RNA-Binding Protein RBM24 Regulates p63 Expression via mRNA Stability

Enshun Xu, Jin Zhang, Min Zhang, Yuqian Jiang, Seong-Jun Cho, and Xinbin Chen

Abstract

p63, a p53 family member, plays pivotal roles in epidermal development, aging, and tumorigenesis. Thus, understanding how p63 expression is controlled has biological and clinical importance. RBM24 is an RNA-binding protein and shares a high sequence similarity with RBM38, a critical regulator of p63. In this study, we investigated whether RBM24 is capable of regulating p63 expression. Indeed, we found that ectopic expression of RBM24 decreased, whereas knockdown of RBM24 increased, the levels of p63 transcript and protein. To explore the underlying mechanism, we found that RBM24 was able to bind to multiple regions in the p63 3’ untranslated region and, subsequently, destabilize p63 transcript. Furthermore, we showed that the 3’ untranslated region in p63 transcript and the RNA-binding domain in RBM24 were required for RBM24 to bind p63 transcript and consequently, inhibit p63 expression. Taken together, our data provide evidence that RBM24 is a novel regulator of p63 via mRNA stability.

Implications: Our study suggests that p63 is regulated by RBM24 via mRNA stability, which gives an insight into understanding how posttranscriptional regulatory mechanisms contribute to p63 expression. Mol Cancer Res; 12(3); 359–69. ©2013 AACR.

Introduction

p63 is a member of the p53 family, including p53, p63, and p73 (1). All 3 proteins are transcriptional factors and share a high sequence similarity, especially in the DNA-binding domain (2). Like other p53 family members, p63 gene has complex expression patterns because of the usage of 2 distinct promoters and alternative splicing at the C-terminus. The usage of 2 promoters results in 2 major p63 isoforms, TAp63 and ΔNp63, and each isoform is alternatively spliced into at least 5 variants, α, β, γ, δ, and ε (3, 4). Importantly, TAp63 isoforms, transcribed from the upstream P1 promoter, contain a transactivation domain similar to that in p53 and thus can induce a number of p53 target genes including p21 and MDM2 (5). By contrast, ΔNp63 isoforms, transcribed from the P2 promoter in intron 3, lack the N-terminal transactivation domain and are presumably thought to be transcriptionally inactive. Interestingly, some studies showed that ΔNp63 carries a ΔN activation domain and retains transcriptional activity under certain circumstances (2, 5, 6).

The biological function of p63 is complex because of the presence of multiple isoforms with opposing functions. Studies suggest that the ΔNp63 isoforms have oncogenic potential (7, 8), whereas the TAp63 isoforms play a role in tumor suppression (9). This apparent conflict was recently addressed by generating isoform-specific p63 knockout mice models. Specifically, total p63 knockout mice have defects in skin, teeth, mammary gland, and limb, and die soon after birth (10, 11), suggesting a critical role of p63 in epidermal development. Interestingly, mice deficient in ΔNp63 isoforms largely phenocopy total p63 knockout mice (12). These mice die shortly after birth because of several developmental defects, such as truncated forelimbs and the absence of hind limbs. By contrast, mice deficient in TAp63 isoforms are born live and tumor prone (9). In addition, these mice develop several phenotypes, including accelerated aging, obesity, insulin resistance, and glucose intolerance (13, 14). Together, these in vivo studies indicate a critical role of p63 in skin development, aging, metabolism, and tumorigenesis. Given the biological importance of p63, studies have been carried out to elucidate how p63 expression is controlled. For instance, upon exposure to various stimuli, the level of p63 transcript is regulated by p53 and several other transcription factors (15–17). Moreover, p63 can be posttranscriptionally regulated by RNA-binding protein (RBP) RBM38 and HuR via mRNA stability and protein translation, respectively (18, 19). In addition, several microRNAs (miRNA), including miR-302, miR-130b, and miR-203, are found to regulate p63 mRNA stability (20–22). Furthermore, p63 protein...
stability is regulated by a set of E3 ligases, such as itch, Pirh2, wwp1, and SCPβ1Cp3 (23–26). Nevertheless, other regulators, which are critical in modulating p63 expression, remain to be elucidated.

