Translational repression of p53 by RNPC1, a p53 target overexpressed in lymphomas

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The p53 pathway is critical for tumor suppression, as the majority of human cancer has a faulty p53. Here, we identified RNPC1, a p53 target and a RNA-binding protein, as a critical regulator of p53 translation. We showed that ectopic expression of RNPC1 inhibited, whereas knockdown of RNPC1 increased, p53 translation under normal and stress conditions. We also showed that RNPC1 prevented cap-binding protein eIF4E from binding p53 mRNA via its C-terminal domain for physical interaction with eIF4E, and its N-terminal domain for binding p53 mRNA. Consistent with this, we found that RNPC1 directly binds to p53 5′ and 3′untranslated regions (UTRs). Importantly, we showed that RNPC1 inhibits ectopic expression of p53 in a dose-dependent manner via p53 5′ or 3′ UTR. Moreover, we showed that loss of *RNPC1* in mouse embryonic fibroblasts increased the level of p53 protein, leading to enhanced premature senescence in a p53-dependent manner. Finally, to explore the clinical relevance of our finding, we showed that RNPC1 was frequently overexpressed in dog lymphomas, most of which were accompanied by decreased expression of wild-type p53. Together, we identified a novel p53–RNPC1 autoregulatory loop, and our findings suggest that RNPC1 plays a role in tumorigenesis by repressing p53 translation.

[*Keywords*: p53; translational control; RNA-binding protein; eIF4E; RNPC1; RBM38]

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The p53 tumor suppressor plays a pivotal role in preserving the integrity of the genome and in preventing cancer development. The importance of p53 in this process is demonstrated by the fact that inactivation of p53 occurs in >50% of human cancers, and loss of p53 function is known to be essential for carcinogenesis (Vogelstein et al. 2000; Vousden and Prives 2009). Upon activation, p53 functions as a transcription factor to induce a number of genes, such as p21 (el-Deiry et al. 1993), MDM2 (Barak et al. 1993; Wu et al. 1993), and TIGAR (Bensaad et al. 2006). These p53 target genes mediate diverse biological functions of p53, including cell cycle arrest and apoptosis (Harms et al. 2004; Riley et al. 2008).

P53 activity is primarily controlled by post-translational modifications, including phosphorylation and acetylation (Kruse and Gu 2009; Vousden and Prives 2009). However, p53 activity is also found to be regulated by other mechanisms, particularly translational regulation. For instance, the rate of p53 protein synthesis is found to be increased in UV-irradiated cells (Maltzman and Czyzyk 1984). Consistent with this, several *cis*-regulatory elements known to regulate protein translation have been identified in p53 mRNA, including a putative IRES element (Ray et al. 2006; Yang et al. 2006) and an AU-rich sequence (Fu and Benchimol 1997; Fu et al. 1999). In addition, several RNA-binding proteins (RBPs), including HuR, ribosomal protein L26 (RPL26), and nucleolin, are found to regulate p53 translation (Galban et al. 2003; Mazan-Mamczarz et al. 2003; Takagi et al. 2005; Ofir-Rosenfeld et al. 2008). However, the role of these RBPs in tumorigensis remains unclear.

The *RNPC1* gene, also called *RBM38*, encodes a RBP and is expressed as two isoforms (Fig. 1A): RNPC1a with 239 amino acids and RNPC1b with 121 amino acids identical to the N-terminal region in RNPC1a (Shu et al. 2006). RNPC1 is a target of the p53 family and is known to regulate p21 and p63 mRNA stability by binding to AU-rich elements in their 3′ untranslatd regions (UTRs) (Shu et al. 2006; Zhang et al. 2010). Phylogenic analysis reveals that RNPC1 belongs to the RNA recognition motif (RRM)-containing RBP family, which also includes HuR and nucleolin. Interestingly, RNPC1 can modulate the RNA-binding activity of, and cooperate with, HuR to regulate p21 mRNA stability (Cho et al.

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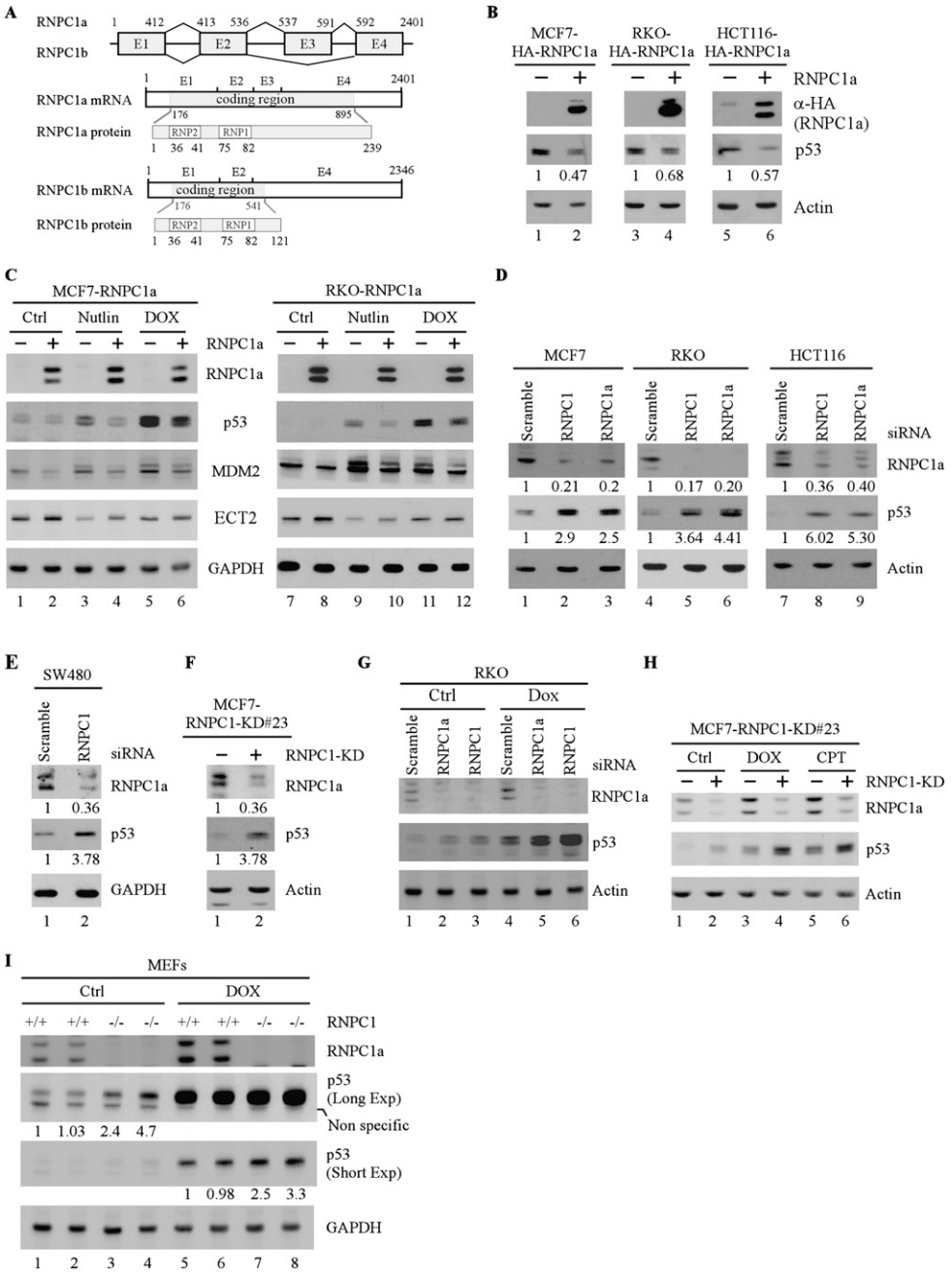

Figure 1. p53 expression is inhibited by ectopic expression of RNPC1a but increased by knockdown or knockout of RNPC1 under normal and stress conditions. (*A*) Schematic illustration of the *RNPC1* locus and the usage of exons for RNPC1a and RNPC1b. (*B*) RNPC1a inhibits p53 expression. Western blots were prepared with extracts from MCF7, RKO, and HCT116 cells uninduced or induced to express HA-tagged RNPC1a for 24 h and probed with antibodies against HA, p53, or actin. The basal levels of p53 were arbitrarily set at 1.0 and the fold change is shown *below* each lane. (*C*) The levels of RNPC1a, p53, MDM2, ECT2, and GAPDH were measured in MCF7 or RKO cells uninduced or induced to express RNPC1a for 12 h, followed by mock treatment or treatment with nutlin-3 or doxorubicin for 12 h. (*D*) The basal level of p53 is increased by total RNPC1 or RNPC1a knockdown. MCF7, RKO, and HCT116 cells were transiently transfected with scrambled siRNA or siRNA against RNPC1a or total RNPC1 for 3 d, and the levels of RNPC1a, p53, and actin were analyzed by Western blot analysis. (*E*) The level of mutant p53 is increased by RNPC1 knockdown in SW480 cells. The experiments were performed as in *C*. (*F*) The levels of RNPC1a, p53, and actin were measured in MCF7 cells uninduced or induced to express shRNA against total RNPC1 for 3 d. (*G*) The levels of RNPC1a, p53, and actin were measured in RKO cells transfected with scrambled siRNA or siRNA against RNPC1a or total RNPC1 for 3 d, followed by treatment with or without doxorubicin for 12 h. (*H*) The levels of RNPC1a, p53, and actin were measured in MCF7 cells uninduced or induced to express shRNA against total RNPC1 for 3 d, followed by mock or doxorubicin or camptothecin treatment for 12 h. (*I*) MEFs isolated from wild-type or *RNPC1*$^{-/-}$ embryos at passage 3 were treated with or without doxorubicin for 12 h, and the levels of RNPC1, p53, and GAPDH protein were analyzed by Western blot analysis.

2010). Consistent with this, RNPC1 is found to play a role in myogenic differentiation via stabilization of the p21 transcript (Miyamoto et al. 2009).

