

Structural analysis of the interaction between FUS/TLS protein and non-coding RNA

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Chapter One

General introduction

1.1 The FET protein family

Fused in sarcoma (FUS), also known as translocated in liposarcoma (TLS), belongs to the FET or TET family (FUS/TLS, Ewing's sarcoma, and TATA-binding protein-associated factor) [1], whose members are multitasking RNA/DNA binding proteins that regulate gene expression, and are implicated in cancer and neurodegenerative diseases. FET family members share domains exhibiting structural similarity (**Fig. 1-1**). FUS was first identified in a human adipose tissue tumor, a myxoid liposarcoma, as a fusion oncogenic protein that results from chromosomal translocation t(12;16)(q13.3;p11.2) [2]. Later, FUS was found to be involved in various devastating neurodegenerative diseases [3]–[7], as well as in a wide variety of biological processes through regulation of gene expression [8]–[16].

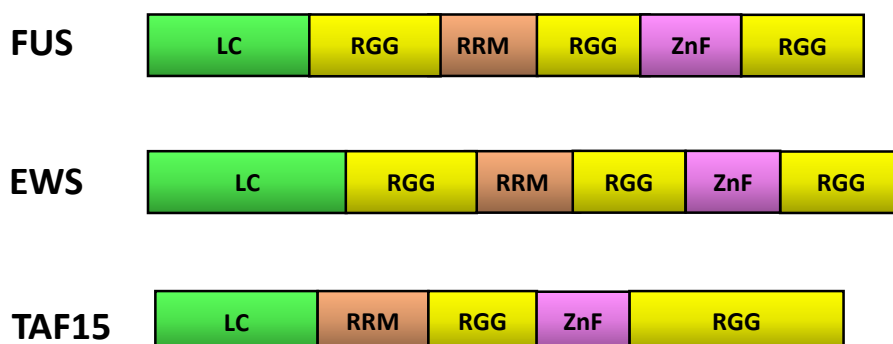


Fig. 1-1. Domain structure similarity of TET proteins. The activation domain (AD), RGG boxes, RBD, and zinc finger (ZF) are depicted for FUS, EWS, and TAF15.

1.2 FUS structure

FUS comprises, from the N- to C- terminal end, a low complexity domain (LC), the first Arginine-Glycine-Glycine-rich motif (RGG1), an RNA recognition motif (RRM), the second RGG (RGG2), a zinc finger domain (ZnF), and the third RGG (RGG3) (**Fig. 1-1, top**). The LC and RGGs are known to be unstructured and, therefore, are called disordered regions. Additionally, RGG, RRM, and ZnF are well known nucleic acid

binding domains, and the corresponding domains of FUS were recently shown to play a central role in RNA and DNA binding. Loughlin *et al.* recently determined the structures of ZnF and RRM, each in a complex with RNA [17]. They showed that ZnF recognizes the GGU sequence, while RRM is more shape-specific than sequence-specific, RRM showing highly degenerate sequence specificity for a NYNY quartet (N=any nucleotide, Y=C or U). It was also shown by isothermal titration calorimetry (ITC) that RRM does not exclusively bind to stem-loop structures but also binds to single-stranded sequences, like AU-rich or GC repeats, as mentioned above. Additionally, the RGG2 portion of RRM-RGG2 was found to interact with the 5' single-stranded loop region of the stem-loop structure and to disrupt the sequential base-base stacking, thereby destabilizing the RNA structure [18]. NMR studies thus have provided a structural basis for RNA recognition by individual nucleic acid-binding domains of FUS.

1.3 The diverse physiological roles of FUS

FUS plays crucial roles in numerous biological processes including transcriptional regulation [8]-[9], mRNA processing [10], microRNA biogenesis [11], nucleocytoplasmic RNA shuttling [19], DNA damage response [20], telomere maintenance [13], gene silencing [15], and regulation of the cell cycle [16], [21]. This impressive diversity of functions enables FUS to integrate several processes in order to accomplish a certain task. For example, FUS can couple transcription to the splicing process through its ability to bind RNA polymerase II (RNAP II) and the splicing factor U1 small nuclear ribonucleoprotein (snRNP) [14].

FUS is reportedly involved in the shortening of the telomere, which is located at the end of the human chromosome, through repression of telomerase-independent telomere-elongation [13]. In this biological process, FUS was found to be included in a ternary complex with telomeric DNA and telomeric repeat-containing RNA (TERRA), whose sequences are d(TTAGGG)_n and r(UUAGGG)_n, respectively. This ternary complex recruits histone trimethyltransferases Suv3-9h and Suv4-20h, by which lysine 20 of H4 (H4K20) and lysine 9 of H3 (H3K9) are trimethylated, resulting in promotion of

heterochromatinization of the telomere, subsequent inhibition of homologous recombination of telomeric DNA, and telomere shortening [13], [22]–[25].

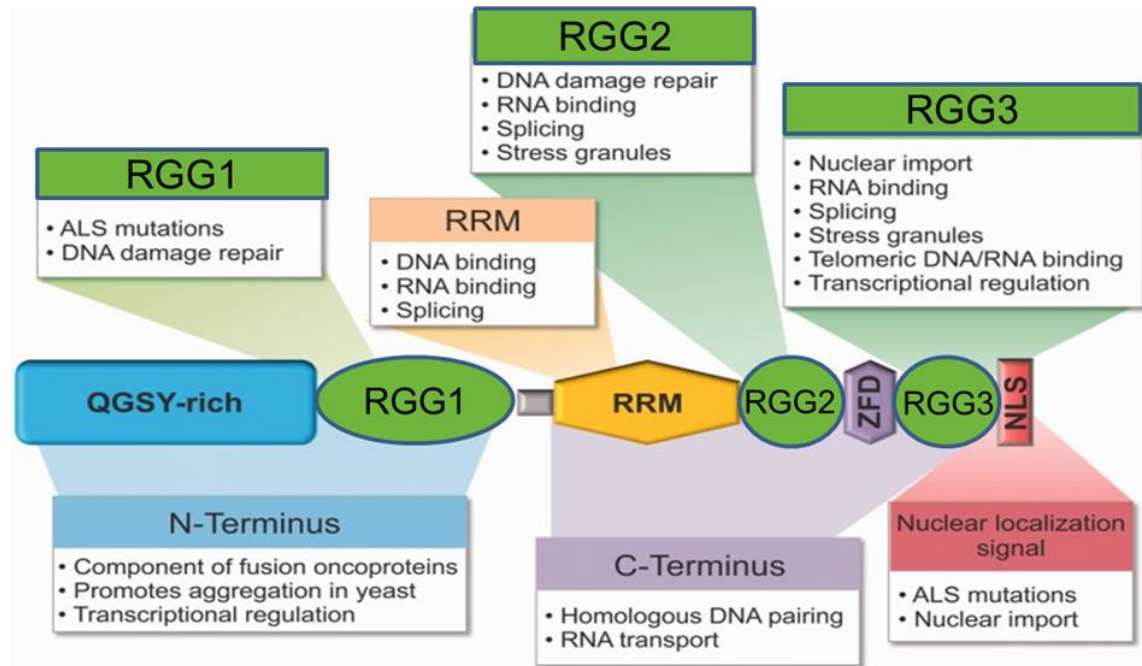


Fig. 1-2. The functional domains within fused in sarcoma (FUS). FUS binds DNA, RNA, and proteins to perform a diverse array of functions. Summarized, here are the known functions of FUS annotated onto the domain structure of the protein.

Note. QGSY-rich = glutamine-glycine-serine-tyrosine-rich or prion-like domain; Gly-rich = glycine-rich; RGG = arginine-glycine-glycine-rich; RRM = RNA recognition motif; ZFD = zinc finger domain; NLS = nuclear localization signal; ALS = amyotrophic lateral sclerosis. Adapted from [26].

It was shown previously that RGG3 of FUS is responsible for the formation of the ternary complex; Tyr residues and Phe residues of RGG3 are involved in the specific recognition of TERRA and telomeric DNA, respectively [25]. Our NMR-based binding assay revealed the interactions in the binary and ternary complexes of RGG3 with telomeric DNA or/and TERRA, which suggested that the plastic roles of tyrosine and phenylalanine are important for RGG3 to efficiently form the ternary complex [27]. It now seems important to investigate the relative roles of the different RNA binding

domains and other regions of FUS in the context of the full-length protein to understand the molecular mechanism by which FUS regulates various biological processes.

1.4 FUS-related diseases

FUS and other RNA binding proteins have been considered as a molecular link between apparently different human diseases such as cancer and neurodegenerative diseases. FUS was found as the major component of nuclear polyglutamine (polyQ) aggregates in a Huntington disease (HD) cell model [4], where it can shift from soluble to insoluble aggregates [5]. Then, it was also found in other PolyQ aggregates, including spinocerebellar ataxia (SCA) type 1, 2, and 3, and dentatorubral-pallidoluysian atrophy (DRPLA) [3]. Concurrently, FUS mutations have been found in amyotrophic lateral sclerosis (ALS) patients [6], [28] and frontotemporal lobar degeneration (FTLD) ones [29]. Although the mentioned diseases have different manifestations, FUS is associated with all these neurodegenerative diseases as inclusion bodies [16], which suggests a common pathway for their neuropathologies. FUS has the ability to undergo liquid-liquid phase separation yielding dynamic liquid-like droplets, which are involved in the assembly of membrane-less structures such as stress granules [30]. In normal physiological conditions, FUS can reversibly shift between dispersed phase, liquid droplet, and reversible gel assemblies. However, through aging or pathological conditions, liquid droplets can be converted into non-reversible gels and fibril assemblies (**Fig. 1-3**) [31], [32]. Mutations causing ALS were found to accelerate the liquid to solid phase transition [33], [34]. RNA was also found to influence the phase transition of FUS [35], [36]. Dysregulation of RNA metabolism is a major cause of several human diseases [37], [38]. The implication of FUS RNA-binding domain mutations in the etiologies of neurodegenerative diseases suggests that the RNA binding ability of FUS is substantial to maintain neuron functionality. RNA binding proteins are considered as emerging therapeutic targets for neurodegenerative diseases as well as cancer prevention and treatment [39]. Therefore, decoding of the FUS-RNA recognition/interaction mechanism is paid much attention.

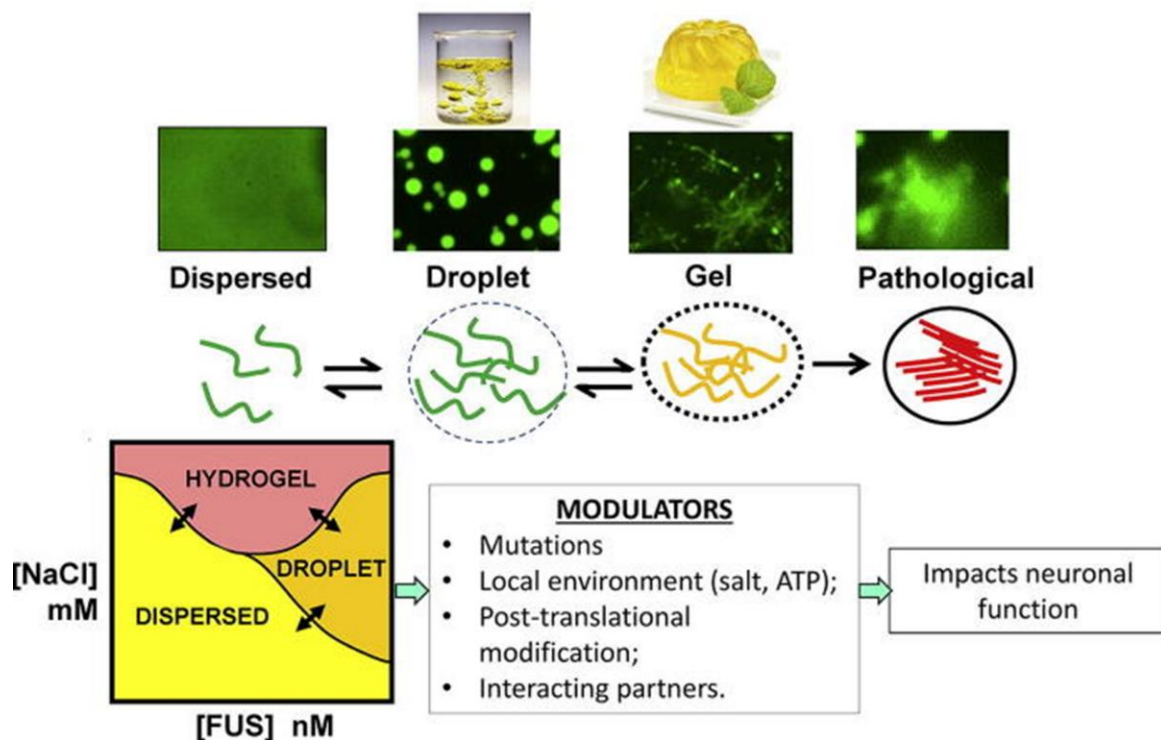


Fig. 1-3. Different physiological and pathological phases of the FUS protein and factors that can govern FUS phase transitions. Adapted from [31].

1.5 FUS-binding specificity

FUS reportedly binds to a wide variety of RNA and DNA sequences, both structured and unstructured, that are involved in transcription, splicing, and so on [10]. Despite the massive accumulative results, the mechanism by which FUS can recognize its target to modulate certain biological process is still in debate. Previously, the systematic evolution of ligands through exponential enrichment (SELEX) showed that FUS recognizes the GGUG motif [40], while a photoactivatable ribonucleoside-enhanced crosslinking immunoprecipitation (PAR-CLIP) study and an RNA-binding competition study identified an AU-rich stem-loop structure and CGCGC sequence, respectively, as additional targets for FUS [41], [42]. More recently, normalization of CLIP-seq (CLIP combined with deep sequencing) data by means of Nascent-seq data showed a combination of six motifs (UGUG, CUGG, UGGU, GCUG, GUGG, and UUGG) are also targeted by FUS [24]. Others showed that FUS binding is not highly specific for RNA sequence or structure [23].

1.6 The role of FUS in *CCND1* repression

Previously, Wang *et al.* reported that FUS downregulates the transcription of *CCND1* in response to DNA damage [8], which is supposed to result in the prevention of DNA replication and cell-cycle progression. DNA damage triggers the transcription of long non-coding RNA (lncRNA) of 602 nucleotides from the 5' upstream region of *CCND1*, named pncRNA (**Fig. 1-4**). Subsequently, FUS is recruited to the 5' upstream region of *CCND1* through binding to pncRNA. Binding of pncRNA also relieves the N-terminal region of FUS from masking by the C-terminal region of FUS, allowing the N-terminal region of FUS to bind to CREB-binding protein (CBP)/E1A-binding protein P300 (p300) and to repress the histone acetyltransferase HAT activity of CBP/p300 allosterically. Accordingly, Wang *et al.* suggested a model where pncRNA serves as a ligand for FUS, causing an allosteric effect to release it from an inactive conformation, which in turn allows gene-specific FUS–CBP/p300 interactions resulting in inhibition of the HAT activity of CBP/p300 and the repression of *CCND1* transcription. Later, we found that a portion of pncRNA (nucleotide numbers 32-62) comprising 31 nucleotides, named pncRNA D1-1, is sufficient for binding of FUS, and demonstrated that the 5' half of pncRNA D1-1 takes on a single-stranded structure and that the 3' half forms a stem-loop structure [18]. Here, we hypothesize that FUS undergoes structural changes, i.e., from a “closed” to an “open” conformation, upon binding to pncRNA (**Fig. 1-4**) and pncRNA D1-1, and investigate this hypothesis experimentally.

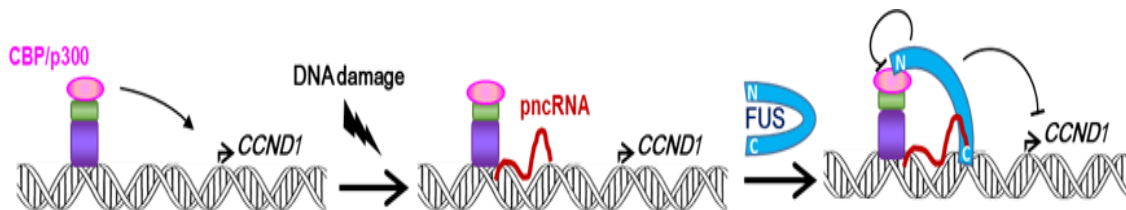


Fig. 1-4. The mechanism of repression of *CCND1* by FUS on DNA damage. A closed-to-open conformational change of FUS is hypothesized on binding pncRNA, which enables FUS to interact with CBP/p300 and repress their histone acetyltransferase activities, resulting in the repression of *CCND1*. Adapted from [8].

1.7 The aim of this study

In this study, we firstly demonstrated by means of FRET assay that FUS really undergoes a conformational change, extension, on binding to pncRNA. The extension is implied to correspond to the closed-to-open conformational change of FUS. We then identified a critical region of pncRNA for the induction of the conformational change of FUS. We also investigated the effect of the length of RNA on the conformational change of FUS. Subsequently, we investigated the effects of other important FUS targets, telomeric DNA and TERRA, both of which form the G-quadruplex structure, on the conformational change of FUS. We examined whether ALS-linked mutations have any effects on either the conformation or conformational change of FUS. We further investigated the conformational change of FUS on a single molecule basis using HS-AFM. Finally, we investigated the effect of a specific sequence of pncRNA on FUS aggregation that could be induced by the shearing effect of pipetting.

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Chapter Two

RNA sequence and length contribute to RNA-induced conformational change of FUS/TLS

2.1 Introduction

To investigate the structural changes of FUS upon the addition of various nucleic acids, we performed fluorescence resonance energy transfer (FRET) assay. FRET is a methodology that can determine the distance between two chromophores. In order to observe efficient FRET, the donor and acceptor fluorophores must meet three requirements: (1) a donor emission spectrum must overlap with the acceptor emission spectrum (**Fig. 2-1a**). (2) The distance between the donor-acceptor pair must be less than 10 nm (**Fig. 2-1b**). (3) The donor and acceptor fluorophore dipoles must not be perpendicular to each other (**Fig. 2-1c**). When all three conditions are satisfied, high FRET efficiency is obtained [1]–[3]. In this case, excitation of the donor would result in a doublet peak at the maximum emission wavelength of both donor and acceptor (**Fig. 2-1 d, blue spectrum**). If any of those conditions is missed, FRET efficiency would be reduced, and only the emission of the donor will be observed upon donor excitation (**Fig. 2-1 d, green spectrum**). The FRET assay is useful to obtain information on the average of all possible conformations at a particular time. The FRET assay has previously been applied to unfolded and intrinsically disordered proteins to characterize the ensembles of disordered states and their transitions to partially or fully ordered states [4], [5].

To undertake the FRET assay, we introduced blue fluorescent protein (BFP) and green fluorescent protein (GFP) to the N- and C-termini of FUS, respectively (**Fig. 2-2**). Additionally, maltose binding protein (MBP) was added to the N-terminus of BFP to enhance the solubility of FUS, and a 6xHis-tag was attached to the C-terminus of GFP for purification. We expect that FUS takes on a closed-form when free, in which GFP and BFP are supposed to be in close proximity, and shows high FRET efficiency (**Fig. 2-3, left**). On the other hand, upon binding with the target RNA, we expect that FUS takes on an open form, in which GFP and BFP are supposed to be far apart, and shows low FRET efficiency (**Fig. 2-3, right**).

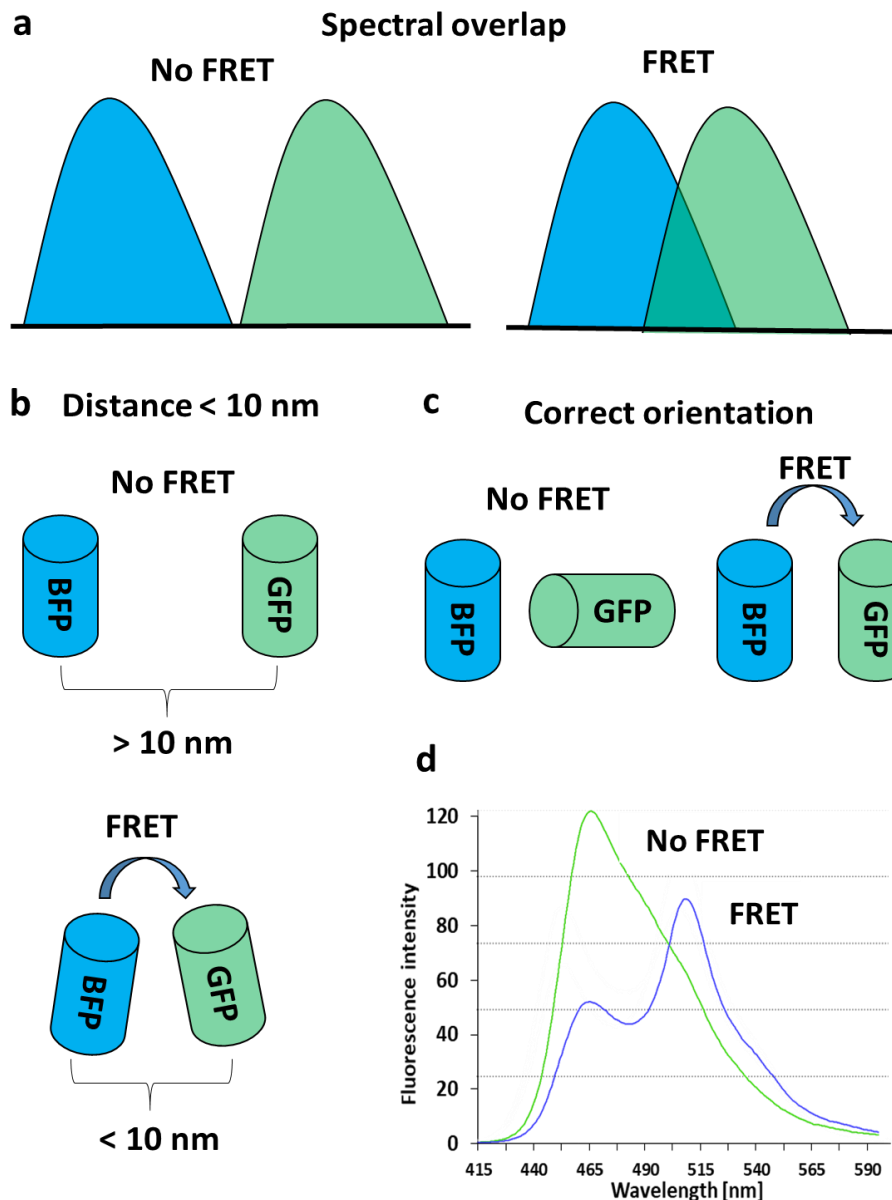


Fig. 2-1. Requirements for efficient FRET using BFP and GFP as an example of FRET pair. **(a)** There must be an overlap between the donor fluorophore emission spectrum and the excitation spectrum of the acceptor fluorophore. **(b)** If the FRET donor and acceptor are more than 10 nm apart, then no FRET occurs, and the donor emits fluorescence. If the donor and acceptor are within ~10 nm of one another, then energy transfer can occur from the donor (BFP) to the acceptor (GFP). **(c)** If the donor and acceptor fluorophore dipoles are perpendicular to one another, then the donor molecule will emit fluorescence. However, if the dipoles are parallel to each other, FRET will occur. **(d)** Representative fluorescence spectra in the case of efficient FRET (blue spectrum) and if no FRET occurs (green). **(a-c)** Adapted from reference [3].

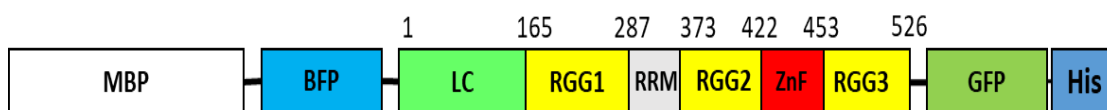


Fig. 2-2. Schematic illustration of the FUS fusion protein used in this study, MBP-BFP-FUS-GFP-6xHis. Residue numbers of FUS are indicated.

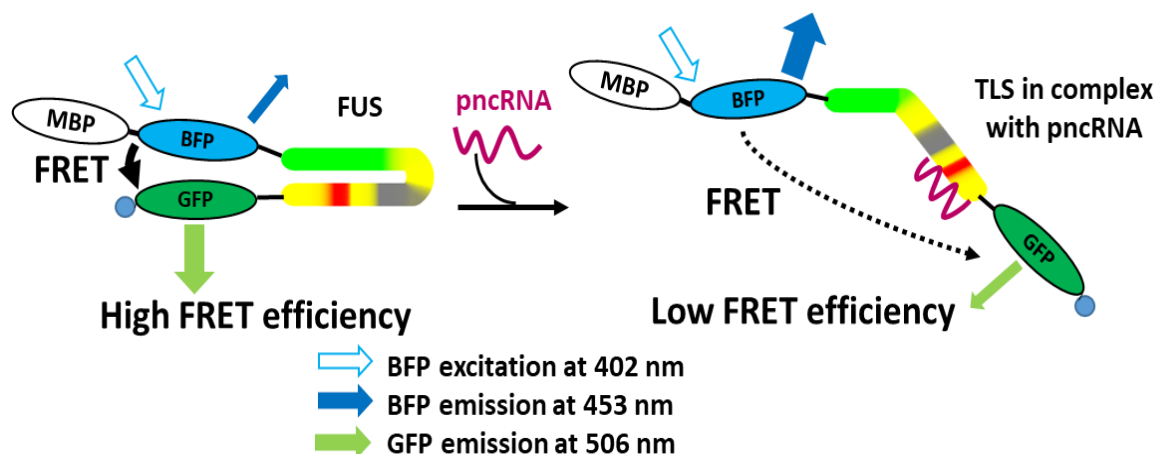


Fig. 2-3. Illustration of FRET methodology to detect the closed-to-open conformational change of FUS. The closed conformation in the absence of pncRNA would result in high FRET efficiency (left), and the open conformation upon the binding of pncRNA would result in low FRET efficiency (right).

2.2 Results

2.2.1 Implication of the closed-to-open conformational change of FUS on binding to pncRNA

The MBP-BFP-FUS-GFP-6xHis protein was successfully expressed in *E. coli* cells and purified by Ni-affinity chromatography, followed by size-exclusion chromatography (SEC). Monomer fractions of the protein in SEC were collected with reference to the elution volume of the molecular marker standard (**Fig. 2-4**). **Fig. 2-5** shows the results of SDS-PAGE of the purified protein. Hereafter, MBP-BFP-FUS-GFP-6xHis protein is simply called FUS.

