

Interplay between PTB and miR-1285 at the p53 3'UTR modulates the levels of p53 and its isoform $\Delta 40p53\alpha$

Aanchal Katoch, Biju George, Amrutha Iyyappan, Debjit Khan and Saumitra Das*

Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560012, India

Received April 28, 2017; Revised July 06, 2017; Editorial Decision July 10, 2017; Accepted July 26, 2017

ABSTRACT

p53 and its translational isoform $\Delta 40p53$ are involved in many important cellular functions like cell cycle, cell proliferation, differentiation and metabolism. Expression of both the isoforms can be regulated at different steps. In this study, we explored the role of 3'UTR in regulating the expression of these two translational isoforms. We report that the *trans* acting factor, Polypyrimidine Tract Binding protein (PTB), also interacts specifically with 3'UTR of *p53* mRNA and positively regulates expression of p53 isoforms. Our results suggest that there is interplay between miRNAs and PTB at the 3'UTR under normal and stress conditions like DNA damage. Interestingly, PTB showed some overlapping binding regions in the p53 3'UTR with miR-1285. In fact, knockdown of miR-1285 as well as expression of p53 3'UTR with mutated miR-1285 binding sites resulted in enhanced association of PTB with the 3'UTR, which provides mechanistic insights of this interplay. Taken together, the results provide a plausible molecular basis of how the interplay between miRNAs and the PTB protein at the 3'UTR can play pivotal role in fine tuning the expression of the two p53 isoforms.

INTRODUCTION

Tumour suppressor p53 protein is one of the most frequently altered genes in human cancers, which plays a key role in maintaining genomic integrity by controlling cell-cycle progression and cell survival (1). TP53 produces different isoforms using alternate promoters, splicing sites and translation initiation sites (2). One of the isoforms of p53 is $\Delta 40p53$, which is a translational isoform of p53. It has been shown earlier that $\Delta 40p53$ is translated from the same mRNA from which full-length p53 is also translated, using an internal ribosome entry site (IRES) (3,4). Because $\Delta 40p53$ is generated by initiation at an internal position, it lacks N-terminus, and is often referred to in literature

as ΔN -p53 (5). p53 is activated in response to a range of stressors such as DNA-damaging agents, radiation, oxidative stress, and proto-oncogenes that regulate cell proliferation, differentiation, cell cycle and metabolism (6).

Earlier studies have also shown that *p53* mRNA stability and translation are strong regulators of p53 expression (7). The translational regulation of p53 involves interaction of various proteins with 5'UTR and 3'UTR of its RNA. RPL26 and nucleolin bind to the 5'UTR and regulate the translation and induction of p53 respectively under DNA damage (8). IRES-mediated translation is also regulated by different IRES *trans* acting factors (ITAFs) such as PTB (9), ANXA2, PSF (10), DAP5 (11), TCP80 (12), SMAR1 (13) under different stress conditions like DNA damage, ER stress and glucose starvation.

Post-translational regulation of p53 is also mediated by its 3'UTR. Proteins like GAPDH, hnRNPD and hnRNPA/B (14), as well as Hzf and HuR (15) interact with p53 3'UTR; similarly several micro RNAs such as miR-125b, miR-504, miR-25, miR-30d (16) and miR-1285 (17) can regulate p53 message. There are RBPs (such as RPL26, RNPC1) that bind to both p53 5' and 3'UTRs, and help in 5'-3'UTR interactions, which are critical for translation regulation of *p53* mRNA (18).

Additionally, the interplay between miRNAs and RNA binding proteins (RBPs) on target 3' UTR can also modulate target expression under different stress conditions. The RBPs that bind to the regions near to the miRNA binding sites can potentially affect miRNA mediated regulation either directly by sterically blocking miRNA binding or indirectly by changing local RNA structure. Apart from one very recent report (19), such interactions are relatively less studied for p53.

Since our laboratory studies had shown earlier that PTB (9) binds to the IRES element of *p53* mRNA, including its 5'UTR, and regulate IRES mediated translation of both p53 and $\Delta 40p53$, we investigated the binding of PTB protein at the 3'UTR to understand the regulation further. Knockdown of PTB leads to significant decrease in translation of p53 isoforms regulated through the 3'UTR. It appears that PTB binding sites overlap with some miRNA

*To whom correspondence should be addressed. Tel: +91 80 2293 2886; Fax: +91 80 2360 2697; Email: sdas@mcbl.iisc.ernet.in
Present address: Debjit Khan, Cellular and Molecular Medicine, Cleveland Clinic Lerner Research Institute, 9500 Euclid Avenue, NC1-104, Cleveland, OH 44195, USA.

binding sites at 3'UTR. Our results suggest that the binding of PTB to 3'UTR regulates the interaction of miR1285 and p53-3'UTR to fine tune translation regulation of the two p53 isoforms. Taken together, our study suggests that such interplay might be more important under stress conditions for the differential regulation of the p53 isoforms.

MATERIALS AND METHODS

Plasmids constructs

p53 3'UTR: p53 3'UTR between EcoRI and NotI sites in pcDNA3 backbone (a generous gift from Dr ParthoSarathi Ray). Luc-p53 3'UTR: Fluc (Firefly luciferase) (between HindIII and EcoRI) followed by p53 3'UTR (between EcoRI and NotI) in pcDNA3 backbone (a generous gift from Dr ParthoSarathi Ray). p53(1-134)-luc(5'UTR-Luc): It consists of 5'UTR region (1-134) and Fluc. p53(1-134)-luc-3'UTR (5'UTR-Luc-3'UTR): It consists of 5'UTR (1-134), Fluc and 3'UTR of p53 mRNA. p53 5'UTR-cDNA: It consists of 5'UTR and cDNA. p53 5'UTR-cDNA-3'UTR: It consists of 5'UTR, cDNA and 3'UTR of p53 mRNA. Luc-3'UTR mut miR-1285 M1(3'UTR mutated for miR-1285 one binding site), Luc-3'UTR mut miR-1285 M2 (3'UTR mutated for miR-1285 second binding site), Luc -3'UTR mut miR-1285 M1+M2 (3'UTR mutated for miR-1285 both binding sites). Luc- p53-3'UTR-SNV 93(G>A), Luc-p53-3'UTR-SNV 287 (G>A), Luc-p53-3'UTR-SNV 737(G>C), Luc-p53-3'UTR-SNV 806(C>T), Luc-p53-3'UTR-SNV93(G>A), Luc-p53-3'UTR-SNV287(G>A), Luc-p53-3'UTR-SNV737(G>C), Luc-p53-3'UTR-SNV806(C>T). p53-3'-UTR-Mut 2a (Mutant:2a = TT to GG 755-756), p53-3'-UTR-Mut 3a (3a = TT to GG 748-749), Luc-3'UTR-Mut 2a and Luc-3'UTR-Mut 3a. GFP expression was measured as transfection efficiency control from a pCDGFP plasmid vector with GFP ORF in pcDNA3 backbone.

Cell lines and transfections

H1299 and A549 cells were maintained in DMEM (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum. For luciferase assays or western blots, 70% confluent monolayer of H1299 or A549 were transfected with various luciferase constructs or cDNA plasmids using Lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, CA, USA) in Opti-MEM (Invitrogen). Four to five hours later, the medium was replaced with DMEM (with antibiotic) and 10% FBS. At the desired time point, the cells were harvested and processed as required. The cells were treated with doxorubicin (2 μ m) for 16 h to induce DNA damage stress.

siRNA anti-miR transfections

H1299 cells were transfected with 50 nM/100nM si PTB RNA. A control scramble siRNA (Dharmacon) was used in the partial silencing experiments as control. Co-transfection of siRNA with various plasmid constructs was performed in H1299 and A549 cells using Lipofectamine 2000 (Invitrogen) transfection reagent in Opti-MEM (Invitrogen). Similarly, anti-miRs were also used (10 nM /20 nM/30 nM anti-miR-1285, anti-miR-30d, anti-miR-504, anti-miR-181 and

anti-miR-125b) to partially silence the respective miRNAs. After 48 h, the cells were harvested in passive lysis buffer for reporter gene assays and in RIPA lysis buffer for western blot analysis.

In vitro transcription

In vitro transcription reactions for UV crosslinking were done. α -³²P-labeled RNAs were transcribed using T7 polymerase and α -³²P-uridine triphosphate (Perkin Elmer) after linearizing the respective plasmids.

Protein purification

Recombinant His-PTB was purified as described earlier (9). Briefly, recombinant PTB was transformed in *Escherichia coli* (BL21 DE3) cells and the expression was induced by 0.6 mM IPTG. For purifying the protein, the lysate was incubated with Ni²⁺-nitrilotriacetic acid-Agarose (Qiagen) under non-denaturing conditions at 4°C and eluted with 250 mM imidazole (9).

UV-induced crosslinking of proteins and RNA and immunoprecipitation assay (UV-IP)

α -³²P RNA probes were *in vitro* transcribed by using T7 RNA polymerase. RNA probes were allowed to complex with purified proteins at 30°C for 30 min in RNA binding buffer, and then UV-crosslinked for 20 min. RNA-protein complexes were digested with RNase A for 30 min at 37°C and then the complexes were resolved on SDS 12% or 10% polyacrylamide gels and analyzed by phosphorimaging.

For immunoprecipitation, the RNaseA-treated reaction mixtures (30 μ g of total protein) were made up to 500 μ l with polysome lysis buffer (100 mM KCl, 5 mM MgCl₂, 10 mM HEPES, pH 7.0, 0.5% NP-40, 1 mM DTT, 100 U/ml RNasin and 1% protease inhibitor cocktail) and precleared with protein G-Sepharose beads for 1 h at 4°C. The samples were spun at 4000 rpm for 2 min to pellet the beads, and the supernatant was removed. Protein G-Sepharose beads were incubated with 2 μ g of anti-PTB antibody (Calbiochem) overnight at 4°C in a total volume of 200 μ l of polysomolysis buffer and added to the precleared lysates, followed by incubation for 4 h with continuous mixing on a rotator device at 4°C. The beads were washed four times with polysome lysis buffer. SDS sample buffer was then added to the beads and boiled to release the immunoprecipitated protein, and the supernatant was electrophoresed on an SDS-12% PAGE gel. The gel was dried, exposed, and developed by autoradiography.

