Integrated Regulation of HuR by Translation Repression and Protein Degradation Determines Pulsatile Expression of p53 Under DNA Damage

HIGHLIGHTS
Systems modeling with experimental validation of p53 translation regulatory network

- Includes miR-125b and HuR in the network as upstream regulators of p53 expression
- Discovers miR-125b and the E3 ligase TRIM21 as new regulators of HuR in the network
- miR-125b and TRIM21 affect cell viability via p53 in response to UV-induced DNA damage

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Integrated Regulation of HuR by Translation Repression and Protein Degradation Determines Pulsatile Expression of p53 Under DNA Damage

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SUMMARY
Expression of tumor suppressor p53 is regulated at multiple levels, disruption of which often leads to cancer. We have adopted an approach combining computational systems modeling with experimental validation to elucidate the translation regulatory network that controls p53 expression post DNA damage. The RNA-binding protein HuR activates p53 mRNA translation in response to UVC-induced DNA damage in breast carcinoma cells. p53 and HuR levels show pulsatile change post UV irradiation. The computed model fitted with the observed pulse of p53 and HuR only when hypothetical regulators of synthesis and degradation of HuR were incorporated. miR-125b, a UV-responsive microRNA, was found to represses the translation of HuR mRNA. Furthermore, UV irradiation triggered protein degradation of HuR mediated by an E3-ubiquitin ligase tripartite motif-containing 21 (TRIM21). The integrated action of miR-125b and TRIM21 constitutes an intricate control system that regulates pulsatile expression of HuR and p53 and determines cell viability in response to DNA damage.

INTRODUCTION
The protein p53 is the hub of a complex regulatory network of incoming stress signals and outgoing effector pathways that plays a crucial tumor suppressor role (Horn and Vousden, 2007). Different stresses such as DNA damage, hypoxia, and oncogene activation upregulate and activate the p53 protein, mainly by inhibiting the interaction between p53 and the E3 ubiquitin ligase Mdm2 (Horn and Vousden, 2007; Michael and Oren, 2003). The enhanced p53 level causes increased Mdm2 expression, which in turn reduces p53 to a low steady-state level, thereby forming a negative feedback loop post DNA damage (Lahav et al., 2004).

Although the core of the p53 regulatory network consists of the p53-Mdm2 negative feedback loop, it is, nevertheless, clear that other mechanisms for regulating p53 protein levels in response to DNA damage are also important. The observation that the protein synthesis inhibitor cycloheximide (CHX) is capable of blocking p53 induction and partially inhibiting G1 arrest in response to DNA damage provided evidence that the control of p53 mRNA translation plays a key role in p53 induction (Fu et al., 1996; Kastan et al., 1991). Translational regulation of p53 is mediated by the interaction of RNA-binding proteins (RBPs) and microRNAs (miRNAs) with both the 5′ and 3′ UTRs of p53 mRNA (Vilborg et al., 2010). The RBP HuR is a major regulator of p53 mRNA stability and translation in response to DNA damage. HuR undergoes nuclear-cytoplasmic translocation and binds to the p53 mRNA 3′ UTR, enhancing p53 protein synthesis in response to DNA-damaging UVC irradiation (Ahuja et al., 2016; Mazan-Mamczarz et al., 2003).

The first report of an miRNA directly regulating p53 was miR-125b, which is an important negative regulator of p53 protein synthesis and p53-mediated apoptosis (Le et al., 2009). A homolog of miR-125b, miR-125a, also interacts with p53 mRNA 3′ UTR and inhibits translation (Zhang et al., 2009). Recent work has shown that cross talk between miR-125b- and HuR-mediated regulation of p53 mRNA translation controls p53 protein synthesis in response to UV radiation (Ahuja et al., 2016).

Previous studies have shown that the p53 level increases dramatically after exposure to DNA-damaging radiation and then declines in a pulsatile manner (Lev-Bar-Or et al., 2000). Interestingly, DNA double-strand breaks, induced by ionizing γ-radiation or radiomimetic drugs such as necrostatin-1, trigger a series of p53 oscillations with fixed amplitude and duration (Batchelor et al., 2011). In contrast, DNA single-strand...
breaks, induced by non-ionizing UV radiation, causes a single pulse of p53, with an amplitude dependent on the UV dose (Batchelor et al., 2011; Collister et al., 1998; Gaglia and Lahav, 2014; Purvis et al., 2012). Moreover, quantitative studies examining p53 expression in individual living cells in response to DNA damage have shown that there is heterogeneity of pulsatile expression of p53 among individual cells in a population (Geva-Zatorsky et al., 2006; Lahav et al., 2004). These observations have introduced new questions regarding the mechanism and function of p53 oscillatory dynamics. A number of studies have employed mathematical modeling approaches to elucidate this behavior (Batchelor et al., 2008; Ma et al., 2005; Proctor and Gray, 2008). All the modeling approaches have only considered the p53-Mdm2 negative feedback loop and its upstream regulators, which regulate p53 protein stability, to explain the oscillatory dynamics of p53 (Ciliberto et al., 2005; Geva-Zatorsky et al., 2006; Lahav, 2008; Lev Bar-Or et al., 2000; Ma et al., 2005; Wagner et al., 2005). However, it is important to consider the regulation of p53 protein synthesis to realistically explain the pulsatile expression pattern of p53 in response to DNA damage. This necessitates the inclusion of regulators of p53 mRNA translation that are induced by DNA damage, such as HuR and miR-125b, in the regulatory model of p53 expression in response to genotoxic stress.

Therefore, to understand the translation regulatory network that controls pulsatile p53 expression in response to DNA damage, we have adopted an approach combining computational modeling and experimental validation in a reiterative manner. We have found that both p53 and HuR levels show a pulse over a 12-h time period after exposure to UV irradiation. However, modeling of the HuR-p53-Mdm2 network failed to show the expression pattern obtained experimentally. Further modeling that fitted the experimental observations suggested the presence of a translation inhibitor and a degradation inducer of HuR. The modeling was validated by the discovery of an miRNA inhibiting translation of HuR mRNA and an E3 ubiquitin ligase inducing HuR protein degradation in response to DNA damage. Together these observations have provided the basis of an approach combining computational modeling and biochemical experimentation, which has allowed the discovery of hitherto unknown regulators of p53 expression in the cellular DNA damage response.

RESULTS
Dynamic Modeling of the Minimal p53-Mdm2 Negative Feedback Circuit Shows a Pulsatile Change in p53 Level
The p53-Mdm2 negative feedback circuit was selected as the minimal network, and a mathematical model of the system dynamics containing the key processes that regulate gene expression was developed. The rate equations representing the expression of p53 and Mdm2 were represented by non-linear differential equations consisting of synthesis and degradation terms. Complete model details, model parameters, and parameter testing are described in Modeling Procedures in Materials and Methods. The numerical integration of the set of differential equations with the parametric values (Table S1) showed a pulsatile change in the p53 level (Figure S1). This suggested that the minimal p53-Mdm2 negative feedback loop was sufficient to explain the oscillatory behavior of p53 expression post DNA damage, as in previous studies (Lahav, 2008; Proctor and Gray, 2008).

Inclusion of HuR and miR-125b as Regulators of p53 Expression Fails to Obtain a Fit between the Model and Experimental Data
The pulsatile change in p53 level corresponded to our experimental observation when we exposed MCF7 breast carcinoma cells to a pulse of UVC radiation and observed a single broad pulse of p53 protein approximately over a period of 12 h post UV exposure (Figure 1A). There was a high positive correlation (Pearson R = 0.68) between the result of the simulation (solid purple line) and the experimental data (bar graphs) obtained from seven independent experiments (Figure S1). However, this feedback circuit only considers the stabilization of p53 and does not consider changes in p53 protein synthesis in response to DNA damage. Therefore HuR and miR-125b were introduced in the model as positive and negative regulators, respectively, of p53 protein synthesis (Figure 1B). Terms representing the effect of HuR and miR-125b were included in the differential rate equation representing p53 expression (Figure 1C). However, in the absence of known regulatory processes for miR-125b expression in response to UV, a curve-fitting equation describing the experimentally observed biphasic miR-125b expression pattern in response to UV was included (Figure 1D) in the model. Remarkably, the simulation of these equations now failed to produce a fit (Pearson R = −0.15) with the experimentally observed pulsatile change in p53 expression (Figure 1E). Moreover, the simulation predicted a linear change in HuR level that did not show any correlation (Pearson R = 0.15) with the experimentally observed change in the cytoplasmic level of HuR, which also
Figure 1. Modeling of the p53 Regulatory Network, Including miR-125b and HuR as Regulators of p53 mRNA Translation, Fails to Obtain a Fit between the Model and Experimental Observations
(A) Representative immunoblot of cytoplasmic lysates of MCF7 cells exposed to a 10 J/m² pulse of UVC radiation and collected at indicated time points post UV exposure, probed with p53 and GAPDH antibodies.
(B) Network diagram of the p53 translation regulation network in response to UV irradiation. The edges in red represent newly added regulatory processes.
(C) Rate equations representing the p53 translation regulation network in response to UV irradiation. The rate equation for miR-125b expression consists of a curve-fitting equation with a decreasing exponential function and a Hill equation-like function describing the experimentally observed biphasic miR-125b expression pattern in (C).

\[
\frac{d[H_uR]}{dt} = \alpha_{H_uR} - \beta_{H_uR}[H_uR]
\]
\[
\frac{d[p53]}{dt} = \alpha_{p53}(1 + K_{p53}[H_uR]) - K_{Mdm2}[Mdm2][p53] - \beta_{p53}[p53]
\]
\[
\frac{d[Mdm2]}{dt} = \alpha_{Mdm2}(1 + K_{p53}[p53]) - \beta_{Mdm2}[Mdm2]
\]
\[
\frac{d[miR-125b]}{dt} = f(t)
\]

(D) Graph showing the relative concentration of miR-125b over time.

