Serine 195 phosphorylation in the RNA-binding protein Rbm38 increases p63 expression by modulating Rbm38’s interaction with the Ago2–miR203 complex

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The p63 transcription factor, a p53 family protein, regulates genes involved in various cellular processes, including cell growth and differentiation. We previously showed that RNA-binding motif protein (Rbm38) is a p63 target and, in turn, regulates p63 expression in an Ago2/miR203-dependent manner. To confirm this, we generated mouse embryo fibroblasts (MEFs) in which Ser-193 in mouse Rbm38 (equivalent to Ser-195 in human Rbm38) was substituted with aspartic acid (Rbm38S193D/S195D) or alanine (Rbm38S193A/S195A). We observed that the p63 transcript level was decreased in Rbm38S193D/S195D MEFs, but increased in Rbm38S193A/S195A MEFs. Mechanistically, we found that WT Rbm38, but not Rbm38S195D, is required for p63 mRNA degradation mediated by microRNA 203 (miR203). Furthermore, we noted that Argonaute 2 (Ago2), a key regulator in microRNA-mediated mRNA decay, associates with WT Rbm38, and this association was reduced by Ser-195 phosphorylation. Together, our results reveal a critical mechanism by which Ser-195 phosphorylation in Rbm38 increases p63 expression by attenuating the association of Rbm38 with the Ago2–miR203 complex.

p63 belongs to the p53 family of transcription factors that include p53, p63, and p73 (1–3). Because of the usage of two distinct promoters, p63 is expressed as two major isoforms, called TAp63 and ΔNp63, both of which have multiple variants through alternative splicing at the C terminus (2). Like p53, p63 functions as a transcription factor and induces many targets involved in various cellular processes, including cell proliferation, differentiation, epithelial development, and cellular senescence (4–6). Interestingly, although p63 shares many functional properties with p53, p63 is not a classic tumor suppressor as p63 is rarely mutated in human cancers. Indeed, p63 exhibits unique functions in the regulation of differentiation and development (6). Consistently, p63-deficient mice exhibit severe developmental defects in limb, skin, hair, teeth, and mammary and salivary glands (5, 6). Thus, exploring how p63 is regulated is critical for understanding the biological function of p63.

The RNA-binding protein Rbm38, also called RNPC1, is a target of the p53 family, including p63, as well as E2F1 (7, 8). Rbm38 is found to regulate expression of genes necessary for several cellular processes, including cell growth and differentiation. For example, Rbm38 is found to regulate Mdm2, p21, p73, and Pten mRNA stability through binding to the AU/U-rich elements in their 3′UTRs (8–11). We also found that Rbm38 decreases p63 mRNA stability by binding to AU/U-rich elements in their 3′UTR (12, 13). By contrast, Rbm38 enhances p63 mRNA stability by binding to a GU-rich element in p63 mRNA 3′UTR, which has a sequence different from that in p63α/β 3′UTR (14). In addition, we found that Rbm38 interacts with p53 mRNA and represses p53 translation (15). Moreover, Rbm38 is capable of regulating gene expression by modulating miRNA activity (16).

GSK3β is a serine/threonine kinase that is required for multiple cellular functions, including metabolism, cell growth, and differentiation (17). Previous studies showed that GSK3β can phosphorylate β-catenin and promotes its degradation (18, 19). Our group also showed that Rbm38 can be phosphorylated by GSK3β at Ser-195, and Ser-195 phosphorylation converts Rbm38 from a repressor to an activator of p53 mRNA translation via altered interaction with elf4E (20). The unique function of Ser-195 phosphorylation in Rbm38 lets us postulate whether Ser-195 phosphorylation alters the ability of Rbm38 to regulate p63 mRNA stability. Indeed, we found that Ser-195 phosphorylation abrogates the ability of Rbm38 to decrease...
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p63α expression. Furthermore, we showed that WT Rbm38, but not phosphomimetic Rbm38-S195D, physically associates with Ago2, which is required for miR203 to target p63α mRNA for degradation. Together, we uncovered a novel mechanism by which Ser-195 phosphorylation in Rbm38 increases p63 expression by attenuating the association of Rbm38 with the Ago2–miR203 complex.

