Cell stress modulates the function of splicing regulatory protein RBM4 in translation control

Jung-Chun Lin*†, Min Hsu*, and Woan-Yuh Tarn*‡

*Institute of Biomedical Sciences, Academia Sinica, Taipei 115, Taiwan; and †Institute of Molecular Medicine, College of Medicine, National Taiwan University, Taipei 100, Taiwan

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RNA-binding motif protein 4 (RBM4) plays a regulatory role in alternative splicing of precursor mRNA. We show here that cell stress such as arsenite exposure induces phosphorylation of RBM4 at serine 309 and also drives its cytoplasmic accumulation and targeting to stress granule via the $MKK_{3/6}$-p38 signaling pathway. Accordingly, RBM4 suppresses cap-dependent translation in a cis-element-dependent manner. However, RBM4 concomitantly activates internal ribosome entry site (IRES)-mediated translation likely by promoting the association of translation initiation factor eIF4A with IRES-containing mRNAs. Overexpression of RBM4 therefore mimics the effect of cell stress-induced signaling on translation initiation control. Whereas arsenite treatment promotes RBM4 loading onto IRES mRNAs and enhances RBM4–eIF4A interactions, a nonphosphorylatable mutant of RBM4 was unresponsive to arsenite stress and failed to activate IRES-mediated translation. Thus, our results uncover a previously unrecognized paradigm for the RNA-binding protein RBM4 in its phosphorylation-modulated dual action as a suppressor of cap-dependent and enhancer of IRES-mediated translation in response to stress signals.

cell stress | eIF4A | internal ribosome entry site | phosphorylation | splicing factor

Posttranscriptional control of eukaryotic gene expression comprises several levels of regulation such as processing, export, turnover, localization, and translation of mRNAs (1). Each regulation step involves various combinations of RNA-binding proteins that form dynamic messenger ribonucleoproteins with the transcript. These messenger ribonucleoproteins may individually play specific roles in mRNA metabolism by forming distinct regulatory complexes (2). Some of them continuously shuttle between the nucleus and cytoplasm and may thus participate in multiple steps of mRNA metabolism in different subcellular compartments. For example, nuclear precursor mRNA splicing factors serine/arginine-rich (SR) proteins were recently implicated in several postsplicing activities including mRNA export, quality control, and translation (1, 3–5).

Cellular signaling pathways may relocate messenger ribonucleoproteins and thereby modulate their function. For example, environmental stimuli such as osmotic shock induce phosphorylation and cytoplasmic accumulation of heterogeneous nuclear ribonucleoprotein (hnRNP) A1 via the MAPK pathway and hence alter its activity in splicing regulation (6, 7). Activation of the ERK signaling pathway can drive cytoplasmic accumulation of hnRNP K; blockade of this pathway attenuates the ability of hnRNP K to inhibit translation (8).

The cellular response to environmental stress immediately leads to global repression of protein synthesis and aggregation of stalled translation complexes in cytoplasmic foci termed stress granules (SGs) (9, 10). However, stress-induced attenuation of global translation is also accompanied by selective translation of mRNAs that possess internal ribosome entry sites (IRES) (11, 12). In particular, IRES-mediated translation promotes the expression of several stress-response genes, which either allows cell survival or conversely leads to cell death during stress (13–16). The efficiency of IRES-mediated initiation could be differentially modulated by IRES trans-acting factors (ITAFs) (11, 14). At present, how ITAFs act in IRES-dependent initiation and how their activity is regulated by cellular signaling cascades still remain to be deciphered. Because at least ≈3–5% of human genes are predicted to undergo IRES-mediated translation (15), it is important to understand more about the mechanisms underlying this process.

RNA-binding motif protein 4 (RBM4) is ubiquitously expressed with higher abundance in heart and muscle (17). RBM4 acts as a precursor mRNA splicing regulatory factor and can modulate cell type-specific exon selection of α-tropomyosin by binding to intronic CU-rich elements (18). It functionally antagonizes the activity of polypyrimidine tract-binding protein (PTB) by competing for overlapping cis-elements to determine α-tropomyosin exon selection. Moreover, RBM4 is a nucleocytoplasmic shuttling protein (17), but its cytoplasmic function was not determined.

Here we report that phosphorylation of RBM4 can be induced by cell stress, which accompanies its subcellular relocalization. More interestingly, RBM4 could suppress cap-dependent translation but, on the other hand, activate IRES-mediated translation under the control of cell stress signaling.

Results

Cell Stress Induces Phosphorylation and Cytoplasmic Accumulation of RBM4. We wondered whether RBM4 is a phosphoprotein and therefore tested this possibility by using ^{32}P to metabolically label the cells that were transiently expressing FLAG-tagged RBM4. Immunoprecipitated FLAG-RBM4 was radiolabeled, indicating its phosphorylation (Fig. 1*B*, WT). Because the sequence $RDRS^{309}P$ within the C-terminal domain of RBM4 (Fig. 1*A*) resembles the SR protein kinase (SRPK) phosphorylation site consensus (19), we tested whether serine 309 is a phosphoacceptor site. Alanine substitution of this serine residue in the RBM4 SA mutant largely abrogated ^{32}P incorporation, suggesting that Ser-309 is the major site of phosphorylation (Fig. 1*B*, SA). To investigate the biological relevance of RBM4 phosphorylation, we generated antibodies (anti-pS309) against an RBM4 peptide bearing phosphorylated Ser-309. Immunoblotting showed that anti-pS309 could recognize RBM4 that had been phosphorylated *in vitro* by purified recombinant SRPK1 (Fig. 1*C*). When phosphorylated RBM4 was further treated with

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Abbreviations: SR, serine/arginine-rich; hnRNP, heterogeneous nuclear ribonucleoprotein; SG, stress granule; IRES, internal ribosome entry site; ITAF, IRES trans-acting factors; EMCV, encephalomyocarditis virus; PTB, polypyrimidine tract-binding protein.