The *RNPC1* gene is located on chromosome *20q13*, a site frequently amplified in breast cancer (Ginestier et al. 2006; Letessier et al. 2006), prostate cancer (Zheng et al. 2001; Bar-Shira et al. 2002), ovarian cancer (Tanner et al. 2000), colorectal cancer (Korn et al. 1999; Knosel et al. 2003), and chronic lymphocytic leukemia (Krackhardt et al. 2002). In addition, RNPC1 overexpression is found to promote the transition of colorectal adenoma to carcinoma (Hermsen et al. 2002; Carvalho et al. 2009). However, it is not clear how RNPC1 exerts its oncogenic activity. As the most frequently inactivated tumor suppressor, p53 may be targeted by RNPC1. In the current study, we found that RNPC1 inhibits p53 translation and plays a role in tumorigenesis by repressing p53 translation.

Results

Ectopic expression of RNPC1a inhibits, whereas knockdown or knockout of RNPC1 increases, p53 translation under normal and stress conditions

To investigate whether RNPC1 regulates p53 expression, the level of p53 protein was examined in HCT116, RKO, and MCF7 cells upon inducible expression of HA-tagged RNPC1a or RNPC1b. We found that the steady-state level of p53 protein was reduced by RNPC1a in HCT116, RKO, and MCF7 cells (Fig. 1B, cf. lanes 1,3,5 and 2,4,6, respectively). We also showed that the level of ectopic RNPC1a was about twofold higher than that of endogenous RNPC1a induced by DNA damage (Supplemental Fig. S1A), indicating that ectopic RNPC1a was not excessively expressed. Conversely, ectopic RNPC1b had no effect on p53 expression in MCF7 and RKO cells (Supplemental Fig. S1B). Similarly, transient expression of RNPC1a, but not RNPC1b, inhibited p53 expression in a dose-dependent manner (Supplemental Fig. S1C). Furthermore, we showed that upon treatment with doxorubicin or nutlin-3, ectopic expression of RNPC1a markedly attenuated p53 accumulation in MCF7 and RKO cells (Fig. 1C, cf. lanes 3,5,9,11 and 4,6,10,12, respectively). Concomitantly, the level of MDM2 was decreased, whereas the level of ECT2, a gene repressed by p53 (Scoumanne and Chen 2006), was increased (Fig. 1C). In contrast, ectopic RNPC1b had no effect on p53 accumulation by DNA damage (Supplemental Fig. S1D).

Next, we determined the effect of endogenous RNPC1 on p53 expression. To this end, we first measured the level of RNPC1a and RNPC1b transcripts in MCF7 cells and found that RNPC1b transcripts were 40% more abundant than that of RNPC1a (Supplemental Fig. S1E). Next, siRNA against RNPC1a (targeting the unique RNPC1a sequence in exon 3) or total RNPC1 (targeting the sequence common to both RNPC1a and RNPC1b) was transiently transfected into MCF7 cells. The specificity of these siRNAs against RNPC1 was confirmed by quantitative RT–PCR (Supplemental Fig. S1F). As expected, the level of RNPC1a protein was decreased by siRNA against total RNPC1 or RNPC1a, but not scrambled siRNA (Fig. 1D, cf. lanes 1 and 2,3). Due to the low reactivity of anti-RNPC1, the level of RNPC1b was undetectable. Consistent with the above observations, the level of p53 protein was markedly increased in MCF7 cells upon transient knockdown of total RNPC1 or RNPC1a (Fig. 1D, cf. lanes 1 and 2,3). To rule out potential cell type-specific effects, the experiment was repeated in RKO, HCT116, MCF10A, and SW480 cells. We showed that the level of p53 protein was markedly increased by total RNPC1 or RNPC1a knockdown in RKO and HCT116 (Fig. 1D, cf. lanes 4,7 and 5,6 and 8,9, respectively) as well as in immortalized, but nontransformed MCF10A (Supplemental Fig. S1G) cells. Similarly, mutant p53 protein in SW480 cells was also significantly increased by RNPC1 knockdown (Fig. 1E). The latter observation is particularly significant since mutant p53 is often highly expressed and has a long half-life (Brosh and Rotter 2009). To confirm this, we generated MCF7 cell line number 23, which can inducibly express shRNA against total RNPC1. We found that upon inducible knockdown of total RNPC1, the level of p53 protein was markedly increased (Fig. 1F). To rule out the possibility that the increased p53 in these RNPC1 konckdown cells is due to an indirect mechanism, such as DNA damage, we showed that RNPC1 knockdown did not alter the formation of 53BP1 foci (Supplemental Fig. S2), an indicator of DNA damage response (Schultz et al. 2000). In addition, the level of p53 induced by DNA damage was further enhanced in RKO cells upon transient knockdown of total RNPC1 or RNPC1a (Fig. 1G, cf. lanes 4 and lanes 5,6) and in MCF7 cells upon inducible knockdown of total RNPC1 (Fig. 1H, cf. lanes 3,5 and 4,6, respectively).

To further confirm the above observations, we isolated primary mouse embryonic fibroblasts (MEFs) from two pairs of *RNPC1*$^{+/+}$ and *RNPC1*$^{-/-}$ littermate embryos. We found that the lack of RNPC1 markedly enhanced p53 expression under normal and DNA damage-induced conditions (Fig. 1I, cf. lanes 1,2,5,6 and 3,4,7,8, respectively). We would like to mention that in *RNPC1*$^{+/+}$ MEFs treated with doxorubicin, p53 was accumulated, leading to increased expression of RNPC1 (Fig. 1I, cf. lanes 1,2 and 5,6), suggesting that p53 induction of RNPC1 is conserved in MEFs.

RBPs are known to regulate gene expression via post-transcriptional mechanisms, including mRNA stability and protein translation (Zhang and Chen 2008). To explore the underlying mechanism by which RNPC1 regulates p53, we showed that both p53 transcript (Supplemental Fig. S3A,B) and p53 protein half-life (Supplemental Fig. S3C,D) were not altered by knockdown of total RNPC1 or RNPC1a. This suggests that RNPC1a likely regulates p53 expression through protein translation. To test this, the level of newly synthesized p53 protein was measured by ^{35}S-metabolic labeling in cells with or without RNPC1a expression. We showed that RNPC1a inhibited p53 translation in HCT116, RKO, and SW480 cells (Fig. 2A,B). Conversely, the level