Results

Ser-195 phosphorylation alters the ability of Rbm38 to inhibit p63 mRNA stability

Previously, we found that Rbm38 negatively regulates p63α mRNA stability through binding to the AU/U-rich elements in p63 3′UTR (12, 13). We also found that phosphorylation of Ser-195 in Rbm38 alters the ability of Rbm38 from a repressor to an activator of p53 mRNA translation (20). Thus, we wanted to determine whether Ser-195 phosphorylation alters the ability of Rbm38 to regulate p63 mRNA stability. To test this, p63 expression was examined in multiple cell lines that inducibly express HA-tagged WT Rbm38, nonphosphorylatable Rbm38-S195A, or phosphomimetic Rbm38-S195D. We would like to mention that Rbm38 protein is expressed as two bands in the SDS-polyacrylamide gel: the upper (slow-migrating) band representing Ser-195–phosphorylated Rbm38; the lower (fast-migrating) band representing unphosphorylated Rbm38 (8, 10, 13, 15, 20). Consistently, we found that upon induction, Rbm38 protein was expressed as two bands in cell lines that inducibly express HA-tagged WT Rbm38 (Fig. 1, A and C, lanes 1 and 2), whereas Rbm38-S195A protein was expressed as the fast-migrating nonphosphorylatable band (Fig. 1, A and C, lanes 3 and 4). In contrast, Rbm38-S195D protein was expressed as the slow-migrating Ser-195 phosphorymimetic Rbm38 (Fig. 1, A and C, lanes 5 and 6). Interestingly, we found that both WT Rbm38 and S195A were capable of suppressing ΔNp63α expression in MCF7 and HaCaT cells (Fig. 1, A and C, lanes 1–4). In contrast, Rbm38-S195D did not suppress ΔNp63α expression in MCF7 cells (Fig. 1A, lanes 5 and 6) and even slightly increased ΔNp63α expression in HaCaT cells (Fig. 1C, lanes 5 and 6). Similar results were observed in ME180 and MIA-PaCa2 cells transiently transfected with a control vector or a vector expressing HA-tagged WT Rbm38, S195A, or S195D (Fig. S1). Consistent with this, we found that the level of p63α transcript was decreased by WT Rbm38 and S195A in MCF7 (Fig. 1B) and HaCaT cells (Fig. 1D). In contrast, upon expression of Rbm38-S195D, the level of p63α transcript was increased highly in HaCaT cells (Fig. 1D) and slightly in MCF7 cells (Fig. 1B).

To rule out potential nonphysiological artifacts of ectopic expression above, we generated Rbm38-KI mice in which Ser-193 (equivalent to Ser-195 in human Rbm38) is substituted with alanine (S193A) or aspartic acid (S193D). Next, these mice were used to generate a set of Rbm38S193A/S193A (A/A) and Rbm38S193D/S193D (D/D) MEFs along with WT and Rbm38−/− MEFs as a control. As shown in Fig. 1E, Rbm38 was detected in WT but not Rbm38−/− MEFs. We also showed that S193A and S193D proteins were expressed as two polypeptides with similar migration patterns as WT Rbm38 (Fig. 1E), suggesting that the slower migrating band of S193A and S193D proteins may be subjected to phosphorylation at other serine/threonine residues. Nevertheless, we found that loss of Rbm38 led to increased expression of p63 mRNA in Rbm38−/− MEFs as compared with that in WT MEFs (Fig. 1F, lanes 1 and 2), which is consistent with a previous report (12). Interestingly, we found that the level of p63 transcript was decreased in A/A MEFs but increased in D/D MEFs (Fig. 1F). These data indicate that the effect of ectopic Rbm38-S195A or Rbm38-S195D on p63 expression in MCF7 and HaCaT cells is recapitulated by endogenous knockin Rbm38-S193A and Rbm38-S193D in MEFs.

To determine whether the increased expression of p63 transcript by Rbm38-S193D is due to increased p63 mRNA stability, the half-life of p63 mRNA was determined in WT and D/D MEFs treated with 5,6-dichlorobenzimidazole-β-d-ribofuranoside (DRB; 100 μM) for various times. We found that the half-life of p63 mRNA was increased from 4.44 h in WT MEFs to 7.06 h in D/D MEFs (Fig. 1G). These data suggest that phosphomimetic S195D abrogates the ability of Rbm38 to decrease p63 mRNA stability.

