Toxic PR poly-dipeptides encoded by the *C9orf72* repeat expansion target Kap β 2 and dysregulate phase separation of low-complexity domains

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ABSTRACT

Low-complexity (LC) domains of proteins are found in about one fifth of human proteome, and a group of LC-domains form labile cross- β polymers and liquid-like droplets. Polymers and droplets formed from LC-domains are dynamically regulated by posttranslational modifications and molecular chaperones including nuclear transport receptors. Repeat expansion in the first intron of a gene designated *C9orf72*, which is the most prevalent form of familial amyotrophic lateral sclerosis (ALS), causes nucleocytoplasmic transport deficit, however, the detailed mechanism remains unsolved. Here we show that the proline:arginine (PR) poly-dipeptides encoded by the *C9orf72* repeat expansion bound nuclear transport receptor Kap β 2 through its nuclear localization signal (NLS) recognition motif, and inhibited the ability of Kap β 2 to melt fused in sarcoma (FUS) droplets by competing interaction with FUS. The findings in this study offer mechanistic insights as to how the *C9orf72* repeat expansion disables nucleocytoplasmic transport and causes neurodegenerative diseases.

Intrinsically disordered low-complexity (LC) protein sequences (LC-domains), also called prion-like domains or amyloid-like domains, are often found in RNA/DNA-binding proteins, intermediate filaments and nuclear pore proteins (1-3). Approximately 20% of human proteome harbors LC-domains, and a group of LC-domains forms labile cross- β polymers and liquid-like droplets in the test tube and RNA granules in the cell (1,4). RNA-binding proteins fused in sarcoma (FUS) and heterogeneous nuclear ribonucleo-proteins (hnRNPs) have been extensively studied in recent years, including studies on their capacity to phase-separate into liquid-like droplets both in the test tube and in the cell (1,4-7).

Labile cross- β polymers formed from LC-domains (hereafter LC polymers) are controlled to be dynamic and not to grow too long at the physiological condition in the cell (8). Once the balance between short and long tilts toward long, LC polymers turn into stable pathogenic fibrils, which are often found in neurodegenerative diseases (8). Human genetics has provided us with some clues to how LC polymers become pathogenic. Specific mutations [D-to-V] in the LC domain of hnRNPs from amyotrophic lateral sclerosis (ALS) and multisystem proteinopathy (MSP) cause stable cross- β polymers (2,9). A hexanucleotide

repeat expansion in *C9orf72*, the most prevalent form of familial ALS and frontotemporal dementia (FTD), produces the most toxic products—poly-dipeptides, proline:arginine (PR) and glycine:arginine (GR) (10,11). These arginine-rich poly-dipeptides bind proteins with LC sequences (2,12) and stabilize LC polymers (2).

Cells have multiple layers of regulatory system to keep LC polymers short and dynamic. Most posttranslational modifications (e.g. phosphorylation, oxidation) are found at LC regions and well known to regulate LC polymerization (1,5-7,10,13-15). Recent works by four different groups similarly conclude that nuclear transport receptors function as a molecular chaperone to keep the LC proteins monomeric (14,16-18).

Three genetic studies revealed the repeat-expansion in *C9orf72* gene disrupts nucleocytoplasmic transport (19-21). One common path for both nuclear import and export is the pore buried in the nuclear envelop called nuclear pores. PR poly-dipeptides derived from the *C9orf72* repeat expansion bind and stabilize polymeric form, but not monomeric form, of phenylalanine:glycine (FG)-rich domains of nuclear pore proteins (3). Nuclear transport receptors are known to be responsible for nucleocytoplasmic transport, but it is still unclear how the repeat expansion in *C9orf72* compromises nucleocytoplasmic transport.

