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Hypoxia-inducible factor 1 alpha is regulated by RBM38, a RNA-binding protein and a p53 family target, via mRNA translation

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ABSTRACT

Hypoxia-inducible factor 1 (HIF1), a heterodimeric transcription factor, consists of HIF1 α and HIF1 β and is necessary for cell growth and survival under a hypoxic condition. Thus, the level and activity of HIF1 α needs to be tightly controlled. Indeed, HIF1 α protein stability is controlled by prolyl hydroxylase and von Hippel-Lindau-mediated proteasomal degradation. However, it remains unclear whether HIF1 α expression is controlled by other pathways. Here, we showed that RNA-binding protein RBM38, a target of the p53 family, regulates HIF1 α expression via mRNA translation. Specifically, we showed that under a hypoxic condition, ectopic expression of RBM38 decreased, whereas knockdown of RBM38 increased, the level of HIF1 α protein. We also showed that the rate of de novo HIF1 α protein synthesis was increased by knockdown of RBM38. Additionally, we showed that RBM38 directly bound to HIF1 α 5' and 3'UTRs. Consistently, we showed that the rate of mRNA translation for a heterologous reporter that carries HIF1 α 5'and/or 3'UTRs was increased upon knockdown of RBM38. Furthermore, we showed that knockdown of RBM38 increased, whereas ectopic expression of RBM38 decreased, the binding of eIF4E to HIF1 α mRNA. Together, our data suggest that RBM38 is a novel translational regulator of HIF1 α under a hypoxic condition.

INTRODUCTION

Hypoxia (low oxygen tension) induces an array of cellular processes to maintain ATP production via glycolysis and other survival pathways [1, 2]. Hypoxia-inducible factor 1 (HIF1), a well-defined hypoxia responsive factor, consists of two distinct subunits, HIF1 α (HIF1 α) and HIF1 β (ARNT). HIF1 belongs to a subfamily of the basic-helix-loop-helix-PAS transcription factors [3]. In response to high levels of oxygen, HIF1 α protein is modified by prolyl hydroxylase and rapidly degraded through the VHL-mediated proteasomal pathway [1]. Once normoxia turns into hypoxia, prolyl hydroxylase is inactivated and subsequently, HIF1 α is rapidly stabilized through decreased degradation [3]. Upon accumulation, HIF1 α induces an array of target genes associated with cell survival (insulin-like growth factor-binding protein-1, Nip3), angiogenesis (vascular endothelial growth factor

A, angiopoietin-2, plasminogen activator inhibitor-1), and energy metabolism (glucose transporter-1, hexokinase-2, glyceraldehyde-3-phosphate dehydrogenase) [1, 3]. Additionally, HIF1 α appears to possess transcription-independent activities through physical interaction with c-Myc and Cdc6 to regulate the cell cycle [4-6].

RBM38, also called RNPC1, is a target of the p53 family and a RNA recognition motif (RRM)-containing RNA binding protein [7]. RBM38 is expressed primarily as RBM38 (239 amino acids) along with a minor isoform, RBM38b (121 amino acids). RBM38b has a sequence identical to the N-terminal region of RBM38. RBM38 is known to regulate mRNA translation of p53 and mRNA stability of p21, HuR, p63, p73, MDM2, and MIC-1 transcripts [7-13].

Although HIF1 α expression is mainly regulated by post-translational modifications and protein stability [3, 14], other pathways have been found to regulate HIF1 α

expression, including transcription and translation [15]. In this study, we showed that ectopic expression of RBM38 decreased, whereas knockdown of RBM38 increased, the level of HIF1 α protein under a hypoxic condition. Moreover, we found that knockdown of RBM38 enhanced HIF1 α mRNA translation via binding to HIF1 α 5' and 3'UTRs. Together, we uncovered a novel mechanism by which HIF1 α is regulated by the p53 pathway via RBM38.

RESULTS

HIF1 α expression is regulated by RBM38 under a hypoxic condition

HIF1 α is necessary for cell survival under a hypoxic condition and its expression is controlled by multiple positive and negative regulators in addition to VHL-mediated proteasomal degradation [16]. Since HIF1 α has