(E) Graphs comparing the computed and experimental relative concentrations of p53 and HuR over time.

(F) Immunoblot of cytoplasmic lysates of MCF7 cells at different time points post UV exposure, probed with HuR and GAPDH antibodies.
Figure 1. Continued

(D) Best-fit curve for miR-125b expression over 12-h period post UV irradiation as obtained previously (Ahuja et al., 2016). The curve-fitting equation representing the biphasic expression pattern is represented. The $r^2$ value for curve fitting of miR-125b expression is 0.918695.

(E) Plots representing simulation and experimental data of change of p53 and HuR levels over a 12-h period post exposure to a 10 J/m$^2$ pulse of UVC irradiation. The simulation plots (purple lines) represent the numerical integration of the rate equations in (B). The experimental plots (bar graphs) are composed of intensity values of p53 and HuR bands obtained from cell lysates collected at the designated time points post UV exposure. Experimental data represent mean ± SD values from seven independent immunoblots, normalized to corresponding GAPDH band intensities. The normalized band intensities are scaled to the 0-h time point band intensity, taken as 1. * Represents significant difference from values at 0 h, and # represents significant difference from values at 12-h time points. * or # signifies a p-value ≤ 0.05, ** or ### signifies a p-value ≤ 0.005. * or ## signifies a p-value ≤ 0.01, *** or ### signifies a p-value ≤ 0.005.

(F) Representative immunoblot of cytoplasmic lysates of MCF7 cells exposed to UVC radiation and collected at indicated time points post UV exposure and probed with HuR and GAPDH antibodies.

See also Figures S1 and S13 and Table S1.

showed a broad pulse similar to that of p53 (bar graphs in Figures 1E and 1F). This indicated the potential presence of other, hitherto unknown, regulatory factors induced by DNA damage in the regulatory network regulating p53 expression.

**Incorporation of miR-125b as a Negative Regulator of HuR Improves the Model but Does Not Fit with the Experimental Data**

The pulsatile change in HuR level in response to UV irradiation suggested the presence of negative regulator(s) of HuR induced by UV radiation. As miR-125b is induced by UV and represses p53 mRNA translation in the same timescale, we considered the possibility of HuR being a target of miR-125b-mediated translation repression. Interestingly, a target site of miR-125b, with a completely complementary seed sequence, was predicted at the 671–692 nucleotide position in the HuR mRNA 3′ UTR by miRNA target prediction algorithms (Figure 2A). miR-125b levels were negatively correlated with HuR levels in the breast carcinoma cell lines MCF7 and MDA-MB-231, and HuR level in MDA-MB-231 cell line was enhanced in a dose-dependent manner by transfection with an antagomiR to miR-125b (Figure S2). Overexpression of miR-125b in MCF7 cells resulted in a dose-dependent decrease in HuR protein level but not in the HuR mRNA level (Figure 2B, quantification in Figure S3A). miR-125b overexpression (Figure S3B) showed a significant dose-dependent decrease in luciferase activity from a reporter gene construct containing the wild-type (WT) HuR 3′ UTR but not from a reporter gene containing the HuR 3′ UTR with a mutant miR-125b target site (Figure 2C). There was no change in firefly luciferase mRNA level on miR-125b overexpression (Figure S3C). Also, transfection of cells overexpressing miR-125b with a miR-125b antagoniR restored luciferase expression from the reporter gene construct containing the HuR 3′ UTR (Figure 2C). UV irradiation of cells transfected with the luciferase reporter constructs showed significant increase of luciferase activity from reporter gene constructs with HuR WT 3′ UTR and HuR 3′ UTR with mutant miR-125b target site at 2 h post UV irradiation, which coincides with the lowest level of miR-125b. However, luciferase activity from the WT 3′ UTR decreased significantly compared with miR-125b target site mutant 3′ UTR at subsequent time points, which coincides with the increase in miR-125b level (Figure S4). Analysis of ribosomal fractions from cells overexpressing miR-125b showed that HuR mRNA was mostly present in the non-translating mRNA-protein complex (mRNP) fractions compared with that in control cells (Figure 2D). HuR mRNA and miR-125b were found to be associated with Ago2, the major component of the RNA-induced silencing complex, by RNA immunoprecipitation of lysates from cells expressing miR-125b (Figure S5). Together, these data established miR-125b as an UV-induced repressor of HuR protein synthesis.

Therefore, we incorporated the miR-125b mediated downregulation of HuR expression in the model of the p53 regulatory network (Figure 2E) with $k_{mtl}$ as the regulatory constant representing the effect of miR-125b on HuR expression (Figure 2F). Simulation of the rate equations showed that p53 protein level attained a plateau after the initial increase. Moreover, HuR expression also exhibited a non-linear increase compared with the continuous linear increase as seen in the previous model. However, in neither case the simulation (solid purple line) matched with the experimentally observed change (bar graphs) in protein levels (Pearson R = −0.10 for p53 and 0.21 for HuR) (Figure 2G). Hence inclusion of miR-125b as a negative regulator of HuR protein synthesis improved the model but failed to match the observed pulsatile change in either HuR or p53. This suggested the presence of yet unknown regulatory factor(s) in the network.
Figure 2. Inclusion of miR-125b As a Negative Regulator of HuR Expression Fails to Obtain a Fit between the Model and Experimental Observations

(A) Schematic representation of the region of the HuR 3’ UTR containing the putative miR-125b target site and Homo sapiens miR-125b sequence.

(B) Immunoblots of lysates of MCF7 cells transfected with three increasing concentrations of pSUPER-EGFP-miR-125b probed with HuR and GAPDH antibodies. Semiquantitative RT-PCR of total RNA isolated from the cell lysates using HuR- and GAPDH-specific primers (lower panels).

(C) MCF7 cells transfected with firefly luciferase constructs without 3’ UTR sequence or containing HuR-WT mRNA 3’ UTR or miR-125b-binding-site-mutated HuR mRNA 3’ UTR were cotransfected with three increasing concentrations of

\[
\frac{d\text{[HuR]}}{dt} = \frac{\alpha_{\text{HuR}}}{1 + K_{\text{up}}[\text{miR-125b}]} - \beta_{\text{HuR}}[\text{HuR}]
\]

\[
\frac{d[p53]}{dt} = \frac{\alpha_{p53}[1 + K_{\text{up}}[\text{HuR}]]}{1 + K_{\text{up}}[\text{miR-125b}]} - \beta_{p53}[p53]
\]

\[
\frac{d[Mdm2]}{dt} = \alpha_{\text{Mdm2}}[1 + K_{\text{p53}}[p53]] - \beta_{\text{Mdm2}}[Mdm2]
\]

\[
\frac{d[\text{miR-125b}]}{dt} = \alpha(t)
\]
Incorporation of a Hypothetical Protein Degradation Factor as a Negative Regulator of HuR in the Model Obtains a Fit with the Experimental Data

Incorporation of miR-125b as an inhibitor of HuR protein synthesis in the model only modified the nature of the expression kinetics but failed to match the pulsatile expression pattern of HuR. We therefore investigated whether the degradation of HuR protein contributed to the pulsatile change in HuR level. HuR protein level was found to remain nearly unaltered for 12 h in cells treated with CHX, an inhibitor of global protein synthesis, demonstrating HuR to be a highly stable protein (Figure 3A, quantification in Figure S6A). However, UV irradiation with CHX treatment led to a 70% reduction in HuR protein by 12 h, suggesting a rapid degradation of HuR protein induced by UV exposure (Figure 3A, quantification in Figure S6B). Therefore we investigated whether UV induced proteasomal degradation of HuR. Treatment of cells with CHX and MG-132, a proteasome inhibitor, prevented the degradation of HuR post UV exposure (Figure 3A, quantification in Figure S6C). Also, immunoprecipitation of HuR, followed by western blot with anti-ubiquitin antibody, showed enhanced poly-ubiquitination of HuR in UV-treated cells when compared with cells not exposed to UV (Figure 3B). The poly-ubiquitination of HuR increased in a time-dependent manner and showed a maximum between 6 and 12 h post UV exposure, coinciding with the decrease in HuR level (Figure 3C). Together, these observations demonstrated that HuR undergoes ubiquitination and proteasomal degradation induced by UV irradiation.