Although the previous studies have reported that PR poly-dipeptides bind and stabilize the cross- β polymer conformation of the LC proteins, the effect of the PR poly-dipeptides to the regulators including nuclear transporter is remained elusive. In this study, we have investigated the interaction between PR poly-dipeptides and nuclear transport receptor Kap β 2 and evaluated the effect of the PR poly-dipeptides to the function of Kap β 2 in regulation of the liquid-liquid phase separation of LC proteins. Our data from in vitro experiments exploiting biophysical methods provide a mechanistic insight into as to how PR poly-dipeptides disable nucleocytoplasmic transport and cause neurodegenerative diseases.

Results

PR poly-dipeptides inhibit chaperone function of Kap β 2

First, we examined the effect of PR poly-dipeptides on Kap β 2 in terms of melting phase-separated full-length FUS droplets. We prepared 18 repeated proline:arginine poly-dipeptide with maltose binding protein (MBP) motif (MBP-PR18). As previously reported (16), FUS forms liquid-like droplets via liquid-liquid phase separation, and the phase separation of FUS is blocked by Kap β 2 observed with fluorescence microscopy (Fig. 1A). In contrast, Kap β 2 loses the ability to melt the FUS droplet in the

presence of MBP-PR18 (Fig. 1A). To better clarify the data, we performed further analysis on the formation and dissolution of FUS droplet by monitoring the turbidity of the solution. Phase-separated droplets floating in the solution demonstrate turbidity and the progress of phase separation can be monitored by tracking turbidity. Full length of FUS formed liquid droplets, and Kap β 2 blocked FUS from phase separating into liquid-like droplets. By contrast, the addition of equimolar of MBP-PR18 inhibited the ability of Kap β 2 (Fig. 1B). These data indicate that PR poly-dipeptides with Kap β 2 inhibited the chaperone function of Kap β 2 on FUS phase separation.

Next, we prepared the LC domain of FUS (residues 2-214), and tested the ability of Kap β 2 in preventing polymerization of the LC domain of FUS using hydrogel binding assay (1). Briefly, the FUS-LC fused to mCherry was purified as described previously (1), and concentrated to self-associate and form cross- β polymers to obtain hydrogel droplets. After that the prepared GFP:FUS-LC was challenged for homotypic polymer extension in the presence of Kap β 2 (see more details in Methods). FUS-LC hydrogel binding was not blocked by Kap β 2 (Fig. S1A), suggesting the importance of proline-tyrosine nuclear localization signal (PY-NLS) for Kap β 2 to recognize FUS as a substrate.

Then, we applied the LC domain of hnRNPA2 (residues 181-341), whose PY-NLS is located in the middle of its LC domain. Kap β 2 (1 μ M) was capable of blocking hydrogel binding of GFP:hnRNPA2-LC (1 μ M) (Fig. 1C), suggesting that Kap β 2 captures hnRNPA2-LC in 1:1 ratio by the interaction between PY-NLS and monomeric state of hnRNPA2-LC. Further, 1 μ M of PR poly-dipeptides inhibited the ability of Kap β 2 from blocking the hydrogel binding of hnRNPA2-LC (Fig. 1D).

Interaction between Kap β 2 and PR poly-dipeptides

Based on two independent interactomes, Karyopherins are found to be potential interactors in the complex with PR poly-dipeptides (2,12). To see the direct interaction between PR poly-dipeptides and Kap β 2, we performed on-column binding assay with purified recombinant proteins (Fig. 2A, B). We found that both GST-PR18 and MBP-PR18 bound to Kap β 2 by the on-column binding assay, while PR8 (eight repeat poly-dipeptide of Pro-Arg) did not bind (Fig. 2A, B). Isothermal titration calorimetry (ITC) showed that MBP-PR18 binds to Kap β 2 in 1:1 ratio at K_d value of 81.3nM (Fig. 2C). The binding assay indicates that Kap β 2 interacts with PR poly-dipeptides at one site with high affinity. Kap β 2 binds PR poly-dipeptides in a similar affinity to PY-NLS of FUS and stronger than full-length FUS (Table 1, Table S1). These results indicate that expanded PR poly-dipeptides directly interact and disrupt Kap β 2 to inhibit droplet formation

of FUS.

