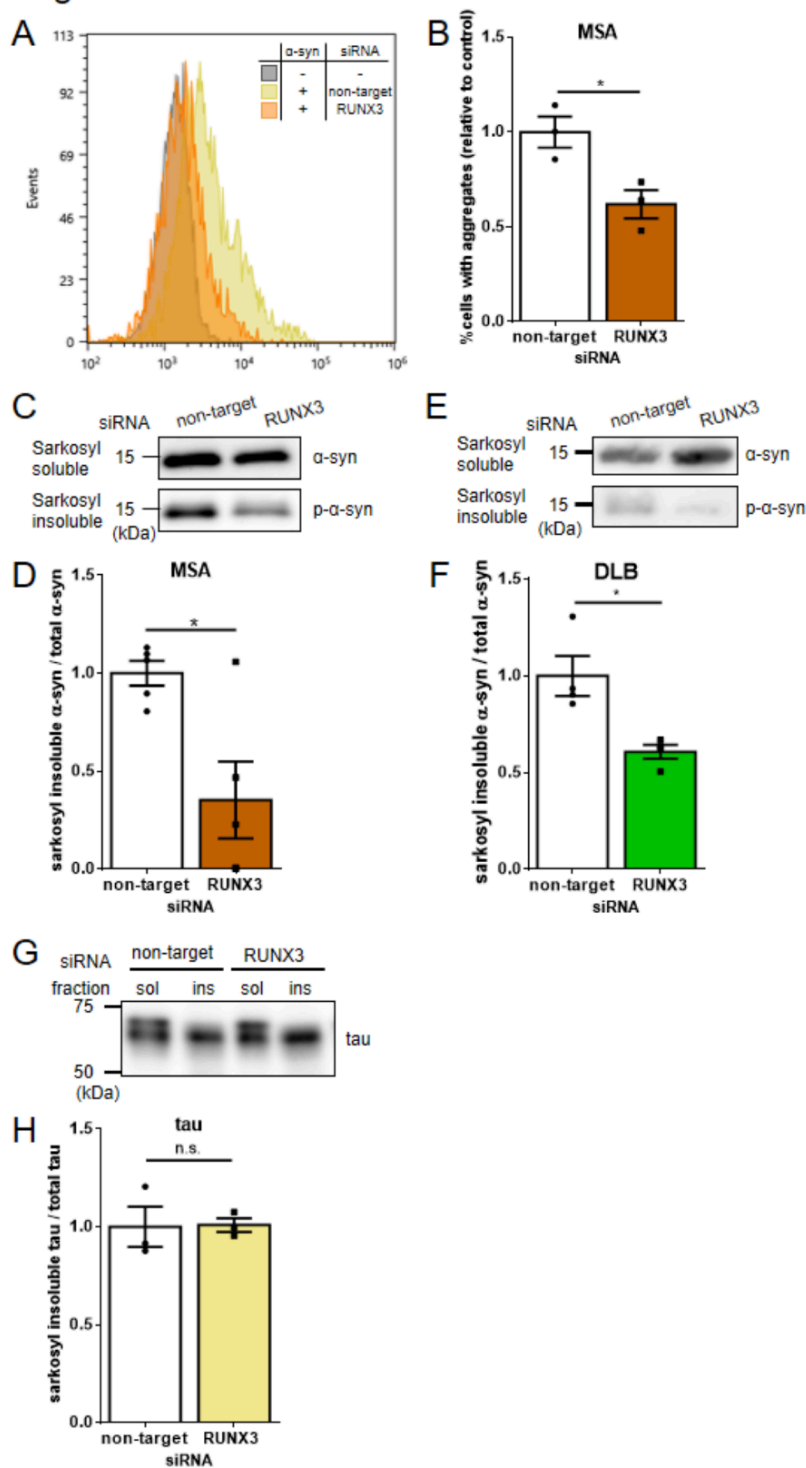


Figure 2

RUNX3 KD decreases α-syn aggregation induced by synucleinopathy patient derived filaments.

(A) Representative histogram of cells treated with non-target siRNA or *RUNX3* siRNA and MSA patient derived α-syn filaments stained by C05-05.

