

RRM but not the Asp/Glu domain of hnRNP C₁/C₂ is required for splicing regulation of Ron exon 11 pre-mRNA

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The Ron proto-oncogene is a human receptor for macrophage-stimulating protein (MSP). The exclusion of exon 11 in alternative splicing generates Δ RON protein that is constitutively activated. Heterogenous ribonucleoprotein (hnRNP) C₁/C₂ is one of the most abundant proteins in cells. In this manuscript, we showed that both hnRNP C₁ and C₂ promoted exon 11 inclusion of Ron pre-mRNA and that hnRNP C₁ and hnRNP C₂ functioned independently but not cooperatively. Moreover, hnRNP C₁ stimulated exon 11 splicing through intron 10 activation but not through intron 11 splicing. Furthermore, we showed that, whereas the RRM domain was required for hnRNP C₁ function, the Asp/Glu domain was not. In conclusion, hnRNP C₁/C₂ promoted exon 11 splicing independently by stimulating intron 10 splicing through RRM but not through the Asp/Glu domain. [BMB Reports 2019; 52(11): 641-646]

INTRODUCTION

Pre-mRNA splicing occurs in a large RNA-protein complex called a spliceosome (1). Spliceosome assembly is a stepwise process in which U1 snRNP basepairs with a 5' splice-site, then U2 snRNP basepairs with a branch-point. Then, the U4/U5/U6 snRNP is loaded into the complex. Through alternative exon inclusion/exclusion, proteins with slightly different, completely different, and opposite functions are

produced (2-4).

Ron is a cell surface-located receptor composed of disulfide-linked α and β subunits, which are produced from a 190 kDa single chain precursor by proteolytic cleavage (5). Δ RON is an uncleaved protein isoform lacking 49 amino acids in the extracellular domain, which is produced by the exclusion of exon 11 in alternative splicing procedures (6). For Δ RON is unable to bind the ligand, it must be constitutively activated by tyrosine phosphorylation through intracellular oligomerization (6, 7). SRSF1, SRSF2, and hnRNP A1 have been demonstrated to regulate exon 11 splicing (8-10).

Heterogeneous nuclear ribonucleoprotein (hnRNP) C₁/C₂ is one of the most abundant proteins in cells (11). hnRNP C₁/C₂ includes an N-terminal RNA recognition motif (RRM), a basic leucine zipper (bZIP)-like motif (bZLM), and an acidic aspartic acid/glutamic acid-rich (Asp/Glu) domain (UniprotKB - P07910) (12). While RRM plays a minimal role in the overall affinity of hnRNP C₁/C₂ for RNA, a highly basic 40 amino acid (aa) domain preceding the leucine zipper motif provides high-affinity for RNA (13, 14). An acidic Asp/Glu domain is necessary for homo- or heterotetramer formation of hnRNP C₁ and C₂ (13). hnRNP C₁/C₂ inhibits splicing of the Alu element in the human genome by interfering with U2AF⁶⁵ binding to the cryptic PPT sequence (15).

Here, we show that hnRNP C₁/C₂ promoted exon 11 inclusion of Ron pre-mRNA through stimulation of intron 10, but not intron 11, splicing. In addition, we found that hnRNP C₁ and hnRNP C₂ functioned independently, not cooperatively. Importantly, although the RRM domain was required for hnRNP C₁/C₂ function, the Asp/Glu domain of hnRNP C₁ was not necessary for promoting Ron exon 11 splicing.

RESULTS

Reduced hnRNP C₁/C₂ expression inhibits exon 11 splicing of Ron pre-mRNA

In order to understand the role of hnRNP C₁/C₂ in the splicing of Ron pre-mRNA, we investigated whether reduced expression of hnRNP C₁/C₂ affected Ron alternative splicing. Fig. 1A shows that hnRNP C₁/C₂-targeted shRNA treatment reduced both hnRNP C₁ and hnRNP C₂ expression analyzed

