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ORIGINAL ARTICLE

Loss of NgBR causes neuronal damage through decreasing KAT7-mediated RFX1 acetylation and FGF1 expression

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Abstract

Parkinson's disease (PD) is a common neurodegenerative movement disorder characterized by dopaminergic neuron loss in the substantia nigra pars compacta and striatal dopamine depletion. The *NUS1* gene, which encodes the neurite outgrowth inhibitor B receptor (NgBR), has been recently identified as a novel risk gene for PD. However, its roles and mechanism in neurodegeneration are still unclear. Here, we demonstrate that NgBR deficiency triggers neuronal damage through a novel KAT7/RFX1/FGF1 axis. RNA sequencing and experimental verification revealed that NgBR depletion downregulates expression and secretion of fibroblast growth factor 1 (FGF1), which led to inactivation of the PI3K/AKT signaling pathway. Mechanistically, NgBR deletion suppresses lysine acetyltransferase 7 (KAT7) expression, impairing KAT7-mediated acetylation of regulatory factor X1 (RFX1), a transcriptional repressor for *FGF1*. This stabilized RFX1 by blocking its proteasomal degradation, thereby suppressing *FGF1* transcription. Crucially, exogenous FGF1 rescued AKT signaling and mitigated neuronal damage in NgBR-deficient models. Our findings establish NgBR-KAT7-RFX1 as a regulatory axis controlling FGF1-dependent neuroprotection, which promotes the understanding of PD pathogenesis and highlights FGF1 supplementation as a potential therapeutic strategy.

Keywords Parkinson's disease · NUS1/NgBR · Neuronal damage · FGF1 · RFX1 · Acetylation

Introduction

Parkinson's disease (PD) is a neurodegenerative disease second only to Alzheimer's disease (AD), and its prevalence increases progressively with age. Epidemiological studies have shown that the fastest growing incidence among neurological disorders is PD, which outpaces AD [1]. The symptoms of PD are mainly motor disorders, including resting tremor, rigidity, bradykinesia, and postural balance disorders [2], which are caused mainly by the selective loss of dopaminergic neurons in the substantia nigra pars compacta and the resulting reduction in dopamine release in the striatum [3, 4]. Currently, the pathogenesis of PD has not been fully elucidated, and clinical strategies for PD treatment

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involve the use of drugs such as dopamine agonists or the dopamine precursor levodopa, which cannot prevent the progressive loss of dopaminergic neurons and are accompanied by severe adverse effects [5]. Therefore, it is important to explore the pathogenesis and therapeutic interventions of PD.

Approximately 15% of PD patients have a family history of the disease, whereas 5–10% have a single-gene form of Mendelian inheritance. To date, more than 20 PD-causative genes have been identified. Moreover, more genetic risk genes and variants of sporadic PD phenotypes have been identified in various association studies [6, 7]. The genetic variations related to PD provide much evidence for understanding the pathogenesis of PD. Recently, the *NUS1* gene, which encodes the neurite outgrowth inhibitor-B receptor (NgBR), was identified as a novel risk gene for PD [8–10]. An in vivo study of Drosophila or zebrafish models revealed that *nus1* deficiency leads to movement phenotypes, a decrease in the number of dopaminergic neurons, and other PD-like phenotypes [8, 11, 12]. In addition to PD, genetic evidence also shows that mutations in the *NUS1* gene are closely related to familial epilepsy, tremor and ataxia [13–15]. These data suggest that NgBR plays important functional roles in the central nervous system.

NgBR is localized mainly to the plasma membrane and endoplasmic reticulum (ER), and its biological functions are limited to the periphery and are related mainly to the promotion of vascular remodeling and formation, the regulation of cholesterol metabolism, protein N-glycosylation, etc [14]. However, the functions of NgBR in neurons, especially in dopaminergic neurons, and how its deficiency contributes to neuronal damage and PD pathology are largely unknown.

Here, we identified FGF1 as a key downstream effector of NgBR. NgBR deficiency inhibits fibroblast growth factor 1 (*FGF1*) transcription, leading to PI3K/AKT inactivation and neuronal death. Loss of NgBR inhibits lysine acetyltransferase 7 (KAT7) expression and KAT7-mediated acetylation of regulatory factor X1 (RFX1), thus enhancing the stability of RFX1, a transcription repressor of *FGF1*. Strikingly, FGF1 administration reversed neuronal damage induced by NgBR deficiency.

Materials and methods

Experimental animals

Nus1$^{flox/flox}$ (C57BL/6JGpt-*Nus1*^{em1Cflox}/Gpt, Strain NO. T010909) were purchased from GemPharmatech Company (Nanjing, China). Specific pathogen-free (SPF) C57BL/6J pregnant mice were purchased from SLAC ANIMAL (Shanghai, China). All animals were raised under a 12-hour light/dark cycle with free access to food and water. All animal experiments performed in this study were in accordance with the institutional guidelines for the use and care of animals, and all procedures were approved by the Institute Animal Welfare Committee of Soochow University.

Cell culture and drug treatment

MES23.5 dopaminergic cells, murine neuroblastoma Neuro-2a (N2a) cells and human embryonic kidney 293 (HEK293) cells were grown in Dulbecco's modified Dulbecco's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, New York, NY, USA), streptomycin (100 μg/mL) and penicillin (100 μg/mL) (Gibco). Human neuroblastoma SH-SY5Y cells were cultured in DMEM/F12 medium supplemented with 10% FBS, penicillin (100 μg/mL) and streptomycin (100 μg/mL). Primary cultured astrocytes and microglia were isolated from 3-day-old C57BL/6J mice, and primary cortical neurons were isolated from the cortex of *Nus1*$^{flox/flox}$ mouse embryos on embryonic day 17. The methods and procedures for obtaining and culturing primary cultured cells were described previously [16, 17].

Reagents

FGF1 (HZ-1327) was purchased from Proteintech (Rosemont, USA). Heparin was a kind gift from Dr. Zhenqing Zhang at Soochow University. MG132 (S1748) was purchased from Beyotime Biotechnology (Shanghai, China). NH_4Cl (326372) and 1-methyl-4-phenylpyridinium ion (MPP^+, D048) were purchased from Sigma (St. Louis, MO, USA). WM-3835 (S9805) was purchased from Selleck Technology (Houston, TX, USA). Nicotinamide (HY-B0150) and trichostatin A (HY-15144) were purchased from MedChem Express. Except for FGF1 and heparin, which were dissolved in PBS, all the reagents were dissolved in dimethyl sulfoxide (DMSO). A Cre recombinase-expressing lentiviral vector (LV-Cre) and a control lentiviral vector (LV-Ctrl) were purchased from Brain VTA (Wuhan, China).

Plasmid transfection

pcDNA3.1-3xFLAG-NgBR, pcDNA3.1-3xFLAG-RFX1, pcDNA3.1-3xFLAG-RFX2, pcDNA3.1-3xFLAG-RFX3 and RFX1-pcDNA3.1-EGFP-N were purchased from YouBio (Changsha, China). For plasmid transfection, cells were transfected with plasmids via Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA interference

Small interfering RNAs (siRNAs) against the human *NUS1*, *RFX1*, *KAT7* and *STUB1* genes, as well as the mouse *Nus1* gene, were synthesized with the sequences shown in Table 1 in the supplemental information. The cells were transfected with siRNAs via Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions.

Cell viability assay

Cell viability was measured with a Cell Counting Kit-8 (CCK8) (APExBIO Technology LLC, Houston, USA) according to the manufacturer's instructions. Briefly, SH-SY5Y cells were treated with two human-derived siRNAs to silence *NUS1* expression for 48 h or silence *NUS1* expression for 36 h, followed by treatment with FGF1 (100 ng/mL)/heparin (10 μg/mL) or PBS for 12 h. Then, the cells were incubated with 10% CCK8 reagent for 2 h at 37 °C. Cell viability was determined by measuring the absorbance at 450 nm with an enzyme marker.

Cell cytotoxicity assay

The cytotoxicity of lactate dehydrogenase (LDH) release was detected via a CytoTox 96 nonradioactive cytotoxicity assay (Promega, Madison, WI, USA). Briefly, SH-SY5Y cells were transfected with two human-derived siRNAs to silence *NUS1* expression for 48 h or to silence *NUS1* expression for 36 h, followed by FGF1 (100 ng/mL)/heparin (10 μg/mL) treatment or PBS for 12 h. Then, 50 μL of cell supernatant was collected, mixed with 50 μL of CellTiter-Glo and shaken for 20 min at room temperature. Cytotoxicity was determined by measuring the absorbance at 490 nm with an enzyme meter.

ELISA

SH-SY5Y cells were inoculated into 24-well plates and treated with siRNAs against *NUS1* for 24 h. Then, the cells were cultured with serum-free medium for 24 h, and 100 μL of the FGF1 content in the culture medium or in cell lysate was measured with an ELISA kit (EK0339, BOSTER, Wuhan, China).

Quantitative real-time PCR

The procedures for RNA extraction and reverse transcription have been described elsewhere [18]. Using a 7500 real–time PCR system (Applied Biosystems), qRT–PCR analysis was performed to measure target RNA abundance quantitatively via Power SYBR Green PCR Master Mix (Vazyme Biotech, Nanjing, China). The sequences of the human primers used for real-time PCR are shown in Table 2 in the supplemental information. The relative mRNA levels of these genes to those of *β-actin* were calculated via the $2^{-\Delta\Delta CT}$ method.

Transcriptome sequencing

First, total RNA was extracted from three technical repeats via TRIzol reagent (Invitrogen). Quality control and library construction were performed by Huada Gene Technology Company, and then transcriptome sequencing was performed via the BGISEQ platform. The raw data were filtered to remove reads of low quality, reads with adaptor sequences and reads with high levels of N bases. The clean reads were aligned to the reference genome via HISAT software. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment was performed, differentially expressed genes (DEGs) were identified, and expression analysis was conducted on the Dr. Tom analysis system (https://biosys.bgi.com).

