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Original Research

Influence of RRM, RGG and Potential Phosphorylated Sites in Cold-Inducible Protein RBM3 on its Subcellular Localization and Neuroprotective Effects

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Abstract

Background: As a potent mediator of hypothermic neuroprotection, the cold-inducible protein RBM3 is characterized with one RNA-recognition motifs (RRM) and one arginine-glycine-rich (RGG) domain. It is known that these conserved domains are required for nuclear localization in some RNA-binding proteins. However, little is known about the actual role of RRM and RGG domains in subcellular localization of RBM3. **Methods**: To clarify it, various mutants of human *Rbm3* gene were constructed. Plasmids were transfected into cells and the localization of RBM3 protein and its varias mutants in cells and role in neuroprotection. **Results**: In human neuroblastoma SH-SY5Y cells, either a truncation of RRM domain (aa 1–86) or RGG domain (aa 87–157) led to an obvious cytoplasmic distribution, compared to a predominant nuclear localization of whole RBM3 protein (aa 1–157). In contrast, mutants in several potential phosphorylated sites of RBM3, including Ser102, Tyr129, Ser147, and Tyr155, did not alter the nuclear localization of RBM3. Similarly, mutants in two Di-RGG motif sites also did not affect the subcellular distribution of RBM3. Lastly, the role of Di-RGG motif in RGG domains was further investigated. The mutant of double arginines in either Di-RGG motif-1 (Arg87/90) or -2 (Arg99/105) exhibited a higher cytoplasmic localization, indicating that both Di-RGG motifs are required for nucleic localization of RBM3. **Conclusions**: Our data suggest that RRM and RGG domains are both required for the nuclear localization of RBM3, with two Di-RGG domain being crucial for nucleocytoplasmic shuttling of RBM3.

Keywords: RBM3; subcellular localization; neuroprotective; RGG; RRM

1. Introduction

Mild hypothermia (32–35 °C) has been frequently used as an effective treatment for various nerve injuries [1]. During hypothermia, a subgroup of proteins including RNA-binding motif protein 3 (RBM3), is substantially induced, while the global protein synthesis is decreased [2,3]. Mild hypothermia is a clinically recognized treatment that is commonly used to relieve nerve damage caused by ischemia and hypoxia. Many studies have shown that RBM3, a cold stress protein, mediates the neuroprotective effects of mild hypothermia largely [4–8]. Thus, it is speculated that RBM3 induction may represent a new strategy for therapy of neural injuries in place of mild hypothermia [9].

RNA-binding protein (RBP) plays an important role in the cell cycle. It is involved in RNA post-transcriptional regulation and translation processes [10,11]. RBM3 is such a kind of RNA-binding protein consisting of one RRM (RNA recognition motif) domains at N-terminus and two arginine/glycine-rich (RGG) domains at C-terminus [12,13]. The RRM motif, also known as RBM (RNA-binding motif) or RNP (ribonucleoprotein domain), is a well-defined RNA-binding domain mainly presented in eukaryotic genes, especially in higher vertebrate. RRM motifs function in post-transcriptional gene expression processes by interacting with nucleic acids or other proteins [14–17]. The RGG domain is also an RNA-binding motif found in many RBPs [18]. Since RGG motifs were first described, they have been identified in many other proteins, often occurring as multiple repeats [19]. Proteins containing RGG motif are involved in mRNA processing [20]. The accumulated data showed RBM3 is predominantly localized in nucleus [21], but sometimes it can translocate to cytoplasm, indicating that RBM3 is a nuclear-cytoplasmic shuttling protein. However, the actual role of RRM and RRG domains in RBM3 subcellular localization remains largely unknown.

In addition to RRM and RGG domains, prediction of RBM3 molecules with the softwares KinasePhos and Uniprot show that RBM3 contains various potential post-translational modification (PTM) sites, including phosphorylation sites on Ser102(S), Tyr129(Y), Ser147(S), Tyr155(Y) amino acids and arginine methylation sites on Arg87 and 90 in Di-RGG1 motif, and Arg99 and 105 in Di-RGG2 motif. It would be interesting to determine whether