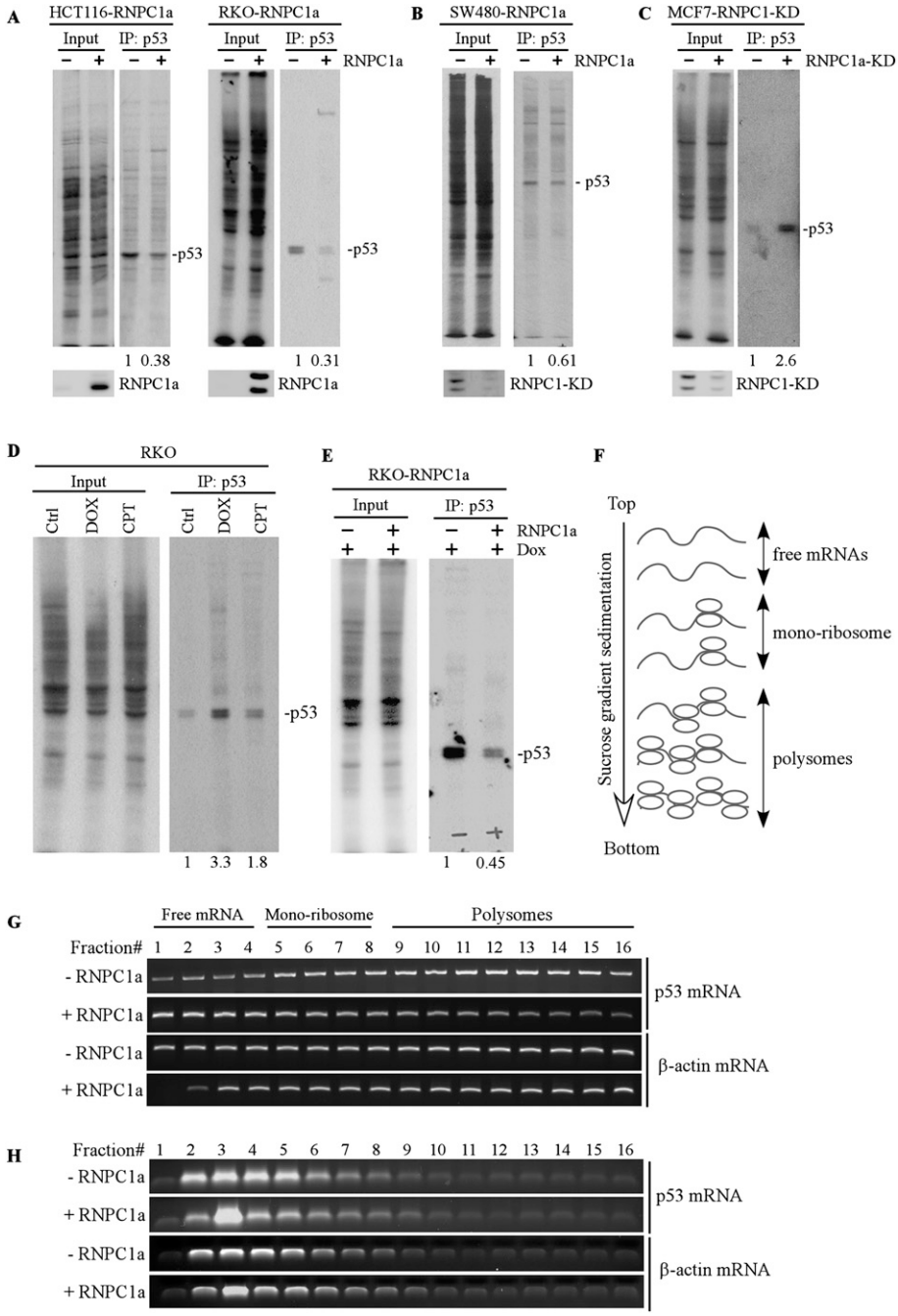

Figure 2. p53 protein translation is inhibited by ectopic expression of RNPC1a but increased by knockdown of RNPC1 under normal and stress conditions. (*A*) The level of newly synthesized p53 protein is decreased by RNPC1a. HCT116 and RKO cells were uninduced or induced to express RNPC1a for 12 h and then ^{35}S-labeled for 5 min, followed by immunoprecipitation with anti-p53. The immunocomplexes were resolved by SDS-PAGE, and p53 was visualized by autoradiography. The input was blotted with anti-RNPC1 to ensure RNPC1a expression, shown *below* each column. (*B*) The level of newly synthesized p53 protein is decreased by RNPC1a in SW480 cells. The experiment was performed as in *A*, except that SW480 cells were used and ^{35}S-labled for 20 min. (*C*) The level of newly synthesized p53 protein is increased by RNPC1 knockdown. The experiment was performed as in *A* with MCF7 cells uninduced or induced to knock down RNPC1a for 3 d and then ^{35}S-labeled for 5 min. (*D*) p53 translation is increased upon DNA damage. RKO cells were treated with or without doxorubicin or camptothecin for 24 h, and the level of newly synthesized p53 protein was measured by ^{35}S-labeling. (*E*) RNPC1a inhibits p53 translation under a stress condition. RKO cells were uninduced or induced to express RNPC1a for 12 h, followed by treatment with doxorubicin for 24 h, and the level of newly synthesized p53 protein was measured by ^{35}S-labeling. (*F*) Schematic presentation of free, monoriobsomal, and polysomal mRNA distribution after sucrose gradient sedimentation. (*G*) RNPC1a inhibits the association of heavy polysomes with p53 mRNA. Polysomes were separated by sucrose density gradient from HCT116 cells uninduced or induced to express RNPC1a for 12 h. p53 and actin transcripts were detected in each fraction by RT–PCR. (*H*) The experiment was performed as in *D*, except that cell extracts were treated with puromycin.

of newly synthesized p53 protein was increased upon RNPC1 knockdown in MCF7 cells (Fig. 2C). Next, we examined whether RNPC1a inhibits p53 translation under a stress condition. We found that p53 translation was increased in RKO cells treated with doxorubicin or camptothecin (Fig. 2D), consistent with a previous report (Takagi et al. 2005). Nevertheless, the level of newly synthesized p53 protein in RKO cells treated with doxorubicin was inhibited by ectopic expression of RNPC1a (Fig. 2E).

To confirm the translational repression of p53 by RNPC1, sucrose gradient sedimentation assay (Fig. 2F) was performed to examine the association of polysomes with p53 mRNA in HCT116 cells with or without RNPC1 expression. We showed that the profile of polysomes was not altered by RNPC1a expression (Supplemental Fig. S4A). However, the extent of heavy polysomes associated with p53, but not actin, transcripts was significantly decreased upon RNPC1a expression (Fig. 2G, fractions 9–16). Next, to verify whether this alteration is polysome-dependent, puromycin was used to dissociate polysomes from RNA transcripts (Blobel and Sabatini 1971). As expected, all of the polysomes were disrupted upon puromycin treatment regardless of RNPC1a expression (Supplemental Fig. S4B). As a result, the association of both p53 and actin mRNAs with polysomes was shifted from heavy to light fractions of the sucrose gradient regardless of RNPC1a expression (Fig. 2H). Together, these data suggest that RNPC1 represses p53 translation under normal and stress conditions.

RNPC1a prevents eIF4E from binding p53 mRNA, which is dependent on its N-terminal RRM for binding p53 mRNA and its C-terminal regulatory domain for physical interaction with eIF4E

In mammalian cells, translation initiation is the rate-limiting step and common target for translational control. Thus, we asked whether eIF4F, the translation initiation complex, is targeted by RNPC1a to repress p53 translation. To address this, we sought to inhibit the formation of the eIF4F complex through knockdown of eIF4E, the cap-binding protein and a key component of eIF4F. We found that upon eIF4E knockdown, the overall basal level of p53 translation was decreased as expected (Fig. 3A,B, lanes 3,4). Nevertheless, knockdown of eIF4E attenuated translational repression of p53 by RNPC1a (Fig. 3A,B, cf. lanes 1,3 and 2,4, respectively). This suggests that eIF4E is involved in RNPC1-mediated p53 repression. As RNPC1a had no effect on eIF4E expression (Fig. 3A,B), we determined whether RNPC1a regulates the binding of eIF4E to p53 mRNA by RNA immunoprecipitation followed by RT–PCR (RNA-ChIP) assay. We showed that upon induction of RNPC1a, p53 mRNA was detected in RNPC1a (anti-HA), but not control IgG, immunoprecipitates (Fig. 3C,D, cf. lanes 3,7 and 4,8, respectively). Based on the amount of input (5%) and that used for RNA-ChIP (95%) versus the intensity of RNA transcripts immunoprecipitated with RNPC1a, we estimated that the relative level of p53 transcripts associated with RNPC1a was 8% in RKO cells and 5% in HCT116 cells (Fig. 3C,D). Interestingly, upon RNPC1a expression, the relative level of p53 transcripts associated with eIF4E was markedly decreased from 12% to 3% in RKO cells and from 11% to 3% in HCT116 cells (Fig. 3C,D, cf. lanes 5 and 6). As a control, RNPC1a neither bound, nor inhibited the binding of eIF4E, to actin mRNA (Fig. 3C,D). Moreover, we showed that upon treatment with RNase A, these interactions were eliminated (Fig. 3E). Furthermore, we showed that RNPC1b was unable to prevent eIF4E from binding p53 mRNA (Fig. 3F, cf. lanes 7 and 8), although it was able to interact with p53 mRNA (Fig. 3F, lane 6). This is consistent with the above observations that RNPC1b was unable to suppress p53 translation (Fig. 1; Supplemental Fig. S1). In contrast, knockdown of eIF4E had no effect on the binding activity of RNPC1a to p53 mRNA (Fig. 3G,H, lane 6). Nevertheless, siRNA against eIF4E, but not scramble siRNA, markedly inhibited the extent of eIF4E associated with p53 mRNA (Fig. 3G,H, cf. lane 7). These data suggest that impaired binding of eIF4E to p53 mRNA is critical for RNPC1a to inhibit p53 expression.

Like other RRM-containing RBPs, the RNA-binding domain in RNPC1 is composed of two RNA-binding submotifs: RNP1 and RNP2 (Shu et al. 2006). Thus, we determined whether the RNA-binding domain in RNPC1 is required RNPC1a-mediated p53 repression by using ΔRNP1 and ΔRNP2 mutants (Fig. 3I) as previously described (Zhang et al. 2010). We showed that these mutants were deficient in binding to p53 mRNA (Fig. 3J,K, cf. lanes 7 and 8) and incapable of preventing eIF4E from binding p53 mRNA (Fig. 3J,K, cf. lanes 5 and 6). Consistent with this, p53 expression was not inhibited by these mutants (Fig. 3L, cf. lanes 1 and 2–4, respectively). Nevertheless, like wild-type RNPC1 (Fig. 3A,B. cf. lanes 1 and 2), ΔRNP1 and ΔRNP2 mutants had no effect on eIF4E expression (Supplemental Fig. S5).

To further explore how RNPC1a prevents eIF4E from binding p53 mRNA, we examined whether RNPC1a inhibits the binding of eIF4E to the cap structure. We found that RNPC1 had no effect (Supplemental Fig. S6). Next, we determined whether RNPC1a physically interacts with eIF4E. To eliminate the possibility that the interaction between RNPC1 and eIF4E is mediated by their binding to a mRNA (e.g., p53 mRNA), cell extracts, recombinant proteins, and in vitro translated proteins were treated with RNaseA prior to immunoprecipitation and GST pull-down assay. We found that eIF4E was detected in RNPC1-containing, but not IgG-containing, immunoprecipitates (Fig. 4A, cf. lanes 3,5 and 4,6, respectively). Conversely, RNPC1a was detected in eIF4E-containing, but not IgG-containing, immunoprecipitates (Fig. 4B, cf. lanes 3,5 and 4,6, respectively). Similarly, in vitro translated RNPC1a and eIF4E were found to form a complex by immunoprecipitation assay (Fig. 4C), whereas recombinant RNPC1 and eIF4E were found to form a complex by GST pull-down assay (Fig. D). Interestingly, ΔRNP1 and ΔRNP2 mutants were still capable

