Self-assembled FUS binds active chromatin and regulates gene transcription

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Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease. Fused in sarcoma (FUS) is a DNA/RNA binding protein and mutations in FUS cause a subset of familial ALS. Most ALS mutations are clustered in the C-terminal nuclear localization sequence of FUS and consequently lead to the accumulation of protein inclusions in the cytoplasm. It remains debatable whether loss of FUS normal function in the nucleus or gain of toxic function in the cytoplasm plays a more critical role in the ALS etiology. Moreover, 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 active chromatin and that the ALS mutations dramatically decreased FUS chromatin binding ability. Functionally, the chromatin binding is required for FUS transcription activation, but not for alternative splicing regulation. The N-terminal QGSY (glutamine-glycine-serine-tyrosine)-rich region (amino acids 1–164) mediates FUS self-assembly in the nucleus of mammalian cells and the self-assembly is essential for its chromatin binding and transcription activation. In addition, RNA binding is also required for FUS self-assembly and chromatin binding. Together, our results suggest a functional assembly of FUS in the nucleus under physiological conditions, which is different from the cytoplasmic inclusions. The ALS mutations can cause loss of function in the nucleus by disrupting this assembly and chromatin binding.

fused in sarcoma | amyotrophic lateral sclerosis | chromatin binding | self-assembly | transcription

Amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease) is a progressive and fatal neurodegenerative disease characterized by motor neuron loss. The etiology underlying the disease is yet to be better understood. Approximately 15–20% of ALS cases are hereditary (familial ALS). Mutations in fused in sarcoma (FUS), which is a DNA/RNA binding protein, are found to be responsible for a subset of familial ALS patients (1, 2). Interestingly, mutations in other RNA binding proteins TAR DNA-binding protein 43 (TDP-43) (3), TAF15 (4), hnRNP2B1, and hnRNP1 (5) have also been reported in familial ALS patients. Cytoplasmic protein inclusions are a common histopathological feature of ALS with mutations in the RNA binding proteins.

FUS is a multifunctional protein and has been reported to play a role in various aspects of RNA metabolism (6), including transcription regulation and alternative splicing. FUS was initially identified in liposarcomas as part of a fusion protein (7, 8) in which the N-terminal domain of FUS (amino acid 1–266) is recombined to transcription factor CHOP at its N terminus. The FUS–CHOP fusion protein activates the transcription of oncogenes and promotes tumorigenesis (9, 10). In familial ALS, most mutations are clustered in the C-terminal nuclear localization sequence (NLS) of FUS and consequently cause the mislocalization of FUS protein from the nucleus to the cytoplasm and the accumulation of protein inclusions (11–13). 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 ALS etiology and the two mechanisms are not necessarily exclusive of each other.

Cytoplasmic FUS inclusions resemble stress granules, indicated by colocalization of FUS with different stress granule components (11, 12). Stress granules are temporary cellular structures containing RNAs and proteins from suspended translation apparatus (14). Stress granule formation promotes cell survival under stressed conditions by redistributing translation resources. Compromised stress granule response in the presence of FUS mutants is considered a contributing factor to motor neuron dysfunction (15).

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 (16). The prion-like domain consists of an intrinsically disordered QGSY (glutamine-glycine-serine-tyrosine)-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 patients with familial ALS and has been reported to cause intranuclear aggregation of FUS (17). 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. It is also unclear how ALS mutations impair the nuclear function of FUS. 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

Significance

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder and mutations in fused in sarcoma (FUS) cause a subset of familial ALS. Mutant FUS forms cytoplasmic inclusions, but it is unclear whether loss of FUS function in the nucleus or toxicity gained in the cytoplasm is more critical in the ALS etiology. The physiological function of FUS is also uncharacterized. We found that a significant portion of FUS was bound to active chromatin and that ALS mutations dramatically reduced FUS chromatin binding. A high order FUS assembly is mediated by the N-terminal QGSY (glutamine-glycine-serine-tyrosine)-rich region and is required for FUS chromatin binding and the transcription activation by FUS. ALS mutations in FUS can cause its loss of function in the nucleus by disrupting this assembly and chromatin binding.

