RND2 attenuates apoptosis and autophagy in glioblastoma cells by targeting p38 MAPK signaling pathway

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Abstract

Background
Inhibition of p38 MAPK signaling leads to glioblastoma multiform (GBM) tumorigenesis. Nevertheless, the molecular mechanism which induces the p38 MAPK signaling silent during GBM genesis is yet to be figured out. Identifying new factors which could regulate p38 MAPK signaling is important for tumor treatment.

Methods
Flow-cytometry, TUNEL assay, Immunofluorescence, JC-1 assays, as well as western blotting analysis were used to detect the apoptosis of GBM cells. The detection devices of autophagy levels in GBM cells were western blotting analysis, immunofluorescence of LC3B protein, LC3B puncture assays and transmission electron microscopy. The functions of these critical molecules are further confirmed by intracranial xenografts in nude mice as in vivo experiments. Tumor tissue samples and clinical information were used to identify the correlation between RND2 and p62, LC3B expression, survival time of patient, tumor volume in clinical patients.

Results
We found that small GTPase RND2 expression significantly increased in human glioblastomas by summarizing the data of TCGA database. Our study demonstrated that the RND2 function is as an endogenous repressor of the p38 MAPK phosphorylation complex. RND2 physically interacted with p38, decreasing p38 phosphorylation, therefore, p38 MAPK signaling activities were inhibited. The forced expression of RND2 repressed the p38 MAPK signaling, which inhibited glioblastoma cell autophagy and apoptosis in vitro and induced the xenograft mice’s tumor growth in vivo. The downregulation of RND2, nevertheless, enhanced p38 MAPK signaling activities and promoted glioma cell autophagy and apoptosis. The inhibition of p38 phosphorylation abolished RND2 deficiency-mediated GBM cell autophagy and apoptosis. Most important, our study found that the RND2 expression was inversely correlated with patient survival time and was positively correlated with tumor size, indicating that RND2 was an oncogene which predicts a poorer clinical outcome of patients.

Conclusions
Our findings revealed RND2’s new function in GBM genesis and offered mechanistic insights into the
inhibitory effects of RND2 in regard of the p38 MAPK activation regulation.

Background
As the most frequently seen primary intracranial tumor, Glioma has the highest fatality rate in the central nervous system of adults, and half of them are glioblastomas (1). It’s difficult to remove the tumor completely. To make things worse, the unclear boundary with normal tissue leads to easily recurrence of tumor (2). And GBM can be categorized into three transcriptional-defined and clinical-related subtypes: proneural (PN), mesenchymal (MES) and classical (CL) according to gene expression profiling studies (3). Despite the fact that standard therapies including surgeries, chemotherapy with TMZ and radiotherapy have developed rapidly, the one-year survival rate remains only 40.6% and the five-year survival rate is only 5.6% (1). The resistance of cell death is one of hallmarks in GBM cells (4), therefore, it’s crucial to clarify the specific molecular mechanism to detect a novel therapeutic target in GBM.

p38 MAPK signaling plays an important role in GBM genesis (5). Human GBM tissues and glioma cells studies have already observed hyper-activation of p38 MAPK. However, how the p38 MAPK signaling becomes to be silent during GBM genesis is unknown. Our study revealed that RND2 (also known as RND7), a small GTPase, was an endogenous inhibitor of p38 MAPK signaling. Upregulation of RND2 detected in human GBM shouldered the responsibilities of the inhibition of p38 MAPK signaling, promoting glioma genesis.

As an atypical member of the Rho GTPase family, RND2 is short of detectable GTPase activity. The most distinctive function of RND2 is the inhibitory effect on Rho kinase-mediated biological functions, including actin cytoskeleton formation and phosphorylation of myosin light chain phosphatase (6). Recent studies also point out that RND2 is a key regulator of neuronal movement in the development of brain and is also essential in regulating the actin cytoskeleton of cells (7, 8). Besides, as a novel and specific effector of Rnd2 GTPase, Rapostlin induces the neurite branching (9). However, RND2’s pathological role in human GBM progression has not been investigated and the associated animal studies are to be explored.

Here, our study has provided evidences to reveal that the RND2 was an oncogene in human
glioblastomas. Firstly, RND2 expression levels were significantly upregulated in human glioblastomas, which were inversely correlated with patient survival time and p38 MAPK signaling and cells apoptosis, meanwhile were positively associated with tumor size. Secondly, our study also demonstrated that RND2 physically interacted with p38 and facilitated their protein phosphorylation, which prevented p38 MAPK signaling from hyper-activation. RND2 overexpression led to the inhibition of the p38 signaling pathway and reduces expression of its downstream substrates, LC3B, p62 (10), and induced expression of its downstream substrates Beclin-1 (11), along with increased autophagy, further inducing apoptosis resistance compared with increased cleaved-casepase3, which was another downstream substrate of p38 signaling pathway (12). On the contrary, the knockdown of RND2 reduced the binding of RND2 to p38 protein, which also led to the increasing of p38 protein phosphorylation, thus promoted the activation of the p38 signaling pathway, then its downstream substrates such as LC3B and p62 related to autophagy were increased while Beclin-1 was reduced, when the expression of cleaved-casepase3, an indicator of apoptosis, increased. In conclusion, our findings revealed a new function which was not previously undescribed of RND2 in GBM genesis and provided a new insight into RND2’s inhibitory effect on activation regulation mechanism of the p38/MAPK.

Methods And Materials
Bioinformation Analysis
The expression of RND2 in different human cancers were downloaded from TCGA database and the profiles in normal human tissues were based on information from HPA database (https://www.proteinatlas.org). RND2 expression profiles in different subtype gliomas were analyzed based on the GlioVis portal (http://gliovis.bioinfo.cnio.es) (13).

Human GBM and control brain tissues
Human control brain tissues and GBM tissues were acquired from the Department of Neurosurgery, Renmin Hospital of Wuhan University. GBM tissues were sampled during surgeries and stored at minus 80°C. Controlled brain tissues were collected from patients during the emergency surgeries with traumatic brain injury. The procurement and use of tissue in this study were approved by Renmin Hospital of Wuhan University’s Institutional Ethics Committee of the Faculty of Medicine (approval
number: 2012LKSZ (010) H). The histological diagnosis of glioma was confirmed by the pathologists of the Department of Pathology which belongs to Renmin Hospital of Wuhan University. All tumor samples were subjected to pathological examination and related molecular testing (MGMT, 1p19q, and IDH1/IDH2), all defined according to the 2016 WHO classification (14). Every piece of clinical information of patients was listed and presented in Supplemental Table S1.

