HnRNPA1 interacts with G-quadruplex in the TRA2B promoter and stimulates its transcription in human colon cancer cells

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The human TRA2B gene consists of 10 exons and 9 introns and produces 5 splice isoforms (TRA2β1 to TRA2β5). TRA2B exon 2 encodes multiple premature termination codons. TRA2β3 lacks exon 2 and is translated into a functional transformer 2β (Tra2β) protein, whereas TRA2β4 contains 10 exons and works as a functional RNA. Overexpressed Tra2β and ectopic expression of TRA2β4 may be oncogenic. We found that heterogeneous nuclear ribonucleoprotein (hnRNP)A1 and hnRNPU interacted with TRA2β4 exon 2. Minigene assays revealed that hnRNPA1 facilitated inclusion of exon 2, whereas hnRNPU promoted its skipping. However, knockdown of hnRNPA1 or hnRNPU reduced both TRA2β1 and TRA2β4 levels, and overexpression of these hnRNPs increased levels of both isoforms, suggesting that hnRNPA1 and hnRNPU mainly regulate the transcription of TRA2B. In fact, hnRNPA1 and hnRNPU positively regulated the promoter activity of TRA2B. Circular dichroism analyses, electrophoretic mobility shift assays and chromatin immunoprecipitation assays demonstrated the presence of G-quadruplex (G4) formation in the promoter of TRA2B. Formation of G4 suppressed TRA2B transcription, whereas hnRNPA1, but not hnRNPU, interacted with the G4 to facilitate transcription. Our results suggest that hnRNPA1 may modulate TRA2B transcription through its regulation of G4 formation in its promoter in colon cancer cells.

The human TRA2B gene encodes transformer 2β (Tra2β) protein, a member of the serine/arginine-rich splicing factor-like family. Tra2β regulates alternative splicing of several genes such as calcitonin/calcitonin gene-related peptide (CGRP), myosin phosphatase targeting subunit 1 (MYPT1), survival motor neuron (SMN) and microtubule-associated protein tau (TAU) in a concentration-dependent manner14-16. Tra2β is overexpressed in breast, cervical, ovarian, prostate and lung cancers15-19. Tra2β levels were greater in high-grade gliomas than low-grade gliomas, and Tra2β knockdown resulted in suppression of cell proliferation and inhibition of cell migration10. In human prostate cancer, overexpression of Tra2β was a significant predictor of recurrence and poor survival20. Thus, TRA2B has been recognized as an oncogene.

The human TRA2B gene consists of 10 exons and 9 introns and generates 5 mRNA isoforms (TRA2β1 to TRA2β5) through alternative splicing and usage of alternative promoters or polyadenylation sites11. Among them, TRA2β1 and TRA2β4 are the main isoforms25. TRA2β1 mRNA consists of exons 1–10 except for exon 2, whereas TRA2β4 isoform contains all 10 exon13. Although TRA2β1 mRNA is translated into Tra2β protein, TRA2β4 does not encode full-length protein because of the presence of premature stop codons in exon 2. We reported that oxidative stress stimulated TRA2B transcription through heat shock factor 114 and increased TRA2β4 production through HuR13. TRA2β4 is preferentially expressed in the nuclei of human colon cancer cells through association with nucleolin15, facilitating abnormal cell growth12. Tra2β protein interacts with the BCL2 3′-UTR and promotes abnormal growth of colon cancer cells16. Based on these findings, both Tra2β and TRA2β4 are considered to be oncogenic.

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new targets for treatment of colon cancer. However, the transcriptional and post-transcriptional regulation of TRA2B are not fully understood.

In the present study, we identified heterogeneous nuclear ribonucleoprotein (hnRNP) A1 and hnRNPU as TRA2β-binding proteins using biotinylated RNA pull-down and RNA immunoprecipitation assays. The hnRNP family functions as major splicing factors as well as transcriptional regulators for various genes. In fact, both hnRNPA1 and hnRNPU could upregulate the promoter activity of TRA2B. However, we found that hnRNPA1, but not hnRNPU, interacted with a G-quadruplex (G4) structure in the TRA2B promoter. G4 reportedly has various functions related to transcription, replication, telomere maintenance, DNA damage repair, posttranscriptional regulation and many others. Notably, recent studies have revealed that G4 resides in the promoters of many oncogenes or tumor suppression genes. In particular, the transcription of K-RAS or C-MYC was reported to be regulated via G4 in their promoter regions. Here, we suggest that hnRNPA1 may regulate TRA2B transcription through interaction with G4 in its promoter region.