‡To whom correspondence should be addressed at: Institute of Biomedical Sciences, Academia Sinica, 128 Academy Road Section 2, Nankang, Taipei 115, Taiwan. E-mail: wtarn@ibms.sinica.edu.tw.

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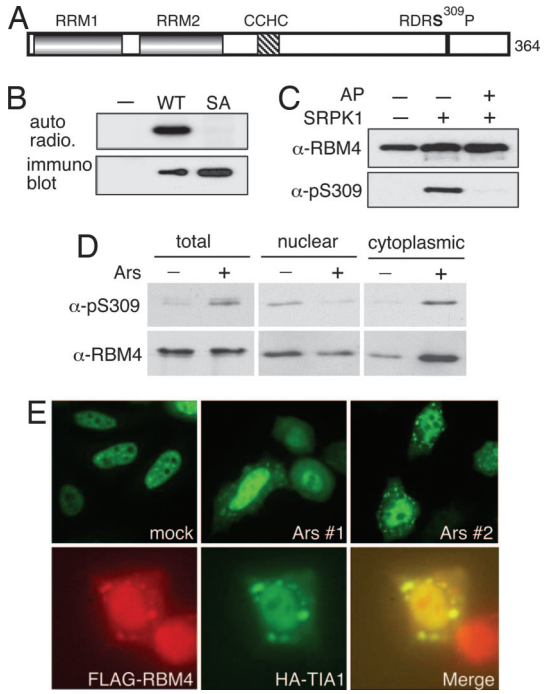

Fig. 1. Arsenite stress induces phosphorylation and subcellular relocalization of RBM4. (*A*) The domain structure of RBM4. RRM and CCHC represent RNA recognition motif and zinc finger, respectively. (*B*) HeLa cells that transiently expressed the wild-type (WT) or SA mutant of FLAG-RBM4 were metabolically labeled with ^{32}P-orthophosphate. Anti-FLAG immunoprecipitates were analyzed by autoradiography and immunoblotting using anti-FLAG. (*C*) MBP-RBM4 was *in vitro* phosphorylated by recombinant SRPK1. The reaction was mock treated or treated with alkaline phosphatase (AP) followed by immunoblotting using anti-RBM4 and anti-pS309. (*D*) HEK 293 cells were treated with 0.5 μM sodium arsenite (Ars) for 1 h. Immunoblotting of total cell lysate and subcellular fractions was performed using anti-pS309 and anti-RBM4. (*E*) HeLa cells were transiently transfected with the GFP-RBM4 expression vector (*Upper*) or cotransfected with vectors for expressing FLAG-RBM4 and HA-TIA-1 (*Lower*), followed by arsenite treatment as in *D*. (*Upper*) Fluorescence of GFP-RBM4 in mock cells and cells treated with arsenite (Ars #1 and Ars #2). (*Lower*) Double immunofluorescence using both anti-FLAG and anti-HA.

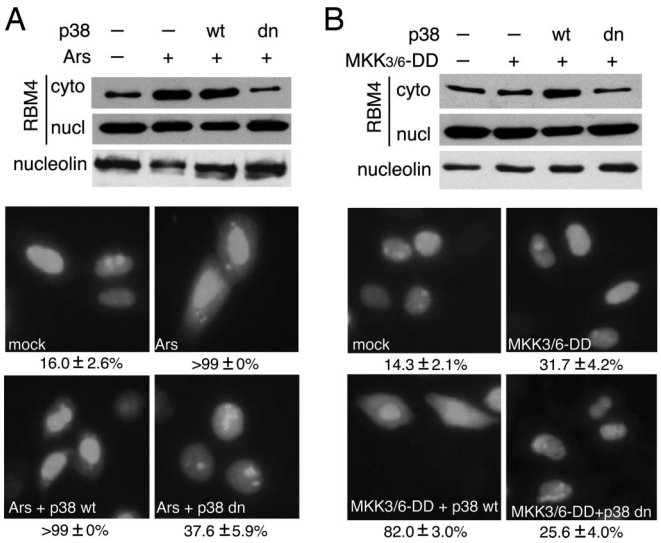

Fig. 2. Arsenite stress transduces signals to RBM4 via the MKK$_{3/6}$-p38 signaling pathway. (*A Upper*) HEK 293 cells were transfected with an empty vector or vector encoding wild-type (wt) or a dominant-negative (dn) mutant of p38 kinase. At 48 h posttransfection, arsenite (Ars) treatment was carried out. RBM4 in the cytoplasmic (cyto) and nuclear factions (nucl) was detected using anti-RBM4; nucleolin was used as a loading control. (*A Lower*) HeLa cells were cotransfected with the GFP-RBM4 expression vector and a p38 kinase vector followed by arsenite (Ars) treatment. Values given are the percentage of the cells expressing cytoplasmic GFP-RBM4 in ≈100 positively transfected cells; average was obtained from three independent experiments. (*B Upper*) Constitutively active MKK$_{3/6}$-DD was transiently expressed in HEK 293 cells alone or together with the wild-type or dominant-negative mutant of p38 kinase. Cell fractionation and immunoblotting were performed as in *A*. (*B Lower*) Transfection of HeLa cells with the GFP-RBM4 vector alone or together with MAPK(s), and data quantitation were as in *A*.

phosphatase, its immunoreactivity was lost, demonstrating the specificity of anti-pS309 (Fig. 1*C*).