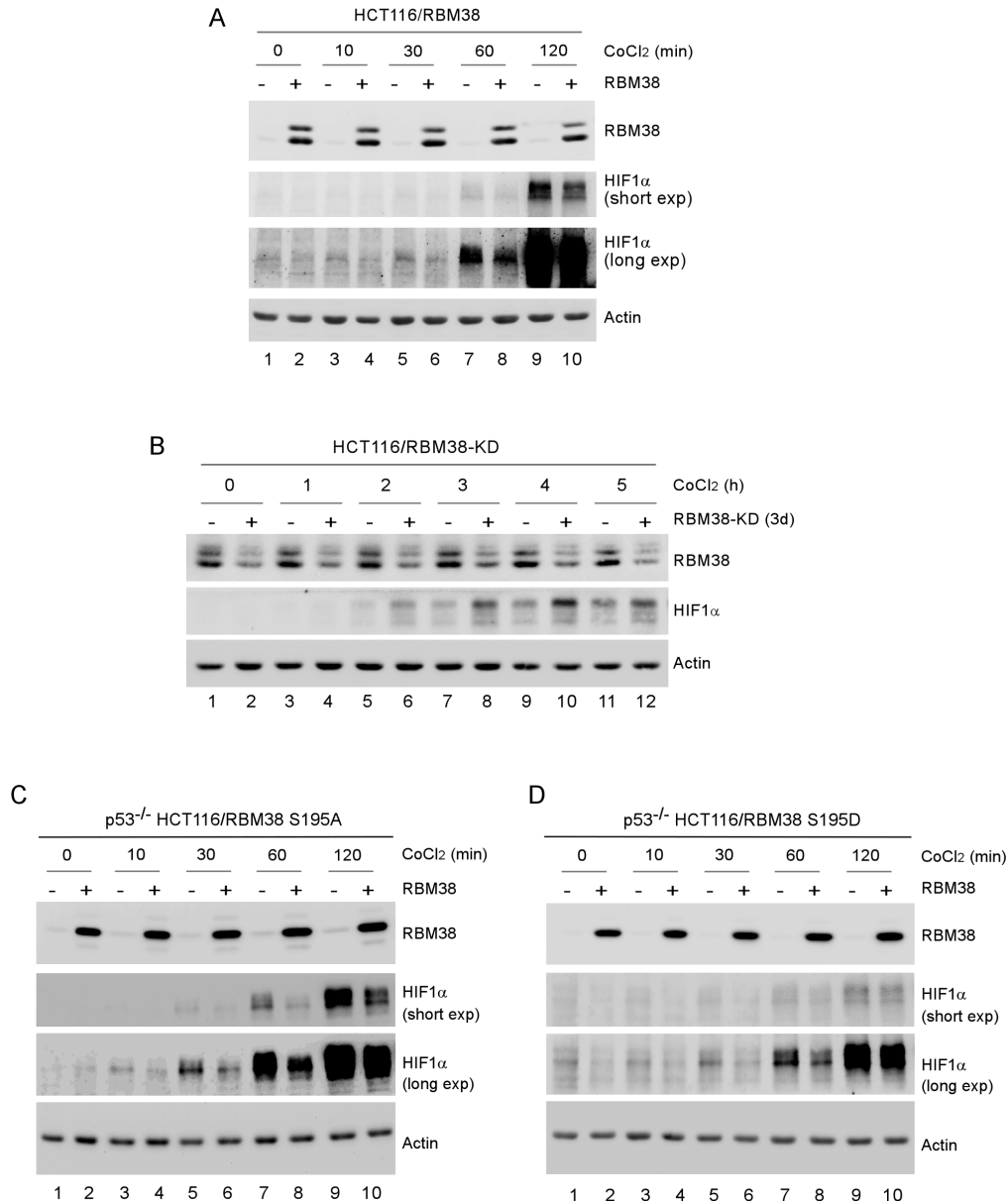


Figure 1: HIF1 α expression is regulated by RBM38 under a hypoxic condition. (A) HCT116 cells were uninduced (-) or induced (+) to express RBM38 with doxycycline for 24 h and then treated with 500 μ M CoCl₂ for the indicated times. The levels of RBM38, HIF1 α , and actin were measured by Western blotting. (B) HCT116 cells were uninduced (-) or induced (+) to knock down RBM38 with doxycycline for 3 days and then treated with 500 μ M CoCl₂ for the indicated times. The levels of RBM38, HIF1 α , and actin were measured by Western blotting. (C-D) p53^{-/-} HCT116 cells were uninduced (-) or induced (+) to express RBM38-S195A (C) or RBM38-S195D for 48 h and then treated with 500 μ M CoCl₂ for the indicated times. The experiments were performed as in (A).

a long 3'UTR along with an AU-rich element (ARE), we examined whether HIF1 α expression is modulated by RNA-binding protein RBM38, a target of the p53 family and a potent regulator of multiple pro-survival and pro-death factors [7-13]. To test this, HCT116 cell line in which RBM38 can be inducibly expressed under the control of a tetracycline-regulated promoter was used. We showed that the level of HIF1 α protein was decreased by RBM38 in HCT116 cells treated with CoCl₂, a hypoxia mimetic (Fig. 1A). Next, we examined whether knockdown of RBM38 has an opposite effect on HIF1 α expression under a hypoxic condition. To test this, HCT116 cell line in which RBM38 can be inducibly knocked down under the control of a tetracycline-regulated promoter was used. We found that under a hypoxia-mimic condition (CoCl₂ treatment), the levels of HIF1 α protein were increased by knockdown of RBM38 in a time-dependent manner (Fig. 1B).

Previously, we showed that phosphorylation of RBM38 modulates RBM38 to regulate p53 expression. To test this, the effect of phosphorylation of RBM38 on HIF1 α expression was measured in p53-null HCT116 cells, which can inducibly express RBM38-S195A,

a non-phosphorylatable form, or RBM38-S195D, a phosphor-mimetic. We found that under a hypoxia-mimic condition (CoCl₂ treatment), the levels of HIF1 α protein were decreased by both RBM38-S195A and RBM38-S195D (Fig. 1C-D), suggesting that RBM38 is capable of regulating HIF1 α regardless of its phosphorylation status.

To confirm the regulation of HIF1 α by RBM38 under a hypoxic condition, MCF7 and HCT116 cells were transduced with a lentivirus expressing RBM38 shRNA or luciferase shRNA for 3 d and then incubated under a hypoxia condition (~0.1% oxygen) for various times. As a control, the levels of p53 protein were measured and found to be increased by knockdown of RBM38 regardless of the condition of oxygen tension (Fig. 2A-B, p53 panels), consistent with our previous studies [9, 17]. Interestingly, we found that the levels of HIF1 α in both MCF7 and HCT116 cells were increased upon knockdown of RBM38 under a hypoxic condition for 6 h, but little if any under the same condition for 3 h (Fig. 2A-B, compare lanes 3 and 5 with lanes 4 and 6, respectively). Since p53 is capable of destabilizing HIF1 α protein through the ubiquitin-dependent proteasomal degradation pathway [9,