Materials and Methods
Reagents
Anti-RBM24, raised in rabbit, was generated by Cocalico Biologicals. Anti-p63, 4A4, was purchased from Santa Cruz Biotechnology. Anti-HA was purchased from Covance. Anti-actin, proteinase inhibitor cocktail, RNase A, and protein A/G beads were purchased from Sigma. Scrambled siRNA (GGC CGA UUG UCA AAU AAU U) and siRNA against RBM24 (CAC UGG AGC UGC AUU ACC G) were purchased from Dharmacon RNA Technologies. Transfection reagent Metafectene was purchased from Bio-tex (Germany). Silencerfect lipid was purchased from Bio-Rad Laboratories. TRizol reagent purchased from Invitrogen. The MMLV reverse transcriptase was purchased from Promega. EST clone, containing a full-length human TAp63 (clone ID 5552611), was purchased from OpenBiosystem. The MEGA. EST clone, containing a full-length human TAp63 and SCF stability is regulated by a set of E3 ligases, such as itch, Pirh2, wwp1, and SCPβ1Cp3 (23–26). Nevertheless, other regulators, which are critical in modulating p63 expression, remain to be elucidated.

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Plasmids
To generate pcDNA3-HA-RBM24, a PCR product was amplified by using cDNA samples from MCF7 cells as a template and then inserted into pcDNA3-HA vector via EcoRI and Xhol sites. The primers were a forward primer, 5'-GGG GCT CGA GCA ATT TCT TAA, and a reverse primer, 5'-GGG CTC GAG CTA TTG CAT TCG GTC TGT CTG-3'. To generate pcDNA3-RBM24, a PCR product was amplified by using cDNA samples from MCF7 cells as a template and then inserted into pcDNA3 vector via HindIII and Xhol sites. The primers were a forward primer, 5'-AAA AAG CTT CAC CAT GAT GCA CAC GAC CAA GAA GGA CAC GAC GTA CA-3', and a reverse primer, 5'-GGG CTC GGT GTG TGG TAG GGC TTG GTG TAC GTC GTC TGC TGT CTG-3'. The primers to amplify fragment 2 were a forward primer, 5'-AGG ACA CGA CGT ACA CCA AGC CCT ACC ACA CCA CCG ACG CAC CCA GCC-3', and a reverse primer, 5'-GGG CTC GAG CTA TTG CAT TCG GTC TGT CTG-3'. The primers for second-step PCR were a forward primer, 5'-AGG ACA CGA CGT ACA CCA AGC CCT ACC ACA CCA CCG ACG CAC CCA GCC-3', and a reverse primer, 5'-GGG CTC GAG CTA TTG CAT TCG GTC TGT CTG-3'.

To generate pGEX-5X-1-RBM24 vector, a DNA fragment was digested from pcDNA3-HA-RBM24 and then inserted into pGEX-5X-1 vector via EcoRI and Xhol sites. To generate pGEX-5X-1-RBM24-ARNP1, a PCR product was amplified by using pcDNA3-RBM24-ARNP1 as a template and then inserted into pGEX-5X-1 vector via EcoRI and Xhol sites. The PCR primers were a forward primer, 5'-CGG AAT TCA TGC ACA CGA CCC AGA AGC CGT ACA-3', and a reverse primer, 5'-GGG CTC GAG CTA TTG CAT TCG GTC TGT CTG-3'. To generate pcDNA3-TAp63 expression vector, a DNA fragment was amplified by using TAp63 α EST clone as a template with a forward primer, 5'-GGG GAA GCT TGC CAC CAT GAA TTT TGA AAC TTC ACG G-3', and a reverse primer, 5'-GGG GAA GCT TGC CAC CAT GAA TTT TGA AAC TTC ACG G-3'. The PCR products were cloned into pcDNA3 via HindIII and BamHI sites. To generate pcDNA3-Tap63α-3'UTR expression vector, a DNA fragment containing the full-length p63 3'UTR was amplified by using TAp63α EST clone as a template and then inserted into pcDNA3 vector via BamHI and Xhol sites. The PCR primers were a forward primer, 5'-GGG GGA GCT TGC CAC CAT GAA TTT TGA AAC TTC ACG G-3', and a reverse primer, 5'-GGG GGA GCT TGC CAC CAT GAA TTT TGA AAC TTC ACG G-3'.