**Cell Culture**

Human renal epithelial cell line (293T) and human GBM cell lines (U87 and U251) were from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The information of U251 and U87 cell lines, generation of U87 stable cell line, and cell culture were described in our previous study (15). Cells were cultivated in DMEM along with 1% Penicillin/Streptomycin and 10% fetal bovine serum, incubating temperature was 37℃, with 5% of CO2. And the STR Authentication was listed in supplemental materials.

**Reagents and Antibodies**

Antibodies used in this experiment included the following items: anti-RND2 (13844-1-AP, Proteintech, USA), anti-Rho7 /Rnd2 (GXT56070, from GeneTex in USA), anti-p-p38(#4511, from Cell Signaling Technology in USA), anti-p38 (#9212, from Cell Signaling Technology in USA), anti-cleaved-caspase3 (ab32042, from Abcam in UK), anti-caspase3 (NB100-56708SS, from Novus in USA), anti-Bax (50599-2-Ig, from Proteintech in USA), anti-GAPDH (#5174, from Cell Signaling Technology), anti-SQSTM1/P62 (M162-3, from Medical Biological Laboratories in Japan), anti-Beclin1 (11306-1-AP, from Proteintech in USA), anti-LC3B (GB11124, from Servicebio in China), USA), anti-DYKDDDK/Flag-tag (ANT102, from Antgene in China), and anti-His-tag (D291-3, from Medical Biological Laboratories in Japan).

Autophagy inhibitor Wortmannin (3-MA) and the autophagy activator Rapamycin (Sirolimus) (S1039, from USA) were bought from Selleck (S2758, USA).

**Quantitative Real-Time PCR (qPCR) and RNA Extraction**

The extraction of the total RNA of tissues and cells was assisted of Trizol reagent (Invitrogen, USA). And the reverse transcription of RNA used PrimeScript RT Reagent Kit (RR047A, from Takara in Japan) in order to synthesize cDNA. Adopting SYBR Premix Ex Taq II (RR820A, from Takara), we performed qPCR to detect mRNA levels under the guidance of specifications provided by manufacturers and
performed in 2.1 real-time PCR Systems of Bio-Rad CFX Manager (Bio-Rad, from USA). The related Ct method was adopted to compare the data of experiment group and the control group, while GAPDH was set as internal control. The sequences of primer are listed in Supplemental TableS3. The clinical information of GBM patients who provided samples listed in Supplemental Table S2.

**shRNA Transfection**
Specific shRNA targeting at human RND2 (shRND2) and negative controlled shRNA (shctrl) were purchased from RiboBio Corporation (from Guangzhou in China). Referring to the specifications, Lipofectamine 3000 transfection reagent (L3000015, Thermo Fisher Scientific) was used in the transfection. The sequences of different shRND2 were mentioned in Supplemental TableS3.

**DNA Construction and Transfection**
RND2 cDNA was subcloned by a Flag tag (Flag-RND2) into the pcDNA3.1 vector. Full-length p38 cDNA was subcloned by a 6xHis tag (His-p38) into the pcDNA3.1 vector. Transfections were carried out with the help of transfection reagent Lipofectamine 3000 (L3000015, Thermo Fisher Scientific) in accordance with the specifications.

**Flow Cytometric Analysis**
Annexin V-PE/7-AAD kit (Becton Dickinson, New Jersey, USA) was used to measure the apoptosis rate of GBM cells. GBM cells were collected and then washed by PBS for three times. And samples were stained with Annexin V-PE/7-AAD for 15 min in the dark and using FACSCalibur flow cytometer (Becton Dickinson) analyzed the specific apoptosis of GBM cells in an hour after staining. When 7-AAD and PE Annexin V were both negative, it suggested that the cells were still viable cells and no apoptosis was found. When PE Annexin V showed positive yet 7-AAD was negative, cells were found in the early stage of apoptosis. Positive 7-AAD and PE Annexin V both positive meant that the cells were in the late stage of apoptosis or even were dead already. To calculate the total apoptosis rates and finish statistical analysis, this experiment adopted the sum of upper right quadrant and low right quadrant.

**Mitochondrial membrane potential (ΔΨm) assay**
For early stage apoptosis, the collapse of the Δψm functions is a hallmark event. Δψm variations were detected by Olympus BX51 microscope (Olympus, in Japan) which captured the images of cells after JC-1 staining (Yeasen, Shanghai, China), all operation of which followed manufacturer's specifications.
We also recorded the ratio of JC-1 which aggregated (red fluorescence) to monomers (green fluorescence). ImageJ software was used to detect the intensity of fluorescence intensity. The drop in the red/green fluorescence intensity ratio showed the loss of ΔΨm.

**TUNEL Assay**
The feature of apoptotic cell is the fragmentation of DNA, which can be measured by TUNEL kit. And we followed the protocol offered by the manufacturer of TUNEL kit (Roche Diagnostics, from Mannheim in Germany). Images of staining cells were collected with the help of the Olympus BX51 microscope (from Olympus in Japan). ImageJ software was used to detect the intensity of fluorescence intensity.

**Western Blotting**
Cells or tissues were lysed in RIPA buffer with protease and phosphatase inhibitors (Cocktail (from Roche) and PMSF (from Beyotime)) for 30 minutes at 4°C. Protein concentration was detected by BCA kit (from Biosharp in China). Protein was separated in SDS-PAGE and then was transferred onto the PVDF membrane which were cultivated in the primary antibodies (including anti-GAPDH, anti-Flag, anti-β-actin, anti-p-p38, anti-Rnd2, anti-p38, anti-His, anti-p62, anti-Caspase3, anti-Lc3B, anti-Beclin1, anti-Bax, anti-Cleaved-Caspase3) for one night and in the secondary antibodies for an hour. The proteins were delineated with imaging system of LI-COR Odyssey Infrared (LI-COR Bioscience, from USA). ImageJ software was used to detect the gray value of blots. Quantity of relative protein was normalized by GAPDH.

**Immunoprecipitation assays**
Cell lines U87 and U251 were co-transfected with plasmids Flag-Rnd2 (from Miaoling Biology in China) and His-p38 (from Miaoling Biology in China). 48 hours after transfection, while cells were lysed in IP buffer containing 1% NP-40, 50 mM NaF, 2 mM Na3VO4, 4 mM Na pyrophosphate and protease inhibitors. 3 µg antibodies (anti-Flag, anti-His or IgG (from Beyotime) are added into cell lysates and incubated with 30 µL Protein A/G (Santa Cruz Biotechnology) at the temperature of 4°C overnight. The precipitates are washed 5 times or more by IP buffer and are boiled for 5 minutes in 40 µL 1.5x loading buffer (from Beyotime) followed by Western Blot.

