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Genetic ablation of Rbm38 promotes lymphomagenesis in the context of mutant p53 by downregulating PTEN

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Abstract

Mutant p53 exerts gain-of-function effects that drive metastatic progression and therapeutic resistance, but the basis for these effects remain obscure. The RNA binding protein RBM38 limits translation of mutant p53 and is often ablated in tumors harboring it. Here we show how loss of Rbm38 significantly alters cancer susceptibility in mutant p53 knock-in mice, by shortening lifespan, altering tumor incidence and promoting T cell lymphomagenesis. Loss of Rbm38 enhanced mutant p53 expression and decreased expression of the tumor suppressor Pten, a key regulator of T cell development. Furthermore, Rbm38 was required for Pten expression via stabilization of Pten mRNA through an AU-rich element in its 3'UTR. Our results suggest that Rbm38 controls T cell lymphomagenesis by jointly modulating mutant p53 and Pten, with possible therapeutic implications for treating T cell malignancies.

Significance—An RNA-binding protein controls T cell lymphomagenesis by jointly modulating mutant p53 and PTEN, with possible therapeutic implications for treating T cell malignancies.

Keywords

Rbm38; Pten; mutant p53; mRNA stability; T-lymphoblastic lymphoma

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Introduction

The tumor suppressor p53 is often referred to as the “guardian of the genome” (1, 2). p53 is the most commonly mutated gene in human cancer and loss of p53 is known to play a central role in tumor development (3). The importance of p53 in tumor suppression is underscored by its ability as a transcription factor to regulate a series of downstream target genes (4). For example, p21(WAF1/CIP1) (5) and GADD45 (6) are induced by p53 to regulate the cell cycle; PUMA (7) and Killer/DR5 (8) are induced by p53 to mediate apoptosis.

Unlike most tumor suppressor genes, which are mainly inactivated as a result of deletion or truncation, the *p53* gene is frequently altered as missense mutations, which are clustered within the central DNA binding domain (9). There are several hotspot mutation sites in p53, such as R175H, R248W, and R273H, which occur frequently in cancers(10). Consequently, these mutations make p53 protein defective to induce its target genes for tumor suppression (11). Notably, some mutants acquire new and distinct oncogenic properties, generally referred to as “gain-of-function”, such as the ability to promote tumor progression and metastasis (12, 13). In support of this notion, mutant p53 knock-in mice exhibit significantly different tumor spectra and high incidence of tumor metastasis when compared with p53-null mice (14, 15). Additionally, clinical studies have shown that a high level of mutant p53 is correlated with more aggressive tumors, poorer outcomes, and enhanced resistance to chemotherapeutic drugs (16). Thus, targeting the mutant p53 pathway will lay a foundation to develop new therapeutic approaches for a broad range of cancers.

Rbm38, an RNA-binding protein, has been implicated in cell cycle regulation and differentiation (17–19). Consistent with its cellular activities, Rbm38 is necessary for normal hematopoiesis and loss of Rbm38 leads to accelerated aging and spontaneous tumors in mice (20). The diverse biological functions of Rbm38 are probably due to its role as an RNA-binding protein to regulate multiple targets. For example, Rbm38 regulates mRNA stability of Mdm2 (21), estrogen receptor (22), and p21 (17). In addition, Rbm38 can regulate both wild-type and mutant p53 translation by modulating the binding of eIF4E, a translation initial factor, to the p53 mRNA (23, 24). Interestingly, Rbm38 is a target of the p53 family proteins, including p53, p63, and p73 (17, 25) Thus, Rbm38 and the p53 family form a feedback regulatory loop. Notably, based on the TCGA database and other studies (26–28), Rbm38 expression is altered together with mutant p53 in several types of human cancers, including breast cancer and acute myeloid leukemia. Thus, it is possible that the Rbm38-mutant p53 axis plays a role in tumorigenesis.

In the current study, by using *Rbm38*-deficient and mutant p53-KI compound mouse models, we showed that *Rbm38* deficiency alters the cancer susceptibility of mutant p53-KI mice. We also showed that loss of Rbm38 promotes the development of T-lymphoblastic lymphomas (T-LBLs) via modulating both mutant p53 and Pten. Additionally, we identified a novel mechanism by which Pten is regulated by Rbm38 via mRNA stability. Together, these data suggest that an exquisite regulation among Rbm38, p53, and Pten is critical for preventing T-cell lymphomagenesis.

Materials and Methods

Mutant mice

Rbm38-knockout mice (on a pure C57BL/6 background) were previously generated (20). The $p53^{+/-}$ (29), $p53^{R270H/+}$ (14) and $p53^{R172H/+}$ (14) mice (on a C57BL/6 background) were purchased from The Jackson Laboratory. $Rbm38^{+/-}$ mice were crossed with $p53^{+/-}$, $p53^{R270H/+}$ and $p53^{R172H/+}$ mice to generate $Rbm38^{+/-};p53^{+/-}$, $Rbm38^{+/-};p53^{R270H/+}$, $Rbm38^{+/-};p53^{R172H/+}$ mice. Next, $Rbm38^{+/-};p53^{+/-}$ mice were crossed with $Rbm38^{+/-};p53^{R270H/+}$ and $Rbm38^{+/-};p53^{R172H/+}$ mice to generate $Rbm38^{-/-};p53^{R270H/-}$ and $Rbm38^{-/-};p53^{R172H/-}$ mutant mice, respectively. The $p53^{+/-}$ mice were also crossed with $p53^{R270H/+}$ and $p53^{R172H/+}$ mice to generate $p53^{R270H/-}$ and $p53^{R172H/-}$ mice, respectively. All animals and use protocols were approved by the University of California at Davis Institutional Animal Care and Use Committee.

Cell culture, cell line generation, and primary mouse embryonic fibroblasts (MEFs) isolation

U2OS, HCT116, and MiaPaCa2 cells were cultured in DMEM (Dulbecco's Modified Eagle's medium, Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA). Raji and Romas cells were cultured in RPMI-1640 supplement with 10% fetal bovine serum. U2OS, HCT116, and MiaPaCa2 were purchased from the American Type Culture Collection (ATCC). Raji and Romas cells were obtained from Dr. Tuscano at UC Davis and were originally purchased from ATCC (30). Cell lines were obtained from ATCC between 2007 and 2016 and used at below passage 20 or within 2 months for this study after reception or thawing. Cells were tested negative for mycoplasma and after thawing and used within two months. Since all cell lines from ATCC have been thoroughly tested and authenticated, we did not authenticate the cell lines used in this study. To generate Rbm38-KO cell lines by CRISPR-Cas9, pSpCas9(BB)-2A-Puro vector expressing Rbm38 guide RNA#1 (5'- GTC GGT ATC TGT CGG GTG GA 3') was transfected into U2OS whereas vector expressing Rbm38 guide RNA#2 (5'-TTT TCT CCA TCA GGG CAC GT 3') was transfected into HCT116 and MiaPaCa2 cells. The cells were selected with puromycin and each individual clone was confirmed by western blot analysis. To isolate $p53^{R270H/-}$ and $Rbm38^{-/-};p53^{R270H/-}$ MEFs, $Rbm38^{+/-};p53^{+/-}$ mice were bred with $Rbm38^{+/-};p53^{R270H/+}$ mice. At 13.5-day, embryos were isolated as described previously (31). The MEFs were cultured in DMEM supplemented with 10% FBS (HyClone), 55 μM β-mercaptoethanol, and 1× non-essential amino acids (NEAA) solution (Cellgro). To isolate $p53^{R172H/-}$ and $Rbm38^{-/-};p53^{R172H/-}$ MEFs, $Rbm38^{+/-};p53^{+/-}$ mice were bred with $Rbm38^{+/-};p53^{R172H/+}$mice and the MEFs were isolated from 13.5-day embryos.

Western Blot Analysis

Western blot analysis was performed as previously described (32). The antibodies used in this study were as follows: anti-p53(1C12) and anti-Pten (Cell signaling), anti-actin (Sigma), anti-HA (Covance), and anti-Rbm38 (customized).

