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Rbfox family proteins make the homo- and hetero-oligomeric complexes

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ABSTRACT

Rbfox family of proteins that consists of Rbfox1, Rbfox2, and Rbfox3 in mammals regulates alternative pre-mRNA splicing in various tissues via direct binding to their RNA binding element. Although many studies have indicated the splicing activity of each member of the Rbfox family, the interactions of Rbfox family proteins are largely unknown. Here, we have investigated interactions among Rbfox family proteins. Co-immunoprecipitation (Co-IP) and GST-pull down assays confirmed that Rbfox proteins form homo and hetero complexes. Moreover, *in vivo* crosslinking using disuccinimidyl suberate treatment indicated that the Rbfox proteins form a dimer which then assembles with other proteins to form a large multiprotein complex. Duolink *in situ* proximity ligation (PLA) assay revealed that neuron specific Rbfox3 protein interacts with other Rbfox family proteins. This study is the first to provide an evidence that Rbfox family proteins form homo- and hetero-oligomeric complexes *in vivo*.

1. Introduction

RNA binding proteins (RBPs) bind to target RNAs and play important roles in the regulation of every aspect of RNA metabolism, such as synthesis, splicing, transcription, translation, and degradation. In particular, the tissue-specific expression of RBPs influences the development and function of specific cells through the control of the diversity and expression of target genes.

The Rbfox family of RBPs is known to specifically bind the RNA element (U)GCAUG to regulate alternative splicing [1–5]. The Rbfox family proteins comprise Rbfox1 (A2BP1), Rbfox2 (RBM9) and Rbfox3 (NeuN): Rbfox1 is expressed in neural tissue, heart, and muscle [3,5]; Rbfox2 is expressed in a broad variety of tissues including stem cells, hematopoietic cells and embryos [6]; and Rbfox3 is expressed exclusively in neurons [7]. Therefore, Rbfox family proteins overlap in most areas of the brain. The mutation and abnormal expression of Rbfox1 are found in patients with autism, epilepsy and mental retardation [8,9]. Rbfox2 plays an important role in cerebellar development [10]. Rbfox3 is important for neurological function; mutations in the gene are a known cause of cognitive impairment, autistic features, epilepsy and delayed nerve development [11–14]. In addition, Rbfox3 plays an important role in the balance and function of the hippocampal circuitry. As the Rbfox family proteins exert a widespread influence on synapse and neurodevelopment functions, it is necessary to study the function of the Rbfox family proteins in each region of the brain.

Most proteins do not function alone, but interact with other proteins complexes within a protein-protein interaction network to perform numerous sophisticated processes that influence the structure and function of various cell types. Proteins play different roles in the cell, which depend on the partner proteins that interact with protein complexes within the protein-protein interaction network, which alter dynamically based on the surrounding environment. Thus, protein-protein interactions are essential for studies of protein function as they play an important role in many biological processes, including cell proliferation, cell-cycle control, differentiation, signal transduction, and apoptosis [15]. Despite the importance of protein-protein interactions, studies on the Rbfox family proteins are mostly limited to the alternative splicing activities of each member; therefore, many of the interactions between the Rbfox family proteins remain unclear.

The interaction of Rbfox3 proteins with polypyrimidine tract binding protein-associated splicing factor (PSF) is known to be essential for the activation of neural cell-specific alternative splicing of the NMHC II-B N30 exon [16]. Rbfox proteins were recently shown to interact with a large assembly of splicing

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regulators (LARS) via the C-terminal domain (CTD), which is essential for splicing activity and has been reported to play a crucial role in alternative splicing [17]. However, the interactions between the Rbfox family proteins in neuronal cells have not yet been studied.

Thus, in this study, we investigated the interactions between the proteins of the Rbfox family. We discovered that the Rbfox family proteins form large multiprotein complexes *in vitro*, in addition to homo- and hetero-complexes. Furthermore, we demonstrated that in the adult mouse brain, as well as in cells where the Rbfox family proteins are commonly expressed, neuron-specific Rbfox3 proteins interact with other Rbfox family proteins.

2. Materials and methods

2.1. Cell culture and transfection

HeLa cervical cancer cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM, WELGENE, Korea) containing 10% heat-inactivated fetal bovine serum (Gibco, USA) at 37 °C in an atmosphere containing 5% CO_2. Amaxa Nucleofector (Lonza, Switzerland) was used for transfection of the plasmid constructs according to the manufacturer's instructions.