Immunoblot analysis and antibodies

After the cells were treated with drugs, siRNAs or lentiviruses, they were lysed in cell lysis buffer (0.5% deoxycholate, 1% NP-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and a protease inhibitor mixture (Roche)). The samples were separated by 8% or 12% SDS–PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were incubated overnight at 4 °C with the primary antibodies shown in Table 3 in the supplemental information. The following secondary antibodies were used: horseradish peroxidase-conjugated sheep anti-mouse and anti-rabbit antibodies (Amersham Pharmacia Biotech, Piscataway, NJ, Sweden). The proteins were visualized with an enhanced chemiluminescence (ECL) detection kit (Thermo Fisher, Waltham, MA, USA) via a chemiluminescence imaging system (Bioshine ChemiQ 4800, Shanghai, China).

PI staining assay

After SH-SY5Y cells were treated with siRNAs against *NUS1* and FGF1/heparin, the cells were incubated with Hoechst 33,342 (Sigma–Aldrich, St. Louis, MO, USA) and PI (Sigma–Aldrich, St. Louis, MO, USA) for 5 min. The cells were then observed with an inverted IX71 microscope system (Olympus, Tokyo, Japan).

Immunofluorescence staining

HEK293 cells and primary neurons were fixed with 4% paraformaldehyde (PFA) for 10 min and permeabilized with 0.1% Triton X-100 for 10 min. After three washes with PBS, the cells were blocked with 1% FBS in PBS for 1 h. Next, the cells were incubated with anti-KAT7 (sc-9996, Santa Cruz) or anti-cleaved-caspase 3 (9661 S, Cell Signaling Technology) and anti-MAP2 (MAB3418, Millipore) antibodies together. Subsequently, the cells were stained with 40,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich) for 5 min and then washed three times with PBS. Finally, the cells were observed with a confocal microscope system (Nikon, Tokyo, Japan).

Immunoprecipitation assay

The cells were lysed in cell lysis buffer at 4 °C, sonicated and centrifuged at high speed to collect the supernatant. The supernatant was incubated with protein G agarose (Thermo Fisher Scientific) as well as anti-Flag (A5882, Sigma–Aldrich) overnight at 4 °C. Protein complexes coupled to protein G agarose were washed three times with lysis buffer and analyzed by immunoblotting.

Flow cytometry assay

After the cells were treated with drugs and siRNAs, the cell precipitate was collected. After the cells were resuspended by adding 195 µL of Annexin V-FITC conjugate, 5 µL of Annexin V-FITC and 10 µL of propidium iodide staining solution were added, mixed well, incubated for 10–20 min at room temperature in the dark, and then detected via a flow cytometer (Cytomics FC 500, Beckman).

Statistical analysis

Quantitative analysis of the immunoblots was performed via Photoshop 7.0 (Adobe, USA). GraphPad Prism 8.0 (GraphPad Software, USA) was used for statistical analysis and plotting. Significant differences were assessed via unpaired t tests or one-way or two-way analysis of variance (ANOVA) for multiple comparisons. The significance criterion was set at $P<0.05$. *$P<0.05$, **$P<0.01$, *** $P<0.001$, ns, not statistically significant. These values are shown as the means ± SDs.

Results

Loss of NgBR leads to severe neuronal damage

To investigate the role of NgBR in neuronal cells, we first transfected two murine-derived siRNAs to silence *Nus1* expression in N2a and MES23.5 cells or two human-derived siRNAs to silence *NUS1* expression in SH-SY5Y cells and then assayed the effects on cell viability and damage. We found that NgBR deficiency significantly reduced cell viability and induced damage in all types of cells (Fig. 1A and F). In addition, a significant increase in activated cleaved-caspase3 was detected in NgBR-deficient cells (Fig. 1G and H). Next, we examined whether NgBR deletion has similar effects on primary neurons. We transfected primary cortical neurons derived from *Nus1*$^{flox/flox}$ fetal mice with lentiviral LV-Cre for 6 days to delete *Nus1* expression. We also found that primary cortical neurons deficient in *Nus1* presented significantly greater levels of cleaved caspase3 protein than did control neurons (Fig. 1I and J). In addition, a significant decrease in the number and branches of primary cortical neurons was found in the *Nus1* deletion group compared with those in the control groups (Fig. 1K and M). As *NUS1* is a risk gene for PD, we then investigated whether the well-known PD-inducing toxins such as MPP^+ affects its expression and found that MPP^+ treatment for 12 and 24 h resulted in a significant decrease in NgBR protein level in SH-SY5Y cells (Fig. S1A). Knockdown of NgBR significantly aggravated MPP^+-induced neuronal damage and decreased cell viability, while NgBR overexpression mitigated MPP^+-induced cytotoxicity (Fig. S1B). These results indicate that loss of NgBR results in severe damage to both cultured neuronal cells and primary neurons, and its deletion makes neuronal cells more sensitive to neurotoxicity induced by neurotoxin.

NgBR deficiency induces inactivation of the PI3K-AKT pathway

To investigate the mechanism of neuronal injury induced by NgBR deletion, we screened for differentially expressed genes (DEGs) via transcriptome sequencing after the transfection of two murine-derived siRNAs to knock down NgBR expression in N2a cells and analyzed the data. With a fold change > 2 and $P<0.001$ as the screening criterion, the expression of 45 genes significantly and synchronously changed in cells in which two different siRNAs were used to silence NgBR (Fig. 2A). The details of these 45 genes were listed in Supplementary File 2. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the 45 genes revealed high enrichment of the PI3K-AKT pathway (Fig. 2B). Considering that NgBR deletion significantly affects neuronal survival, and PI3K-AKT pathway is pivotal for neuronal survival, we focused on whether NgBR deletion affects the activity of the PI3K-AKT pathway to mediate neuronal damage. We detected AKT phosphorylation and found that the levels of phosphorylated AKT were significantly reduced by NgBR deficiency in SH-SY5Y

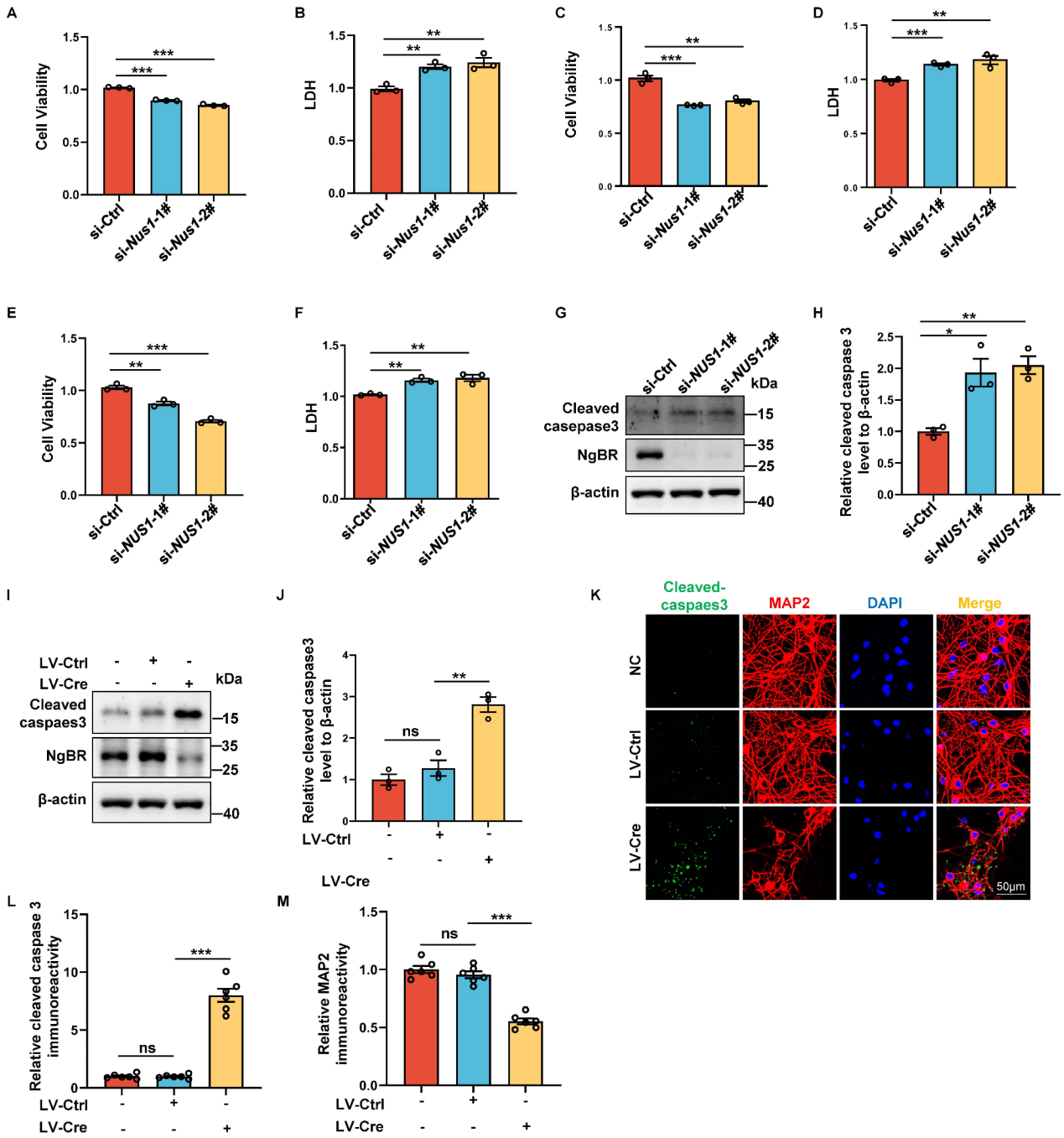


Fig. 1 Loss of NgBR leads to severe neuronal damage. **A-F** N2a (**A**, **B**), MES23.5 (C, D) or SH-SY5Y (E, F) cells were transfected with the indicated siRNAs for 48 h. Then, the cell viability was determined via the CCK8 assay, and cytotoxicity was detected via the LDH assay, $n=3$, ** $P<0.01$, *** $P<0.001$. **G** SH-SY5Y cells were transfected with the indicated siRNAs for 48 h. Then, the cell lysates were subjected to immunoblot analysis. **H** The relative protein level of cleaved caspase3 to that of β-actin in (**G**) was analyzed, $n=3$, * $P<0.05$, ** $P<0.01$. **I-M** Primary cortical neurons derived from *Nus1*$^{flox/flox}$ fetal mice were infected with LV-Ctrl or LV-Cre and cultured for 6 days. Then, the cell lysates were subjected to immunoblot analysis (**I**), or the cells were subjected to immunofluorescence (**K**). The relative protein level of cleaved caspase3 to that of β-actin in (**I**) was analyzed as shown in J; $n=3$; ns, not statistically significant; ** $P<0.01$. Quantitative analysis of the fluorescence intensity of cleaved-caspase3 and MAP2 in (**K**) is shown in L and M, respectively; scale bar, 50 μm. $n=6$, ns, not statistically significant, *** $P<0.001$

