

RESEARCH PAPER

 OPEN ACCESS

Interplay between RNA-binding protein HuR and microRNA-125b regulates p53 mRNA translation in response to genotoxic stress

Deepika Ahuja , Ashish Goyal *, and Partho Sarothi Ray

Department of Biological Sciences, Indian Institute of Science Education and Research, Kolkata, Mohanpur, Nadia, West Bengal, India

ABSTRACT

Tumor suppressor protein p53 plays a crucial role in maintaining genomic integrity in response to DNA damage. Regulation of translation of p53 mRNA is a major mode of regulation of p53 expression under genotoxic stress. The AU/U-rich element-binding protein HuR has been shown to bind to p53 mRNA 3'UTR and enhance translation in response to DNA-damaging UVC radiation. On the other hand, the microRNA miR-125b is reported to repress p53 expression and stress-induced apoptosis. Here, we show that UVC radiation causes an increase in miR-125b level in a biphasic manner, as well as nuclear cytoplasmic translocation of HuR. Binding of HuR to the p53 mRNA 3'UTR, especially at a site adjacent to the miR-125b target site, causes dissociation of the p53 mRNA from the RNA-induced silencing complex (RISC) and inhibits the miR-125b-mediated translation repression of p53. HuR prevents the oncogenic effect of miR-125b by reversing the decrease in apoptosis and increase in cell proliferation caused by the overexpression of miR-125b. The antagonistic interplay between miR-125b and HuR might play an important role in fine-tuning p53 gene expression at the post-transcriptional level, and thereby regulate the cellular response to genotoxic stress.

Abbreviations: RBP, RNA-binding protein; miRNA, microRNA; UTR, untranslated region; ARE, AU-rich element; ncRNA, non-coding RNA; UVC, ultra violet C; RISC, RNA-induced silencing complex; PAR-CLIP, Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation

ARTICLE HISTORY

Received 9 May 2016
Revised 9 August 2016
Accepted 24 August 2016

KEYWORDS

Cancer; DNA damage; HuR; translation regulation; microRNA; miR-125b; p53; RNA-binding protein

Introduction

The tumor suppressor protein p53 plays a crucial role in maintaining genomic integrity by governing cell-cycle progression, DNA repair and apoptosis in response to genotoxic stress.¹ On exposure to stress stimuli such as DNA damage, hypoxia or nutrient deprivation, p53 is mostly activated through post-translational modifications and altered subcellular localization, resulting in increased protein stability and activity.² However, increasing evidence has shown that p53 expression is also controlled by the regulation of p53 mRNA stability and translation.³ Both RNA-binding proteins (RBPs) and non-coding RNAs (ncRNAs) have been implicated in such regulation.⁴ RBPs Ribosomal protein (RPL26), poly-pyrimidine tract binding protein (PTB) and nucleolin are reported to bind to the 5'-untranslated region (5'-UTR) of p53 mRNA and regulate cap-dependent and Internal Ribosome Entry site (IRES)-mediated translation.^{5,6} Similarly, Wig1 and HuR bind to the 3'-untranslated region (3'UTR) of p53 mRNA and enhance mRNA stability and translation.⁷⁻⁹ HuR is an RBP belonging to the Hu/ELAV (embryonic lethal abnormal vision) family of RBPs which interacts with AU- and U-rich elements mostly present in the 3'UTR of target mRNAs to regulate stability and/or

translation.¹⁰ HuR has been shown to bind to the p53 mRNA 3'UTR and enhance p53 translation in response to ultraviolet C (UVC) radiation.⁸ It has been suggested that translation regulation of p53 by HuR in response to genotoxic stress allows the synthesis of p53 protein while cellular transcription is paused during a period of DNA repair.

p53 translation is also negatively regulated by 2 miRNAs, miR-125a and miR-125b, which target the same miRNA response element in the p53 mRNA 3'UTR.^{11,12} miR-125b has been shown to be an important negative regulator of p53 protein synthesis, and p53-mediated apoptosis, during stress response in human cells and development of zebrafish embryos.¹² miR-125b is reported to act as a tumor suppressor in some cancers but as an oncogene in other cancer types.¹³ Negative regulation of p53 translation by miR-125b might be an important determinant of the latter's oncogenic activity.

While RBPs and miRNAs are known to independently regulate the stability and/or translation of specific mRNAs, a number of recent reports have demonstrated functional interplay between specific RBPs and miRNAs in regulating translation of multiple genes.¹⁴ This interplay can either be antagonistic, where the binding of the RBP prevents the binding of the

CONTACT Partho Sarothi Ray  psray@iiserkol.ac.in

*Current address: Division of RNA Biology & Cancer, German Cancer Research Center (DKFZ), Im Neuenheimer Feld, Heidelberg, Germany

 Supplemental data for this article can be accessed on the [publisher's website](#).

Published with license by Taylor & Francis Group, LLC © Deepika Ahuja, Ashish Goyal, and Partho Sarothi Ray

This is an Open Access article distributed under the terms of the Creative Commons Attribution-Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. The moral rights of the named author(s) have been asserted.

miRNA, such as in the case of HuR preventing the translation repression of the CAT1 mRNA by miR-122 during amino acid starvation or DND1 alleviating the miR-221 and miR-222-mediated repression of cyclin-dependent kinase inhibitor 1B (CDKN1B).^{15,16} It can also be cooperative, such as in the case of the RBP tristetraprolin (TTP) enabling the miR-16-mediated translation repression of tumor necrosis factor (TNF α) and cyclooxygenase 2 (COX2) mRNAs.¹⁷ Interplay between RBPs and miRNAs appear to be an important mode of fine-tuning gene expression in various cellular processes, especially cancer. Recently, a long non-coding RNA, 7SL, was found to repress p53 translation by competing with HuR, but no such interplay between miRNAs and RBPs in regulating p53 mRNA translation is known.¹⁸

In this study we have shown the antagonistic interplay between HuR and miR-125b in regulating p53 mRNA translation under genotoxic stress. Binding of HuR to the p53 mRNA 3'UTR prevented miR-125b mediated translation repression, and counteracted the cell growth-promoting and anti-apoptotic activity of miR-125b. UVC irradiation caused nuclear-cytoplasmic translocation of HuR, which interacted with the p53 mRNA 3'UTR, allowing high level of p53 expression. HuR-mediated de-repression of p53 translation might therefore be a means of maintaining p53 level in presence of high concentration of miR-125b under genotoxic stress.

Results

HuR reverses miR-125b-mediated repression of p53 mRNA translation

Three sequences within the p53 3'UTR have been reported to be potential HuR binding sites (Fig. 1A). One, an U-rich sequence, is present exactly adjacent to the miR-125b binding site, and is predicted to form an independent single-stranded region by the Mfold RNA folding program (Fig. S1) suggesting that HuR binding might influence miR-125b mediated translation repression of p53. Overexpression of miR-125b inhibited p53 expression in MCF7 human breast carcinoma cells in a dose-dependent manner (Fig. 1B). However, exogenous expression of HuR in presence of the highest concentration of miR-125b, reversed the inhibition of p53 expression and restored p53 expression to near normal level. Overexpression of HuR alone caused a moderate increase in p53 expression (Fig. 1C). However, there was no significant change in p53 mRNA level either on miR-125b or HuR overexpression, indicating that HuR reversed the miR-125b-mediated translation repression of p53 mRNA without affecting the mRNA level (Fig. S2A). Also, overexpression of HuR did not reduce miR-125b level, suggesting that the effect of HuR was not mediated by the degradation or inhibition of synthesis of the miRNA (Fig. S2B). In order to check whether the effect of HuR was mediated via the p53 3'UTR, miR-125b was coexpressed with a reporter gene construct consisting of the p53 3'UTR inserted downstream of firefly luciferase gene. miR-125b overexpression showed a dose-dependent decrease in luciferase activity, which was rescued by overexpression of HuR, whereas no effect was observed on a firefly reporter construct lacking the p53 3'UTR (Fig. 1D). We also investigated whether interaction with HuR could dissociate the

p53 mRNA from the RISC complex. p53 mRNA was found to be associated with Ago2 by RNA-immunoprecipitation of lysates from cells expressing miR-125b (Fig. 1E). Conversely, HuR overexpression, even in presence of miR-125b, resulted in significantly enhanced association of p53 mRNA with HuR, suggesting that HuR binding could dissociate the p53 mRNA from the RISC complex. In order to confirm whether HuR reversed the translation repression of p53 mRNA by miR-125b, ribosomal fractionation was performed with cells overexpressing miR-125b, and miR-125b together with HuR. Analysis of the ribosomal fractions showed that p53 mRNA was mostly present in the non-translating and lighter polysomal fractions of the cells expressing miR-125b compared to the control cells, in which it was mostly associated with the heavier polysomal fractions (Fig. 1F and quantitation). However, overexpression of HuR caused the shifting of p53 mRNA to the heavier polysomal fractions, showing that HuR could reverse the translation repression of p53 caused by miR-125b. Overexpression of HuR alone also caused association of p53 mRNA with heavy polysomes, supporting the role of HuR in enhancing p53 mRNA translation.