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physiological conditions is likely different from cytoplasmic inclusions found in cells harboring ALS mutations. These mutations can cause 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 units/mL benzonase (Fig. S1A). A significant amount of endogenous FUS was in the chromatin-bound (CB) fraction (Fig. 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 result, we used 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 (Fig. 1B). The nuclear soluble protein Sp1 and the chromatin-bound histone H3 were used 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 [Arg-521 mutated to Gly (R521G) and Arg-495 mutated to stop codon (R495X), resulting in the deletion of the C-terminal 32 amino acids] were expressed in HEK cells at a comparable level to the endogenous FUS (Fig. S1B) and the cell lysates were subjected to the fractionation as described above. The ALS mutations significantly decreased the chromatin-bound FUS compared with the nuclear soluble FUS (Fig. 1C). Quantitative analysis confirmed that the ratio of chromatin-bound and nuclear soluble fractions decreased for the FUS ALS mutants ($P < 0.02$; Fig. 1C). 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 which chromatin domain FUS binds to. Chromatin domains can be fractionated by limited nuclease digestion and gradient salt elution based on different architectural levels (18) (flowchart in Fig. S1C). Transcriptionally active chromatin domains are loosely packed, therefore are easily digested by nuclease and eluted at lower salt concentration (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 (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 (Fig. 1D). The DNA electrophoresis of S1, S2, E1, and E2 fractions are also shown in Fig. 1D to confirm that E1 and E2 are active and inactive chromatin, respectively (18). 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 compared with wild-type FUS (Fig. 1E). The quantitative results of the E1/S2 ratio are shown in Fig. 1E ($P < 0.05$ for R521G and $P < 0.005$ for R495X). The expression level of GFP-FUS was comparable to that of the endogenous FUS in this experiment (Fig. S1D).

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 (Fig. S2). The chromatin-bound fraction was prepared with the SDS and benzonase extraction method as in Fig. S1A. All N-terminal fragments (FUS 1–164, 1–284, 1–370, and 1–494) were detected in the chromatin-bound fraction (Fig. 2A). In contrast, none of the C-terminal fragments lacking the N-terminal QGSY-rich region (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 (amino acids 1–164) 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 (Fig. 2B) and is excluded from nucleoli (arrows in Fig. 2B and Fig. S3), which is consistent with a previous report (19). With the N-terminal 1–164 deletion, the punctate pattern disappeared and the FUS protein was evenly distributed in the entire nucleus including nucleoli. The results indicate that the punctate pattern inside the nucleus observed under the confocal microscope may be related with FUS chromatin binding.

Fig. 1. ALS mutations reduce FUS binding with active chromatin. (A) The soluble (S) and chromatin-bound (CB) fractions were subjected to SDS/PAGE and Western blot with FUS and histone H3 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. Quantification of FUS in each fraction out of the total FUS amount is shown as percentage values. (C) The distribution of GST-tagged wild-type FUS and ALS mutants R521G and R495X was examined by the fractionation method as in B. Percentage values represent the relative abundance of FUS in each fraction out of the total FUS amount. The ratio of CB and NS was quantified and the results from three independent experiments are presented. $*P < 0.02$. (D) Active and inactive chromatin domains were separated as shown in the flowchart in Fig. S1C. 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). (E) 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 D. 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: copper-zinc superoxide dismutase (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.

Fig. 2. The QGSY-rich region is required for FUS chromatin binding. (A) The full-length (FL) and the truncated FUS proteins were subjected to the chromatin-bound protein isolation protocol by SDS and benzonase extraction as shown in Fig. S1A. The chromatin-bound and soluble fractions were subjected to SDS/PAGE and Western blot with indicated antibodies. (B) The intranuclear distribution of the EGFP-tagged full-length FUS and FUS 165–526 lacking the QGSY-rich region was examined by confocal microscopy. Green, GFP-tagged FUS; blue, DAPI staining of DNA; and arrows, nucleoli.

The Role of FUS Chromatin Binding in Transcription Regulation and Alternative Splicing. FUS has been shown to regulate gene transcription (20–22) and alternative splicing (20, 23). 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, which is deficient in chromatin binding. We used the manganese superoxide dismutase (MnSOD) reporter assay (Fig. S4A) because FUS was shown to activate the transcription of MnSOD (21). The full-length FUS or FUS 165–526 construct 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-fold, compared with the nontransfected control ($P < 0.01$; Fig. 3A). In contrast, the truncated FUS 165–526 lost the ability of increasing the MnSOD reporter gene activity. In addition, we tested the transcription activation of another gene, histone-lysine *N*-methyltransferase, SMYD3, which was previously reported (23). Similarly, the truncated FUS 165–526 failed to activate the transcription of the endogenous SMYD3 gene (Fig. 3B). Because 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 the minigene splicing assay (24). The full-length FUS or FUS 165–526 construct along with the E1A or insulin receptor 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 (Fig. 3C). Overexpression of the truncated FUS 165–526 had a similar effect on the splicing of both minigenes as the full-length FUS. Quantitative analysis showed that the ratio of inclusion and exclusion transcripts changed in a similar fashion by the overexpression of either the full-length FUS or the truncated FUS 165–526, which is incapable of binding to chromatin (Fig. 3C), 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) in the minigene assay (Fig. S4B). This negative control supports the specificity of the splicing results in Fig. 3C. The protein levels of the exogenous full-length FUS, FUS 165–526, and endogenous FUS were comparable (Fig. S4C). The results suggest that chromatin binding is not required for FUS function in alternative splicing, although it is required for FUS regulation of gene transcription.