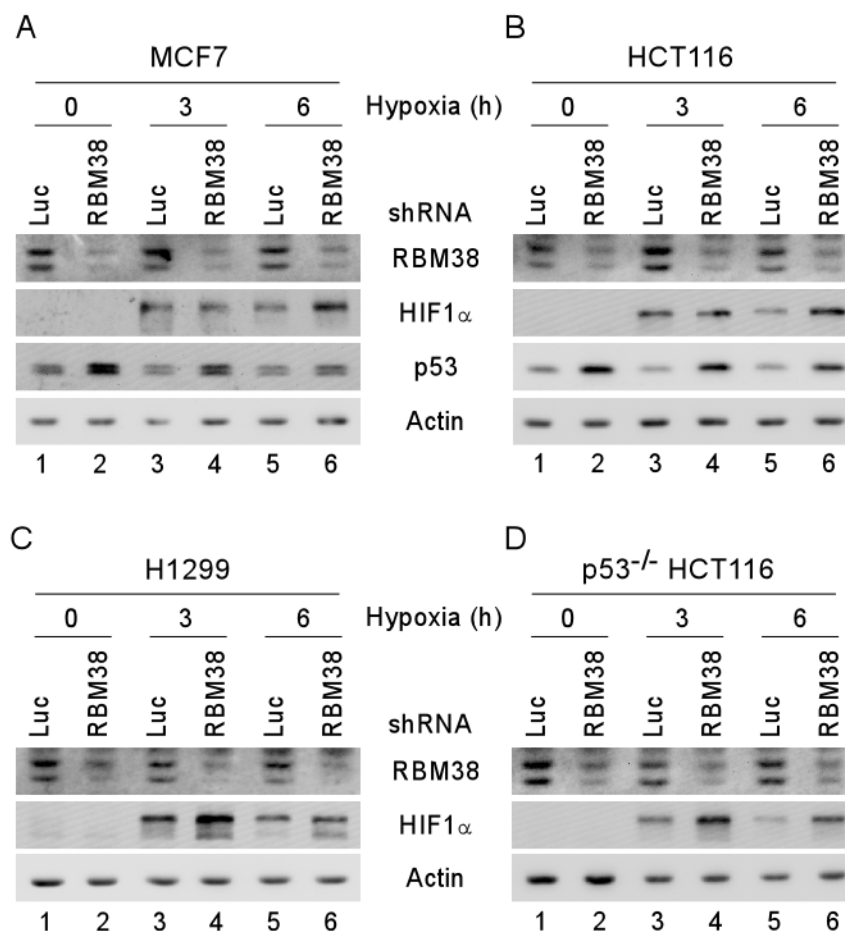


Figure 2: HIF1 α expression is increased by knockdown of RBM38 under a hypoxic condition. MCF7 (A), HCT116 (B), H1299 (C), and p53^{-/-} HCT116 (D) cells were transduced with a lentivirus expressing a control luciferase (Luc) shRNA or RBM38 shRNA, selected by puromycin for 3 d, and then exposed to hypoxia for 0, 3, or 6 h. Whole cell lysates were collected and the levels of RBM38, HIF1 α , p53, or actin were determined by Western blot analysis.

14], p53-null HCT116 and H1299 cells were used to rule out potential effects of wild-type p53 on RBM38-mediated HIF1 α regulation. Indeed, we found that the levels of HIF1 α protein were markedly increased by knockdown of RBM38 under the same hypoxic condition for both 3 and 6 h (Fig. 2C-D, compare lanes 3 and 5 with 4 and 6, respectively). Together, these data suggest that RBM38 is necessary for maintaining proper expression of HIF1 α under a hypoxic condition.

RBM38 regulates HIF1 α mRNA translation

As an RNA-binding protein, RBM38 is known to regulate gene expression through post-transcriptional mechanisms, including mRNA stability and translation [9]. To explore how RBM38 regulates HIF1 α expression under a hypoxic condition, RT-PCR was performed to measure the level of HIF1 α transcript in H1299 and p53^{-/-} HCT116

cells upon knockdown of RBM38. We showed that the levels of RBM38 transcript were decreased by shRNA against RBM38 in H1299 and p53^{-/-} HCT116 cells exposed to hypoxia for various times (Fig. 3A-B). However, the levels of HIF1 α transcript were not significantly altered by knockdown of RBM38 under both normoxic and hypoxic conditions (Fig. 3A-B). Similarly, under a hypoxia-mimic condition, the levels of HIF1 α transcript were not significantly altered by knockdown of RBM38 in H1299 cells (Fig. 3C). Thus, we postulate that RBM38 regulates HIF1 α expression potentially through mRNA translation. To test this, we measured the levels of newly synthesized HIF1 α protein in ³⁵S-labeled H1299 and p53^{-/-} HCT116 cells treated with CoCl₂ for 3 h. Indeed, we found that the levels of newly synthesized HIF1 α protein were markedly increased (2.37 and 2.86 fold) by knockdown of RBM38 in H1299 and p53^{-/-} HCT116 cells (Fig. 4A-B). Together, these data suggest that RBM38 regulates HIF1 α mRNA translation under a hypoxic condition.