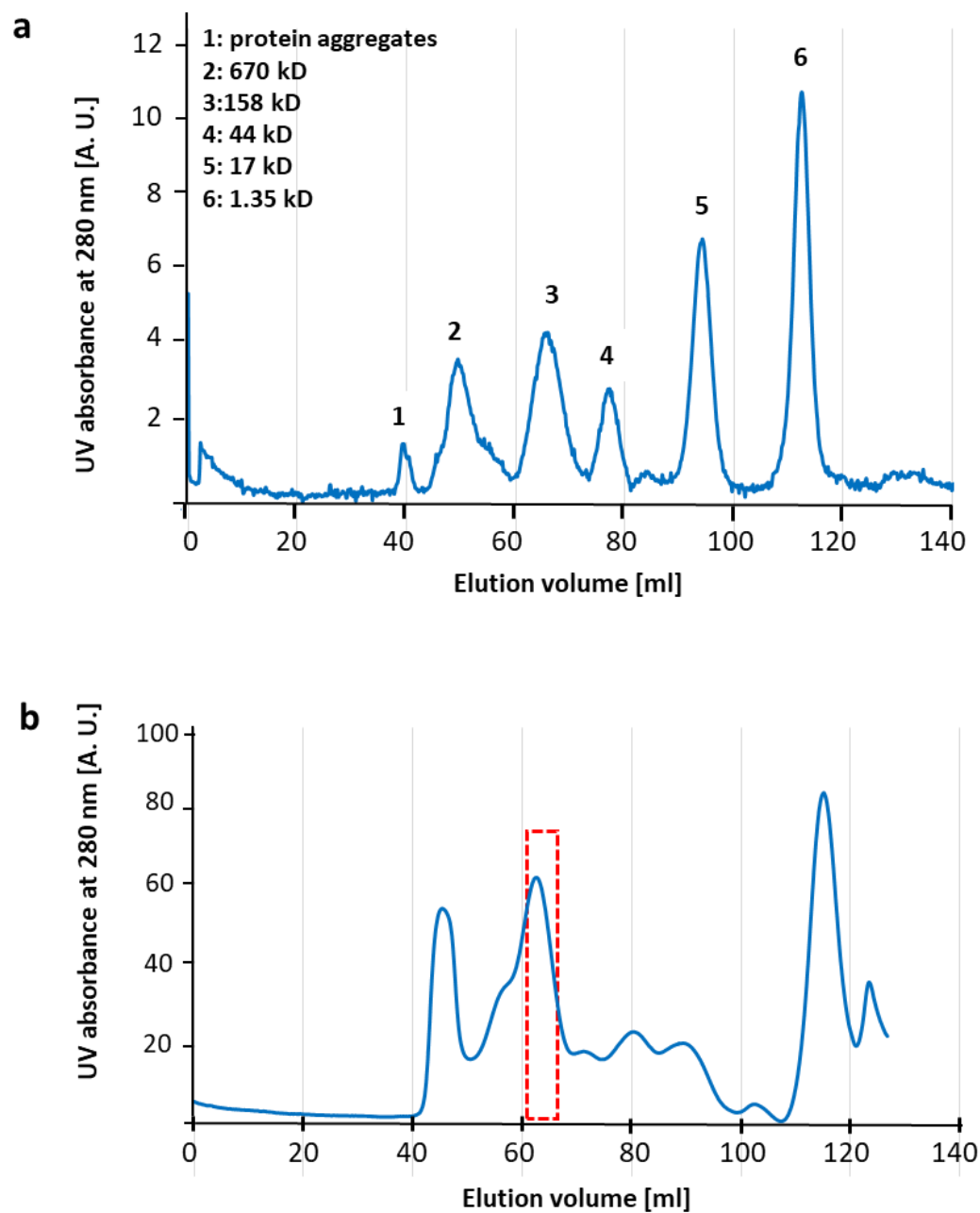


Fig. 2-4. Purification of FUS as a monomer form. **(a)** Size-exclusion chromatography (SEC) of molecular size marker proteins. The theoretical molecular weights of proteins are also indicated. **(b)** SEC of FUS. Fractions corresponding to monomer FUS indicated by the red rectangular were collected.

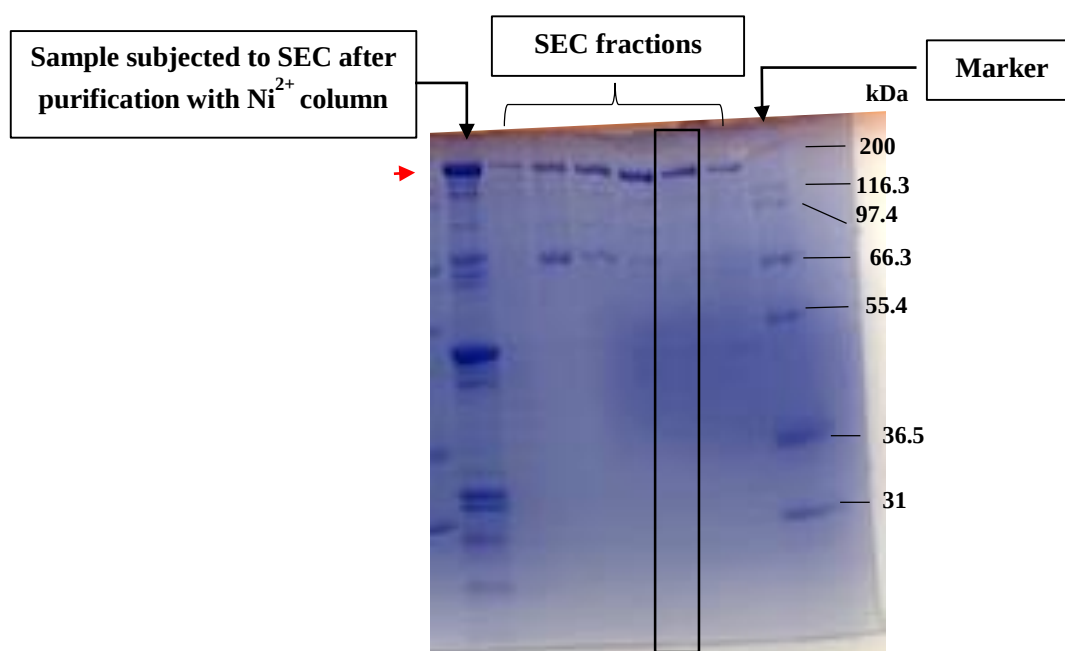


Fig. 2-5. The uncropped full-length 10% SDS-PAGE gel for fractions of MBP-BFP-FUS-GFP-6xHis eluted on size exclusion chromatography (SEC) and the molecular mass markers. The lane boxed in black was used for FRET experiments. The red arrow indicates the position of MBP-BFP-FUS-GFP-6xHis.

The full-length pncRNA was determined to be 602 nucleotides. However, it was reported that a 31-mer fragment (32-62) of pncRNA is critical for FUS binding [6] (**Fig. 2-6**). Firstly, the conformational change of FUS caused by binding of the full-length pncRNA (602 nucleotides) was examined by FRET. **Fig. 2-7** shows the fluorescence spectrum of FUS excited at 402 nm. In addition to the peak of BFP at around 453 nm, due to FRET, a peak of GFP at about 506 nm was observed. On the addition of the full-length pncRNA, the fluorescence intensity at 506 nm decreased, while that at 453 nm increased (**Fig. 2-7a**). This reflects a reduction in FRET, which indicates the increase in the BFP-GFP distance. The increase in the BFP-GFP distance means some extension of FUS. It is implied that FUS undergoes the closed-to-open conformational change on binding to pncRNA, which enables FUS to interact with CBP/p300 for the repression of expression of the *CCND1* gene.

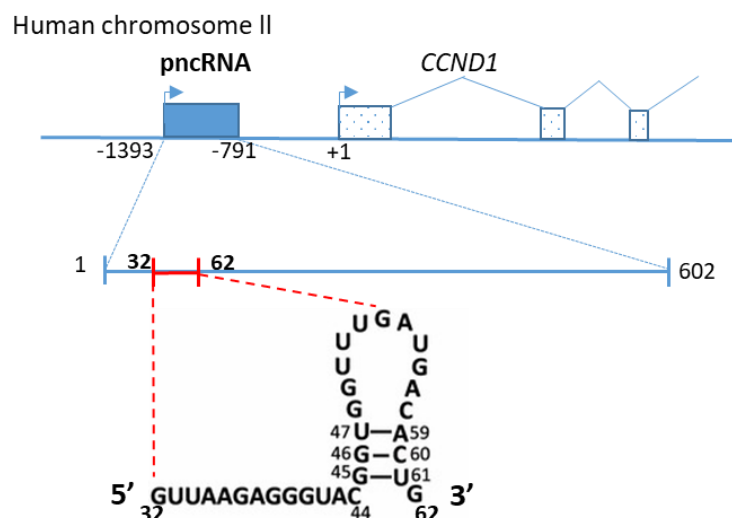


Fig. 2-6. The localization, length, sequence and secondary structure of pncRNA. Adapted from reference [6].

Secondly, the conformational change of FUS caused by binding of pncRNA D1-1 (31 nucleotides, **Fig. 2-6**), R31 (**Table 2-1 in Materials and Methods**), was examined by FRET. The spectral change of FUS caused by R31 turned out to be very similar to that caused by the full-length pncRNA (**Fig. 2-7a and b**). This indicates that R31 is sufficient to produce the same extent of conformational change of FUS as the full-length pncRNA. Thus, it was revealed that R31 is sufficient not only for binding of FUS but also for causing the conformational change of FUS.

The remaining protein after the FRET experiments was subjected again to SEC, which ensured that the protein exists in the monomer state over the experimental time. This indicates that the observed FRET is intramolecular-one, not intermolecular-one. MBP is fused to increase the solubility. No RNA binding activity was observed for the MBP protein [7], [8]. The effect of MBP on the FRET results was examined. MBP was cleaved by thrombin, and the resultant protein was purified by SEC. The fluorescence spectrum and its change on the addition of R31 were very similar for FUS with and without MBP (**Fig. 2-8**). These results indicated that the presence of MBP does not affect the FRET results for FUS.

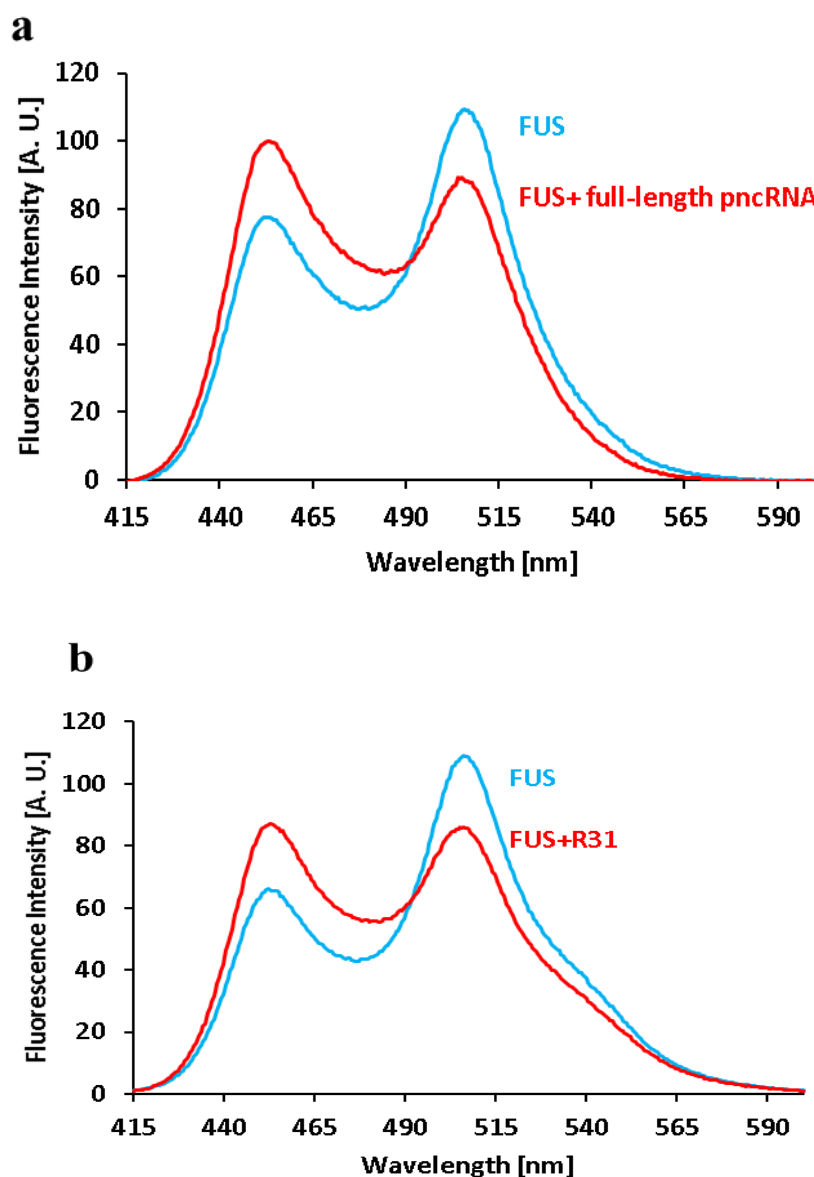


Fig. 2-7. Detection of the closed-to-open conformational change of FUS on binding of pncRNA by FRET. **(a)** Fluorescence spectrum of FUS (100 nM), excited at 402 nm, in either the absence (blue) or presence (red) of an equimolar amount of full-length pncRNA of 602 nt. **(b)** The names and sequences of fragments of R31 of pncRNA. d, Fluorescence spectrum of FUS in either the absence (blue) or presence (red) of an equimolar amount of R31 of pncRNA.

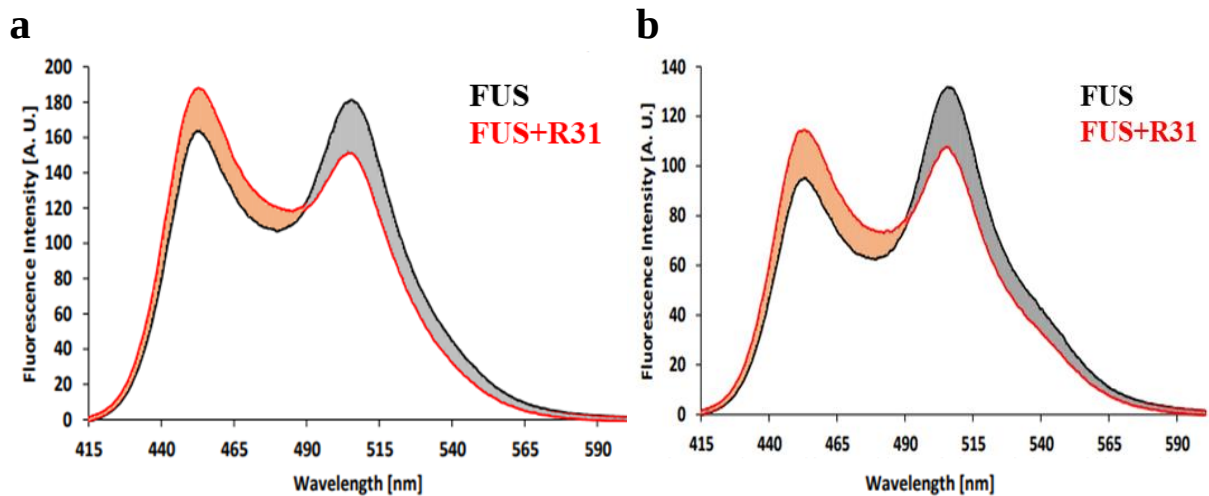


Fig. 2-8. Little effect of the fusion of MBP on the FRET results for BFP-FUS-GFP. Fluorescence spectra of BFP-FUS-GFP with and without pncRNA (R31) in the presence (a) and absence (b) of the MBP fusion. The areas corresponding to the increase (orange) and decrease (gray) in fluorescence by the addition of R31 are colored.

2.2.2 Identification of a critical region of pncRNA for induction of the conformational change of FUS

In order to identify a critical region of pncRNA for induction of the conformational change of FUS, various fragments of R31 (**Fig. 2-9a**) were tested. The relative FRET efficiency, also known as the proximity ratio, $E = I_{\text{GFP}} / (I_{\text{GFP}} + I_{\text{BFP}})$, was calculated in either the presence or absence of each fragment and the change in E caused by each RNA fragment was obtained. $|\Delta E|$ can be regarded as a measure to estimate the extent of the conformational change of FUS caused by RNA. It should be noted that R6 caused a more considerable conformational change of FUS than either R7 or R10, although R6 is shorter (**Fig. 2-9b**). It is supposed that the GAGGGU region of pncRNA is critical for induction of the conformational change of FUS. When a single mutation was introduced at R6, reduction in $|\Delta E|$ was observed for GAGAGU and AAGGGU (mutations are underlined) (**Fig. 2-10a**). This indicated that R6 causes a conformational change of FUS in a sequence-specific manner.

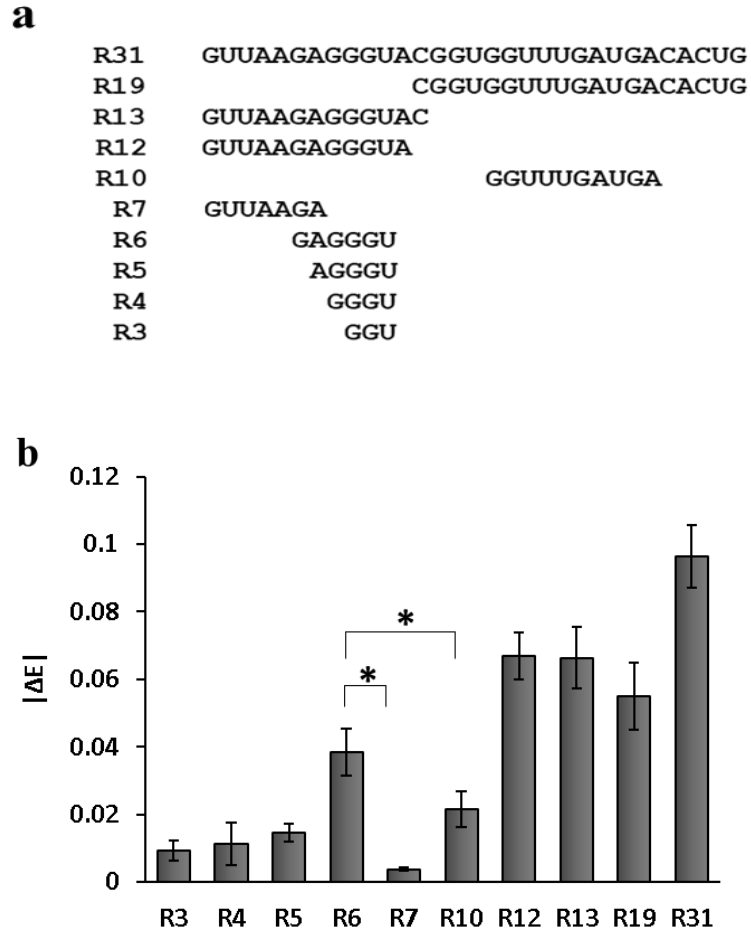


Fig. 2-9. Identification of key portions of pncRNA for the structural change of FUS. **(a)** The names and sequences of fragments of R31 of pncRNA. **(b)** Absolute values of the change in the relative FRET efficiency, ΔE , where $E = \text{IGFP} / (\text{IGFP} + \text{IBFP})$, on the addition of each fragment of R31 of pncRNA. Three independent results were averaged for each fragment. $|\Delta E|$ is presented as a mean $\pm$ SD. * indicates p value < 0.05 .

It is remarkable that $|\Delta E|$ of R19 is much larger than that of R10. R19 can form the stem-loop structure shown in **Fig. 2-6**, while R10 cannot due to the lack of the residues that form the stem structure. It is suggested that the stem-loop structure in the 3' region of R31 is also essential for the induction of the conformational change of FUS.

It was seen that R12 caused a more substantial conformational change of FUS than R19, although R12 is shorter (**Fig. 2-9**). This indicates that the 5' single-stranded region of R31 has a slightly more pronounced effect on the conformational change of FUS than the 3' stem-loop region.

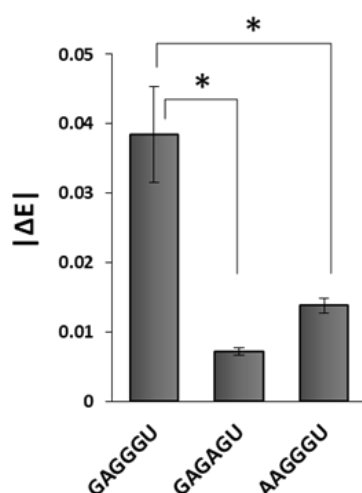


Fig. 2-10. The presence of G residues influences the extent of the FUS conformational change. Absolute values of the change in the relative FRET efficiency, ΔE , where $E = I_{\text{GFP}} / (I_{\text{GFP}} + I_{\text{BFP}})$, on the addition of R6, GAGGGU, and its mutants, GAGAGU and AAGGGU. * indicates p value < 0.05 . Three independent results were averaged for each fragment. $|\Delta E|$ is presented as a mean $\pm$ SD.

2.2.3 The DNA counterpart of pncRNA causes less conformational change of FUS

D13 is a DNA counterpart of R13 of pncRNA (see Table 2-2 in Materials and Methods). $|\Delta E|$ of D13, 0.0175 ± 0.0018 (Fig. 2-11a), is rather smaller than that of R13, 0.0663 ± 0.0091 (Fig. 2-9b). Similarly, $|\Delta E|$ of D19, 0.0309 ± 0.0025 (Fig. 2-11a), is somewhat smaller than that of R19, 0.0550 ± 0.0099 (Fig. 2-9b), and $|\Delta E|$ of D31, 0.0594 ± 0.0039 (Fig. 2-11a), is slightly smaller than that of R31, 0.0964 ± 0.0092 (Fig. 2-9b). The conformational change of FUS on the binding of the DNA counterpart of pncRNA turned out to be much less. A consecutive addition of D13 followed by D19 and then D31 showed an increasing extension in FUS conformation as indicated by the reduction in GFP/BFP ratio that was obtained from the FRET assay (Fig.2-11b).

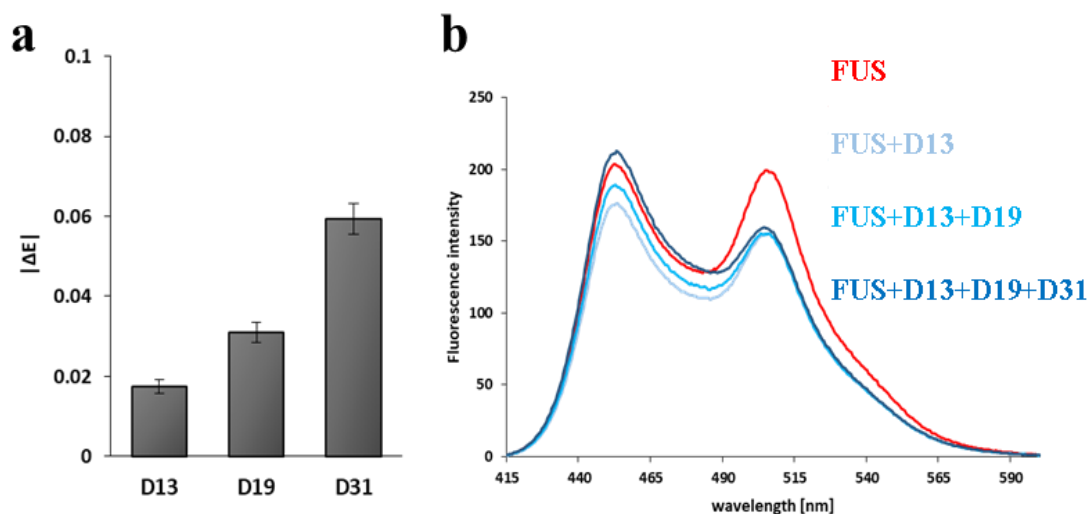


Fig. 2-11. The conformational change of FUS caused by DNA counterparts of the fragments of pncRNA. **(a)** $|\Delta E|$ of DNA counterparts, D13, D19 and D31. **(b)** Overlaying of fluorescence spectra shows the effect of consecutive addition of DNA counterparts, D13, D19 and D31. Three independent results were averaged for each fragment. $|\Delta E|$ is presented as a mean $\pm$ SD.

2.2.4 The effect of the length of RNA on the conformational change of FUS

It was reported that the affinity of FUS to RNA is affected not only by the presence of a specific sequence but also by the length of non-specific RNAs [7]. Therefore, we examined the effect of the length of non-specific RNAs on the conformational change of FUS. It was revealed from the results in **Fig. 2-11** that G residues play a specific role in the conformational change of FUS on the binding. Thus, oligomers of U residues were chosen as representative non-specific RNAs. It was found that the longer the non-specific RNA is, the larger the $|\Delta E|$ value is (**Fig. 2-12**). That is, a long non-specific RNA causes a more considerable conformational change of FUS than a short non-specific RNA.

R13 is a part of pncRNA (R31) and thus is regarded as a specific sequence (**Fig. 2-9a**). U₁₃ is a non-specific version of R13 of the same length. $|\Delta E|$ of U₁₃ (0.0233 ± 0.0043) is smaller than that of R13 (0.0663 ± 0.0091) (**Fig. 2-9b and 2-12**). The R13/U₁₃ ratio of $|\Delta E|$ is 2.8. Similarly, R19 and U19 are regarded as specific and non-specific sequences of the same length. $|\Delta E|$ of U₁₉ (0.0361 ± 0.0051) is smaller than that of R19 (0.0550 ± 0.0099) (**Fig. 2-9b and 2-12**), the ratio being 1.5. R31 and U₃₁ are also regarded as

specific and non-specific sequences of the same length. $|\Delta E|$ of U₃₁ (0.0743 ± 0.0103) is smaller than that of R31 (0.0964 ± 0.0092) (**Fig. 2-9b and 2-12**), the ratio being 1.3. It should be noted that the specific/non-specific ratio of $|\Delta E|$ becomes smaller when the length of RNA becomes greater. That is, the difference in the extent of the conformational change of FUS caused by RNA between specific and non-specific sequences becomes smaller when the length of RNA increases.

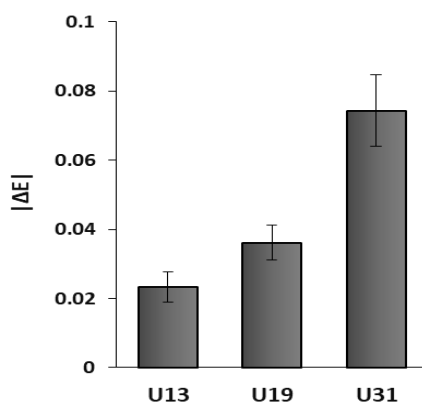


Fig. 2-12. The conformational change of FUS caused by oligomers of U residues of various lengths. Three independent results were averaged for each fragment. $|\Delta E|$ is presented as a mean $\pm$ SD

2.2.5 Comparison of the affinity of FUS toward specific and non-specific RNAs

The affinity of FUS toward specific R31 and non-specific U₃₁ was compared. Firstly, the affinity was compared through a titration of FUS against either TAMRA-labeled R31 or TAMRA-labeled U₃₁ using the fluorescence anisotropy of the complex. First of all, it should be noted that on the basis of the excitation/emission spectra of both BFP [9] and GFP [10], the light of 546 nm used for the excitation of TAMRA does not result in excitation of either BFP or GFP. Due to the complex formation between FUS and either R31 or U₃₁ during the titration, the fluorescence anisotropy increased (**Fig. 2-13a**). The difference in affinity between R31 and U₃₁ was not evident under the experimental conditions applied.

