

# The E3 Ubiquitin Protein Ligase HERC2 Modulates the Activity of Tumor Protein p53 by Regulating Its Oligomerization\*

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Monica Cubillos-Rojas, Fabiola Amair-Pinedo, Roser Peiró-Jordán, Ramon Bartrons, Francesc Ventura, and Jose Luis Rosa<sup>1</sup>

From the Departament de Ciències Fisiològiques II, Campus de Bellvitge, Institut d'Investigació Biomèdica de Bellvitge (IDIBELL), Universitat de Barcelona, L'Hospitalet de Llobregat, Barcelona 08907, Spain

**Background:** HERC2 has been implicated in DNA repair mechanisms and neurological disorders.

**Results:** HERC2 binds p53 and regulates its transcriptional activity, affecting cellular processes modulated by p53 such as cell growth or DNA damage response.

**Conclusion:** HERC2 modulates p53 activity by regulating its oligomerization.

**Significance:** HERC2 is a novel regulator of p53 signaling.

The tumor suppressor p53 is a transcription factor that coordinates the cellular response to several kinds of stress. p53 inactivation is an important step in tumor progression. Oligomerization of p53 is critical for its posttranslational modification and its ability to regulate the transcription of target genes necessary to inhibit tumor growth. Here we report that the HECT E3 ubiquitin ligase HERC2 interacts with p53. This interaction involves the CPH domain of HERC2 (a conserved domain within Cul7, PARC, and HERC2 proteins) and the last 43 amino acid residues of p53. Through this interaction, HERC2 regulates p53 activity. RNA interference experiments showed how HERC2 depletion reduces the transcriptional activity of p53 without affecting its stability. This regulation of p53 activity by HERC2 is independent of proteasome or MDM2 activity. Under these conditions, up-regulation of cell growth and increased focus formation were observed, showing the functional relevance of the HERC2-p53 interaction. This interaction was maintained after DNA damage caused by the chemotherapeutic drug bleomycin. In these stressed cells, p53 phosphorylation was not impaired by HERC2 knockdown. Interestingly, p53 mutations that affect its tetramerization domain disrupted the HERC2-p53 interaction, suggesting a role for HERC2 in p53 oligomerization. This regulatory role was shown using cross-linking assays. Thus, the inhibition of p53 activity after HERC2 depletion can be attributed to a reduction in p53 oligomerization. Ectopic expression of HERC2 (residues 2292–2923) confirmed these observations. Together, these results identify HERC2 as a novel regulator of p53 signaling.

p53 plays a central role in coordinating cellular responses to stress by determining whether cells respond to various types and levels of stress with apoptosis, cell cycle arrest, senescence, DNA repair, cell metabolism, or autophagy. p53 functions as a

transcription factor that activates and represses a wide range of genes. Although some effects of p53 may be independent of transcription, the p53-controlled transactivation of target genes is an essential feature of each stress response pathway. p53 is normally kept at low levels through ubiquitination and proteasomal degradation mediated by several E3 ubiquitin ligases. Activation of p53 in response to stress classically consists of three sequential steps: p53 stabilization, sequence-specific DNA binding, and recruitment of the general transcriptional machinery to activate the transcription of p53 target genes. During these steps, p53 activation is controlled by several posttranslational modifications, such as ubiquitination, phosphorylation, and acetylation, and by interactions with other proteins. Genetic studies suggest that p53 activation *in vivo* is even more complex, and an antirepression step involving the release of p53 from repression factors such as MDM2 and MDMX has been proposed to reconcile these models (1–5).

p53-interacting proteins may regulate p53 activation at different levels, and, thus, the identification of new p53 interactors and analysis of their biological relevance is likely to shed more light on p53-dependent cellular processes. The proteins Cullin 7 (CUL7), Parkin-like cytoplasmic (PARC), and HECT- and RCC1-like domains 2 (HERC2) contain a common domain named CPH (a conserved domain within Cul7, PARC, and HERC2 proteins) (6, 7). Both CUL7 and PARC are known to bind cytoplasmic p53 through their CPH domains, and both promote cell growth by antagonizing p53 function (6–8). Consistent with this, *in vivo* studies clearly implicate CUL7 in growth regulation, and CUL7 germ line mutations were found in patients with autosomal recessive 3-M and Yakuts short stature syndromes, which are characterized by profound growth retardation (9).