RNA Interference

Scrambled siRNA (GGC CGA UUG UCA AAU AAU U) and siRNAs against Rbm38 (5′-CAC CUU GAU CCA GCG GAC UUA-3′) were purchased from Dharmacon (Chicago, IL). For siRNA transfection, RNAiMax (Life Technology) was used according to the user's manual. Both Raji and Romas cells were transfected with scrambled or Rbm38 siRNA at 100 nM for 3 days.

RNA Isolation, RT-PCR, and Quantitative PCR (qPCR)

Total RNA was isolated with Trizol reagent as described (32). cDNA was synthesized with Reverse Transcriptase (promega) and used for RT-PCR. The PCR program used for amplification was (i) 94 °C for 5 min, (ii) 94 °C for 45 s, (iii) 58 °C for 45 s, (iv) 72 °C for 30 s and (v) 72 °C for 10 min. From steps 2–4, the cycle was repeated 22 times for actin or 28 times for Rbm38 and Pten. For qPCR analysis, 20-μl reactions were set up using 2X qPCR SYBR Green Mix (ABgene, Epsom, UK) along with 5 μM primers. The reactions were run on a StepOne plus (Invitrogen) using a two-step cycling program: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 68 °C for 30 s. A melt curve (57–95 °C) was generated at the end of each run to verify the specificity. The primers for human Pten were forward primer, 5'- ACC CAC CAC AGC TAG AAC TT-3', and reverse primer, 5'- GGG AAT AGT TAC TCC CTT TTT GTC-3'. The primers for mouse Pten were forward primer, 5'- TGT AAA GCT GGA AAG GGA CGG ACT G −3' and reverse primer, 5'- AGC AGT GCC ACG GGT CTG TAA TCC −3'. The primers for 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 for human actin were forward primer, CTG AAG TAC CCC ATC GAG CAC GGC A-3', and reverse primer, 5'-GGA TAG CAC AGC CTG GAT AGC AAC G-3'. The primers for mouse Rbm38 were forward primer 5'-GAC GCA TCG CTC AGA AAG T-3', and reverse primer, 5'-GAG GAG TCA GCC CGT AGG T-3'.

Plasmids

pcDNA3-Rbm38 and pcDNA3-HA-EGFP expression vector was previously generated (17, 33). To generate HA-tagged EGFP expression vector carrying various regions of Pten 3'-UTR, cDNAs from HCT116 cells were used as template to amplify various regions of Pten 3'UTR. The PCR products were then clone into pcDNA3-HA-EGFP vector via N*ot*I and X*ho*I. The primers to amplify 3'UTR-A were forward primer, 5'- TTG CGG CCG CAT AGA TAT TCT GAC ACC ACT GAC TCT GAT CCA G-3', and reverse primer, 5'- TTG CGG CCG CAT AGA TAT TCT GAC ACC ACT GAC TCT GAT CCA G-3'. The primers to amplify 3'UTR-B were forward primer, 5'- TTG CGG CCG CAG TTG GGA CTA GGG CTT CAA TTT CAC TTC-3', and reverse primer, 5'- CCC TCG AGT TAA ACA GTA GGC TTT GAA GGA CAG CAG G-3'. The primers to amplify 3'UTR-C were forward primer, 5'- TTG CGG CCG CGA TAA TGC CTC ATC CCA ATC AGA TGT CC-3', and reverse primer, 5'- CCC TCG AGA CAT GTA ATC TGC ATC TGT GGC ATT AGA AAG-3'. The primers to amplify 3'UTR-D were forward primer, 5'- TTG CGG CCG CAC TCA TAA TCC TAT CAC CTG GAG ACA TAG CC-3', and reverse primer, 5'- CCC TCG AGG GAA AAA TAC TCT CCA TAG GAA GGC ATT TC-3'. The primers to amplify

3’UTR-C1 were forward primer, 5’- TTG CGG CCG CGA TAA TGC CTC ATC CCA ATC AGA TGT CC-3’, and reverse primer, 5’- CCC TCG AGC TTG AAA TAA TAA GTC CCA CTG TAA CAT TGT CTA CT-3’. The primers to amplify 3’UTR-C2 were forward primer, 5’- TTG CGG CCG CAT AAA GTA GAC AAT GTT ACA GTG GGA CT-3’, and reverse primer, 5’- CCC TCG AGG ACT GGC TAC CCA CAT TCT GAT AT-3’. The primers to amplify 3’UTR-C3 were forward primer, 5’- TTG CGG CCG CAG AAT GTG GGT AGC CAG TCA GAC AAA TTC-3’, and reverse primer, 5’- CCC TCG AGA CAT GTA ATC TGC ATC TGT GGC ATT AGA AAG-3’. To generate HA-EGFP expression vector with point mutation for ARE#1, two steps PCR were used. The first round PCR were to amplify two DNA fragments (#1 and #2) using pcDNA3-HA-EGFP-3’UTR-C as template. The forward primer for fragment#1 was the same as the forward primer of 3’UTR-C and the reverse primer was 5’-CAC CAA AAT GTC TTC CCC GGG CCC CGA ATA CTT TTA GAC T-3’. The forward primer for fragment#2 was 5’- AGT CTA AAA GTA TTC GGG GCC CGG GGA AGA CAT TTT GGT G-3’ and the reverse primer was the same as the reverse primer of 3’UTR-C. The second round PCR was performed using a mixture of Fragment #1 and #2 as template with forward and reverse primer of 3’UTR-C. The PCR products were then clone into pcDNA3-HA-EGFP via *Not*I and *Xho*I. To generate HA-EGFP expression vector with point mutation for ARE#2, similar strategy was used except that different primers were used to amplify fragment #1 and #2. The primers for fragment #1 were the forward primer of 3’UTR-C and the reverse primer, 5’-TTA AAC ACT GGT GAG CCC CGG GCC CTC AAT GGA AAT GGT-3’. The primers for fragment #2 were the forward primer, 5’- ACC ATT TCC ATT GAG GGC CCG GGG CTC ACC AGT GTT TAA-3’ and the reverse primer of 3’UTR-C. To generate HA-EGFP expression vector with point mutation for both ARE#1 and #2, two steps PCR was performed by using HA-EGFP expression vector with ARE2 point mutation as a template with the primers for generating ARE1 point mutation. To generate a vector expressing Rbm38 guide RNA, a short oligo was inserted into the pSpCas9(BB)-2A-Puro (PX459) vector (34) via *Bbs*I site. To generate *Rbm38*-KO HCT116 and Mia-PaCa2 cells, the Rbm38 guide RNA sequence is 5’-TTT TCT CCA TCA GGG CAC GT 3’. To generate *Rbm38*-KO U2OS cells, the Rbm38 guide RNA sequence is 5’- GTC GGT ATC TGT CGG GTG GA-3’.

Histological Analysis

Mouse tissues were fixed in 10% (wt/vol) neutral buffered formalin, routinely processed, and embedded in paraffin blocks. Tissue sections (5 μm) were sectioned and stained with H&E.