We next asked whether RBM4 phosphorylation can be modulated upon changes in cell growth conditions or environments. Using anti-pS309, we found that phosphorylation of RBM4 was significantly enhanced when cells were exposed to 0.5 μM sodium arsenite for 1 h (Fig. 1*D*, total). Arsenite stress-induced phosphorylation of RBM4 occurred primarily at Ser-309, because transiently expressed SA mutant remained unlabeled when cells were fed with ^{32}P (data not shown). We next examined the subcellular distribution of RBM4 under stress conditions. Fig. 1*D* shows that arsenite treatment increased RBM4 protein levels in the cytoplasm, with a concomitant decline of nuclear RBM4 (α-RBM4). Similar results were obtained using anti-pS309, indicating that phosphorylated RBM4 relocates from the nucleus to the cytoplasm upon arsenite treatment.

To verify arsenite-induced relocation of RBM4, RBM4 fusion to GFP was transiently expressed in HeLa cells. Like endogenous RBM4, GFP-RBM4 could be detected by anti-pS309 (data not shown) and localized primarily in the nucleus (Fig. 1*E*). Upon cell exposure to arsenite, GFP-RBM4 appeared in the cytoplasm (Fig. 1*E*, Ars #1). Notably, in ≈50% of the transfectants, RBM4 showed evident signals in cytoplasmic foci (Ars #2). When coexpressed with TIA-1 (Fig. 1*E*) or PABP1 (data not shown), robust colocalization of RBM4 with these SG-associated proteins was observed. In conclusion, upon various stress treatments such as arsenite (this study) and heat shock (data not shown), RBM4 could become phosphorylated and translocate to the cytoplasm and SGs.

Cell Stress Targets RBM4 via the MKK3/6-p38 Signaling Pathway. We next examined cellular signaling pathways that are involved in arsenite-induced cytoplasmic accumulation of RBM4. Pretreatment of HeLa cells with the p38 MAPK inhibitor SB203580 could diminish cytoplasmic signals of GFP-RBM4 even under stress conditions [supporting information (SI) Fig. 7]. Accordingly, cell fractionation experiments showed that transient expression of a dominant-negative p38 mutant, which is defective in its kinase activity, prevented the arsenite-induced cytoplasmic increase of RBM4 (Fig. 2*A Upper*). Consistently, fluorescent images revealed that cytoplasmic GFP-RBM4 was barely detected when the mutant p38 kinase was coexpressed in arsenite-treated HeLa cells (Fig. 2*A Lower*).

When cells expressed both constitutively active MKK$_{3/6}$-DD and wild-type p38 kinases, the level of RBM4 in the cytoplasmic fraction was substantially increased (Fig. 2*B Upper*). Indeed, GFP-RBM4 became detectable in the cytoplasm of ≈30% of the transfected cells upon overexpression of MKK$_{3/6}$-DD alone (Fig. 2*B Lower*). Coexpression of MKK$_{3/6}$-DD and wild-type p38 kinase allowed the majority of the cells showing GFP-RBM4 signals in the cytoplasm, albeit with fewer foci compared with arsenite treatment (Fig. 2*B Lower*). Nevertheless, the dominant-negative p38 kinase failed to enhance the effect of MKK$_{3/6}$-DD upon relocalization of RBM4 to the cytoplasm (Fig. 2*B Lower*). Thus, activation of the MKK$_{3/6}$-p38 pathway could mimic the effect of arsenite-induced stress on driving cytoplasmic relocal-