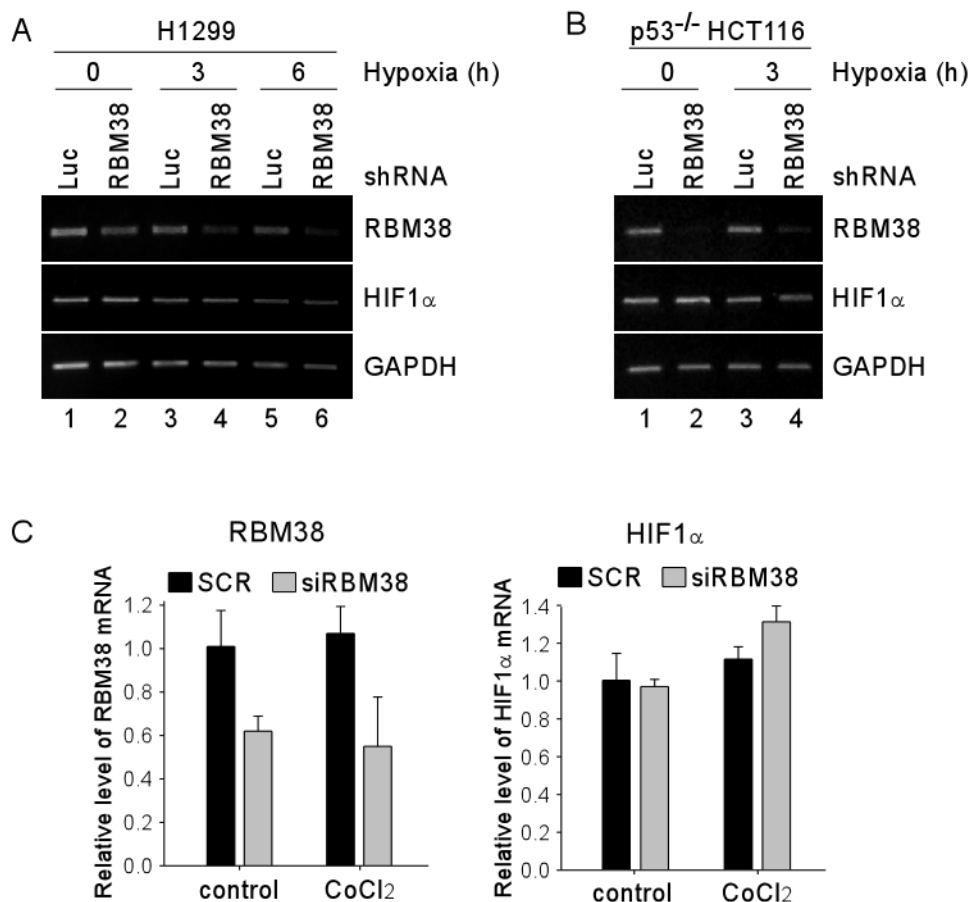


Figure 3: Knockdown of RBM38 has no effect on the level of HIF1 α mRNA. (A-B) H1299 (A) and p53^{-/-} HCT116 (B) cells were transduced with a lentivirus expressing a control luciferase (Luc) shRNA or RBM38 shRNA for 3 d, followed by exposure to hypoxia for 0-6 h for H1299 (A), and 0-3 h for p53^{-/-} HCT116 (B) cells. Total RNAs were isolated and RT-PCR was performed to measure the levels of RBM38, HIF1 α , and GAPDH transcripts. (C) H1299 cells were transiently transfected with scramble siRNA (SCR) or siRNA against Rbm38 (SiRBM38) for 48 h, followed by treatment with 500 μ M CoCl₂ for 3 h. Total RNAs were isolated and quantitative RT-PCR was performed in triplicates to measure the levels of RBM38, HIF1 α , and GAPDH transcripts. The levels of RBM38 and HIF1 α transcripts were normalized to that of the GAPDH transcript. The relative fold change for RBM38 (left panel) and for HIF1 α (right panel) is the ratio of the transcript level in cells with knockdown of RBM38 versus that in cells transfected with a control scrambled siRNA.

RBM38 directly binds to HIF1 α transcript

Considering that RBM38 is an RNA-binding protein, we postulate that the binding of RBM38 to HIF1 α mRNA is required for regulating HIF1 α expression. To test this, RNA immunoprecipitation was performed and showed that HIF1 α mRNA was highly enriched in anti-RBM38-immunocomplexes, (Fig. 5A, HIF1 α panel, compare lane 2 with 3). In addition, RBM38 was found to interact with p21 transcript (Fig. 5A, p21 panel), consistent with previous reports [7, 18]. In contrast, no interaction was found between GAPDH transcript and RBM38 (Fig. 5A). Next, a set of HIF1 α RNA probes were generated and used for RNA Electrophoretic Mobility Shift Assay (REMSA) to map the binding sites of RBM38 in HIF1 α transcript (Fig. 5B). We showed that recombinant RBM38 protein bound strongly to HIF1 α 5'UTR (Fig. 5C) and 3'UTR (Fig. 5E, compare lanes 3-4). The binding of RBM38 to a probe derived from p21 3'UTR, which is known to carry a RBM38-response element [18], was performed and used as a positive control (Fig. 5E, compare lanes 1-2). To confirm the specificity of RBM38 binding to HIF1 α transcript, RNA competition assay was performed and showed that the binding of RBM38 to HIF1 α 5'UTR was abrogated by an excess amount of cold HIF1 α 5'UTR or p21 probe (Fig. 5D, compare lanes

2 with 3-4, respectively). Similarly, the binding of RBM38 to HIF1 α 3'UTR was abrogated by an excess amount of cold HIF1 α 3'UTR (Fig. 5E, compare lanes 4-5) or cold p21 probe (Fig. 5F, compare lanes 2-3). To define the RBM38-binding site in HIF1 α 3'UTR, three additional RNA probes, fragments A-C, were generated (Fig. 5B). We showed that RBM38 bound strongly to probe B, but not to A and C (Fig. 5G). Additionally, the binding of RBM38 to HIF1 α 3'UTR probe was markedly inhibited by an excess amount of cold probe B and 3'UTR, but not by probe A (Fig. 5H, compare lanes 2 with 3-5, respectively). To further map the RBM38-binding site in fragment B, two sub-fragments, B1 and B2, were generated (Fig. 5B). We showed that RBM38 bound strongly to probes B and B1, but only weakly to B2 (Fig. 5I, compare lanes 1, 3, and 5 with 2, 4, and 6, respectively). These data suggest that RBM38 can directly bind to both HIF1 α 5' and 3' UTRs.

RBM38 regulates HIF1 α mRNA translation through HIF1 α 5' and 3' UTRs

To determine whether HIF1 α 5' and/or 3'UTRs are necessary and sufficient for RBM38 to regulate HIF1 α mRNA translation, we generated five reporter vectors (Fig. 6A): EGFP reporter coding region alone; EGFP along with

