RNF38 encodes a nuclear ubiquitin protein ligase that modifies p53

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ABSTRACT

The RNF38 gene encodes a RING finger protein of unknown function. Here we demonstrate that RNF38 is a functional ubiquitin protein ligase (E3). We show that RNF38 isoform 1 is localized to the nucleus by a bipartite nuclear localization sequence (NLS). We confirm that RNF38 is a binding partner of p53 and demonstrate that RNF38 can ubiquitinate p53 in vitro and in vivo. Finally, we show that overexpression of RNF38 in HEK293T cells results in relocalization of p53 to discrete foci associated with PML nuclear bodies. These results suggest RNF38 is an E3 ubiquitin ligase that may play a role in regulating p53.

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1. Introduction

The p53 tumor suppressor gene (TP53) has been termed "the guardian of the genome" [1] and it is one of the most frequently mutated genes in human cancers [2–5]. Mutations in the regulators of p53, such as Arf, Mdm2 and Pirh2, are also commonly seen in cancer cells [6–8]. Considering the extensive involvement of p53 in tumorigenesis, understanding p53 regulation is important to the continued development of anti-cancer strategies.

In a search for new p53 interactors, Lunardi et al. employed an affinity purification approach with *Drosophila* p53 in which they identified many novel p53 binding proteins, including the little studied protein, RING finger protein 38 (RNF38) [9]. Although human *RNF38* has been previously cloned [10], its function is currently unknown.

RNF38 mRNA is widely expressed in a variety of human tissues [10], and evolutionary conservation suggests an important cellular function. Interestingly, *RNF38* is located on an area of chromosome 9 (9p13) that is frequently deleted in multiple cancers such as ganglioglioma [11], lung cancer [12,13], hepatocellular carcinoma [14], and in the 9p13-24 deletion seen in the lymphoid blast transformation of chronic myeloid leukemia [15].

Many proteins containing a RING finger motif act as ubiquitin protein ligases [16,17]. We wanted to explore the possibility that RNF38 might have E3 activity and whether or not p53 might be a substrate. p53 is known to be regulated by several ubiquitin protein ligases, the best studied of which is Mdm2/Hdm2. [18–20]. Other E3 ligases that can modify p53 include WWP1, Pirh2 and Cop1 [21–23]. Here we find that RNF38 does possess E3 ubiquitin ligase activity and can recognize p53 as a substrate for ubiquitination. In addition, overexpression of RNF38 alters the nuclear localization of p53.

2. Materials and methods

2.1. Cell culture and transfections

All cell lines were obtained from the Tissue Culture Shared Resource at the University of Colorado Cancer Center. Human embryonic kidney 293 (HEK293) and HEK293T cells were maintained in DMEM (Hyclone) supplemented with 10% fetal bovine serum and antibiotics. H1299 cells were grown in RPMI (Hyclone) supplemented with 10% fetal bovine serum and antibiotics. Transfections were performed with Lipofectamine (Invitrogen).

2.2. Constructs

cDNA for *RNF38* isoform 1 was obtained by RT-PCR from HEK293 mRNA and cloned into pcDNA 3.1(+) (Invitrogen). The *RNF38* cDNA insert and all other constructs derived by PCR were fully sequenced. GFP fusions were made in pEGFP-C1 (Clontech). PCR primers and cloning details are provided in the supplementary

materials. The mammalian expression vector pEBG-SrfI was obtained from Yusen Liu. Vector pMT 123 expressing HA-ubiquitin was obtained from Mathias Treier.

2.3. Expression and purification of RNF38 from baculovirus

A six histidine tag was added to the amino terminus of RNF38 by PCR and cloned into the baculovirus recombination plasmid pVL1393. The Baculovirus Core Facility at the University of Colorado Cancer Center performed the recombination into baculovirus and growth in SF9 cells. RNF38 was purified from extracted nuclei using immobilized Co^{++} ("Talon", Clontech) affinity chromatography. Full details are provided in the supplementary data.