Akt–GSK3 pathway modulates Ser-195 phosphorylation of Rbm38 and consequently p63 expression

Glycogen synthase kinase-3β (GSK3β), a Ser/Thr kinase, is a master regulator for many cellular activities, including gene expression, cell cycle, and apoptosis (21). It is well known that Akt kinase phosphorylates GSK3β at Ser-9 and then inhibits GSK3β activity (22). Previously, we found that upon inhibition of the phosphatidylinositol 3-kinase (PI3K)–protein kinase B (Akt) pathway, GSK3β is activated and then phosphorylates Rbm38 at Ser-195, leading to increased p53 expression (20). Because Ser-195 phosphorylation alters the ability of Rbm38 to regulate p53 expression, we sought to determine whether p63 expression is regulated by the Akt–GSK3 pathway via Rbm38. To test this, MCF7 and ME180 cells were treated with MK2206, a potent inhibitor of Akt kinase (23). We showed that upon treatment with MK2206, the level of Ser-9–phosphorylated GSK3β, but not total GSK3β, was decreased in MCF7 and ME180 cells (Fig. 2, A and C, p-GSK3β and GSK3β panels), concomitantly with an increase in phosphorylated Rbm38 as detected by a specific antibody against Ser-195–phosphorylated Rbm38 (Fig. 2, A and C, p-Rbm38 panels). Importantly, we found that the levels of p63 protein and transcript were highly increased in MCF7 cells (Fig. 2, A and B) and ME180 cells (Fig. 2, C and D) by MK2206 in a dose-dependent manner.

To confirm that Rbm38 is necessary for enhanced p63 expression by the Akt inhibitor, Rbm38-knockdown MCF7 cells were treated with or without MK2206. We showed that the levels of p63 protein and transcript were increased in MCF7 cells upon treatment with MK2206 in a dose-dependent manner (Fig. 2, E and F, compare lanes 1 with 2 and 3). However, upon knockdown of Rbm38, MK2206 had little if any effect on the levels of p63 protein and transcript (Fig. 2, E and F, compare lanes 4 with 5 and 6). Additionally, we would like to note that the levels of p63 protein and transcript were increased in MCF7 cells upon knockdown of Rbm38 (Fig. 2, E and F, ΔNp63 panels), consistent with our previous study (13). These results sug-
Suggest that p63 expression is increased by Ser-195 phosphorylation of Rbm38 via the Akt–GSK3 pathway.

**U-rich element in p63 3’UTR is required for Rbm38 to regulate p63 expression**

Previously, we reported that endogenous Rbm38 destabilizes p63 mRNA through binding to an AU/U-rich element in p63 3’UTR (13). To determine whether p63 3’UTR is recognized by S193D to regulate p63 expression, RNA ChIP assay was performed with anti-Rbm38 antibody using extracts from WT, A/A (Rbm38S193A/S193A) and D/D (Rbm38S193D/S193D) MEFs, followed by RT-PCR. IgG was used as an isotype control, and actin mRNA was measured as a negative control. We found that p63 mRNA was present in Rbm38/S193A/S193D, but not in control IgG, immunocomplexes (Fig. 3, A and B, p63 panels, compare lanes 3 and 4 with 5 and 6, respectively). These data suggest that like Rbm38, S193A and S193D are capable of binding to p63 mRNA. As a control, we found that actin mRNA was not detected in Rbm38, S193A, and S193D immunocomplexes (Fig. 3, A and B, Actin panel, compare lanes 3 and 4 with 5 and 6, respectively).
Next, to determine the Rbm38-binding site in p63 3′ UTR, multiple reporters were generated (Fig. 3C). First, a reporter carrying the GFP-coding region plus p63 5′ UTR (Fig. 3, D and E) was transfected into MCF7 cells together with an empty vector or a vector expressing HA-tagged Rbm38 or Rbm38-S195D. We found that both Rbm38 and S195D had no effect on the level of p63 protein in mock-treated MCF7 cells (without or with Rbm38-KD) was arbitrarily set as 1.0, and the relative fold change is shown below each lane. F, levels of Rbm38, ΔNp63, and actin transcripts were measured in MCF7 cells uninduced or induced to knock down Rbm38 for 48 h, followed by treatment with 0–2 μM MK2206 for 2 h. The relative level of ΔNp63 transcript in mock-treated MCF7 cells was arbitrarily set as 1.0, and the relative fold change is shown below each lane.

