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RNA binding motif 4 inhibits the replication of ebolavirus by directly targeting 3′-leader region of genomic RNA

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ABSTRACT

Ebola virus (EBOV) belongs to *Filoviridae* family possessing single-stranded negative-sense RNA genome, which is a serious threat to human health. Nowadays, no therapeutics have been proven to be successful in efficiently decreasing the mortality rate. RNA binding proteins (RBPs) are reported to participate in maintaining cell integrity and regulation of viral replication. However, little is known about whether and how RBPs participate in regulating the life cycle of EBOV. In our study, we found that RNA binding motif protein 4 (RBM4) inhibited the replication of EBOV in HEK293T and Huh-7 cells by suppressing viral mRNA production. Such inhibition resulted from the direct interaction between the RRM1 domain of RBM4 and the "CU" enrichment elements located in the PE1 and TSS of the 3′-leader region within the viral genome. Simultaneously, RBM4 could upregulate the expression of some cytokines involved in the host innate immune responses to synergistically exert its antiviral function. The findings therefore suggest that RBM4 might serve as a novel target of anti-EBOV strategy.

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KEYWORDS Ebola virus; RNA binding proteins; RBM4; RRM1; 3′-leader region

Introduction

Ebola virus disease (EVD) is a severe and frequently lethal disease caused by Ebola virus (EBOV) infection [1,2]. There have been a number of outbreaks of EDV since its discovery, resulting in over 10,000 deaths [3]. Currently, only a vaccine and a few monoclonal antibodies are approved by the FDA for the prevention and treatment of EBOV [4–6]. Nonetheless, existing antiviral treatment strategies cannot significantly reduce the mortality rate of Ebola patients [1]. The high fatality rate of EVD indicates that the continuous development of antivirals is necessary.

EBOV belongs to the *Ebolavirus* genus of the *Filoviridae* family, and its genome is a single-stranded, non-segmented, negative-sense RNA, with a length of approximately 19 kb, and encodes seven viral proteins including nucleoprotein (NP), viral protein (VP) 35, VP40, envelope glycoprotein (GP), VP30, VP24, and polymerase L protein [7,8]. The genome organization of EBOV is 3′-leader-NP-VP35-VP40-GP-VP30-VP24-L-5′-trailer. The genomic 3′-leader region harbours replication promoter and transcription start sequence (TSS) of EBOV. The replication promoter consists of two elements including PE1 (1–55 nt) and PE2 (81–128 nt), whereas TSS was located between PE1 and PE2 [9]. Filovirus genes are flanked by highly conserved transcription start and stop signals. The TSS comprises a stretch of 12 nucleotides and varies slightly in EBOV (3′-$CU^{C}/_{A}$-CUUCUAAUU) [10]. The PE1, TSS and PE2 jointly participate in regulating replication and transcription in the Ebola lifecycle.

RNA binding proteins (RBPs) were reported to interact with multiple RNA species and hundreds of individual transcripts, potentially regulating RNA

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splicing, polyadenylation, mRNA stability, and mRNA localization through interacting with coding and non-coding RNAs [11,12], interfering with a multitude of regulatory networks and maintenance of cellular integrity [13]. RBPs are divided into many subfamilies according to different functions, including Hu-antigen R (HuR), heterogeneous nuclear ribonucleoprotein family (hnRNP) and RNA binding motifs protein family (RBM), etc [14]. Furthermore, RBM was reported to participate in virus replication processes and regulate bacterial adaptive responses [15–18].

As a member of the RBM family, RBM4 has been reported to be involved in tumour repression and antiviral therapies. RBM4 could inhibit the activity of the MAPK signalling pathway, which results in inhibiting the proliferation of gastric cancer cells [19]. Additionally, RBM4, also known as LARK, showed antiviral activities in shrimp by upregulating the expression of many components of the NF-κB and JAK-STAT pathways [20]. However, there are limited reports on the role of RBM4 in the replication process of viruses.

In this study, using EBOV transcription- and replication-competent virus-like particle (EBOV-trVLPs) which could simulate the life cycle of the EBOV virus, we confirmed that overexpression of RBM4 could inhibit the replication of EBOV both in HEK293T and Huh-7 cells. Moreover, we also found that RBM4 could exert its antiviral function by directly interacting with "CU" enrichment elements located in the PE1 and TSS of the 3′-leader region within the viral genome *via* its RRM1 subdomain, and such interaction could lead to the decreased viral mRNA production and the reduced replication level of EBOV. Finally, RBM4 was found to upregulate the expression of some cytokines involved in innate immune response, which may synergistically exert its antiviral function. These findings suggest that RBM4 might serve as a novel target of anti-EBOV strategy.

Materials and methods

Cell culture and antibodies

Human embryonic kidney 293 T (HEK293T) cells and Huh-7 human liver cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, C11965500BT) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO_2.

The antibodies used in this study include: rabbit polyclonal anti-RBM4 (Proteintech, 11614-1-AP), rabbit monoclonal anti-HA (Sigma, H6908), rabbit monoclonal anti-FLAG (Sigma, F7425), mouse monoclonal anti-α-Tubulin (MBL, M175-3), mouse monoclonal anti-GAPDH (Abcam, ab9484), rabbit control IgG (ABclonal, AC005), IRDye 680RD goat anti-rabbit (LI-COR, 926-68071), IRDye 800CW goat anti-mouse (LI-COR, 926-32210).

Plasmids

All plasmids encoding Zaire ebolavirus (strain Mayinga) viral proteins (pCAGGS NP, VP35, VP30 and L), pCAGGS T7 polymerase, EBOV minigenome p4cis-vRNA-RLuc (encoding *Renilla* luciferase, VP40, GP, and VP24), and pCAGGS *firefly* luciferase are stored in our lab and have been described previously [21,22]. Plasmids expressing HA-tagged NP, VP35, VP40, GP, VP30, and VP24 (Zaire ebolavirus) were constructed previously and maintained in our lab. RBM4 cDNA was reverse transcribed from the total RNA of HEK293T cells, and was further cloned into pCAGGS-vector carrying FLAG tag by ClonExpress II kit (Vazyme, C112) according to the manufacturer's instructions. Plasmids containing different RBM4 subdomains were further constructed with the corresponding primers (Supplementary Table 1). Sequences of all the plasmid constructs were confirmed by DNA sequencing.

siRNA transfection

HEK293T cells and Huh-7 cells were transfected with 50 nM of negative control (siNC) or RBM4-speicfic siRNA (siRBM4-1 target sequence: 5′-CAAAGUUGCAUGUGGGCAACATT-3′) (Guangzhou Ruibo Biotechnology) with Lipofectamine 2000 (Invitrogen, 11668019) according to the manufacturer's instructions. The cells were then transfected with plasmids for EBOV-trVLPs assay at 24 h after siRNA transfection.

Western blotting (WB)

HEK293T cells were collected and lysed with NP40 lysis buffer (Beyotime, P0013F) supplemented with protease inhibitor cocktail (Sigma Aldrich, P9599) on ice for 30 min, followed by centrifugation with the speed of 12,000 ×g at 4°C for 10 min. The supernatant was collected and boiled with loading buffer (final concentration of 1×) at 100°C for 10 min, and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membrane (Pall, 66,485) by semi-dry transfer method. The membranes were then blocked with 5% non-fatty milk for 2 h at room temperature (RT) and incubated with primary antibodies overnight at 4°C. Then the membrane was washed with PBST 3 times (10 min each) and was then incubated with IRDye secondary antibodies (LI-COR) for 45 min at RT, followed by washing 3 times with PBST and then the membrane was scanned with the Odyssey infrared imaging system (LI-COR).

Co-immunoprecipitation (Co-IP) assay

HEK293T cells were seeded on 6 cm plates and transfected with corresponding plasmids. 48 h after transfection, cells were collected and lysed with 400 μL NP40 lysis buffer supplemented with protease inhibitor cocktail on ice for 30 min. Lysates were clarified by 12,000 ×g centrifugation with 3 min, and 40 μL of the lysates was taken as an input control. The remaining lysates were incubated with anti-FLAG magnetic beads (Thermo Scientific, A36798) overnight at 4°C. The beads were then washed 10 times with 500 μL NETN buffer (5 mmol/L M NaCl, 0.5 mmol/L EDTA, 1 mmol/L Tris-HCl (pH 8.0), 0.5% NP-40). Proteins were eluted with loading buffer and denatured by boiling at 100°C for 10 min. Input control and IP samples were then analysed by WB.