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 more than 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 a 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 (Fig. S5). 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 (25) (Fig. 4A, Left). Whereas the GST-tagged FUS 165–526 was not found in the chromatin-bound fraction (Fig. 2A), the DsRed2-tagged FUS 165–526 was found in the chromatin-bound fraction (Fig. 4B). As a control, a monomeric DsRed variant (DsRed-M)

Fig. 3. The QGSY-rich region is required for FUS transcription activation but not splicing regulation. (A) The transcription activation by FUS was monitored by a dual luciferase reporter assay. The ratio of Firefly and Renilla luciferase activities in the presence of full-length FUS or FUS 165–526 lacking the QGSY-rich 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) The mRNA splicing regulation by FUS was monitored by minigene splicing assay. (Left) The diagram of alternative splicing of the E1A and insulin receptor 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 between full-length FUS and FUS 165–526.

Fig. 4. The QGSY-rich region mediates FUS self-assembly. (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 Fig. S1A. The chromatin-bound and soluble fractions were subjected to SDS/PAGE followed by Western blot. (C) DsRed2–FUS 165–526 showed a punctate 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; and arrows, nucleoli. (D) Native gel electrophoresis of FUS in the chromatin-bound (CB) and soluble (S) fractions. The slow mobility of FUS suggests a high order assembly of FUS in the CB fraction.

(26) was tagged to FUS 165–526 (Fig. 4A, Right). The monomeric DsRed-tagged FUS 165–526 was not detected in the chromatin-bound fraction (Fig. 4B). 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 (Fig. 4C, Top, compare with Fig. 2B), whereas the monomeric DsRed-tagged FUS 165–526 was evenly distributed in the nucleus (Fig. 4C, Bottom). As a control, DsRed2 alone showed an even distribution throughout the cell (Fig. 4C, Middle).

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 Fig. S1A and chromatin-bound proteins were released to the solution by sonicating the resuspended pellet (Fig. S6). The soluble and chromatin-bound fractions were subjected to native gel electrophoresis followed by Western blot with the FUS antibody. The chromatin-bound FUS migrated as a much slower band compared with the soluble FUS that is not associated with chromatin (Fig. 4D), 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 in the freshly made HEK cell lysate before the centrifugation and SDS and benzonase

incubation. The levels of FUS in the chromatin-bound fraction decreased dramatically with increasing amounts of RNase A (Fig. 5A). At the highest RNase A concentration (100 $\mu\text{g}/\text{mL}$), 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 (Fig. 5B). Because 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 hypothesis, 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 (Fig. 5C). 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 (Fig. 1). Specifically, the chromatin-bound FUS is associated with transcriptionally active chromatin and much less with the condensed inactive chromatin (Fig. 1D). A recently published study showed the colocalization of FUS with the activated form of RNA polymerase II in the nucleus (27), supporting our biochemical association of FUS with active chromatin as well as the punctate pattern of FUS inside the nucleus (Fig. 2B). Moreover, the association of FUS with active chromatin was significantly reduced by the ALS mutations R521G and R495X (Fig. 1C and E). 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 (21, 22). This study shows that the truncated FUS 165–526 lacking the N-terminal QGSY-rich region is incapable of binding to chromatin.

Fig. 5. RNA dependence of FUS self-assembly and chromatin binding. (A) FUS chromatin binding is dependent on RNA. 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 Fig. S1A. The amount of chromatin-bound FUS in the presence of RNase A was examined by Western blot. (B) RNA dependence of chromatin binding of the DsRed2-tagged FUS 165–526. 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. (C) RNA dependence of FUS self-assembly. The chromatin-bound fraction was incubated with indicated amounts of RNase A and subjected to native gel electrophoresis. The soluble fraction was included as a control.

Moreover, the truncated FUS 165–526 did not activate MnSOD and SMYD3 gene transcription (Fig. 3*A* and *B*), supporting that the chromatin-binding property of FUS is required for its gene transcription regulation function. This conclusion is also supported by a previous report that knockdown of FUS in mouse brain decreased expression of hundreds of genes (23). 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 (21).