Figure 2. p63 expression is increased by Ser-195–phosphorylated Rbm38 via the Akt–GSK3 pathway. A and C, levels of p-GSK3β, GSK3β, p-Rbm38, Rbm38, p63α, and actin proteins were measured in MCF7 cells (A) and ME180 cells (C) treated with 0–2 μM MK2206 for 2 h. The relative level of ΔNp63α is shown below each lane. B and D, levels of ΔNp63 and actin proteins were measured in MCF7 cells (B) and ME180 cells (D) treated with 0–2 μM MK2206 for 2 h. The relative level of ΔNp63 transcript is shown below each lane. E, levels of p-GSK3β, GSK3β, Rbm38, p63α, and actin proteins were measured in MCF7 cells uninduced or induced to knock down Rbm38 for 48 h, followed by treatment with 0–2 μM MK2206 for 2 h. The relative level of p63α protein in mock-treated MCF7 cells (without or with Rbm38-KD) was arbitrarily set as 1.0, and the relative fold change is shown below each lane. F, levels of Rbm38, ΔNp63, and actin proteins were measured in MCF7 cells uninduced or induced to knock down Rbm38 for 48 h, followed by treatment with 0–2 μM MK2206 for 2 h. The relative level of ΔNp63 transcript in mock-treated MCF7 cells was arbitrarily set as 1.0, and the relative fold change is shown below each lane.
miRNAs are known to regulate RNA stability by binding to the 3' UTR of a target mRNA (24–27). miR203 has been identified as a skin-specific microRNA, and previous studies showed that miR203 regulates p63 expression by directly binding to p63 3' UTR (28, 29). Interestingly, the binding site for miR203 in p63 3' UTR is adjacent to the one for Rbm38. Because Rbm38 is known to modulate the access of miRNAs to its targets (16), we wanted to determine whether miR203 is involved in the differential regulation of p63 mRNA stability by Rbm38 and S195D. To test this, multiple cell lines were used and transfected with a control miRNA or miR203 mimic. miR203 mimic is a small, chemically modified dsRNA that mimics endogenous miR203.
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Upon ectopic expression of HA-tagged Rbm38, p63 expression was markedly inhibited by Rbm38 (Fig. 4B, p63 panel, compare lanes 1 and 3), which was further inhibited by co-expression of miR203 (Fig. 4B, p63 panel, compared lane 3 with 4; also compare lanes 1 and 4). These data suggest that the ability of miR203 to suppress p63 expression is Rbm38-dependent. In contrast, p63 expression was increased by S195D in Rbm38-KO cells (Fig. 4C, compare lanes 1 and 3). Moreover, miR203 had no effect on the ability of S195D to increase p63 expression (Fig. 4C, compare lanes 3 and 4).

To confirm the above observation, miR203 inhibitor (anti-miR203), which specifically inhibits endogenous miR203, was used to examine its effect on p63 expression. We found that the levels of p63 protein and transcript were increased by anti-miR203 in MCF7 cells (Fig. 4D, top and lower panels) and ME180 and Mia-PaCa2 cells (Fig. S2, C and D), consistent with the previous report (28). Next, Rbm38-KO MCF7 cells were transiently transfected with a control or anti-miR203 along with or without Rbm38 expression. We found that in the absence of Rbm38, anti-miR203 had very little effect on p63 expression in Rbm38-KO MCF7 cells (Fig. 4E, compare lanes 1 and 2). In the presence of Rbm38, anti-miR203 was able to counter the inhibition of p63 expression by Rbm38 (Fig. 4E, compare lanes 3 and 4; also compare lanes 1 and 4). By contrast, we found that anti-miR203 was not able to further elevate p63