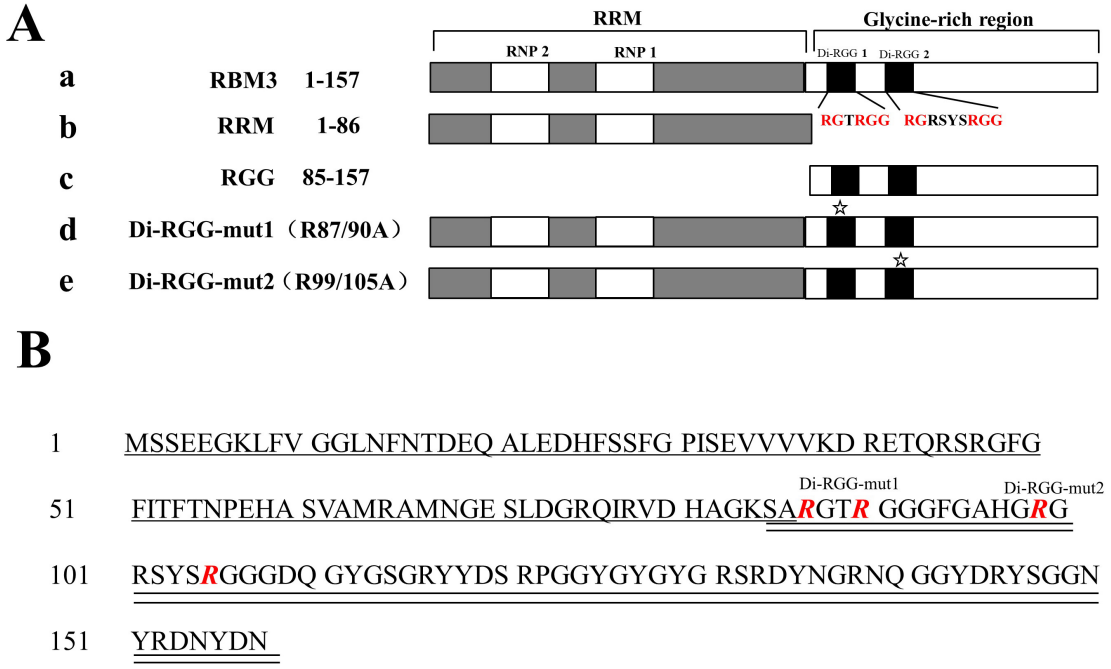

Fig. 1. RBM3 domain deletion and RGG domain arginine mutant. (A) Mutant pattern diagram, wild type RBM3 (a), RBM3 with RRM domain only (b), RBM3 with RGG domain only (c), RBM3 with the first Di-RGG motif mutation (d), RBM3 with mutation in the second Di-RGG motif (e) The mutation position is marked as "☆". (B) Schematic structure of RBM3 with RGG domain sequence: the Di-RGG motif is indicated by double underline, and the alanine mutated arginine in the R-A mutant is indicated by red italic bold.

these potential PTM sites affect the nuclear-cytoplasmic shuttling of RBM3.

Using traditional gene mutagenesis technique, we constructed various mutants of RBM3, including truncation, point mutation or deletion. With these mutants of RBM3, we attempted to determine the effects of different domains and potential PTM sites on RBM3 subcellular localization. In addition, we would examine the influence of these mutants on the protective effect of RBM3 using staurosporine-insulted SH-SY5Y as a cell model.

2. Materials and Methods

2.1 Materials

Antibody against RBM3 (ab134946) was purchased from Abcam (Cambridge, MA, USA), and the antibodies against cleaved PARP (#9541), mCherry (#43590), and β-actin (#4970) were purchased from Cell Signaling Technologies (Beverly, MA, USA). Antibodies against LaminB1 (66095) and Myc-tag (16286) were purchased from Proteintech Group Inc. (Boston, MA, USA). Staurosporine (STS) (STS, AM-2282) was purchased from MedChemExpress (New, NJ, USA).

2.2 Cell Culture and Drug Treatment

Human neuroblastoma cell line SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C. For immunoblotting of cleaved poly ADP-ribose polymerase (PARP), cells were harvested 24 h after addition of 0.5 μM STS.

2.3 Plasmids and Transient Transfection

RBM3 transcript from SH-SY5Y cells was amplified using RT-PCR, sequenced, and then subcloned into the pXJ40-Myc or pmCherry vector. RRM or RGG-truncated constructs were generated using PCR with RBM3 as templates and inserted into pXJ40-Myc or pmCherry vector. Di-RGGmut1 (R87/90A) Di-/RGGmut2 (R99/105A) point mutations were generated using a PCR-based site-directed mutagenesis. Cells cultured in six-well plate were transfected with plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4 Cell Viability Assay

Cell viability was determined with Cell Counting Kit-8 (CCK-8) assay following the manufacturer's procedures. In brief, cells transfected with different plasmids were seeded into 96-well plates and incubated with fresh medium containing STS or dimethyl sulfoxide (DMSO) for 24 hours at 37 °C. The CCK-8 was then added to the medium and the absorbance was measured at a wavelength of 450 nm by a SepctraMax Plus Absorbance Microplate Reader (Molecular Devices, San Jose, CA, USA).