Cell culture and cell line generation
HaGaT, ME180, MIA-PaCa2, MCF10A, HCT116, and MCF7 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS as previously described (18, 27). Mouse Embryo Fibroblasts (MEFs) were cultured in DMEM supplemented with 10% FBS, 1X nonessential amino acids, and 55 μmol/L β-mercaptoethanol. HCT116 and MCF7 stable cell lines that can inducibly express RBM24 were generated as described previously (28). Briefly, pcDNA4-RBM24 was transfected into HCT116 and MCF7 parental cells, which express a tetracycline repressor (pcDNA6; ref. 28). The RBM24 expressing cells were selected with zeocin (150
μg/mL) and confirmed by Western blot analysis. To induce RBM24 expression, tetracycline (1 μg/mL) was added to medium for various times.

MEF isolation

RBM38−/−; p53−/− MEFs were isolated as previously described (29). To generate TAp63α−/− MEFs, mice heterozygous for TAp63 (14), a gift from Dr. Elsa R. Flores’ lab, were bred. MEFs were isolated from 13.5-day-old embryos as previously described (30). All animals are housed at the University of California at Davis CLAS vivarium facility. All animals and use protocols were approved by the University of California at Davis Institution Animal Care and Use Committee.

Western blot analysis

Western blot analysis was performed as previously described (5). Briefly, cells lysates were collected and resuspended with 1× SDS sample buffer. Proteins were then resolved in an 8% to 12% SDS-PAGE gel and transferred to a nitrocellulose membrane, followed by ECL detection. The level of protein was quantified by densitometry. The data are representative of 3 independent experiments.

Recombinant protein purification, RNA probe generation, and REMSA

Bacteria BL21 was transformed with a pGEX-5X-1 vector expressing glutathione S-transferase (GST)-tagged RBM24, ΔRNPI, or ΔRNPD and positive clones were selected. The recombinant proteins were then purified by glutathione sepharose beads (Amersham Biosciences). RNA probes containing various regions of p63 3′UTR were generated as previously described (18). The p21 probe was generated as previously described (31). RNA electrophoretic mobility assay (REMSA) was performed as described previously (18). Briefly, 32P-labeled probes were incubated with recombinant protein in a binding buffer [10 mmol/L HEPES-KOH at pH 7.5, 90 mmol/mL potassium acetate, 1.5 mmol/L

Figure 1. Ectopic expression of RBM24 suppresses p63 expression. A, sequence similarity between human RBM38 and RBM24. The RNP1 and RNP2 submotifs are shown in boxes. B to E, ectopic expression of RBM24 inhibits ΔNp63α expression. ME180 (B), HaCaT (C), and MCF10A (D) cells were transiently transfected with a control vector or a vector expressing HA-tagged RBM24 for 48 hours, and the level of HA-tagged RBM24, ΔNp63α, and actin was determined by Western blot analysis. The level of ΔNp63α protein was normalized to that of actin and arbitrary set as 1.0 in control cells. The relative fold changes were shown below each lane. E, RBM24 inhibits TAp63α expression. MIA-PaCa2 cells was transiently transfected with a control vector or a vector expressing HA-tagged RBM24 for 48 hours, followed by Western blot analysis to determine the level of HA-tagged RBM24, TAp63α, and actin. The level of TAp63α protein was normalized to that of actin and arbitrary set as 1.0 in control cells. The relative fold changes were shown below each lane.

p63 Expression Is Regulated by RBM24 through mRNA Stability
magnesium acetate, 2.5 mmol/L dithiothreitol, 40 U of RNase inhibitor (Ambion)] at 30°C for 30 minutes. RNA–protein complexes were resolved on a 6% acrylamide gel and radioactive signals were detected by autoradiography.