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 (Fig. 3*C*), 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 (Fig. 6).

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 (28). 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 (29). Prion was originally coined to describe a pathogenic protein (PrP^{Sc}), which can use itself as template to convert native protein (PrP^C) into a misfolded conformation and subsequently form amyloid-like aggregates. The prion-like activity has been described in yeast (30) as well as multicellular organisms, involving various processes such as antiviral signaling (31, 32) and memory formation (33). The prion-like domain in RNA binding proteins is involved in the formation of dynamic and reversible structures such as stress granules (34) and processing bodies (35). 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 (Fig. 24). Using native gel electrophoresis and the substitution of the QGSY-rich region with monomeric and tetrameric DsRed (Fig. 4), we demonstrated

that this region is required for high order assembly of FUS and the binding of FUS to chromatin. This is a previously unidentified function of the QGSY-rich region.

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 (Fig. 1*D*) supports this notion. In addition, the interaction between FUS and the C-terminal domain (CTD) of RNA polymerase II has been demonstrated in vitro (20, 36–38). 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. Because the N-terminal domain (1–266) is reported to be responsible for interacting with RNA polymerase II (37), 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 because RNase A treatment disrupted FUS self-assembly (Fig. 5*C*) and reduced FUS chromatin binding to a undetectable level (Fig. 5*A*). It is noted in Fig. 24 that the 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 compared with those containing the zinc finger domain (FUS 1–494 and full-length FUS). An additional examination of DsRed2-tagged FUS 165–370 and FUS 165–526 also suggested that the zinc finger domain facilitated FUS chromatin binding (Fig. S7). Because 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 co-existence of the two pools of FUS (assembled/chromatin-bound and soluble) in the nucleus (Fig. 6). 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 such RNA, FUS remains soluble and carries out other functions such as regulating splicing. Indeed, a previous study demonstrated that noncoding RNAs recruit FUS to chromatin to regulate gene expression (39). A more recent study showed RNA molecules seeded high-order assembly of FUS in vitro (38), supporting the proposed model in Fig. 6.

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 related 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 in our previous studies (21). FUS chromatin binding may also be crucial for DNA damage repair. FUS is among one of the early response proteins in DNA damage repair (40, 41). Deleting the N-terminal amino acids 1–285 significantly reduced FUS recruitment to DNA damage sites induced by laser microirradiation (40). Deficient binding of FUS to chromatin can result in increased DNA damage and genome instability, which is especially harmful to the terminally differentiated nondividing neurons. This notion is also supported by a recent study showing that the ALS mutation R521C caused DNA damage in a transgenic mouse model (42).

Fig. 6. 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.

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.

Materials and Methods

Reagents, plasmids, oligonucleotide primers, and general methods for cell culture and transfection, gene transcription reporter assay, real-time RT-PCR, minigene splicing assay, immunostaining, and confocal microscopy are described in *SI Materials and Methods*. Three critical protocols are briefly described below and more details can be found in *SI Materials and Methods*. Data are presented as means from three independent experiments and *P* values were calculated with the Student *t* test.

Chromatin-Bound Protein Separation. Cells were suspended in radioimmuno-precipitation assay (RIPA) buffer with protease inhibitors and homogenized with a 23G needle. After 20 min incubation on ice, cell lysates were centrifuged at $1,000 \times g$ for 10 min at 4 °C. The supernatants were removed and the pellet was resuspended with RIPA buffer supplemented with 0.3% SDS and 250 units/mL benzonase. Pellet suspensions were incubated on ice for 10 min and centrifuged again at $1,000 \times g$ for 10 min 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.

Separation of Active Chromatin. Active and inactive chromatin domains were separated following a previously published salt elution protocol (18). Briefly, nuclei were precipitated by $1,300 \times g$ centrifugation, and purified nuclei were resuspended in separation buffer containing 2,000 gel units/mL micrococcal nuclease and 1 mM CaCl_2 . After incubation at 37 °C for 10 min, the digested nuclei were eluted with 150 mM NaCl at 4 °C for 2 h (E1, active chromatin) and with 600 mM NaCl at 4 °C overnight (E2, inactive chromatin) sequentially.

Native Gel Electrophoresis. Cells were suspended in detergent-free lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA) and homogenized by either passing through a 23G needle or sonication. Cell lysates were mixed with 6X loading buffer and loaded on a 8% (wt/vol) polyacrylamide gel soaked in detergent-free running buffer. After denaturation, the gel was ready for transferring and Western blotting.

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