Figure 4. Rbm38 but not Rbm38-S195D recruits miR203 to target p63 mRNA for degradation. A, MCF7 cells were transfected with a control miRNA or miR203 mimic for 48 h. The levels of p63 and actin proteins (upper panel) and transcripts (lower panel) were measured in MCF7 cells. The relative level of p63 protein and transcript is shown below each lane. B and C, Rbm38-KO MCF7 cells were transiently transfected with a control (Ctrl) vector or a vector that expresses HA-tagged Rbm38 (B) or HA-S195D (C), along with a control miRNA or miR203 mimic for 48 h. Cell lysates were collected and subjected to Western blot analysis with anti-HA (HA-Rbm38 or HA-S195D), anti-p63, and anti-actin. The relative level of p63 was determined, and the relative ratio is shown below each pair. D, MCF7 cells were transfected with a control anti-miRNA or anti-miR203 for 48 h. The levels of p63 protein and actin proteins and transcripts were measured in MCF7 cells. The relative level of p63 is shown below each lane. E and F, Rbm38-KO MCF7 cells were transiently transfected with a control vector or a vector that expresses HA-tagged Rbm38 (E) or HA-S195D (F), along with a control anti-miRNA or anti-miR203 for 48 h. Cell lysates were collected and subjected to Western blot analysis with anti-HA (HA-Rbm38 or HA-S195D), anti-p63, and anti-actin. The relative level of p63 was determined, and the relative ratio was shown below each pair.
expression increased by S195D (Fig. 4F, compare lanes 3 and 4), which is consistent with the above observation that miR203 did not abrogate the ability of S195D to increase p63 expression (Fig. 4C, compare lanes 3 and 4).

**Rbm38 recruits Argonaute2 (Ago2) for miR203-mediated degradation of p63 transcript**

Previously, Rbm38 was found to modulate the ability of several miRNAs to target their RNA substrates Ago2 (16), which is known to be required for miRNA-mediated mRNA decay (30). Additionally, we found that miR203 was capable of inhibiting p63 expression in the presence of Rbm38 but not S195D (Fig. 4, B and C and E and F). This let us speculate that S195D prevents miR203 from binding to the p63 mRNA, leading to enhanced p63 expression. To test this, we generated HCT116-S195D stable cell lines in that endogenous Ser-195 in Rbm38 was substituted with aspartic acid. Next, HCT116-S195D cells along with isogenic control cells were used to examine the association of endogenous Ago2 with endogenous Rbm38 or Rbm38-S195D by immunoprecipitation followed by Western blot analysis. We found that Ago2 was detectable in Rbm38 immunoprecipitation complexes in HCT116 cells (Fig. 5A, lane 3). However, the association between Ago2 and Rbm38-S195D was weaker in HCT116-S195D cells (Fig. 5A, lane 6), suggesting that phosphorylation of Ser-195 reduces the association between Rbm38 and Ago2. Together, these data suggest that the association between Rbm38 and Ago2 is required for miR203-mediated degradation of p63 transcript.

**Discussion**

Although Rbm38 regulates both p53 and p63 expression, the mechanisms by which p53 and p63 are regulated differently (13, 15, 20). Previously, we found that Rbm38 binds to p63 3'UTR and inhibits p63 expression via decreased mRNA stability (13). In contrast, Rbm38 interacts with eIF4E and represses eIF4E binding to p53 5'UTR, leading to inhibition of p53 mRNA translation (15). Interestingly, Ser-195 phosphorylation of Rbm38 abrogates the interaction between Rbm38 and eIF4E, resulting in an increased p53 translation (20). In this study, we found that phosphomimetic S195D abrogates the ability of Rbm38 to decrease p63 mRNA stability (Fig. 1). Additionally, activation of GSK3 leads to increased Rbm38 phosphorylation along with...
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increased p63 expression in an Rbm38-dependent manner (Fig. 2). Mechanistically, we found that phosphomimetic S195D attenuates the association of Rbm38 with Ago2, which prevents miR203 from targeting p63 mRNA for degradation. Together, we uncovered a novel mechanism by which Ser-195 phosphorylation modulates its ability of Rbm38 to regulate p63 mRNA stability via miR203 as summarized in Fig. 5B.