**RNA isolation and reverse transcription-PCR analysis**

Total RNAs were isolated using TRIzol reagent. cDNA was synthesized using MMLV reverse transcriptase according to the user’s manual. The PCR program used for amplification was (i) 94°C for 5 minutes, (ii) 94°C for 45 seconds, (iii) 58°C for 45 seconds, (iv) 72°C for 1 minute, and (v) 72°C for 10 minutes. From step 2 to 4, the cycle was repeated 20 times for human and mouse actin or 30 times for RBM24 and P63. To amplify human actin, 2 pairs of primers were used. The first pair of primers was used for the RT-PCR analysis in Figs. 2A and C, 3A, and 4A whereas the second pair of primers were used for all other RT-PCR analysis. The first pair of primers were forward primer, 5'-CTG GAC GAC AGG AAC AAT GCC-3', and reverse primer, 5'-GGA CTG GTG GAC GAG ACC-3'. The second pair of primers were forward primer, 5'-AGC GCG GCT ACA GCT TCA-3', and reverse primer, 5'-CGT AGC ACA GCT TCC CCT TAA TG-3'. These primers were used for the rest of RT-PCR analysis. The primers to amplify mouse actin were forward primer, 5'-CCC ATC TAC GAG GGC TAT-3', and reverse primer, 5'-AGA AGG AAG GCT GGA AAA-3'. The primers to amplify human RBM24 were forward primer, 5'-ACC CAG AAG GAC AGC AGG TA-3', and reverse primer, 5'-TCC ATG ATG GGG TTG GGA T-3'. The primers to amplify human ΔNp63 were forward primer, 5'-TAC CTG GAA AAT GCC-3', and reverse primer, 5'-ACT GCT GGA AGG ACA CGA-3'. The primers to amplify mouse TAp63 were forward primer, 5'-AGC CCA TTG ACT TGA ACT-3', and reverse primer, 5'-GGA CTG GTG GAC GAG-3'. The primers to amplify human RBM24 were forward primer, 5'-ACC CAG AAG GAC AGC AGG TA-3', and reverse primer, 5'-TCC ATG ATG GGG TTG GGA T-3'. The primers to amplify human ΔNp63 were forward primer, 5'-TAC CTG GAA AAT GCC-3', and reverse primer, 5'-ACT GCT GGA AGG ACA CGA-3'. The primers to amplify mouse TAp63 were forward primer, 5'-AGC CCA TTG ACT TGA ACT-3', and reverse primer, 5'-GGA CTG GTG GAC GAG-3'. The primers to amplify human TAp63 were forward primer, 5'-ACC CAG AAG GAC AGC AGG TA-3', and reverse primer, 5'-TCC ATG ATG GGG TTG GGA T-3'. The primers to amplify human ΔNp63 were forward primer, 5'-TAC CTG GAA AAT GCC-3', and reverse primer, 5'-ACT GCT GGA AGG ACA CGA-3'. The primers to amplify human TAp63 were forward primer, 5'-AGC CCA TTG ACT TGA ACT-3', and reverse primer, 5'-GGA CTG GTG GAC GAG-3'. The primers to amplify human ΔNp63 were forward primer, 5'-TAC CTG GAA AAT GCC-3', and reverse primer, 5'-ACT GCT GGA AGG ACA CGA-3'.

Quantitative PCR (qPCR) was performed using 2× qPCR SYBR Green Mix (ABgene) with 5 μmol/L primers. Reactions were run on a realplex using a 2-step cycling program: 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, 68°C for 30 seconds. A melting curve (57–95°C) was generated at the end of each run to verify the specificity. The primers for human actin were forward primer, 5'-TCC ATC ATG AAG TGT GAC GT-3', and reverse primer, 5'-TGA TCC ACA TCT GCT GGA AG-3'. The primers for human TAp63 were forward primer, 5'-AGC CCA TTG ACT TGA ACT-3', and reverse primer, 5'-GGA CTG GTG GAC GAG-3'. The primers to amplify human ΔNp63 were forward primer, 5'-TAC CTG GAA AAT GCC-3', and reverse primer, 5'-ACT GCT GGA AGG ACA CGA-3'.

