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**Corresponding Author:** Haihong Shen

**Authors:** Haihong Shen<sup>1,\*</sup>, Heegyum Moon<sup>1,2</sup>, Ha Na Jang<sup>1</sup>, Yongchao Liu<sup>1</sup>, Namjeong Choi<sup>1</sup>, Jagyeong Oh<sup>1</sup>, Jiyeon Ha<sup>1</sup>, Hyeon Ho Kim<sup>3</sup>, Xuexiu Zheng<sup>1</sup>

**Institution:** <sup>1</sup>School of life Sciences, Gwangju Institute of Science and Technology,

<sup>2</sup>New Drug Development Center, Daegu-Gyeongbuk Medical Innovation Foundation,

<sup>3</sup>Department of Health Sciences and Technology, Samsung Advanced Institute for Health Sciences and Technology,

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**Author's name:** Heegyum Moon<sup>a, 1</sup>, Ha Na Jang<sup>a</sup>, Yongchao Liu<sup>a</sup>, Namjeong Choi<sup>a</sup>, Jagyeong Oh<sup>a</sup>, Jiyeon Ha<sup>a</sup>, Hyeon Ho Kim<sup>b</sup>, Xuexiu Zheng<sup>a,\*</sup> and Haihong Shen<sup>a,\*</sup>

**Affiliation:**

<sup>a</sup>School of Life Sciences, Gwangju Institute of Science and Technology, Gwangju 500-712, Korea.

<sup>1</sup>Current affiliation: New Drug Development Center, Daegu-Gyeongbuk Medical Innovation Foundation, 88 Dongnae-ro, Dong-gu, Daegu 41061, Korea.

<sup>b</sup>Department of Health Sciences and Technology, Samsung Advanced Institute for Health Sciences and Technology, Sungkyunkwan University, Seoul, Korea

**Running Title:** hnRNP C<sub>1</sub>/C<sub>2</sub> promotes exon 11 inclusion of Ron

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**Corresponding Author's Information:**

\*Corresponding author: Phone: 82-62-715-2507, Fax: 82-62-715-2484, E-mail

address:haihongshen@gist.ac.kr

\*Corresponding author: Phone: 82-62-715-2520, Fax: 82-62-715-2484, E-mail

address:xuexiuzheng@gist.ac.kr

**ABSTRACT**

The Ron proto-oncogene is a human receptor for macrophage-stimulating protein (MSP). The exclusion of exon 11 in alternative splicing generates  $\Delta$ RON protein that is constitutively activated. Heterogenous ribonucleoprotein (hnRNP) C<sub>1</sub>/C<sub>2</sub> is one of the most abundant proteins in cells. In this manuscript, we showed that both hnRNP C<sub>1</sub> and C<sub>2</sub> promoted exon 11 inclusion of Ron pre-mRNA and that hnRNP C<sub>1</sub> and hnRNP C<sub>2</sub> functioned independently but not cooperatively. Moreover, hnRNP C<sub>1</sub> 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 C<sub>1</sub> function, the Asp/Glu domain was not. In conclusion, hnRNP C<sub>1</sub>/C<sub>2</sub> promoted exon 11 splicing independently by stimulating intron 10 splicing through RRM but not through the Asp/Glu domain.

## 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  $\alpha$  and  $\beta$  subunits, which are produced from a 190 kDa single chain precursor by proteolytic cleavage (5).  $\Delta$ 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  $\Delta$ 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) C<sub>1</sub>/C<sub>2</sub> is one of the most abundant proteins in cells (11). hnRNP C<sub>1</sub>/C<sub>2</sub> 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 C<sub>1</sub>/C<sub>2</sub> 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 C<sub>1</sub> and C<sub>2</sub> (13). hnRNP C<sub>1</sub>/C<sub>2</sub> inhibits splicing of the Alu element in the human genome by interfering with U2AF<sup>65</sup> binding to the cryptic PPT sequence

(15).

Here, we show that hnRNP C<sub>1</sub>/C<sub>2</sub> promoted exon 11 inclusion of Ron pre-mRNA through stimulation of intron 10, but not intron 11, splicing. In addition, we found that hnRNP C<sub>1</sub> and hnRNP C<sub>2</sub> functioned independently, not cooperatively. Importantly, although the RRM domain was required for hnRNP C<sub>1</sub>/C<sub>2</sub> function, the Asp/Glu domain of hnRNP C<sub>1</sub> was not necessary for promoting Ron exon 11 splicing.