Rbm38 is known to recognize AU/U-rich elements, but the precise region to which Rbm38 binds has not been located in the p63 transcript. Here, using a reporter assay and mutational analysis, a U-rich element (nt 4071–4098) in p63 3’UTR is found to be recognized by Rbm38 and necessary for Rbm38 to regulate p63 mRNA stability (Fig. 3). It is of note that RNA-binding proteins often modulate miRNA activity by enhancing or blocking the binding of miRNAs to their targets (31). Indeed, Rbm38 is found to selectively inhibit the accessibility of miRNAs to the 3’UTRs of p21, LAT52, and Sirt1 transcripts (16). Although the p63 transcript is recognized and regulated by miR203 (28, 32, 33), it remains unclear whether Rbm38 and miR203 are coordinated to regulate p63 mRNA stability. Here, we showed that Rbm38 is required for miR203 to target p63 mRNA for degradation (Figs. 3 and 4). Interestingly, phosphomimetic S195D attenuates Rbm38 from interacting with Ago2 and thus reduces miR203 to target p63 mRNA for degradation, although Rbm38–S195D is still capable of binding to p63 3’UTR (Figs. 3–5). These observations prompted us to speculate that upon activation of GSK3 kinases and/or inhibition of Akt kinases, Rbm38 is phosphorylated at Ser-195, which would attenuate the ability of Rbm38 to interact with Ago2 as well as the ability of Rbm38 to recruit miR203 to target p63 mRNA for degradation (Fig. 5B).

Our previous study showed that GSK3β phosphorylates Rbm38, which is consistent with a previous report that GSK3β is activated by endoplasmic reticulum stress to phosphorylate p53 (20, 34) and by DNA damage to phosphorylate TIP60 (35). In this study, we found that blocking the PI3K–Akt pathway leads to activation of the GSK3β kinase, which in turn increases p63 expression in an Rbm38-dependent manner, consistent with the report that inhibition of GSK3β kinase activity decreases ΔNp63α expression under a non-stress condition (36). Thus, further understanding of how GSK3β regulates Rbm38 phosphorylation and p63 activity under both basal and stress conditions is warranted, especially considering that GSK3 inhibitors are currently being explored as a therapeutic agent for cancer and cardiovascular and neurodegenerative diseases.

As an inhibitor of p63 mRNA stability, Rbm38 can be targeted to modulate p63 expression. Indeed, we found recently that compound Rbm38 5’UTR–TAp63+/-; mice, wherein the Rbm38 deficiency restores p63 expression to near normal levels, have a longer life span along with reduced tumor incidence as compared with TAp63+/- or Rbm38-/- mice (12). Given that Ser-195 phosphorylation of Rbm38 enhances p63 expression, future experiments are warranted to determine whether Rbm38 phosphorylation and/or phosphomimetic S195D modulates the ability of Rbm38 to regulate p63-dependent tumor suppression and premature aging.

Materials and methods

Reagents

Proteinase inhibitor mixture, RNase A, 5,6-dichlorobenzimidazole-β-D-ribofuranoside, and protein A/G beads were purchased from Sigma. MK2206 was purchased from SelleckChem (Houston, TX). RevertAid First Strand cDNA Synthesis kit was purchased from Thermofisher Scientific™ (Carlsbad, CA).