Generation of RBM4 knockout (RBM4-KO) HEK293T cell lines

Firstly, the guide RNA (gRNA) sequence targeting RBM4 mRNA sites was designed by zlab guide-design-resources website (https://portals.broadinstitute.org/gpp/public/ analysis-tools/sgrna-design). Then, lentivirus was packaged with HEK293T cells of 10 cm plate, transfecting plasmids 3 μg pLentiCRISPRV2 containing gRNA of RBM4, 0.75 μg psPAX2 and 0.75 μg pMD2.G. Simultaneously, pLentiCRISPRV2 was used as control. Moreover, HEK293T cells were infected with lentivirus and screened with puromycin. Furthermore, the RBM4 knockout efficiency was preliminarily confirmed by DNA sequencing and WB. Finally, the monoclonal cell was picked up and continuously cultured.

Cell viability assay

HEK293T (LentiV2 or RBM4-KO) cells were seeded into 96-well plates. Cells were cultured for 6–8 h and were added with 10 μL CCK-8 solution using the cell counting Kit 8 (CCK-8) (Beyotime, C0038) according to the manufacturer's instructions to assess the cell ability. After 2 h, the absorbance at OD450 nm was measured by a Multimode reader Synergy H1 (BioTek).

EBOV-trVLPs assay

The assay was described previously [21,22]. In brief, HEK293T cells (producer cells) were seeded in 6-well plate and transfected with 125 ng pCAGGS-NP, 125 ng pCAGGS-VP35, 75 ng pCAGGS-VP30, 1μg pCAGGS-L, 250 ng EBOV minigenome (encoding *Renilla* Luciferase, VP40, GP and VP24) and 250 ng pCAGGS-T7 by Mirus TransIT-LT1 (Invitrogen, MIR2300). After 12 h, the cell culture medium was discarded and replaced with a fresh medium. After 72 h of transfection, supernatants containing EBOV-trVLPs were collected, filtered through a 0.22 μm filter (Millipore, SLGPR33RB) and stored at −80°C. To support the infection of the EBOV-trVLPs, HEK293T cells (target cells) were seeded in a 12-well plate and transfected with helper plasmids (50 ng pCAGGS-NP, 50 ng pCAGGS-VP35, 30 ng pCAGGSVP30, 400 ng pCAGGS-L, and 100 ng pCAGGS-Tim-1). Simultaneously, 5 ng pCAGGS-*firefly* luciferase was used as transfection control. After 24 h of transfection, the medium was discarded, and cells were infected with supernatants containing EBOV-trVLPs. After 12 h of infection, the medium was discarded and replaced with fresh medium. After 48 h of infection, target cells were lysed and analysed with a dual-luciferase assay kit (Promega, E2920) according to the manufacturer's instructions.

RNA immunoprecipitation (RIP) assay

Briefly, HEK293T cells were seeded on 6 cm plates and transfected with pCAGGS- RBM4-FLAG plasmids. After 24 h of transfection, cells were continuously transfected with pCAGGS-NP, pCAGGS-VP35, pCAGGS-VP30, pCAGGS-L, EBOV minigenome, and pCAGGS-T7 for 48 h. Forty-eight hours later, the cells were lysed in polysome lysis buffer (NP-40 lysis buffer, 1 mmol/L DTT, 80 units/mL ribolock RNase inhibitor, complete protease inhibitor cocktail) for 30 min on ice, and the cell lysate was then centrifuged at 12,000 ×g for 5 min to remove cell debris. The supernatants were incubated with protein A/G beads pre-coated with rabbit anti-FLAG or a non-specific IgG control antibody at 4°C for 12∼16 h. After washing 5 times with NT-2 buffer (50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L $MgCl_2$, and 0.05% NP-40), precipitated RNA was extracted with TRIzol (Takara, 9109) for subsequent analysis.

RNA isolation and quantitative RT–PCR

The total RNA was extracted from cells using the Purelink^{TM} RNA Mini kit (Invitrogen, 12,183,025). The reverse transcription (RT) and quantitative PCR (qPCR) for measurement of EBOV RNA were performed according to previous methods [23]. Briefly, for cDNA synthesis, 500 ng RNA was reverse transcribed with M-MLV Kit (Invitrogen, 28025-013) using random primer pd(N)9 for total RNA, oligo dT primer for EBOV mRNA, a specific primer (-vRNA-RT) for EBOV vRNA, and a specific primer (+cRNA-RT) for EBOV cRNA. The quantitative PCR primers targeting RBM4, β-actin, VP40 for mRNA, and 5′-trailer for cRNA and vRNA were listed in Supplementary Table 1. Real-time quantitative PCR was performed using the MagicSYBR mixture

(CWBIO, CW3008H) on QuantStudio 5 Real-time PCR system (Thermo Fisher Scientific), with the program as follows: 95°C 30 s and then 40 cycles of 95°C 5 s and 60°C 30 s. The experiment data was analysed with QuantStudio™ Design & Analysis Software.

Sequence analysis

RBM4 sequences were analysed by DNASTAR software. All RBM4 sequences of different species were obtained from GenBank, including *Homo sapians* (NM_00289), *Pan troglodytes* (NM_001246641), *Sus scrof* (DQ917635), *Bos Taurus* (NM_001077010), *Rattus norvegicus* (NM_001170484), and *Mus musculus* (BC144949).

Statistical analysis

All data were presented as mean ± standard error of mean (S.E.M). Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software, San Diego, California, USA). The differences between groups were analysed by two-tailed Student's *t*-tests, and a *P* value less than 0.05 was considered statistically significant.

Results

RBM4 inhibits the replication of EBOV

It has been reported that RBM4 was able to regulate the expression of many components of the NF-κB and JAK-STAT pathways in shrimp and played positive roles in both antiviral and antibacterial responses [20]. In this study, we found that the replication level of EBOV-trVLPs reflected by relative luciferase activity was decreased in HEK293T and Huh-7 cells transfected with RBM4 expression plasmid compared with vector control (Figure 1(A, B)), indicating that RBM4 may also be able to inhibit the replication of EBOV. To further investigate the possibility of RBM4 being an extensive anti-EBOV target, we analysed the amino acid sequences of RBM4 within *homo sapiens*, *pan troglodytes*, *sus scrofa*, *bos taurus*, *rattus norvegicus* and *mus musculus*, which are all the susceptible hosts of EBOV [24], and found that the homology of RBM4 in different species was more than 95% (Figure 1(C)), demonstrating that RBM4 could be an extensive target for anti-EBOV strategy. To investigate the anti-EBOV role of RBM4, two small interfering RNA (siRNA) targeting endogenous RBM4 mRNA (siRBM4-1 and siRBM4-2) were transfected into HEK293T and Huh-7 cells. It was found that siRBM4-1 could effectively knock down the levels of endogenous RBM4 compared with the siNC-transfected group (Supplementary Figure 1A–D). We can also find that the relative EBOV-trVLPs luciferase activity was significantly increased when endogenous RBM4 was knocked down in HEK293T and Huh-7 cells (Figure 1(D, E)).

To further confirm the role of RBM4 in inhibiting the replication of EBOV, *RBM4* gene was knocked out by CRISPR/Cas9 genome editing approach in HEK293T cell (Supplementary Figure 1E), and one cell clone (RBM4-KO) with an insertion frameshift mutation was harvested (Supplementary Figure 1F), and the endogenous RBM4 level was further examined by WB. It showed that the endogenous RBM4 was significantly knocked out compared to the control group (Supplementary Figure 1G). Cell viability assay suggested that there was no difference between RBM4-KO HEK293T cells and control cells (Supplementary Figure 1H), indicating that knocking down the endogenous RBM4 did not have any influence on cell viability. Furthermore, the EBOV-trVLPs assay showed that the relative luciferase activity was significantly increased in RBM4-KO HEK293T cells compared with the control group (Figure 1(F)). Overall, our data indicated that the conservative RBM4 could inhibit the replication of EBOV.