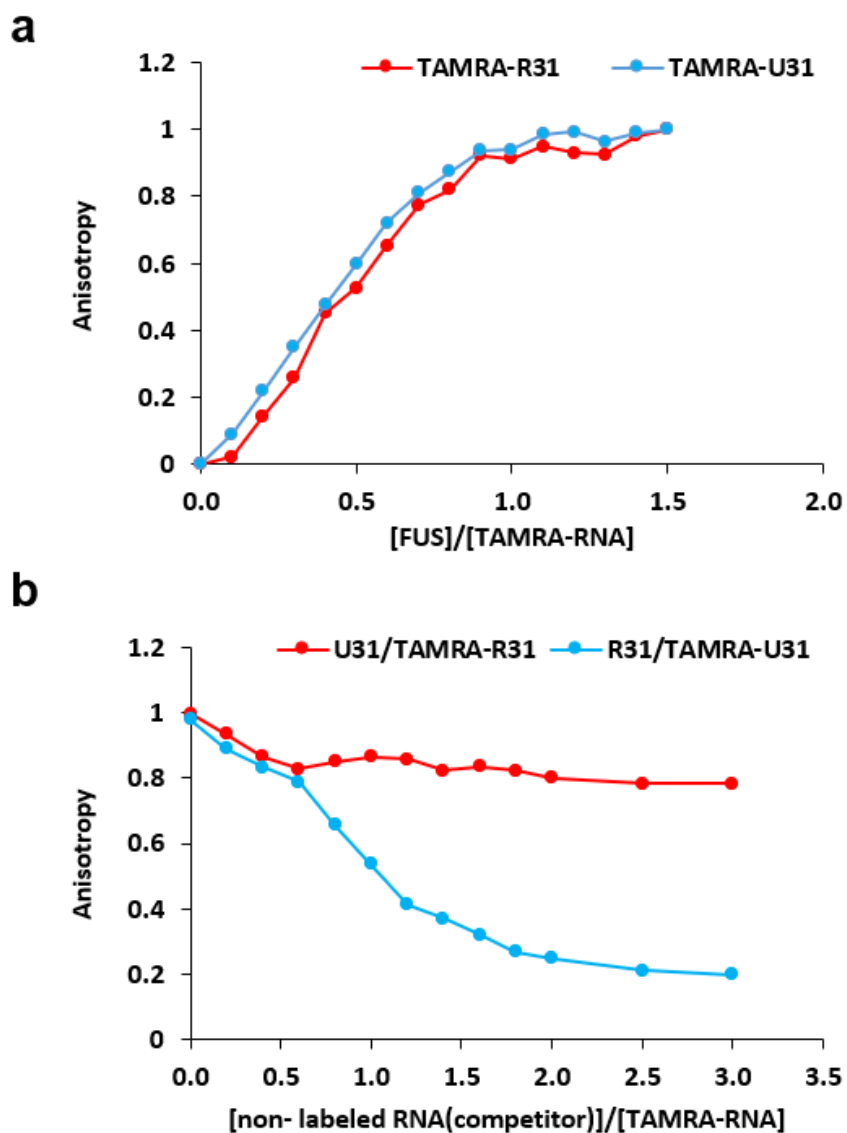


Fig. 2-13. The difference in affinity toward FUS between a specific RNA, R31 of pncRNA, and non-specific RNA, U₃₁. **(a)** Fluorescence anisotropy of TAMRA-labeled R31 (100 nM) (red) and TAMRA-labeled U₃₁ (100 nM) (blue) in the course of the addition of FUS. **(b)** Fluorescence anisotropy of TAMRA-labeled R31 (100 nM) (red) in the presence of a 1.5 molar ratio of FUS in the course of the addition of non-labeled U₃₁, and that of TAMRA-labeled U₃₁ (100 nM) (blue) in the presence of a 1.5 molar ratio of FUS in the course of the addition of non-labeled R31.

Then, the difference in affinity was examined by means of competition experiments. When R31 was added to the solution containing the FUS-U₃₁ complex, the anisotropy greatly decreased (**blue line in Fig. 2-13b**). This indicates that U₃₁ that was bound to FUS was mostly replaced by the added R31. When U₃₁ was added to the solution containing the FUS-R31 complex, on the other hand, the anisotropy decreased just a little (**red line in Fig. 2-13b**). This indicates that R31 that was bound to FUS was not replaced by the added U₃₁, but that was still mostly bound to FUS. These competition results demonstrated that the affinity of FUS toward specific R31 is higher than that toward non-specific U₃₁.

2.2.6 The effect of the G-quadruplex structure of DNA/RNA on the conformational change of FUS

It was reported that FUS interacts with either telomeric DNA or TERRA, both of which form the G-quadruplex structure [11]–[13]. Therefore, the conformational changes of FUS caused by this DNA and RNA were examined. The sequences of TERRA and telomeric DNA (Htelo) are shown in **Table 2-1**. It was revealed that both TERRA and telomeric DNA cause the implicated closed-to-open conformational change of FUS (**Fig. 2-14a, b**), $|\Delta E|$ being 0.0776 ± 0.0029 and 0.0614 ± 0.0059 , respectively (**Fig. 2-14e**).

Then, the necessity of the presence of the G-quadruplex structure for the conformational change of FUS caused by TERRA and telomeric DNA was examined. Two G residues of TERRA were replaced by U residues to produce a mutant TERRA, Mut_TERRA (**Table 2-2 in Materials and Methods**). Similarly, two G residues of telomeric DNA were replaced by T residues to produce a mutant telomeric DNA, Mut_Htelo (**Table 2-1 in Materials and Methods**). It has already been revealed that both mutants do not form the G-quadruplex [14]. It was found that both mutant TERRA and telomeric DNA can cause a similar conformational change of FUS (**Fig. 2-14c, d**), $|\Delta E|$ being 0.0748 ± 0.0013 and 0.0845 ± 0.0124 , respectively (**Fig. 2-14e**). These results indicate that the presence of the G-quadruplex structure is not critical for the conformational change of FUS caused by either TERRA or telomeric DNA.

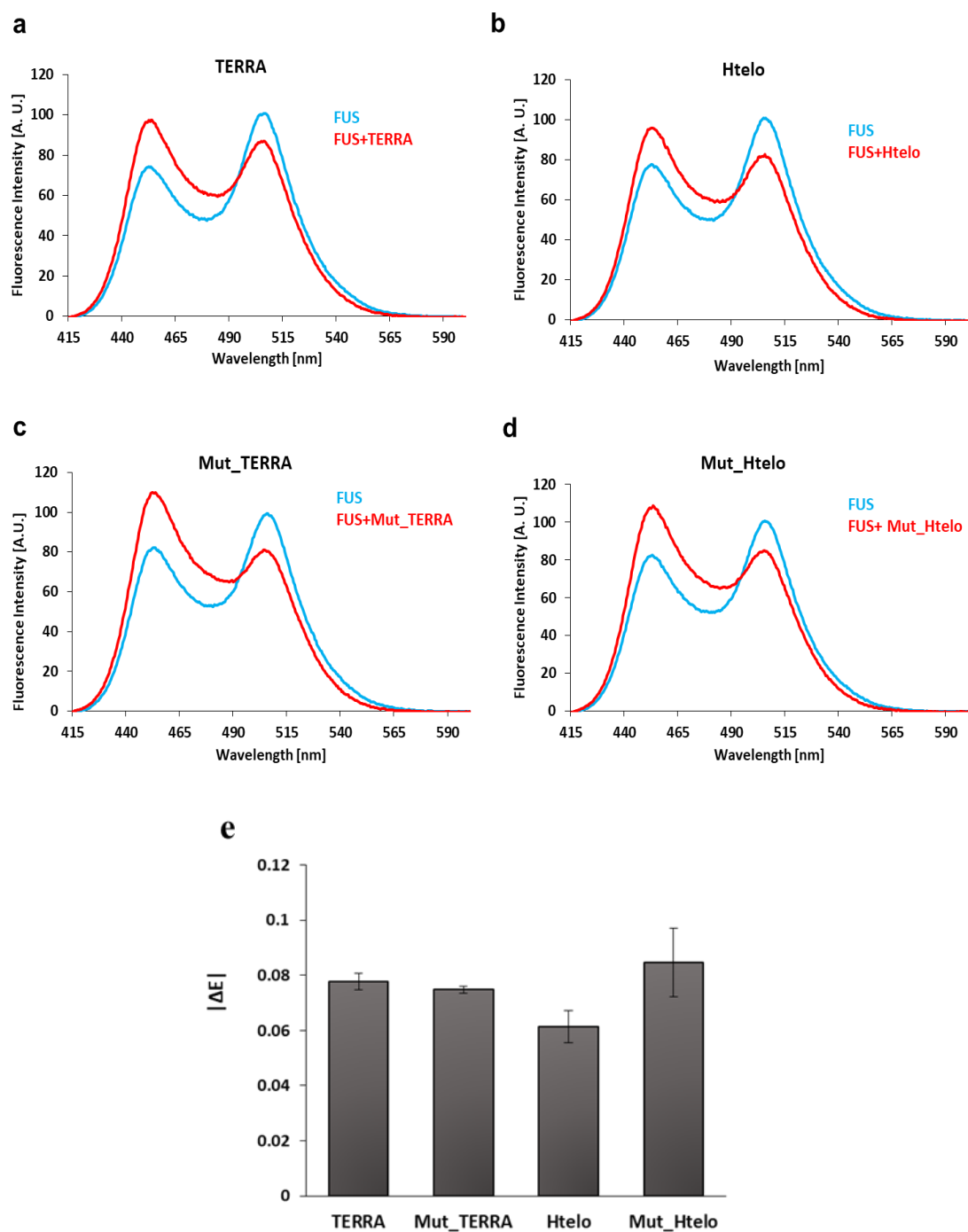


Fig. 2-14. The conformational changes of TLS caused by TERRA and telomeric DNA. Fluorescence spectrum of TLS in either the absence (blue) or presence (red) of TERRA (a), telomeric DNA (b), mutant TERRA (c), or mutant telomeric DNA (d). (e) $|\Delta E|$ of each RNA or DNA.

2.2.7 ALS-linked mutation does not affect either the conformation or the conformational change of FUS

FUS mutations are found in ~5% of familial ALS patients [15]. The effect of each mutation on either the conformation of FUS in the absence of RNA or the extent of the conformational change induced by pncRNA was examined. Two mutants reported in ALS patients, P525L and K510R [16], [17], were tested. The mutant proteins were successfully expressed and purified in the same way as the wild-type protein. The fluorescence spectrum of either P525L or K510R was similar to that of the wild-type. This indicated that both the mutants take on a structure that is similar to that of the wild-type protein. $|\Delta E|$ of either P525L or K510R on binding of R31 was also close to that of the wild-type (**Fig. 2-15**). Thus, it was found that ALS-linked mutations do not affect either the conformation of FUS or the extent of the conformational change induced by pncRNA.

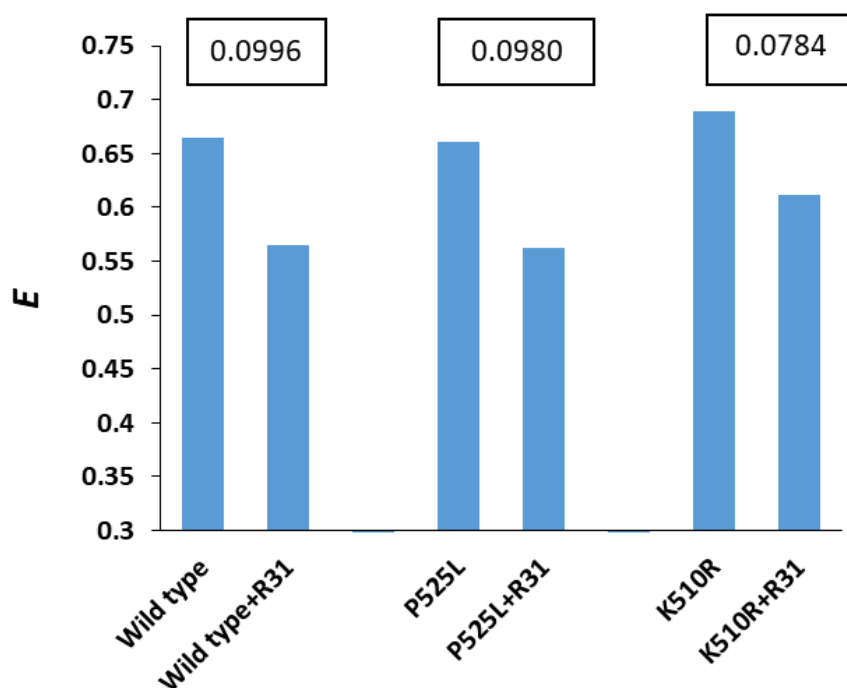


Fig. 2-15. The conformation of FUS and its change caused by pncRNA (R31) are not affected by ALS-linked mutations of FUS. The E values in the absence and presence of R31 for wild-type and mutant FUSs are shown, each $|\Delta E|$ value being indicated in a box.

2. 3 Discussion

In order to explain the biological function of FUS, the closed-to-open conformational change of FUS on binding of pncRNA was suggested (**Fig. 2-3**) [18], although no experimental proof was available. Here, FRET results indicated for the first time that FUS actually undergoes a conformational change, extension, on binding of the full-length pncRNA of 602 nucleotides (**Fig. 2-7a**). It is implied that this extension corresponds to the closed-to-open conformational change.

We assume that the driving force for the formation of the closed conformation is the electrostatic interactions between the LC domain and RGG domains. The isoelectric point (pI) of the LC domain estimated with the ExPASy program [19] is 3.56, indicating that the LC domain is negatively charged at neutral pH. On the other hand, the pI values of RGG1, RGG2, and RGG3 are 9.30, 12.13, and 11.02, respectively, indicating that all the RGG domains are positively charged under neutral pH. Thus, electrostatic interactions between the LC domain and RGG domains are likely. The Π -cation interaction between tyrosine residues in the LC domain and arginine residues in the RGG domains [36] may also contribute to the formation of the closed conformation. As RNA is negatively charged, we assume that pncRNA binds to the positively charged RGG domains and disrupts the interactions between the LC domain and RGG domains, resulting in a disruption of the closed conformation and the transformation to the open conformation.

We revealed that the RRM domain does not interact with pncRNA, while the RGG3 domain does [6]. The interaction of the ZnF domain with a certain RNA was reported, in which the involvement of N435, F438, and R441 was suggested [20]. Therefore, mutants as to these residues were examined. The $|\Delta E|$ values of the mutants for these residues turned out to be almost the same as the $|\Delta E|$ value of the wild-type for R31. This may support the importance of the interactions involving RGG domains with pncRNA for the conformational transformation, which is described above.

It should be added that the extension of FUS in which the N-terminal region of FUS is relieved from masking by the C-terminal region of FUS can account for the experimental transcriptional repression, even if the view of the closed-to-open

conformational change of FUS may be oversimplified. It should also be added that the conformational change may not be a simple two-state transition but that the FRET results may indicate that the population of the extended conformations of FUS increase on binding of the pncRNA.

It is remarkable to find that a small portion of pncRNA, pncRNA D1-1 of 31 nucleotides (R31), is sufficient to cause this complete conformational change of FUS (**Fig. 2-7b**), although it has already been shown that R31 is enough for binding of FUS [6].

The dissection of R31 revealed that the GAGGU sequence located in the 5' half of R31 is critical for the conformational change of FUS (**Fig. 2-9b**). In particular, the importance of G residues for the conformational change of FUS was revealed by mutagenesis (**Fig. 2-10**). The fact that the sequences recognized by FUS are mostly rich in G residues [6], [12], [20]–[23] may be biologically relevant in the context of the importance of G residues for causing the conformational change of FUS. The stem-loop structure in the 3' half of R31 was also determined to be critical for the conformational change of FUS (**Figs. 2-6 and 2-9b**).

It was revealed that the DNA counterpart of pncRNA causes less conformational change of FUS than pncRNA (**Fig. 2-11**), which indicates that the difference in the sugar type affects the conformational change of FUS. This may be related to the previous report that RNAs bind to FUS more strongly than DNA counterparts [7].

It was found that the extent of the conformational change of FUS caused by RNA is governed not only by a specific sequence but also by the length of a non-specific sequence. Long oligo(U) RNA causes a larger conformational change of FUS than shorter oligo(U) RNA (**Fig. 2-12**). Apparently, in addition to the presence of the specific RNA sequence, the length of non-specific RNA is another factor that determines the extent of the conformational change of FUS. This finding may be related to the previous report that regardless of the presence of a particular sequence or structure, the length of RNA/DNA dominates the affinity toward FUS in some cases [7].

When the RNA is short, the extent of the caused conformational change of FUS greatly differs between specific and non-specific sequences. When the RNA is long, on

the other hand, the extent of the caused conformational change of FUS does not differ so much between specific and non-specific sequences (**Fig. 2-9 and 2-12**). This can be interpreted by that for the conformational change of FUS, the RNA sequence factor is dominant when the RNA is short, while the RNA length factor is dominant when the RNA is long. Thus, the extent of the conformational change of FUS is supposed to be governed by two factors, sequence and length.

In order to address the biological significance of specific R31 over non-specific U₃₁, the affinity toward FUS was compared between R31 and U₃₁. A competition experiment indicated that specific R31 exhibits a higher affinity toward FUS than non-specific U₃₁ (**Fig. 2-13b**). Therefore, it is assumed in cells that a specific RNA such as R31 can interact with FUS due to high affinity, while a non-specific RNA such as U₃₁ cannot due to low affinity. Then, as a matter of fact, the conformational change of FUS can be caused only by a specific RNA, but not by a non-specific RNA. Thus, practically, only a specific RNA may exert the biological function.

There could be two models regarding the relationship between the binding of RNA and the RNA-induced conformational change. In the first model, the binding and the conformational change are in concert. In the second model, the binding is not sufficient to trigger the conformational change. All the data presented can be basically explained by the first model. Nonetheless, we do not completely rule out the contribution of the second model in a certain case, in order to consider the greater biological significance of a specific RNA.

Induction of the conformational change of FUS was examined not only for pncRNA but also for other targets of FUS, TERRA, and telomeric DNA, that are known to form the G-quadruplex structure. It was found that TERRA and telomeric DNA cause a similar extent of conformational change of FUS as pncRNA (**Fig. 2-14a, b, and e**). It is suggested that the conformational change of FUS might be generally caused by target DNA/RNA and that the conformational change may be essential for FUS to exert certain biological functions through the target DNA/RNA.

It was also found that the formation of the G-quadruplex structure is not needed for either TERRA or telomeric DNA to cause the conformational change of FUS (**Fig. 2-**

14c, d, and e). It was reported that FUS binds to the G-quadruplex structure more strongly than the single-stranded structure [12], [24], although this is controversial [7]. Therefore, it may still be the case that the conformational change of FUS caused by either TERRA or telomeric DNA having the G-quadruplex structure is biologically significant if only the interaction with high affinity can occur in cells.

The possible correlation between the occurrence of ALS and the change in either the conformation of FUS or the extent of the conformational change of FUS caused by RNA was examined. FRET studies did not show any correlation between them.

2.4 Conclusion

We demonstrated by means of the FRET assay that FUS indeed undergoes a conformational change, extension, on binding to pncRNA. The extension is implied to correspond to the closed-to-open conformational change of FUS. A critical region of pncRNA for induction of the conformational change of FUS was identified. Data showed the effect of the length of RNA in addition to sequence specificity on the conformational change of FUS. We found that other important FUS targets, telomeric DNA and TERRA, can induce a conformational change of FUS. Under our experimental conditions, we did not observe any effect of ALS-linked mutations on either the conformation or conformational change of FUS.

2.5 Materials and Methods

2.5.1 Preparation of oligonucleotides

The sequences of the oligonucleotides used in FRET and fluorescence anisotropy analyses are shown in **Table 2-1**. Full-length pncRNA was in vitro transcribed with a MEGAscript Kit (AM1334, Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, the pncRNA sequence was inserted into the pcDNA 3.1 (+) vector (V790-20, Thermo Fisher Scientific), and RNA was transcribed by T7 transcription and purified by phenol/chloroform extraction, followed by isopropanol precipitation. The deletion mutants of pncRNA (TAMRA-labeled and non-labeled) and their DNA counterparts (non-labeled) were synthesized, purified, and de-salted by FASMAC Co., Ltd. TERRA, mutant TERRA, Htelo, mutant Htelo, A₁₂, G₁₂, U₁₂, and C₁₂ were obtained

from Hokkaido System Science Co., Ltd. The sequences of the primers used for subcloning are shown in **Table 2-2**. The primers were synthesized and purified by Invitrogen.

Table 2-1. RNA/DNA sequences used in this study.

Name	Sequence
full length pncRNA	r (UUUUUCUAUCAGUUUUUCUUUGAGCUUUUACUGUUAAGAGGGUACGG UGGUUUGAUGACACUGAACUAUAUUCAAAAGGAAGUAAAUGAACAGUU UUCUUAUUUUGGGGCAGGUACUGUAAAAAUA AAAACAAAAGUU AAGAC AGUAAAAUGUCCUUUUUUUUUUUUAAUGCACC AAAGAGACAGAACCUGU AAUUUUUAAAAACUGUGUAUUUUUAAUUUACAU CUGCUUAAGUUUGCGAU AAUAUUGGGGACCCUCUCAUGUAACCACGAACACCUAUCGAUUUUGCU AAAAAUCAGAU CAGUACACUCGUUUGUUUAAUUGAUAAUUGUUCUGAA UUAUGCCCGGCUCCUGCCAGCCCCCUCACGCUCACGAAUUCAGUCC CAG GGCAA AUUCUAAAGGUGAAGGGACGUCUACACCCCCAACAAAACCAAU UAGGAACCUUCGGUGGUCUUGUCCCAGGCAGAGGGGACUAAUAUUUC AGCAAUUUAAUUUCUUUUUUUAAUUUAAAAAAAUGAGUCAGAAUGGAGA UCACUGUUUCUCAGCUUUC CAUUCAGAGGUGUGUUUCUCCCGGUUAAA UUGCCGGCACGGGAAGGGAGGGGGUGCA)
pncRNA (R31)	r (GUUAAGAGGGUACGGUGGUUUGAUGACACUG)
5'-pncRNA (R13)	r (GUUAAGAGGGUAC)
3'-pncRNA (R19)	r (CGGUGGUUUGAUGACACUG)
R3	r (GGU)
R4	r (GGGU)
R5	r (AGGGU)
R6	r (GAGGGU)
R7	r (GUUAAGA)
R10	r (GGUUUGAUGA)
R12	r (GUUAAGAGGGUA)
U ₁₃	r (UUUUUUUUUUUUUU)
U ₁₉	r (UUUUUUUUUUUUUUUUUUUU)
U ₃₁	r (UU)
D31	d (GTTAAGAGGGTACGGTGGTTTGATGACACTG)
D13	d (GTTAAGAGGGTAC)
D19	d (CGGTGGTTTGATGACACTG)
TERRA	r (UUAGGGUUAGGGUUAGGGUUAGGG)
Mut_TERRA	r (UUAGGGUUAGUGUUAGUGUUAGGG)
Htelo	d (TTGGGTTAGGGTTAGGGTTAGGGA)
Mut Htelo	d (TTGGGTTAGTGTTAGTGTTAGGGA)

2.5.2 Plasmid construction

The DNA sequence coding for the FUS was inserted between the gene encoding the maltose binding protein (MBP) and the hexahistidine tag (6xHis) of the pMAL-p2x vector. Then the genes encoding a blue fluorescent protein (TagBFP) and green fluorescent protein (TagGFP2) were inserted between the genes encoding MBP and FUS, and between the genes encoding FUS and 6xHis, respectively. The DNA sequences coding for the TagBFP and TagGFP2 were obtained from the pCasper3-BG plasmid (Clontech) by means of polymerase chain reaction (PCR) using the primers indicated in **Table 2-2**. The obtained DNA sequence encoding MBP-BFP-FUS-GFP-6xHis was confirmed by DNA sequencing. The ALS-linked FUS mutants, K510R FUS and P525L FUS, were constructed by following the QuickChange Site-directed Mutagenesis Kit protocol (Agilent Technologies) using the primers shown in **Table 2-2** and the above-obtained plasmid containing the MBP-BFP-FUS-GFP-6xHis gene as a template.

Table 2-2. Primer pairs used for PCR amplification (TagBFP and TagGFP2) and site-directed mutagenesis (P525L and K510R).

TagBFP	forward	5'	CGCGGATCCGGTAGCGGCAGCGGTAGCATGAGCGAGCTGATTAAGGAGAAC	3'
	reverse	5'	CGCACCGGTAAGCTTGTGCCCCAGTTTGCTAG	3'
TagGFP2	forward	5'	CGGGGTACCATGAGCGGGGCGAGGAG	3'
	reverse	5'	CTAGTCTAGACCTGTACAGCTCGTCCATGCCGTG	3'
P525L	forward	5'	TGTATGGTAGCGGCAGCGGTACCATGAGCG	3'
	reverse	5'	CGCTACCATAACAGCCTCTCCCTGCGATCC	3'
K510R	forward	5'	GGATGGATTCCAGGGGTGAGCACAGACAGG	3'
	reverse	5'	GGAATCCATCCTGCCAGGGCCAAAGCCACC	3'

2.5.3 Expression of MBP-BFP-FUS-GFP-6xH and its solubilization

The MBP-BFP-FUS-GFP-6xHis protein (FUS) and the two mutants, FUS K510R and FUS P525L, were expressed using BL21 (DE3) Gold Escherichia coli cells. A single colony was inoculated into 10 mL LB medium containing 100 mg/L ampicillin and grown overnight at 37°C.

The overnight culture was transferred to 1 L LB medium containing the same antibiotics and incubated at 37°C until the OD₆₀₀ reached ~0.6. Then the temperature of the culture was lowered to 20°C, and the expression of the protein was induced by the addition of 0.1 mM isopropylthio-β-D-galactopyranoside (IPTG). The culture was incubated for an additional 20 h. Subsequently, the bacterial cells were pelleted at 1500 g and stored at -20°C until purification.

2.5.4 Protein purification

The stored cells were resuspended in lysis buffer comprising 50 mM Tris-HCl, pH 7.6, 25 mM glucose, 1% CHAPS (Wako Pure Chemical Industries, Ltd.), 10 mM benzamidine, 5 U/mL DNase I (Invitrogen), 1 mg/L RNase (Nippon Gene), and 0.2 g/L lysozyme. All the following procedures were carried out at 4°C unless otherwise stated. Cells were lysed by sonication (Astrason ultrasonic processor, Misonix Inc.) on ice. The cell lysates were centrifuged at 50,000 g for 30 min. The supernatants were purified by nickel-affinity column chromatography using Ni Sepharose beads (GE Healthcare Bio-Sciences). The column was washed with a wash buffer comprising 50 mM Tris-HCl, pH 7.6, 1 M NaCl, 25 mM glucose, 1% CHAPS, and 25 mM imidazole. Protein was eluted with elution buffer comprising 50 mM Tris-HCl, pH 7.6, 25 mM glucose, 1% CHAPS, and 500 mM imidazole. 10% SDS-PAGE was performed to identify the fractions containing FUS. The pooled fractions were further purified by size exclusion chromatography (SEC) using a Hiload 16/60 Superdex 200 prep grade column (GE Life Sciences). The column was equilibrated with a buffer comprising 50 mM Tris-HCl, pH 7.6, 250 mM NaCl, 25 mM glucose, and 1% CHAPS. The fractions containing monomer FUS were collected. The purity was confirmed by 10% SDS-PAGE. The protein concentration was determined from the absorbance at 280 nm using a molar extinction coefficient (ϵ) value of 185,770. MBP and 6xHis tags were kept attached; MBP enhances the solubility. MBP and His tag cleavage was performed once to ensure that their presence did not affect the conformational change of FUS. A final concentration of 5 mM fresh dithiothreitol (DTT) was added to the purified FUS solution, which was then kept at 4°C. All the experiments were carried out within one week after purification.

2.5.5 Fluorescence resonance energy transfer (FRET) assay

A final concentration of 50 nM FUS dissolved in 5 mM Tris-HCl, pH 7.6, 0.1% CHAPS, 2.5 mM glucose, 25 mM NaCl, and 0.5 mM DTT was prepared in a total volume of 140 μ L as a FUS sample by diluting the FUS stock solution with RNase-free water. An equimolar amount of RNA was also dissolved. Fluorescence spectra were collected with a steady-state photon counting spectrofluorometer (JASCO FP-8500 spectrometer, Japan Spectroscopic Co.) using a standard quartz cuvette with an optical path length of 1 cm. The excitation wavelength was 402 nm. The spectra slit width of 5 nm was used for excitation and emission with an integration time of 1 nm/s from 415 nm to 650 nm. All the measurements were carried out at 25°C. A blank was measured and subtracted from all the spectra. Data were processed using a JASCO Spectra Manager for the FP-8000 series.

2.5.6 FRET calculations

The relative FRET efficiency (E) for FUS, also known as proximity ration, was calculated using the formula, $E = I_{\text{GFP}}/(I_{\text{GFP}}+I_{\text{BFP}})$, where I_{BFP} and I_{GFP} are the intensities at 453 nm for BFP and 506 nm for GFP, respectively. Background due to the buffer was subtracted from the obtained fluorescence data. The effects on I_{GFP} of direct excitation of GFP at 402 nm and leakage of the BFP emission into the GFP emission, bleed through, were corrected following the method described in the literature [25]. The change in E value upon addition of RNA was calculated using the equation, $\Delta E = |E_{+\text{RNA}} - E_{-\text{RNA}}|$, where $E_{-\text{RNA}}$ and $E_{+\text{RNA}}$ are the E values in the absence and presence of RNA, respectively.