Plasmids

pcDNA4 and pcDNA3 vectors expressing HA-tagged Rbm38, HA-tagged Rbm38–S195A, or HA-tagged Rbm38–S195D were generated as described previously (20). The pcDNA3-TAp63-CDS and pcDNA3-TAp63–3’UTR expression vectors were generated as described previously (37). pcDNA3 vector expressing GFP was generated as described previously (20). pcDNA3 vector expressing HA-tagged p53-R175H was generated as described previously (14). The pcDNA3 p53-R175H–3’UTR reporter was generated by cloning p63 3’UTR into pcDNA3-HA-p53-R175H downstream of p53-R175H-CDS. p63 3’UTR was amplified with forward primer, 5’–GCG CTC GAG GCC TCA CCA TGT GAG CCT TTC C-3’, and reverse primer, 5’–CTC ATT CTC TTT AAC ATA CCT TTC CCT TCC-3’. To generate pcDNA3 p53-R175H–3’UTR-Dpolypoly(U), which lacks a U-rich region (nt 4071–4098), a two-step PCR strategy was used. The first-step PCR was performed to separately amplify two DNA fragments by using pcDNA3–p63–3’UTR as a template. Fragment 1 was amplified with forward primer, 5’–GGG CTC GAG GCC TCA CCA TGT GAG CCT TTC C-3’, and reverse primer, 5’–GAA GAA AAA GGA GAT TGT AAA GAG AAT-3’, and a reverse primer, 5’–CTC ATT CCT TTT AAC ATA CCT TTC CCT TCC CTC-3’. The second-step PCR was performed using a mixture of fragments 1 and 2 as a template with forward primer, 5’–GGG CTC GAG GCC TCA CCA TGT GAG CCT TTC C-3’, and reverse primer, 5’–CTC ATT CCT TTT AAC ATA CCT TTC CCT TCC CTC-3’. The second PCR was performed using a mixture of fragments 1 and 2 as a template with forward primer, 5’–GGG CTC GAG GCC TCA CCA TGT GAG CCT TTC C-3’, and reverse primer, 5’–GAA GAA AAA GGA GAT TGT AAA GAG AAT-3’, and a reverse primer, 5’–TCT AGA GCA TGT CCT GCC AAA CAA AAA G-3’. This PCR product was then inserted into pcDNA3-HA-p53-R175H vector by BamHI and XhoI sites to generate pcDNA3-HA-p53-R175H–3’UTR-Dpolypoly(U). Rbm38 guide RNAs (gRNAs) were designed using CRISPR design tool. To generate a vector expressing a single-guide RNA (sgRNA) targeting Rbm38, two 25-nt oligonucleotides were annealed and then cloned into pSpCas9 (BB) sgRNA expression vector (41). The sgRNA sequences for Rbm38–KO is 5’–ACA CTA CCA CGC ACG CCT GC–3’, the sgRNA sequence for Rbm38–S195D–KI is 5’–CGA GGC GCC GTA TGG GTA G-3’. To generate the S195D–KI HR template, an 800-bp DNA fragment was amplified using HCT116 genomic DNA with a forward primer, 5’–GGC GGC GTA TGG GTA CTG GTC AT-3’, and a reverse primer, 5’–GAA TGG TGC CCC AGC GGT CCT CG-3’. This DNA fragment was then cloned into pGEMT vector and used as a template for two-step PCR. The first step was to amplify two DNA fragments #1 and #2. The DNA fragment #1 was amplified using forward primer #1, 5’–GGC GTA TGG GTA CTG GTC AT-3’, and reverse primer #1, 5’–CTG TAG CCC ACG AAC GAC GCA GCC GTG GCA GGA TCC GGC GCG TAT GGG TAC TGA.
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DNA synthesis

TCA TAT GTG GCC GG-5' and reverse primer, 5'-TGC GGA TAC AAT CCA TG-3'.

Cell culture

Rbm38S193A/S193A and Rbm38S193D/S193D MEFs were generated as described previously (11). Rbm38-S193A and Rbm38-S193D mice were generated by HR strategy (42). HaCaT cells expressing HA-tagged Rbm38, Rbm38-S195A, Rbm38-S195D were generated as described previously (20). Rbm38-KO MCF7 cell lines were generated by using CRISPR/Cas9 technology, as described previously (38). HCT116-S195D cells were generated by using CRISPR-Cas9 technology. Briefly, HCT116 cells were transfected with Rbm38-KI gRNA vector and Rbm38-KI HR template. Cells were selected with puromycin for 3 weeks. Individual clones were picked and then confirmed by sequence analysis. MCF7, HaCaT, ME180, Mia-PaCa2, HCT116, and their derivatives were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT).

