Microenvironment and Immunology

Cancer Research

Human NK Cells Are Alerted to Induction of p53 in Cancer Cells by Upregulation of the NKG2D Ligands ULBP1 and ULBP2

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Abstract

Natural killer (NK) cells are immune cells sensing and eliminating foreign, stressed, transformed, and senescent cells through specialized surface receptors, such as NKG2D, that interacts with several virus- or stress-inducible ligands, including ULBP1 and -2, which are expressed on target cell surfaces. For example, induction of DNA damage or cellular senescence pathways in tumor cells led to upregulation of NKG2D ligands that activate NK cells. Although, both pathways activate p53, the relationship of p53 activation to upregulation of NKG2D ligands has not been addressed. In this study, we report that induction of wild-type p53, but not mutant p53, strongly upregulated mRNA and cell surface expression of ULBP1 and -2, whereas expression of other NK cell ligands was not affected. We defined intronic p53-responsive elements in these two novel p53 target genes. Coculture of wild-type p53-induced human tumor cells with primary human NK cells enhanced NKG2D-dependent degranulation and IFN-γ production by NK cells. Accordingly, treatment of certain wild-type p53-expressing tumor cell lines with the p53-reactivating small molecular compound RITA resulted in upregulation of ULBP2 mRNA and cell surface protein expression. Taken together, our findings define the involvement of p53 in the regulation of specific NKG2D ligands that enhance NK cell–mediated target recognition. One implication of our work is that activating p53 after adoptive transfer of NK cells might constitute an effective combinatorial strategy of NK cell–based immunochemotherapy in cancers in which wild-type p53 function is preserved. *Cancer Res; 71(18); 5998–6009. ©2011 AACR.*

Introduction

The transcription factor p53 plays a central role in cell-cycle regulation, DNA repair, senescence, and apoptosis (1–3). Consistent with a prominent role as a tumor suppressor, p53 is mutated or deleted in approximately 50% of cancers. In the remaining tumors, p53 function is frequently impaired, for example, by overexpression of its cellular inhibitor murine double minute 2 (MDM2; ref. 4). Recently, it has been shown that reactivation of p53 in mouse cancer models induced rapid tumor regression by the induction of apoptosis or cellular senescence depending on the tumor type (5). Furthermore, the restoration of p53 expression in mouse liver carcinoma not only resulted in cellular senescence of the tumor cells but also in the production of inflammatory cytokines and the recruitment of innate immune cells (6). The depletion of macrophages, neutrophils, or natural killer (NK) cells accelerated tumor progression (6). Thus, innate immune cells, including NK cells, play an important role in the regression of p53-expressing tumors. The exact mechanism of the observed NK cell–dependent tumor cell elimination, however, remains unknown.

NK cell activation is determined by a delicate balance of signals delivered by inhibitory and activating receptors (7). Inhibitory receptors mainly recognize self-MHC class I molecules. Activating receptors, such as natural cytotoxicity receptors, NKG2D, or DNAM-1, interact with virus-, stress-, transformation-, or senescence-inducible ligands (7–9). These ligands are normally not expressed on the cell surface of healthy cells. Tumor cells frequently express high levels of activating NK cell receptor ligands and low levels of MHC class I rendering these cells highly susceptible to NK cell–mediated lysis (10).

In humans, the activating NK cell receptor NKG2D is expressed on NK cells and subsets of T cells, including NKT cells, activated $CD8^+$ T cells and $\gamma\delta^+$ T cells (11, 12). So far, several ligands for human NKG2D including MICA, B, and ULBP1, 2, 3, 4, 5, and 6 were identified (11, 13–17). To date, the regulation of the expression of NKG2D ligands is only

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partially understood. Depending on the cell type and stress stimulus, NKG2D ligand expression can be regulated at transcriptional and posttranscriptional levels. Posttranscriptional mechanisms include MICA/B downregulation by microRNAs targeting their 3′-untranslated regions (18) and proteolytic shedding of MICA/B, ULBP2, and ULBP4 from the cell surface (19–21). Furthermore, ULBP1 expression was upregulated in head and neck squamous cell cancer by proteasome inhibitors (22). ULBP1–3 expression was enhanced in epithelial tumor cells upon treatment with histone deacetylase 3 (HDAC3) inhibitors (23). In addition, heat shock factor 1 enhanced mRNA transcription of MICA/B after activation upon heat shock (24). Several reports showed that triggering of the DNA damage response by agents that activate ATM (ataxia telangiectasia, mutated) and ATR (ATM- and Rad3-related) protein kinases led to elevated mRNA expression of certain NKG2D ligands (9, 25, 26). It is well established that the ATM/ATR pathway activates p53. To date, it is unknown, whether the regulation of certain NKG2D ligands depends on p53.

In this study, we investigated whether the transcription factor p53 is involved in the regulation of human NKG2D ligands. Here, we show that the induction of wild-type (wt) p53, but not mutant (mut)p53, in the p53-null non–small cell lung cancer (NSCLC) cell line H1299, resulted in pronounced selective upregulation of the human NKG2D ligands ULBP1 and -2 leading to NK cell activation. Furthermore, our study shows that *ULBP1* and *-2* are direct p53 target genes. Finally, the treatment of certain cancer cells with the small molecular compound, RITA (Reactivation of p53 and Induction of Tumor cell Apoptosis) that reactivates wtp53 resulted in the upregulation of ULBP2 expression. Thus, our study uncovers a novel mechanism of NKG2D ligand regulation by p53 and might help to design novel, more effective regimens to treat cancer.

Materials and Methods

Cell culture

The NSCLC cell line H1299 carrying tetracycline-inducible wtp53 or mutp53 (R175H, defective in DNA binding; ref. 27) was maintained in RPMI containing 4.5 g/mL glucose (Sigma-Aldrich) with 2 mmol/L L-glutamine, 10% fetal calf serum (FCS), 10 mmol/L HEPES, 1 mmol/L sodium pyruvate (all Invitrogen), 22 μg/mL Zeocin, and 2.9 μg/mL blasticidin (both USBiological). For wtp53 or mutp53 induction, H1299-derived cell lines were treated for 16 hours with 5 ng/mL or 1 μg/mL doxycyclin (Sigma-Aldrich), respectively (27). For inhibition of transcription, H1299-derived cell lines were treated with 10 μg/mL actinomycin D or the respective amount of dimethyl sulfoxide (DMSO; both Sigma-Aldrich) 10 hours after doxycycline treatment. Primary human NK cells were purified from peripheral blood mononuclear cells of healthy donors by magnetic cell separation by using the NK cell isolation Kit (Miltenyi Biotec). NK cells were maintained for 48 hours in RPMI containing 4.5 g/mL glucose with 2 mmol/L L-glutamine, 10% human serum (PAA), 200 U/mL human IL-2 (Chiron Corporation), and 10 ng/mL human IL-15 (Peprotech) before they were used for coculture assays. NK cell purity was determined by flow cytometry by gating on $CD56^+CD3^-$ cells and was higher than 95%. The osteosarcoma cell line U2OS, the fibrosarcoma cell line HT1080, the colorectal carcinoma cell line HCT116, and the hepatocellular carcinoma cell line HepG2 that all express wtp53 were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/mL glucose and 2 mmol/L L-glutamine with 10% FCS. For p53 reactivation, tumor cells were cultured in the presence of 1 μmol/L RITA (Cayman Chemical) dissolved in DMSO for 24 hours. In parallel, cells were cultured with DMSO only. All cell cultures were conducted in the presence of 100 U/mL penicillin G and 100 μg/mL streptomycin (Invitrogen). All cell lines were tested by the Multiplex cell Contamination Testing (McCT) Service of the German Cancer Research Center within 1 month before conducted experiments (28).

