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PHYSIOLOGICAL FUNCTION OF FUS: AN RNA BINDING PROTEIN IN MOTOR NEURON DISEASE

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PHYSIOLOGICAL FUNCTION OF FUS: AN RNA BINDING PROTEIN IN MOTOR NEURON DISEASE

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine at the University of Kentucky

By

Liuqing Yang

Lexington, Kentucky

Director: Dr. Haining Zhu, Professor of Molecular and Cellular Biochemistry

Lexington, Kentucky

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ABSTRACT OF DISSERTATION

PHYSIOLOGICAL FUNCTION OF FUS:
AN RNA BINDING PROTEIN IN MOTOR NEURON DISEASE

FUS is an RNA binding protein implicated in the motor neuron disease—amyotrophic lateral sclerosis (ALS, also called Lou Gehrig's disease). ALS is a fatal neurodegenerative disease characterized by progressive motor neuron death. Mutations in the FUS gene cause about 4% of familial ALS (FUS ALS). Mutated FUS protein mislocalizes from the motor neuron nucleus to the cytoplasm and forms inclusions in the cytoplasm. It is unclear how FUS mislocalization induces motor neuron dysfunction and degeneration. This dissertation research was designed to investigate the physiological functions of FUS in the nucleus, with a purpose to shed light on the pathogenesis of FUS ALS. Using biochemical and cell biology approaches, we revealed that there are two functionally distinct pools of FUS inside the nucleus. A portion of FUS is bound to active chromatin domains and is involved in gene transcription regulation. ALS mutations significantly decrease FUS chromatin binding. We further discovered chromatin binding requires FUS oligomerization, which is mediated by an intrinsically disordered QGSY (glutamine-glycine-serine-tyrosine) -rich region in FUS.

Using confocal microscopy and an *in vitro* FUS oligomerization assay, we identified chromatin-associated nuclear RNAs as the trigger of FUS oligomerization. We further discovered that the RNA binding ability of FUS is also required for the cytoplasmic inclusion formation, which does not require the QGSY-rich region. By exchanging localizations of wild-type FUS and mutant FUS, we demonstrated that subcellular localization and RNAs play a more important role than ALS mutations in determining distinct FUS distribution and organization in different cellular compartments.

By knocking down protein arginine methyltransferase gene and using methylation inhibitor treatment, we found that a post-translational modification of FUS—arginine

methylation—can regulate FUS chromatin binding. Suppression of arginine methylation restored mutant FUS binding to active chromatin domains. Altogether, we revealed the distribution-related FUS physiological functions in the nucleus and identified a potential way to reverse the destructive effect of ALS mutations on wild-type FUS.

KEYWORDS: Fused in Sarcoma, Amyotrophic Lateral Sclerosis, Chromatin Binding, Oligomerization, Arginine Methylation

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PHYSIOLOGICAL FUNCTION OF FUS:

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Fused in Sarcoma (FUS) is an RNA binding protein implicated in the motor neuron degenerative disease—amyotrophic lateral sclerosis (ALS). Mutations in the FUS gene cause a subset of familial ALS (hereditary ALS) [1, 2]. The name of FUS comes from a fusion gene, which contains part of the FUS gene, identified in 1993 to cause liposarcoma, a mesenchyme-derived cancer [3, 4]. The physiological function of the FUS protein itself is not fully understood. This dissertation research was designed to investigate the physiological role of FUS, with a purpose to shed light on the pathogenesis of ALS, by using biochemical and cell biology approaches.

Amyotrophic lateral sclerosis

ALS, also called Lou Gehrig's disease in the United States, is a fatal motor neuron degenerative disease. Although categorized as a rare disease, ALS is the most common motor neuron disease. The prevalence of ALS in the United States is 3.9 in 100,000 people in 2011 [5]. ALS is a very devastating disease and the patients typically pass away in 2-5 years after diagnosis [6]. The peak age of the diagnosed patients is around 60 years [6]; thus ALS is a late onset disease. Because of its fatality and late onset, ALS puts a heavy burden on the families of ALS patients and the society. In the summer of 2014, ALS caught public attention through a viral social media phenomenon, the ice-bucket challenge.

ALS is currently not curable. Thesole medication approved by the FDA for the ALS treatment is Riluzole, but it extends the life of ALS patients only for several months [7]. Other treatments for ALS are simply relieving the symptoms and improving the quality of life for the patients. These symptomatic treatments include medications to alleviate pain and emotional instability, physiotherapies to improve weakened muscles, and nutrition adjustment to compensate reduced food intake and hypermetabolism [6].

ALS is characterized by both upper and lower motor neuron degeneration. Pure upper or lower motor neuron degeneration is diagnosed as different diseases, for example primary lateral sclerosis (PLS) that mainly involves upper motor neuron degeneration [8]

and spinal muscular atrophy (SMA) that primarily affects lower motor neurons [9]. Upper motor neurons carry information from the motor cortex to the spinal cord and lower motor neurons carry information from the spinal cord to voluntary muscles. Spasticity is a typical ALS symptom resulting from upper motor neuron degeneration. Muscle weakness, the most publicly recognized ALS symptom, is a consequence of lower motor neuron degeneration [6]. Reduced motor neuron numbers in the motor cortex and the spinal cord were detected in postmortem ALS patients.

Motor neuron degeneration in ALS

What causes motor neuron death in ALS is still a mystery. Both genetic and environmental factors contribute to the ALS pathogenesis. There are about 10% of all ALS cases are familial ALS [10], meaning gene mutations are the culprits for them. The majority of ALS cases, about 90%, are sporadic, meaning their disease-causing factors are not clear. Environmental factors are the main suspects in sporadic ALS cases. These environmental factors include life styles, e.g., smoking, unhealthy dietary, and lack of exercise; occupational risk factors, e.g., brain trauma in athletes and military personnel and toxins or viruses exposure in certain professions; and medical conditions such as metabolic diseases, cancer, and neuroinflammation [11]. It is noted that the genetic factors also play an important role in many cases of sporadic ALS. Further investigation is needed to determine the specific mechanisms by which the above causal or risk factors induce motor neuron dysfunction and degeneration.

At the cellular level, an increasing amount of evidence supports that motor neurons die in a non-cell-autonomous fashion in ALS. The neighboring cells of motor neurons, most of the time glial cells, also contribute to their demise. Those glial cells are also susceptible to the causal or risk factors of ALS. The evidence of the non-cell-autonomous mechanism mainly comes from the study of cell-type specific expression of mutant proteins in mice and the co-culture of healthy motor neurons with sick glial cells. In transgenic mice, expression of mutant SOD1, an ALS-causing protein, in motor neurons alone did not produce ALS-like phenotypes [12, 13] whereas expression in all cell types did [14, 15]. In the co-culture experiments, mutant SOD1 carrying or ALS patients derived astrocytes killed healthy motor neurons [16, 17]. In mutant SOD1

expressing mice, motor neuron-specific silencing of the mutant gene delayed the disease onset but had no effect on the disease progression [18-20], suggesting that the effects of ALS causal factors on motor neurons and glial cells have different contributions to the course of motor neuron degeneration.

For the motor neuron itself, the direction of its degeneration, from the cell body to the axon (dying forward) or from the axon to the cell body (dying back), is still under debate. The main evidence of the dying back mechanism comes from the observation that the impaired neuromuscular junctions were observed prior to motor neuron loss in ALS mouse models [21, 22]. But the prevailing mechanism is dying forward, mainly because of the experimental observation that motor neurons die through programmed cell death in ALS mouse model [23] and that misfolded proteins in motor neurons spread "forward" along the anterograde axonal transport [24]. Another piece of evidence arguing against the dying back mechanism is the fact that both upper and lower motor neurons die at about the same rate in ALS [6].

At the molecular level, a variety of mechanisms for motor neuron loss have been proposed. These include glutamate receptor-mediated excitotoxicity, oxidative stress induced structural damage, protein aggregation, impaired axonal transport, neuroinflammation, and aberrant RNA metabolism [25]. Various therapeutic interventions based on these molecular mechanisms are currently under development [26, 27]. Riluzole, the only drug in the market, is a suppressor of glutamate receptors to dampen their excitability [28, 29]. But the overall drug development for ALS is much slower than for other diseases, largely due to the still-limited knowledge about the molecular pathways towards motor neuron degeneration.

Since the mutated genes in more than half of familial ALS cases have been identified so far, a good start point to unravel the molecular pathogenesis of familial ALS is to study their physiological functions and pathological properties. Because of the shared disease phenotypes of familial ALS and sporadic ALS, the finding in familial ALS study will also be of great value in understanding the pathogenesis of sporadic ALS.

Genes underlying familial ALS

The first ALS gene encoding Cu/Zn superoxide dismutase (SOD1) was identified in 1993 [30]. Mutations in the SOD1 gene cause about 20% of all familial ALS cases [10]. The second major ALS gene encoding TAR DNA-binding protein 43 kDa (TDP-43) was identified in 2008 [31]. Mutations in the TDP-43 gene cause about 4% of familial ALS cases [10]. A year later, the FUS gene, mutations in which also cause about 4% of familial ALS cases [10], was identified [1, 2]. Both TDP-43 and FUS are RNA binding proteins. The identification of them opened a new window to study ALS pathogenesis: RNA metabolism. In 2011, the ALS gene accounting for the largest portion of familial ALS cases was identified: C9orf72 [32, 33]. Mutations in C9orf72 cause about 40% of all familial ALS cases [10]. The function of the protein product of C9orf72 is yet to be identified.

Mutations in the SOD1, TDP-43, and FUS genes mostly take place in the coding region and result in single amino acid substitutions in their protein products. A small number of mutations also result in carboxyl-terminal (C-terminal) deletions of FUS and TDP-43 [34]. In contrast, mutations in C9orf72 do not change its coding sequence, but increase the number of hexanucleotide (GGGGCC) repeats in the first intron [32, 33]. In normal control, the first intron of C9orf72 contains less than 23 GGGGCC repeats. In ALS patients, the number of repeats increases to hundreds or more [32, 33]. The expanded GGGGCC repeats in ALS patients can be transcribed into repetitive RNAs [32, 33], thus C9orf72 mutation also involves RNA metabolism.

Three potential disease-causing mechanisms have been proposed for C9orf72 mutation: 1) the reduction of C9orf72 protein level [32, 33] may be harmful for motor neurons; 2) repetitive GGGGCC RNAs sequester essential RNA binding proteins that are critical for motor neuron health; and 3) GGGGCC RNA repeats can be translated through an unconventional repeats-associated non-ATG (RAN) translation mechanism [35, 36] and the translated dipeptide repeats are toxic to motor neurons [37, 38]. Interestingly, the toxicity of dipeptide repeats involves their accumulation in the nucleoli and the disturbance of pre-mRNA splicing and ribosomal RNA biogenesis [38]. The study of

C9orf72 disease-causing mechanism further strengthened the idea that aberrant RNA metabolism may play a critical role in ALS pathogenesis.

Additional genes have also been identified in rare forms of familial ALS. Four such genes also involve RNA metabolism: SETX encodes a potential DNA/RNA helicase [39]; ANG encodes a ribonuclease which cleaves tRNA and stimulates rRNA synthesis [40]; HNRNPA1 encodes a heterogeneous nuclear ribonucleoprotein which functions in various RNA metabolism processes [41]; and TAF15 encodes TATA box binding protein associated factor which plays a role in transcription regulation [42]. Therefore, RNA metabolism is an emerging field for multiple forms of familial ALS.

ALS genes and sporadic ALS

ALS genes are also implicated in sporadic ALS. Mutations in ALS genes such as SOD1, TDP-43, FUS and C9orf72 were also found in sporadic ALS [43-45], indicating that familial and sporadic ALS may share the same pathological pathways. More importantly, wild-type protein products of the ALS genes with abnormal properties or behaviors have been widely observed in sporadic ALS. For example, oxidized and misfolded wild-type SOD1, which possesses many mutant SOD1 properties, has been detected in a large number of sporadic ALS cases [46-48]. Wild-type or truncated TDP-43 are the major components of ubiquitin-positive inclusions observed in the motor neurons of most of sporadic ALS patients [49, 50]. FUS positive inclusions have also been reported in sporadic ALS [51] and FUS protein is involved in multiple forms of sporadic ALS mediated by SOD1, TDP-43, or C9orf72 [52-54]. The implication of ALS genes in sporadic ALS strengthened the importance of familial ALS research in revealing the overall etiology of ALS.

FUS in ALS

FUS is an RNA binding protein predominantly localized in the nucleus. ALS mutations result in FUS retention in the cytoplasm and the formation of FUS inclusions in the cytoplasm [1, 2]. FUS has multiple functions in the nucleus [44]. However it is unclear how FUS is deployed to fulfill its multiple roles or what are the consequences of ALS mutations on FUS nuclear functions. FUS inclusions colocalize with stress granule

proteins [55, 56] in the cytoplasm. Stress granules are temporary RNA/protein complexes that ensure cell survival under harsh conditions [57]. But it is still unknown whether mutant FUS affects cell stress response. The Holy Grail question: is nuclear loss of FUS or cytoplasmic accumulation of FUS inclusions responsible for motor neuron degeneration is still under debate.

i) Loss-of-function mechanism

Most of ALS mutations in FUS are clustered in its C-terminal nuclear localization sequence (NLS) [34]. Mutations reduce the binding affinity of FUS NLS to transportin1 [58, 59], the nuclear envelop protein that is responsible for importing cytoplasmic synthesized proteins into the nucleus. Therefore a direct consequence of most FUS ALS mutations is FUS cytoplasmic retention, which subsequently results in nuclear FUS deficiency.

The reported roles of FUS in the nucleus include DNA damage repair [60-62], transcription regulation [63-65], and splicing regulation [63, 64, 66]. The immediate suspicion of the consequences of a nuclear FUS deficiency is the disturbance of these cellular activities that FUS participates in. Indeed, FUS knock-out induced a high incidence of genomic instability in mice [62]. In mouse brain and cultured cells, FUS depletion changed the expression level of hundreds of genes and the splicing pattern of hundreds of mRNAs [63, 64]. However, it is unknown whether these consequences of nuclear FUS deficiency contribute directly to motor neuron degeneration. The FUS knock-out mice died shortly after birth with no clear neuron-related phenotypes [62].

Loss of RNA metabolism-regulating protein has been proved able to cause motor neuron death. SMA is a lower motor neuron degenerative disease caused by deletion or nonsense mutations of a gene named survival of motor neuron (SMN) [9]. SMN regulates small nuclear RNPs assembly which is crucial for mRNA splicing in the nucleus [67].

Another potential loss-of-function mechanism involves cytoplasmic FUS. Although wild-type FUS is primarily localized in the nucleus, a small amount of wild-type FUS also exists and carries out certain functions in the cytoplasm. This is

particularly true in the neuronal cells [68-70]. FUS has been reported to be important for mRNA transport in the dendrites and protein local translation in the synapsis [71, 72]. In this scenario, ALS mutations may change the conformation of FUS and impair its function in local translation regulation [73]. Disturbed dendritic local translation has also been proved to cause neuronal disorders. Fragile X syndrome is a neural developmental disease caused by mutations in fragile X mental retardation (FXR) gene [74, 75]. The protein product of the FXR gene is critical for mRNA transport in the dendrites [76, 77].

ii) Gain-of-function mechanism

The cytoplasmic-retained nonresident FUS mutants may cause motor neuron dysfunction and degeneration through gain-of-toxic-function mechanisms. Cytoplasmic FUS inclusions are a convenient suspect because proteinaceous inclusions are commonly observed in a spectrum of neurodegenerative diseases. Inoculation of the components of neurodegenerative diseases related proteinaceous inclusions, for instance β-amyloid [78], Tau [79], or α-synuclein [80], induced similar disease phenotypes in mice. These results further intrigue researchers to incline to the gain-of-function mechanism.

However, most of the toxic inclusions are composed of misfolded proteins in a cross-β-sheet structure [81-85], the structural basis of amyloidal aggregation. No amyloidal aggregation has been detected in FUS inclusions [86, 87]. An intermediate product of amyloidal aggregation, amyloidal oligomers, are considered the real culprit of neuronal death in various neurodegenerative diseases in recent years [88, 89]. But till now no toxic FUS oligomers has been reported. These facts require researchers to be cautious when interpreting FUS mutants toxicity in model organisms.

A distinct property of FUS inclusions compared to the amyloidal aggregates is its colocalization with stress granule proteins [55, 56]. Stress granules contain mRNAs and RNA binding proteins from the suspended protein translation apparatus [57]. It is still a mystery how mutant FUS is incorporated into stress granules and whether mutant FUS disturbs normal stress granule response. It is conceivable that persistent FUS inclusions sequester and immobilize essential stress granule proteins, thus weakening the normal stress response and resulting in motor neuron degeneration in the long run.

Transgenic expression of FUS mutants in rodents also gave confusing results. Most of FUS transgenic models reproduced some ALS phenotypes, including low viability, motor defects, and alteration of motor neuron dendritic structure [73, 90, 91]. But the most prominent pathological features of ALS with FUS mutations—FUS inclusions and motor neuron loss—were not detected in these models [73, 90, 91].

Two recent reports of FUS gain-of-function in the nucleus further complicated the understanding. One reported that a point mutation in FUS resulted in its intranuclear aggregation and caused ALS [92]. The other reported that over-expressing FUS in cultured cortical neuron nucleus, not cytoplasm, induced neuron death [93].

At this time, it remains debatable whether a loss-of-function mechanism or a gain-of-function mechanism alone is the sole culprit of motor neuron degeneration in familial ALS with FUS mutations. It is possible that both can contribute to the pathogenesis. Before we make further speculation, it is better to re-examine the physiological function of FUS in details and rethink how ALS mutations in FUS cause such a detrimental effect on motor neurons. Below I will describe the domain structure of the FUS protein and briefly discuss their potential involvement in the physiological or pathological activities of FUS.

FUS domain structure

The amino-terminus (N-terminus) of FUS contains an intrinsically disordered sequence [94]. The composition of amino acids in this sequence is simple and limited to certain types; thus it is also called a low complexity domain [95]. The disordered sequence can be divided into two regions (Figure 1.1). From amino acid 1 to amino acid 164 is located a QGSY-rich region which is mainly comprised of glutamine, glycine, serine, and tyrosine. From amino acid 165 to amino acid 284 a glycine-rich region, which contains mainly glycine, is located.

FUS N-terminus was also predicted as a prion-like domain based on its glutamine/glycine-rich feature [96]. The word prion was coined to describe a misfolded protein that can convert normal protein conformation to a pathological conformation and drive the formation of fibrilar aggregates. Accordingly, one of the properties of the prion-

like domain is aggregation-prone [97]. Indeed, purified FUS protein formed fibrilar aggregates and the prion-like domain played an essential role in this process [98]. A study of FUS low complexity domain found that the FUS N-terminus itself formed fibrilar aggregates at high concentration [95, 99, 100]. Because misfolded protein aggregation has long been blamed for neuronal death in many neurodegenerative diseases, the aggregation-prone property of the FUS N-terminus caught much attention when discussing gain-of-function mechanisms.

After the disordered sequence, a RNA recognition motif (RRM) is located from amino acid 285 to amino acid 370 (Figure 1.1). The structure and RNA binding properties of FUS RRM have been characterized by our lab [101]. A zinc finger domain is located from amino acid 371 to amino acid 494, which contains a zinc finger motif (ZnF) flanked by two arginine-glycine-glycine (RGG) repeat regions. Both the RRM and zinc finger domain can bind RNA; they define the RNA binding protein identity of FUS. In a FUS pull-down and RNA sequencing study, RNAs from more than 5,000 human genes and RNAs from more than 8,000 mouse genes were identified as targets for FUS binding [64]. The two gene pools are largely overlapped (69%) [64], suggesting FUS plays fundamental role in RNA metabolism.

At the very C-terminus of FUS is located a NLS (Figure 1.1). FUS NLS is a non-classical NLS which is called PY-NLS because of its proline/tyrosine pair [102]. The PY-NLS of FUS was identified by our lab [55] and other labs [103-105]. We and others determined the structure of FUS PY-NLS and its complex with transportin 1 [58, 59]. Both studies found that ALS mutations dramatically reduced the binding affinity of FUS NLS to transportin 1 [58, 59].

Besides these major sequence features, there are three RGG repeat regions interspersed between functional motifs (Figure 1.1). The first RGG repeat region (RGG1) is located in the glycine-rich region. The second RGG repeat region (RGG2) is located between the RRM and the ZnF and the third one (RGG3) is located after the ZnF and before the NLS. Because of the positive charges of arginine side chain and the negative charges of nucleic acid, it is believed that RGG repeats can significantly enhance the binding affinity of RRM or ZnF to RNA. Additionally, RGG repeats are typical

substrates of protein arginine methyltransferases (PRMTs) [106]. FUS has been reported to be arginine-methylated by PRMT1 [107, 108].

In this dissertation research, we revealed how nuclear FUS is deployed for its multiple functions inside the nucleus and how ALS mutations disturb this process (Chapter 3). The nuclear FUS is separated into two functionally distinct pools: chromatin-bound and non-chromatin-bound. Only chromatin-bound FUS can regulate gene transcription. The requirement for FUS chromatin binding is oligomerization (or self-assembly). Interestingly, FUS oligomerization is mediated by the QGSY-rich region, part of the predicted prion-like domain. ALS mutations significantly decrease FUS chromatin binding. We identified RNA molecules that are essential for FUS organization in different cellular compartments: nucleus and cytoplasm (Chapter 4). In the nucleus, chromatin-associated RNAs initiate FUS oligomerization; in the cytoplasm, RNA binding ability is required for FUS inclusion formation. We also discovered arginine methylation in the RGG repeats that can regulate FUS chromatin binding (Chapter 5). Suppression of arginine methylation restored mutant FUS chromatin binding. Together, we revealed the distribution-related FUS physiological functions and their regulation in the nucleus and identified a potential way to reverse the effect of ALS mutations on FUS.

Figure 1.1 FUS domain structure

QGSY, QGSY-rich region; G, glycine-rich region; RRM, RNA recognition motif; zinc finger, zinc finger domain; NLS, nuclear localization sequence; RGG, arginine-glycine-glycine repeats.

Figure 1.1 FUS domain structure

Chapter 2: Materials and Methods

Plasmids

The pEBG-FUS (GST-FUS) and pEGFP-C3-FUS plasmids were constructed in a previously published study [55]. Truncated FUS coding sequences were amplified by PCR from pEBG-FUS or pEGFP-C3-FUS R521G plasmid template and subcloned into pEBG (restriction sites: *Bam* HI and *Kpn* I), pEGFP-C3 (restriction sites: *Bgl* II and *Kpn* I), pDsRed-Monomer-C1 (Clontech, restriction sites: *Kpn* I and *Bam* HI), and pDsRed2-C1 (Clontech, restriction sites: *Bgl* II and *Kpn* I) vectors. pEGFP-C3-FUS-NES and pEGFP-C3-FUS R495X-NLS were constructed in another previously published study [109]. Firefly luciferase reporter plasmid was constructed by subcloning the MnSOD promoter region (-2947-+24) into pGL3 reporter plasmid at the site upstream of luciferase coding sequence [110]. Renilla luciferase reporter plasmid was purchased from Promega. E1A minigene plasmid was constructed by cloning of the E1A genomic sequence into the pcDNA1.1 eukaryotic expression vector [111]. Insulin receptor minigene was constructed by cloning the insulin receptor genomic sequence into pSG5 vector [112]. LMNA Mut minigene was constructed by cloning the LMNA genomic sequence from HGPS patients, which have a point mutation in exon 11 of the LMNA gene, into βglo3S vector [113]. SMN2 minigene plasmid was constructed by cloning the SMN2 genomic sequence into pCI vector and Tau minigene plasmid was constructed by cloning the Tau genomic sequence into pSVIRB vector. pCMV2B-PRMT1 plasmid is a gift from Dr. Weimin Gong at the Institute of Biophysics, Chinese Academy of Sciences.

Antibodies and reagents

FUS antibody (sc47711), Sp1 antibody (sc-59), SOD1 antibody (sc-11407), Histone H1 antibody (sc-10806), GST antibody (sc-139), DsRed antibody (sc-33353), DsRed2 antibody (sc-101526), and GFP antibody (sc-8334) were purchased from Santa Cruz Biotechnology. Histone H3 antibody (catalog no. 9715) was purchased from Cell Signaling Technology. Methylarginine antibody (catalog no. ab412) was purchased from Abcam. PRMT1 antibody (catalog no. 07-404) and Benzonase (catalog no. 70746) were purchased from EMD Millipore. Micrococcal nuclease was purchased from New England

Biolabs (catalog no. M0247). 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB, catalog no. 1916) and Adenosine, periodate oxidized (AdOx, catalog no. 7154) were purchased from Sigma-Aldrich. RNase-free DNase (catalog no. M6101) was purchased from Promega. Proteinase K (catalog no. P8102) was purchased from New England BioLabs. RNase A (catalog no. 19101) and QIAquick PCR Purification Kit (catalog no. 28104) were purchased from QIAGEN. PRMT1 siRNA (SMARTpool: siGENOME PRMT1 siRNA) was purchased from GE Healthcare Dharmacon.

Cell culture and transfection

HEK cells were grown in Dulbecco's Modified Eagle's Medium (Life Technologies, catalog no. 11965) plus 10% fetal bovine serum (Sigma-Aldrich, catalog no. F2442) and 1% Penicillin-Streptomycin (Sigma-Aldrich, catalog no. P4333). Cell transfection was performed using 0.5 μg plasmid for cells cultured in 6-well plates (unless specifically noted) following the standard protocol as previously described [55]. Cells were subjected to experiments 48 hours after transfection unless otherwise noted. For chromatin salt elution assay, we used HEK cells with doxycycline-inducible GFP-FUS, GFP-FUS R521G, and GFP-FUS R495X expression as described in [114]. Briefly, inducible HEK cells were cultured, induced with doxycycline and subjected to experiments 48 hours after induction.