Immunoprecipitation of RNP complexes and RT-PCR

H1299 cells were co-transfected with either Luc-p53-3'UTR WT or Luc-p53-3'UTR mut-miR-1285 M1+M2 plasmid and si PTB or anti-miR-1285/ anti-miR-30d. The RNA-protein complexes were then immunoprecipitated with Ago-2/IgG/PTB anti-antibody. The p53 3'UTR was detected by using specific primers (forward primer: 5'-TAATACGACTCACTATAGGGGGGAGTGG CGTGAT-3', reverse primer: 5'-TGCAGATGTGCTTGC

AGAATGTAAAAGATG-3') for 3'UTR region by RT-PCR. For Ago-2 immunoprecipitation the transfected cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM KCl, 5 mM MgCl₂, 10 mM sodium orthovanadate, 1 mM DTT, 1% Triton X-100, 100-U/ml RNasin and 1% protease inhibitor cocktail). Similarly, A549 cells, treated with doxorubicin (16 h) or no treatment cells, were also lysed in same buffer for immunoprecipitating Ago-2. Supernatants were pre-cleared with Fast-Flow protein-G sepharose beads (Sigma-Aldrich) for 45 min at room temperature. Pre-cleared lysates containing equal total protein (1 mg) were incubated with anti-Ago-2 antibody (Cell Signaling Technology) or rabbit IgG antibody (Sigma-Aldrich) saturated protein-G sepharose beads overnight at 4°C. RNP complexes conjugated to antibody-bound beads were spun down at 8000 rpm for 2 min; subsequently, the beads were washed twice with IP buffer (20 mM Tris-HCl, pH 7.5, 150 mM KCl, 5 mM MgCl₂, 10 mM sodium orthovanadate, 1 mM DTT), and the bound beads were then processed for western blot and RNA isolation for RT-PCR. For immunoprecipitation of PTB polysomal lysis buffer (100 mM KCl, 5 mM MgCl₂, 10 mM HEPES at pH 7.0, 0.05% NP-40, 1 mM DTT, 100-U/ml RNasin and 1% protease inhibitor cocktail) was used, and same buffer was used for the washes also. Rest of the protocol used was same as that for Ago-2 immunoprecipitation, except that the pre-cleared lysate was incubated with anti-PTB antibody (Calbiochem) or rabbit IgG antibody saturated protein-G sepharose beads for 4 h at 4°C.

Western blot analysis

Protein concentrations of the extracts were assayed by Bradford (Bio-Rad) and equal amounts of cell extracts were separated by 12% SDS-PAGE and transferred to nitrocellulose membrane (Sigma). Samples were then analyzed by western blotting using rabbit-raised anti-p53 polyclonal antibody (CM1, kind gift from Dr Robin Fahraeus, INSERM, France), sheep polyclonal antibody (Sapu), mouse raised 1801 (kind gift from Dr Jean-Christophe Bourdon, University of Dundee, Dundee) and DO1, mouse monoclonal anti PTB antibody, rabbit polyclonal anti Ago-2 antibody (C34C6, Cell Signaling Technology) followed by secondary antibody (horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG; Sigma) or anti-sheep, and mouse-monoclonal anti-β-actin antibody (Sigma) was used as control for equal loading of total cell extracts.

Dual-luciferase assay

The transfected cells were harvested in PLB lysis buffer after 12 h/24 h/48 h of transfection and luciferase activity was measured, using Luciferase assay kit from Promega.

RNA isolation and real-time PCR

Total RNA was extracted using TRIzol reagent (Sigma). RNA amount was quantified with Nanospectro photometer and reverse transcribed (ABI). miRNAs were quantified with Taqman-based method. Following are the primers used:

Actin_forward:5'TCACCCACACTGTGCCCA3',
Actin_reverse:5'TGAGGTAGTCAGTCAGGT3';
GAPDH_forward:5'CAGCCTCAAGATCATCAGCA
AT3', GAPDH_reverse:5'GGTCATGAGTCCTTCCAC
GA3'; p21_forward:5'CCTCAAATCGTCCAGCGAC3',
p21_reverse:5'CATTGTGGGAGGAGCTGTG3'; 14-
3-3σ_forward:5'TGAGAACTGGACATGGGCAG-3',
14-3-3σ_reverse:5'GAGGAAACATGGTCACACC3';
Fluc_forward:5'GATTGACAAGGATGGATGGC3',
Fluc_reverse:5'CGTCATCGTCCGGAAGACCT3';
p53_forward:5'TGGGCTTCTTGCATTCTGG3',
p53_reverse:5'GCTGTGACTGCTTGTAGATGGC3';
3F4:5'TAATACGACTCACTATAGGGGGGAGTGGC
GTGAT3', 3R4:5'TGCAGATGTGCTTGCAGAATGT
AAAAGATG3'.

Toe-printing assay

In vitro transcribed full length p53 3'UTR RNA was incubated in the presence or absence of purified recombinant PTB or BSA for toe-printing assay. The RNAs in the ribo-nucleoprotein complexes were reverse transcribed by using AMV-RT enzyme (NEB) and 3R3 reverse primer; (3R3: 5'-CTGGGCAACAAAGCGAGACCCAG TCTCAAAG-3'). The resulting cDNAs were resolved on 8% acrylamide-8 M urea PAGE in parallel with a sequencing reaction. The results were analyzed using a phosphorimager.

mFOLD software

For predicting the structure of p53 3'UTR and different regions of p53 3'UTR Mfold software was used.

Statistical analysis

The data were expressed as mean ± SD. Statistical significance was determined using two-sided Student's *t*-test. The criterion for statistical significance was $P \leq 0.05$ (*) or $P \leq 0.01$ (**) or $P \leq 0.001$ (***) or $P \leq 0.0001$ (****)

RESULTS

PTB binds to 3'UTR of p53 mRNA

In order to investigate the binding of PTB with the p53 3'UTR, UV-crosslinking experiments were performed using recombinant PTB protein. The purity of recombinant PTB protein was checked using silver staining and by western blot analysis using anti-PTB antibody (Supplementary Figure S1A and B). Radiolabeled RNA corresponding to the p53 3'UTR was allowed to complex with increasing concentrations of recombinant PTB. Phosphor imaging analyses indicated that PTB interacts with both the 5' and 3'UTRs of p53 RNA (Figure 1A). In order to study the specificity of the interaction, competition UV cross-linking assays were performed using 100- and 200-fold molar excess of either unlabeled p53 3'UTR RNA (self-RNA), unlabeled p53 5'UTR RNA (non-self RNA) or a non-specific RNA. We observed that unlabeled p53 3'UTR RNA could efficiently compete out the binding, hence, confirming the

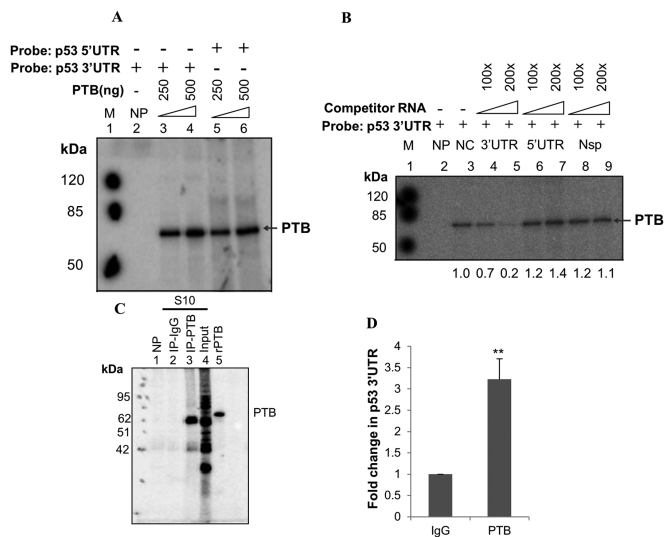


Figure 1. PTB binds specifically to p53 3'UTR. (A) α - 32 P-labeled 3'UTR (Lanes 3,4) and α - 32 P-labeled 5'UTR (lanes 5 and 6) were UV cross-linked with increasing concentration of PTB (250 ng, 500 ng), lane 2 is no protein (NP) and lane 1 represents the MW marker in kDa. (B) Competition UV crosslinking of PTB and α - 32 P-labeled 3'UTR with 100- and 200-fold molar excess of either unlabeled p53 3'UTR RNA (lanes 4 and 5), unlabeled p53 5'UTR (lanes 6 and 7) or non-specific RNA (Nsp RNA) (lanes 8 and 9) showing the specificity of PTB and 3'UTR binding. Lane 1 represents the MW marker in kDa, lane 2 is no protein (NP) and lane 3 represents binding in the absence of competitor RNA (NC). (C) After UV cross-linking of α - 32 P-labeled p53 3'UTR RNA with A549 cell S10 extract, immunoprecipitation was carried out using the respective antibodies, followed by incubation with protein G-Sepharose beads. The beads were either used after saturation with IgG isotype control antibody (lane 2) or anti-PTB antibody (lane 3). Lane 1, no protein (NP); lane 4, 25% of the UV-cross-linked S10 extract (input). Lane 5 is UV-crosslinked recombinant PTB (500 ng) protein. Numbers to the left represent relative mobilities of the molecular mass markers. (D) H1299 cells were transfected with Luc-p53 3'UTR construct. After 48 h, the cells were lysed with lysis buffer. RNP complexes were immunoprecipitated with anti-PTB antibody. p53 3'UTR was detected by RT-PCR analysis ($n = 3$).

specificity of the interaction of PTB with 3'UTR of p53 mRNA (Figure 1B). We also used UV cross-linking immunoprecipitation studies to establish that PTB interacts with the p53 3'UTR. For this experiment, cytosolic (S10) extract prepared from A549 cells was incubated with α - 32 P-labeled p53 3'UTR RNA, followed by UV-crosslinking, and the complexes were immunoprecipitated and resolved in SDS-12% PAGE. PTB was specifically precipitated from the cross-linked cytosolic extracts using anti-PTB antibody but not with the IgG isotype control antibody (Figure 1C).