2.5.7 Fluorescence anisotropy assay

100 nM TAMRA-labeled RNA (TAMRA-RNA) in 5 mM Tris-HCl, pH 7.6, 0.1% CHAPS, 2.5 mM glucose, 25 mM NaCl, and 0.5 mM DTT was prepared in a total volume of 140 μ L. Then the purified protein was titrated against TAMRA-RNA. For the competition assay, the non-labeled RNA competitor was titrated against a solution containing FUS and TAMRA-RNA, whose molar ratio was 1:1.5. Fluorescence anisotropy measurements were conducted using a FP-8500 spectrofluorometer (JASCO) with excitation at 546.0 nm and emission at 577.5 nm at 25°C. For each titration point, fluorescence anisotropy spectra were recorded, seven times, which were automatically averaged. JASCO Spectra Manager software was used for spectral measurement. Data were exported as Excel files for analyses.

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Chapter Three

Direct visualization of the conformational change of FUS/TLS upon binding to promoter-associated non-coding RNA using HS-AFM

3.1 Introduction

In Chapter 2, we could detect the conformational change of FUS upon binding pncRNA as well as other RNA/DNA sequences using the FRET assay. Although FRET is very sensitive to distance changes between chromophores that are attached to protein termini, it was challenging to accurately measure the distance change using FRET efficiency (E) calculations. That is because, in ensemble FRET, the estimated E would reflect the average distance change for all the molecules in the measured sample. If there is a considerable distance variation in the sample, the measured distance can hardly reflect the actual distance between FUS termini for each molecule [1], [2]. Hence, FRET provided qualitative information about the FUS conformational change, which was very useful for comparing the effects of different nucleic acids on the FUS structure. However, the single molecule detection of FUS structural changes was essential to get quantitative data.

High-speed atomic force microscopy (HS-AFM) is a nano-dynamic technique that is very useful for observing the structural dynamics of biological molecules in physiological solutions [3], [4]. An AFM generates images by scanning the surface of a sample by a small cantilever. A sharp tip at the end of the cantilever is put in contact with the surface, bending the cantilever and thus changing the amount of laser light reflected into the photodiode. The height of the cantilever is then adjusted to restore the response signal resulting in the measured cantilever height tracing the surface (**Fig. 3-1**). HS-AFM has been successfully used to visualize the conformation, movement, and interaction of proteins [5]–[8]. Therefore, we aimed to directly visualize the conformational change of a single molecule of the FUS fusion protein using HS-AFM.

FUS comprises an N-terminal low complexity domain (LC), followed by an Arg-Gly-Gly-rich domain (RGG1), an RNA recognition motif (RRM), a second RGG domain (RGG2), a zinc-finger domain (ZnF), and a third RGG domain (RGG3). Most domains of FUS are intrinsically disordered regions (IDRs), the exception being the RRM and ZnF ones [9]. Therefore, the visualization of FUS by high-speed atomic force microscopy (HS-AFM) was supposed to be difficult, particularly when FUS takes on an extended flexible structure, although it is not impossible [10]. In this chapter, by means of HS-AFM, we succeeded in visualizing the hypothesized compact-to-extended conformational change of FUS caused by pncRNA.

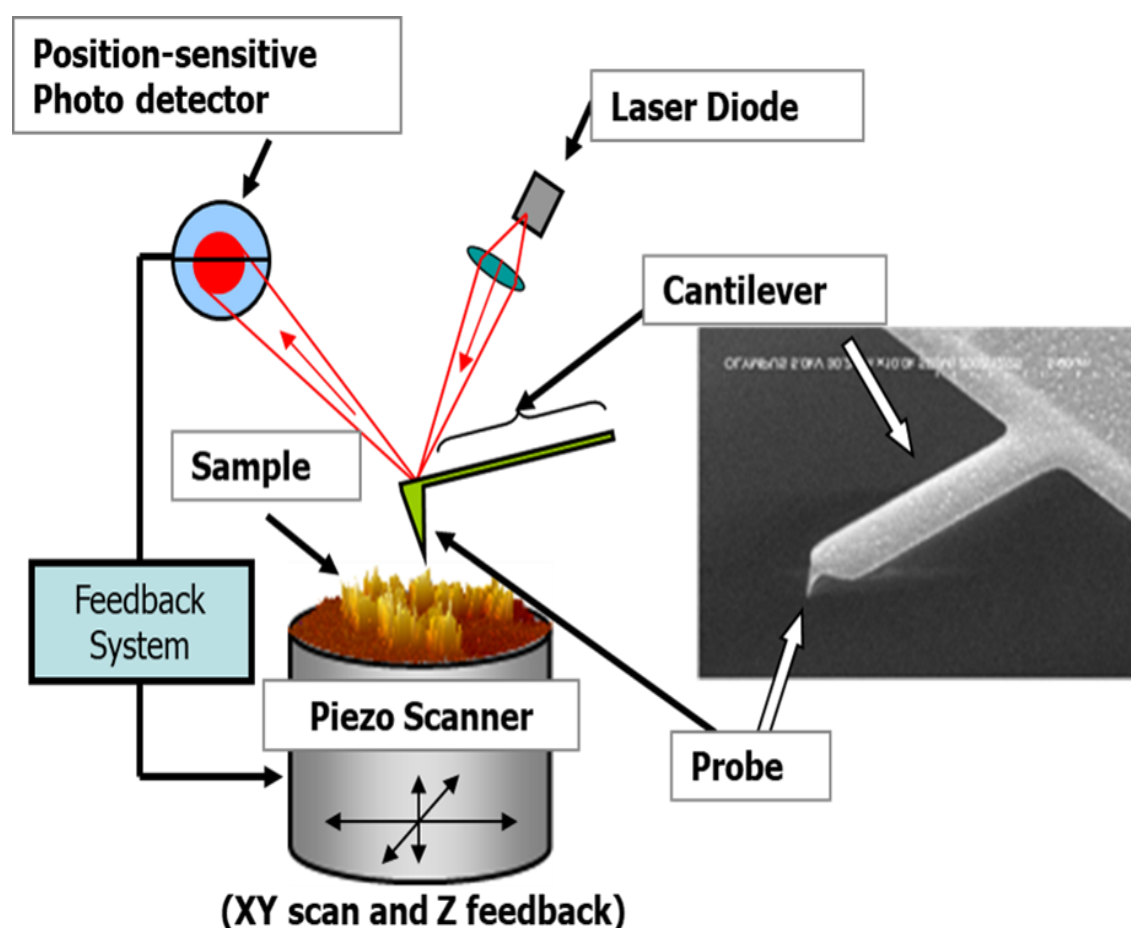


Fig. 3-1. Illustration of high-speed atomic force microscopy (HS-AFM). Adapted from [11].

3.2 Results

3.2.1 Experimental set up for FUS fusion protein

In order to facilitate visualization of the conformational change of FUS, a maltose-binding protein (MBP) and a blue-fluorescent protein (BFP) were attached to the N-terminus of FUS, and a green-fluorescent protein (GFP) was attached to its C-terminus, respectively, to obtain a fusion protein (**Fig. 3-2**). These three proteins are known to form a globular structure (Protein Data Bank (PDB) IDs for MBP, BFP, and GFP are 1OMP, 3M24, and 1EMA, respectively), and thus are expected to be visualized as spheres on HS-AFM. Therefore, when FUS takes on a compact globular structure, visualization of maximally four spheres is expected. When FUS takes on an extended flexible structure and visualized just faintly due to its IDR characteristics, two spheres in proximity in HS-AFM must be MBP and BFP located at the N-terminus of FUS as MBP and BFP are directly linked, and the remaining visible sphere must be GFP located at the C-terminus of FUS. As shown below, the attachment of the three proteins facilitates understanding of the conformational behavior of FUS.

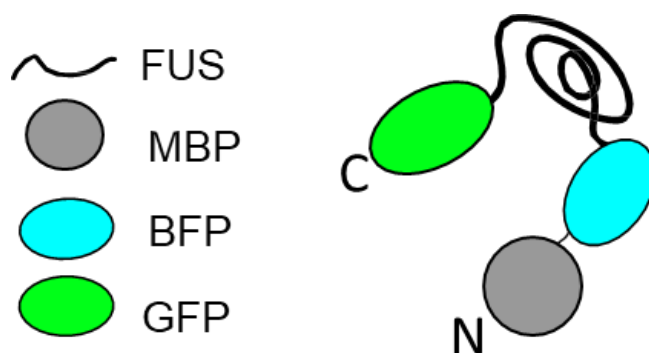


Fig. 3-2. Schematic drawing of the FUS fusion protein; MBP [gray] - BFP [cyan] - FUS [black] -GFP [green], designed as a compact form. N and C indicate the N- and C-termini, respectively.

The C-terminal region of BFP is supposed to be flexible because it lacks any secondary structure [12]. Additionally, a flexible linker, Gly-Ser-Gly-Ser-Gly-Thr, was inserted between FUS and GFP. Thus, FUS is isolated from MBP-BFP and GFP by flexible regions, respectively. Therefore, the effect of the attachment of MBP-BFP and GFP on the conformational behavior of FUS is supposed to be moderate, if any. It should also be noted that MBP has no RNA binding activity [13].

The fusion protein was expressed in BL21 (DE3) Gold *Escherichia coli* cells and purified by nickel-affinity column chromatography followed by size exclusion chromatography. The fusion protein was immobilized on a mica surface modified with 0.05% (3-aminopropyl) triethoxysilane (APTES). All HS-AFM experiments were performed using a laboratory-built high-speed atomic force microscope [6]. Igor pro software was used for data analysis.

3.2.2 *FUS is mainly in a compact form in the absence of RNA*

Although the full-length pncRNA comprises 602 nucleotides, it was shown that a fragment of 31 nucleotides, named fragment 1-1, is enough for appropriate binding to FUS [14]. Hereafter, fragment 1-1 is just called pncRNA.

In the absence of pncRNA, an AFM image indicated that most FUS molecules (and most fusion protein molecules also) form a compact structure (**Fig. 3-3**). The C-terminal region of FUS (166-526) comprising three RGG domains, RRM and ZnF, is responsible for binding DNA/RNA [15]–[17]. The C-terminal region is supposed to be positively charged ($pI=9.88$), and the N-terminal region (1-165) to be negatively charged ($pI=3.56$) [18]. Therefore, the electrostatic interaction between the N- and C-terminal regions of FUS would be a driving force for the formation of the compact structure. It was reported that the cation- π interaction between arginine residues of the RGG domains of the C-

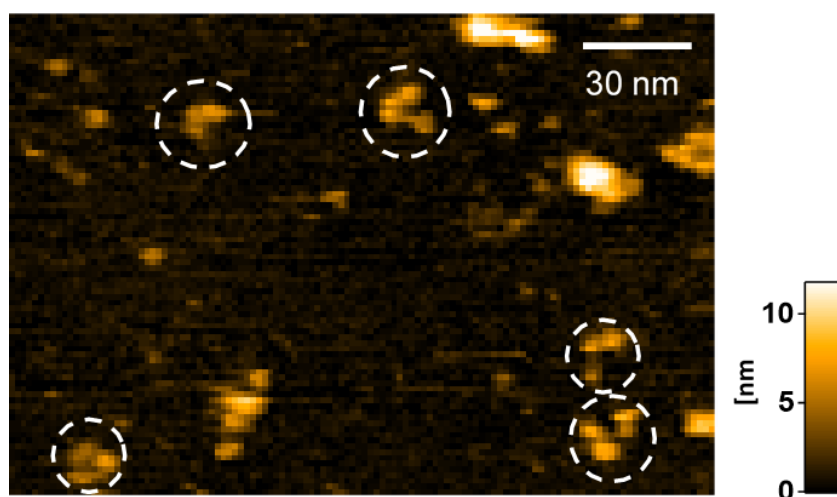


Fig. 3-3. A representative HS-AFM image of the fusion protein (10 nM) in the absence of pncRNA in the measurement buffer (10 mM phosphate buffer, pH 6.8). Molecules in the compact form are indicated by broken white circles. 0.3 s/frame and 120×85 pixels².

terminal region and tyrosine residues of the N-terminal region is a driving force of the liquid-liquid phase separation of FUS [19]. The cation- π interaction may also contribute to the formation of the compact structure.

3.2.3 The moment of FUS structural change could be visualized by HS-AFM

The moment of the compact-to-extended conformational change of FUS was successfully captured by continuous monitoring of the behavior of a molecule before and after the addition of pncRNA (**Fig. 3-4a**). In **Fig. 3-4a**, pncRNA was added at time 0 s. A FUS molecule (and a fusion protein molecule also) forms a compact structure for a moment and then, at a certain time, FUS quickly transformed into an extended structure. The distance between BFP and GFP is a measure of the extension of a FUS molecule.

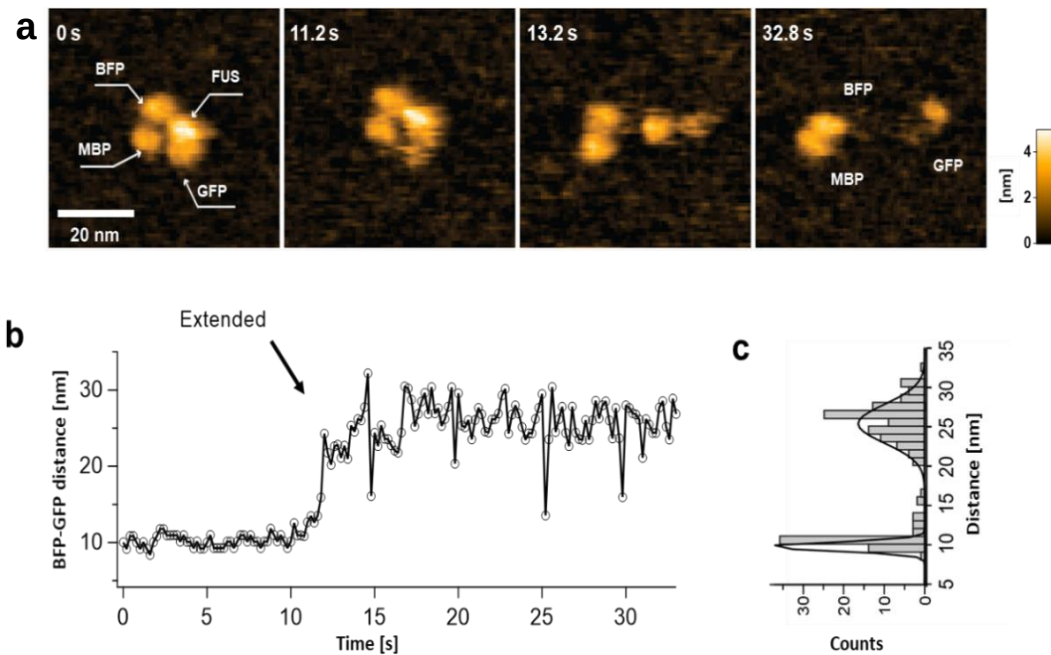


Fig. 3-4. HS-AFM observation of the moment of the conformational transition of FUS. **(a)** Consecutive HS-AFM images showing the moment of the conformational transition of FUS in the presence of pncRNA. 0.2 s/frame. **(b)** Time course of the distance between BFP and GFP. FUS is transformed from a compact structure into an extended structure, as indicated by a black arrow. **(c)** The corresponding histogram of the BFP-GFP distance being fitted to a Gaussian distribution with center values of 9.8 ± 0.8 nm (compact structure) and 25.4 ± 3.5 nm (extended structure), respectively.

When the compact-to- extended conformational change of FUS was caused by pncRNA, the BFP-GFP distance dramatically changed from ca. 10 nm to 27 nm (**Fig. 3-4b**). A histogram corresponding to the distribution of BFP-GFP distance shows two peaks that correspond to the compact and extended forms of FUS, respectively (**Fig. 3-4c**)

3.2.4 FUS is extended and more flexible in the presence of pncRNA

In order to assess the difference in FUS behavior in the absence and presence of pncRNA, ten fusion molecules were tracked for at least 20 seconds in the absence and presence of pncRNA, respectively. **Fig. 3-5a** presents the behavior of one molecule that was monitored for ~ 40 s in the absence of pncRNA. A schematic drawing of the observed compact structure is shown in **Fig. 3-5b**. A time course for a representative FUS molecule in the absence of RNA is shown in **Fig. 3-5c**. In the presence of pncRNA, monitoring of a FUS molecule suggested that FUS takes on an extended structure, although FUS can be mostly seen just faintly (**Fig. 3-5d**). A schematic drawing of the extended structure is shown in **Fig. 3-5e**. It is reasonable that FUS can be seen just faintly in the extended structure because most of FUS comprises IDRs. The formation of the extended structure of FUS can be deduced more solidly on the basis of the locations of MBP, BFP and GFP (**Fig. 3-5d and e**). A time course corresponding to the molecule shown in **Fig. 3-5d**, as a representative FUS molecule in the absence of RNA, is shown in **Fig. 3-5f**.

It was revealed that the third RGG domain of the C-terminal region of FUS is important for binding to pncRNA [14]. Therefore, it is supposed that pncRNA binds to RGG3 and thus interferes with the interaction between the N- and C-terminal regions, which results in destruction of the compact structure and the transition to the extended structure. It is supposed that this conformational change makes the N-terminal region of FUS, which was masked with the C-terminal region before, exposed for the inhibitive interaction with CBP/p300.

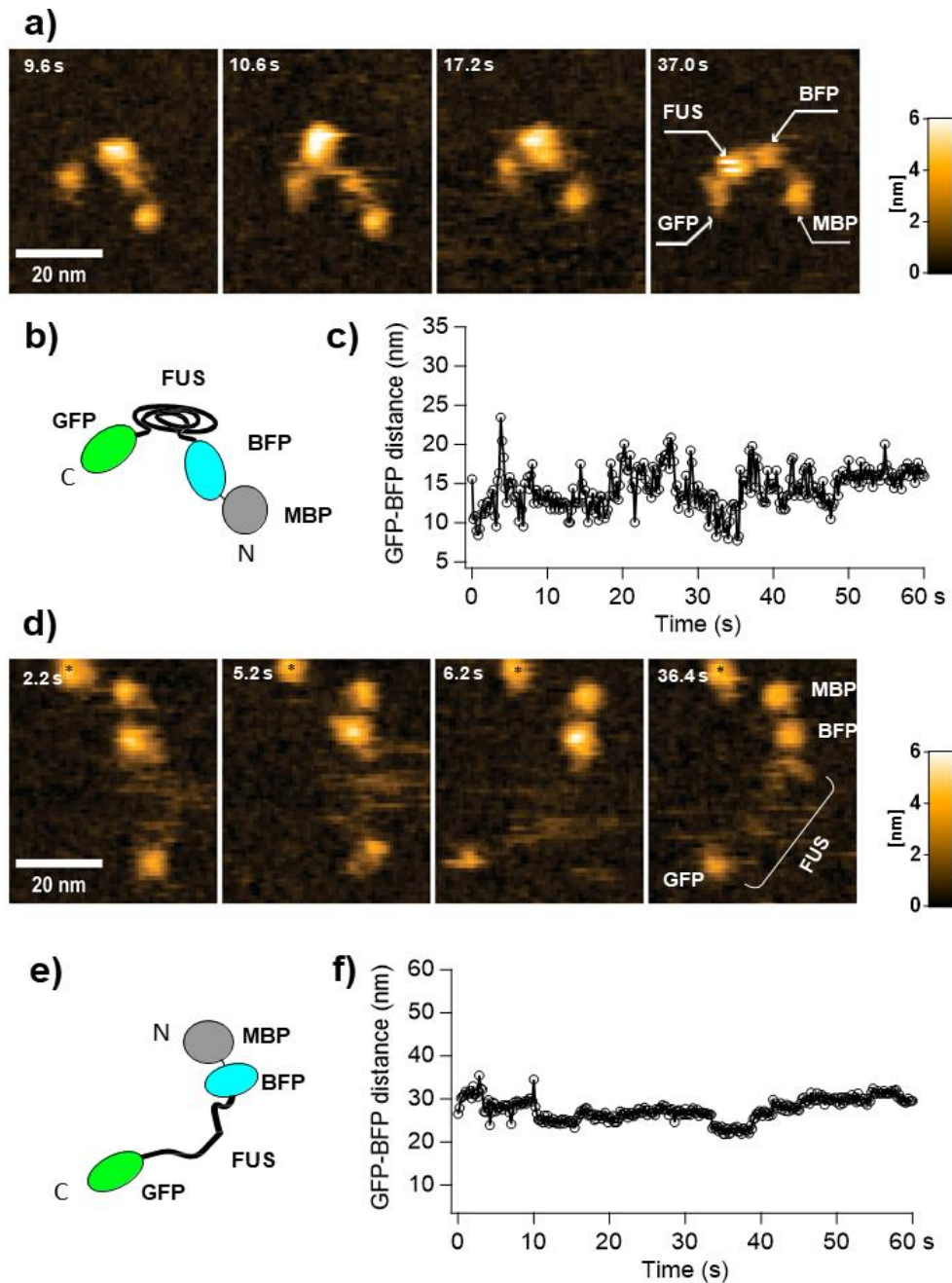


Fig. 3-5. Tracking of FUS in the absence and presence of pncRNA. **(a)** Monitoring of the fusion protein in the absence of pncRNA. A 2 μ L droplet of the 10 nM fusion protein was placed on the APTES-mica surface. 0.2 s/frame. **(b)** Schematic drawing of the observed compact FUS fusion protein in **(a)**. **(c)** Time course of the distance between BFP and GFP in the absence of pncRNA for the molecule shown in **(a)**. **(d)** Monitoring of the FUS fusion protein in the presence of pncRNA. An equimolar amount of pncRNA as to the fusion protein was added to the solution in the chamber. Tracking was started from time 0, while pncRNA was added earlier. 0.2 s/frame. *: Another fusion protein. **(e)** Schematic drawing of the extended FUS fusion protein in **(d)**. **(f)** Time course of the distance between BFP and GFP in the presence of pncRNA for the molecule shown in **(d)**.

Most magnified AFM images of the fusion protein in the absence of pncRNA exhibited four compact regions with different heights from the mica surface (**Fig. 3-6a**). It is supposed that they correspond to MBP, BFP, FUS, and GFP, as indicated in **Fig. 3-6b**. In the presence of pncRNA, on the other hand, the compact region that corresponds to FUS is missing, and the distance between BFP and GFP rather increased due to extension of FUS (**Fig. 3-6c and d**).

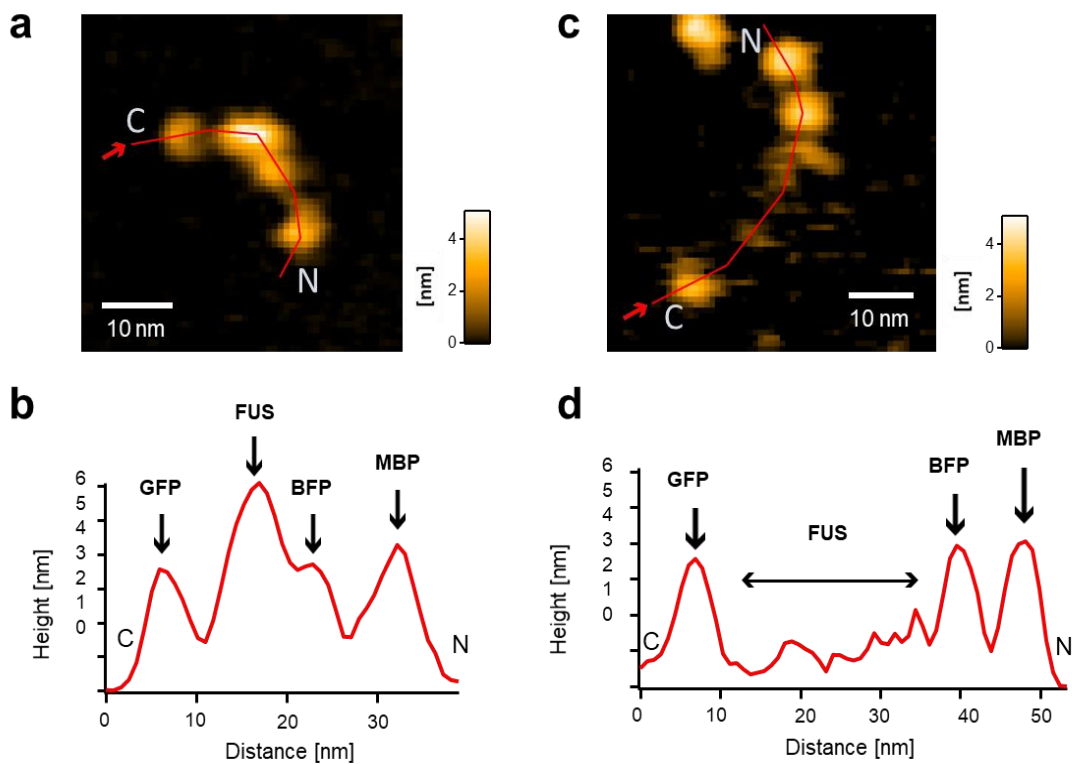


Fig. 3-6. Length and height of the FUS fusion protein in the absence and presence of pncRNA. **(a)** Magnified HS-AFM image of the fusion protein in the absence of pncRNA. Frame rate: 0.2 s/frame and 55×55 pixels². **(b)** Height profile along the red line in the HS-AFM image of **(a)**. **(c)** Magnified HS-AFM image of the fusion protein in the presence of pncRNA. Frame rate: 0.2 s/frame and 60×60 pixels². **(d)** Height profile along the red line in the HS-AFM image of **(c)**.

3.2.5 Analysis of FUS structural changes before and after the addition of pncRNA

3.2.5.1 Analysis of the radius of gyration (R_g)

In order to address the extension of FUS and the increased conformational flexibility of FUS in the extended structure, the radius of gyration (R_g) (**Fig. 3-7a**) of the fusion molecule was analyzed both in the absence and presence of pncRNA. The R_g analysis was carried out for ten molecules in consecutive frames in both the absence and presence of pncRNA, the total numbers of images being 3,204 and 3,998, respectively. Each molecule was traced for 20 to 60 seconds, depending on its visibility in the imaging frame. The histogram of R_g in the absence of pncRNA well fitted to the Gaussian distribution with a center value of 5.7 ± 0.1 nm and a width value of 2.6 ± 0.1 nm (**Fig. 3-7b**).

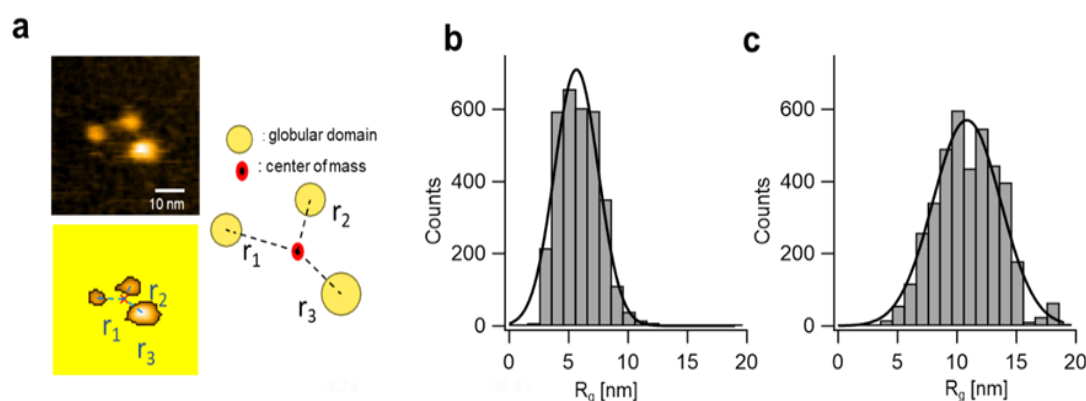


Fig. 3-7. Analysis of distribution of the radius of gyration of the FUS fusion protein. **(a)** The method to obtain the radius of gyration (R_g). The average distance between the center of mass and the globular domains is calculated as $(r_1 + r_2 + r_3)/3$ and defined as R_g . **(b)** A histogram of the radius of gyration of the fusion protein in the absence of pncRNA ($n=3204$) fitted to a Gaussian distribution with a center value of 5.7 ± 0.1 nm and a width value of 2.6 ± 0.1 nm. **(c)** A histogram of the radius of gyration of the fusion protein in the presence of pncRNA ($n=3998$) fitted to a Gaussian distribution with a center value of 10.8 ± 0.2 nm and a width value of 4.0 ± 0.2 nm.