2.2. Preparation of myc-tagged proteins

The full-length coding regions of the Rbfox1, Rbfox2 and Rbfox3 cDNAs were introduced into the plasmid pCS3+MT. The expression plasmids for Rbfox1 and Rbfox2 have been described previously as A016 and F011, respectively [18]. Expression constructs encoding myc-Rbfox3 in the pCS3+MT plasmid have also been described previously [7]. Myc-RBPs were synthesized *in vitro* from the pCS3+MT construct using the TNT Coupled Reticulocyte Lysate System (Promega, USA) according to the manufacturer's protocol.

2.3. Preparation of the extract, Co-immunoprecipitation (Co-IP), and immunoblot analysis

Whole cell extracts were prepared by lysis in M-PER mammalian protein extraction reagent (ThermoFisher Scientific, USA), supplemented with a protease inhibitor cocktail (Roche Applied Science, Switzerland). For immunoprecipitation, the mouse brain whole cell extracts containing 1 mg protein were incubated overnight in 0.5 ml M-PER buffer with either anti-Rbfox3 antibody or control IgG at 4 °C. Further, Dynabeads Protein G (Invitrogen, USA) were added to the lysate and antibody mixture and further incubated for 2 h with rotation. The Dynabeads Protein G/antibody/protein complexes were washed with M-PER buffer six times. For DNase treatment, the beads coated with lysate and antibody mixture were incubated in 200 U/ml Turbo DNase (Ambion, USA) at 25 °C for 10 min and washed again with M-PER buffer.

For immunoblot analysis, the protein samples were solubilized in Laemmli sample buffer (Bio-rad, USA) and heated at 95 °C for 5 min. 20–50 μg of protein was subjected to SDS-PAGE. Protein concentration was determined using the Qubit® protein assay kit (ThermoFisher Scientific, USA) according to the manufacturer's protocol. Samples were separated on a NuPAGE 4–20% Bis-Tris Gel (ThermoFisher Scientific, USA) followed by their transfer to nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in Phosphate Buffered Saline (PBS) containing 0.05% Tween-20 for 1 h at RT and further incubated with primary antibodies overnight at 4 °C. After washing the membranes, immune complexes were detected using the horseradish peroxidase-conjugated secondary antibody (Abcam, USA) and SuperSignal system (ThermoFisher Scientific, USA). The primary antibodies used in this study were rabbit polyclonal anti-Rbfox2 (Bethyl Laboratories, USA), mouse monoclonal anti-Rbfox3 (anti-NeuN, Millipore, USA), and mouse monoclonal anti-myc (Invitrogen, USA).

2.4. GST pull-down assay

GST–Rbfox3 fusion proteins were expressed in KRX bacterial cells and purified on glutathione sepharose beads (Amersham Pharmacia, USA). For the GST pull-down assay, the *in vitro* synthesized myc-tagged protein and GST-fusion protein bound to glutathione beads were incubated in 5–10 ml of PP-300 buffer (20 mM Tris–HCl pH 8.0, 300 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 0.2% NP-40, protease inhibitors) for 1 h at 4 °C with end-over-end rotation. The protein complexes were eluted by boiling the sample buffer in sodium dodecyl sulfate (SDS) and further analyzed by immunoblot method.

2.5. In-cell crosslinking

Disuccinimidyl suberate (DSS) along with desiccant (ThermoFisher Scientific, USA) was stored at 4 °C and solubilized in DMSO before use. Cells were harvested by scraping, washed twice with ice-cold PBS to remove culture medium and proteins from the cells, then resuspended in PBS with EDTA-free protease inhibitor cocktail (Roche Applied Science, Switzerland). Samples with the crosslinker were incubated under rotating for 30 min at 37 °C. The reaction was quenched by the addition of 1 M Tris, pH 7.5 to a final concentration of 50 mM in the reaction mixture and incubated further for 15 min at RT. The cells were then lysed in M-PER buffer supplemented with a protease inhibitor cocktail. Insoluble particles were pelleted by centrifugation at 16,000 × g for 10 min at 4 °C and the supernatant was saved for immunoblot analysis.