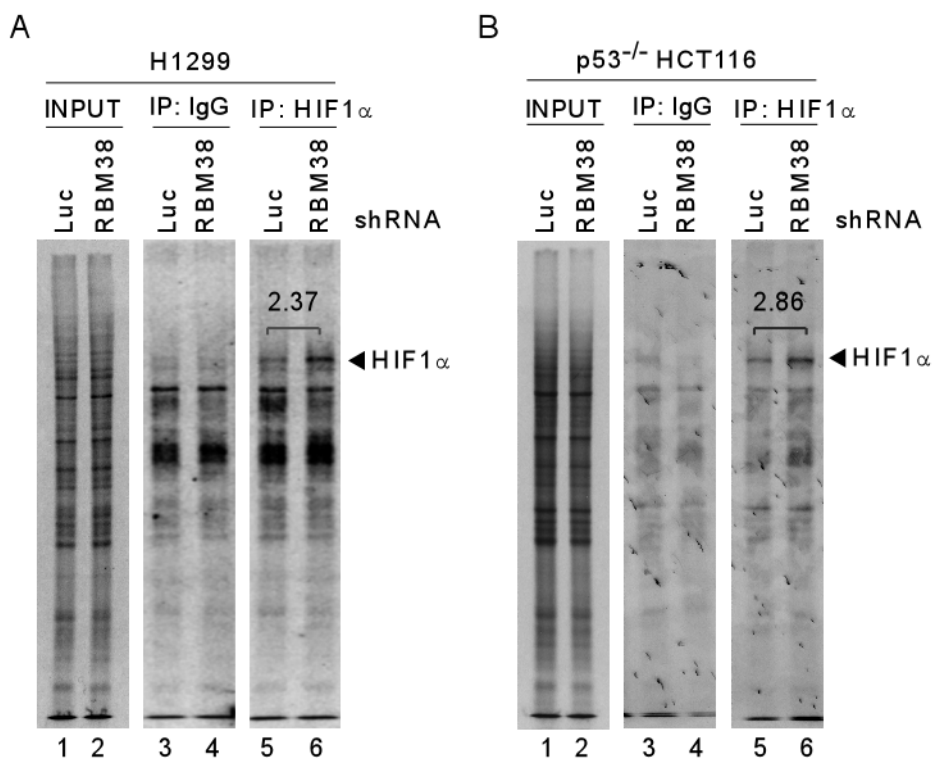


Figure 4: Knockdown of RBM38 enhances HIF1 α expression through mRNA translation. ³⁵S-metabolic labeling assay was performed with H1299 (A) or p53^{-/-} HCT116 (B) cells. Cells were transduced with a lentivirus expressing a control luciferase (Luc) shRNA or RBM38 shRNA, selected by puromycin for 3 d, and then treated with 500 μ M CoCl₂ for 3 h, followed by labeling with ³⁵S-methionine and ³⁵S-leucine. Cell lysates were isolated and used for immunoprecipitation with anti-HIF1 α (H1 α 67, Sigma) or non-immune mouse IgG. The samples from immunoprecipitation were separated in 8% SDS/PAGE and the protein signals were captured by autoradiography.