**Results**

**Effects of knockdown or overexpression of hnRNPA1 or hnRNPU on TRA2β expression.** First, we prepared 3 cDNA probes (full-length TRA2β, full-length TRA2β4, and TRA2β4 exon 2) and used them to identify TRA2β-binding proteins through exon 2 (Fig. 1a). After biotinylated RNA pull-down assays using each probe, purified proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluoride membrane, and visualized by silver staining. As shown in Supplementary Fig. S1a, 2 protein bands with molecular masses of ~100 kDa and ~40 kDa preferentially bound to exon 2 and exon 2-containing TRA2β4. As described in a previous study, hnRNPU was present in the 100-kDa proteins. Liquid chromatography/mass spectrometry (LC-MS/MS) was employed to identify hnRNPA1 as one of the 40-kDa proteins (Supplementary Fig. S1a). We were particularly interested in hnRNPA1 and hnRNPU as exon 2-binding proteins, since these RNA-binding proteins are known to be associated with accelerated cell growth. Additional biotinylated RNA pull-down assays (Supplementary Fig. S1b) and RNA immunoprecipitation assays (Supplementary Fig. S1c,d) demonstrated that both hnRNPA1 and hnRNPU were preferentially associated with TRA2β, compared with TRA2β1 and GAPDH.

Next, we evaluated how hnRNPA1 and hnRNPU modified the expression of TRA2β1 and TRA2β4. After knockdown of hnRNPA1 or hnRNPU, real-time quantitative PCR (RT-qPCR) was employed to measure TRA2β1 and TRA2β4 using primer sets that had been designed to specifically amplify these 2 transcripts. To avoid off-target effects, we used 2 commercially available sets of siRNAs for each target protein. All of the siRNAs effectively reduced their target transcripts (Fig. 1b,e). Unexpectedly, silencing of hnRNPA1 or hnRNPU significantly decreased the levels of both TRA2β1 and TRA2β4 (Fig. 1c,d,f,g). Tra2β3 protein levels were also reduced after knockdown of hnRNPA1 or hnRNPU (Fig. 1h,i).

We also examined the effects of transient overexpression of hnRNPA1 or hnRNPU using the pEBMulti vector system. Overexpressed mRNA and protein levels of hnRNPA1 or hnRNPU are shown in Fig. 2a,d,g,h. Densitometric analysis showed that hnRNPA1 and hnRNPU increased 1.60 ± 0.12 and 1.47 ± 0.13-fold, respectively (mean ± SD, n = 4). The overexpressed hnRNPA1 and hnRNPU significantly increased not only TRA2β1 but also TRA2β4 levels (Fig. 2b,c,e,f) in association with increases in Tra2β3 protein levels (Fig. 2g,h). Thus, both hnRNPA1 and hnRNPU seemed to positively regulate TRA2β transcription.

**Roles of hnRNPA1 and hnRNPU in alternative splicing of TRA2β pre-mRNA.** TRA2β4 exon 2-binding proteins, hnRNPA1 and hnRNPU, are thought to regulate TRA2β pre-mRNA processing. To test this, we employed minigene assays. As shown in Fig. 1, siRNAs #1 and #2 for hnRNPA1 or hnRNPU showed similar knockdown efficacies. Therefore, only siRNA #1 for hnRNPA1 or hnRNPU was used in the following experiments. The minigene contains the entire sequence from TRA2B exon 1 to exon 3 in a pcDNA3.1 vector (Supplementary Fig. S2a). After HCT116 cells were treated with hnRNPA1, hnRNPU or control siRNA for 48 h, an empty vector (mock) or a vector containing the TRA2B minigene was transfected into the cells. After reverse transcription using a gene-specific primer, transcribed products were amplified by PCR and separated by agarose gel electrophoresis.

Treatment with hnRNPA1 siRNAs decreased TRA2β3 by 50% (Fig. 1c) and TRA2β4 by 30% (Fig. 1d), whereas hnRNPA1 siRNA decreased the TRA2β4/TRA2β3 ratio by 20% compared with control siRNA treatment (Supplementary Fig. S2b), suggesting that hnRNPA1 may facilitate inclusion of exon 2. Treatment with hnRNPU siRNAs decreased both TRA2β1 and TRA2β4 by 20 to 30% (Fig. 1f,g), whereas hnRNPU siRNA increased the TRA2β4/TRA2β3 ratio over 3-fold (Supplementary Fig. S2c), indicating that hnRNPU may facilitate skipping of exon 2. Thus, although hnRNPA1 and hnRNPU could modify the alternative splicing of TRA2B exon 2, the effects were minimal when compared to the effects on the transcriptional regulation of TRA2B.

We also evaluated whether the interaction of hnRNPA1 or hnRNPU affected the turnover of TRA2β1 and TRA2β4. We found that knockdown of hnRNPA1 or hnRNPU did not change the stability of TRA2β1 and TRA2β4 (Supplementary Fig. S3).