Furthermore, to validate the interactions *ex vivo*, RNP-IP experiments were performed. p53 null H1299 cells were transfected with reporter constructs expressing luciferase RNA fused with the p53 3'UTR or canonical p53 5'UTR (positive control). The RNA-protein complexes were immunoprecipitated using anti-PTB antibody and the p53 3'UTR/5'UTR was detected by RT-PCR. Results demonstrate that PTB is associated with the p53 3'UTR within cells (Figure 1D and Supplementary Figure S1C and D).

PTB knockdown decreases p53 and Δ 40p53 expression mediated through 3'UTR

In order to investigate the role of PTB in 3'UTR mediated regulation of p53, three different reporter gene constructs were used, namely, Luc-3'UTR, 5'UTR-Luc and 5'UTR-Luc-3'UTR. H1299 cells were individually transfected with these constructs in presence and absence of si PTB or non-specific siRNA (si Nsp). Rluc (Renilla luciferase) was used as transfection control in all sets. Results show that partial silencing of PTB decreased luciferase activity in all the constructs compared to control (si Nsp) (Figure 2A).

Furthermore, the role of PTB in p53 3'UTR mediated regulation was investigated using p53 cDNA constructs having either 5'UTR (p53 5'UTR-cDNA) or both 5'UTR and 3'UTR (p53 5'UTR-cDNA-3'UTR). H1299 cells were co-transfected individually with these constructs with si PTB or with non-specific si Nsp. As we observed with the reporter construct, the partial silencing of PTB resulted in the reduction of expression of both p53 and Δ 40p53 from the respective cDNA constructs (Figure 2B). In order to check whether this decrease in the expression was due to the decrease in protein stability (proteasomal degradation), a proteasomal degradation pathway inhibitor, MG132 was used. Upon MG132 treatment (60 min) after 47 h of PTB knockdown, no significant de-repression in the expression of p53 isoforms from either constructs was observed. The results suggest that the role of PTB is possibly not at the level of protein degradation (Supplementary Figure S2A and B). Interestingly, significant reductions in the expression of luciferase reporter gene, p53 and Δ 40p53 were observed in presence of 3'UTR in all the constructs. Further repression was observed upon partial knockdown of the *trans* acting factor (Figure 2A and B). This indicates a functional role of PTB in 3'UTR mediated regulation of p53 isoforms.

Similar experiment was done with A549 cells. Cells were transfected with either si PTB or non-specific si Nsp. Partial silencing of PTB decreased the endogenous levels of both p53 and Δ 40p53 and this was confirmed with three different antibodies (CM1, Sapu and DO1) (Figure 2C, Supplementary Figure S2C and D) and also their respective target mRNA levels (*p21* and *14-3-3 σ* respectively) (Figure 2D). Further, to understand the role of PTB in a stress condition like DNA damage where it translocates from nucleus to cytoplasm as shown in Supplementary Figure S2E, which is in accordance with our earlier laboratory studies (9), we examined the effect of partial silencing of PTB after 16 h doxorubicin (2 μ M) treatment. Results indicate that PTB silencing can prevent the enhancement of the steady-state levels of p53 isoforms and their target mRNA levels upon doxorubicin treatment (Figure 2E, Supplementary Figure S2F-H). We also observed similar effects in H1299 cells co-transfected with p53 5'UTR-cDNA-3'UTR and si PTB/si Nsp. GFP expression from a pCDGFP plasmid vector was measured as transfection efficiency control. p53 and Δ 40p53 bands were detected using four different anti p53 antibodies (CM1, Sapu, 1801 and DO1) (Figure 2F and G, Supplementary Figure S2I-L).

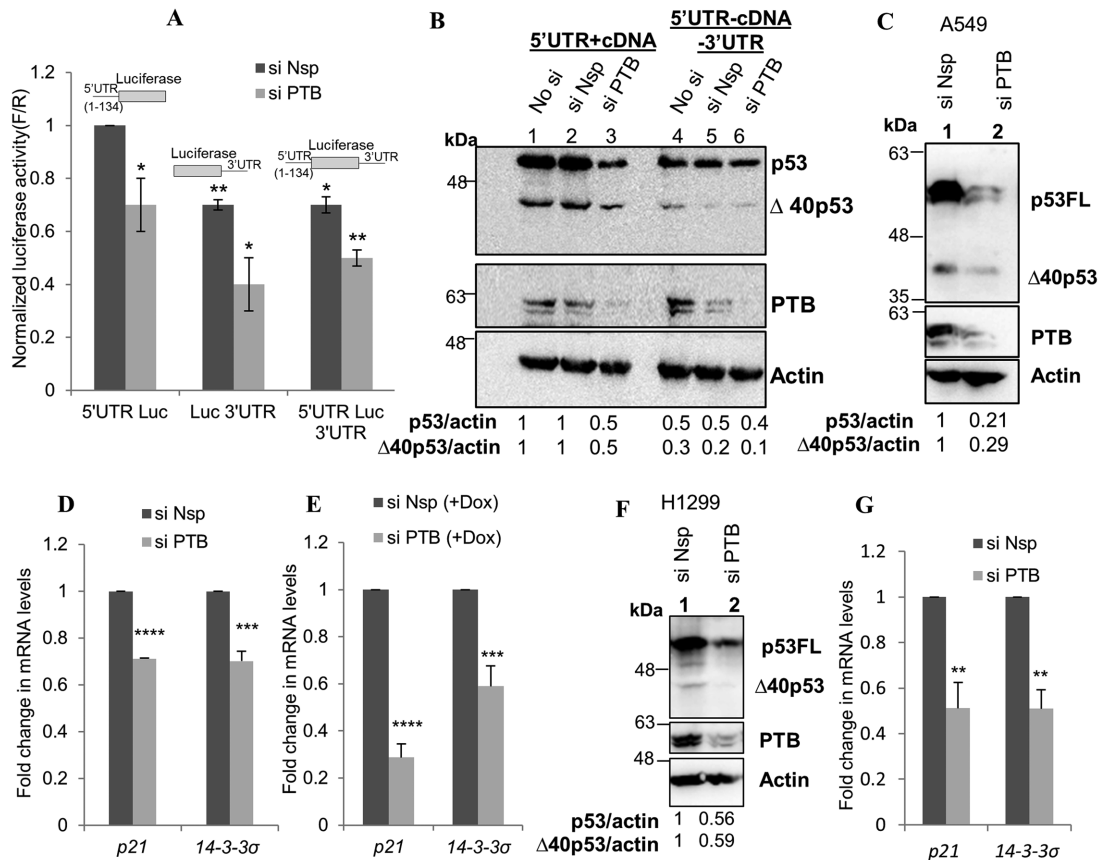


Figure 2. Partial silencing of PTB decreases p53 expression and target mRNA levels. (A) H1299 (p53) null cell line was co-transfected with luciferase (Firefly luciferase) constructs having only 5'UTR (5'UTR Luc), 3'UTR (Luc 3'UTR), or both 5' and 3'UTR (5'UTR Luc 3'UTR), and either non specific si (si Nsp) or si PTB. (25 nM) Rluc (Renilla luciferase) was used as transfection control in all sets. Cells were harvested after 48 h in passive lysis buffer and processed for luciferase assay. The graph represents the normalized luciferase activity, i.e. Fluc/Rluc (F/R) ($n = 3$). (B) H1299 cells were co-transfected with p53 cDNA construct having 3'UTR (5'UTR + cDNA + 3'UTR) or no 3'UTR (5'UTR + cDNA), and either non specific si (si Nsp) or si PTB (50nM). No siRNA (No si) transfection is the control lane. After 48 h, the cells were lysed and processed for immunoblotting. Upper panel: p53 and $\Delta 40p53$; middle panel: PTB; lower panel: actin. (C) Western blot analysis of cell extracts from A549-cells transfected with either non specific si (si Nsp) or si PTB (100 nM), probed with CM1 after 48 h. Upper panel: p53 and $\Delta 40p53$; middle panel: PTB; lower panel: actin. (D) Quantitative PCR of *p21* and *14-3-3 σ* mRNA levels normalized to actin in A549 cells transfected with either non specific si (si Nsp) or si PTB (100 nM) for 48 h ($n = 4$). (E) Quantitative PCR of *p21* and *14-3-3 σ* mRNA levels normalized to Actin in A549 cells transfected with either non specific si (si Nsp) or si PTB (100 nM) for 48 h and treated with doxorubicin (2 μ M) for last 16 h ($n = 5$). (F) Western blot analysis of cell extracts from H1299 cells co-transfected with p53 cDNA construct having 3'UTR (5'UTR + cDNA + 3'UTR) and either non specific si (si Nsp) or si PTB (100 nM), probed with CM1 after 48 h post transfection. (G) Quantitative PCR of *p21* and *14-3-3 σ* mRNA levels normalized to GAPDH in H1299 cells co-transfected with p53 cDNA construct having 3'UTR (5'UTR + cDNA + 3'UTR) and either non specific si (si Nsp) or si PTB (100 nM) for 48 h ($n = 3$).

