FUS functions in coupling transcription to splicing by mediating an interaction between RNAP II and U1 snRNP

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Edited by Tom Maniatis, Columbia University Medical Center, New York, NY, and approved May 27, 2015 (received for review March 30, 2015)

Pre-mRNA splicing is coupled to transcription by RNA polymerase II (RNAP II). We previously showed that U1 small nuclear ribonucleoprotein (snRNP) associates with RNAP II, and both RNAP II and U1 snRNP are also the most abundant factors associated with the protein fused-in-sarcoma (FUS), which is mutated to cause the neurodegenerative disease amyotrophic lateral sclerosis. Here, we show that an antisense morpholino that base-pairs to the 5′ end of U1 snRNA blocks splicing in the coupled system and completely disrupts the association between U1 snRNP and both FUS and RNAP II, but has no effect on the association between FUS and RNAP II. Conversely, we found that U1 snRNP does not interact with RNAP II in FUS knockdown extracts. Moreover, using these extracts, we found that FUS must be present during the transcription reaction in order for splicing to occur. Together, our data lead to a model that FUS functions in coupling transcription to splicing via mediating an interaction between RNAP II and U1 snRNP.

Significance

The protein fused-in-sarcoma (FUS) is mutated to cause the neurodegenerative disease amyotrophic lateral sclerosis, but its normal cellular role remains to be understood. Previous work showed that FUS associates with both RNA polymerase II (RNAP II) and the essential splicing factor U1 small nuclear ribonucleoprotein (snRNP). Here we were able to directly investigate the functional significance of these interactions using an in vitro system. We show that FUS is essential for the interaction between U1 snRNP and RNAP II and that FUS must be present during the RNAP II transcription reaction in order for splicing to occur. Together, these data indicate that FUS mediates an interaction between RNAP II and U1 snRNP, thereby physically and functionally coupling transcription to splicing.

Author contributions: Y.Y. and R.R. designed research; Y.Y. performed research; Y.Y. and R.R. analyzed data; and Y.Y. and R.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1506282112/-/DCSupplemental.
analysis revealed that the U1 AMO specifically inhibited splicing, but not transcription, and in a dose-dependent manner (Fig. S1). Moreover, the U1 AMO inhibited spliceosome assembly, and, instead, a faster migrating complex was detected (Fig. S1). Continued incubation did not allow proper spliceosome assembly from this complex (Fig. S1). Furthermore, the U1 AMO blocked both splicing and spliceosome assembly only when the AMO was added to the coupled system before, but not after, transcription (Fig. S2). Finally, when CMV–Ftz was transcribed in the presence of the U1 AMO, followed by dilution into a chase reaction in normal nuclear extract, splicing did not occur (Fig. S2). Together, these data using the U1 AMO indicate that U1 snRNP must be loaded onto pre-mRNA during the transcription reaction in order for efficient spliceosome assembly and splicing to occur. In previous work, we found that SR proteins, which associate with U1 snRNP, also must be recruited to pre-mRNA during the RNAP II transcription reaction in order for splicing to occur (19). In addition, we and others found that U1 snRNP/SR proteins are the only essential splicing factors that associate with RNAP II, and this association occurs even when RNAP II is not present at transcription promoters (18–20). Because U1 snRNP/SR proteins are recruited to pre-mRNA during the earliest steps in the spliceosome assembly pathway (21), the data lead to a model in which U1 snRNP/SR proteins bound to RNAP II are recruited to promoters, and then, while the nascent pre-mRNA is being synthesized, these splicing factors are recruited to the 5′ splice site and adjacent exon to initiate spliceosome assembly. This close physical and functional relationship between RNAP II and U1 snRNP/SR proteins provides a likely explanation for the potent enhancement of spliceosome assembly and splicing observed both in vivo and in vitro when pre-mRNAs are transcribed by RNAP II vs. other polymerases (12–15, 36, 37).