RBM4 inhibits the replication of EBOV by suppressing viral mRNA production

We next sought to explore the mechanism of RBM4 in inhibiting the replication of EBOV. As is known that three types of viral RNAs were produced during the EBOV life cycle, including viral genome RNA (vRNA), complementary RNA (cRNA), and mRNA. To determine whether the RNA production was influenced by RBM4 during its inhibiting the EBOV replication, we use different reverse-transcription primers to distinguish vRNA, cRNA and mRNA in HEK293T cells infected by EBOV-trVLPs. The results showed that there were no differences in the levels of vRNA and cRNA when overexpressing RBM4 in HEK293T cells, but the mRNA level was significantly decreased after RBM4 was overexpressed compared with the control group (Figure 2(A)). Whereas the mRNA level of EBOV was significantly increased when endogenous RBM4 was knocked down or knocked out, the levels of vRNA and cRNA remained unchanged (Figure 2(B,C)). Therefore, the RBM4 was able to suppress viral mRNA production rather than vRNA and cRNA.

RBM4 interacts with the genome of EBOV

Given that RBM4 belongs to RNA binding protein superfamily, we wonder whether RBM4 could interact with the RNA genome of EBOV. Therefore, the RIP experiment was conducted and it was found that the result indicated that RBM4 interacted with the genome of EBOV, IgG as a non-specific control (Figure

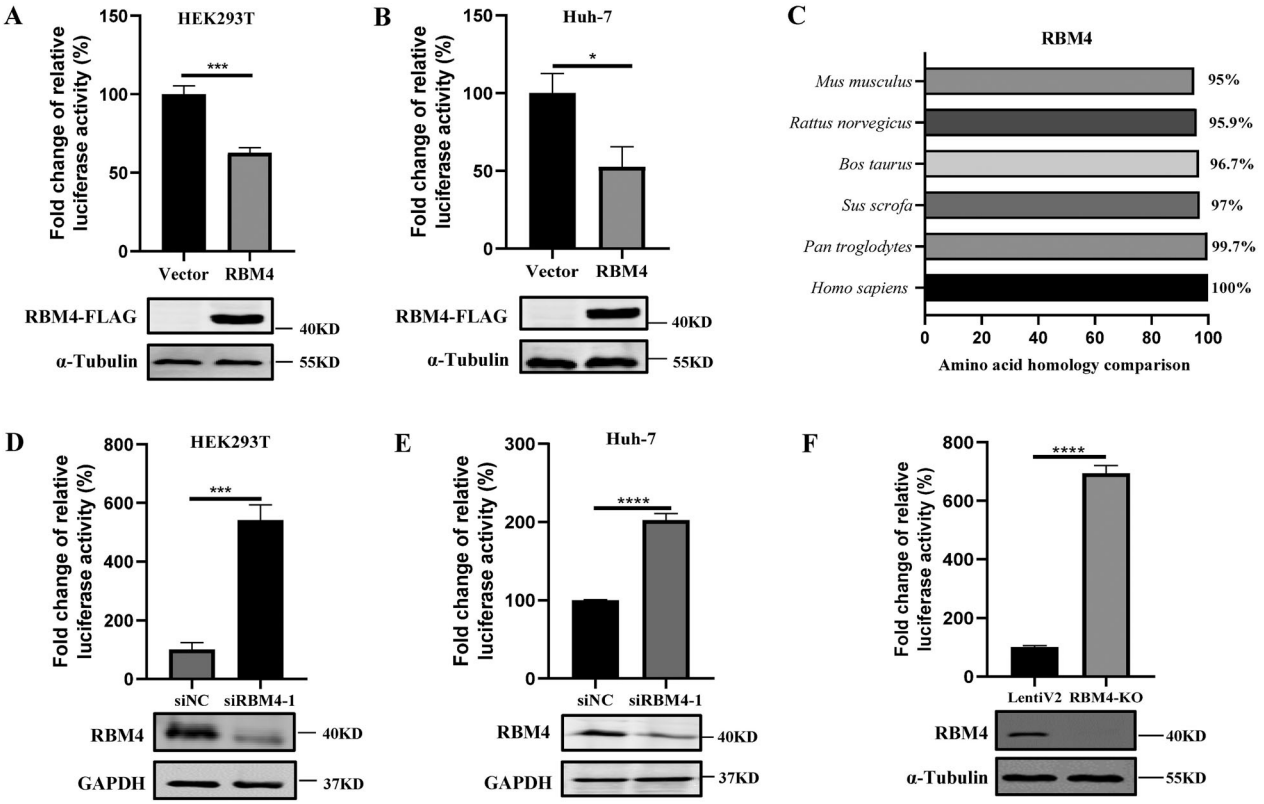

Figure 1. RBM4 could inhibit EBOV replication in both HEK293T and Huh-7 cells. (A–B) HEK293T cells and Huh-7 cells were transfected with pCAGGS-RBM4-FLAG and empty vector. Twenty-four hours later, cells were transfected with plasmids encoding NP, VP35, VP30, L and Tim-1. At 24 h post-transfection (h p.t.), medium was discarded, and cells were infected with EBOV-trVLPs. At 12 h post-infection (h p.i.), supernatants were discarded and replaced with fresh medium. At 48 h p.i., expression of RBM4 in cells was detected by WB, and the viral replication was measured by dual-luciferase assay. (C) Homology analysis of RBM4 in Ebola virus susceptible hosts. (D–E) HEK293T cells and Huh-7 cells were transfected with siRNA NC and si345. Twenty-four hours later, EBOV-trVLPs assay were performed as described in (A–B). At 48 h p.i., expression of RBM4 in cells was detected by WB, and the viral replication was measured by dual-luciferase assay. (F) EBOV-trVLPs assay were conducted with RBM4-KO and control LentiV2 cell lines. At 48 h p.i., expression of RBM4 in cells was detected by WB, and the viral replication was measured by dual-luciferase assay. The mean and SEM from one representative experiment ($n = 3$) of 3 independent experiments are indicated. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$ (two-tailed Student t-test).

3(A,B)). The quantitative RT–PCR results also indicated that RBM4 could bind to the viral genome compared with control (Figure 3(C)). Since a recent study reported that a series of proteins, including RBM4, may interact with EBOV VP35 [25], we wonder whether RBM4 interacts with the viral proteins of EBOV. Co-IP experiment was conducted and the result showed that RBM4 was not able to interact with any EBOV viral proteins including VP35, VP24, VP30, VP40, GP and NP (Figure 3(D)). The above results indicated that RBM4 could interact with the viral genome of EBOV instead of viral proteins.

N-terminal RRM1 domain of RBM4 is the key region that interacts with the genome of EBOV

We further aim to identify the subdomains of RBM4 that could interact with EBOV RNA genome. As an RNA binding protein, RBM4 was reported to consist of three main structural domains, which are RNA recognition motif (RRM), zin-fingers and alanine-rich region [26]. To describe the key region of RBM4 interacting with the EBOV RNA genome, two different truncated forms of RBM4 were generated. RBM4a contains 145–364 aa and RBM4b contains 1–200 aa, with the Zinc-finger domain overlapped (Figure 4(A)). Both the two truncated forms of RBM4 and the full-length RBM4 were used in the RIP assay, and we found that RBM4b could interact with the genome of EBOV rather than RBM4a (Figure 4(B)). The quantitative RT–PCR results further support the interaction between RBM4b and the virus genome (Figure 4(C)). Thereby, RBM4b, the N-terminal domain of RBM4, was the main region interacting with the EBOV RNA genome.