2.6. Duolink in situ proximity ligation (PLA) assays

Duolink PLA assays were performed on human cervical cancer cells HeLa that over-expressed Rbfox3 protein and 8-week-old C57BL/6 female mouse brain. HeLa cells transfected with Rbfox3 expression plasmids were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.5% Triton X-100 in PBS for 15 min. 8-week-old C57BL/6 female mice were sacrificed by CO_2 asphyxiation. Isolated mice brains were fixed in 4% paraformaldehyde overnight, followed by overnight immersion in PBS containing 30% sucrose before being embedded in Tissue-Tek® O.C.T. Compound (Sakura® Finetek, USA) for cryo-sectioning. Frozen samples were sectioned at 12-μm thickness.

The samples were then blocked with 5% BSA for 1 h in a humidity chamber to prevent evaporation. Slides were incubated overnight at 4 °C with rabbit anti-Rbfox1, anti-Rbfox2 or anti-Rbfox3 and mouse anti-Rbfox3 primary antibodies. Primary antibodies were discarded and the slide was washed 3 times with wash buffer for 5 min each. Two PLA probes (anti-mouse and anti-rabbit) were added and incubated for 1 h at 37 °C in a humidity chamber. Slides were washed with wash buffer twice for 5 min each, and ligation solution was added. Subsequently, bound antibody-oligonucleotide conjugates were ligated together for 30 min at 37 °C. The slides were washed again with wash buffer, amplification solution was added, and ligated templates were amplified for 2 h at 37 °C. Slides were then washed twice with wash buffer for 10 min each and mounted with mounting medium. Images were acquired using a Zeiss LSM 510 Meta confocal laser-scanning microscope.

3. Results and discussion

3.1. The interactions among Rbfox family proteins in vitro

To investigate the interactions between the Rbfox family proteins, we performed Co-IP with an anti-Rbfox3 antibody in mouse brain lysate, which is known to express the Rbfox family proteins (Fig. 1A). The immunoprecipitated protein complexes were analyzed by immunoblotting with anti-Rbfox1, anti-Rbfox2, and anti-Rbfox3 antibodies. Rbfox1 and Rbfox2 were not detected in control IgG, but were clearly detected in a complex formed by the anti-Rbfox3 antibody. These Co-IP results indicated that Rbfox3 proteins interacted with the Rbfox family proteins and were present in the same complex.

Next, we performed an *in vitro* GST pull-down assay to evaluate the potential direct interaction between Rbfox3 and the Rbfox family proteins (Fig. 1B). In this study, to make the fusion proteins more suited to the native system of mammalian proteins, myc-tagged Rbfox family proteins were synthesized by *in vitro* transcription and translation using rabbit reticulocyte lysate instead of *E. coli*. Myc-tagged Rbfox family proteins were mixed with GST alone or glutathione beads coated with purified GST-Rbfox3, the bound proteins were collected, and immunoblotting was performed by using an anti-myc antibody. In this GST-pull-down assay, myc-Rbfox 1, 2, and 3 were all detected to bind with purified GST-Rbfox3. Through this, we confirmed that Rbfox3 and the Rbfox family proteins displayed direct physical interaction, without the assistance of other proteins, to form homo- or hetero-complexes.

In order to examine whether the direct interaction between the Rbfox family proteins formed a structural assembly at the intracellular level, we captured the native Rbfox2 and Rbfox3 protein assemblies by crosslinking with DSS (Fig. 1C). DSS is a homobifunctional membrane-permeable crosslinker containing an amine-reactive *N*-hydroxysuccinimide on both ends of an 8-carbon spacer arm. After the overexpression of empty vector (control), myc-Rbfox2 or myc-Rbfox3 in HeLa cells, the cells were treated with DSS and the assembly state of myc-Rbfox2 or myc-Rbfox3 was analyzed by immunoblotting. The Myc antibody-originated immunoglobulin heavy chain proteins are indicated by IgG(H) in Fig. 1C. We observed the monomers and dimers at the expected molecular weights in the DSS-treated myc-Rbfox2. In addition to myc-Rbfox3, a band with the expected molecular weight of the monomer and a band with the expected size of the dimer were identified. Further, large complexes that included myc-Rbfox2 and myc-Rbfox3 were identified, which demonstrated that the Rbfox family proteins formed large multiprotein complexes that contain various proteins, including dimeric forms.