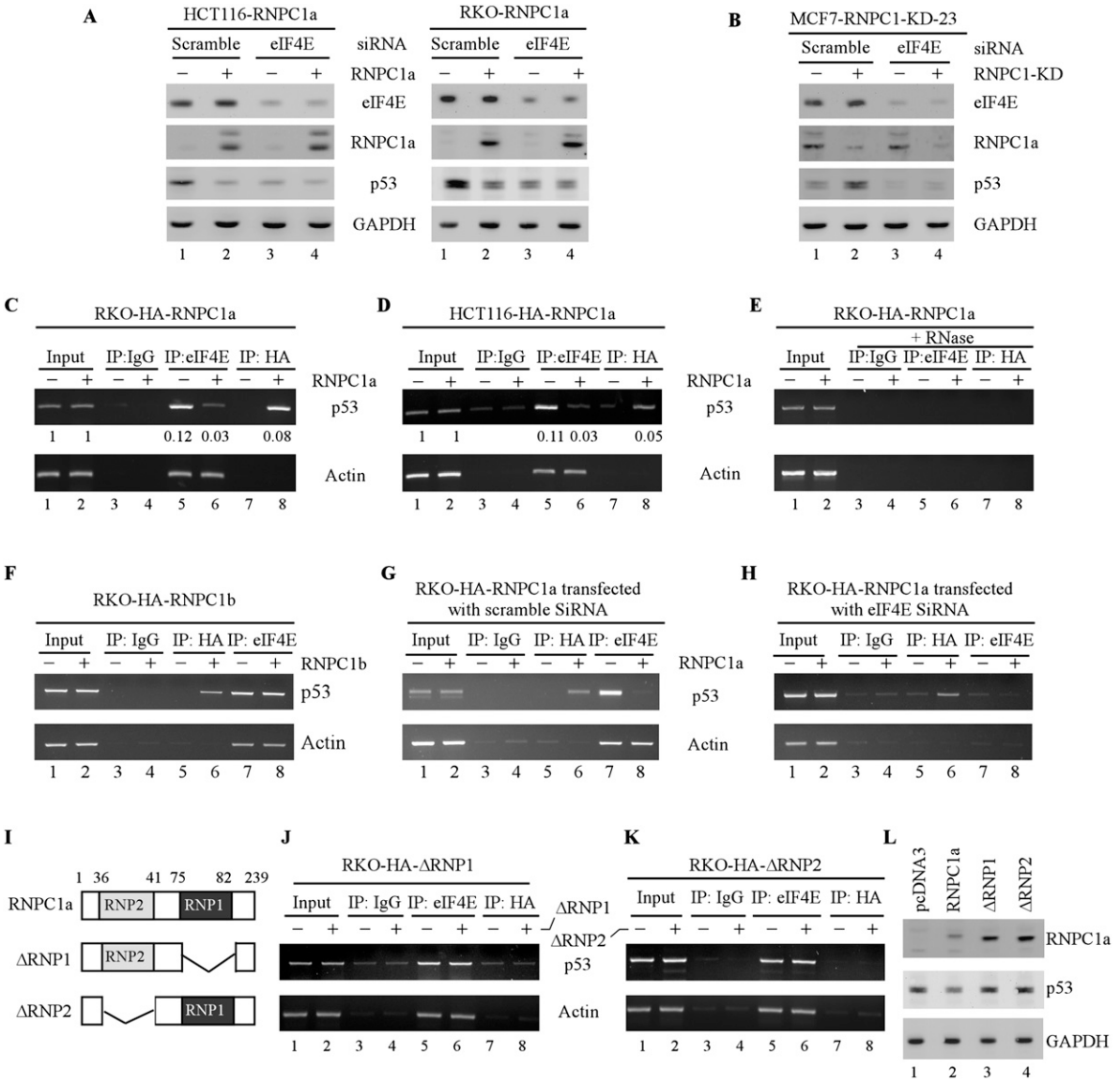

Figure 3. RNPC1a prevents eIF4E from binding p53 mRNA, which is dependent on its N-terminal RRM for binding p53 mRNA. (*A*) The level of eIF4E, RNPC1, p53, and actin was measured in HCT116 and RKO cells transfected with scrambled siRNA or siRNA against eIF4E for 3 d, followed with or without RNPC1a induction for 12 h. (*B*) The experiment was performed as in *A*, except that MCF7 cells were transfected with scrambled siRNA or siRNA against eIF4E along with or without RNPC1 knockdown for 3 d. (*C*,*D*) RNPC1a prevents eIF4E from binding p53 mRNA. RKO (*C*) and HCT116 (*D*) cells were uninduced or induced to express RNPC1a for 18 h, followed by immunoprecipitation with antibodies against HA-tagged RNPC1a or eIF4E, or with mouse IgG. Total RNAs were purified from immunocomplexes and subjected to RT–PCR to measure the level of p53 and actin mRNAs associated with RNPC1a or eIF4E. (*E*) The association of p53 and actin transcripts with RNPC1a or eIF4E is RNA-dependent. The same experiment was performed as described in *C*, except that cell lysates were treated with RNase prior to immunoprecipitation. (*F*) RNPC1b is unable to prevent eIF4E from binding p53 mRNA. The experiment was performed as described in *C* with RKO cells uninduced or induced to express RNPC1b. (*G*,*H*) RNPC1a is able to bind to p53 mRNA regardless of eIF4E knockdown. The experiment was performed as described in *C*, except that RKO cells were transfected with scrambled siRNA (*G*) or siRNA against eIF4E (*H*) for 3 d, followed with or without RNPC1a induction for 12 h. (*I*) Schematic illustration of RNP1 and RNP2 deletion mutants. (*J*,*K*) The RNA-binding domain is required for RNPC1 to prevent eIF4E from binding p53 mRNA. The experiment was performed as in *C* with RKO cells uninduced or induced to express ΔRNP1 (*J*) or ΔRNP2 (*K*). (*L*) ΔRNP1 and ΔRNP2 are unable to inhibit p53 expression. RNPC1, p53, and GAPDH were examined in RKO cells transfected with empty pcDNA3 or a vector expressing RNPC1a, ΔRNP1, or ΔRNP2 for 24 h.

of interacting with eIF4E (Fig. 4E,F), although both mutants were inert in binding p53 mRNA and in suppressing p53 translation (Fig. 3J–L). In contrast, RNPC1b was unable to interact with eIF4E (Fig. 4G). As a control, eIF4E was unable to interact with an unrelated HA-tagged ferredoxin reductase (FR) (Fig. 4H,I). Together, these data suggest that RNPC1a physically interacts with eIF4E via its C-terminal region.

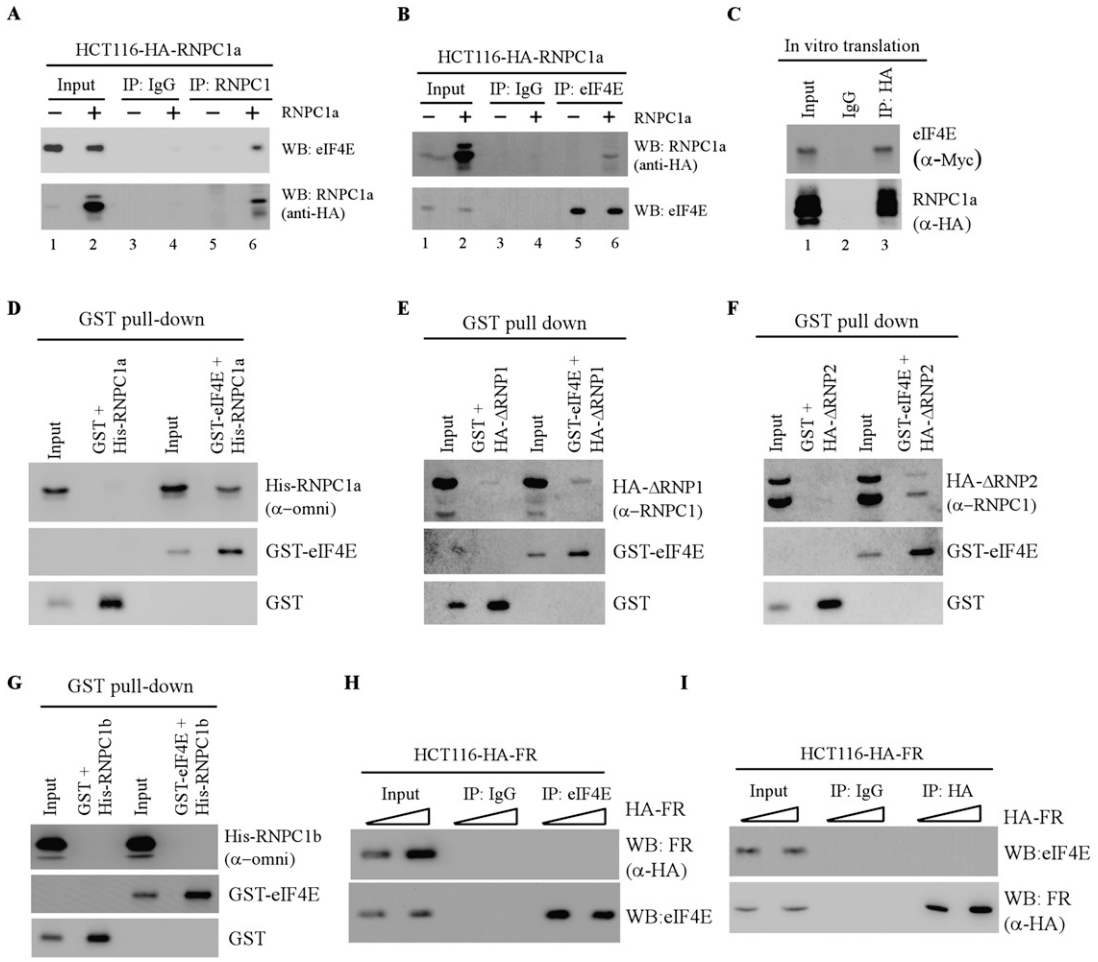

Figure 4. RNPC1a interacts with eIF4E. (*A*,*B*) Cell lysates were prepared from HCT116 cells uninduced or induced to express RNPC1a for 18 h, treated with RNase A, and then immunoprecipitated with anti-HA (*A*) or anti-eIF4E (*B*) along with a control IgG. The immunocomplexes were examined with anti-eIF4E (*A*, *top* panel) or anti-HA (*B*, *top* panel). The blots were then stripped and reblotted with anti-HA (*A*, *bottom* panel) or anti-eIF4E (*B*, *bottom* panel). (*C*) HA-tagged RNPC1a physically interacts with Myc-tagged eIF4E proteins in vitro. HA-tagged RNPC1a or Myc-tagged eIF4E expression vector (1 μg) was used for coupled in vitro transcription/translation reactions as described in the Materials and Methods. Equal volumes of in vitro translated RNPC1 and eIF4E protein lysates were treated separately with RNaseA and then mixed, followed by immunoprecipitation with 1 μg of HA antibody or control IgG. The immunocomplexes were examined by Western blot analysis with anti-myc (*top* panel), and the blots were then stripped and reblotted with anti-HA (*bottom* panel). (*D*) GST pull-down assays were performed with recombinant GST-eIF4E and histidine-tagged RNPC1a. Equal amounts of GST or GST-fused eIF4E were incubated with his-tagged RNPC1a along with glutathione sepharose for 3 h. Complexes were then washed, followed by Western blot analysis using antibody against histidines (anti-omini) or GST. (*E*,*F*) GST pull-down assays were performed with recombinant GST-eIF4E and HA-tagged ΔRNP1 (*E*) or ΔRNP2 (*F*). The experiments were performed as in *D*, except that recombinant HA-tagged ΔRNP1 or ΔRNP2 protein was used. (*G*) GST pull-down assays were performed with recombinant GST-eIF4E and His-tagged RNPC1b. The experiments were performed as in *D*, except that recombinant His-tagged RNPC1b protein was used. (*H*,*I*) eIF4E does not interact with HA-tagged FR. The experiments were performed as in *A* and *B*, except that HCT116 cells were uninduced or induced to express HA-tagged FR.