As no regulator of HuR degradation in response to UV irradiation was known, we postulated “X” as a novel negative regulator of HuR that is induced as a result of UV irradiation and leads to degradation of HuR protein (Figure 3D). X was incorporated as a negative regulator of HuR in the differential equation describing the expression of HuR (Figure 3E). Remarkably, simulation of the rate equations generated a pulsatile expression pattern for both p53 and HuR (solid purple lines), which matched satisfactorily (Pearson R = 0.76 for p53 and R = 0.94 for HuR) with the experimentally observed change in HuR and p53 levels (bar graphs) post DNA damage (Figure 3F). This suggested that the pulsatile change in HuR level, and its effect on p53, may be a result of the combination of translation repression of HuR mRNA by miR-125b and degradation of HuR protein by an unknown factor(s) induced by UV radiation.

The Hypothetical HuR Degradation Factor “X” Is the E3 Ubiquitin Ligase TRIM21

To identify the hypothetical HuR protein degradation factor “X,” myc-tagged HuR was overexpressed in MCF7 cells. Cells exposed to UV irradiation and treated with MG132 were lysed 6 h after UV exposure, and lysates were immunoprecipitated with anti-HuR antibody and IgG. The immunoprecipitate was analyzed by mass spectrometry for proteins specifically co-immunoprecipitated with HuR when compared with IgG (Table S2). Two proteins, Serpin B3 and the E3 ubiquitin protein ligase tripartite motif-containing 21 (TRIM21), along with HuR itself, were found to be specifically present in the HuR immunoprecipitate. Serpin B3 was not considered to play a role in HuR degradation as it is a serine protease inhibitor. However, as HuR was found to undergo proteasomal degradation in response to UV irradiation, interaction with an E3
A Time post CHX treatment (h): 0 2 4 6 8 12

Immunoblot, HuR

Immunoblot, GAPDH

B Ctrl. UV Input lysate Ctrl. UV IP Ab: IgG HuR IgG HuR

Immunoblot, Ubiquitin

Immunoblot, HuR

Immunoblot, GAPDH

C Time post UV exposure (h): 0 2 4 6 8 12

HuR [Ub]n

Immunoblot, Ubiquitin

Immunoblot, HuR

Immunoblot, GAPDH

D UV miR-125b HuR X

K_{K_x} \frac{d[H][HuR]}{dt} = K_{K_{HuR}}[HuR][X] - \beta_{HuR}[HuR]

\frac{d[p53]}{dt} = \alpha_{IU}[p53] + \beta_{IU}[p53]

\frac{d[Mdm2]}{dt} = \alpha_{IU}[Mdm2] + \beta_{IU}[Mdm2]

\frac{d[H]}{dt} = f(t)

E

\frac{d[Ub]}{dt} = \alpha_{X} - \beta_{X}[X]

\frac{d[Ub]}{dt} = \alpha_{Mdm2}[Ub] - \beta_{Mdm2}[Ub]

\frac{d[H]}{dt} = \alpha_{HuR} - \beta_{HuR}[HuR]

\frac{d[p53]}{dt} = \alpha_{IU}[p53] - \beta_{IU}[p53]

\frac{d[Mdm2]}{dt} = \alpha_{IU}[Mdm2] - \beta_{IU}[Mdm2]

F Relative concentration

p53

Computational

Experimental

Time (hours)

HuR

Relative concentration

Computational

Experimental

Time (hours)

** **

***###

# #
Ubiquitin ligase was highly significant. Therefore the E3 ubiquitin ligase TRIM21 was considered as a potential candidate for the unknown factor “X.”

**TRIM21 Interacts with HuR in Response to UV Irradiation and Causes Poly-ubiquitination and Degradation of HuR**

To confirm the interaction between TRIM21 and HuR, TRIM21 was immunoprecipitated from MCF7 cells treated with MG132, and HuR was found to associate with TRIM21 by immunoblotting with HuR antibody, and vice versa (Figure 4A). This interaction between HuR and TRIM21 was found to be enhanced in UV-exposed cells when compared with cells not exposed to UV (Figure 4B) and was independent of RNA binding (Figure S7). Also, the interaction between TRIM21 and HuR was found to increase in a time-dependent manner over the period of 12 h post UV exposure and was maximum between 4 and 12 h post UV exposure, which coincided with the previously observed decrease in HuR level (Figure 4C). We also checked whether the knockdown of TRIM21 affected the poly-ubiquitination of HuR post UV irradiation. Cells transfected with a small interfering RNA (siRNA) against TRIM21 (Figure 4D, lower panel) showed reduced poly-ubiquitination of HuR post UV exposure, compared with cells transfected with a control siRNA, and the level of poly-ubiquitination of HuR in siTRIM21-transfected cells exposed to UV was similar to that in cells not exposed to UV (Figure 4D, right panel, lanes 1 and 4). Overexpression of TRIM21 in MCF7 cells caused the degradation of HuR upon UV irradiation only, confirming that UV irradiation induced the interaction between TRIM21 and HuR and the subsequent degradation of the latter (Figure 4E). The breast carcinoma cell line MDA-MB-231, which has lower level of HuR compared with MCF7, was found to have higher level of TRIM21, and siRNA-mediated knockdown of TRIM21 in MDA-MB-231 could enhance the level of HuR in these cells (Figure 4F). Together, these observations showed TRIM21 as the E3 ubiquitin ligase responsible for the UV-induced poly-ubiquitination and degradation of HuR.

**UV-Induced Ubiquitination of Lys-182 by TRIM21 Causes HuR Degradation**

Previously ubiquitination of the Lys-182 (K182) residue in the RNA recognition motif 2 (RRM2) of HuR (Figure 5A) has been shown to be responsible for HuR degradation induced by heat shock, but the E3 ubiquitin ligase that ubiquitinates the K182 residue was not demonstrated (Abdelmohsen et al., 2009). Therefore we investigated whether the UV-induced degradation of HuR involved ubiquitination of K182 and TRIM21 was the E3 ubiquitin ligase responsible. Exogenous expression of Myc-tagged wild-type HuR (HuR-WT) and HuR in which the Lys-182 was mutated to Arg (K182R) in MCF7 cells followed by UV irradiation led to degradation of HuR-WT during the 12-h period post UV irradiation, whereas HuR-K182R was refractory to degradation (Figure 5B). Determination of the half-life of the WT and K182R mutant HuR post UV-irradiation showed that HuR-WT had a half-life of around 7 h, whereas the half-life of the K182R mutant was greater than 12 h (Figure 5C). Overexpression of hemagglutinin (HA)-tagged ubiquitin in cells showed a 2-fold decrease in HuR level compared with cells not overexpressing ubiquitin 6 h post UV exposure (Figure S8). Cells expressing HA-tagged ubiquitin, when transfected with constructs expressing HuR-WT or K182R mutant HuR and exposed to UV, showed significantly higher ubiquitination of HuR-WT compared with K182R mutant HuR (Figure 5D). The overall level of ubiquitination remained nearly unchanged in cells
The modeling of p53 and HuR expression post UV-mediated DNA damage led to the discovery of miR-125b and TRIM21 as regulators of HuR synthesis and degradation, respectively. We therefore investigated the combined contributions of miR-125b and TRIM21 in generating the pulsatile change of cytoplasmic HuR and p53 levels in response to UV irradiation. MCF7 cells treated with a control oligo showed the pulsatile pattern of cytoplasmic HuR during the 12-h period post UV irradiation with a peak at around 4 h post UV exposure (Figure 6B). However, treatment with an antagonomiR against miR-125b abolished the pulse and resulted in a plateau in HuR level from around 6 h post UV exposure (Figure 6B and best-fit curve in Figure 6C). This demonstrated that antagonizing miR-125b function resulted in de-repression of HuR protein synthesis. Similarly, inhibition of HuR degradation by siRNA-mediated knockdown of TRIM21 resulted in a
A

| NLS | RRM1 | RRM2 | HNS | RRM3 |
|-----|------|------|-----|------|
|     |      |      |     |      |

HuR WT

| NLS | RRM1 | RRM2 | HNS | RRM3 |
|-----|------|------|-----|------|
|     |      |      |     |      |

HuR K182R

B

Time post UV exposure (h): 0 2 4 6 8 12

HuR WT-Myc

Immunoblot, Myc

Immunoblot, β Actin

HuR K182R-Myc

Immunoblot, Myc

Immunoblot, β Actin

C

C

Relative Myc:Tag HuR Levels

Time Post UV Irradiation (Hours)