Punch skin biopsies were obtained after informed consent from symptomatic familial ALS patients carrying the R521G FUS mutation as previously published [110]. The study was approved by the Institutional Review Board of the University of Kentucky and details of the patients were described in [110]. Fibroblast cells were generated from the skin biopsies and cultured in Minimum Essential Medium Eagle (Sigma-Aldrich, catalog no. M5650) plus 20% FBS, 2 mM L-glutamine (Sigma-Aldrich, catalog no. G7513) and 1% Penicillin-Streptomycin.

Chromatin-bound protein isolation

Cells were suspended in radioimmunoprecipitation assay (RIPA) buffer (EMD Millipore, catalog no. 20-188) containing protease inhibitor cocktail (Sigma-Aldrich, catalog no. P8340, 1:1000), 200 μM phenylmethanesulfonylfluoride (PMSF) and 1 mM

sodium orthovanadate. Cells were homogenized with a 23G needle. After 20 min incubation on ice, cell lysates were centrifuged at 1,000 x g for 10 minutes at 4°C. The supernatants were removed and the pellet was resuspended with RIPA buffer supplemented with 0.3% SDS and 250 U/ml Benzonase. Pellet suspensions were incubated on ice for 10 minutes and centrifuged again at 1,000 x g for 10 minutes at 4°C. The supernatant from the second centrifugation contained most of the chromatin-bound proteins.

Alternatively, the Subcellular Protein Fractionation Kit from Pierce (catalog no. 78840) was used following the manufacturer's instructions. Cell lysates were separated into cytoplasmic, membrane, nuclear soluble, chromatin bound and cytoskeletal fractions.

Isolation of active chromatin using salt elution

Active and inactive chromatin domains were separated following a previously published salt elution protocol [115]. Briefly, cells were harvested, washed and incubated in nucleus isolation buffer (10 mM HEPES, pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 0.34 M Sucrose; 10% Glycerol; 1 mM DTT; 0.1% Triton X-100; 1:1000 protease inhibitor cocktail) on ice for 8 minutes. After 1,300 x g centrifugation, nuclei were precipitated in the pellet and cytoplasm was retained in supernatant (S1). Purified nuclei were resuspended in separation buffer containing 2,000 gel units/ml Micrococcal nuclease and 1 mM $CaCl_2$. The nuclei suspension was incubated at 37°C for 10 minutes and the reaction was stopped by adding 2 mM EGTA. The supernatant (S2) was removed and the digested nuclei were eluted with 150 mM NaCl at 4 °C for 2 hours (E1) and with 600 mM NaCl at 4 °C overnight (E2) sequentially. The remnant (P) from 600 mM NaCl elution was resuspended with 2% SDS loading buffer. The S1, S2, E1, E2 and P fractions were subjected to SDS-PAGE followed by Western blot.

SDS polyacrylamide gel electrophoresis (SDS/PAGE) and Western blot

Cell extracts, fractions, and eluates were mixed with loading buffer containing 2% SDS and heated at 95 °C for 5 minutes. SDS-PAGE was performed using 10% or 4-15% polyacrylamide gel to resolve proteins. After being transferred to a nitrocellulose membrane, the membrane was blotted with appropriate primary antibodies in TBST with

5% milk, target proteins were visualized using HRP-linked secondary antibodies (GE Healthcare) and ECL HRP substrate (Thermo Scientific, catalog no. 34078).

Immunostaining and fluorescence confocal microscopy

Cells were seeded on gelatin-covered 18 mm glass coverslips in 12-well plates and transfected with 0.25 μg plasmid. 24 hours after transfection, cells were fixed with 4% paraformaldhyde at 37 °C for 15 minutes and permeabilized with 0.25% Triton at room temperature for 5 minutes. Permeabilized cells and nuclei were blocked with 10% bovine serum albumin (BSA) at 37 °C for 1 hour and immunostaining was performed with primary antibodies at 4 °C overnight and secondary antibodies at 37 °C for 1 hour. Both primary and secondary antibodies were diluted in 3% BSA in 1x PBS. During secondary antibody incubation, 2 μg/ml DAPI was added to stain nuclear DNA. Cells were subsequently mounted with Vectashield mounting medium (Vector Laboratories, catalog no. H-1000) and observed under a Nikon A1 confocal microscope.

Luciferase reporter gene assay

0.05 μg firefly and 0.005 μg renilla luciferase reporter plasmids were transfected along with pEGFP-C3-FUS into HEK cells. 48 hours later, luciferase assay was performed with the Dual-Luciferase Reporter Assay System from Promega (catalog no. E1910) following the manufacturer's instructions.

Reverse transcription and real-time PCR

Total RNA was extracted with TRIzol LS Reagent (Life Technologies, catalog no. 10296-010) following the manufacturer's instructions. Total RNA concentration was determined by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). 5 μg total RNA was incubated with 1 μl RNase-Free DNase (Promega, catalog no. M6101) in 20 μl reactions at 37°C for 1 hour. 2 μl DNase-treated total RNA was used to synthesize first strand cDNA with SuperScript III First-Strand Synthesis System (Life Technologies, catalog no. 18080-051) using oligo(dT) primer. 2 μl cDNA was used for real-time PCR with Power SYBR Green PCR Master Mix (Life Technologies, catalog no. 4367659) using a Mx3005P qPCR System (Agilent Technologies, model no. 401513). Primers used

for SMYD3 real-time PCR are: sense: 5'-GGCAAACTGCAGCTACATCA-3'; anti-sense: 5'-GTTGGCGTCGCATTCTTCTA-3'.

Minigene splicing assay

Total RNA extraction and first strand cDNA synthesis were carried as described above except the primers used here were gene-specific anti-sense oligos instead of oligo(dT). Primers used are: E1A sense: 5'- AGGTTGTTCACGTAGGCCGC-3', anti-sense 5'- AGCATGTTGAGCCGGGCAAGT-3'; insulin receptor sense: 5'-TAATACGACTCACTATAGGGC-3', anti-sense: 5'-GCTGCAATAAACAAGTTCTGC-3'; LMNA Mut sense: 5'-GCTTCTGACACAACTGTGTTCACTAGC-3', anti-sense: 5'-GCAGTTCTGGGGGCTCTGG-3'; SMN2 sense: 5'-GGTGTCCACTCCCAGTTCAA-3', anti-sense: 5'-GCCTCACCACCGTGCTGG-3', special anti-sense for the first strand cDNA synthesis: 5'-TGGTTTGTCCAAACTCATCAA; and Tau sense: 5'-CAGCTACAGTCGGAAACCATCAGCAAGC-3', anti-sense: 5'-CACCTCCAGTGCCAAGGTCTGAAGGTCA-3'. The splicing products were resolved by 1% agarose gel electrophoresis followed by ethidium bromide staining.

GST pull-down and mass spectrometry

GST-tagged FUS 1-164, FUS 165-526, and full-length FUS were expressed in HEK cells. GST pull-down was performed using glutathione Sepharose 4 B (GE Healthcare, cat no. 17-0756-01) as previously described [116]. The pull-down samples were digested with trypsin and the tryptic peptides were subjected to shot-gun proteomics analysis as previously described [117]. Briefly, the tryptic peptides were analyzed by LC-MS/MS using an LTQ Velos Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with a Nano-LC Ultra/cHiPLC-nanoflex HPLC system (Eksigent, Dublin, CA) through a nano- electrospray ionization source. Tandem MS/MS data were acquired using CID fragmentation of selected peptides during the information-dependent acquisition. The LC-MS/MS results were subjected to protein identification and acetylation sites determination using ProteomeDiscoverer 1.3 software (Thermo Fisher Scientific) and MASCOT server.

Chromatin-bound protein isolation for native gel electrophoresis

To preserve the native state of proteins, HEK cells were lysed in a detergent-free lysis buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA) plus protease inhibitor cocktail (1:1000), 200 μM PMSF, and 1 mM sodium orthovanadate by passing through a 23G needle several times. After centrifugation at 1,000 x g at 4 °C, chromatin and cell debris were in the pellet and non-chromatin-bound proteins were in the supernatant. The pellet was resuspended in the detergent-free lysis buffer and chromatin-bound proteins were released by breaking chromatin DNA with sonication (Sonic Vibra-Cell Ultrasonic Processor VCX130PB, amplitude 40, 15 x 1 second cycles). After centrifugation again at 1,000 x g at 4 °C, chromatin-bound proteins were retained in the supernatant. Both chromatin-bound proteins and non-chromatin-bound proteins were subjected to 8% native polyacrylamide gel electrophoresis and Western blot as described below.

Native gel electrophoresis

The chromatin-bound and non-chromatin-bound proteins (50 μl) were mixed with 10 μl of 6X loading buffer (350 mM Tris-HCl, pH 6.8, 30% Glycerol, 0.24% SDS, 0.02% Bromphenol blue). Samples were loaded on a 8% polyacrylamide gel soaked in detergent-free running buffer (25 mM Tris, 192 mM Glycine). The stacking was performed at 75 Volts and the separating was performed at 120 Volts. Subsequently, the gel was incubated with transfer buffer (25 mM Tris, 192 mM Glycine, 10% Methanol) supplemented with 0.25% SDS at 70 °C for 10 minutes. After the denaturation step, the gel was ready for transferring and Western blot following the same procedure as described earlier.

Initiation of FUS oligomerization by chromatin content

Different amount of chromatin content, which was released by sonicating the re-suspended chromatins in the detergent-free lysis buffer, was incubated with the non-chromatin-bound sample for 20 minutes on ice. The mixture was then subjected to native gel electrophoresis and Western blot.

During the incubation of the chromatin content and the non-chromatin-bound sample, 50 U/ml RNase-free DNase or 1 ng/ml RNase A was added in the mixture. After incubation, the effect of DNase or RNase on the oligomerization of non-chromatin-bound FUS was assessed by native gel electrophoresis. The nucleic acid contents in each sample were purified with QIAquick PCR Purification Kit after proteinase K digestion and subjected to agarose gel electrophoresis.

Formaldehyde crosslinking

The chromatin-bound and non-chromatin-bound proteins were prepared as above and separately incubated with formaldehyde at a final concentration of 1% at room temperature with rocking for 10 minutes. Crosslinking was quenched by adding glycine to a final concentration of 137.5 mM. The crosslinked samples were subjected to 12% SDS/PAGE followed by Western blot.

Nuclear RNA extraction and the effect of nuclear RNA on FUS oligomerization

Cells were harvested, washed and incubated in nucleus isolation buffer (10 mM HEPES, pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 0.34 M Sucrose; 10% Glycerol; 0.1% Triton X-100) on ice for 8 minutes. Nuclei were isolated by 1,300 x g centrifugation and resuspended in 0.25 ml nucleus isolation buffer. Total nuclear RNA was extracted by 0.75 ml TRIzol LS Reagent. 2 or 20 μg nuclear RNA was incubated with 50 μl non-chromatin-bound extract at 4 °C overnight. These samples were subsequently subjected to native gel electrophoresis and Western blot.

***In vitro* RNA binding assay**

FUS peptide coding sequences were subcloned into the pET22b plasmid (Novagen). FUS peptides were expressed in *E.coli* Rosetta cells (EMD Millipore) and purified with Ni2+ affinity chromatography and cation exchange column (GE Healthcare, catalog no. 17-5157-01). The RNA probe (5'-UUAGGGUUAGGGUUAGGGUUAGGG-3') was labeled with 5'-Cy3 (Invitrogen). Different concentrations of FUS peptides were incubated with 5 pmol RNA probes in 10 μl reaction buffer (20 mM Tris-HCl, pH 7.5; and 50 mM NaCl) on ice for 30 minutes. The mixtures were loaded on a 6%

polyacrylamide gel and run in ice cold TBE buffer at 100 Volts for 45 minutes. The gel was analyzed by ProteinSimple gel imaging system.

FUS immunoprecipitation

AdOx-treated, PRMT1 knocked-down, or PRMT1 over-expressing HEK cells were lysed in RIPA buffer containing protease inhibitor cocktail (1:1000), 200 μM PMSF and 1 mM sodium orthovanadate by passing through a 23S needle several times. After 1,000 g centrifugation at 4 °C, the supernatants were pre-cleared with 50 μl of Protein G slurry at 4°C for 1 hour. Immunoreaction was performed with 2 μg FUS antibody in 500 μl of supernatant at 4°C for 1 hour. The antigen-antibody complexes were enriched by 50 μl Protein G at 4°C overnight, purified by RIPA buffer washing, and released by SDS loading buffer at 95 °C for 5 minutes.

Chapter 3: Self-assembled FUS Binds Active Chromatin and Regulates Gene Transcription

Modified from the manuscript "Self-assembled FUS binds active chromatin and regulates gene transcription", published in the Proceedings of the National Academy of Sciences of the United States of America. 2014. 111(50): 17809-17814. doi: 10.1073/pnas.1414004111. The introduction was simplified for fluidity and the figures were reorganized.

Introduction

ALS is a progressive and fatal neurodegenerative disease characterized by motor neuron loss. The etiology underlying the disease is yet to be better understood. About 10% of ALS cases are hereditary (familial ALS). Mutations in FUS, which is an RNA binding protein, are found to be responsible for a subset of familial ALS patients [1, 2].

FUS is a multifunctional protein and has been reported to play a role in various aspects of RNA metabolism [44], including transcription regulation and alternative splicing. In familial ALS, most mutations are clustered in the C-terminal NLS of FUS and consequently cause the mislocalization of FUS protein from the nucleus to the cytoplasm and the accumulation of protein inclusions [55, 103, 104]. Such observations suggest two potential disease-causing mechanisms: loss of FUS normal function in the nucleus and gain of toxic function in the cytoplasm. It remains to be determined which mechanism plays a more critical role in the ALS etiology and the two mechanisms are not necessarily exclusive of each other.

Although the mutations in the C-terminal NLS are critical to the cytoplasmic accumulation of mutant FUS, the N-terminal prion-like domain has been reported to be crucial for FUS aggregation *in vitro* and in yeast cells [98]. The prion-like domain consists of a QGSY-rich region (amino acids 1-164) and a glycine-rich region (amino acids 165-239). A missense mutation (G156E) in the QGSY-rich region has been found in familial ALS patients and has been reported to cause intranuclear aggregation of FUS [92]. However, the role of the QGSY-rich region in maintaining FUS intranuclear distribution and function under physiological conditions is unknown.

The physiological function of FUS in the nucleus remains to be fully understood. In this study, we found that a significant portion of nuclear FUS was bound to chromatin and that the ALS mutations dramatically decreased FUS chromatin binding ability. Functionally, chromatin binding is required for FUS transcription activation, but not for the regulation of alternative splicing. We further determined that the N-terminal QGSY-rich region mediates FUS self-assembly in the nucleus of mammalian cells and that FUS self-assembly is essential for chromatin binding and transcription activation. In addition, RNA binding is also required for FUS self-assembly and chromatin binding. Together, our results suggest that a functional assembly of FUS in the nucleus under physiological conditions is different from the cytoplasmic inclusions found in cells harboring ALS mutations. These mutations cause of loss-of-function in the nucleus by disrupting FUS assembly and chromatin binding as well as transcriptional activities.

Results

FUS is bound to chromatin and ALS mutations reduce chromatin binding

The wild-type FUS protein is known to be predominantly localized in the nucleus. We asked whether FUS is associated with chromatin. Chromatin-associated proteins in human embryonic kidney (HEK) cells were prepared by extraction with 0.3% SDS and 250 U/ml Benzonase (see flow chart in Figure 3.1A). A significant amount of endogenous FUS was in the chromatin-bound (CB) fraction extracted by SDS and Benzonase (Figure 3.1A). As a control, histone H3 was also present in the CB fraction. The result provided the initial evidence that FUS may bind to chromatin. To confirm this, we employed an independent protocol to fractionate HEK cell lysate into cytoplasmic, membrane, nuclear soluble, chromatin bound and cytoskeletal fractions. The amount of chromatin-bound FUS was comparable to that in the nuclear soluble (NS) fraction (Figure 3.1B). Western blots of the nuclear soluble protein Sp1 and the chromatin-bound protein H3 are included to demonstrate the effectiveness of the fractionation method.

Next, we tested whether the ALS mutations had an effect on FUS chromatin binding. Wild-type FUS and ALS mutants (R521G, Arg 521 mutated to Gly; R495X, Arg 495 mutated to the stop codon, resulting in the deletion of the C-terminal 32 amino acids)

were expressed in HEK cells and the cell lysates were subjected to the fractionation as described above. The ALS mutations significantly decreased the chromatin-bound FUS as compared to the nuclear soluble FUS (Figure 3.2). Quantitative analysis confirmed that the ratio of chromatin-bound and nuclear soluble fractions decreased for the FUS ALS mutants ($p < 0.02$; Figure 3.2). Consistent with previous reports, the ALS mutations also increased the cytoplasmic portion of FUS.

To shed light on the functional role of the chromatin-bound FUS, we further fractionated chromatin to determine to which chromatin domain FUS binds. Chromatin domains can be fractionated by limited nuclease digestion and gradient salt elution based on different architectural levels [115]. Transcriptionally active chromatin domains are loosely packed, therefore are easily digested by nuclease and eluted at lower salt concentration (Figure 3.3A; E1, 150 mM NaCl elution). Transcriptionally inactive chromatin domains are densely packed, therefore are more difficult to be digested and can only be eluted at a higher salt concentration (Figure 3.3A; E2, 600 mM NaCl elution). A significant amount of endogenous FUS was detected in the nuclear soluble (S2) and active chromatin (E1) fractions, whereas a lesser amount of FUS was detected in the inactive chromatin (E2) fraction indicated by Histone H1 immunoblot (Figure 3.3A). The DNA electrophoresis of S1, S2, E1 and E2 fractions are also shown in Figure 3.3A to confirm that E1 and E2 are active and inactive chromatin, respectively [115]. The results suggest that FUS may play a regulatory role in the transcriptionally active chromatin.

The effect of the ALS mutations was also examined on the association of FUS with active chromatin. The level of FUS in active chromatin domains (E1) significantly decreased for the ALS mutations R521G and R495X as compared to wild-type FUS (Figure 3.3B). The quantitative results of the E1/S2 ratio are also shown in Figure 3.3B ($p < 0.05$ for R521G and $p < 0.005$ for R495X).

The N-terminal QGSY-rich region is responsible for FUS chromatin binding

To determine which domains of FUS are responsible for chromatin binding, we generated a series of GST-tagged FUS truncation constructs (Figure 3.4A). The chromatin-bound fraction was prepared with the SDS and Benzonase extraction method

as in Figure 3.1A. All N-terminal fragments (FUS 1-164, 1-284, 1-370, and 1-494) were detected in the chromatin-bound fraction (Figure 3.4B). In contrast, none of C-terminal fragments lacking the N-terminal QGSY-rich domain (FUS 165-526, 285-526, 371-526, and 495-526) was detected in the chromatin-bound fraction. The results suggest that the N-terminal QGSY-rich region is both sufficient and required for FUS chromatin binding.

Interestingly, deleting the N-terminal QGSY-rich region also significantly changed the intranuclear distribution of FUS. Full-length FUS displays a punctate pattern inside the nucleus (Figure 3.5A) and is excluded from nucleoli (arrows in Figures 3.5A), which is consistent with a previous report [118]. With the N-terminal 1-164 deletion, the punctate pattern disappeared and the FUS protein was evenly distributed in the entire nucleus including nucleoli (Figure 3.5B). The results indicate that the punctate pattern inside the nucleus observed under the confocal microscope may be related with FUS chromatin binding.

The role of FUS chromatin binding in transcription regulation and alternative splicing

FUS has been shown to regulate gene transcription [63, 110, 114] and alternative splicing [63, 64]. We next tested the relationship between chromatin binding and FUS function in gene transcription and alternative splicing using the full-length FUS and the truncated FUS 165-526 that is deficient in chromatin binding. We used a manganese superoxide dismutase (MnSOD) reporter assay since FUS was shown to activate the transcription of MnSOD [110]. The full-length FUS or FUS 165-526 along with a reporter plasmid carrying the MnSOD promoter were transfected into HEK cells. MnSOD reporter gene activities showed that the full-length FUS increased reporter gene activity approximately two folds, as compared to the non-transfected control ($p < 0.01$; Figure 3.6A). In contrast, the truncated FUS 165-526 lost the ability of increasing MnSOD reporter gene activity. In addition, we tested the transcription activation of another gene histone-lysine N-methyltransferase SMYD3 that was previously reported to be regulated by FUS [64]. Similarly, truncated FUS 165-526 failed to activate the transcription of the endogenous SMYD3 gene (Figure 3.6B). The protein levels of exogenous full-length FUS, FUS 165-526, and endogenous FUS were comparable

(Figure 3.6C). Since FUS 165-526 is deficient in chromatin binding, the results suggest that chromatin binding is required for FUS function in regulating gene transcription.

The role of FUS in regulating mRNA splicing was examined using a minigene splicing assay [111]. The full-length FUS or FUS 165-526 construct along with the minigene plasmids were transfected into HEK cells. Reverse transcription PCR was used to detect alternative splicing products. Overexpression of the full-length FUS decreased exon inclusion in E1A and insulin receptor transcripts (Figure 3.7A). Overexpression of the truncated FUS 165-526 had a similar effect on the splicing of both minigenes as full-length FUS. Quantitative analysis showed that the ratio of inclusion and exclusion transcripts changed in a similar fashion by the overexpression of either full-length FUS or truncated FUS 165-526 that is incapable of binding to chromatin (Figure 3.7A), i.e. the truncated FUS 165-526 was as effective as the full-length FUS in regulating alternative splicing. We also showed that FUS overexpression did not affect the splicing of lamin A/C (LMNA Mut) in the minigene assay (Figure 3.7A bottom). This negative control supports the specificity of the splicing results. The results suggest that chromatin binding is not required for FUS to function in alternative splicing, although it is required for FUS regulation of gene transcription. In Figure 3.7B, we showed a similar trend of regulation with SMN2 and Tau minigenes.

The N-terminal QGSY-rich region mediates FUS self-assembly

We next determined why the N-terminal amino acids 1-164 of FUS are required for FUS chromatin binding. The N-terminal QGSY-rich region is so named based on the fact that about 80% of the amino acid residues in this region are glutamine, glycine, serine, or tyrosine. This region is intrinsically disordered, we thus reasoned that a binding partner may cooperatively mediate FUS chromatin binding. To identify binding partner(s), we expressed GST-tagged FUS 1-164, FUS 165-526, and full-length FUS in HEK cells and did a GST pull-down. Next we used mass spectrometry analysis to determine interacting proteins. The criteria for a putative partner are: it should interact with FUS 1-164 and full-length FUS, but not with FUS 165-526. No other proteins were identified to qualify for the above criteria, with the exception of endogenous FUS (Figure 3.8). The results suggest that FUS may interact with itself through the QGSY-rich region.

We hypothesized that self-oligomerization of FUS through the N-terminal QGSY-rich region is critical to FUS chromatin binding ability. To test this hypothesis, we substituted the QGSY-rich region with a DsRed variant (DsRed2) that can form tetramers [119] (Figure 3.9A left). Whereas the GST-tagged FUS 165-526 was not found in the chromatin-bound fraction (Figure 3.4B), the DsRed2-tagged FUS 165-526 was found in the chromatin-bound fraction (Figure 3.9B). As a control, a monomeric DsRed variant (DsRed-M) [120] was tagged to FUS 165-526 (Figure 3.9A right). The monomeric DsRed-tagged FUS 165-526 was not detected in the chromatin-bound fraction (Figure 3.9B). Thus, we conclude that oligomerization of FUS through the N-terminal QGSY region (1-164) is essential for FUS chromatin binding. The tetrameric DsRed2 tag also restored the punctate distribution and nucleolar exclusion of FUS 165-526 (top of Figure 3.10, compare to Figure 3.5 A and B) whereas the monomeric DsRed tagged FUS 165-526 was evenly distributed in the nucleus (bottom of Figure 3.10). As a control, DsRed2 alone showed an even distribution throughout the cell (middle of Figure 3.10), supporting that the punctate distribution of DsRed2-FUS 165-526 is caused by its chromatin binding.

We next used native gel electrophoresis to examine the self-assembly of endogenous FUS in HEK cells. We prepared the soluble and chromatin-bound FUS in a similar fashion as in Figure 3.1 and chromatin-bound proteins were released to the solution by sonicating the resuspended pellet (Figure 3.11). The soluble and chromatin-bound fractions were subjected to native gel electrophoresis followed by Western blot analysis with the FUS antibody. The chromatin-bound FUS migrated as a much slower band as compared to the soluble FUS that is not associated with chromatin (Figure 3.11), supporting that the chromatin-bound FUS indeed forms a high order assembly. Combined with the results obtained from substituting the N-terminal QGSY-rich region with the monomeric or tetrameric DsRed tag, we conclude that FUS binds to chromatin in a self-assembled complex and the self-assembly is mediated by the N-terminal QGSY-rich region.

RNA is also required for FUS self-assembly

Because FUS is an RNA binding protein, we tested whether RNA plays a role in FUS chromatin binding. RNase A was added to a freshly made HEK cell lysate prior to

centrifugation and SDS and Benzonase incubation. The levels of FUS in the chromatin-bound fraction decreased dramatically with increasing amounts of RNase A (Figure 3.12). At the highest RNase A concentration (100 μg/ml), no FUS was detected in the chromatin-bound fraction, suggesting that FUS chromatin binding is RNA dependent. We also tested the RNA dependency of chromatin binding of DsRed2-tagged FUS 165-526 that lacks the N-terminal QGSY-rich region. Surprisingly, the binding of DsRed2-tagged FUS 165-526 to chromatin did not decrease in the presence of increasing amounts of RNase A (Figure 3.13). Since DsRed2 oligomerization does not require RNA, the difference of RNA dependency between the full-length FUS and the DsRed2-tagged FUS 165-526 suggest that RNA may be required for the assembly of the full-length FUS. To test this, RNase A was added in the sonication lysate containing the chromatin-bound FUS. Native gel electrophoresis showed that the slower-migrating FUS band, which is the assembled and chromatin-bound FUS, shifted toward the nuclear soluble FUS in the presence of RNase A (Figure 3.14). The results consistently support that RNA is required for FUS self-assembly.