**Interaction between hnRNPA1 and G-quadruplex in the TRA2B promoter.** To reveal how hnRNPA1 regulated the transcription of TRA2B, we first assessed the promoter activity of TRA2B using a dual-luciferase reporter assay. We previously characterized the basal promoter region (~166 to ~21 bp) of TRA2B, the transcription start site is located 107 bp upstream from the translational start site. HCT116 cells were transfected with the pGL3 basic vector containing −398 to +107 bp of the 5′-flanking region of TRA2B and used for assessment of the TRA2B promoter activity. Knockdown of hnRNPA1 resulted in a small but significant decrease of the luciferase activity (Fig. 3a). Those results were consistent with the finding that knockdown of hnRNPA1 reduced the levels of both TRA2β1 (Fig. 1c) and TRA2β4 (Fig. 1d).
Figure 1. Effects of knockdown of hnRNPA1 or hnRNPU on TRA2B expression. (a) Schematic diagrams of TRA2B, TRA2β1 and TRA2β4. Exons are indicated by Arabic numbers in the boxes. Dotted lines show alternative splicing of exon 2. (b) After HCT116 cells were treated with 20 nM control (ctl) siRNA or one of the 2 hnRNPA1 siRNAs (#1 and #2) for 48 h, hnRNPA1 mRNA levels were measured by RT-qPCR using GAPDH mRNA as an internal control for normalization. Values are expressed as relative changes, compared with those in control siRNA-treated cells (means ± SD, n = 4). (c,d) After knockdown of hnRNPA1, TRA2β1 and TRA2β4 levels were measured by RT-qPCR and compared with those in control siRNA-treated cells. Values are means ± SD, n = 4. (e) After treatment of HCT116 cells with 20 nM control siRNA or one of the 2 hnRNPU siRNAs (#1 and #2) for 48 h, hnRNPU mRNA levels were measured by RT-qPCR. The relative values are means ± SD, n = 3. (f,g) Changes in the expression levels of TRA2β1 and TRA2β4 after hnRNPU knockdown. (h) After hnRNPA1 knockdown, hnRNPA1 and Tra2β levels were measured by Western blot analysis using GAPDH as a loading control. Immunoreactive signals of corresponding bands were quantified by densitometry. Relative values (means ± SD, n = 4) are indicated below each figure. (i) Changes in hnRNPU and Tra2β proteins after hnRNPU knockdown were measured by Western blot analysis. Values are means ± SD, n = 4. *Significantly different, compared with control siRNA or mock (p < 0.05 by two-tailed Student’s t-test).
To elucidate the mechanism for \( \text{hnRNPA1} \)-dependent regulation of promoter activity, we were particularly interested in the presence of a latent G-quadruplex (G4) sequence (\( 5'\)-GGGGG\( G \)GGGGC\( C \)CGAGG\( G \)GGGG\( G \)-3') in the \( \text{TRA2B} \) gene promoter region (\(-214 \to -188 \) bp) (Fig. 3b). Circular dichroism analysis indicated that this sequence had a unique spectrum with the highest peak at 265 nm and the lowest peak at 240 nm, results characteristic of a parallel G4 structure. Introduction of the mutation (\( 5'\)-GGAGG\( T \)GGAGC\( C \)CGAGG\( G \)GGGG\( G \)-3') abolished the peaks characteristic of the G4 structure (Fig. 3c). To confirm the formation of the G4 structure, we also employed electrophoretic mobility shift assays (EMSA) using an IRDye700-labeled G4 sequence and a G4 specific antibody (BG4). EMSA showed a shifted G4-BG4 complex band that disappeared after adding an excess amount of non-labeled wild-type G4, but not mutant G4 nucleotides (Fig. 3e). To further validate the formation of the G4 structure, we employed chromatin immunoprecipitation (ChIP)-PCR assays. As shown in Fig. 3d, BG4 bound to a chromatin region that included the G4 sequence of the \( \text{TRA2B} \) promoter. Moreover, \( \text{hnRNPA1} \) associated with the same region (Fig. 3d).
Considering these results, hnRNPA1 likely interacted with the G4 sequence to promote TRA2B gene transcription. To confirm this hypothesis, we further demonstrated the involvement of hnRNPA1 in G4 formation of the TRA2B promoter. As shown in Fig. 3f, the BG4-G4 complex was abolished by addition of purified hnRNPA1.
protein, suggesting that G4 formation was inhibited by hnRNPA1. In addition, ChIP assay with BG4 antibody showed that the G4 in the TRA2B promoter increased in hnRNPA1 knockdown cells compared to control cells (Fig. 3g). These results might suggest that hnRNPA1 bound to G4 sequence in the TRA2B promoter and unfolded its G4 structure.

For further analysis of interaction between hnRNPA1 and G4 in the TRA2B promoter, we treated HCT116 cells with a well-known G4 stabilizer, pyridostatin (PDS). Treatment with PDS decreased the mRNA levels of TRA2β1 (Fig. 4a) and TRA2β4 (Fig. 4b), and Tra2β protein (Fig. 4c). Furthermore, overexpression of hnRNPA1 significantly increased both TRA2β1 and TRA2β4 (Fig. 4d,e). In contrast, these increases were completely blocked by treatment with PDS (Fig. 4f,g). After PDS treatment of untreated, hnRNPA1-overexpressing or mock-transfected HCT 116 cells, the TRA2B promoter activity was measured by a dual-luciferase reporter assay. Values (fold-changes) are means ± SD, n = 4. *Significantly different by two-tailed Student’s t-test (p < 0.05).