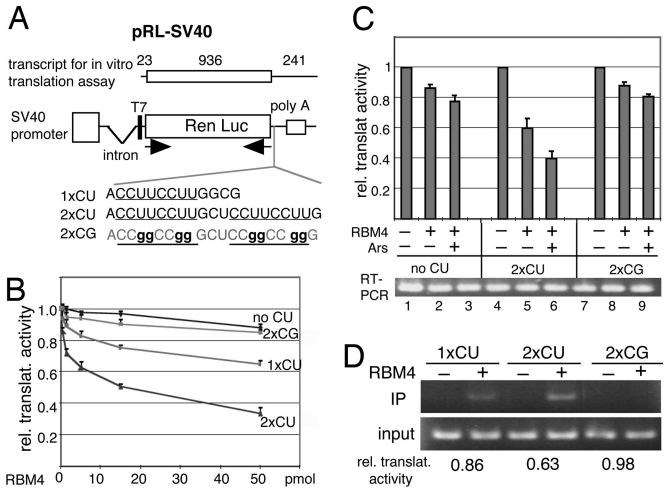

Fig. 3. RBM4 suppresses cap-dependent translation in a CU-rich element-dependent manner. (*A*) The luciferase reporter pRL-SV40 and its derivatives containing the wild-type or mutant CU-rich elements (details can be found in the *SI Materials and Methods*). Nucleotides represent the length of the luciferase coding region and UTRs. (*B*) *In vitro* translation was performed in rabbit reticulocyte lysates with increasing amounts of recombinant His-tagged RBM4. The effect on translation was measured as luciferase activity relative to that in the reaction lacking recombinant RBM4. (*C*) The pRL-SV40 (no CU) vector or its derivatives (2×CU and 2×CG) was transfected alone or together with the FLAG-RBM4 expression vector into HEK 293 cells; the pGL3-control vector encoding firefly luciferase was included in all transfections. The relative luciferase activity (*Renilla* vs. firefly) of individual treatments was compared with that of the mock. RT-PCR was performed using the primers as depicted in *A* (arrows) to detect reporter mRNAs. (*D*) HEK 293 cells were cotransfected with pRL-SV40 (1×CU, 2×CU, or 2×CG) and the FLAG-RBM4 or mock expression vector. Reporter mRNA associated with FLAG-RBM4 was detected by immunoprecipitation-RT-PCR. Values below the gel represent relative translation activity of the reporters; the data were obtained from three independent experiments and normalized to that of the control, pRL-SV40-no CU.

ization of RBM4. Together, the results in Fig. 2 indicated that the effect of arsenite stress signaling on RBM4 relocalization likely involves the $MKK_{3/6}$-p38 signaling pathway.

RBM4 Suppresses Cap-Dependent Translation. Arsenite signals targeting RBM4 to SGs infers its cytoplasmic function in either translation or in other mRNA metabolic event. We thus performed an *in vitro* translation assay to explore the possible function of RBM4 in translation. In this assay, luciferase reporter mRNA was translated in rabbit reticulocyte lysates followed by measurement of the luciferase activity. Because RBM4 has a tendency to bind CU-rich sequences (18), we inserted a CU-rich element derived from human α-tropomyosin intron 9a immediately downstream of the luciferase coding region (Fig. 3*A*). Recombinant His-tagged-RBM4 inhibited translation of the CU-rich element-containing luciferase mRNA in a dose-dependent manner, and the transcript with the duplicated CU element yielded lower luciferase activities than that with one copy (Fig. 3*B*). However, translation of the reporter mRNA containing a mutated CU element (2×CG; ref. 18) was not significantly suppressed by RBM4, indicating that the CU-rich element confers RBM4-mediated translation inhibition.

To assess the effect of RBM4 on translation under stress conditions, we cotransfected a cytomegalovirus-driven luciferase reporter with the FLAG-RBM4 expression vector. RBM4 minimally reduced the expression of the luciferase reporter lacking an engineered CU-rich element or containing a mutated element but significantly suppressed the reporter with the wild-type CU elements (Fig. 3*C*). Association of RBM4 with the reporter mRNAs, likely through the CU-rich element, was correlated well with its translation inhibitory effect (Fig. 3*D*). Moreover, arsenite treatment furthered RBM4-mediated translation suppression, yielding a relative maximal inhibition to ≈50% (Fig. 3*C*, compare lane 6 with lane 3). The level of cytoplasmic luciferase mRNA was similar between the transfectants (Fig. 3*C*, RT-PCR), indicating that RBM4 had no apparent effect on reporter mRNA export or on mRNA stability. Together, RBM4 potentially exerts a suppressive activity on translation via binding to responsive elements within the 3′ UTR of mRNAs.

RBM4 Modulates IRES-Mediated Translation. Although cellular stresses largely inhibit cap-dependent translation, some mRNAs are instead translated via their IRES under this condition (12, 15). We therefore tested whether RBM4 could modulate IRES-mediated translation. A CMV-driven dicistronic reporter was used for the *in vivo* translation assay; expression of the two cistrons, firefly and *Renilla* luciferases, involves cap- and encephalomyocarditis virus (EMCV) IRES-dependent translation, respectively (Fig. 4*A*). Translation initiation factor eIF4A is essential but limiting for the EMCV IRES-mediated translation (20). Therefore, when eIF4A was overexpressed, the activity of *Renilla* luciferase was elevated by ≈4-fold (Fig. 4*B*, lane 6). Interestingly, overexpression of RBM4 markedly activated this IRES-mediated translation of *Renilla* luciferase, but the activity of cap-dependent translation of firefly luciferase encoded by the same dicistronic mRNA was still suppressed (lane 2). PTB exhibited various effects on EMCV IRES translation (SI Fig. 8). Optimal levels of PTB could merely activate *Renilla* luciferase activity (by at most 1.4-fold; ref. 21), but excess PTB slightly suppressed EMCV translation (Fig. 4*B*, lane 3). Nonetheless, neither ASF/SF2 nor hnRNP A1 had any significant effect on the EMCV IRES activity (lanes 4 and 5).

Translation of the luciferase reporter mRNA bearing the EMCV IRES in the 5′ UTR (Fig. 4*A*) was assayed *in vitro* by using the reticulocyte lysate translation system. Consistent with the *in vivo* observation, recombinant RBM4 stimulated IRES-dependent translation of the *Renilla* luciferase mRNA in a dose-dependent manner (Fig. 4*C*). Note that, under similar conditions, RBM4 inhibited cap-dependent translation to different extents upon the presence or absence of the CU elements (Fig. 3*B*). Therefore, activation of this luciferase reporter by RBM4 was likely through the EMCV IRES.