PTB binds at different regions in the p53 3'UTR

We have predicted the unconstrained structure of 1.2 kb long p53 3'UTR (GenBank: AB082923.1) using the ZukerMfold programme (20). As shown in Figure 3A, the 3'UTR has distinct stem-loop structures. To locate PTB binding sites within the 3'UTR, we divided the 3'UTR into four regions (R-I–R-IV, Figure 3B). These regions were individually amplified using different sets of primer followed by *in vitro* transcription using T7 RNA polymerase to generate RNAs corresponding to these four regions. UV-crosslinking assay was performed using α - 32 P-labeled 3'UTR and purified recombinant PTB protein in presence and absence of competing unlabeled RNAs corresponding to different regions of 3'UTR (Figure 3C). Comparing the relative band intensities, the probable binding sites for PTB in the 3'UTR were assigned (Table 1). PTB appeared to in-

Table 1. Binding regions in p53 3'UTR for PTB

REGIONS	I	II	III	IV
PTB		++	+++	

teract predominantly with R-III followed by R-II of the p53 3'UTR.

Interplay between the ITAFs and miRNAs at 3'UTR

In our experiments with the reporter constructs, significant reduction in luciferase expression was observed in the presence of the p53 3'UTR. We hypothesized that this reduction might be due to the binding of miRNAs to the 3'UTR. In fact, regions within 3'UTR, where miRNAs are known to bind, were found to overlap with the puta-

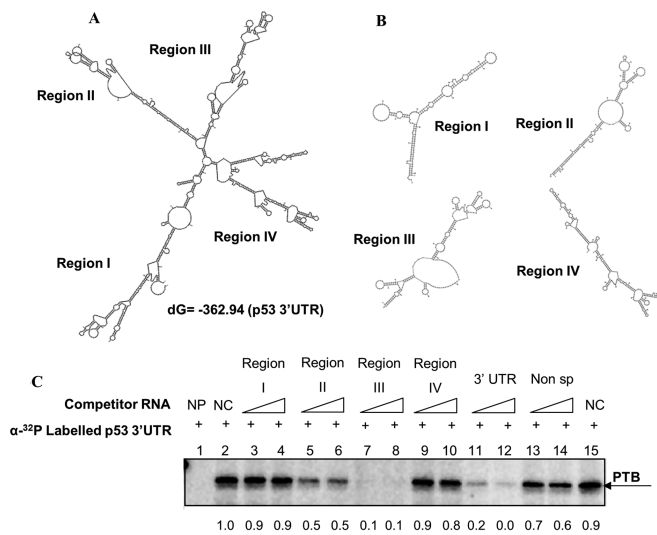


Figure 3. PTB binding regions in p53 3'UTR. (A) Mfold-predicted structure of complete p53 3'UTR. (B) Different regions of p53 3'UTR used in panel C are shown separately. (C) Competition UV crosslinking of α -³²P-labeled 3'UTR and unlabeled cold RNA of different regions of 3'UTR with PTB. Lane 1: no protein (NP), lane 2: no competition (NC), lane 3 and 4: 100- and 200-fold molar excess of either unlabeled p53 3'UTR region I RNA, lanes 5 and 6: 100- and 200-fold molar excess of either unlabeled p53 3'UTR region II RNA, lanes 7 and 8: 100- and 200-fold molar excess of either unlabeled p53 3'UTR region III RNA, lanes 9 and 10: 100- and 200-fold molar excess of either unlabeled p53 3'UTR region IV RNA, lanes 11 and 12: 100- and 200-fold molar excess of either unlabeled p53 3'UTRRNA, lanes 13 and 14: non-specific RNA (Nsp RNA).

tive binding regions of PTB (ITAF) (Table 2). To investigate the possible interplay between miRNAs and PTB/at the p53 3'UTR, we performed immunoprecipitation experiments (RNP-IP). H1299 cells were co-transfected with the luciferase-p53 3'UTR RNA construct and siRNA against PTB. The RNA protein complexes were then immunoprecipitated with anti-Ago2 antibody and p53 3'UTR was detected by RT-qPCR. Results indicated that the association of p53 3'UTR with Ago-2 increases upon partial silencing of PTB (Figure 4A, Supplementary Figure S3A and B), suggesting a possible interplay between the protein and miRNAs.

We were curious regarding the effect of increased abundance of PTB in the cytoplasm under DNA damage stress on the interplay. For this purpose, H1299 cells were transfected individually with the reporter constructs Luc, Luc-3'UTR, 5'UTR-Luc and 5'UTR-Luc-3'UTR along with GFP plasmid as transfection control. These cells were treated with doxorubicin (2 μ M) for 16 hours, harvested, and checked for luciferase activity; luciferase activity increased in presence of doxorubicin in all the constructs compared to the basal Fluc-construct that lacks any regulatory region (Figure 4B, Supplementary Figure S3C). In a similar experiment, the RNA protein complexes were immunoprecipitated from Fluc-p53 3'UTR transfected H1299 cells with antiAgo-2 antibody and p53 3'UTR was detected by RT-qPCR. Results indicate that p53 3'UTR interaction with Ago-2 decreases in presence of doxorubicin (Figure 4C and Supplementary Figure S3D). Further, to detect endogenous p53 3'UTR-Ago2 association, Ago2 immunoprecip-

itation was performed in A549 cell line after doxorubicin treatment (Figure 4D, Supplementary Figure S3D). As earlier, we detected a decreased association of Ago-2 with p53 3'UTR upon doxorubicin treatment, whereas association of PTB with 3'UTR increased in presence of doxorubicin in both H1299 and A549 cells (Figure 4E and F, Supplementary Figure S3E). A decrease in p53 3'UTR-Ago2 interaction after doxorubicin induced DNA-damaging stress can occur if p53-targeting miRNA levels concomitantly decrease. However, we observed a modest increase in the levels of these miRNAs (miR-1285 and miR-30d) in both A549 and H1299 cells with doxorubicin treatment (Supplementary Figure S3 F-G). This indicates that under stress, cytoplasmic PTB may displace Ago2 from p53 3'UTR even at elevated levels of miRNAs that target p53.

In order to check the effect of specific miRNAs (that could have overlapping binding sites with PTB) on the expression of luciferase activity, H1299 cells were co-transfected with Luc-3'UTR and anti-miRs for miR-30d/miR-1285/miR-504/miR-181 and Rluc as transfection control. An increase in the luciferase activity with anti-miR-30d and anti-miR-1285 was observed when compared to the others (Figure 5A, Supplementary Figure S3H). Similarly, co-transfection was also carried out for anti-miR-1285/miR-30d and p53 5'UTR-cDNA/p53 5'UTR-cDNA-3'UTR in H1299 cells. We observed an increase in the expression of both the p53 isoforms with knockdown of miR-30d and miR-1285 (Figure 5B, Supplementary Figure S3I). As expected, this increase was observed only in the construct having p53 3'UTR and not in the construct that lacks the 3'UTR. In order to examine the effect of these miRNAs on the endogenous levels of both the p53 isoforms, similar experiment was performed in A549 cells. The presence of anti-miR for miR-1285 and miR-30d increased the expression of p53 and Δ 40p53, which was confirmed with four different anti p53 antibodies (Figure 5C, Supplementary Figure S3J-L). The p53 and Δ 40p53 target mRNA levels (*p21* and *14-3-3 σ* respectively) also increased, which was more significant in presence of anti-miR-1285 as compared to anti-miR-30d (Figure 5D). We also observed similar effects in H1299 cells co-transfected with p53 5'UTR-cDNA-3'UTR, anti-miRs. GFP expression from a pCDGFP plasmid vector was measured as transfection efficiency control (Supplementary Figure S3M-Q).

Since the immunoprecipitation experiments (Figure 4A-F) with Ago-2 and PTB suggested a possible interplay between PTB and the miRNAs, we further investigated the interplay between PTB and the miRNAs (miR-30d and miR-1285). For this, the H1299 cells were co-transfected with plasmid expressing luciferase-p53 3'UTR (Luc-3'UTR) and anti-miR-30d/ anti-miR-1285. RNA protein complexes were then immunoprecipitated with anti-PTB antibody. The p53 3'UTR was detected by RT-qPCR. With the partial silencing of miR-1285, an increase in the association of p53 3'UTR with PTB was observed, whereas no such increase was seen with anti-miR-30d (Figure 5E, Supplementary Figure S4A and B). Hence, results suggest a direct, reciprocal interplay between PTB and miR-1285 at the p53 3'UTR. In an alternate approach, to validate this interplay, H1299 cells were transfected with Fluc constructs having either wild type p53 3'UTR or 3'UTR mutant for miR-1285