Discussion

FUS is associated with transcriptionally active chromatin and the association is impaired by the ALS mutations

FUS is predominantly localized in the nucleus, however the distribution and function of FUS inside the nucleus remain to be fully understood. We found that there are two pools of FUS inside the nucleus: nuclear soluble and chromatin bound (Figures 3.1-3.3). Specifically, the chromatin-bound FUS is associated with transcriptionally active chromatin and much less with the condensed inactive chromatin (Figure 3.3A). A recently published study showed the co-localization of FUS with the activated form of RNA polymerase II in the nucleus [121], supporting our biochemical association of FUS with active chromatin. Moreover, the association of FUS with active chromatin was significantly reduced by the ALS mutations R521G and R495X (Figure 3.2 and 3.3B). The reduced chromatin binding by the ALS mutants suggests that the disease-causing mutations may result in a loss of function in the nucleus.

Chromatin binding of FUS is required for its function of regulating gene transcription, but not for its role in alternative splicing

The observation that FUS binds to active chromatin is consistent with earlier reports that FUS can regulate gene transcription [110, 114]. This study shows that the truncated FUS 165-526 lacking the N-terminal QGSY-rich region is incapable of binding to chromatin. Moreover, the truncated FUS 165-526 did not activate MnSOD and SMYD3 gene transcription (Figures 3.6A and 3.6B), supporting that the chromatin-binding property of FUS is required for its gene transcription regulation function. This significance of FUS chromatin binding in gene transcription regulation is supported by a previous report that knock-down of FUS in mouse brain decreased expression of hundreds of genes [64]. The requirement of chromatin binding for its gene transcription activation, along with the finding that ALS mutations impair chromatin binding, provides a mechanism for the previous observation that the ALS mutant FUS lost its transcription activation capability [110].

In contrast to gene transcription, the splicing of E1A and insulin receptor minigenes was largely unchanged between the full-length FUS and the truncated FUS 165-526 (Figure 3.7), suggesting that FUS regulation of alternative splicing does not require its chromatin binding property. Combined together, these results suggest that two different pools of FUS in the nucleus, i.e. chromatin-bound and nuclear soluble FUS, regulate gene transcription and alternative splicing, respectively (Figure 3.15).

A prion-like domain is also found in another RNA binding protein TDP-43 that is also implicated in ALS. Interestingly, the prion-like domain of TDP-43 is required for TDP-43 splicing regulation, but not for transcription activation [122]. Our study reveals a significant difference between the functional relevance of the prion-like domain of FUS and that of TDP-43, suggesting that FUS and TDP-43 may function differently in the nucleus.

The role of the QGSY-rich region in FUS self-assembly and chromatin binding

The N-terminal domain of FUS (amino acids 1-239) has been predicted to be a prion-like domain [96]. Prion was originally coined to describe a pathogenic protein

(PrP^{sc}) which can use itself as template to convert native protein (PrP) into a misfolded conformation and subsequently form amyloid-like aggregates. The prion-like activity has been described in yeast [97] as well as multicellular organisms, involving various processes such as antiviral signaling [123, 124] and memory formation [125]. The prion-like domain in RNA binding proteins is involved in the formation of dynamic and reversible structures such as stress granules [126] and processing bodies [127]. In this study, we determined that the QGSY-rich region (1-164) within the prion-like domain is essential and sufficient for chromatin binding of FUS (Figure 3.4). Using native gel electrophoresis and the substitution of the QGSY-rich region with monomeric and tetrameric DsRed (Figures 3.9-3.11), we demonstrated that this region is required for high order assembly of FUS and the binding of FUS to chromatin. This is a new function of the QGSY-rich region identified in this study.

It remains unclear exactly how the high order assembly of FUS binds to chromatin. We propose that FUS chromatin binding is mediated by the interaction between FUS and RNA polymerase II. Our finding that FUS is preferentially associated with active chromatin (Figure 3.3) supports this notion. In addition, the interaction between FUS and the C-terminal domain (CTD) of RNA polymerase II has been demonstrated *in vitro* [63, 100, 128, 129]. In this study, tetrameric DsRed can restore chromatin binding of the truncated FUS 165-526, indicating that the QGSY-rich region (1-164) is only responsible for self-assembly but not the physical interaction with RNA polymerase II. Since the N-terminal domain (1-266) is reported to be responsible for interacting with RNA polymerase II [100], the physical interaction is likely mediated by the glycine-rich region (165-266).

RNA dependence of FUS self-assembly and chromatin binding

This study shows that RNA molecules are also required for FUS self-assembly and chromatin binding since RNase A treatment disrupted FUS self-assembly (Figure 3.14) and reduced FUS chromatin binding to a undetectable level (Figure 3.12). It is noted in Figure 3.4 that truncation mutants of FUS lacking the zinc finger domain (FUS 1-164, 1-284 and 1-370) showed lower abundance in the chromatin-bound fraction as compared to those containing the zinc finger domain (FUS 1-494 and full-length FUS).

Since the zinc finger domain and the RNA recognition motif (RRM) are both nucleic acid binding domains, our interpretation is that the zinc finger domain can contribute to FUS RNA binding and subsequently chromatin binding. The results combined together support the critical role of RNA dependence of FUS chromatin binding.

We further propose that RNA molecules initiate FUS self-assembly and chromatin binding. This model explains the coexistence of the two pools of FUS (assembled/chromatin-bound and soluble) in the nucleus (Figure 3.15). In the presence of appropriate RNA molecules, FUS assembles, binds to active chromatin and carries out its gene transcription regulation function. In the absence of RNA, FUS remains soluble and carries out other functions such as regulating splicing. Indeed, a previous study demonstrated that non-coding RNAs recruit FUS to chromatin to regulate gene expression [130]. A more recent study showed RNA molecules seeded high-order assembly of FUS *in vitro* [129], supporting the proposed model in Figure 3.15.

Significance of FUS chromatin binding in ALS

This study shows that the N-terminal QGSY-rich region is required for self-assembly of FUS under physiological conditions. This physiological assembly is essential for FUS intranuclear distribution and correlated functions such as transcription activation. The ALS mutations disrupt this assembly and chromatin binding, which could result in several potentially adverse consequences. The ALS mutations R521G and R495X, which significantly decreased FUS chromatin binding, were found to impair the gene transcription of a critical mitochondrial antioxidant protein MnSOD [110]. FUS chromatin binding may also be crucial for DNA damage repair. FUS is among one of the early response proteins in DNA damage repair [60, 61]. Deleting the N-terminal amino acids 1-285 significantly reduced FUS recruitment to DNA damage sites induced by laser micro-irradiation [61]. Deficient binding of FUS to chromatin can result in increased DNA damage and genome instability which is especially harmful to the terminally-differentiated non-dividing neurons. This notion is also supported by a recent study showing that the ALS mutation R521C caused DNA damage in a transgenic mouse model [91].

In summary, FUS self-assembly and binding to chromatin in the nucleus under physiological conditions are critical to its proper function. Disrupting FUS assembly and chromatin binding can cause perturbations in multiple cellular processes and ultimately lead to motor neuron dysfunction and degeneration in ALS.

Figure 3.1 A portion of nuclear FUS is chromatin-bound.

(A) Flowchart of chromatin-bound protein isolation by SDS and benzonase extraction. Cell lysates were prepared by passing through a 23-gauge needle and subjected to 1,000 × g centrifugation for 10 min. Chromatin-bound proteins were released from the pellet by incubation with RIPA buffer containing 0.3% SDS and 250 units/mL benzonase. The soluble (S) and chromatin-bound (CB) fractions were subjected to SDS/PAGE and Western blot with indicated antibodies. (B) HEK cell lysates were separated into cytoplasmic (C), membrane (M), nuclear soluble (NS), chromatin-bound (CB), and cytoskeletal (Sk) fractions using a Pierce Subcellular Protein Fractionation kit. Each fraction was subjected to SDS/PAGE and Western blot with indicated antibodies.

Figure 3.1 A portion of nuclear FUS is chromatin-bound

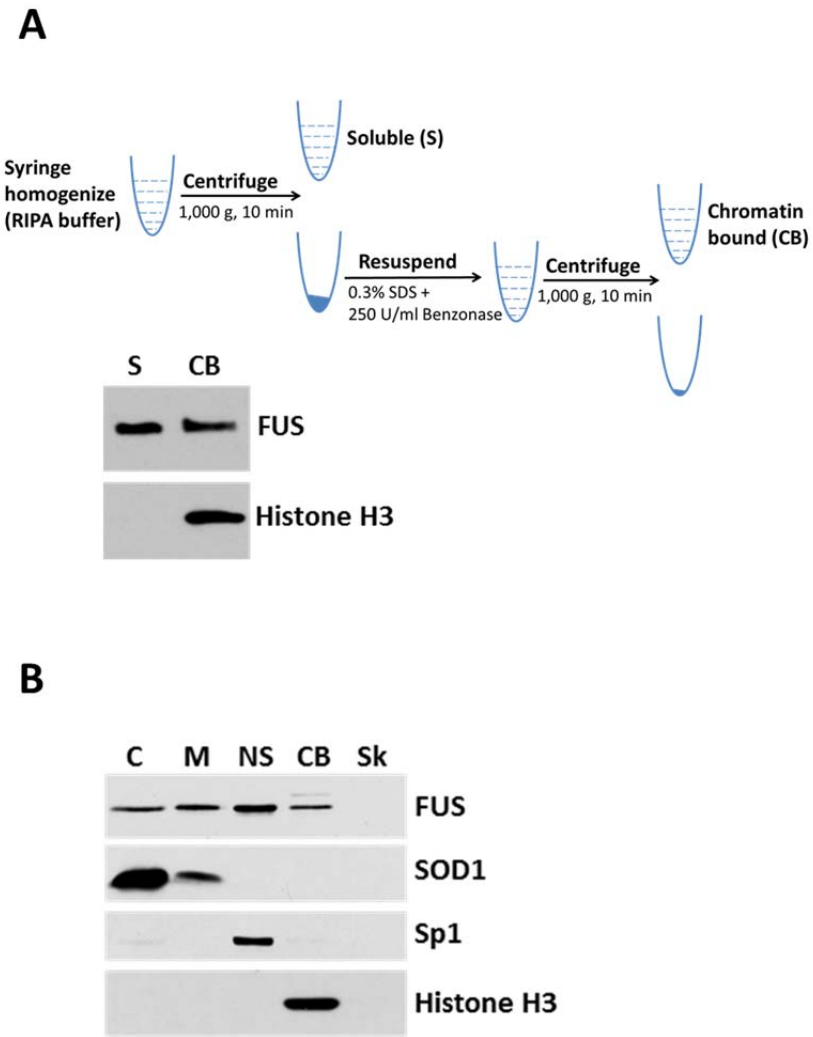
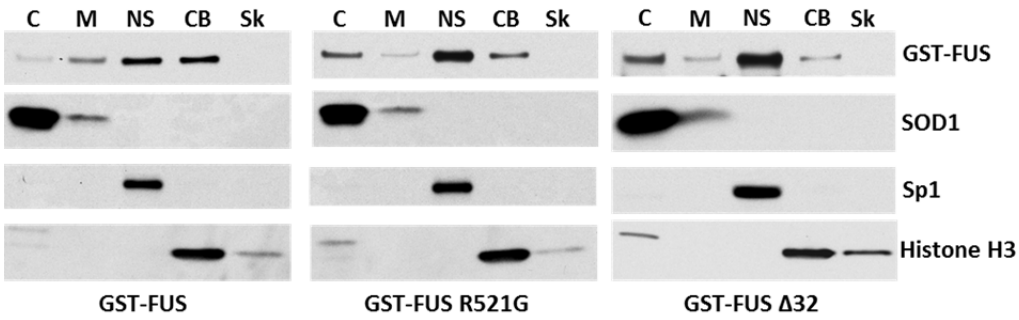


Figure 3.2 ALS mutations decrease FUS chromatin binding.

The distribution of GST-tagged wild-type FUS and ALS mutants R521G and R495X was examined by the fractionation method as in Figure 3.1B. The ratio of CB and NS was quantified and the results from three independent experiments are presented. *P < 0.02.

Figure 3.2 ALS mutations decrease FUS chromatin binding



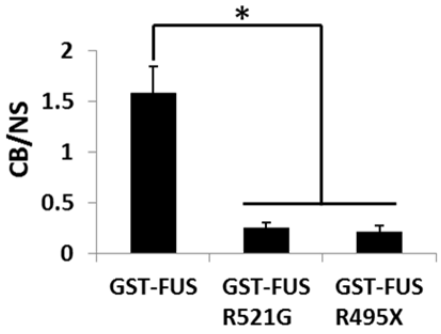


Figure 3.3 FUS is bound to active chromatin.

(A)The flowchart shows the preparation of active and inactive chromatin domains using a previously published salt elution protocol [115]. HEK cell nuclei were separated from cytoplasm (S1) and washed with separation buffer (W1) before micrococal nuclease (MNase) treatment to release nuclear soluble proteins (S2). After second time wash (W2), nuclei were extracted with 150 mM NaCl (E1) and 600 mM NaCl (E2) sequentially. The remnant pellet was resuspended in 2% SDS loading buffer (P). All fractions (S1, W1, S2, W2, E1, E2, and P) were subjected to SDS/PAGE and Western blot with indicated antibodies (Upper) or agarose gel electrophoresis and ethidium bromide staining (Lower). (B) The association of GFP-tagged wild-type FUS and ALS mutants R521G and R495X with active chromatin was examined using the salt elution protocol as in A. The ratio of FUS in E1 and S2 was quantified and the results from three independent experiments are presented. *$P < 0.05$. The antibodies used in Western blot were: SOD1, a primarily cytoplasmic protein; transcription factor Sp1, a nuclear soluble protein; histone H3, a chromatin-bound protein; and histone H1, a protein associated with inactive chromatin.

Figure 3.3 FUS is bound to active chromatin

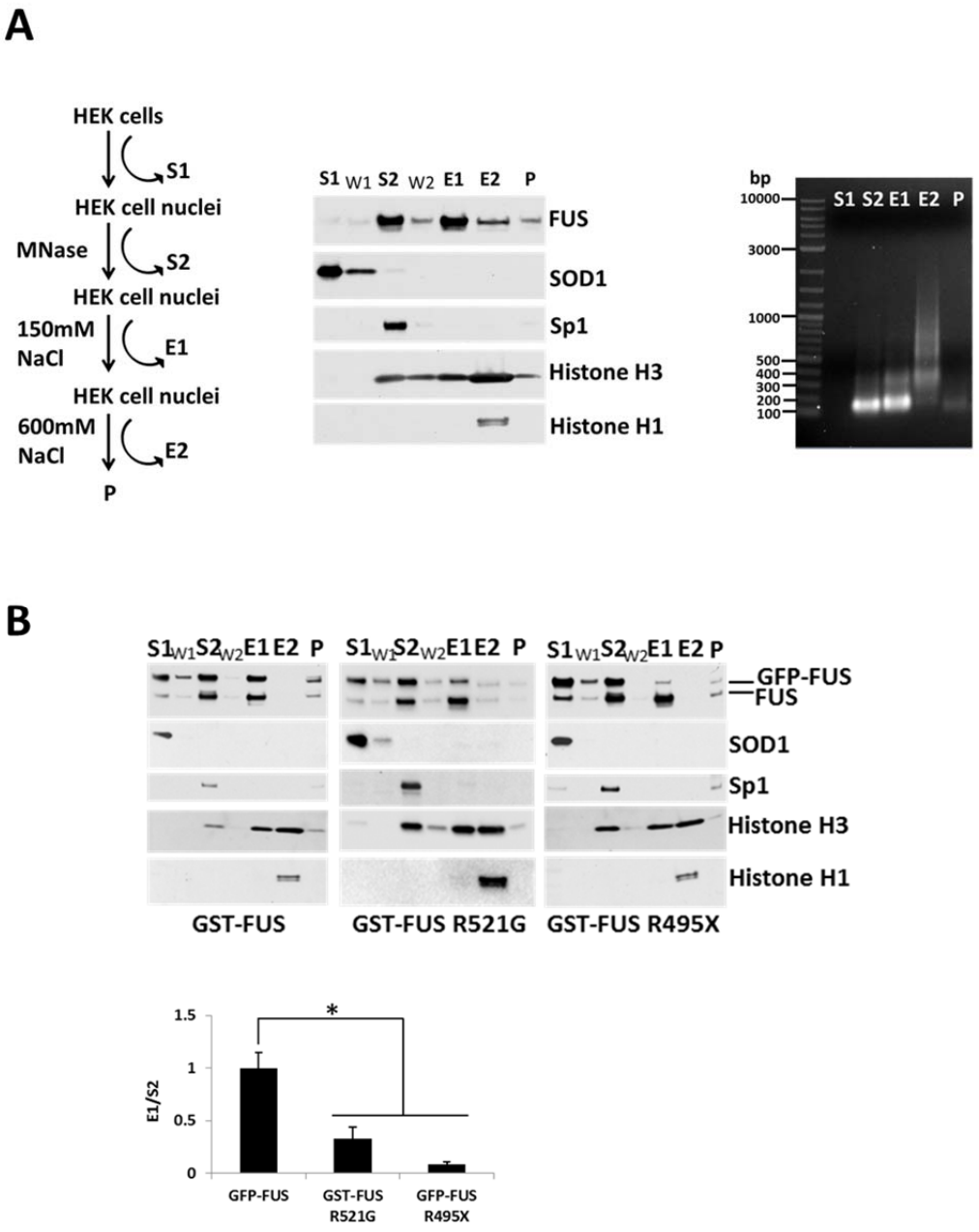


Figure 3.4 QGSY-rich region is responsible for FUS chromatin binding.

(A) Diagram of GST-tagged full-length (FL) FUS and truncated mutants used in the study. (B) The QGSY-rich region is required for FUS chromatin binding. HEK cells expressing FUS FL and truncated mutants were subjected to the chromatin-bound protein separation protocol by SDS and benzonase extraction as shown in Figure 3.1A. The chromatin-bound and soluble fractions were subjected to SDS/PAGE and Western blot with indicated antibodies.

Figure 3.4 QGSY-rich region is responsible for FUS chromatin binding

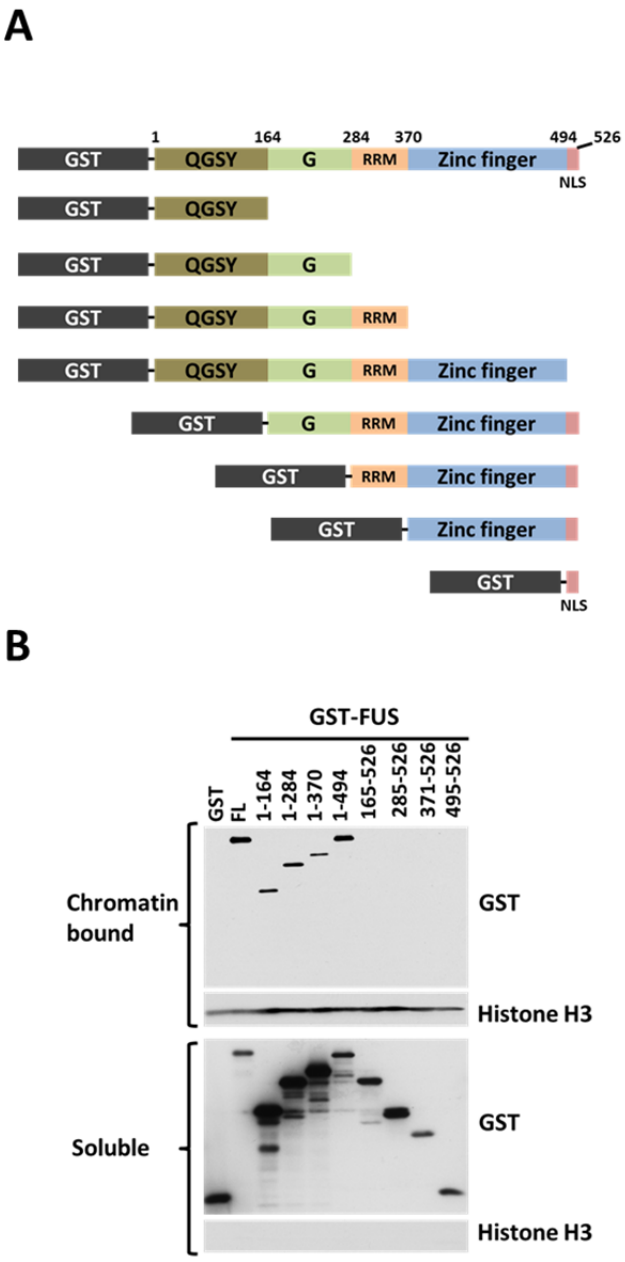


Figure 3.5 QGSY-rich region is required for FUS intranuclear distribution.

(A) The intranuclear distribution of EGFP-FUS. Green, EGFP-tagged FUS; red, Nop56; blue, DAPI staining of DNA; arrows, nucleoli. Confocal microscopic analysis showed a granular punctate distribution of FUS inside the nucleus. Moreover, FUS was not colocalized with the nucleolar marker Nop56, suggesting that FUS is excluded from nucleoli under physiological conditions. The confocal images were acquired using a Nikon A1 confocal microscope as described in Chapter 2. (B) QGSY-rich region deletion abolished FUS characteristic distribution pattern. Green, EGFP-tagged FUS; blue, DAPI staining of DNA; arrows, nucleoli.

Figure 3.5 QGSY-rich region is required for FUS intranuclear distribution

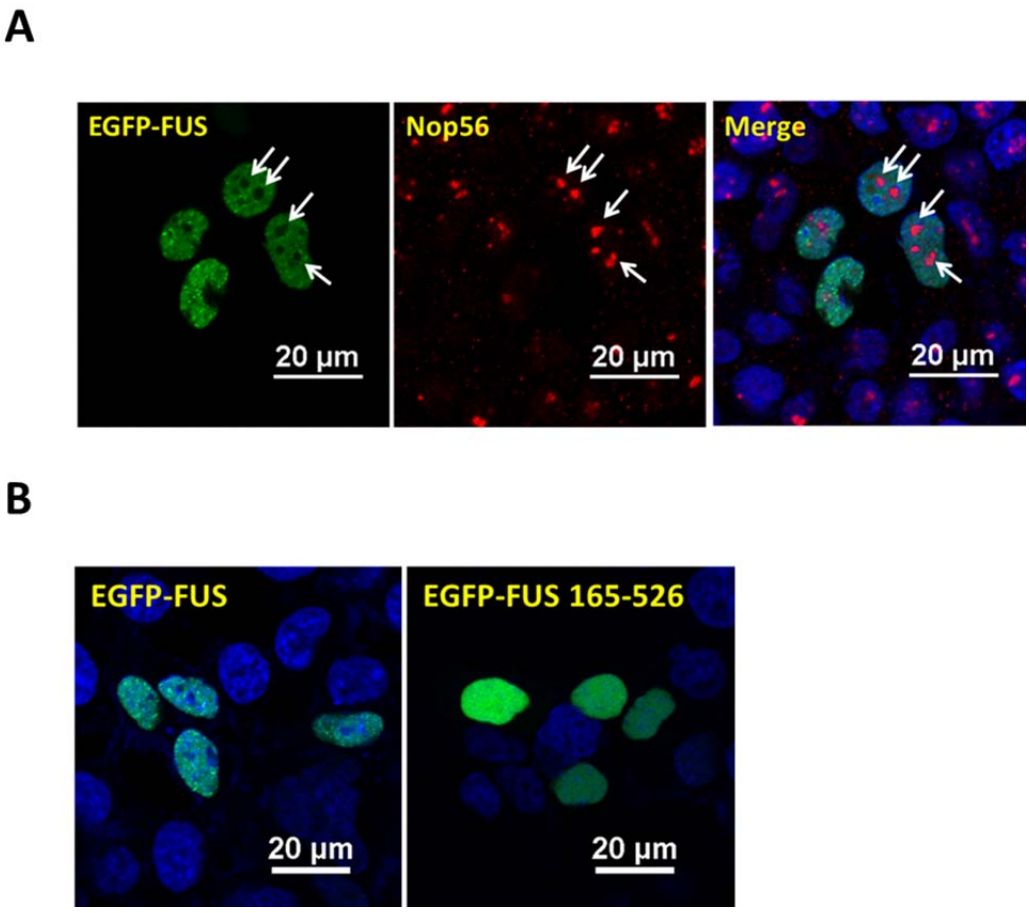


Figure 3.6 Chromatin binding is required for FUS transcription regulation.

(A) The transcription activation by FUS was monitored by a dual luciferase reporter assay as described in Chapter 2. Diagram shows Firefly luciferase gene is driven by the MnSOD promoter and Renilla luciferase gene is driven by the HSV TK promoter as a control. Bar graph shows the ratio of Firefly and Renilla luciferase activities in the presence of full-length FUS or FUS 165–526 lacking the QGSYrich region. The results from three independent experiments are presented. NT, only reporter plasmid transfected. *P < 0.01. (B) The transcription activation of endogenous SMYD3 gene by FUS as measured by real-time PCR. The results from three independent experiments are presented. *P < 0.05. (C) Western blot of lysates from HEK cells transfected with 0.5 μg pEGFP-C3-FUS as described in Chapter 2. Cell lysates were collected 48 h after transfection and the membrane was blotted with FUS antibody. * indicates endogenous FUS.