Interaction between Kap β 2 and PR poly-dipeptides investigated by solution NMR

The presence of a direct interaction between Kap β 2 and PR poly-dipeptides was further validated by solution nuclear magnetic resonance (NMR) (Fig. 3). Despite large size of Kap β 2 (100 kDa) in the solution NMR, the use of advanced NMR techniques including methyl-selective isotope labeling (Fig. 3A) as well as methyl-transverse relaxation-optimized spectroscopy (TROSY) (22,23), achieved high-quality NMR spectra (Fig. 3B). Although the NMR resonances are only derived from the methyl groups of Ile, Leu, Val, and Met, these methyl groups are found to be widely distributed in the structure of Kap β 2 and thus, expected to sensitively detect the binding of poly-dipeptides to any region in Kap β 2. The NMR spectra of the isotopically labeled Kap β 2 were acquired in the absence and presence of PR20 (Fig. 3C). The addition of the PR poly-dipeptides induced significant perturbations to several resonances of Kap β 2 (Fig. 3C), indicating that the PR poly-dipeptides directly bind to the specific regions of Kap β 2.

Interaction between Kap β 2 and PR20/M9M/FUS(1-500) investigated by solution NMR

In order to see if the perturbed resonances by the addition of PR20 are derived from the NLS-binding site of Kap β 2, we also performed NMR measurement for Kap β 2 in complex with M9M peptide; it has been shown to bind to NLS-binding site of Kap β 2 (24). We found that the binding of the M9M peptide induced significant perturbations to the many of the resonances of Kap β 2 (Fig. 4 and Fig. S2A). The significant perturbations suggest tight binding of the M9M peptide to Kap β 2, which is consistent with the high affinity reported in the previous study (24). Interestingly, several of the perturbed resonances by the addition of the PR poly-dipeptide were also caused by the binding of the M9M peptide (Fig. 3C and 4, Fig. S2A). Peaks overlapped between PR20 and M9M, indicating that the binding site for PR poly-dipeptide partially overlaps with the binding site for M9M peptide. Less significant perturbations by the addition of the PR-peptide can be explained by lower binding affinity for Kap β 2 (Fig. 2C, Table 1, Table S1).

Perturbed resonances by the binding of M9M are expected to be derived around the NLS-binding site, as seen in the crystal structure of Kap β 2 in complex with PY-NLS (PDB code: 4FDD) (25). Here, we focused on the resonances that were perturbed both by the binding of PR20 and M9M (Fig. 3C and Fig. 4, Fig. S2A). The most significant perturbations were seen for the resonances numbered 40 (Ile), 49 (Met), 64 (Val), 150 (Leu), and 155 (Leu). The amino acid types were deduced from the chemical shift statistics of the methyl

resonances (Fig. 3B). Our NMR data indicate that Ile, Leu, Val, and Met residues are involved in the interactions with PR20 and M9M. Given the fact that M9M binds to the PY-NLS-binding site of Kap β 2, these perturbed resonances can be attributed to the Kap β 2 residues close to PY-NLS as seen in the crystal structure of Kap β 2 in complex with PY-NLS (PDB code: 4FDD). These include the isoleucine residues (I457, I540, I642, I722, I773, I804), the leucine residues (L419, L539, L767), the valine residues (V643, V724), and the methionine residue (M308) (Fig. 5A, B). Among them, L539, I540, I642, V643 were found to be located at the negatively charged cavity of Kap β 2 (Fig. 5A). Taking into account the positive charge of the PR poly-dipeptide, these four residues located at the negatively charged cavity of Kap β 2 may be important for its interaction with PR poly-dipeptide. Strikingly, the molecular dynamics (MD) calculation demonstrated that PR poly-dipeptides are recognized by the negatively charged cavity and L419, I457, L539, I540, I642, V643, I722, are shown to be located in the binding site, which is highly consistent with the NMR data (Fig. 5C). Thus, NMR experiments corroborated by MD simulation indicated that the PR poly-dipeptide partially binds to the PY-NLS-binding site on Kap β 2.