(B) Quantification of the percentage of cells with aggregates in (A) relative to control (n=3, mean  $\pm$  SEM, Student's t-test, \*: p<0.05).

(C) Representative immunoblotting of sarkosyl-soluble  $\alpha$ -syn and sarkosyl-insoluble p- $\alpha$ -syn within cells transfected with MSA patient derived filaments after siRNA treatment.

(D) Quantitative analysis of sarkosyl insoluble p- $\alpha$ -syn in (C) normalized by total  $\alpha$ -syn (n=5, mean  $\pm$  SEM, Student's t-test, \*: p<0.05).

(E) Representative immunoblotting of sarkosyl-soluble  $\alpha$ -syn and sarkosyl-insoluble p- $\alpha$ -syn within cells transfected with DLB patient derived filaments after siRNA treatment.

(F) Quantitative analysis of sarkosyl insoluble p- $\alpha$ -syn in (E) normalized by total  $\alpha$ -syn (n=4, mean  $\pm$  SEM, Student's t-test, \*: p<0.05).

(G) Representative immunoblotting of sarkosyl-soluble and sarkosyl-insoluble tau within cells transfected with tau filaments after siRNA treatment.

(H) Quantitative analysis of sarkosyl insoluble tau in (G) normalized by total tau (n=3, mean  $\pm$  SEM, Student's t-test, n.s.: no significance).