Figure 3.6 Chromatin binding is required for FUS transcription regulation

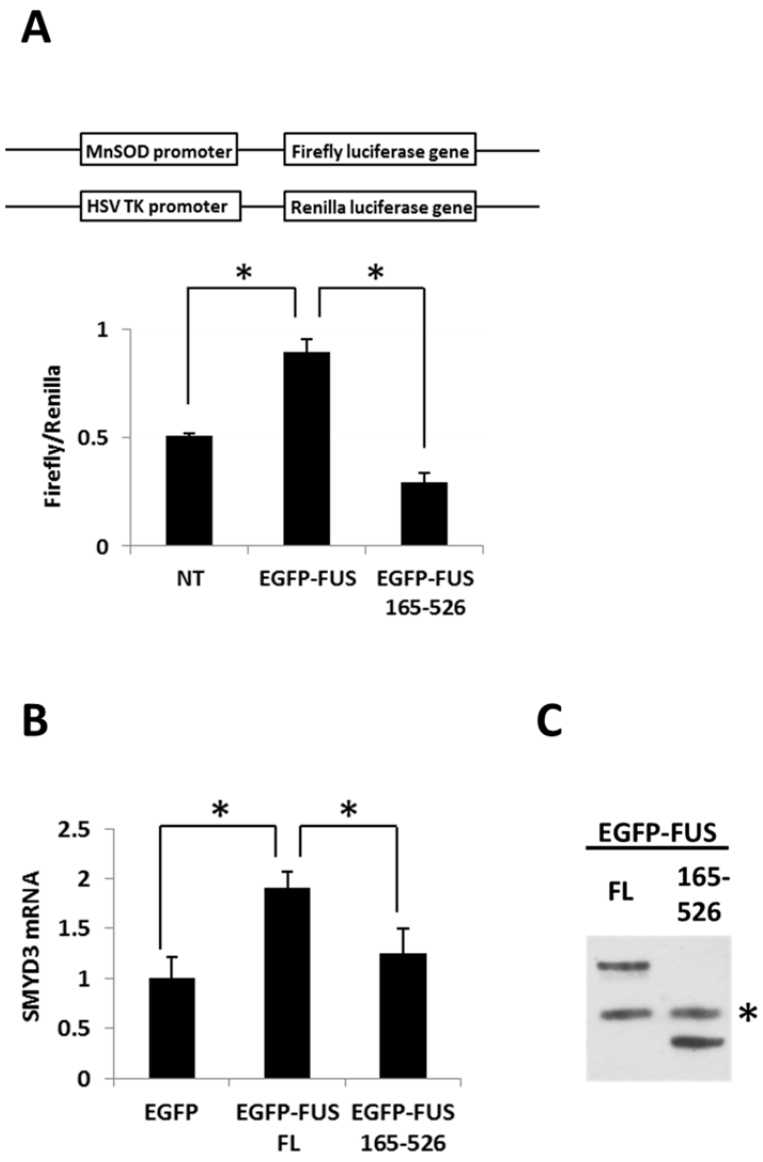


Figure 3.7 Chromatin binding is not required for FUS splicing regulation.

(A) The mRNA splicing regulation by FUS was monitored by minigene splicing assay. (Left) The diagram of alternative splicing of the E1A, insulin receptor, and LMNA Mut minigenes. Dash lines indicate exon inclusion, whereas solid lines indicate exon exclusion in splicing products. (Middle) Images of the ethidium bromide-stained gels show minigene transcript variants in HEK cells expressing full-length FUS or FUS 165–526 lacking the QGSY-rich region. The major exon inclusion and exon exclusion transcripts are indicated, respectively. (Right) The ratio of exon inclusion and exon exclusion transcripts was quantified and results from three independent experiments are presented in the bar graph. *$P < 0.01$. N.S., no significant difference. (B) SMN2 and Tau minigenes splicing are also regulated by FUS. Shown are the representative results from two independent experiments.

Figure 3.7 Chromatin binding is not required for FUS splicing regulation

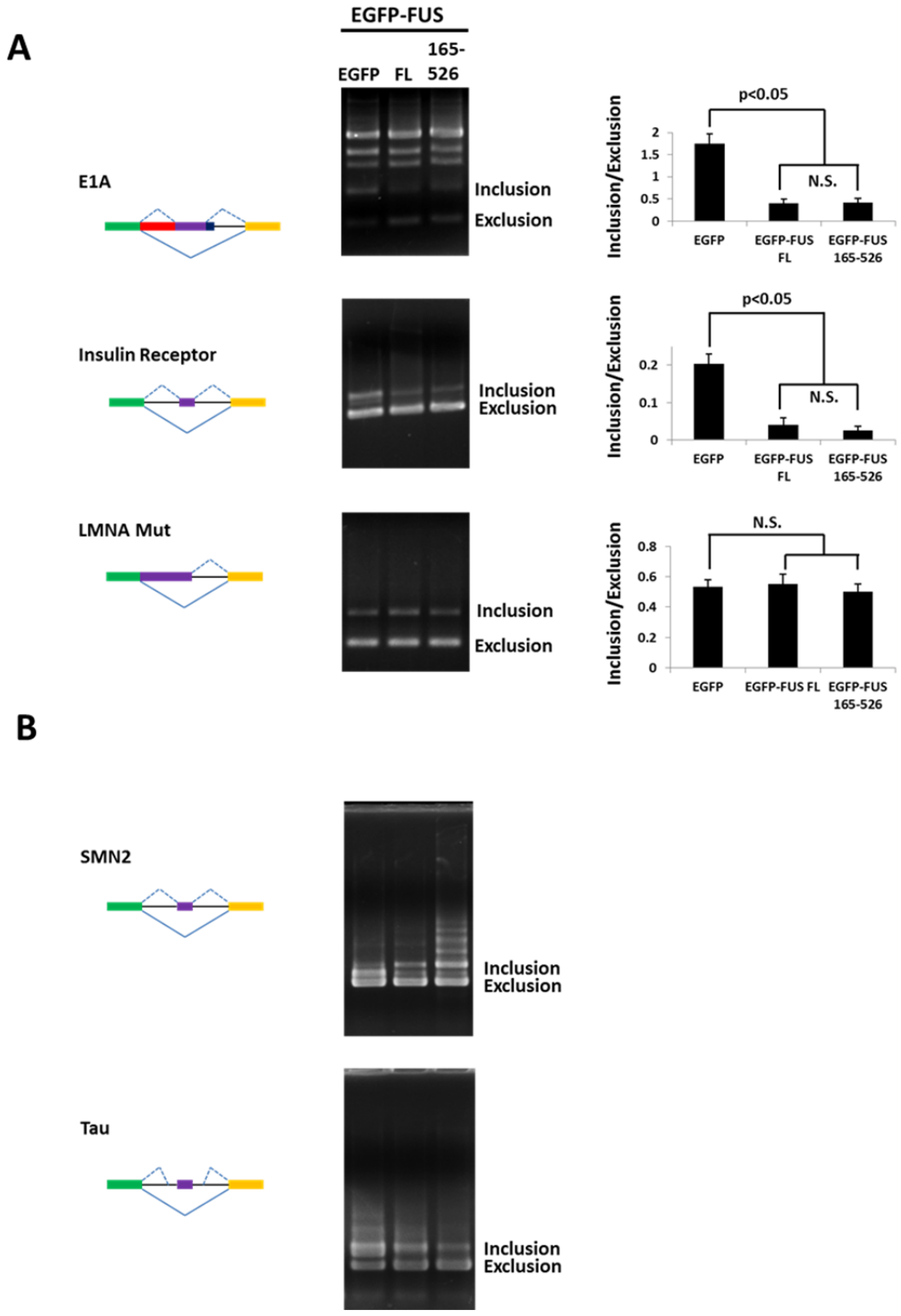


Figure 3.8 Self-interaction of FUS through the QGSY-rich region

Identification of the endogenous FUS in GST-FUS pull-down samples. GST-tagged FUS 1-164, FUS 165-526, and full-length FUS were expressed in HEK cells. GST pull-down was performed and the pull-down samples were subjected to shot-gun proteomics analysis using LC-MS/MS as described in Chapter 2. The endogenous FUS was identified as an interacting protein with GST-FUS 1-164 and GST-FUS full length, but not with GST-FUS 165-526. Four and three unique peptides were identified in the GST pull-down samples of GST-FUS 1-164 and GST-FUS full length, respectively. In the GST-FUS 1-164 pull-down sample, all four peptides are from FUS sequences beyond the N-terminal 1-164 residues, thus the endogenous FUS must be an interacting protein with truncated FUS 1-164. The mass spectrometric experiments were performed by Dr. Jing Chen in our lab.

Figure 3.8 Self-interaction of FUS through the QGSY-rich region

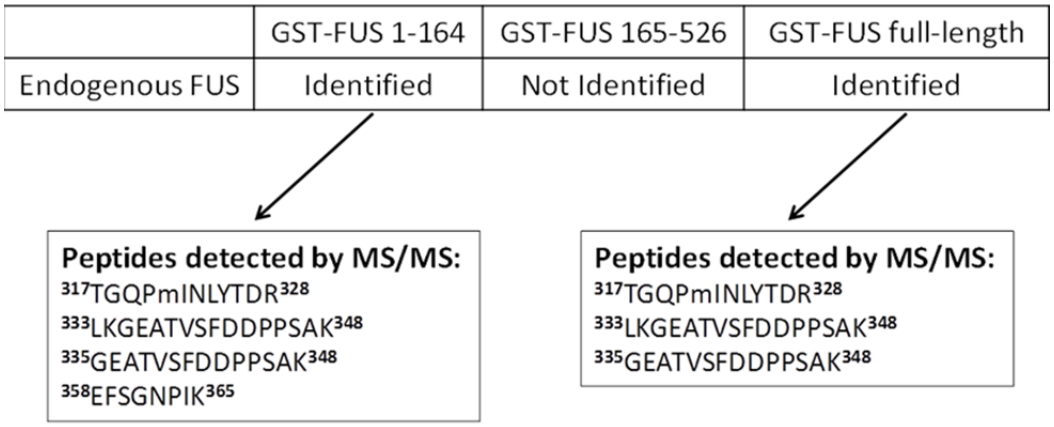


Figure 3.9 Oligomerization restored FUS 165-526 chromatin binding.

(A) Diagram of FUS 165-526 tagged by tetrameric DsRed2 or monomeric DsRed-Monomer. (B) Tetrameric DsRed2 restored the binding of FUS 165-526 to chromatin, whereas monomeric DsRed-tagged FUS 165-526 was not detected in the chromatin-bound fraction. HEK cells were transfected with DsRed2-FUS 165-526 or DsRed-Monomer-FUS 165-526 or the corresponding DsRed control. The chromatin-bound proteins were prepared by SDS and benzonase extraction as shown in Figure 3.1A. The chromatin-bound and soluble fractions were subjected to SDS/PAGE followed by Western blot.

Figure 3.9 Oligomerization restored FUS 165-526 chromatin binding

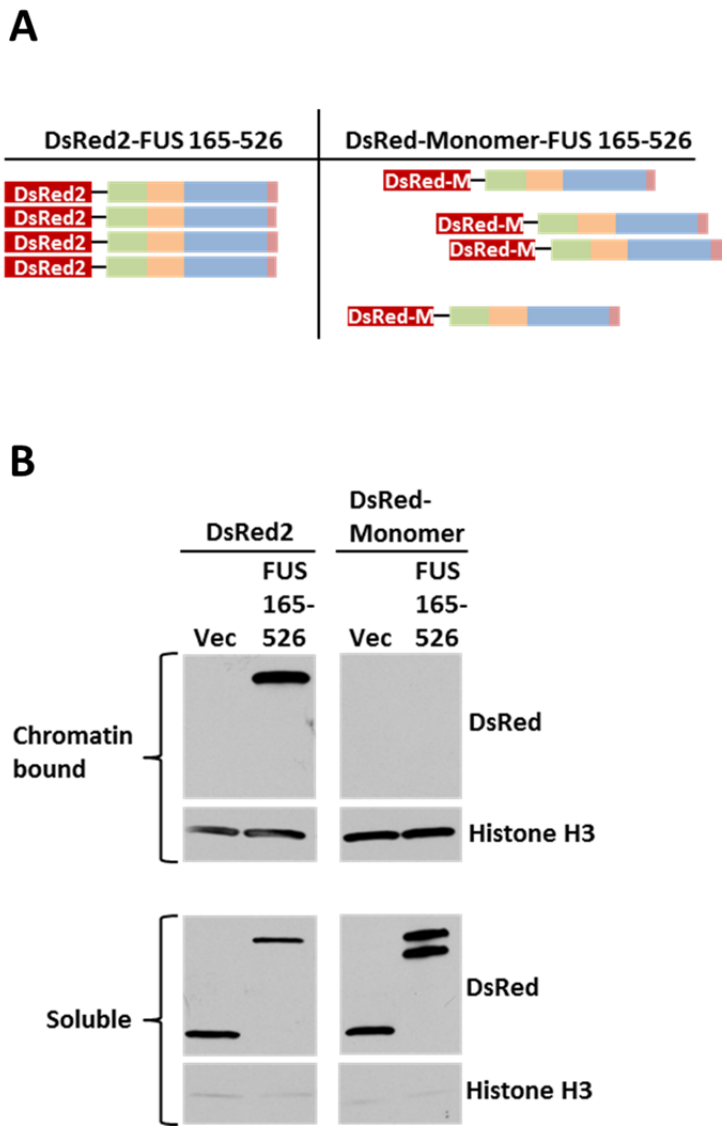


Figure 3.10 Oligomerization restored FUS 165-526 characteristic distribution.

DsRed2-FUS 165-526 showed granular distribution and nucleolar exclusion inside the nucleus, similar to that of the full-length FUS. The monomeric DsRed-tagged FUS 165-526 was evenly distributed in the nucleus. Cells were fixed 24 h after transfection and subjected to confocal microscopic analysis. Red, DsRed and DsRed-tagged FUS; blue, DAPI staining of DNA; arrows, nucleoli.

Figure 3.10 Oligomerization restored FUS 165-526 characteristic distribution

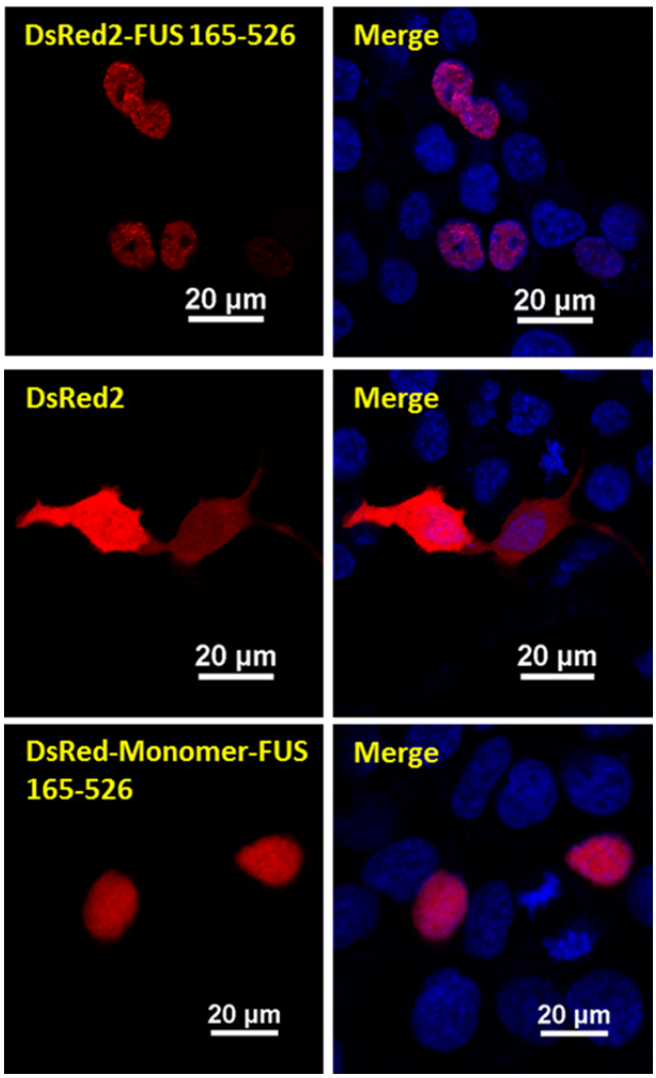


Figure 3.11 Endogenous FUS self-assembly

The chromatin-bound (CB) and soluble (S) fractions were prepared similarly as in Figure 3.1A, except the cells were lysed in detergent-free buffer instead of RIPA buffer and the pellet was resuspended by sonication instead of SDS and benzonase treatment. CB and S fractions were subjected to native gel electrophoresis and Western blot as described in Chapter 2. The slow mobility of FUS suggests a high order assembly of FUS in the CB fraction.

Figure 3.11 Endogenous FUS self-assembly

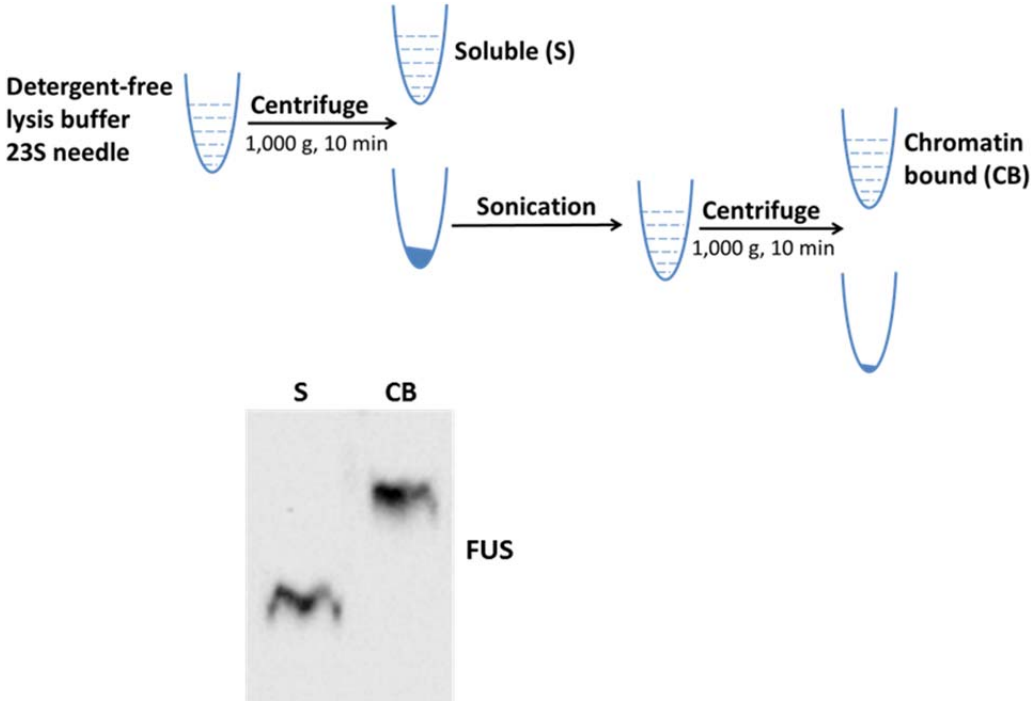


Figure 3.12 FUS chromatin binding is RNA dependent.

HEK cell lysates were incubated with indicated amounts of RNase A for 20 min on ice before separation of the chromatin-bound and soluble fractions using the protocol as in Figure 3.1A. The amount of chromatin-bound FUS in the presence of RNase A was examined by Western blot.

Figure 3.12 FUS chromatin binding is RNA dependent

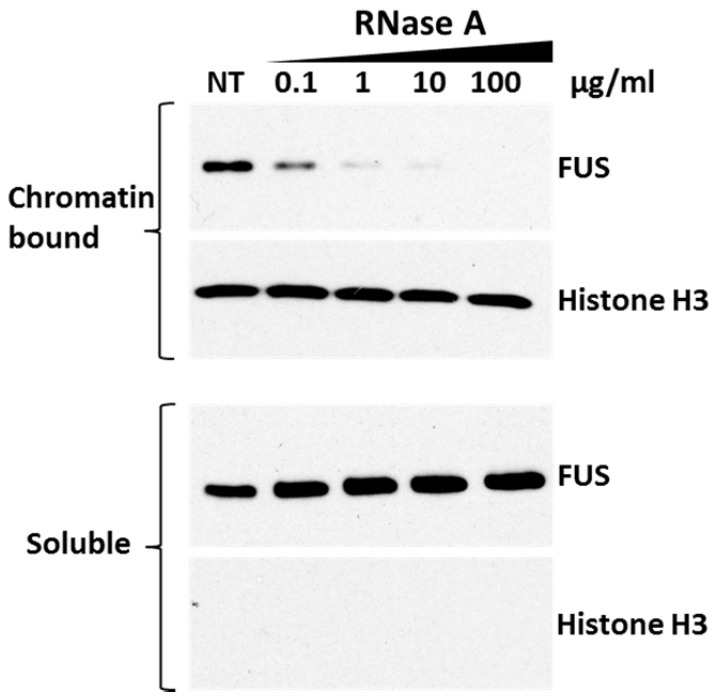


Figure 3.13 DsRed2-FUS 165-526 chromatin binding is independent of RNA.

HEK cells were transfected with DsRed2-FUS 165-526 and harvested 48 h after transfection. Cell lysates were incubated with indicated amounts of RNase A for 20 min on ice and separated to the chromatin-bound and soluble fractions. The amount of chromatin-bound DsRed2-FUS 165-526 in the presence of RNase A was examined by Western blot.

Figure 3.13 DsRed2-FUS 165-526 chromatin binding is independent of RNA

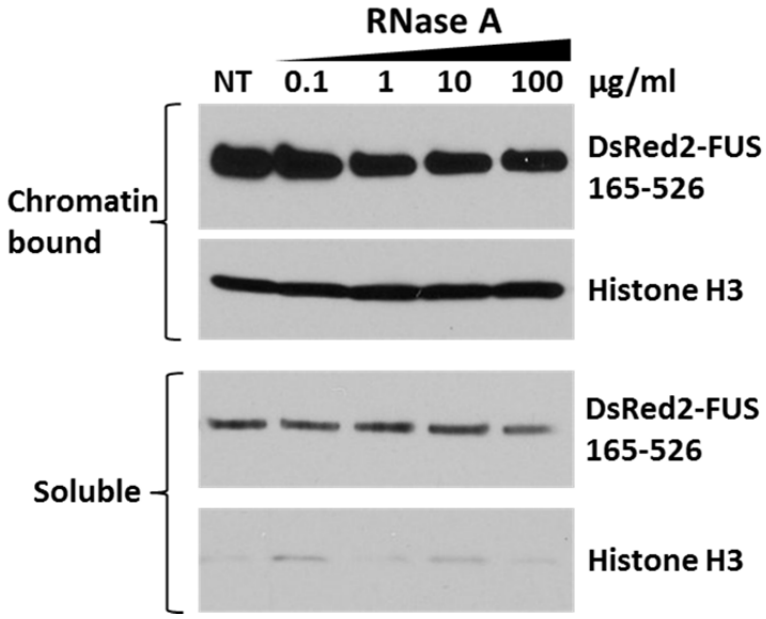


Figure 3.14 RNA is required for FUS self-assembly.

The chromatin-bound fraction as in Figure 3.11 was incubated with indicated amounts of RNase A and subjected to native gel electrophoresis. The soluble fraction was included as a control.

Figure 3.14 RNA is required for FUS self-assembly

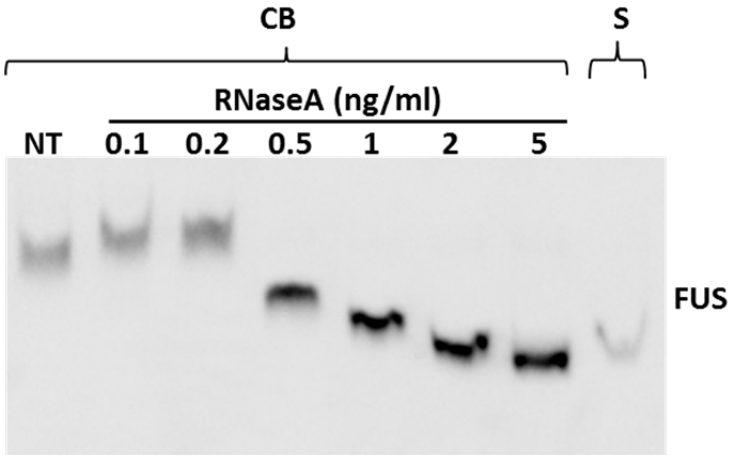
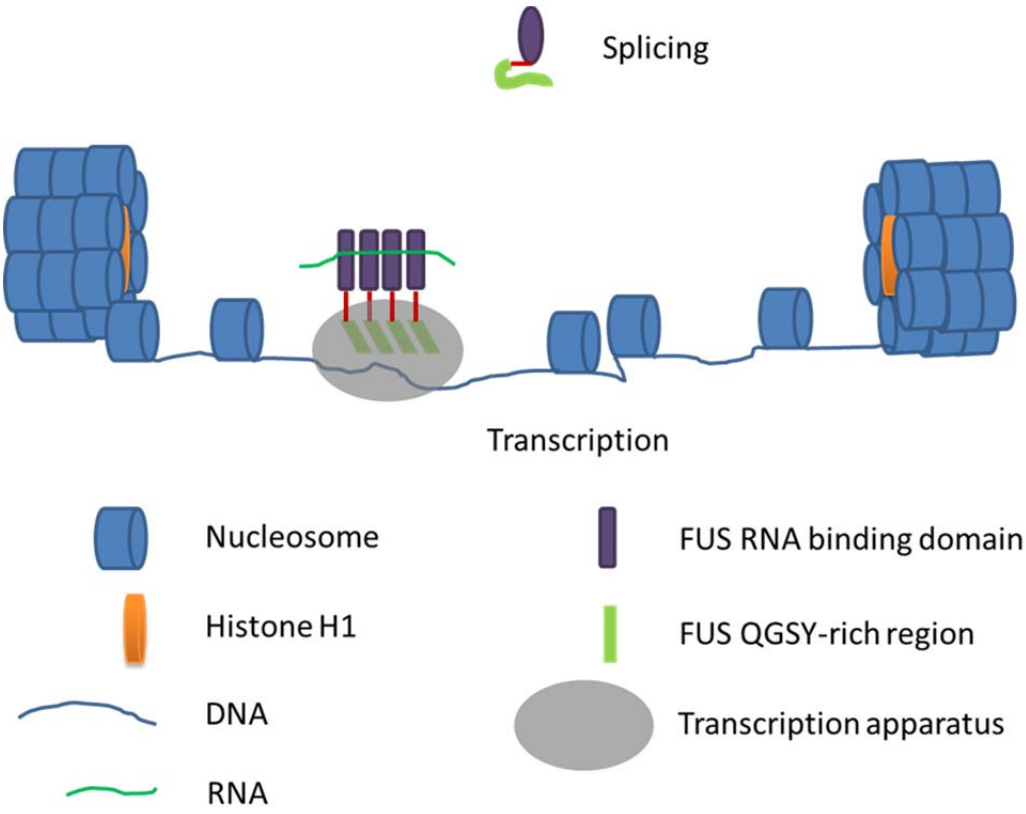


Figure 3.15 Proposed model of FUS self-assembly and chromatin binding.