On the other hand, the histogram of R_g in the presence of pncRNA well fitted to a Gaussian distribution with a center value of 10.8 ± 0.2 nm and a width value of 4.0 ± 0.2 nm (**Fig. 3-7c**). The increase in the central value of R_g of the fusion protein on the binding of pncRNA quantitatively indicates the compact-to-extended conformational change of

FUS caused by pncRNA. Additionally, an increase in the width value of R_g on binding of pncRNA was also noted. This indicates that the conformational flexibility of FUS increased on binding pncRNA.

3.2.5.2 BFP-GFP distance analysis

The compact-to-extended conformational change and the conformational flexibility of FUS caused by pncRNA were confirmed by the analysis of the BFP-GFP distance (**Fig. 3-8a**). The BFP-GFP distance histograms in the absence and presence of pncRNA fitted the Gaussian distribution with a center value of 13.2 ± 0.3 nm and a width value of 7.3 ± 0.4 nm, and ones of 25.1 ± 0.7 nm and 13.1 ± 1.0 nm, respectively (**Fig. 3-8b and c**). The extension of FUS and the increase in the conformational flexibility on binding of pncRNA were confirmed.

In the presence of pncRNA, the existence of a minor distribution of the BFP-GFP distance with a center value of ca. 14 nm (**Fig. 3-8c**), which is close to the value of 13.2 nm, was observed for the distribution in the absence of pncRNA (**Fig. 3-8b**). The minor

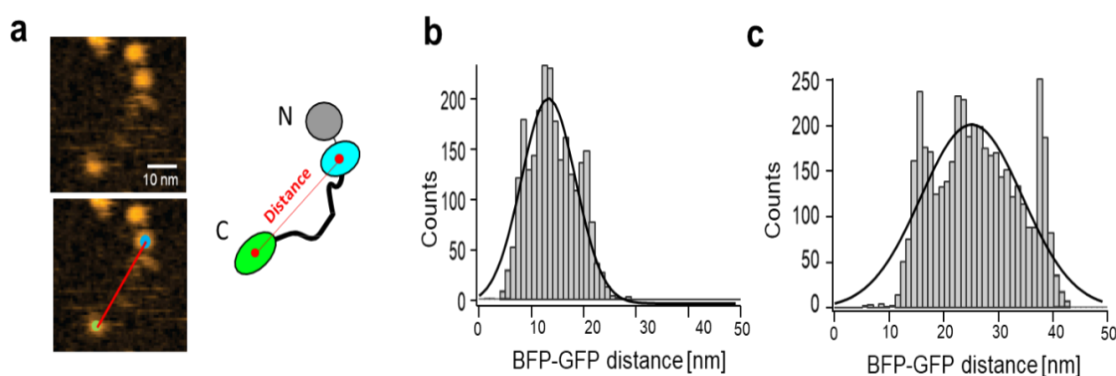


Fig. 3-8. Analysis of BFP-GFP distance of the FUS fusion protein. **(a)** The method to obtain the BFP-GFP distance. Globular domains representing BFP and GFP are shown (blue and green circles). The distance between the centers of the globular domains is measured. **(b)** A histogram of the BFP-GFP distance in the absence of pncRNA ($n=2502$) fitted a Gaussian distribution with a center value of 13.2 ± 0.3 nm and a width value of 7.3 ± 0.4 nm. **(c)** A histogram of the BFP-GFP distance in the presence of pncRNA ($n=4472$) fitted to a Gaussian distribution with a center value of 25.1 ± 0.7 nm and a width value of 13.1 ± 1.0 nm.

distribution is supposed to correspond to the fraction of FUS in the free state, even in the presence of pncRNA due to the equilibrium.

3.3 Discussion

In this study, we have validated the assumption of a FUS structural change in response to pncRNA binding. We succeeded in visualizing the compact-to-extended conformational transition of FUS on binding pncRNA. Data showed an extension, dynamics, and more flexibility of the FUS fusion protein upon binding pncRNA. In BFP-GFP analysis, the measured distance between the center of BFP and GFP in the extreme closed form was ~ 5 nm (**Fig. 3-4a, 0s, and 3-8b**). The GFP X-ray crystal structure (Protein Data Bank ID 1EMA) showed that it is folded into a cylinder shape with a length of 4.2 nm and a diameter of 2.3 nm [20]. The crystal structure of tag BFP (Protein Data Bank ID 3M24) [12] is very similar to that of GFP. Thus, the minimum BFP-GFP distance that was observed corresponds to the minimum distance that is possible between the buried fluorophores. Although the size of MPB is larger than that of either BFP or GFP (45 kD vs. 26 kD and 27 kD, respectively), that difference was not clear in the AFM image. Considering the crystal structure of MPB (Protein Data Bank ID 1OMP), the distance between the N and C termini of MBP is 4.35 nm, which explains why MBP appeared to be at similar height to GFP and BFP (**Fig. 3-6**). In the compact form, FUS (53 kD) appeared higher, probably due to its larger loose structure (**Fig. 3-6a, b**).

The destruction of the attraction interaction between the N- and C-terminal regions of FUS on binding of pncRNA to the C-terminal region rationally explains both the conformational extension of FUS and the increase in the conformational flexibility of FUS. In the extended structure, the N-terminal domain of FUS is not masked by the C-terminal domain of FUS anymore but is free for the interaction. Thus, the N-terminal domain of FUS can interact with histone acetyltransferases, CBP/p300, and interfere with their acetyltransferase activities. It is also supposed that the increased conformational flexibility of FUS in the presence of pncRNA is helpful for FUS to efficiently search for and bind to CBP/p300 for the interference.

3.4 Conclusion

In summary, we succeeded in visualizing the compact-to-extended conformational transition of FUS on binding pncRNA. This conformational transition can make the N-terminal domain of FUS become exposed, and interact with and interfere with histone acetyltransferases, CBP/p300. The detected increase in the conformational flexibility of FUS on binding pncRNA is supposed to allow an efficient search for CBP/p300. Thus, this study has provided an insight into the mechanism by which FUS represses the transcription of *CCND1* through the interaction with pncRNA in response to DNA damage.

3.5 Materials and Methods

3.5.1 Sample preparation

The FUS fusion protein was immobilized on a mica surface modified with 0.05% (3-aminopropyl) triethoxysilane (APTES) (Shin-Etsu Silicone, Tokyo, Japan). The mica surface was cleaved so as to have a clean and smooth surface in each experiment. After treatment with APTES, a sample droplet of ~2 μ L was placed on the APTES-mica surface and incubated for 3 min. The remaining FUS molecules were removed by rinsing with observation buffer (10 mM phosphate buffer, pH 6.8). After removing residual molecules, the sample stage was immersed in a chamber containing 70 μ L of measurement buffer.

3.5.2 High speed- AFM measurements

All HS-AFM experiments were performed using a laboratory-built high-speed atomic force microscope (HS-AFM) [2]-[8] at room temperature. For HS-AFM measurements, small cantilevers (BL-AC7DS: Olympus, Tokyo, Japan) with a resonance frequency of approximately 0.8 MHz, a quality factor of ~2, and a spring constant of ~0.2 were used in the tapping mode. All properties of small cantilevers were estimated in liquid. To reduce the tip-sample loading force, the amplitude of cantilever oscillation was adjusted to 1-2 nm. Additionally, the set-point of amplitude for feedback control was set at ~90% of the free amplitude.

3.5.3 Image analysis

Image analysis was carried out using laboratory-built software based on IgorPro V 8 (Wavemetrics, Inc., Lake Oswego, OR). All HS-AFM images were first processed by Gaussian-filter smoothing and background-tilt compensation using first-order plane fitting. For analysis of the distance between BFP and GFP, as shown in **Fig. 3-8a**, the center position of BFP or GFP was independently determined by means of a tracking algorithm using a two-dimensional correlation coefficient [5], and then the distance between the center positions was calculated for each frame. To calculate the radius of gyration (R_g), we manually set the threshold to remove the background and identify the regions of globular domains, as shown by the yellow-filled region. The center of mass of three globular domains was calculated based on the height distribution within each domain and the center position of each domain. Finally, R_g was estimated using the distance between the center position of each domain and the center of mass for all domains. Data represent three independently purified protein samples.

All histograms fitted with a normal Gaussian curve described as $f(x)=A\exp\{-(x-x_0)^2/2\sigma^2\}$, where x , x_0 , σ^2 and A correspond to the measured distance, average, dispersion and a constant, respectively. The fitting was carried out using a method of regression analysis built into IgorPro.

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Chapter Four

Long non-coding RNA can diminish the aggregation of FUS/TLS caused by shearing stress

4.1 Introduction

Most proteins have a certain folded structures that are required for certain biological functions. However, intrinsically disordered proteins (IDPs) and proteins that are rich in intrinsically disordered regions (IDRs), such as FUS protein, can adopt different conformations, which enables those proteins to regulate different biological functions. IDPs typically contain multiple charged side chains and few hydrophobic residues [1]. Previous studies showed that IDPs are not typically found in extended forms, but rather compact forms due to hydrogen bonds and electrostatic charges [2], [3]. In our study, Chapters 2 and 3, we showed that FUS is also tends to form a compact structure in the absence of RNA/DNA [4], [5]. Improper regulation of IDPs usually results in protein aggregation, which is associated with cell malfunctioning.

FUS has the ability to undergo liquid-liquid phase separation to form dynamic liquid-like droplets, which is involved in the assembly of membraneless structures such as stress granules [13]. In normal physiological conditions, FUS can reversibly shift between dispersed phase, liquid droplet, and reversible gel assemblies. However, with aging and/or pathological conditions, liquid droplets can be converted into non-reversible gels and fibril assemblies [14]. The implication of FUS aggregation in various devastating neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) [9], [10], Huntington disease (HD) [6], [7], and frontotemporal lobar degeneration (FTLD) [11], is attracting increasing attention to study the mechanism(s) of neuropathogenesis that is/are mediated by FUS aggregation. *In vitro*, other factors such as pH, salt concentration, protein concentration, and RNA presence were found to affect the FUS tendency for various assemblies. In ALS patients, mutations causing ALS were found to accelerate the liquid to solid phase transition [15], [16]. So, factors that control the aggregation tendency of FUS can be excellent therapeutic targets for FUS-associated neuropathology.

4.2 Results

4.2.1 *Shearing stress caused by pipetting can induce FUS aggregation*

Throughout our study of the effect of pncRNA on the conformational change of FUS, we observed that upon few strokes of sample pipetting, which is regularly performed to ensure sample homogeneity, reductions in both the BFP and GFP fluorescence intensities were observed. We supposed that this observed reduction in the whole FRET spectrum is caused by aggregation of FUS protein. Pipetting can subject protein molecules to shear stress due to the difference in the velocity of molecules adjacent to the tips and molecules in the middle. Additionally, the tension force on the molecules at the liquid/air interface is different from that in the liquid phase. The unequal force distribution on a protein molecule might induce some conformational change that is favored for aggregation assembly (**Fig. 4-1**). We checked whether aggregation could be induced by incubating a sample in the fluorescence spectrophotometer cell without pipetting. No reduction in fluorescence intensity was observed when pipetting was not performed over the same time course (~ 15 minutes). A longer incubation time rather caused slight increase in the BFP and GFP fluorescence intensities due to sample evaporation, which results in a higher concentration of FUS fusion protein in the cuvette.

To verify our assumption that the observed reduction in the fluorescence spectrum of the FUS fusion protein is caused by a kind of FUS aggregation, we prepared four protein samples, for which different numbers of strokes of pipetting were performed. Samples were prepared by diluting the stock protein 5 times with pure water with 3 gentle strokes of pipetting to get 100 nM protein. For the P0 sample no further pipetting was performed after the pipetting that was required for dilution. P15, P30, and P45 are samples with a further 15, 30, and 45 strokes of pipetting, respectively. First, the FRET spectra of those samples were measured to confirm the reduction in the fluorescence spectrum of each sample in response to pipetting. Then, all samples were imaged by fluorescence microscopy to observe any aggregation. Whereas FUS can aggregate in different phases, we had to set the parameters for the observed particles. For phase separated particles, a perfect circular shape is observed (circularity=1). For other kinds of aggregation, the circularity is set to < 0.8. In our observation, we focused on aggregation rather than phase separation.

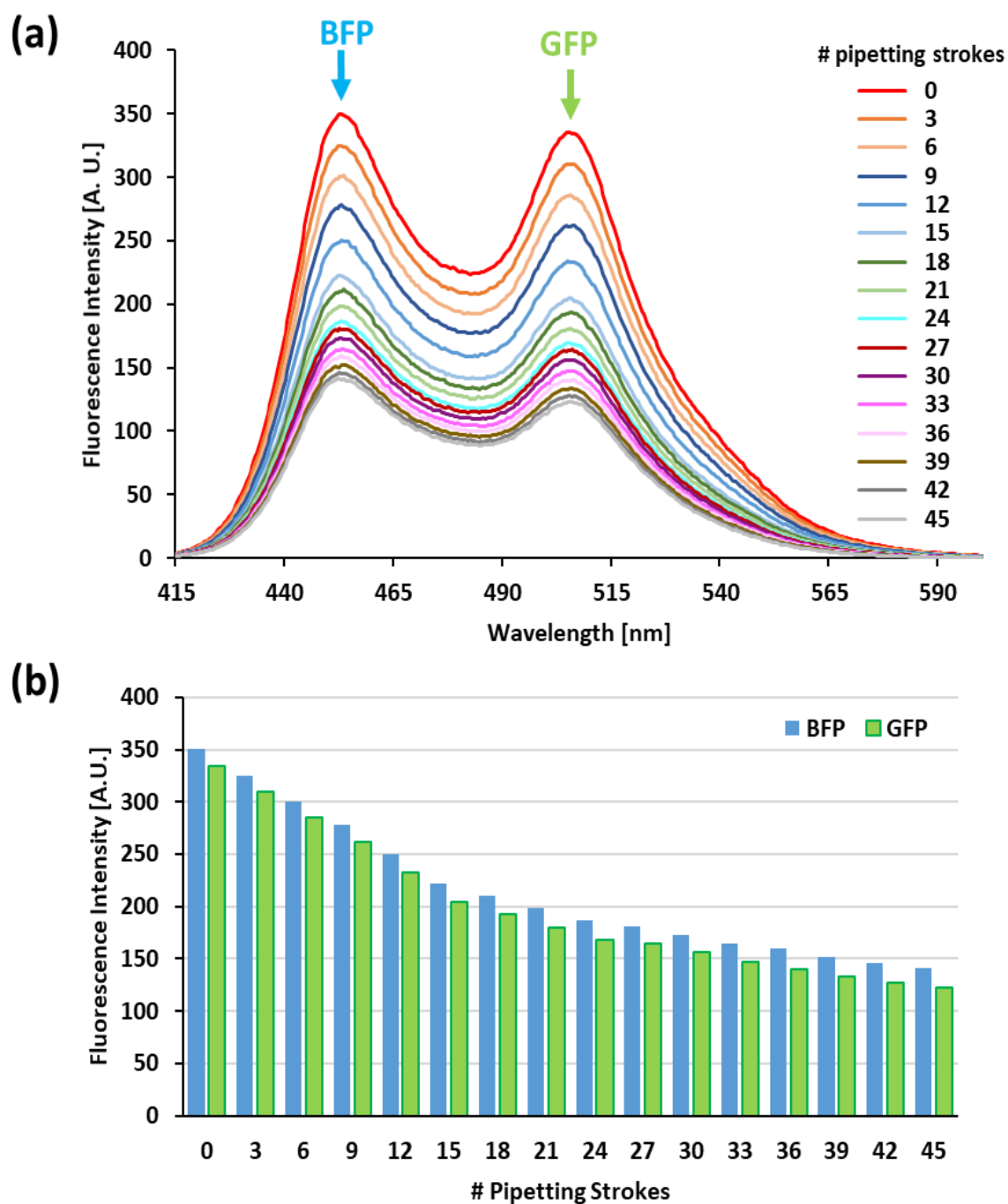


Fig. 4-1. Effect of pipetting strokes on the FUS fluorescence spectrum. **(a)** Overlaid fluorescence spectra. Each spectrum was measured after 3 successive strokes of pipetting with a fixed time interval (60 seconds). Pipetting was performed until saturation, i.e; where no further reduction could be observed. The total number of pipetting strokes is indicated on the right. **(b)** Bar graph showing BFP and BFP fluorescence intensities corresponding to fluorescence spectra that are shown in (a).

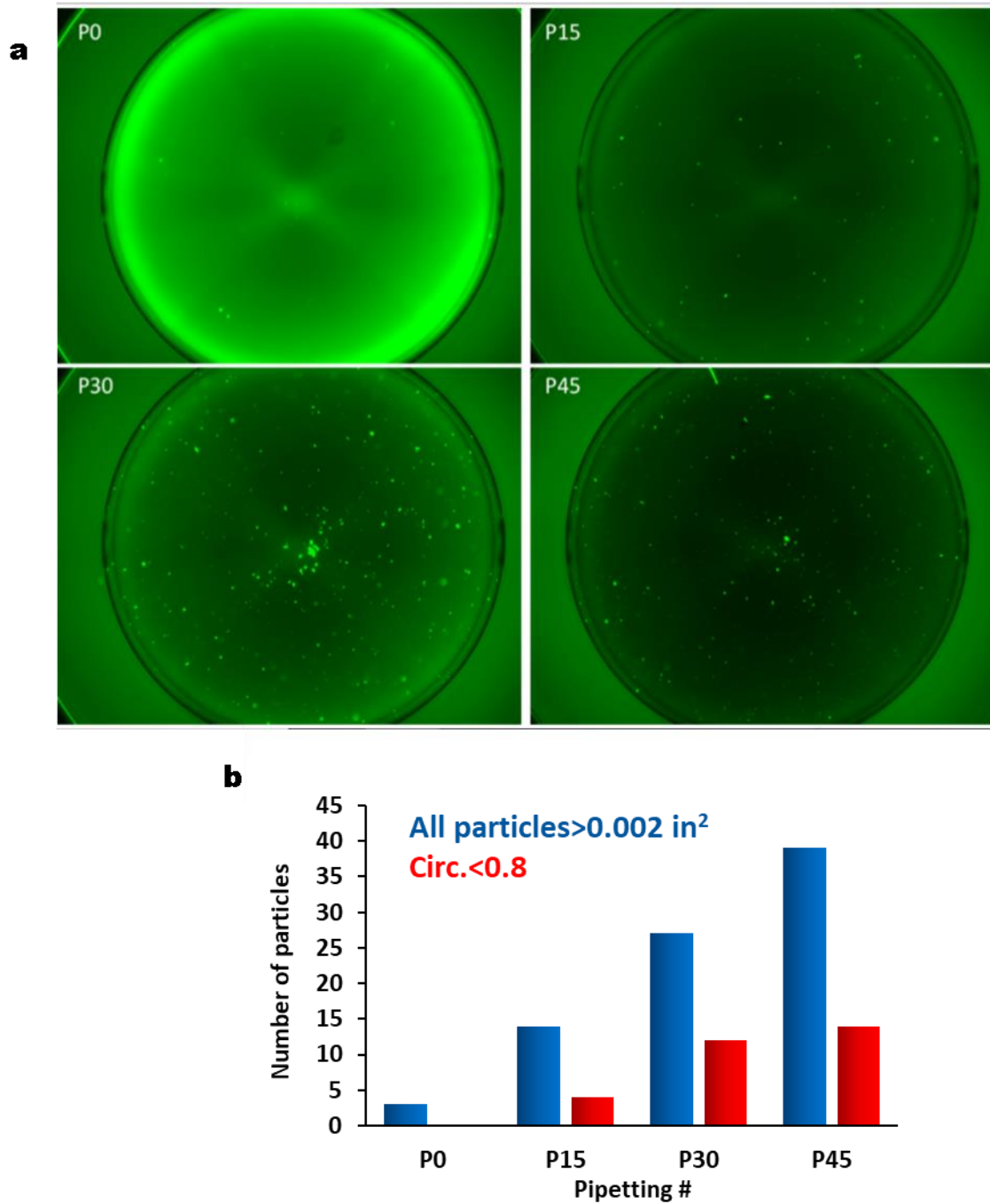


Fig. 4-2. Effect of pipetting on FUS aggregation. (a) Fluorescence microscope imaging of 100 nM FUS fusion protein (MBP-BFP-FUS-GFP-6xH) after different number of pipetting strokes; P0, P15, P30, and P45. (b) Particle counts for images shown in (a). Two parameters are used for particles counting: all particles with size $> 0.002 \text{ in}^2$, and particles with a non-smooth circular shape ($\text{Circ} < 0.8$) to exclude phase separated particles.

Therefore, all particles with size $>0.002 \text{ in}^2$ were counted (**Fig. 4-2b, blue bars**), and we also counted particles with circularity <0.8 , which excluded phase separated particles (**Fig. 4-2 b, orange bars**). In both cases, the number of strokes of pipetting showed a correlation with increase in the number of the observed particles (**Fig. 4-2b**), which supports our assumption that the reduction in the FRET spectrum is due to FUS aggregation.

FUS is known to assemble into various aggregation forms in normal physiological as well as pathological conditions. By fluorescence microscopy, we could exclude the phase separated FUS. Next, we investigated FUS aggregation that was induced by pipetting using transmission electron microscopy (TEM) to determine whether the formed aggregations are amorphous or have a certain structure such as amyloid B fibrils, which have a pathological implication in various neurodegenerative diseases. TEM imaging showed that FUS aggregations that were induced by pipetting were mostly amorphous (**Fig. 4-3**).

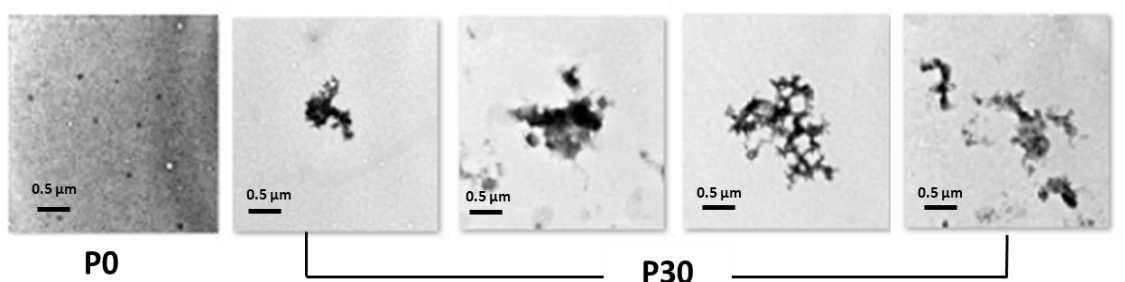


Fig. 4-3. TEM imaging for 10 nM FUS fusion protein (MBP-BFP-FUS-GFP-6xH) before pipetting (P0) and after applying 30 strokes of pipetting (P30).

MBP is usually used to improve the solubility of a fused protein. BFP and GFP are easily soluble proteins that might also improve the solubility of other proteins when attached together. However, there was a tiny possibility that the complex fusion protein might influence FUS aggregation behaviour. To exclude this possibility, a more simple fusion protein, Strep-GFP-FUS, was constructed. The effect of pipetting on Strep-GFP-FUS was investigated by fluorescence microscopy using the same buffer conditions. The results showed a similar tendency of an aggregation increase with pipetting, which showed that MBP and BFP do not influence the aggregation tendency of FUS protein (**Fig. 4-4**).

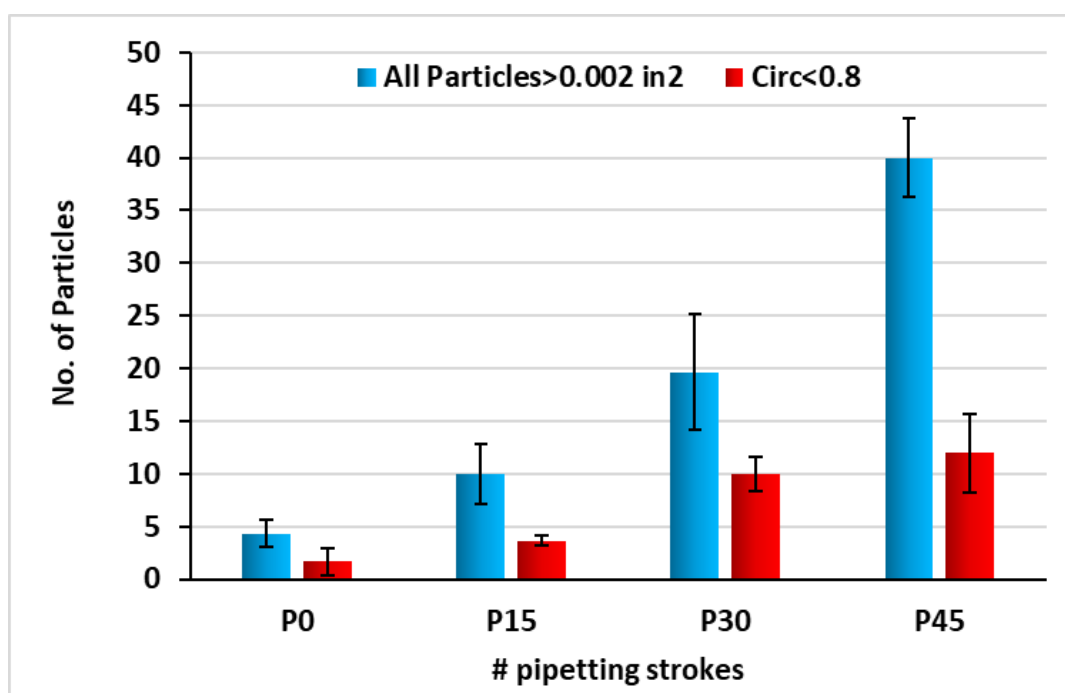


Fig. 4-4. Effect of pipetting on Strep-GFP-FUS aggregation. Analysis of fluorescence microscope imaging of 100 nM Strep-GFP-FUS protein after different numbers of pipetting strokes; P0, P15, P30, and P45. Two parameters are used for particle counting: all particles with size $> 0.002 \text{ in}^2$, and particles with a non-smooth circular shape ($\text{Circ} < 0.8$) to exclude phase separated particles.

4.2.2 PncRNA can stop the effect of shearing stress

Interestingly, the effect of shear stress caused by pipetting was not observed when pncRNA (R31) was added to FUS fusion protein. In a fresh protein sample, R31 addition causes a change in the GFP/BFP ratio; then pipetting does not have a further effect on the GFP and BFP intensities. On the contrary, for samples stored for few days at 4 °C, pipetting after the addition of pncRNA raised both the BFP and GFP intensities slightly, which indicated dissociation of some previously existing FUS aggregates. We found that pncRNA fragments (R31, R19, and R13) could stop the observed spectrum reduction (**Fig. 4-5**).