Since RBM4b mainly consisted of RRM1 and RRM2 subdomains, and the RRMs subdomains were reported to be the main RNA binding region, we next investigated RRM subdomain which was the key region that supported the interaction between RBM4 and EBOV RNA genome. The two RRM subdomains were individually truncated (Figure 4(D)), and the results of RIP assay showed that the truncation

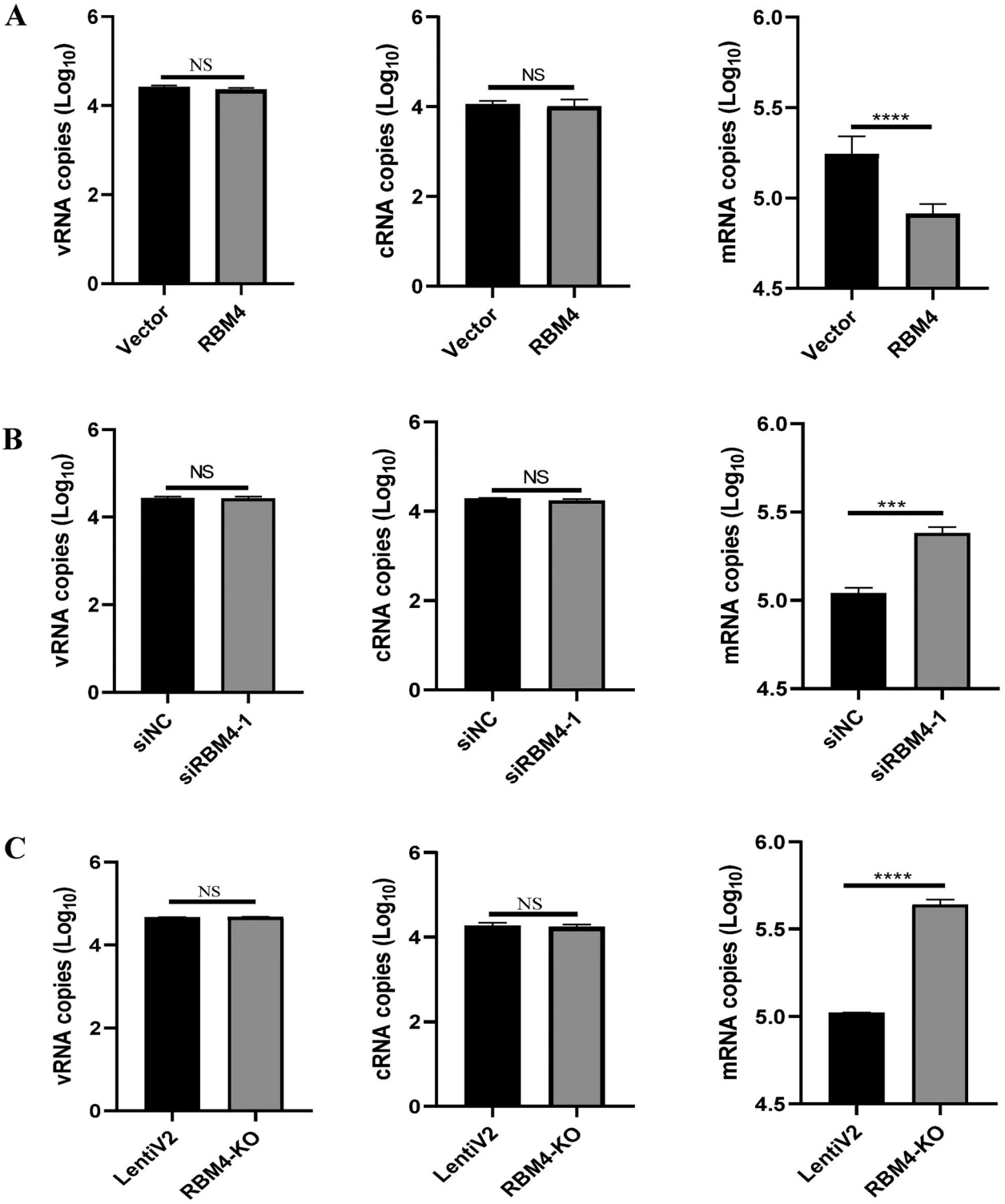

Figure 2. The RBM4 inhibits mRNA production of EBOV. (A) HEK293T cells were transfected with pCAGGS-RBM4-FLAG and empty vector. At 24 h p.t., EBOV-trVLPs assay were performed. At 72 h p.t., cells were collected and detected mRNA level by quantitative RT-PCR. (B) HEK293T cells were transfected with negative control siRNA (NC) and RBM4 specific siRNA named siRBM4-1. At 24 h p.t., EBOV-trVLPs assay were performed. At 72 h p.t., cells were collected and detected mRNA level by quantitative RT-PCR. (C) EBOV-trVLPs assay were performed with RBM4-KO and control LentiV2 cell lines. Forty-eight hours later, cells were collected and detected mRNA level by quantitative RT-PCR. The mean and SEM from one representative experiment ($n = 3$) of 3 independent experiments are indicated. ***$P < 0.001$, ****$P < 0.0001$ (two-tailed Student t-test). NS, not significant.

form which lacked RRM2 (Del-RRM2) could still interact with the genome of EBOV, rather than the truncation form that lacked RRM1 (Del-RRM1) (Figure 4(E)). The quantitative RT–PCR results further support the interaction between delRRM2 and the virus genome (Figure 4(F)). These above results demonstrated that RRM1 subdomain, located at the N-terminal of RMB4b, was the key region that supported the interaction between RBM4 and EBOV RNA genome.