D

Input lysate
HuR WT HuR K182R

IP Ab: IgG HA IgG HA

Immunoblot, Myc

Immunoblot, β Actin

E

Input lysate IP Ab: IgG TRIM21

HuR WT: + -

HuR K182R: - +

TRIM21: + +

IgG TRIM21

MITM: + +

Immunoblot, Myc

Immunoblot, TRIM21

F

HuR WT

TRIM21

Immunoblot, Myc

Immunoblot, TRIM21

Immunoblot, β Actin

G

Time post UV exposure (h): 0 4 12

HuR WT

HuR K182R

Immunoblot, Myc

Immunoblot, β Actin
Similar effects of antagomiR-125b and TRIM21 siRNA were observed on p53 levels, except that the pulsatile expression pattern of HuR post DNA damage to a linear increase, as seen in our initial model demonstrated that the combination of translation de-repression and inhibition of degradation converted like HuR level (Figure 6B and best-fit curve in Figure 6C). This is likely due to the effect of Mdm2-mediated upon treatment with combination of antagomiR-125b and TRIM21 siRNA, p53 level attained a plateau un-factor, resulted in both p53 and HuR levels attaining plateaux (Figure S11), corresponding to the experimental observations on antagomiR-125b transfection. This shows that the abrogation of either translation repression or protein degradation will result in the loss of the pulsatile change in HuR and p53 levels.

To investigate the effect of the miR-125b-TRIM21-HuR-p53 regulatory network on cell behavior in response to DNA damage, we checked the viability of the UV-treated cells, which have been subjected to inhibition of miR-125b or TRIM21 function, or both. Prolonged increase in p53 level in response to DNA damage will block the cell cycle and decrease cell viability, whereas the pulsatile decrease in p53 level will allow the cells to re-enter the cell cycle and resume cell proliferation. Analysis of the viability of UV-treated MCF7 cells showed that cells transfected with a control oligo did not show a significant change in cell viability, except at 4 h post UV treatment, when the p53 level is the highest (Figure 7A). However, cells that have been transfected with either antagomiR-125b or TRIM21 siRNA or both, in all of which cases p53 levels attains a high plateau from 8–12 h post UV treatment, showed significant reduction in cell viability when compared with control oligo-transfected cells (Figure 7A). We also checked whether the change in cell viability on treatment with antagomiR-125b or TRIM21 siRNA was due to the effect of changes in p53 level. MDA-MB-231 cells, which have non-functional p53, were similarly transfected with a control oligo, antagomiR-125b or TRIM21 siRNA, or a combination of both. Interestingly, MDA-MB-231 cells transfected with control oligo showed significantly reduced viability from 8–12 h post UV irradiation, suggesting that the absence of DNA repair activity of p53 in these cells led to reduced viability (Figure 7B). Also, there was no difference in viability between cells treated with the control oligo and with antagomiR-125b or TRIM21 siRNA or both, unlike in MCF7 cells, indicating that the absence of functional p53 resulted in no effect of inactivation of miR-125b and TRIM21 (Figure 7B). These observations demonstrated that the crucial effect of the miR-125b-TRIM21-HuR regulatory network on cell survival is mediated by its effect on the regulation of p53 expression.

**DISCUSSION**

Dynamic modeling, coupled with experimental validation, of the regulatory system controlling p53 mRNA translation in response to UV-induced DNA damage revealed the role of miR-125b and TRIM21 as regulators of protein synthesis and degradation, respectively, of the RNA-binding protein HuR. HuR undergoes nuclear-cytoplasmic translocation in response to a variety of stress stimuli such as UV, bacterial
We have investigated the role of the p53 mRNA translation regulatory network, specifically the translation of both cell proliferation and apoptosis (Le et al., 2011). The demonstration of miR-125b as a regulator of p53 protein synthesis mediated by HuR appears to be crucial in attaining an increased steady-state level of p53, together with the stabilization of p53 protein by inhibition of its interaction with Mdm2. HuR increases p53 protein synthesis not only by directly enhancing p53 mRNA translation but also by preventing the repression of p53 translation by the UV-induced miRNA, miR-125b (Ahuja et al., 2016). The biphasic expression of miR-125b in response to UV allows an enhancement of p53 expression at early time points after UV exposure, but causes repression of p53 mRNA translation at later time points, thereby contributing to the pulsatile nature of p53 expression (Ahuja et al., 2016).

The decrease in HuR and p53 levels in the later time period post UV exposure is partially due to translation repression by miR-125b. However, as inclusion of miR-125b as a repressor of HuR protein synthesis failed to generate the pulsatile behavior of HuR and p53, we considered degradation of HuR protein contributing to oscillatory behavior of p53. Despite its stability, HuR protein destabilization by ubiquitin-mediated proteolysis or caspase-mediated cleavage is observed in response to stresses such as moderate heat shock, glycolysis inhibition, DNA damage, and CoCl2-induced hypoxia (Abdelmohsen et al., 2009; Chu et al., 2016). The biphasic expression of miR-125b in response to UV allows an enhancement of p53 expression at early time points after UV exposure, but causes repression of p53 mRNA translation at later time points, thereby contributing to the pulsatile nature of p53 expression (Ahuja et al., 2016).

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The integration of these incoherent and coherent feedforward loops with the p53-Mdm2 negative feedback loop causes the pulsatile change in p53 level in response to genotoxic stress and gives a window of opportunity to the cells to re-enter the cell cycle and resume cell division.

These two C-FFLs act in concert to enhance cellular p53 level only in response to persistent DNA damage. The C-FFLs in the p53 regulatory network include a type 1 C-FFL, which involves damaged DNA, HuR, and p53, and a type 2 C-FFL consisting of miR-125b, HuR, and p53 (Figure S16). Together, these observations have provided the basis of a systematic approach consisting of computational modeling and biochemical experimentation, which has allowed further elucidation of the intricate regulatory network controlling p53 expression and cell behavior in response to genotoxic stress. The translation regulatory network of p53 in response to UV-induced DNA damage consists of two overlapping incoherent and coherent feedforward loops and negative feedback loop with HuR and p53 as the central axis. See also Figure S16.

Limitations of the Study

This study develops and validates a mathematical model for the regulation of p53 expression in response to DNA damage that includes the regulators of p53 protein synthesis and stabilization. However, the model excludes the dynamics of the upstream regulators of p53 activation, such as the sensor and effector kinases.
(Maréchal and Zou, 2013), assuming these to be reflected in the dynamics of Mdm2, their downstream target. The proposed mathematical model can be said to capture the major contributing factors of the modeled phenomena if the model results are close to the experimental observations. However, it is a partial model, and the regulatory system, owing to its high level of complexity, will have aspects that are unaccounted for in the model (Aris and Penn, 1980). Explicit inclusion of the dynamics of the upstream regulators of p53 activation would lead to further refinement of the model and may lead to the discovery of other novel regulators and mediators of the cellular DNA damage response.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.05.002.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.S.R. and B.P.; Methodology, P.S.R., B.P., S.D.M., and A. Ghosh; Investigation, A. Guha, D.A., B.P., S.D.M., K.D., and B.W.; Resources, D.R. and V.S.; Writing, P.S.R., A. Guha, and S.D.M.; Supervision, P.S.R.; Funding Acquisition, P.S.R.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

Abdelmohsen, K., Srikantan, S., Yang, X., Lal, A., Kim, H.H., Kuwano, Y., Galban, S., Becker, K.G., Kamara, D., de Cabo, R., et al. (2009). Ubiquitin-mediated proteolysis of HuR by heat shock. EMBO J. 28, 1271–1282.

Abdelmohsen, K., Kim, M.M., Srikantan, S., Mercken, E.M., Brennan, S.E., Wilson, G.M., de Cabo, R., and Gorospe, M. (2010). miR-519 suppresses tumor growth by reducing HuR levels. Cell Cycle 9, 1354–1359.

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.

Aris, R., and Penn, M. (1980). The mere notion of a model. Math. Model. 1, 1–12.

Batchelor, E., Mock, C.S., Bhan, I., Loewer, A., and Lahav, G. (2008). Recurrent initiation: a mechanism for triggering p53 pulses in response to DNA damage. Mol. Cell 30, 277–289.