HERC family proteins contain two characteristic domains: HECT and RCC1-like. Proteins with HECT domains have been reported to function as E3 ubiquitin ligases, and those containing RCC1-like domains have been reported to function as GTPase regulators. These two activities are essential in a number of important cellular processes, such as the cell cycle, cell signaling, and membrane trafficking (10–14). HERC proteins can be classified into two subgroups: large (HERC1–2) and

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<sup>1</sup> To whom correspondence should be addressed: Dept. de Ciències Fisiològiques II, Campus de Bellvitge, Universitat de Barcelona, L'Hospitalet de Llobregat, Barcelona 08907, Spain. E-mail: joseluisrosa@ub.edu.

small (HERC3–6 in humans and HERC3–5 in mice). Structurally, small HERC proteins contain little more than the two characteristic domains, whereas, functionally, they have been related to ubiquitination and ISGylation processes associated with membrane traffic and the immune response (15–19). Large HERC proteins contain additional domains, including several RCC1-like domains. HERC1 has been implicated in membrane trafficking and cell proliferation/growth through its interactions with the ARF, Rab, clathrin, M2-pyruvate kinase, and TSC2 proteins (20–23). *HERC2* is one of two major genes responsible for eye color in humans (24). Several radiation and ethylnitrosourea-induced mutations at the mouse *Herc2* locus cause neuromuscular tremor, runting, juvenile lethality, and sperm defects (25–27). In humans, a single-base mutation in the *HERC2* gene has also been implicated in a syndrome similar to Angelman syndrome that causes neurodevelopmental delay (28, 29). Other mutations affecting members of the HERC family have been associated with sterility, growth retardation, and neurodegeneration (30, 31). More recently, *HERC2* has also been implicated in cell cycle and DNA damage responses. Thus, it has been reported that *HERC2* may function as an assembly factor for the RNF8-Ubc13 complex, which promotes Lys-63-linked polyubiquitination at sites of DNA damage in response to DNA double strand breaks (32). Furthermore, *HERC2* interacts with claspin, a protein essential for G<sub>2</sub>/M checkpoint activation and replication fork stability, suggesting that *HERC2* regulates the progression of DNA replication (33).

*HERC2* may also function as an E3 ubiquitin ligase for degradation of the xeroderma pigmentosum A protein during circadian control of nucleotide excision repair and of the breast cancer suppressor BRCA1 during the cell cycle, respectively (34, 35). More recently, it has been reported that NEURL4 is also a substrate of *HERC2*, participating in the ubiquitin-dependent regulation of centrosome architecture (36). Additionally, *HERC2* can stimulate the ubiquitin-protein ligase activity of other E3 ligases such as E6AP (37). All of these observations suggest a multifunctional role of *HERC2* in cell biology.

In this study, we report a new function for *HERC2*. We show that *HERC2* binds to p53 and that the silencing of *HERC2* alters p53 activity as a transcriptional factor, affecting the expression of genes regulated by p53 such as *p21*, *p53R2*, and *p53AIP1*. Under these conditions, we observed increased cellular growth and focus formation in clonogenic assays. The *HERC2*-p53 interaction is disrupted by p53 mutations in the tetramerization domain. Additionally, cross-linking experiments with glutaraldehyde indicated that *HERC2* regulates the oligomerization of p53. These findings demonstrate that *HERC2* is a key component in p53 regulation.