2.4. Antibodies

Mouse monoclonal anti-p53 (1C12) was purchased from Cell Signaling. Rabbit anti-p53 (sc-6243), mouse monoclonal anti-p53 (sc-126), rabbit anti-RNF38 (sc-102096), rabbit anti-GFP (sc-8334) and mouse monoclonal anti-PML (sc-966) were purchased from Santa Cruz Biotechnology. A second rabbit anti-RNF38 antibody (AP12816a) was acquired from Abgent. Mouse anti-NR1 (#05–432) was obtained from Upstate Biotechnology. Horseradish peroxidase (HRP) conjugated rat anti-HA antibody (3F10) was from Roche Applied Science.

2.5. Binding studies

GST and GST-p53 fusion proteins were purified from *Escherichia coli* BL21 transformed with pGEX-4T-2-(p53). Either 2 μg of purified RNF38 in GST binding buffer (50 mM NaCl, 20 mM Tris pH 7.5, 1 mM $MgCl_2$, 1 mM DTT, 0.1% Thesit and 1.0 mg/mL BSA) or 850 μg of 293T whole cell extract in lysis buffer (50 mM NaCl, 20 mM Tris pH 7.5, 1 mM $MgCl_2$, 1.0% Trition X-100, 1 mM Na_3VO_4, 1 mM DTT and 1 mM PMSF plus protease inhibitors) were incubated at 4 °C for 30 min with equal amounts (2 μg) of GST or GST-p53. Preblocked (5% BSA) glutathione-Sepharose beads (G.E. Healthcare Lifesciences) were then added to the mixtures and incubated for 2 h at 4 °C with mixing. The beads were then washed with GST wash buffer (50 mM NaCl, 20 mM Tris pH7.5, 1 mM $MgCl_2$, 1 mM DTT and 0.1% Thesit) and subjected to western blot analysis.

2.6. Ubiquitination assay – in vitro

Purified RNF38 was mixed with 50 μg/ml E1, E2 and 0.5 mg/ml HA-ubiquitin in ubiquitin buffer (50 mM KCl, 20 mM HEPES pH 7.4, 5 mM $MgCl_2$, 1 mM DTT and 1 mM ATP) and incubated for 30 min at 30 °C with mixing at 600 rpm. The reaction products were analyzed by western blot with anti-RNF38 (sc-102096) and anti-HA antibodies.

For *in vitro* p53 ubiquitination reactions, p53 was immunoprecipitated from HEK293T cells lysed in RIPA+ buffer (150 mM NaCl, 20 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 1.0% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF plus protease inhibitors). Mouse anti-p53 (1C12) or mouse anti-NR1 control (0.75 μg) was added to 500 μg of precleared whole cell lysate and incubated overnight. Antibody complexes were then bound to preblocked (5% BSA) protein G-agarose (Sigma–Aldrich P-4691) for two hours followed by extensive washing: twice with RIPA+, twice with SNNTE (0.5 M NaCl, 50 mM Tris pH 7.5, 5 mM EDTA, 5% sucrose, 1% NP-40), twice with 200 mM glycine pH 2.5, and twice with ubiquitin buffer. The washed beads were then used in *in vitro* ubiquitination reactions with or without purified RNF38 as described above. Following the ubiquitination reactions, the beads were washed twice with RIPA+ and then analyzed by western blot with anti-p53 and anti-HA antibodies.

2.7. Ubiquitination assay – in vivo

HEK293T cells were transfected with pMT 123 (HA-ubiquitin) plus pEBG or pEBG-RNF38. 24 h post-transfection cells were passaged and allowed to grow for 48 h. At 72 h post-transfection the cells were incubated for 4 h in 25 μM MG132 (Sigma–Aldrich) then collected in lysis buffer (100 mM NaCl, 20 mM Tris pH 7.5, 5 mM EDTA, 10% glycerol, 1% NP-40, 1 mM DTT, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 10 mM NEM plus protease inhibitors). Immunoprecipitation of p53 was performed using 1 μg of mouse anti-p53 (sc-126) antibody to 1.5 mg of precleared whole cell extract followed by binding to preblocked protein G-agarose. Bound complexes were washed with lysis buffer then analyzed by western blot with rat anti-HA antibody.