3.2. The interactions among Rbfox family proteins in their native state

Prior to the Duolink *in situ* proximity ligation (PLA) assay to study the interactions between the Rbfox family proteins in the native cellular environment, we confirmed the intracellular localization of the proteins after the overexpression of Rbfox3 in HeLa cells (Fig. 2A). HeLa cells showed a high expression of Rbfox2, but Rbfox1 and Rbfox3 expression was hardly detected. We confirmed that endogenous Rbfox2 (red) was expressed in the nucleus, as previously shown, and that Rbfox3 (green) was also localized in the nucleus in the Rbfox3-overexpressed cells. After confirmation of the intranuclear localization of Rbfox2 and Rbfox3, the PLA assay was used to identify the Rbfox2-Rbfox3 protein interaction. This method visualized the interactions of the Rbfox family proteins in the native cellular environment, and provided direct evidence of the interaction between the two proteins in the cell. We performed an experiment to validate whether this was a suitable assay method for the confirmation of the Rbfox family protein interactions prior to use in tissue samples. First, Rbfox2 and Rbfox3 proteins were immunolabeled by primary antibodies of different species (rabbit and mouse) and a complementary oligonucleotide-conjugated secondary PLA probe (anti-rabbit antibody plus sense and anti-mouse antibody minus sense) was allowed to interact with each primary antibody [19,20]. If two antibody molecules are in close proximity (<40 nm), the complementary DNA strand is ligated and amplified to produce distinct fluorescent dots, which can be visualized using a fluorescence microscope. Each fluorescent dot corresponds to the protein-protein interaction of Rbfox2 and Rbfox3 in HeLa cells. After the overexpression of Rbfox3 in HeLa cells, the

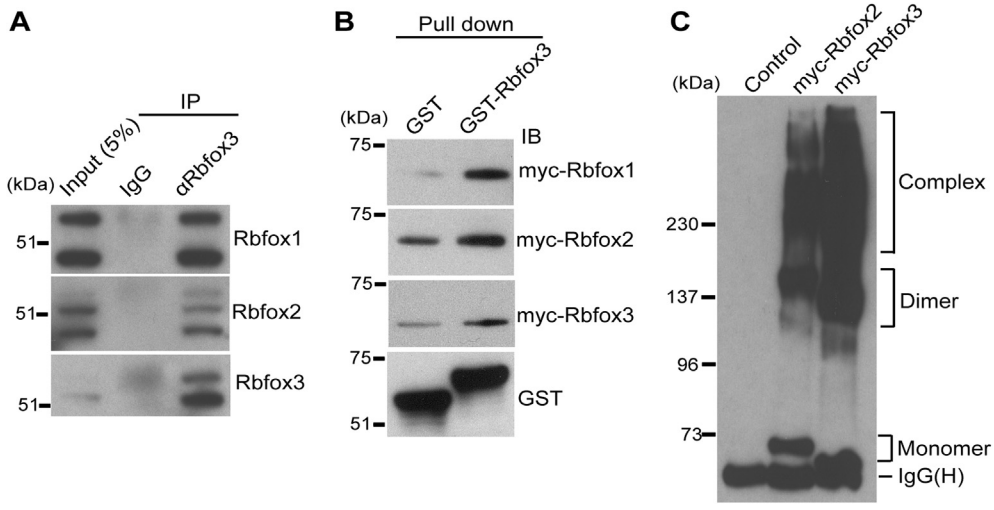

Fig. 1. Rbfox family proteins form homo- and hetero-oligomeric complexes *in vitro*. (A) Co-IP from the mouse brain lysate using the anti-Rbfox3 mouse monoclonal antibody. The resulting Co-IP were subjected to SDS–PAGE, followed by immunoblotting with anti-Rbfox1, anti-Rbfox2 or anti-Rbfox3 antibodies. Five percent of the amount of protein used in the IP was used as a control. (B) *In vitro* pull-down analysis of GST-Rbfox3 with *in vitro* synthesized myc-Rbfox1, myc-Rbfox2 or myc-Rbfox3. Immunoblotting of pull-down products with the anti-myc antibody. GST served as control. (C) Equivalent soluble lysates from HeLa cells transfected with empty vector (Control), myc-Rbfox2 or myc-Rbfox3 were treated with the cell-permeable NHS-ester crosslinker DSS. Myc-tagged proteins were detected by immunoblot analysis with anti-myc antibody. The corresponding position of myc-tagged protein monomer, dimer and complex are indicated. Myc antibody originated immunoglobulin heavy chain is shown as IgG(H).