Wild-type FUS forms high order assemblies and binds to active chromatin where FUS regulates gene transcription. FUS regulation of mRNA splicing does not require self-assembly or chromatin binding, thus is mediated by the pool of soluble FUS.

Figure 3.15 Proposed model for FUS self-assembly and chromatin binding



Chapter 4: Subcellular Localization and RNAs Determine FUS Architecture in Different Cellular Compartments

Introduction

Fused in sarcoma (FUS) is a DNA/RNA binding protein. Mutations in FUS cause a subset of familial amyotrophic lateral sclerosis (ALS) [1, 2]. ALS is a progressive neurodegenerative disease characterized by the loss of motor neurons [131].The ALS mutations result in the mislocalization of FUS from the nucleus to the cytoplasm and the formation of FUS-containing inclusions in the cytoplasm [1, 2]. It is unknown how the mislocalization of mutant FUS causes motor neuron dysfunction and degeneration. Cytoplasmic FUS inclusions are immune-positive of stress granule markers [55, 104], a temporary cellular structure important for cell survival under a variety of stresses [57]. The endogenous FUS has also been reported to participate in stress granule formation under various stresses [132, 133]. Mutant FUS has been shown to alter stress response dynamics, which is hypothesized to cause motor neuron degeneration [134-137]. While proteinaceous inclusions involved in neurodegenerative diseases are often composed of amyloidal-aggregated misfolded proteins, FUS inclusions are structurally different [86, 87]. Specifically, FUS inclusions in the brain of frontal temporal dementia (FTD) patients were negative of thioflavin-S staining that specially stains cross-β-sheet structures in amyloidal aggregates [87]. In addition, SDS-resistant oligomers were not detected in cells expressing various FUS mutants [86]. The architecture of FUS protein in the cytoplasmic inclusions remains to be determined.

We previously reported that a sub-population of nuclear FUS is associated with active chromatin and participates in gene transcription regulation [110, 114, 138]. The ALS mutations reduce FUS chromatin binding, disrupting FUS function in regulating gene transcription. We found that the chromatin-bound FUS was oligomerized and showed a granular pattern inside the nucleus. FUS oligomerization is mediated by the N-terminal QGSY-rich region (glutamine, glycine, serine, and tyrosine-rich region) and requires RNA binding [138]. The QGSY-rich region is part of a predicted prion-like domain [96] that is required for FUS aggregation *in vitro* [98]. Therefore we examined whether the QGSY-rich-region mediated oligomerization is also the structural basis of

FUS cytoplasmic inclusions. The results will help us understand the nature of FUS cytoplamic inclusions. In addition, better understanding of the architectures of FUS proteins in the nuclear and cytoplasmic compartments will help determine whether redirecting cytoplasmic FUS mutants into the nucleus would be a potential therapeutic intervention for ALS as proposed in recent studies [107, 108, 139-141]. Moreover, the requirement of RNA binding in FUS binding to chromatin raised the question whether RNAs directly mediate FUS protein organization in the nuclear granules and cytoplasmic inclusions.

In this study, we revealed that, in contrast to nuclear granules of FUS, the formation of FUS cytoplasmic inclusions does not require the QGSY-rich region mediated oligomerization. By examining wild-type and mutant FUS proteins in different cellular compartments, we demonstrated that the subcellular localization plays an important role in determining the FUS protein architecture. We also discovered that chromatin-associated RNAs are critical to initiate the oligomerization of nuclear FUS. Moreover, the RNA binding ability was also required for FUS cytoplasmic inclusion formation. These results collectively suggest that subcellular localization and local RNA species are the determining factors for FUS distinct architectures and distribution patterns in different cellular compartments.

Results

FUS proteins are organized differently in nuclear granules and cytoplasmic inclusions.

We have recently reported that the QGSY-rich region mediated FUS oligomerization is essential for the formation of nuclear granules of FUS [138]. When the QGSY-rich region (amino acids 1-164) was deleted, the granular distribution pattern disappeared and FUS molecules diffused in the entire nucleus (Figure 4.1A). We examined whether the QGSY-rich region mediated oligomerization was also the structural basis of cytoplasmic inclusions of mutant FUS. FUS R495X (arginine 495 to stop codon, a deletion mutant causing aggressive ALS [142, 143]) formed cytoplasmic inclusions that were co-localized with the stress granule maker G3BP1 (Figure 4.1B). We

deleted the QGSY-rich region from FUS R495X and found that the truncation protein still formed cytoplasmic inclusions co-localized with G3BP1 (Figure 4.1B). The results suggest that the QGSY-rich region is not required for the inclusion formation of ALS mutant FUS in the cytoplasm. The above results provide the initial evidence that FUS proteins in the nuclear granules and cytoplasmic inclusions are organized differently.

Subcellular compartment determines distinct local distribution patterns of FUS.

The above results also raised an interesting question: what causes different local distribution patterns of FUS protein in different cellular compartments? Two factors can potentially contribute to it: subcellular compartment (nucleus versus cytoplasm) and protein mutation (wild-type versus ALS mutations). We rationalized that simultaneous examination of the local distribution pattern of nuclear and cytoplasmic subpopulations of mutant FUS protein can help delineating which factor plays a more important role. We took advantage of ALS point mutations (e.g. R521G) that cause partial retention of FUS protein in the cytoplasm while maintaining a significant portion in the nucleus. FUS R521G exhibited granular distribution and was excluded from nucleoli (arrows in Figure 4.2A), which is very similar to wild-type FUS in the nucleus (compare to Figure 4.1A). This pattern was also observed in the fibroblast cells derived from familial ALS patient carrying the R521G mutant FUS (Figure 4.2B). The cytoplasmic retained FUS R521G mutant formed inclusions (Figure 4.2A, left image, arrow heads). We deleted the QGSY-rich region of FUS R521G and examined how it changed the distribution of FUS protein in the nucleus and cytoplasm. The granular pattern of FUS R521G in the nucleus disappeared and FUS R521G was evenly distributed in the entire nucleus after the QGSY-rich region was deleted (Figure 4.2A, right). The changes of FUS R521G in the nucleus were similar to the effect of QGSY-rich region deletion on wild-type FUS (Figure 4.1A, bottom row). In contrast, cytoplasmic inclusions of FUS R521G were still observed upon the deletion of the QGSY-rich region (Figure 4.2A, right, arrow heads), which is similar to the effect of QGSY-rich region deletion on FUS R495X (Figure 4.1B, bottom row). These observations suggest that the subcellular compartment (i.e. nuclear or cytoplasmic) plays an important role in determining the local distribution pattern of FUS.

To further test this notion, we generated two chimeric proteins: FUS R495X fused with a nuclear localization sequence (NLS) from hnRNPD and wild-type FUS tagged with a typical nuclear export sequence (NES) (Figure 4.3A). The chimeric protein R495X + hnRNPD NLS was largely localized inside the nucleus and showed a punctate pattern with nucleolar exclusion (Figure 4.3B), resembling wild-type FUS (compare to Figure 4.1A). Tagging the NES to wild-type FUS brought the protein outside the nucleus and the chimeric protein formed inclusions in the cytoplasm (Figure 4.3C), which is similar to R495X (compare to Figure 4.1B). These results solidify the notion that the subcellular compartment is the critical factor determining the local distribution pattern of FUS.

The ALS mutant FUS protein in the nucleus responds to transcription inhibition in a similar fashion to wild-type FUS.

The above results suggest that ALS mutations have little effect on the local distribution pattern of the FUS protein localized in the nucleus. To further test this notion, we treated cells with transcription inhibitor 5,6-dichloro-1-β-D-ribofuranosyl-1H-benzimidazole (DRB) and examined how wild-type and mutant FUS proteins responded to this stress. The wild-type FUS protein accumulated into areas close to nucleoli upon DRB treatment (Figure 4.4A), consistent with previous reports [118, 144]. Similarly, FUS R521G and FUS R495X (Figure 4.4B) mutant proteins also accumulated next to nucleoli upon DRB treatment. The result again suggests that the nuclear localized mutant FUS responds to transcription inhibition similarly to wild-type FUS.

Chromatin-associated mutant FUS oligomerizes similarly to wild-type FUS.

Our recently published results suggest that there are two pools of FUS inside the nucleus (chromatin-bound and nuclear soluble) and the granular distribution is attributed to the chromatin-bound FUS [138]. In this study, we used two biochemical approaches, native gel electrophoresis and chemical crosslinking, to examine the nuclear granules and the cytoplasmic inclusions observed in Figure 1. The chromatin-bound (CB) and non-chromatin-bound (NCB) fractions were prepared from lysates of cells expressing wild-type, R521G, or R495X FUS as previously described [138] (Figure 4.5A). The chromatin-bound FUS represents the portion of FUS producing nuclear granules and the

non-chromatin-bound FUS includes the cytoplasmic FUS that can form inclusions. The chromatin-bound wild-type FUS migrated slower than the non-chromatin-bound wild-type FUS (Figure 4.5B), which we previously interpreted that the chromatin-bound FUS was oligomeric whereas the non-chromatin-bound was largely monomeric [138]. Interestingly, R521G and R495X mutants in both chromatin-bound and non-chromatin-bound fractions migrated similarly to wild-type FUS in the corresponding fraction (Figure 4.5B). The results supported that the mutant FUS proteins behaved similarly to wild-type FUS, i.e. mutant FUS also existed as oligomeric form when bound to chromatin and as monomeric when in cytoplasm. Although mutant FUS showed reduced ability of binding to chromatin [138], the chromatin-bound mutant FUS protein formed oligomers as wild-type FUS. The lack of oligomeric mutant FUS in the cytoplasmic fraction suggest that the cytoplasmic FUS protein does not need to oligomerize in the process of forming inclusions, which is consistent with the earlier results that the QGSY-rich region is not required for cytoplasmic inclusion formation (Figure 4.1B bottom and 4.2A, right).

We performed chemical crosslinking to further examine the oligomeric status of FUS proteins. The chromatin-bound wild-type and mutant FUS proteins all formed oligomeric species as evidenced by the crosslinked signals (Figure 4.5C). In contrast, no crosslinked species were detected for either wild-type or mutant FUS proteins in the non-chromatin-bound fraction. The above results consistently support that, regardless wild-type of ALS mutants, the chromatin-bound FUS protein oligomerizes whereas the cytoplasmic FUS protein does not.

Chromatin content initiates oligomerization of wild-type and mutant FUS.

We next examined what triggers FUS oligomerization. Since the non-chromatin-bound FUS did not oligomerize no matter how long it was incubated in the cell lysate deprived of chromatin content (data not shown), we hypothesized that certain constituents in the chromatin-bound fraction initiated FUS oligomerization. We incubated the chromatin-bound fraction with the non-chromatin-bound fraction and analyzed them with native gel electrophoresis. With increasing amounts of chromatin content in the mixture, the wild-type FUS protein shifted from the monomeric position towards the oligomeric

position (Figure 4.6A). The result demonstrated that the chromatin content was able to initiate oligomerization of non-chromatin-bound FUS. Interestingly, a single band was observed in each sample with a position between the monomeric and oligomeric bands (Figure 4.6A), suggesting that FUS protein reached a new equilibrium during incubation. Similarly, the chromatin content initiated oligomerization of mutant FUS in the non-chromatin-bound fraction as well (Figure 4.6B).

Chromatin-associated RNAs are responsible for initiating FUS oligomerization.

The chromatin fraction contains DNAs, RNAs and proteins, thus we asked what triggers FUS oligomerization. Since FUS can bind nucleic acids, we specifically tested whether DNA or RNA triggered FUS oligomerization. We incubated RNase-free DNase or RNase A along with the chromatin-bound and non-chromatin-bound fractions. The RNase treatment abolished mutant FUS oligomerization whereas the DNase treatment had no effect on mutant FUS oligomerization (Figure 4.7A). The RNase treatment did not change DNA content in the chromatin-bound fraction whereas the DNase treatment digested DNAs (Figure 4.7B). The result suggests that RNA molecules associated with chromatin are responsible for initiating FUS oligomerization. To confirm this notion, we extracted nuclear RNAs with Trizol reagent and incubated them with chromatin-deprived cell extract. The Trizol-extracted nuclear RNAs indeed triggered mutant FUS oligomerization in a dose-dependent manner (Figure 4.7C). As a control, RNAs extracted from the cytoplasmic fraction induced little oligomerization of FUS. The results support the notion that the nuclear RNAs associated with chromatin are responsible for initiating oligomerization of FUS protein.

RNA binding is also required for cytoplasmic FUS inclusions.

We next examined whether RNAs are also involved in inclusion formation of cytoplasmic FUS. The RNA recognition motif (RRM) binds to RNA with low affinity [101] and the flanking RGG1, RGG2, Zinc-finger (ZnF) and RGG3 motif can potentially enhance the RNA binding. We thus generated a series of FUS truncation mutants with different RNA binding motifs (Figure 4.8A). R495X mutant FUS formed large inclusions in most cells (Figure 4.8B, lower right panel). However, after deleting RGG2/ZnF/RGG3

domains, the truncated protein was evenly distributed in the cytoplasm with no inclusions (Figure 4.8B, upper left panel). The ΔZnF/RGG3 truncation containing both RRM and RGG2 domains formed cytoplasmic inclusions but the proportion of inclusion-containing cells is significantly lower than R495X (Figure 4.8B, upper right panel; Figure 4.8C). The ΔRGG3 truncation containing RRM, RGG2 and ZnF domains formed inclusions in more cells than ΔZnF/RGG3 (Figure 4.8B, lower left panel; Figure 4.8C). The quantitative results of the percentage of cells containing cytoplasmic inclusions are shown in Figure 4.8C.

We also examined the RNA binding abilities of the relevant domains using a gel-shift titration assay. Under the conditions tested, the RRM domain alone did not bind to the RNA probe, which is consistent with our previous publication that the binding affinity between the RRM domain and RNA was weak (K_d in hundreds μM range) [101]. With addition of RGG2, ZnF and RGG3 domains, the binding affinity between the truncated FUS and the RNA probe significantly increased as evidenced by the shift of the FUS protein band by the RNA probe (Figure 4.8D). The correlation between the stronger RNA binding affinity and the increased formation of cytoplasmic FUS inclusions suggests that RNA binding is also required for cytoplasmic FUS inclusion formation.

Discussion

The architecture of the FUS protein is different in nuclear granules and cytoplasmic inclusions.

Since the cytoplasmic inclusions of mutant FUS are a hallmark of familial ALS caused by the FUS mutations, it is critical to understand the structural basis of such inclusions. No cross-β-sheet structure, which is typical in amyloidal aggregates, was identified in FUS inclusions [86, 87]. The purified FUS protein formed fibrils *in vitro* and the N-terminal prion-like domain (amino acids 1-266) was required for the fibril formation [98]. Another study also demonstrated that the FUS N-terminal domain itself (amino acids 2-214) formed hydrogel at high concentrations *in vitro* [95]. We also found that the N-terminal QGSY-rich region (amino acids 1-164) was required to form functional oligomers in the nucleus, which can be demonstrated by a granular pattern

under confocal microscope and a slower motility in native gel electrophoresis [138]. In this study, we started testing whether FUS cytoplasmic inclusions are mediated by the N-terminal domain of FUS.

We first deleted the QGSY-rich region from R495X mutant FUS and examined its distribution under confocal microscope. To our surprise, deletion of the QGSY-rich region did not change cytoplasmic inclusions of R495X (Figure 4.1B), suggesting that the N-terminal domain is not required for FUS cytoplasmic inclusion formation. This finding is in contrast to the observation that FUS nuclear granules disappeared after the deletion of the QGSY-rich region (Figure 4.1A). Similar observations were found in cells expressing R521G point mutant FUS that was present in both the nucleus and cytoplasm (Figure 4.2A). Moreover, native gel electrophoresis (Figure 4.5B) and crosslinking (Figure 4.5C) results provided additional evidence that the FUS protein in cytoplasmic inclusions mostly existed as the monomeric form. This is in contrast to mutant SOD1 inclusions where oligomeric SOD1 was readily found [145, 146]. Our results suggest that the cytoplasmic FUS inclusions are not disordered-region-mediated fibrillar aggregates (Figure 4.9). It is conceivable that FUS inclusions can be amorphous aggregates that can either become amyloidal aggregation through a common intermediate [147] or be disaggregated by heat shock proteins [148].

Subcellular compartment determines FUS local distribution pattern and behavior.

We asked the question whether subcellular compartment or ALS mutation is more important to determine FUS protein architecture and local distribution pattern. By forcing mutant FUS into the nucleus (Figure 4.3B) and wild-type FUS out of the nucleus (Figure 4.3C), we found that swapping subcellular compartments completely changed the local distribution patterns of FUS protein, regardless wild-type or mutant. The results suggest that the subcellular localization plays a critical determining the architecture and distribution of FUS in different cell compartments. The ALS-causing mutations clearly change the subcellular localization of FUS, but mutations appear to have less influence on the local distribution pattern of FUS protein inside the specific compartment. An additional implication is that the ALS mutations within the C-terminal NLS have no significant impact on the overall conformation of FUS.

Moreover, we demonstrated that mutant FUS protein localized in the nucleus behaves similarly to the wild-type FUS protein. Specifically, the nucleus-localized mutant FUS protein formed granules (Figure 4.2 and 4.3), oligomerized (Figure 4.5) and responded to transcription inhibition (Figure 4.4) in a similar fashion to the wild-type FUS. The results support the critical significance to restore the nuclear localization of mutant FUS that accumulates in cytoplasm aberrantly. Several studies have aimed to redirect mutant FUS into the nucleus as a potential therapeutic intervention [107, 108, 139-141] and this intervention showed beneficial effects in model organisms [108, 139, 141]. Additional work is needed to test whether this approach can be an effective therapy in mammalian models.

Local RNAs are critical for FUS protein architecture.

We demonstrated that chromatin-associated RNAs initiated FUS oligomerization (Figures 4.6 and 4.7), which is essential to FUS chromatin binding and the appearance of granular distribution in the nucleus. However, we do not know the identities of RNAs that trigger FUS oligomerization. A previous study using a chimeric protein of the N-terminal prion-like domain of FUS and a DNA binding domain of a transcription factor FLI showed that microsatellite DNA fragments with repetitive sequence significantly enhanced the oligomerization of the prion-like domain of FUS [100]. It is conceivable that the proximal binding of multiple FUS chimeric proteins increased the local concentration of N-terminal FUS and triggered its oligomerization. This observation suggests that nascently transcribed non-coding RNAs, which are still attached on chromatin, from microsatellite sites may trigger the oligomerization of full-length FUS in the nucleus. Indeed, chromatin-associated non-coding RNA was reported to recruit FUS to chromatin to regulate gene transcription [130]. A separate genome-wide study identified a substantial portion of FUS binding RNAs as long non-coding RNAs [64]. However the specific nuclear RNAs initiating oligomerization of FUS in the nucleus remain to be experimentally determined.

Interestingly, we also found that the formation of FUS inclusions in the cytoplasm also required RNA binding capability (Figure 4.8). FUS has been reported to play a role in the formation of stress granules [55, 104, 132, 133], a dynamic cytoplasmic structure

containing proteins and messenger RNAs when translation halts in response to a variety of stresses. Different from the dynamic stress granules that are normally temporary under stresses, the cytoplasmic inclusions of FUS were observed persistently in this study. Thus, the RNAs sequestered in the persistent inclusions could result in a loss of function of the RNAs and the corresponding proteins. Knowing their identities can help us determine what cellular processes might be impaired by mutant FUS.

Furthermore, we speculate that the RNAs required for nuclear FUS oligomerization and cytoplasmic FUS inclusions are likely two separate cohorts, although a partial overlap of these two pools is conceivable. In summary, the subcellular localization and the local pool of RNAs play a critical role in determining the special architecture of FUS protein (Figure 4.9). Such protein architecture may be crucial in distinguishing RNA binding protein pathogenic mechanism from non-RNA-binding protein misfolding/aggregation mechanism in ALS. Moreover, the findings from this study support the feasibility of restoring the nuclear localization of mutant FUS as a potential therapeutic strategy of ALS.

Figure 4.1 FUS proteins are organized differently in nuclear granules and cytoplasmic inclusions.

(A) Full-length wild-type FUS was expressed in HEK cells and formed nuclear granules and was excluded from nucleoli in the nucleus (the top row). These two features were lost in the truncated FUS ΔQGSY that lacks the QGSY-rich region (the bottom row). Nop56 (red) is a marker for nucleoli. (B) The R495X mutant FUS formed cytoplasmic inclusions that are co-localized with the stress granule marker G3BP1 (the top row). Deleting the QGSY-rich region from R495X did not change the formation of G3BP1-positive cytoplasmic inclusions (the bottom row). All images were taken with a Nikon A1 confocal microscope. Green: EGFP-tagged wild-type or mutant FUS. Blue: DAPI staining of DNA.

Figure 4.1 FUS proteins are organized differently in nuclear granules and cytoplasmic inclusions

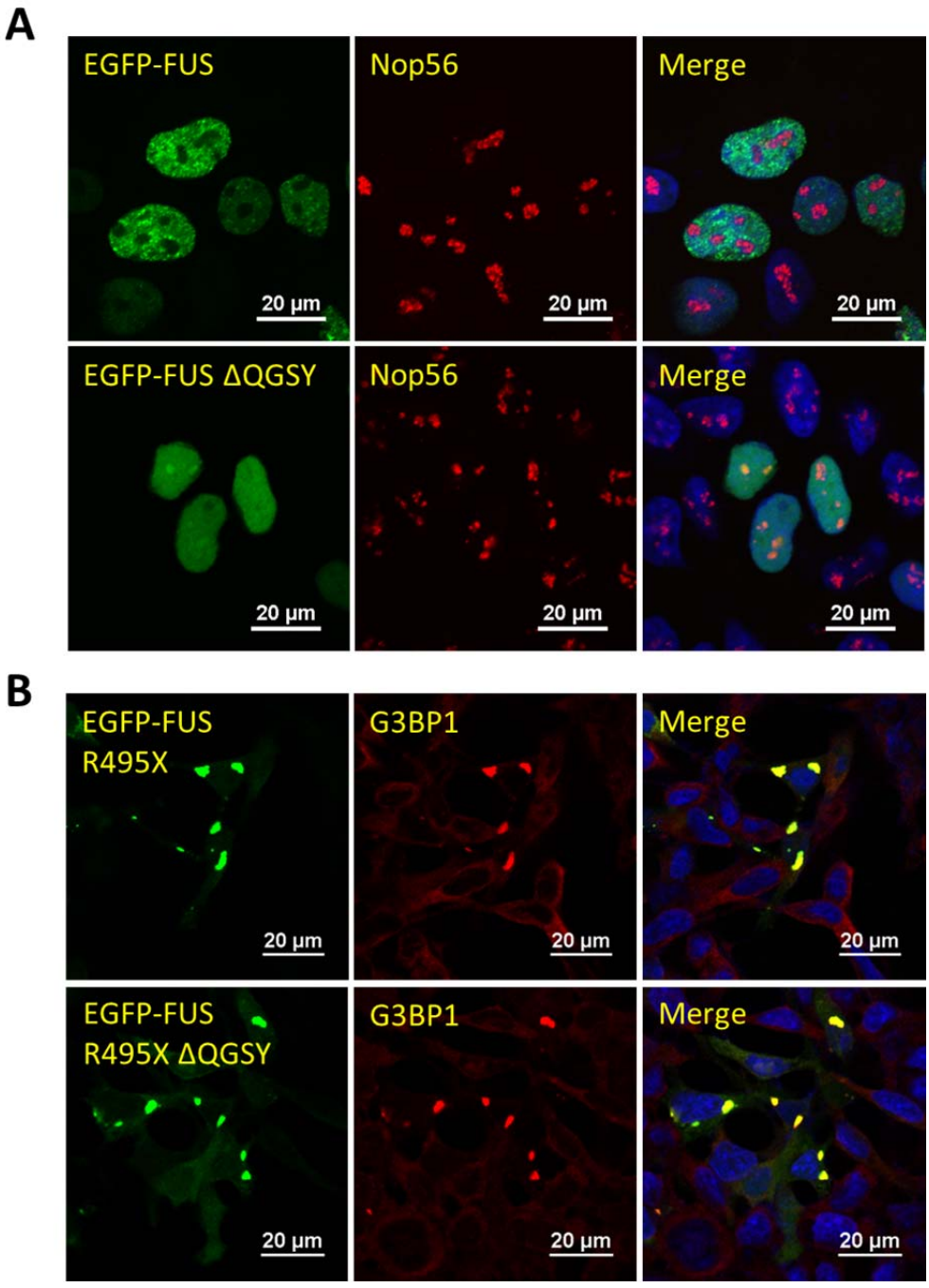


Figure 4.2 Nucleus-localization plays important role in determining FUS granular distribution.