The U1 AMO Disrupts RNAP II Interaction with U1 snRNP, but Not with FUS. To further investigate the significance of the association between U1 snRNP and RNAP II, we next asked whether the U1 AMO affected this interaction. In addition, we investigated the effect of the U1 AMO on the association of FUS with RNAP II, because FUS interacts with both U1 snRNP and RNAP II. Nuclear extracts were treated with the control or U1 AMO, followed by immunoprecipitations (IPs) with a control antibody or an antibody against RNAP II. As expected, analysis of the proteins revealed that both FUS and U1 snRNP components (U1-70K, U1A, and U1C) were specifically communoprecipitated with RNAP II from extracts treated with the control AMO (Fig. 1B, lanes 1 and 2).
However, when the same IPs were carried out by using extracts treated with the U1 AMO, the U1 snRNP components were specifically lacking (Fig. 1B, lanes 3 and 4). In contrast, FUS levels were the same in nuclear extract treated with either the control or U1 AMOs (Fig. 1B, lanes 2 and 4). These results, showing loss of U1 snRNP components in the presence of the U1 AMO and no effect on FUS, were also observed on Western blots (Fig. 1C). snRNAs and 5S rRNA are indicated. (D–F) Same as A–C, except that FUS antibody was used for the IPs.

Fig. 2. U1 AMO disrupts the interaction between FUS and U1 snRNP. (A) Coomassie-stained gel showing the proteins immunoprecipitated by a control antibody (lanes 1 and 3) or an antibody against U1C (lanes 2 and 4) after nuclear extract was incubated with the control (lanes 1 and 2) or U1 (lanes 3 and 4) AMO. FUS, the U1 snRNP proteins, and molecular-mass markers (in kDa) are shown. (B and C) Same as A, except that proteins were analyzed by Western (B) or RNA was analyzed by ethidium bromide staining (C). snRNAs and 5S rRNA are indicated. (D–F) Same as A–C, except that FUS antibody was used for the IPs.

Interaction of FUS with U1 snRNP Is Blocked by the U1 AMO. We next used the AMOs to investigate the interaction between U1 snRNP and FUS. To undertake this investigation, we first used antibodies against U1C to carry out an IP of U1 snRNP, followed by analysis of the proteins on a Coomassie-stained gel (Fig. 2D). This analysis revealed that the U1 snRNP proteins and
FUS were immunoprecipitated in extracts treated with the control AMO (Fig. 2A, lane 2). In marked contrast, FUS was not immunoprecipitated when extracts were treated with the U1 AMO (Fig. 2A, lane 4). We also observed a decrease in levels of U1-70K and U1A when U1 snRNP was immunoprecipitated in the U1 AMO–treated extracts, but no effect was detected on the levels of the Sm snRNP core components. The same results were observed on Western blots of the U1 snRNP IP in the AMO–treated extracts. Importantly, these data revealed that FUS was associated with U1 snRNP when the control AMO was used, but was specifically and completely dissociated from U1 snRNP when the U1 AMO was used (Fig. 2B, compare lanes 3 and 5). As expected from the Coomassie data (Fig. 2A), U1 snRNA was detected on an ethidium bromide–stained gel in the U1C IP when extracts were treated with either the control or U1 AMO (Fig. 2C).

To further examine the effect of the U1 AMO on the U1 snRNP–FUS interaction, we carried out FUS IPs from extracts treated with the AMOs and examined the IPs on a Coomassie–stained gel. This analysis also revealed that the U1 AMO causes a striking loss of association between FUS and U1 snRNP (Fig. 2D). The same results were obtained by Western analysis of the FUS IPs (Fig. 2E). Moreover, U1 snRNA was present in the FUS IP when carried out in the extract treated by the control AMO, but was completely lost when the FUS IPs were carried out in extracts treated with the U1 AMO (Fig. 2F, lanes 4 and 6). We conclude that the interaction between FUS and U1 snRNP is completely blocked in extracts treated with the U1 AMO. This AMO base-pairs to the 5′ portion of U1 snRNA (Fig. L4), and it is possible that this base pairing affects the conformation of U1 snRNP. This conformational change may be responsible for the complete dissociation of FUS from U1 snRNP, as well as lead to the decreased levels of U1-70K and U1A that we observed after treatment with this AMO. Whether the splicing block observed with the U1 AMO in the previous work (34, 35) and in our work reported here is due to disruption of base pairing between U1 snRNA and the 5′ splice site and/or due to the disruption of the proteins associated with U1 snRNP remains to be established.