Figure 3

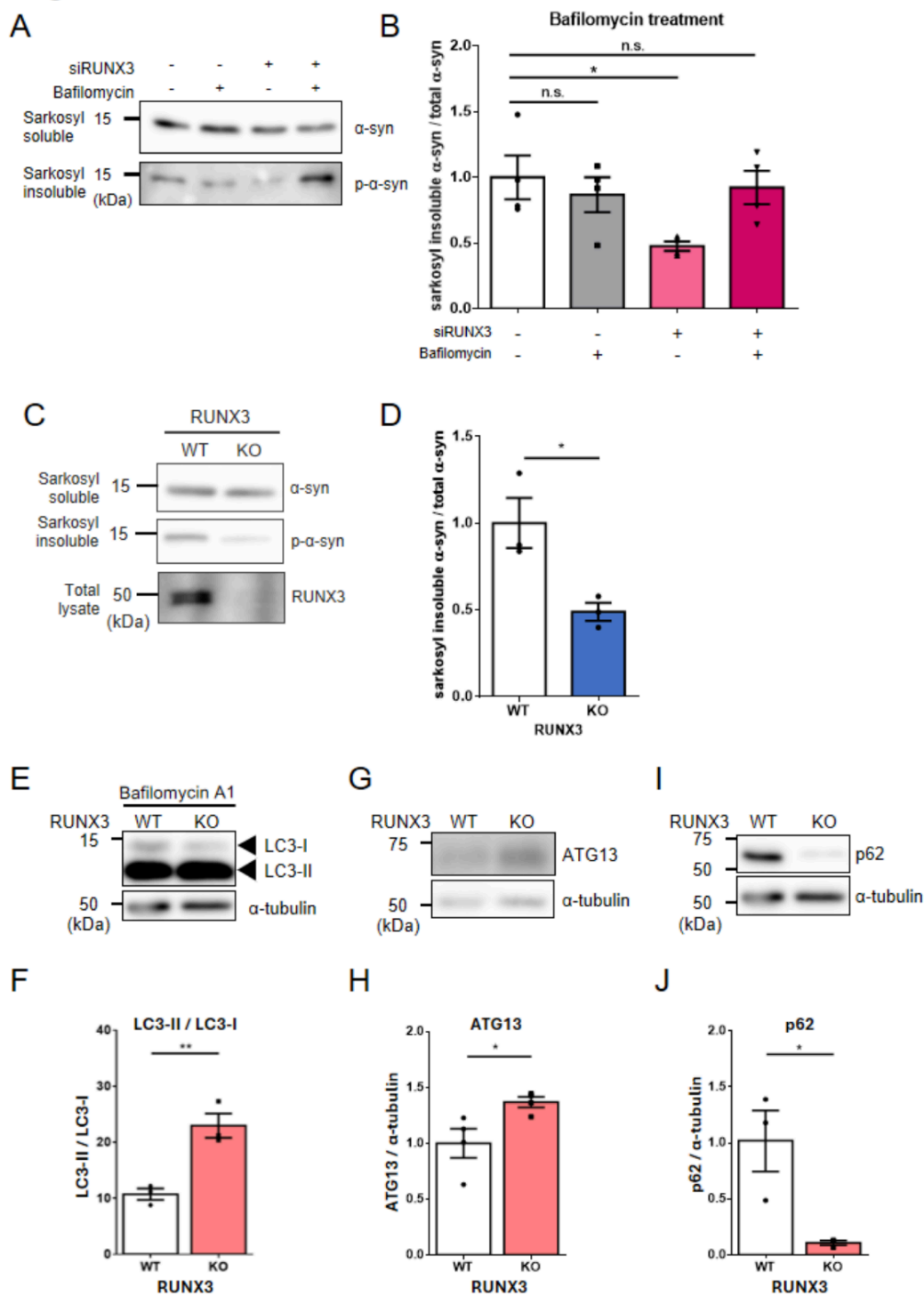


Figure 3

RUNX3 regulates  $\alpha$ -syn aggregation by altering autophagic activity.

(A) Representative immunoblotting of sarkosyl-soluble  $\alpha$ -syn and sarkosyl-insoluble p- $\alpha$ -syn within cells transfected with PFFs with and without bafilomycin A1 treatment after siRNA treatment.

- (B) Quantitative analysis of sarkosyl insoluble p- $\alpha$ -syn in (A) normalized by total  $\alpha$ -syn (n=4, mean  $\pm$  SEM, Dunnett's multiple comparison, \*: p<0.05, n.s.: no significance).
- (C) Representative immunoblotting of sarkosyl-soluble  $\alpha$ -syn, sarkosyl-insoluble p- $\alpha$ -syn and RUNX3 in the total lysate of Cas9/ $\alpha$ -syn HEK and RUNX3 KO Cas9/ $\alpha$ -syn HEK transfected with PFFs.
- (D) Quantitative analysis of sarkosyl insoluble p- $\alpha$ -syn in (C) normalized by total  $\alpha$ -syn (n=5, mean  $\pm$  SEM, Student's t-test, \*: p<0.05).
- (E) Representative immunoblotting of LC3 in Cas9/ $\alpha$ -syn HEK and RUNX3 KO Cas9/ $\alpha$ -syn HEK under bafilomycin A1 treatment.
- (F) Quantitative analysis LC3-II to LC3-I in (E) (n=3, mean  $\pm$  SEM, Student's t-test, \*\*: p<0.01).
- (G) Representative immunoblotting of ATG13 in Cas9/ $\alpha$ -syn HEK and RUNX3 KO Cas9/ $\alpha$ -syn HEK under bafilomycin A1 treatment.
- (H) Quantitative analysis of ATG13 expression in (G) normalized by  $\alpha$ -tubulin (n=4, mean  $\pm$  SEM, Student's t-test, \*: p<0.05).
- (I) Representative immunoblotting of p62 in Cas9/ $\alpha$ -syn HEK and RUNX3 KO Cas9/ $\alpha$ -syn HEK.
- (J) Quantitative analysis of p62 expression in (I) normalized by  $\alpha$ -tubulin (n=3, mean  $\pm$  SEM, Student's t-test, \*: p<0.05).