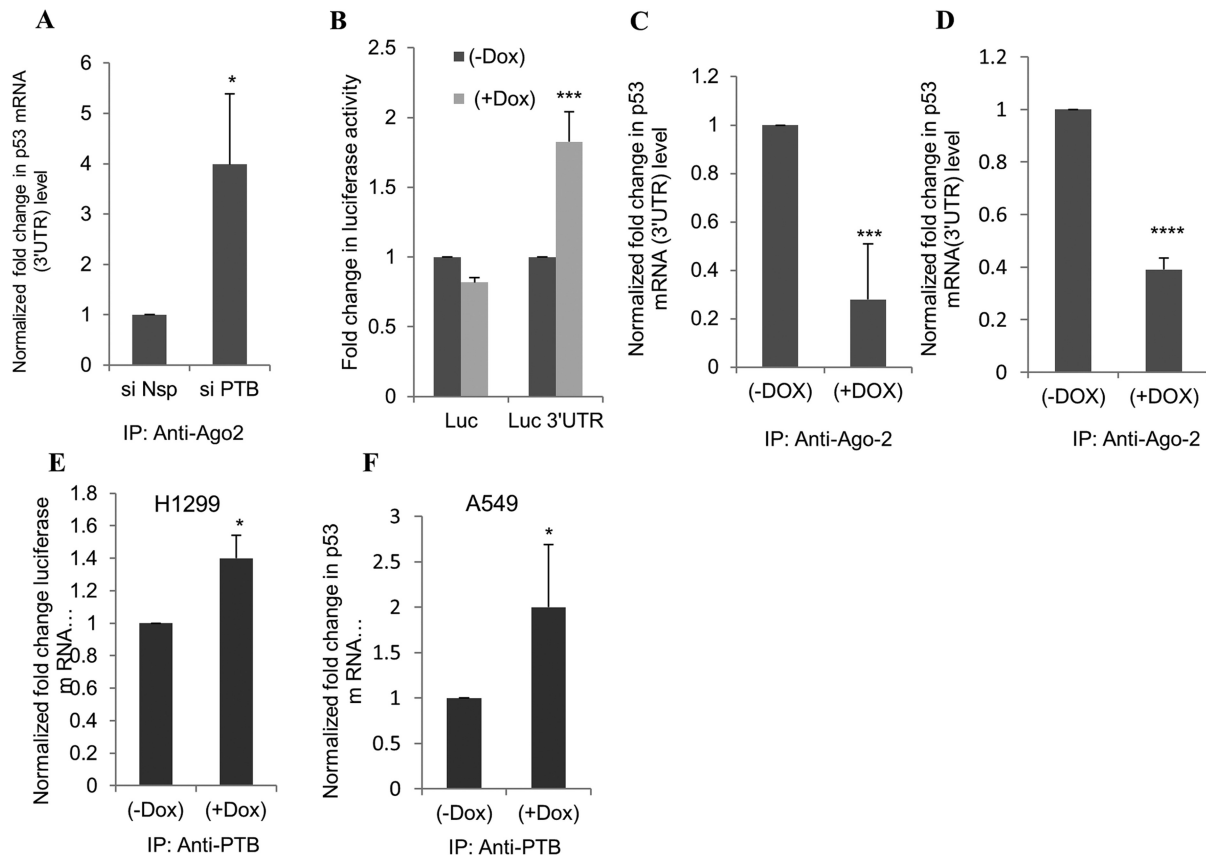


Figure 4. Interplay between the ITAFs and miRNAs at 3'UTR. (A) H1299 cells were co-transfected with Luc-3'UTR and either si PTB/non specific siRNA (50 nM). After 48 h, the cells were lysed with lysis buffer. RNP complexes were immunoprecipitated with anti- Ago-2/IgG antibody. p53 3'UTR was detected by RT-PCR analysis. The values for RNA immunoprecipitated with antiAgo-2 antibody in different conditions (si Nsp and si PTB) were first normalized with its respective values for RNA immunoprecipitated with IgG control antibody and then the fold change was calculated using the value for 3'UTR pulled down with anti-Ago-2 upon si Nsp transfection as the basal value ($n = 4$). (B) H1299 cells were transfected individually with the reporter constructs Fluc (labeled as Luc), Fluc-3'UTR (labeled as Luc3'UTR). These cells were treated with doxorubicin (2 μ M) for 16 h. After 24 h, the cells were harvested and checked for luciferase activity ($n = 5$). (C) H1299 cells were transfected with plasmid expressing luciferase-p53 3'UTR (Luc 3'UTR). The cells were treated with doxorubicin (2 μ M) for 16 hours. After 24 hours the cells were harvested. RNP complexes were immunoprecipitated with anti-Ago-2 antibody. p53 3'UTR was detected by RT-PCR analysis. The values for RNA immunoprecipitated with anti Ago-2 antibody in different conditions (+/- DOX) were first normalized with its respective values for RNA immunoprecipitated with IgG control antibody and then the fold change was calculated using the value for 3'UTR pulled down with anti-Ago-2, without doxorubicin treatment as the basal value ($n = 5$). (D) RNP complexes were immunoprecipitated with anti-Ago-2 antibody and endogenous p53 3'UTR was detected by RT-PCR analysis from A549 cells in presence and absence of doxorubicin (2 μ M) for 16 h. The values for RNA immunoprecipitated with anti Ago-2 antibody in different conditions (+/- DOX) were first normalized with its respective values for RNA immunoprecipitated with IgG control antibody and then the fold change was calculated as in Figure 4C ($n = 5$). (E) H1299 cells were transfected with plasmid expressing luciferase-p53 3'UTR (Luc 3'UTR) or luciferase (Fluc) alone. The cells were treated with doxorubicin (2 μ M) for 16 h. After 24 h, the cells were harvested. RNP complexes were immunoprecipitated with anti-PTB antibody. Luciferase mRNA was detected by RT-PCR analysis. The values for RNA immunoprecipitated with anti PTB antibody under different conditions (+/- DOX) were first normalized with its respective values for RNA immunoprecipitated with IgG control antibody, and then the fold change was calculated using the value for 3'UTR pulled down with anti-PTB, without doxorubicin treatment as the basal value ($n = 3$). (F) RNP complexes were immunoprecipitated with anti-PTB antibody and endogenous p53 3'UTR was detected by RT-PCR analysis from A549 cells in the presence and absence of doxorubicin (2 μ M) for 16 h. The values for RNA immunoprecipitated with anti PTB antibody in different conditions (+/- DOX) were first normalized with its respective values for RNA immunoprecipitated with IgG control antibody and then the fold change was calculated as in D ($n = 4$).

binding sites and GFP plasmid as transfection control. The constructs with mutant 3'UTRs showed increased luciferase activity as compared to the wild type. Luciferase expression was highest in the construct in which both miR-1285 binding sites were mutated (Figure 5F), hence, further experiments were performed with this construct. Mutations in miR-1285 binding sites were also confirmed by using the anti-miR 1285 in the cells transfected either with wild type or double-mutant 3'UTR (M1 + M2). The luciferase activity of wild type construct doubled with anti-miR but not with the mutant constructs (Supplementary Figure S4 C-

D). After validating the effect of mutations in the 3'UTR, IP-RT experiment with anti PTB/IgG antibody was performed with H1299 cells transfected with Luc 3'UTR-WT or -M1M2. Results showed that PTB interaction was significantly higher for M1 + M2 3'UTR that is deficient in miRNA binding as compared to the WT 3'UTR (Figure 5G, Supplementary Figure S4E). Similar experiment was also done with anti-Ago-2/IgG antibody to examine the level of association of wild type and mutant 3'UTR. In comparison with wild type 3'UTR, decreased association of the mutant 3'UTR was observed with Ago-2 (Figure 5H, Sup-