RNA isolation, RT-PCR, and quantitative RT-PCR

Total RNA was isolated with TRIzol reagent (Invitrogen). cDNA was synthesized using RevertAid First Strand cDNA Synthesis kit according to the manufacturer’s protocol (ThermoFisher ScientificTM). The primers used to amplify actin (human) were forward primer, 5'-CTG AAG TAC CCC ATC GAG CAC GCC A-3' and reverse primer, 5'-GGG TAG CAC AGC CTG GAT AGC AAC G-3'. The primers used to amplify human p63 were forward primer, 5'-CAT GGG ATT CCC ATC GCA CCA GCAA G-3' and reverse primer, 5'-GGT CAT CAC CTG GAT GA-3'. The primers used to amplify human Rbm38 were forward primer, 5'-TTA TTA TTA CCG ATC CAT GTC-3' and reverse primer, 5'-TGC GGA TAC AGT CCA TGC TA-3'. The primers used to amplify human ΔNp63 were forward primer, 5'-AAG GAA ATG AAT TTT GA-3' and reverse primer, 5'-TGC GGA TAC AGT CCA TGC TA-3'. The primers used to amplify human p63α were forward primer, 5'-CAT GGA CAA GCT GCC TCC ATG-3' and reverse primer, 5'-AGG AGA ATT GGT GGA GCT G-3'. The primers used to amplify human Rbm38 were forward primer, 5'-TCT ACC GAC GCC TCG CTC AG-3' and reverse primer, 5'-CCC AGA TAT GCC AGG TTC AC-3'. The primers used to amplify murine p63 were forward primer, 5'-AGC GAG ACC AGG CAG AT-3' and reverse primer, 5'-CAT CAT CTG GGG ATC TCC GT-3'. The primers used to amplify murine TAp63 were forward primer, 5'-TAC AGA TCT GCC ATG TCG CA-3' and reverse primer, 5'-GCA TGC GGA TAC AAT CCA TG-3'.

mRNA half-life assay

To measure the stability of p63 mRNA, WT or D/D MEFs were treated with 5,6-dichlorobenzimidazole-β-d-ribofuranside (DRB; 100 μM) for various times. The relative level of p63 mRNA was determined by qRT-PCR and then normalized to the level of actin mRNA from three separate experiments. The half-life of p63 mRNA was plotted versus time.

Western blot analysis and immunoprecipitation–Western blot analysis

Western blotting was performed as described previously (39). Briefly, cell lysates was resolved in 8–12% SDS-polyacrylamide gel and then transferred to nitrocellulose membrane. The blots were then incubated with a primary and then a secondary antibody, followed by detection with enhanced chemiluminescence. To perform the IP-WB analysis, cells were lysed in 1.0% Triton lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1.0% Triton X-100) supplemented with the protease inhibitor mixture (100 μg/ml), followed by incubation with 1 μg of antibody or control IgG. The immunocomplexes were brought down by protein A/G beads and subjected to Western blot analysis. Antibodies against p63 (4A4) and GFP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GSK3β, anti-p-GSK3β (specific to serine 9), and anti-Ago2 were purchased from Cell Signaling Technology (Beverly, MA). Anti-Rbm38 antibody was made as described previously (20). Anti-phospho-Ser-195 Rbm38 antibody (anti-p-Rbm38) was generated by OpenBiosystems by using a phosphopeptide spanning serine 195 (I188DQYPYAAS(P)PATA199). Anti-HA was purchased from Covance (San Diego). Anti-actin and horseradish peroxidase-conjugated secondary antibodies against rabbit and mouse IgG were purchased from Bio-Rad. The immunoreactive bands were visualized by enhanced chemiluminescence (ThermoFisher Scientific) and quantified by densitometry with the BioSpectrum 810 Imaging System (UVP LLC, Upland, CA).

RNA-IP assay

RNA immunoprecipitation was carried out as described previously (40). Briefly, cells extracts were prepared with immunoprecipitation buffer (10 mM HEPES, pH 7.0, 100 mM KCl, 5 mM MgCl2, 0.5% Nonidet P-40, and 1 mM DTT) and then incubated with 2 μg of anti-Rbm38 or an isotype control IgG overnight at

Reverse primer, 5'-AGC GAG ACC AGG CAG AT-3' and reverse primer, 5'-CAT CAT CTG GGG ATC TCC GT-3'.
Ser-195 phosphorylation in Rbm38 decreases p63 expression

4 °C. The RNA–protein immunocomplexes were brought down by protein A beads. RT–PCR analysis was carried out to determine the levels of p63 and actin transcripts.

**Micro-RNA transfection**

Micro-RNA transfections were performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. The miRNAs used were mirVana™ miRNA negative control and mirR203 mimic (ID: MC10152, Ambion–ThermoFisher Scientific) with a sequence of 5 ’-GUG AAA UGU UUA GGA CCA CUA G-3’ (Double-stranded RNA). miRNA inhibitors (anti-miRNA) used in this study were mirVana™ miRNA control and mirR203-specific inhibitor (ID: MH10152, Ambion–ThermoFisher Scientific) with a sequence of 5’-GUG AAA UGU UUA GGA CCA CUA G-3’ (single-stranded RNA).

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