The p53 5′ UTR or a poly(U) element in the p53 3′ UTR is required for RNPC1 to inhibit p53 expression

To identify RNPC1-binding sites in p53 mRNA, RNA electrophoretic mobility shift assay (REMSA) was performed using radiolabeled RNA probes containing the entire p53 5′ UTR or 3′ UTR (Fig. 5A). We showed that recombinant GST fusion protein containing HA-tagged RNPC1a, but not GST protein, formed a complex with the p53 5′ UTR (Fig. 5B, cf. lanes 1 and 2) or 3′ UTR probe (Fig. 5C, cf. lanes 1 and 2). Moreover, the complex formation was inhibited by an excess amount of cold p21 probe (Fig. 5B, lane 3). The p21 3′ UTR is known to contain a RNPC1-binding site (Shu et al. 2006). Similarly, RNPC1b was able to bind to both the p53 5′ UTR (Fig. 5B, lanes 4,5) and 3′ UTR (data not shown), albeit to a lesser extent. Next, to further delineate the binding sites of RNPC1 in the p53 3′ UTR, two radiolabeled RNA probes (probes A and B), spanning the entire p53 3′ UTR, were generated. We showed that probe B, but not probe A,

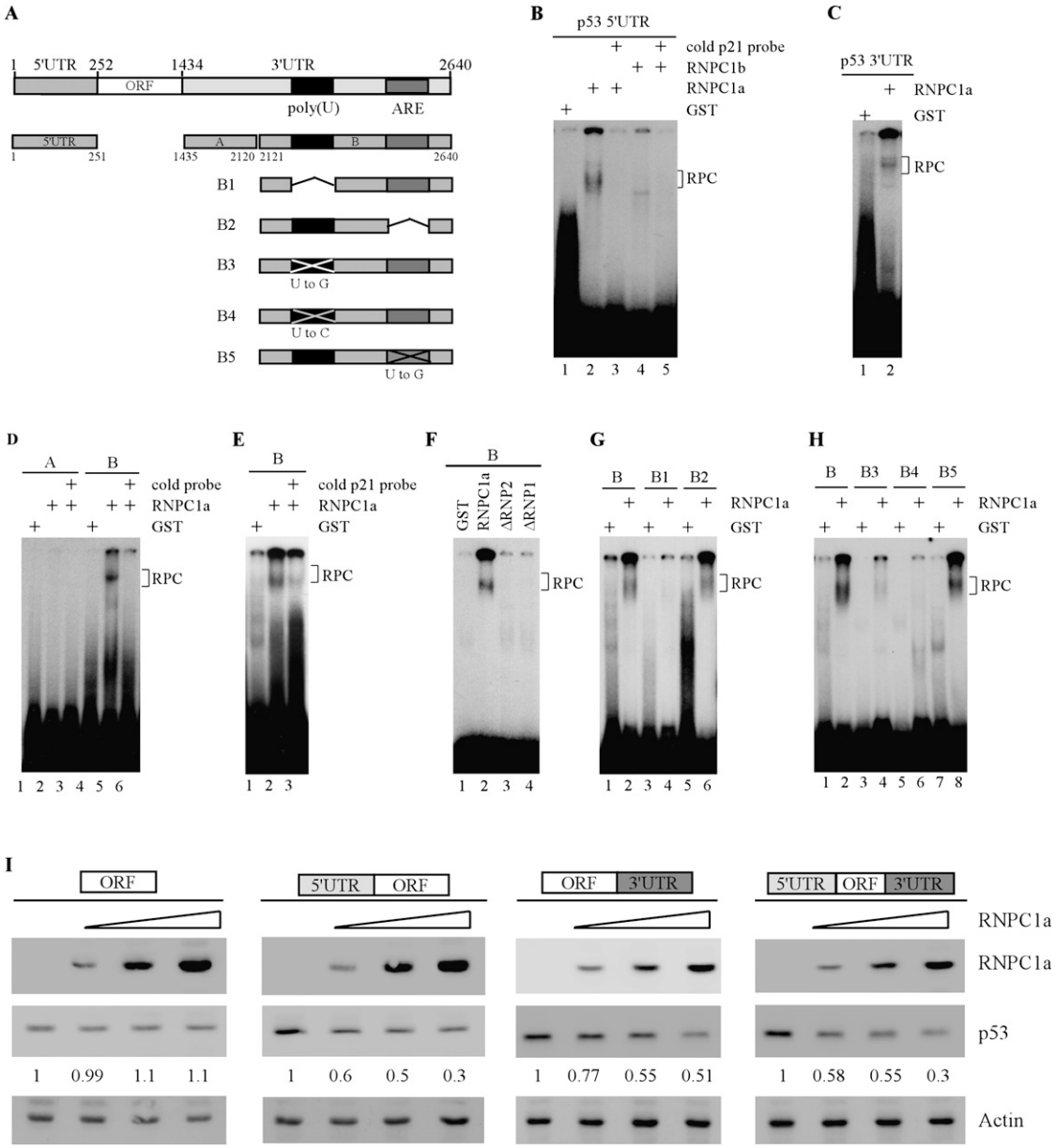

Figure 5. The p53 5′ UTR or a poly(U) element in the p53 3′ UTR is required for RNPC1 to inhibit p53 expression. (*A*) Schematic presentation of p53 mRNA and the location of probes. Poly(U) and AU-rich elements are indicated. (*B*) RNPC1 directly binds to the p53 5′ UTR. ^{32}P-labeled RNA probes were mixed with recombinant GST or HA-RNPC1a-GST or HA-RNPC1b-GST fusion protein. For competition assay, cold p21 probe was added to the reaction run in lanes *3* and *5*, respectively. The bracket indicates RNA–protein complexes (RPC). (*C*) RNPC1 directly binds to the p53 3′ UTR. REMSA assay was performed as in *B* using the p53 3′ UTR as a probe. (*D*,*E*) RNPC1a directly binds to probe B but not probe A. REMSA assay was performed as in *B* with probes A and B. (*F*) The RNA-binding domain in RNPC1 is required for RNPC1a to bind p53 mRNA. REMSA assay was performed as in *B* by incubating ^{32}P-labeled probe B with GST, GST-HA-RNPC1a, GST-HA-ΔRNP1, or GST-HA-ΔRNP2. (*G*,*H*) The poly(U) element in the p53 3′ UTR is required for RNPC1a to bind p53 mRNA. REMSA assay was performed as in *B* with probes B and B1–B5. (*I*) The presence of the p53 5′ UTR or 3′ UTR is sufficient for RNPC1a to inhibit p53 expression. Various amounts of RNPC1a expression vector were transfected into H1299 cells along with a fixed amount of p53 expression vector that contains the coding region (ORF) alone or in combination with the 5′ UTR, the 3′ UTR, or both. The levels of p53, RNPC1a, and actin were analyzed by Western blot analysis. The fold change of p53 is shown *below* each lane.

formed a complex with RNPC1a (Fig. 5D,E) but not ΔRNP1 and ΔRNP2 mutants (Fig. 5F). In addition, the binding of RNPC1a to probe B was blocked by an excess amount of cold p21 probe (Fig. 5E, lane 3) or cold probe B (Fig. 5D, lane 6). To further map the binding sites of RNPC1a in fragment B, we showed that RNPC1a was able to bind to probe B2 (Fig. 5G, lane 6), which lacks an AU-rich element, but not probe B1 (Fig. 5G, lane 4), which

lacks a poly(U) element. In line with this, point mutations from U to G or C in the poly(U) element (nucleotides 2188–2205) significantly disrupted the binding with RNPC1a (Fig. 5H, cf. lanes 2 and 4,6, respectively). In contrast, point mutations from U to G in the AU-rich element (nucleotides 2579–2583 and nucleotides 2598–2601) had no effect on RNPC1a binding (Fig. 5H, cf. lane 8).

To explore the role of the p53 5′ and 3′ UTRs in RNPC1a-mediated repression of p53 translation, we generated various expression vectors that contain the p53 coding region (ORF) alone or in combination with the 5′ UTR, the 3′ UTR, or both. We showed that ectopic expression of p53 was markedly inhibited by RNPC1 in a dose-dependent manner as long as the p53 expression vector carries the 5′ UTR, the 3′ UTR, or both (Fig. 5I, three right panels). In contrast, RNPC1 had no effect on ectopic p53 expression from an expression vector that only contains the p53 coding region (Fig. 5I, left panel).

Loss of RNPC1 induces p53 expression, leading to p53-dependent senescence in primary MEFs

To explore the biological effect of RNPC1a on p53, we performed colony formation assay and found that the ability of MCF7 cells to form colonies was significantly inhibited by RNPC1a knockdown (Supplemental Fig. S7A,B). We also showed that this inhibited colony formation was dependent on p53, since RNPC1a knockdown had no effect on colony formation in p53 knockdown MCF7 (Supplemental Fig. S7C,D) or in $p53^{-/-}$ HCT116 (Supplemental Fig. S7E,F) cells. Next, to explore the physiological relevance of RNPC1a-mediated repression of p53 translation, we examined whether RNPC1 is involved in p53-mediated premature cellular senescence with primary MEFs generated from $RNPC1^{+/+}$, $RNPC1^{+/-}$, and $RNPC1^{-/-}$ littermate embryos. We showed that p53 was highly induced by RNPC1 deficiency, especially upon treatment with doxorubicin (Fig. 6A, cf. lanes 4 and 5,6), consistent with the observation in Figure 1H. We also showed that the p53 target gene PAI-1 and p130, both of which are associated with p53-induced senescence (Kortlever et al. 2006; Helmbold et al. 2009), were induced by RNPC1 deficiency in the presence and absence of treatment with doxorubicin (Fig. 6A, cf. lanes 1,4 and 2,3 and 5,6, respectively). In addition, we showed that the number of cells stained positive with senescence-associated β-galactosidase (SA-β-gal) was markedly increased by lack of RNPC1 regardless of DNA damage (Fig. 6B). Quantitative analysis indicated that SA-β-gal-positive cells were increased by threefold to eightfold upon loss of RNPC1 (47.5% in $RNPC1^{-/-}$ MEFs vs. 5.5% in $RNPC1^{+/+}$ MEFs and 6.5% in $RNPC1^{+/-}$ MEFs in the absence of doxorubicin; 64.5% in $RNPC1^{-/-}$ MEFs vs. 18% in $RNPC1^{+/+}$ MEFs and 19.5% in $RNPC1^{+/-}$ MEFs in the presence of doxorubicin) (Fig. 6C). Furthermore, to determine whether the increased senescence in $RNPC1^{-/-}$ MEFs is p53-dependent, we generated $RNPC1^{-/-}$; $p53^{-/-}$ MEFs by crossing $RNPC1^{+/-}$; $p53^{+/-}$ mice. We found that p53 deficiency abrogated the increased level of PAI-1 and p130 expression induced by lack of RNPC1 in the presence and absence of treatment with doxorubicin (Fig. 6D, cf. lanes 2,5 and 3,6, respectively). Similarly, p53 deficiency abrogated the increased number of SA-β-gal-positive cells induced by lack of RNPC1 regardless of treatment with doxorubicin (Fig. 6E,F). In fact, $RNPC1^{-/-}$; $p53^{-/-}$ MEFs exhibited an immortal phenotype in the absence of treatment with doxorubicin (Fig. 6E, bottom left panel). These data suggest that p53 plays a critical role in RNPC1-induced senescence.