UVC irradiation causes nuclear-cytoplasmic translocation of HuR and prevents miR-125b-mediated translation repression of p53

UVC-induced DNA damage is known as a potent stimulus for translational upregulation of p53.⁸ Exposure of MCF7 breast carcinoma cells to a 10 J/m² pulse of UVC radiation, followed by monitoring p53 protein level in the cytoplasm over a period of 12 hours, showed an increase of p53 from 2 hour onwards, reaching a maximum at around 4 hours and then decreasing to near-basal level by 12 hours post-UVC exposure (Fig. 2A). Interestingly, miR-125b expression showed a 90% decrease at 2 hour post-UVC exposure and then gradually increased by more than 10-fold in the next 10 hours (Fig. 2B). In order to validate whether the decreased p53 level at 8–12 hours post UVC-exposure was due to the increase in miR-125b, cells were transfected with an antisense oligo to miR-125b (miR-125b antagomiR) prior to UVC exposure. Cells transfected with miR-125b antagomiR showed increased p53 expression at 8–12 hours post UVC exposure, suggesting that the decrease in p53 level was due to high levels of miR-125b (Fig. S3). The increased level of p53 at 2 hour post UVC exposure might therefore be due to the large decrease of miR-125b at this time point. However, as p53 level was high at 4–6 hours post UVC exposure in presence of increased miR-125b, we checked whether HuR contributed to this upregulation of p53 protein by preventing miR-125b-mediated translation repression. Cytoplasmic HuR level showed a similar pattern as p53, increasing from 2 hour post UVC irradiation, reaching a maximum at 4 hours and subsequently decreasing, coinciding with the decrease in the p53 level (Fig. 2C). RNA immunoprecipitation using anti-HuR antibody, followed by semi-quantitative RT-PCR using p53-specific primers, showed enhanced association of p53 mRNA with HuR protein at 4 hour post-UVC treatment, which decreased at 12 hour (Fig. 2D). Immunofluorescence of UVC-irradiated cells using HuR-specific antibody showed nuclear-cytoplasmic

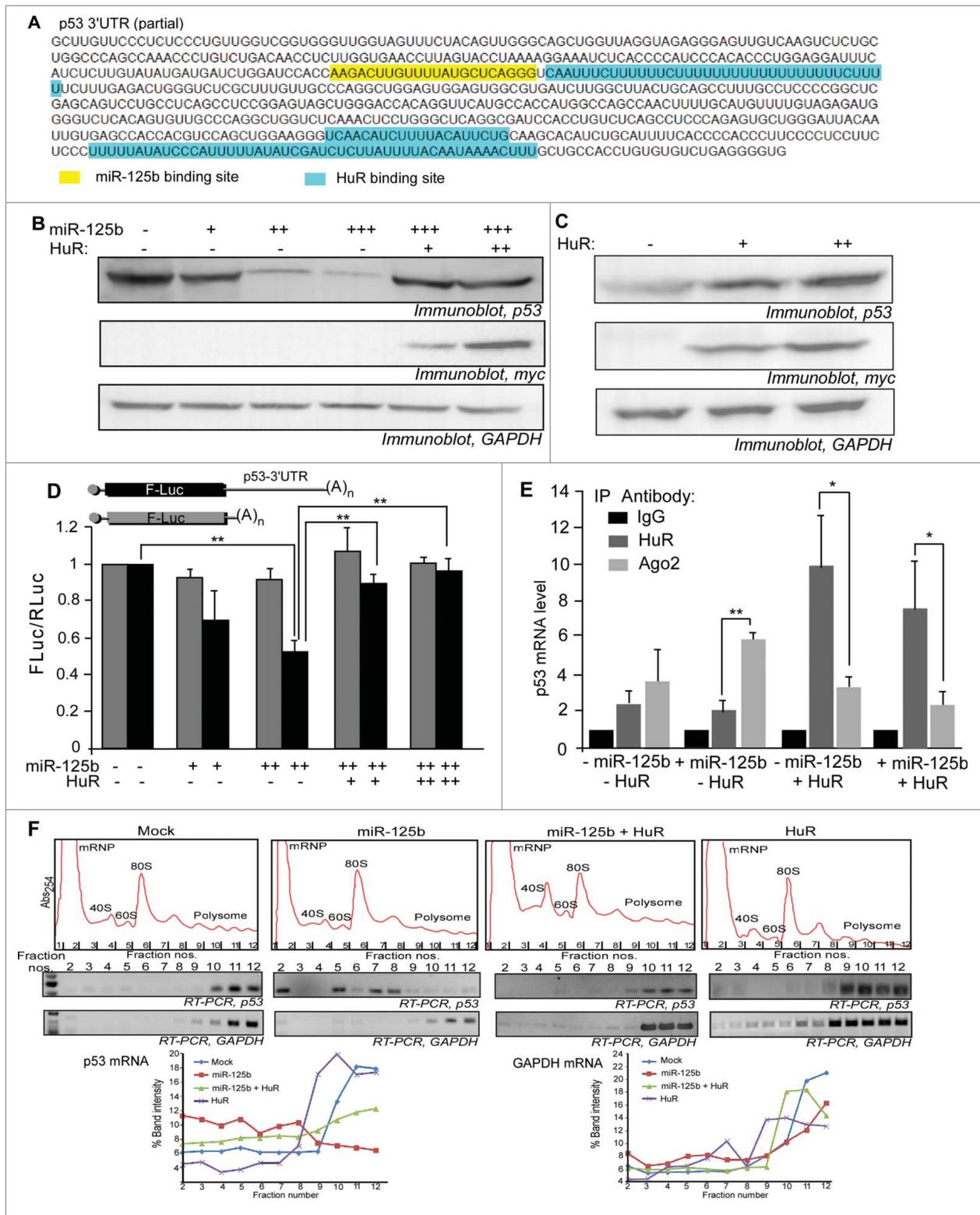


Figure 1. HuR reverses miR-125b-mediated translation repression of p53 mRNA. (A) Partial sequence of p53 3'UTR (nt. 512 to 1190) indicating the miR-125b binding site and the 3 HuR binding sites. (B) Immunoblots of lysates of MCF7 cells transfected with 3 increasing concentrations of pSUPER-miR-125b and cotransfected with 2 increasing concentrations of pCI-neo-myc-HuR probed with p53, myc, and GAPDH antibodies. (C) Immunoblots of lysates of MCF7 cells transfected with 2 increasing concentrations of pCI-neo-myc-HuR probed with p53, myc, and GAPDH antibodies. (D) Cells transfected with Fluc-p53'UTR and Fluc reporter gene constructs and pCMV-Rluc were cotransfected with 2 increasing concentrations of pSUPER-miR-125b and 2 concentrations of pCI-neo-myc-HuR. Fluc values are normalized to Rluc values as transfection control. (E) MCF7 cells were either untransfected (-miR-125b/-HuR) or transfected with pSUPER-miR-125b together with (+miR-125b/+HuR) or without pCI-neo-myc-HuR (+miR-125b/-HuR) or with pCI-neo-myc-HuR alone (-miR-125b/+HuR). Cell lysates were immunoprecipitated with HuR and Ago2 antibody and control IgG. RNA associated with the immunoprecipitates was subjected to qRT-PCR using p53 and GAPDH primers, and p53 mRNA levels were normalized to GAPDH mRNA levels. (F) Ribosomal fractions from MCF7 cells, either mock transfected or transfected with pSUPER-miR-125b or pSUPER-miR-125b and pCI-neo-myc-HuR, or with pCI-neo-myc-HuR alone were analyzed by sucrose density gradient fractionation. rRNA content, measured at 254 nm, is plotted against fraction numbers. RNA isolated from selected fractions was analyzed by semi-quantitative RT-PCR using p53 and GAPDH primers. The level of mRNA content in each fraction, represented as band intensity of PCR products of each lane as % of total band intensity of all lanes, are plotted against respective fraction numbers below.

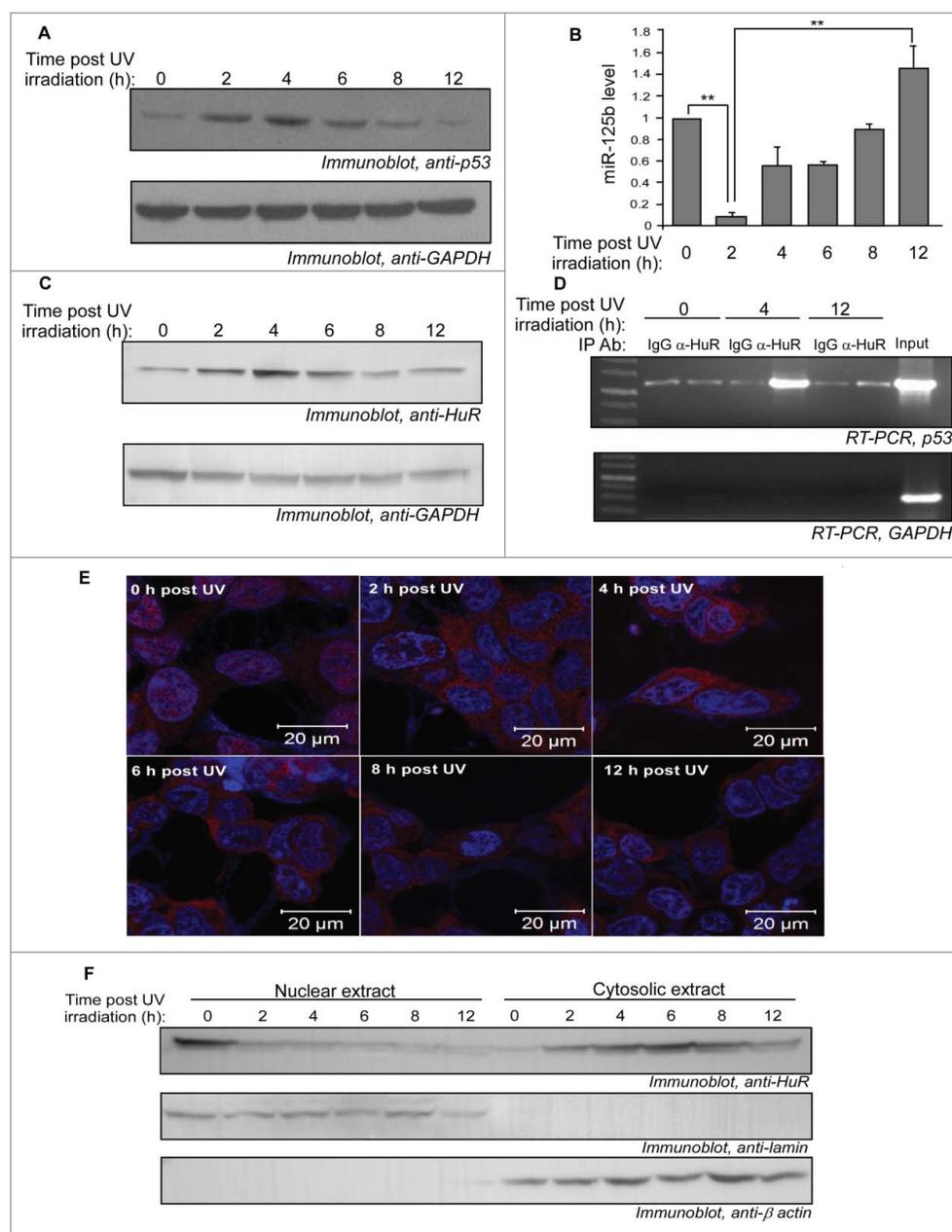


Figure 2. UVC irradiation causes nuclear-cytoplasmic translocation of HuR and prevents miR-125b-mediated translation repression of p53. (A) Immunoblots of lysates of MCF7 cells exposed to UVC radiation and collected at indicated time points post UVC-exposure, probed with p53 and GAPDH antibodies. (B) qRT-PCR of total RNA isolated from UVC-treated MCF7 cells collected at indicated time points using miR-125b specific primers. miR-125b RNA levels were normalized to U6 snRNA levels. Data represents fold change of normalized miR-125b RNA level in UVC-treated cells over UVC-untreated cells. (C) Immunoblots of lysates of cells exposed to UVC radiation and collected at indicated time points post UVC-exposure, probed with HuR and GAPDH antibodies. (D) Lysates of MCF7 cell collected post UVC exposure were immunoprecipitated with HuR antibody or control IgG. RNA associated with the immunoprecipitate was subjected to RT-PCR using p53 or GAPDH primers. Input represents total RNA isolated from cell lysate. (E) MCF7 cells were UVC treated and immunofluorescence of cells collected at various time points post UVC treatment was observed using anti-HuR primary and AlexaFluor568-conjugated secondary antibodies (red). Nucleus was visualized using DAPI staining (blue). (F) MCF7 cells were UVC irradiated following which cells collected at various time points were fractionated into nuclear and cytoplasmic fractions. Fractions were immunoblotted with anti-HuR, anti-lamin (nuclear marker) and anti- β actin (cytoplasmic marker) antibodies.

translocation of HuR from 2 hours post-UVC exposure, reaching a maximum cytoplasmic level at 4 hours and subsequently decreasing, suggesting that the UVC-induced cytoplasmic translocation of HuR allowed its specific interaction with the p53 mRNA, and corresponded with high level of p53 expression, even in the presence of enhanced miR-125b (Fig. 2E). Fractionation of the UVC-irradiated cells into nuclear and cytosolic fractions, followed by immunoblotting for HuR, also showed the gradual nuclear-cytoplasmic

translocation of HuR, reaching a maximum cytosolic level at around 4–6 hours, and subsequently decreasing by 12 hours post UVC exposure (Fig. 2F).