2.8. Immunofluorescence

Cultured cells were seeded on glass coverslips, transfected and 24 h later fixed with 4% para-formaldehyde in PBS. Cells were permeabilized with cold methanol, rinsed with PBS, and blocked with 2% nonfat milk, 0.1% Tween-20 in PBS. Primary antibody rabbit anti-p53 (sc-6243) was used at 2 μg/ml in 1% BSA, 0.5% Triton X-100 in PBS and incubated for 1 h at room temperature. Primary antibody rabbit anti-RNF38 (AP12816a) was used at 5 μg/ml and anti-PML (sc-966) was used at 4 μg/ml incubated overnight at 4 °C. Cells were washed with 0.05% Tween-20 in PBS and incubated with the appropriate fluorescent secondary (Alexa Fluor 568 goat anti-rabbit or Alexa Fluor 488 goat anti-mouse, Invitrogen) at 2 μg/ml for 1 h. Nuclei were counterstained with 0.2 μg/ml Hoechst 33258 (Kodak). Coverslips were mounted in gelvatol and examined with a Nikon Eclipse Ti-S microscope fitted with the appropriate fluorescence filters, DS-Qi1 camera and NIS-Elements imaging software.

3. Results

3.1. RNF38 is a nuclear protein

In their study identifying p53-interacting proteins in *Drosophila*, Lunardi et al. also examined the intracellular localization of 41 mammalian orthologs, including RNF38 [9]. Immunofluorescence of epitope-tagged RNF38 overexpressed in U2OS cells revealed diffuse nuclear staining. However, the data presented was a supplementary figure containing an image of a single cell with no mention of which RNF38 isoform was used or if any other staining patterns were observed. As a first step toward understanding RNF38 function, we sought to confirm and expand this result.

We first examined RNF38 in HEK293T cells. Endogenous RNF38 was detectable by immunofluorescence with only one of two commercially available anti-RNF38 antibodies (AP12816a) under the conditions employed. Similar to the results of Lunardi et al., we find endogenous RNF38 is also localized to the nucleus in HEK293T cells (Fig. 1A). RNF38 is predicted to have three isoforms. Isoform 1 is the largest, containing 515 amino acids, whereas isoform 2 lacks amino acids 5–54, and isoform 3 lacks amino acids 1–84. According to the manufacturer, the AP12816a antibody was raised against amino acids 47–77 of RNF38, a region present in isoform 1 but partially or entirely lacking in the other isoforms. We chose to focus on isoform 1, henceforth referred to as simply "RNF38".

The endogenous RNF38 signal was fairly weak, so to further investigate RNF38 nuclear localization, we constructed a
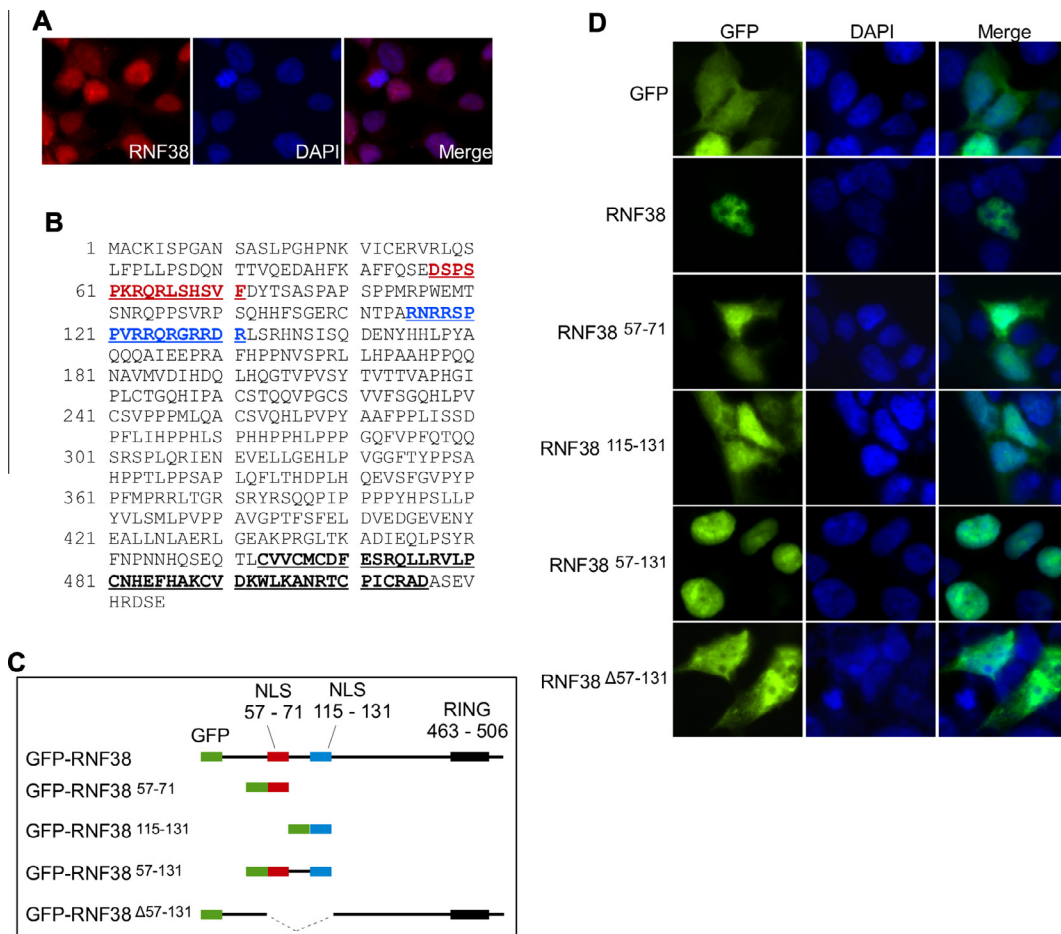
A