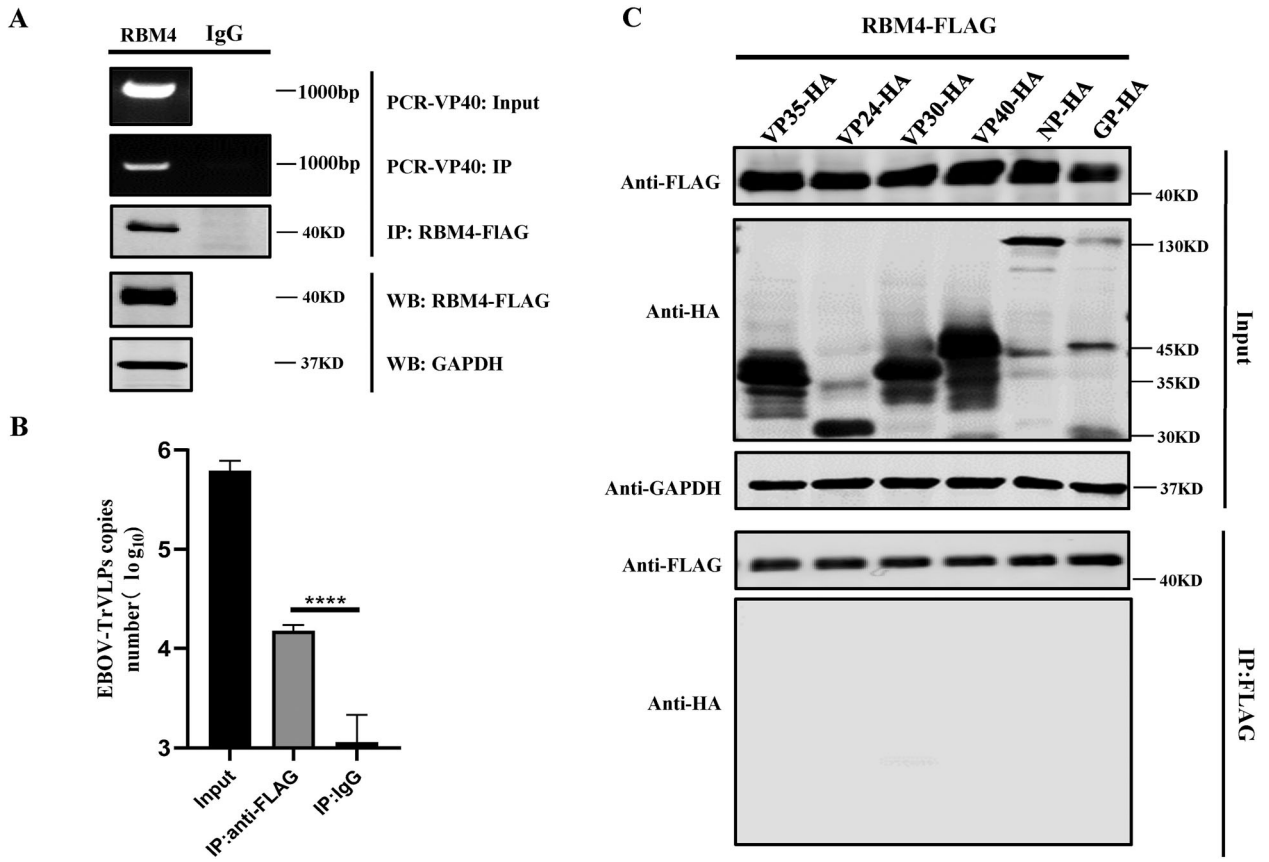

Figure 3. RBM4 interacts with EBOV genomic RNA instead of viral proteins. (A–B) RBM4-FLAG interacted with EBOV genome. HEK293T cells were transfected with RBM4-FLAG. Twenty-four hours later, EBOV-trVLPs assay were performed. Seventy-two hours later, expression of RBM4-FLAG and viral genome in cells were detected by WB and PCR, respectively. Then, the interaction between RBM4-FLAG and viral genome was detected using RIP assay. (C) C-terminal FLAG-tagged RBM4 no interacted with other viral proteins. Plasmids encoding VP35-HA, VP24-HA, VP30-HA, VP40-HA, NP-HA, and GP-HA were co-transfected separately with RBM4-FLAG into HEK293T cells. At 48 h p.t., cells were collected, followed by anti-FLAG Co-IP assay and WB. The mean and SEM from one representative experiment ($n = 3$) of 3 independent experiments are indicated. ****$P < 0.0001$ (two-tailed Student *t*-test).

"CU" enrichment region within the 3′-leader of EBOV RNA genome is the key region that interacts with RBM4

It was reported that RBM4 can specifically bind to the "CUCU" or "CCUUCU" elements which was so-called the "CU" enrichment region [27,28]. To identify the key region in EBOV RNA genome that supports the interaction between RBM4 and EBOV genome, "CU" enrichment regions were screened in the EBOV RNA genome. We found two "CU" enrichment regions, one was "CUUCUU" in PE1 and the other was "CUCCUUCU" in TSS. Both of them were located in the 3′-leader of the EBOV genome. To investigate whether such "CU" enrichment regions were the key components mediating the interaction between the RRM1 domain of RBM4 and EBOV genome, we constructed PE1 mutated, TSS mutated, and PE1/TSS mutated viral genomes by replacing the PE1 sequence (CUUCUU) and the TSS sequence (CUCCUUCU) with "AGAGAG" and "GAAAGGAG," respectively (Figure 5(A)). RIP assay results showed that RBM4 was able to interact with wildtype, PE1 mutated, and TSS mutated. However, no interaction was observed between RBM4 and the viral genome with PE1/TSS mutant (Figure 5(B)). The quantitative RT–PCR results were consistent with the RIP results, which showed that RBM4 interacted with the wildtype virus genome and a single mutation of "CU" enrichment regions but not the double mutant (Figure 5(C)). Further experiments verified that such "CU" enrichment regions also mediated the interaction between the RRM1 domain and EBOV genome (Figure 5(D,E)). Collectively, our data showed that the two "CU" enrichment elements within the 3′-leader of EBOV genome were the key region for its interaction with the RRM1 domain of RBM4.

Interaction between RBM4 and EBOV RNA genome via RRM1 is responsible for the reduced viral mRNA production and EBOV replication

Since the key region that supports the interaction between RBM4 and EBOV RNA genome was

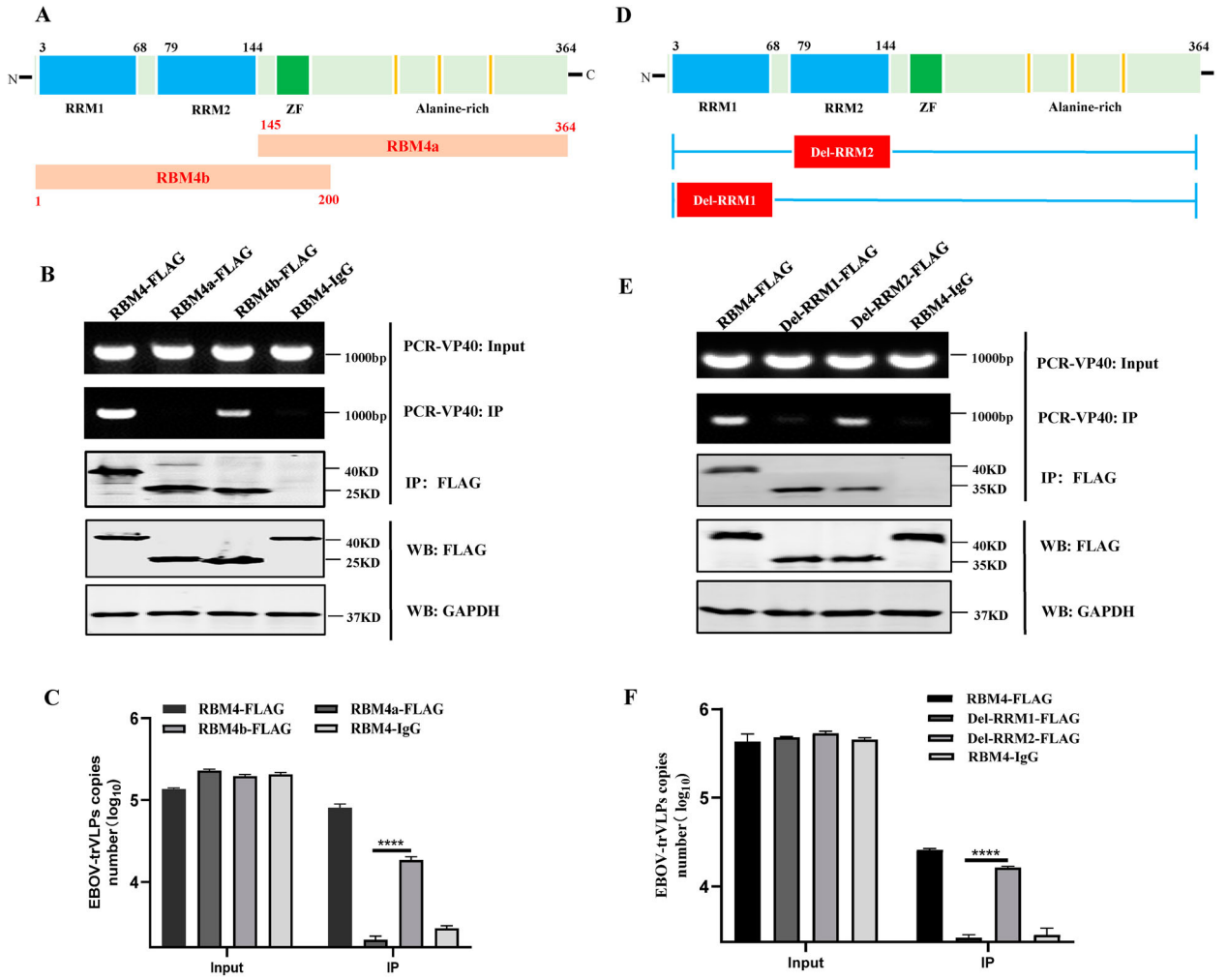

Figure 4. The N-terminal region RRM1 of RBM4 is the key domain in interacting with EBOV genome. (A) Schematic of two RBM4 mutants including N-terminal RBM4b and C-terminal RBM4a. (B–C) The plasmids encoding RBM4-FLAG, RBM4a-FLAG and RBM4b-FLAG were transfected with HEK293T cells. At 24 h p.t., EBOV-trVLPs assay were performed. At 72 h p.t., expression of RBM4 or its truncations and viral genome in cells were detected by WB and PCR, respectively. Then, the interaction between RBM4 or its truncations and viral genome was detected using RIP assay. (D) Schematic of two RBM4 mutants including delRRM1 with deleting RRM1 domain and delRRM2 with deleting RRM2 domain. (E–F) The plasmids encoding RBM4-FLAG, delRRM1-FLAG and delRRM2-FLAG were transfected with HEK293T cells. At 24 h p.t., EBOV-trVLPs assay was performed. At 72 h p.t., expression of RBM4 or its truncations and viral genome in cells were detected by WB and PCR, respectively. Then, the interaction between RBM4 or its truncations and viral genome was detected using RIP assay. The mean and SEM from one representative experiment ($n = 3$) of 3 independent experiments are indicated. ****$P < 0.0001$ (two-tailed Student *t*-test).