HuR causes the UVC-mediated enhancement of p53 mRNA translation by competing with miR-125b

In order to confirm that the enhancement of p53 post UVC irradiation was at least in part due to HuR, MCF7 cells were

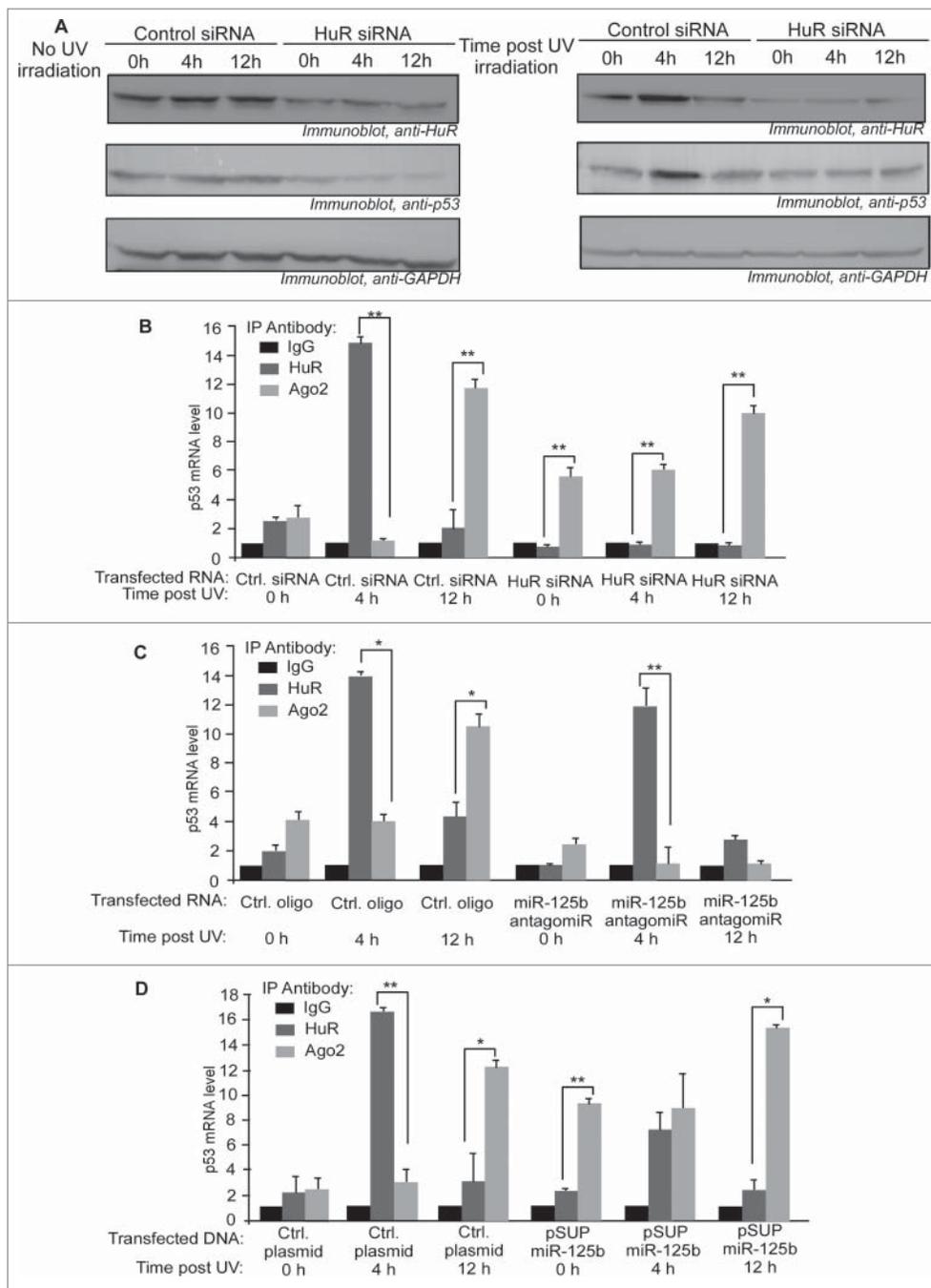


Figure 3. HuR competes with miR-125b to cause the UVC-mediated enhancement of p53 mRNA translation. (A) MCF7 cells were transfected with HuR siRNA or control siRNA and either exposed or not exposed to UVC. Lysates of cells collected at indicated time points were immunoblotted with HuR, p53 and GAPDH antibodies. (B) MCF7 cells were transfected with HuR siRNA or control siRNA and UVC irradiated. Lysates of cells collected at indicated time points were immunoprecipitated with control IgG, HuR and Ago2 antibodies. RNA isolated from immunoprecipitates was reverse transcribed and p53 mRNA level was estimated by qPCR. (C) MCF7 cells were transfected with miR-125b antagomiR or a control oligonucleotide and UVC irradiated. Lysates of cells collected at indicated time points were immunoprecipitated with control IgG, HuR and Ago2 antibodies. RNA isolated from immunoprecipitates was reverse transcribed and p53 mRNA level was estimated by qPCR. (D) MCF7 cells were transfected with pSUPER-miR-125b plasmid or a control plasmid and UVC irradiated. Lysates of cells collected at indicated time points were immunoprecipitated with control IgG, HuR and Ago2 antibodies. RNA isolated from immunoprecipitates was reverse transcribed and p53 mRNA level was estimated by qPCR.

transfected with siRNA against HuR and control siRNA, in presence and absence of UVC irradiation (Fig. 3A). In absence of UVC irradiation, siRNA against HuR efficiently knocked down HuR level, together with a decrease in p53 level, compared to cells treated with the control siRNA (left panel). However, on UVC irradiation, cells treated with the control siRNA showed an increase in HuR level after 4 hours, with a subsequent decrease at 12 h as observed

earlier. HuR level remained uniformly low in presence of siRNA against HuR. p53 protein level also showed an increase at 4 hours in control siRNA-treated cells followed with a decrease at 12 hours upon UVC exposure, but in cells treated with HuR siRNA, p53 level remained unchanged (right panel). Together these observations indicate that HuR is responsible for enhanced expression of p53 post UVC exposure.

In order to investigate whether HuR prevented the loading of the RISC complex on the p53 mRNA post UVC exposure, RNA immunoprecipitation using HuR and Ago2 antibodies was done from cells which have been transfected with HuR siRNA or control siRNA prior to UVC irradiation (Fig. 3B). In cells transfected with control siRNA, p53 mRNA was found to be associated with HuR at 4 h post UVC irradiation, when HuR is mostly present in the cytoplasm. However at 12 h post UVC exposure, when HuR level is low and miR-125b level is high, p53 mRNA was mostly associated with Ago2. Conversely, in cells transfected with HuR siRNA, p53 mRNA was always associated with Ago2, with maximum association at 12 h when miR-125b is expressed at the highest level, suggesting that in absence of HuR, miR-125b is efficiently loaded on p53 mRNA post UVC exposure. This was further verified by RNA immunoprecipitation from cells transfected with an antagomiR against miR-125b, and a control oligonucleotide, prior to UVC exposure (Fig. 3C). In cells transfected with the control oligo, p53 mRNA was found to be mostly associated with HuR at 4 h post UVC irradiation and with Ago2 at 12 h post UVC irradiation. However, in cells transfected with the miR-125b antagomiR, p53 mRNA was associated with HuR at 4 h post UVC exposure, but not associated with Ago2 at 12 h post UVC exposure, showing that the RISC complex is not loaded on the p53 mRNA post UV irradiation if miR-125b is antagonized, even when HuR level is low. These observations were further supported by RNA immunoprecipitation from cells in which miR-125b was overexpressed prior to UVC exposure (Fig. 3D). In control cells, p53 mRNA was mostly associated with HuR at 4 h and with Ago2 at 12 h post UV exposure. However, in cells overexpressing miR-125b, there was high association with Ago2 at all time points post UVC exposure, with nearly equal binding of p53 mRNA with HuR and Ago2 at 4 h post UV exposure, when cytoplasmic HuR level is high. Together, these observations show that HuR is responsible for the UVC-mediated enhancement of p53 translation by competing with miR-125b-RISC binding to the p53 mRNA.

The binding site of HuR proximal to the miR-125b target site is necessary and sufficient for preventing translation repression

A PAR-CLIP experiment to detect transcriptome-wide binding sites for HuR had suggested 3 binding sites for HuR in p53 mRNA 3'UTR.¹⁹ The first site, a U-rich sequence, is adjacent to the miR-125b binding site whereas the other 2 sites are located more distally. We have referred to the 3 sites as proximal (P), middle (M) and distal (D) (Fig. 4A and Fig. S1). In order to determine the contribution of the 3 potential HuR-binding sites to HuR binding and reversal of miR-125b-mediated translation repression, we have deleted the 3 binding sites singly or in combination, and evaluated the binding to purified HuR. Deletion of any of the 3 sites resulted in decreased HuR binding, with the maximum decrease observed in case of removal of the P site and the minimum decrease seen on removal of the M site (Fig. 4B). Deletion of both the P and D sites resulted in further decrease of HuR binding whereas removal of all the 3 sites resulted in near abrogation of HuR binding. We also

found that a 200 nucleotide region of the p53 3'UTR (nt. 597–796) consisting of the miR-125b target site and the P site could bind with HuR (Fig. S4).