B

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  1 MACKISPGAN SASLPGHPNK VICERVRLQS
    LFPLLPSDQN TTVQEDAHFK AFFQSEDSPS
 61 PKRQRLSHSV FDYTSASPAP SPPMRPWEMT
    SNRQPPSVRP SQHHFSGERC NTPARNRRSP
121 PVRRQRGRRD RLSRHNSISQ DENYHHLPYA
    QQQAIEEPRA FHPPNVSPRL LHPAAHPPQQ
181 NAVMVDIHDQ LHQGTVPVSY TVTTVAPHGI
    PLCTGQHIPA CSTQQVPGCS VVFSGQHLPV
241 CSVPPPMLQA CSVQHLPVPY AAFPPLISSD
    PFLIHPPHLS PHHPPHLPPP GQFVPFQTQQ
301 SRSPLQRIEN EVELLGEHLP VGGFTYPPSA
    HPPTLPPSAP LQFLTHDPLH QEVSFGVPYP
361 PFMPRRLTGR SRYRSQQPIP PPPYHPSLLP
    YVLSMLPVPP AVGPTFSFEL DVEDGEVENY
421 EALLNLAERL GEAKPRGLTK ADIEQLPSYR
    FNPNNHQSEQ TLCVVCMCDF ESRQLLRVLP
481 CNHEFHAKCV DKWLKANRTC PICRADASEV
    HRDSE
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C

D

Fig. 1. RNF38 is a nuclear protein with a bipartite nuclear localization signal. (A) Endogenous RNF38 is located in the nucleus. HEK293T cells were stained with anti-RNF38 antibody, nuclei were counterstained with Hoechst 33258 and cells examined by fluorescence microscopy. (B) RNF38 contains two potential nuclear localization signals. *In silico* searches identified amino acids 57–71 (red) and 115–131 (blue) as possible nuclear localization signals. The RING finger domain is also indicated (463–506). (C) GFP-RNF38 fusion constructs used to test candidate nuclear localization signals. (D) Amino acid sequence 57–131 is sufficient for nuclear localization. At 24 h post-transfection with the constructs shown in (C), HEK293T cells were fixed and treated as in (A). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

GFP-tagged RNF38 expression vector. As shown in Fig. 1, transient expression (Fig. 1D, second row) resulted in nuclear localization of GFP-RNF38. Note also that the stronger signal from overexpressed GFP-RNF38 allows more detail to be observed, and the protein appears to be excluded from nucleoli.

3.2. Identification of nuclear localization signals

To identify the motif(s) responsible for RNF38 nuclear localization we analyzed its amino acid sequence with several nuclear localization signal (NLS) prediction programs. cNLS Mapper [24] identified amino acids 57–71 as a possible NLS, and amino acids 115–131 were identified as a possible NLS by NLStradamus [25]. We then created a series of GFP fusions to examine the role these sequences might play in RNF38 localization (Fig. 1C).