Results

Loss of Rbm38 shortens the lifespan of, and alters tumor incidence in, mutant $p53^{R270H/-}$ knockin mice

To determine the role of Rbm38-mutant p53 axis in tumorigenesis *in vivo*, mutant $p53^{R270H}$ (equivalent to human R273H, a contact mutant) knock-in mice were crossed with *Rbm38*-KO mice to generate a cohort of $p53^{R270H/-}$ and $Rbm38^{-/-}; p53^{R270H/-}$ MEFs. We found that the level of mutant p53(R270H) protein was markedly increased by loss of Rbm38 (Fig. 1A), consistent with our previous report that mutant p53 expression was repressed by

Rbm38 (23). Next, a cohort of $p53^{R270H/-}$ (n=31) and $Rbm38^{-/-}; p53^{R270H/-}$ (n=26) mice was generated and monitored throughout their lifespan. We would like to note that $p53^{R270H/-}$ mice has been analyzed for another tumor study, which is recently published (35). We found that except one $p53^{R270H/-}$ mouse, all the $p53^{R270H/-}$ and $Rbm38^{-/-}; p53^{R270H/-}$ mice succumbed to spontaneous tumors (Supplemental Table S1–2). The median lifespan for $Rbm38^{-/-}; p53^{R270H/-}$ mice was 19 weeks, which was significantly shorter than that for $p53^{R270H/-}$ mice (26 weeks) (p=0.002 by Log Rank Test) (Fig. 1B). Moreover, loss of Rbm38 significantly altered the tumor incidence in $Rbm38^{-/-};p53^{R270H/-}$ mice (Fig. 1C). $Rbm38^{-/-}$; $p53^{R270H/-}$ mice were prone to lymphomas (p=0.0262 by Fisher's exact test) whereas $p53^{R270H/-}$ mice were prone to sarcomas (p=0.0323 by Fisher's exact test). We also noticed that the lymphomas developed in $Rbm38^{-/-}; p53^{R270H/-}$ mice were mostly T-lymphoblastic lymphomas (TLBL) (Supplemental Fig. S1A), which was significantly increased in $Rbm38^{-/-}; p53^{R270H/-}$ mice as compared to $p53^{R270H/-}$ mice (p=0.0027 by Fisher's exact test). The affected mutant mice usually suffered with short breath due to enlarged thymus in the thoracic cage (Supplemental Fig. S1B). We would like to mention that while this manuscript is in preparation,

Loss of Rbm38 shortens the lifespan of, and alters tumor incidence in, mutant $p53^{R172H/-}$ knockin mice

Recent studies indicate that not all p53 mutants have the same oncogenic activities and some p53 mutants have unique activities in terms of the ability to inhibit wild-type p53 and gain-of-functions (36). In this regard, mutant $p53^{R172H/-}$ (equivalent to human R175H, a conformation mutant) knock-in mice were crossed with $Rbm38^{-/-}$ mice to generate a cohort of $p53^{R172H/-}$ and $Rbm38^{-/-}$; $p53^{R172H/-}$ MEFs. We showed that the level of mutant p53(R172H) protein in $Rbm38^{-/-}$; $p53^{R172H/-}$ MEFs was increased by loss of Rbm38 as compared to that in $p53^{R172H/-}$ MEFs (Fig. 2A). Next, a cohort of $p53^{R172H/-}$ (n=19) and $Rbm38^{-/-}$; $p53^{R172H/-}$ (n=19) mice was generated and monitored throughout their lifespan. We found that the median survival time was 24.3 weeks for $p53^{R172H/-}$ mice and 21 weeks for $Rbm38^{-/-}$; $p53^{R172H/-}$ mice (Fig. 2B). The difference in survival time between $p53^{R172H/-}$ and $Rbm38^{-/-}$; $p53^{R172H/-}$ mice was statistically significant (p=0.012 by LogRank test). We also found that both $p53^{R172H/-}$ and $Rbm38^{-/-}$; $p53^{R172H/-}$ mice developed similar types of tumors including TLBL, angiosarcoma, and spindle cell sarcoma (Supplemental Fig. S2). However, loss of *Rbm38* altered the tumor incidence in $p53^{R172H/-}$ mice (Fig. 2C and Supplemental Tables S3–4). Specifically, the incidence of TLBL was significantly higher in $Rbm38^{-/-}$; $p53^{R172H/-}$ mice than that in $p53^{R172H/-}$ mice (p=0.0033 by Fisher's exact test). By contrast, the incidence of diffuse large B cell lymphoma (DLBCL) was significantly lower in $Rbm38^{-/-}$; $p53^{R172H/-}$ mice than that in $p53^{R172H/-}$ mice (p=0.0182 by Fisher's exact test).

Loss of Rbm38 increases mutant p53 expression but decreases Pten expression in TLBLs

TLBL is originated from developing thymocytes due to abnormal differentiation and proliferation in the thymus. The increased incidence of TLBL in the compound mutant mice (Fig. 1C and 2C) indicates that loss of *Rbm38* may alter the regulatory mechanism for T cell differentiation and proliferation. Thus, the expression of several T-cell regulators, including NFAT1, NFAT2 and Tbet, was examined in the thymi of WT and *Rbm38*-KO mice. NFAT1

and NFAT2 play a critical role in T-cell development and activation (37–39) whereas Tbet is expressed in $CD4^+$ T lymphocytes committed to Th1 T-cell development (40). We showed that loss of *Rbm38* did not alter their expression (Supplemental Fig. S3A). We also examined IFN-γ, which plays a role in inducing Th1 cells (41), and IL6, which controls T cell proliferation, survival, and commitment (42). Again, these cytokines were not altered by Rbm38-KO (Supplemental Fig. S3A). Consistent with this, we showed that the level of NFAT1 and T-bet was not altered by loss of Rbm38 in the TLBLs from $p53^{R270H/-}$ and $Rbm38^{-/-}; p53^{R270H/-}$ mice (Supplemental Fig. S3B) or from $p53^{R172H/-}$ and $Rbm38^{-/-}; p53^{R172H/-}$ mice (Supplemental Fig. S3C).

The above data let us speculate that loss of Rbm38 may affect the T-progenitor cell development. To test this, we examined expression of Pten (Phosphatase and TENsin homolog deleted on chromosome 10), a key regulator of T-progenitor cells (43). Notably, $Pten^{+/-}$ mice are prone to TLBLs (44). In addition, loss or reduced expression of Pten was found to be an early event in TLBLs developed in p53-null mice (45). In this regard, the levels of mutant p53(R270H) and Pten were analyzed in TLBLs from $p53^{R270H/-}$ and $Rbm38^{-/-}; p53^{R270H/-}$ mice. We found that the level of mutant p53(R270H) protein was increased by loss of Rbm38 in TLBLs (Fig. 3A), consistent with the above observation in $Rbm38^{-/-};p53^{R270H/-}$ MEFs (Fig. 1A). Interestingly, we found that the level of Pten protein was decreased in all four TLBLs from $Rbm38^{-/-}; p53^{R270H/-}$ mice as compared to the ones from $p53^{R270H/-}$ (Fig. 3A). Consistent with this, the level of Pten transcript was also decreased in these TLBLs from $Rbm38^{-/-}; p53^{R270H/-}$ mice as compared to the ones from $p53^{R270H/-}$ mice (Fig. 3B). Next, we determined the effect of Rbm38-KO on the expression of mutant p53 and Pten in the TLBLs from $Rbm38^{-/-};p53^{R172H/-}$ and $p53^{R172H/-}$ mice. As expected, the level of mutant p53(R172H) was also increased by loss of Rbm38 (Fig. 3C). Importantly, the levels of Pten protein and transcript were decreased in the TLBLs from $Rbm38^{-/-}; p53^{R172H/-}$ mice as compared to the ones from $p53^{R172H/-}$ (Fig. 3C–D). Together, these data suggest that loss of Rbm38 promotes the development of TLBLs in $Rbm38^{-/-}; p53^{R270H/-}$ and $Rbm38^{-/-}; p53^{R172H/-}$ mice by concomitantly modulating both mutant p53 and Pten.