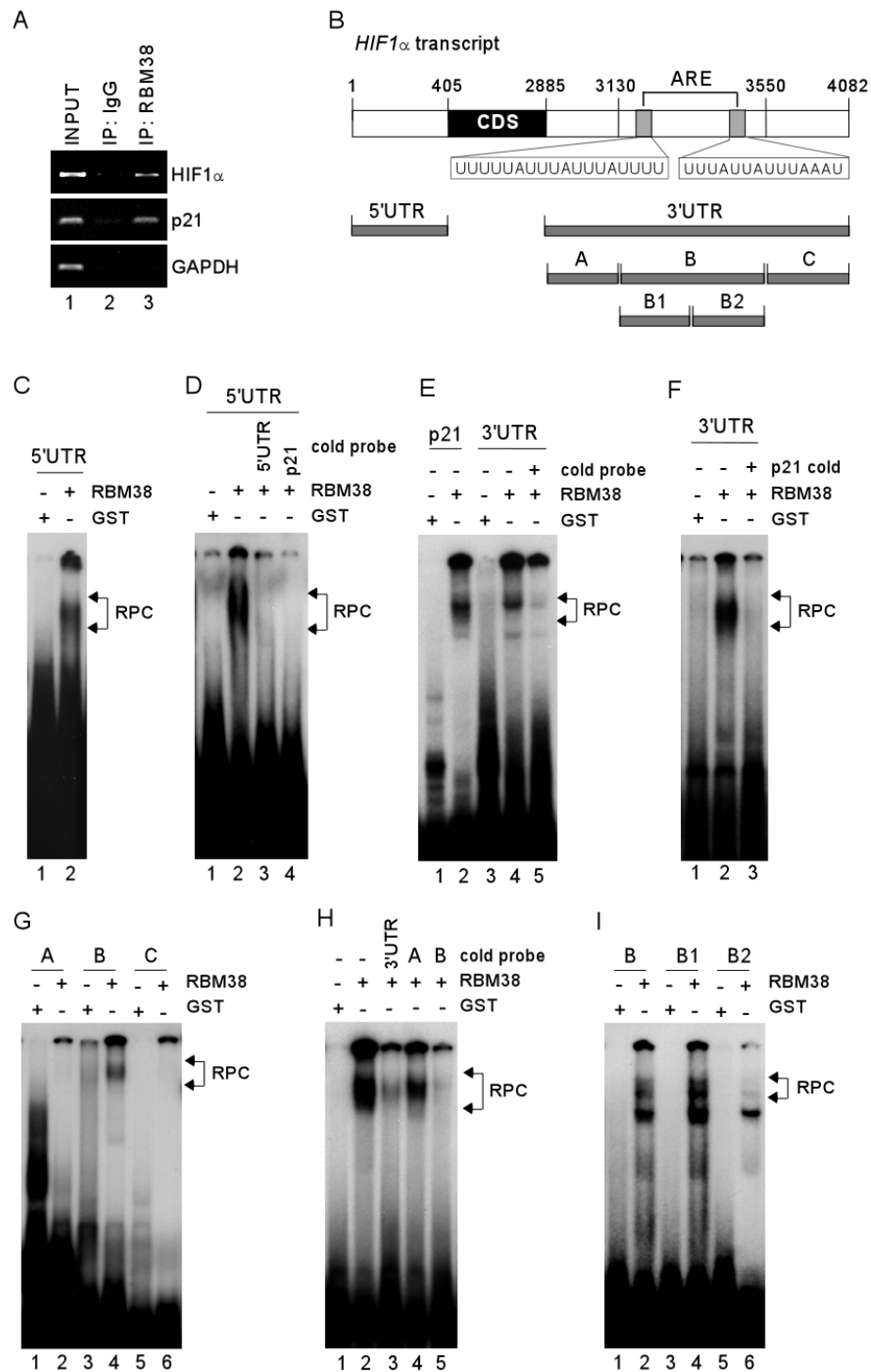


Figure 5: RBM38 directly binds to HIF1 α 5' and 3' UTRs. (A) Whole cell lysates from H1299 cells were collected and immunoprecipitated with anti-RBM38 antibody or control IgG, followed by RT-PCR to determine the level of HIF1 α transcripts in control IgG and anti-RBM38 immunocomplexes. The levels of p21 and GAPDH transcripts were measured as positive and negative controls, respectively. (B) Schematic presentation of HIF1 α transcript and the location of probes. Putative ARE regions are shown in shaded box. (C) REMSA was performed by mixing 32 P-labeled RNA probe, HIF1 α 5'UTR, with recombinant GST or GST-fused RBM38. (D) REMSA assay was performed by mixing 32 P-labeled RNA probe, HIF1 α 5'UTR, along with or without an excess amount (50-fold) of unlabeled HIF1 α 5'UTR or p21 probe. (E-F) REMSA was performed by mixing 32 P-labeled RNA probe (p21 or HIF1 α 3'UTR probe) with recombinant GST or GST-fused RBM38 along with or without an excess amount (50-fold) of unlabeled HIF1 α 3'UTR (E) or p21 probe (F). (G) REMSA was performed by mixing 32 P-labeled RNA probe (fragment A, B, or C) with recombinant GST or GST-fused RBM38. (H) REMSA was performed as described in (F) except that cold HIF1 α 3'UTR, fragment A, or fragment B was used. (I) REMSA was performed as described in (G) except that probes B, B1, and B2 were used. The arrow indicates RNA-protein complexes.

HIF1 α 5' UTR; EGFP along with HIF1 α 3' UTR; EGFP along with HIF1 α 5' and 3' UTRs; and EGFP along with mutant HIF1 α 3' UTR, which lacks the RBM38-binding site in the B1 segment as showed in Fig. 5I. We showed that in p53^{-/-} HCT116 cells, knockdown of RBM38 had no effect on EGFP expression for a vector that does not carry any sequence from HIF1 α transcript (Fig. 6B). Interestingly, the levels of EGFP protein were increased by 1.4-fold for the vector that carries HIF1 α 5'UTR (Fig. 6C), 1.3-fold for the vector that carries HIF1 α 3'UTR (Fig. 6D), and 2.2-fold for the vector that carries both HIF1 α 5' and 3' UTRs (Fig. 6E). Most importantly, we showed that the level of EGFP protein was not significantly increased by knockdown of RBM38 for the vector that carries mutant HIF1 α 3'UTR (3'UTRmut) (Fig. 6F-G, compare lanes 3-4). Again, as a control, knockdown of RBM38 by siRNA and shRNA led to increased expression of EGFP for the vector carries HIF1 α 3'UTR (Fig. 6F-G, compare lanes 1-2).

RBM38 modulates the binding of eIF4E to the cap structure on HIF1 α mRNA

To explore the mechanism by which RBM38 regulates HIF1 α mRNA translation, we examined whether RBM38 modulates the binding of eIF4E, a key component of translation initiation complex eIF4F, to the cap structure of HIF1 α mRNA in p53^{-/-} HCT116 cells. RNA immunoprecipitation followed by RT-PCR assay was performed and showed that the level of eIF4E associated with HIF1 α mRNA was increased (2.86-fold) upon knockdown of RBM38 in p53^{-/-} HCT116 cells at a low oxygen condition (Fig. 7A). In contrast, the level of eIF4E associated with HIF1 α mRNA was decreased (0.68-fold) upon ectopic expression of RBM38 in p53^{-/-} HCT

116 cells treated with 500 μ M CoCl₂ for 2 hours (Fig. 7B). These results suggest that RBM38 prevents eIF4E from binding to HIF1 α transcripts, and thus inhibits HIF1 α mRNA translation.

DISCUSSION

HIF1 α plays a critical role in hypoxia to improve glycolysis, oxygen delivery, and angiogenesis for tumor cells [1, 3]. Although HIF1 α is mainly regulated by VHL-mediated proteasomal degradation, it can be regulated by other post-transcriptional mechanisms [16]. Indeed, we found a novel mechanism by which HIF1 α expression is regulated by RBM38 via mRNA translation. We also found that RBM38 directly binds to HIF1 α 5' and 3'UTRs. Additionally, an ARE element in HIF1 α 3'UTR is recognized by RBM38. Importantly, we showed that both HIF1 α 5' and 3' UTRs are necessary and sufficient for RBM38 to regulate HIF1 α mRNA translation. Since RBM38 inhibits the binding of eIF4E to HIF1 α cap structure, we postulate that upon binding to HIF1 α 5' and/or 3' UTRs, RBM38 may physically hinder the binding of eIF4E to HIF1 α 5' cap structure. Alternatively, since RBM38 physically interacts with eIF4E [9]. We hypothesize that upon binding to HIF1 α transcript, RBM38 and eIF4E get close together and interact with each other on the HIF1 α transcript, which then prevents eIF4E from associating with HIF1 α 5'-cap.