**FUS Is Required for the Interaction Between RNAP II and U1 snRNP.** Our data presented above reveal that FUS still interacts with RNAP II in the presence of the U1 AMO, whereas U1 snRNP does not interact with either FUS or RNAP II in the presence of the U1 AMO. Thus, we next investigated the possibility that FUS might mediate the interaction between RNAP II and U1 snRNP (Fig. 3A). To test this hypothesis, we knocked down FUS in HeLa cells with shRNA, using a scrambled shRNA as a control. Tubulin was a loading control. (C) IP/Westerns were carried out with control (Cntl; lanes 3 and 4) or FUS (lanes 5 and 6) KD small-scale nuclear extracts. The respective inputs are shown in lanes 1 and 2. (D) Same as C, except that total RNA was isolated from the IPs with the indicated antibodies and end-labeled with 32pCp. Bands were detected by phosphorimager.

**A Role for FUS in Coupling Transcription to Splicing.** We next used the FUS KD nuclear extracts to investigate the role of FUS in the coupled txn/splicing reaction. When CMV–Ftz (Fig. S1A) was transcribed in the FUS or control KD extracts, pre-mRNA was efficiently generated by the 10-min time point (Fig. 4A, lanes 1 and 3). By 30 min of incubation, the pre-mRNA was efficiently converted into spliced mRNA in the control nuclear extract (lane 2). In contrast, in the FUS KD extract, splicing was inhibited, which can be seen by comparing the level of unspliced pre-mRNA to spliced mRNA (Fig. 4A, lane 4). In addition, the splicing intermediates (exon 1 and lariat–exon 2) accumulate to higher levels in the FUS KD extract than in the control extract (Fig. 4A, compare lanes 2 and 4), suggesting that the low levels of FUS remaining in the KD extract allow some splicing, but with delayed kinetics. To further investigate the role of FUS in the coupled txn/splicing reaction, we performed a chase assay in which the CMV–Ftz DNA template was first transcribed in the control or FUS KD nuclear extracts, followed by an eightfold dilution into normal nuclear extract and continued incubation. As shown in Fig. 4B, when pre-mRNA was transcribed in the control KD extract, it was efficiently spliced when chased in normal nuclear extract (lanes 1 and 2). In contrast, when pre-mRNA was transcribed in FUS KD nuclear extract, it was not spliced when chased in normal nuclear extract (lanes 3 and 4). We also carried out the reciprocal experiment in which pre-mRNA was transcribed in normal nuclear extract (Fig. 4C, lane 2). In marked contrast, FUS was not immunoprecipitated when extracts were treated with the U1 AMO (Fig. 2A, lane 4). We also observed a decrease in levels of U1-70K and U1A when U1 snRNP was immunoprecipitated in the U1 AMO–treated extracts, but no effect was detected on the levels of the Sm snRNP core components. The same results were observed on Western blots of the U1 snRNP IP in the AMO–treated extracts. Importantly, these data revealed that FUS was associated with U1 snRNP when the control AMO was used, but was specifically and completely dissociated from U1 snRNP when the U1 AMO was used (Fig. 2B, compare lanes 3 and 5). As expected from the Coomassie data (Fig. 2A), U1 snRNA was detected on an ethidium bromide–stained gel in the U1C IP when extracts were treated with either the control or U1 AMO (Fig. 2C).