Loss of Rbm38 decreases Pten expression in the TLBLs from $Rbm38^{-/-};p53^{-/-}$ mice

Previously, we showed that *Rbm38* deficiency led to increased incidence of lymphoma in $Rbm38^{-/-};p53^{-/-}$ mice (16 lymphomas out of total 17 tumors) as compared to that in $p53^{-/-}$ mice (20 lymphomas out of total 33 tumors) (p=0.0183 by Fisher's exact test) (Fig. 4A) (20). In terms of TLBLs, the incidence was not statistically higher in $Rbm38^{-/-};p53^{-/-}$ mice than that in $p53^{-/-}$ mice (p=0.1305 by Fisher's exact) (Fig. 4A). However, the median survival time for mice succumbed to TLBLs was significantly shorter for $Rbm38^{-/-};p53^{-/-}$ mice (18 weeks) than that for $p53^{-/-}$ mice (25 weeks) (p=0.002 by LogRank Test) (Fig. 4B), suggesting that the TLBLs in $Rbm38^{-/-};p53^{-/-}$ mice are more aggressive than the ones in $p53^{-/-}$ mice. As Pten expression was decreased by loss of Rbm38 in $Rbm38^{-/-};p53^{R270H/-}$ and $Rbm38^{-/-};p53^{R172H/-}$ mice, we thus asked whether Pten expression is altered in the TLBLs from $Rbm38^{-/-};p53^{-/-}$ mice. We would like to note that one $p53^{-/-}$ (ID: 10-26-5) and one $Rbm38^{-/-}; p53^{-/-}$ (ID: 10-26-5) mice were generated for current study. All other mice were generated and used in a previous study (20). Indeed, we found that the levels of

both Pten proteins and transcripts were markedly decreased in the TLBLs from *Rbm38*$^{-/-}$;*p53*$^{-/-}$ mice as compared to that from *p53*$^{-/-}$ mice (Fig. 4C–D).

Rbm38 is required for Pten expression

Loss of Rbm38 leads to decreased expression of Pten in the TLBLs from mutant p53 knock-in and *p53*-null mice (Figs. 3–4). These results let us speculate that Rbm38 directly regulates Pten expression. In this regard, we examined Pten expression in both WT and *Rbm38*$^{-/-}$ mouse thymi and found that the level of Pten transcript was reduced by loss of Rbm38 (Fig. 5A). Next, to examine whether knockdown of Rbm38 alters Pten expression in human lymphoma cells, Rbm38 siRNA along with a control siRNA were transiently transfected into Raji and Romas cells, both of which are lymphoma cell lines. We found that Rbm38 siRNA led to decreased expression of Rbm38 protein, which was accompanied with a reduction of Pten protein (Fig. 5B–C). Next, to test whether Rbm38 regulates Pten expression in non-lymphoma tissues, various cancer cell lines, including U2OS (osteosarcoma), HCT116 (colon cancer), and Mia-PaCa2 (pancreatic cancer), were used to generate *Rbm38*-KO cell lines by CRISPR-Cas9 technology. We would like to mention that HCT116 and U2OS cells contain wild-type p53 whereas Mia-PaCa2 cells contain mutant p53(R248W). Sequencing analysis indicated that *Rbm38*-KO U2OS cells had a 4-nt out of frame deletion in Exon 1 of the *Rbm38* gene. *Rbm38*-KO HCT116 cells had a 4-nt deletion, whereas *Rbm38*-KO Mia-PaCa2 cells had 1-nt deletion, in exon 4 of the *Rbm38* gene. As expected, the level of Rbm38 protein was undetectable in these *Rbm38*-KO HCT116, U2OS, and Mia-PaCa2 cells as compared to their respective isogenic control cells (Fig. 5D–F, Rbm38 panel). Importantly, the level of Pten protein was decreased by loss of Rbm38 (Fig. 5D–F, Pten panel). Conversely, we found that ectopic expression of Rbm38 increased Pten expression in Mia-PaCa2 cells (Fig. 5G, Pten panel). Next, qRT-PCR analysis was performed to examine whether loss of Rbm38 alters the level of Pten transcripts. Indeed, we found that the level of Pten transcript was reduced by loss of Rbm38 in HCT116, U2OS, and Mia-PaCa2 cells (Fig. 5H–J). Together, these data suggest that Rbm38 is required for Pten expression regardless of p53 genetic status.

Rbm38 stabilizes Pten mRNA via an AU-rich element in its 3'UTR

As a RNA binding protein, Rbm38 is known to regulate gene expression via mRNA stability (46, 47). Thus, we examined whether Pten mRNA turnover is regulated by Rbm38. To test this, isogenic control and Rbm38-KO MiaPaCa2 cells were treated with DRB (5,6-Dichlorobenzimidazole-β-D-ribofuranoside) for various times and the half-life of Pten mRNA was determined by qRT-PCR. DRB inhibits the elongation step during RNA polymerase II transcription and subsequently, de novo mRNA synthesis (48). Thus, DRB can be used to measure the half-life of a given mRNA, which is defined as the time required to degrade 50% of the mRNA. We found that the half-life of Pten mRNA was decreased from 3.55 h in isogenic control cells to 1.85 h in Rbm38-KO cells (Fig. 6A). As Rbm38 is known to preferentially bind to AU-rich elements (AREs) (17), we searched for and found several AREs in Pten 3'UTR (Fig. 6B). To test this, we generated several EGFP reporters that contain HA-tagged EGFP alone, or together with a fragment from various regions of Pten 3'UTR (Fig. 6B). We found that Rbm38 had no effect on EGFP expression from EGFP reporter vectors that carried only EGFP (Fig. 6C) or the EGFP reporter fused with fused

with A (Fig. 6D) or B (Fig.6E) fragment of Pten 3'UTR. Moreover, we found that ectopic expression of Rbm38 led to increased expression of EGFP when the EGFP reporter was fused with the C fragment of Pten 3'UTR (Fig. 6F), but not the one fused with D fragment (Fig. 6G). To further delineate Rbm38 binding site(s) in the C fragment, we generate three additional EGFP reporters that contain C1, C2, or C3 fragment from Pten 3'UTR (Fig. 6B). We found that the reporter that contained the C2 fragment, but not the C1 and C3 fragments, was responsive to Rbm38 (Fig. 6H). Upon close examination, we found two AREs (ARE1 and ARE2) located in the C2 fragment (Fig. 6B). To determine whether these AREs are responsive to Rbm38, we generated three mutant C2 fragments in that the A and U in the ARE1, ARE2, or both were substituted with G and C, respectively (Fig. 6B). We found that mutation of ARE1 nearly abolished, whereas mutation of ARE2 had no effect on, EGFP expression induced by Rbm38 (Fig. 6I). Similarly, Rbm38 was unable to induce EGFP expression when both ARE1 and ARE2 were mutated (Fig. 6I). Together, these data indicated that the ARE1 in Pten 3'UTR is likely responsible for Rbm38 to stabilize Pten mRNA.

Discussion

Recently, we showed that loss of Rbm38 promotes mutant p53 translation (23). Interestingly, loss of Rbm38 occurs frequently in tumors with mutant p53 (27, 28). Thus, we hypothesize that loss of Rbm38 is necessary for maintaining high levels of mutant p53, which then promotes tumor development. To test this, we generated compound mutant mice containing *Rbm38* deficiency and knock-in mutant $p53^{R270H}$ or $p53^{R172H}$. Indeed, we found that *Rbm38* deficiency significantly enhances the cancer susceptibility of $p53^{R270H/-}$ or $p53^{R172H/-}$ mice by shortening the lifespan and altering the tumor incidence (Figs. 1–2). Interestingly, we showed previously that in a p53-heterozygous background, *Rbm38* deficiency protects mice from developing tumors at least in part via enhanced wild-type p53 expression (20). These data indicate that *Rbm38* deficiency may have differential effects on tumorigenesis depending on p53 status. Thus, p53 status needs to be considered when exploring Rbm38 as a target for cancer treatment.

In this study, we showed that $p53^{R270H/-}$ and $p53^{R172H/-}$ mice had a median survival of 24–26 weeks (Fig. 1B and2B) and were prone to lymphomas (Fig. 1C and2C), consistent with a previous report (14). Importantly, in an *Rbm38*-null background, the incidence of the TLBLs was markedly increased in both $p53^{R270H/-}$ and $p53^{R172H/-}$ mice along with increased expression of mutant p53 (Figs. 1C, 2C and Fig. 3). These data let us speculate that enhanced mutant p53 expression by loss of Rbm38 is needed to promote the formation of TLBLs, likely through its gain-of-function. In support of this notion, it was reported previously that $p53^{R270/R270H}$ mice developed substantially more TLBLs than $p53^{R270H/-}$ animals owing to increased levels of mutant p53 (42). Similarly, previous study indicated that $p53^{R172H/R172H}$ mice showed a high incidence of lymphomas when compared to $p53^{R172H/+}$ animals (49). Together, these data indicates that *Rbm38* deficiency cooperates with mutant p53 to promote proliferation of T cells with improper pre-TCR signaling and subsequently increases the incidence of TLBLs.