In order to evaluate the contribution of the HuR binding sites to the rescue of miR-125b mediated translation repression induced by HuR, we made reporter gene constructs containing deletion fragments of the p53 3'UTR lacking each of the 3 binding sites. Reporter gene assay following cotransfection with miR-125b and HuR expressing constructs showed that absence of the P site resulted in failure of rescue of miR-125b-mediated translation repression (Fig. 4C). Lack of the D site resulted in partial rescue of translation repression, although the restoration of reporter gene activity was greater than that in the case of absence of the P site. Removal of the M site did not affect the rescue of translation by HuR. Removal of both P and D sites, or all the 3 sites, resulted in failure of rescue of translation. Finally, the construct containing the 200 nucleotide fragment consisting of the miR-125b binding site and only the P site was able to completely restore translation on HuR overexpression. Together, these observations suggest that the proximal HuR binding site is necessary and sufficient for the reversal of miR-125b-mediated translation repression by HuR.

HuR reverses the anti-apoptotic and cell proliferative effect of miR-125b

miR-125b has been shown to regulate both apoptosis and cell proliferation in various cellular contexts.¹³ Specifically, miR-125b has been shown to suppress p53-induced apoptosis.¹² Therefore we investigated in what way the antagonistic relationship between miR-125b and HuR in regulating p53 expression affects proliferation and apoptosis of cells. MCF7 cells overexpressing miR-125b, when subjected to serum deprivation, an apoptotic stimulus, showed reduced level of apoptosis (4%) compared to mock transfected cells (33%) as observed by Annexin-V-FITC and PI staining (Fig. 5A). However, on co-expression of HuR, the proportion of apoptotic cells increased to 37%, indicating that HuR could reverse the suppression of apoptosis induced by miR-125b. Expression of HuR alone moderately increased the proportion of apoptotic cells (Fig. S5). Comparison of proliferation rates of the mock-transfected and miR-125b-overexpressing cells over a 72 hour period showed a significantly higher rate of proliferation of the miR-125b-expressing cells (Fig. 5B). Overexpression of HuR in the miR-125b-expressing cells reduced the proliferation rate to that of mock-transfected cells. Overexpression of HuR alone did not cause a change in rate of cell proliferation compared to mock-transfected cells. Exposure of miR-125b-expressing cells to UVC irradiation also reduced the cell proliferation rate to that of mock-transfected cells (Fig. 5C). The effect of HuR in the UVC-induced reversal of cell proliferation was investigated by exposing miR-125b-expressing cells transfected with siRNA against HuR to UVC, in which case the cell proliferation rate increased significantly. This indicated that the reduction in the proliferation rate of miR-125b-expressing cells on UVC exposure was mediated by HuR. siRNA-mediated knockdown of HuR, both in absence and presence of UVC irradiation, also caused significant increase in cell proliferation, suggesting an important role of HuR in controlling the proliferative potential of cells.

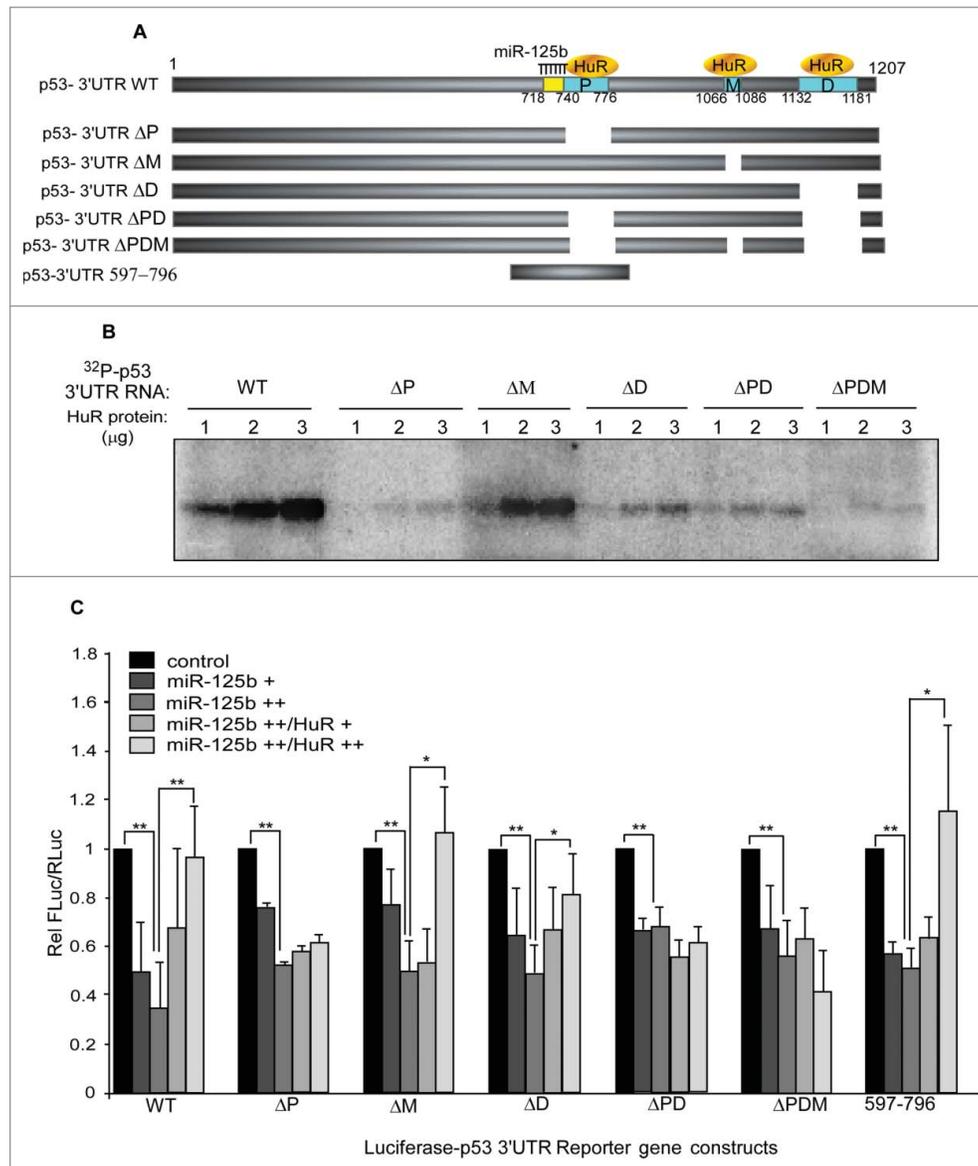


Figure 4. The binding site of HuR proximal to the miR-125b target site is necessary and sufficient for preventing translation repression. (A) Schematic diagram of wild type (WT) p53 3'UTR indicating miR-125b and HuR-binding sites and various deletions mutants. (B) 32 P-UTP labeled full length and various deletion mutants of p53 3'UTR RNA were incubated with purified HuR protein, UV-crosslinked, digested with RNase A and resolved on 10% SDS-PAGE. (C) Reporter gene constructs containing wild type and various deletion mutants of p53 3'UTR were transfected into MCF7 cells together with pSUPER-miR-125b or pCI-Neo-myc-HuR or both.

Evaluation of colony forming potential showed significantly increased colony formation by the miR-125b-overexpressing cells, which was reduced by HuR overexpression as well as by UVC-irradiation (Fig. 5D, left and right panels). HuR overexpression or UVC irradiation only also caused reduction in colony formation compared to mock transfected cells (Fig. S6). Together, these observations showed that miR-125b overexpression can reduce apoptosis and enhance cell proliferation and colony formation in MCF7 cells, which can be reversed by HuR overexpression or UVC-irradiation.

The antagonistic effect of HuR and miR-125b on cell proliferation and apoptosis is mediated via p53

HuR and miR-125b were found to exert opposing effects on the tumorigenic potential of cells, with miR-125b expression enhancing cell proliferation and reducing apoptosis, and HuR

expression reversing the above effects of miR-125b. As HuR and miR-125b were shown to act antagonistically in regulating p53 expression, we therefore investigated whether the opposing effects of HuR and miR-125b in the process of oncogenesis is mediated via p53. Cells transfected with a non-specific siRNA together with the miR-125b-expressing construct, when subjected to an apoptotic stimuli, showed a reduced proportion of apoptotic cells (17%) compared to cells only transfected with the non-specific siRNA (24%) (Fig. 6A). The percentage of apoptotic cells was enhanced when HuR was overexpressed alone (39%), or together with miR-125b (46%) or when miR-125b expressing cells were exposed to UVC (27%). Cells transfected with a siRNA against p53 showed a much reduced level of apoptosis (4%) which remained nearly unchanged when subjected to miR-125b or HuR overexpression, or on UVC irradiation. This indicated that the effects of miR-125b and HuR on cellular apoptosis are mediated via p53. Comparison of proliferation