Figure 2

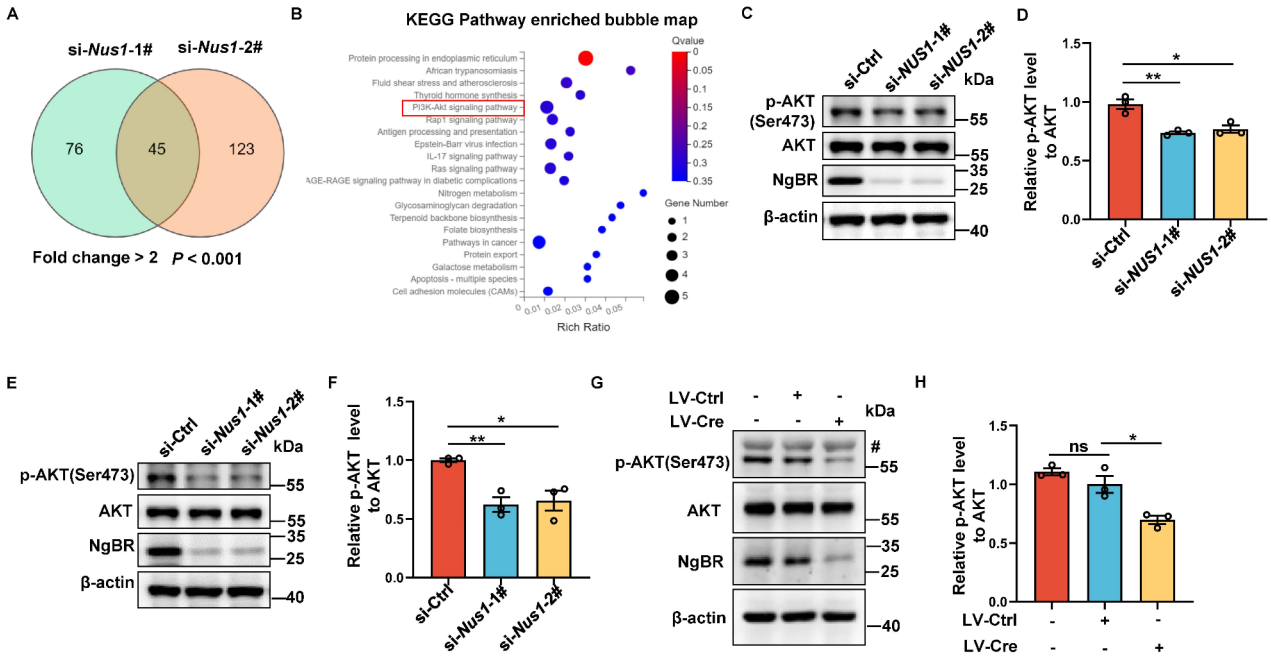


Fig. 2 NgBR deficiency induces inactivation of the PI3K–AKT pathway. **A** N2a cells were transfected with two kinds of targeted siRNAs for 48 h, after which three duplicate samples were subjected to transcriptome sequencing. The Venn diagram represents the number of DEGs identified in each group and both groups (fold change > 2, $P < 0.001$). **B** KEGG pathway enrichment bubble map of the 45 common differentially expressed genes in (**A**). **C-F** SH-SY5Y and N2a cells were transfected with the indicated siRNAs for 48 h. Then, the cell lysates were subjected to immunoblot analysis. The relative protein levels of p-AKT and AKT in (**C**) and (**E**) are shown in D and F, respectively. $n = 3$, * $P < 0.05$, ** $P < 0.01$. **G** *Nus1*$^{flox/flox}$ primary cortical neurons were infected with LV-Ctrl or LV-Cre and cultured for 6 days. Then, the cell lysates were subjected to immunoblot analysis. **H** The protein level of p-AKT relative to that of AKT was analyzed. $n = 3$, ns, no statistical significance, * $P < 0.05$, # indicates the nonspecific band

cells, MES23.5 cells and primary cortical neurons (Fig. 2C and H). These results suggest that inactivation of the PI3K–AKT pathway may be involved in the process of neuronal damage caused by NgBR deficiency.

NgBR deficiency results in the downregulation of FGF1 expression and secretion

We found that four genes whose expression significantly changed were related to the PI3K-AKT pathway via expression clustering heatmap analysis, including *Hsp90b1*, *Lama4*, *Ngfr*, and *Fgf1*, in which *Hsp90b1* and *Lama4* were upregulated, whereas *Ngfr* and *Fgf1* were downregulated (Fig. 3A). Heat shock protein 90 beta family member 1 (HSP90B1) and laminin subunit alpha 4 (LAMA4) positively regulate the activity of the PI3K-AKT pathway [19, 20], and NGFR has both proapoptotic and antiapoptotic effects in association with different ligands [21]. In addition, FGF1 is highly expressed in the brain, including dopaminergic neurons, and plays an important role in neuronal survival [22, 23]; therefore, we next focused on whether NgBR affects PI3K-AKT activity by regulating FGF1 expression. We first validated that *FGF1* expression was downregulated by NgBR knockdown through qRT–PCR in SH-SY5Y cells (Fig. 3B). We also confirmed that the protein level of FGF1 was significantly decreased after targeted knockdown of NgBR expression (Fig. 3C and D). Conversely, overexpression of Flag-NgBR in SH-SY5Y cells significantly increased FGF1 protein levels (Fig. 3E and F). In addition, knockdown or knockout of NgBR markedly reduced the FGF1 protein level in MES23.5 cells, N2a cells and primary cortical neurons (Fig. 3G and L). To investigate whether FGF1 expression is affected by NgBR deletion in two other important cell types in the brain, microglia and astrocytes, we first detected NgBR expression abundance in primary microglia, neurons and astrocytes and found that NgBR expression is high in primary neurons and microglia, but low in astrocytes (Fig. S2A). We then knocked down NgBR in the microglia cell line BV2 and primary astrocytes and found that the FGF1 protein was barely detected in BV2 microlial cells (Fig. S2B), whereas NgBR deletion did not significantly affect FGF1 expression in astrocytes (Fig. S2C). These results suggest that NgBR deletion reduces FGF1 expression in a neuron-specific manner. As FGF1 is a secretory growth factor, we then measured the intracellular content of FGF1 and its secretion into the extracellular