**RNA-Chip analysis**

RNA-Chip analysis was performed as previously described (32). Briefly, cells (2 × 10^7) were lysed with an immunoprecipitation buffer (100 mmol/L KCl, 5 mmol/L MgCl2, 10 mmol/L Heps, 1 mmol/L DTT, and 0.5% NP-40), and then incubated with 2 μg of anti-RBM24 or rabbit immunoglobulin G (lgG) at 4°C overnight. The RNA–protein immunocomplexes were brought down by protein A/G beads, followed by RT-PCR analysis.
Statistical analysis
All experiments were performed at least 3 times. Numerical data were expressed as mean ± SDs. Two group comparisons were analyzed by 2-sided Student t test. P values were calculated, and a P of <0.05 was considered significant.

Results
Ectopic expression of RBM24 suppresses, whereas knockdown of RBM24 increases, p63 expression
In an effort to understand the underlying mechanisms by which p63 expression is controlled, we showed previously that RBM38, also called RNPC1, is able to destabilize p63

transcript and plays a critical role in p63-mediated keratinocyte differentiation (18). Interestingly, a search of gene database revealed that RBM38 has a paralogue, named RBM24, which shares a high degree of sequence similarity with that of RBM38 (Fig. 1A). The RBM24 gene encodes 236 aa and is located on chromosome 6. Structure analysis shows that RBM24 contains one RNA-binding domain, which is composed of 2 submotifs, RNP1 and RNP2. Most remarkably, the RNA-binding domain in RBM24 is identical to the one in RBM38 (Fig. 1A). Therefore, it is plausible that RBM24 may regulate p63 expression.

To determine whether RBM24 regulates p63 expression, a control vector or a vector expressing HA-tagged RBM24 was transiently transfected into ME180 cells. The level of p63 transcript is decreased by ectopic expression of RBM24. A–C, ME180 cells (A), HaCaT cells (B), MCF10A cells (C), and RBM38–/–; p53–/– MEFs (F) were transiently transfected with a control vector or a vector expressing HA-tagged RBM24 for 48 hours. Total RNAs were isolated and subjected to RT-PCR analysis to determine the level of RBM24, ΔNp63, and actin transcripts. The level of ΔNp63 transcript was normalized to that of actin and arbitrary set as 1.0 in control cells. The relative fold changes were shown below each lane. D and E, HCT116 (D) and MCF7 (E) cells were uninduced or induced to express RBM24 for 48 hours. Total RNAs were isolated and subjected to RT-PCR analysis to determine the level of RBM24, ΔNp63, and actin transcripts. G, the level of ΔNp63 transcript in HaCaT cells, which were transfected with a control vector or RBM24-expressing vector, was measured by qRT-PCR. The level of actin mRNA was measured as an internal control. H, the experiment was performed as in G except that MCF7 cells, which were uninduced or induced to express RBM24, were used. I–J, MIA-PaCa2 cells were transiently transfected with a control vector or a vector expressing HA-tagged RBM24 for 48 hours. Total RNAs were isolated and subjected to RT-PCR analysis (I) or qRT-PCR (J) to determine the level of RBM24, TAp63, and actin transcripts.
RBM24 was detectable upon transfection (Fig. 1B, RBM24 panel). Interestingly, we found that the ΔNp63α protein was markedly inhibited by RBM24 (Fig. 1B, ΔNp63α panel). Similarly, we found that RBM24 inhibited ΔNp63α expression in HaCaT and MCF10A cells (Fig. 1C and D, ΔNp63α panels). Furthermore, we tested whether RBM24 has an effect on TAp63 expression in HaCaT and MCF10A cells (Fig. 1C and D, panel). Similarly, we found that TAp63 expression was markedly decreased by ectopic expression of RBM24 (Fig. 1E, TAp63 panel). Together, these data suggest that p63 expression is repressed by ectopic expression of RBM24.