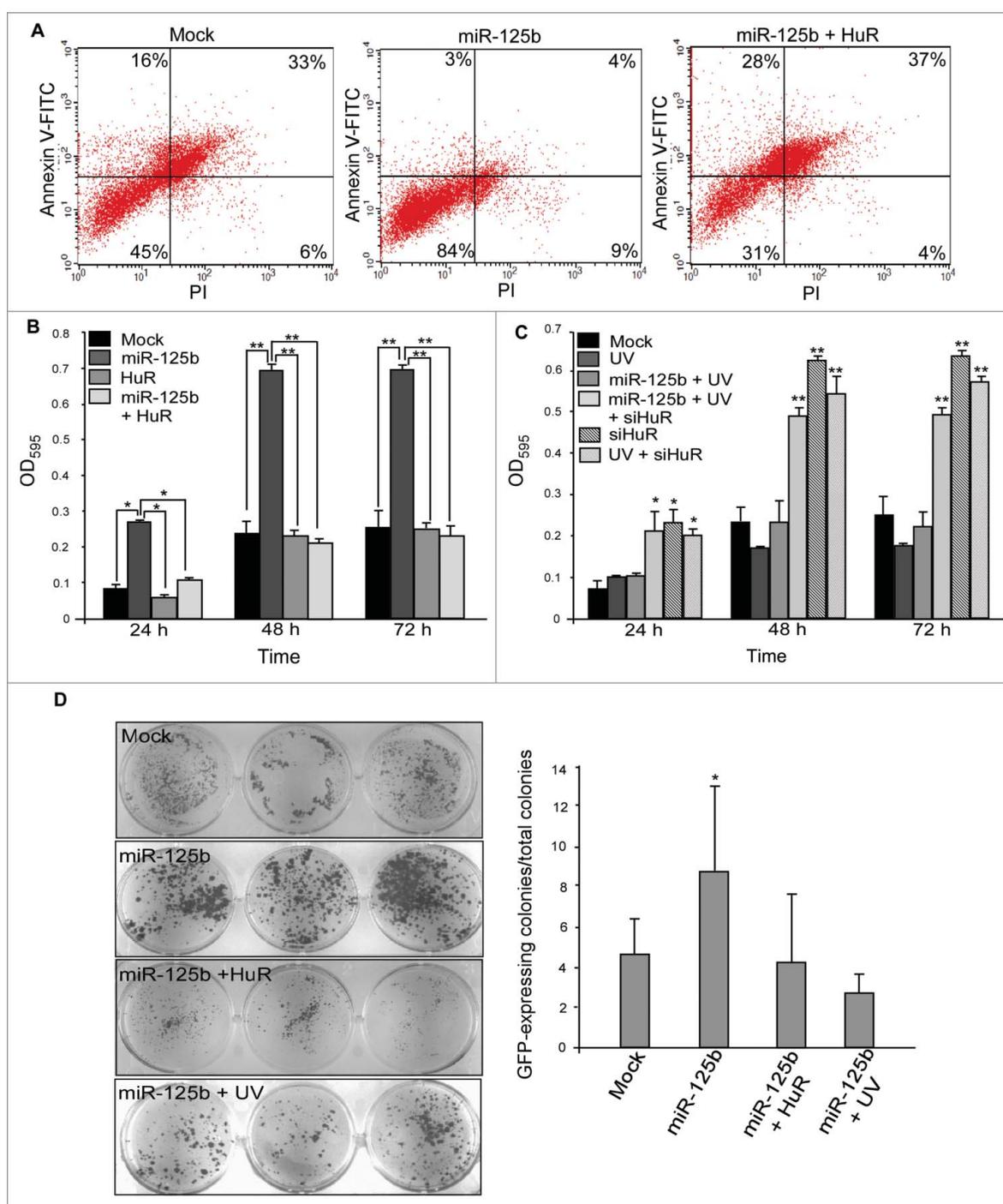


Figure 5. HuR reverses the anti-apoptotic and cell proliferative effect of miR-125b. (A) MCF7 cells were either mock transfected, transfected with pSUPER-miR-125b alone or together with pCI-neo-myc-HuR and then serum starved for 48 hours to induce apoptosis. Cells were stained with AnnexinV-FITC and PI to detect apoptosis by flow cytometry. The x-axis and y-axis represents PI and Annexin staining respectively. The percentage of cells in each quadrant is indicated. (B) MCF7 cells mock transfected, or transfected with pSUPER-miR-125b or pCI-neo-myc-HuR alone or in with both pSUPER-miR-125b and pCI-neo-myc-HuR or UVC treatment were allowed to grow post-transfection and MTT assay was performed at indicated time points. (C) MCF7 cells mock transfected, or UVC irradiated or transfected with pSUPER-miR-125b alone or in combination with UVC and with UVC + HuR siRNA were allowed to grow post-transfection and MTT assay was performed at 24, 48 and 72 hours. (D) MCF7 cells were mock transfected, or transfected with pSUPER-miR-125b alone or in combination with pCI-neo-myc-HuR or UVC treatment. 10^3 cells were seeded and colonies were counted after 14 d for GFP expression and by crystal violet staining. Ratios of GFP-expressing colonies/total number of colonies from 3 experiments are plotted.

rates of control siRNA and p53 siRNA transfected cells, subjected to miR-125b or HuR overexpression or both, over a 72 hour period also showed that the effects of miR-125b and HuR on cell proliferation was mediated by p53 (Fig. 6B). miR-125b enhanced cell proliferation in control siRNA-transfected cells, but failed to do so in cells transfected with p53 siRNA.

The proliferation rate of p53 siRNA-transfected cells was significantly higher than that of control siRNA-transfected cells, and was refractory to either miR-125b or HuR expression or both, as well as to UV irradiation. Evaluation of colony forming potential of control siRNA and p53 siRNA transfected cells showed that knockdown of p53 significantly enhanced the

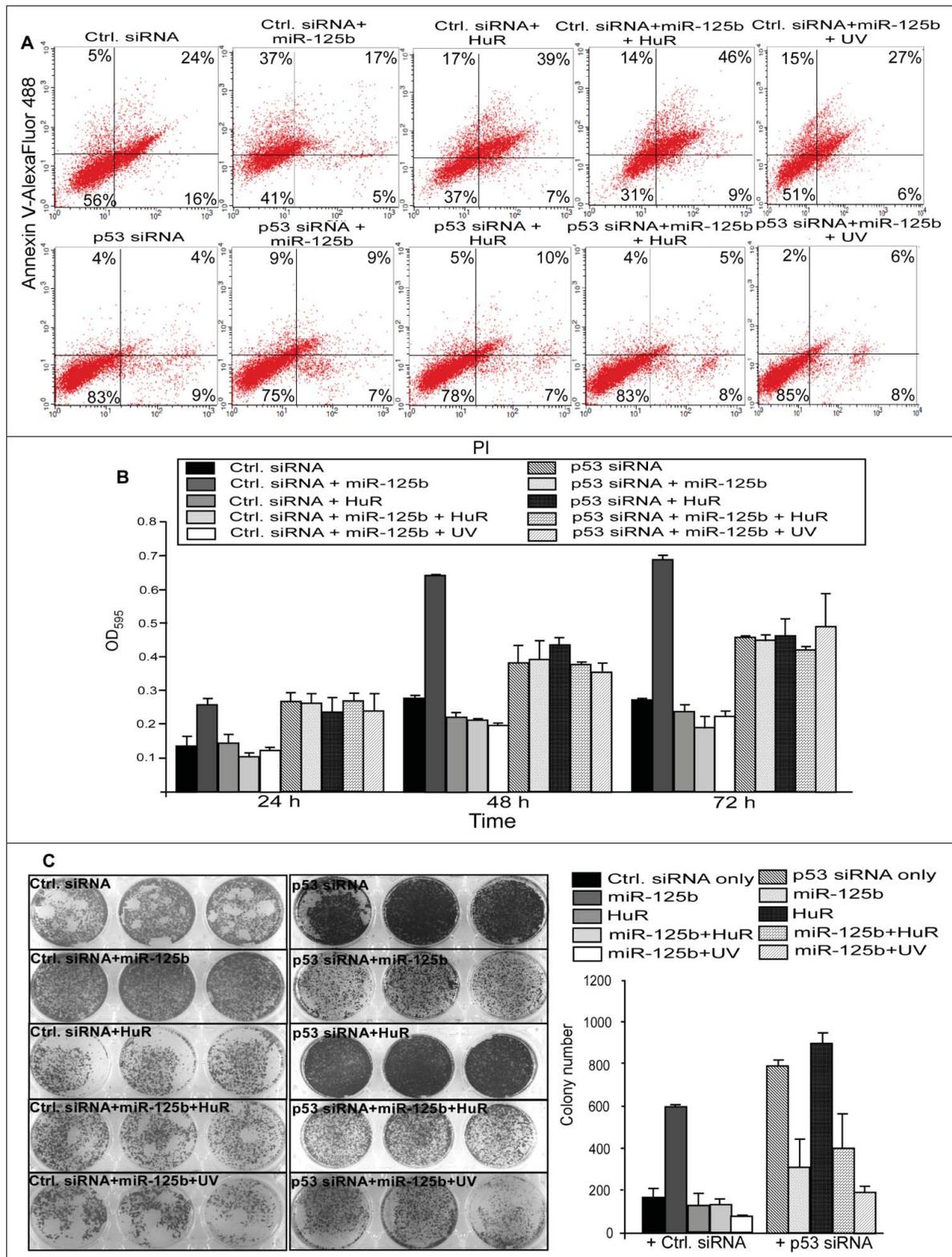


Figure 6. The antagonistic effect of HuR and miR-125b on cell proliferation and apoptosis is mediated via p53. (A) MCF7 cells were either transfected with control siRNA or p53 siRNA, in combination with miR-125b, HuR, miR-125b and HuR and miR-125b and UVC. Cells were subjected to apoptotic stimulus (48 hour serum starvation) following which cells were stained with AlexaFluor 488-Annexin V and PtdIns to detect apoptosis by flow cytometry. The x-axis and y-axis represents PI and Annexin staining respectively. The percentage of cells in each quadrant of representative flow cytograms is indicated. (B) MCF7 cells were either transfected with control siRNA or p53 siRNA, in combination with miR-125b, HuR, miR-125b and HuR and miR-125b and UVC. Cells were allowed to grow post-transfection or UVC exposure and MTT assay was performed at indicated time points. (C) MCF7 cells were either transfected with control siRNA or p53 siRNA, in combination with miR-125b, HuR, miR-125b and HuR and miR-125b and UVC. 10^3 cells were seeded and colonies were counted after 14 d by crystal violet staining. Average number of colonies in each treatment from 2 independent experiments is plotted.

colony formation by cells (Fig. 6C, right and left panels). However, remarkably, when miR-125b was overexpressed in the cells in which p53 has been knocked down, the colony numbers decreased compared to control siRNA-transfected cells in which miR-125b was overexpressed. The reduction in colony formation by miR-125b overexpression in p53 knocked down cells was not reversed by HuR overexpression or UVC irradiation. This points to the possibility that the cell proliferative effects of miR-125b is mediated by its translation repressive effect on p53 and when p53 is silenced over a prolonged period, the antiproliferative effect of miR-125b becomes dominant. Similarly, HuR overexpression in p53 knocked down cells also showed enhanced colony formation, suggesting that the pro-oncogenic function of HuR dominates in absence of p53.