To explore the mechanism by which RBM4 activates IRES-mediated translation, we investigated the interaction between RBM4 and several translation initiation factors. Coimmunoprecipitation of transiently expressed RBM4 with eIF4A (Fig. 4*D*) and eIF4G (SI Fig. 9*A*) in the presence of RNase indicated that their interaction was through direct contact or protein–protein interactions. However, the interactions between RBM4 and three other translation factors tested, i.e., eIF4E, eIF2α, and PABP1, were likely RNA dependent (SI Fig. 9*A*). Thus, RBM4 may associate with translation initiation complexes or subcomplexes that contain eIF4A and/or eIF4G. Because eIF4A binds stably to the EMCV IRES together with eIF4G (22), we therefore examined whether overexpression of RBM4 has any effect on the eIF4A-IRES RNA interaction. Transiently expressed HA-eIF4A was immunoprecipitated from cell lysates followed by RT-PCR analysis of the coprecipitated RNAs. Fig. 4*E* shows that association of eIF4A with the EMCV IRES reporter mRNA was greatly enhanced by coexpressed RBM4 but not by any other effectors.

Because IRES-mediated translation often occurs in response to cellular stress, we examined whether arsenite-triggered RBM4 phosphorylation at Ser-309 is critical for its activity in IRES translation. The *in vivo* translation assay showed that the non-phosphorylatable RBM4 mutant (SA) failed to promote IRES-

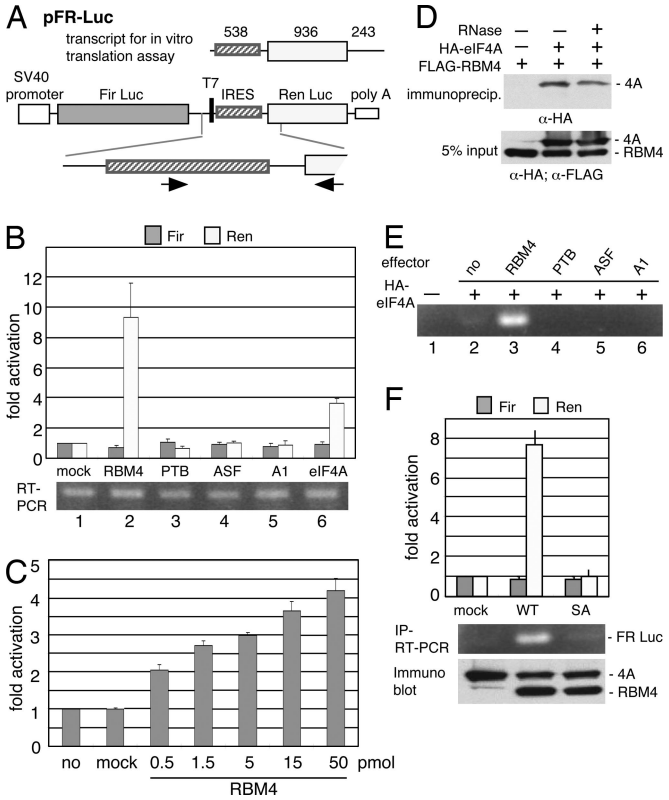

Fig. 4. RBM4 activates EMCV IRES-mediated translation. (*A*) The dicistronic luciferase reporter pFR-Luc contains firefly (Fir) and *Renilla* (Ren) luciferase ORFs; between two ORFs is the EMCV IRES. (*B*) pFR-Luc was mock-transfected or cotransfected with an effector expression vector into HEK 293 cells. Both firefly and *Renilla* luciferase activities were measured in individual transfectants and normalized to that of the respective mock transfectants. Reporter mRNA was detected by RT-PCR using the primers (arrows) as depicted in panel *A*. A1 represents hnRNP A1. (*C*) *In vitro* translation of the *Renilla* luciferase RNA that harbors the EMCV IRES at the 5′ UTR (*A*) was performed as in Fig. 3*B*. No, buffer only; mock, nickel resin eluates of the bacterial lysate without recombinant RBM4. (*D*) For coimmunoprecipitation, FLAG-RBM4 alone (lane 1) or together with HA-eIF4A was transiently expressed in HEK 293 cells. Anti-FLAG immunoprecipitates were further treated with RNase A or mock treated, followed by immunoblotting (antibodies used as indicated below the gel); anti-FLAG was not used for probing the immunoprecipitates because eIF4A signals would be disturbed by comigrated Ig heavy chains. (*E*) Transfection was analogous to *B* except that HA-eIF4A was additionally included in lanes 2–6. Immunoprecipitation was performed using anti-HA followed by RT-PCR detection of the reporter transcript as in *B*. (*F*) The pFR-Luc vector was cotransfected with the wild-type or mutant FLAG-RBM4 expression vector or with an empty vector (mock) into HEK 293 cells. Activation fold was measured as in *B*. Immunoprecipitation (IP)-RT-PCR was as in *E*, and immunoblotting was using both anti-HA and anti-FLAG.