Transient expression of GFP in HEK293T cells results in a uniform staining pattern throughout the nucleus and cytoplasm (Fig. 1D). In contrast, the GFP-RNF38 fusion protein appears restricted to the nucleus. GFP fusion to either amino acids 57–71 or 115–131 is insufficient to confer complete nuclear localization, though a larger GFP fusion to amino acids 57–131 is completely localized to the nucleus. This region contains both of the basic residue-rich sequences identified as putative NLSs and thus appears to function as a bipartite NLS. Deletion of this region results in loss of
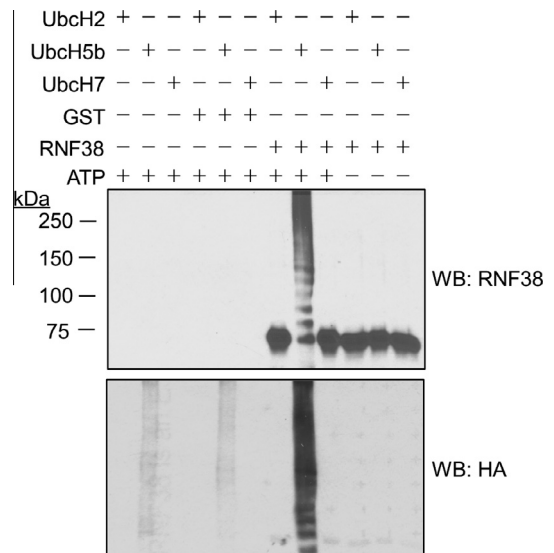

Fig. 2. RNF38 is capable of autoubiquitination *in vitro*. Reactions contained recombinant RNF38, E1, HA-ubiquitin, and either UbcH2, UbcH5b or UbcH7 with or without 1 mM ATP. Purified GST was used as a control. Reactions were analyzed by immunoblot with anti-RNF38 (sc-102096) and anti-HA antibodies.

exclusive nuclear localization with the GFP-RNF38Δ57–131 fusion protein localized in both cytoplasm and nucleus.

3.3. RNF38 is an E3 ubiquitin ligase

In addition to ubiquitination of their target proteins, many E3 ubiquitin ligases undergo autoubiquitination *in vitro* [16,17]. Fig. 2 shows *in vitro* ubiquitination assays using His-tagged RNF38 purified from baculovirus infected SF9 cells. RNF38 clearly undergoes robust autoubiquitination and shows specificity in interacting with E2s. Of the three E2 proteins tested (UbcH2, UbcH5b and UbcH7), only UbcH5b supported RNF38 autoubiquitination. These findings demonstrate RNF38 is an active ubiquitin protein ligase (E3).

3.4. The RING domain limits RNF38 overexpression

We found RNF38 to be resistant to overexpression by transfection despite using a variety of expression plasmids and cell lines (including MDA-MB-231, H1299 and HEK293, data not shown). Since an active RING domain might limit expression through autoubiquitination or down-regulation of some other cellular component, we removed the C-terminal RING domain from RNF38 (RNF38 ΔRING) to explore the issue.