Discussion

The response of p53 to genotoxic stress is crucial for maintaining the integrity of the genome by causing cell cycle arrest, while allowing for the repair of damaged DNA and ultimately causing programmed cell death if the DNA damage is too severe to be repaired.² Although post-translational modification and protein stabilization play a major role in the upregulation of p53 protein in response to genotoxic stress, a substantial number of studies have suggested a critical role for the enhanced translation of p53 mRNA after DNA damage.^{5,6,20,21} It has been suggested that stimulation of translation of specific genes, such as p53, in response to DNA damage would allow transcription to pause while DNA repair can proceed so as to prevent aberrant transcripts from being generated but permitting the synthesis of proteins specifically required for the genotoxic stress response.⁸ We have now demonstrated that this enhanced translation of p53 in response to UVC irradiation is, at least in part, mediated by HuR preventing the translation repression of p53 by the UVC-induced miRNA, miR-125b.

miR-125b, a miRNA belonging to the miR-125a/b family in vertebrates and homologous to *lin-4* in *C. elegans*, is a known negative regulator of p53.¹² Overexpression of miR-125b has been shown to reduce p53 level, and suppress apoptosis, in human neuroblastoma and lung fibroblast cells. Interestingly, miR-125b was found to be downregulated when zebrafish embryos were treated with DNA-damaging agents, which corresponded to the rapid increase in p53 protein level in response to DNA damage.¹² Remarkably, in our study we found a biphasic pattern of miR-125b expression in response to UVC irradiation, with miR-125b level initially decreasing after UVC exposure following which there was a substantial increase over the next 10 hours. The initial decrease in miR-125b level corresponded to an increase in p53 protein. However, subsequently when miR-125b level again increased, p53 mRNA was protected from translation repression by HuR, which was found to interact with the p53 mRNA and cause its dissociation from the RISC complex. This function of HuR appears to reverse the decrease in apoptosis and increase in cell proliferation induced by miR-125b.

miR-125b has been shown to regulate both cell proliferation and apoptosis, in a cellular context-dependent manner and can potentially act as both a tumor suppressor and an oncogene.¹³ miR-125b expression is down-regulated in ovarian and thyroid

cancers^{22,23} but upregulated in pancreatic cancer, prostate cancer, megakaryoblastic leukemia and acute myeloid leukemia (AML).²⁴⁻²⁸ Although miR-125b downregulates apoptosis in many cases, by repressing genes such as p53, Bak 1 and PUMA,^{12,29} it has also been shown to downregulate proliferation of various cancer cell lines.³⁰⁻³² Therefore, the role of miR-125b in regulating 2 apparently opposing processes, cell proliferation and apoptosis, allows it to buffer and fine tune the subtle balance between apoptosis and cell cycle regulators in complex cellular responses. In fact, miR-125b has been found to directly target 20 genes in the p53 network, which includes regulators of both apoptosis and cell proliferation.¹³ Therefore, the delayed increase of miR-125b in response to UVC irradiation might be part of the complex cellular response to DNA damage which causes a decrease in cell proliferation and increase in programmed cell death if the DNA damage is not repaired. However, exogenous overexpression of miR-125b causes downregulation of p53, and thereby increases cell proliferation as we observe in our experiments. Remarkably, our observations suggest that p53 plays a crucial role in this dual function of miR-125b, as miR-125b overexpression causes a decrease in colony formation when p53 is absent. This is not observed in the cell proliferation assay which is done over a period of 72 hours, but is observed in the colony formation assay which is done over a period of 14 days. This gives rise to the interesting possibility that miR-125b targets p53 and causes increased cell proliferation and reduced apoptosis, thereby acting as an oncogenic miRNA. However, when p53 is lost or repressed over a prolonged period, miR-125b targets other regulators of cell proliferation and thereby acts as a tumor suppressor. Thus, the miR-125b-mediated regulation of p53 appears to play an important role in the complex process of maintaining the balance between cell proliferation and apoptosis.

HuR, which undergoes nuclear cytoplasmic translocation in response to UVC irradiation, counteracts the translation repressive effect of miR-125b on p53 mRNA, and thereby maintains p53 expression in response to genotoxic stress even when miR-125b level is high. This allows the cell to undergo p53-mediated cell cycle arrest and apoptosis if the DNA damage repair fails. This growth suppressive and pro-apoptotic effect suggests an anti-oncogenic function of HuR. However, a number of reports have demonstrated the role of HuR in inhibiting apoptosis, enhancing cell proliferation and facilitating invasion and metastasis, suggesting the function of HuR as a pro-oncogenic factor.³³ Therefore our observations also suggest a dual role for HuR as both a pro-oncogenic and anti-oncogenic factor, with the genotoxic stress induced nuclear-cytoplasmic translocation of HuR causing inhibition of cell proliferation and increased apoptosis via enhanced translation of p53 mRNA. Apoptotic stimuli has been shown to shift HuR from being a promoter of cell survival to caspase-mediated programmed cell death, suggesting that apoptotic or genotoxic stimuli might activate the anti-oncogenic function of HuR.³⁴ Interestingly our observations also suggest that HuR enhance tumor cell colony formation when p53 is knocked down, indicating that the anti-proliferative effect of HuR is dependent on p53.

The interplay between HuR and miR-125b which regulates p53 expression is therefore a new feature of the DNA

damage response in cells. Multiple examples of competitive and cooperative interplay between RBPs and miRNAs are now known in cancer.¹⁴ HuR appears to play a major role among RBPs involved in such crosstalk with miRNAs.³⁵ HuR has been shown to function antagonistically with miRNAs such as miR-122, miR-548c, miR-494, miR-16, miR-331-3p and miR-1192 in response to different stimuli.^{15,36-40} On the other hand HuR recruits the miRNA let-7 to repress c-Myc expression.⁴¹ HuR also competes with a long non coding RNA, 7SL, to bind with the p53 3'UTR and regulate p53 mRNA translation.¹⁸ Recently, we have also shown that HuR may act as a "miRNA sponge" by binding with and sequestering miR-21, thereby reversing the miR-21-mediated translation repression of the tumor suppressor protein PDCD4.⁴² Based on our observations in the case of the p53 mRNA, we propose a model in which HuR, on cytoplasmic localization, binds to the 3 binding sites in the p53 3'UTR (Fig. 7). Binding to the site proximal to the miR-125b target site causes the displacement of miR-125b and dissociation of the p53 mRNA from the RISC. Further studies are required to investigate whether the displacement of miR-125b is due to direct steric hindrance or due to a conformational change of the RNA which makes the binding site of miR-125b unavailable. It is interesting to note that the HuR binding site is adjacent to, but non-overlapping with, the miR-125b target site, which is similar to what has been seen in the case of many mRNAs in studies investigating the entire mRNA interactome of HuR.^{19,43} This suggests conformational switching, rather than steric hindrance, as the possible

mechanism for the antagonism between HuR and miRNA binding. Recently it has been shown that HuR can prevent the miR-125b-induced recruitment of the PARN deadenylase to the p53 mRNA and its subsequent degradation.⁴⁴ However, our study, together with earlier studies, have shown that miR-125b causes translational repression, rather than destabilization, of the p53 mRNA¹² and we have shown that DNA-damage induced nuclear-cytoplasmic translocation of HuR reverses the miR-125b-mediated translation repression by dissociating the p53 mRNA from the RNA-induced silencing complex. Therefore the crosstalk between HuR and miR-125b might take place by different molecular mechanisms in different contexts to regulate the translation repression or degradation of p53 mRNA in order to fine tune p53 expression in response to multiple stress stimuli.

Materials and methods

Plasmid constructs

The full length (1190 nt) p53 3'UTR was isolated from human leukocyte RNA by RT-PCR and cloned into pCDNA3.1 (Thermo Fisher Scientific, V790-20) and downstream of firefly luciferase gene in pCDNA3-Fluc vector. Different 3'UTR deletion constructs were cloned by PCR from pCDNA3.1-p53 3'UTR construct. Double-stranded DNA oligo encoding miR-125b was cloned into pSUPER vector (Oligoengine, VEC-PBS-0002) containing the EGFP gene which was transcribed to produce shRNA corresponding to miR-125b. pCI-neo-myc-HuR

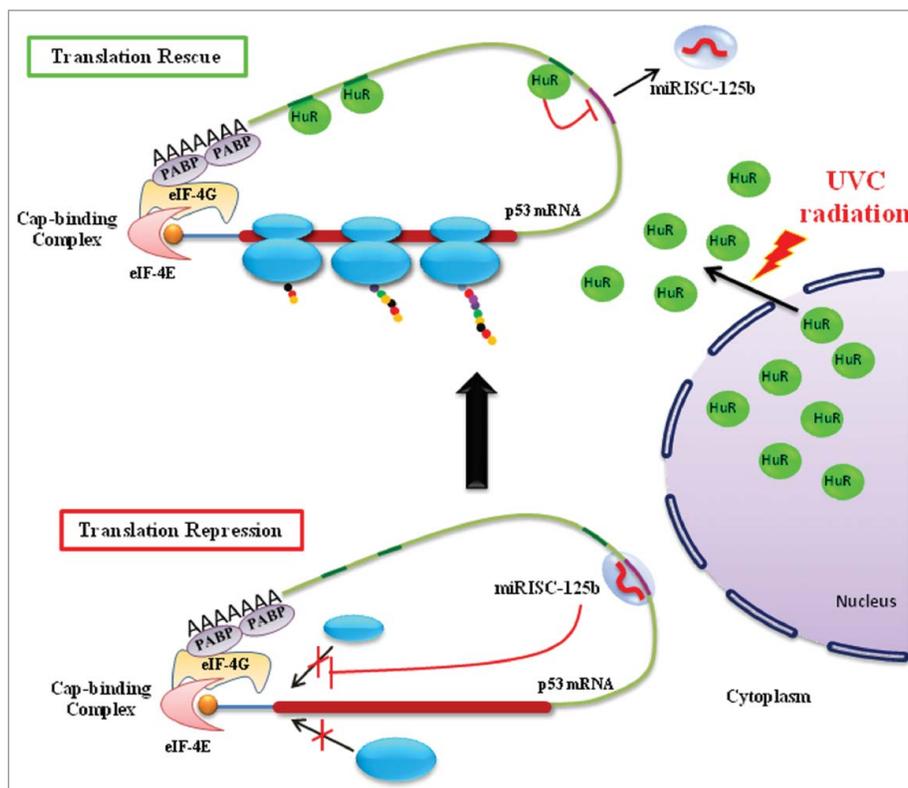


Figure 7. Antagonistic interplay between HuR and miR-125b regulates p53 expression in response to UVC irradiation. Proposed model showing nuclear-cytoplasmic translocation of HuR on UVC irradiation resulting in HuR binding to p53 3'UTR and causing dissociation of miR-125b-RISC complex from p53 RNA allowing rescue of translation.

(gift from S N Bhattacharya, CSIR-IICB) and pET-28b-HuR (gift from S Das, IISc) were used for mammalian and bacterial expression of HuR respectively.