## EXPERIMENTAL PROCEDURES

**Reagents**—The following reagents were used: anti-*HERC2* monoclonal, anti-clathrin heavy chain (BD Biosciences); anti-mTOR, anti-TSC1, anti-phospho-Ser-15-p53, and anti-Lamin A/C (4C11, Cell Signaling Technology); anti-TSC2 (C-20), anti-p21 (C-19), and anti-p53 (FL-393) (Santa Cruz Biotechnology, Inc.); anti-p53 Ab-5 (DO-7) (Neo Markers); anti-MDM2 (2A10) (Abcam); anti-GST monoclonal (GenScript); anti-Ran and anti-*HERC1* (22); anti- $\alpha$ -tubulin (Ab-1), bleomycin sulfate

and nutlin-3a (Calbiochem); Z-Leu-Leu-Leu-al (MG132) (Sigma-Aldrich); horseradish peroxidase-conjugated secondary antibodies (Invitrogen); protein A-Sepharose, protein G-Sepharose, and glutathione-Sepharose (GE Healthcare); Talon metal affinity resin (BD Bioscience); Immobilon-P PVDF transfer membrane (Millipore Corp.); cycloheximide (Applchem); luciferase assay system (Promega); and luminescent  $\beta$ -galactosidase detection kit II (Clontech Laboratories).

**Plasmids, Antibodies, and siRNAs**—pEGB-p53 constructs (WT,  $\Delta$ N200,  $\Delta$ N300, and  $\Delta$ N300 $\Delta$ C43), p53 constructs (WT, NLS, NES, R337C, and L344P), His-p53 constructs (1–360 and 1–320), luciferase reporters (p21WAF1, p53AIP1, and p53R2), and the Myc-*HERC2* F3 construct (residues 2292–2923) were provided by Dr. Y. Xiong (6), Dr. Y. Zhang (38), Dr. C. H. Arrowsmith (39), Dr. Y. Taya (40), and Dr. T. Ohta (35), respectively. The Gateway system (Invitrogen) was used to generate *HERC2* and *HERC1* plasmids. cDNAs encoding the amino terminus (residues 1–199), the CPH domain (residues 2547–2640), and the carboxyl terminus (residues 4785–4834) of the human *HERC2* protein and the amino terminus (residues 1–155) of the human *HERC1* were amplified by PCR from human fetal brain or HeLa cDNA libraries using specific oligonucleotides. Amplified fragments were used to generate entry clones with pDONR221 and expression vectors with pDEST15 (GST fusion) or pDEST17 (His fusion) following standard Gateway protocols. The plasmids were sequenced. Fusion proteins were expressed in bacteria, purified, and used for pull-down experiments and to generate anti-*HERC2* (named bvg3 and bvg4 antibodies against residues 1–199 and bvg1 and bvg2 antibodies against residues 4785–4834) and anti-*HERC1* (bvg5 and bvg6 antibodies against residues 1–155) polyclonal antibodies, as described previously (15). Two siRNAs targeting the human sequence of *HERC2* (H2.2, GACUGUAGCCAGAUUGAAA and H2.4, GGAAAGCACUGGAUUCGUU) were purchased from Ecogen or GenePharma. Similar results were obtained with both. siRNAs for *HERC1* (Q1, CGGCAUGGAUGAACAAAUU), p53 (p53, GACUCCAGUGGUAUUCUAC) and a non-targeting siRNA (NT,<sup>2</sup> UAGCGACUAAACACAUCAA) were purchased from Ecogen or GenePharma.

**Cell Culture and Transfections**—HeLa, HEK-293, U2OS, and H1299 cells were cultured at 37 °C in DMEM (Invitrogen) with 10% fetal bovine serum. Transfection of cells (plasmids and siRNAs) was carried out using calcium phosphate. Briefly, the day before transfection, the cells were seeded into 6-well plates at 30% confluence. On the day of transfection, cells were at 60% confluence. For a final volume of 100  $\mu$ l, 50  $\mu$ l of CaBES solution (500 mM CaCl<sub>2</sub> and 100 mM BES (pH 6.95)) was mixed with 50  $\mu$ l of MilliQ water containing 4  $\mu$ g of plasmid DNA or siRNA to reach a final concentration of 100 nM. Then, 100  $\mu$ l of BES solution (280 mM NaCl, 0.75 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.75 mM NaH<sub>2</sub>PO<sub>4</sub>, and 50 mM BES) at the appropriate pH was slowly added while aerating the mix. The final mix was incubated for 15 min at room temperature and then added to the cells. Bleomycin or cycloheximide was added to a final concentration of 10  $\mu$ g/ml or 15  $\mu$ g/ml, respectively, at the indicated times. MG132 was

<sup>2</sup>The abbreviations used are: NT, non-targeting; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; IP, immunoprecipitation.