Figure 4

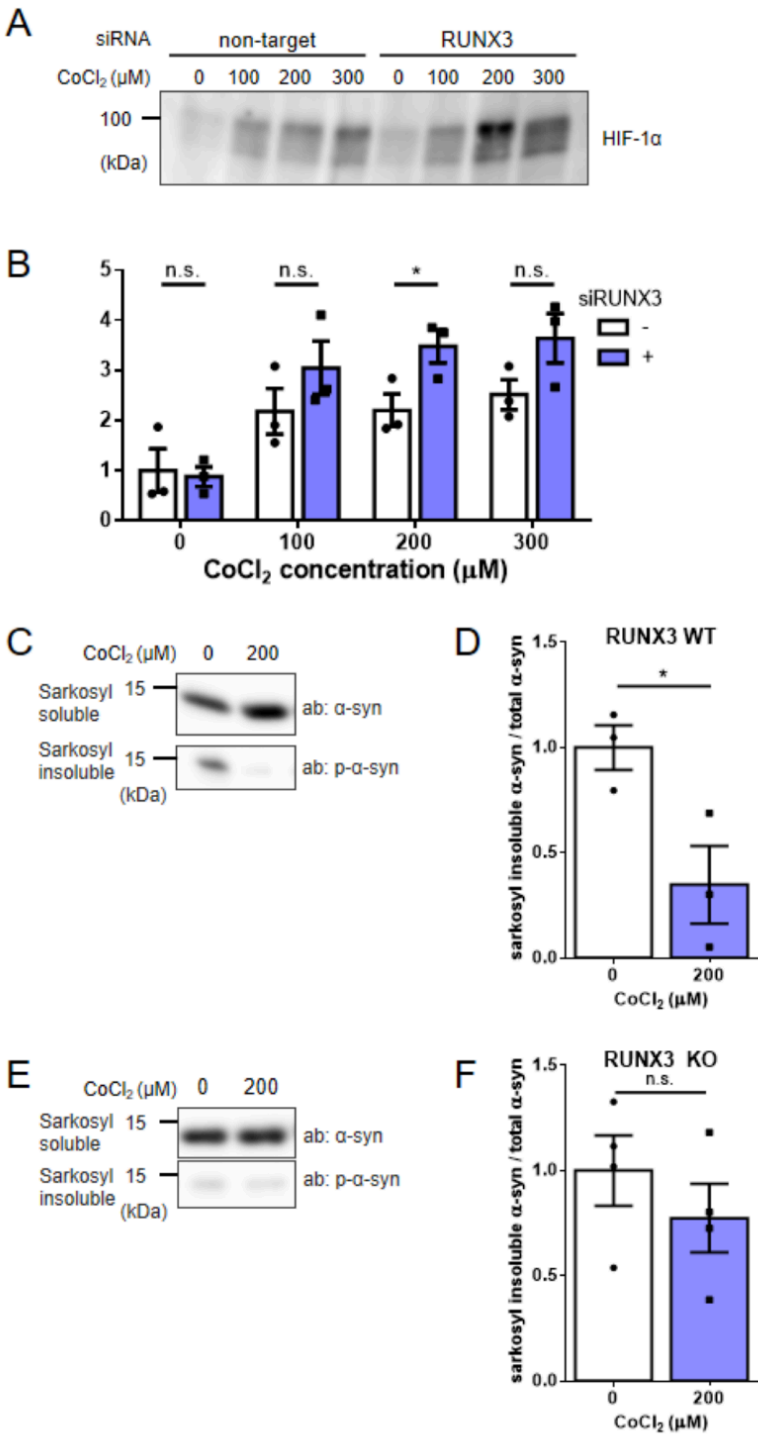


Figure 4

RUNX3 alters HIF-1α levels, resulting in autophagy dependent α-syn degradation.

(A) Representative immunoblotting of HIF-1α in cells treated with the indicated concentrations of CoCl<sub>2</sub>.

- (B) Quantitative analysis of HIF-1 $\alpha$  expression in (A) (n=3, mean  $\pm$  SEM, Student's t-test, \*: p<0.05, n.s.: no significance).
- (C) Representative immunoblotting of sarkosyl-soluble  $\alpha$ -syn and sarkosyl-insoluble p- $\alpha$ -syn within Cas9/ $\alpha$ -syn HEK treated with CoCl<sub>2</sub> after transfection with PFFs.
- (D) Quantitative analysis of sarkosyl insoluble p- $\alpha$ -syn in (C) normalized by total  $\alpha$ -syn (n=3, mean  $\pm$  SEM, Student's t-test, \*: p<0.05).
- (E) Representative immunoblotting of sarkosyl-soluble  $\alpha$ -syn and sarkosyl-insoluble p- $\alpha$ -syn within RUNX3 KO Cas9/ $\alpha$ -syn HEK treated with CoCl<sub>2</sub> after transfection with PFFs.
- (F) Quantitative analysis of sarkosyl insoluble p- $\alpha$ -syn in (E) normalized by total  $\alpha$ -syn (n=3, mean  $\pm$  SEM, Student's t-test, n.s.: no significance).