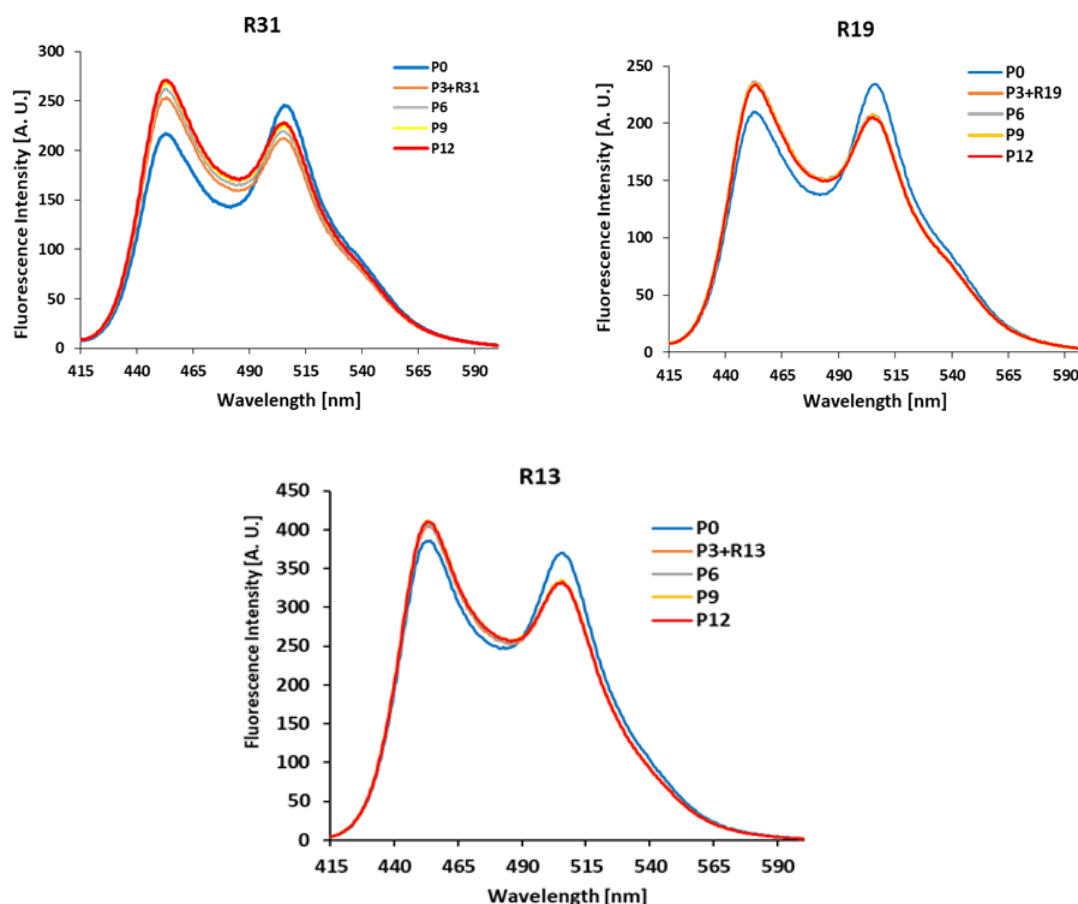


Fig. 4-5. Fluorescence spectrum of FUS fusion protein is not reduced by the pipetting effect in the presence of pncRNA fragments R31, R19, or R13. The initial spectrum was measured in the absence of RNA, then each RNA was added with 3 strokes of pipetting, and then several strokes of pipetting were applied as indicated by P#.

4.2.3 Shorter pncRNA fragments (<12 mer) cannot stop the shearing effect on FUS

By observing the effects of shorter pncRNA fragments, we found that fragments of the 10-mer or less could not keep the fluorescence spectrum at its initial level after pipetting (**Fig. 4-6**). To find the minimum sequence that can hinder the effect of shearing stress on FUS fusion protein, a sequential addition experiment was performed for pncRNA

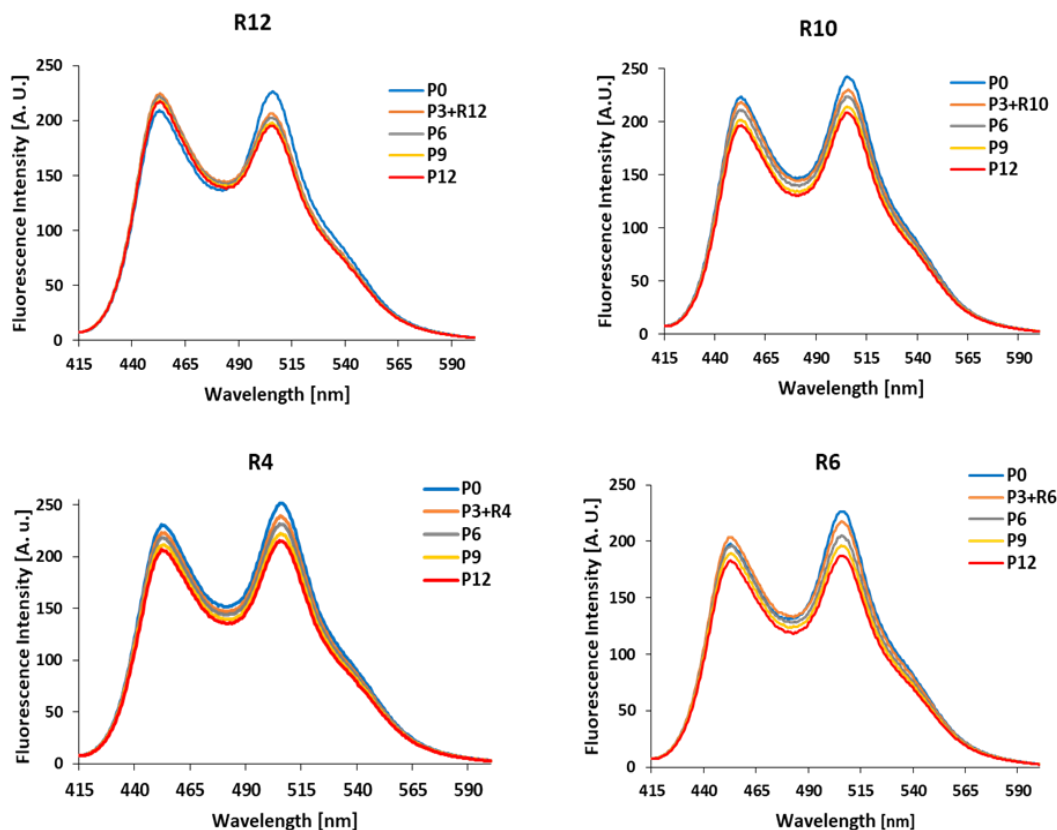


Fig. 4-6. Effect of shearing stress in the presence of shorter pncRNA fragments. Fluorescence spectra of FUS fusion protein show the effect of pipetting in the presence of different pncRNA fragments, R12, R10, R6, and R4.

fragments starting from the addition of the shortest sequence (R3) to the longer sequences (R4, R5, R6, ... R31) (**Fig. 4-7 a**). Reduction in the whole spectrum with a slight change in the GFP/BFP ratio was observed on the addition of R3, R4, R5, R6, R7, and R10. When R13 was added to the protein in the presence of the mentioned shorter fragments, the reduction in the whole spectrum was hindered. Pipetting after R13 addition raised up the whole spectrum. Pipetting after the addition of R19 and R31 also caused a raise of the whole spectrum. To check whether the hindrance of the shearing effect that is caused by pipetting is related to the conformational change of FUS, which is caused by different pncRNA fragments, the corresponding relative FRET efficiency (E) was calculated after each pncRNA fragment addition (**Fig. 4-7 b**). The observed coupling between the conformational change of FUS and its aggregation hindrance might reflect some structural prerequisites for FUS aggregation.

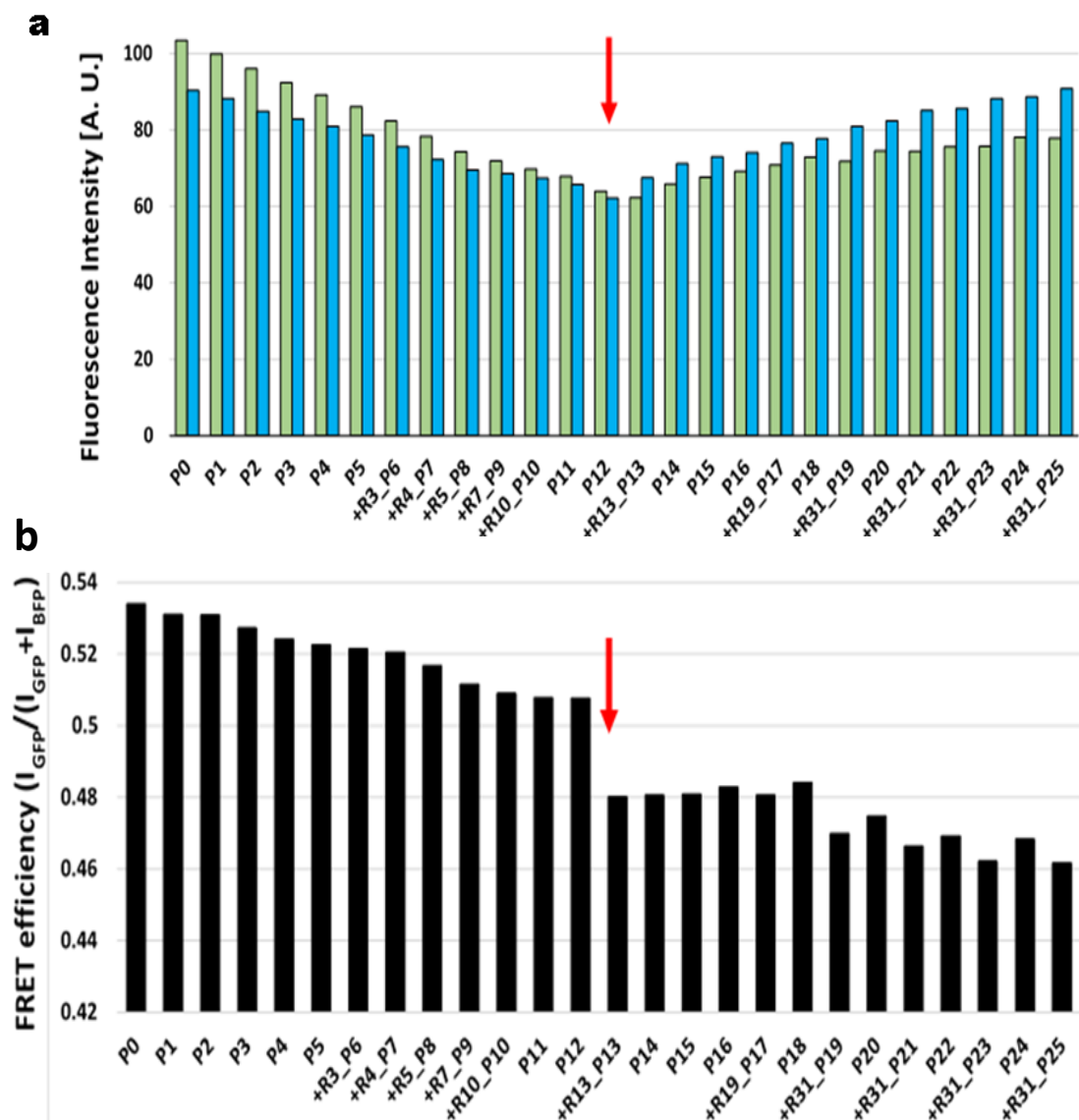
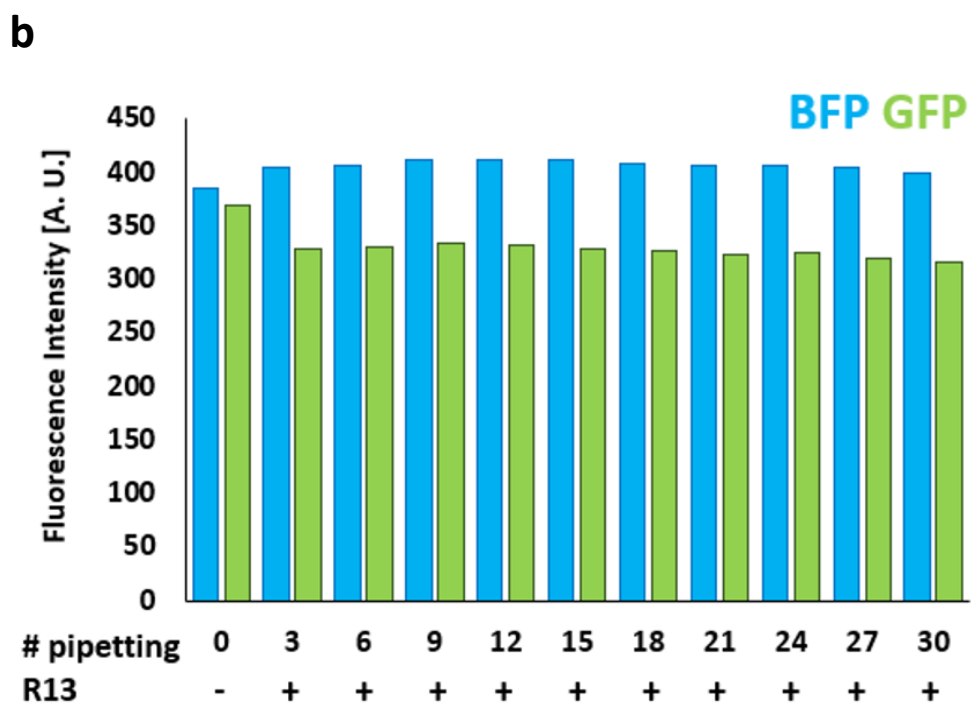
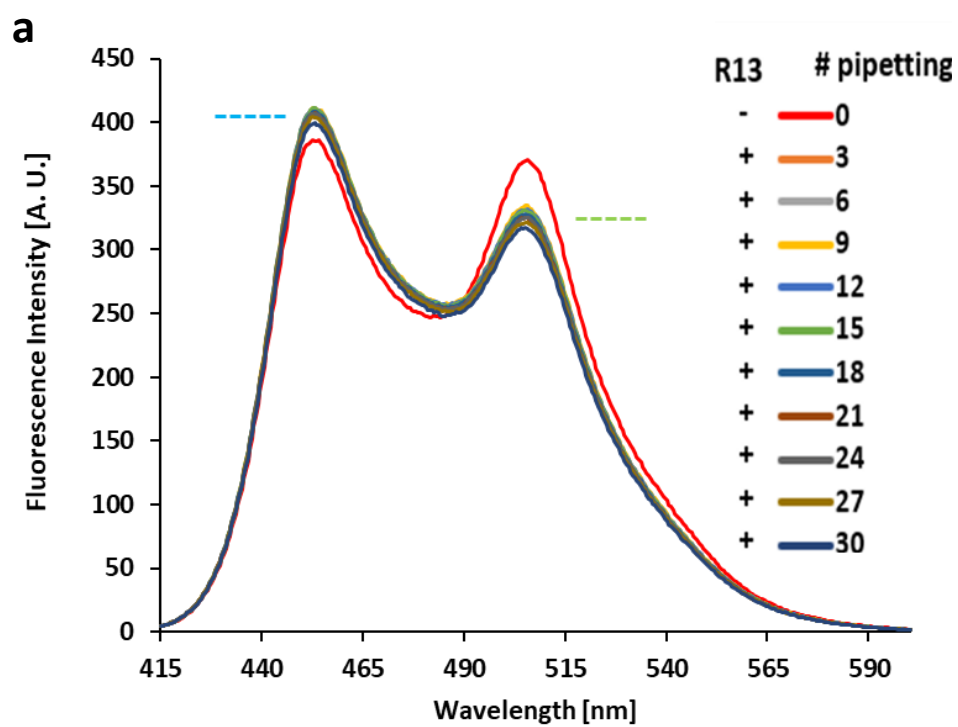


Fig. 4-7. Aggregation tendency of FUS protein in the presence of different pncRNA fragments and its correlation with the FUS conformational change. Several strokes of pipetting were applied to a protein sample followed by a sequential addition starting from R3, followed by longer fragments (R4, R5, R7, R10, R13, R19, and finally R31) (a) A bar graph shows BFP and GFP fluorescence intensities in response to each indicated condition. (b) A bar graph presents the calculated relative FRET efficiency for each condition corresponding to (a). The numbers of pipetting strokes are indicated as P.

4.2.4 The effect of R13 is sequence-specific

Our first thought was that sequence length affects the aggregation tendency. To validate this assumption, the effect of R13, which is considered to be a specific sequence, was compared to the effect of U₁₃, which is regarded as a non-specific sequence. Two means of comparison were performed using the FRET assay. In the first experiment, R13 was directly added to FUS fusion protein, and then 30 strokes of pipetting were performed (**Fig. 4-8 a and b**). In the second experiment, 15 strokes of pipetting were applied to FUS protein to observe the reduction of the fluorescence spectrum, and then an equimolar amount of R13 was added. After R13 addition, 15 more strokes of pipetting were applied (**Fig. 4-8c and d**). The same experimental conditions were repeated using an equimolar amount of U₁₃ instead of R13 (**Fig. 4-9 a, b, c, and d**). Thus, the effect of R13 was directly compared with that of U₁₃. Fluorescence spectra measured after R13 addition were hardly affected by pipetting (**Fig. 4-8a and b**). The GFP and BFP fluorescence intensities that were obtained after several strokes of pipetting before and after the addition of R13 showed that R13 could stop the reduction caused by pipetting, and that it could even increase the fluorescence intensities slightly (**Fig. 4-8c and d**). In contrast, U₁₃ could not hinder the reduction of the fluorescence spectrum caused by pipetting in both experiments (**Fig. 4-9**).

We further confirmed the difference between R13 and U₁₃ as to the reduction of FUS aggregation by fluorescence microscopy (**Fig. 4-10**). Thirty strokes of pipetting were performed on 100 nM Strep-GFP-FUS protein in the presence of either R13 or U₁₃. Both samples were imaged by fluorescence microscopy (**Fig. 4-10a**). Particles more than 0.002 μm^2 were counted as total aggregation (**Fig. 4-10b, blue bars**). Phase separated particles were excluded by setting the circulatory parameter to (Circ <0.8) (**Fig. 4-10b, red bars**). Both counts showed that FUS aggregations were significantly less in the presence of R13. This data implies that a specific sequence can affect the aggregation tendency of FUS.



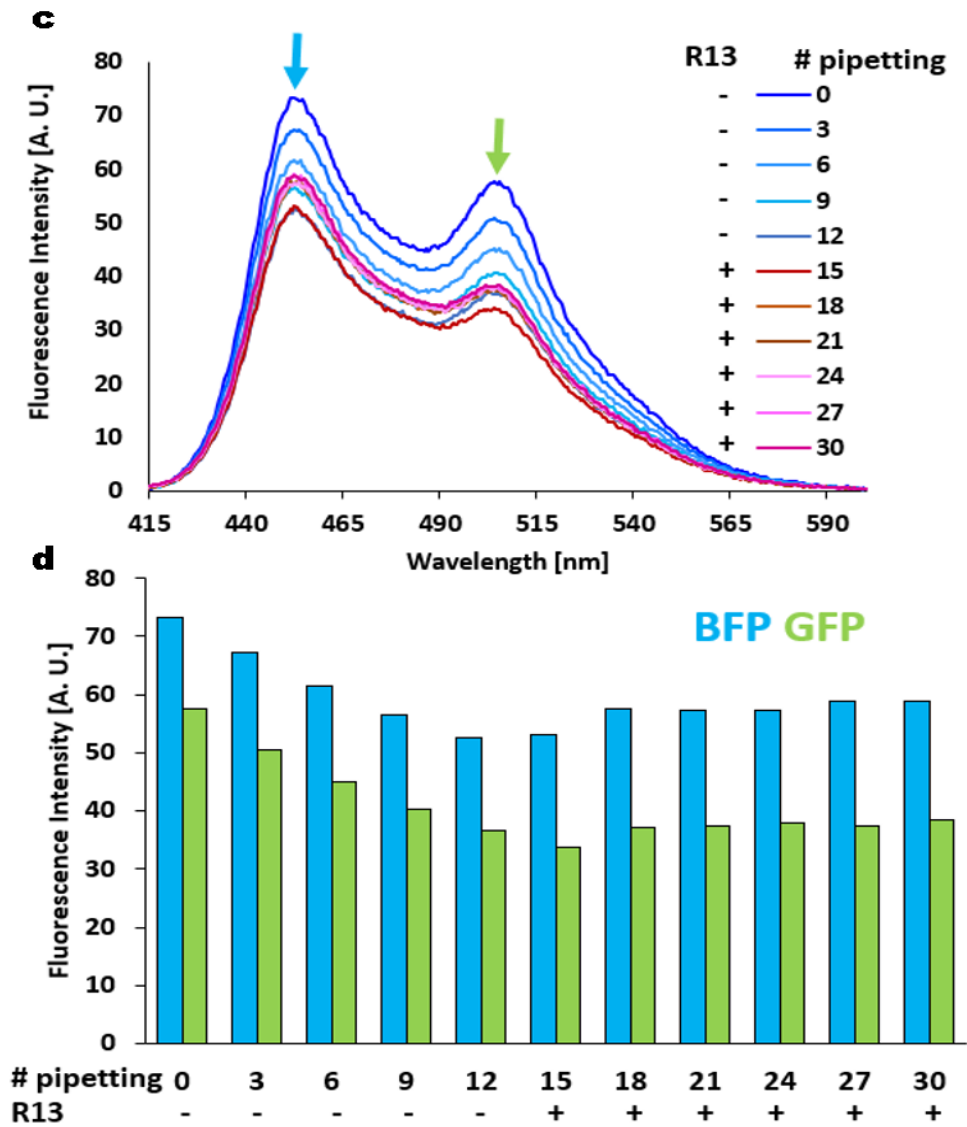
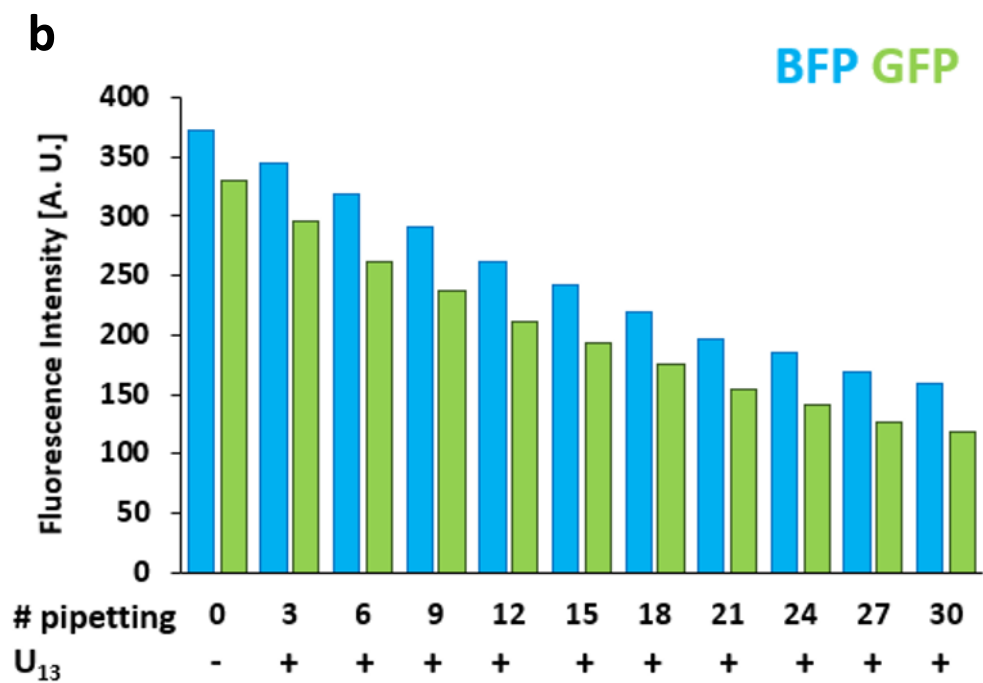
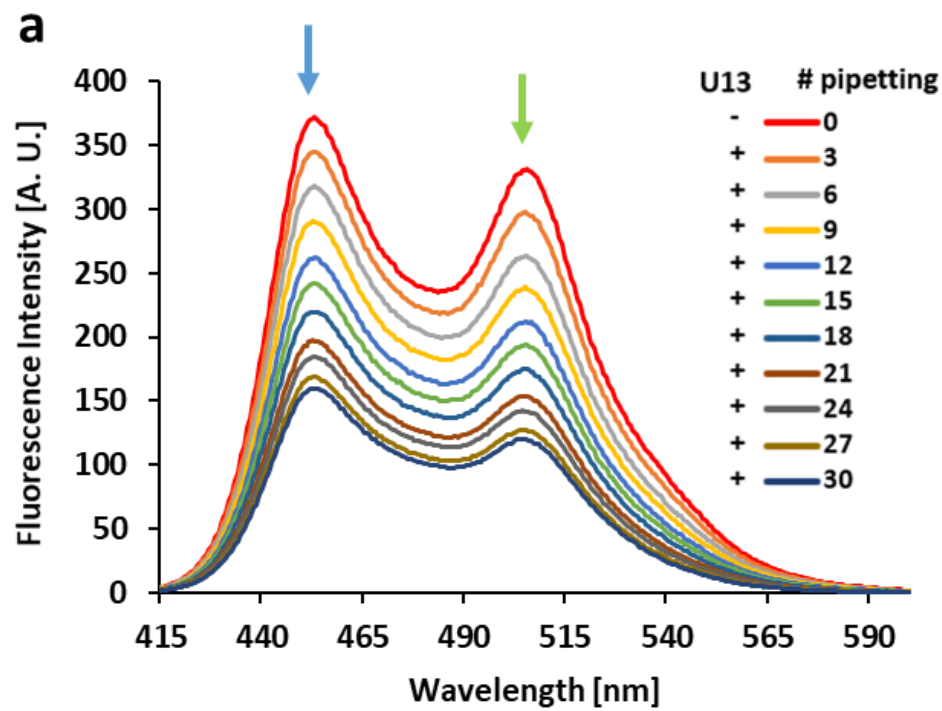


Fig. 4-8. R13 can hinder the shearing effect caused by pipetting of FUS. (a) Overlaid fluorescence spectra where an equimolar of R13 was added to 100 nM FUS fusion protein, and then 30 strokes of pipetting were applied. (b) A bar graph for GFP and BFP fluorescence intensities corresponding to the fluorescence spectra shown in (a). (c) Overlaid fluorescence spectra where 12 strokes of pipetting were applied to 50 nM FUS fusion protein, and then an equimolar amount of R13 was added with 3 strokes of pipetting for mixing. Fifteen more strokes of pipetting were applied after R13 addition. The accumulative number of strokes of pipetting is indicated as p numbering. (d) A bar graph for GFP and BFP fluorescence intensities corresponding to the fluorescence spectra shown in (c).