Cell culture, treatment and transfection

MCF7 human breast carcinoma cells were maintained in Dulbecco's modified Eagle's Medium (Thermo Fisher Scientific, 12100-046) with 10% FBS (Thermo Fisher Scientific, 10500-064) and 1% Pen-Strep (Thermo Fisher Scientific, 15140-122). Cells were exposed to 10 J/m² short wavelength UV (UVC) irradiation in UVC crosslinker (CL 1000, UVP). Cells were transfected with different vectors, siRNAs (siGENOME SMART pool, ELAVL1 (M-003773-04-0005), p53(M-003329-03) and Non Targeting siRNA pool (D-001206-13-05) GE-Dharmacon) and antagomiR against miR-125b (Trilink Biotechnologies, C33-B02A) using Lipofectamine 2000 (Thermo Fisher, 11668019) in DMEM low glucose medium (Thermo Fisher, SH30021.01). DNA amount for transfection was equalised by pGEMT plasmid (Promega, A3600).

Immunoblotting

Cells were lysed in S10 lysis buffer (10 mM HEPES, 15 mM KCl, 1 mM PMSF, 1 mM DTT, 0.1% Triton X100) and centrifuged at 10,000 g for 20 minutes for cytoplasmic lysate preparation. Nuclear and cytosolic extracts were prepared as described previously.⁴⁵ Cell lysates were quantified using Bradford reagent (Amresco, E530-1L), resolved on 12% SDS-PAGE and were immunoblotted using anti HuR (3A2, Santa Cruz Biotechnology), p53 (DO-1, Santa Cruz Biotechnology), Ago2 (C34C6, Cell Signaling), Lamin A/C (4C11, Cell Signaling), β -actin (A00730, Genscript) and GAPDH (FL-335, Santa Cruz Biotechnology) antibodies. Chemiluminescent signal was detected using Femtolucent Plus HRP (Geno Biosciences, 786-003).

RNA Immunoprecipitation

A 50% slurry of pre-swelled Protein A Sepharose beads (Sigma Aldrich, P3391) was incubated with anti HuR (Santa Cruz Biotechnology, 3A2), anti Ago2 (Cell Signaling, C34C6) and control IgG (Santa Cruz Biotechnology, sc-2027) overnight at 4°C. 300 μ g of precleared lysate was added to the bead-antibody mix and incubated for 4 hrs at 4°C and washed 5 times with NT2 (50 mM Tris Chloride (pH7.4), 150 mM NaCl, 1 mM MgCl₂, 0.05% NP-40) buffer. RNA was isolated from the immunoprecipitated complexes by Trizol (Thermo Fisher, 15596-018) followed by semiquantitative RT-PCR or qPCR with p53 3'UTR specific primers and GAPDH primers as control.

Quantitative PCR

Total cellular RNA was extracted using Trizol and polyadenylated using Poly A polymerase (New England Biolabs, M0276S). cDNA was synthesized from polyadenylated RNA using oligo(dT)-adapter primer by MuMLV reverse transcriptase (Thermo Fisher, 28025-013). An adapter-specific primer and microRNA-125b specific primer (miScript primer assay

kit, Qiagen, MS00006629) with Power SYBR Green master mix (Applied Biosystems) were used for qPCR reactions in Step One Plus Real time PCR system (Thermo Fisher, 4367659). U6B snRNA and GAPDH primers were used for miRNA and mRNA quantity normalization respectively.

Immunofluorescence

Cells exposed or unexposed to UVC were fixed with 10% PFA at different time points post exposure. After blocking, cells were incubated in anti-HuR antibody (Santa Cruz Biotechnology, 3A2) at 1:500 dilution followed by 1:500 diluted Alexafluor 568-conjugated anti-mouse secondary antibody (Life Technologies, A11004) and DAPI at 0.5 ng/ml (Thermo Fisher, D1306) for nuclear staining. Images were taken in a laser scanning confocal microscope (Zeiss LSM 710) at 40X magnification.

in vitro RNA-protein UV crosslinking assay

³²P-UTP labeled RNA probes were synthesized by *in vitro* transcription from linearized plasmid DNA templates using Maxiscript T7 *in vitro* transcription kit (Thermo Fisher, AM1312). Bacterially expressed and purified 6X His-tagged HuR protein was preincubated in binding buffer (5 mM HEPES, 25 mM KCl, 2 mM MgCl₂, 2 mM DTT, 0.1 mM EDTA, 1.5 mM ATP, 1 μ g/ μ l tRNA) and then with radiolabelled RNAs of equal specific activity. The RNA-protein complexes were crosslinked with UVC radiation (254 nm) followed by RNase A digestion and were electrophoresed on 12% SDS-PAGE. Gels were dried in gel drier (BioRad) followed by phosphorimaging using Typhoon Trio+ variable mode imager (GE healthcare).

Polysome analysis

Transfected cells were treated with Cycloheximide (100 μ g/ml) for 30 minutes and lysed with polysome lysis buffer (20 mM Tris-chloride (pH 7.4), 5 mM MgCl₂, 150 mM NaCl, 1 mM DTT, 0.2 mM PMSF, 0.5% NP40, 1X protease inhibitor, 100 U/ml RNase Inhibitor) containing cycloheximide. Cytosolic extract was obtained by centrifugation at 10,000 x g for 20 min. 50 OD (260 nm) of cell lysate was loaded on 10–50% (w/v) sucrose gradient followed by centrifugation at 100,000 x g, at 4°C for 4 hours. Fractions were collected using a programmable gradient fractionator (Biocomp Instruments) and absorbance of fractions was measured at 254 nm. RNA was isolated from the fractions by phenol-chloroform extraction and ethanol precipitation.

Cell proliferation, colony formation and apoptosis assays

48 hours post transfection with pSUPER-miR-125b and pCI-neo-Myc-HuR vectors, 10³ cells were seeded and cell proliferation was determined at 24, 48 and 72 hours using the cell growth determination kit, MTT based (Sigma Aldrich, CGD1). Similarly transfected cells, with or without UVC exposure, were seeded in 6 well plates and after 14 d of growth, colonies were counted for GFP expression or stained with crystal violet. For apoptosis assay, 24 hours post transfection cells were exposed

to apoptotic stimulus (48 hours serum starvation). Treated cells were stained using Annexin V FITC-Propidium iodide apoptosis detection kit (Sigma Aldrich, APOAF) or AlexaFluor 488-AnnexinV/Dead cell apoptosis kit (Thermo Fisher, V13241) and analyzed using flow cytometry (FACScalibur, BD Biosciences).

Statistical analysis

All graphical data represent mean \pm standard deviation of at least 3 independent experiments (biological replicates) each done in duplicate (technical replicates). * signifies a p-value \leq 0.05 and ** signifies a p-value \leq 0.01 (Paired one-tailed Students t test) between controls and samples indicated in the figures.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank S. Das (IISc, Bangalore) and S. N. Bhattacharyya (CSIR-IICB, Kolkata) for providing reagents. We thank Abhishek Guha for help with illustration. This work was supported by a Wellcome Trust-DBT India Alliance intermediate fellowship grant (WT500139/Z/09/Z) to PSR and UGC senior research fellowship to DA.

ORCID

Deepika Ahuja  <http://orcid.org/0000-0002-3991-6089>

Ashish Goyal  <http://orcid.org/0000-0002-9830-3987>

References

- Giaccia AJ, Kastan MB. The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev* 1998; 12:2973-83; PMID:9765199; <http://dx.doi.org/10.1101/gad.12.19.2973>
- Horn HF, Vousden KH. Coping with stress: multiple ways to activate p53. *Oncogene* 2007; 26:1306-16; PMID:17322916; <http://dx.doi.org/10.1038/sj.onc.1210263>
- Ray PS, Grover R, Das S. Two internal ribosome entry sites mediate the translation of p53 isoforms. *EMBO Rep* 2006; 7:404-10; PMID:16440000; <http://dx.doi.org/10.1038/sj.embor.7400623>
- Vilborg A, Wilhelm MT, Wiman KG. Regulation of tumor suppressor p53 at the RNA level. *J Mol Med (Berl)* 2010; 88:645-52; PMID:20306257; <http://dx.doi.org/10.1007/s00109-010-0609-2>
- Takagi M, Absalon MJ, McLure KG, Kastan MB. Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. *Cell* 2005; 123:49-63; PMID:16213212; <http://dx.doi.org/10.1016/j.cell.2005.07.034>
- Grover R, Ray PS, Das S. Polypyrimidine tract binding protein regulates IRES-mediated translation of p53 isoforms. *Cell Cycle* 2008; 7:2189-98; PMID:18635961; <http://dx.doi.org/10.4161/cc.7.14.6271>
- Vilborg A, Glahder JA, Wilhelm MT, Bersani C, Corcoran M, Mahmoudi S, Rosenstierne M, Grandér D, Farnebo M, Norrild B, et al. The p53 target Wig-1 regulates p53 mRNA stability through an AU-rich element. *Proc Natl Acad Sci U S A* 2009; 106:15756-61; PMID:19805223; <http://dx.doi.org/10.1073/pnas.0900862106>
- Mazan-Mamczarz K, Galbán S, López de Silanes I, Martindale JL, Atsuo Y, Keene JD, Gorospe M. RNA-binding protein HuR enhances p53 translation in response to ultraviolet light irradiation. *Proc Natl Acad Sci U S A* 2003; 100:8354-9; PMID:12821781; <http://dx.doi.org/10.1073/pnas.1432104100>
- Zou T, Mazan-Mamczarz K, Rao JN, Liu L, Marasa BS, Zhang AH, Xiao L, Pullmann R, Gorospe M, Wang JY. Polyamine depletion increases cytoplasmic levels of RNA-binding protein HuR leading to stabilization of nucleophosmin and p53 mRNAs. *J Biol Chem* 2006; 281:19387-94; PMID:16690610; <http://dx.doi.org/10.1074/jbc.M602344200>
- Hinman MN, Lou H. Diverse molecular functions of Hu proteins. *Cell Mol Life Sci* 2008; 65:3168-81; PMID:18581050; <http://dx.doi.org/10.1007/s00018-008-8252-6>
- Zhang Y, Gao J-S, Tang X, Tucker LD, Quesenberry P, Rigoutsos I, Ramratnam B. MicroRNA 125a and its regulation of the p53 tumor suppressor gene. *FEBS Lett* 2009; 583:3725-30; PMID:19818772; <http://dx.doi.org/10.1016/j.febslet.2009.10.002>
- Le MTN, Teh C, Shyh-Chang N, Xie H, Zhou B, Korzh V, Lodish HF, Lim B. MicroRNA-125b is a novel negative regulator of p53. *Genes Dev* 2009; 23:862-76; PMID:19293287; <http://dx.doi.org/10.1101/gad.1767609>
- Le MTN, Shyh-Chang N, Khaw SL, Chin L, Teh C, Tay J, O'Day E, Korzh V, Yang H, Lal A, et al. Conserved regulation of p53 network dosage by microRNA-125b occurs through evolving miRNA-target gene pairs. *PLoS Genet* 2011; 7(9): e1002242; PMID:21935352; <http://dx.doi.org/10.1371/journal.pgen.1002242>
- van Kouwenhove M, Kedde M, Agami R. MicroRNA regulation by RNA-binding proteins and its implications for cancer. *Nat Rev Cancer* 2011; 11:644-56; PMID:21822212; <http://dx.doi.org/10.1038/nrc3107>
- Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W. Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell* 2006; 125:1111-24; PMID:16777601; <http://dx.doi.org/10.1016/j.cell.2006.04.031>
- Kedde M, Strasser MJ, Boldajipour B, Oude Vrielink JAF, Slanchev K, le Sage C, Nagel R, Voorhoeve PM, van Duijse J, Ørom UA, et al. RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. *Cell* 2007; 131:1273-86; PMID:18155131; <http://dx.doi.org/10.1016/j.cell.2007.11.034>
- Jing Q, Huang S, Guth S, Zarubin T, Motoyama A, Chen J, Di Padova F, Lin S-C, Gram H, Han J. Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell* 2005 [cited 2014 Apr 30]; 120:623-34; PMID:15766526; <http://dx.doi.org/10.1016/j.cell.2004.12.038>
- Abdelmohsen K, Panda AC, Kang M-J, Guo R, Kim J, Grammatikakis I, Yoon J-H, Dudekula DB, Noh JH, Yang X, et al. 7SL RNA represses p53 translation by competing with HuR. *Nucleic Acids Res* 2014; 42:10099-111; PMID:25123665; <http://dx.doi.org/10.1093/nar/gku686>
- Mukherjee N, Corcoran DL, Nusbaum JD, Reid DW, Georgiev S, Hafner M, Ascano M, Tuschl T, Ohler U, Keene JD. Integrative Regulatory Mapping Indicates that the RNA-Binding Protein HuR Couples Pre-mRNA Processing and mRNA Stability. *Mol Cell* 2011; 43:327-39; PMID:21723170; <http://dx.doi.org/10.1016/j.molcel.2011.06.007>
- Fu L, Minden MD, Benchimol S. Translational regulation of human p53 gene expression. *EMBO J* 1996; 15:4392-401; PMID:8861966
- Chen J, Kastan MB. 5'-3'-UTR interactions regulate p53 mRNA translation and provide a target for modulating p53 induction after DNA damage. *Genes Dev* 2010; 24:2146-56; PMID:20837656; <http://dx.doi.org/10.1101/gad.1968910>
- Nam EJ, Yoon H, Kim SW, Kim H, Kim YT, Kim JH, Kim JW, Kim S. MicroRNA expression profiles in serous ovarian carcinoma. *Clin Cancer Res* 2008; 14:2690-5; PMID:18451233; <http://dx.doi.org/10.1158/1078-0432.CCR-07-1731>
- Visone R, Pallante P, Vecchione A, Cirombella R, Ferracin M, Ferraro A, Volinia S, Coluzzi S, Leone V, Borbone E, et al. Specific microRNAs are downregulated in human thyroid anaplastic carcinomas. *Oncogene* 2007; 26:7590-5; PMID:17563749; <http://dx.doi.org/10.1038/sj.onc.1210564>
- Bloomston M, Frankel WL, Petrocca F, Volinia S, Alder H, Hagan JP, Liu C-G, Bhatt D, Taccioli C, Croce CM. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA* 2007; 297:1901-8; PMID:17473300; <http://dx.doi.org/10.1001/jama.297.17.1901>
- Shi X-B, Xue L, Yang J, Ma A-H, Zhao J, Xu M, Tepper CG, Evans CP, Kung H-J, deVere White RW. An androgen-regulated miRNA suppresses Bak1 expression and induces androgen-independent growth