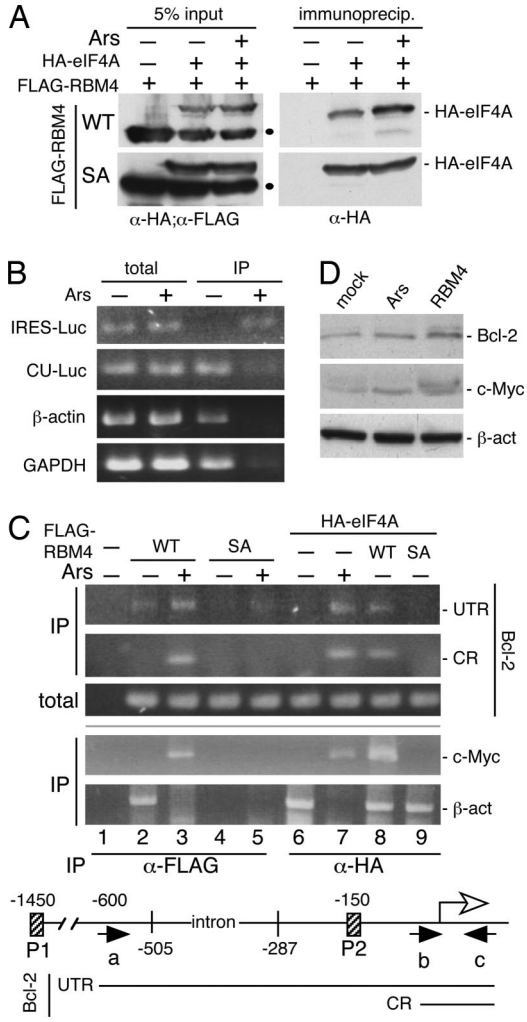

Fig. 5. Arsenite-induced signaling modulates the function of RBM4 in translation. (*A*) HEK 293 cells were transiently transfected with the wild-type (WT) or mutant (SA) FLAG-RBM4 vector alone or together with HA-eIF4A, followed by arsenite treatment. Immunoprecipitation and immunoblotting were as in Fig. 4*D*; dots represent FLAG-RBM4. (*B*) The FLAG-RBM4 expression vector was transfected alone (for detection of endogenous transcripts) or cotransfected with pFR-Luc (IRES-Luc) or pRL-SV40–2×CU (CU-Luc) into HEK 293 cells. RNA obtained from anti-FLAG immunoprecipitates was analyzed by RT-PCR using the primers as in Figs. 3 and 4. (*C*) HEK 293 cells were transfected with the expression vector for wild-type or mutant FLAG-RBM4 alone (lanes 2–5) or were mock transfected (lane 1). Lanes 6–9 show transfection of the HA-eIF4A vector alone (lanes 6 and 7) or together with FLAG-RBM4 (WT, lane 8; SA, lane 9). The transfectants were further treated with arsenite (Ars) or were mock treated. Immunoprecipitation (IP) was performed using anti-FLAG (lanes 1–5) or anti-HA (lanes 6–9) followed by RT-PCR detection. The scheme shows the promoters (P1 and P2) and 5′ UTR of Bcl-2 and the RT-PCR products (UTR and CR, representing coding region) generated by two sets of the primers (a/c and b/c, respectively). The nucleotides given are the position relative to the translation start site (white arrow). (*D*) Cell lysate was prepared from mock-treated or arsenite-treated HEK 293 cells or from cells that transiently expressed FLAG-RBM4. Immunoblotting was performed using antibodies for c-Myc, Bcl-2, and β-actin.

mediated translation (Fig. 4*F Upper*). Although the SA mutant still interacted with eIF4A, it was much less effective at promoting the eIF4A-IRES interaction as compared with the wild type (Fig. 4*F Lower*).

The above results indicated that RBM4 can facilitate IRES-mediated translation, perhaps by stabilizing eIF4A or eIF4A-containing initiation complexes on IRES RNA, and moreover provided a clue to how the RBM4 SA mutant lost its IRES-activation effect. The detailed mechanisms still remain to be studied.

Arsenite-Induced Signaling Modulates the Function of RBM4 in Translation. The above results indicated that arsenite-induced signaling may impact the differential activity of RBM4 in translation control. We thus evaluated the effect of arsenite on RBM4 interactions with eIF4A as well as with other initiation factors. The coimmunoprecipitation assay revealed that arsenite treatment could disrupt the interactions of RBM4 with eIF4E and PABP1 but had no significant effect on its interaction with either eIF4G or eIF2α (SI Fig. 10). Interestingly, this treatment appeared to enhance the interaction of eIF4A with the wild-type

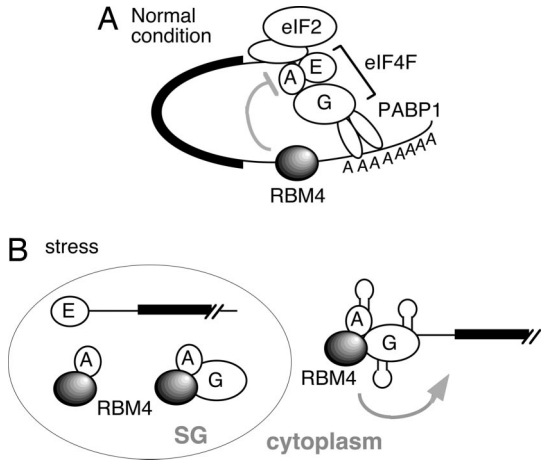

Fig. 6. Model for RBM4 function in translation control. (*A*) RBM4 constantly shuttles between the nucleus and cytoplasm under normal conditions. We hypothesize that as RBM4 reaches the cytoplasm, it participates in gene-specific translation inhibition via binding to CU-rich elements in the target mRNAs. Whether RBM4 suppresses translation via its interaction with eIF4A still remains to be investigated. Thick line, protein coding region. (*B*) When cells encounter stress, MAPK signaling induces RBM4 phosphorylation and translocation to the cytoplasm and SGs. RBM4 may sequester eIF4A or the eIF4A/4G complex to suppress global protein synthesis in SGs. Concomitantly, RBM4 activates IRES-mediated translation by promoting its own and eIF4A interactions with IRES mRNAs. The IRES translation may take place outside the SGs.