Pten is necessary for maintaining the hematopoietic stem cells and known to inhibit leukemia-initiating cells by suppressing the PI3K-AKT pathway (50). Additionally, both Pten and Rbm38 were found to be decreased in human leukemia patients (27, 51). Here, we showed that Pten expression was reduced by loss of Rbm38 in the TLBLs from mutant p53 knock-in mice (Fig. 3). Thus, it is possible that in a mutant p53 context, a reduced level of Pten by *Rbm38* deficiency would activate the PI3K-Akt pathway, which alters T cell differentiation and proliferation, leading to TLBLs. Interestingly, Pten was reduced by loss of Rbm38 in the TLBLs from *p53*-null mice. Since *p53*-null mice were prone to TLBLs, it is not surprising that loss of Rbm38 did not significantly increase the incidence of TLBLs in *p53*-null mice (Fig. 4A). Nevertheless, $Rbm38^{-/-};p53^{-/-}$ mice succumbed to TLBLs had a shorter lifespan than that for $p53^{-/-}$ mice (Fig. 4B). Thus, we speculate that reduced Pten expression in $Rbm38^{-/-};p53^{-/-}$ mice would promote proliferation of T cells that are lymphomagenic, leading to more aggressive tumors.

We showed that Rbm38 is required for Pten expression (Fig. 5). We also showed that Rbm38 binds to an AU-rich element in Pten 3′ UTR and subsequently, stabilizes Pten mRNA (Fig. 6). To our knowledge, this is the first report that Pten is regulated by a RNA-binding protein via mRNA stability. Of note, several miRNAs were found to inhibit Pten expression, including miR-21(52), miR-214 (53) and miR-19 (54). In addition, it was found that several Pten competing endogenous RNAs (ceRNAs), such as Pten pseudogene transcripts (55), can compete for miRNAs that targeted sequences common to both Pten mRNA and its pseudo-mRNAs (56). Thus, it will be interesting to examine whether and how Rbm38 play a role in modulating Pten expression via these miRNAs or ceRNAs. Moreover, as Pten 3'UTR contains several AU-rich elements, it would be interesting to examine whether other RNA-binding proteins, such as HuR (19), known to bind an AU-rich element, can cooperate or antagonize with Rbm38 to regulate Pten expression and the biological significance of these regulations.

Our data suggest that Rbm38 controls two "hits" during the development of T-cell lymphomagenesis by concomitantly modulating mutant p53 and Pten. However, several questions remain to be addressed. First, although the TLBLs in $Rbm38^{-/-};p53^{R270H/-}$ and $Rbm38^{-/-};p53^{R172H/-}$ mice were histologically indistinguishable from the ones in $p53^{R270H/-}$ and $p53^{R172H/-}$ mice, it would be interesting to examine the pre-TCR signaling pathway in these tumors and further elucidate the role of Rbm38, Pten, and mutant p53 in T-cell development. Second, the compound mice in this study are genetically predisposed to mutant p53 throughout their lifespan. As a result, most of compound mice die early due to TLBLs, which may not fully mimic the development of T-cell malignancies in humans. Thus, it would necessary to use conditional knockout mouse models to elucidate the role of Rbm38-mutant p53 axis in tumor development. Third, as TLBLs have similar clinical presentations and cytogenetic characteristics as T-cell acute lymphoblastic leukemia (T-ALL), these compound mutant mice may be used as a model to test the efficacy of preclinical drugs for treatment of T-ALL.

In summary, we have uncovered a novel mechanism by which Pten is regulated by Rbm38 via mRNA stability. Our data indicate that loss of Rbm38 synergizes with mutant p53 to

promote lymphomagenesis though down-regulation of Pten and that the Rbm38-mutant p53/Pten axes may be explored for the treatment of T cell malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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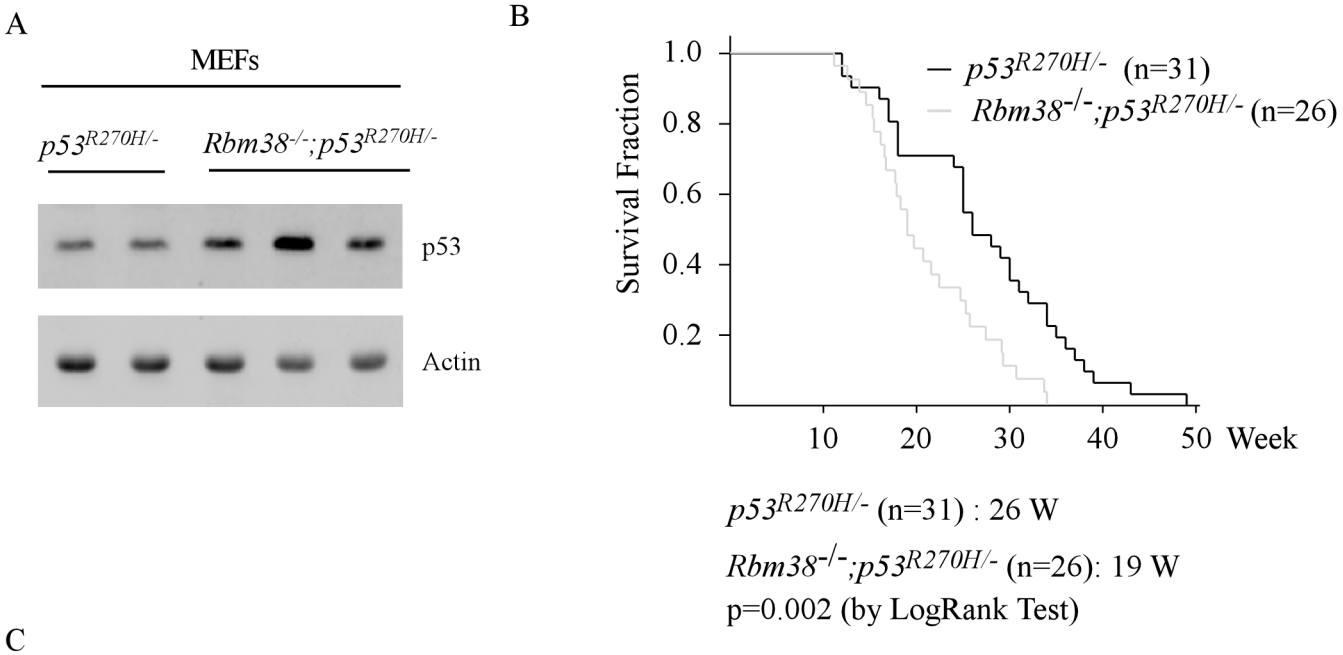