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Fig. 4. FUS is required during the transcription reaction to promote splicing. (A) CMV–Ftz was incubated under txn/splicing conditions in the control (Cntl; lanes 1 and 2) or FUS (lanes 3 and 4) KD nuclear extracts for 10 and 30 min. Splicing intermediates and products are indicated. (B) CMV–Ftz was incubated under txn/splicing conditions for 10 min to allow transcription in control (lane 1) or FUS (lane 3) KD nuclear extracts followed by addition of α-amanitin. An aliquot of the transcription reaction was then diluted eightfold into fresh nuclear extract and incubated under txn/splicing conditions for an additional 20 min to allow splicing (lanes 2 and 4). (C) Same as B, except that transcription was carried out for 10 min in the normal nuclear extract (lanes 1 and 3) followed by dilution and continued incubation in the control (lane 2) or FUS (lane 4) knockdown nuclear extracts. (D) CMV–Ftz DNA template was incubated for 5 min to allow transcription, followed by continued incubation for 20 min to allow splicing, whereas naked T7–Ftz pre-mRNA was incubated for 0 and 20 min.

1and 3) and chased in the control or FUS KD nuclear extract. This analysis revealed that the pre-mRNA was efficiently spliced when chased in either the control or FUS KD nuclear extract (Fig. 4C, lanes 2 and 4). Thus, if the pre-mRNA is transcribed in the presence of available FUS, then splicing occurs efficiently in normal nuclear extract. However, if the pre-mRNA is transcribed in the absence of available FUS, then it does not splice in normal nuclear extract. These data indicate that the function of FUS in splicing has to take place during the transcription reaction. This conclusion is further supported by the observation that FUS KD has no significant effect on uncoupled splicing using naked T7–Ftz pre-mRNA, whereas coupled splicing of CMV–Ftz is inhibited in the same FUS KD extract (Fig. 4D).

The data presented here, together with previous observations showing that FUS associates with both RNAP II and U1 snRNP, lead to the model that FUS functions in coupling transcription to splicing via mediating an interaction between RNAP II and U1 snRNP. Considering that transcription-coupled splicing is such a fundamental cellular process, it is possible that its disruption by mutant FUS contributes to the pathogenesis of ALS. In support of this possibility, many of the mutations in FUS that are ALS-causative are located in the nuclear localization signal (NLS), which leads to mislocalization of FUS to the cytoplasm in ALS patient cells (29, 38, 39). Moreover, we recently obtained evidence that the U1 snRNP core complex (U1 snRNA and the Sm proteins) is comislocalized to the cytoplasm with FUS in ALS patient fibroblasts harboring mutations in the NLS (40). Thus, decreased levels of both FUS and U1 snRNP in the nucleus may lead to the aberrant splicing that has been reported in transfected cells and ALS patient cells expressing FUS with NLS mutations (29, 30, 38, 39). In previous work, FUS was shown to bind to the C-terminal domain of RNAP II and regulate phosphorylation of serine-2 (23, 25, 27). Moreover, when FUS is knocked down, RNAP II accumulates at the start site of transcription, and premature polyadenylation is observed (23, 25). Notably, U1 snRNP has been found to play other important roles in addition to its function in splicing. One of these roles is known as telescripting, in which binding of U1 snRNP to nascent pre-mRNAs is required to prevent premature polyadenylation during transcription (34, 41). The lack of telescripting, which was found to occur on antisense transcripts due to the presence of more polyA sites and fewer 5’ splice sites, is thought to be a mechanism for suppressing transcription of antisense RNAs (35). Thus, the observation that premature polyadenylation occurs with FUS
KD (23, 25), together with our observation that FUS mediates an interaction between U1 snRNP and RNAPII, suggests that the RNAPII–FUS–U1 snRNP complex is the entity that functions in telomere signaling. The data also raise the possibility that defective telomere signaling may contribute to ALS pathogenesis.

Materials and Methods

Plasmids and Antibodies

The plasmid encoding CMV–Ftz was described (13). The templates for RNAPII transcription were prepared as described (13, 16). Antibodies to U1-70K (9C4.1) were from Millipore; U1C (H1C12) was from Sigma; U1A (Bj-7), HA, and tubulin were from Santa Cruz; RNAPII II (BWG16) was from Covance; FUS (293A) was from Bethyl; and SmB/B (Y12), mouse IgG, and rabbit IgG were from Abcam. Mouse IgG, rabbit IgG, and HA were used as negative controls for polyclonal and monoclonal antibodies, respectively. The FUS rabbit polyclonal antibody was described (22).