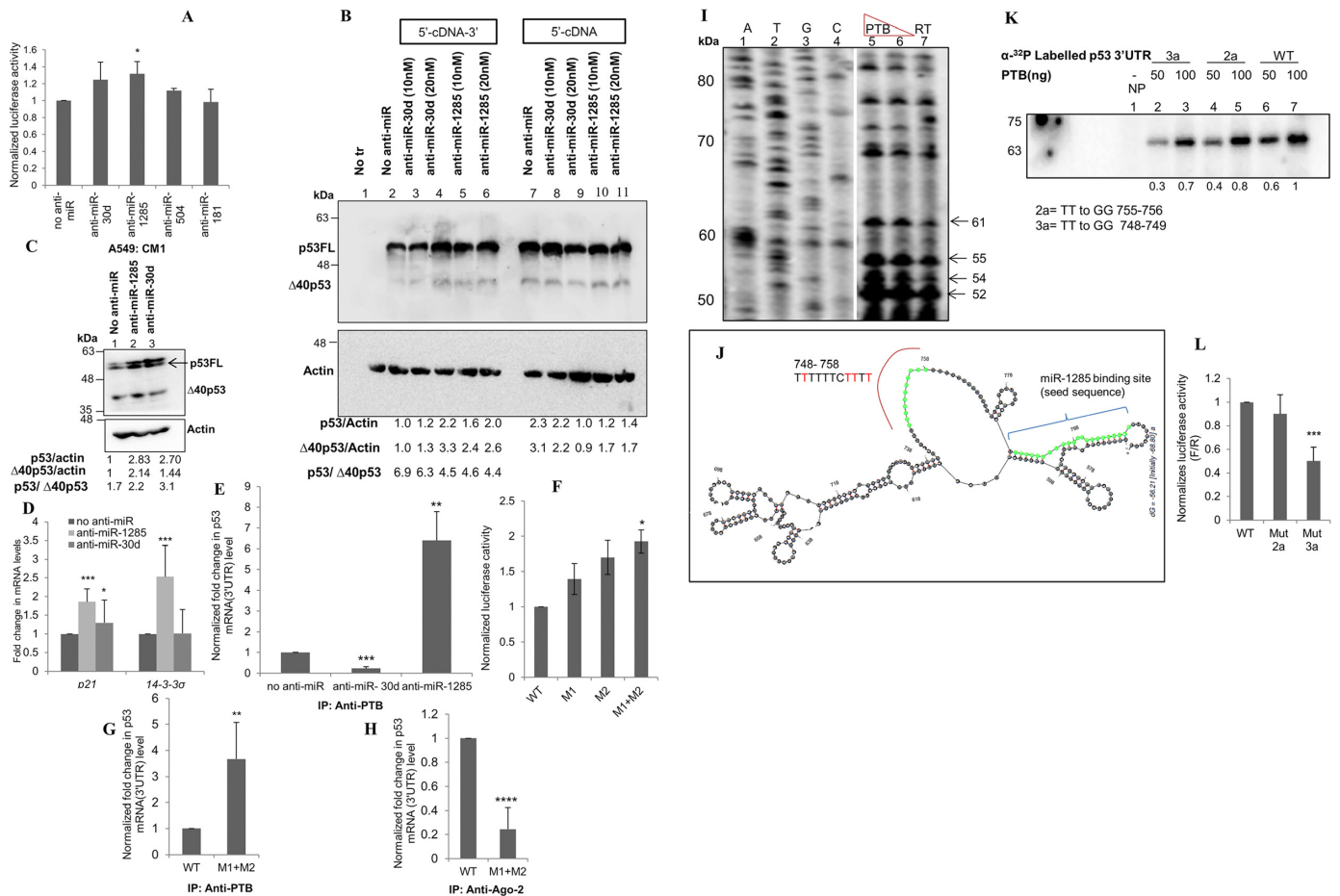


Figure 5. Effect of knockdown of miRNAs on the expression of p53 isoforms and miRNAs interplay with PTB. (A) H1299 cells were co-transfected with Luc-3'UTR and anti-miRNAs for miR-30d/miR-1285/miR-504/miR-181 (10 nM) and Rluc (Renilla luciferase) as transfection control. After 48 h, the cells were lysed and processed for luciferase activity. The graph represents the normalized luciferase activity, i.e. Fluc/Rluc (F/R) ($n = 3$). (B) H1299 cells were co-transfected with p53 cDNA construct having 3'UTR (5'UTR + cDNA + 3'UTR) or no 3'UTR (5'UTR + cDNA) and anti-miR-30d/anti-miR-1285 in two concentrations (10 nM and 20 nM). After 48 h, the cells were lysed and processed for immunoblotting. (C) Western blot analysis of cell extracts from A549-cells transfected with anti-miRNAs for miR-1285/miR-30d (30 nM), probed with CM1 after 48 h. Upper panel: p53 and Δ40p53; lower panel: actin. (D) Quantitative PCR of *p21* and *14-3-3σ* mRNA levels normalized to Actin in A549 cells transfected with anti-miRNAs for miR-1285/ miR-30d (30 nM) for 48 h ($n = 3$). (E) H1299 cells were co-transfected with Luc-3'UTR and anti-miRNAs for miR-30d/miR-1285 (20 nM). After 48 h, the cells were lysed and RNP complexes were immunoprecipitated with anti-PTB antibody. RT PCR was performed for checking the levels of 3'UTR associated with PTB. The values for RNA immunoprecipitated with anti PTB antibody in different conditions (no anti-miR, anti-miR-30d and anti-miR-1285) were first normalized with its respective values for RNA immunoprecipitated with IgG control antibody and then the fold change was calculated using the value for 3'UTR pulled down with anti-PTB, without anti-miR treatment as the basal value ($n = 5$). (F) H1299 cells were transfected with wild type Fluc -3'UTR (labeled as WT), Fluc -3'UTR mut miR-1285 M1 (3'UTR mutated for miR-1285 one binding site, labeled as M1), Fluc -3'UTR mut miR-1285 M1+M2 (3'UTR mutated for miR-1285 both binding sites, labeled as M1 + M2) and GFP expression from a GFP plasmid vector was used as transfection efficiency control. After 24 h, the cells were lysed and processed for luciferase activity. The graph shows normalized Fluc activity of different constructs ($n = 3$). (G) H1299 cells were transfected with either wild type Fluc -3'UTR (labeled as WT) or Fluc -3'UTR mut miR-1285 M1+M2 (3'UTR mutated for miR-1285 both binding sites, labeled as M1 + M2). After 24 h, the cells were lysed and RNP complexes were immunoprecipitated with anti-PTB antibody. RT PCR was performed for checking the levels of 3'UTR associated with PTB. The values for RNA (WT and M1 + M2) immunoprecipitated with anti PTB antibody were first normalized with its respective values for RNA immunoprecipitated with IgG control antibody, and then the fold change was calculated using the value for 3'UTR pulled down with anti-PTB from WT-3'UTR transfected cells as the basal value ($n = 6$). (H) H1299 cells were transfected with either wild type Fluc -3'UTR (labeled as WT) or Fluc -3'UTR mut miR-1285 M1+M2 (3'UTR mutated for miR-1285 both binding sites, labeled as M1 + M2). After 24 h, the cells were lysed and RNP complexes were immunoprecipitated with anti-Ago-2 antibody. q-RT PCR was performed for checking the levels of 3'UTR associated with Ago-2. The values for RNA (WT and M1+M2) immunoprecipitated with anti Ago-2 antibody were first normalized with its respective values for RNA immunoprecipitated with IgG control antibody and then the fold change was calculated using the value for 3'UTR pulled down with anti-Ago-2 from WT-3'UTR transfected cells as the basal value ($n = 6$). (I) Toe-printing analysis to map the contact points of PTB on p53 3'UTR. p53 3'UTR RNA was incubated in absence (lane 7) and presence (lanes 5 and 6) of increasing concentrations of purified recombinant PTB (200 ng, 400 ng). The RNA in the ribonucleoprotein complexes were reverse transcribed using 3R3 reverse primer (p53 3'UTR region 3 reverse primer) and the resulting cDNAs were resolved in 8% acrylamide-8 M urea PAGE in parallel with a sequencing reaction. The cDNA products terminated at the sites due to protein binding is marked by the arrows. The toe-prints at positions 61 (749T), 55 (755T), 54 (756T), 52 (758T) in the 3' UTR are indicated. (J) Schematic representation of the toe-prints of PTB protein on putative predicted secondary structure of p53 3'UTR R-III (third region) RNA (nucleotides 759–809 in 3'UTR) generated by MFOLD. Nucleotides 748 to 758 bind PTB and are pointed out in red. The curly bracket region shows the miR-1285 binding site (seed sequence). (K) α-³²P-labeled 3'UTR (wild type: WT, mutant: 2a = TT to GG 755–756, 3a = TT to GG 748–749) were UV crosslinked with increasing concentration of PTB (50 ng, 100 ng), lane 1 is no protein (NP). (L) H1299 cells were transfected with wild type Luc -3'UTR (labeled as WT), Luc -3'UTR Mut2a and Luc -3'UTR Mut3a (Mutant: 2a = TT to GG 755–756, 3a = TT to GG 748–749) and Rluc was used as transfection control. After 24 h, the cells were lysed and processed for luciferase activity. The graph shows the normalized Fluc activity (F/R) of different constructs ($n = 6$).

Table 2. Binding regions in p53 3'UTR for PTB, and miRNAs. The positions of SNVs present at the miRNA binding sites in the p53 3'UTR are also shown

REGIONS	I	II	III	IV	SNVs
PTB		++	+++		
miR-30-5p (Kumar <i>et al.</i> , 2011)		✓		✓	287–292
miR-25-3p (Kumar <i>et al.</i> , 2011)				✓	93–98
miR-125b (Le <i>et al.</i> , 2009)	✓		✓		734–739
miR-504 (Hu <i>et al.</i> , 2010)	✓		✓		736–741 and 1065–1070
miR-1285 (Tian <i>et al.</i> , 2010)			✓	✓	804–809
miR-380-5p			✓		93–98
miR-92a	✓				
miR-141				✓	
miR-200a				✓	
miR-15a				✓	1084–1089
miR-16				✓	1084–1089

plementary Figure S4F). Therefore, both the approaches suggest the interplay between PTB and miR-1285 at the 3'UTR.

Since our results provide evidence of interplay between PTB and miR-1285 at the 3'UTR, further, putative contact points of PTB were examined using primer-extension inhibition (toe-printing) assay to determine the distance between PTB and miR-1285 binding site at the 3'UTR. In this assay, *in vitro* transcribed full length p53 3'UTR mRNA was incubated with increasing concentrations of PTB protein. A reverse primer within R-III (as described previously) was used in this assay because it lies close to the miR-1285 binding site. PTB toe-prints were determined by comparison with corresponding DNA sequencing reaction. We observed distinct toe-prints at positions 61 (749T), 55 (755T), 54 (756T), 52 (758T) in the 3' UTR (Figure 5I). Interestingly, these toe-prints were within 30 nucleotides from the seed sequence of miR-1285 binding site. The results of the toe-printing assays are schematically represented in Figure 5J. Further these results were validated by generating mutants of the toe-prints by performing UV-crosslinking assay with α -³²P-labeled wild type/mutant (Mutant: 2a = TT to GG 755–756, 3a = TT to GG 748–749) 3'UTR and recombinant PTB protein. Decreased binding was observed in both mutants as compared to the wild type 3'UTR (Figure 5K). Effect of this decreased binding was checked by luciferase assay with luc 3'UTR construct having either the wild type or mutant (2a, 3a) 3'UTR. Decreased luciferase activity was observed in both the mutants as compared to wild type, but the decrease was significant in case of mutant 3a (TT to GG 748–749) (Figure 5L).

SNVs in the p53 3'UTR can affect 3'UTR function

Furthermore, we curated single nucleotide variations (SNVs) in the p53 3'UTR, at the miRNA binding sites from literature (21), as shown in Table 3. We investigated the effect of these SNVs on the 3'UTR mediated regulation of p53 expression by using reporter gene constructs containing wild type 3'UTR (Luc-3'UTR WT) or 3'UTRs harbouring individual SNVs (Fluc-3'UTR SNV 93/287/737/806) in H1299 and A549 cells. Changes observed in the luciferase activity due to the SNVs were not significant compared to wild type, except SNV 93 and SNV 806; SNV 806 displayed highest reporter activity compared to the WT or

Table 3. Positions of SNVs present in the 3'UTR for some of the miRNAs are listed

miRNAs	Position of SNVs
hsa-miR-30d	287 G>A
hsa-miR-25/92a	93 G>A
hsa-miR-504	737 G>C
hsa-miR-1285	806 C>T

other SNVs and there were no significant changes in mRNA level of SNV 806–3'UTR as compared to wild type (Figure 6A and B, Supplementary Figure S5).

To check if SNV 806 affected the protein binding to the 3'UTR, UV-cross linking experiment was performed with increasing concentration of cytoplasmic S10 extract and recombinant PTB protein, using α -³²P-labeled 3'UTR WT or α -³²P-labeled 3'UTR SNV 806 probe. There was a significant increase in binding of a protein (approximately 63 kDa) with the SNV 806 as compared to the WT 3'UTR. From our earlier studies, this protein was anticipated to be PTB. Further, to verify this observation, we also found a similar increase in the binding with recombinant PTB protein with SNV 806 as compared to the WT 3'UTR (Figure 6C). Taken together, these results demonstrate the molecular basis of miR1285 and PTB interplay in 3'UTR-mediated regulation of p53 gene expression under physiological and stress conditions.