**Immunohistochemistry**
The tissues are embedded in paraffin after being fixed in 4% paraformaldehyde and cut into slides. After hydrated, the slides are treated with 3% H2O2 for 10 minutes and blocked by 1% BSA for 1 hour. The samples are incubated in primary antibodies (anti-Lc3B, anti-Cleaved-Caspase, anti-p-p38, anti-Bax, anti-Rnd2) for one night and in HRP-labelled secondary antibodies (from Servicebio in China). DAB (from Servicebio in China) is used for dyeing and hematoxylin is used to stain the nuclei. Pictures are snapped with the Olympus BX51 microscope (from Olympus). A semiquantitative score was applied to describe the distribution and intensity of RND2 staining (0 = negative, 1 = weak, 2 = moderate, 3 = strong, and 4 = strong and widely distributed).

Assay of Green Fluorescent Protein-LC3 Puncta
RND2 plasmids were used in the transfection into GBM cell which expressed stably green fluorescent protein (GFP)-LC3. Transfected cells after 2 days were fixed with paraformaldehyde whose concentration was 4% and then confocal laser scanning microscope (from Olympus in Japan) was used to obtain GFP-LC3 dots in the cells. The dots in GFP-LC3 were calculated and at least 100 cells were calculated in this process.

Transmission Electron Microscopy (TEM)
Electron fixation solution with 2.5% glutaraldehyde fixed cells transfected with CTRL or RND2 plasmids. And the cells were post-fixed in 1% osmic acid. First, a graded series of ethanol was used to dehydrate the specimen was and the specimen were placed in capsules contained embedding medium and heated at 70°C for about 9 hours. The specimen sections were stained by uranyl acetate and alkaline lead citrate. Next, the stained sections were observed with a TEM (HitachiHT7700, Tokyo, Japan).

Intracranial Xenograft Model
PBS was used to suspend U87-MG cells which had stable lentivirus RND2 or CTRL expressions at the concentration of $1 \times 10^5$ cells/μL and the cells were then injected into Balb/c nude mice’s right striatum which were 6 weeks old by stereotactic implantation. Referring to the analysis on survival, the mice were under periodical monitoring and they were sacrificed when serious neurological symptoms appeared and/or evident loss of weight (more than 20% of their body weight) occurred. We removed the whole mouse brains and fixed them in paraformaldehyde whose concentration was 4%.
The brains were also kept for further analysis and were embedded in paraffin. Institutional Animal Care and Use Committee subordinate to Renmin Hospital of Wuhan University approved all experiments mentioned above on animals.

Statistical Analysis
All experiments were triplicate and were replicated at least once more. All expressed data showed within mean ± standard deviations. Statistical analyses were carried out with GraphPad Prism 7 and SPSS version 19.0. The comparison of the means between the two groups was used Unpaired Student t tests. p values which were less than 0.05 were considered to be significant statistics. One-way analysis of variance (short for ANOVA) was performed to determine the differences between groups. When analysis showed significant variance, post hoc testing which targeted on the differences between groups was carried out by Student-Newman-Keuls test. Pearson experiment was adopted in order to analyse the correlation of RND2 and other genes. *P < 0.05, **P < 0.01, ***P < 0.001 was considered significant statistics.

Results
Rnd2 was upregulated in human GBM.
Firstly, from public TCGA database, we studied the RND2’s expression profiles in diverse human cancers(http://cancergenome.nih.gov/). The results showed that the RND2 expression was evidently higher in glioblastomas among 14 categories of human cancers (Fig. 1A). Besides, we also analyzed RND2 expression profiles in diversified human tissues based on Human Protein Atlas database (http://www.proteinatlas.org/). We found that RND2 expressed obviously higher in brain and testis than other tissues (Fig.S1A). Furthermore, RND2 significantly upregulated in GBM compared with normal brain tissue according to TCGA database (Fig. 1B). Consistently, we also carried out the western blot and RT- PCR to analysis protein and the mRNA of RND2 from different organs of C57 mouse and found that RND2 were highly expressed in brain (Fig.S1B, C). Further we detected the expression of RND2 in three sub-class glioblastomas and it was found out that, compared with PN or CL GBM subtypes, the expression was at a significantly lower level in MES GBM (Fig. 1C). To determine whether the protein levels of RND2 elevated or not in clinical samples, we analyzed the expression of RND2 in human glioblastoma samples, including 14 normal brain tissue, 31 WHO grade
II gliomas, 41 WHO grade III gliomas and 52 glioblastomas, we found that RND2 expressed significantly higher in gliomas compared with normal brain tissues (Fig. 1D, E). All the information of patients was listed in Supplementary Table S1. Furthermore, we found that mRNA level of RND2 was significantly increased in gliomas, no matter low-grade glioma or high-grade glioma (Fig. 1F). All these data suggested that RND2 was upregulated and acts as a potential oncogene in GBM, hence, we furtherly explored the function of RND2 in GBM.

**RND2 knockdown induced GBM cell apoptosis in vitro**

We conducted function studies to detect apoptosis in U87 and U251 cells in order to track the function of RND2 in GBM. Different shRNAs (shRND2-1 and shRND2-2) targeting RND2 were designed and efficacies of knockdown were ensured by western blot (Fig. 2 I and Fig. S2F). Firstly, to evaluate cell death, we performed cleaved-caspase3 immuno-fluorescence and immune-blotting and found that RND2 knockdown groups expressed a significantly higher level of cleaved-caspase3 contrasted to control group both in U87 cells and U251 cells (Fig. 2A, B, Fig. 2I). It was known that one of the hallmarks events of the early stage of apoptosis was the loss of ΔΨm (16). The JC-1 staining manifested that knockdown of RND2 persuaded GBM cells’ loss of ΔΨm (Fig. 2C, D). Furthermore, we performed TUNEL staining and it was demonstrated by the result that the rate of TUNEL-positive cells elevated significantly in RND2 knockdown groups (Fig. 2E, F). Annexin V-PE/7-AAD staining which was detected through flow cytometry showed that RND2 knockdown decreased apoptosis both in U87 and U251 cells (Fig. 2G, H and Fig. S2A, B).

To further support these data, immunoblotting was also used to examine the expression of the BCL-2-associated X protein (BAX) when RND2 was up or down regulated in U87 and U251 cells. The result showed that the expression of BAX was decreased when RND2 was overexpressed in U87 and U251 cells, and reduced levels of RND2 resulted in the opposite effects (Fig. 2I, Fig S2C).

Collectively, all these data suggested the apoptosis of glioblastoma cells was negatively regulated by RND2 expression level.

**RND2 overexpression reduced GBM cell autophagy in vitro.**

Because autophagy was one of the most important mechanism of cell death, further, we want to
explored whether RND2 played a key role in autophagy regulation in glioblastoma cells. First, we found that RND2 was negative with LC3B but positive with p62, which are both the markers of autophagy (17), in the same location of human glioblastoma samples by immunochemistry staining (Fig. 3A and Fig.S3C, D). Furthermore, we detected mRNA level of RND2 was negative with LC3B but positive with p62 by RT-PCR (Fig. S3A, B). In a word, all these data suggested that RND2 had a negative relationship with autophagy.