Truncation of the RING domain greatly increased RNF38 expression (Fig. S1A). In two cell types (HEK293T and H1299), transfection with GFP alone or GFP-RNF38ΔRING yielded many GFP-positive cells, whereas with full-length GFP-RNF38, only rare GFP-positive cells were observed. Western blot analysis of these cells confirmed the results (Fig. S1B).
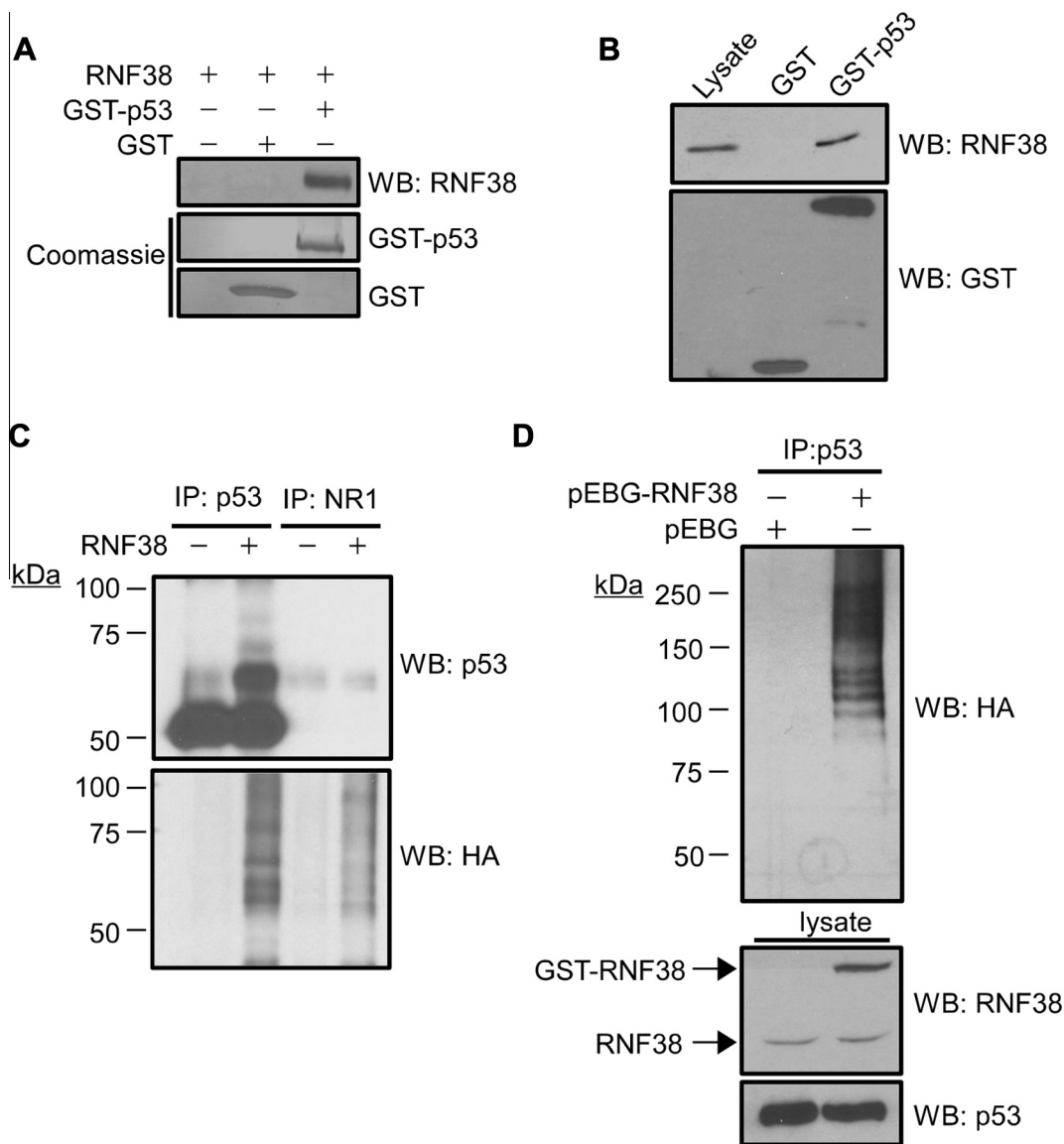

Fig. 3. RNF38 binds and ubiquitinates p53. (A) RNF38 binds to p53 *in vitro*. Binding interactions were tested using recombinant RNF38 incubated either alone, with GST, or GST-p53 followed by binding to glutathione-Sepharose. Bound proteins were analyzed by immunoblot with anti-RNF38 antibody (sc-102096). (B) Endogenous RNF38 binds p53. 293T cell lysate was incubated with purified GST or GST-p53. Complexes were isolated and analyzed as in (A). Lane one (Lysate) contains 3.5% of the cell lysate used for comparison. (C) p53 is ubiquitinated by purified RNF38 *in vitro*. p53 was immunoprecipitated from 293T cell lysate using mouse anti-p53 or a control antibody (anti-NR1). Isoloated immune complexes were added to *in vitro* ubiquitination reactions with recombinant HA-ubiquitin, E1, UbcH5b and with or without RNF38, as indicated. Reaction products were analyzed by immunoblot with antibodies to p53 and HA (ubiquitin). (D) Overexpression of RNF38 increases p53 ubiquitination. HEK293T cells were transfected with pMT 123 (HA-ubiquitin) plus either the pEBG expression vector or pEBG-RNF38. 72 h post-transfection, cells were treated with 25 μM MG132 for 4 h, lysed, and p53 recovered by immunoprecipitation. Precipitated proteins were analyzed by western blot with anti-HA antibody.