Batchelor, E., Loewer, A., Mock, C., and Lahav, G. (2011). Stimulus-dependent dynamics of p53 in single cells. Mol. Syst. Biol. 7, 1–8.

Ben-Chetrit, E., Fox, R.I., and Tan, E.M. (1990). Dissociation of immune responses to the SS-A (Ro) 52-kd and 60-kd polypeptides in systemic lupus erythematosus and Sjögren’s syndrome. Arthritis Rheum. 33, 349–355.

Chu, P.-C., Chuang, H.-C., Kulp, S.K., and Chen, C.-S. (2012). The mRNA-stabilizing factor HuR protein is targeted by β-TrCP protein for degradation in response to glycolysis inhibition. J. Biol. Chem. 287, 43639–43650.

Ciliberto, A., Novak, B., and Tyson, J.J. (2005). Steady states and oscillations in the p53/Mdm2 network. Cell Cycle 4, 488–493.

Collister, M., Lane, D.P., and Kuehl, B.L. (1998). Differential expression of p53, p21(waf1/cip1) and hdm2 dependent on DNA damage in Bloom’s syndrome fibroblasts. Carcinogenesis 19, 2115–2120.

Espinosa, A., Zhou, W., Ek, M., Hedlund, M., Brauner, S., Popovic, K., Horvath, L., Wallerskog, T., Oukka, M., Nyberg, F., et al. (2006). The Sjögren’s syndrome-associated autoantigen Ro52 is an E3 ligase that regulates proliferation and cell death. J. Immunol. 176, 6277–6285.

Espinosa, A., Dardalhon, V., Brauner, S., Ambrosi, A., Higgs, R., Quintana, F.J., Sjöstrand, M., Eloranta, M.-L., Ni Gabhann, J., Winqvist, O., et al. (2009). Loss of the lupus autoantigen Ro52/Trim21 induces tissue inflammation and systemic autoimmunity by disregulating the IL-23-Th17 pathway. J. Exp. Med. 206, 1661–1671.

Fu, L., Minden, M.D., and Benchimol, S. (1996). Translational regulation of human p53 gene expression. EMBO J. 15, 4392–4401.
Gagliani, G., and Lahav, G. (2014). Constant rate of p53 tetramerization in response to DNA damage controls the p53 response. Mol. Syst. Biol. 10, 1–8.

Gallouzi, I.E., Brenner, C.M., and Stetiz, J.A. (2001). Protein ligands mediate the CRM1-dependent export of HuR in response to heat shock. Mol. Syst. Biol. 7, 1348–1361.

Geva-Zatorsky, N., Rosenfeld, N., Itzkovitz, S., Milo, R., Sigal, A., Dekel, E., Yarnitzky, T., Liron, Y., Polak, P., Lahav, G., et al. (2006). Oscillations and variability in the p53 system. Mol. Syst. Biol. 2, 1–13.

Guo, X., Wu, Y., and Hartley, R.S. (2009). MicroRNA-125b represses cell growth by targeting HuR in breast cancer. RNA Biol. 6, 575–583.

Higgs, R., Nigabhandh, J., Ben Larbi, N., Breen, E.P., Fitzgerald, K.A., and Jeffreys, C.A. (2008). The E3 ubiquitin ligase Ro52 negatively regulates IFN-beta production post-pathogen recognition by polylubiquitin-mediated degradation of IRF3. J. Immunol. 181, 1780–1786.

Horn, H.F., and Vousden, K.H. (2007). Coping with stress: multiple ways to activate p53. Oncogene 26, 1306–1316.

Ikeda, K., and Inoue, S. (2012). TRIM proteins as Participation of p53 protein in the cellular mechanisms. Prostate 72, 6311.

Kojima, K., Fujita, Y., Nozawa, Y., Deguchi, T., and Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R.W. (1991). Negative regulation of RNA-binding protein HuR by tumor suppressor ECRG2. Oncogene 6, 28–38.

Kastan, M.B., Oryekwore, O., Sdiransky, D., Vogelstein, B., and Craig, R.W. (1991). Participation of p53 protein in the cellular response to DNA damage. Cancer Res. 51, 6304–6311.

Kojima, K., Fujita, Y., Nozawa, Y., Deguchi, T., and Ito, M. (2010). MiR-34a attenuates paclitaxel-resistance of hormone-refractory prostate cancer PC3 cells through direct and indirect mechanisms. Prostate 70, 1501–1512.

Lahav, G. (2008). Oscillations by the p53-Mdm2 feedback loop. Adv. Exp. Med. Biol. 647, 28–38.

Lahav, G., Rosenfeld, N., Sigal, A., Geva-Zatorsky, N., Levine, A.J., Elowitz, M.B., and Alon, U. (2004). Dynamics of the p53-Mdm2 feedback loop in individual cells. Nat. Genet. 36, 167–150.

Lazzarini, E., Karzowieniwoska, J., Nigabhandh, J., Smith, S., Barnes, B.J., and Jeffreys, C.A. (2014). TRIPartite motif 21 (TRIM21) differentially regulates the stability of interferon regulatory factor 5 (IRFS) isoforms. PloS One 9, e103609.

Le, M.T.N., Teh, C., Shyh-Chang, N., Xie, H., Zhou, B., Korzh, V., Lodish, H.F., and Lim, B. (2009). MicroRNA-125b is a novel negative regulator of p53. Genes Dev. 23, 862–876.

Le, M.T.N., Shyh-Chang, N., Khaw, S.L., Chin, L., Teh, C., Tay, J., O’Day, E., Korzh, V., Yang, H., Lal, A., et al. (2011). Conserved regulation of p53 network dosage by microRNA-125b occurs through evolving miRNA-target gene pairs. PLoS Genet. 7, e1002242.

Lev Bar-Or, R., Maya, R., Segel, L.A., Alon, U., Levine, A.J., and Oren, M. (2000). Generation of oscillations by the p53-Mdm2 feedback loop: a theoretical and experimental study. Proc. Natl. Acad. Sci. U S A 97, 11250–11255.

Lucchesi, C., Sheikh, M.S., and Huang, Y. (2016). Negative regulation of RNA-binding protein HuR by tumor suppressor ECRG2. Oncogene 35, 2565–2573.

Ma, L., Wagner, J., Rice, J.J., Hu, W., Levine, A.J., and Stolovitzky, G.A. (2005). A plausible model for the digital response of p53 to DNA damage. Proc. Natl. Acad. Sci. U S A 102, 14266–14271.

Mangan, S., and Alon, U. (2003). Structure and function of the feed-forward loop network motif. Proc. Natl. Acad. Sci. U S A 100, 11980–11985.

Maréchal, A., and Zou, L. (2013). DNA damage sensing by the ATM and ATR kinases. Cold Spring Harb. Perspect. Biol. 5, a012716.

Mazan-Mamczarz, K., Galbán, S., López 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.

Michael, D., and Oren, M. (2003). The p53-Mdm2 module and the ubiquitin system. Semin. Cancer Biol. 13, 49–58.

Morse, H.C., Ozato, K., and Ozato, K. (2009). Regulation of tumor suppressor p53 at the RNA level. J. Mol. Med. (Berl) 88, 645–652.

Wagner, J., Ma, L., Rice, J.J., Hu, W., Levine, A.J., and Stolovitzky, G.A. (2005). p53-Mdm2 loop controlled by a balance of its feedback strength and effective dampening using ATM and delayed feedback. Syst. Biol. (Stevenage) 152, 109–118.

Wang, W., Furnameaux, H., Cheng, H., Caldwell, M.C., Hutter, D., Liu, Y., Holbrook, N., and Gorospe, M. (2000). HuR regulates p21 mRNA stabilization by UV light. Mol. Cell. Biol. 20, 760–769.

Xu, F., Zhang, X., Lei, Y., Liu, X., Liu, Z., Tong, T., and Wang, W. (2010). Loss of repression of HuR translation by miR-16 may be responsible for the elevation of HuR in human breast carcinoma. J. Cell. Biochem. 111, 727–734.

Yoshimi, R., Chang, T.-H., Wang, H., Asumi, T., Morse, H.C., Ozato, K., and Ozato, K. (2009). Gene disruption study reveals a nonredundant role for TRIM21/Ro52 in NF-kappaB-dependent cytokine expression in fibroblasts. J. Immunol. 182, 7527–7536.

Zhang, T., Bzathnik, P., and Tyson, J.J. (2007). Exploring mechanisms of the DNA-damage response: p53 pulses and their possible relevance to apoptosis. Cell Cycle 6, 85–94.