Further, we detected the effect of RND2 in glioblastoma cells by up or down regulated in U87 and U251 cells, and the LC3B immunofluorescence results showed that the cells overexpressing RND2 had lower fluorescence intensity (Fig. 3B and Fig.S2E). Additionally, GFP-LC3B expressed stably in U87 cells to facilitate the visualization of autophagy, and we found that the overexpression of RND2 inhibited GFP-LC3 puncta formation compared with control cells (Fig. 3C and Fig. S2D).

Besides, it was also showed that the amount of autophagic vacuoles per cell was evidently lowered in the RND2 overexpression group compared with control group (Fig. 3F, G). Moreover, the RND2 overexpression decreased LC3B and Beclin-1 levels but increased p62 levels (Fig. 3D), inversely, the proteins expressed totally different when RND2 knocked down (Fig. 3E).

In a summary, RND2 could inhibit autophagy inhibited cell death, as well as reduce both apoptotic and autophagic cell death.

**RND2 Overexpression Reduced Cell apoptosis and Autophagy in an Intracranial Xenograft Model**

We established U87 through stable overexpression of RND2 and constructed an intracranial model of xenograft for the purpose of investigating the potential effects of RND2 in vivo. In the beginning, we tested efficacy of the RND2 overexpression and the process was ensured by western blot and RT-PCR (Fig. S7A, B). Next, we created an intracranial xenograft model by implanting U87 cells intracranially.

According to Kaplan-Meier curves, mice in the control group survived significantly longer than the RND2 overexpression group mice (Fig. 4A). Within expectation, the mice which were implanted with the RND2 overexpression U87 cells had bigger tumors than the mice with the U87 cells with control group (Fig. 4B, C), and the weight of the tumor in the RND2 overexpression group was significantly
higher than that of the control group (Fig. 4D). Immuno-histochemical assays showed that RND2 led to higher expression levels of P62 and Bcl-2 and lower the expression levels of cleaved caspase-3, and LC3B (Fig. 6E). This results also proved that RND2 could weaken autophagy and apoptosis in vivo.

RND2 Interacted with p38 Physically and Inhibited p38 Phosphorylation.
Notch signaling, NF-kb signaling, p53 signaling and Snail1 signaling also played a key role in glioblastoma cells death (18–20), we explored the activity of the signaling by detecting the target gene expression level by RT-PCR, and result showed that there was no significant difference when RND2 was overexpressed (Fig. S4A-G). The interaction between proteins was the basis to active signaling pathway, therefore, we performed co-immunoprecipitation of MDM2, P53 and Snail1 with RND2 in GBM. But the result showed that there was no direct interaction between them (Fig. S4H-G).

It’s also reported in previous studies that the activated p38 MAPK signaling pathway could induce cell death in cancer(5). To determine whether RND2 regulated p38 MAPK signaling pathway, we detected the p-p38 and p38 levels. We found that p38 levels weren’t influenced by RND2, however, p-p38 was induced when RND2 was overexpressed in U87 and U251 cells (Fig. 5C, D and Fig.S5G), meanwhile increased when RND2 was knocked down (Fig. 5C and Fig.S5G). As is known that p38 phosphorylation is an indicator of p38 MAPK signal activation (21), so RND2 could reduce p38 MAPK signal activation in glioblastoma cells.

In order to detect how RND2 decrease p-p38 expression level, co-immunoprecipitation and immunofluorescence assays, which could ensure the association between proteins, was used to explore the potential mechanism. Firstly, the subcellular localization of endogenous RND2 in GBM cells were examined by immunofluorescence and the results showed that the endogenous RND2 was expressed not only mainly in cytoplasm but also in cellular membrane (Supplementary Fig.S5E).

Furthermore, the co-localization of RND2 and p38 in GBM patient tissues was observed mainly existing in the cytoplasm by immunofluorescence (Fig. S5A). Consistently, these results of co-immunoprecipitation assays in U87 cells determined the physical interaction between RND2 and p38 in U87 and in U251 cells (Fig. 5B and Fig. S5D). Distribution of p38 were found mainly in cytoplasm and were detected in nucleus as well, but in U87 cells, we found that the p38 co-localized with RND2
was in cytoplasm (Fig. 5A and Fig. S5B, C), which was in accordance with our former results. Notably, the overexpression of RND2 resulted in a huge decrease in the levels of nuclear p-p38 (Fig. S5E). In summary, these results indicated that activity of p38 MAPK signaling pathway was downregulated by RND2 and then p38 regulated its own downstream target genome.

p38 induced autophagy in GBM cells and rescued RND2-mediated autophagy and apoptosis. p38 MAPK signaling pathway can regulate autophagy and cell death, however, its regulation of autophagy response sometimes has both-side regulation, including positive and negative (11, 22). Therefore, in the next step, we are going to explore the role of p38 MAPK signaling in autophagy of GBM cells. BIRB796, the inhibitor of phosphorylation of p38, was used block the activity of p38 MAPK signaling and the result showed that the cells had lower fluorescence intensity when cells were treated by BIRB796 (Fig. 6A, B). Besides, it was also showed that the amount of autphagic vacuoles per cell was evidently lowered in BIRB796 group compared with DMSO group (Fig. 6C, D). The western blot results showed BIRB796 decreased LC3B and Beclin-1 levels but increased p62 levels (Fig. 6E). All these data demonstrated that p38 MAPK signaling increased autophagy in human glioblastoma cells.

As demonstrated previously, RND2 was able to target p38 MAPK signaling pathway in GBM cells, furtherly, we also speculated that p38 could be involved in RND2-mediated autophagy and apoptosis. The number of autophagic vacuoles in one cell on average was calculated through TEM and it was found that the number soared in U87 cells caused by overexpressed p38, which could rescue the inhibition effects resulted from RND2 (Fig. 7A, B). Furthermore, we detected the autophagy-mediated protein and found that overexpressing p38 could rescue the inhibition of autophagy caused by RND2 (Fig. 7C). And 3-MA, the inhibitor of autophagy, decreased apoptosis caused by knockdown of RND2 in U87-MG (Fig. 7E, F) and the relative apoptosis rate decreased significantly between DMSO and 3-MA group (Fig. 7G). Additionally, western blotting analysis results revealed that 3-MA was significantly decreased the RND2-mediated GBM cells apoptosis (Fig. 7D).

In summary, all of the results demonstrated that RND2 could inhibit autophagy and decrease apoptosis through inhibiting p38 MAPK signaling pathway. Furthermore, RND2 associated autophagy inhibiting was part reason of GBM cells apoptosis resistance.
RND2 Predicted Poor Prognosis of Patients Suffering GBM.