## RESULTS

### Reduced hnRNP C<sub>1</sub>/C<sub>2</sub> expression inhibits exon 11 splicing of Ron pre-mRNA

In order to understand the role of hnRNP C<sub>1</sub>/C<sub>2</sub> in the splicing of Ron pre-mRNA, we investigated whether reduced expression of hnRNP C<sub>1</sub>/C<sub>2</sub> affected Ron alternative splicing. Figure 1A shows that hnRNP C<sub>1</sub>/C<sub>2</sub>-targeted shRNA treatment reduced both hnRNP C<sub>1</sub> and hnRNP C<sub>2</sub> expression analyzed by RT-PCR and immunoblotting analysis (left panel). The results in Figure 1A show that hnRNP C<sub>1</sub>/C<sub>2</sub>-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 C<sub>1</sub>/C<sub>2</sub> expression inhibited exon 11 splicing in HCT 116 cells. Similarly, as shown in Figure 1B (left panel), shRNA-mediated knockdown also successfully reduced hnRNP C<sub>1</sub>/C<sub>2</sub> expression in MDA MB 231 cells (lane 3) and reduced hnRNP C<sub>1</sub>/C<sub>2</sub> expression suppressed exon 11 inclusion (~18%). Taken together, these results demonstrate that reduced expression of hnRNP C<sub>1</sub>/C<sub>2</sub> inhibited exon 11 inclusion of Ron pre-mRNA.

### hnRNP C<sub>1</sub> and C<sub>2</sub> promotes exon 11 of Ron pre-mRNA independently

Next, we investigated whether increased expression of hnRNP C<sub>1</sub> or hnRNP C<sub>2</sub> had the opposite effect of reduced hnRNP C<sub>1</sub>/C<sub>2</sub> expression on exon 11 splicing. In order to address the question, an hnRNP C<sub>1</sub> or C<sub>2</sub> expression plasmid and Ron exon 10-12 mini-gene were co-transfected into MDA MB 231 cells. Figure 2A shows that hnRNP C<sub>1</sub> and C<sub>2</sub> promoted an increase in exon 11 inclusion (~22% or ~17% independently, respectively) to similar levels, which was the opposite effect of hnRNP C<sub>1</sub>/C<sub>2</sub> knockdown (lane 3 and 4). In addition, the results also indicate that the 13 amino acids included only in hnRNP C<sub>2</sub> did not play a significant role in this activity.

In addition to forming homo-tetramers by themselves, hnRNP C<sub>1</sub> and C<sub>2</sub> have also been shown to form a hetero-tetramer with a 3:1 ratio (16). We, therefore, asked if hnRNP C<sub>1</sub> and C<sub>2</sub> could promote Ron exon 11 splicing cooperatively. To answer this question, we introduced both hnRNP C<sub>1</sub> and C<sub>2</sub> expression plasmids with the Ron mini-gene into cells. The results in Figure 2A show that the co-expression of hnRNP C<sub>1</sub> and C<sub>2</sub> had similar effects as the individual expression of either the hnRNP C<sub>1</sub> or C<sub>2</sub> plasmid (lane 5). Thus, we conclude that these two proteins did not synergistically increase **either hnRNP C<sub>1</sub> or C<sub>2</sub>** expression. By combining the results in Figures 1 and 2A, we conclude that hnRNP C<sub>1</sub> and C<sub>2</sub> functioned similarly to promote exon 11 inclusion of Ron pre-mRNA and that the 13 amino acids in hnRNP C<sub>2</sub> were not required for the function. To simplify our studies, we decided to examine only the roles of hnRNP C<sub>1</sub> in this report.