Figure 3

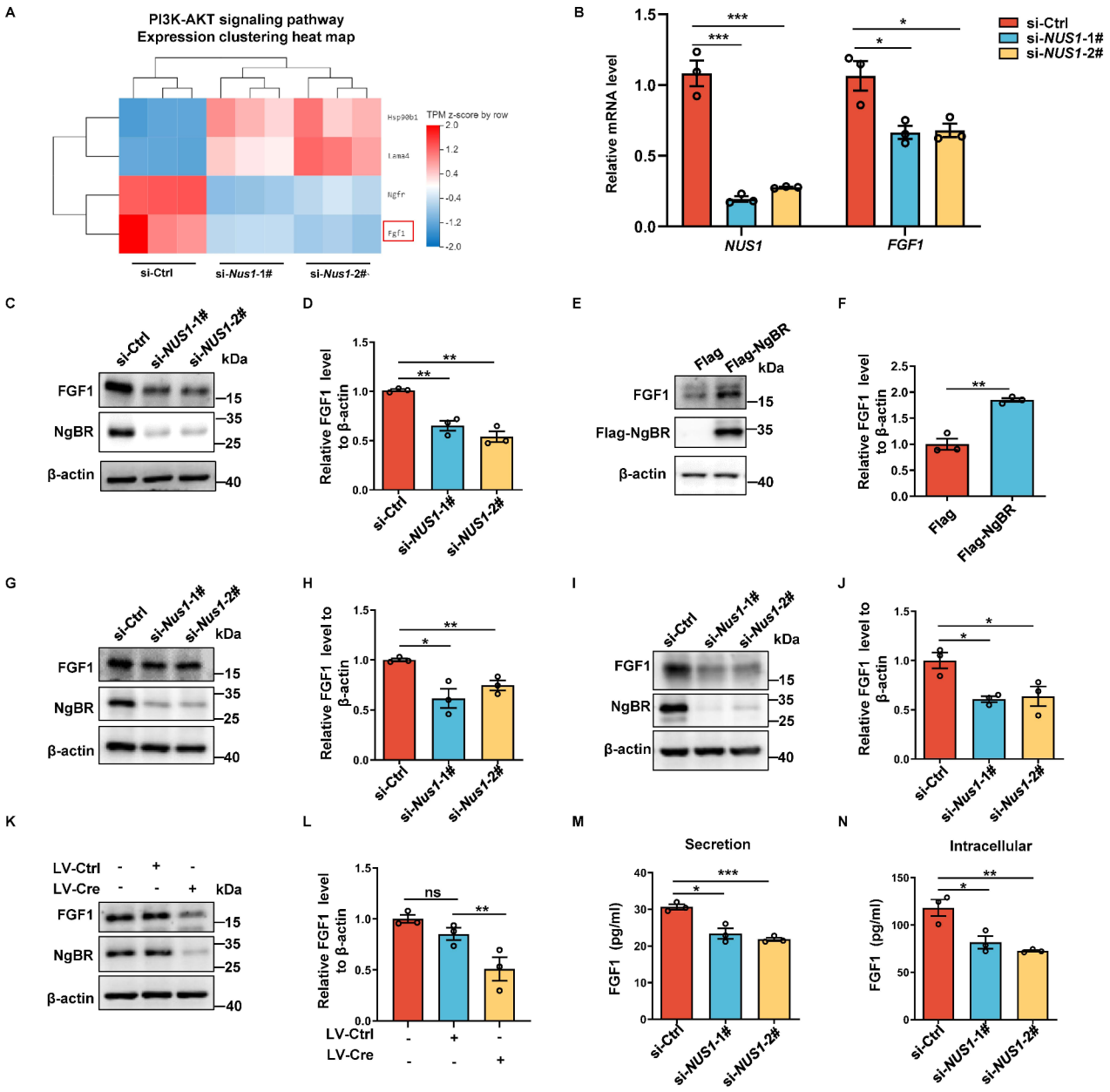


Fig. 3 NgBR deficiency leads to the downregulation of FGF1 expression and secretion. **A** Expression clustering heatmap analysis of differentially expressed genes related to the PI3K-AKT pathway shown in Fig. 2B. **B–D** SH-SY5Y cells were transfected with the indicated siRNAs for 48 h. Then, the samples were subjected to qRT–PCR (**B**) or immunoblotting (**C**). The relative protein level of FGF1 to that of β-actin in (**C**) was analyzed as shown in D. $n=3$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$. **E** SH-SY5Y cells were transfected with Flag or Flag-NgBR for 48 h, after which the cell lysates were subjected to immunoblot analysis. **F** The protein levels of FGF1 relative to those of β-actin in (**E**) were analyzed. $n=3$, ** $P<0.01$. **G** N2a cells were transfected with the indicated siRNAs for 48 h. Then, the cell lysates were subjected to immunoblot analysis. **H** The protein level of FGF1 relative to that of β-actin in (**G**) was analyzed. $n=3$, * $P<0.05$, ** $P<0.01$. **I** MES23.5 cells were transfected with the indicated siRNAs for 48 h. Then, the cell lysates were subjected to immunoblot analysis. **J** The protein level of FGF1 relative to that of β-actin in (**I**) was analyzed. $n=3$, * $P<0.05$. **K** *Nus1*$^{flox/flox}$ primary cortical neurons were infected with LV-Ctrl or LV-Cre and cultured for 6 days. Then, the cell lysates were subjected to immunoblot analysis. **L** The protein level of FGF1 relative to that of β-actin in (**K**) was analyzed. $n=3$, ** $P<0.01$. **M-N** SH-SY5Y cells were transfected with the indicated siRNAs for 24 h. Then, the cells were cultured with serum-free medium for 24 h, and the FGF1 content in the culture medium (**M**) and cell lysate (**N**) was detected via an ELISA kit. $n=3$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$

compartment after NgBR deletion. We found that both the secretion of FGF1 outside cells and the protein content of FGF1 in cells were significantly reduced after the deletion of NgBR (Fig. 3M and N). These results demonstrated that loss of NgBR downregulates *FGF1* transcription in neurons and leads to decreases in both the intracellular FGF1 content and its secretion.

Supplementation with exogenous FGF1 alleviates the neuronal damage caused by NgBR deletion

FGF1 regulates multiple cellular functions, including migration, proliferation, differentiation, and survival, by binding to FGF receptors (FGFRs) and triggering the activation of downstream signaling pathways, such as the PI3K-AKT pathway, which can be enhanced by either heparin acetylsulfate ((heparan sulfate (HS)) or heparan sulfate proteoglycans (HSPGs) [24]. Next, we investigated whether exogenous FGF1 supplementation can ameliorate or reverse the inactivation of PI3K-AKT signaling and neuronal damage caused by NgBR deficiency. Consistent with previous studies, we also found that FGF1 treatment significantly increased AKT activity and that combination with heparin further enhanced FGF1-mediated AKT activation (Fig. S3A and S3B). Furthermore, the administration of FGF1/heparin completely reversed the inactivation of AKT induced by NgBR deficiency (Fig. 4A and B). Exogenous FGF1/heparin treatment significantly reversed the decrease in cell viability caused by NgBR deletion and inhibited the death of SH-SY5Y cells (Fig. 4C and F). We also examined apoptosis via Annexin V/PI staining via flow cytometry and found that exogenous FGF1/heparin significantly inhibited the apoptosis induced by NgBR deletion (Fig. S4A and S4B). Similarly, exogenous FGF1 supplementation significantly suppressed the increase in cleaved caspase-3 protein levels caused by NgBR deletion in primary cortical neurons (Fig. 4I and J). Moreover, we detected the morphology of primary neurons characterized by MAP2 expression and apoptosis characterized by activated caspase-3 by immunofluorescence and found that exogenous FGF1 significantly improved the morphological defects and alleviated neuronal apoptosis caused by NgBR deletion (Fig. 4K-M). These results demonstrate that supplementation with exogenous FGF1 can completely restore AKT activity and significantly inhibit neuronal cell death caused by NgBR deletion.

NgBR regulates FGF1 expression through RFX1

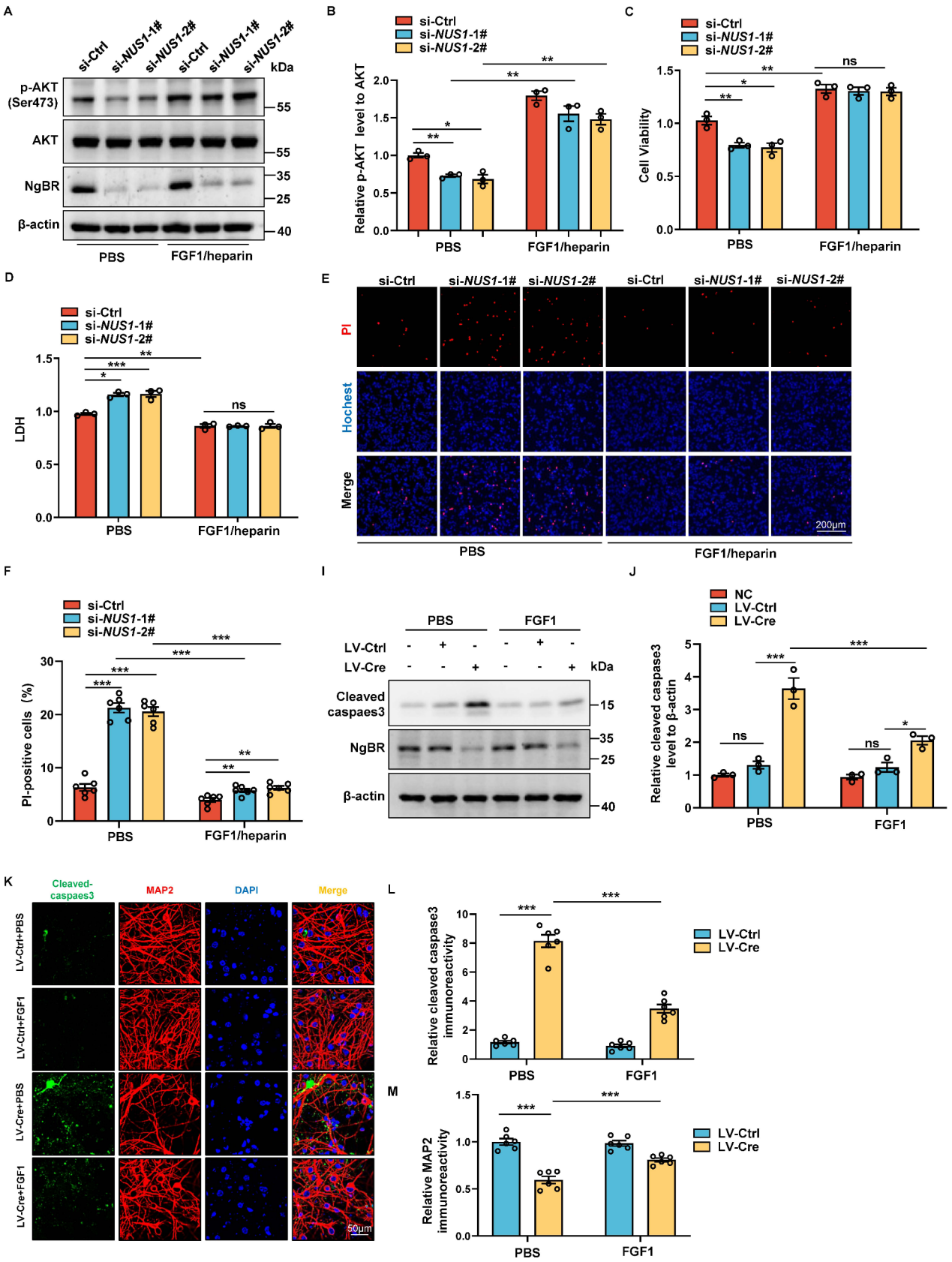
We next investigated how NgBR regulates *FGF1* expression at the transcriptional level. The promoter of the