In order to further evaluate RND2’s role in clinical samples, we performed RT-PCR assays to examine levels of the RND2 expression as shown before (Fig. 1F) in tumor tissues sampled from patients suffering primary GBM. In the experiment, we identified low-expression and high-expression of RND2 by RND2 mRNA expression levels, and we carry out Kaplan-Meier analysis (Table 1), which all the information is mentioned in TableS2. Kaplan-Meier curves showed that the patients in low-expression group survived significantly longer than the patients in the RND2 high-expression group (Fig. 8A). Additionally, the expression of RND2 had a positive correlation with tumor volume (Fig. 8B).

In a word, all these data revealed that RND2 could be defined as a biomarker of glioblastomas and would indicate the poor prognosis confronting with GBM patients.

Discussion

For the first time, our results showed that the RND2/p38/MAPK signaling axis regulated cell death including autophagic activities and apoptosis in GBM. The expression of RND2 upregulated in GBM was defined as a predictor of patients. Constitutively expressed or induced RND2 decreased the phosphorylation of p38 by interacting physically in this process, inhibiting GBM cell autophagy and apoptosis (Fig. 7H).

GBM is the most frequently seen malignant primary tumor in the central nervous system of adults. In ever-accelerated treatments, including radical surgery, radiotherapy and chemotherapy, the overall survival time of patients suffering from GBM only remains about 18 months (1). The evasion and resistance of apoptosis are hallmarks of malignant tumors (23), which inspires that apoptosis might be one therapeutic strategy of antitumor drugs. Besides, GBM cells are always in lack of intrinsic pathway apoptosis, which are results leads to chemo-resistance and treatment failure (24, 25).

Furthermore, autophagy is identified as type II programmed cell death, especially in cells with apoptosis deficiencies (26). However, other studies show that autophagy can inhibit the development of GBM in the early stage but promote the survival of GBM cells in the late stage. In recent years, clinical studies of autophagy inhibitors in the glioblastomas don’t get expected results, which means that the role of autophagy plays in cell death is complicated (27). Consequently, it is necessary to
explore more specific targets that regulate autophagy to inhibit the development of GBM. Hence, how to induce glioblastoma cell death by autophagy and apoptosis is an urgent problem to solve and is significant for clinical treatment.

RND2 is a member of RND subfamily, which is a subfamily of Rho GTPases. The main feature of RND2 is lack of intrinsic and GAP-stimulated GTPase activities (28). Besides, its function relies on not GDP/GTP exchange but transcriptional, post-translational, and post-transcriptional mechanisms (29). The activities of RND2 have been explored not only in normal tissue development but also in disease states (7). However, the activities of RND2 in cancer hitherto haven’t been demonstrated. What’s more, the mechanistic and direct role of RND2 in GBM tumorogenesis is totally unexplored. Up to now, only a few proteins including Rapo1lin, MgcRacGAP and Vps4-A have been identified as RND2 binding partner (9, 30, 31). Our study is the first time to advance the knowledge of RND2 with GBM and cell death. Our data showed that the expression levels of RND2 dramatically decreased in GBM compared with normal brain tissues. Besides, RND2 has negative correlation with patient prognosis while RND2 was positively correlated with the tumor size of tumors, which suggested RND2 as a potential target for treating GBM.

Our study identified and validated p38 as a RND2 substrate. The activity of p38 MAPK was related to the enhanced expression of autophagic markers (such as ATG5/ATG12 and LC3B) and apoptotic markers (such as PARP and caspase-3) (32, 33), thus, p38 MAPK is quite critical in regulating the balance of survival and cell death (21). As we all know, there are a lot of downstream substrates which could be regulated by p38 mitogen-activated protein kinase regulating various cellular processes through a cascade of complex phosphorylation (34). p38 signaling suppresses tumorigenesis and promotes apoptosis in various types of cancers. Consequently, it is important to figure out the mechanisms how specific substrates are recognized through regulating p38. There may be some factors, such as availability, concentration, and subcellular localization of upstream proteins (35), influencing the substrate selection. It’s found that p38 expresses in both the cytoplasm and the nucleus but mainly in the cytoplasm, this demonstrates that the distribution of p38 might be critical in regulating substrates. Hence, the potential binding partner of p38 may regulate p38 in different ways
such as phosphorylation sites and subcellular localizations (36). In our study, firstly, we identified that p38 was the substrate for RND2. Secondly, RND2 decreased the phosphorylation of p38 by directly binding with p38 in the cytoplasm of cancer cells. Third, RND2-mediated p38 MAPK signaling was critical for autophagy activities.

Our study further identified RND2/p38/MAPK signaling axis downregulating cell death, owing to that the inhibition of autophagy coincided with the inhibition of apoptosis and autophagy mediated cell death, in which the autophagy inhibition attenuated apoptosis. However, p38 played a dual role in autophagy. For example, phosphorylation of p38 could promote the expression of the key protein in autophagy which is Beclin-1, leading to the cell death (11), inversely, p38 MAPK inhibited autophagy by phosphorylating ULK1 (22).

Our data provided the significant evidence that RND2 inhibited autophagy through dephosphorylation of p38 specifically. Autophagy had significant functions under different pathological conditions but the crosstalk with apoptosis was still controversial (37). It has been pointed out that autophagy occurs before apoptosis, which was necessary condition. Autophagy suppressed the development of tumors by inhibiting the expression of the oncogenes and promoted pro-apoptotic factors to induce the cell death (38). However, chemo-resistance was one of the most common problem during anti-cancer therapy and autophagy was closely related to this process by avoiding apoptosis induced by chemotherapy in different cancers. Moreover, autophagy provided nutrients for cells in hypoxia or starvation environment to protect tumor cells from apoptosis in GBM (39). Besides, autophagy and apoptosis were transformed or synergistic under certain conditions. Apoptosis and autophagy could occur simultaneously to trigger cell death, and apoptosis could also accelerate the transformation of cells to autophagic cell death (40, 41). Above all, our study revealed that RND2/p38/MAPK signaling axis downregulated the autophagy and apoptosis at the same time and apoptosis could be influenced by RND2-mediated autophagy to improve the survival of GBM cells. Our results enriched the knowledge of the mechanism of how could autophagy be precisely regulated in glioblastomas, which may provide potential solution of chemo-resistance. In a summary, our data not only suggested RND2 as an alternative therapeutic target of malignant human cancers such as GBM,
but also provided a solid foundation for the development of compound targeting RND2, which could be transformed into clinical applications combined with chemotherapies.

Conclusion
In summary, our study showed that RND2 regulated the p38 MAPK pathway along with cell death in GBM. RND2 binds with p38 directly and inhibited the phosphorylation of p38 protein, leading to the inhibition of the p38 MAPK signaling pathway, which downregulated apoptosis and autophagy in GBM cells. In clinical GBM samples, RND2 was defined as an oncogene which predicted a poorer clinical outcome of patients. The new discoveries helped us comprehend the regulation of p38 MAPK signaling pathway in GBM. As a result, RND2 could be a potential therapeutic target to be used during the treatment of GBM.