A previous study demonstrated that Kap β 2 recognizes FUS regions other than NLS (16). In order to investigate the additional binding sites on Kap β 2 for FUS delta-NLS, we used NMR to investigate the interaction between Kap β 2 and FUS- Δ NLS(FUS1-500) in the presence of M9M peptide. Given that M9M peptide occupies the NLS-binding site on Kap β 2 (24), our data indicates that Kap β 2 recognizes FUS(1-500) using the sites other than NLS-binding site. The perturbed resonances induced by the addition of FUS are distinct from those perturbed by the binding of PR poly-dipeptide, indicating that the binding site for FUS(1-500) does not overlap with that for PR poly-dipeptide.

Discussion

Neurodegenerative diseases are often linked to complications in nucleocytoplasmic transport (26,27). Nuclear pore complexes are composed of outer ring scaffold and centrally located LC-domains enriched with phenylalanine and glycine, generally called FG-domains that form permeability barrier between nucleus and cytoplasm. FG-domains form labile cross- β polymers and are the targets of toxic PR poly-dipeptides derived from *C9orf72* repeat expansions (3). Macro molecules go through nuclear pores upon exit and into the cellular nucleus. PR poly-dipeptides clog nuclear pores by binding to FG-domains and stabilizing FG-domains as polymeric, leading to nucleocytoplasmic transport defect. However, this does not fully account for the results from the genetic studies (19-21).

In this study, we show that PR poly-dipeptides disrupt chaperone activity of nuclear transport receptor Kap β 2 to FUS. We investigated the interactions between the PR poly-dipeptides and Kap β 2. In vitro binding assay showed that Kap β 2 exclusively binds to extended PR (PR18) poly-dipeptide. This result agrees with the toxicity of *C9orf72* expansions reported in familial ALS. Results from ITC experiments provided support for the inhibition of Kap β 2 by extended PR poly-dipeptide. The binding constant of PR18 and Kap β 2 was two folds stronger than that of full-length FUS and Kap β 2. Binding partners of Kap β 2 share PY-NLS and RGG domains (24), but detailed mechanisms driving Kap β 2 recognition of cargos remain to be elucidated.

Despite large size of Kap β 2 (100 kDa) for solution NMR, the use of the advanced NMR techniques including methyl-selective isotope labeling as well as methyl-transverse relaxation-optimized spectroscopy (TROSY) (22,23) achieved high-quality NMR spectra. This is one of the largest class of the proteins that are analyzed by high-resolution solution NMR. A series of the interaction analyses based on high-resolution NMR spectra have revealed that the PR poly-dipeptide binds to Kap β 2.

The binding sites on Kap β 2 partially overlaps with that used for the recognition of PY-NLS interactions, but does not overlap with that used for the recognition of FUS- Δ NLS (Fig. 3 and 4). Previous studies have shown that PY-NLS of FUS binds to the large center cavity of Kap β 2 that has negatively-charged regions (16,24). Negatively charged surface of the cavity may be targeted by arginines of PR poly-dipeptides. The binding of PR poly-dipeptide to the negatively-charged center cavity of Kap β 2 are further corroborated by MD calculation (Fig. 4). The amino acids within 5 Å around the PR poly-dipeptide suggested by MD calculation were in good agreement with those indicated by NMR experiments. These data imply the possible mechanism of the toxicity of the PR poly-dipeptide toward Kap β 2-mediated nuclear transport, in which the PR poly-dipeptide interferes with the interaction between Kap β 2 and FUS NLS.