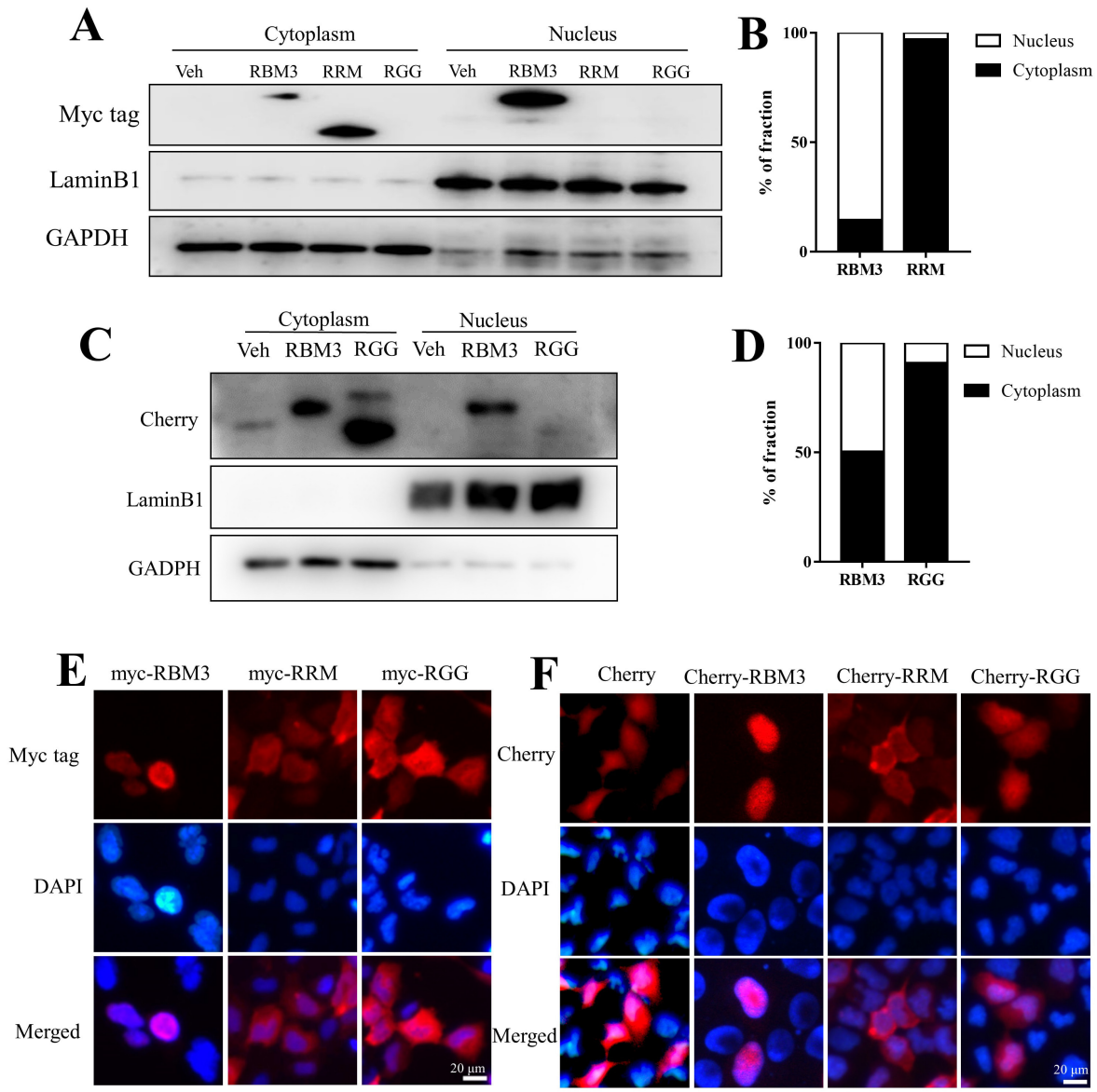

Fig. 2. The effect of RBM3 domain deletion on subcellular localization. (A,C) Subcellular localization of RBM3, RRM mutants and RGG mutants were determined by Western blots with myc tag antibody or cherry tag antibody. LaminB1 and GAPDH were used as nuclear loading control and cytosolic loading control, respectively. (B,D) Blots density was quantified and normalized to LaminB1 or GAPDH, respectively. (E) Distribution of myc-RBM3 fusion protein, RRM mutants (myc-RRM) and RGG mutants (myc-RGG) in cells. (F) Distribution of Cherry alone, Cherry-RBM3 fusion protein, RRM mutants (Cherry-RRM) and RGG mutants (Cherry-RGG) in cells. Scale bar, 20 μm.

2.5 Cellular Fluorescence

Cells were seed onto cell slides in a six-well plate and cultured for 24 h, the RBM3 mutant plasmids were transfected into the cells for 2 d, and images were collected using a fluorescent inverted microscope (Carl Zeiss, White Plains, NY, USA).