identified, we further wonder whether the interaction between RBM4 and EBOV RNA genome was responsible for the inhibition of EBOV replication by RBM4. It was found that both the full-length RBM4 and RBM4b could inhibit the replication of EBOV instead of RBM4a (Figure 6(A)). However, such inhibition could not be observed when RRM1 subdomain was deleted (Figure 6(B)), indicating that the interaction between RRM1 subdomain and EBOV RNA genome may be responsible for the inhibition of EBOV replication by RBM4. Besides, it was also found that the interaction between RBM4 and EBOV RNA genome *via* RRM1 domain was responsible for the reduced EBOV mRNA production, since RBM4 could not inhibit the EBOV mRNA production when its RRM1 domain was deleted (Figure 6(C)). The above results indicated that interaction between RBM4 and EBOV RNA genome *via* RRM1 domain was responsible for the reduced viral mRNA production and EBOV replication by RBM4, making the RRM1 domain of RBM4 a novel target for anti-EBOV therapy. Therefore, the homology of RRM1 subdomain within different species that could be infected with EBOV was further investigated, and it was found that there was almost 100% homology of RRM1 subdomain within different species (Figure 6 (D)). Collectively, the RRM1 subdomain of RBM4 was the key region that participated in the suppression of EBOV replication, and the nearly 100% homology of RRM1 subdomain within different susceptible species for EBOV infection made it a potential antiviral target.

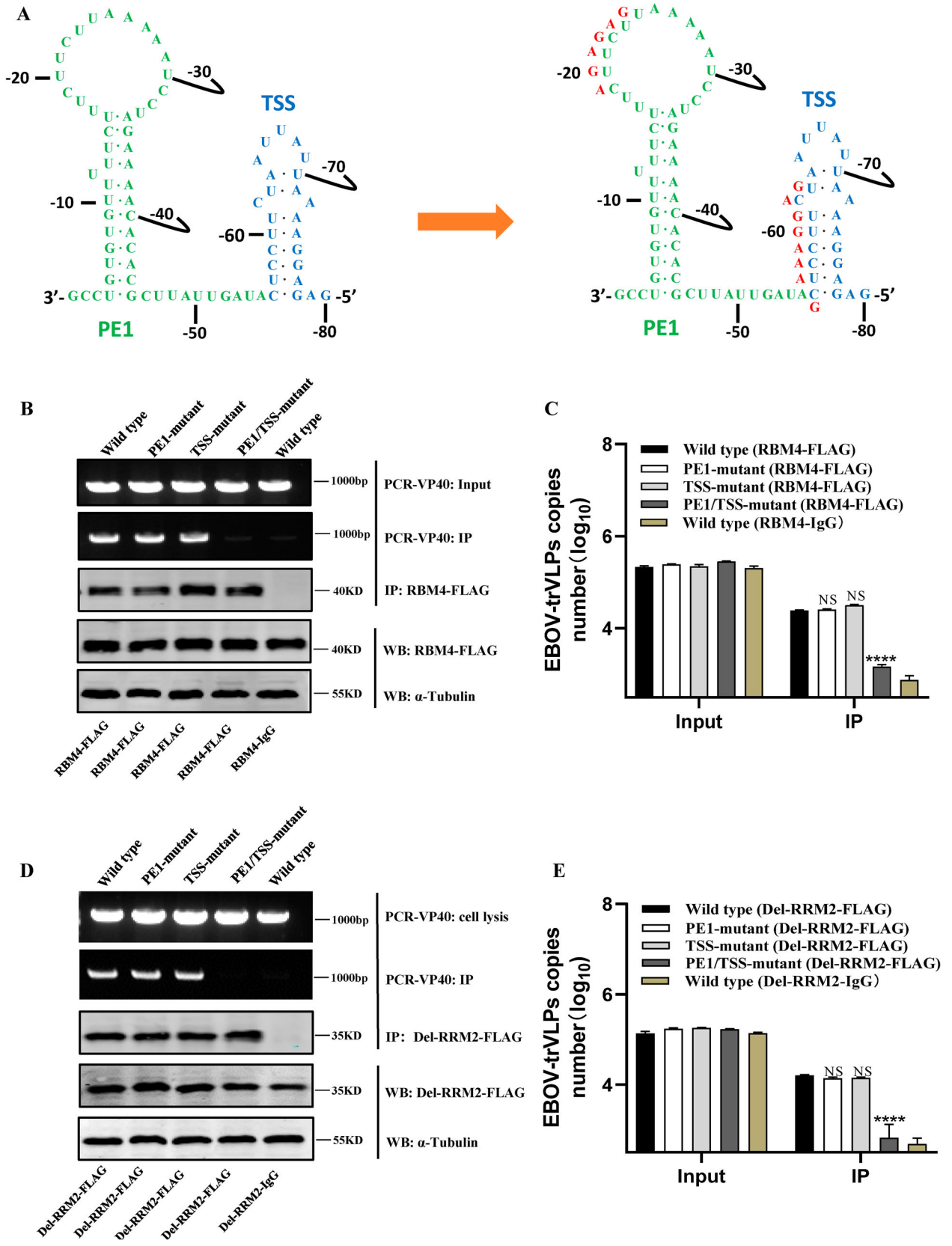

Figure 5. The "CU" enrichment elements of 3′-leader region of viral genome are crucial for interacting with RBM4. (A) Schematic of viral genome 3′-leader region, and its mutant which replacing the "CUUCUU" sequence of PE1 and "CUCCUUCU" sequence of TSS with "AGAGAG" and "GAAAGGAG." (B–C) HEK293T cells were transfected with pCAGGS-RBM4-FLAG. Twenty-four hours later, cells were transfected with plasmids encoding NP, VP35, VP30, L, p4cis-vRNA-RLuc or p4cis-vRNA-RLuc-mutant. At 24 h p.t., supernatants were discarded and replaced with fresh medium. At 48 h p.i., expression of RBM4-FLAG and viral genome or its mutants in cells were detected by WB and PCR, respectively. Then, the interaction between RBM4-FLAG and viral genome or its mutants was detected using RIP assay. (D–E) HEK293T cells were transfected with pCAGGS-Del-RRM2-FLAG. Twenty-four hours later, cells were transfected with plasmids encoding NP, VP35, VP30, L, p4cis-vRNA-RLuc or p4cis-vRNA-RLuc-mutant. At 24 h p.t., supernatants were discarded and replaced with fresh medium. At 48 h p.i., expression of Del-RRM2-FLAG and viral genome or its mutants in cells were detected by WB and PCR, respectively. Then, the interaction between Del-RRM2-FLAG and viral genome or its mutants was detected using RIP assay. The mean and SEM from one representative experiment ($n = 3$) of 3 independent experiments are indicated. ***$P < 0.001$, ****$P < 0.0001$ (two-tailed Student t-test). NS, not significant.