Zhang, Y., Gao, J.-S., Tang, X., Tucker, L.D., Quesenberry, P., Rigoutsos, I., and Ramratnam, B. (2009). MicroRNA-125b and its regulation of the p53 tumor suppressor gene. FEBS Lett. 583, 3725–3730.

Zhang, Z., Mao, M., Lu, N., Weng, L., Yuan, B., and Liu, Y.-J. (2012). The E3 ubiquitin ligase TRIM21 negatively regulates the innate immune response to intracellular double-stranded DNA. Nat. Immunol. 14, 172–178.

Reddy, B.A., van der Knaap, J.A., Bot, A.G.M., Mohd-Sarip, A., Dekkers, D.H.W., Timmermans, M.A., Martens, J.W.M., Demmers, J.A.A., and Venniger, C.P. (2014). Nucleotide biosynthetic enzyme GMP synthase is a TRIM21-controlled relay of p53 stabilization. Mol. Cell 53, 448–470.

Shwetha, S., Sharma, G., Raheja, H., Goel, A., Aggarwal, R., and Das, S. (2018). Interaction of miR-125b-Sp with Human antigen R mRNA: mechanism of controlling HCV replication. Virus Res. 258, 1–8.

Stewart-Ornstein, J., and Lahav, G. (2017). p53 dynamics in response to DNA damage vary across cell lines and are shaped by efficiency of DNA repair and activity of the kinase ATM. Sci. Signal. 10, eaah6671.

Talwar, S., Jin, J., Carroll, B., Liu, A., Gillespie, M.B., and Palanisamy, V. (2011). Caspase-mediated cleavage of RNA-binding protein HuR regulates c-Myc protein expression after hypoxic stress. J. Biol. Chem. 286, 32333–32343.

Vilborg, A., Wilhelm, M.T., and Wiman, K.G. (2010). Regulation of tumor suppressor p53 at the DNA repair and activity of the kinase ATM. Sci. Signal. 3, a012716.
Supplemental Information

Integrated Regulation of HuR by Translation
Repression and Protein Degradation Determines
Pulsatile Expression of p53 Under DNA Damage

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Modelling of the p53-Mdm2 negative feedback circuit shows a pulse of p53 in response to UV. (A) Network diagram of the minimal p53-Mdm2 negative feedback circuit in response to UV irradiation. (B) Rate equations representing the p53-Mdm2 negative feedback circuit in response to UV irradiation. (C) Plot representing simulation and bar graphs representing experimental data of change of p53 level over a 12 h period post-exposure to a 10 J/m² pulse of UVC irradiation. The simulation plot represents the numerical integration of the rate equations in (B). The bar graphs represent intensity values of p53 bands obtained from immunoblots of cell lysates collected at the designated time points post UV exposure, normalized to corresponding GAPDH band intensities. Data represents mean ± SD values from 7 independent immunoblots. The normalized band intensities are scaled to the 0 hour time point band intensity, taken as 1.
Figure S2. Related to Figure 2.

(A) Immunoblot of lysates from MCF7 and MDA-MB-231 cells with anti-HuR and anti- β-actin antibodies. (B) qRT-PCR of total RNA from MCF-7 and MDA-MB-231 with miR-125b specific primers. Data represents mean ± SD from two biological replicates, each with two technical replicates. (C) Immunoblot of lysates from MDA-MB-231 cells transfected with two increasing concentrations of antagoniR-125b with anti-HuR and anti- β-actin antibodies. * signifies a p-value ≤ 0.05 and ** signifies a p-value ≤ 0.01.
Figure S3. Related to Figure 2

(A) Immunoblot of lysates from MCF-7 cells transfected with three increasing concentrations of miR-125b-expressing plasmid. HuR band intensities are normalized to corresponding GAPDH band intensities. (B) qRT-PCR of total RNA from MCF-7 cells cotransfected with three increasing concentrations of miR-125b-expressing plasmid and a plasmid containing HuR mRNA 3’UTR downstream of firefly luciferase gene with miR-125b specific primers. (C) qRT-PCR of total RNA from the same cells with firefly luciferase specific primers. Data represents mean ± SD from three independent experiments. ** signifies a p-value <= 0.01.
MCF-7 cells transfected with Firefly luciferase constructs without 3’UTR sequence or containing wild-type HuR mRNA 3’UTR or miR-125b binding site-mutated HuR mRNA 3’UTR was exposed to UV irradiation following which cells were collected at the designated time points and luciferase assay performed. Fluc values are normalized to RLuc values as transfection control. Data represents mean ± SD from three independent experiments each with two replicates. *signifies a p-value \( \leq 0.05 \), ** signifies a p-value \( \leq 0.01 \) and *** signifies a p-value \( \leq 0.005 \).
Figure S5. Related to Figure 2

Lysates of MCF7 cells either mock transfected or transfected with miR-125b expressing construct were immunoprecipitated with anti-Ago2 antibody or non-immune IgG. RNA associated with the immunoprecipitates was isolated and associated HuR mRNA and miR-125b RNA levels were determined by qRT-PCR. Data represents mean ± SD from two biological replicates, each with two technical replicates. * signifies a p-value ≤ 0.05.
Immunoblots of lysates of MCF7 cells treated with cycloheximide (CHX) (A) or CHX and UV irradiation (B) and CHX and MG132 and UV irradiation (C) and collected at designated time points over a 12 hour period. Lysates were probed with HuR and GAPDH antibodies. HuR band intensities were normalized to GAPDH band intensities. Data represents mean ± SD from three independent experiments. * signifies a p-value \( \leq 0.05 \), ** signifies p-value \( \leq 0.005 \).
(A) Lysates of MCF7 cells were either treated or not treated with RNase A and immunoprecipitated with anti-HuR antibody or non-immune IgG. Immunoprecipitates and input lysates were immunoblotted with anti-TRIM21, HuR and GAPDH antibodies. (B) Total RNA isolated from input lysate without and with RNase A treatment was electrophoresed on agarose gel and stained with ethidium bromide.
Figure S8. Related to Figure 5.

(A) Lysates of MCF7 cells either mock transfected or transfected with a construct expressing HA-tagged ubiquitin and treated with MG132 was immunoblotted with anti-HA and anti-ubiquitin antibodies. (B) MCF7 cells transfected as in (A) were collected 6 hours after UV irradiation and immunoblotted with anti-HuR and anti-GAPDH antibodies.
Figure S9. Related to Figure 5.

(A) Immunoblots of cell lysates expressing Myc-tagged HuR WT or HuR K182R transfected with three increasing concentrations of a construct expressing TRIM21. 48 hours post transfection cells were treated with CHX for 30 min and UV irradiated. Lysates were immunoblotted with anti-Myc and anti-β Actin antibodies. Myc-tagged HuR band intensities were normalized to β Actin band intensities. (B) Immunoblots of cytoplasmic lysates of cells expressing Myc-tagged HuR WT or HuR K182R and UV irradiated. Myc-tagged HuR band intensities were normalized to β Actin band intensities. Data represents mean ± SD from three independent experiments. * signifies a p-value ≤ 0.05.
(A) Whole cell lysates of MCF7 cells transfected with control siRNA or TRIM21 siRNA and treated with CHX and collected at designated time points post exposure to 10 J/m² pulse of UV were immunoblotted with TRIM21, HuR and GAPDH antibodies. (B) Quantification of HuR levels, normalized to GAPDH, from three independent experiments showing Mean ± SD and best fit curves.
Figure S11. Related to Figure 6.

(A) Rate equations representing the p53 translation regulation network in response to UV irradiation. The rate equation for HuR expression contains a term representing the effect of X ($K_{XH}$) on HuR degradation. Terms representing the effect of miR-125b have been omitted from all equations. 

(B) Plots representing simulation and experimental data of change of p53 and HuR levels over a 12 hour period post exposure to a 10 J/m² pulse of UVC irradiation. The computed plots (purple lines) represent the numerical integration of the rate equations in (A). The experimental data is represented as bar graphs. Experimental data represents mean ± SD values from 7 independent immunoblots, normalized to corresponding GAPDH band intensities.
Estimation of robustness of Model 1 (*Figure S1*) by varying assumed parameters (initial conditions: p53 concentration (PV16) = 0.55 and Mdm2 concentration (PV17) = 0.05) between 0-2
Figure S13. Related to Figure 1.