2.6 Western Blot

After rinsing twice with cold phosphate buffer saline (PBS), cells were collected in cold lysis buffer by centrifuging at 15,000 g for 15min. Protein concentration was determined using Bradford assay. 40 μg of protein sample was resolved by electrophoresis on 8–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, CA, USA). The membrane was blocked with 5% skim milk and then incubated with different primary antibodies overnight. After washing with PBS for 30min, the membrane was further incubated with HRP-conjugated secondary antibodies for 1 h and visualized with enhanced chemiluminescence (ECL) kits (Wanleibio Biotechnology Co, Shenyang, China) by a luminescence

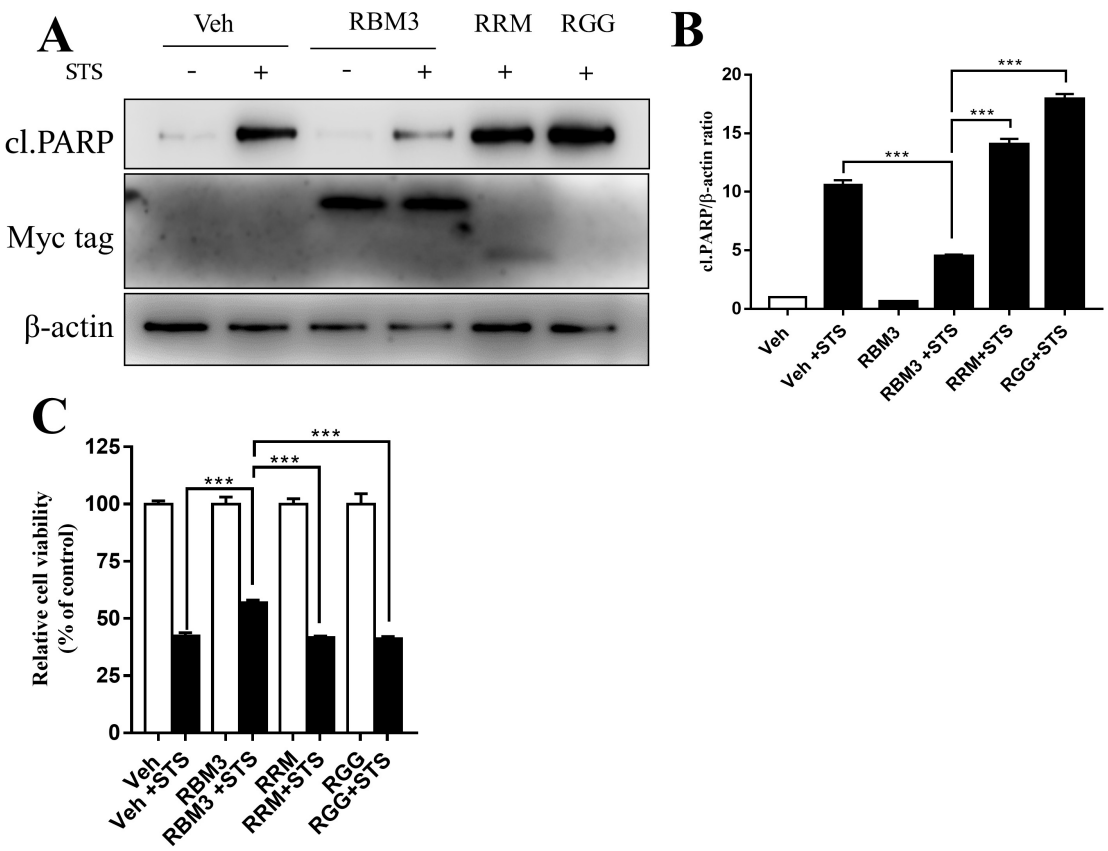

Fig. 3. The effect of RBM3 domain deletion on neuroprotection. (A) Cleavage of PARP (cl.PARP) in SH-SY5Y cells transfected with plasmids (pXJ40-myc (Veh), pXJ40-myc-RBM3 (RBM3), pXJ40-myc-RRM (RRM) and pXJ40-myc-RGG (RGG)) were detected by western blot with anti-PARP after treatment with STS (0.5 μM) for 24 h. β-actin was used as a loading control. (B) Quantitative analysis of the cleavage of PARP (cl.PARP) levels in the cells. (C) Cell viability was detected with CCK-8 assay. Values were expressed as mean $\pm$ SD obtained from three different experiments performed in triplicate. ***$p < 0.001$, compared to cells RBM3 overexpressing cell treated with STS (RBM3 + STS).

imager, Amersham Imager 600 (GE Healthcare, Chicago, IL, USA). Finally, it was quantified and analyzed by ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.7 Statistical Analysis

All the experiments were performed at least three times. Data were examined by Student's t test and expressed as mean $\pm$ standard deviation (SD). p value < 0.05 was considered statistically significant.