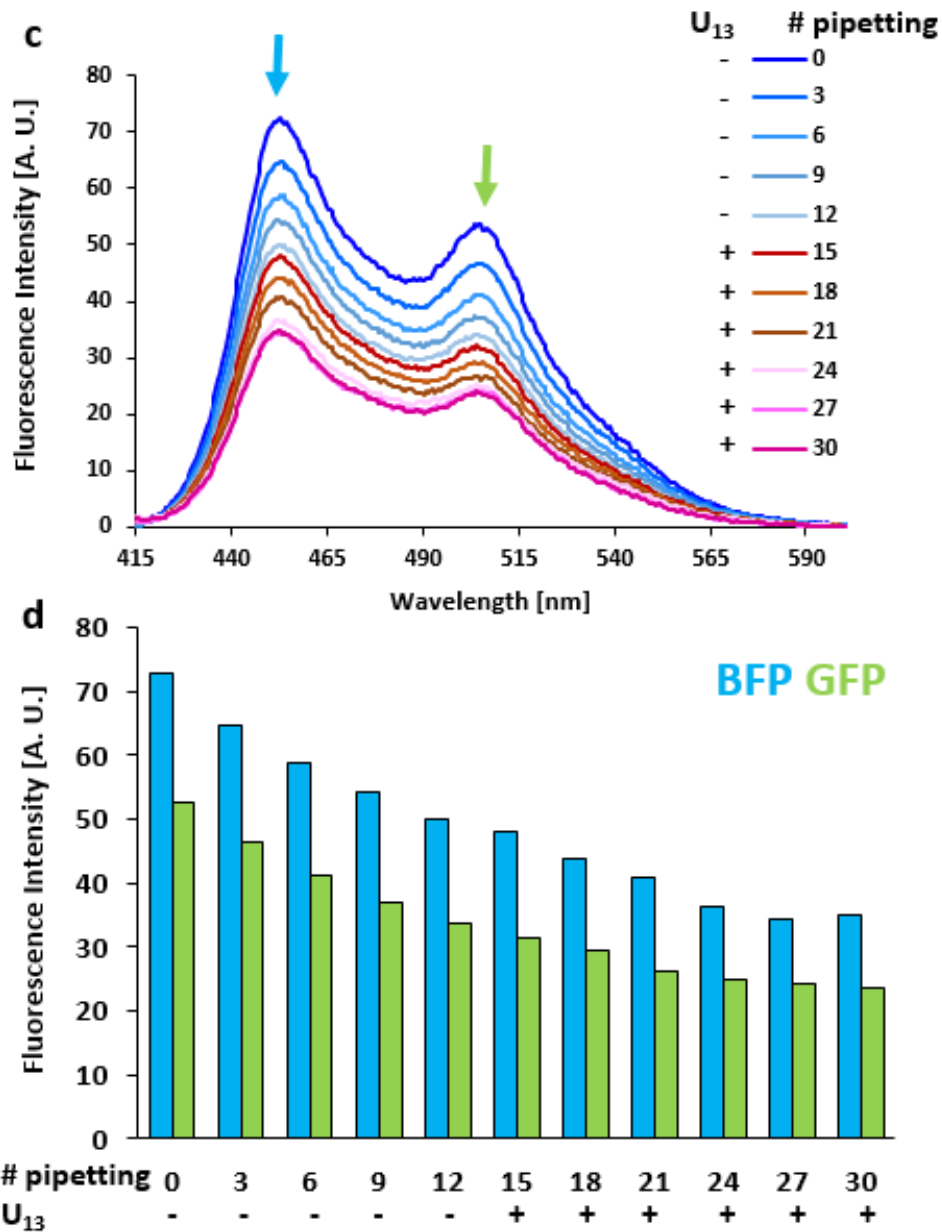


Fig. 4-9. PolyU₁₃ cannot hinder the shearing effect caused by pipetting of FUS. (a) Overlaid fluorescence spectra where an equimolar of U₁₃ was added to 100 nM FUS fusion protein, and then 30 strokes of pipetting were applied. (b) A bar graph for GFP and BFP fluorescence intensities corresponding to the fluorescence spectra shown in (a). (c) Overlaid fluorescence spectra where 12 strokes of pipetting were applied to 50 nM FUS fusion protein, and then an equimolar amount of U₁₃ was added with 3 strokes of pipetting for mixing. Fifteen more strokes of pipetting were applied after U₁₃ addition. (d) A bar graph for GFP and BFP fluorescence intensities corresponding to the fluorescence spectra shown in (c). The accumulative number of strokes of pipetting is indicated as p numbering.

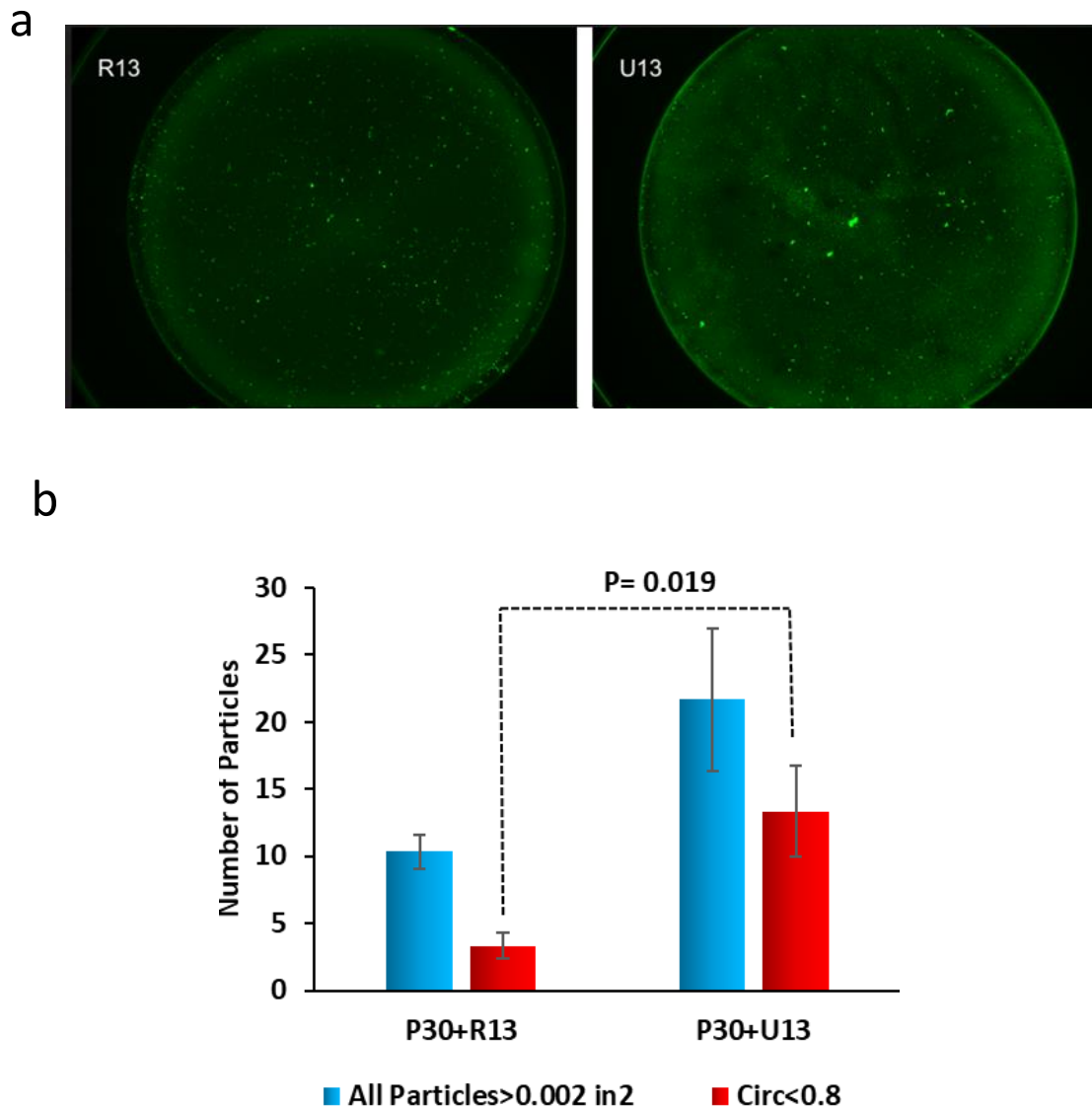


Fig. 4-10. Effect of pipetting on FUS aggregation. (a) Representative fluorescence microscope images of 100 nM Strep-GFP-FUS after applying 30 strokes of pipetting in the presence of R13 or U₁₃. (b) Particle counts for 3 independent fluorescence microscope imaging experiments presented in (a). Two parameters are used for particles counting: all particles with size $> 0.002 \text{ in}^2$, and particles with a non-smooth circular shape (Circ < 0.8) to exclude phase separated particles.

4.2.5 Salt effect on FUS aggregation

Next, the effect of salt on FUS aggregations that were induced by pipetting was examined. Thirty strokes of pipetting were applied to 100 nM FUS fusion protein at 50 mM NaCl concentration to induce FUS aggregation. Then, NaCl was added to FUS protein up to 300 mM final concentration. An additional 30 strokes were applied at 300 mM NaCl. Later, the NaCl concentration was raised to 500 mM with an additional 15 strokes of pipetting. The fluorescence intensities of BFP and GFP (I_{BFP} and I_{GFP} , respectively) were measured after each 3 strokes of pipetting with different salt concentrations (50, 300 and 500 mM NaCl). At 50 mM NaCl, the reduction in I_{BFP} and I_{GFP} increased by pipetting. Interestingly, raising NaCl to 300 mM not only stopped the observed aggregation, but also it gradually restored higher I_{BFP} and I_{GFP} on pipetting. The pipetting was performed at 300 mM until no further change in I_{BFP} and I_{GFP} could be observed. Further addition of NaCl (500 mM) slightly increased I_{BFP} and I_{GFP} even more.

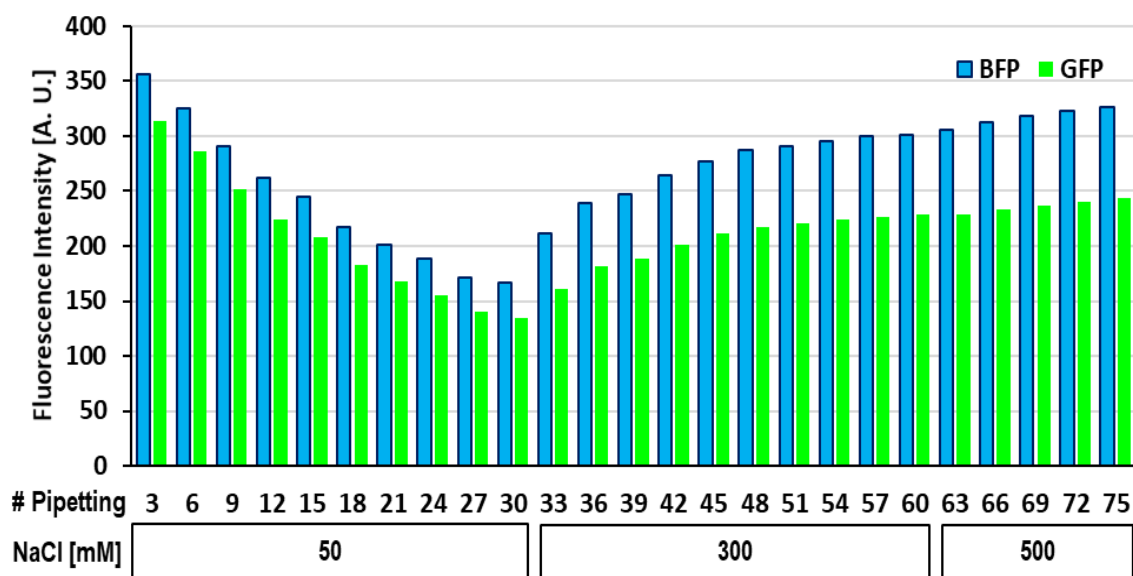


Fig. 4-11. Salt effect on FUS aggregation induced by pipetting. A fluorescence spectrum of 100 nM FUS fusion protein was measured at an initial NaCl concentration of 50 mM. Pipetting was performed at 3 strokes intervals as indicated by # pipetting to induce FUS aggregation. After 30 strokes of pipetting, the NaCl concentration was increased to 300 mM, and a further 30 strokes of pipetting were applied at the same NaCl concentration (300 mM). Then NaCl was added to 500 mM, and a further 15 strokes of pipetting were applied at 500 mM NaCl concentration. Blue and green bars represent the BFP and GFP fluorescence intensities, respectively.

That reversion of the fluorescence spectrum levels was accompanied by a reduction of GFP/BFP, which indicates a more extended FUS conformation (**Fig. 4-11**). The change in FUS conformation in the presence of a higher salt concentration could be due to the disruption of an electrostatic force between the negatively charged N-terminal and positively charged C-terminal of FUS protein as predicted from their amino acid structures in Chapter 2 [5].

4.2.6 Temperature effect on FUS aggregation

Next, we investigated the effect of temperature on FUS fusion protein. Usually, a protein is stored at 4 °C for few days after purification. Fluorescence measurements are made at room temperature (25 °C). When the fluorescence spectrum is measured at 4 °C and 50 °C, different FRET patterns are observed. At 4 °C, the GFP/BFP ratio is higher. Whereas, raising the temperature to 50 °C reduces the GFP/BFP ratio, which is expected from the extension of FUS molecules due to the thermal stress. Furthermore, the whole spectrum level is reduced, as observed as the pipetting effect. Rounds of cooling and heating between 4 and 50 °C showed a reversible effect with almost identical spectra after each round (**Fig. 4-12**). This suggests that changing the temperature of FUS between 4 to 50 °C, for a short time, might not cause deterioration of the FUS protein. Then, the fluorescence spectrum of FUS was monitored at higher temperatures by heating a 0.5 μ M protein sample from 4 to 70 °C with a 3 degrees' interval. A change in the GFP/BFP ratio was observed on heating. Again, the change in FUS conformation is accompanied by a reduction in the fluorescence spectrum level. A sharper conformational change is observed at temperature > 40 degrees. The reduction in the fluorescence spectrum was continuous on heating up to 70 °C (**Fig. 4-13a and c**). Cooling from 70 to 4 °C could partially restore both the FUS conformation and fluorescence spectrum levels. That reversion was observed only if cooling was applied immediately after reaching 70 °C (**Fig. 4-13b and d**). However, if heating at 70 °C is applied for about 5 minutes, an irreversible change in the FUS fluorescence spectrum is observed (**Fig. 4-14**), which indicates destruction to the FUS confirmation that cannot be restored by cooling.

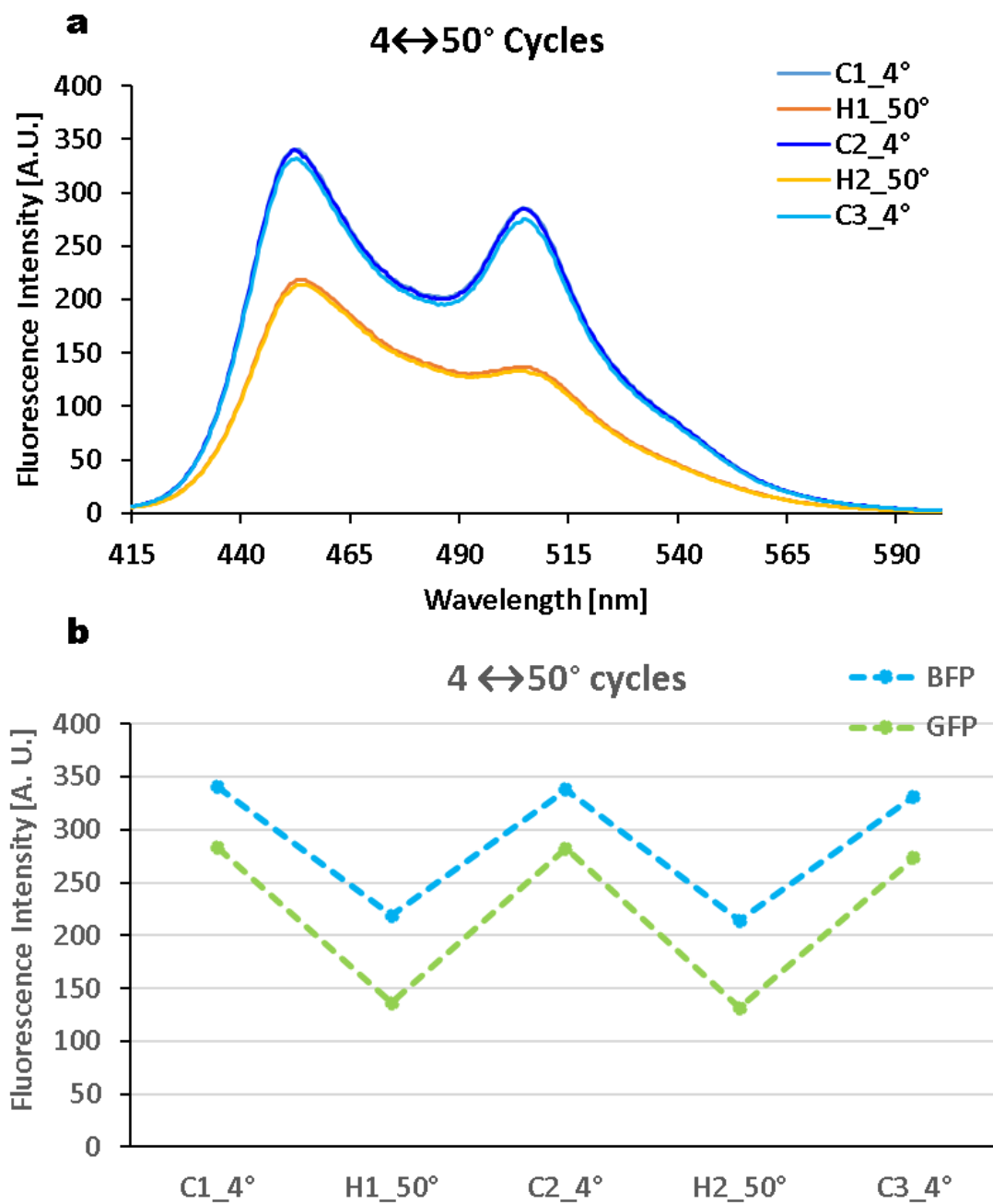
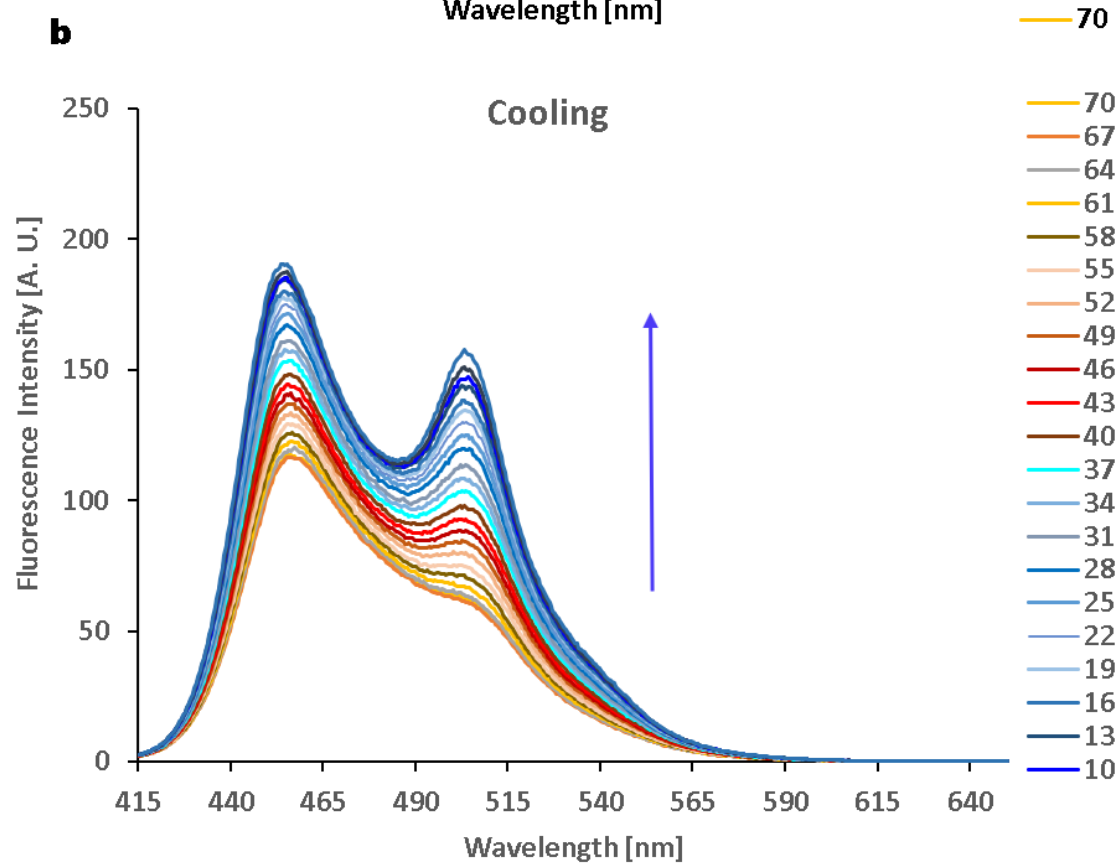
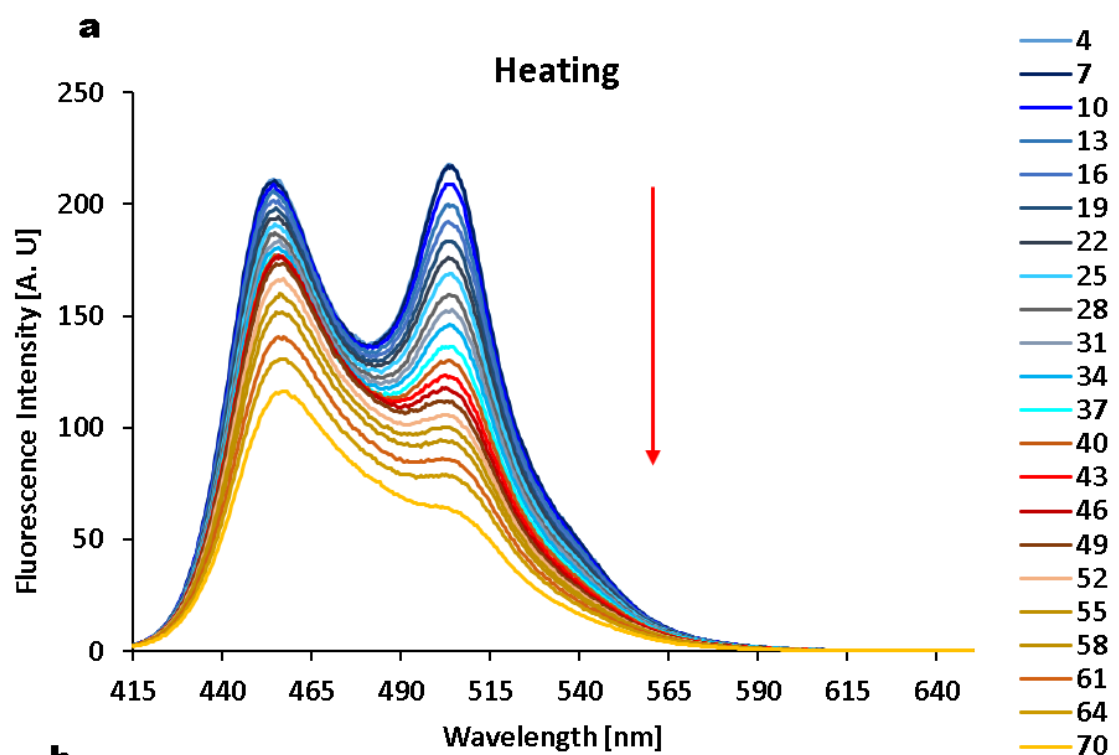


Fig. 4-12. Rounds of heating and cooling from 4 to 50 °C. **(a)** Fluorescence spectrum of successive rounds of heating to 50 °C and cooling back to 4 °C. **(b)** A graph shows the reversibility of the BFP and GFP fluorescence intensities during the indicated heating- cooling rounds.



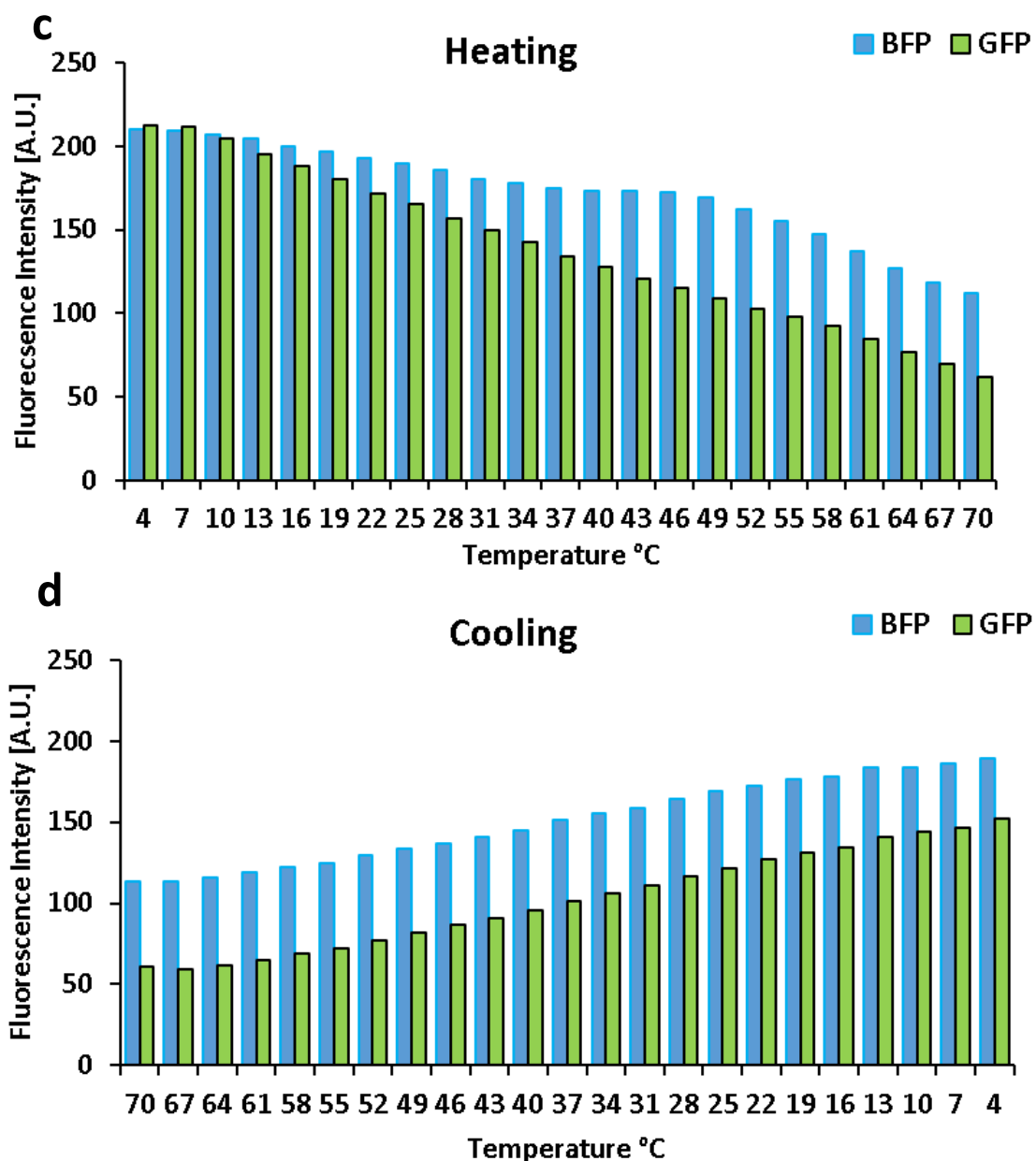


Fig. 4-13. Temperature effect on FUS fusion protein. (a) Overlaid fluorescence spectra for FUS fusion protein in the case of heating with a 3 degrees' difference between each pair of successive spectra. (b) Overlaid fluorescence spectra for FUS fusion protein shows a reversible effect of cooling after the heating indicated in (a). The corresponding temperature for each spectrum is shown on the right. (c) and (d) Bar graphs for GFP and BFP fluorescence intensities corresponding to the fluorescence spectra shown in (a) and (b), respectively.