Transfection with p53 expression plasmid

For transfection, 6×10^5 HT1080 or U2OS cells were plated per 10 cm dish. Twenty-four hours later, cells were cotransfected with 500 ng pEGFP-C1 (Clontech) and 2 μg wtp53 expression plasmid (*TP53* gene cloned in pCMV-3Tag-1; Agilent Technologies) or 2 μg vector control (pcDNA3.1+; Invitrogen). U2OS cells were transfected with FuGENE 6 transfection reagent (Promega) and HT1080 cells with Lipofectamine 2000 reagent (Invitrogen) according to manufacturers' instructions. Both transfection reagents were used at a 3:1 (reagent: DNA) ratio. Twenty-four hours after transfection, cells were harvested for analysis of transfection efficiencies by gating on $7\text{-AAD}^-\text{EGFP}^+$ cells by flow cytometry and ULBP1/2 mRNA expression levels were determined by quantitative real-time PCR.

Luciferase reporter assay

For luciferase reporter assays, in Figure 4A and 5A, the depicted intronic sequences of the ULBP1/2 genes containing the potential p53REs were cloned into the pGL3-Basic plasmid (Promega) 5′ of the luciferase gene. The pGL3-Basic plasmid contains no internal promoter. For the assays, 2×10^5 H1299 cells per well were plated in a 6-well plate. Twenty-four hours later, cells were cotransfected by using Lipofectamine 2000 reagent (Invitrogen) with 500 ng of the respective luciferase reporter plasmids and 0 to 40 ng p53 expression plasmid filled up to 500 ng with pcDNA3.1 plasmid. Each transfection also contained 500 ng of the internal standard RSV-β-Gal plasmid to correct for possible variations in transfection efficiencies. Cells were harvested 18 to 24 hours after transfection by using the Luciferase Assay System with Reporter Lysis Buffer from Promega following the manufacturer's instructions. Luciferase activities were quantified by using a Lucy 1 microplate luminometer (Anthos). Beta-galactosidase activity was measured by cleavage of *O*-nitrophenyl-b-D-galactopyraniside (ONPG). For all samples, relative luciferase activity was calculated as luciferase activity/beta-galactosidase activity. Fold induction was calculated by setting the relative luciferase activity of the transfection without p53 expression plasmid as 1.

Chromatin immunoprecipitation

For chromatin immunoprecipitation (ChIP) experiments, 1×10^7 untreated or doxycyclin-treated H1299 cells

with either wtp53 or mutp53 were harvested and processed with the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology) according to the manufacturer's instructions. The amount of input chromatin per ChIP was 10 μg. For p53-specific ChIP, chromatin was immunoprecipitated with 2 μg monoclonal antibody (mAb) clone 1C12 (Cell Signaling Technology) or 2 μg of a respective isotype control mAb (clone 11711; R&D Systems). The relative amounts of chromatin immunoprecipitated by either isotype control or α-p53 mAb was determined by real-time PCR with the respective p53RE-specific primers as described in the Supplementary Material.

IFN-γ ELISA

H1299-derived cell lines were treated for 16 hours with doxycyclin as described before. Subsequently, cells were harvested, washed twice, and the coculture with NK cells was set up. A total of 2.5×10^5 target cells per well were incubated in a 48-well plate for 24 hours with 2.5×10^5 NK cells in a final volume of 500 μL in RPMI containing 4.5 g/mL glucose with 2 mmol/L L-glutamine, 10% human serum, and 100 U/mL human IL-2. In NKG2D-blocking experiments, α-NKG2D mAb (clone BAT221; Miltenyi Biotec) or isotype control $mIgG_1$ (clone 11711; R&D Systems) were added to NK cells at a final concentration of 10 μg/mL 30 minutes before coculture with target cells. IFN-γ release was determined by the Human IFN-γ ELISA MAX Standard Set (BioLegend) according to the manufacturer's instructions.

Degranulation assay

The coculture of H1299-derived cell lines with NK cells was done as described for IFN-γ ELISA. A total of 1×10^5 target cells per well were cocultured in a 96-well plate for 4 hours with 1×10^5 NK cells in a final volume of 200 μL in RPMI containing 4.5 g/mL glucose with 2 mmol/L L-glutamine and 10% human serum in the presence of BD GolgiStop (BD Biosciences), fluorescein isothiocyanate (FITC)–anti-human CD107a (clone H4A3) and FITC–anti-human CD107b (clone H4B4) or FITC–mIgG1 isotype control (clone MOPC-21). Afterwards, cells were stained with PE–anti-human CD3 (clone HIT3a) and APC–anti human CD56 (clone HCD56) followed by staining with 7-AAD. All antibodies were purchased from BioLegend. The percentage of $CD107a^+/b^+$ NK cells was determined by gating on 7-$AAD^-CD56^+CD3^-$ cells. Blockade of NKG2D was done as described for IFN-γ ELISA.

Statistical analyses

For the calculation of statistical significances of differences between experimental groups, 2-tailed Student's *t* tests for unpaired data were used. *P* values of less than 0.05 were considered as significant.

Additional Material and Methods

Additional Material and Methods can be found in the Supplements.

Results

Induction of wtp53 expression in H1299 cells upregulates cell surface expression of the NKG2D ligands ULBP1 and -2

To investigate the impact of wtp53 induction on NK cell ligand regulation, we used the p53 null NSCLC cell line H1299 with doxycyclin-inducible wtp53 expression (27). As control, H1299 cells with a doxycyclin-inducible DNA-binding defective mutp53 (R175H) were analyzed. Figure 1A shows that doxycyclin treatment induced wtp53 or mutp53 expression in the H1299-derived cell lines. Furthermore, in wtp53-expressing cells, but not in mutp53-expressing cells, the classical p53 target gene, the cyclin-dependent kinase inhibitor p21, was expressed after wtp53 induction. Importantly, induction of wtp53, but not mutp53, in H1299 cells significantly upregulated the cell surface expression of the NKG2D ligands, ULBP1 (3.9-fold increase in MFI) and ULBP2 (5.3-fold increase in MFI), and the binding of a NKG2D-F_c fusion protein (Fig. 1B and C). Expression of other NKG2D ligands was not significantly changed after p53 induction (Fig. 1B and C). In addition, expression of MHC class I, ICAM-1, NKp30-, NKp44-, NKp46 ligands, and the DNAM-1 ligands, CD112 and CD155, was not altered by wtp53 expression in H1299 cells (data not shown). All stainings were done upon gating on viable, 7-AAD negative cells. On average, 80% of nonfloating cells were viable after the 16-hour doxycyclin treatment as determined by staining with 7-AAD (data not shown). Taken together, our data indicate that the reintroduction of wtp53 in H1299 tumor cells lacking p53 expression led to a pronounced upregulation of the activating NK cell ligands ULBP1 and ULBP2.