Fig. 4 Exogenous FGF1 alleviates the neuronal damage caused by NgBR deletion. **A** SH-SY5Y cells were transfected with the indicated siRNAs for 36 h and then treated with PBS or FGF1 (100 ng/mL)+heparin (10 μg/mL) for 12 h. Then, the cell lysates were analyzed by immunoblotting with the indicated antibodies. **B** The protein level of p-AKT relative to that of AKT in (**A**) was analyzed. $n=3$, * $P<0.05$, ** $P<0.01$. **C-D** SH-SY5Y cells were transfected with the indicated siRNAs for 36 h and then treated with PBS or FGF1 (100 ng/mL)/heparin (10 μg/mL) for 12 h. Then, the cell viability was determined via the CCK8 assay (**C**), and the cytotoxicity was detected via the LDH assay (**D**), $n=3$; ns, no statistical significance; * $P<0.05$; ** $P<0.01$; *** $P<0.001$. **E** SH-SY5Y cells were treated in the same way as those in C. Then, the cells were stained with PI and Hoechst. Representative images of PI staining are shown. **F** PI-positive cells were counted and quantified by Hoechst-labeled cells. $n=6$, *** $P<0.001$. **I***Nus1*$^{flox/flox}$ primary cortical neurons were infected with LV-Ctrl or LV-Cre and cultured for 3 days. Then, the neurons were treated with PBS or 100 ng/mL FGF1 for 3 days. Then, the cell lysates were subjected to immunoblot analysis. **J** The protein levels of cleaved caspase3 relative to those of β-actin in (**I**) were analyzed. $n=3$, ns, not statistically significant, * $P<0.05$, *** $P<0.001$. **K–M***Nus1*$^{flox/flox}$ primary cortical neurons were treated in the same way as those in (**I**), after which the cells were subjected to immunofluorescence (**K**). Quantitative analysis of the fluorescence intensity of cleaved caspase3 (**L**) and MAP2 (**M**) is shown. Scale bar, 50 μm. $n=6$, *** $P<0.001$

FGF1 gene has four alternative tissue-specific promoters named A to D, and the brain-specific promoter is *FGF1B* [25]. The regulatory factor of X-box gene family (RFX) has been reported to be closely related to the transcriptional regulation of *FGF1B*; RFX1 is the transcriptional repressor of *FGF1B*, and RFX2 and RFX3 are its transcription factors [26, 27]. Therefore, we first measured whether NgBR affects the transcription level of RFX family proteins. We found that knockdown of NgBR had no significant effect on the mRNA levels of *RFX1*, *RFX2* or *RFX3* (Fig. S5A) but specifically increased the protein levels of both endogenous RFX1 and exogenous Flag-RFX1 without affecting Flag-RFX2 or Flag-RFX3 protein levels (Fig. 5A and D, Fig. S5B and S5C). RFX1 protein levels were also significantly increased in primary cortical neurons in which NgBR was deleted (Fig. 5E and F). These results indicate that NgBR regulates FGF1 transcription by affecting RFX1 stability rather than transcription. Ubiquitination is a process of covalently binding ubiquitin (Ub) to target proteins by E3 ubiquitin ligase, thus regulating their stability and degradation [28]. And RFX1 has been reported that is a protein that can be ubiquitinated and degraded [29]. So, we tested whether NgBR deficiency has an effect on RFX1 ubiquitination. As shown in Fig.S6, knockdown of NgBR significantly decreased the modification of RFX1 ubiquitination, suggesting that NgBR regulates RFX1 degradation process mediated by its ubiquitination.

Next, we verified whether RFX1 also regulates *FGF1* expression in neuronal cells and found that RFX1 overexpression significantly reduced FGF1 protein levels (Fig. 5G

Figure 4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FGF1/heparin
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Cell Viability
D
LDH
E
PI
Hochest
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F
PI-positive cells (%)
I
PBS
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LV-Ctrl
LV-Cre
Cleaved caspaes3
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Relative cleaved caspase3 level to β-actin
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Cleaved-caspaes3
MAP2
DAPI
Merge
LV-Ctrl+PBS
LV-Ctrl+FGF1
LV-Cre+PBS
LV-Cre+FGF1
50μm
L
Relative cleaved caspase3 immunoreactivity
M
Relative MAP2 immunoreactivity

Figure 5

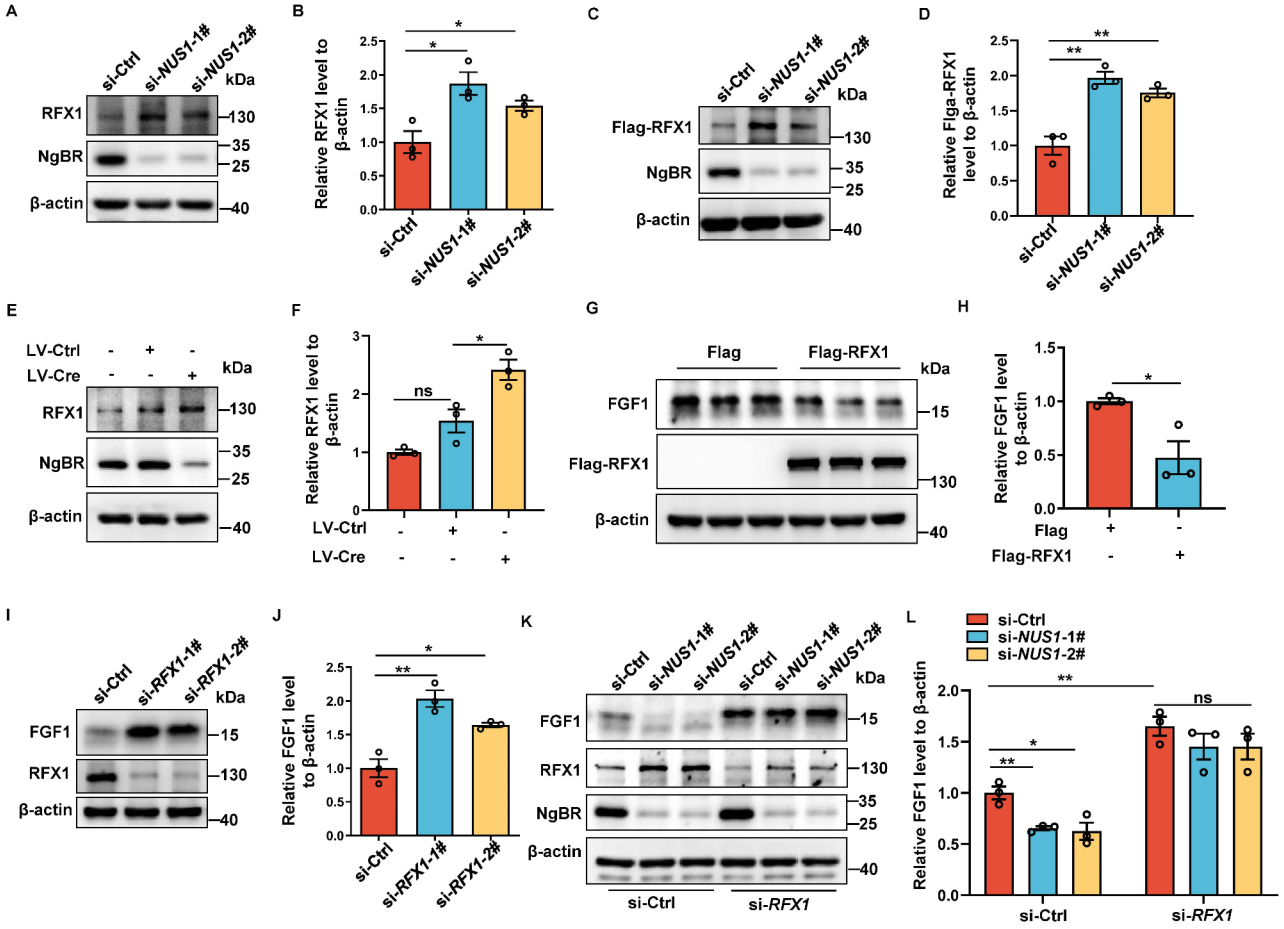

Fig. 5 NgBR regulates FGF1 expression through RFX1. **A** SH-SY5Y cells were transfected with the indicated siRNAs for 48 h. Then, the cell lysates were subjected to immunoblot analysis. **B** The relative protein level of RFX1 to that of β-actin in (**A**) was analyzed. $n=3$, * $P<0.05$. **C** SH-SY5Y cells harboring Flag-RFX1 were transfected with the indicated siRNAs for 48 h. Then, the cell lysates were subjected to immunoblot analysis. **D** The relative protein level of Flag-RFX1 to that of β-actin in (**C**) was analyzed. $n=3$, ** $P<0.01$. **E** *Nus-1*$^{flox/flox}$ primary cortical neurons were infected with LV-Ctrl or LV-Cre and cultured for 6 days. Then, the cell lysates were subjected to immunoblot analysis. **F** The relative protein levels of RFX1 and β-actin in (**E**) were analyzed. $n=3$, ns, not statistically significant, * $P<0.05$. **G** SH-SY5Y cells were transfected with Flag-RFX1 for 24 h, after which the cell lysates were subjected to immunoblot analysis. **H** The protein level of FGF1 relative to that of β-actin in (**G**) was analyzed. $n=3$, * $P<0.05$. **I** SH-SY5Y cells were transfected with the indicated siRNAs for 72 h. Then, the cell lysates were subjected to immunoblot analysis. **J** The protein level of FGF1 relative to that of β-actin in (**I**) was analyzed. $n=3$, * $P<0.05$, ** $P<0.01$. **K** SH-SY5Y cells were transfected with the indicated siRNAs for 72 h. Then, the cell lysates were subjected to immunoblot analysis. **L** The protein level of FGF1 relative to that of β-actin in (**K**) was analyzed. $n=3$, ns, not statistically significant, * $P<0.05$, ** $P<0.01$

and H), whereas RFX1 knockdown resulted in significant upregulation of FGF1 protein levels in SH-SY5Y cells (Fig. 5I and J). Moreover, we further explored whether the regulation of FGF1 by NgBR was dependent on RFX1. We found that silencing RFX1 expression blocked the downregulation of FGF1 expression caused by NgBR deletion (Fig. 5K and L). Thus, these results suggest that loss of NgBR enhances the protein stability of the transcriptional repressor RFX1 of FGF1 to repress FGF1 expression in neuronal cells.