## HERC2 Regulates p53 Oligomerization

added to the cells for 6 h to a final concentration of 10  $\mu\text{M}$ . For the treatment with nutlin, the cells were incubated for 16 h to a final concentration of 10  $\mu\text{M}$ .

**Cell Lysate and Immunoblotting**—Cells were lysed and processed as described previously (41). Giant and small proteins were analyzed simultaneously using the LAG (42) or the Tris acetate PAGE systems (43, 44). Band intensities were analyzed using a gel documentation system (LAS-3000, Fujifilm). Protein levels were normalized and expressed as a percentage of controls.

**Immunoprecipitations and Pulldowns**—Cells lysed with CHAPS buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, and 0.3% CHAPS) containing 50 mM  $\beta$ -glycerophosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 5  $\mu\text{g/ml}$  leupeptin, 5  $\mu\text{g/ml}$  aprotinin, 1  $\mu\text{g/ml}$  pepstatin A, and 100  $\mu\text{g/ml}$  benzamidin were centrifuged for 10 min at 13,000  $\times g$ . Animal tissues were processed and analyzed as described previously (30). For immunoprecipitation (IP), supernatants (input) were incubated with preimmune serum or with anti-HERC2 (bvg3 or bvg4), anti-HERC1 (410 or bvg6), or anti-p53 antibodies for 2 h at 4  $^{\circ}\text{C}$  and immunoprecipitated with protein A-Sepharose or protein G-Sepharose for 1 h at 4  $^{\circ}\text{C}$ . In these experiments, HERC2 was detected with anti-HERC2 monoclonal antibody. For pulldowns, supernatants (input) were incubated with 5  $\mu\text{g}$  of purified GST fusion proteins bound to a glutathione resin for 1 h at 4  $^{\circ}\text{C}$ . Pellets were washed three times with CHAPS buffer and analyzed by PAGE and immunoblot analysis. For direct interaction, His-p53 (wild-type, amino acids 1–393), deleted His-p53 (amino acids 1–320), and GST-CPH fusion proteins were expressed in bacteria and purified using Talon or glutathione resins, similarly as described previously (15). 4  $\mu\text{g}$  of purified His-p53 (1–393) or deleted His-p53 (1–320) bound to a resin was incubated with 2  $\mu\text{g}$  of purified GST-CPH protein in 0.2 ml of binding buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2.5 mM  $\text{MgCl}_2$ , 5 mM imidazole, 0.5% Nonidet P-40, 2 mM DTT, and 1 mg/ml BSA) for 1 h at 4  $^{\circ}\text{C}$ . Pellets were washed four times with binding buffer and analyzed by PAGE and immunoblot analysis.

**Subcellular Fractionation**—Cells were resuspended in buffer A (10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM  $\text{MgCl}_2$ , 0.34 M sucrose, 10% glycerol, 1 mM DTT, and 0.1% Triton X-100) containing 50 mM NaF, 20 mM  $\beta$ -glycerophosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 5  $\mu\text{g/ml}$  leupeptin, 5  $\mu\text{g/ml}$  aprotinin, 1  $\mu\text{g/ml}$  pepstatin A, and 100  $\mu\text{g/ml}$  benzamidin and incubated on ice for 5 min for permeabilization. Total cell lysates were prepared by resuspending the lysate directly in sample buffer. After permeabilization, the cells were centrifuged at 4500 rpm for 5 min at 4  $^{\circ}\text{C}$ . Supernatants were collected and considered cytoplasmic fraction. Pellets were washed with buffer A and centrifuged at 4500 rpm for 5 min at 4  $^{\circ}\text{C}$ . This step was repeated three times. The final pellet (nuclear fraction) was resuspended in the initial volume half of buffer A. All fractions were sonicated before analysis by PAGE.

**Luciferase Assay**—U2OS or H1299 cells were transfected with the corresponding reporter. Luciferase activity was quantified using a luciferase assay system (Promega). Luciferase values were normalized using  $\beta$ -galactosidase activity measured

using the Luminescent  $\beta$ -Galactosidase Detection Kit II (Clontech Laboratories). Luminescence levels are expressed as a percentage of controls.