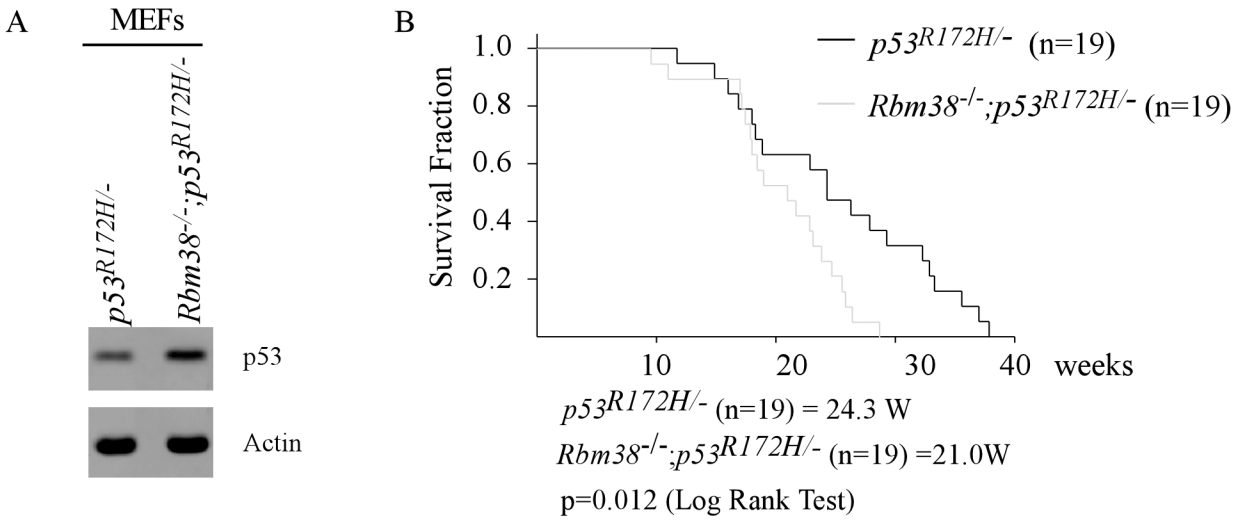
C

Tumor spectrum in $p53^{R270H/-}$ and $Rbm38^{-/-};p53^{R270H/-}$ mice

Tumors	$p53^{R270H/-}$ (n=31)	$Rbm38^{-/-};p53^{R270H/-}$ (n=26)
Lymphoma		
Diffuse large B-cell lymphoma (DLBCL)	2	0
T-lymphoblastic lymphoma (TLBL)	15	22
Unclassified lymphoma	5	2
Sarcoma		
Fibrosarcoma	1	0
Spindle cell sarcoma	7	1
Unclassified sarcoma	1	0
Carcinoma		
Squamous cell carcinoma	2	1
Total tumors	33	26

Figure 1.

(A) The level of p53 and actin was examined in $p53^{R270H/-}$ and $Rbm38^{-/-};p53^{R270H/-}$ MEFs. (B) Kaplan Meier curves of $p53^{R270H/-}$ (n=31) and $Rbm38^{-/-};p53^{R270H/-}$ (n=26) mice. The median survival time was 26 weeks for $p53^{R270H/-}$ mice and 19 weeks for $Rbm38^{-/-};p53^{R270H/-}$ mice (p=0.002 by LogRank test). (C) The tumor spectrum of $p53^{R270H/-}$ (n=31) and $Rbm38^{-/-};p53^{R270H/-}$ (n=26) mice. The incidence of TLBLs is significantly higher in $Rbm38^{-/-};p53^{R270H/-}$ mice than that in $p53^{R270H/-}$ mice (p=0.0027 by Fisher's exact test). The incidence of sarcomas is significantly lower in $Rbm38^{-/-};p53^{R270H/-}$ mice than that in $p53^{R270H/-}$ mice (p=0.0323 by Fisher's exact test).



C

Tumor spectrum in *p53*$^{R172H/-}$ and *Rbm38*$^{-/-}$;*p53*$^{R172H/-}$ mice

Tumors	*p53*$^{R172H/-}$ (n=19)	*Rbm38*$^{-/-}$;*p53*$^{R172H/-}$ (n=19)
Lymphoma		
Diffuse large B-cell lymphoma (DLBCL)	10	2
T-lymphoblastic lymphoma (TLBL)	5	14
Unclassified lymphoma	2	2
Sarcoma		
Angiosarcoma	5	2
Spindle cell sarcoma	1	2
Teratoma	1	0
Total tumors	24	22

Figure 2.

(A) The level of p53 and actin was examined in *p53*$^{R172H/-}$ and *Rbm38*$^{-/-}$; *p53*$^{R172H/-}$ MEFs. (B) Kaplan Meier curves of *p53*$^{R172H/-}$ (n=19) and *Rbm38*$^{-/-}$; *p53*$^{R172H/-}$ (n=19) mice. The median survival time was 24 weeks for *p53*$^{R172H/-}$ mice and 21 weeks for *Rbm38*$^{-/-}$; *p53*$^{R172H/-}$ mice (p=0.012 by LogRank test). (C) The tumor spectrum of *p53*$^{R172H/-}$ (n=19) and *Rbm38*$^{-/-}$; *p53*$^{R172H/-}$ (n=19) mice. The incidence of TLBLs is significantly higher in *Rbm38*$^{-/-}$;*p53*$^{R172H/-}$ mice than that in *p53*$^{R172H/-}$ mice (p =0.0033 by Fisher's exact test). The incidence of diffuse large B cell lymphomas is significantly lower in *Rbm38*$^{-/-}$;*p53*$^{R172H/-}$ mice than that in *p53*$^{R172H/-}$ mice (p = 0.0182 by Fisher's exact test).

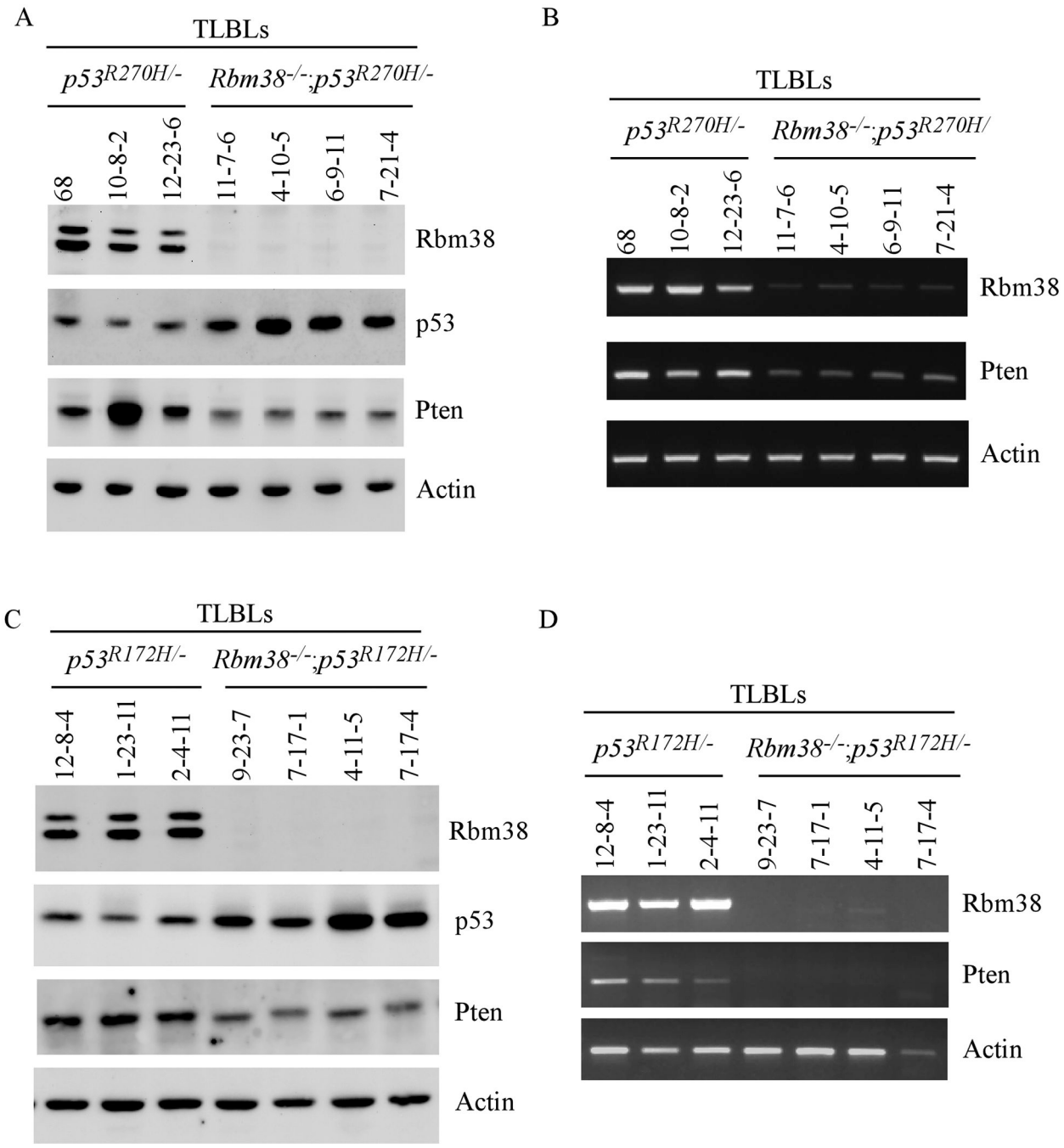


Figure 3.