Loss of NgBR inhibits RFX1 acetylation and stability

The regulation of protein stability mainly occurs post-translationally through various enzyme-catalyzed chemical modifications of proteins, such as methylation, acetylation, phosphorylation, ubiquitination, and glycosylation, which alter the structural and chemical properties of proteins [30]. Several articles have reported that RFX1 is a protein that can be modified by acetylation and that acetylation affects its protein stability [31, 32].

Consistent with previous studies, we also found that RFX1 is an acetylated protein in cells (Fig. 6A). To explore whether acetylation affects RFX1 protein stability, we treated SH-SY5Y cells with the pan-histone deacetylase (HDAC) family inhibitor trichostatin A (TSA) and the sirtuin (SIRT) inhibitor nicotinamide (NAM), as well as their combination to inhibit the deacetylation process of proteins. The results showed that TSA alone or the TSA/NAM combination, but not NAM alone, resulted in a significant decrease in RFX1 protein levels (Fig. 6B and C), whereas TSA did not significantly affect the mRNA level of *RFX1* (Fig. S7A). The level of proteins is inextricably linked to their degradation processes, and the degradation pathways are mainly categorized into two pathways: the lysosomal system and the ubiquitin–proteasome system (UPS) [30]. We wondered which degradation pathway participates in the degradation of the RFX1 protein, especially acetylated RFX1. Compared with the lysosomal inhibitor NH_4Cl, the proteasome inhibitor MG132 significantly increased the Flag-RFX1 protein level, suggesting that RFX1 is mainly degraded through the UPS (Fig. S7B and S7C). Moreover, pretreatment with MG132 but not with NH_4Cl significantly blocked TSA-induced RFX1 protein reduction (Fig. 6D and E). Then we measured whether TSA treatment influence RFX1 ubiquitination, and found that TSA treatment significantly increased the ubiquitination of Flag-RFX1 (Fig.S7D). A previous study reported that STUB1, an E3 ubiquitin ligase, mediates RFX1 degradation in systemic lupus erythematosus [29]. However, our results indicated that the knockdown of STUB1 did not affect RFX1 stability with or without TSA treatment in neuronal cells (Fig. S7E).

These results suggest that enhancing the acetylation of RFX1 promotes its degradation mainly through the UPS pathway. Therefore, we next wondered whether NgBR affects the acetylation of RFX1 and its degradation. Although the total protein level of Flag-RFX1 was significantly

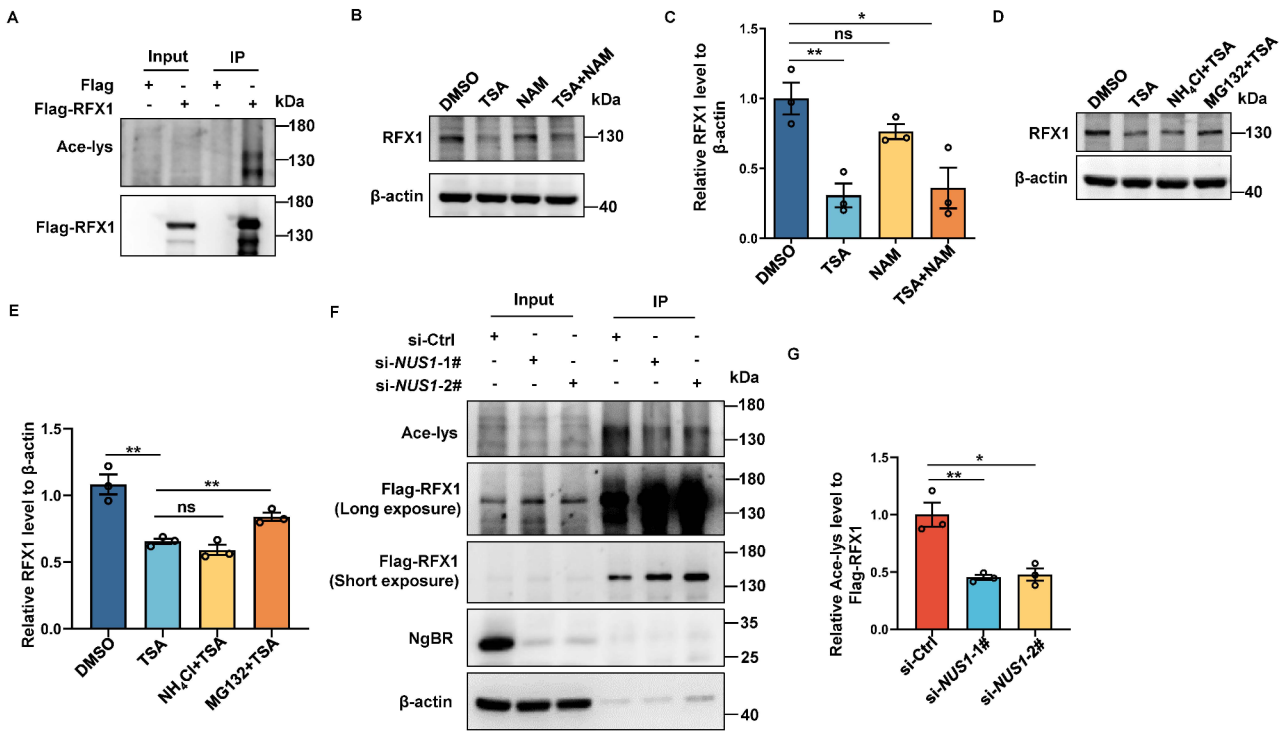

Fig. 6 Loss of NgBR inhibits RFX1 acetylation and stability. **A** The cell lysate supernatants of HEK293 cells transiently transfected with Flag or Flag-RFX1 were immunoprecipitated using an anti-Flag antibody. The samples were subsequently subjected to immunoblot analysis. **B** SH-SY5Y cells were treated with TSA (1 μM), NAM (10 mM) or their combination for 18 h. Then, the cell lysates were subjected to immunoblot analysis. **C** The relative protein level of RFX1 to that of β-actin in (B) was analyzed. $n=3$, ns, not statistically significant, * $P<0.05$, ** $P<0.01$. **D** SH-SY5Y cells were pretreated with NH_4Cl (10 mM) or MG132 (5 μM) for 2 h, then the cells were treated with TSA (1 μM) for 18 h as indicated. Then, the cell lysates were subjected to immunoblot analysis. **E** The relative protein levels of RFX1 and β-actin in (**D**) were analyzed. $n=3$, ns, not statistically significant, ** $P<0.01$. **F** HEK293 cells harboring Flag-RFX1 were transfected with the indicated siRNAs for 48 h. Then, the cell lysates were immunoprecipitated with an anti-Flag antibody, and the samples were subjected to immunoblot analysis. **G** The protein level of Ace-lys relative to that of Flag-RFX1 in (**F**) was analyzed. $n=3$, * $P<0.05$, ** $P<0.01$

increased, its acetylation level was significantly decreased after NgBR deletion (Fig. 6F and G). Together, these results suggest that loss of NgBR enhances the protein stability of RFX1 by inhibiting its acetylation.

Loss of NgBR inhibits KAT7-mediated RFX1 acetylation

Lysine deacetylases (KDACs) and lysine acetyltransferases (KATs) dynamically regulate the acetylation of substrates [33] RFX1 has been shown to interact with and be deacetylated by HDAC1 and inhibit the expression of *COL1A2* [31]. RFX1 was also reported to bind to and be deacetylated by SIRT1, which regulates its protein stability [32]. In addition, HDAC2 and HDAC3 have also been reported to be involved in the acetylation of RFX family proteins [31]. Therefore, we first examined whether NgBR affects the levels of these proteins. However, there were no significant changes in the protein levels of HDAC1, HDAC2, HDAC3, SIRT1 or SIRT2 after NgBR was knocked down (Fig. S8A), suggesting that these KDACs are not involved in NgBR-mediated acetylation of RFX1.

Recently, loss of NgBR was shown to regulate the expression of the downstream protein CCM1/2 by decreasing the expression of KAT7, also known as HBO1, a lysine acetyltransferase, thereby causing cerebrovascular lesions [34]. Consistent with this finding, we also found that knockdown of NgBR resulted in a significant decrease in KAT7 protein levels in neuronal cells (Fig. 7A and B). In NgBR-deficient primary cortical neurons, KAT7 protein levels were significantly lower than those in control neurons (Fig. 7C and D). Therefore, we speculated that KAT7 may be involved in the acetylation regulation of RFX1 by NgBR. We first detected whether KAT7 interacts with RFX1 in cells. As shown in Fig. 7E and F, KAT7 was pulled down by Flag-RFX1 but not empty Flag in the co-immunoprecipitation experiment, and KAT7 was found to be recruited to and colocalized with EGFP-RFX1 in the nucleus by immunofluorescence. We then treated cells harboring Flag-RFX1 with the KAT7-specific inhibitor WM-3835 and detected the acetylation level of Flag-RFX1. We found that the acetylation level of Flag-RFX1 significantly decreased with WM-3835 treatment (Fig. 7G and H), suggesting that KAT7 could regulate the acetylation process of RFX1. We next investigated whether KAT7 could regulate RFX1 protein stability. The protein levels of both endogenous RFX1 and exogenous Flag-RFX1 increased significantly after WM-3835 treatment in SH-SY5Y cells (Fig. 7I and L), whereas the transcriptional level of *RFX1* did not change significantly (Fig. S8B). To exclude the nonspecific effects of WM-3835, we used two siRNAs to silence KAT7 expression and found that the RFX1 protein level was significantly increased after KAT7 was knocked down in SH-SY5Y cells (Fig. 7M and N), which was similar to the effect of the KAT7 inhibitor. These results indicate that the acetyltransferase KAT7 can bind to RFX1 in the nucleus and mediate its acetylation, thus regulating RFX1 stability.