### **hnRNP C<sub>1</sub> promotes intron 10 but not intron 11 splicing of Ron pre-mRNA**

We asked if hnRNP C<sub>1</sub> 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, Figure 2B). Using this mini-gene, we

performed RT-PCR with primer pairs corresponding to exon 10 and the downstream vector sequence. The results in Figure 2B demonstrate that hnRNP C<sub>1</sub> expression increased the intron 10-spliced isoform significantly (~15%, lane 3). Thus, hnRNP C<sub>1</sub> 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 (Figure 2C, lower panel). The results in Figure 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 Figure 2C show that intron 11 splicing was not altered by the hnRNP C<sub>1</sub> treatment. The results in Figure 2B and 2C indicate that hnRNP C<sub>1</sub> affected intron 10 but not intron 11 splicing. Therefore, we conclude that hnRNP C<sub>1</sub> promoted exon 11 inclusion through activation of intron 10 splicing.

### **Conserved splice-site sequences of exon 11 do not affect hnRNP C<sub>1</sub> function**

We further asked if splicing sites flanking exon 11 would regulate the effect of hnRNP C<sub>1</sub> 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, Figure 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 Figures 3B and 3C). However, the results in Figure 3B demonstrate that a mutation of the 5' splice-site in a conserved sequence did not disrupt hnRNP C<sub>1</sub> function on Ron exon 11 splicing



because hnRNP C<sub>1</sub> 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 C<sub>1</sub> in exon 11 splicing.

#### **The Asp/Glu domain is dispensable for hnRNP C<sub>1</sub> function in Ron exon 11 splicing.**

hnRNP C<sub>1</sub> 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 C<sub>1</sub>. To determine if these two domains were necessary for hnRNP C<sub>1</sub>-mediated Ron exon 11 splicing, we produced two hnRNP C<sub>1</sub> mutants, in which either the RRM domain or the Asp/Glu domain was deleted ( $\Delta$ RRM,  $\Delta$ Asp/Glu) (Figure 4A). The results in Figure 4B show that the  $\Delta$ RRM mutant protein was not able to promote exon 11 inclusion (lane 4). Thus, the RRM domain was required for the role of hnRNP C<sub>1</sub> 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 that the  $\Delta$ Asp/Glu mutant of hnRNP C<sub>1</sub> 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 C<sub>1</sub> in Ron exon 11 splicing. The results indicate that tetramer formation was not required for the role of hnRNP C<sub>1</sub> 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 C<sub>1</sub>/C<sub>2</sub> promoted Ron exon 11 splicing by

stimulating intron 10 but not intron 11 splicing. Moreover, hnRNP C<sub>1</sub> and C<sub>2</sub> 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 C<sub>1</sub> 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 (23-25). Whereas SRSF1 and hnRNP A1 target exon 12 RNA and SRSF2 targets exon 11 RNA, here, we demonstrated that hnRNP C<sub>1</sub> targeted exon 10 sequences. However, in spite of these studies, it is still unclear if SRSF1, hnRNP A1, SRSF2, and hnRNP C<sub>1</sub> 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 C<sub>1</sub>/C<sub>2</sub> 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 C<sub>1</sub>/C<sub>2</sub> was not necessary for viability (18). Thus, it is likely that hnRNP C<sub>1</sub>/C<sub>2</sub> played redundant roles in splicing in the cells. In this study, we presented direct evidence that hnRNP C<sub>1</sub>/C<sub>2</sub> regulated alternative splicing using cells in which hnRNP C<sub>1</sub>/C<sub>2</sub> was either overexpressed or suppressed. Our results, as well as a few other reports, demonstrated that hnRNP C<sub>1</sub>/C<sub>2</sub> was an essential regulatory protein of alternative splicing (19-21). However, previous studies of hnRNP C<sub>1</sub>/C<sub>2</sub> 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 C<sub>1</sub>/C<sub>2</sub>

expression induced a much more significant change in exon 11 splicing compared to hnRNP C<sub>1</sub>/C<sub>2</sub> overexpression. The difference can be explained by the fact that hnRNP C<sub>1</sub>/C<sub>2</sub> is one of the most abundant proteins in cells (11). Thus, shRNA treatment induced a significant decrease in hnRNP C<sub>1</sub>/C<sub>2</sub> expression. Although we used various approaches, we were not able to show that the endogenous Ron exon 11 splicing was affected by hnRNP C<sub>1</sub>/C<sub>2</sub> overexpression. Nonetheless, the transient expression of hnRNP C<sub>1</sub>/C<sub>2</sub>, 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 C<sub>1</sub> and C<sub>2</sub> are able to form homo- or heterotetramers (13). However, it seems that the role of hnRNP C<sub>1</sub>/C<sub>2</sub> in Ron splicing was not required for tetramer formation, based on two pieces of evidence. First, hnRNP C<sub>1</sub> and C<sub>2</sub> 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 C<sub>1</sub>, 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 C<sub>1</sub>, C<sub>2</sub> was inserted into a pcDNA6/myc-His A (Invitrogen) plasmid. The  $\Delta$ Asp/Glu and  $\Delta$ RRM mutants of hnRNP were produced by overlapping PCR using the hnRNP C<sub>1</sub> expression plasmid as a template.