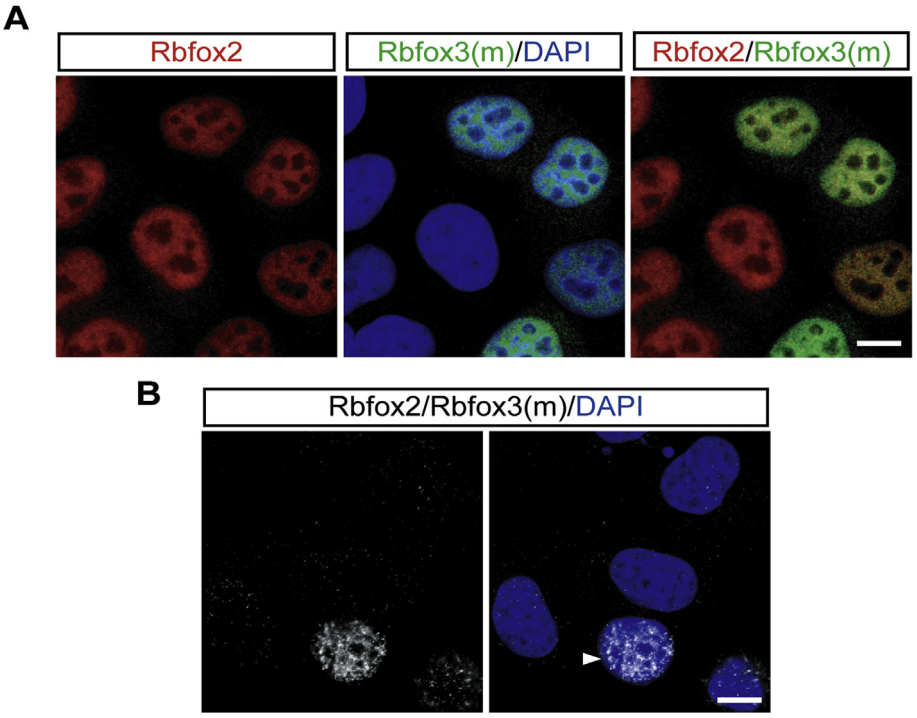

Fig. 2. Rbfox2 and Rbfox3 proteins assemble with each other in the nucleus. (A) HeLa cells were transfected with the expression constructs of myc-Rbfox3. The fixed and permeabilized cells immunostained with Rbfox3 (green) antibody together with Rbfox2 (red) antibody. Nuclei are stained with DAPI (blue). Scale bar, 10 μm. (B) HeLa cells were transfected with the expression constructs of myc-Rbfox3. Duolink proximity ligation assay for protein interactions between Rbfox2 proteins and Rbfox3 proteins. White dots indicate interaction of Rbfox2-Rbfox3 proteins (white arrow); and nuclei are stained with DAPI (blue). Scale bar, 10 μm. m, mouse monoclonal antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

binding between Rbfox2 and Rbfox3 proteins was identified by a white dot-like probe in the PLA assay (Fig. 2B, white arrow). These results suggested that Rbfox3 proteins interact with each other to form a complex in the nucleus of the overexpressed cells and provided confirmation that this experimental method was suitable for the identification of Rbfox family protein interactions.