(A) R521G mutant FUS was expressed in HEK cells and formed both nuclear granules excluded from nucleoli and cytoplasmic inclusions (the left panel). Arrows: nucleoli. Arrow heads: cytoplasmic EGFP-FUS R521G inclusions. Deleting the QGSY-rich region did not change the cytoplasmic inclusions, but changed the distribution of R521G FUS inside the nucleus (the right panel). The granular pattern disappeared and FUS was evenly distributed in the entire nucleus. Green: EGFP-FUS R521G. Blue: DAPI staining of DNA. (B). Immunostaining of FUS in fibroblast cells derived from a familial ALS patient carrying the R521G FUS mutation. The nuclear population of FUS showed granular pattern and was excluded from nucleoli. Green: FUS; red: Nop56; and blue: DAPI staining of DNA. All images were taken with a Nikon A1 confocal microscope.

Figure 4.2 Nucleus-localization plays important role in determining FUS granular distribution

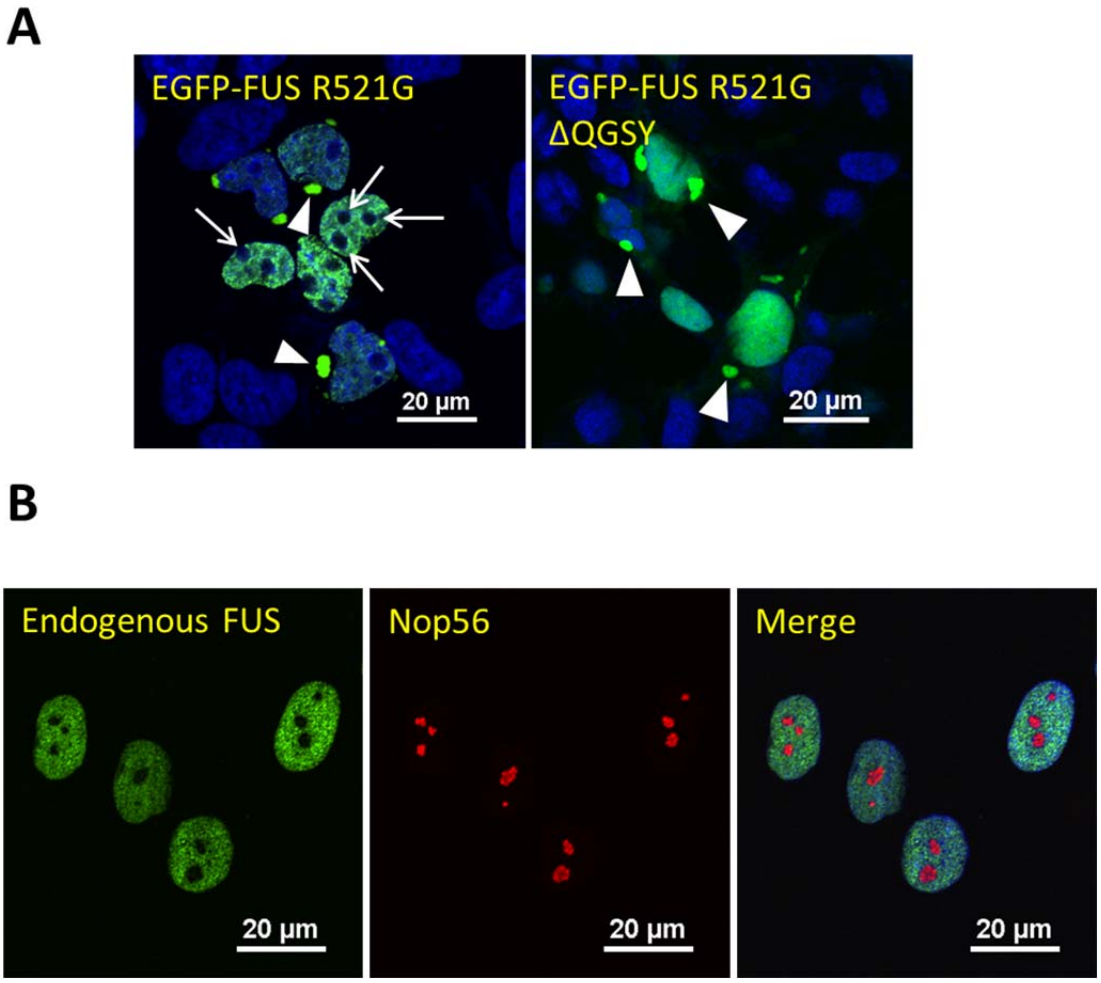


Figure 4.3 Subcellular localization plays fundamental role in determining FUS different organization.

(A). Domain structure of FUS and various FUS constructs used in this study. (B) FUS R495X formed inclusions in the cytoplasm (the left panel). Tagging FUS R495X with a nuclear localization sequence (NLS) targeted the chimeric protein in the nucleus (the right panel). The granular pattern of the chimeric protein in the nucleus was similar to wild-type FUS. (C) Tagging the wild-type FUS (the left panel) with a nuclear export sequence (NES) caused the cytoplasmic localization of the chimeric protein (the right panel). The wild-type FUS targeted to the cytoplasm also formed inclusions in a similar fashion to mutant FUS. All images were taken with a Nikon A1 confocal microscope. Green: EGFP-tagged wild-type or mutant FUS. Blue: DAPI staining of DNA.

Figure 4.3 Subcellular localization plays fundamental role in determining FUS different organization

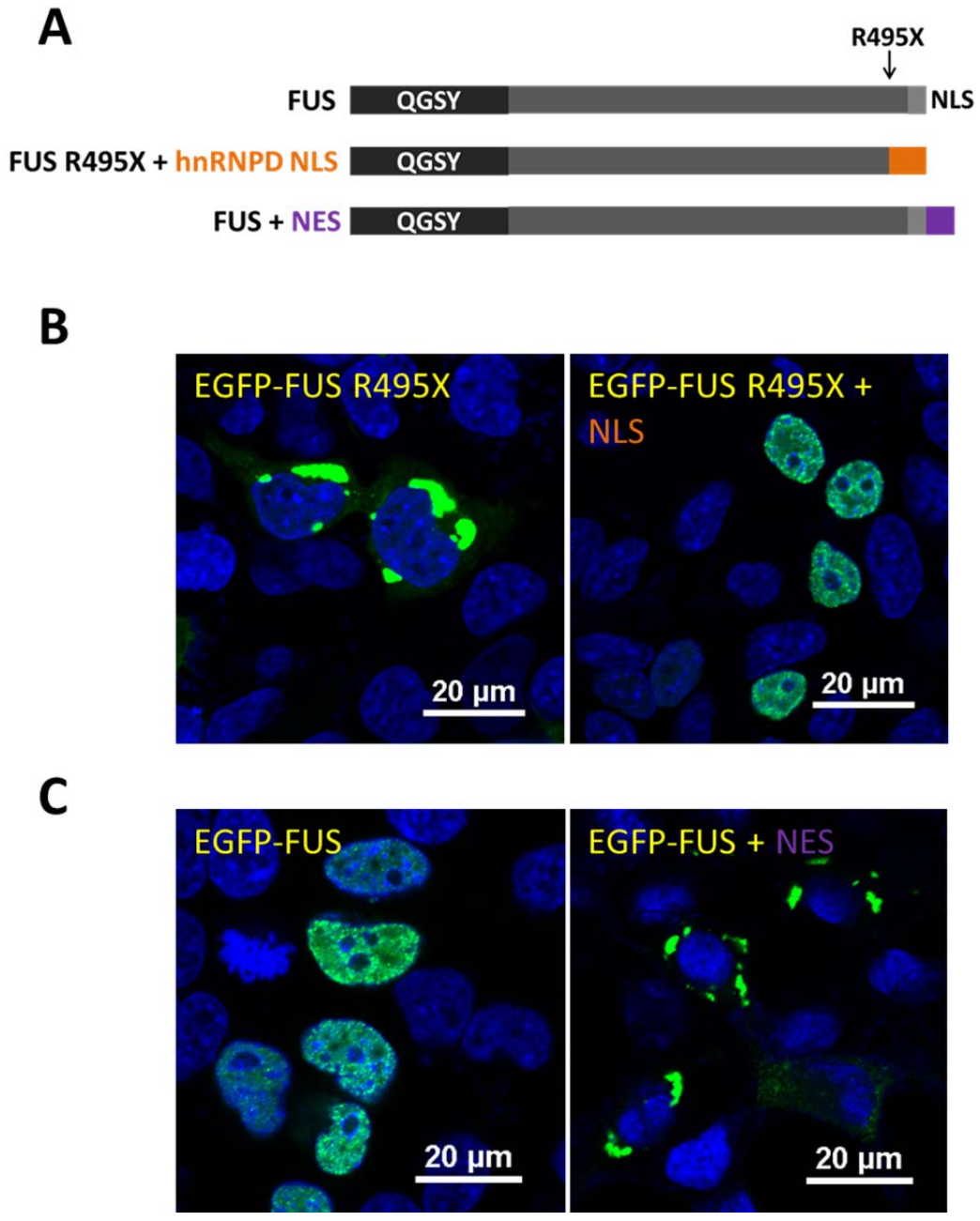


Figure 4.4 FUS mutants responded to transcription inhibition similarly as wild-type FUS.

(A) Wild-type FUS accumulated in areas close to nucleoli in the presence of transcription inhibitor DRB. Cells expressing FUS were treated with 25 μM DRB for 2 h followed by formaldehyde fixation and Nop56 immunostaining. (B) FUS mutants R521G and R495X also accumulated in the peri-nucleolus areas in the presence of DRB. Cells expressing R521G or R495X FUS were treated with 25 μM DRB for 2 h before formaldehyde fixation. All images were taken with a Nikon A1 confocal microscope. Green: EGFP-tagged FUS. Red: Nop56. Blue: DAPI staining of DNA.

Figure 4.4 FUS mutants responded to transcription inhibition similarly as wild-type FUS

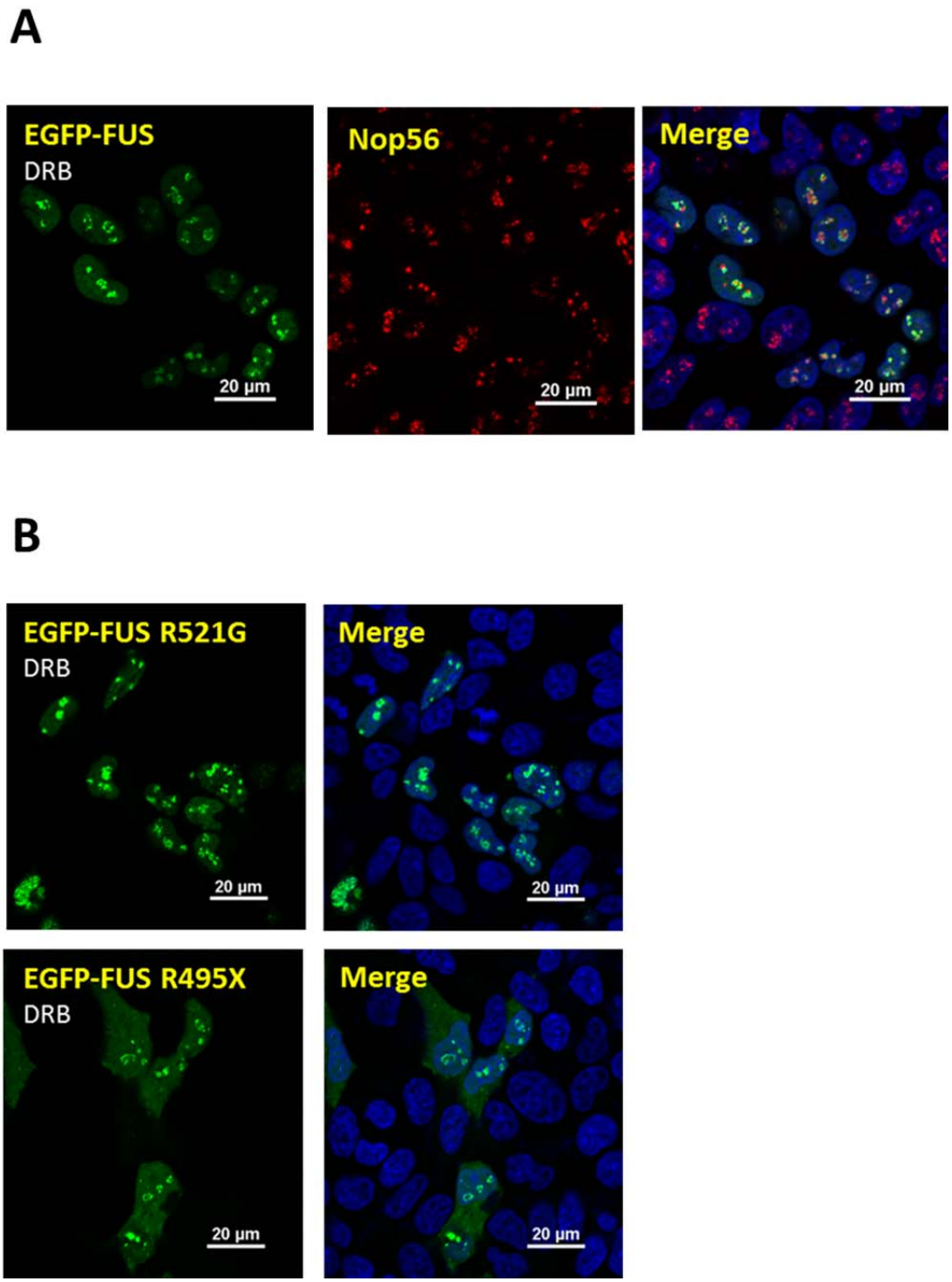


Figure 4.5 FUS mutants oligomerized similarly as wild-type FUS.

(A) Flow chart of preparing chromatin-bound and non-chromatin-bound samples. (B) R521G and R495X mutant FUS proteins migrated similarly as wild-type FUS in native gel in both chromatin-bound (CB) and non-chromatin-bound (NCB) fractions. In the CB fraction, both mutant and wild-type FUS migrated as oligomers. In the NCB fraction, both mutant and wild-type FUS migrated as monomers. (C) The CB and NCB samples were subjected to crosslinking by 1% formaldehyde at room temperature for 10 min. SDS/PAGE analysis showed crosslinked oligomers for both wild-type and mutant FUS in the CB fraction.

Figure 4.5 FUS mutants oligomerized similarly as wild-type FUS

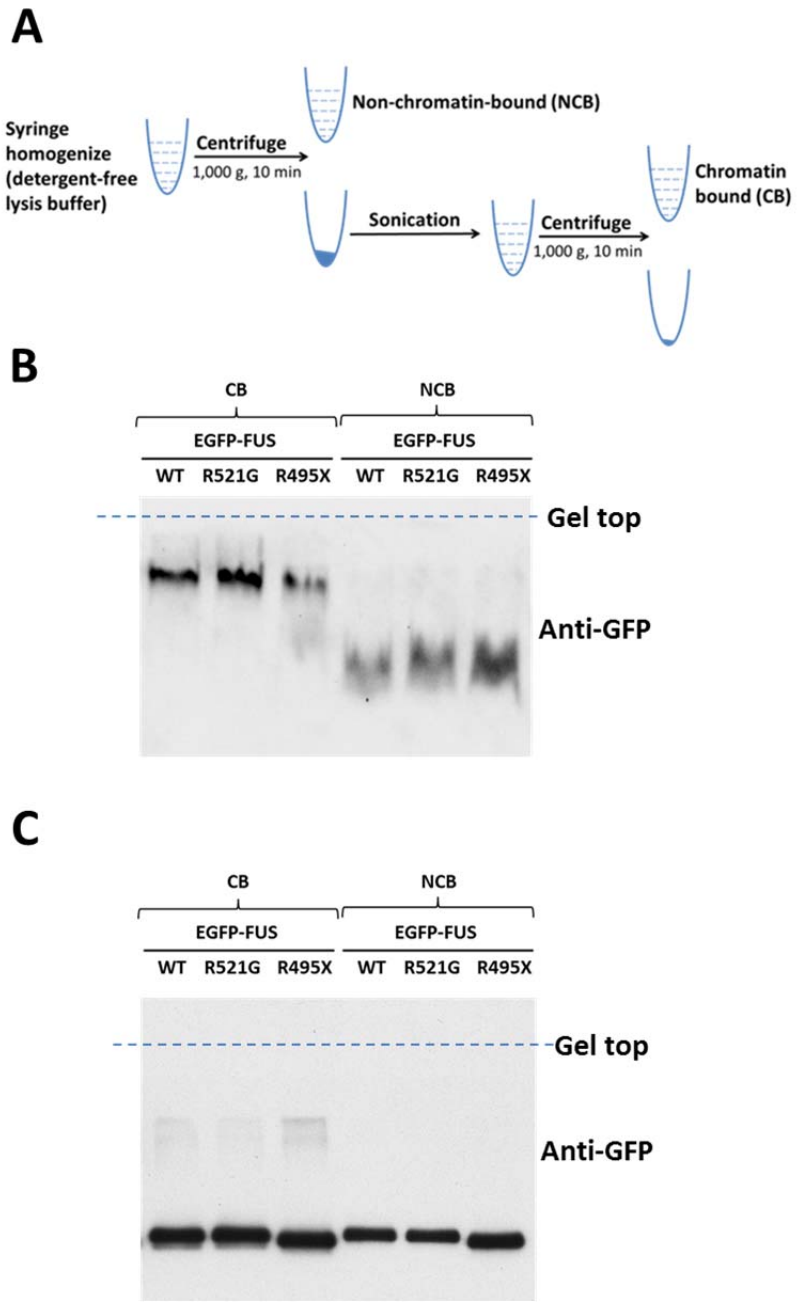


Figure 4.6 The chromatin-bound fraction induced oligomerization of FUS in the non-chromatin-bound fraction.

(A) Incubation of the CB and NCB fractions induced oligomerization of wild-type FUS in the NCB fraction. Different amounts of chromatin-bound samples were incubated with the NCB samples on ice for 20 min. The mixtures were subjected to native gel electrophoresis followed by Western blot with a FUS antibody. The individual CB and NCB fractions were shown as control. The slower mobility of FUS suggested a high order assembly of FUS. (B) The R495X mutant FUS also oligomerized when incubated with the CB fraction.

Figure 4.6 The chromatin-bound fraction induced oligomerization of FUS in the non-chromatin-bound fraction

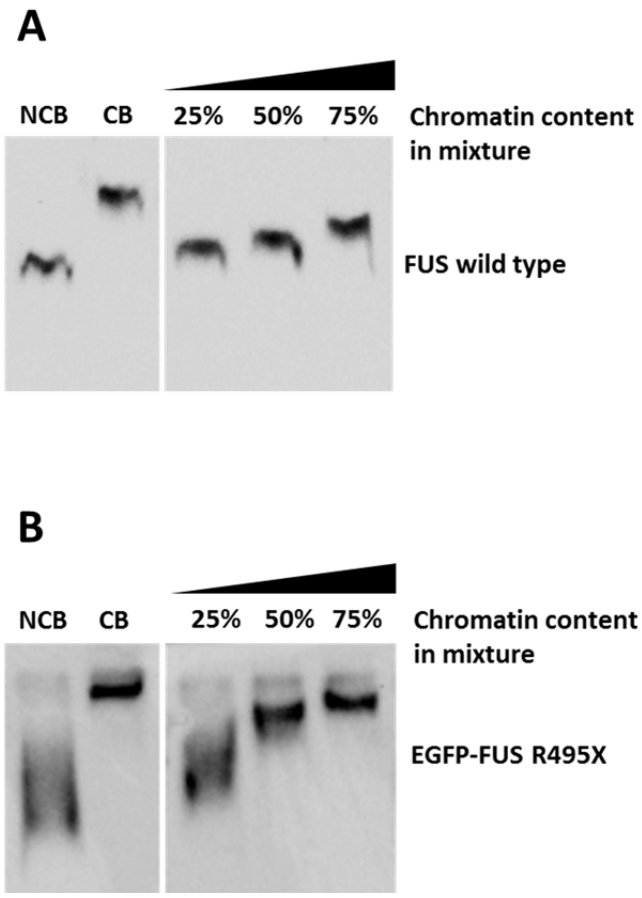


Figure 4.7 Nuclear RNAs triggered FUS oligomerization.

(A) Addition of RNase A in the mixture of CB and NCB fractions inhibited the oligomerization of FUS. In contrast, DNase did not have any effect on FUS oligomerization. 50 U/ml RNase-free DNase or 1 ng/ml RNase A was added into mixture of the NCB sample isolated from cells expressing R495X FUS and the CB fraction (1:1 ratio). After 1 hr incubation at room temperature, the samples were subjected to native gel electrophoresis and Western blot with GFP antibody. (B) Agarose gel electrophoresis followed by ethidium bromide staining was performed to examine the nucleic acid contents in the mixtures from (A). DNA (500-10,000 bp smear) and RNA (visible in the top part of NT lane, > 10,000 bp, and in the full DNase lane) was evidently degraded in the presence of DNase and RNase, respectively. (C) The NCB fraction (50 μl) isolated from cells expressing R495X FUS was incubated with 2 or 20 μg of nuclear RNAs extracted with the Trizol reagent. After incubation at 4 °C overnight, the samples were subjected to native gel electrophoresis and Western blot.

Figure 4.7 Nuclear RNAs triggered FUS oligomerization

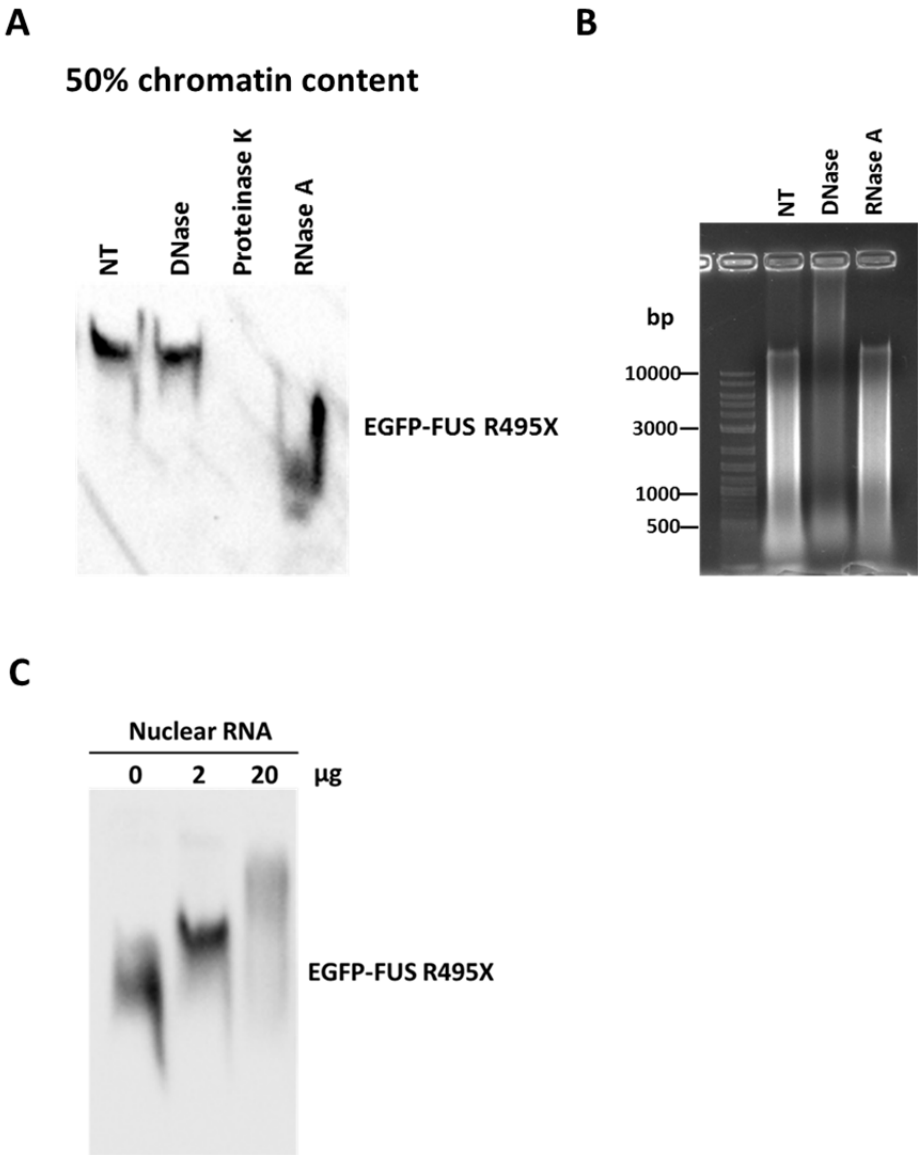


Figure 4.8 RNA binding was also required for the formation of FUS cytoplasmic inclusions.

(A) Diagram of GST tagged R495X FUS and a series of truncations lacking RNA binding motifs. QGSY, the QGSY-rich region; G, the glycine-rich region; RRM, the RNA recognition motif; RGG, arginine-glycine-glycine repeat regions; and ZnF, the zinc finger motif. (B) Inclusion formation of the GST tagged R495X FUS and the RNA binding motif truncation mutants in HEK cells. GST-FUS mutants were immunostained with a GST antibody and the images were acquired with a Nikon A1 confocal microscope. The insets are magnified areas indicated by yellow squares. Green: GST-FUS. Blue: DAPI staining of DNA. (C) The percentages of inclusion-containing cells with different FUS R495X truncation mutants. More than a hundred cells in three random view fields were counted for each different truncation mutant. Data shown represent mean +/- SD and p values were calculated using Student t-test. (D) In vitro RNA binding of FUS truncation mutants with different RNA binding motifs. Indicated concentrations of purified FUS truncation proteins were incubated with 5'-Cy3-labeled RNA probe and the samples were subjected to polyacrylamide gel electrophoresis (see details in Chapter 2). The images were acquired with a ProteinSimple gel imaging system. The gel shift of the RNA probe illustrates the binding of FUS protein to the RNA probe. The work in (D) was done by Chunyan Niu in Dr. Weimin Gong's lab in the Chinese Academy of Science in Beijing, China.