![Figure 4](https://doi.org/10.1038/s41598-019-46659-x)

**Figure 4.** Changes in TRA2B transcription by Pyridostatin treatment. (a) and (b) After a 24 h treatment of HCT116 cells with 20 μM pyridostatin (PDS), a G4 stabilizer, TRA2β1 and TRA2β4 levels were measured by RT-qPCR. Values (fold-changes) are means ± SD, n = 4. *Significantly different by two-tailed Student’s t-test (p < 0.05). (c) TRA2β3 levels in PDS-treated cells were measured by Western blot analysis. (d,e) HCT116 cells were transfected with the pEBMulti vector carrying full-length hnRNPA1 or empty vector (mock). After these cells were treated with 20 μM PDS for 24 h, TRA2β1 and TRA2β4 levels were measured by RT-qPCR. Values (fold-changes) are means ± SD, n = 4. *Significantly different by two-tailed Student’s t-test (p < 0.05). (f,g) After PDS treatment of untreated, hnRNPA1-overexpressing or mock-transfected HCT 116 cells, the TRA2B promoter activity was measured by a dual-luciferase reporter assay. Values (fold-changes) are means ± SD, n = 4. *Significantly different by two-tailed Student’s t-test (p < 0.05).

To further confirm the regulation of G4 structure formation by hnRNPA1, we performed double-immunofluorescence staining with anti-hnRNPA1 and BG4 antibodies. As shown in Fig. 5, confocal laser microscopy revealed that only small numbers of G4 structure were detected with BG4 antibody in control siRNA-treated HCT116 cells, whereas the numbers of G4 structure formation were substantially increased when hnRNPA1 was silenced. These results suggest that hnRNPA1 may associate with the G4 sequence and interfere with G4 formation, allowing TRA2B transcription. The reduction of hnRNPA1 may facilitate G4 formation and downregulate TRA2B transcription.

**Regulation of TRA2B transcription by hnRNPU.** Although we could not detect any interaction between hnRNPU and the G4 structure, hnRNPU knockdown decreased the luciferase reporter activity of the TRA2B promoter (−398 to +107 bp) by 50% (Fig. 3a and Supplementary Fig. S5a). To determine the hnRNPU-responsive region in the promoter, serially truncated segments of the promoter were prepared and used for the dual-luciferase
colon cancer cell growth by hnRNPA1 and hnRNPU.

Fig. 3d. However, ChIP assays with different sets of primers did not show any binding of hnRNPU to the region of (Supplementary Fig. S5b). This functional promoter region was included in the primer for ChIP (−231 to +107 bp) region was roughly doubled (Supplementary Fig. S5b). This functional promoter region was included in the primer for ChIP (−231 to +107 bp) region was roughly doubled (Supplementary Fig. S5b). However, ChIP assays with different sets of primers did not show any binding of hnRNPU to the region of (Supplementary Fig. S5b), suggesting that hnRNPU might interact with the region via other proteins or RNAs to promote transcription.

Expression of hnRNPA1, hnRNPU, TRA2β1, and TRA2β3 in colon cancers and regulation of colon cancer cell growth by hnRNPA1 and hnRNPU. Using Tissue Scan RT Colon Cancer Disease Panels III HCRT103 (OriGene, MD, USA), mRNA levels of hnRNPA1, hnRNPU, or TRA2β1 and TRA2β3 levels in 24 patients with colon cancers were measured by RT-qPCR. As shown in Fig. 6a,b, hnRNPA1 mRNA levels were positively correlated with the expression levels of TRA2β1, whereas expression levels of hnRNPA1 showed weak correlation with those of TRA2β3 in the colon cancers. HnRNPU mRNA levels were positively correlated to the expression levels of TRA2β1 and TRA2β3 (Fig. 6c,d). We further examined the expression levels of these mRNAs in normal colonic epithelial cells (HCEC-1CT) and colon cancer cells (HCT116). HCT116 cells expressed higher levels of TRA2β1, TRA2β3, hnRNPA1, and hnRNPU mRNAs than those in HCEC-1CT cells (Fig. 6e). In addition, HCT116 cells expressed significantly higher amounts of Tra2β1, hnRNPA1, and hnRNPU proteins compared with HCEC-1CT cells (Fig. 6f). Although this is the limited survey, these results suggest that hnRNPA1 and hnRNPU may contribute Tra2β3 overexpression in colon cancer cells.

We also examined the effects of hnRNPA1 and hnRNPU on the growth of HCT116 cells. Cell growth assessed by the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) revealed that knockdown of hnRNPA1 or hnRNPU significantly decreased the growth rate of HCT116 cells (Fig. 6g).

Discussion

In this study, we identified hnRNPA1 and hnRNPU as TRA2β3 exon 2-binding proteins. Our data demonstrated that these 2 hnRNPs regulated alternative splicing of exon 2. The minigene assay indicated that hnRNPA1 facilitated inclusion of exon 2, whereas hnRNPU promoted skipping of the exon. In addition to regulating the splicing reaction, we found that hnRNPA1 and hnRNPU could upregulate TRA2B transcription in different ways.