## Figure 5

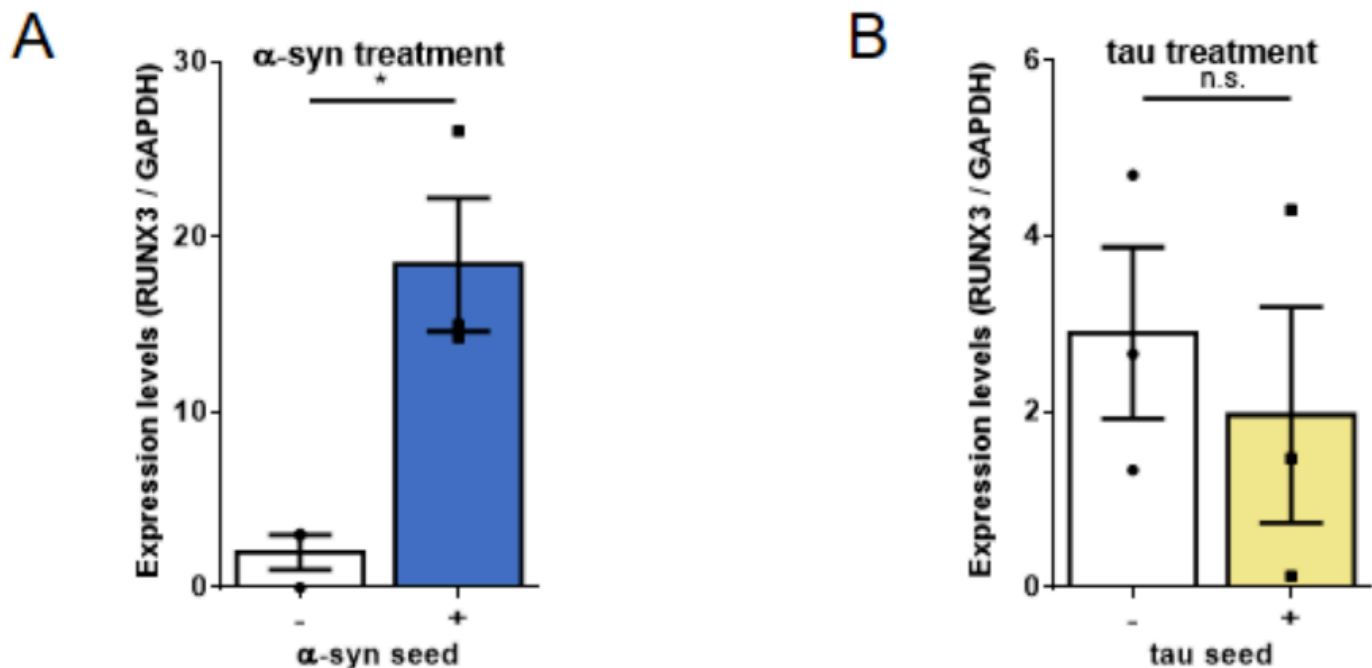


**Figure 5**

Treatment with  $\alpha$ -syn filaments alters RUNX3 expression.

(A) qRT-PCR analysis of *RUNX3* mRNA levels after  $\alpha$ -syn filaments treatment normalized by *GAPDH* (n=3, mean  $\pm$  SEM, Student's t-test, \*: p<0.05).

(B) qRT-PCR analysis of *RUNX3* mRNA levels after tau filaments treatment normalized by *GAPDH* (n=3, mean  $\pm$  SEM, Student's t-test, n.s.: no significance).