DISCUSSION

Interaction of different RNA binding proteins (RBPs) with 5'UTR and 3'UTR of p53 mRNA is necessary for regulating the expression of p53. Some of these proteins such as RPL26 bind to both the UTRs and regulate its translation under DNA damage (18). Our earlier studies have reported PTB to be an important ITAF for p53-IRES mediated translation under DNA damage (9). Current study reveals that PTB interacts specifically with 3'UTR of p53 mRNA as confirmed by *in vitro* and *ex vivo* experiments, and functionally regulates the expression of p53 and Δ 40p53 and their targets.

miRNAs are known to repress p53 gene expression through interaction with 3'UTR as described by Kumar *et al.* (16). Therefore, the decrease in expression of luciferase as well as p53 isoforms in cDNA constructs in the pres-

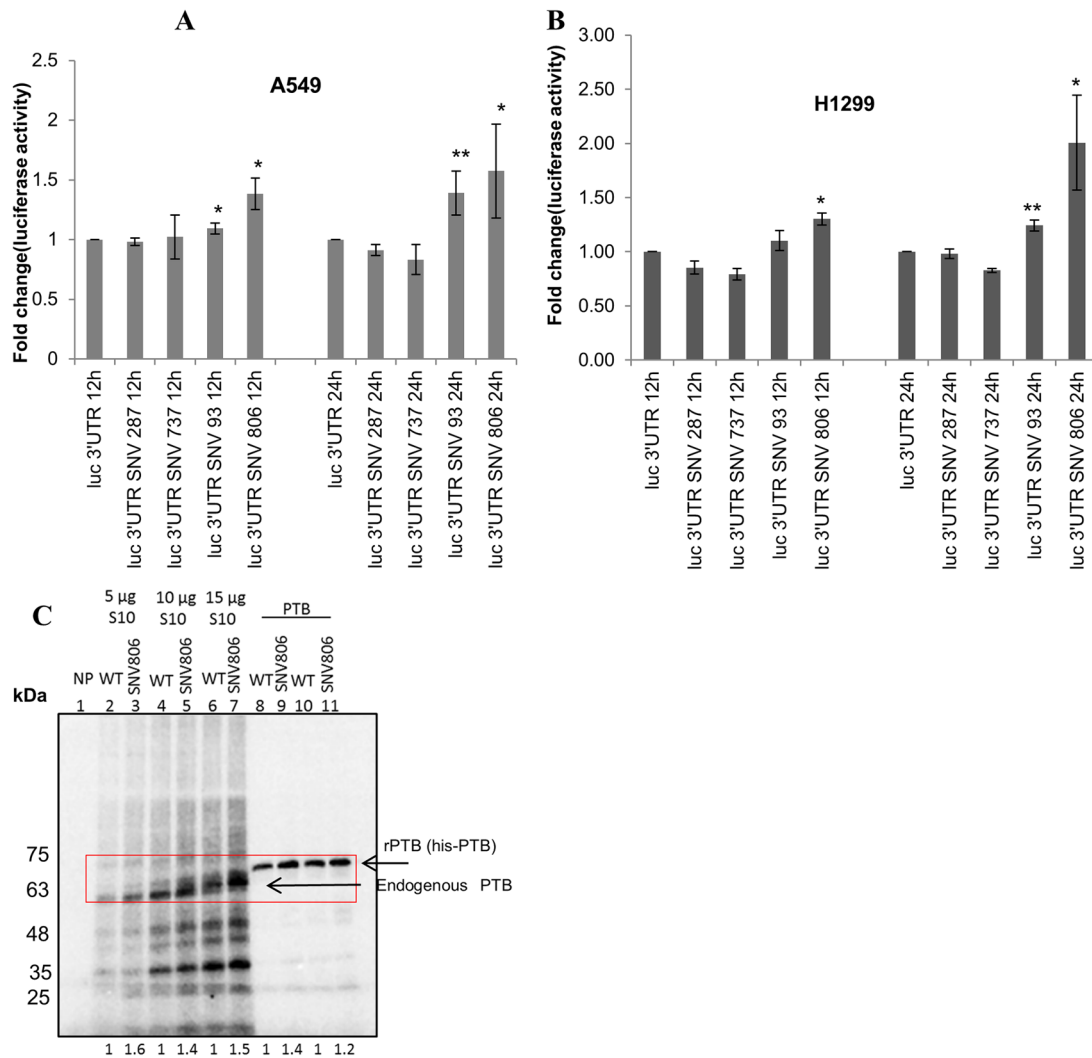


Figure 6. SNVs in the p53 3'UTR can affect 3'UTR function. (A) A549 cells were transfected with luciferase constructs having either wild type 3'UTR or 3'UTR with the SNVs (SNV 287, SNV 737, SNV 93 and SNV 806). After 12 h and 24 h, the cells were lysed and processed for luciferase activity ($n = 6$). (B) H1299 cells were transfected with luciferase constructs having either wild type 3'UTR or 3'UTR with the SNVs (SNV 287, SNV 737, SNV 93 and SNV 806). After 12 h and 24 h, the cells were lysed and processed for luciferase activity ($n = 6$). (C) Cytoplasmic-S10 extract from A549 cells at three different concentrations) was incubated with α - 32 P UTP labeled p53 3'UTR WT and p53 3'UTR having the SNV 806 (C-T) RNA for UV cross-linking. Protein bands in the box show that PTB binding in both S10 and recombinant protein is more with p53 3'UTR having the SNV 806 (C-T) as compared to WT 3'UTR.

ence of 3'UTR, as shown in this study, can be attributed to the interaction between miRNAs and 3'UTR. Further, miRNAs and the RBPs together play a crucial role in the regulation of gene expression. For example, miR-297 or miR-299-mediated repression of VEGF mRNA was shown to be relieved by HNRNPL (22). Other studies have reported IGFBP2 to alleviate miR-183 mediated repression of BTRC mRNA (23) and miR-340-mediated repression of the 3' UTR of microphthalmia-induced transcription factor (MITF) mRNA (24). In addition, it has also been shown that HuR inhibits miR-21-mediated translation repression of *PDCD4* mRNA by binding to its 3'UTR in MCF7 breast carcinoma cells (25). Surprisingly, such studies are scarce for critical tumor suppressor p53. One recent study reports the interplay of HuR and miR125b in regulating p53 translation in genotoxic stress (19). In the current study, *in vitro*

experiments identified the possible binding regions of PTB in p53 3'UTR, that overlap with the reported miRNA binding sites. The increased association of p53 3'UTR with Ago-2 after partial silencing of PTB provides evidence of such an interplay.

The interplay between the miRNAs and PTB can modulate and fine-tune the expression of p53 isoforms under different conditions like DNA damage. In this study, the experiments in presence of doxorubicin showed an increase in reporter activity when p53 3'UTR was present, increased endogenous p53 and $\Delta 40$ p53 levels, and also their respective target (Supplementary Figure S2G–H); at the same time, upon doxorubicin treatment there was decreased association of p53 3'UTR with Ago-2, and in contrast, an increased association with PTB, even at slightly elevated p53 targeting miRNA levels. This points towards the physiological rele-

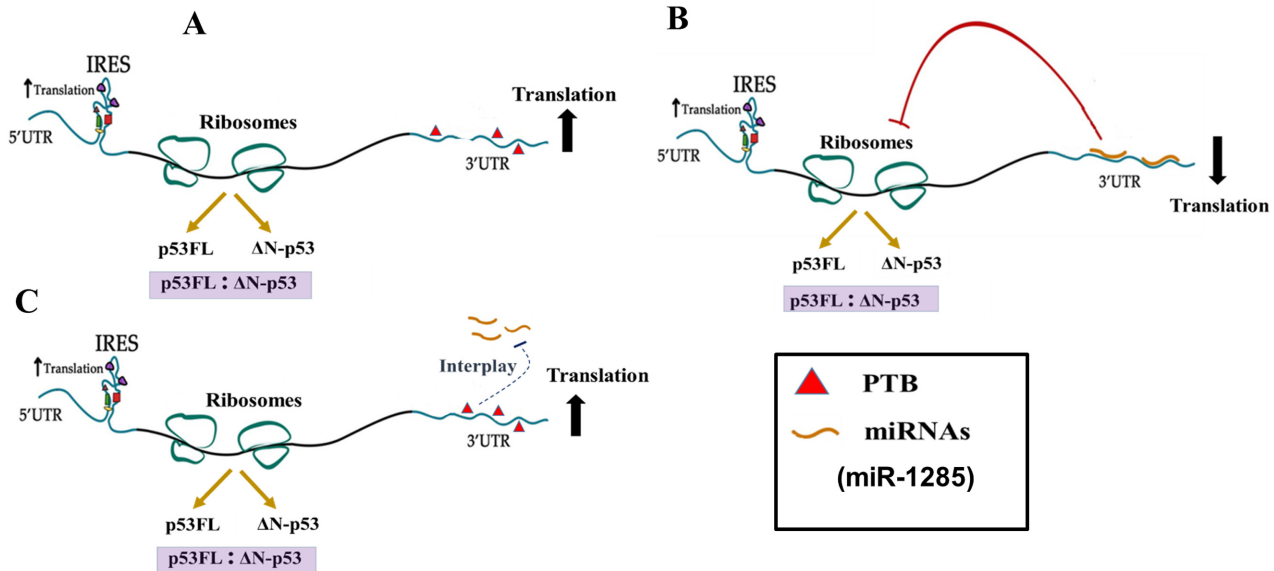


Figure 7. Model depicting the interplay between the proteins and miRNAs. (A–C) Model showing the interplay between the proteins (PTB) and miRNAs (miR-1285) at the p53 3'UTR resulting in the fine tuning of the expression of p53 isoforms.

vance of proteins and miRNA interplay under stress conditions (like DNA damage). Further, silencing of miRNAs using anti-miRs leads to an increase in the luciferase activity in the Fluc-3'UTR construct as expected. This increase was higher for anti-miR-30d and anti-miR-1285 compared to anti-miRs against other miRNAs, while anti-miR-181, used as a negative control, did not show any significant change. Therefore, miR-30d and miR-1285 were investigated to gain further understanding. Decreased levels of miR-1285 also increased endogenous expression of both the p53 isoforms, followed by increase in their respective targets in A549 cells. p53 3'UTR association increased with PTB on silencing of miR-1285 but not miR-30d. A p53 3'UTR, which lacks miR1285 binding sites, showed higher degree of PTB association compared to WT 3'UTR. On the contrary, the mutant 3'UTR showed a decreased interaction with Ago-2, which further strengthened and provided evidence of the possible interplay among PTB, miR1285 and p53 3'UTR *ex vivo*. Interplay between a protein and miRNA can occur if their binding sites are common or neighboring to each other. Toe-printing results showed that PTB binds close to the binding site of miR-1285, which is another evidence of interplay between the two.