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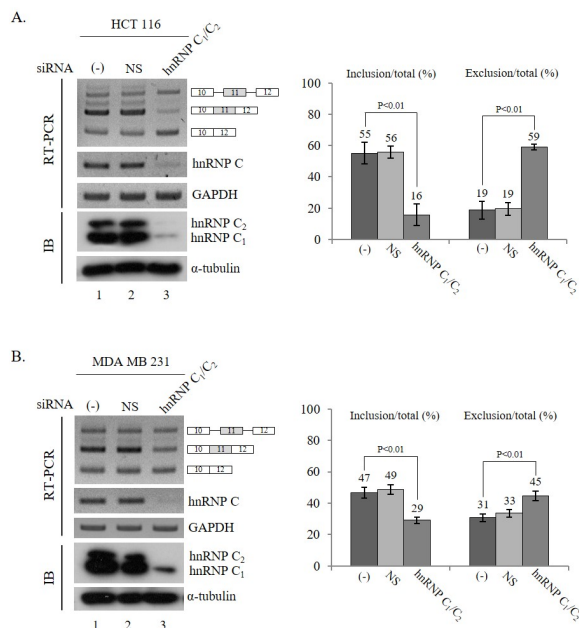


Fig. 1. Reduced hnRNP C₁/C₂ expression inhibits exon 11 splicing of Ron pre-mRNA. RT-PCR analysis of Ron exon 11 splicing was performed with RNAs extracted from untreated, non-silencing shRNA, and hnRNP C₁/C₂ shRNA lentivirus-treated HCT 116 (A) and MDA MB 231 (B) cells. The exon 11 included, skipped, and unspliced isoforms are shown as indicated. The reduced expression of hnRNP C₁/C₂ is demonstrated by RT-PCR and immunoblotting using anti-hnRNP C₁/C₂ antibody, with GAPDH and α -tubulin as controls. The quantitation results of exon 11 inclusion or exclusion in total RNA are shown in the right panel.

by RT-PCR and immunoblotting analysis (left panel). The results in Fig. 1A show that hnRNP C₁/C₂-targeting shRNA virus treatment induced a significant decrease in the exon 11-included isoform (~39%) and an increase in the exon 11-skipped isoform (~40%) in HCT 116 cells. Thus, reduced hnRNP C₁/C₂ expression inhibited exon 11 splicing in HCT 116 cells. Similarly, as shown in Fig. 1B (left panel), shRNA-mediated knockdown also successfully reduced hnRNP C₁/C₂ expression in MDA MB 231 cells (lane 3) and reduced hnRNP C₁/C₂ expression suppressed exon 11 inclusion (~18%). Taken together, these results demonstrate that reduced expression of hnRNP C₁/C₂ inhibited exon 11 inclusion of Ron pre-mRNA.

hnRNP C₁ and C₂ promotes exon 11 of Ron pre-mRNA independently

Next, we investigated whether increased expression of hnRNP C₁ or hnRNP C₂ had the opposite effect of reduced hnRNP C₁/C₂ expression on exon 11 splicing. In order to address the question, an hnRNP C₁ or C₂ expression plasmid and Ron exon 10-12 mini-gene were co-transfected into MDA MB 231 cells. Fig. 2A shows that hnRNP C₁ and C₂ promoted an