Successful overexpression of full-length RNF38 constructs was detectable on western blots using derivatives of the vector pEBG in HEK293T cells (Fig. S1C). The pEBG plasmid contains an SV40 origin of replication, allowing amplification of the plasmid in HEK293T cells which express SV40 large T antigen. Treatment of cells with the proteasome inhibitor MG132 did not increase RNF38 expression, nor was a prominent ladder of ubiquitinated RNF38 species observed, suggesting that autoubiquitination may not be the mechanism limiting overexpression of full-length RNF38 (Fig. S1C). Additional experiments will be required to identify the mechanism(s) regulating RNF38 expression.

3.5. RNF38 is a p53 binding protein

We have examined the p53-RNF38 binding interaction first reported by Lunardi et al. using a different affinity purification technique employing a fusion protein between human p53 and glutathione S-transferase (GST). Purified RNF38 binds specifically to GST-p53 but not GST alone (Fig. 3A, upper panel). Similarly, endogenous RNF38 in lysates of HEK293T cells specifically binds GST-p53 but not GST alone (Fig. 3B).

3.6. RNF38 ubiquitinates p53 in vitro and in vivo

To test whether p53 is a substrate for ubiquitination by RNF38, endogenous p53 was immunoprecipitated from HEK293T lysate and added to in vitro ubiquitination reactions. Control immunoprecipitates using an unrelated antibody (anti-NR1) were treated similarly. We found that p53 was ubiquitinated in an RNF38-dependent manner under these conditions (Fig. 3C).

Next, we asked whether we could detect RNF38-dependent ubiquitination of p53 in cells. HEK293T cells were transfected with an expression vector for HA-tagged ubiquitin and either GST or GST-RNF38. p53 was recovered by immunoprecipitation and analyzed by immunoblotting for HA-ubiquitin (Fig. 3D). GST-RNF38 clearly increased p53 ubiquitination in vivo, as evidenced by the ladder of higher molecular weight species reacting with HA (ubiquitin) antibody. We note that the p53 complexes isolated from cells appear more extensively modified than those produced in vitro,

suggesting there may be additional factors or conditions involved in p53 ubiquitination in cells.

Investigation of possible effects of RNF38 on p53 turnover has been hindered by the resistance of RNF38 to overexpression. As mentioned earlier, detection of biochemical levels of exogenous RNF38 has only been achieved in HEK293T cells, in which SV40 large T antigen prevents normal p53 turnover [26]. Experiments in other systems, including H1299 cells (which lack p53 expression), have been inconclusive due to insufficient/undetectable RNF38 expression.

3.7. Overexpression of RNF38 alters p53 localization

Since biochemical analysis of possible RNF38 effects on p53 turnover has been uninformative due to the technical issues described above, we turned to fluorescence microscopy to allow visualization of any effects on intracellular localization of p53. HEK293T cells were transfected with GFP-RNF38 or GFP alone, then fixed and stained for p53. In untransfected and GFP-transfected HEK293T cells, p53 is distributed diffusely throughout the nucleus with a small minority of cells (13%) showing punctate staining (Fig. 4). In cells transfected with GFP-RNF38, p53 redistributed to large punctate structures in the nucleus in the majority of GFP-RNF38 positive cells (69%).

Since p53 has been previously identified in PML (promyelocytic leukemia-associated protein) nuclear bodies [27,28], we analyzed the localization of PML and p53 in RNF38 transfected cells. A typical cell with a punctate p53 phenotype is shown in Fig. 4C. In these cells many, though not all, p53 foci associate directly adjacent to PML nuclear bodies. The number of p53 and PML foci vary from cell to cell, and while many p53 foci colocalize with PML foci, a few of each type do not. These data suggest that overexpression of RNF38 can change the distribution of p53 within the nucleus from diffuse to punctate, and many of these foci are associated with PML nuclear bodies.