3. Results

3.1 Both RRM and RGG Domains are Required for Nuclear Localization of RBM3

To determine the influence of RGG and RRM domains on the nuclear localization of RBM3, truncations encoding RRM (AA1-86) and RGG (AA87-157) were cloned into pXJ40-myc, respectively (Fig. 1A). After transfection, the cytoplasmic protein and the nuclear protein were separated. As shown in Fig. 2A,B, RRM protein is almost completely localized in the cytoplasm (96.95%), compared with full-length RBM3 (14.46%). However, RGG could not be detected by Western blot due to its small molecular weight (9 kD). Hence, we cloned RGG DNA to pmCherry, and the Western blot showed RGG protein is largely localized in the cytoplasm (77.75%), compared with full-length RBM3 (Fig. 2C,D). Immunofuorescence double labelling showed that RRM and RGG protein are also largely localized in the cytoplasm (Fig. 2E,F). These data suggest that both RRM and RGG domains are required for RBM3 nuclear localization.

3.2 Both RRM and RGG Domains are Required to Prevent STS-Induced Apoptosis

It has been reported that RBM3 can inhibit STS-induced apoptosis in neural cells [21]. To determine the role of RGG and RRM domains in neuroprotective effects of RBM3, SH-S5Y neural cells were transfected with different RBM3 mutant plasmids mentioned above. Western blot showed that STS-induced PARP cleavage was significantly increased after overexpression of RRM and RGG mutant plasmids compared with wild-type RBM3 (Fig. 3A,B). Consistently, CCK-8 assays obtained same re-