ULBP1 and ULBP2 are upregulated at the transcriptional level

Next, we analyzed, whether ULBP1 and -2 were upregulated at the transcriptional level by quantitative real-time PCR. Indeed, ULBP1 and -2 mRNAs (relative ULBP1 mRNA levels by 11-fold and ULBP2 mRNA levels by 20-fold) were strongly upregulated upon induction of wtp53, but not mutp53, after 16 hours in H1299 cells (Fig. 2A and B). In a next step, we investigated whether wtp53 induction affected the stability of ULBP1/2 mRNA. After 10-hour treatment of wtp53 H1299 with doxycyclin, the transcription inhibitor actinomycin D was added. ULBP1/2 mRNA levels were analyzed by quantitative real-time PCR at different time points (Fig. 2C and D). Blockade of transcription diminished ULBP1/2 mRNA expression levels to the same extent in nontreated and doxycyclin-treated H1299 cells (Fig. 2C and D). These data indicate that the increase of ULBP1/2 mRNA levels was not a result of increased mRNA stability, suggesting that enhanced mRNA levels are caused by *de novo* transcription. Next, we tested whether expression of wtp53 enhances ULBP1/2 mRNA levels in additional tumor cell lines. To this end, we cotransfected the 2 sarcoma cell lines, U2OS and HT1080, with a wtp53 and an enhanced green fluorescent protein (EGFP) expression plasmid. Twenty-four hours after transfection, total RNA was isolated for quantification of ULBP1/2 mRNA levels by real-time PCR, and transfection efficiencies were determined

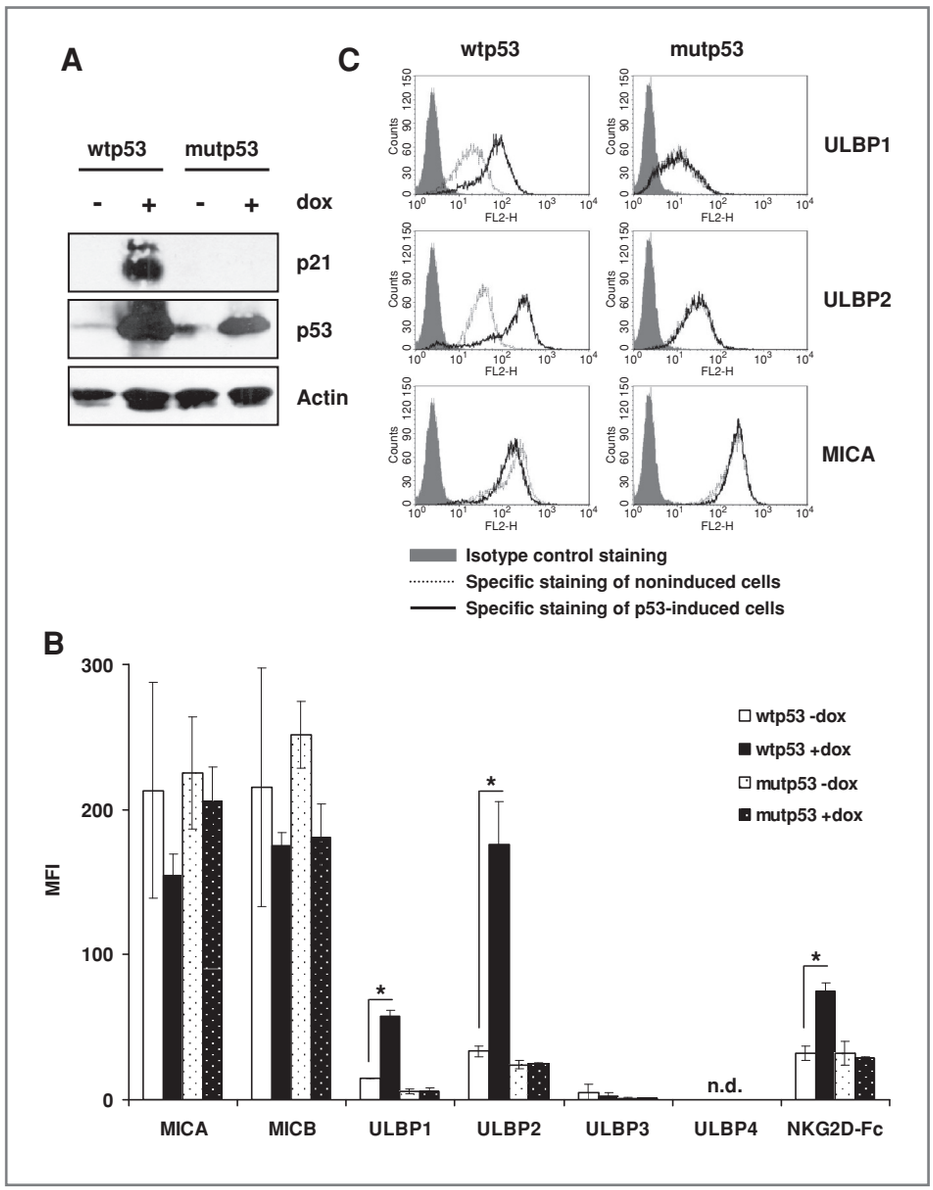

Figure 1. Induction of wtp53 expression in H1299 cells upregulates the cell surface expression of the NKG2D ligands ULBP1 and -2. Expression of wt/mutp53 was induced in H1299 cells by treatment with doxycyclin for 16 hours. A, Western Blot analysis of p21, p53, and actin expression in wt/mutp53 H1299 cells after ± doxycyclin treatment is depicted. B, cell surface expression of NKG2D ligands was monitored on wt/mutp53 H1299 cells after ± doxycyclin treatment. MFI was calculated as geometrical mean of specific staining minus isotype control staining. The mean of 3 independently conducted experiments ± SD is indicated. C, representative histograms show cell surface expression of ULBP1, -2, and MICA in noninduced and doxycyclin-treated wt/mutp53 H1299 cells. B and C, dead cells were excluded by gating on 7-AAD–negative cells. *, $P < 0.05$ determined by Student's *t* test; n.d. = not detectable; dox, doxycycline.

by flow cytometric analysis gating on living EGFP-positive cells. Transfection efficiencies of HT1080 cells were at least 45% and of U2OS cells at least 30% (data not shown). Figure 2E shows that in both cell lines, the p53 target gene p21 was induced on protein level by overexpression of wtp53. Notably, relative ULBP2 mRNA amounts significantly increased in both cell lines after wtp53 transfection, whereas ULBP1 mRNA levels were not detectable (Fig. 2F). In summary, these data indicate that ULBP1/2 mRNA levels increase in several tumor cell lines after overexpression of wtp53 and that the increased ULBP1/2 mRNA levels in H1299 cells after dox treatment are not because of enhanced mRNA stability.

ULBP1 and ULBP2 are direct p53 target genes

Furthermore, we investigated whether the transcriptional upregulation of ULBP1 and -2 involved direct p53 binding to p53-responsive elements (p53REs) in the *ULBP1* and *-2* genes. Therefore, we carried out an *in silico* screen for potential p53REs (TRANSFAC number: T00671) in *MICA, -B, ULBP1*, *-2, -3*, and *-4* genes by using the Transcription Element Search System (TESS: http://www.cbil.upenn.edu/cgi-bin/tess/tess). Typical p53REs are composed of 2 half sites of 10 bp separated by a spacer of 0 to 21 bp (29). Potential p53REs with a suitable spacer length were only found in the last 530 bp of the first introns of the *ULBP1* and *-2* genes (Fig. 3A). ChIP with a p53-specific mAb revealed binding of wtp53, but not of mutp53, to both p53REs in the *ULBP1/2* genes in H1299 cells upon doxycyclin treatment (Fig. 3B and C). Of note, no binding using the p53-specific mAb on a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence (30) was detected by ChIP ruling out nonspecific precipitation. Binding of wtp53 to p53RE1 of the