HIF1 α accumulation in tumors can be induced by various stress signals, including hypoxia in tumor microenvironment, loss of a tumor suppressor, or oncogene activation [14, 19]. Increased HIF1 α abundance promotes tumor growth and angiogenesis [1, 20]. In this study, we showed that RBM38 deficiency leads to increased expression of HIF1 α . Thus, an obvious question

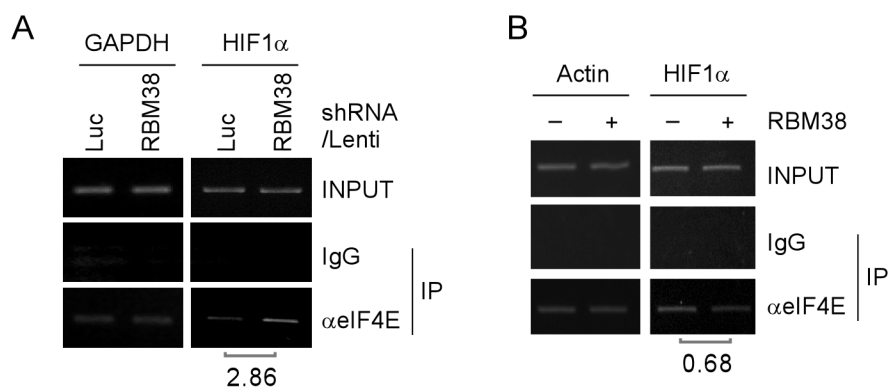


Figure 7: RBM38 prevents eIF4E from binding HIF1 α transcript. (A) p53^{-/-}HCT116 cells were transduced with lentivirus particles expressing a control luciferase (Luc) shRNA or RBM38 shRNA for 3 days and then cultured at a low oxygen condition, followed by immunoprecipitation with a control IgG or anti-eIF4E. Total RNAs were purified from immunocomplexes and subjected to RT-PCR analysis to measure the level of HIF1 α and GAPDH mRNAs. The relative level of HIF1 α mRNA was measured by densitometry, and the relative fold change was shown below each pair. (B) p53^{-/-}HCT116 cells were uninduced (-) or induced (+) to express HA-tagged RBM38 for 48 h and then treated with 500 μ M CoCl₂ for 2 hours, followed by immunoprecipitation with a control IgG or anti-eIF4E. Total RNAs were purified from immunocomplexes and subjected to RT-PCR analysis to measure the level of HIF1 α and actin mRNAs. The relative level of HIF1 α mRNA was measured by densitometry, and the relative fold change was shown below each pair.

would be: is there a functional connection between RBM38 and HIF1 α ? RBM38 is found to be overexpressed in several types of cancers [9, 21-29]. In addition, loss of RBM38 in mouse embryonic fibroblasts leads to premature senescence through activation of p53 [9]. Here, we showed that RBM38 deficiency leads to increased accumulation of HIF1 α under a hypoxic condition. These results suggest that both RBM38 overexpression and deficiency lead to tumor promotion. Thus, further studies are needed to address the functional link between RBM38 and HIF1 α under the hypoxic tumor microenvironment, which may explain how tumors thrive under a hypoxic condition. Additionally, since RBM38 expression and phosphorylation may be altered under a hypoxic condition, future studies are needed to address whether the binding of RBM38 to HIF1 α UTRs is affected by hypoxia.

EXPERIMENTAL PROCEDURES

Plasmids

pGEX vector expressing GST or GST-tagged RBM38 was used for producing recombinant RBM38 protein as previously described [18]. Lentiviral vectors (pLKO.1-puro) expressing shRNA against RBM38 and luciferase were prepared as previously described [8].

To generate EGFP expression vector carrying HIF1 α 5' and/or 3'UTRs, a DNA fragment containing EGFP coding region was amplified using pEGFP-N2 vector as a template with forward primer including HA-tag, EGFP-BamHI-HA-F, and reverse primer, EGFP-R. The primers for cloning are listed in Table 1. The PCR product was digested with *Bam*HI and *Not*I and cloned into pcDNA3 vector (Invitrogen). The vector was designated as pcDNA3-HA-EGFP. A fragment containing HIF1 α 5' or 3'UTR was amplified using cDNA from H1299 cells as template with forward primer, HIF1 α -5'UTR-KpnI-F or HIF1 α -3'UTR-NotI-F, and reverse primer, HIF1 α -5'UTR-BamHI-R or HIF1 α -3'UTR-XhoI-R. The PCR products were digested with *Kpn*I and *Bam*HI for HIF1 α -5'UTR or *Not*I and *Xho*I for HIF1 α -3'UTR and cloned into pcDNA3/HA-EGFP vector. The vectors were designated as pcDNA3/HIF1 α -5'UTR/HA-EGFP and pcDNA3/HA-EGFP/HIF1 α -3'UTR. To generate pcDNA3/HIF1 α -5'UTR/HA-EGFP/HIF1 α -3'UTR, pcDNA3/HIF1 α -5'UTR/HA-EGFP vector was digested with *Kpn*I and *Bam*HI. The digested DNA fragment containing HIF1 α -5'UTR was cloned into pcDNA3/HA-EGFP/HIF1 α -3'UTR. The HIF1 α -3'UTR deletion mutation was generated from two cDNA fragments using two-steps PCRs with HIF1 α -3'UTR-NotI-F and HIF1 α -3'UTR-XhoI-R, followed by subcloning into pcDNA3-HA-EGFP. The fragment 1 was amplified using primers HIF1 α -3'UTR-NotI-F and HIF1 α -3'UTR-mut-R whereas the fragment 2 was produced using

primers HIF1 α -3'UTR-mut-F and HIF1 α -3'UTR-XhoI-R.

Cell culture

Human breast cancer MCF7 cell line, Human colorectal carcinoma HCT116 cell line, and human non-small cell lung carcinoma H1299 cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA). p53^{-/-} HCT116 cell line was used as described [30]. Cell lines were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and maintained at 37°C in a humidified 5% CO₂. p53-null HCT116 cell lines, in which RBM38 can be inducibly knocked down or in which RBM38, RBM38-S195A, or RBM38-S195D can be inducibly expressed, were generated and cultured as previously described [7, 10, 17]. Cells were subjected to hypoxia (0.1 to 1%) by exposure to 10% H₂/5% CO₂/balanced N₂ at 37°C in Forma 1025/1029 Anaerobic Chamber (Thermo Scientific).