To determine whether endogenous RBM24 regulates p63 expression, ME180 and HaCaT cells were transiently transfected with a control siRNA or a siRNA against RBM24. Again, we found that the level of RBM24 transcript was markedly reduced by RBM24, but not by control, siRNA (Fig. 2A and C, RBM24 panels). Importantly, we found that the level of ΔNp63α proteins was increased by RBM24 knockdown (Fig. 2B and D, ΔNp63α panels). Furthermore, we tested whether TAp63α expression is regulated by endogenous RBM24 and found to be increased upon RBM24 knockdown in MIA-PaCa2 cells (Fig. 2E and F). Together, these data suggest that knockdown of RBM24 increases p63 expression.

Ectopic expression of RBM24 decreases, whereas knockdown of RBM24 increases, the level of p63 transcript

RBPs are known to posttranscriptionally regulate their targets mainly through mRNA stability or protein translation. Thus, to explore how RBM24 regulates p63 expression, the level of p63 transcript was measured in ME180 cells transiently transfected with a control or RBM24 expression vector. We found that upon transient expression of RBM24, the level of ΔNp63α transcript was decreased in ME180 cells (Fig. 3A, ΔNp63α panel). Similarly, ectopic expression of RBM24 was able to reduce the level of ΔNp63α transcript in HaCaT and MCF10A cells (Fig. 3B and C, ΔNp63α panels). To verify this, HCT116 and MCF7 cells that can inductively express RBM24 were used. We found that the level of ΔNp63α transcript was decreased upon RBM24 induction (Fig. 3D and E, ΔNp63α panels). Next, we determined whether RBM24 regulates p63 expression in the absence of p53 and RBM38. To address this, RBM38−/−:p53−/− MEFs were transiently transfected with a control or RBM24 expression vector and the level of p63 transcript was measured. We found that RBM24 was able to significantly decrease the level of p63 transcript in the absence of p53 and RBM38 (Fig. 3F, ΔNp63α panel). Consistently, qPCR analysis showed that the level of ΔNp63α transcript was decreased by ectopic expression of RBM24 in HaCaT and MCF7 cells (Fig. 3G and H). Furthermore, we determined whether RBM24 regulates TAp63 transcript by RT-PCR and qPCR. We showed that the level of TAp63 transcript was markedly decreased by ectopic expression of RBM24 in MIA-PaCa2 cells (Fig. 3I–J). Together, these data suggest that ectopic expression of RBM24 decreases the level of p63 transcript.

Next, to determine whether endogenous RBM24 regulates p63 transcript, ME180 cells (A), HaCaT cells (B), and RBM38−/−:p53−/− MEFs (C) were transiently transfected with a control or RBM24 siRNA for 72 hours. Total RNAs were purified and subjected to RT-PCR analysis to determine the level of RBM24, ΔNp63α, and actin transcripts. The level of ΔNp63α transcript was normalized to that of actin and arbitrary set as 1.0 in control cells. The relative fold changes were shown below each lane. D and E, the level of TAp63α transcript is increased by knockdown of RBM24. MIA-PaCa2 cells were transiently transfected with a control or RBM24 siRNA for 72 hours. Total RNAs were purified and subjected to RT-PCR (D) or qRT-PCR (E) to determine the level of RBM24, TAp63α, and actin transcripts. The relative fold of TAp63α transcript was normalized to that of actin and arbitrary set as 1.0 in control cells. The relative fold changes were shown below each lane.