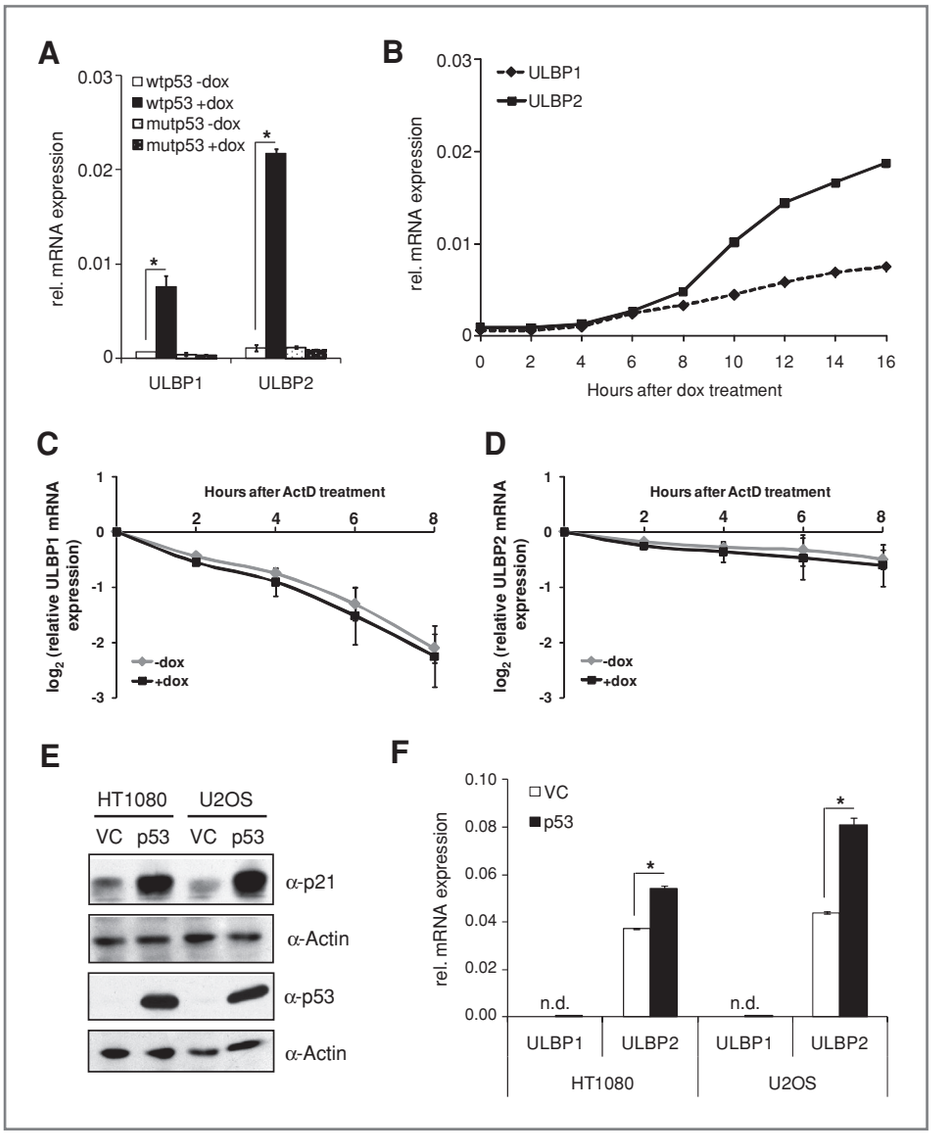

Figure 2. ULBP1 and -2 are upregulated at the transcriptional level. A, expression of ULBP1/2 mRNAs was monitored in wt/mutp53 H1299 cells 16 hours after ± doxycyclin treatment by quantitative real-time PCR. B, kinetics of ULBP1/2 mRNA expression in wtp53 H1299 cells after doxycyclin treatment is shown. A and B, ULBP1/2 mRNA expression levels are depicted relative to GAPDH mRNA expression levels (set as 1). The mean of 2 independently conducted experiments ± SD is shown. C and D, wtp53 H1299 cells were either left untreated or treated for 10 hours with doxycyclin, and ULBP1/2 mRNA expression was monitored by quantitative real-time PCR at different time points after the addition of 10 μg/mL actinomycin D (ActD). Log_2 of relative ULBP1/2 mRNA expression compared with the relative ULBP1/2 mRNA expression before the addition of ActD (set as 1) is shown. Expression of ULBP1/2 was normalized to GAPDH. E and F, HT1080 and U2OS cells were transfected with either 2 μg of a wtp53 expression plasmid (p53) or 2 μg of pcDNA3.1+ (VC). E, expression of p53, p21, and actin was analyzed by Western blot 24 hours after transfection. F, relative ULBP1/2 mRNA expression 24 hours after transfection was monitored as described in (A, B). B–F, one representative experiment of 2 independently conducted experiments is shown. The mean of duplicates ± SD is depicted. *, $P < 0.05$ determined by Student's *t* test; n.d. = not detectable; VC = vector control; dox, doxycycline.

ULBP1 gene was increased by 5.2-fold and to p53RE2 by 7.6-fold compared with untreated cells (Fig. 3B). In the *ULBP2* gene, wtp53 binding to p53RE1 was enhanced by 3.5-fold and to p53RE2 by 4.1-fold (Fig. 3C). Altogether, these data show that ULBP1 and -2 mRNAs are upregulated by induction of wtp53 in H1299 cells and that wtp53 binds to p53REs in the *ULBP1* and *-2* genes.

One intronic p53RE in the *ULBP1* and *-2* genes is sufficient to induce ULBP1 and ULBP2 transcription

To determine the involvement of the identified p53REs in p53-mediated *ULBP1* and *ULBP2* transcription, we generated luciferase reporter constructs by cloning of 530 bp long fragments of the first introns of the *ULBP1* and *-2* genes harboring the potential p53REs into a luciferase reporter