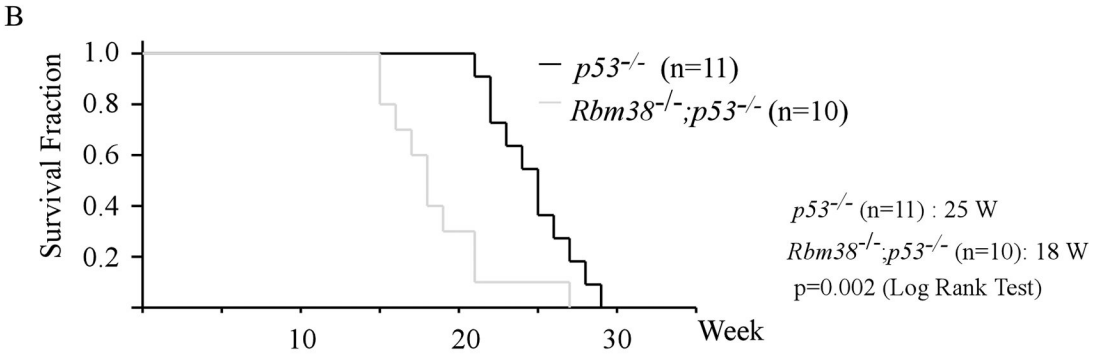
(A-B) The levels of Rbm38, p53, Pten proteins (A) and transcripts (B) were examined in T-lymphoblastic lymphomas from $p53^{R270H/-}$ and $Rbm38^{-/-}; p53^{R270H/-}$ mice. (C-D) The levels of Rbm38, p53, Pten proteins (C) and transcripts (D) were examined in TLBLs from $p53^{R172H/-}$ and $Rbm38^{-/-}; p53^{R172H/-}$ mice.

A

Tumor spectrum in *p53*$^{-/-}$ (n=24) and *Rbm38*$^{-/-}$;*p53*$^{-/-}$ mice (n=19)

	p53$^{-/-}$ (n=24)	*Rbm38*$^{-/-}$;*p53*$^{-/-}$ (n=19)
Lymphoma		
Diffuse large B-cell lymphoma (DLBCL)	4	4
T-lymphoblastic lymphoma (TLBL)	11	10
Unclassified lymphoma	5	2
Other tumors	13	1

Data adapted from *PNAS*, 111(52):18637-42



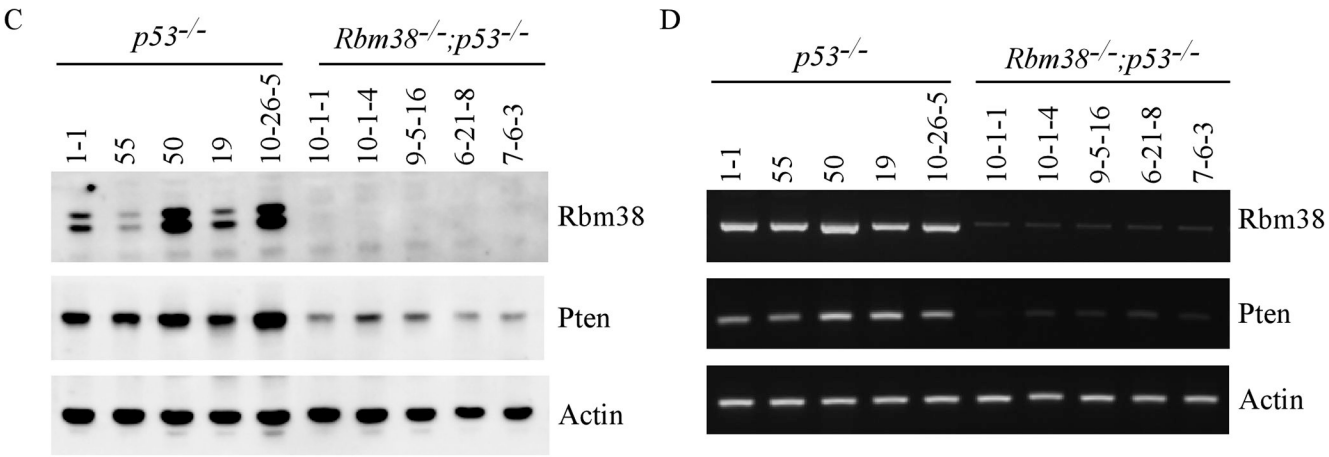


Figure 4.

(A) The tumor spectrum of *p53*$^{-/-}$ (n=24) and *Rbm38*$^{-/-}$; *p53*$^{-/-}$ (n=19) mice. These data were adapted from a previous tumor study. (B) Kaplan Meier curves of *p53*$^{-/-}$ (n=11) and *Rbm38*$^{-/-}$; *p53*$^{-/-}$ (n=10) mice dying from T-lymphoblastic lymphoma. The median survival time was 25 weeks for *p53*$^{-/-}$ mice and 18 weeks for *Rbm38*$^{-/-}$; *p53*$^{-/-}$ mice (p=0.002 by LogRank test). (C-D) The levels of Rbm38, p53, Pten proteins (C) and transcripts (D) were examined in TLBLs from *p53*$^{-/-}$ and *Rbm38*$^{-/-}$; *p53*$^{-/-}$ mice. Both *p53*$^{-/-}$ (ID: 10-26-5) and *Rbm38*$^{-/-}$; *p53*$^{-/-}$ (ID: 10-26-5) mice were generated for current study.