Through the use of this established experimental method, we also performed the Duolink PLA assay in each region of the adult mouse brain to observe the interaction between Rbfox3 and the Rbfox family proteins in many different types of cells within tissue samples (Fig. 3). We have previously reported the immunofluorescence staining patterns of the Rbfox family proteins in the mouse brain [16]. In the brain stem region and cerebrum, a mixture of cells expressed only Rbfox3, Rbfox3 and Rbfox1 or Rbfox2, and Rbfox1 or Rbfox2. The Duolink PLA assay revealed that Rbfox3 and the Rbfox family proteins formed assemblies, which appeared as red dots in most cells of the cerebrum (Fig. 3A). Although the expression of the Rbfox family proteins overlapped in most regions of the brain, the expression of the Rbfox family proteins in the cerebellum is known to show a different pattern. According to previously reported immunofluorescence results, Rbfox3 was restricted to the internal granular layer (IGL), where it was strongly expressed, whereas Rbfox1 and Rbfox2 were weakly expressed. Rbfox2 protein expression was not only strongly detected in the inner layer and the migrating granular layer of the outer granule layer (EGL), but also in the molecular layer (ML) [10]. In the IGL, where the expression level of Rbfox3 was high, the number of red dots that represented the Rbfox3-Rbfox3 protein complex was higher in comparison with the ML and the EGL, as shown in the Duolink PLA assay (Fig. 3B). Rbfox1, which was weakly expressed in the IGL, rarely showed an intracellular proximity to Rbfox3. In general, a few red dots, representing the Rbfox2-Rbfox2 protein complex, were observed in the cerebellum. This was consistent with previous studies that showed the expression patterns of Rbfox2 and Rbfox3 were not uniform within the cerebellum [16]. As shown in the cerebrum, the Rbfox family proteins were uniformly expressed across the brain stem region. We observed the Rbfox2-Rbfox3 protein complex in the brain stem by using the Duolink PLA assay. In particular, the Rbfox3-Rbfox3 and the Rbfox2-Rbfox3 proteins formed robust complexes, as evidenced by a cluster of distinct red dots (Fig. 3C). Finally, we confirmed the interaction between the Rbfox family proteins in the olfactory bulb. Rbfox3 proteins were expressed in mature neural cells in the olfactory bulb area of neonatal mice, but Rbfox family proteins were not expressed in the olfactory bulb of adult mice [21,22]. This is consistent with the results of our Duolink PLA assay, in which the red dots that represented the interaction between Rbfox3 and the Rbfox family proteins were hardly observed in the olfactory bulb (Fig. 3D).

In this study, we used Co-IP and GST pull-down assays to confirm that the neuron-specific protein Rbfox3 interacted directly with the Rbfox family proteins and formed homo- or hetero-complexes *in vitro*. In addition, the presence of homodimers and the large multiprotein complexes of Rbfox2 and Rbfox3 were confirmed using DSS crosslinking. The PLA assay, which visualized the interactions between the Rbfox family proteins in the native state, clearly demonstrated that when the Rbfox family proteins were commonly expressed in regions of the adult mouse brain region, Rbfox3 interacted with the Rbfox family proteins. Therefore, we used various experimental methods to confirm that the Rbfox family proteins formed complexes through interactions with each other. The diversity of the RBP structure is linked to the diversity of RNA recognition, which subsequently confers a variety of RBP functions. This implied the possibility of functional interplay in cases when two or more Rbfox family proteins bind to the same