### **RT-PCR**

Total RNA was extracted using RiboEx (GeneAll) following the manufacturer's protocol. Reverse transcription was performed using 0.5  $\mu$ g RNA with oligo (dT) primer and ImProm-II<sup>TM</sup> reverse transcriptase (Promega). The reaction mixture (0.5  $\mu$ l) was amplified by PCR using G-Taq polymerase (Cosmo Genetech).

### **Purification of hnRNP C<sub>1</sub> protein**

Total protein was extracted from HEK293 cells transfected with the pcDNA6/myc-His A-hnRNP C<sub>1</sub> plasmid by 30 min incubation with lysis buffer (50mM NaH<sub>2</sub>PO<sub>4</sub>, 500mM NaCl, 5mM imidazole, 0.5% Tween-20, and 1mM 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 NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl, 0.5% Tween-20, and 1 mM PMSF). After washing, the hnRNP C<sub>1</sub> protein was eluted from the Ni-NTA agarose beads using elution buffer (250mM imidazole in binding buffer) for 20 min at 4 °C.

### **Knockdown of hnRNP C<sub>1</sub>/C<sub>2</sub> 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  $\mu$ m filter. To knock down the hnRNP C<sub>1</sub>/C<sub>2</sub> expression, lentivirus-containing supernatants were added to the cells supplemented with 10  $\mu$ g/ml polybrene. After 72 h infection, the RNAs were extracted for RT-PCR.

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## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

## FIGURE LEGENDS

**Figure 1. Reduced hnRNP C<sub>1</sub>/C<sub>2</sub> 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 C<sub>1</sub>/C<sub>2</sub> 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 C<sub>1</sub>/C<sub>2</sub> is demonstrated by RT-PCR and immunoblotting using anti-hnRNP C<sub>1</sub>/C<sub>2</sub> antibody, with GAPDH and  $\alpha$ -tubulin as controls. The quantitation results of exon 11 inclusion or exclusion in total RNA are shown in the right panel.

**Figure 2. (A) hnRNP C<sub>1</sub> and hnRNP C<sub>2</sub> promote exon 11 splicing of Ron pre-mRNA independently.** (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 C<sub>1</sub>, hnRNP C<sub>2</sub>, or both hnRNP C<sub>1</sub> and C<sub>2</sub>. 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 C<sub>1</sub> 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 C<sub>1</sub> 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.

**Figure 3. Conserved splice-site sequences of exon 11 do not affect hnRNP C<sub>1</sub> 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 C<sub>1</sub>-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 C<sub>1</sub>-expressing cells. GAPDH was used as a loading control. The quantitation of exon 11 inclusion in total RNA is shown.

**Figure 4. The Asp/Glu domain is dispensable for the function of hnRNP C<sub>1</sub> in Ron exon 11 splicing.** (A) Scheme of the hnRNP C<sub>1</sub> protein domains is shown. RRM and Asp/Glu domain are shown with gray boxes. Parts deleted in the  $\Delta$ RRM and  $\Delta$ Asp/Glu mutants are shown in dotted line-framed boxes. (B) RT-PCR analysis of exon 11 splicing using RNAs extracted from cells expressing  $\Delta$ RRM and  $\Delta$ Asp/Glu mutant proteins. Expression of myc-tagged wild-type hnRNP C<sub>1</sub>,  $\Delta$ RRM, or  $\Delta$ Asp/Glu mutant protein is confirmed with anti-myc antibody. The quantitation of exon 11 inclusion in total RNA is demonstrated in the lower panel.

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Figure 1.