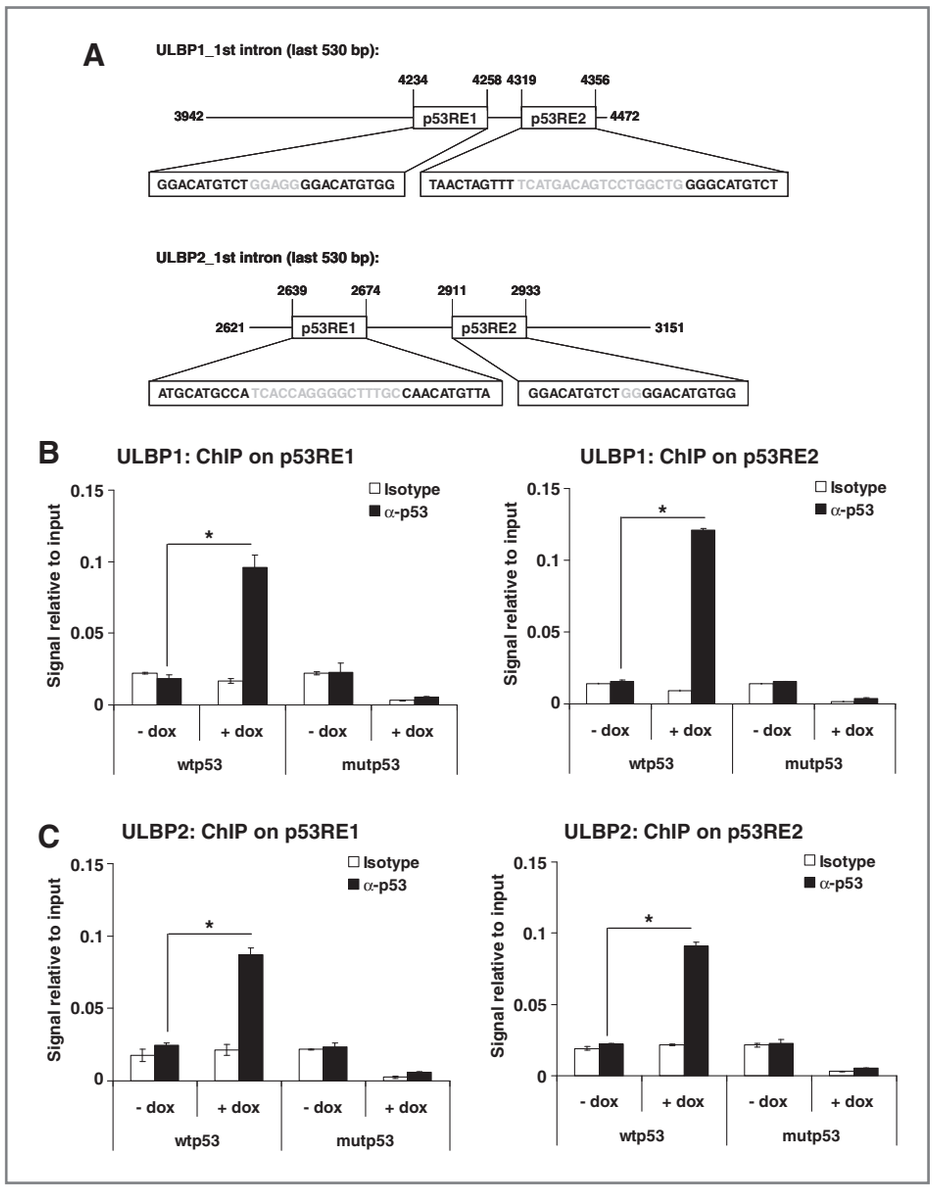

Figure 3. p53 binds to intronic p53REs in *ULBP1* and *ULBP2* genes. A, schematic picture of the potential p53REs in the *ULBP1/2* genes. B and C, binding of p53 to the potential p53REs in the *ULBP1/2* genes was analyzed in wt/mutp53 H1299 cells ± doxycyclin treatment by ChIP. The amount of immunoprecipitated chromatin bound by either isotype control or α-p53 mAb was quantified by real-time PCR with the respective p53RE-specific primers. Specific signals were set relative to signals obtained for the input chromatin (set as 1). One representative experiment of 2 independently conducted experiments is shown. The mean of duplicates ± SD is depicted. *, $P < 0.05$ determined by Student's *t* test. dox, doxycycline.

plasmid. In addition, we also prepared constructs in which p53RE half sites of all potential p53REs (ULBP1/2_Δp53RE1/2) or single potential p53REs (ULBP1/2_Δp53RE1, ULBP1/2_Δp53RE2) were deleted to determine the p53RE(s) that are essential for ULBP1/2 transcription. The cloned intronic sequences of the *ULBP1* and *-2* genes and the p53RE deletion constructs are shown in Figures 4A and 5A, respectively. Luciferase reporter analyses showed that luciferase activity was strongly increased upon transfection with a p53 containing plasmid in a dose-dependent manner when the luciferase constructs containing the 530 bp long fragments of the first introns of the *ULBP1* and *-2* genes were present (Figs. 4B, 5B). Deletion of all potential p53REs (ULBP1/2_Δp53RE1/2) from these constructs resulted in a reduction of luciferase activity to the levels of the empty vector control (Figs. 4B, 5B). At relatively low levels of p53 (transfection with 0–10 ng plasmid), transfection of constructs in which the first p53RE from both constructs was deleted (ULBP1/2_ Δp53RE1) resulted in high luciferase activity similar to constructs containing 530 bp long fragments of the first introns of the *ULBP1* and *-2* genes (ULBP1/2). A minor effect on p53-induced luciferase activity was observed when high amounts of p53 (transfection with 40 ng of plasmid) were present. Importantly, deletion of the second p53REs from both constructs (ULBP1/2_ Δp53RE2) strongly reduced luciferase activity to background levels (Figs. 4B and 5B). Thus, although p53 bound to both p53RE1/2 in the *ULBP1* and *-2* genes as determined by ChIP (Fig. 3), luciferase reporter assays revealed an essential role of ULBP1/2 p53RE2 and only a minor contribution of ULBP1/2 p53RE1 in p53-induced *ULBP1* and *ULBP2* transcription.

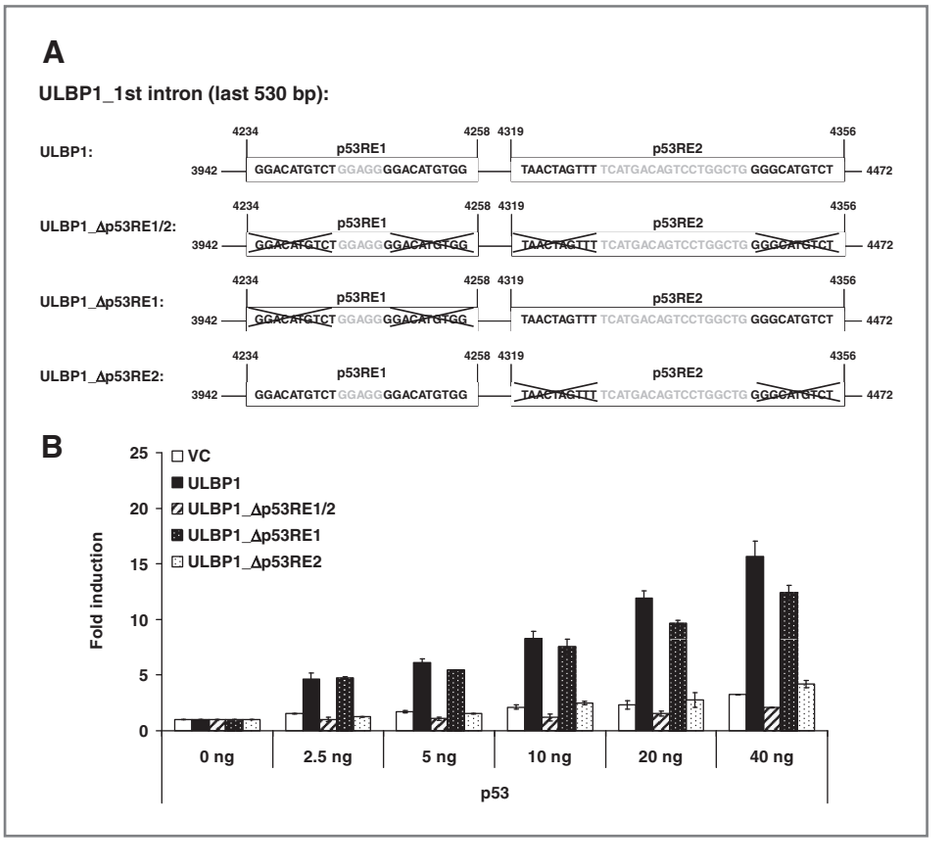

Figure 4. One intronic p53RE in the *ULBP1* gene is sufficient to induce expression of a luciferase reporter gene. A, the construct containing the 530 bp long fragment of the first intron of the *ULBP1* gene (ULBP1) and the constructs with deleted p53RE half sites of both potential p53REs (ULBP1_Δp53RE1/2) or single potential p53REs (ULBP1_Δp53RE1, ULBP1_Δp53RE2) are depicted. B, luciferase reporter assays after transfection of the ULBP1 p53RE(s) deletion constructs are shown. H1299 cells were cotransfected with the luciferase reporter plasmids, a β-galactosidase expression vector and increasing amounts of a wtp53 expression vector. Eighteen hours after transfection, luciferase and β-galactosidase activities were measured. Luciferase activity was normalized to β-galactosidase activity (set as 1) and fold induction was calculated by setting the relative luciferase activity of the transfection without p53 expression vector as 1. One representative experiment of 2 independently conducted experiments is shown. The mean of duplicates ± SD is depicted. VC, vector control.