The current study revealed that PR poly-dipeptides bind nuclear import receptor Kap β 2 through PY-NLS recognition motif and compromise the ability of Kap β 2 to bind cargo proteins. This finding is an additional evidence how PR poly-dipeptides compromise protein-protein interaction within the cell. In addition to the ability of PR poly-dipeptides to bind and stabilize LC-polymers, the binding of PR poly-dipeptides to Kap β 2 explains why PR poly-dipeptides disrupt Kap β 2 to melt liquid-like droplets of FUS. The findings in this study offers mechanistic insights as to how *C9orf72* repeat expansion disables nucleocytoplasmic transport and causes neurodegenerative diseases.

Experimental procedures

Constructs, protein expression and purification

Kap β 2 was expressed from GST-fusion constructs using pGEX6P-1 vector. FUS and PRn proteins were expressed from MBP-fusion constructs using the pMAL-TEV vector. All recombinant proteins were expressed individually in BL21(DE3) *E.coli* cells induced with 0.5 mM isopropyl- β -D-thiogalactoside (IPTG) for 12 hours at 20°C. Bacteria expressing Kap β 2 was lysed with sonicator in buffer containing 50 mM Tris pH 7.5, 200 mM NaCl, 20%(v/v) glycerol, 2 mM DTT. Kap β 2 was purified using GSH Spharose beads (GS4B, GE Healthcare), cleaved with HRV3C protease, anion exchange chromatography (HiTrap Q HP, GE Healthcare) and gel filtration chromatography (Superdex200 16/60, GE Healthcare) in buffer containing 20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM DTT, 2 mM Mg(OAc)₂ and 10% glycerol. MBP-FUS and MBP-PRn proteins were lysed in 50 mM Tris pH7.5, 1.5 M NaCl, 10% glycerol, 2 mM DTT. MBP-fusion proteins were purified by affinity chromatography using amylose resin eluted with buffer containing 50 mM Tris pH7.5, 150 mM NaCl, 10% glycerol, 2 mM DTT and 20 mM Maltose. It was then further purified for MBP-PRn by cation-exchange chromatography (HiTrap SP HP, GE Healthcare) and gel filtration chromatography (Superdex200 16/60, GE Healthcare).

Turbidity assay and Imaging of turbid solution

Prior to adding TEV protease, 8 μ M MBP-FUS, $\pm$ 8 μ M Kap β 2 $\pm$ MBP-PR18 were mixed buffer containing 20 mM Hepes pH7.4, 150 mM NaCl, 10% Glycerol, 2 mM Mg(Oac)₂, 20 μ M Zn(Oac)₂ and 2 mM DTT to reaction volumes of 100 μ L. TEV protease was added to the premixture, to a final concentration of 40 μ g/mL then incubated at 30°C for 60 min to digest all MBP-fusion protein. The solution let cool down to 20°C then measure OD 395 nm using plate reader. For the imaging experiment, turbid solutions were imaged with a fluorescence microscopy (Olympus BX53).

Hydrogel binding assay

GFP and mCherry fusion LC domain of FUS (residue 2-214) and hnRNPA2 (residue 181-341) were expressed in *E.coli* BL21(DE3) cells with 0.5 mM IPTG at 20°C for overnight and purified as previously described (1). Expression plasmids for recombinant proteins were obtained from Steven L. McKnight Laboratory. Hydrogel droplets of mCherry:LC domain of FUS and hnRNPA2 were prepared as previously reported (1). For hydrogel binding assays, purified GFP-fused proteins were diluted to 1 μ M in the buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 20mM BME, 0.1mM PMSF, 0.5mM EDTA) and pipetted into the hydrogel dish. After overnight incubation, horizontal

sections of the droplets were scanned with excitation wavelengths on a confocal microscope (FLUOVIEW FV3000, OLYMPUS). PR20-HA peptides were synthesized by SCRUM Inc. (Japan).

Pull-down binding assay

In-vitro pull-down binding assays were performed using GST-PR18 or GST-Kap β 2 immobilized on GSH sepharose beads (GE Healthcare). Four μ g GST proteins were immobilized on beads. Thirty μ L of GST-proteins beads are incubated with equal molar Kap β 2, MBP-PRn for 20-30 min and washed three times with buffer containing 20mM Hepes pH7.4, 150 mM NaCl, 10% Glycerol, 2mM Mg(OAc)₂ and 2mM DTT. Bound proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue.