- of prostate cancer cells. *Proc Natl Acad Sci U S A* 2007; 104:19983-8; PMID:18056640; <http://dx.doi.org/10.1073/pnas.0706641104>
26. Bousquet M, Harris MH, Zhou B, Lodish HF. MicroRNA miR-125b causes leukemia. *Proc Natl Acad Sci U S A* 2010; 107:21558-63; PMID:21118985; <http://dx.doi.org/10.1073/pnas.1016611107>
 27. Klusmann JH, Li Z, Böhmer K, Maroz A, Koch ML, Emmrich S, Godinho FJ, Orkin SH, Reinhardt D. miR-125b-2 is a potential oncomiR on human chromosome 21 in megakaryoblastic leukemia. *Genes Dev* 2010; 24:478-90; PMID:20194440; <http://dx.doi.org/10.1101/gad.1856210>
 28. Bousquet M, Quelen C, Rosati R, Mansat-De Mas V, La Starza R, Bastard C, Lippert E, Talmant P, Lafage-Pochitaloff M, Leroux D, et al. Myeloid cell differentiation arrest by miR-125b-1 in myelodysplastic syndrome and acute myeloid leukemia with the t(2;11)(p21;q23) translocation. *J Exp Med* 2008; 205:2499-506; PMID:18936236; <http://dx.doi.org/10.1084/jem.20080285>
 29. Chen X, Liu J, Feng W, Wu X, Chen S. MiR-125b protects against ethanol-induced apoptosis in neural crest cells and mouse embryos by targeting Bak 1 and PUMA. *Exp Neurol* 2015; 271:104-11; PMID:26024858; <http://dx.doi.org/10.1016/j.expneurol.2015.04.026>
 30. Shi L, Zhang J, Pan T, Zhou J, Gong W, Liu N, Fu Z, You Y. MiR-125b is critical for the suppression of human U251 glioma stem cell proliferation. *Brain Res* 2010; 1312:120-6; PMID:19948152; <http://dx.doi.org/10.1016/j.brainres.2009.11.056>
 31. Hofmann MH, Heinrich J, Radziwill G, Radziwil G, Moelling K. A short hairpin DNA analogous to miR-125b inhibits C-Raf expression, proliferation, and survival of breast cancer cells. *Mol Cancer Res* 2009; 7:1635-44; PMID:19825990; <http://dx.doi.org/10.1158/1541-7786.MCR-09-0043>
 32. Mizuno Y, Yagi K, Tokuzawa Y, Kanesaki-Yatsuka Y, Suda T, Katagiri T, Fukuda T, Maruyama M, Okuda A, Amemiya T, et al. miR-125b inhibits osteoblastic differentiation by down-regulation of cell proliferation. *Biochem Biophys Res Commun* 2008; 368:267-72; PMID:18230348; <http://dx.doi.org/10.1016/j.bbrc.2008.01.073>
 33. Abdelmohsen K, Gorospe M. Posttranscriptional regulation of cancer traits by HuR. *Wiley Interdiscip Rev RNA* 1:214-29; PMID:21935886; <http://dx.doi.org/10.1002/wrna.4>
 34. von Roretz C, Lian XJ, Macri AM, Punjani N, Clair E, Drouin O, Dormoy-Raclet V, Ma JF, Gallouzi I-E. Apoptotic-induced cleavage shifts HuR from being a promoter of survival to an activator of caspase-mediated apoptosis. *Cell Death Differ* 2013; 20:154-68; PMID:22955946; <http://dx.doi.org/10.1038/cdd.2012.111>
 35. Srikantan S, Tominaga K, Gorospe M. Functional interplay between RNA-binding protein HuR and microRNAs. *Curr Protein Pept Sci* 2012; 13:372-9; PMID:22708488; <http://dx.doi.org/10.2174/138920312801619394>
 36. Srikantan S, Abdelmohsen K, Lee EK, Tominaga K, Subaran SS, Kuwano Y, Kulshrestha R, Panchakshari R, Kim HH, Yang X, et al. Translational control of TOP2A influences doxorubicin efficacy. *Mol Cell Biol* 2011; 31:3790-801; PMID:21768308; <http://dx.doi.org/10.1128/MCB.05639-11>
 37. Tominaga K, Srikantan S, Lee EK, Subaran SS, Martindale JL, Abdelmohsen K, Gorospe M. Competitive regulation of nucleolin expression by HuR and miR-494. *Mol Cell Biol* 2011; 31:4219-31; PMID:21859890; <http://dx.doi.org/10.1128/MCB.05955-11>
 38. Epis MR, Barker A, Giles KM, Beveridge DJ, Leedman PJ. The RNA-binding protein HuR opposes the repression of ERBB-2 gene expression by microRNA miR-331-3p in prostate cancer cells. *J Biol Chem* 2011; 286:41442-54; PMID:21971048; <http://dx.doi.org/10.1074/jbc.M111.301481>
 39. Young LE, Moore AE, Sokol L, Meisner-Kober N, Dixon DA. The mRNA stability factor HuR inhibits microRNA-16 targeting of COX-2. *Mol Cancer Res* 2012; 10:167-80; PMID:22049153; <http://dx.doi.org/10.1158/1541-7786.MCR-11-0337>
 40. Dormoy-Raclet V, Cammas A, Celona B, Lian XJ, van der Giessen K, Zivojnovic M, Brunelli S, Riuzzi F, Sorci G, Wilhelm BT, et al. HuR and miR-1192 regulate myogenesis by modulating the translation of HMGB1 mRNA. *Nat Commun* 2013; 4:2388; PMID:24005720; <http://dx.doi.org/10.1038/ncomms3388>
 41. Kim HH, Kuwano Y, Srikantan S, Lee EK, Martindale JL, Gorospe M. HuR recruits let-7/RISC to repress c-Myc expression. *Genes Dev* 2009; 23:1743-8; PMID:19574298; <http://dx.doi.org/10.1101/gad.1812509>
 42. Poria DK, Guha A, Nandi I, Ray PS. RNA-binding protein HuR sequesters microRNA-21 to prevent translation repression of proinflammatory tumor suppressor gene programmed cell death 4. *Oncogene* 2016; 35:1703-15; PMID:26189797; <http://dx.doi.org/10.1038/onc.2015.235>
 43. Lebedeva S, Jens M, Theil K, Schwanhäusser B, Selbach M, Landthaler M, Rajewsky N. Transcriptome-wide analysis of regulatory interactions of the RNA-binding protein HuR. *Mol Cell* 2011; 43:340-52; PMID:21723171; <http://dx.doi.org/10.1016/j.molcel.2011.06.008>
 44. Zhang X, Devany E, Murphy MR, Glazman G, Persaud M, Kleiman FE. PARN deadenylase is involved in miRNA-dependent degradation of TP53 mRNA in mammalian cells. *Nucleic Acids Res* 2015; 43:10925-38; PMID:26400160; <http://dx.doi.org/10.1093/nar/gkv959>
 45. Baldwin AS. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* 1996; 14:649-83; PMID:8717528; <http://dx.doi.org/10.1146/annurev.immunol.14.1.649>