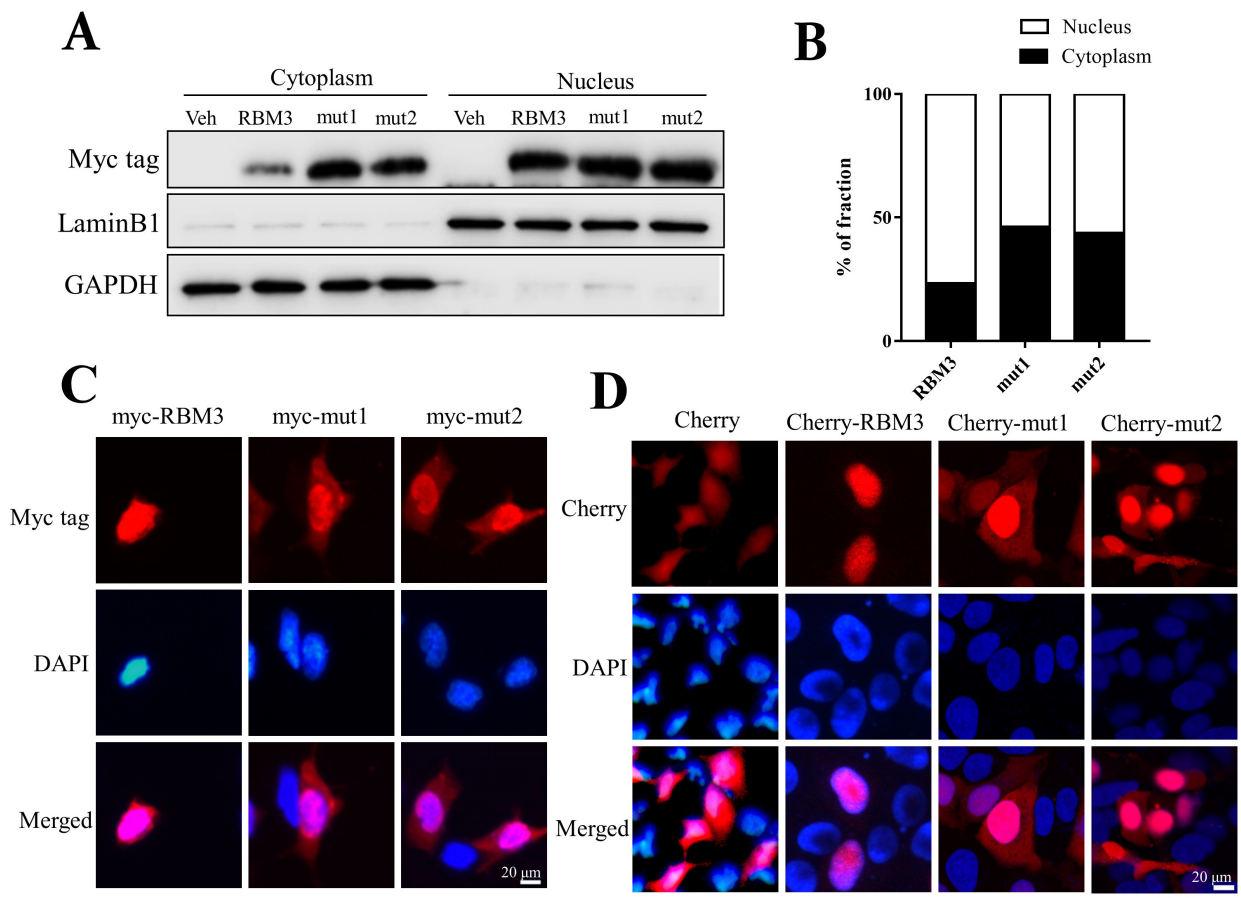

Fig. 4. The effect of mutation of Di-RGG motif in RBM3 on localization. (A) Subcellular localization of RBM3, Di-RGG mut 1 (mut1) and Di-RGG mut 2 (mut2) were determined by Western blots with myc tag antibody. LaminB1 and GAPDH were used as nuclear loading control and cytosolic loading control, respectively. (B) Blots density was quantified and normalized to LaminB1 or GAPDH, respectively. (C) Distribution of myc-RBM3 fusion protein, Di-RGG mut 1 (myc-mut1) and Di-RGG mut 2 (myc-mut2) in cells. (D) Distribution of Cherry alone, Cherry-RBM3 fusion protein, Di-RGG mut 1 (Cherry-mut1) and Di-RGG mut 2 (Cherry mut2) in cells. Scale bar, 20 μm.

sults (Fig. 3C). Together, the results indicate that both RGG and RRM domains are required for exerting the neuroprotective process of RBM3.

3.3 The Di-RGG Motif is Indispensable for RBM3 Nuclear Localization

To determine the influence of the two Di-RGG motifs on the nuclear localization of RBM3, mutations (two Arg were replaced by two Ala) encoding R87/90A of mut1 and R99/105A of mut2 were cloned into pXJ40-myc, respectively (Fig. 1B). After transfection, the cytoplasmic protein and the nuclear protein were separated. As shown in Fig. 4A, the signals for Di-RGG mut1 and Di-RGG mut2 detected in the cytoplasm were increased by 22.8% and 20.3%, respectively, compared to wild type RBM3 (Fig. 4B). Immunofuorescence double labelling showed that Di-RGG motif mutations proteins are also largely localized in the cytoplasm (Fig. 4C,D). These data suggest that two Di-RGG are required for RBM3 nuclear localization.

3.4 Neuroprotection of STS by RBM3 does not Require the Di-RGG Motif

Next, we determine the potential role of Di-RGG motif in RBM3-conferred neuroprotective effcts unclear. The Di-RGG motif mutant plasmids Di-RGG mut1 and Di-RGG mut2 were transiently transfected into cells. After STS insult, the cleaved PARP was detected by Western blot (Fig. 5A). It was found that compared with the wild-type, the two Di-RGG motif mutants had no significant difference in the levels of cleaved PARP (Fig. 5B). Similarly, the same results were observed in CCK-8 detection (Fig. 5C). Therefore, the role of arginine in the Di-RGG motif may be limited to nuclear localization and has no contribution for neuroprotective effect of RBM3.

3.5 Mutants at the Potential Phosphorylation Site of RBM3 did not Alter the Nuclear Localization of RBM3

Protein phosphorylation is the most important way of PTM [22,23]. Being a molecular switch, protein phosphorylation regulates the biological activity of various pro-