Isothermal titration calorimetry (ITC)

ITC experiments were performed with a Malvern iTC200 calorimeter (Malvern Instruments). Proteins were dialyzed overnight against buffer containing 20mM Hepes pH7.4, 150mM NaCl, 10% Glycerol and 2mM β -mercaptoethanol. 200 μ M MBP-PR18 was titrated into the sample cell containing 20 μ M Kap β 2(Δ Loop, residues 321-371 were replaced with GGSGSGS linker). ITC experiments were performed at 25°C with 19 rounds of 2 μ L injections. Data was analyzed using Origin software.

Expression and purification of isotopically labeled Kap β 2

The Kap β 2 expression plasmid was transformed into BL21(DE3) cells. The protein sample with ¹H,¹³C-labeled methyl groups in deuterium background were prepared as described previously (22). The cells were grown in medium with ¹⁵NH₄Cl (2 gL⁻¹) (CIL) and ²H₇-glucose (2 gL⁻¹) (CIL) in 99.9% ²H₂O (Isotec). The precursors for methyl groups of Ile, Val, and Leu, α -ketobutyric acid (50 mg L⁻¹) and α -ketoisovaleric acid (80 mg L⁻¹), and [¹³CH₃] methionine (50 mg L⁻¹) were added to the culture one hour before the addition of IPTG. Protein expression was induced by the addition of 0.5 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG) at OD₆₀₀ ~0.6, followed by ~16 hours of incubation at 25°C. Cells were harvested and resuspended in the lysis buffer containing 50mM HEPES pH 7.4, 150mM NaCl, 20 % Glycerol, 2mM DTT, 2mM EDTA. Cells were disrupted by sonicator and centrifuged at 18,000rpm for 45 min. Proteins were purified using GS4B resin (GE healthcare) and eluted with the buffer containing 20mM HEPES pH7.4, 20mM NaCl, 2 mM EDTA, 10% Glycerol, 30mM GSH. The GST tag was removed by HRV 3C protease at 4°C for ~16 h. The protein was further purified by anion exchange using HiTrap Q FF (GE healthcare) with the buffer containing 20mM Imidazole pH 6.5, 20-1000mM NaCl, 2mM EDTA,

2mM DTT, 20% Glycerol, followed by gel filtration using Superdex 200 16/60 (GE Healthcare) with the buffer containing 20mM HEPES pH7.4, 150mM NaCl, 2mM MgCl₂, 2mM DTT, 2% Glycerol. Protein concentration was determined spectrophotometrically at 280 nm using the corresponding extinction coefficient.

NMR experiments

The isotopically labeled Kap β 2 was prepared in the NMR buffer containing 20 mM ²H-Tris pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 2mM DTT, 2% glycerol, and concentrated to 20 μ M. NMR experiments were performed on Bruker 600 MHz NMR at 20°C. Perturbation of the side chain methyl resonances were monitored using ¹H-¹³C heteronuclear multiple quantum coherence (HMQC), respectively. Spectra were processed using the NMRPipe software (28). Data analysis was performed by Olivia software (<http://fermi.pharm.hokudai.ac.jp/olivia/>). The perturbations of the resonances were evaluated by chemical shift change or intensity change. Chemical shift perturbations (CSPs) for the methyl groups were calculated by the equation;

$$\Delta\delta = \sqrt{\left(\frac{\Delta\delta_H}{\alpha}\right)^2 + \left(\frac{\Delta\delta_C}{\beta}\right)^2} \quad (\text{Eq. 1})$$

where $\Delta\delta_H$ and $\Delta\delta_C$ are the chemical shift changes of ¹H and ¹³C, respectively, by the addition of the ligand, and α and β are chemical shift distribution of ¹H and ¹³C, respectively, of methyl groups as reported in the Biological Resonance Data Bank (<http://www.bmrb.wisc.edu>) (29). Errors for the intensity ratio were estimated based on the peak intensity and noise level.