Declarations

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Authors’ contributions
Baohui Liu and Qianxue Chen designed research. Yang Xu, Qian Sun and Fan’en Yuan carried out the experimental work. Huimin Dong, Yangzhi Qi, Huikai Zhang and Xiaoxing Xiong analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials
The data used to support the findings of this study are available from the corresponding author upon request.

Ethics approval and consent to participate
The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):
Institutional Ethics Committee of the Faculty of Medicine at Renmin Hospital of Wuhan University approval (2012LKSZ (010) H) to carry out the study within its facilities.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no conflict of interest.

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References

1. Ostrom QT, Gittleman H, Truitt G, Boscia A, Kruchko C, Barnholtz-Sloan JS. CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2011–2015. Neuro-oncology. 2018;20(suppl_4).
2. Aldape K, Zadeh G, Mansouri S, Reifenberger G, von Deimling A. Glioblastoma: pathology, molecular mechanisms and markers. Acta Neuropathol. 2015;129(6):829–48.
3. Wang Q, Hu B, Hu X, Kim H, Squatrito M, Scarpace L, et al. Tumor Evolution of Glioma-Intrinsic Gene Expression Subtypes Associates with Immunological Changes in the Microenvironment. Cancer cell. 2018;33(1):152.
4. Kornienko A, Mathieu V, Rastogi SK, Lefranc F, Kiss R. Therapeutic agents triggering nonapoptotic cancer cell death. Journal of medicinal chemistry. 2013;56(12):4823–39.
5. Wagner EF, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer. 2009;9(8):537-49.
6. Heng JI-T, Qu Z, Ohtaka-Maruyama C, Okado H, Kasai M, Castro D, et al. The zinc
finger transcription factor RP58 negatively regulates Rnd2 for the control of neuronal migration during cerebral cortical development. Cereb Cortex. 2015;25(3):806-16.

7. Heng JI-T, Nguyen L, Castro DS, Zimmer C, Wildner H, Armant O, et al. Neurogenin 2 controls cortical neuron migration through regulation of Rnd2. Nature. 2008;455(7209):114-8.

8. Chardin P. Function and regulation of Rnd proteins. Nat Rev Mol Cell Biol. 2006;7(1):54-62.

9. Fujita H, Katoh H, Ishikawa Y, Mori K, Negishi M. Rapostlin is a novel effector of Rnd2 GTPase inducing neurite branching. J Biol Chem. 2002;277(47):45428-34.

10. Xu Y, Jagannath C, Liu X-D, Sharafkhaneh A, Kolodziejska KE, Eissa NT. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity. 2007;27(1):135–44.

11. Tan P, He L, Xing C, Mao J, Yu X, Zhu M, et al. Myeloid loss of Beclin 1 promotes PD-L1hi precursor B cell lymphoma development. J Clin Invest. 2019;129(12):5261–77.

12. Trempolec N, Dave-Coll N, Nebreda AR. SnapShot: p38 MAPK signaling. Cell. 2013;152(3).

13. Bowman RL, Wang Q, Carro A, Verhaak RGW, Squatrito M. GlioVis data portal for visualization and analysis of brain tumor expression datasets. Neurooncology. 2017;19(1):139-41.

14. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. Acta Neuropathol. 2016;131(6):803-20.

15. Liu B, Dong H, Lin X, Yang X, Yue X, Yang J, et al. RND3 promotes Snail 1 protein degradation and inhibits glioblastoma cell migration and invasion. Oncotarget. 2016;7(50):82411-23.
16. Ricci JE, Munoz-Pinedo C, Fitzgerald P, Bailly-Maitre B, Perkins GA, Yadava N, et al. Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain. Cell. 2004;117(6):773–86.

17. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy. 2016;12(1).

18. Liu B, Lin X, Yang X, Dong H, Yue X, Andrade KC, et al. Downregulation of RND3/RhoE in glioblastoma patients promotes tumorigenesis through augmentation of notch transcriptional complex activity. Cancer Med. 2015;4(9):1404–16.

19. Sun Q, Dong H, Li Y, Yuan F, Xu Y, Mao S, et al. Small GTPase RH0E/RND3, a new critical regulator of NF-kappaB signalling in glioblastoma multiforme? Cell proliferation. 2019;52(5):e12665.

20. Mai WX, Gosa L, Daniels VW, Ta L, Tsang JE, Higgins B, et al. Cytoplasmic p53 couples oncogene-driven glucose metabolism to apoptosis and is a therapeutic target in glioblastoma. Nat Med. 2017;23(11):1342–51.

21. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science. 2002;298(5600):1911–2.

22. He Y, She H, Zhang T, Xu H, Cheng L, Yepes M, et al. p38 MAPK inhibits autophagy and promotes microglial inflammatory responses by phosphorylating ULK1. J Cell Biol. 2018;217(1):315–28.

23. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646–74.

24. Krakstad C, Chekenya M. Survival signalling and apoptosis resistance in glioblastomas: opportunities for targeted therapeutics. Mol Cancer. 2010;9:135.
25. Mao H, Lebrun DG, Yang J, Zhu VF, Li M. Deregulated signaling pathways in glioblastoma multiforme: molecular mechanisms and therapeutic targets. Cancer Invest. 2012;30(1):48–56.

26. Kim M, Jung J-Y, Choi S, Lee H, Morales LD, Koh J-T, et al. GFRA1 promotes cisplatin-induced chemoresistance in osteosarcoma by inducing autophagy. Autophagy. 2017;13(1):149–68.

27. Ulasov IV, Lenz G, Lesniak MS. Autophagy in glioma cells: An identity crisis with a clinical perspective. Cancer Lett. 2018;428:139–46.

28. Riou P, Villalonga P, Ridley AJ. Rnd proteins: multifunctional regulators of the cytoskeleton and cell cycle progression. Bioessays. 2010;32(11):986–92.

29. Jaffe AB, Hall A. Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol. 2005;21:247–69.

30. Naud N, Touré A, Liu J, Pineau C, Morin L, Dorseuil O, et al. Rho family GTPase Rnd2 interacts and co-localizes with MgcRacGAP in male germ cells. Biochem J. 2003;372(Pt 1):105–12.

31. Tanaka H, Fujita H, Katoh H, Mori K, Negishi M. Vps4-A (vacuolar protein sorting 4-A) is a binding partner for a novel Rho family GTPase, Rnd2. Biochem J. 2002;365(Pt 2):349–53.