**RT Quantitative PCR Analysis**—Total RNA was isolated from transfected cells using the Ultraspec RNA isolation system (Biotecx) according to the protocol of the manufacturer. 2  $\mu\text{g}$  of total RNA was reverse-transcribed using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCR was carried out using the ABI Prism 7900 HT fast real-time PCR system, and commercially available human TaqMan assays (Applied Biosystems) were used to quantify gene expression of *CDKN1A* (p21) (Hs00355782\_m1). The housekeeping gene *GAPDH* (HS99999905\_m1) was used for normalization. PCR data were captured and analyzed using the Sequence Detector software (SDS version 2.3, Applied Biosystems).

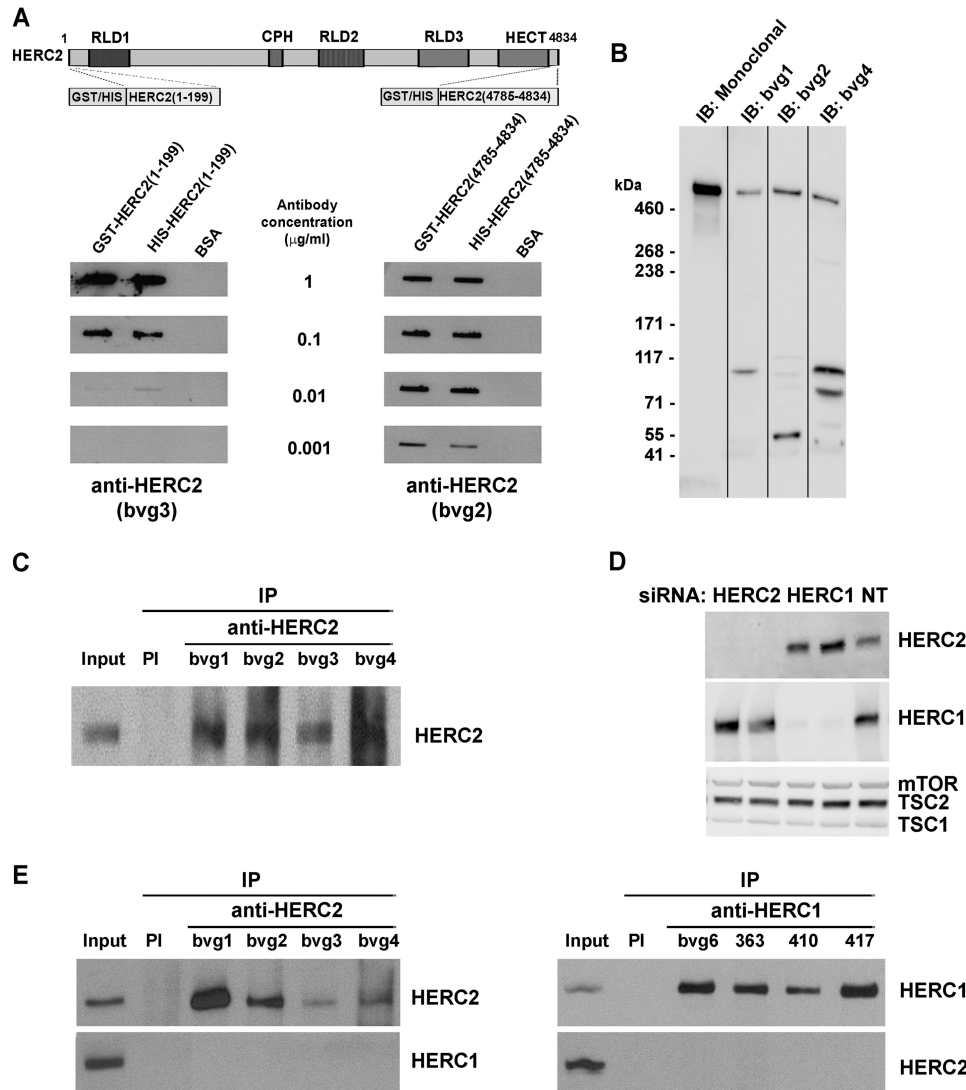
**Proliferation and Clonogenic Assays**—For proliferation assays, 24 h after siRNA transfection, cells were reseeded at 3000 cells/well in triplicate in 6-well dishes for 7 days in DMEM (Invitrogen) with 5% calf serum. The cells were stained with crystal violet (containing 2% ethanol) after dissolving the dye in 1% SDS. The absorbance at 550 nm was quantified and plotted proportional to cell number. For clonogenic assays, 500 cells were reseeded and grown until cells formed sufficiently large colonies ( $\sim 15$  days). The colonies were stained with crystal violet and quantified using ImageJ software.