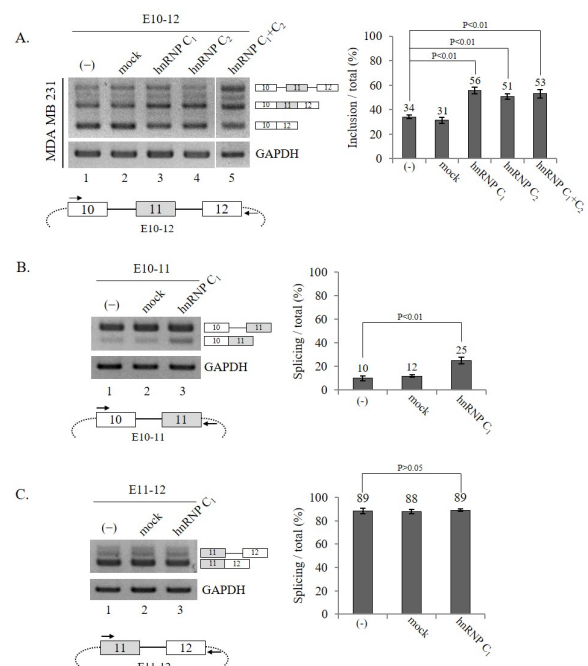


Fig. 2. hnRNP C₁ and hnRNP C₂ promote exon 11 splicing of Ron pre-mRNA independently. (A) (Upper panel) Shown are RT-PCR results of Ron exon 11 splicing from the E10-12 mini-gene in MDA MB 231 cells expressing hnRNP C₁, hnRNP C₂, or both hnRNP C₁ and C₂. The quantitation results are shown on the right. (Lower panel) The scheme of Ron mini-gene (E10-12) is shown. The intronic RNA is represented with solid lines and the vector sequence is shown as dot lines. The primers used to detect the mini-gene splicing are shown with arrows. (B, C) hnRNP C₁ promotes intron 10 but not intron 11 splicing of Ron pre-mRNA. (B) (Upper panel) RT-PCR analysis of intron 10 splicing from cells expressing hnRNP C₁ is shown with GAPDH as a loading control. The quantitation results are shown on the right. (Lower panel) The scheme of the E10-11 mini-gene is shown. The intronic RNA is represented with solid lines and the vector sequence is shown as dotted lines, and the primers used to detect intron 10 splicing are shown as arrows. (C) (Upper panel) RT-PCR analysis of intron 11 splicing is shown, with GAPDH as a loading control. The quantitation results are shown in the right panel. (Lower panel) The scheme of the E11-12 mini-gene is shown. The primers used to detect intron 11 splicing are shown with arrows.

increase in exon 11 inclusion (~22% or ~17% independently, respectively) to similar levels, which was the opposite effect of hnRNP C₁/C₂ knockdown (lane 3 and 4). In addition, the results also indicate that the 13 amino acids included only in hnRNP C₂ did not play a significant role in this activity.

In addition to forming homo-tetramers by themselves, hnRNP C₁ and C₂ have also been shown to form a hetero-tetramer with a 3:1 ratio (16). We, therefore, asked if hnRNP C₁ and C₂ could promote Ron exon 11 splicing cooperatively. To answer this question, we introduced both hnRNP C₁ and C₂ expression plasmids with the Ron mini-gene

into cells. The results in Fig. 2A show that the co-expression of hnRNP C₁ and C₂ had similar effects as the individual expression of either the hnRNP C₁ or C₂ plasmid (lane 5). Thus, we conclude that these two proteins did not synergistically increase either hnRNP C₁ or C₂ expression. By combining the results in Fig. 1 and 2A, we conclude that hnRNP C₁ and C₂ functioned similarly to promote exon 11 inclusion of Ron pre-mRNA and that the 13 amino acids in hnRNP C₂ were not required for the function. To simplify our studies, we decided to examine only the roles of hnRNP C₁ in this report.

hnRNP C₁ promotes intron 10 but not intron 11 splicing of Ron pre-mRNA

We asked if hnRNP C₁ affected splicing of intron 10 or intron 11 in Ron pre-mRNA. To detect intron 10 splicing, we applied a mini-gene in which only exon 10 to exon 11 sequences were included (E10-11, lower panel, Fig. 2B). Using this mini-gene, we performed RT-PCR with primer pairs corresponding to exon 10 and the downstream vector sequence. The results in Fig. 2B demonstrate that hnRNP C₁ expression increased the intron 10-spliced isoform significantly (~15%, lane 3). Thus, hnRNP C₁ promotes intron 10 splicing. We next analyzed intron 11 splicing using another mini-gene, which includes exons 11-12 (E11-12). The primer pairs that corresponded to exon 11 and the vector sequences were used to detect intron 11 splicing (Fig. 2C, lower panel). The results in Fig. 2C show that intron 11 was almost completely spliced in the E11-12 mini-gene (lane 1). Thus, we expect that a further increase in exon 11-spliced products would be hard to detect. The results in Fig. 2C show that intron 11 splicing was not altered by the hnRNP C₁ treatment. The results in Fig. 2B and 2C indicate that hnRNP C₁ affected intron 10 but not intron 11 splicing. Therefore, we conclude that hnRNP C₁ promoted exon 11 inclusion through activation of intron 10 splicing.