RBM24 destabilizes p63 transcript

To investigate the underlying mechanism by which RBM24 regulates p63 expression, we first determined whether RBM24 regulates p63 transcript. Specifically, the level of p63 pre-mRNA was measured in HaCaT cells, transiently transfected with a control or RBM24 expression vector. We found that overexpression of RBM24 had no effect on the level of p63 pre-mRNA in HaCaT cells (Fig. 5A, pre-p63 panel). Consistent with this, the levels of p63 pre-mRNA was not altered by ectopic expression of RBM24...
in ME180 and MIA-PaCa2 cells (Fig. 5B and C, pre-p63 panels). Similarly, RBM24 knockdown had no effect on p63 pre-mRNA in HaCaT, ME180, and MIA-PaCa2 cells (Fig. 5D–F, pre-p63 panels). These results suggest that p63 is regulated by RBM24 via posttranscriptional mechanisms, such as mRNA stability. Thus, the half-life of ΔN and TA p63 transcripts was measured in cells treated with actinomycin D, which inhibits de novo RNA synthesis. Specifically,
MCF7 cells were uninduced or induced to express RBM24 for 48 hours, followed by actinomycin D treatment for various times. Similarly, MIA-PaCA2 cells were transfected with a control vector or a vector expressing RBM24, followed by actinomycin D treatment for various times. The level of DN and TA p63 transcripts was determined by RT-PCR analysis and the relative half-life of DN and TA p63 transcripts was calculated. We showed that the half-life of DNp63 mRNA was deceased from 3.7 hours in the control cells to 2.7 hours in cells with RBM24 expression (Fig. 5G), and the half-life of TAp63 mRNA was deceased from 9.3 hours in the control cells to 7.2 hours in cells with RBM24 expression (Fig. 5H). Together, these data suggest that RBM24 shortens the half-life of p63 mRNA.

RBM24 binds to multiple regions in the 3' UTR of p63 transcript

To further decipher the underlying mechanism by which RBM24 destabilizes p63 mRNA, we determined whether RBM24 associates with p63 transcript in vivo by performing an RNA-Chip analysis. We found that p63 transcript was present in RBM24, but not control IgG, immunoprecipitates (Fig. 6A, compare lane 2 with lane 3). As a control, RBM24 was unable bind to actin mRNA (Fig. 6A, lane 3). Next, the binding site(s) of RBM24 in p63 transcript was mapped by performing REMSA. Specifically, radiolabeled probes (A−C), spanning the entire p63 3' UTR (Fig. 6B), were incubated with recombinant GST or GST-fused RBM24 protein along with a TAp63α expression vector along with a TAp63α expression vector containing the coding region alone (D) or in combination with a full-length p63 3' UTR (E). Cell lysates were collected and the level of RBM24, TAp63α, and actin proteins was determined by Western blot analysis. The relative level of TAp63α was normalized to that of actin and arbitrary set as 1.0 in control cells. The relative fold changes were shown below each lane.
expression vector that contains TAp63α coding sequence alone or together with a full-length p63 3′UTR. Next, these TAp63α expression vectors were transiently transfected into TAp63+/− MEFs along with a control or RBM24 expression vector. We found that RBM24 had no effect on TAp63α expression from an expression vector that only contains TAp63α coding sequence (Fig. 6D). By contrast, TAp63α expression was significantly inhibited by RBM24 when the TAp63α expression vector contains a full-length p63 3′UTR (Fig. 6E). Together, these results suggest that the p63 3′UTR is necessary for RBM24 to repress p63 expression.

The RNA-binding domain is required for RBM24 to inhibit p63 expression

The RNA-binding domain in RBM24 is composed of 2 RNA recognition submotifs, RNP1 and RNP2 (Fig. 7A). Thus, to determine whether the RNA-binding domain is required for RBM24 to inhibit p63 expression, we generated 2 RBM24 deletion mutants, which lack RNP1 (ΔRNP1) and RNP2 (ΔRNP2), respectively (Fig. 7A). Next, the ability of ΔRNP1 or ΔRNP2 to bind to p63 3′UTR was determined by REMSA. We found that neither ΔRNP1 nor ΔRNP2 were capable of binding to the p63 3′UTR (Fig. 7B). Furthermore, we found that unlike wild-type RBM24, neither ΔRNP1 nor ΔRNP2 were capable of inhibiting ΔNp63α expression in HaCaT cells (Fig. 7C). Taken together, these data suggest that the RNA-binding domain is required for RBM24 to bind p63 transcript and consequently, inhibits p63 expression.