HnRNPA1 and hnRNPU are multifunctional proteins that have both DNA- and RNA-binding abilities. These 2 hnRNPs are splicing factors as well as transcription-regulating factors. HnRNPA1 regulates the transcription of genes in a variety of ways. It has been shown that hnRNPA1 interacts with the promoter region of the genes for thymidine kinase32, γ-fibrinogen33 chain and APOE34, and activates transcription of these genes in immortalized or cancer cell lines. The reduction of hnRNPA1 likely changed alternative splicing of TRA2B exon 2. However, it decreased expression levels of both TRA2β1 and TRA2β3. In fact, the reduction of hnRNPA1 significantly decreased the promoter activity of TRA2B.

It was recently shown that hnRNPA1 regulates the stability of G-quadruplex (G4) structures in certain promoters and regulates the expression of cancer-related genes, such as KRAS35,36. G4 is now recognized as a crucial regulator of cancer-related genes36 and is considered a potential target for cancer treatment37. Paramasivam et al. reported that hnRNPA1 unwound G4 and promoted KRAS gene expression36. Several studies suggested that the interaction between hnRNPA1 and G4 in telomeric repeat-containing RNA (TERRA) was important to maintain telomeres. HnRNPA1 associated with TERRA and altered the binding of protection of telomeres 1 (POT1) to
Figure 6. Relationship between TRA2B transcript levels and hnRNPA1 or hnRNPU mRNA levels in colon cancer. (a–d) Amounts of hnRNPA1, hnRNPU or TRA2β1 and TRA2β4 were measured by RT-qPCR in 24 patients with colon cancer. ACTB mRNA was used as an internal control for normalization. The correlations between hnRNPA1 and TRA2β1 (a), hnRNPA1 and TRA2β4 (b), hnRNPU and TRA2β1 (c), or hnRNPU and TRA2β4 (d) were analyzed by Pearson’s correlation analysis. (e) Comparison of mRNA levels of hnRNPA1, hnRNPU or TRA2β1 and TRA2β4 in normal colon cell line (HCEC) and colon cancer cell line (HCT116). (f) Comparison of protein levels of Tra2β, hnRNPA1, and hnRNPU in HCEC and HCT116. (g) Effects of hnRNPA1 or hnRNPU knockdown on growth of HCT116 cells. After treatment of HCT116 cells with control, hnRNPA1, or hnRNPU siRNA, growth of these cells was monitored by counting the number of cells using CellTiter-Glo Luminescent Cell Viability Assay (Promega). Values are means ± SD, n = 4. *Significantly different compared with control siRNA-treated cells (p < 0.05) by two-tailed Student’s t-test.
telomeric single-stranded DNA, resulting in the prevention of TERRA-mediated telomerase inhibition\(^{38,39}\). Liu et al. showed that telomere RNA G-quadruplex played a crucial role in the association with hnRNPA1 in vitro and in living cells\(^{40}\). These results indicated that G4-dependent binding of hnRNPA1 to telomere RNA might regulate telomere extension by mediating telomerase activity. Based on these findings, we were particularly interested in the presence of the putative G4 sequences (5′-GGGGTGGGGCCCTGCGAGGGCGGG-3′) in the TRA2B gene promoter region (−214 to −188 bp). This sequence structure displayed a G4-specific spectrum (the highest peak at 265 nm and the lowest peak at 240 nm) by circular dichroism analysis. The presence of a G4 structure was confirmed by EMSA with a G4-specific antibody (BG4) as well as a ChIP-PCR assay. However, further experiments are needed to characterize structure and dynamics of G4 in the TRA2B promoter in colon cancer cells. Our results suggest that hnRNPA1 may associate with the G4 sequence and modify the G4 structure to promote TRA2B gene transcription. Notably, treatment with a G4 stabilizer, pyridostatin (PDS), decreased the TRA2B transcript levels in association with the decrease in the TRA2B promoter activity. Furthermore, overexpression of hnRNPA1 significantly increased both TRA2/31 and TRA2/34 levels, whereas the increases were completely cancelled with PDS. Thus, our results suggest that hnRNPA1 may regulate TRA2B transcription mainly through the G4 structure. Consistent with these findings, the results of immunofluorescence staining with an anti-G4 antibody (BG4) showed that G4 was masked and only small amounts of G4 formation were observed under ordinary conditions. However, G4 structure formation was likely facilitated once hnRNPA1 was silenced. These data are consistent with several reports that have shown that hnRNPA1 could un-wind G4 structure formation in telomeres\(^{41-43}\).

HnRNPU also binds to promoters and regulates the transcription of several genes such as Apod\(^{44}\), Klf4\(^{45}\), Oct4\(^{46}\), and Opn\(^{47}\) in murine cells including fibroblast cells, ES cells and macrophage cells. Furthermore, Nozawa et al. reported that hnRNPU interacted with chromatin and changed its structure via chromatin-associated RNAs as a function of transcription\(^{48}\). In addition, knockdown of hnRNPU reportedly reduced global transcription levels after 72 h, but not after 48 h\(^{49}\). In our experimental conditions, we examined gene expression 48 h after treatment with hnRNPU siRNAs. Although the TRA2B promoter possessed an hnRNPU-responsive region, we could not detect any direct association of hnRNPU with the promoter. In addition, it did not interact with G4 in the TRA2B promoter. Thus, it is possible that hnRNPU functionally regulates the promoter by interacting with certain RNAs or proteins. Past studies reported that hnRNPU formed a protein complex with RNA polymerase II at the promoter regions to promote transcription\(^{6,49,50}\). The same mechanism could exist in the TRA2B promoter.