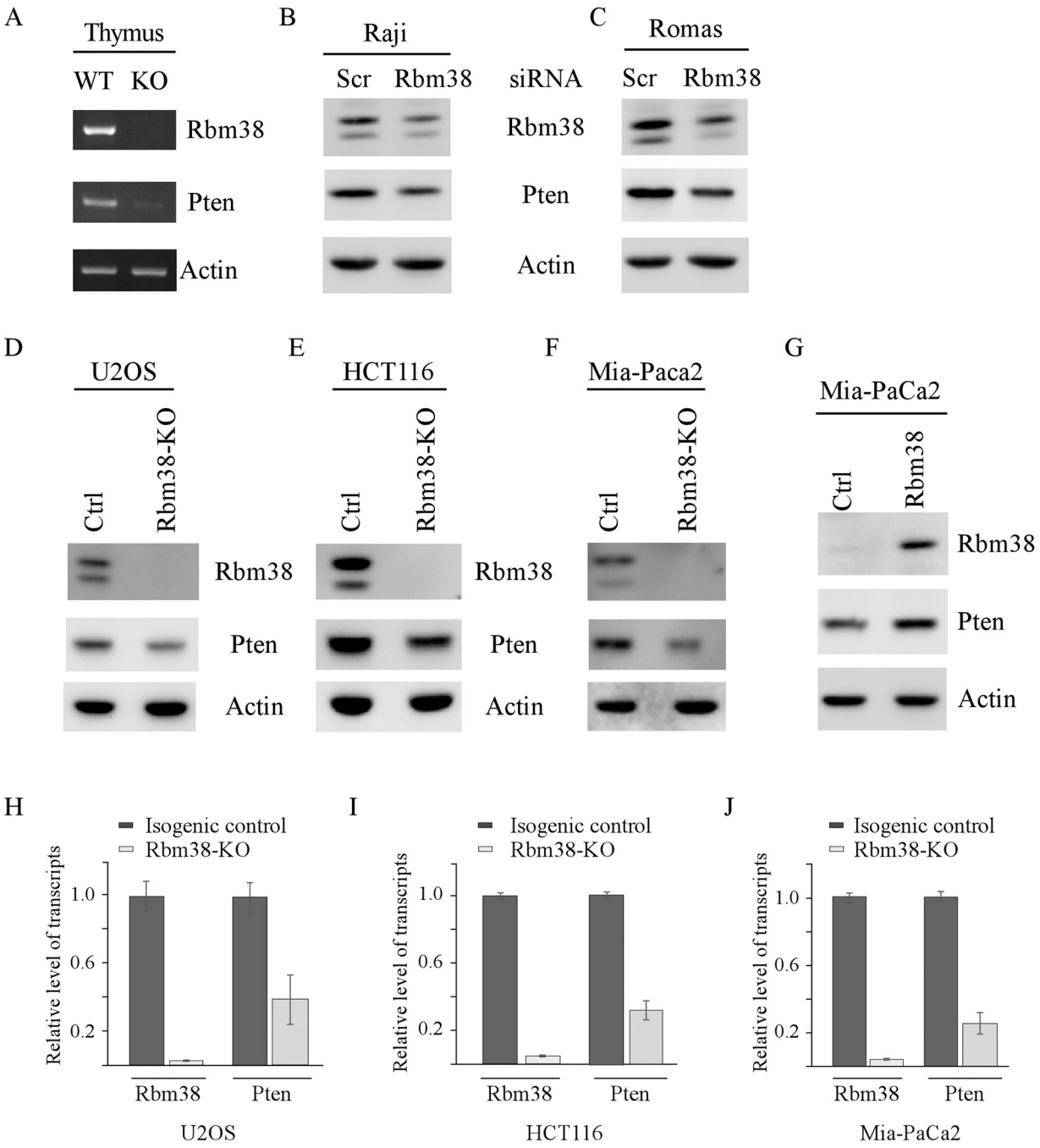


Figure 5.

(A) The levels of Rbm38, Pten, and actin transcripts were examined in the thumus from WT and *Rbm38*$^{-/-}$ mice. (B-C) The levels of Rbm38, Pten, and actin proteins were examined in Raji (B) or Romas (C) cells transiently transfected with scrambled or Rbm38 siRNA at 100 nM for 3 days. (D-F) The levels of Rbm38, Pten, and actin proteins were examined in isogenic control and Rbm38-KO U2OS (D), HCT116 (E), and Mia-Paca2 (F) cells. (G) The level of Rbm38, Pten and actin prpteins were examined in Mia-PaCa2 cells transfected with a control or Rbm38 expression vector for 24 h. (H-J) The levels of Pten and actin transcripts

were examined by qRT-PCR in isogenic control and Rbm38-KO U2OS (H), HCT116 (I), and Mia-Paca2 (J) cells.

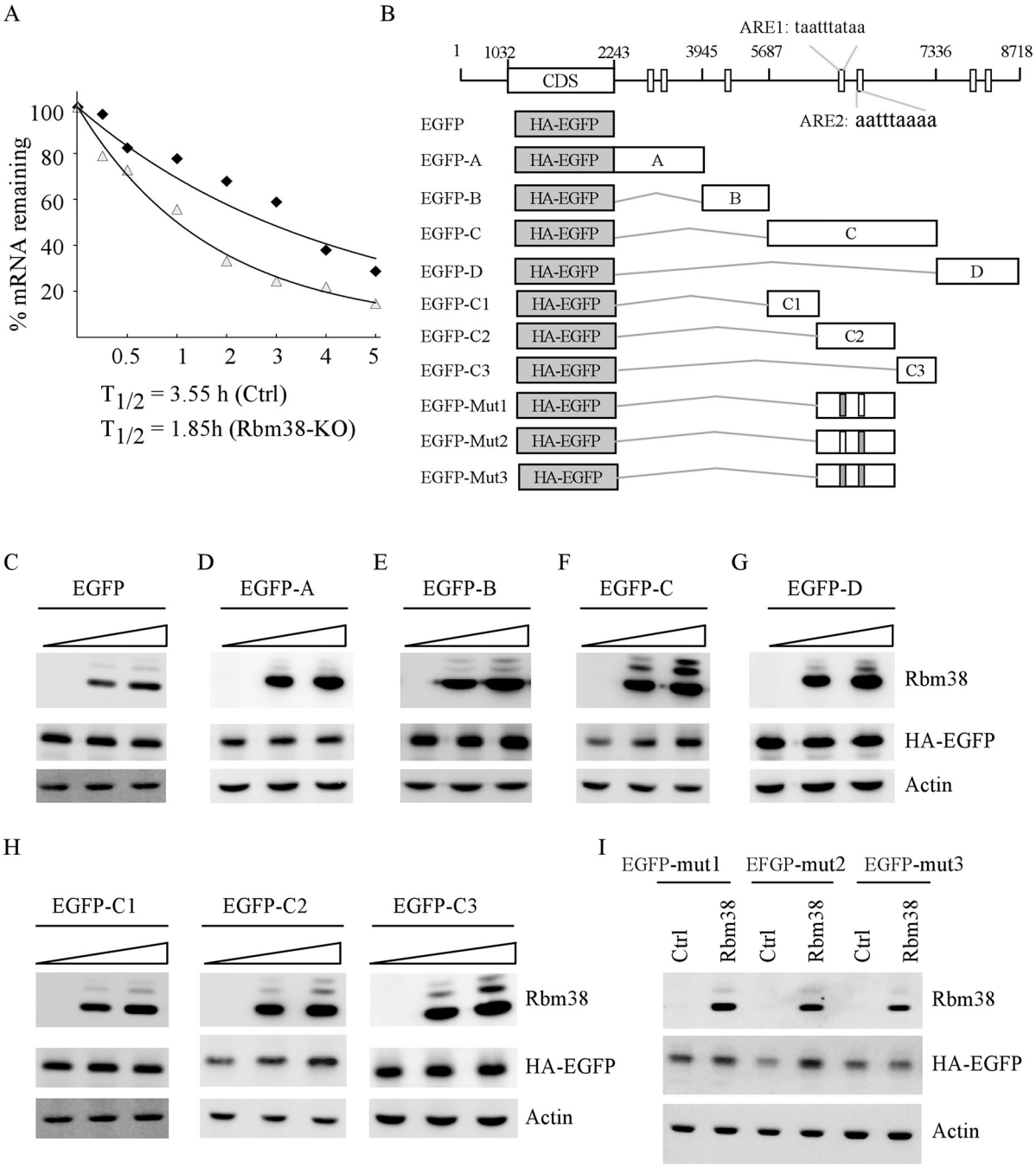


Figure 6.

(A) Rbm38 is required for maintaining Pten mRNA stability. The level of Pten transcript was measured by qRT-PCR in isogenic control and Rbm38-KO Mia-Paca2 cells treated with DRB for various times. (B) Schematic representation of Pten transcript and the locations of 3'UTR fragments for reporter assay. AU-rich elements were shown in boxes. The grey box indicates mutation in ARE1 or ARE2. (C-G) Various amounts of Rbm38 expression vector were transfected into $p53^{-/-}$ HCT116 cells along with a fixed amount of EGFP (C) or EGFP expression vectors that contain Pten 3'UTR-A (D), 3'UTR-B (E), 3'UTR-C (F), or 3'UTR-

D (G). The levels of Rbm38, HA-EGFP, and actin were examined by western blot analysis. (H) The levels of Rbm38, HA-EGFP, and actin proteins were examined in $p53^{-/-}$ HCT116 cells transfected with various amount of Rbm38 expression vector along with a fixed amount of EGFP vectors that contain Pten 3'UTR-C1, C2, or C3. (I) HA-EGFP vector that contains point mutation in ARE1, ARE2 or both was transfected into $p53^{-/-}$ HCT116 cells together with or without Rbm38 expression vector, followed by western blot analysis to examine the level of Rbm38, HA-EGFP, and actin.