RNAPII Transcription and Splicing

CMV–Ftz DNA was incubated under in vitro splicing conditions as described (13, 17). For AMO assays, control or U1 AMOs (34, 35) were added to HeLa cell nuclear extract at a final concentration of 12 μM. Control or FUS KD nuclear extracts were prepared by using the small-scale nuclear extract procedure (17, 33). For spliceosome assembly, the CMV–Ftz DNA template was incubated under in vitro splicing conditions for 10 min. G-50 columns were used to remove unincorporated [3H]-UTP. A total of 1 μL of heparin (6.5 g/L) was added to 10 μL of G-50 column-purified reactions before loading on 1.2% low-melting-point agarose gels (42). Uncoupled splicing of naked T7–pre-mRNA shown in Fig. 4D was carried out in control or FUS KD nuclear extracts side-by-side under identical conditions as used for transsplicing with the CMV–DNA template.

IPs

Antibodies were coupled to protein A Sepharose and then covalently cross-linked beads. After rotation overnight at 21°C, the unbound antibodies were washed with washing buffer (1X PBS, 0.1% Triton X-100, 0.2 mM PMFS, protease inhibitor EDTA-free (Roche), spun at 4°C for 5 min, and added to 40 μL antibody–cross-linked beads. After rotation overnight at 4°C, six washes (1.5 mL each) were performed by using wash buffer (1X PBS containing 0.1% Triton X-100, 0.2 mM PMFS, pH 8.0). Proteins were eluted by adding 60 μL of SDS sample loading buffer, followed by incubation for 20 min at room temperature. DTT was added to a final concentration of 5 μM, and samples were boiled for 10 min and loaded on 4–12% SDS gel. For RNA IPs, total RNA was isolated and analyzed on 6.5% denaturing gels stained with ethidium bromide.

Acknowledgments

We thank Binkai Chi, Shanye Yin, Edward Adams, and other members of the R.R. laboratory for useful discussion. This work was supported by National Institutes of Health Grant GM043372 (to R.R.) and ALS Therapy Alliance Grant 2013-3-006 (to R.R.). Hela cells were obtained from the National Cell Culture Center C. Biovec International.

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PNAS | July 14, 2015 | vol. 112 | no. 28 | 8613
Fig. S1. An AMO targeting U1 snRNA inhibits splicing and spliceosome assembly in the coupledtxn/splicing system. (A) Schematic of CMV–Ftz DNA template. The black boxes indicate the exons, and the line indicates the intron. The sizes of the exons and introns are shown. (B) The indicated amounts of control (Cntl; lanes 1–6) or U1 AMOs (lanes 7–12) were added to HeLa cell nuclear extract before carrying out the coupled txn/splicing reaction. (C and D) Cntl or U1 AMO (12 μM) were incubated in nuclear extract for 10 (lanes 1 and 2) or 30 (lanes 3 and 4) minutes and then analyzed on a nondenaturing gel (C) or on a native agarose gel (D). The asterisks in D indicate the faster-migrating complex that was generated in the presence of the U1 AMO.
**Fig. S2.** The U1 AMO inhibits splicing and spliceosome assembly when added before, but not after, transcription. (A) No AMO (lanes 1 and 2), control AMO (Cntl, lanes 2–5) or U1 AMO (lanes 7–10) was added to HeLa cell nuclear extract before or after 10 min of transcription of CMV–Ftz, followed by continued incubation for 20 min. The splicing intermediates and products are indicated. The line below the lariat intron marks breakdown of the lariat intron. (B) Same as A, except that spliceosome assembly (10-min time point) was analyzed on an agarose gel. The asterisk indicates the faster migrating complex that was generated in the presence of the U1 AMO. (C) The CMV–Ftz DNA template was transcribed in the presence of the Cntl or U1 AMO, followed by a 10-fold dilution in normal nuclear extract to allow a chase for 20 additional minutes.