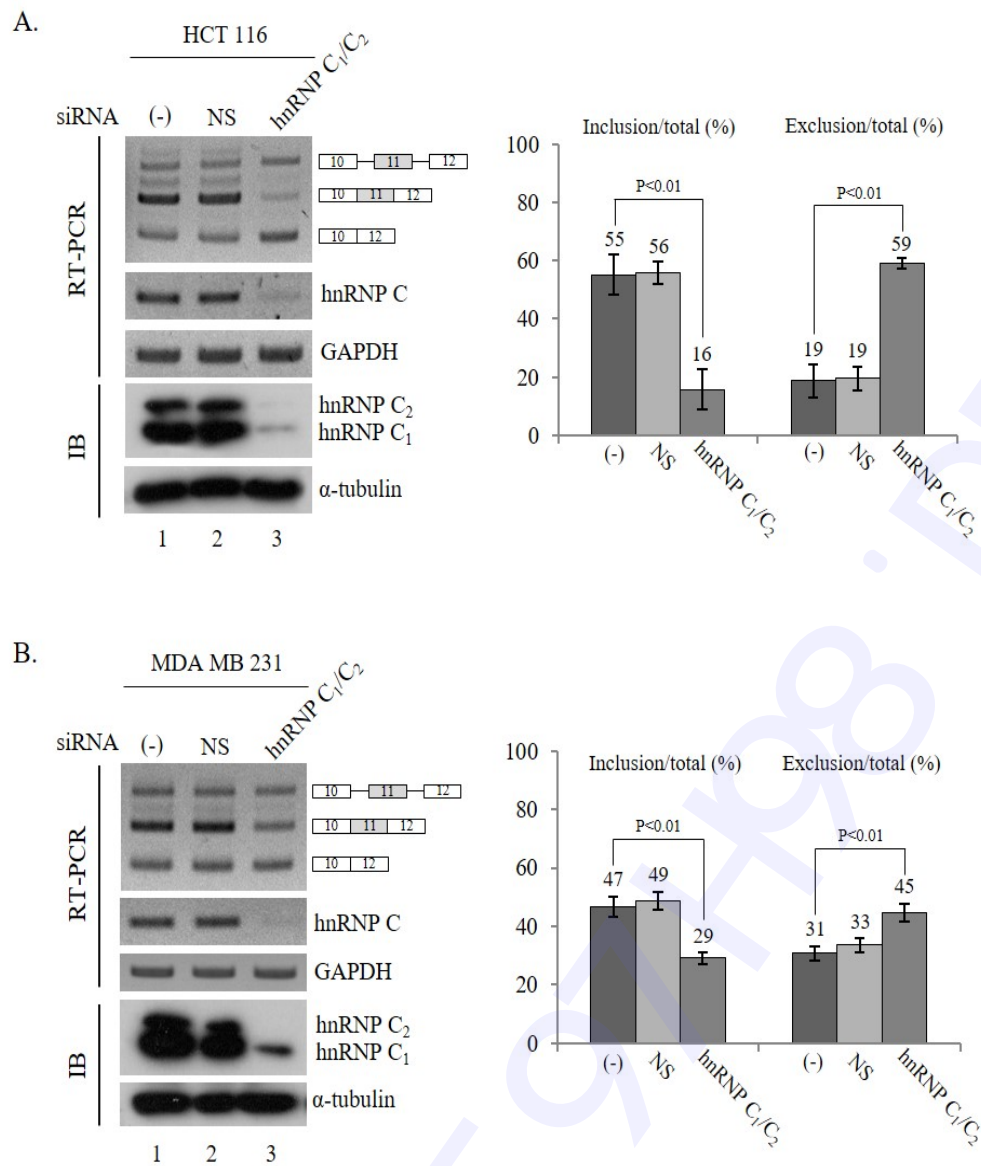


Fig. 1.

Figure 2.