Conserved splice-site sequences of exon 11 do not affect hnRNP C₁ function

We further asked if splicing sites flanking exon 11 would regulate the effect of hnRNP C₁ on the exon 11-inclusion of Ron pre-mRNA. To test this possibility, we used two previously described mutant mini-gene constructs (10) in which either the 5' splice-site or the 3' splice-site was mutated into conserved sequences (5'-cons or 3'-cons, Fig. 3A). Consistent with our previous reports, more conserved splice-sites sequences in exon 11 facilitated exon 11 inclusion significantly (10), leading to the predominant production of exon 11-included isoforms by these two mutants (lane 1 of Fig. 3B and 3C). However, the results in Fig. 3B demonstrate that a mutation of the 5' splice-site in a conserved sequence did not disrupt hnRNP C₁ function on Ron exon 11 splicing because hnRNP C₁ still promoted exon 11 inclusion in the 5'-cons mutant. In the 3'-cons mutant, since the exon 11-excluded

isoform was not detectable, a decrease in the exon 11-skipped form was not observable. Taken together, we conclude that conserved splice-site sequences of exon 11 did not affect the role of hnRNP C₁ in exon 11 splicing.

The Asp/Glu domain is dispensable for hnRNP C₁ function in Ron exon 11 splicing

hnRNP C₁ includes RRM, bZLM, and Asp/Glu domains. While the RRM domain is required for RNA binding, the Asp/Glu domain is required for the formation of tetramers of hnRNP C₁. To determine if these two domains were necessary for hnRNP C₁-mediated Ron exon 11 splicing, we produced two hnRNP C₁ mutants, in which either the RRM domain or the Asp/Glu domain was deleted (Δ RRM, Δ Asp/Glu) (Fig. 4A). The results in Fig. 4B show that the Δ RRM mutant protein was not able to promote exon 11 inclusion (lane 4). Thus, the RRM domain was required for the role of hnRNP C₁ in exon 11 splicing. This was not unexpected because the RRM domain is required for the binding target RNA in exon 10. In contrast, we found

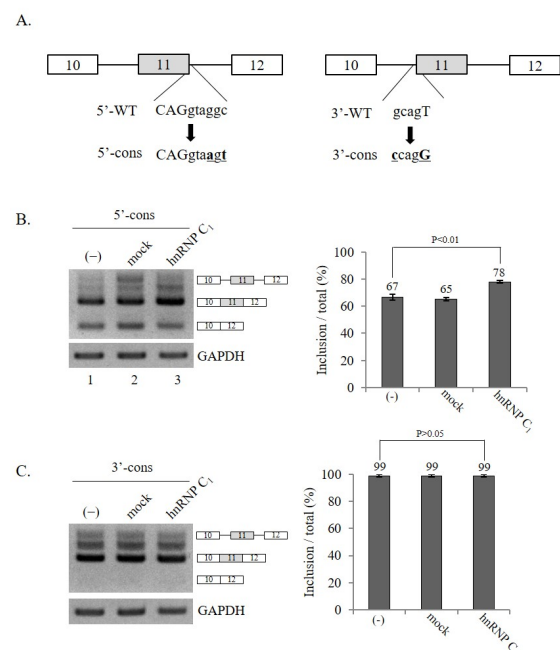


Fig. 3. Conserved splice-site sequences of exon 11 do not affect hnRNP C₁ function. (A) (Left panel) 5' splice-site sequence of Ron exon 11 (5'-WT) and its conserved splice-site mutant sequence are shown (5'-cons). (Right panel) 3' splice-site sequence of Ron exon 11 (3'-WT) and its conserved splice-site mutant sequence are shown (3'-cons). (B) RT-PCR analysis of exon 11 splicing using 5'-cons mini-gene in hnRNP C₁-expressing cells. GAPDH was used as a loading control. The quantitation of exon 11 inclusion in total RNA is shown. (C) RT-PCR analysis of exon 11 splicing using 3'-cons mini-gene in hnRNP C₁-expressing cells. GAPDH was used as a loading control. The quantitation of exon 11 inclusion in total RNA is shown.