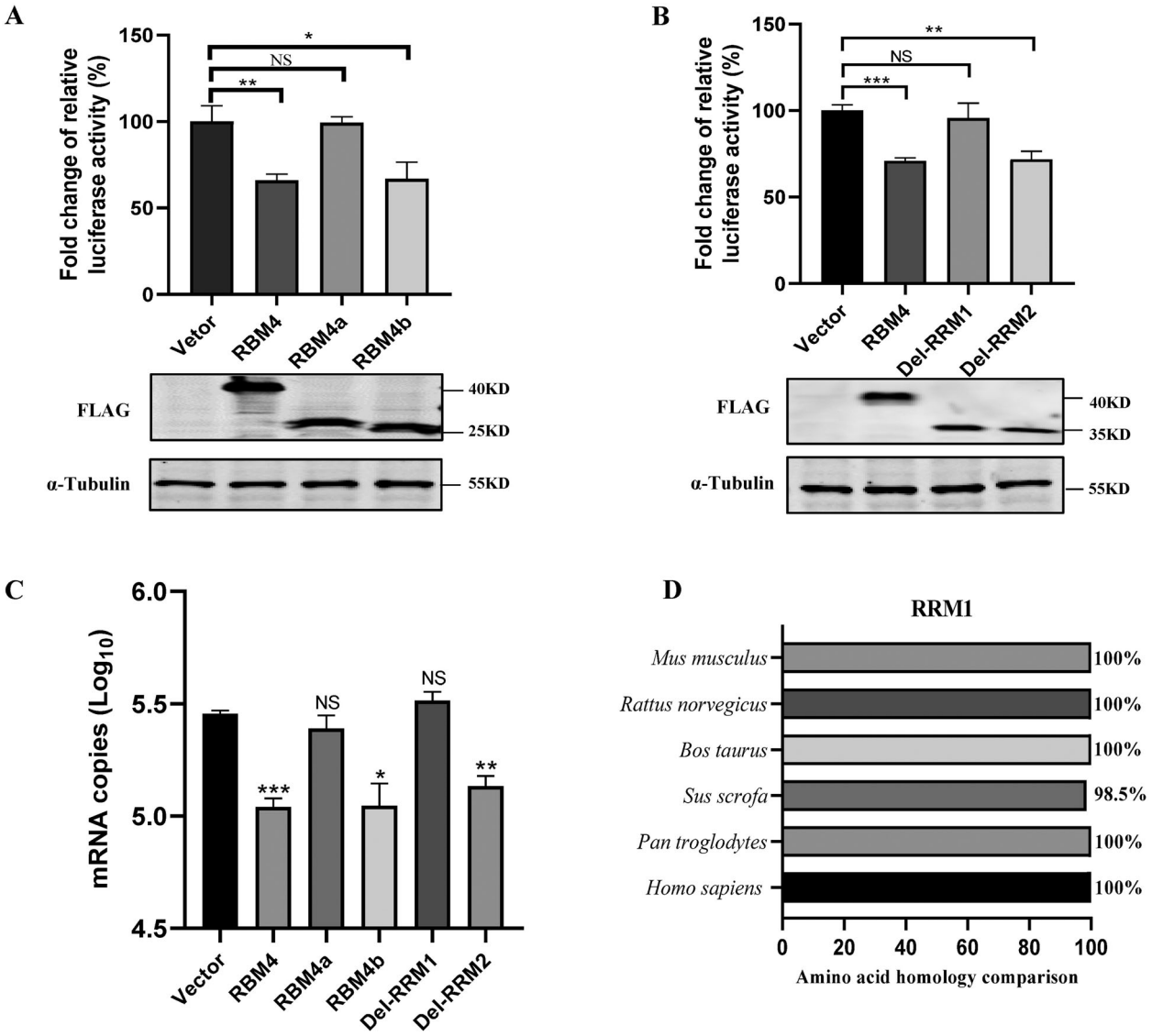

Figure 6. The N-terminal region RRM1 of RBM4 is responsible for inhibiting the replication of EBOV. (A) The plasmids vector, and encoding RBM4-FLAG, RBM4a-FLAG and RBM4b-FLAG were transfected with HEK293T cells. At 24 h p.t., EBOV-trVLPs assay were performed. At 72 h p.t., expression of RBM4 or its mutants in cells was detected by WB, and the viral replication was measured by dual-luciferase assay. (B) The plasmids vector, and encoding RBM4-FLAG, delRRM1-FLAG and delRRM2-FLAG were transfected with HEK293T cells. At 24 h p.t., EBOV-trVLPs assay were performed. At 72 h p.t., expression of RBM4 or its mutants in cells was detected by WB, and the viral replication was measured by dual-luciferase assay. (C) The plasmids vector, and encoding RBM4-FLAG, RBM4a-FLAG, RBM4b-FLAG, delRRM1-FLAG, and delRRM2-FLAG were transfected into HEK293T cells. At 24 h p.t., EBOV-trVLPs assay were performed. At 72 h p.t., cells were collected to detect mRNA level by quantitative RT-PCR. (D) Homology analysis of RRM1 of RBM4 in EBOV susceptible hosts. The mean and SEM from one representative experiment ($n = 3$) of 3 independent experiments are indicated. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (two-tailed Student t-test). NS, not significant.

RBM4 could upregulate the expression of cytokines involved in innate immune responses

Apart from the direct interactions between RBM4 and EBOV RNA genome, we wonder whether RBM4 could regulate the innate immune pathway since RBM4 was reported to be involved in antiviral responses in shrimp by regulating humoral immunity [20]. Therefore, we transfected the RBM4 expression plasmid into HEK293T cells followed by poly(I:C) stimulation, and the levels of several cytokines including *IFNB1*, *CXCL10* and *TNFA* were measured by quantitative RT–PCR. It was found that poly(I:C) could successfully stimulate the expression level of such cytokines and RBM4 could further increase their expressions (Figure 7(A–C)). Meanwhile, the levels of these cytokines were obviously downregulated in HEK293T cells with endogenous RBM4 knocked out compared with the control group, followed by poly(I:C) stimulation (Figure 7(D–F)). Therefore, RBM4 could also regulate the innate immune pathway and may exert its antiviral function synergistically.

Discussion

The interaction between EBOV infection is actually a complex conflict between the host and the virus. On

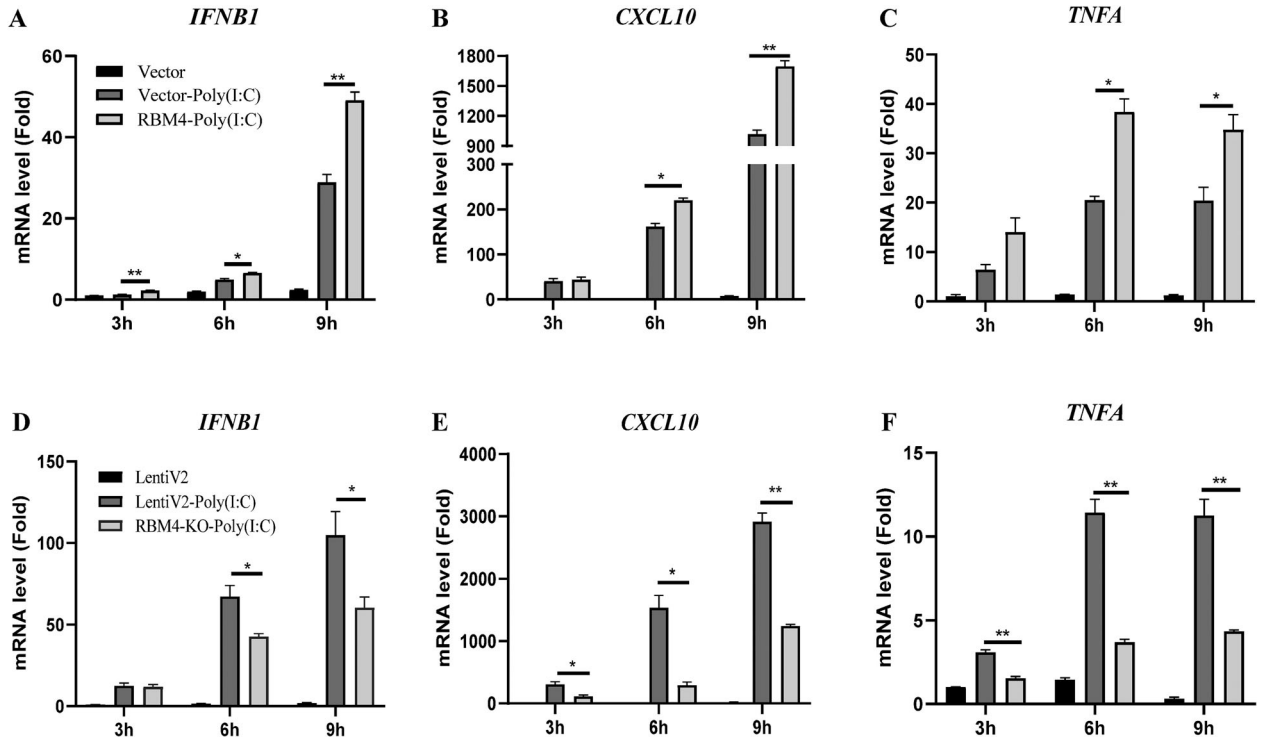

Figure 7. The upregulation of cytokine may be synergically involved in the anti-EBOV role of RBM4. (A–C) HEK293T cells were transfected with pCAGGS-RBM4-FLAG and empty vector. Twenty-four hours later, cells were then transfected with poly(I:C). All cells were collected, and different cytokines were detected by quantitative RT-PCR at 3 h p.t., 6 h p.t. and 9 h p.t.. (D–F) RBM4-KO and control LentiV2 HEK293T cells were transfected with pCAGGS-RBM4-FLAG and empty vector. Twenty-four hours later, cells were then transfected with poly(I:C). All cells were collected, and different cytokines were detected by quantitative RT-PCR at 3 h p.t., 6 h p.t. and 9 h p.t.. The mean and SEM from one representative experiment ($n = 3$) of 3 independent experiments are indicated. *$P < 0.05$, **$P < 0.01$ (two-tailed Student *t*-test).