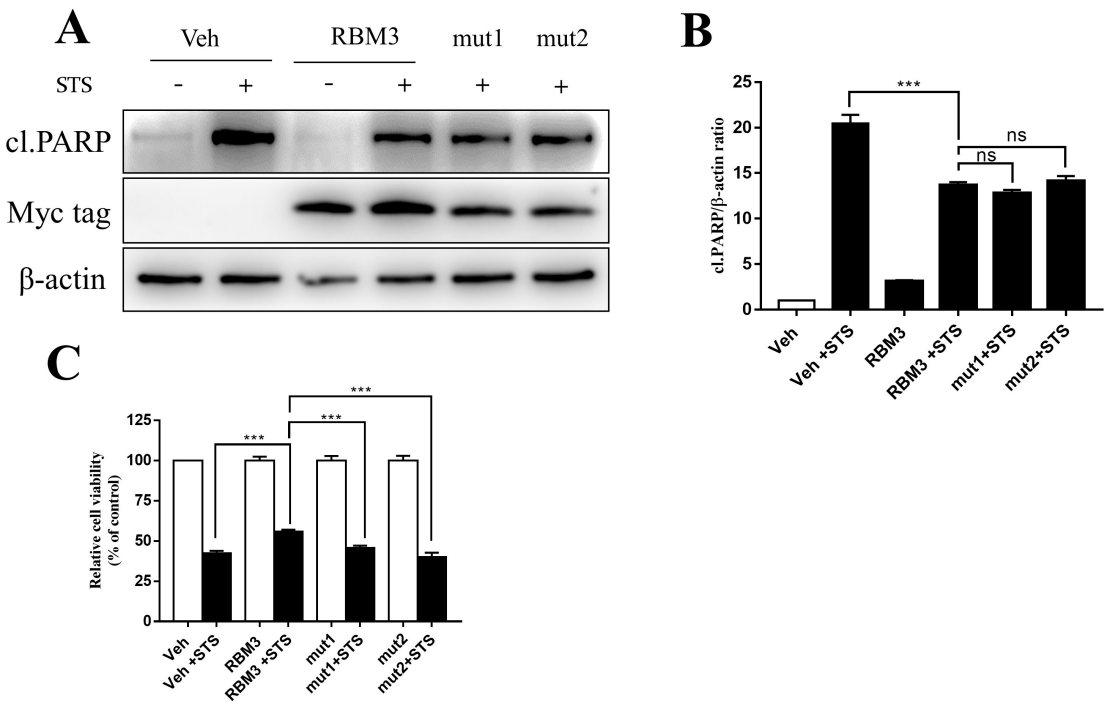

Fig. 5. The effect of mutation of Di-RGG motif in RBM3 on neuroprotection. (A) Cleavage of PARP (cl.PARP) in SH-SY5Y cells transfected with plasmids (pXJ40-myc (Veh), pXJ40-myc-RBM3 (RBM3), pXJ40-myc-Di-RGG mut1 (mut1) and pXJ40-myc- Di-RGG mut2 (mut2)) were detected by western blot with anti-PARP after treatment with STS (0.5 μM) for 24 h. β-actin was used as a loading control. (B) Quantitative analysis of the cleavage of PARP (cl.PARP) levels in the cells. (C) Cell viability was detected with CCK-8 assay. Values were expressed as mean $\pm$ SD obtained from three different experiments performed in triplicate. n.s. not significant and ***$p < 0.001$, compared to cells RBM3 overexpressing cell treated with STS (RBM3 + STS).

tein [24]. Uniprot analysis shows RBM3 has four potential phosphorylation sites (Ser102, Tyr129, Ser147, and Tyr155), but their role in RBM3 subcellular localization is to be investigated. In the way, we constructed plasmids with four phosphorylation sites mutated respectively. After transfection, the cytoplasmic protein and the nuclear protein were separated. Unfortunately, no obvious change in RBM3 subcellar localization could be observed between wild type RBM3 and the four mutants of RBM3 (Fig. 6A–D). In conclusion, these mutants in phosphorylation site may does not contribute in RBM3 subcellular localization.

4. Discussion

RBM3 was first identified as a gene linked to X-chromosome linked gene tlocated at region Xp11.23 on the short arm. RBM3 was found to be up-regulated even when the total protein decreased during hibernation, and up-regulation of RBM3 was also found under conditions such as ischemia and radiation [25]. Up-regulation of RBM3 in extreme cases is to some extent a protective measure for cells. In Diego Peretti's research [6], it was found that RBM3 can affect the repair of nerve cell synapses, and once RBM3 is missing or RBM3 cannot function normally, it will accelerate the damage of related pathological models and accelerate the occurrence of diseases. Numerous experiments have shown that RBM3 is important for cellular stress, but the subcellular localization of RBM3 remains unclear [1,6,13,26–28]. Smart *et al.* [29] discovered two distinct RBM3 spliceosomes, "Arg-" and "Arg+", in their research. In a follow-up study, it was found that the "Arg-" isoform of RBM3 exhibited higher dendritic localization in primary cortical neurons, compared to the "Arg+" isoform. Si *et al.* [30] found that RBM3 can localize in the cytoplasm and inhibit oxygen-glucose deprivation-induced apoptosis by regulating the formation of stress granules. Our experimental results show that the two domains of RBM3 protein, RRM and RGG, both play a decisive role in the localization of RBM3 protein to the nucleus.

The RRM is widely presented in eukaryotes and composed of two a-helices and four β-sheets. Bhatter *et al.* [31] found that deletion of RRM domains compromised the ability of Sbp1 to induce growth defects in yeast by preventing localization of Sbp1 to RNA granules upon glucose starvation. Eulalio *et al.* [32] showed that the RRM in GW182 protein contributes to miRNA-mediated gene silencing through protein-RNA interaction. In addition, the RRM domain can also stabilize the structure of the protein [33]. Our experimental results confirmed that the RRM domain in RBM3 plays a decisive role in the localization of RBM3 in the nucleus. RBM3 without the RRM domain