Discussion

Although regulation of p63 expression has been extensively studied, very little is known about the posttranscriptional regulation of p63 by either RBPs or miRNAs. RBPs are key regulators in posttranscriptional control of RNAs and altered expression of RBPs is implicated in several kinds of human diseases including cancer (33–36). In this study, we identified RNA-binding protein RBM24 as a novel regulator of p63 via mRNA stability. Specifically, we showed that the levels of p63 protein and transcript are decreased by ectopic expression of RBM24. Consistent with this, knockdown of endogenous RBM24 increases the levels of p63 transcript and protein. Moreover, we showed that RBM24 inhibited p63 expression by reducing the half-life of p63 transcript. Consistently, RBM24 is able to bind to multiple regions in the 3′UTR of p63 transcript and subsequently, destabilize p63 transcript. Furthermore, we showed that both the 3′UTR in p63 transcript and the RNA-binding domain in RBM24 are required for regulating p63 expression.

The biological function of RBM24 and its downstream targets remain largely unknown. To date, RBM24 is suggested to be involved in skeletal muscle differentiation by regulating MyoD (37) and myogenin (38). More recently, RBM24 is found to be involved in sarcomeric assembly and cardiac contractility (39, 40), suggesting a critical role of RBM24 in heart development. However, as a RBM24 paralogue, RBM38 was found to be critical in tumorigenesis (41, 42). Therefore, it is likely that RBM24 and RBM38 have their own distinct functions, although both proteins share high degree of sequence similarity. In our study, we found that p63 is a novel downstream target of RBM24. Although the biologic significance of this regulation remains unknown, it is likely that RBM24 participates in the p63 network by regulating p63 expression via various pathways. For example, RBM24 may play a role in TAp63-mediated tumor suppression or in ΔNp63-mediated epidermal development. Moreover, RBM38 is a target of the p53 family and forms a feedback or feed-forward regulatory loop with the p53 family proteins (18, 29, 43, 44). As a RBM38 closely related protein, RBM24 may participate in the p53 family-RBM38 autoregulatory loop, including regulation of p53 and p73 expression. Thus, future studies to address these questions will help us better understand the biological function of RBM24.

We have previously reported that RBM38 is able to regulate p63 mRNA stability by binding to the 3′UTR of p63 transcript (18). In this study, we found that like RBM38, RBM24 was able to bind to the p63 3′UTR and destabilize p63 transcript. Of note, the binding sites of RBM24 to p63 transcripts are located in the same regions as that of RBM38 (Fig. 6 and ref. 18). We postulate that the
similar regulation to p63 by RBM38 and RBM24 is because of their high degree of sequence similarity (Fig. 1A). Interestingly, RBM24 can regulate p63 expression in the absence of RBM38 (Figs. 3F and 4C), suggesting that the function of these 2 proteins may not be redundant. Nevertheless, it still remains to be elucidated whether RBM24 and RBM38 cooperatively or antagonistically regulate p63 mRNA stability. First, RBM24 and RBM38 may compete to bind to p63 transcript. Second, it is likely that RBM24 enhances the RNA-binding activity of RBM38 to p63 transcript and vice versa, resulting in destabilized p63 transcript. Third, because of their high degree of sequence similarity, RBM24 and RBM38 may form a heterodimer and negatively regulate p63 mRNA stability. These issues need to be addressed in the future studies.

Of note, several miRNAs, including miR-130b (21), miR-302 (20), and miR-203 (22), are found to posttranscriptionally regulate p63 expression. These miRNAs are also found to cooperatively or antagonistically downregulate p63 and consequently, affect the p63 network.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: E. Xu, J. Zhang, X. Chen
Development of methodology: E. Xu, J. Zhang, M. Zhang, Y. Jiang, S.-J. Cho
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Chen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Xu, J. Zhang, X. Chen
Writing, review, and/or revision of the manuscript: E. Xu, J. Zhang, X. Chen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Xu, Y. Jiang, S.-J. Cho
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**References**


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