In order to investigate the interaction between Kap β 2 and PR poly-dipeptide by NMR, the NMR spectra of the isotopically labeled Kap β 2 in the absence and presence of the synthetic peptide of (Pro-Arg)₂₀-HA were measured. In order to investigate the interaction between Kap β 2 and the M9M peptide, the Kap β 2-M9M complex was prepared as follows: The purified GST-M9M protein was added to the isotopically labeled Kap β 2, followed by the removal of the GST tag by TEV protease at RT for 2h and then 4°C for ~16 h before it was buffer exchanged into the NMR buffer using Amicon Ultra-4 50k (Merck). In order to investigate the interaction between Kap β 2 and FUS(1-500) by NMR, the NMR spectra of the isotopically labeled Kap β 2 in complex with the M9M peptide in the absence and presence of FUS(1-500) were measured.

MD simulation

Molecular dynamics (MD) simulations were performed by Maestro (Schrödinger) with Desmond program (D. E. Shaw Research). The input structure

for MD simulations were prepared by taking chain A of the crystallographic dimer from the crystal structure of Kap β 2 in complex with FUS (PDB: 5YVG), and mutating side chains of chain X to PR. The protein and PR poly-dipeptide were solvated with a rectangular box of TIP3P water molecules and neutralized by adding sodium ions. The simulation system was

equilibrated with the default protocol of the Desmond program. A 15ns MD simulation was performed under the following conditions: equilibration of simulation was performed using the isothermal–isobaric ensemble (NPT), the van der Waals interaction between atoms separated by over 10Å were cut off.

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FOOTNOTES

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The abbreviations used are: LC, low-complexity; ALS, amyotrophic lateral sclerosis; NLS, nuclear localization signal; PY-NLS, proline-tyrosine nuclear localization signal; FUS, fused in sarcoma; hnRNP, heterogeneous nuclear ribonucleo-protein; LC polymer, labile cross- β polymer formed from LC-domains; MSP, multisystem proteinopathy; FTD, frontotemporal dementia; PR, proline:arginine; GR, glycine:arginine; FG, phenylalanine:glycine; MBP, maltose binding protein; ITC, isothermal titration calorimetry; NMR, nuclear magnetic resonance; TROSY, methyl-transverse relaxation-optimized spectroscopy; MD, molecular dynamics;

Table 1. Kd value of MBP-tagged of PR18 and FUS fragments against Kap β 2 determined by ITC

Ligand	Kd (nM)	Reference
PR18	81.3	this work
FUS(476-526)	71.5	Yoshizawa 2018
FUS(453-526)	173	Yoshizawa 2018
FUS(371-526)	209	Yoshizawa 2018
FUS(1-526)	158.7	Yoshizawa 2018

Figure 1. PR poly-dipeptides inhibit chaperone function of Kapβ2. (A) Mixture of 7.2μM MBP-FUS and 0.8μM MBP-FUS-EGFP were treated with TEV for 1 hour in presence or absence of Kapβ2 and MBP-PR18. 50μm scale bars are shown in the images. (B) Turbidity of 8μM MBP-FUS in the presence of buffer, ± 8μM Kapβ2, ± 8μM MBP-PR18. Abs_{395 nm} is normalized to measurement of MBP-FUS + buffer + Tev. Mean of 3 technical replicates, ±SD. (C) Hydrogel droplets made from mCherry:LC domain of hnRNPA2 (lower images) were incubated with 1μM of GFP (left panel) and GFP:hnRNPA2-LC (right panel) and visualized by confocal microscopy. GFP:hnRNPA2-LC was challenged for homotypic polymer extension in the presence of different concentration of Kapβ2 (left to right: 0.1μM, 0.3μM, 1μM, 3μM, 10μM, respectively). (D) GFP:hnRNPA2-LC containing Kapβ2 (1.0μM) were applied to hydrogel droplets in the presence of diverse concentration of PR20-HA (left to right: 0.1μM, 0.3μM, 1μM, 3μM, 10μM, respectively).