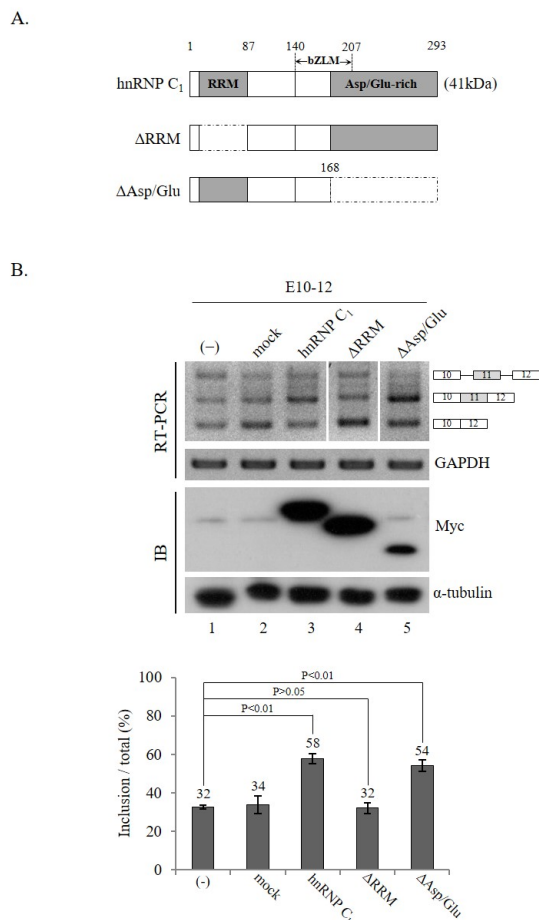


Fig. 4. The Asp/Glu domain is dispensable for the function of hnRNP C₁ in Ron exon 11 splicing. (A) Scheme of the hnRNP C₁ protein domains is shown. RRM and Asp/Glu domain are shown with gray boxes. Parts deleted in the ΔRRM and ΔAsp/Glu mutants are shown in dotted line-framed boxes. (B) RT-PCR analysis of exon 11 splicing using RNAs extracted from cells expressing ΔRRM and ΔAsp/Glu mutant proteins. Expression of myc-tagged wild-type hnRNP C₁, ΔRRM, or ΔAsp/Glu mutant protein is confirmed with anti-myc antibody. The quantitation of exon 11 inclusion in total RNA is demonstrated in the lower panel.

that the ΔAsp/Glu mutant of hnRNP C₁ was still capable of promoting exon 11 inclusion (lane 5). Therefore, we conclude that the Asp/Glu domain was not needed for the role of hnRNP C₁ in Ron exon 11 splicing. The results indicate that tetramer formation was not required for the role of hnRNP C₁ in Ron exon 11 alternative splicing.

DISCUSSION

We previously demonstrated that SRSF2 regulated Ron exon 11 splicing by contacting exon 11 sequences (10). In this study, by using shRNA-mediated knockdown and

overexpression, we showed that hnRNP C₁/C₂ promoted Ron exon 11 splicing by stimulating intron 10 but not intron 11 splicing. Moreover, hnRNP C₁ and C₂ played roles in Ron exon 11 splicing independently but not synergistically, demonstrated by experiments using cells co-expressing both proteins. Furthermore, the acidic Asp/Glu domain required for tetramer formation was not necessary for the role of hnRNP C₁ in Ron splicing.

Ron pre-mRNA splicing is regulated by multiple proteins, including SRSF1, hnRNP A1, and SRSF2, through various sequences on Ron pre-mRNA (8-10). Whereas SRSF1 and hnRNP A1 target exon 12 RNA and SRSF2 targets exon 10 sequences. However, in spite of these studies, it is still unclear if SRSF1, hnRNP A1, SRSF2, and hnRNP C₁ function cooperatively in Ron exon 11 splicing, spliceosome assembly, and/or splice-site selection. Moreover, how and why these different proteins only target their respective sequences on Ron RNA, but not other potential binding sequences at other locations, need to be answered.