It is known that several hnRNP family proteins interact together at alternative splice sites. Moreover, the hnRNP family regulates both inclusion and skipping of exons depending on the type of genes\(^{51}\). According to our results, hnRNPA1 and hnRNPU promoted and decreased the splicing TRA2/3 pre-mRNA. However, both TRA2/31 and TRA2/34 were decreased after knockdown of either hnRNPA1 or hnRNPU. Thus, hnRNPA1 and hnRNPU mainly regulate TRA2B transcription rather than TRA2/3 pre-mRNA processing.

Trα2\(^{3}\), TRα2\(^{3}\), hnRNPA1 and hnRNPU were reportedly significantly associated with accelerated cell growth. Knockdown of hnRNPA1 arrested cell cycle arrest in oral squamous cancer cells and lung adenocarcinoma\(^{6,31}\). Knockdown of hnRNPU induced cell senescence and increased CDKN1A\(^{46}\). Notably, we also reported that TRA2/3 knockdown stimulated CDN1A transcription and increased p21 levels in colon cancer cells, resulting in cell senescence\(^{32}\). TRA2/3 interacted with Sp1 through a Sp1-binding sequence (485-GGGG-488) in a stem-loop structure of exon 2\(^{21}\). TRA2/3 associated with nucleolin and was preferentially localized in the nuclei of human colon cancer cells\(^{33}\). Overexpression of TRA2/3 significantly decreased CDN1A mRNA levels and accelerated cell growth\(^{32}\). As to Trα2\(^{3}\), this protein could bind to the BCL2 3′-UTR and disrupt miR-204-binding to the BCL2 3′-UTR. Overexpressed Trα2\(^{3}\) could increase Bcl-2 expression through competition with miR-204, facilitating colon cancer cell growth\(^{46}\). Thus, ectopic overexpression of Trα2\(^{3}\) protein and TRA2/3 mRNA causes abnormal growth of colon cancer cells. A pathological study showed that hnRNPA1 and hnRNPU were expressed significantly higher in the nuclei of colorectal cancer tissue than normal colon tissue\(^{52}\). Considering these results, TRA2B gene transcripts and hnRNPA1 or hnRNPU may be closely interacting regulators of the cell cycle or cell growth, which is a crucial phenotype of cancer cells. The mechanisms of regulation of cell cycle by the hnRNP family are not fully elucidated. However, the mechanism may involve TRA2B products in the downstream pathway.

In summary, we identified hnRNPA1 and hnRNPU as TRA2/3 exon 2-binding proteins. These two hnRNPs regulated TRA2B transcription and alternative splicing of the exon 2. HnRNPA1, but not hnRNPU, interacted with a G4 sequence in the TRA2B promoter and facilitated TRA2B transcription. Trα2\(^{3}\) protein and TRA2/3 play crucial roles in abnormal colon cancer cell growth, and G4 is now recognized as a crucial regulator for cancer-related genes. These results suggest that hnRNPA1/G4 may participate in ectopic overexpression of TRA2B products in colon cancer cells.

Materials and Methods
Cell culture and knockdown experiments. Human HCT116 colon cancer cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Nacalai Tesque, Tokyo, Japan) supplemented with 5% heat-inactivated fetal bovine serum (FBS) at 37 °C in 5% CO2. HCT116 cells were treated with 20 nM negative control siRNA (AllStars Negative; Qiagen), hnRNPA1 or hnRNPU siRNAs using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA) for 48 h.
Western blot analysis. Whole cell lysates were prepared using RIPA buffer (10 mM Tris-HCl, pH 7.4; 1% Nonidet P-40; 1 mM EDTA; 0.1% SDS; 150 mM NaCl) containing a complete protease inhibitor cocktail and a phosphatase inhibitor cocktail (Roche Diagnostics, Basal, Switzerland). The extracted proteins (10 μg protein) were separated by SDS-PAGE and then transferred to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA). The membranes were incubated with a mouse monoclonal anti-GAPDH antibody (Santa Cruz Biotechnology), anti-hnRNPA1 antibody (Santa Cruz Biotechnology), anti-hnRNPU antibody (Bethyl Laboratories, TX, USA), or anti-Tra2β antibody (Abcam, Cambridge, UK) at 1:1000 dilution. The bound antibodies were detected with Pierce Western Blotting Substrate (Thermo Fisher Scientific).