Figure 2. Interaction between Kap β 2 and PR poly-dipeptides. (A) Pull-down binding assay showing interaction between GST-PR18 and Kap β 2. (B) Pull-down binding assay showing interaction between GST-Kap β 2 and MBP-PR18/MBP-PR8. (C) Dissociation constants (K_d) measured by ITC of Kap β 2(Δ Loop) binding to MBP-PR18.

Figure 3. Interaction between Kapβ2 and PR poly-dipeptides investigated by solution NMR. (A) The crystal structure of Kapβ2 in complex with FUS PY-NLS (PDB ID: 4FDD). FUS PY-NLS is shown as magenta ribbon and Kapβ2 is shown as gray ribbon with spheres representing methyl groups of Ala (pink), Ile (green), Leu (blue), Met (orange), and Val (yellow). Kapβ2 is enriched in the methyl-bearing residues, indicating that sufficient information can be collected from the ^1H - ^{13}C -correlated methyl NMR spectra. (B) ^1H - ^{13}C -correlated methyl NMR spectra of [$\text{U}-^2\text{H}$; Ile-1- $^{13}\text{CH}_3$; Leu,Val- $^{13}\text{CH}_3/\text{C}^2\text{H}_3$]-labeled Kapβ2. (C) ^1H - ^{13}C -correlated methyl NMR spectra of [$\text{U}-^2\text{H}$; Ile-1- $^{13}\text{CH}_3$; Leu,Val- $^{13}\text{CH}_3/\text{C}^2\text{H}_3$]-labeled Kapβ2 in the absence (blue) and presence (red) of the PR poly-dipeptides. Significant perturbations were observed for several resonances. The significant representative perturbations are indicated by peak numbers.

Figure 4. Interaction between Kapβ2 and PR20/M9M/FUS(1-500) investigated by solution NMR. (A) ^1H - ^{13}C -correlated methyl NMR spectra of [U- ^2H ; Ile-1- $^{13}\text{CH}_3$; Leu, Val- $^{13}\text{CH}_3/\text{C}^2\text{H}_3$]-labeled Kapβ2 in the absence (blue) and presence (red) of the PR poly-dipeptides. (top panel), in the absence (blue) and presence (green) of the M9M (middle panel), or in the presence of the M9M (green), and the presence of M9M and FUS(1-500) (orange) (bottom panel). The significant representative perturbations are indicated by arrow heads. The perturbations that are only seen for PR20 are indicated by red arrow heads. The perturbations that are only seen for M9M are indicated by green arrow heads. The perturbations that are common to PR and M9M are indicated by the purple arrow heads. For clarity, only the region of the Ile methyl resonances is shown. The full-range spectra are shown in Figure 3C (for top panel) and Figure S2 (for middle and bottom panels). Chemical shift difference (B) and intensity ratio (C) between the methyl resonances of Kapβ2 by the interaction with the PR poly-dipeptides (top panel), the M9M (middle panel), and FUS(1-500) (bottom panel). In panel (B), the resonances disappeared by the addition of the binding partner are indicated by the asterisks. In the bottom panels of (B) and (C), the resonances that are disappeared by complex formation with M9M are indicated by gray bars.

Figure 5. Structural model of PR interaction to Kapβ2. (A) Electrostatic potential of Kapβ2 (PDB code: 4FDD). PY-NLS colored magenta. Blue and red color shows positive charged and negative charged region, respectively. (B) Residues that are located on negative charged cavity. Leucine, isoleucine, Methionine and Valine are colored green, blue, orange and yellow, respectively. (C) MD calculated model of PR poly-dipeptides inside of Kapβ2 cavity.