RBM4 (by ≈3-fold on average) but not with the mutant (Fig. 5*A*). Therefore, arsenite signaling could modulate RBM4–eIF4A interaction, probably by induction of RBM4 phosphorylation. We next examined the effect of arsenite on RBM4 binding to IRES-bearing mRNAs. Immunoprecipitation-RT-PCR analysis showed that arsenite promoted RBM4 binding to coexpressed IRES-containing mRNA but disrupted its interaction with the mRNAs that undergo cap-dependent translation, including the *Renilla* luciferase reporter and cellular β-actin and GAPDH mRNAs (Fig. 5*B*). These results suggested that RBM4 switches its binding between different sets of mRNA substrates in response to environmental stress.

A number of cellular mRNAs encoding stress responsive proteins use IRES for translation initiation during cell stress (15). To examine whether RBM4 is also involved in cellular IRES-mediated translation, HEK 293 cells were transiently transfected with epitope-tagged RBM4 or eIF4A expression vectors followed by arsenite treatment. RT-PCR was then performed to detect RBM4 or eIF4A-associated mRNAs. For Bcl-2 mRNAs, two sets of the primers were used; one was specific to its IRES region. Indeed, IRES-containing transcripts of Bcl-2 were the major products yielded from promoter P1 in nonneuronal cells, including HEK 293 cells (23). Arsenite treatment induced association of RBM4 and eIF4A with the IRES of Bcl-2 (Fig. 5*C*, lanes 2, 3, 6, and 7), but the SA mutant was not responsive to arsenite (lanes 4 and 5); neither of the treatments altered the level or splicing pattern of Bcl-2 mRNAs (total). Moreover, we observed that the wild-type RBM4, but not the SA mutant, could stimulate eIF4A binding to Bcl-2 IRES (lanes 8 and 9). A similar result was observed with other IRES mRNAs, such as c-Myc (Fig. 5*C*) and BiP (data not shown). Therefore, overexpression of RBM4 could mimic the effect of arsenite on IRES translation. However, both RBM4 and eIF4A dissociated from β-actin mRNA upon arsenite signaling (lanes 2, 3, 6, and 7), which is in sharp contrast to the result with the IRES mRNAs and may reflect global inhibition of translation. As further revealed by immunoblotting, Bcl-2 and c-Myc protein levels were elevated by overexpression of RBM4; such a moderate increase was observed with arsenite treatment (Fig. 5*D*), as reported in ref. 23. Therefore, RBM4 probably modulates IRES-mediated gene expression at the translation level in response to cellular stress.

Discussion

Cell Stress Induces Phosphorylation and Subcellular Relocalization of RBM4. RBM4 is a phosphoprotein, and phosphorylation at its serine 309 can be induced upon cell stress signaling, which leads to alter its subcellular translocation and likely modulates its activity on translation control. Our data suggest that RBM4 is targeted by at least one of the kinases acting downstream of the $MKK_{3/6}$-p38 kinase pathway (Fig. 2). SRPK1 can *in vitro* phosphorylate Ser-309 of RBM4 (Fig. 1), but whether this occurs *in vivo* remains to be investigated. Previous reports indicate that casein kinase II may relay the MAPK signaling to SRPK1 and that SRPK1 can be activated early during cell apoptosis (24–26); these signaling pathways could also be tested by future experiments. The PI3K/Akt pathway induces phosphorylation of several SR proteins and modulates their activity in splicing and even in translation (27, 28). However, PI3K inhibitor LY294002 failed to block arsenite-induced RBM4 phosphorylation and cellular relocalization (SI Fig. 7). Therefore, it is possible that cellular signaling pathways play individual roles in regulating mRNA metabolism through different sets of RNA-binding proteins.

RBM4 primarily localizes to the nucleus with higher concentration in nucleoli and continuously shuttles between the nucleus and the cytoplasm (17). Upon nuclear import, RBM4 is transiently colocalized with SR proteins in nuclear speckles (17). This study shows that cell stress-mediated signaling caused RBM4 accumulation in the cytoplasm and targeting to SGs, further suggesting dynamic localization of RBM4 even under the control of cellular signaling. Thus, like many other RNA processing factors such as SR proteins, HuR, and PTB (1, 14), RBM4 operates as a multifunctional regulator of mRNA metabolism in different cellular compartments and particularly in response to cell stress.