the one hand, EBOV increases its replication, evades the host immune response and accelerates disease progression by interacting with host factors including Protein Phosphatase 1 (PP1) [29], SET and MYND domain-containing protein (SMYD3) [30]; and carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD) [31]. On the other hand, the host restricts or even inhibits the replication of EBOV by interacting with the virus [32]. Therefore, uncovering the interactions between EBOV and the host was of significance in the prevention, diagnosis and treatment of EBOV infection [33]. In this study, we found that RBM4, a member of RBP family, could inhibit EBOV replication *by* directly interacting with the viral RNA genome.

Previous studies have reported that several members of RBM protein family were able to restrict cancer progression. RNA binding motif single-stranded interacting protein 2 (RBMS2) could increase the stability of p21 mRNA by binding to its 3′-UTR and inhibit the proliferation of breast cancer cells [34]. Besides, recent studies have found that some members of RBM protein family could participate in the regulation of viral replication. RBM24 inhibits the translation of SARS-CoV-2 polyproteins by interacting with the 5′-UTR [35]. RBM38 interacts with the intronic splicing enhancer 2 (ISE2) element of B19V pre-mRNA and enhances 11-kDa protein expression to promote viral replication [36]. In our study, we found that RBM4, as a member of RBPs, significantly inhibited the replication of EBOV by suppressing the production of viral mRNA rather than influencing the production of vRNA and cRNA (Figures 1 and 2), indicating the potential anti-EBOV role of RBM4.

As is known that EBOV could infect a wide range of organs and tissue cells, and the major target cells during its early infection were macrophages and dendritic cells. An early study found that RBM4 is expressed in liver, spleen, thymus and other tissues in the human body (Supplementary Figure 2A) [37]. Simultaneously, *via* detecting the expression level of RBM4 in different tissues of mice, we found that many tissues of mice could detect the mRNA level expression of RBM4 (Supplementary Figure 2B). RBM4 is broadly expressed in different tissues and may play an important role in EBOV infection.

Here, the mechanism by which RBM4 inhibited the replication of EBOV was investigated. RBM4 can bind to the “CU” enrichment region in PE1 and TSS of the EBOV genome 3′-leader through its RRM1 domain, resulting in the inhibition of viral mRNA production and replication of EBOV (Figures 4 and 5). The findings suggest that RBM4 plays a significant role in inhibiting EBOV replication. Since other studies have

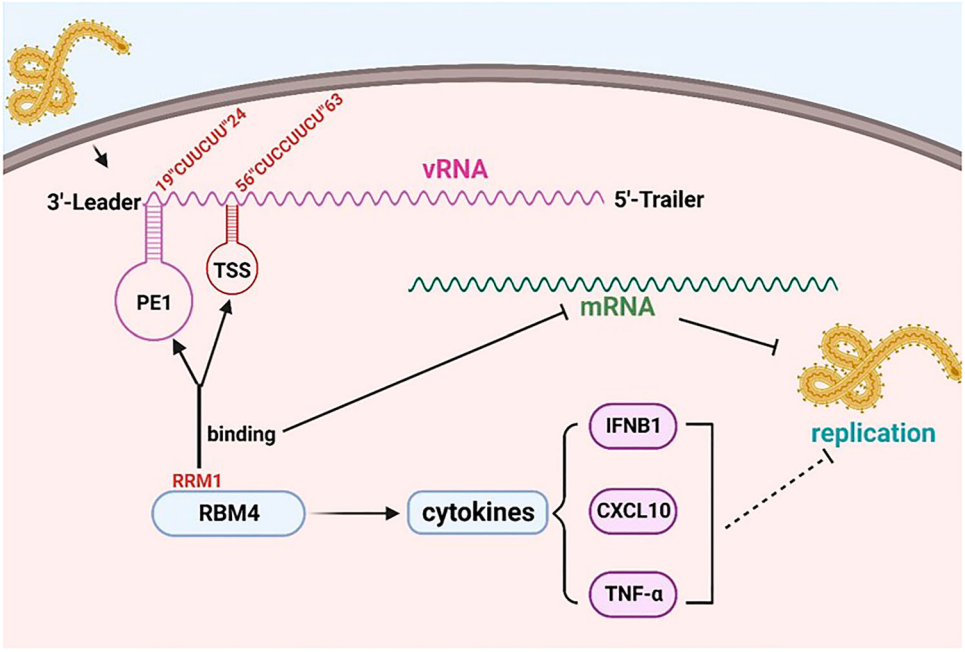

Figure 8. Schematic diagram of RBM4 inhibiting the replication of EBOV. After EBOV infected with cells, RBM4 binds to "CU" enrichment elements of viral genome 3′-leader region to suppress mRNA production to inhibit the replication of EBOV. The RBM4 also upregulates some cytokines and may synergistically exert its antiviral function.

also proved the antiviral role of RBM4 in white spot syndrome virus [20] and human endogenous retroviruses [38], our study provided further evidence of highly lethal EBOV virus for RBM4 to exert its antiviral function, which would enlarge its antiviral spectrum.

Ebola virus disease is a zoonotic disease, with occasional spillovers to humans, non-human primates, and possibly other mammal animals and rodent animals [2,39]. Fruit bats are thought to be the natural hosts of the EBOV [40]. Therefore, analysing the conservation of RBM4, especially RRM1, among different species, is crucial for evaluating the anti EBOV potential of RBM4 and its future practical application in antiviral therapy. In this study, we found that the homology of amino acid sequence of RBM4 in different susceptible animals of EBOV was over 95%, especially for the curial domain RRM1, the homology of which was almost 100% within different species. This might imply that RBM4 had the potential to be an anti-EBOV candidate in animals susceptible to EBOV infection.

The multiple cytokines and chemokines could be produced after viral infection as the host immune responses, in which type I IFNs are the principal cytokines involved in the antiviral responses [41]. Accumulating evidence show that many RNA binding proteins, including RBM4, rely on their RNA binding capacity to enhance host immune responses. For example, RBM4 is able to activate the NF-κB signalling pathway via interacting and stabilizing RelA mRNA [42]. Additionally, RBM47 amplified IFN downstream signalling by binding to the 3'-UTR of IFNAR1 mRNA, increases mRNA stability, and retards the degradation of IFNAR1 [43]. Therefore, we surmise that RBM4 increases the expression of *IFNB1*, *TNFA*, and *CXCL10* via similar mechanisms, which needs to be further explored.

In conclusion, our present study demonstrated that the RBM4 could directly bind to the "CU" enrichment elements of PE1 and TSS located in the 3′-leader region of the viral genome *via* its RRM1 domain to suppress viral mRNA production and further inhibit the replication of EBOV (Figure 8). Simultaneously, RBM4 may also regulate innate immune pathways to synergistically exert its antiviral function. Taken together, the results imply that RBM4 might serve as a novel target of anti-EBOV strategy. Further investigation is still needed to reveal how to apply RBM4 in the treatment of EBOV infection.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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