Induction of wtp53 expression in H1299 cells leads to their enhanced recognition by primary NK cells in a NKG2D-dependent manner

In a next step, we investigated, whether the enhanced expression of ULBP1 and -2 in wtp53-expressing H1299 cells affected the IFN-γ production and degranulation of primary NK cells. Figure 6A shows that significantly higher levels of IFN-γ were produced by NK cells that were cocultured with doxycyclin-induced wtp53 as compared with doxycyclin-induced mutp53 H1299 cells. In addition, degranulation of NK cells was significantly increased in a coculture with wtp53-, but not mutp53-, expressing H1299 cells (Fig. 6B). The IFN-γ production and degranulation of NK cells were significantly reduced by addition of a blocking mAb directed against NKG2D but not by an isotype-matched control mAb (Fig. 6C and D). Thus, wtp53-induced H1299 cells that expressed increased levels of ULBP1 and -2 led to an enhanced activation of NK cells in a NKG2D-dependent manner.

The small molecular compound RITA upregulates ULBP2 in certain wtp53-expressing cancer cells

Targeting the p53/MDM2 interaction by small molecular compounds that reactivate p53 is a novel therapeutic strategy for cancers expressing wtp53 (4, 31, 32). In our study, the small molecular compound RITA (31) that reactivates p53 in different wtp53-expressing cancer cell lines was used. RITA binds to p53 and blocks the p53/MDM2 interaction leading to p53 accumulation, cell-cycle arrest, and apoptosis of cancer cells (31). To investigate the impact of RITA on NKG2D ligand expression, we treated 2 sarcoma cell lines, U2OS and HT1080, and 2 carcinoma cell lines, HCT116 and HepG2, with 1 μmol/L RITA for 24 hours and monitored ULBP1/2 cell surface and mRNA expression (Fig. 7 and data not shown). All cell lines expressed wtp53. Flow cytometric analyses were done upon gating on viable, 7-AAD negative cells. On average, 80% to 90% of nonfloating cells were viable after the 24 hours RITA treatment as determined by staining with 7-AAD (data not shown). RITA treatment led to high levels of p53 expression in

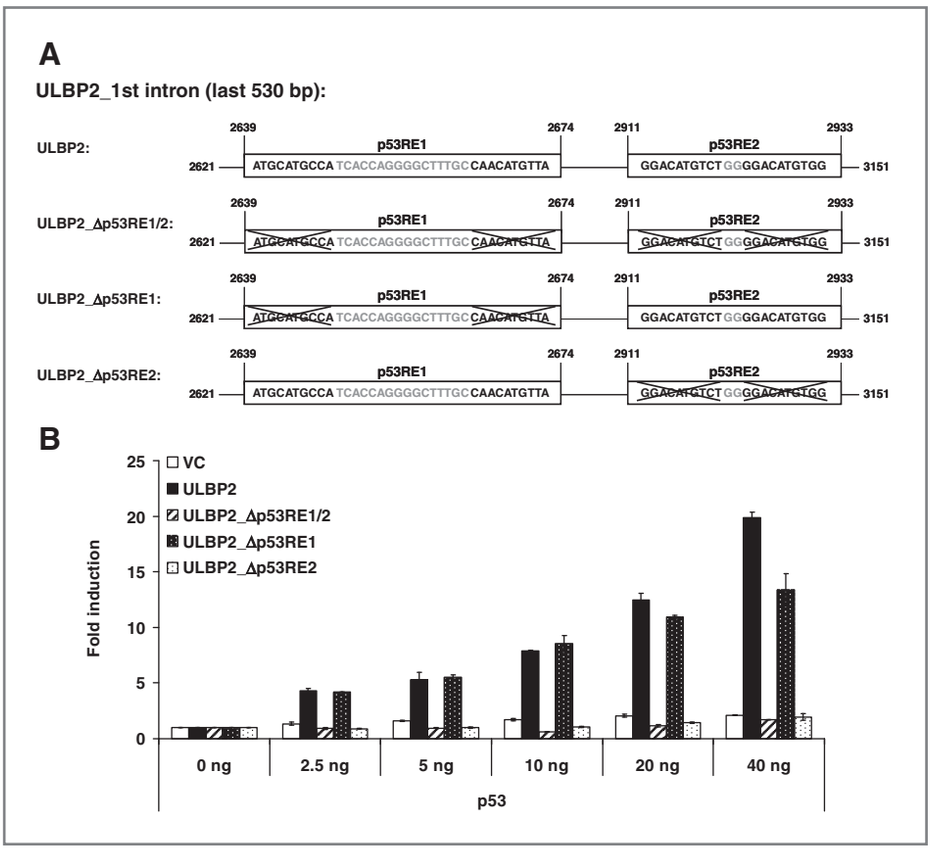

Figure 5. One intronic p53RE in the *ULBP2* gene is sufficient to induce expression of a luciferase reporter gene. A, the construct containing the 530 bp long fragment of the first intron of the *ULBP2* gene (ULBP2) and the constructs with deleted p53RE half sites of all potential p53REs (ULBP2_Δp53RE1/2) or single potential p53REs (ULBP2_Δp53RE1, ULBP2_Δp53RE2) are depicted. B, luciferase reporter assays after transfection of the ULBP2 p53RE (s) deletion constructs are shown. H1299 cells were cotransfected with the luciferase reporter plasmids, a β-galactosidase expression vector and increasing amounts of a wtp53 expression vector. Experiments were conducted as described in Figure 4. One representative experiment of 2 independently conducted experiments is shown. The mean of duplicates ± SD is depicted. VC, vector control.

all 4 wtp53-expressing cancer cell lines (Fig. 7A). In addition, RITA treatment led to induction of apoptosis in all tested cell lines as shown by cleavage of the caspase-3 target PARP (Fig. 7A). Importantly, RITA treatment significantly upregulated ULBP2 cell surface and mRNA expression in the 2 sarcoma cell lines HT1080 and U2OS (Fig. 7B–D). However, RITA treatment did not alter or induce ULBP2 expression in the 2 carcinoma cell lines HCT116 or HepG2 (Fig. 7B–D). ULBP1 mRNA and cell surface expression were not detectable and not induced upon RITA treatment in the tested cell lines (data not shown). Of note, MICA/B mRNA and cell surface expression was slightly downregulated in the majority of experiments and expression of total NKG2D ligands, as determined by cell surface staining with a NKG2D-Fc fusion protein, was unchanged (data not shown). These results indicate that RITA treatment upregulates the cell surface expression of the activating NK cell ligand ULBP2 in a subset of wtp53-expressing cancer cell lines.

Discussion

Our study reveals a novel mechanism of the transcriptional regulation of human NKG2D ligands by p53. Our results show that wtp53 induction in the NSCLC cell line, H1299, resulted in a pronounced upregulation of the human NKG2D ligands ULBP1 and -2 (Fig. 1) and led to NK cell activation (Fig. 6). In addition, reactivation of wtp53 in 2 sarcoma cell lines, U2OS and HT1080, by the small molecular compound RITA, significantly increased ULBP2 mRNA and cell surface expression (Fig. 7). The observed increase in ULBP1/2 mRNA levels after wtp53 induction in H1299 cells was not because of enhanced ULBP1/2 mRNA stability (Fig. 2C and D). By using 2 approaches, namely ChIP and luciferase reporter assays, we show that *ULBP1* and *ULBP2* are direct p53 target genes. Our ChIP analysis showed that p53 was capable of binding to both p53REs in the *ULBP1* and *ULBP2* promoters (Fig. 3). Of note, our luciferase reporter assays revealed an essential role of the second p53REs and a minor involvement of the first p53REs in p53-mediated expression of *ULBP1* and *ULBP2* genes (Figs. 4 and 5). Because the deletion of the second p53REs was sufficient to reduce the p53-induced luciferase activity to background levels, we assume that the first p53REs might only play a subordinate role in p53-induced *ULBP1/2* transcription. It was reported that binding of p53 to p53REs is not sufficient for transcription of p53 target genes (reviewed in ref. 29). In this context, a recent analysis of the *ULBP1* 5′ promoter revealed that steady-state transcription of *ULBP1* is mainly dependent on the transcription factors Sp1 and Sp3 (33). Because Sp1 and Sp3 can serve as cofactors for p53-mediated transcriptional activation of p53 target genes, such as $p21^{Cip1}$ (34), it is possible that p53 also interacts with Sp1 or -3 for the efficient transcriptional regulation of the *ULBP1* gene and maybe also of the *ULBP2* gene.