We would like to mention that the level of p21 protein was not significantly induced, although the level of p53 protein was highly increased in $RNPC1^{-/-}$ MEFs (Fig. 6A,D, p53 and p21 panels). Previously, we showed that RNPC1 is required for p21 mRNA stability (Shu et al. 2006). These data led us to speculate that p21 mRNA in $RNPC1^{-/-}$ MEFs may be less stable, although p21 transcription is increased by p53. To test this, precursor and mature p21 mRNAs in $RNPC1^{+/+}$, $RNPC1^{-/-}$, and $p53^{-/-}$; $RNPC1^{-/-}$ MEFs treated with or without doxorubicin were measured separately by specific primers (Supplemental Fig. S8A). We showed that p21 transcription was mostly p53-dependent, as the level of precursor and mature p21 mRNAs was much lower in $RNPC1^{-/-}$; $p53^{-/-}$ MEFs than that in $RNPC1^{+/+}$ and $RNPC1^{-/-}$ MEFs (Supplemental Fig. S8B,C). We also showed that the level of precursor p21 mRNA was about twofold higher in $RNPC1^{-/-}$ MEFs than that in $RNPC1^{+/+}$ MEFs (Supplemental Fig. S8B) regardless of doxorubicin treatment, suggesting that p21 transcription is increased upon p53 accumulation induced by lack of RNPC1. Nevertheless, the level of mature p21 mRNA was similar in both $RNPC1^{+/+}$ and $RNPC1^{-/-}$ MEFs (Supplemental Fig. S8C). Furthermore, the half-life of p21 mRNA was decreased from 3.8 h in $RNPC1^{+/+}$ MEFs to 2.8 h in $RNPC1^{-/-}$ MEFs (Supplemental Fig. S8D). This is consistent with our previous report (Shu et al. 2006) and suggests that RNPC1 is required for maintaining the expression level of p21 induced by p53.

RNPC1 is frequently overexpressed along with decreased expression of p53 in dog lymphoma

Lymphoma occurs frequently in dogs, representing ~6% of all dog malignant cancers (MacVean et al. 1978; Galli et al. 1990). Unlike lymphoma in mice experimentally induced by genetic manipulations, lymphoma in dogs occurs spontaneously. Most importantly, dog lymphoma is clinically and morphologically similar to its human counterpart and has been used as an alternative clinical model for cancer treatment and prevention (Greenlee et al. 1990; Paoloni and Khanna 2008). Thus, to investigate the biological and potential clinical importance of our findings, we sought to examine whether RNPC1 is deregulated in dog lymphoma. First, we showed that dog RNPC1a was up-regulated by DNA damage in MDCK and D-17 cells, which carry wild-type p53, but not in Cf2Th cells, which carry a mutant p53 (Supplemental Fig. S9A). In addition, we found that upon knockdown of total RNPC1 or RNPC1a in D-17, Cf2Th, and dog lymphoma cells, the level of p53 protein was increased (Supplemental

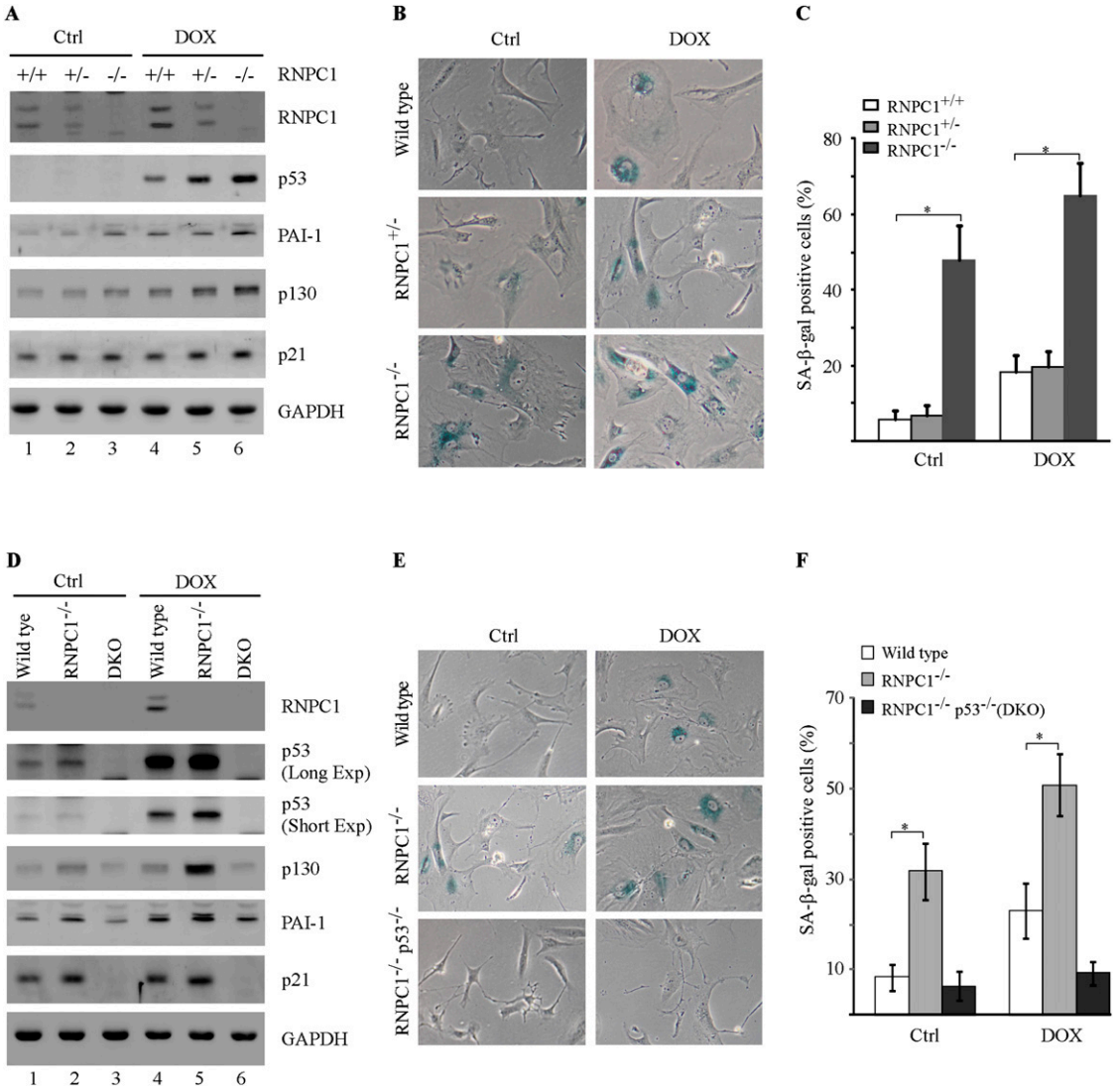

Figure 6. Loss of *RNPC1* triggers p53-dependent senescence in primary MEFs. (*A*) Primary *RNPC1*$^{+/+}$, *RNPC1*$^{+/-}$, and *RNPC1*$^{-/-}$ MEFs at passage 5 were treated with or without doxorubicin for 12 h, and the levels of RNPC1, p53, PAI-1, p130, p21, and GAPDH were analyzed by Western blot analysis. (*B*) Primary *RNPC1*$^{+/+}$, *RNPC1*$^{+/-}$, and *RNPC1*$^{-/-}$ MEFs at passage 5 were treated with or without 50 ng/mL doxorubicin for 2 d, followed by SA-β-gal staining assay as described in the Materials and Methods. (*C*) Quantification of the percentage of SA-β-gal-positive cells as shown in *B*. (*D*) Primary wild-type, *RNPC1*$^{-/-}$, and *RNPC1*$^{-/-}$; *p53*$^{-/-}$ MEFs at passage 5 were treated with or without doxorubicin for 12 h, and the levels of RNPC1, p53, PAI-1, p130, p21, and GAPDH were analyzed by Western blot analysis. (*E*) Primary wild-type, *RNPC1*$^{-/-}$, and *RNPC1*$^{-/-}$ *p53*$^{-/-}$ MEFs at passage 5 were treated with or without 50 ng/mL doxorubicin for 2 d, followed by SA-β-gal staining assay. (*F*) Quantification of the percentage of SA-β-gal-positive cells as shown in *E*.

Fig. S9B,C). Together, these data suggest that the RNPC1-p53 regulatory loop is conserved in dogs.