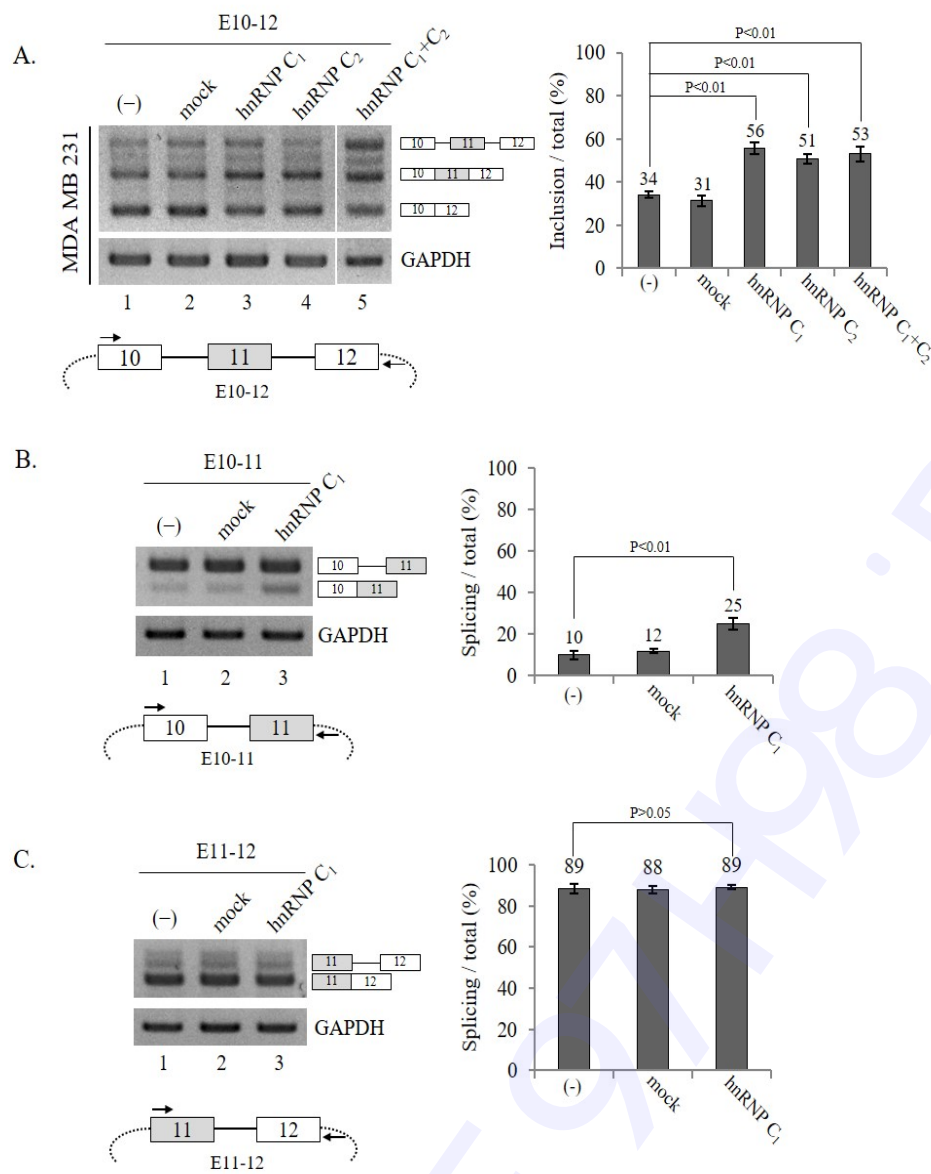


Fig. 2.

Figure 3.