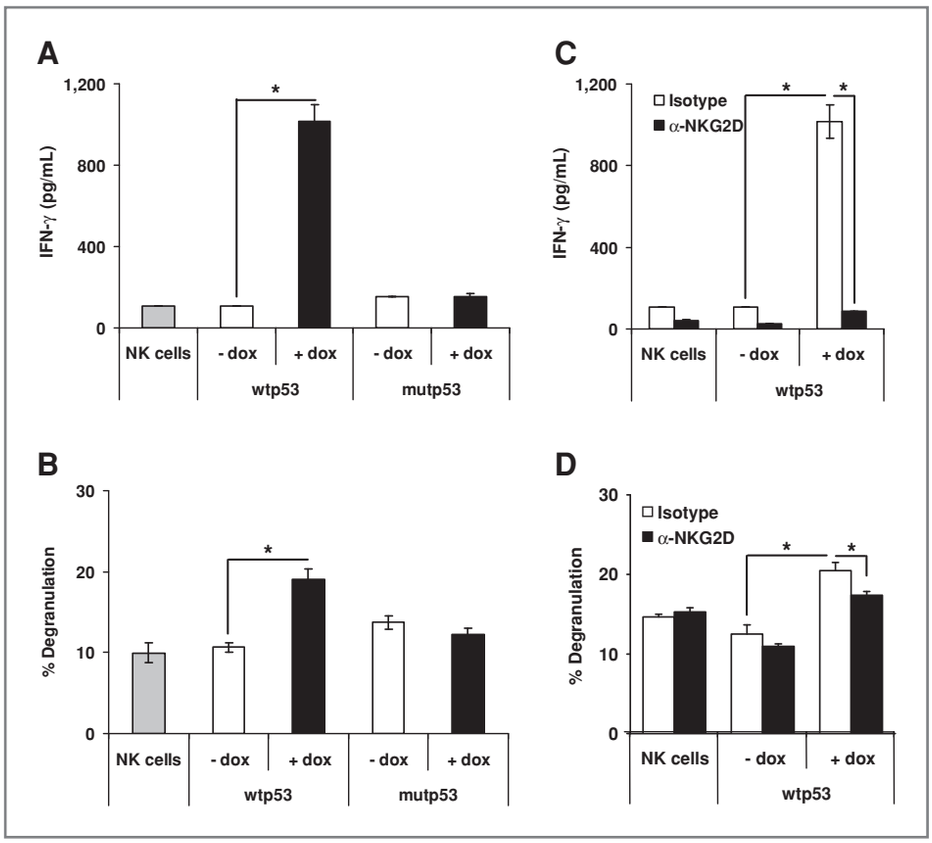

Figure 6. Induction of wtp53 expression in H1299 cells leads to enhanced recognition by primary NK cells in a NKG2D-dependent manner. A and C, IFN-γ production by primary NK cells after 24 hours coculture with noninduced (-dox) or wt/mutp53-expressing (+dox) H1299 cells was assessed. One representative experiment of 3 independently conducted experiments is shown. The mean of duplicates ± SD is indicated. B and D, degranulation of primary NK cells after 4-hour coculture with noninduced or wt/mutp53-expressing H1299 cells was analyzed. One representative experiment of 3 independently conducted experiments is shown. The mean of triplicates ± SD is indicated. A–D, primary NK cells were expanded for 48 hours with IL-2 and IL-15 before they were used for coculture experiments. C and D, α-NKG2D mAb or isotype control were added to cocultures at a final concentration of 10 μg/mL. *, $P < 0.01$ determined by Student's *t* test; VC, vector control; dox, doxycycline.

The constructs used in our luciferase reporter assays that contained the 530-bp long intronic *ULBP1/2* gene fragments upstream of the luciferase gene led to strong induction of luciferase activity upon transfection with a p53-containing plasmid. Because no additional promoter was present in our construct, the intronic *ULBP1/2* gene fragments by themselves might work as promoters. By definition, a promoter, in contrast to an enhancer, functions in an orientation-dependent manner. Thus, we flipped the *ULBP1/2* gene fragments in front of the luciferase gene from their 5′→3′ orientation to a 3′→5′ orientation and luciferase assays were carried out. Our results revealed that the luciferase activity was not affected by flipping the orientation of the *ULBP1/2* gene fragments (data not shown). Because the ULBP1/2 p53REs have palindromic properties and do not change their core sequence with the most conserved nucleotides when flipped, the intronic ULBP1/2 p53REs may still function as promoters that may be bidirectional. Whether p53-induced ULBP1/2 transcripts initiate at the site of the p53REs and whether transcripts initiating at this site encode intact proteins is currently unknown. Alternatively, the intronic ULBP1/2 p53REs may work as enhancers. In this context, it was observed that some enhancers occasionally exhibit promoter activity in reporter assays that may not be physiologically significant. Further analyses are needed to clarify these issues.

Recently, it has been shown that activation of the ATM/ATR protein kinases by DNA damage–inducing agents resulted in the upregulation of certain NKG2D ligands including ULBP1-3 in tumor cells and normal human fibroblasts (25, 26). It is well established that p53 can be activated downstream of ATM/ATR protein kinases resulting in the induction of p53 target genes. Our data indicate the involvement of p53 in the upregulation of the human NKG2D ligands ULBP1 and 2. In addition, in a mouse model of liver carcinoma, p53 reactivation resulted in tumor cell senescence and NK cell–dependent tumor regression (6). The mechanism of NK cell–dependent elimination of tumor cells was not addressed in the study. It is possible that activating NK cell ligands such as NKG2D ligands were induced in the tumor cells upon p53 reactivation. Of note, by using *K-ras*- and *c-myc*–transformed $p53^{-/-}$ ovarian epithelial cells, Gasser and colleagues observed that the induction of mouse NKG2D ligand expression by DNA damage–inducing agents did not require p53 (26). We assume that both p53-dependent and p53-independent pathways regulate the expression of NKG2D ligands after induction of DNA damage and/or cellular senescence. It is also possible that certain mouse and human NKG2D ligands are differentially regulated by p53. Our *in silico* analysis of the mouse *RAET1* genes, however, revealed potential intronic p53REs. Future studies will investigate whether certain mouse RAE1 family members are regulated by p53 in a similar manner as their human homologues ULBP1 and -2. In a recent study, not only certain human NKG2D ligands but also the DNAM-1 ligands CD155 and CD112 were upregulated by DNA