Estimation of robustness of Model 2 (Figure 1B) by varying assumed parameters (initial conditions: p53 concentration (PV16) = 1, HuR concentration (PV15) = 1, and Mdm2 concentration (PV17) = 0.05) between 0-2.
Estimation of robustness of Model 3 (Figure 2E) by varying assumed parameters (initial conditions: p53 concentration (PV16) = 1, HuR concentration (PV15) = 1, and Mdm2 concentration (PV17) = 0.05) between 0-2.
Estimation of robustness of Model 4 (Figure 3D) by varying of initial conditions (p53 concentration (PV16) = 1, HuR concentration (PV15) = 1, Mdm2 concentration (PV17) = 0.05 and X concentration (PV18) = 0.1 ) between 0-2 and assumed parameters ($K_{XH}$(PV14) = 0.5, $\alpha_X$(PV4) = 0.2 and $\beta_X$(PV8) = 0.15) over a 10-fold range of values.
Schematic diagrams of the three Type 1 and one Type 2 incoherent and coherent feed forward loops in the translation regulatory network regulating p53 expression in response to UV-induced DNA damage. The outputs of the individual feed forward loops show expression patterns of HuR, p53, miR-125b and TRIM21 in response to DNA damage, taken as a step function.
Table S1. Related to Figures 1, 2, and 3

Model details: Parameters and initial conditions

| PV | Parameter | Description | Values  | Source   |
|----|-----------|-------------|---------|----------|
| 1  | $\alpha_{HuR}$ | Cytoplasmic HuR accumulation rate | 0.6     | Experimental |
| 2  | $\alpha_{p53}$ | p53 synthesis rate | 0.4     | Experimental |
| 3  | $\alpha_{Mdm2}$ | Mdm2 synthesis rate | 0.2     | Literature¹ |
| 4  | $\alpha_X$ | X synthesis rate | 0.3     | Assumed |
| 5  | $\beta_{HuR}$ | HuR degradation rate | 0.008   | Experimental |
| 6  | $\beta_{p53}$ | p53 degradation rate | 0.22    | Experimental |
| 7  | $\beta_{Mdm2}$ | Mdm2 degradation rate | 1       | Literature¹ |
| 8  | $\beta_X$ | X degradation rate | 0.25    | Assumed |
| 9  | $k_{mH}$ | miR-125b dependent HuR repression rate | 0.309   | Experimental |
| 10 | $k_{mp}$ | miR-125b dependent p53 repression rate | 0.271   | Experimental |
| 11 | $k_{Hp}$ | HuR dependent p53 production rate | 1.185   | Experimental |
| 12 | $k_{pM}$ | p53 dependent mdm2 production rate | 0.9     | Literature¹ |
| 13 | $k_{Mp}$ | Mdm2 dependent p53 degradation rate | 1.4     | Literature¹ |
| 14 | $k_{XH}$ | X dependent HuR degradation rate | 0.25    | Assumed |
| 15 | $HuR_0$ | Initial HuR concentration | 1       | Assumed |
| 16 | $p53_0$ | Initial p53 concentration | 0.55    | Assumed¹ |
| 17 | $Mdm2_0$ | Initial Mdm2 concentration | 0.05    | Assumed |
| 18 | $X_0$ | Initial X concentration | 0.1     | Assumed |

† For minimal model, initial concentration of p53 was taken as 0.55 for simulation and then scaling was done to 1 for initial concentration of p53 for plotting.

References

[1] Eric Batchelor, Caroline Mock, Irun Bhan, Alexander Loewer, and Galit Lahav. Recurrent Initiation: A Mechanism for Triggering p53 Pulses in Response to DNA Damage. Molecular Cell, Volume 30, Issue 3, 277 – 289.
Table S2. Related to Figure 4

Comparison of Proteins identified in LC-MS analysis.

| Protein                                           | accession  | Mass Da | Spectral Counts | SC ratio |
|----------------------------------------------------|------------|---------|-----------------|----------|
| 78 kDa glucose-regulated protein precursor         | 16507237   | 72402   | 20              | 0.00     |
| actin, cytoplasmic 1                               | 4501885    | 42052   | 13              | 0.00     |
| ATP synthase subunit beta, mitochondrial precursor | 32189394   | 56525   | 6               | 0.00     |
| cold-inducible RNA-binding protein                 | 4502847    | 18637   | 6               | 0.00     |
| DNA damage-binding protein 1                       | 148529014  | 128142  | 11              | 0.00     |
| fructose-bisphosphate aldolase A isoform 1         | 4557305    | 39851   | 3               | 0.00     |
| hemoglobin subunit alpha                           | 4504345    | 15305   | 7               | 0.00     |
| lanC-like protein 1                                | 5174445    | 45995   | 19              | 0.00     |
| PREDICTED: probable ATP-dependent RNA helicase DDX46 isoform X1 | 530380277 | 117902  | 11              | 0.00     |
| serine/arginine-rich splicing factor 2             | 47271443   | 25461   | 5               | 0.00     |
| S-phase kinase-associated protein 1 isoform a      | 25777711   | 18223   | 8               | 0.00     |
| splicing factor U2AF 65 kDa subunit isoform a      | 6005926    | 53809   | 10              | 0.00     |
| stress-70 protein, mitochondrial precursor         | 24234688   | 73920   | 27              | 1        |
| putative RNA-binding protein Luc7-like 2 isoform 1 | 116812577  | 46942   | 18              | 1        |
| serine/arginine-rich splicing factor 1 isoform 1   | 5902076    | 27842   | 18              | 1        |
| tubulin beta chain                                 | 29788785   | 50095   | 52              | 6        |
| splicing factor U2AF 35 kDa subunit isoform a      | 5803207    | 28368   | 8               | 1        |
| ATP synthase subunit alpha, mitochondrial isoform a precursor | 4757810 | 59828   | 7               | 1        |
| serine/arginine-rich splicing factor 3             | 4506901    | 19546   | 12              | 2        |
| tubulin alpha-1B chain                             | 57013276   | 50804   | 27              | 5        |
| 40S ribosomal protein S5                           | 13904870   | 23033   | 5               | 1        |
| annexin A2 isoform 2                               | 4757756    | 38808   | 5               | 1        |
| galectin-7                                         | 4504598    | 15123   | 4               | 1        |
| protein-L-isoaspartate(O-aspartate) O-methyltransferase isoform 1 | 226530908 | 30524   | 8               | 2        |
| serum albumin preproprotein                        | 4502027    | 73117   | 20              | 5        |
| protein S100-A8                                    | 21614544   | 10885   | 7               | 2        |
| alpha-enolase isoform 1                            | 4503571    | 47481   | 34              | 10       |
| signal recognition particle 14 kDa protein         | 149999611  | 14675   | 3               | 1        |
| protein S100-A9                                    | 4506773    | 13291   | 16              | 6        |
| RNA-binding protein FUS isoform 1                  | 4826734    | 53622   | 16              | 6        |
| RNA-binding motif protein, X chromosome isoform 1   | 56699409   | 42306   | 13              | 5        |
| Protein Name | Accession Number | Fold Change | p-Value | Relative Expression |
|--------------|------------------|-------------|---------|---------------------|
| Glyceraldehyde-3-phosphate dehydrogenase isoform 1 | 7669492 | 36201 | 13 | 0.39 |
| Activated RNA polymerase II transcriptional coactivator p15 | 217330646 | 14386 | 4 | 0.40 |
| Desmocollin-1 isoform Dsc1b preproprotein | 4826702 | 94916 | 4 | 0.40 |
| RNA-binding protein EWS isoform 2 | 4885225 | 68721 | 2 | 0.40 |
| Heat shock 70 kDa protein 1A/1B | 167466173 | 70294 | 43 | 0.40 |
| Protein S100-A7 | 115298657 | 11578 | 8 | 0.42 |
| Peroxiredoxin-1 | 4505591 | 22324 | 7 | 0.44 |
| Desmoplakin isoform I | 58530840 | 334021 | 15 | 0.44 |
| Elongation factor 1-alpha 1 | 4503471 | 50451 | 6 | 0.46 |
| 40S ribosomal protein S19 | 4506695 | 16051 | 107 | 0.46 |
| 60S ribosomal protein L37a | 4506643 | 10497 | 2 | 0.50 |
| Nucleolin | 55956788 | 76625 | 2 | 0.50 |
| Desmoglein-1 preproprotein | 119703744 | 114702 | 22 | 0.55 |
| Small nuclear ribonucleoprotein Sm D3 | 4759160 | 14021 | 5 | 0.56 |
| Macrophage migration inhibitory factor | 4505185 | 12639 | 4 | 0.57 |
| Heterogeneous nuclear ribonucleoprotein U isoform b | 14114161 | 89665 | 3 | 0.60 |
| 60S ribosomal protein L23a | 17105394 | 17684 | 5 | 0.63 |
| 60S ribosomal protein L12 | 4506597 | 17979 | 11 | 0.65 |
| 60S ribosomal protein L21 | 18104948 | 18610 | 4 | 0.67 |
| Prolactin-inducible protein precursor | 4505821 | 16847 | 5 | 0.71 |
| Dermcidin preproprotein | 16751921 | 11391 | 8 | 0.73 |
| Junction plakoglobin | 4504811 | 82434 | 5 | 0.83 |
| THO complex subunit 4 | 238776833 | 27541 | 25 | 0.86 |
| 40S ribosomal protein S12 | 14277700 | 14905 | 6 | 1.00 |
| Malate dehydrogenase, mitochondrial isoform 1 precursor | 21735621 | 35937 | 3 | 1.00 |
| Small nuclear ribonucleoprotein Sm D1 | 5902102 | 13273 | 6 | 1.00 |
| High mobility group protein B1 | 4504425 | 25049 | 10 | 1.11 |
| 40S ribosomal protein S25 | 4506707 | 13791 | 8 | 1.14 |
| 60S ribosomal protein L23 | 4506605 | 14970 | 14 | 1.17 |
| 60S ribosomal protein L38 | 4506645 | 8270 | 11 | 1.38 |
| 60S acidic ribosomal protein P2 | 4506671 | 11658 | 30 | 1.43 |
| 60S acidic ribosomal protein P1 isoform 1 | 4506669 | 11621 | 10 | 1.67 |
| Histone H2B type 1-C/E/F/G/I | 4504257 | 13898 | 8 | 2.00 |
| ELAV-like protein 1 | 38201714 | 36240 | 6 | HuR only |
| Protein                                      | Accession | Fold Change | HuR | Remarks          |
|----------------------------------------------|-----------|-------------|-----|------------------|
| serpin B3                                    | 5902072   | 44565       | 0   | 2                |
| E3 ubiquitin-protein ligase TRIM21           | 15208660  | 54170       | 0   | 4                |