Figure 4.8 RNA binding was also required for FUS cytoplasmic inclusion formation

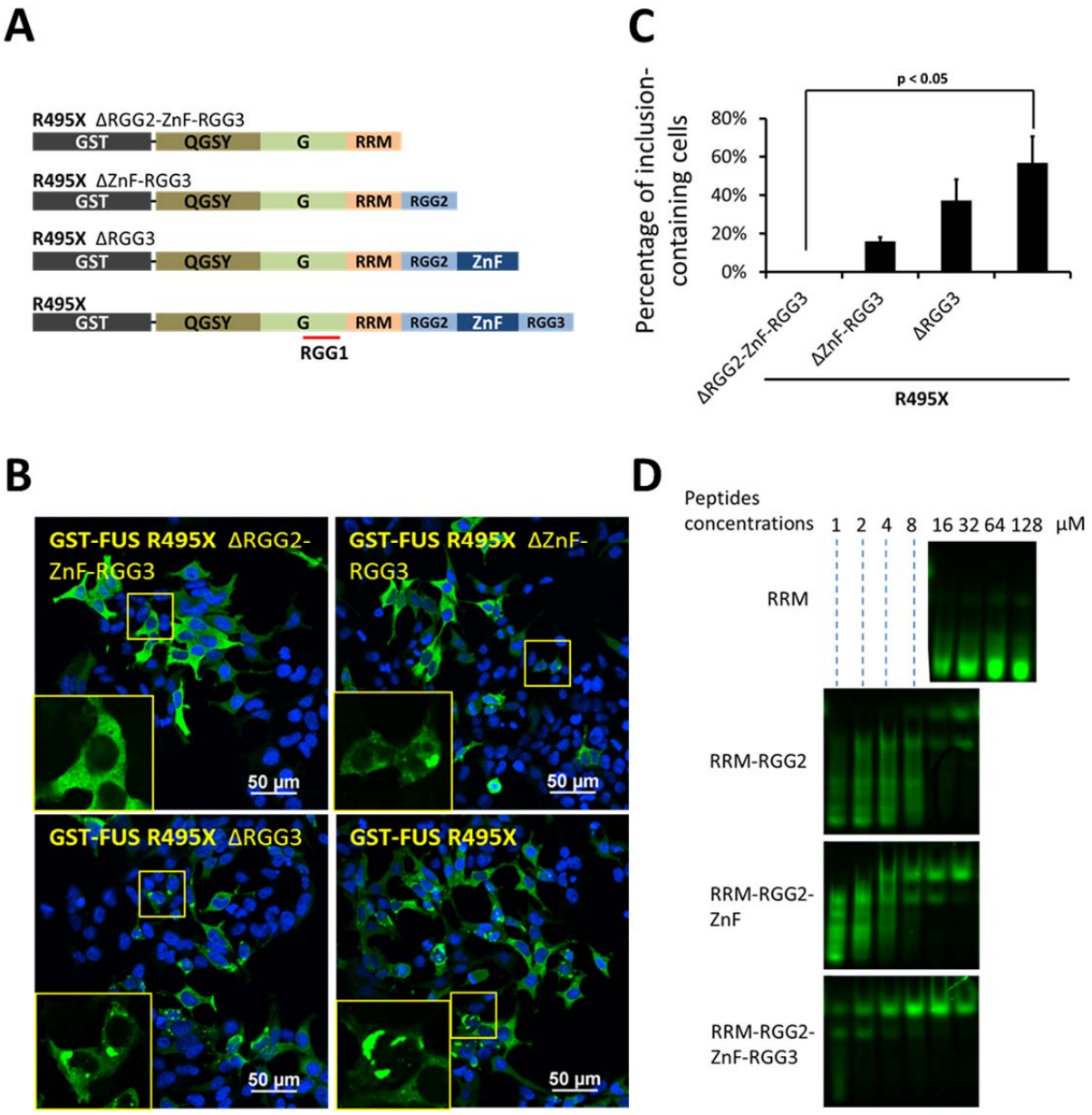
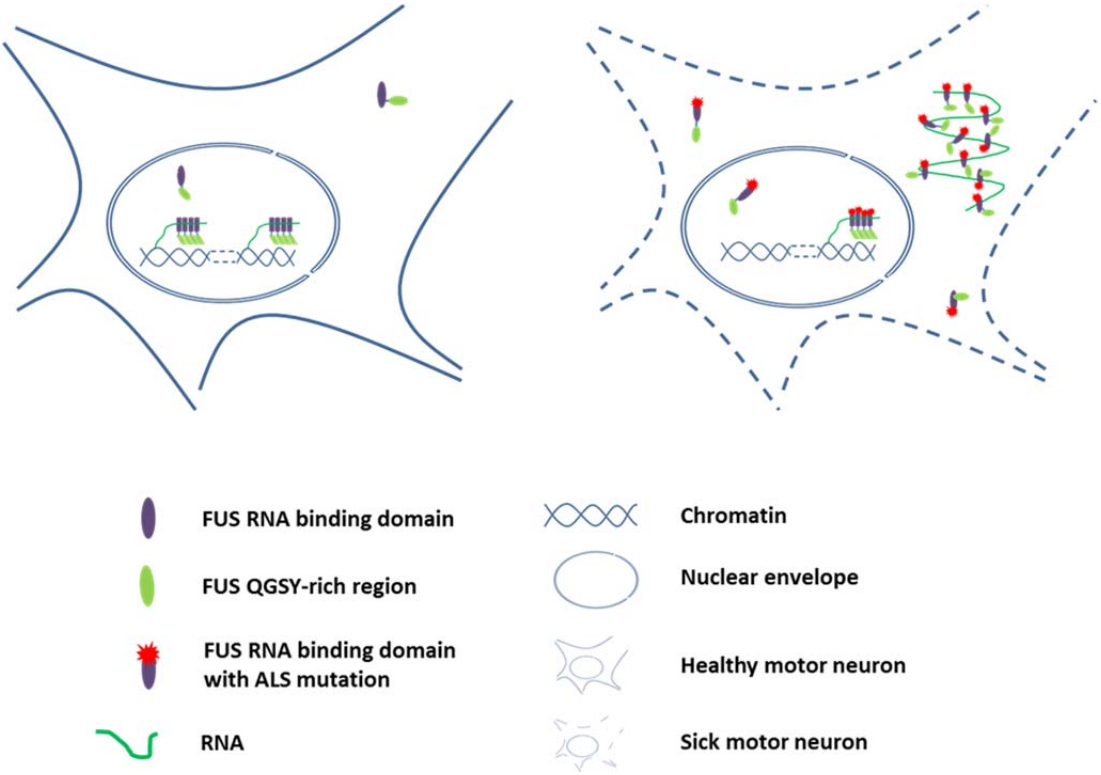


Figure 4.9 The proposed model of FUS distribution and architecture in different cellular compartments.

The left side shows that chromatin-associated RNAs initiate FUS oligomerization through the QGSY-rich region in the nucleus. Nuclear soluble and cytoplasmic FUS normally exists as monomers under physiological conditions. The right side shows that ALS mutations cause cytoplasmic retention of FUS. However, mutant FUS in the nucleus was still able to oligomerize similarly to wild-type FUS. The cytoplasm-retained FUS forms relatively disordered inclusions that require binding cytoplasmic RNAs.

Figure 4.9 The proposed model of FUS distribution and architecture in different cellular compartments



Chapter 5: Arginine Methylation Regulates FUS Chromatin Binding and Transcription Regulation Ability

Introduction

FUS plays a fundamental role in regulating gene transcription since it can directly interact with RNA polymerase II [63, 100, 128] and depletion of it changed the mRNA level of hundreds of genes in mouse brain and cultured cells [63, 64]. However, the exact mechanism through which FUS regulate gene transcription is not clear. In our previous study [138], we found that transcription regulation mediated by FUS requires its oligomerization and chromatin binding through the QGSY-rich region in the N-terminus of FUS. We also found mutations, which cause familial ALS, significantly reduce FUS chromatin binding. As a consequence, those ALS mutations may also impair FUS-mediated transcription regulation. Indeed, the expression of MnSOD gene, a FUS regulated gene encoding the primary antioxidant enzyme in the mitochondria, is significantly reduced in ALS patient fibroblasts as compared to in normal controls [110].

Post-translational modifications of proteins play important roles in regulating their distribution and function. Phosphorylation [149, 150] and arginine methylation [151] are the two post-translational modifications on FUS that have been reported. Phosphorylation of FUS plays an important role for its participation in DNA damage repair [152, 153]. Methylation on the arginine residues in FUS has been reported to regulate its nucleus/cytoplasm localization [107, 108, 140, 141]. Arginine methylation is catalyzed by protein arginine methyltransferases (PRMTs) which transfer methyl groups from S-adenosyl methionine to the guanidino nitrogen of arginine residues [106]. The primary enzyme for FUS arginine methylation is PRMT1 [107, 108].

FUS has three arginine-glycine-glycine (RGG) repeat regions interspersed between different functional motifs. Two of them are critical for FUS RNA binding ability [Chapter 2]. The RGG repeats are typical substrates of PRMTs [106]. It is important to know whether FUS arginine methylation occurs in the RGG repeats, since any of the modifications in these regions may affect FUS RNA binding ability.

In this study, we found that FUS arginine methylation can regulate its chromatin binding and transcription regulation ability. We first confirmed that FUS arginine methylation mainly takes place in the RGG-repeat regions. We then discovered methylation inhibition increased the level of chromatin-bound FUS. By knocking-down and over-expression of PRMT1, we found that arginine methylation can regulate FUS chromatin binding. With cells expressing mutant FUS, we found that methylation inhibition restored mutant FUS binding to active chromatin. With reporter gene plasmid containing MnSOD gene promotor, we found that arginine methylation regulated FUS transcription activation ability. Together, our results suggest arginine methylation can be used as handle to reverse ALS mutations-caused FUS physiological function loss.

Results

FUS is arginine-methylated in the RGG repeat regions

To probe arginine methylation regions in FUS, we made a series of constructs expressing FUS truncation mutants (Figure 5.1A left) and transfected them in HEK cells. GST-tagged FUS truncation mutants were pulled-down by glutathione beads and subjected to SDS/PAGE and Western blot with methylarginine antibody. As shown in Figure 5.1A right, full length FUS arginine methylation was confirmed as no methylarginine detected in the GST tag itself. By comparing methylarginine level of FUS 1-164 to FUS 165-284 and FUS 1-370 to FUS 1-494, we detected that the glycine-rich region (165-284) and zinc finger domain (371-494) are two main arginine methylation regions in FUS (Figure 5.1A right).

Glycine-rich region contains the first RGG repeat region (RGG1); zinc finger domain contains the other two RGG repeat regions (RGG2 and RGG3) (Figure 5.1B left). We further truncated FUS in zinc finger domain (Figure 5.1B left) and did the same GST pull-down and Western blot. As shown in Figure 5.1B right, while the deletion of either RGG2 (370-409) or RGG3 (472-494) resulted in methylarginine level decrease, the deletion of zinc finger motif did not change methylarginine level. The result indicates zinc finger domain arginine methylation mainly takes place in RGG2 and RGG3.

Inhibition of arginine methylation enhanced FUS chromatin binding

Adenosine dialdehyde (AdOx) is an inhibitor of S-adenosylhomocysteine hydrolase [154], an enzyme critical for the regeneration of the methyl group donor--S-adenosyl methionine. We found AdOx treatment significantly increased FUS protein level isolated together with chromatin (Figure 5.2A). HEK cells were grown in the medium containing 20 μM AdOx before we isolated the chromatin-bound proteins using the protocol described previously. A corresponding decrease of FUS protein levels in the soluble fraction was also detected (Figure 5.2A). The result indicates methylation inhibition enhanced FUS chromatin binding.

To see how FUS arginine methylation was affected by AdOx, we immunoprecipitated FUS out of soluble fractions in Figure 5.2A and did Western blot with methylarginine antibody. As shown in Figure 5.2B, at high AdOx concentrations FUS arginine methylation was not detectable, indicating high concentration AdOx inhibited FUS arginine methylation.

PRMT1 regulates FUS arginine methylation and chromatin binding

The results of methylation inhibition experiment indicate there is a correlation between FUS chromatin binding and arginine methylation. To test whether FUS arginine methylation is a causal factor of its chromatin binding, we knocked-down PRMT1, the primary enzyme for FUS arginine methylation, with siRNA in HEK cells and did FUS immunoprecipitation and chromatin-bound protein isolation. As shown in Figure 5.3A, PRMT1 knock-down reduced FUS arginine methylation level significantly. As a consequence, chromatin-bound FUS from PRMT1 knock-down samples increased significantly comparing to chromatin-bound FUS from control siRNA samples (Figure 5.3B). On the opposite direction, we over-expressed PRMT1 in HEK cells and did FUS immunoprecipitation and chromatin-bound protein isolation. Increasing amounts of pCMV2B-PRMT1 plasmids were transfected into HEK cells. As shown in Figure 5.4A, over-expression of PRMT1 increased FUS arginine methylation level in a dose-dependent manner. With higher arginine methylation level, less FUS was detected in

chromatin-bound fraction (Figure 5.4B). Together, the results suggest that FUS chromatin binding is likely regulated by its own arginine methylation.

Suppression of arginine methylation restored mutant FUS binding to active chromatin domains.

Since ALS mutations significantly reduce FUS chromatin binding and loss of FUS on chromatin may cause extensive transcription dysregulation [63, 64, 138], we wanted to test whether suppression of arginine methylation can reverse mutation-caused FUS loss on chromatin. For this purpose, we treated HEK cells expressing mutant FUS with AdOx and separated chromatin-bound and non-chromatin-bound proteins with chromatin salt elution method (Figure 5.5A). As described in Chapter 1, chromatin salt elution can separate active chromatin domains from inactive chromatin domains. The reason we fractionated chromatin was to make sure that the restored chromatin-bound mutant FUS was in the transcriptionally active chromatin domains. As shown in Figure 5.5, R521G mutation resulted in a decrease of FUS protein level in the E1 fraction, which mainly contains active chromatin domains as indicated by histone immunoblots, comparing with wild-type FUS fractionation (Figure 3.3). With AdOx treatment, which suppresses FUS arginine methylation, a substantial increase of the amount of FUS in the E1 fraction was detected (Figure 5.5C). We also showed the DNA contents in each fraction in Figure 5.5B agarose gel image. The result suggests suppression of arginine methylation can reverse the effect of ALS mutation on FUS chromatin binding.

Arginine methylation regulates FUS-mediated transcription activation

We next asked whether arginine methylation can regulate the gene transcriptions mediated by FUS. For this purpose, we used a dual-luciferase assay as described in Chapter 3 with a firefly luciferase gene driven by the MnSOD promoter (Figure 5.6A). We cotransfected the luciferase plasmids with either PRMT1 siRNA or plasmid into HEK cells and did the luciferase assay as described in Chapter 2. We found PRMT1 knock-down significantly increased MnSOD promoter activity, whereas PRMT1 over-expression significantly decreased MnSOD promoter activity (Figure 5.6B). The result is strongly correlated with PRMT1-regulated FUS chromatin binding ability (Figure 5.3 and

5.4). Since the MnSOD promoter is bound and activated by FUS [110], the result suggests FUS arginine methylation can regulate its transcription activation ability through adjusting its chromatin binding. We also tested how FUS transcription regulation ability was affected by a series of compounds that are inhibitors of methionine S-adenosyltransferase 2A and expected to reduce S-adenosyl methionine levels in cells [155] (Figure 5.6C). The VMS compounds were generously provided by Dr. David Watt in our department.

Discussion

In this study, we found arginine methylation can regulate FUS chromatin binding and transcription regulation ability. This funding suggests that arginine methylation can be used as a handle to reverse ALS-mutation-caused FUS chromatin dissociation and transcription activation deficiency. Indeed, we showed evidence that suppression of arginine methylation can restore mutant FUS binding to the active chromatin domains.

Both AdOx and PRMT1 knock-down involves more targets than just FUS. Cautions should be given when interpreting the relation between FUS chromatin binding and its own arginine methylation. This situation can be worse in the case of transcription regulation since a lot of transcription factors are also the substrate of PRMT1 [106]. With our own data we cannot exclude the possibility that other transcription factors also participated in PRMT1-regulated MnSOD promotor activity. However, our primary goal is to reverse FUS chromatin-dissociation caused by ALS mutations. Although other proteins may be also involved or even play primary roles in this process, we can also achieve our goal, but with a caution of the side effects. From this comes our motivation to test arginine methylation modulators with our reporter gene assay. Our ultimate objective is to identify arginine methylation modulators which can reverse ALS mutation effects on FUS while have no or very little toxic effects on cells.

Chromatin binding may also be required for FUS DNA damage repair [61]. In the paper published by Mastrocola, A.S., *et al*., the authors showed that FUS was recruited to DNA damage sites shortly after laser-induced micro-irradiation. But if N-terminal disordered region of FUS was deleted, the recruitment of FUS to DNA damage sites was

almost abolished. Since FUS N-terminus is responsible for its chromatin binding, it is conceivable that chromatin binding may also be important for the response of FUS to DNA damages. Therefore the restoration of mutant FUS chromatin binding driven by arginine methylation suppression may show beneficial effects in a broader range than just transcription regulation.

We do not know the exact mechanism by which arginine methylation regulates FUS chromatin binding. But we detected that arginine methylation mainly takes place in RGG repeat regions (Figure 5.1). Two of them, RGG2 and RGG3, are very important for FUS RNA binding ability (Figure 4.8). The mechanism is considered to be that the charge-charge interaction--the positive charges of arginine guanidinium groups in the RGG repeats and the negative charges of phosphates in RNA--enhances the binding affinity of FUS to RNA. Methylation on guanidino nitrogen may alter the charge distribution in guanidinium group or disturb the charge-charge interaction by steric effects. Therefore arginine methylation may reduce the binding affinity of FUS to RNA. With regard to the role of RNA in FUS oligomerization and chromatin binding, it is very likely that arginine methylation regulates FUS chromatin binding through alteration of its binding affinity to RNAs.

Arginine methylation suppression has shown beneficial effects in model organisms with transgenic expression of FUS or FUS mutants [108, 139, 141]. The mechanism is probably the relocation of mutant FUS from the cytoplasm to the nucleus, driven by arginine methylation suppression. In ALS patients, both cytoplasmic FUS accumulation and nuclear FUS loss happen. Our finding that arginine methylation suppression can increase FUS chromatin binding together with its reported toxicity-reducing effect with mutant FUS suggest suppression of arginine methylation may serve as an effective approach to save motor neurons from FUS ALS mutations.

Figure 5.1 FUS arginine methylation occurs in the RGG-repeat regions.

(A) FUS arginine methylation was mainly detected in the glycine-rich region and zinc finger domain. Diagram shows the GST-tagged FUS and FUS truncation mutants and the RGG-repeat regions. Western blot of GST pull-down products was performed with methylarginine antibody and GST antibody. (B) Zinc finger domain arginine methylation was mainly detected in RGG2 and RGG3. Diagram shows FUS zinc finger domain truncation mutants and RGGs. Western blot was done as described in A.

Figure 5.1 FUS arginine methylation occurs in the RGG-repeat regions

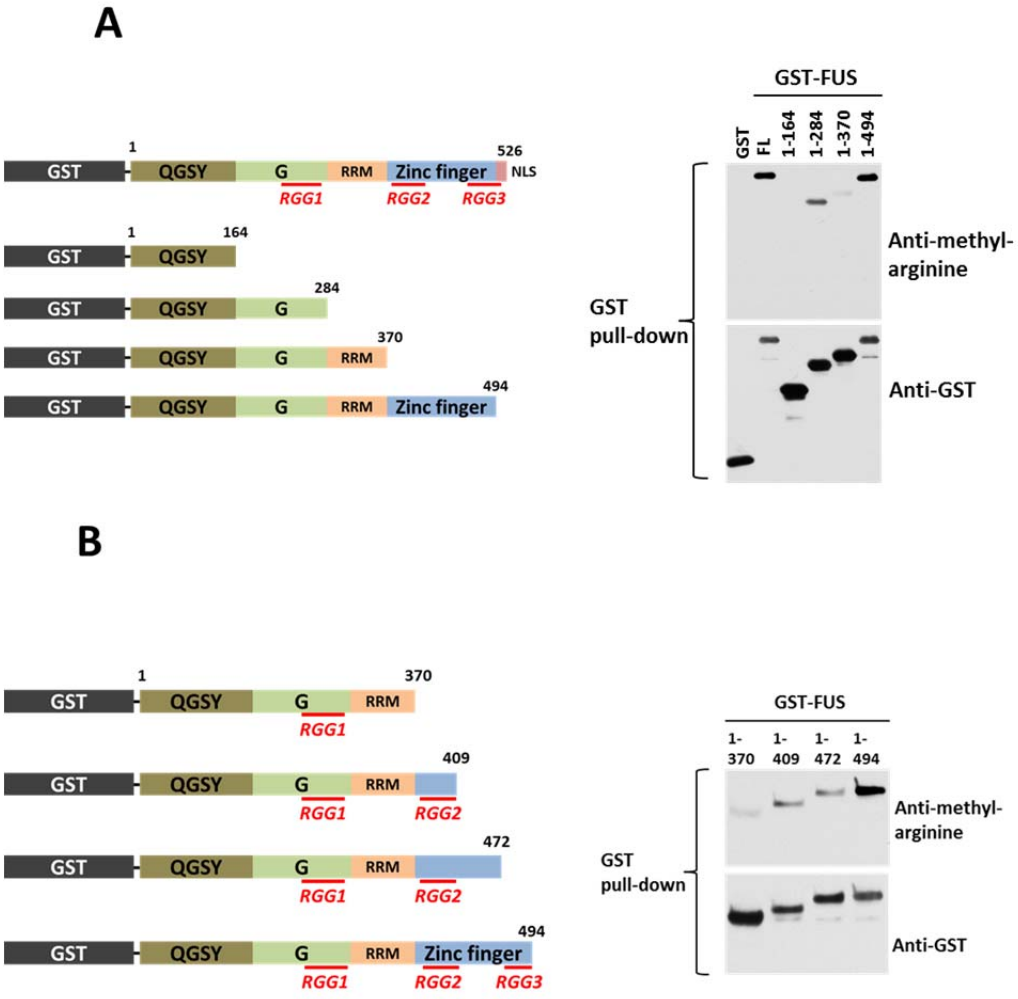


Figure 5.2 Methylation inhibition enhanced FUS chromatin binding.

(A) The chromatin-bound FUS was increased by methylation inhibition. Cells were grown in media containing methylation inhibitor AdOx at indicated concentrations. Chromatin-bound proteins were isolated as in Figure 3.1A and Western blot was performed with FUS and Histone H3 antibodies. (B) FUS arginine methylation was inhibited by AdOx. FUS was immunoprecipitated from soluble fractions in A and and Western blot was performed with methylarginine and FUS antibodies.

Figure 5.2 Methylation inhibition enhanced FUS chromatin binding

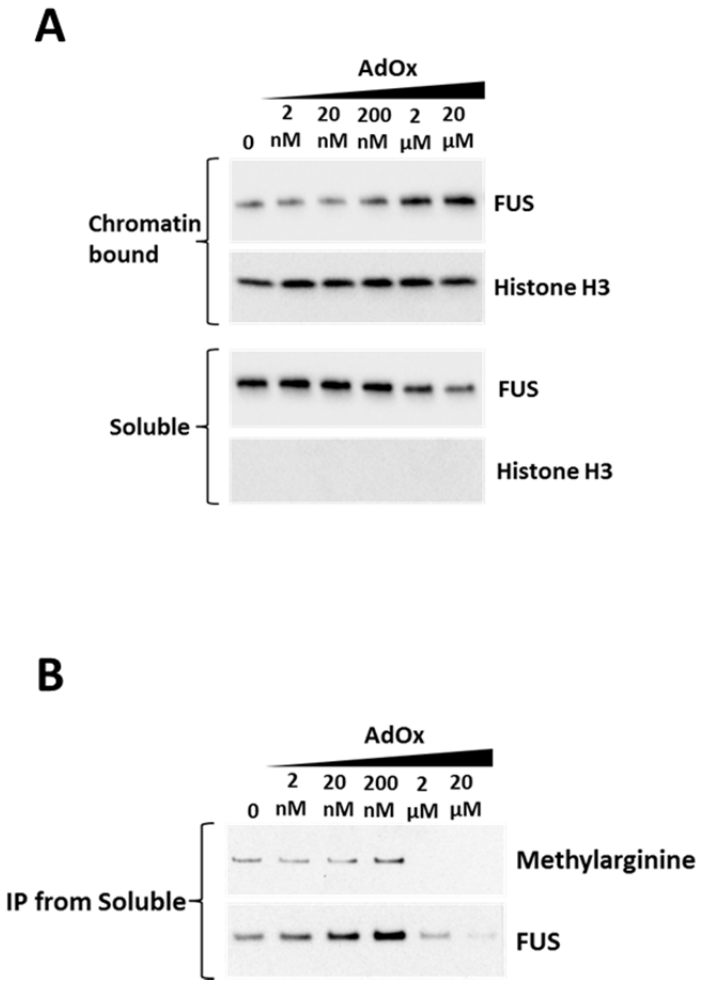


Figure 5.3 Knock-down of PRMT1 suppressed FUS arginine methylation and enhanced FUS chromatin binding.

(A) PRMT1 knock-down decreased FUS methylarginine level. Western blot was performed with FUS immunoprecipitation product and methylarginine and FUS antibodies. The ratio of methylarginine and FUS was quantified and the results from three independent experiments are presented. (B) PRMT1 knock-down increased FUS protein level in the chromatin-bound fraction. Western blot was performed with FUS, Histone H3, and PRMT1 antibodies. The ratio of chromatin-bound and soluble FUS was quantified and the results from three independent experiments are presented.