Next, we analyzed the level of RNPC1a mRNA and protein in six normal dog lymph nodes and 28 dog lymphomas. The clinical information for each dog patient is shown in Supplemental Table S1. We showed that among the six normal lymph nodes, number 3 had the highest, whereas number 6 had a lower, expression level of RNPC1 protein (Supplemental S9D) and mRNA (Supplemental S9E). Therefore, number 3 and number 6 normal lymph nodes were used as a control for lymphomas. Strikingly, we found that the level of RNPC1 protein was significantly increased for 1.8-fold or more in 17 out of 28 lymphomas (~60.7%) as compared with normal tissues (Fig. 6A,C,E). In addition, we found that *RNPC1* transcript was overexpressed in 12 out of 17 (~70.5%) lymphomas with overexpressed RNPC1 protein (Fig. 6B,D,F). Furthermore, we found that among 17 lymphomas with overexpressed RNPC1 protein, 15 lymphomas showed a reduced level of p53 protein (Fig. 6A,C,E). We would like to mention that the entire coding region of the p53 gene in all 15 lymphomas was sequenced and found to be wild-type (Supplemental Table S1). Together, these data suggest that *RNPC1* is frequently overexpressed in dog lymphomas and may play a role in lymphomagenesis by inactivating p53.

Discussion

How p53 activity is regulated is one of the main focuses in cancer research, as the majority of human cancers have a defective or faulty p53 pathway. In the current study, we identified RNPC1 as a critical regulator of p53 translation under both normal and stress conditions. In addition, we showed that loss of RNPC1 in primary MEFs leads to increased expression of p53, resulting in premature cellular senescence. These findings suggest that under normal conditions, RNPC1 expression is maintained by p53, whereas in response to stimuli, p53 is activated and then induces RNPC1, which in turn regulates p53 expression. Thus, the translational regulation of p53 by RNPC1 represents a novel feedback loop for the p53 pathway (Fig. 7G). However, when RNPC1 expression is

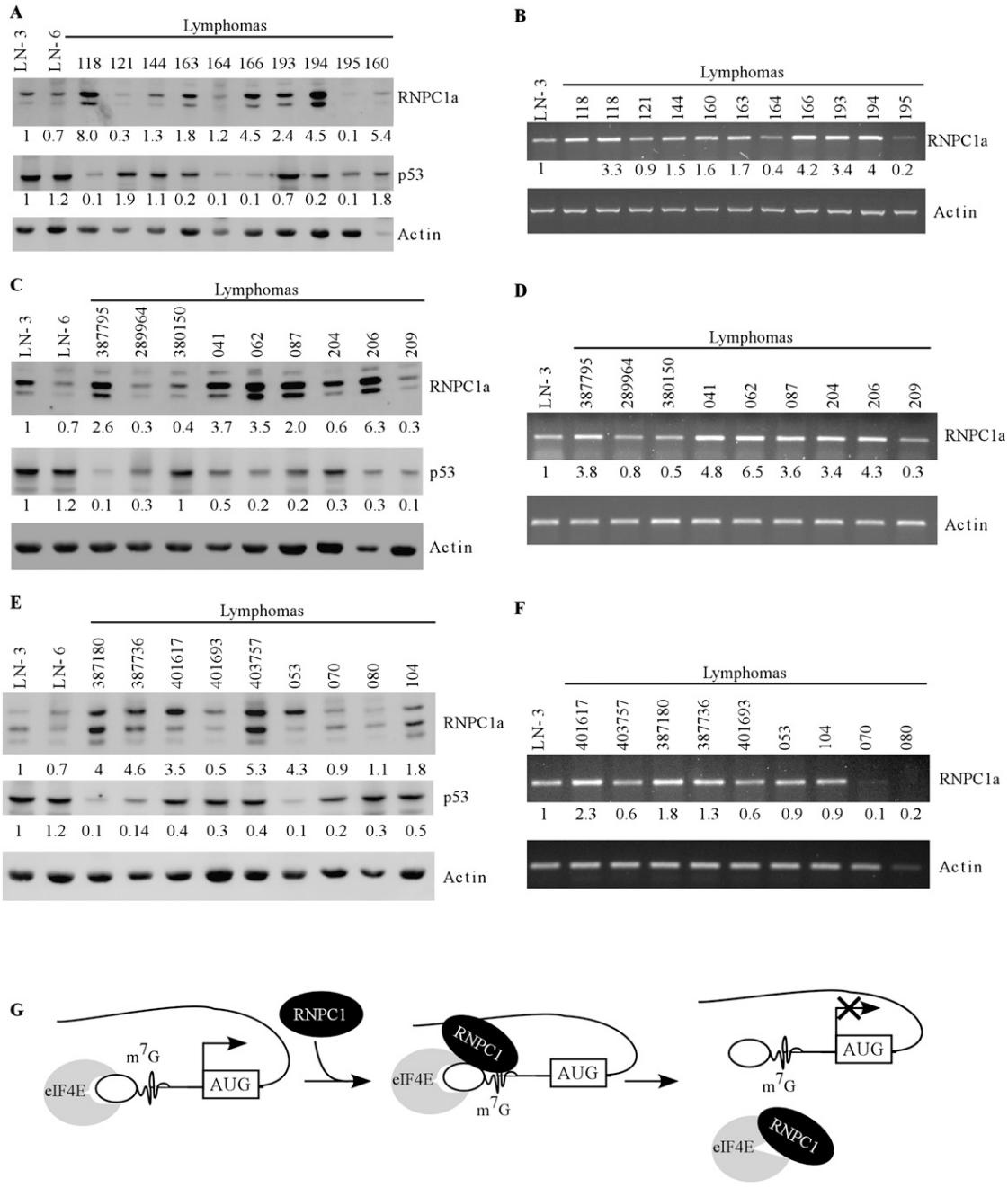

Figure 7. RNPC1 mRNA and protein are overexpressed in dog lymphomas. (*A*,*C*,*E*) The levels of RNPC1, p53, and actin in two normal dog lymph nodes and 28 dog lymphomas were measured by Western blot analysis. The levels of p53 and RNPC1a proteins in LN-3 (normal lymph node) were arbitrarily set at 1.0, and the fold change is shown *below* each lane. (*B*,*D*,*F*) The levels of RNPC1 and actin transcripts in normal and lymphoma tissues were measured by RT–PCR. The level of RNPC1a transcript in LN-3 (normal lymph node) was arbitrarily set at 1.0, and the fold change is shown *below* each lane. (*G*) A model for RNPC1 to regulate p53 translation.

deregulated and consequently overexpressed, as observed in various cancers (Korn et al. 1999; Tanner et al. 2000; Zheng et al. 2001; Bar-Shira et al. 2002; Hermsen et al. 2002; Krackhardt et al. 2002; Knosel et al. 2003; Ginestier et al. 2006; Letessier et al. 2006; Carvalho et al. 2009) and dog lymphoma (this study), p53 translation would be suppressed. As a result, the pressure for genetic alteration of the p53 gene necessary for tumor development and progression would be alleviated. This may be responsible for some tumors, such as lymphoma, wherein p53 mutation is rare but p53 activity is deficient.

Potential mechanism by which RNPC1 inhibits p53 translation

We found that overexpression of RNPC1a inhibited, whereas knockdown of RNPC1a increased, p53 translation under normal and stress conditions. Consistent with this, the association of p53 mRNA with heavy polysomes was inhibited by RNPC1a. To uncover the mechanism, we showed that RNPC1a prevented eIF4E from binding p53 mRNA. Of note, the ability of RNPC1a to repress p53 translation requires the binding of RNPC1 to p53 mRNA, since ΔRNP1 and ΔRNP2 mutants, which were still capable of interacting with eIF4E but unable to bind to p53 mRNA, were unable to prevent eIF4E from binding p53 mRNA. In addition, the ability of RNPC1a to repress p53 translation requires the interaction of RNPC1 with eIF4E, since RNPC1b, which contains an intact RNA-binding domain and can bind to p53 mRNA, was incapable of interacting with eIF4E and unable to prevent eIF4E from binding p53 mRNA. Based on these findings, we propose a model for RNPC1 to regulate p53 translation (Fig. 7G). We speculate that upon binding p53 mRNA, RNPC1a interacts with eIF4E and prevents eIF4E from binding p53 mRNA. As a result, the process of forming a translation-competent initiation complex is blocked, leading to decreased p53 translation.

Despite the above observations, many mechanistic details remain to be elucidated. For instance, it remains unclear whether the interaction between eIF4E and RNPC1a modulates the association between eIF4E and eIF4G, since 4E-binding proteins (4E-BPs) act by competing with eIF4E for binding to eIF4G, thereby blocking eIF4F assembly. However, the mechanism by which RNPC1 represses p53 translation is different from that by 4E-BPs, the latter of which have a global effect on protein translation (Haghighat et al. 1995). Instead, RNPC1 may have an effect on protein translation similar to that by maskin- or cup-type 4E-BPs, since these proteins only affect a specific set of mRNAs for translation through interaction with a RNA element in the target mRNAs and/or through interaction with other RBPs (Stebbins-Boaz et al. 1999; Wilhelm et al. 2003). Interestingly, several RBPs, including HuR, GLD-1, RPL26, and nucleolin (Mazan-Mamczarz et al. 2003; Schumacher et al. 2005; Takagi et al. 2005), which are not known to be directly regulated by p53, have been reported to translationally regulate p53 expression. In particular, a recent study by Chen and Kastan (2010) showed that p53 mRNA is capable of forming a region of dsRNA containing complementary sequences of the 5′ UTR (nucleotides −34 to −54) and 3′ UTR (nucleotides +335 to +352). Importantly, the dsRNA region is bound by RPL26 and is critical for translational regulation of p53 (Chen and Kastan 2010). Interestingly, RNPC1 is found to interact with both the p53 5′ UTR and a poly(U) element in the p53 3′ UTR. Therefore, future studies are needed to identify other RNPC1-interacting proteins. Moreover, whether RNPC1 cooperates with other RBPs, especially RPL26, to regulate p53 translation is warranted.