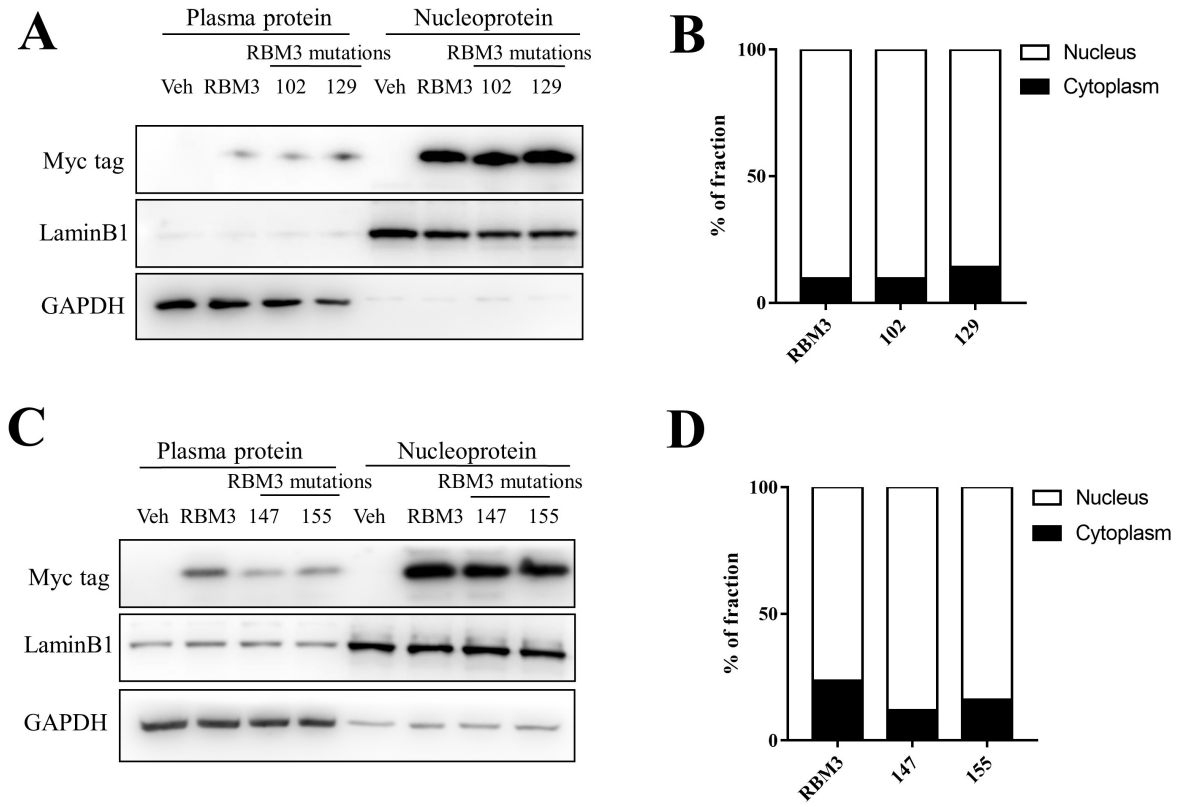

Fig. 6. The effect of potential phosphorylation sites on RBM3subcellular localization. (A,C) Subcellular localization of RBM3, and RBM mutants (presented as 102, 129, 147 and 155) were determined by Western blots with myc tag antibody. LaminB1 and GAPDH were used as nuclear loading control and cytosolic loading control, respectively. (B,D) Blots density was quantified and normalized to LaminB1 or GAPDH, respectively.

will be localized in the cytoplasm, and the deletion of the RRM domain also affects the function of the RBM3 protein.

The arginine/glycine-rich region, known as the RGG domain/motif, is an RNA-binding motif found in many RBPs [34]. RGG motifs have been shown to have multiple biological functions, including regulating protein localization in cells, mRNA translation, synaptic plasticity, and repair of DNA damage [19]. In order to study the biological function of RGG motifs, Palaniraja *et al.* [19] divided RGG motifs into four types, including Tri-RGG, Di-RGG, Tri-RG and Di-RG. For two adjacent RGG sequences separated by 0–4 amino acid residues, Palaniraja *et al.* [19] propose the term Di-RGG. Studies have found that arginine methylation in the RGG domain can affect the transfer of protein CIRP to stress granules (SGs) [35]. In our experiments, we found that the RGG domain is also important for the localization of RBM3, which is similar to the RRM domain. In studies of the neuroprotective function of the RBM3 domain, RGG is more important than RRM. By mutating the arginine within the RGG domain, we found that the arginine within RGG1 and RGG2 is also necessary for the cellular localization of RBM3, but not for the neuroprotective function of RBM3.

5. Conclusions

In summary, our results showed that both RGG domain and the RRM domain are important for the RBM3 nuclear translocation, but only RRM domain are involved in its neuroprotective effect. Since there is no currently recognized nuclear localization signal in the RBM3 molecule, it is important to investigate the molecular mechanism underlying the nuclear translocation of RBM3 in the future.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

LW and TCS contributed equally to this work; BFC and LW designed the research; LW and TCS performed the experiments; CYW, JJL, SQJ and DW analyzed and interpreted the data; and TCS and LW wrote and reviewed the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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