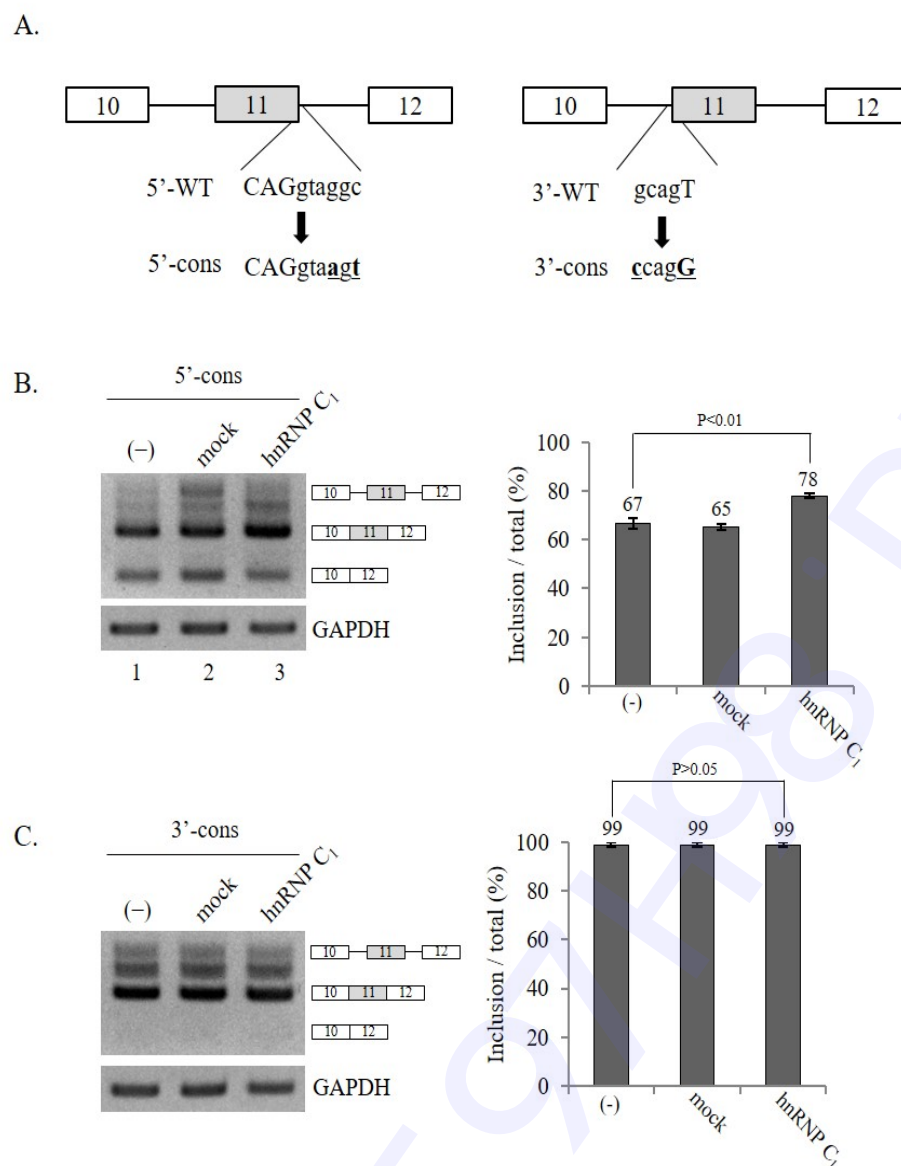
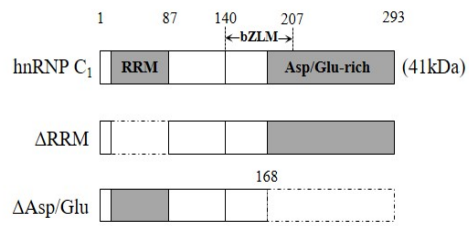


Fig. 3.

Figure 4.

A.



B.

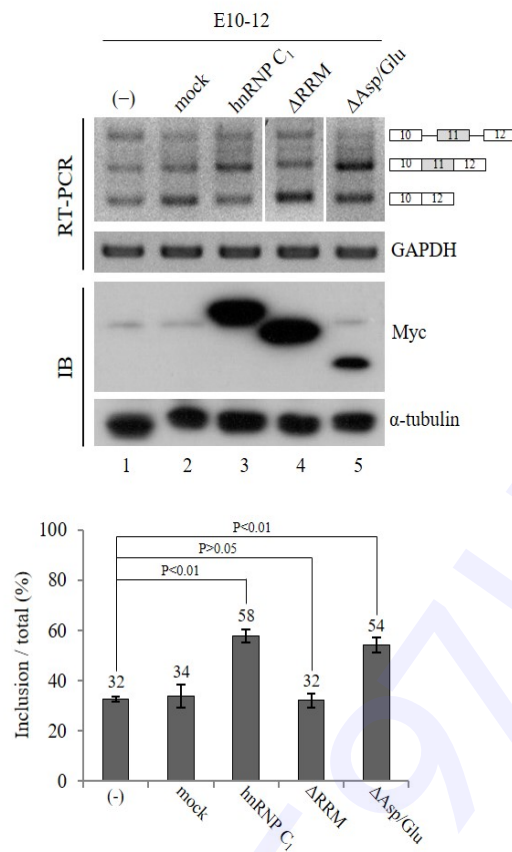


Fig. 4.