We would like to mention that despite p53–RNPC1 feedback control, p53 accumulates in cells by DNA damage, hypoxia, and other types of stress. This suggests that other mechanisms are involved that allow p53 to escape from the translational repression by RNPC1. One potential explanation is that under a stress condition, eIF4E can be inactivated by p53 via multiple mechanisms. For example, p53 has been found to transcriptionally repress eIF4E expression, which led to a decreased expression of eIF4E in response to ionizing radiation (IR) (Zhu et al. 2005). Consistent with this, we showed that upon doxorubicin or campotothecin treatment, the level of eIF4E was decreased concomitantly with an increased expression of p53 and RNPC1a (Supplemental Fig. S10). As a result, RNPC1 repression of p53 translation via eIF4E would be alleviated. In addition, p53 activation can result in rapid dephosphorylation of the eIF4E-binding protein 4E-BP1 (Horton et al. 2002; Constantinou and Clemens 2007), thus sequestering 4E-BP from inhibiting the cap-dependent protein translation. Similarly, activated p53 may also regulate RNPC1 phosphorylation and temporally escape RNPC1-mediated translational repression. Nevertheless, these multiple feedback regulations are worth investigation in the future.

The role of RNPC1 in tumorigenesis

Although RNPC1 is found to be overexpressed in various cancers (Korn et al. 1999; Tanner et al. 2000; Zheng et al. 2001; Bar-Shira et al. 2002; Hermsen et al. 2002; Krackhardt et al. 2002; Knosel et al. 2003; Ginestier et al. 2006; Letessier et al. 2006; Carvalho et al. 2009), its oncogenic activity has not been defined. Our molecular studies indicate that RNPC1 inhibits p53 translation and, consequently, p53 protein expression. To explore the biological consequences, we found that knockdown of RNPC1a inhibited cell proliferation in a p53-dependent manner. Consistent with this, loss of RNPC1 in MEFs leads to increased expression of p53 and, subsequently, enhanced premature cellular senescence under normal and DNA damage-induced conditions. In addition, the enhanced cellular senescence in *RNPC1*$^{-/-}$ MEFs can be fully rescued by loss of p53, indicating that the effect of RNPC1 on cellular senescence is p53-dependent. Furthermore, we found that RNPC1 is frequently overexpressed in dog lymphomas, most of which have a corresponding decreased level of wild-type p53 expression. These data suggest that RNPC1 has an oncogenic potential and deregulation of RNPC1 in dog lymphoma may contribute to p53 inactivation, consistent with the fact that p53 is

infrequently mutated in lymphoma (Matsushima et al. 1994; Newcomb 1995; Oka et al. 1998).

Spontaneous lymphoma is a frequently occurring disease in canines. Dog lymphoma is remarkably similar to its human counterpart, including histopathology, anatomic sites of the disease, and biological and therapeutic behaviors. Previously, we found that the p53 family pathway in dog cells is similar to that in human cells (Zhang et al. 2009). Therefore, the dog may act as an environmental sentinel and serve as a model to address cancer etiology and treatment in humans. Indeed, our finding that overexpression of RNPC1 along with concomitant decreased expression of p53 frequently occurs in dog lymphoma is consistent with gene expression profiles from the Oncomine Cancer Microarray Database, which showed that RNPC1 is highly expressed in human lymphoma. Thus, further studies are warranted to analyze the expression pattern of RNPC1 in human tumor tissues along with the expression pattern and genetic status of p53. Together, our study indicated that RNPC1 may represent an independent biomarker and a potential therapeutic target for the diagnosis and treatment of lymphoma.

Materials and methods

Reagents

The list of supplies is provided in the Supplemental Material.

Cell culture

RKO, MCF7, HCT116, H1299, and SW480 cells were cultured as described (Zhang and Chen 2007). Primary MEFs were cultured in DMEM supplemented with 10% fetal bovine serum. RKO, MCF7, and HCT116 cell lines, which inducibly express HA-tagged RNPC1a or RNPC1b or shRNA against RNPC1a, were used as described (Shu et al. 2006). The stable inducible cell lines were generated by using a Tet-on inducible system as described previously (Harms and Chen 2007). To generate stable cell lines that inducibly express RNPC1a in SW480 cells, pcDNA4-HA-RNPC1a, where RNPC1a expression was driven by CMV promoter and two tetracycline operator 2 sites, was transfected into SW480 expressing a tetracycline repressor (pcDNA6). The RNPC1a-expressing cells were selected with zeocin and confirmed by Western blot analysis. To generate stable cell lines that inducibly express HA-tagged ΔRNP1 and ΔRNP2 in RKO cells, the same strategy was used except that pcDNA4-HA-ΔRNP1 or pcDNA4-HA-ΔRNP2 was transfected into RKO cells expressing a tetracycline repressor. To generate an inducible total RNPC1 knockdown cell line, pBabe-H1-siRNPC1 was transfected into MCF7 cells expressing tetracycline repressor (Harms and Chen 2007). The total RNPC1 knockdown cell lines were selected with puromycin and confirmed by Western blot analysis. For induction, tetracycline (250–500 ng/mL) was added to culture medium.

Generation of RNPC1 knockout mice and isolation of primary MEFs

Conditional RNPC1 knockout mice were generated by the University of California at Davis murine targeted genomics laboratory. Specifically, lox P sites were inserted in the proximal promoter region and the intron (Supplemental Fig. S11A) and floxed mice were bred to a cre deleter strain (Jackson Laboratories, stock no. 003314) expressing cre recombinase in the germline to generate RNPC1 knockout mice. Mice were genotyped (Supplemental Fig. S11B) by PCR using primers as indicated in Supplemental Fig. S11A. To generate RNPC1$^{-/-}$ MEFs, mice heterozygous for RNPC1 were bred and MEFs were isolated from 13.5-d-old embryos as described previously (Scoumanne et al. 2011). To generate RNPC1$^{-/-}$; p53$^{-/-}$ MEFs, mice heterozygous for RNPC1 and mice heterozygous for p53 were bred to generate mice heterozygous for RNPC1 and p53, and these mice were bred to isolate MEFs. All animals were 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.

^{35}S metabolic labeling, Western blot analysis, and immunoprecipitation

These assays were performed as previously described (Bonifacino 2001; Zhang and Chen 2007).

In vitro translation, recombinant protein purification, and GST pull-down assay

In vitro translation was carried out using the TNT Quick-Coupled in vitro transcription/translation system (Promega) according to the users' guide. Specifically, 1 μg of pcDNA3-Myc-eIF4E or pcDNA3-HA-RNPC1a plasmid was used as a template to produce in vitro translated proteins. The recombinant His-tagged RNPC1 and GST proteins were expressed in bacteria BL21 and purified by using NiTA-agarose and glutathione sepharose beads, respectively. For GST pull-down assay, 500 ng of His-tagged RNPC1 and 500 ng of GST-fused eIF4E or GST protein were incubated in E1A-binding buffer (50 mM HEPES at pH 7.6, 50 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 10% glycerol) for 4 h at 4°C, followed by precipitation with glutathione sepharose 4B beads. After three washes, beads were resuspended in 1× SDS loading buffer and subjected to Western blot analysis.

Recombinant protein purification, probe labeling, and REMSA

Recombinant proteins were expressed in bacteria BL21 and purified by glutathione sepharose beads. RNA probes were generated and ^{32}P-labeled by in vitro transcription using PCR products containing T7 promoter and various regions from the p53 5′ UTR or 3′ UTR as a template. The primers to amplify the RNA probes are listed in the Supplemental Material. REMSA assay was performed as described previously (Zhang et al. 2010). Briefly, ^{32}P-labeled probes were incubated with recombinant protein in a binding buffer (10 mM HEPES-KOH at pH 7.5, 90 mM potassium acetate, 1.5 mM magnesium acetate, 2.5 mM DTT, 40 U of RNase inhibitor [Ambion]) at 30°C for 30 min. For supershift, 1 μg of anti-HA was added to the reaction mixture and incubated for an additional 30 min. RNA–protein complexes were resolved on a 5% acrylamide gel and radioactive signals were detected by autoradiography.

Generation of expression and reporter vectors, RNA isolation, RT–PCR, and quantitative PCR

The cloning strategy and primers used are listed in the Supplemental Material.

RNA-ChIP

The RNA-ChIP was performed as described (Peritz et al. 2006).

Polysome profile analysis

HCT116 cells were uninduced or induced to express RNPC1a for 24 h, treated with 0.1 mM cycloheximide for 30 min, and then lysed in a buffer containing 0.5% NP40, 0.1 M NaCl, 10 mM $MgCl_2$, 2 mM DTT, 50 mM Tris-HCl (pH 7.5), 200 U/mL SUPERase·In, 100 μg/mL cycloheximide, and 200 μg/mL heparin. Nuclei were precipitated at 10,000*g* for 10 min. The resulting supernatants were layered on a 10%–50% (w/v) sucrose gradient containing 0.15 M NaCl, 5 mM MgCl2, and 25 mM Tris-HCl (pH 7.5), and centrifuged in a SW40 rotor (Beckman Coulter) at 35,000 rpm for 140 min. Gradients were analyzed by using ISCO fractionator with UV_{254nm} detector, and RNAs were extracted from RNA–protein complexes with phenol-chloroform-isoamyl alcohol and recovered by ethanol precipitation. One microgram of total RNAs from each fraction was used for RT–PCR to detect p53 and actin transcripts. The primers to amplify p53 were 5′-CCCAGCCAAAGAAGAAACCA-3′ and 5′-GTTC CAAGGCCTCATTCAGCT-3′. The primers to amplify actin were 5′-CTGAAGTACCCCATCGAGCACGGCA-3′ and 5′-GGA TAGCACAGCCTGGATAGCAACG-3′.

SA-β-gal staining

This assay was performed as described previously (Qian et al. 2008). Specifically, primary MEFs at passage 5 were treated with or without doxorubicin (50 nM) for 48 h. Cells were then washed with 1× phosphate-buffered saline and fixed with 2% formaldehyde and 0.2% glutaraldehyde for 10–15 min at room temperature, followed by staining with fresh β-gal staining solution overnight at 37°C without CO_2. The β-gal staining solution contains 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside, 40 mm citric acid/sodium phosphate (pH 6.0), 5 mm potassium ferrocyanide, 5 mm potassium ferricyanide, 150 mm NaCl, and 2 mm $MgCl_2$. β-gal-positive cells were counted by light microscopy.

Tissue collection

Fresh frozen dog lymph nodes from clinical samples were provided by the University of California at Davis Small Animal Clinic with the owner's permission. Samples were homogenized in Trizol, followed by RNA and protein purification according to the users' manual.

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