Real-time quantitative PCR (RT-qPCR). Total RNAs were extracted from HCT 116 cells using RNaiso Plus (Takara, Otsu, Japan). Isolated RNA (1 μg) was reverse-transcribed using a PrimeScript RT Master Mix (Takara). RT-qPCR was conducted using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The following primer sets were used; 5′-CCGGAAGTTTCAGGTGGCTGGA-3′ (forward) and 5′-GGTACGTTGTCGTCATC-3′ (reverse) for TRA2β; 5′-GGTACGTTGTCGTCATC-3′ (forward) and 5′-GGTACGTTGTCGTCATC-3′ (reverse) for hnrnpu mRNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were measured as an internal control for normalization. The following primer sets were used; 5′-TCGAGAAGGTCAGTTTGAAGAGGATG-3′ (forward) and 5′-GGGATTCGACGGTACCCCAAT-3′ (reverse). Levels of mRNAs were measured by the comparative ΔΔCt method using GAPDH mRNA and expressed as values relative to the normal samples.

Overexpression of hnRNPA1 and hnRNPU. Full-length hnrnpa1 and hnrnpu mRNAs were cloned into the pEBMulti-Bsd vector (Fujifilm Wako Pure Chemical, Osaka, Japan). For full-length hnrnpu, cDNA was amplified by PCR using the following primer set: 5′-GGATCCTCCGCTGCACCCGCT-3′ (forward) and 5′-GGTACGTTGTCGTCATC-3′ (reverse). A full-length hnrnpa1 was amplified using the following primer set; 5′-GGATCCTCCGCTGCACCCGCT-3′ (forward) and 5′-GGTACGTTGTCGTCATC-3′ (reverse). The amplified products were subcloned into the pCMV-HIS vector (Thermo Fisher Scientific) were added and incubated at room temperature for 1 h. The proteins bound to the beads were isolated with magnetic beads separator and followed by western blot assay or liquid chromatography-mass spectrometry assay (nanoLC-MS/MS, CapLC, Q-Tof).

Biotinylated RNA Pull-down assay. The assays were performed by the method as previously described. Briefly, each target mRNA fragment, template DNA was synthesized by PCR with gene specific primer sets containing T7 RNA polymerase promoter sequence. Biotinylated RNA fragments were generated by in vitro transcription using MAXiScrip T7 Transcription Kit (Thermo Fisher Scientific) and Biotin-11-CTP (PerkinElmer, Waltham, CT, USA). After incubation of 2.5 μg biotinylated RNA with 80 μg of whole cell lysates in TENT buffer (10 mM Tris–HCl buffer, pH 8.0, 250 mM NaCl, 0.5% Triton X-100, and 1 mM EDTA) for 30 min, Dynabeads M-280 Streptavidin (Thermo Fisher Scientific) were added and incubated at room temperature for 1 h. The proteins bound to the beads were isolated with magnetic beads separator and followed by western blot assay or liquid chromatography-mass spectrometry assay (nanoLC-MS/MS, CapLC, Q-Tof).

Ribonucleoprotein immunoprecipitation (RIP) analysis. The assays were performed using the method as previously described. Briefly, 20 μl of protein A Sepharose (Sigma-Aldrich) and 3 μg of anti-hnRNPU or anti-hnRNPA1 antibody were added to 1 mL of NT-2 buffer (50 mM Tris HCl, pH 7.4; 150 mM NaCl; 1 mM MgCl2; 0.05% (v/v) Nonidet P-40). Cells were lysed in lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, and 0.5 mM PMSF). The lysates were mixed with protein A Sepharose, for 2 h with rotation. Subsequently, those mixtures were treated with Turbo DNase (Thermo Fisher Scientific) for 10 min at 37 °C. Bound RNA was extracted using RNeasy Plus kit and subjected to RT-qPCR.

Promoter activity assay. The 5′-flank of the human TRA2B gene was cloned into the pGL3-basic luciferase reporter vector (Promega). The first PCR was performed using genomic DNA from HCT116 cells as a template. The TRA2B proximal promoter region (from −398 to +107 bp) was amplified using the following primer set: 5′-AAATTGGGGAGAAGGATAATGAGTTTGGGGA-3′ (forward) and 5′-GACTCTCTGCGGTCGGCCTGGATCATCAG-3′ (reverse). The amplified products were subcloned into the pGL3-basic vector using SalI and XhoI restriction sites. After treatment with control or hnrnpu siRNA for 24 h, pGL3-luciferase constructs (200 ng) were co-transfected with pRL-CMV vector (100 ng) using Lipofectamine 2000 (Thermo Fisher Scientific) for 24 h. These cells were harvested, and firefly and Renilla luciferase activities were measured by luminometer using the Dual-Luciferase Reporter Assay System (Promega).