Although the role of hnRNP C₁/C₂ was demonstrated in an *in vitro* study (17), their functions in alternative splicing have not yet been elucidated in great detail. In addition, a previous study found that hnRNP C₁/C₂ was not necessary for viability (18). Thus, it is likely that hnRNP C₁/C₂ played redundant roles in splicing in the cells. In this study, we presented direct evidence that hnRNP C₁/C₂ regulated alternative splicing using cells in which hnRNP C₁/C₂ was either overexpressed or suppressed. Our results, as well as a few other reports, demonstrated that hnRNP C₁/C₂ was an essential regulatory protein of alternative splicing (19-21). However, previous studies of hnRNP C₁/C₂ were primarily based on large scale sequencing and screening, therefore, mechanistic insight from those studies was limited. The results in our study showed that reduced hnRNP C₁/C₂ expression induced a much more significant change in exon 11 splicing compared to hnRNP C₁/C₂ overexpression. The difference can be explained by the fact that hnRNP C₁/C₂ is one of the most abundant proteins in cells (11). Thus, shRNA treatment induced a significant decrease in hnRNP C₁/C₂ expression. Although we used various approaches, we were not able to show that the endogenous Ron exon 11 splicing was affected by hnRNP C₁/C₂ overexpression. Nonetheless, the transient expression of hnRNP C₁/C₂, along with the Ron mini-gene, demonstrated that it affected exon 11 splicing but to a much lesser extent than the effect seen from the knockdown.

hnRNP C₁ and C₂ are able to form homo- or heterotetramers (13). However, it seems that the role of hnRNP C₁/C₂ in Ron splicing was not required for tetramer formation, based on two pieces of evidence. First, hnRNP C₁ and C₂ did not function cooperatively in alternative splicing of Ron pre-mRNA, but rather independently. Second, the acidic Asp/Glu domain that is essential for tetramer formation was dispensable in Ron exon 11 splicing. In addition, we showed that the RRM

domain was required for the function of hnRNP C₁, which was not surprising. However, what was striking is that the long Asp/Glu domain was not necessary for Ron splicing, although it was previously shown to be essential for tetramer formation and that the hnRNP C tetramer was important for mRNA transport (22). Therefore, the role of Asp/Glu in Ron pre-mRNA splicing cannot be established. However, whether the Asp/Glu domain is required for other pre-mRNA splicing is still unknown. It is also possible that the Asp/Glu domain plays regulatory roles in alternative splicing.

MATERIALS AND METHODS

Plasmid construction

The coding region of hnRNP C₁, C₂ was inserted into a pcDNA6/myc-His A (Invitrogen) plasmid. The ΔAsp/Glu and ΔRRM mutants of hnRNP were produced by overlapping PCR using the hnRNP C₁ expression plasmid as a template.

RT-PCR

Total RNA was extracted using RiboEx (GeneAll) as previously described (23). Reverse transcription was performed using 0.5 μg RNA with oligo (dT) primer and ImProm-II™ reverse transcriptase (Promega). The reaction mixture (0.5 μl) was amplified by PCR using G-Taq polymerase (Cosmo Genetech).

Purification of hnRNP C₁ protein

Total protein was extracted from HEK293 cells transfected with the pcDNA6/myc-His A-hnRNP C₁ plasmid by 30 min incubation with lysis buffer (50 mM NaH₂PO₄, 500 mM NaCl, 5 mM imidazole, 0.5% Tween-20, and 1 mM PMSF). Prewashed Ni-NTA agarose beads (QIAGEN) were added to the lysates and the mixture was incubated overnight at 4°C in the binding buffer (50 mM NaH₂PO₄, 500 mM NaCl, 0.5% Tween-20, and 1 mM PMSF). After washing, the hnRNP C₁ protein was eluted from the Ni-NTA agarose beads using elution buffer (250 mM imidazole in binding buffer) for 20 min at 4°C.

Knockdown of hnRNP C₁/C₂ with shRNA

To generate shRNA lentivirus, 293T cells were transfected with an shRNA-harboring plasmid (Open Biosystems) and PSPAX2 and PMD2G helper plasmids using PEI reagent. The media was changed after 12 h and incubated for another 24 h. The lentivirus-containing supernatants were harvested with a 0.45 μm filter. To knock down the hnRNP C₁/C₂ expression, lentivirus-containing supernatants were added to the cells supplemented with 10 μg/ml polybrene. After 72 h infection, the RNAs were extracted for RT-PCR.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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