32. Trempolec N, Dave-Coll N, Nebreda AR. SnapShot: p38 MAPK substrates. Cell. 2013;152(4).

33. Kamel WA, Sugihara E, Nobusue H, Yamaguchi-Iwai S, Onishi N, Maki K, et al. Simvastatin-Induced Apoptosis in Osteosarcoma Cells: A Key Role of RhoA-AMPK/p38 MAPK Signaling in Antitumor Activity. Mol Cancer Ther. 2017;16(1):182–92.

34. Bardwell L. Mechanisms of MAPK signalling specificity. Biochem Soc Trans. 2006;34(Pt 5):837–41.
35. Ono K, Han J. The p38 signal transduction pathway: activation and function. Cell Signal. 2000;12(1).

36. Coulthard LR, White DE, Jones DL, McDermott MF, Burchill SA. p38(MAPK): stress responses from molecular mechanisms to therapeutics. Trends Mol Med. 2009;15(8):369–79.

37. Strickland M, Stoll EA. Metabolic Reprogramming in Glioma. Frontiers in cell developmental biology. 2017;5:43.

38. Edinger AL, Thompson CB. Defective autophagy leads to cancer. Cancer cell. 2003;4(6):422–4.

39. Hombach-Klonisch S, Mehrpour M, Shojaei S, Harlos C, Pitz M, Hamai A, et al. Glioblastoma and chemoresistance to alkylating agents: Involvement of apoptosis, autophagy, and unfolded protein response. Pharmacol Ther. 2018;184:13–41.

40. Doherty J, Baehrecke EH. Life, death and autophagy. Nat Cell Biol. 2018;20(10):1110–7.

41. Denton D, Kumar S. Autophagy-dependent cell death. Cell Death Differ. 2019;26(4):605–16.

Tables
Table 1 Kaplan-Meier analysis of 41 clinical GBM patients
| Clinicopathologic variables | Number | Average survival time | P value |
|-----------------------------|--------|-----------------------|---------|
| All cases                   | 41     | 16.475±2.600          | 0.382   |
| Age at diagnosis (years)    |        |                       |         |
| <55                         | 19     | 12.888±1.908          |         |
| ≥55                         | 22     | 20.189±4.422          |         |
| Gender                      |        |                       | 0.618   |
| Male                        | 22     | 18.556±4.335          |         |
| Female                      | 19     | 14.289±2.814          |         |
| KPS                         |        |                       | 0.736   |
| ≤70                         | 8      | 15.000±4.979          |         |
| ≥70                         | 33     | 17.698±3.306          |         |
| Headache                    |        |                       | 0.904   |
| Yes                         | 18     | 12.752±1.692          |         |
| No                          | 23     | 17.195±3.676          |         |
| Intracranial infection      |        |                       | 0.052   |
| Yes                         | 3      | 6.000±4.041           |         |
| No                          | 38     | 17.352±2.759          |         |
| Multiple lesions            |        |                       | 0.746   |
| 1                           | 33     | 16.708±2.740          |         |
| ≥2                          | 8      | 10.250±1.980          |         |
| Volume                      |        |                       | 0.192   |
| ≥42                         | 20     | 12.075±2.216          |         |
| ≤42                         | 21     | 19.473±4.138          |         |
| Course time[d]              |        |                       | 0.584   |
| ≥30                         | 33     | 15.996±2.907          |         |
| ≤30                         | 8      | 17.469±4.948          |         |
| RND2 expression             |        |                       | 0.151   |
| High                        | 14     | 10.399±1.738          |         |
| Low                         | 27     | 18.820±3.488          |         |
|                            | 20     | 9.307±1.189           | 0.017*  |
|                            | 21     | 21.296±3.981          |         |

Supplemental Figure Legends

**Figure S1. The expression of RND2 in normal tissue.** (A) Expression of RND2 in normal human tissues according to HPA database. (B-C) mRNA and protein levels of RND2 from different organs of C57 mouse; (D-E) The immunofluorescent staining showed the relationship between RND2 and GFAP, NSE. Scale bar 50μm.

**Figure S2. RND2 knockdown induced apoptosis and autophagy in U251 cells.** (A) U251 cells were transfected with negative control shRNA (shCtrl) or shRNAs against RND2 (shRND2-1 and shRND2-2), followed by Annexin V-PE/7-AAD staining and flow cytometric analysis. Cell apoptosis was calculated by FACS. **P < 0.01, *P < 0.05. (C) Effects of RND2 overexpression and knockdown on the levels of apoptosis-related proteins in U251 cells. (F) Effects of RND2 overexpression and knockdown on the levels of autophagy-related proteins in U251 cells shCtrl: negative control shRNA. shRND2-1 and shRND2-2: two shRNAs against RND2. pcDNA3.1: the control group. Flag-RND2: RND2 overexpression group. All bar plot data are the means ± SD. The data and graphs are representative of three independent experiments with similar results.
**Figure S3.** RND2 inhibited autophagy in clinical samples. (A-B) Correlation of mRNA levels between RND2 and tumor volume in clinical GBM patients. (C-D) Correlations of IHC data for high or low RND2 expression relative to level of p62 or LC3B, and high or low p-ATG4B expression relative to level of LC3B.

**Figure S4.** RND2 overexpression didn’t regulate key proteins in GBM. (A-G) RND2 overexpression didn’t change Hes1, IL-8, KRas, MMP-2, P21, Slug and Smad4 in mRNA level. (H-G) RND2 didn’t interact with MDM2, Snail and TP53 in GBM cells.

**Figure S5.** RND2 interacts with p38 physically and inhibits p38 phosphorylation in vitro. (A) The co-localization of RND2 and p38 in GBM patient tissues was observed by immunofluorescence, Scale bar 50μm. (B-C) The statistical descriptions of RND2 and p38 co-localization in U87 cells. (D) Co-immunoprecipitation assays in U251 cells determined the physical interaction between RND2 and p38 in U251 cells. (E) The endogenous RND2 was expressed not only mainly in cytoplasm but also in cellular membrane; (F) Effects of RND2 on p-p38 expression in U87 cells according to immunofluorescence. Scale bars, 10μm. (G) Effects of RND2 overexpression and knockdown on the protein levels of p-p38 and p38 in U251 cells.

**Figure S6.** p38 rescued RND2 mediated apoptosis and autophagy in U251 cells. (A) Overexpressing p38 rescued the inhibition of autophagy-related protein caused by RND2 in U251 cells; D. 3-MA decreases the apoptosis-related protein caused by RND2; (B) 3-MA downregulated the RND2-mediated apoptosis in U251 cells and the relative apoptosis rate significant downregulated by 3-MA.

**Figure S7.** Detecting RND2 in RND2 stable overexpression cells. A, B. Efficacy of RND2 overexpression and the process was ensured by western blot and RT-PCR.