HuR only
**Transparent Methods**

Modelling procedure

Rate equations representing the rate of change of concentration of network components over time were represented by non-linear differential equations consisting of synthesis and degradation terms and regulatory terms representing the effect of other network components. Complete model details, initial conditions and parameters are provided in Table S1. The set of differential equations for each model were numerically integrated with the parametric values which were either obtained experimentally, or from literature or assumed. Differential equations were solved by ODE45 module MATLAB (Version R2010b. MathWorks). Curve fitting was done using Curve Fitting Toolbox.

Plasmid constructs

The 1208 nt HuR mRNA 3’UTR containing the putative miR-125b target site (nt 671-693) was isolated from human leukocyte RNA by RT-PCR and cloned downstream of firefly luciferase gene in pCDNA3-Fluc vector. The miR-125b target site was mutated using site directed mutagenesis and cloned into the same vector. The double-stranded DNA oligo encoding miR-125b was cloned into pSUPER vector (Oligoengine) containing the EGFP gene to produce shRNA corresponding to miR-125b. HuR cDNA cloned with a myc-tag in pCDNA3.1 vector and TRIM21 expression construct (gift from Sunit. K. Singh, BHU, Varanasi, India) were used for mammalian expression of HuR and TRIM21 respectively. Expression construct for Haemagluttinin (HA)-tagged ubiquitin was a kind gift from S.N. Bhattacharyya, CSIR-IICB, Kolkata, India.
Cell culture, treatment and transfection

MCF7 and MDA-MB-231 human breast carcinoma cells were maintained in Dulbecco’s modified Eagle’s Medium (Thermo Fisher Scientific) with 10% FBS and 1% Pen-Strep. Cells were exposed to a 10 J/m² pulse of short wavelength UV (UVC) irradiation in UVC crosslinker. Cells were treated with 100 μg/ml cycloheximide (Amresco) or 5 μM MG132 (Sigma Aldrich). Cells were transfected with different vectors, siRNAs (siGENOME SMART pool, TRIM21) and Non Targeting siRNA pool (Dharmacon) and antagomiR against miR-125b (Trilink Biotechnologies) using Lipofectamine 2000 (Thermo Fisher Scientific) in DMEM low glucose medium (Thermo Fisher Scientific). DNA amount for transfection with plasmid constructs was equalised by pGEMT plasmid (Promega).

Reporter assay

Cells transfected with firefly luciferase HuR 3’UTR reporter gene constructs and a Renilla luciferase construct were lysed with passive lysis buffer 48 hours post transfection. Luciferase assay was performed using Dual-Glo Luciferase assay system (Promega) following manufacturer’s protocol. Luminescence was measured in a Plate Chameleon V (Hidex) multilabel microplate reader.

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 x g for 20 minutes for cytoplasmic lysate preparation. Lysates were quantified using Bradford reagent (Amresco), resolved on 12% SDS-PAGE and were immunoblotted using anti HuR (3A2, Santa Cruz Biotechnology), p53 (DO-1, Santa Cruz Biotechnology), TRIM21 (E-11, Santa Cruz Biotechnology), Ubiquitin (P4D1, Cell
Signaling Technologies), Myc (71D10, Cell Signaling Technologies), HA (6E2, Cell Signaling Technologies) β-actin (A00730, Genscript) and GAPDH (FL-335, Santa Cruz Biotechnology) antibodies. Chemiluminescent signal was detected using Femtolucent Plus HRP (Geno Biosciences).

RNA Immunoprecipitation

A 50% slurry of pre-swelled Protein A Sepharose beads (Sigma Aldrich) was incubated with specific antibodies overnight at 4°C. 500 µg of pre-cleared lysate was added to the bead-antibody mix and incubated for 4hrs at 4°C and washed five 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 Scientific) followed by qPCR with HuR 3’UTR specific primers and GAPDH primers as control.

Coimmunoprecipitation

Cells treated with UV and MG132 were lysed with Pierce Direct IP kit IP lysis buffer (Thermo Fisher Scientific). Lysates were immunoprecipitated with anti-HuR, anti-TRIM21 or anti-HA antibody using Pierce Direct IP kit using manufacturer’s protocol. The bound antigen was eluted with elution buffer and immunoblotting was performed with specific antibodies to detect co-immunoprecipitated proteins.

Quantitative PCR

Total cellular RNA was extracted using Trizol and polyadenylated using Poly A polymerase (New England Biolabs). cDNA was synthesised using oligo(dT)-adapter primer by MuMLV reverse transcriptase (Thermo Fisher). An adapter-specific primer and microRNA-125b specific primer (miScript primer assay kit, Qiagen) with Power SYBR Green master mix (Applied
Biosystems) were used for qPCR reactions in Step One Plus Real time PCR system (Applied Biosystems). 3’UTR-specific primers were used for detection of HuR mRNA. Firefly luciferase specific primers were used for detection of Firefly luciferase mRNA having HuR 3’UTR. U6B snRNA (miScript primer assay kit, Qiagen) and GAPDH primers were used for miRNA and mRNA quantity normalisation respectively.

Polysome analysis

Transfected cells were treated with Cycloheximide (100ug/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 254nm. RNA was isolated from the fractions by phenol-chloroform extraction and ethanol precipitation and subsequently used for RT-PCR using gene-specific primers.

Mass spectrometry

Cells overexpressing HuR were exposed to UV irradiation and treated with MG132 were lysed 6 hours after UV exposure and lysates were immunoprecipitated with anti-HuR antibody and IgG. The IP eluates were diluted with 6M urea, 100 mM Tris pH 8.0. Trypsin digestion was carried out by adding approximately 10 μl of 0.1 μg/μl trypsin in 100 mM Tris and incubating overnight at room temperature. LC-MS/MS was performed on a Finnigan LTQ-Orbitrap Elite hybrid mass spectrometer system (Thermo Fisher Scientific). The data were analyzed by using all CID
spectra collected in the experiment to search the human reference sequence database with the search program Mascot. The total number of spectra, termed spectral counts, was compared for proteins in the IP and control samples.

Ubiquitination assay

Cells treated with UV and MG132 and/or transfected with siRNAs or cotransfected with HA-ubiquitin and Myc-tagged HuR constructs were lysed and lysates (supplemented with 0.5mM ATP) were immunoprecipitated with anti-HuR antibody or anti-HA antibody. Immunoprecipitates were resolved on SDS-10%PAGE and immunoblotted with anti-Ubiquitin antibody or anti-Myc antibody.

Cell viability assay

24 hours post transfection with control oligo, antagomiR-125b, TRIM21 siRNA or both, $10^4$ cells were UV irradiated and cell viability was determined at designated time points using MTT assay reagent (Sigma Aldrich).

Statistical analysis

All graphical data represent mean ± standard deviation of at least three independent experiments (biological replicates). The fit of simulated plots to experimentally obtained plots was tested by Pearson's R test. * or # signifies a p-value ≤ 0.05, ** or ## signifies a p-value ≤ 0.01, *** or ### signifies a p-value ≤ 0.005 (Paired two-tailed or one-tailed Students t test as applicable) between controls and samples indicated in the Figures.