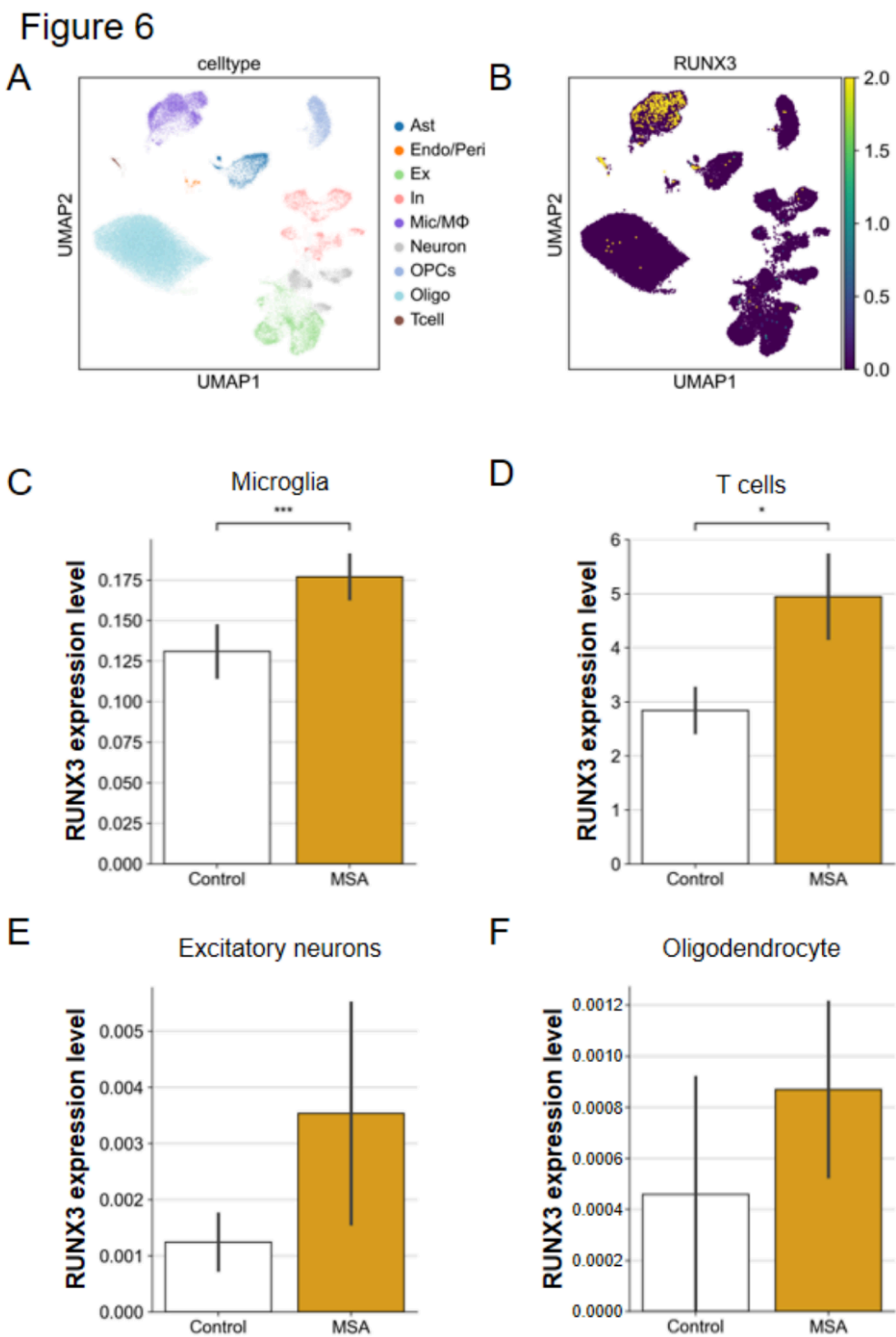


**Figure 6**

RUNX3 expression is altered in synucleinopathy patients.

(A) UMAP plot and cell type annotation of the single nucleus RNA sequencing results for brain samples of MSA patients and non-patient controls.

(B) Expression of *RUNX3* in cell clusters. *RUNX3* expression is high in the microglia cluster, but is also seen in other clusters including neuronal clusters.

(C) *RUNX3* expression in the microglia of MSA and control brains (expression levels per cell, mean  $\pm$  SEM, Mann-Whitney U test, \*\*\*:  $p < 0.001$ ).