RNA interference

For lentiviral shRNA transduction, a lentiviral vector (10 μ g) expressing shRNA against luciferase or RBM38 [17], along with packaging plasmids, pRSV-REV (5 μ g), pMDL g/p RRE (5 μ g), and VSVG (5 μ g), was cotransfected into HEK 293T cells (8 x 10⁶) with Expressfect transfection reagent (Denville Scientific). After 48 h, the supernatant containing shRNA-expressing lentiviral particles was harvested, filtered and concentrated by ultracentrifugation (25,000 rpm, 4°C, 2 h). The concentrated lentiviral particles were then used to transduce cells, followed by puromycin selection (1 μ g/ml) for 3 days to remove un-transduced cells. RBM38 siRNA was used as described [9, 17].

Western blot analysis

Cells were cultured in various conditions and whole cell lysates were prepared with 2X SDS sample buffer. Whole cell lysates were separated in 8 to 12% SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with primary and secondary antibodies, followed by enhanced chemiluminescent detection. The antibodies used in this study are anti-HIF1 α (BD Biosciences), anti-RBM38 (purified rabbit polyclonal), anti-p53 (monoclonal anti-serum, DO-1), and anti-Actin (Sigma).

RNA isolation, RT-PCR

Total RNA was isolated by using Trizol reagent (Invitrogen). cDNA was synthesized using MMLV reverse transcriptase (Promega). PCR was performed with primers

Table 1: Primers for RT-PCR and cloning

Primer Name	Sequence
RBM38-RT-F	5'-cgcagaaggacaccacgttcacca-3'
RBM38-RT-R	5'-tgtagtgcggggtcagcccgtct-3'
HIF1 α -RT-F	5'-cacaggaatggccttgtgaa-3'
HIF1 α -RT-R	5'-ccaagcaggtcataggtggt-3'
GAPDH-RT-F	5'-agcctcaagatcatcagcaatg-3'
GAPDH-RT-R	5'-atggactgtggtcatgagtcctt-3'
HIF1 α -5UT-Kpn-F	5'-gggGGTACCgcgcgccggcctgggcag-3'
HIF1 α -5UT-Bam-R	5'-gggGGATCCGGTGAATCGGTCCCCGCGAT-3'
HIF1 α -3UT-Not-F	5'-gGCGGCCGCgcttttcttaatttcattccttttttggac actg -3'
HIF1 α -3UT-Xho-R	5'-gggCTCGAGGCCTGGTCCACAGAAGATG-3'
HIF1 α -3UT mut-F	CAGTAGCATCGTTTATCCCTTTTTCGAATTATTT TAAGAAGATGCCAATATAATTTTGTAAGAAGGC
HIF1 α -3UT mut-R	GGGATAAACGATGCTACTGCAATGCAATGGTTTAA ATACCAAAAACTGAGAAAATGAG
EGFP-HA-Bam-F	5'-ggggGGATCCgccaccatgTACCCATACGATGTT CCAGATTACGCTgtgagcaaggcgaggagctg-3'
EGFP-R	5'-GTATGGCTGATTATGATCTAG-3'

listed in Table 1.

RNA-immunoprecipitation (RNA-IP)

RNA-IP was carried out as previously described [7, 31]. Briefly, cells (4×10^6) were lysed with 1 ml of lysis buffer (10 mM HEPES, pH7.0, 100 mM KCl, 10 mM MgCl₂, 0.5% NP-40, 1 mM DTT) supplemented with RiboLock Ribonuclease inhibitor (Fermentas) for 15 min on ice. Cell lysates were collected following centrifugation (13,000 rpm, 4°C, 10 min). The RNA-protein immunocomplexes were formed by incubating 0.4 ml of cell lysates with 2 μ g of anti-RBM38 (purified rabbit polyclonal), anti-eIF4E (Santa Cruz, CA), or isotype control IgG at 4°C for 4 h and brought down by 20 μ l of protein G bead (50% slurry). RT-PCR analysis was carried out to measure the RNA-protein interaction. The primers to amplify p21 were used as described in [9].

Probe preparation and RNA Electrophoretic Mobility Shift Assay (REMSA)

All probes were labeled by *in vitro* transcription using a DNA fragment containing T7 promoter and various region of HIF1 α 5' or 3'UTR. Briefly, 500 ng of purified PCR product was incubated with 50 μ Ci of α -³²P-UTP, 0.5 mM each of NTP (A, G, C), 20 unit of T7 RNA polymerase (Ambion) in 20 μ l of reaction at

37°C for 1 h, followed by DNase I (1 unit) treatment for 15 min. The reaction mixture was purified by Sephadex G-50 column to remove unlabeled free nucleotides and the radioactivity of probes was measured by a scintillation counter. REMSA was carried out with a modified protocol as previously described [8]. Briefly, 250 nM of RBM38 recombinant protein, 100 μ g/ml of yeast tRNA, and 50,000 CPM ³²P-labeled RNA probe were mixed in 20 μ l of reaction buffer (10 mM Tris-Cl, pH 8.0, 25 mM KCl, 10 mM MgCl₂, 1 mM DTT) at 25°C for 25 min. RNA/protein complexes were digested with 100 U RNase T1 at 37°C for 15 min and then separated in 7% native PAGE gel. RNA-protein complexes were visualized by autoradiography.

³⁵S-Metabolic labeling Assay

Cells seeded in a 6-cm plate (6×10^6 cells) were washed twice with PBS and incubated in DMEM without L-methionine and L-cysteine for 1 h. Cells were then labeled with 100 μ Ci/ml Easy Tag EXPRESS ³⁵S Protein Labeling Mix (PerkinElmer) for 30 min. Cell lysates were isolated and used for immunoprecipitation with anti-HIF1 α (H1 α 67, Sigma) or non-immune mouse IgG. The samples from immunoprecipitation were separated in 8% SDS/PAGE. The gel was dried on 3-MM paper and the protein signals were captured by autoradiography.

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Authors' contribution

Cho SJ, Teng IF, Zhang M, Yin T, and Jung YS did the experiments. Cho SJ, Teng IF, Zhang M, Yin T, Jung YS and Zhang J analyzed the data; Chen X supervised the project and analyzed the data; Cho SJ, Teng IF, and Chen X wrote the manuscript. All authors read and commented on the draft version of the manuscript and approved the final version.

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