RBM4 Suppresses Cap-Dependent Translation. RBM4 may participate in cellular metabolism of mRNA ligands that bear CU-rich elements because of its preferential binding to such elements (18). Our present data show that RBM4 suppressed translation of CU element-containing reporters (Fig. 3); this result suggests that RBM4 negatively regulates translation of a selective set of cellular mRNAs via binding to their CU-rich sequences under normal cell conditions (Fig. 6*A*). On the other hand, stress signaling could enhance the interaction between RBM4 and eIF4A but dissociate RBM4 from eIF4E-containing complexes and from some mRNAs that are engaged in cap-dependent translation (Fig. 5). A recent report shows that arsenite-induced signaling increases abundance of the eIF4A/4G complex by dissociating eIF4E from the eIF4F complex (29). Thus, under stress conditions, RBM4 may sequester released eIF4A(/4G) in SGs (Fig. 6*B*). Together with the evidence that RBM4 dissociates from non-IRES mRNAs under stress conditions, we suspect that it may act through sequestering released eIF4A(/4G) instead of binding to cis-elements of mRNAs in SGs (Fig. 6*B*)

RBM4 Regulates IRES-Mediated Translation. Overexpression of RBM4 markedly activated EMCV IRES-mediated translation (Fig. 4). RBM4 not only bound to the EMCV IRES but also promoted eIF4A association with this IRES (Fig. 4). In contrast, for the hepatitis C virus (HCV) IRES, RBM4 neither interacted nor activated its translation (data not shown). It is previously known that translation of the EMCV IRES requires eIF4A/4G for 40S ribosomal subunit recruitment whereas activation of HCV IRES-mediated translation is dispensable for eIF4A/4G

(30, 31). Therefore, our observation argues that RBM4 acts in conjunction with eIF4A on IRES-mediated translation initiation. Our data also revealed that arsenite stress induced RBM4 binding to cellular mRNAs encoding Bcl-2 and c-Myc. Although whether translation of these two mRNAs is mediated by authentic IRES is still being debated (23, 32), RBM4 may activate their expression either through IRES or via a not-yet-known mechanism but also involving eIF4A in response to cell stresses.

At present, how ITAFs modulate IRES-mediated translation remains at the periphery of our knowledge. Some ITAFs, such as La and PTB, may act as RNA chaperones to remodel IRES RNA conformation (16, 33). In addition, PTB can promote 48S complex assembly at the initiation codon of IRES (34). This report provides some clues to the mechanism of RBM4 in IRES-mediated translation initiation. We observe here that cell stress augmented the association of RBM4 with eIF4A and that overexpression of RBM4 promoted loading of eIF4A onto different IRES-containing mRNAs (Figs. 4 and 5). Thus, RBM4 participates in IRES-mediated translation control largely through the activity of eIF4A and perhaps in a cis-element-independent manner. To our knowledge, this is perhaps a previously unrecognized mechanism for an ITAF that activates IRES translation.

The question as to where IRES-mediated translation occurs is not directly addressed. Our results show that RBM4 bound to several IRES mRNAs and activated IRES translation as did eIF4A. Although RBM4 is colocalized with eIF4A in SGs, a part of these two proteins still remained in the cytoplasm under stress conditions (data not shown). SGs are thought to act as sites for stalled translation initiation (35). Yet, we observed that SG protein TIA-1 failed to bind any IRESs tested (data not shown). Thus, IRES translation may take place outside the SGs in the cytoplasm under stress conditions (Fig. 6*B*).

Materials and Methods

Stress Treatment. Cells were cultured at a confluency of ≈70–80% and treated with 0.5 μM sodium arsenite for 1 h before harvest.

***In Vivo* Translation Assay.** The cap-dependent translation assay was performed by cotransfection of *Renilla* luciferase reporter pRL-SV40 (or derivatives thereof) as well as firefly luciferase control vector pGL3-control (Promega, Madison, WI) with the FLAG-RBM4 expression vector into HEK 293 cells. For the IRES translation assay, the dicistronic pFR-Luc vector [a kind gift of Y. Chern (Academia Sinica)] containing EMCV IRES nucleotides 363–859 (Fig. 4*A*; similar to GW34 in 4) was used as a reporter. HEK 293 cells were cotransfected with pFR-Luc and an effector expression vector for 48 h and were then lysed in Passive Lysis Buffer (Promega). The reporter transcripts were examined by RT-PCR; no spurious splicing products (5) were detected (data not shown). Firefly and *Renilla* luciferase activities were measured by using the Dual-Luciferase Reporter Assay system (Promega) on a luminometer (Berthold, Nashua, NH). The data were collected from at least three independent experiments.

***In Vitro* Translation Assay.** Plasmid pRL-SV40 (and derivatives thereof) and pFR-Luc were linearized and used as templates for *in vitro* transcription; these transcripts were diguanosine-capped but had no poly(A) tail. *In vitro*-transcribed RNA was recovered by precipitation with 7 M LiCl and quantified by UV spectrophotometry. The 25-μl *in vitro* translation reaction contained 10 ng of *in vitro*-transcribed RNA, 0.5–50 pmol of His-tagged mouse RBM4 (3), 25 μM amino acids, and 12.5 μl of reticulocyte lysate (Promega). The reaction was incubated at 30°C for 2 h, and 10% of the mixture was subjected to the luciferase assay as described above. Each reaction was independently performed at least three times.

Plasmids, Recombinant Proteins, Antibodies, Phosphorylation, Immunofluorescence, Immunoprecipitation, and RT-PCR. Complete details are provided in *SI Materials and Methods*.

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