**Protein Cross-linking Assay**—Cells were transfected with the indicated plasmids and siRNAs and lysed in CHAPS buffer 72 h after transfection. After lysis, the cells were centrifuged, and the supernatant was recovered. Glutaraldehyde was added to the supernatant at the indicated concentrations and incubated on ice for 30 min, as described previously (38). The reaction was stopped with sample buffer 4 $\times$ , and the samples were analyzed by Western blot analysis.

**Statistical Analysis**—Results are expressed as mean  $\pm$  S.E. Data for multiple variable comparisons were analyzed by one-way analysis of variance. For comparison of significance, Dunnett's test or Tukey's test were used according to the statistical program GraphPad Prism.

## RESULTS

**The Ubiquitin Ligase HERC2 Interacts with p53**—To detect endogenous HERC2 protein, we generated polyclonal antibodies against the carboxyl terminus (named bvg1 and bvg2) and the amino terminus (named bvg3 and bvg4) of the human HERC2 protein (Fig. 1A). Immunopurified antibodies specifically detected purified HERC2 fragments (Fig. 1A) and endogenous HERC2 protein (Fig. 1B, electrophoretic mobility of around 500 kDa) by immunoblotting. A commercial monoclonal anti-HERC2 antibody confirmed the band detected by polyclonal antibodies. Polyclonal antibodies were also able to immunoprecipitate endogenous HERC2 protein (Fig. 1C). The HERC2 homolog HERC1 has a similar electrophoretic mobility. To show the specificity of the above antibodies against HERC2, we performed knockdown experiments. Thus, in the presence of HERC2 siRNA, anti-HERC2 antibodies did not recognize HERC1 (Fig. 1D). Likewise, in the presence of HERC1 siRNA, anti-HERC1 antibodies did not recognize HERC2 pro-