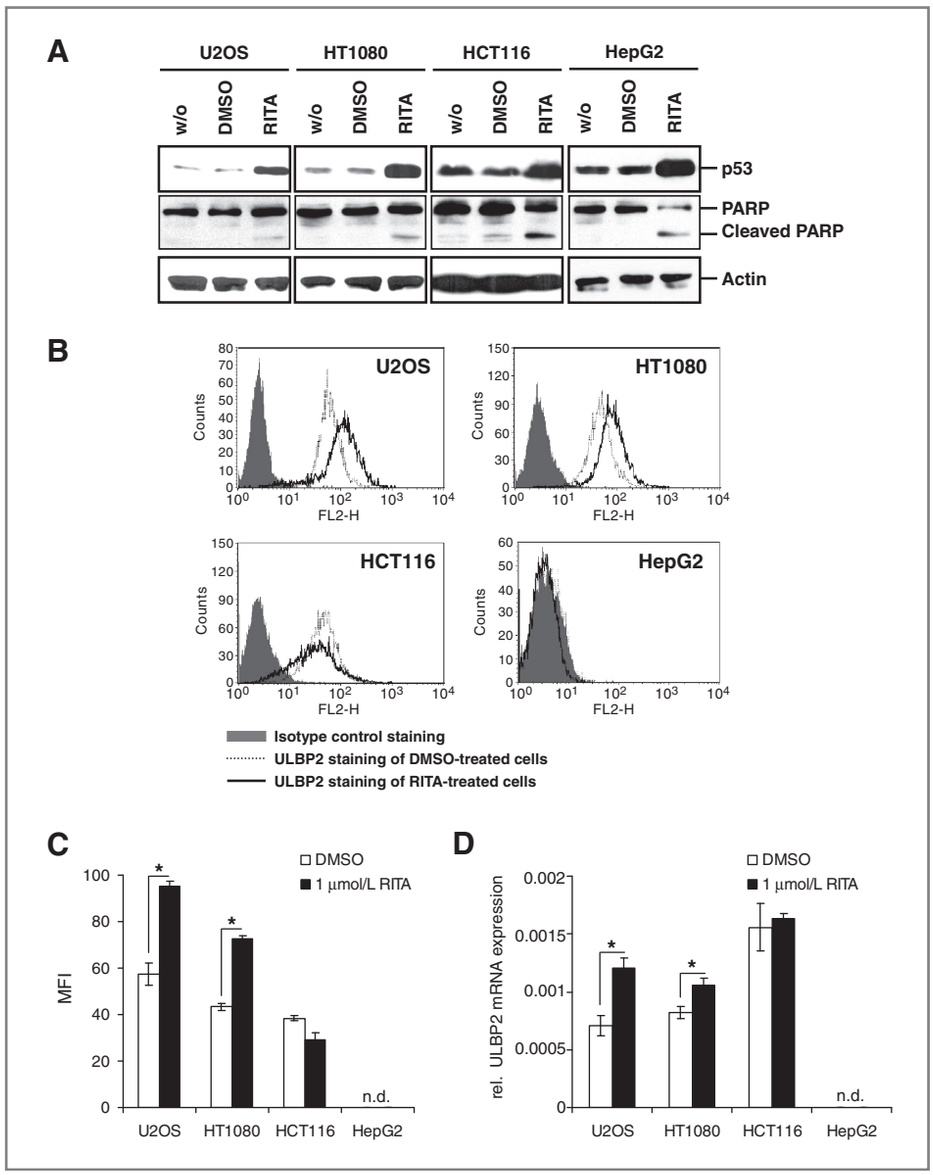

Figure 7. The small molecular compound RITA upregulates ULBP2 in certain wtp53-expressing tumor cell lines. A, Western blot analysis of p53, PARP, and actin expression in the depicted cell lines left untreated (w/o) or after 24 hours of treatment with the solvent control DMSO or 1 μmol/L RITA. B, representative histogram overlays showing cell surface expression of ULBP2 on the depicted cell lines after 24-hour treatment with 1 μmol/L RITA or DMSO. C, cell surface expression of ULBP2 on the depicted cell lines after 24 hours of treatment with 1 μmol/L RITA or DMSO. MFI was calculated as geometrical mean of specific staining minus isotype control staining. B and C, dead cells were excluded by gating on 7-AAD–negative cells. D, expression levels of ULBP2 mRNA were monitored in the depicted cell lines after 24-hour treatment with DMSO or RITA by quantitative real-time PCR. ULBP2 mRNA expression levels are depicted relative to GAPDH mRNA expression levels (set as 1). A–D, one representative experiment of 2 independently conducted experiments is shown. C and D, the mean of duplicates ± SD is depicted. *, $P < 0.05$ determined by Student's *t* test; n.d., not detectable.

damage–inducing reagents on myeloma cells (9). In our study, no effect of p53 induction was observed on the expression of DNAM-1 ligands (data not shown), indicating differential regulation of certain NKG2D ligands and DNAM-1 ligands by p53.

Approximately 50% of human cancers mutate or delete p53 or impair its function to gain a proliferation and survival advantage (4). Our study reveals that wtp53, but not mutp53, induction in cancer cells increased the expression of certain NKG2D ligands resulting in enhanced NKG2D-dependent degranulation (Fig. 6). Thus, lack of p53 function might enable cancer cells not only to promote their proliferation and survival but also to escape from recognition by NK cells and, possibly, other NKG2D-bearing effector cells such as $CD8^+$ T cells, NKT cells, and $\gamma\delta^+$ T cells by defective upregulation of certain NKG2D ligands. In addition, coculture of NK cells and wtp53-, but not mutp53-, expressing cancer cells resulted in the NKG2D-dependent production of IFN-γ (Fig. 6). Because NK cell–derived IFN-γ is important for the induction of Th1 and Tc1 effector responses (35), lack of p53 function might also impair the NK cell–dependent priming of T cells. Thus, not only cancer cell intrinsic but also extrinsic, immune cell–mediated pathways are likely to be affected by the impairment of p53 function in tumor cells.

Targeting the p53/MDM2 interaction by small molecular compounds that reactivate p53 is a novel therapeutic strategy for cancers containing wtp53 (4, 31, 32). In our study, one of these compounds, RITA (31), was used to reactivate wtp53 in different cancer cell lines (Fig. 7). RITA treatment upregulated ULBP2 mRNA and cell surface expression in 2 of 4 tested

cancer cell lines (Fig. 7B–D). The cancer cell lines HT1080 and U2OS cells that upregulated ULBP2 after RITA treatment were sarcomas. The cancer cell lines HCT116 and HepG2 that did not upregulate ULBP2 were carcinomas. In this context, it was reported that different epigenetic mechanisms repress the expression of certain NKG2D ligands in epithelial tumor cells (23, 36). HDAC3 repressed the expression of ULBP1–3 in different epithelial tumors (23) and the *ULBP2* promoter was shown to be heavily methylated in the colon carcinoma cell line HCT116 (36). Therefore, we assume that the cell type–specific DNA methylation or histone deacetylation of the *ULBP1* or *-2* promoters might determine their accessibility to transcription factors such as p53.

Expression of NKG2D ligands on tumor cells is regulated at different levels, including epigenetic, transcriptional, and posttranscriptional mechanisms. Thus, it is tempting to speculate that highest levels of NKG2D ligand expression could be achieved by treatment with a combination of compounds targeting different mechanisms of NKG2D ligand regulation. Such a combination therapy could combine p53-reactivating small molecular compounds with, for example, DNA damage–inducing drugs or HDAC inhibitors. In future studies, different drug combinations will be evaluated not only to achieve high levels of inhibition of tumor cell growth but also to upregulate NKG2D ligands on tumor cells resulting in NK cell activation. Thus, NKG2D ligand inducing treatments including application of p53-reactivating compounds and subsequent adoptive transfer of NK cells could be exploited as an effective regimen of NK cell–based immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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