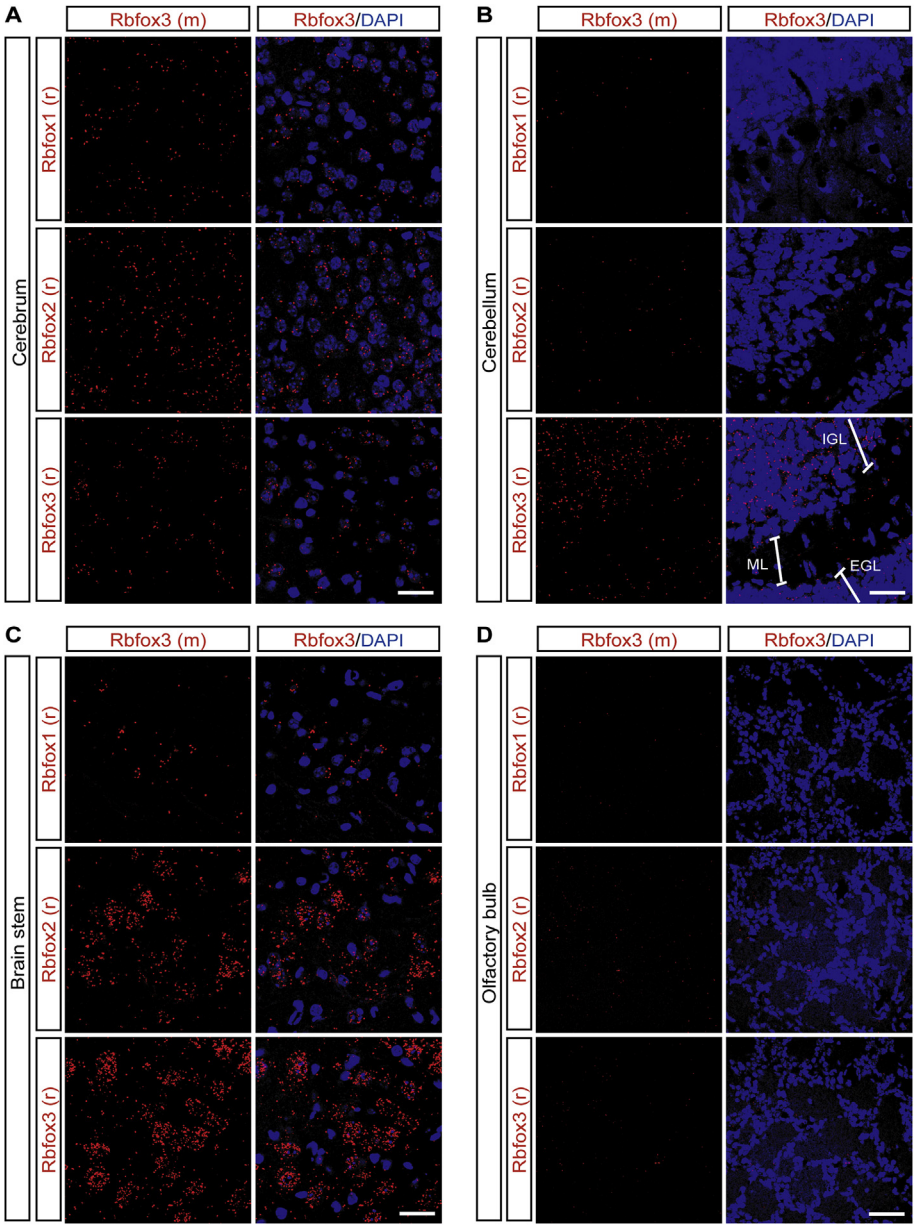

Fig. 3. Rbfox3 proteins interact with Rbfox family proteins in the adult mouse brain. Duolink PLA assay of mouse brain for protein interactions between Rbfox3 proteins and Rbfox family proteins. (A) Cerebrum, (B) Cerebellum, (C) Brain stem, (D) Olfactory bulb. Duolink signals are visualized in red. Each red dot represents a protein interaction event. DAPI stains nuclei (blue). IGL: internal granule layer, ML: molecular layer, EGL: external granule layer. Scale bar, 20 μm; m, mouse monoclonal antibody; r, rabbit polyclonal antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

target RNA, which may lead to the cooperative or competitive regulation of translation or stability. Indeed, it was reported that the number of genes with altered splicing patterns in the brain of the central nervous system (CNS)-specific Rbfox1 knockout mice is relatively small compared with the CNS-specific alternative splicing regulator (Nova) or neural polypyrimidine tract-binding proteins (nPTB) knockout mice [8,9,23]. Rbfox1 knockout mice showed a 60% increase in the expression of Rbfox2 compared with normal mice, which suggested that the Rbfox2 protein complements the function of the Rbfox1 protein. In addition, when proteins form a complex, their specificity and affinity for target RNA is higher compared with that of the individual proteins, which suggests that higher-level protein complexes may be clustered more locally in the cell and allow more rapid recognition of nascent RNA.

Recently, it has been reported that self-aggregation through a tyrosine-rich domain is essential for splicing activation by the Rbfox family proteins [17]. Rbfox proteins interact with large assembly of splicing regulators (LASR) via their C-terminal domain (CTD), which initiates the higher-order assembly of Rbfox and LASR required for the activation of Rbfox-mediated splicing. The low-complexity (LC) sequence within the CTD contains tyrosine repeats, which cause the aggregation of Rbfox proteins. These assemblies are similar to the insoluble content of cells, such as amyloid-like fibrils formed by FUS RNA binding protein (FUS) or the TAR DNA binding protein (TARDBP) of amyotrophic lateral sclerosis (ALS) or other neurodegenerative diseases [24,25]. Unexpectedly, in contrast to these proteins, the Rbfox CTD assembly occurs independently of pathological aggregation and plays an essential role in normal alternative splicing functions.

The complex formation between the Rbfox family proteins

identified in this study is assumed to occur as a result of the tyrosine in the Rbfox CTD; therefore, we plan to identify the precise region of the interaction between the Rbfox family proteins in future studies. In addition, we will also investigate various functional differences, including the alternative splicing activity of the Rbfox family proteins after complex formation. This study has demonstrated the formation of homo- or hetero-oligomeric complexes between the Rbfox family proteins, which could help understand the mechanism of the Rbfox family protein function and regulation in the regions where the Rbfox family proteins are commonly expressed.

Conflicts of interest

The authors have no financial conflicts of interest.

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