4. Discussion

This study has begun the functional characterization of the evolutionarily conserved protein, RNF38. We have shown that RNF38
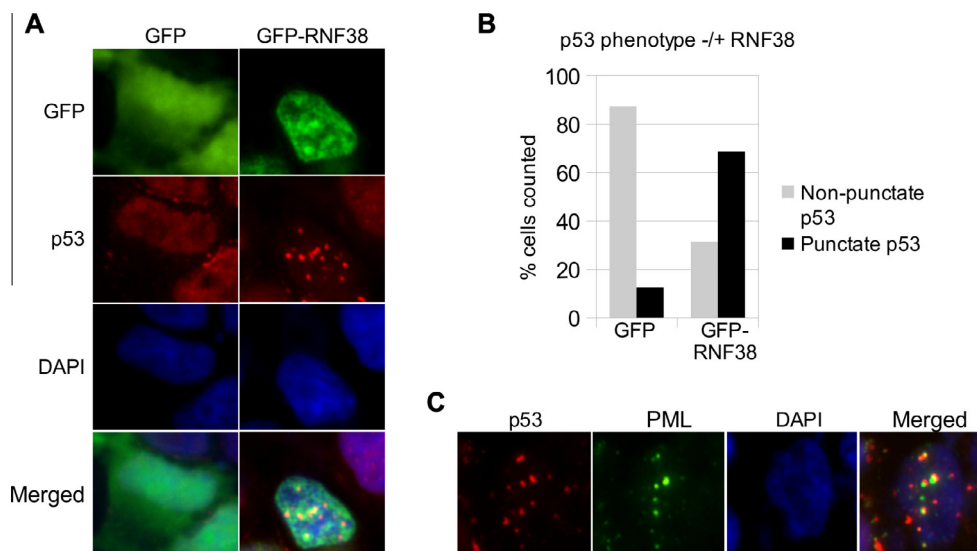

Fig. 4. RNF38 overexpression alters p53 localization. (A) HEK293T cells were transfected with pEBG-GFP or pEBG-GFP-RNF38. At 24 h post-transfection, cells were fixed and stained for p53. A representative image of p53 punctate structure is shown. (B) The p53 phenotype was quantified as punctate or non-punctate in GFP(RNF38) expressing cells. Approximately 200 cells each were counted. (C) RNF38 overexpression induces p53 association with PML nuclear bodies. HEK293T cells were transfected with pEBG-RNF38. 24 h post-transfection cells were fixed and stained for p53 (sc-6243) and PML.

(isoform 1) is localized to the nucleus in several cell types and have identified a bipartite NLS capable of mediating this localization. We have expressed and purified RNF38 from baculovirus-infected SF9 cells and found that purified RNF38 protein is an active ubiquitin protein ligase that exhibits specificity with the E2 protein with which it interacts. We have confirmed Lunardi et al.'s findings that RNF38 is a p53-binding protein, shown that RNF38 can ubiquitinate p53 *in vitro*, and demonstrated that overexpression of RNF38 increases p53 ubiquitination *in vivo*.

Although our efforts to elucidate the dynamics of the p53-RNF38 interaction biochemically were hindered by limited RNF38 overexpression, fluorescence microscopy revealed that overexpression of RNF38 in HEK293T cells alters the intracellular distribution of p53 from a diffuse nuclear pattern to punctate foci, many of which are associated with PML nuclear bodies. Association of p53 with PML nuclear bodies has been previously described in a number of instances [27–30], and it has been suggested that PML nuclear bodies may be sites of functional regulation of p53 [31]. In this regard, our data indicating altered p53 localization upon RNF38 overexpression are intriguing. While insufficient to illuminate the exact functional interplay between these two proteins, these results do provide additional evidence for a regulatory relationship between RNF38 and p53. Taken together with our other findings, a case can be made that RNF38 may be a biologically significant regulator of p53. It remains to future studies to elaborate the full functional details of such regulation.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.031.

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