Minigene assay. To assess the alternative splicing of TRA2B pre-mRNA, genomic sequences of exon 1 to exon 3 were cloned as a minigene and inserted into the multi-cloning site of pcDNA3.1 using restriction enzymes of XhoI and BamHI as previously reported. After HCT116 cells were treated with control, hnrnpu or hnrnpa1 siRNA for 24 h, 2 μg of pcDNA3.1 with minigene or mock vector was transfected for 24 h and then total RNAs were extracted using an RNeasy mini kit (Qiagen). Complementary DNA was obtained by reverse transcription using SuperScriptIII (Thermo Fisher Scientific) and gene-specific primers (5′-TGCGAGATCCCGAGCTGGCGCGTCTGTCGGGGTGCT-3′). PCR was performed for the minigene-originated complementary DNA with the following primer set: 5′-GGGGATCCGCCGAGCTGGCGCGTCTGTCGGGGTGCT-3′.
region was amplified by PCR using the following primer set: 5′-GGGTGGAAGGGTCCCTGCGAGGCCGGG-3′ (forward) and 5′-GGGCTCGAGTACCCGATTCCCAACATGACG-3′ (reverse). PCR products were separated by 1.5% agarose gel electrophoresis. Amounts of each band in the gel were quantified and analyzed by Image J software (NIH).

**Chromatin immunoprecipitation (ChIP) assay.** ChIP assays were conducted using the EZ-Magna ChiP A (Millipore, MA, USA). HCT116 cells in 100-mm dishes at 80–90% confluence (approximately 2 × 10⁶ cells) were fixed in 10 mL DMEM containing 1% formaldehyde for 10 min at room temperature. Cells were washed with ice-cold PBS containing a protease inhibitor cocktail, and the chromatin was isolated according to the manufacturer’s instruction. Isolated chromatin was broken into 200–500 bp sizes using a sonicator (Digital Sonifire; BRANSON, Danbury, CT, USA). Chromatin suspensions (500 μL) were sonicated for 4 cycles (10 s on/60 s off) with a 20% amplitude. After centrifuging the samples at 800 × g for 5 min at 4°C, the supernatants were collected. Fragmented chromatin was diluted in 10 × ChIP dilution buffer and incubated overnight with one of the following: 5 μg of anti-hnRNP A1 antibody, normal mouse IgG, anti-hnRNP B1 antibody, normal rabbit IgG, or normal goat IgG. After bound DNAs were immunoprecipitated with magnetic beads, the G4-containing region was amplified by PCR using the following primer set: 5′-TAGCTTTCCCCGCTCATAGA-3′ (forward) and 5′-CGACGGGGCTCTAATCTCCT-3′ (reverse).

**Circular dichroism (CD) measurement.** CD spectra were measured using the J-820 spectropolarimeter (JASCO, Tokyo, Japan) with a 1-mm path length quartz cuvette. The single strand DNA sequences of G4 (5′-GGGGTGGGAGCCCTGCGAGGCCGGG-3′) and the sequence with mutation (5′-GGAGGTGGAGCCCTGCGAGCGCGAGG-3′) were purchased from Fasmac Co. (Kanagawa, Japan). Single strand DNAs at a concentration of 10 μM were dissolved in 50 mM Tris-HCl (pH 7.4) containing 100 mM KCl, heated at 95°C for 5 min and then slowly cooled to 25°C. CD spectra from 220 to 320 nm were scanned at 25°C as follows: 100 nm/min scanning speed, 1-nm band width, 0.5-nm data interval and 1-s response.

**Electrophoretic mobility shift assay (EMSA).** An IRDye700-labeled G4 sequence of the TRA2B promoter (5′-TGCGGGTTGCGCGCTGCGAGGGCGGG-3′) was obtained from I-L COR Bioscience (Lincoln, NE, USA). The labeled oligonucleotides were dissolved in 10 mM Tris-HCl buffer (pH 7.4) containing 100 mM KCl, heated at 95°C for 5 min and then slowly cooled to 25°C. The labeled oligonucleotides were incubated with BG4 antibody (Absolute Antibody; Oxford, Oxfordshire, UK) in 20 mM HEPES buffer (pH 7.5) containing 100 mM KCl, 0.01% (v/v) glycerol, and 5 mM MgCl₂ for 1 h. Human recombinant hnRNP A1 protein was purchased from ATGen (Gyeonggi, South Korea). The complexes were resolved on a 4% polyacrylamide gel and imaged by ODYSSEY (LI-COR Bioscience).

**Immunofluorescence staining.** After HCT116 cells were treated with control or hnRNP A1 siRNA for 48 h, they were fixed with 4% paraformaldehyde phosphate buffer solution (Nacalai Tesque, Kyoto, Japan) and treated with 0.5% (v/v) Triton X. The cells were further treated with 200 μg/mL RNase A for 1 h at 37°C and then incubated overnight with 0.4 ng/μL of hnRNP A1 antibody or 4 ng/μL of BG4 antibody at 4°C. After washing with PBS, the signals were visualized with Alexa Fluor 488 anti-mouse IgG or Alexa Fluor 555 anti-goat IgG (Thermo Fisher Scientific). The nuclei were stained with TO-PRO-3 (Thermo Fisher Scientific) using a coverslip with Vectashield (Thermo Fisher Scientific).

**Data Availability**

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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Author Contributions

This study was conceived and supervised by T.N., Y.K., K.N. and K.R. T.N. and Y.T. performed most of the experiments. Y.K. performed the biotinylated RNA pull-down assays and RNA immunoprecipitation assay in Supplementary Figure S1. T.N., Y.K. and K.R. wrote the manuscript.

Additional Information

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