Figures
Figure 1
Rnd2 is upregulated in human GBM. (A) Expression of RND2 in 14 categories of human cancers according to TCGA database. (B) Comparison of RND2 expression levels between GBM and normal brain tissues. (C) Comparison of RND2 expression levels between GBM MES, PN, or CL subtypes. Boxplots indicate the median quartiles, with whiskers extending the minimum and maximum range. (D) Representative IHC staining images for RND2 in clinical tissues. Grade II, Grade III and grade IV indicate the pathologic grades of the glioma samples. Scale bars, 20 μm. (E) IHC score of RND2 in clinical tissues. The IHC scores were graded as 0, 1, 2, 3 and 4. Non-tumor tissue, n = 14; WHO II, n = 31; WHO III, n=41 and WHO IV, n = 54. **P < 0.01, ***P < 0.001, ****P < 0.0001. (F) Real-time qPCR indicated significantly higher RND2 mRNA levels in GBM (n = 41) tissues and LGG (n=14) compared to non-tumor (n = 4) tissues. RND2 mRNA expression was normalized to GAPDH using the 2-ΔΔCt method. **P < 0.01, ****P < 0.0001.
RND2 knockdown induced GBM cell apoptosis in vitro. (A-B) Effects of RND2 knockdown on cleaved Caspase-3 expression in U87 cells according to immunofluorescence. Scale bars, 100μm. **P < 0.01. (C-D) Effects of RND2 knockdown on the ΔΨm in U87 cells according to JC-1 staining. A decrease in the ratio of red (aggregates)/green (monomers) fluorescence intensity indicates the loss of ΔΨm. Scale bars, 50 μm. **P < 0.01. (E-F) U87 cell death were detected by TUNEL staining. Scale bars, 50 μm. Statistical analysis of positive rate was showed. **P < 0.01. (G-H) U87 cells were transfected with negative control shRNA (shCtrl) or shRNAs against RND2 (shRND2-1 and shRND2-2), followed by Annexin V-PE/7-AAD staining and flow cytometric analysis. Cell apoptosis was calculated by FACS. **P < 0.01. (E) Effects of RND2 overexpression and knockdown on the levels of apoptosis-related proteins in GBM cells. shCtrl: negative control shRNA. shRND2-1 and shRND2-2: two shRNAs against RND2. pcDNA3.1: the control group. Flag-RND2: RND2 overexpression group. All bar plot data are the means ± SD. The data and graphs are representative of three independent
experiments with similar results.
RND2 overexpression reduces GBM cell autophagy in vitro. (A) Representatives of IHC staining showed RND2 expression in the same location inversely correlates with LC3B but positively correlates with p62. Scale bars, 50 μm. (B) Effects of RND2 knockdown on LC3B expression in U87 cells according to immunofluorescence. Scale bars, 100μm. (C) The numbers of GFP-LC3 puncta were quantified using confocal laser scanning microscopy in U87-MG cells transfected with GFP-LC3. Scale bars, 10 μm. (D) Effects of RND2 overexpression on the levels of autophagy-related proteins in GBM cells. (E) Effects of RND2 knockdown on the levels of autophagy-related proteins in GBM cells. (F-G) Transfected cells were prepared for transmission electron microscopy analysis. The number of autophagic vacuoles was analyzed and the red arrows indicated autophagic vacuoles. **P < 0.01.

shCtrl: negative control shRNA. shRND2-1 and shRND2-2: two shRNAs against RND2. pcDNA3.1: the control group. Flag-RND2: RND2 overexpression group. All bar plot data are the means ± SD. The data and graphs are representative of three independent experiments with similar results.
RND2 overexpression attenuates autophagy and autophagy in an Intracranial Xenograft Model. (A) Mouse survival is shown by Kaplan-Meier curves. GFP groups, n=12. GFP-RND2 groups, n = 12. *P < 0.05. P values were calculated using the log-rank test. (B). Representative images of mouse brain sections from mice intracranially implanted with the GFP and GFP-RND2 with indicated modification and quantification of tumor volume. Scale bars, 1.0 mm. (C) Representative images of mouse brains. (D) The weight of tumors (GFP/GFP RND2 group – PBS group) were analyzed. **P < 0.01. (E) IHC analyses of RND2, p62, LC3B, cleaved caspase-3 and Bcl-2 in mouse tumor sections.
RND2 interacts with p38 physically and inhibits p38 phosphorylation in vitro and in vivo. (A) Immunofluorescent staining (IF) analyses of co-localization of exogenous RND2 and p38 in U87 cells, Scale bar 10μm. (B). Co-immunoprecipitation assays in U87 cells determined the physical interaction between RND2 and p38. (C) Effects of RND2 overexpression and knockdown on the protein levels of p-p38 and p38 in U87 cells. (D) IHC analyses of RND2, p-p38 and p38 in mouse tumor sections. shCtrl: negative control shRNA. shRND2-1 and shRND2-2: two shRNAs against RND2. pcDNA3.1: the control group. Flag-RND2: RND2 overexpression group. All bar plot data are the means ± SD. The data and graphs are representative of three independent experiments with similar results.
Figure 6

Inhibiting p38 phosphorylation reduced autophagy in vitro. (A-B) Effects of p38 de-phosphorylation on LC3B expression in U87 cells according to immunofluorescence. Scale bars, 100μm. **P < 0.01. (C-D) Cells were prepared for transmission electron microscopy analysis. The number of autophagic vacuoles was analyzed and the red arrows indicated autophagic vacuoles. **P < 0.01. (E) Effects of p38 de-phosphorylation on the levels of autophagy-related proteins in U87 cells.
RND2 attenuates autophagy through inhibiting the p38 MAPK signaling pathway activation.
(A-B) p38 rescued cell autophagy induced by RND2 expression. Cells were prepared for transmission electron microscopy analysis. The number of autophagic vacuoles was analyzed and the red arrows indicated autophagic vacuoles. **P < 0.01. C. Overexpressing p38 rescued the inhibition of autophagy-related protein caused by RND2 in U87 cells; D. 3-MA decreases the apoptosis-related protein caused by RND2; (E-G). 3-MA downregulated the RND2-mediated apoptosis in U87MG cells and the relative apoptosis rate significant downregulated by 3-MA. Cell apoptosis was calculated by FACS. **p<0.01, ***p<0.001; H. Mechanistic model for the RND2 regulation of cell autophagy and apoptosis in GBM.

![Graph](image)

**Figure 8**

RND2 Predicted Poor Prognosis of Patients with GBM. A. Kaplan-Meier analyses for GBM patients with tumors expression high or low level of RND2. p<0.05; B. Correlation of expression levels between RND2 and tumor volume in clinical GBM patients.

**Supplementary Files**

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

- u87STR.pdf
- U251STR.pdf
- SupplementaryTableS2.xlsx
- SupplementaryTableS3.xlsx