(D) *RUNX3* expression in the T cells of MSA and control brains (expression levels per cell, mean  $\pm$  SEM, Mann-Whitney U test, \*:  $p < 0.05$ ).

(E) *RUNX3* expression in the excitatory neurons of MSA and control brains (expression levels per cell, mean  $\pm$  SEM, Mann-Whitney U test).

(F) *RUNX3* expression in oligodendrocytes of MSA and control brains (expression levels per cell, mean  $\pm$  SEM, Mann-Whitney U test).

Figure 7

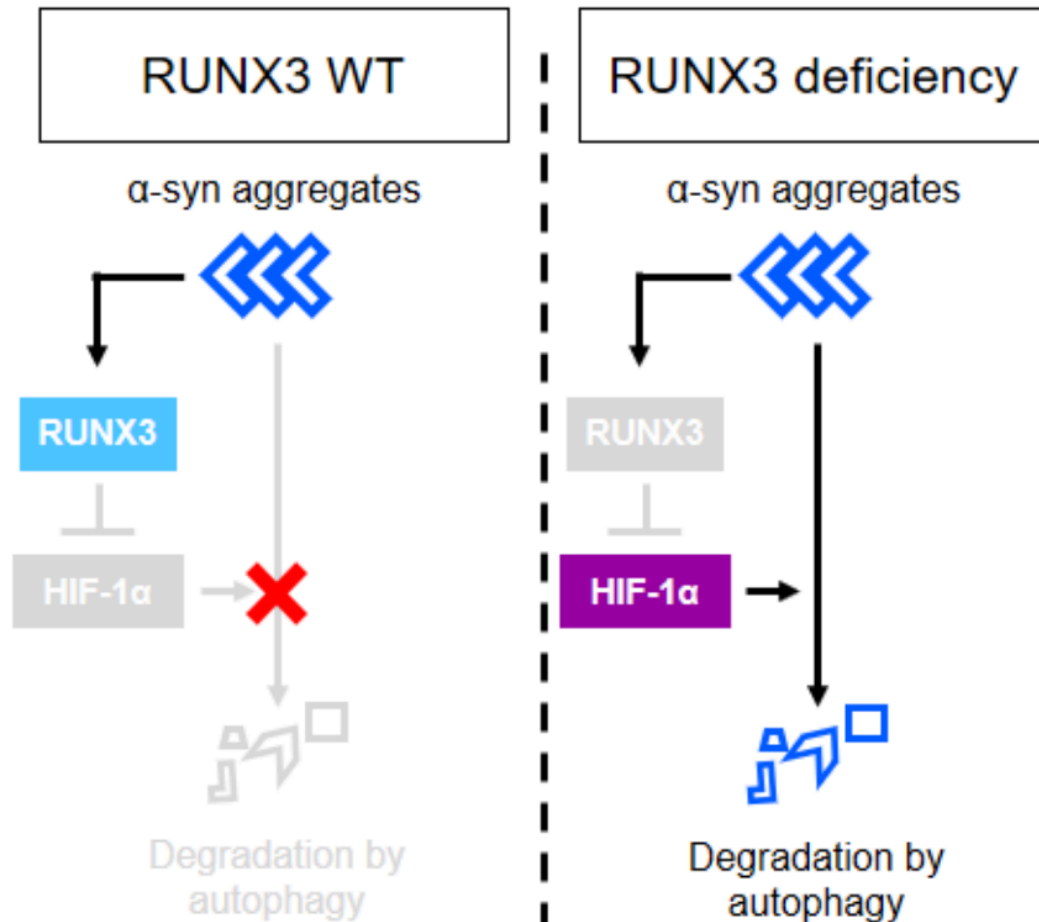


Figure 7

Schematic diagram of RUNX3 regulating  $\alpha$ -syn aggregation.

In normal cells,  $\alpha$ -syn aggregates induces the expression of RUNX3, which inhibits the degradation of  $\alpha$ -syn by autophagy. In RUNX3 deficient cells, the degradation of  $\alpha$ -syn, possibly mediated by HIF-1 $\alpha$ , is not interfered.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TomizawaetalRUNX3MNfinsupple.pdf](#)