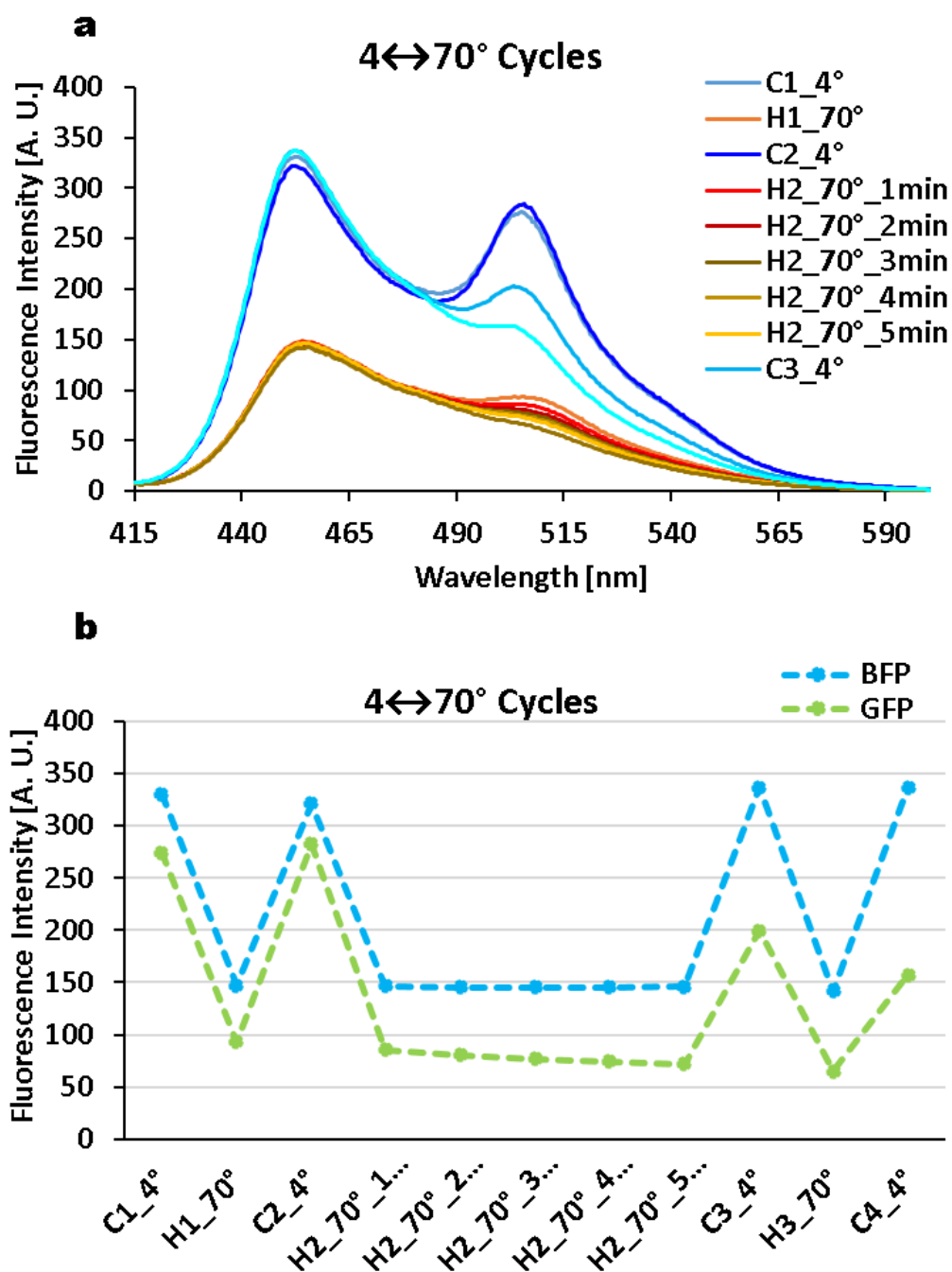


Fig. 4-14. Rounds of heating and cooling from 4 to 70 °C. (a) Fluorescence spectrum of successive rounds of heating to 70 °C and cooling back to 4 °C. In the second round, a fluorescence spectrum was measured every 1 minute after reaching 70 °C for 5 minutes. (b) A graph shows the BFP and GFP fluorescence intensities during the indicated heating-cooling rounds.

4.3 Discussion

A reduction in the fluorescence spectrum was observed on pipetting of FUS protein in the absence or presence of some RNA fragments, whereas in the static state, no change was observed. That was interpreted as a kind of FUS aggregation, which was accompanied by a slight reduction in the FRET signal. FUS aggregations were visualized by fluorescence microscopy and TEM as amorphous aggregates (**Fig. 4-2 and 4-3**). FUS tends to aggregate or form higher order assemblies under certain conditions [17]. The presence of some RNAs or ssDNA sequences can induce the formation of FUS assemblies at lower FUS concentration. All FUS domains are intrinsically prone to aggregation [17]–[19]. However, RNA is not required for assemblies caused by the LC domain [20]. Presumably, RNA that specifically binds to the aggregation surface might block the formation of a higher assembly. In contrast, RNAs that bind somewhere else cannot inhibit the aggregation formation, or on the contrary, they can seed higher order protein structures [17]. Denaturation of FUS at 95°C implied that the FUS structure is required for aggregation assembly [17].

Pipetting strokes can have a shearing effect on a protein, and molecules adjacent to the tip surface are the most affected, and the lowest shearing effect occurs in the middle of the solution. The shearing force can provide the energy required to overcome weak bonds in the intrinsically disordered regions of the protein and thereby change its conformation. Shear has been reported to induce fibril aggregation of the whey protein beta-lactoglobulin [21], [22]. It was suggested that the force at air-water interfaces is about 140 pN, which is similar to the 20–150 pN required to unfold proteins using atomic force microscopy [23]. A rheo NMR study of superoxide dismutase 1 (SOD1) showed that shearing can induce the formation of amyloid fibrils from SOD1 monomers, while with static conditions there is no change in the monomer state [24].

In vivo, blood flow in narrow capillaries can induce considerable shear stress in the circulatory system [25]. The biological significance of shear stress can be represented by the conformational change of a human blood plasma protein, von Willebrand (vWF), which has an important function in coagulation. It was suggested that high shear stress at the site of bleeding injury could induce a structural change in vWF that is critical for

platelet adhesion and thrombus formation at the wound site [26]. At the cellular level, the velocity of organelles inside cells is not uniform. Therefore, a velocity gradient arises, which in turn creates shear stress on proteins inside the cells. Shear can result in protein deformation or a change in alignment so molecules can stick together, causing aggregation. The observed FRET change was gradual, and more aggregation was observed with increasing number of pipetting strokes. As the shearing effect is maximized at the tip interface, several strokes of pipetting might be required to exert shear on most of the molecules in the solution. The shearing effect on protein structure and the subsequent induction of aggregation such as amyloid fibril formation have been studied for decades [22], [27]–[31]. We found that some RNAs can inhibit the aggregation caused by the shearing effect while others cannot. Since RNA fragments with the highest effect on the *E* value are the same as those that can prevent FUS aggregation, we suggest a correlation between FUS conformation and its aggregation tendency. In a very compact form, FUS protein might not be aggregated. Whereas pipetting induces shearing that slightly extends the protein conformation. This partial extension could be favorable for protein aggregation [32]. The more shear is applied, the more aggregation is induced. A gradual change might occur due to the extra shearing effect of each pipetting. Shorter RNA/DNA fragments (less than 12 mer) could induce little change in the conformation of FUS, which may still be favorable for aggregation assembly, or it can not bind to the interface of aggregation assembly. Upon addition of R13 or other longer fragments, a significant conformational change is observed, which might not be favorable for the aggregation assembly. Another possibility is that the binding of a particular region of a protein by RNA/DNA fragment blocks the assembly interface.

At higher temperatures, more extension of the FUS conformation occurred as expected. Interestingly, the change in FUS conformation was accompanied by a reduction in the whole spectrum, as observed on pipetting, although no pipetting was performed in that experiment. There could be two reasons for the reduction in the fluorescence intensity. 1- Fluorescence intensity can be reduced by increasing the temperature. 2- Thermal stress can induce a conformational change in proteins, which might expose hydrophobic regions. Hydrophobic interaction can lead to protein aggregation. The complete reversion in

fluorescence spectrum between 4 and 50 °C suggests that FUS aggregation is not induced in this temperature range, and the observed change is solely due to the effect of temperature on the fluorescence efficiency. At higher temperature (~70 °C), FUS structure is destroyed, which induces aggregations, that in turn, induce further reduction in the fluorescence spectrum. The incomplete reversion of the fluorescence spectrum may reflect the precipitated FUS aggregations.

We found that NaCl can dissolve the aggregated FUS, at least partially, and raise up the fluorescence spectrum after the reduction caused by several strokes of pipetting. Precipitated proteins are usually not easy to dissolve, which suggests that the observed reduction in the fluorescence spectrum is caused by reversible aggregation. In our case, NaCl could prevent FUS aggregation caused by shearing stress at 250-300 mM. Previously, it was found that raising NaCl from 50 mM to 150 mM did not affect full-length FUS phase separation obviously [33]. However, at 300 mM NaCl, phase separation was significantly reduced, which suggests a common role of electrostatic interaction in phase separation as well as other kinds of FUS aggregation.

4.4 Conclusion

We found that FUS protein tends to aggregate in response to shearing stress that is applied by pipetting. R13 could protect FUS from the pipetting effect, while a non-specific sequence does not have the same protective effect on the FUS protein. The degree of conformational change and its correlation with FUS aggregation tendency upon shearing stress, suggest that FUS-RNA interaction also modulate phase transition in either physiological or pathological conditions. Our data revealed pncRNA as a prospective inhibitor for FUS aggregation. This finding might pave the way for more research on the role of lncRNAs as aggregation inhibitors, which could be therapeutic targets for neurodegenerative diseases.

4.5 Materials and Methods

4.5.1 Protein preparation

MPB-BFP-FUS-GFP-6xH protein was expressed and purified as described in Chapter 2. The essence of the preparation is as follows. The fusion protein was expressed in BL21 (DE3) Gold Escherichia coli cells. The protein was induced by the addition of 0.1 mM isopropylthio- β -D-galactopyranoside (IPTG) for 20 hours at 20 °C. Cell pellets were sonicated in lysis buffer comprising 50 mM Tris-HCl, pH 7.6, 25 mM glucose, 1% CHAPS, 10 mM benzamidine, 5 U/mL DNase I, 1 mg/L RNase, and 0.2 g/L lysozyme. The supernatants were purified by nickel-affinity column chromatography using Ni-sepharose beads (GE Healthcare Bio-Sciences), followed by size exclusion chromatography (SEC) using a HiloadTM 16/60 SuperdexTM 200 prep grade column (GE Life Sciences). Protein was stored at 4°C. 5 mM fresh dithiothreitol (DTT) was added to the purified fusion protein solution on the same day as the experiment. The protein sample was diluted five times to get ~ 100 nM FUS fusion protein in buffer (10 mM Tris-HCl, pH 7.6, 5 mM glucose, 0.2% CHAPS, 50 mM NaCl). The fusion protein was used for FRET assay, fluorescence microscopy, and TEM imaging.

Strep-GFP- FUS protein was expressed using BL21. The cells were collected and frozen in -80 °C overnight, and then resuspended in WCE buffer (25mM HEPES, 150mM NaCl, 1.5mM MgCl₂, 0.2mM EDTA, 0.05% Triton, 10% glycerol). Cells were lysed by sonication on ice. The cell lysates were centrifuged at 110,000 g for 40 min. The supernatants were purified on a Strep-Tactin Sepharose Column (gravity flow). The column was washed with Wash buffer (100 mM Tris-HCl, pH 8.0 150 mM NaCl, 1 mM EDTA), and protein was eluted with Elution buffer (100 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin). The protein was concentrated with Amicon Ultra-0.5 Centrifugal Filters to a final concentration of around 5uM. For the aggregation assay, 100 nM sample was added to buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl). The fusion protein was used for fluorescence microscopy.

4.5.2 FRET assay

A final concentration of 100 nM FUS dissolved in 10 mM Tris-HCl, pH 7.6, 0.2% CHAPS, 5 mM glucose, 50 mM NaCl, and 1 mM DTT was prepared in a total volume of 140 μ L as a FUS sample by diluting the FUS stock solution with RNase-free water. An equimolar amount of RNA was also dissolved. Fluorescence spectra were collected with a steady-state photon counting spectrofluorometer (JASCO FP-8500 spectrometer, Japan Spectroscopic Co.) using a standard quartz cuvette with an optical path length of 1 cm. The excitation wavelength was 402 nm. The spectra slit width of 5 nm was used for excitation and emission with an integration time of 1 nm/s from 415 nm to 650 nm. Unless the temperature is prescribed, all the measurements were carried out at 25°C. A blank was measured and subtracted from all the spectra. Data were processed using a JASCO Spectra Manager of the FP-8000 series.

4.5.3 Fluorescence microscopy

100 μ L of 100 nM protein was placed in a 96-well plate and imaged under different pipetting conditions. GFP fluorescence was observed under a fluorescence microscope (Keyence BZ-X710). Images were converted to black and white. Fiji software (RRID: SCR_002285) was used to set particle size parameters to count particles $> 0.002 \text{ in}^2$ to exclude background noise. To distinguish LLPS particles from aggregations, particles with circularity < 0.8 were considered as aggregations.

4.5.4 Transmission electron microscopy

A sample solution was diluted 10-fold up to 10 nM and then spotted onto a collodion-coated copper grid (Nisshin EM Co., Tokyo, Japan). After 1 min, the remaining solution was removed with filter paper and 5 μ L of 1% (w/w) phosphotungstic acid (PTA) was spotted onto a collodion-coated copper grid. The solution was removed immediately with filter paper. Then, the grid was washed with 5 μ L pure water to remove excess PTA. After 1 min, the remaining solution was removed in the same manner. Images were obtained using H-7650 TEM (Hitachi, Tokyo, Japan) operating at 80 kV.

4.6 References

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Chapter Five

General conclusions

Fused in sarcoma/ translocated in liposarcoma (FUS/TLS) is a multifunctional DNA/RNA binding protein that regulates various RNA metabolic processes, including transcription, splicing, transport, translation, and decay. FUS is being paid much attention due to its association with numerous fatal neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD), and Huntington disease (HD). Several studies identified RNA sequence/structure motifs that bind FUS while others showed that FUS binding is not highly specific as to RNA sequence or structure. However, the mechanism of action by which FUS binds to various nucleic acids and exerts its function is still controversial.

FUS plays a role in the DNA damage response through the suppression of *cyclin D1* gene (*CCND1*) transcription, which results in cell cycle arrest to allow DNA repair. In that transcription repression process, the C-terminal region of FUS was reported to interact with long non-coding RNA (lncRNA) that is transcribed from the 5' upstream region of *CCND1*, named promoter-associated non-coding RNA (pncRNA). Although it was proposed that the interaction between FUS and pncRNA can allosterically convert FUS from a closed to an open conformation, this hypothesis has not been experimentally confirmed so far. In this study, we investigated the conformational change of FUS in response to pncRNA binding by means of fluorescence resonance energy transfer (FRET) and high-speed atomic force microscopy (HS-AFM). Then, we studied the effects of temperature and shear stress caused by pipetting on aggregation of the FUS protein. We found that pncRNA can protect FUS from the shearing stress caused by pipetting, which can be linked to the resultant conformational change of FUS upon binding to pncRNA.

In Chapter 1, firstly, a general introduction to the FUS protein family and its structure was given. Then, the diverse physiological and pathological roles of FUS were introduced. Finally, the background and the aim of this study were described.

In Chapter 2, FUS fusion protein construction and FRET assaying were explained. We designed a fusion construct, in which blue fluorescent protein (BFP, donor) and green fluorescent protein (GFP, acceptor) are linked to the N- and C-termini of FUS,

respectively. A change in the FUS conformation was detected as the change in FRET efficiency before and after the addition of pncRNA. The FRET spectrum indicated the presence of FUS in a closed (compact) conformation in the absence of pncRNA. Binding of FUS to pncRNA induced a conformational change of FUS to an extended structure that was detected as the change in the relative FRET efficiency upon the addition of pncRNA. We showed that a short fragment of pncRNA (31-mer), which is a critical sequence for FUS-pncRNA binding, can induce a similar conformational change in FUS to that caused by the full-length pncRNA (601-mer). We further identified a minimum portion of pncRNA, GAGGGU, which can induce a conformational change of FUS. FRET data showed that both sequence and length can influence the degree of FUS conformational change. Then, we showed that other specific sequences, Telomeric repeat-containing RNA (TERRA) and Telomere DNA (Htelo), which have a crucial role in telomere shortening, can also induce a conformational change of FUS that is comparable to the pncRNA effect. FRET assaying for mutated TERRA and Htelo implied that their G-quadruplex secondary structures are not critical for induction of the FUS conformational change. Under our experimental conditions, ALS-linked FUS mutations have no remarkable effect on FUS initial conformation or FUS ability to change its conformation in response to pncRNA binding.

In Chapter 3, HS-AFM was applied to directly visualize the conformation of FUS protein before and after the addition of pncRNA. The moment of the FUS structural change from a compact to an extended form was captured. To investigate the distribution of various conformers in the absence/presence of pncRNA, about 7,000 HS-AFM frames were analyzed by two methods, BFP-GFP distance analysis and radius of gyration (R_g). Histograms for both analyses showed that FUS is mainly observed in a compact conformation in the absence of pncRNA. Upon the addition of pncRNA, FUS becomes more extended and flexible, as estimated from the center and width of histograms.

In Chapter 4, first, we showed that shear stress caused by pipetting can induce aggregation of FUS, which was indicated by the reduction in the whole FRET spectrum after pipetting. That reduction is not observed if no pipetting is applied. The induction of FUS aggregation by pipetting was visualized by fluorescence microscopy and transmission electron microscopy. Then, we showed that a specific pncRNA fragment

(13-mer) can hinder the shear stress effect on FUS aggregation, while a non-specific poly(U) fragment (U₁₃) cannot. Finally, the effect of temperature on FUS conformation and aggregation was shown.

In Chapter 5, a general conclusion as to the research background, results outcomes, importance of the study, and prospective future research is given.

FUS protein is being paid much attention due to its crucial role in several biological processes as well as its implication in cancer and several fatal neurodegenerative diseases. Therefore, understanding of the mechanisms of FUS physiological roles is needed to determine the etiology of FUS-related diseases. In this study, by means of FRET and HS-AFM, we provided a mechanistic insight into the role of FUS in the DNA damage response. We proved that FUS undergoes a conformational change upon binding to pncRNA, which is transcribed from the promotor region of the *CCND1* gene in response to DNA damage. The conformational change releases the N-terminal FUS from C-terminal masking, allowing the N-terminal region to bind transcription coactivators CBP/p300, which results in *CCND1* transcription repression. We further investigated factors that may affect the FUS conformational change. Our data showed that both sequence and length can influence the extent of the FUS conformational change. Interestingly, we showed that shear stress caused by pipetting can induce FUS aggregation in the absence, but not in the presence, of pncRNA. This indicates a possible role of lncRNA in FUS protection from aggregation, which is a hot topic of study due its direct implication in the etiologies of fatal neurodegenerative diseases.

In this dissertation research, we revealed that FUS undergoes a conformational change upon binding pncRNA and other target sequences. We assumed that electrostatic binding between the N-terminal LC domain and C-terminal RGG domains is the main driving force for the compact conformation of FUS. Mutation analysis of both domains could reveal the critical amino acids for the interaction between FUS termini. It was reported that post-translation modifications of FUS alter its ability to bind DNA/RNA. We are going to check the effects of FUS methylation and/or phosphorylation on its initial conformational state and FUS ability to change its conformation in response to lncRNA binding. FUS can exist in different kinds of aggregation, which varies between normal

and pathological conditions. We are curious to check the effects of target lncRNAs on phase separation, gel-like aggregation, and fibril aggregation, which are linked to neurodegenerative diseases. Our study may provide new insights not only into the change of the overall architecture of FUS, but also the roles of lncRNAs as prospective therapeutic targets for protein aggregation-related diseases.

List of Publications & Presentations

List of Journal papers that constitute the thesis:

1. **Nesreen Hamad**, Tsukasa Mashima, Yudai Yamaoki, Keiko Kondo, Ryoma Yoneda, Takanori Oyoshi, Riki Kurokawa, Takashi Nagata & Masato Katahira. “RNA sequence and length contribute to RNA-induced conformational change of TLS/FUS.” *Scientific Reports* (2020), **10**, 2629.
2. **Nesreen Hamad**, Hiroki Watanabe, Takayuki Uchihashi, Takashi & Masato Katahira. “Direct visualization of the conformational change of FUS/TLS upon binding to promoter-associated non-coding RNA using HS-AFM.” *ChemComm*, (2020), **56**, 9134.
3. **Nesreen Hamad**, Ryoma Yoneda, Masatomo So, Riki Kurokawa, Takashi Nagata & Masato Katahira. “Long non-coding RNA can diminish the aggregation of FUS/TLS caused by shearing stress” (**in preparation**).

List of other Journal papers:

1. Masatoshi Aida, **Nesreen Hamad**, Andre Stanlie, Nasim Begum & Tasuku Honjo. “Accumulation of the FACT complex, as well as histone H3.3, serves as a target marker for somatic hypermutation”. *Proc. Natl. Acad. Sci. U. S. A.* (2013), **110**, 7784–7789.
2. Riki Kurokawa, Reina Komiya, Takanori Oyoshi, Yoko Matsuno, Hidenori Tani, Masato Katahira, Keisuke Hitachi, Yuji Iwashita, Takefumi Yamashita, Keiko Kondo, Ryoma Yoneda, Yudai Yamaoki, Naomi Ueda, Tsukasa Mashima, Naohiro Kobayashi, Takashi Nagata, Ayaka Kiyoshi, Masayuki Miyake, Fumi Kano, Masayuki Murata, **Nesreen Hamad**, Kohei Sasaki & Naoyuki Shoji. “Multiplicity in Long Noncoding RNA in Living Cells”. *Biomedical Sciences* (2018), **4**, 18-23

List of presentations (○Presenter):

1. ○**Nesreen Hamad**, Masatoshi Aida, Nasim A. Begum & Tasuku Honjo. “Role of FACT complex in Somatic Hypermutation.” 84th Annual meeting of the Japanese Biochemical Society, Sep. 21st -24th, 2011, Kyoto, Japan. (**Poster**)

2. ○Tsukasa Mashima, Shunsuke Ozawa, **Nesreen Hamad**, Ryoma Yoneda, Riki Kurokawa, Takashi Nagata & Masato Katahira. “Study of RNA-recognition by TLS/FUS and its conformational change via binding.” The 17th Annual Meeting of the Protein Science Society of Japan, Sendai, June 20th -23rd, 2017, Sendai, Japan. **(Poster)**
3. ○**Nesreen Hamad**, Tsukasa Mashima, Yudai Yamaoki, Keiko Kondo, Riki Kurokawa, Takashi Nagata & Masato Katahira. “Conformational change detection of translocated in liposarcoma, TLS, upon binding to various RNAs”. The 19th Annual Meeting of the RNA Society of Japan, July 19th -21st, 2017, Toyama, Japan. **(Poster)**
4. ○Tsukasa Mashima, Shunsuke Ozawa, **Nesreen Hamad**, Ryoma Yoneda, Riki Kurokawa, Takashi Nagata & Masato Katahira. “Structural study of the recognition of non-coding RNA by TLS/FUS.” The 19th Annual Meeting of the RNA Society of Japan, Jul. 19th -21st, 2017, Toyama, Japan. **(Oral)**
5. ○**Nesreen Hamad**, Tsukasa Mashima, Yudai Yamaoki, Keiko Kondo, Takashi Nagata & Masato Katahira. “Analysis of conformational changes of translocated in liposarcoma, TLS, upon binding to promoter-associated non-coding RNA, pncRNA.” The 3rd International Conference on New Horizons in Basic and Applied sciences, Aug. 5th -7th, 2017, Hurghada, Egypt. **(Oral)**
6. Daniel Morrall, ○**Nesreen Hamad**, Masafumi Shimasaki, Huyen Dinh, Wenjin Zhang, Farzaneh Hooman & Hideaki Ohgaki. “Study of Energy Supply Systems of Rural Villages in Developing Nations: Case study of Ti Pin Kan Pauk, Magway, Myanmar.” The 8th International Symposium of Advanced Energy Science, GCOE Group Study, Sep. 5th -7th, 2017, Kyoto, Japan. **(Poster)**
7. ○Tsukasa Mashima, Takashi Nagata, **Nesreen Hamad**, Shunsuke Ozawa, Yudai Yamaoki, Keiko Kondo, Hiroki Watanabe, Ryoma Yoneda, Takayuki Uchihashi, Riki Kurokawa & Masato Katahira. “Interaction between non-coding RNA and TLS/FUS protein, and structural change”. The 44th International Symposium on Nucleic Acids Chemistry, Nov. 14th-16th, 2017, Tokyo, Japan. **(Oral)**
8. ○**Nesreen Hamad**, Tsukasa Mashima, Hiroki Watanabe, Takayuki Uchihashi, Riki Kurokawa, Takashi Nagata & Masato Katahira. “The effect of different target

nucleic acids on the structural change of TLS”. The 40th Annual Meeting of the Molecular Biology Society of Japan, Dec. 6-9th, 2017, Kobe, Japan. **(Oral & Poster)**

9. ○**Nesreen Hamad**, Tsukasa Mashima, Yudai Yamaoki, Hiroki Watanabe, Takayuki Uchihashi, Riki Kurokawa, Takashi Nagata & Masato Katahira. “Detection of different conformational changes of Translocated in liposarcoma, TLS, upon its binding to various nucleic acids.” The 18th Annual Meeting of the Protein Science Society of Japan, Jun. 26-28th, 2018, Niigata, Japan. **(Poster)**
10. ○**Nesreen Hamad**, Tsukasa Mashima, Yudai Yamaoki, Keiko Kondo, Hiroki Watanabe, Takayuki Uchihashi, Riki Kurokawa, Takashi Nagata & Masato Katahira. “Detecting conformational changes of translocated in liposarcoma, TLS, upon binding to various non-coding nucleic acids by FRET and HS-AFM.” The 2nd Annual Meeting of the Long Noncoding RNA Study Group. May 24th, 2018, Tokyo, Japan. **(Oral)**
11. ○**Nesreen Hamad**, Tsukasa Mashima, Yudai Yamaoki, Keiko Kondo, Hiroki Watanabe, Takayuki Uchihashi, Riki Kurokawa, Takashi Nagata & Masato Katahira. “Conformational change detection of translocated in liposarcoma, TLS, upon binding to promoter-associated non-coding RNA, pncRNA.” The International Conference on Biochemistry, Molecular Biology and Biotechnology (ICBMBB), Aug. 15th-16th, 2018, Puchong, Malaysia. **(Oral)**
12. ○**Nesreen Hamad**, Wan Hasnidah Wan Osman, Peng Song, Zhang Zhengxiao, Zhang Wenjin & Shunsuke Tajima. “Study of plastic waste and disposal methods.” The 9th International Symposium of Advanced Energy Science, Sep. 3rd-5th, 2018, Kyoto, Japan. **(Poster)**
13. ○**Nesreen Hamad**, Tsukasa Mashima, Yudai Yamaoki, Keiko Kondo, Hiroki Watanabe, Takayuki Uchihashi, Riki Kurokawa, Takashi Nagata & Masato Katahira. “Detecting conformational changes of translocated in liposarcoma, TLS, upon binding to various non-coding nucleic acids by FRET and HS-AFM.” The 2nd Ajou-Kyoto-Zhejiang Joint Symposium on Energy Science, Jan. 17th-19th, 2019, Ajou, South Korea. **(Oral)**
14. ○**Nesreen Hamad**, Wan Hasnidah Wan Osman, Peng Song, Zhang Zhengxiao, Zhang Wenjin & Shunsuke Tajima. “Study of plastic waste and disposal

methods.” The 2nd Ajou-Kyoto-Zhejiang Joint Symposium on Energy Science, Jan. 17th-19th, 2019, Ajou, South Korea. (**Poster**)

15. ○ **Nesreen Hamad**, Tsukasa Mashima, Yudai Yamaoki, Keiko Kondo, Riki Kurokawa, Takashi Nagata & Masato Katahira. “FRET study reveals factors that influence the conformational change of TLS upon binding non-coding RNAs.” The 4th International Conference on New Horizons in Basic and Applied sciences, July 26th-29th, 2019; Hurghada, Egypt. (**Oral**)
16. ○ **Nesreen Hamad**, Peng Lin, Yuanyuan Zhao, Seyed Mostafa Mortazavi & Esmaeil Ahmadi. “Characterization of household energy services in China, Egypt and Iran”. The 10th International Symposium of Advanced Energy Science, Sep. 3rd-5th, 2019, Kyoto, Japan. (**Poster**)
17. ○ **Nesreen Hamad**, Tsukasa Mashima, Yudai Yamaoki, Keiko Kondo, Ryoma Yoneda, Riki Kurokawa, Takashi Nagata & Masato Katahira “Promotor associated non-coding RNA, pncRNA, can diminish the aggregation of TLS caused by shearing stress.” The 42nd Annual Meeting of the Molecular Biology Society of Japan, Dec. 3rd-6th, 2019, Fukuoka, Japan. (**Oral and Poster**)

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