Figure 5.3 Knock-down of PRMT1 suppressed FUS arginine methylation and enhanced FUS chromatin binding

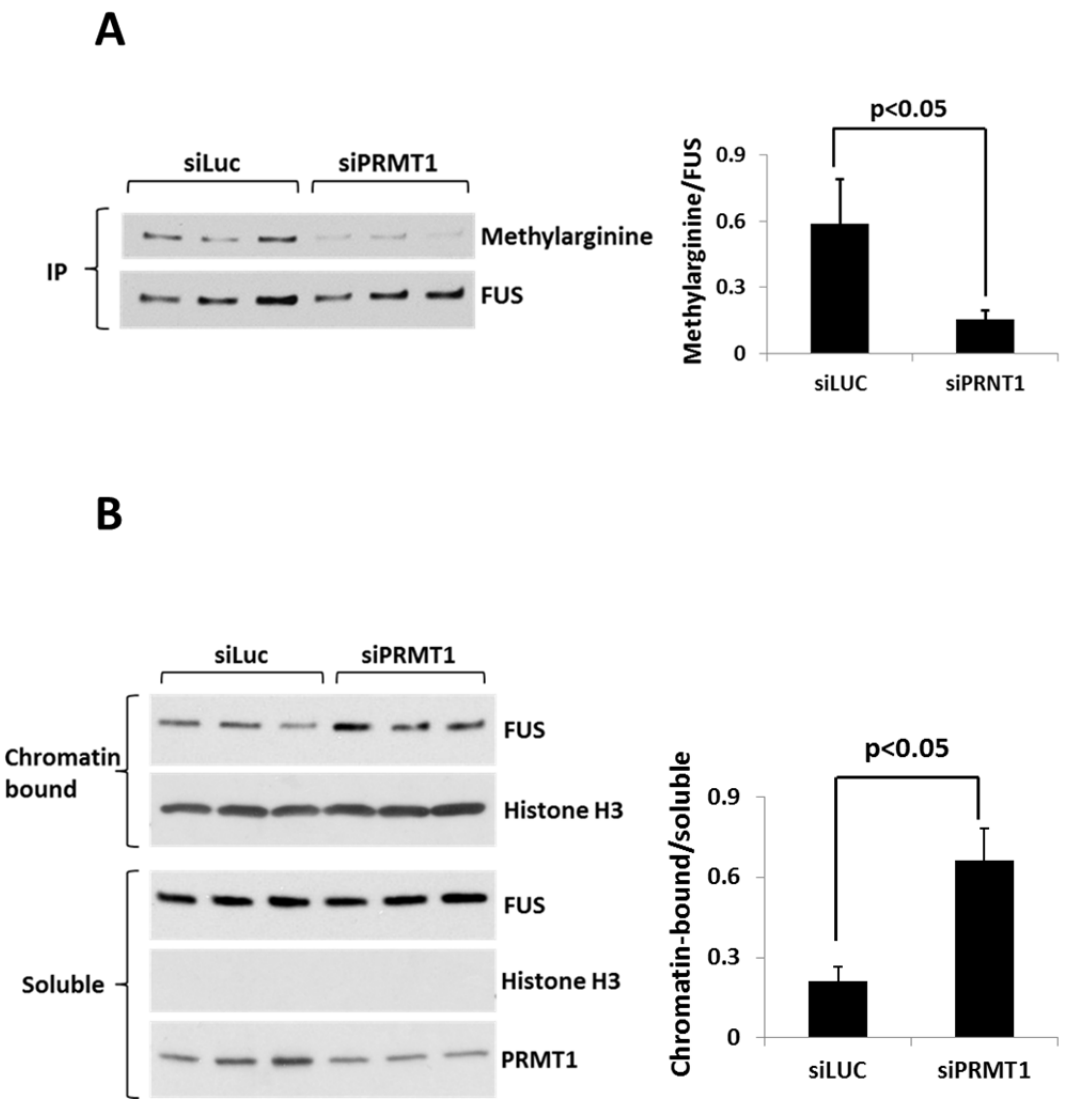


Figure 5.4 PRMT1 over-expression increased FUS arginine methylation and weakened FUS chromatin binding.

(A) PRMT1 over-expression increased FUS arginine methylation. Western blot was performed with methylarginine and FUS antibodies. The ratio of methylarginine and FUS with 1μg FLAG or FLAG-PRMT1 plasmid transfected was quantified and the results from three independent experiments are presented. (B) PRMT1 over-expression reduced FUS protein level in the chromatin-bound fraction. Western blot was performed with FUS, Histone H3, and FLAG antibodies. The ratio of chromatin-bound and soluble FUS with 1μg FLAG or FLAG-PRMT1 plasmid transfected was quantified and the results from three independent experiments are presented.

Figure 5.4 PRMT1 over-expression increased FUS arginine methylation and weakened FUS chromatin binding

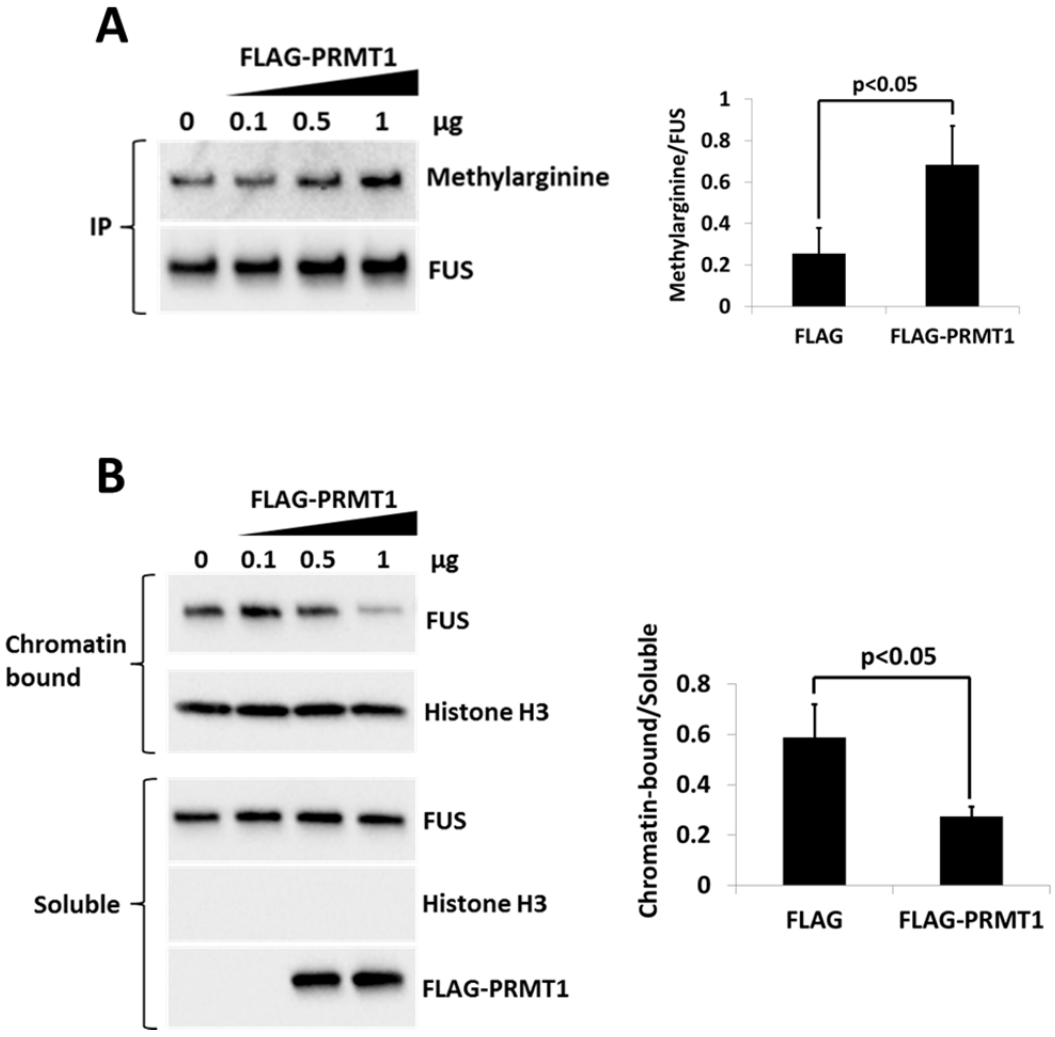


Figure 5.5 Methylation suppression restored mutant FUS binding to active chromatin domains.

(A) Flowchart of chromatin fractionation as in Figure 3.3A. (B) DNA fragments from each fraction. DNA fragments were purified with proteinase K treatment and PCR product purification kit and separated by agarose gel electrophoresis. (C) AdOx treatment restored the level of EGFP-FUS R521G in the E1 fraction. Chromatin from the cells expressing wild-type or mutant FUS with or without AdOx treatment was fractionated. Western blot was performed with indicated antibodies.

Figure 5.5 Methylation suppression restored mutant FUS binding to active chromatin domains

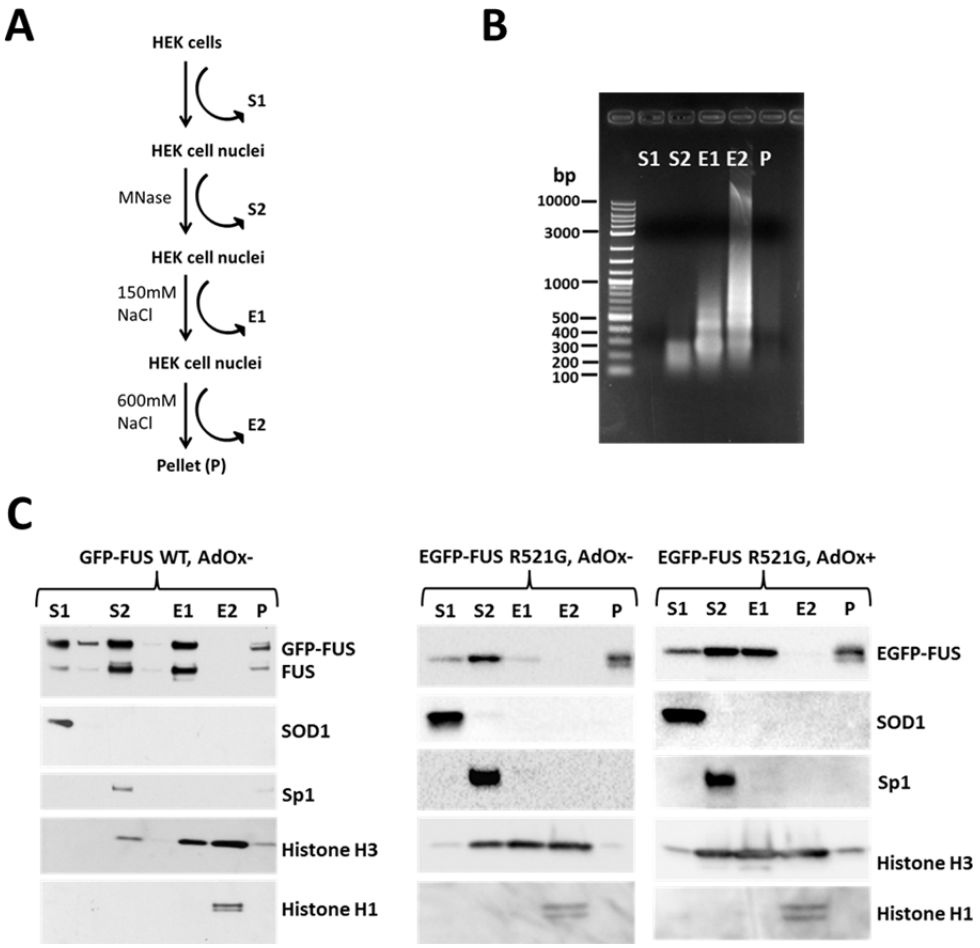
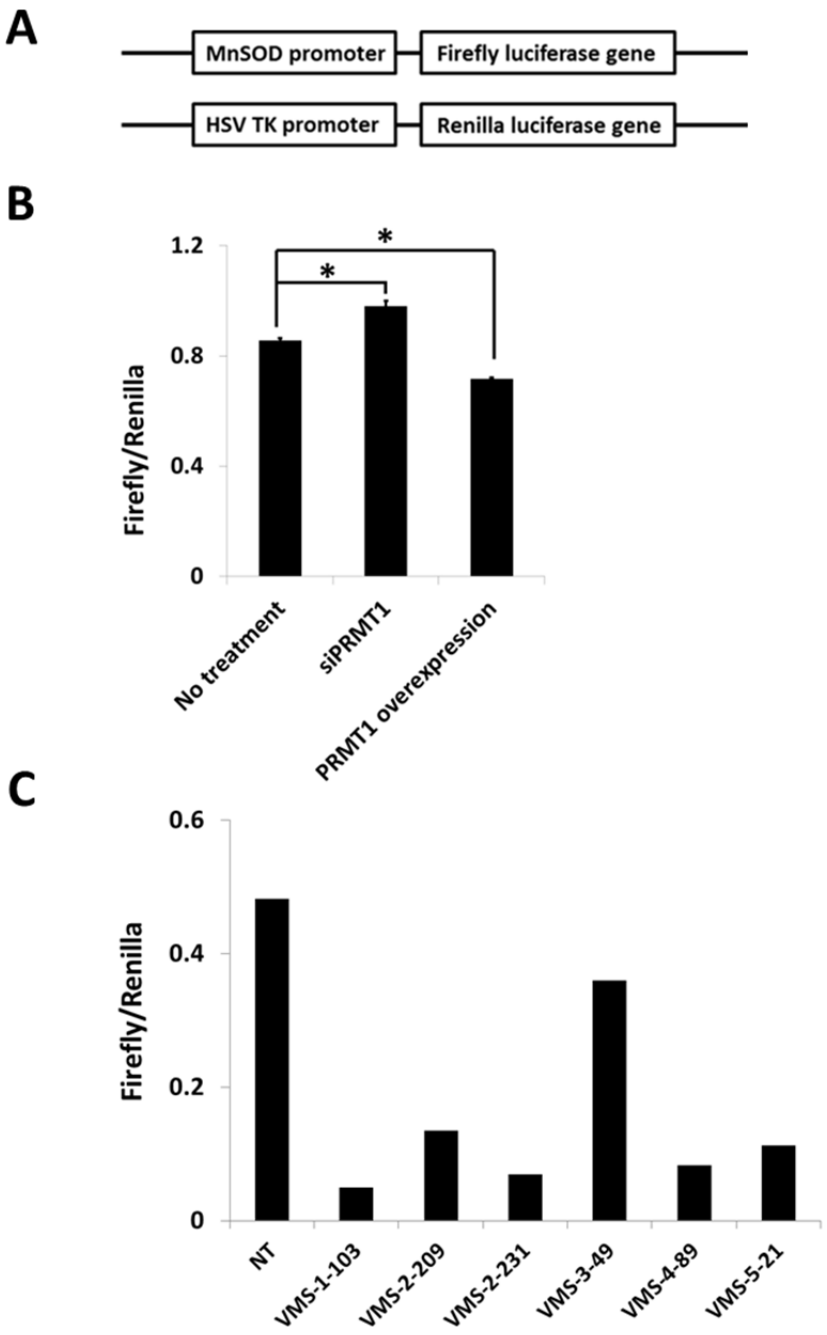


Figure 5.6 Arginine methylation regulates MnSOD promotor activity.

(A) Diagram of the dual-luciferase assay plasmids. Firefly luciferase gene is driven by MnSOD promoter and renilla luciferase gene is driven by HSV TK promoter. (B) MnSOD promoter activity was regulated by PRMT1. HEK cells were cotransfected with the luciferase plasmids and PRMT1 siRNA or FLAG-PRMT1 plasmid two days before lysis and dual luciferase assay. The results from three independent experiments are presented. *: $p<0.05$. (C) MnSOD promoter activity was affected by methionine S-adenosyltransferase 2A inhibitors. The VMS compounds were generously provided by Dr. David Watt in our department. HEK cells transfected with the luciferase plasmids were treated with 20 μM of each compound two days before lysis and dual luciferase assay.

Figure 5.6 Arginine methylation regulates MnSOD promotor activity.



Chapter 6: Summary and Future Directions

In this dissertation research, we revealed how wild-type FUS is organized in the nucleus for its multiple functions and discovered that disassociation of FUS from chromatin is a common consequence of ALS mutations. Considering the necessity of chromatin binding in FUS-mediated transcription regulation, the finding suggests that disturbed transcription regulation can be a consequence of the ALS mutation-caused FUS loss-of-function in the nucleus. Moreover we identified a posttranslational modification, namely arginine methylation, can reverse the reduced association between mutant FUS and chromatin. We further identified RNAs are a critical factor determining FUS organization in the nucleus and the cytoplasm. In the nucleus, RNA triggers FUS oligomerization, providing a potential mechanism of the coexistence of two functionally distinctive pools of FUS. In the cytoplasm, mutant FUS inclusion formation is also RNA-dependent, supporting the notion that the pathogenic mechanism of mutant FUS is different from the widely-accepted pathogenic mechanism of non-RNA-binding protein misfolding/aggregation.

Below, I will discuss the potential implications of the findings in this dissertation and propose future directions which may solidify the conclusions and further extend our understanding of FUS physiology and pathology.

FUS oligomerizes in the nucleus to regulate gene transcription

FUS has multiple functions in the nucleus but it is unknown how nuclear FUS is deployed for its multiple roles. We found that a portion of nuclear FUS is bound to active chromatin and the remainder of FUS is free in the nucleoplasm. Chromatin-bound FUS is designated for gene transcription regulation whereas free nuclear FUS can regulate mRNA alternative splicing. This finding suggests that cells ensure the fulfilment of FUS' multiple roles by allocating FUS molecules into different pools, instead of coupling different cellular processes that FUS participates in.

We identified oligomerization as a way through which cells allocate FUS molecules for chromatin binding and nucleoplasm diffusing. Only oligomerized FUS can bind chromatin. Interestingly, the oligomerization domain of FUS is part of a prion-like

domain which was previously considered bad because of its aggregation-prone property. Regarding the fact that RNA binding proteins have the highest odds to possess a prion-like domain [96], our finding indicates a prion-like domain in RNA binding proteins is physiologically important, rather than a mere evolutionary accident.

However, with this notion we still do not know how cells initiate FUS oligomerization to execute its transcription regulation function. Besides the QGSY-rich region, which is an intrinsic determinant of FUS oligomerization, RNA was identified as an extrinsic determinant of FUS oligomerization. In Chapter 3, we showed RNA is required for FUS oligomerization. In Chapter 4, we confirmed the RNA dependency of FUS oligomerization and discovered chromatin-associated RNAs are the trigger of FUS oligomerization. The identification of the RNA-triggered oligomerization explains the coexistence of chromatin-bound oligomerized FUS and free FUS in the nucleus. We therefore speculate that cells may determine the proportion of chromatin-bound FUS and free FUS by transcribing certain RNAs when it is necessary.

We found the significant loss of chromatin-bound FUS as a consequence of ALS mutations, suggesting ALS mutations can subsequently cause interference in FUS mediated transcription regulation. This notion is supported by the fact that MnSOD gene expression is significantly decreased in fibroblasts from ALS patients compared to in control fibroblasts [110]. FUS plays a fundamental role in gene transcription regulation. In liposarcoma-causing oncogenic fusion protein, where FUS was first identified and named, the transcription activation capability is carried by the FUS part [156, 157]. FUS has been reported as a direct interaction partner of RNA polymerase II [63, 100, 128, 129]. In FUS knock-out mouse brain, the mRNA level of hundreds of genes was changed to a significant level [64]. In FUS knock-down HEK cells, the transcription regulation of hundreds of genes were disturbed [63]. An interesting observation in these two genome-wide studies was that the average change of the mRNA levels of FUS-regulated genes is about two folds—a level considered low compared to the genes regulated by other transcription factors. We detected about the same level of mRNA change in FUS over-expressing cells (Chapter 3). We speculate that FUS plays a fundamental role in fine tuning gene transcription in a large scale rather than certain specific genes. Considering

the late onset of ALS, it is conceivable that the accrued broad-range transcription dysregulation can eventually kill motor neurons.

RNA determines FUS organization in different cellular compartments

Nuclear FUS appears granular under confocal microscope, so does the nucleus-localized mutant FUS. The primary function of FUS nuclear granules is transcription regulation. The structural basis of FUS nuclear granules is the QGSY-rich region mediated oligomerization. In Chapter 3 and Chapter 4, we showed FUS oligomerization was triggered by chromatin-associated RNAs. An immediate question following this discovery is what the identities of the trigger RNAs are.

Mutant FUS forms inclusions in motor neuron cytoplasm. This histopathological feature can be recapitulated in cultured cells (Chapter 4). In Chapter 4, we showed cytoplasmic inclusion formation also requires FUS RNA binding ability. Indeed RNA and RNA binding proteins have been frequently reported in FUS inclusions [55, 56, 87, 104, 158]. The finding that RNA plays a critical role in mutant FUS inclusion formation raises two interesting questions. First, what is the exact role of RNA in FUS inclusions? Or whether cytoplasmic RNAs trigger FUS inclusion formation? The answer can help us better understand why FUS inclusions are so structurally different from inclusions formed by other misfolded proteins. The future studies will also examine the effect of mutant FUS on normal stress granule formation. Second, what are the identities of FUS inclusion-trapped RNAs? Not like stress granules, which are temporary structures, FUS inclusions are persistent. When the stress ebbs away, stress granule RNAs, which are typically messenger RNAs, are released and subjected to translation [57, 159]. However, FUS inclusion-trapped RNAs are persistently sequestered, thus functional loss will likely occur in cellular processes involving the participation of these RNAs or their protein products. Therefore, the identification of FUS inclusion-trapped RNAs can help us discover potential new pathways that might me impaired by mutant FUS.

To identify these FUS-bound RNAs, we plan to do FUS immunoprecipitation and RNA sequencing. The strategy is first to crosslink FUS and targeting RNAs with ultraviolet light, and then to isolate chromatin from wild-type FUS expressing cells or

cytoplasm from mutant FUS expressing cells. Chromatin will be fragmented and dissolved by sonication. The targeting RNAs will be enriched by FUS immunoprecipitation and purified for sequencing. We will compare our sequencing results with the published FUS-bound RNA database to identify specific RNA species.

There are attractive applications if we know the identities and sequences of FUS organization-determining RNAs. First, for nuclear regulatory RNAs, we will search (if they are known RNA species) or investigate (if they are unknown RNA species) how their transcription is regulated and target their transcription-regulating factors to nudge FUS intranuclear distribution to a favorable way. Second, after we know the sequences of nuclear regulatory RNAs, we can design RNA probes to alter mutant FUS distribution or inclusion formation in the cytoplasm, since nuclear RNAs can trigger mutant FUS oligomerization (Chapter 4). Third, the identities of FUS inclusion-trapped RNAs can help us identify new potential pathways that are disturbed by mutant FUS.

Arginine methylation regulates FUS chromatin binding

We found that a posttranslational modification—methylation on arginine—can regulate FUS chromatin binding. Arginine methylation was studied less comprehensive than phosphorylation due to the late identification of arginine methyltransferases [106]. The main cellular activities regulated by arginine methylation are gene expression, DNA damage repair, and signal transduction [106]. Therefore the finding that arginine methylation can regulate FUS chromatin binding supports the notion that chromatin binding plays an important role in FUS-mediated gene transcription regulation and genome stability maintenance.

In Chapter 5, we narrowed down arginine methylation areas in FUS mainly to three short sequences: the glycine-rich region and two arginine-glycine-glycine rich regions (RGG2 and RGG3) flanking zinc finger motif. In Chapter 4, we demonstrated RGG2 and RGG3 were critical for FUS RNA binding *in vitro*. We also discussed the potential mechanism of arginine methylation regulated FUS chromatin binding in Chapter 5. Methylation on arginine residues in RGG2 and RGG3 can alter their affinity to RNA, which plays critical role in FUS oligomerization and chromatin binding. This is

our speculation and we plan to test it experimentally. The strategy is to use the same *in vitro* RNA binding assay, but with different FUS fragments expressing cells. We used *E. coli* in the experiment in Chapter 4; we plan to use mammalian cells to express FUS fragments in the proposed experiment. The advantage of mammalian cells is that we can control FUS fragments arginine methylation level by adding methylation inhibitors or over-expressing PRMT1. The potential implication of the result of the proposed experiment can be very important because RNA binding ability is critical not only in FUS chromatin binding but also in many other FUS-participated cellular activities.

We showed evidence that both methylation inhibitor and PRMT1 knock-down can suppress FUS arginine methylation and increase FUS chromatin binding. PRMT1 enzymatic activity can be pharmacologically inhibited [160]. These facts suggest small compounds modulating either methylation or PRMT1 activity may be applied to restore chromatin binding of mutant FUS. We hope that by screening methylation modulators we can find candidates with beneficial effects of a low arginine methylation level while avoiding cell toxicity. We can use a FUS chromatin binding assay to do the screening either in HEK cells or in primary neurons.

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Manuscripts in preparation

Liuqing Yang, Jiayu Zhang, Marisa Kamelgarn, Chunyan Niu, Jozsef Gal, Weimin, Gong, and Haining Zhu. *Subcellular localization and RNAs determine FUS architecture in different cellular compartments.*

Liuqing Yang, Marisa Kamelgarn, Haining Zhu. *Arginine methylation regulates FUS chromatin binding.*

Presentations:

Oral

2014 *A new function of "prion-like domain" in ALS protein FUS.* Department Student Seminar

2012 *ALS-related protein FUS: post-translational modification and subcellular localization.* Department Retreat

Poster

2013 *Biochemical and functional study of a nuclear RNA binding protein FUS in Lou Gehrig's disease.* Department Retreat

2012 *Arginine methylation modulates FUS binding with chromatin*. International Symposium on ALS/MND

2011 *Investigation of axonal transport in primary motor neuron model of amyotrophic lateral sclerosis*. ASBMB Annual Meeting