NgBR regulates the RFX1-FGF1 axis through KAT7

We further investigated whether KAT7 mediated the regulatory effect of NgBR on the RFX1/FGF1 axis. We found that knockdown of KAT7 significantly reduced the FGF1 protein level in SH-SY5Y cells (Fig. 8A and B), and treatment with the KAT7 inhibitor WM-3835 had a similar effect (Fig. 8C and D). Moreover, WM-3835 treatment significantly downregulated *FGF1* mRNA levels (Fig. 8E). Interestingly, after KAT7 activity was inhibited, NgBR deficiency no longer caused the downregulation of *FGF1* expression or the upregulation of RFX1 protein levels (Fig. 8F and H). These results suggest that loss of NgBR increases the stability of RFX1 and subsequently inhibits the expression of FGF1 in a KAT7-dependent manner.

Discussion

Although the functions of NgBR in the brain are largely unknown, genetic evidence shows that variants in its encoding gene *NUS1* are closely associated with a series of neurological disorders, including PD, tremor, epilepsy, cerebellar ataxia, intellectual disability, dystonia, and congenital disorders of glycosylation [8, 9, 13, 15, 35, 36], suggesting that NgBR has essential physiological functions in the CNS. Moreover, animal models such as zebrafish or Drosophila with *nus1* knockout or deficiency exhibit dopaminergic neuron death and motor-deficient phenotypes [8, 12, 37]. In this study, we used RNA-seq and experimental evidence to reveal the mechanism of neuronal damage and death caused by NgBR deficiency, which involves the downregulation of KAT7-mediated acetylation of RFX1 and its degradation, subsequently inhibiting FGF1 expression.

Current functional studies reveal that NgBR regulates angiogenesis, cholesterol trafficking, dolichol synthesis, and tumorigenesis in peripheral tissues [15]. However, the expression, distribution and function of NgBR in the brain are still unclear. Owing to the limitations of commercial antibodies against NgBR, we could not provide accurate expression pattern data for NgBR in the mouse brain in the current study. NgBR was initially identified as a receptor for neurite outgrowth inhibitor B (Nogo-B) and binds

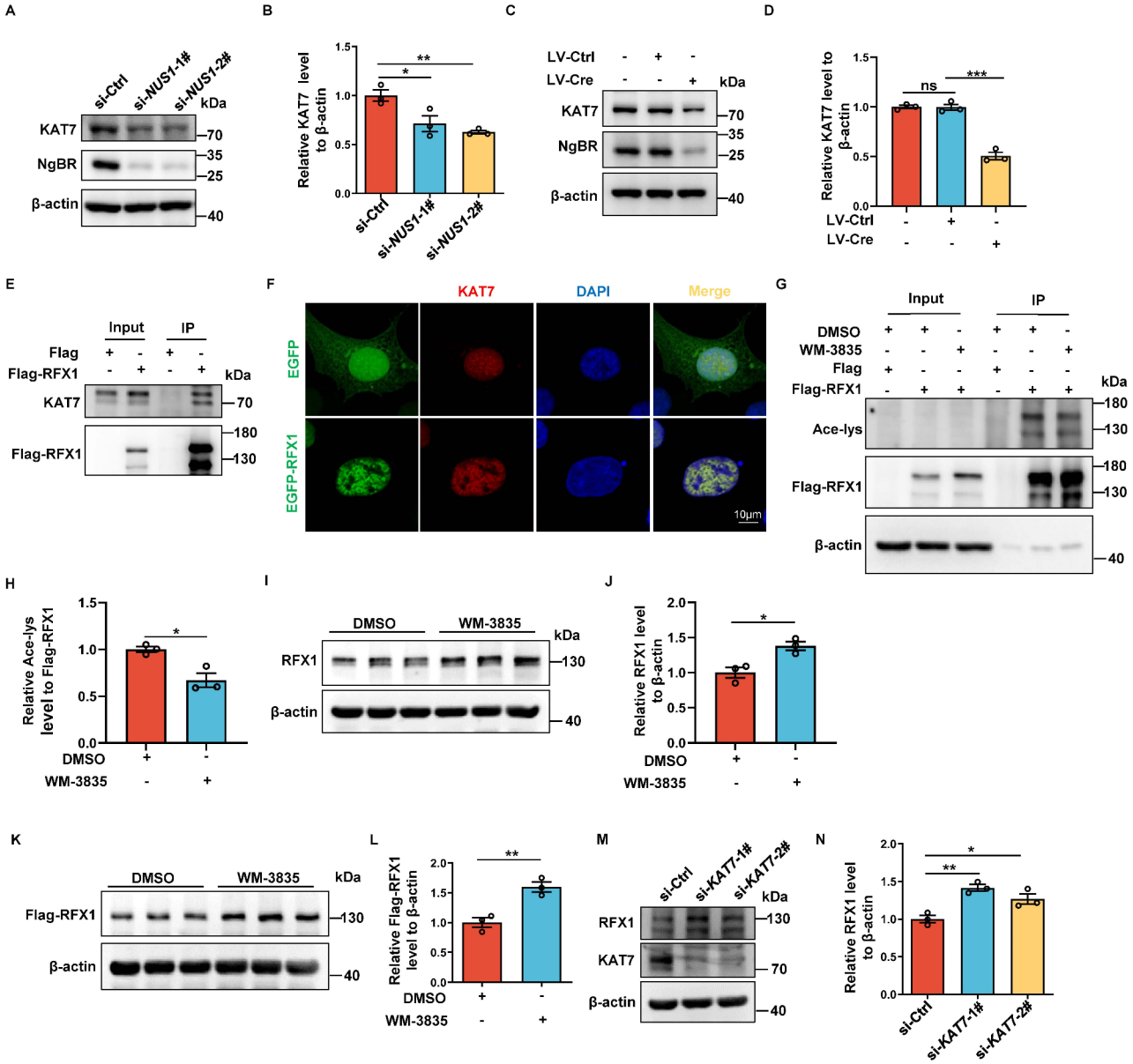

Fig. 7 Loss of NgBR inhibits KAT7-mediated RFX1 acetylation. **A** SH-SY5Y cells were transfected with the indicated siRNAs for 48 h. Then, the cell lysates were subjected to immunoblot analysis. **B** The relative protein level of KAT7 to that of β-actin in (**A**) was analyzed. $n=3$, * $P<0.05$, ** $P<0.01$. **C** *Nus1*$^{flox/flox}$ primary cortical neurons were infected with LV-Ctrl or LV-Cre and cultured for 6 days. Then, the cell lysates were subjected to immunoblot analysis. **D** The relative protein level of KAT7 to that of β-actin in (**C**) was analyzed. $n=3$, *** $P<0.001$. **E** HEK293 cells were transfected with Flag or Flag-RFX1 for 48 h, after which the supernatants of the cell lysates were immunoprecipitated with an anti-Flag antibody. The samples were subsequently subjected to immunoblot analysis. **F** HEK293 cells transiently transfected with EGFP or EGPF-RFX1 were subjected to immunocytochemistry. Scale bar, 10 μm. **G** HEK293 cells were transfected with Flag or Flag-RFX1 for 24 h and then treated with DMSO or WM-3835 (10 μM) for 24 h. Then, the supernatants of the cell lysates were subjected to immunoprecipitation with an anti-Flag antibody. **H** The protein level of Ace-lys relative to that of Flag-RFX1 in (**G**) was analyzed. $n=3$, * $P<0.05$. **I** SH-SY5Y cells were treated with WM-3835 (10 μM) for 24 h. Then, the cell lysates were subjected to immunoblot analysis. **J** The relative protein level of RFX1 to that of β-actin in (**I**) was analyzed. $n=3$, * $P<0.05$. **K** SH-SY5Y cells harboring Flag-RFX1 were treated with WM-3835 (10 μM) for 24 h. Then, the cell lysates were subjected to immunoblot analysis. **L** The relative protein level of Flag-RFX1 to that of β-actin in (**K**) was analyzed. $n=3$, ** $P<0.01$. **M** SH-SY5Y cells were transfected with the indicated siRNAs for 48 h. Then, the cell lysates were subjected to immunoblot analysis. **N** The relative protein level of RFX1 to that of β-actin in (**M**) was analyzed. $n=3$, * $P<0.05$, ** $P<0.01$