An earlier study has reported single nucleotide variations (SNVs) in p53 3'UTR in diffuse large B-cell lymphoma (DLBCL) tumor DNA samples (21). Most of the 3'UTR SNVs were located in validated or putative miRNA-binding sites. Interestingly, SNV806 (C>T), was found to be present at the miR-1285 binding site in the 3'UTR. Luciferase reporter with p53 3'UTR harboring SNV 806 showed higher luciferase activity as compared to the wild type 3'UTR, whereas there was no significant change in its RNA level. The change in luciferase activity could also be partly due to the interaction of different RNA binding proteins with 3'UTR. Interestingly, *in vitro* experiments in the current study indicates that PTB binding to the 3'UTR with SNV

806 is higher compared to WT 3'UTR, thus, also suggesting a possibility of miR-1285 and PTB having common binding regions. The interplay between miR-1285 and PTB might be enhanced under DNA damage stress, and play a critical role in regulating the expression of p53 isoforms.

In summary, we have shown that PTB, which is an established p53 ITAF, interact with the 3'UTR of p53 mRNA, thus regulating the 3'UTR mediated p53 isoform expression. Our study also provides an evidence for the possible interplay between the miRNAs and 3'UTR-binding ITAF, i.e. between miR-1285 and PTB (Figure 7A–C). Overall, the current study provides key insights into the understanding of the translation regulation of p53 mRNA by miRNA and ITAF through 3'UTR. It would also be interesting to investigate other miRNAs, which show such an interplay with PTB or any other ITAF under physiological and different stress conditions at the p53 3'UTR.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We are grateful to Prof. J.C.Bourdon, of University of Dundee, UK and Dr Robin Fahraeus, INSERM, France, for providing us the anti-p53 antibody; Prof. Partho Sarothi Ray, IISER, Kolkata, for sharing p53 3'UTR-Luciferase constructs. Dr Suvendra Bhattacharya and Shwetha S are acknowledged for critical reading of the manuscript. We also thank the present and past SD lab-members for helpful discussion of the work.

Author contributions: A.K., D.K. and S.D.: conception and design of studies, analysis and interpretation, and writing the article; A.K., B.G. and A.A.: generation of constructs, performing experiments and data analysis.

FUNDING

Department of Biotechnology (DBT), Govt. of India (to S.D.); J.C. Bose fellowship for research support (to S.D.); UGC-Kothari (to B.G.); DBT-partnership program with the Indian Institute of Science. DST Fund for Improvement of Science and Technology Infrastructure (FIST) level II infrastructure and University Grants Commission Centre of Advanced Studies support is acknowledged. Funding for open access charge: Department of Biotechnology, Government of India.

Conflict of interest statement. None declared.

REFERENCES

- Levine, A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323–331.
- Marcel, V., Dichtel-Danjoy, M.L., Sagne, C., Hafsi, H., Ma, D., Ortiz-Cuaran, S., Olivier, M., Hall, J., Mollereau, B., Hainaut, P. *et al.* (2011) Biological functions of p53 isoforms through evolution: lessons from animal and cellular models. *Cell Death Differ.*, **18**, 1815–1824.
- Ray, P.S., Grover, R. and Das, S. (2006) Two internal ribosome entry sites mediate the translation of P53 isoforms. *EMBO Rep.*, **7**, 404–410.
- Candéias, M.M., Powell, D.J., Roubalova, E., Apcher, S., Bourougaa, K., Vojtesek, B., Bruzzoni-Giovanelli, H. and Fahraeus, R. (2006) Expression of p53 and p53/47 are controlled by alternative mechanisms of messenger RNA translation initiation. *Oncogene*, **25**, 6936–6947.
- Courtois, S., Verhaegh, G., North, S., Luciani, M.G., Lassus, P., Hibner, U., Oren, M. and Hainaut, P. (2002) DeltaN-p53, a natural isoform of P53 lacking the first transactivation domain, counteracts growth suppression by wild-type p53. *Oncogene*, **21**, 6722–6728.
- Takwi, A. and Li, Y. (2009) The p53 pathway encounters the microRNA world. *Curr. Genomics*, **10**, 194–197.
- Mazan-Mamczarz, K., Galban, S., Lopez de Silanes, I., Martindale, J.L., Atasoy, U., Keene, J.D. and Gorospe, M. (2003) RNA-binding protein HuR enhances P53 translation in response to ultraviolet light irradiation. *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 8354–8359.
- Takagi, M., Absalon, M.J., McLure, K.G. and Kastan, M.B. (2005) Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. *Cell*, **123**, 49–63.
- Grover, R., Ray, P.S. and Das, S. (2008) Polypyrimidine tract binding protein regulates IRES-mediated translation of p53 isoforms. *Cell Cycle*, **7**, 2189–2198.
- Sharathchandra, A., Lal, R., Khan, D. and Das, S. (2012) Annexin A2 and PSF proteins interact with p53 IRES and regulate translation of p53 mRNA. *RNA Biol.*, **9**, 1429–1439.
- Weingarten-Gabbay, S., Khan, D., Liberman, N., Yoffe, Y., Bialik, S., Das, S., Oren, M. and Kimchi, A. (2014) The translation initiation factor DAP5 promotes IRES-driven translation of p53 mRNA. *Oncogene*, **33**, 611–618.
- Halaby, M.J., Harris, B.R., Miskimins, W.K., Cleary, M.P. and Yang, D.Q. (2015) Dereglulation of internal ribosome entry site-mediated p53 translation in cancer cells with defective p53 response to DNA damage. *Mol. Cell. Biol.*, **35**, 4006–4017.
- Khan, D., Katoch, A., Das, A., Sharathchandra, A., Lal, R., Roy, P., Das, S., Chattopadhyay, S. and Das, S. (2015) Reversible induction of translational isoforms of p53 in glucose deprivation. *Cell Death Differ.*, **22**, 1203–1218.
- Rosenstierne, M.W., Vinther, J., Mittler, G., Larsen, L., Mann, M. and Norrild, B. (2008) Conserved CPEs in the p53 3' untranslated region influence mRNA stability and protein synthesis. *Anticancer Res.*, **28**, 2553–2559.
- Nakamura, H., Kawagishi, H., Watanabe, A., Sugimoto, K., Maruyama, M. and Sugimoto, M. (2011) Cooperative role of the RNA-binding proteins Hzf and HuR in p53 activation. *Mol. Cell. Biol.*, **31**, 1997–2009.
- Kumar, M., Lu, Z., Takwi, A.A., Chen, W., Callander, N.S., Ramos, K.S., Young, K.H. and Li, Y. (2011) Negative regulation of the tumor suppressor p53 gene by microRNAs. *Oncogene*, **30**, 843–853.
- Tian, S., Huang, S., Wu, S., Guo, W., Li, J. and He, X. (2010) MicroRNA-1285 inhibits the expression of p53 by directly targeting its 3' untranslated region. *Biochem. Biophys. Res. Commun.*, **396**, 435–439.
- Chen, J. and Kastan, M.B. (2010) 5'-3'-UTR Interactions Regulate p53 mRNA translation and provide a target for modulating p53 induction after DNA damage. *Genes Dev.*, **24**, 2146–2156.
- Ahuja, D., Goyal, A. and Ray, P.S. (2016) Interplay between RNA-binding protein HuR and microRNA-125b regulates p53 mRNA translation in response to genotoxic stress. *RNA Biol.*, **13**, 1152–1165.
- Zuker, M. (2003) Mfold web server For nucleic acid folding and hybridization prediction. *Nucleic Acids Res.*, **31**, 3406–3415.
- Li, Y., Gordon, M.W., Xu-Monette, Z.Y., Visco, C., Tzankov, A., Zou, D., Qiu, L., Montes-Moreno, S., Dybkaer, K., Orazi, A. *et al.* (2013) Single nucleotide variation in the TP53 3' untranslated region in diffuse large B-cell lymphoma treated with rituximab-CHOP: a report from the international DLBCL rituximab-CHOP consortium program. *Blood*, **121**, 4529–4540.
- Jafarifar, F., Yao, P., Eswarappa, S.M. and Fox, P.L. (2011) Repression of VEGFA by CA-rich element-binding microRNAs is modulated by hnRNP L. *EMBO J.*, **30**, 1324–1334.
- Elcheva, I., Goswami, S., Noubissi, F.K. and Spiegelman, V.S. (2009) CRD-BP protects the coding region of betaTrCP1 mRNA from miR-183-mediated degradation. *Mol. Cell*, **35**, 240–246.
- Goswami, S., Tarapore, R.S., Teslaa, J.J., Grinblat, Y., Setaluri, V. and Spiegelman, V.S. (2010) MicroRNA-340-mediated degradation of microphthalmia-associated transcription factor mRNA is inhibited by the coding region determinant-binding protein. *J. Biol. Chem.*, **285**, 20532–20540.
- Poria, D.K., Guha, A., Nandi, I. and Ray, P.S. (2015) RNA-binding protein HuR sequesters microRNA-21 to prevent translation repression of proinflammatory tumor suppressor gene programmed cell death 4. *Oncogene*, **35**, 1703–1715.