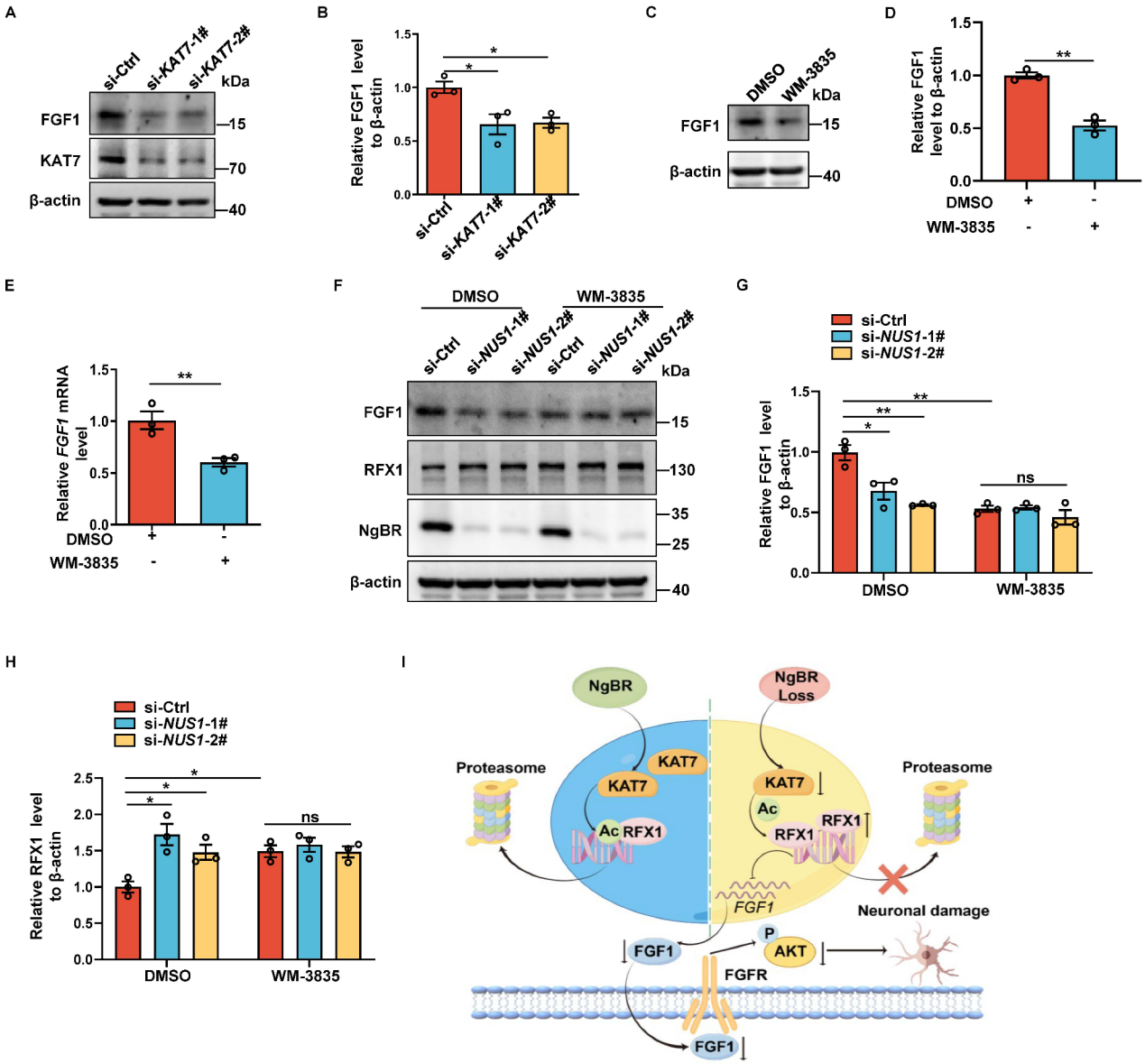

Fig. 8 NgBR regulates the RFX1-FGF1 axis through KAT7. **A** SH-SY5Y cells were transfected with the indicated siRNAs for 48 h. Then, the cell lysates were subjected to immunoblot analysis. **B** The protein level of FGF1 relative to that of β-actin in (**A**) was analyzed. $n=3$, * $P<0.05$. **C** SH-SY5Y cells were treated with WM-3835 (10 μM) for 24 h. Then, the cell lysates were subjected to immunoblot analysis. **D** The protein level of FGF1 relative to that of β-actin in (**C**) was analyzed. $n=3$, ** $P<0.01$. **E** SH-SY5Y cells were treated with WM-3835 (10 μM) for 24 h. Then, the samples were subjected to qRT–PCR. $n=3$, ** $P<0.01$. **F** SH-SY5Y cells were transfected with the indicated siRNAs for 48 h and then treated with DMSO or WM-3835 (10 μM) for 24 h. Then, the cell lysates were subjected to immunoblot analysis. **G-H** The relative protein level of FGF1 or RFX1 to that of β-actin in (**F**) was analyzed. $n=3$, ns, not statistically significant, * $P<0.05$, ** $P<0.01$. **I** Diagram of NgBR regulating FGF1 expression and its deficiency causing neuronal damage

specifically to the amino terminus of Nogo-B [14]. Nogo-B is extensively expressed in multiple organs, including the CNS and peripheral nervous system [38], suggesting that NgBR, as its receptor, may be widely distributed in the nervous system.

FGF1 is also known as acid FGF (aFGF) and is a member of the FGF family, which has a wide range of biological functions in angiogenesis, embryonic development, cell proliferation, and wound healing. FGF1 is expressed at the highest levels in the brain, including dopaminergic neurons, as well as in the peripheral nervous system [23, 39–41]. FGFs are closely associated with the development of PD and are able to increase DA neuron survival and exert neuroprotective effects [42].

FGF1 not only exerts its biological activity through three secretory pathways, namely, autocrine, endocrine, and paracrine pathways [43–45] but also activates all FGFRs by inducing receptor dimerization and juxtaposition of the structural domains of intracellular kinases, thus inducing the activation of downstream signaling, including the PI3K-AKT pathway [46]. NgBR has also been reported to activate PI3K-AKT signaling by binding to farnesylated Ras, thereby promoting tumor cell survival, proliferation and invasion [47]. In addition, NgBR deficiency inhibits vascular endothelial growth factor (VEGF)-stimulated AKT phosphorylation and VEGF-induced chemotaxis and morphogenesis of endothelial cells in a zebrafish model [48]. Our study revealed that NgBR deficiency inactivates PI3K-AKT signaling through decreasing FGF1 expression. In addition to its ability to activate FGFRs, FGF1 is also reported to have neurotrophic and antiapoptotic functions in cells and nuclei. In various types of neuronal cells, FGF1 nuclear translocation is required for both its neurotrophic activity and p53-dependent protection against apoptosis [49–51]. Consistent with our findings, exogenous FGF1 activated AKT activity and alleviated the damage induced by NgBR deficiency in neuronal cells and primary neurons. In PD animal models, FGF1 attenuated the neurotoxicity of 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), activated PI3K/AKT, promoted the survival of TH-positive cells, and significantly restored motor function by inhibiting ER stress and apoptotic processes in PD mice or rats [52–54].

We also revealed that the mechanism by which NgBR deficiency leads to a decrease in FGF1 transcription in neuronal cells is dependent on RFX1, an FGF1 transcriptional repressor. This may also explain why NgBR deletion in astrocytes does not affect FGF1 expression, as that it has been reported that RFX1 is mainly expressed in the nuclei of neurons and microglia in the brain, but it is difficult to detect in astrocytes [55]. NgBR deficiency did not affect the transcription of RFX1 but influenced its protein stability and degradation. RFX1 was reported to be modified by acetylation, and acetylation can affect its protein stability [31, 32]. We found that acetylation of exogenous Flag-RFX1 is decreased but that its total protein level is increased after NgBR silencing. Due to the commercial RFX1 antibodies we used in this study were found not to be suitable for IP experiments, we did not further detect the acetylation and ubiquitination of endogenous RFX1. We did not find changes in the levels of any reported RFX1-related KDACs, including HDAC1/2/3 and SIRT1, after NgBR deletion, suggesting that these KDACs may not be involved in NgBR-mediated acetylation of RFX1 in neuronal cells. However, which KDACs are involved in the deacetylation of RFX1 mediated by NgBR deficiency needs further study. NgBR has been shown to modulate the expression of downstream genes by affecting the expression of KAT7 and its histone acetylation, which can cause cerebrovascular lesions [34]. Consistent with this finding, we also found that knockdown of NgBR resulted in a significant reduction in the protein level of KAT7, a typical member of the MYST (MOZ, Ybf1/Sas3, Sas2, and Tip60) family of acetyltransferases with a wide range of biological functions, which are not only involved in protein acetylation, propionylation, and ubiquitination but also in the regulation of gene transcription [56–59]. We found that KAT7 interacts with RFX1 in the nucleus and mediates its acetylation. Inhibition of KAT7 expression or activity eliminated the influence of NgBR deficiency on RFX1 protein levels and FGF1 transcription. Interestingly, *FGF1* transcription is also controlled by epigenetic regulation, including H3 and H4 acetylation [60, 61], and KAT7 is a major chromatin-modifying enzyme responsible for H3 and H4 acetylation [59]. However, whether KAT7 regulates FGF1 expression by directly affecting histone acetylation needs further study.

In summary, under physiological conditions, NgBR maintains the steady state of the KAT7/RFX1/FGF1 axis and neuronal survival. However, when NgBR is deficient, the expression of KAT7 is inhibited, which limits the KAT7-mediated acetylation of RFX1 and thus inhibits RFX1 degradation, subsequently decreasing FGF1 expression and resulting in neuronal damage (Fig. 8I). Supplementation with exogenous FGF1 significantly ameliorated the neuronal damage caused by NgBR deficiency.

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Author contributions HR, YC and PY designed the research. YH, LL, YM and YH performed the research and analyzed the data. WG, BT and JG supervised the study. The manuscript was written by YH and HR, and all the authors revised the manuscript. All the authors read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this article and its supplementary information files.

Declarations

Ethical approval and consent to participate All animal experiments performed in this study were in accordance with the institutional guidelines for the use and care of animals, and all procedures were approved by the Institute Animal Welfare Committee of Soochow University.

Consent for publication All authors reviewed the results and contributed to the final manuscript. All authors approved this manuscript for publication.

Competing interests The authors declare that they have no conflicts of interest with the contents of this article.

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