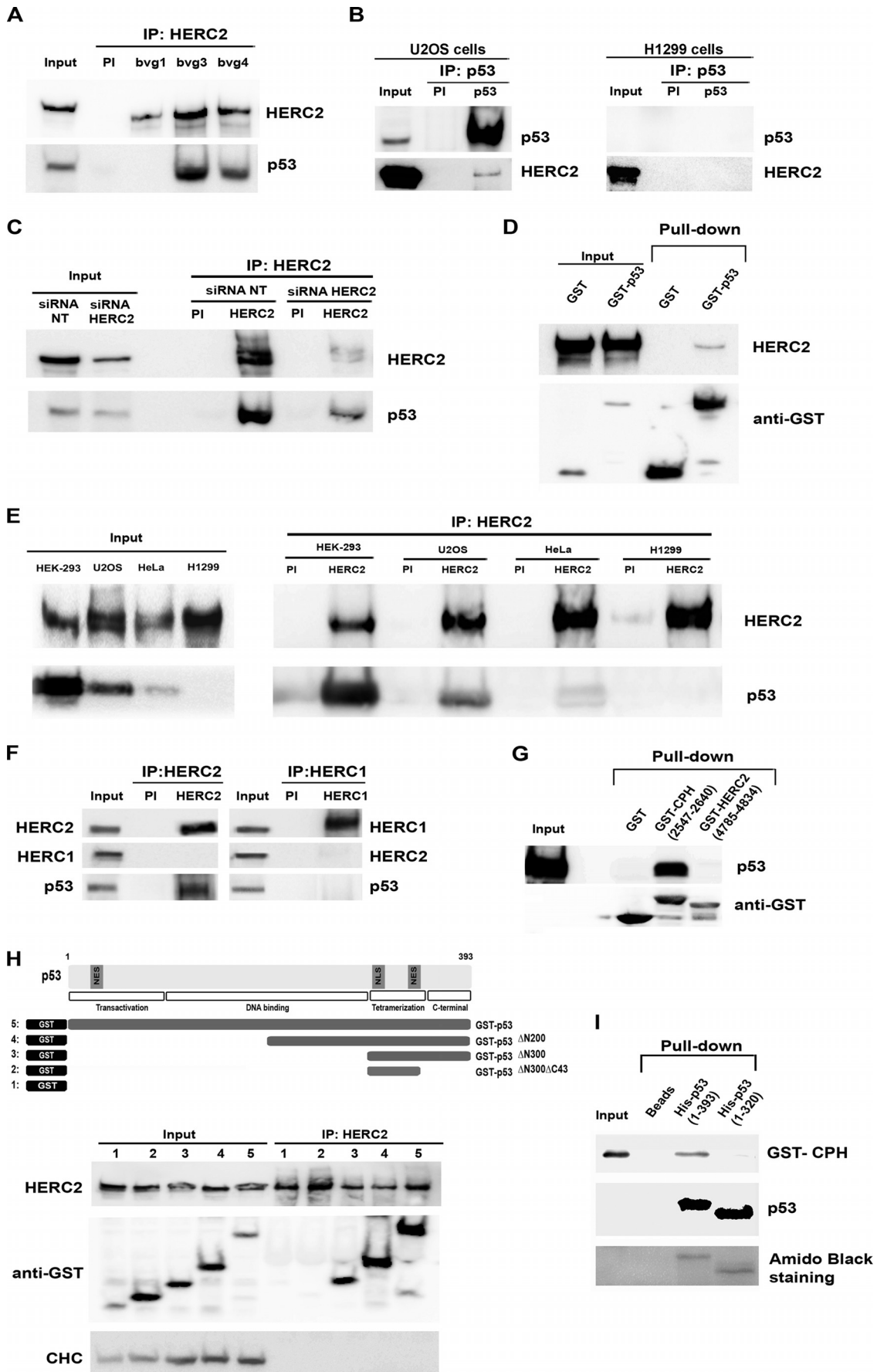
**FIGURE 1. Characterization of the HERC2 protein.** *A*, structure of the HERC2 protein. The RCC1-like domains 1–3 (*RLD1*–3) and the CPH and HECT domains are indicated. Fusion proteins against the indicated amino acid residues were purified and used to generate anti-HERC2 antibodies. Regarding the sensitivity of antibodies, different concentrations of affinity-purified antibodies were tested on slot blots containing 100 ng of fusion proteins or BSA as a negative control. *B*, specificity of anti-HERC2 antibodies. Lysates of HEK-293 were analyzed by immunoblotting (IB) with the indicated antibodies. *C*, anti-HERC2 polyclonal antibodies (bvg1–4) were tested in IP assays with lysates (*Input*) of HEK-293 cells, followed by immunoblotting with anti-HERC2 monoclonal antibody. *PI*, preimmune serum. *D*, lysates from transfected HEK-293 cells with the indicated siRNAs were analyzed by immunoblotting with antibodies against the indicated proteins. *mTOR*, mammalian target of rapamycin. *E*, HEK-293 cell lysates (*Input*) were immunoprecipitated with the indicated antibodies and analyzed by immunoblotting with antibodies against the indicated proteins. Input represents 5% of the extract used.

tein. It has been shown previously that small HERC proteins can form heteromeric complexes (17). To determine whether the giant proteins HERC2 and HERC1 can also form heteromeric complexes, we performed coimmunoprecipitation experiments. Fig. 1*E* shows that HERC2 and HERC1 did not form such complexes.

Following identification of the endogenous HERC2 protein, we used anti-HERC2 antibodies to check the interaction between HERC2 and p53. Immunoprecipitation experiments with two different antibodies against HERC2 (bvg3 and bvg4) showed the coimmunoprecipitation of endogenous p53 with HERC2 in U2OS cells, a human osteosarcoma cell line expressing wild-type p53 (Fig. 2*A*). Reverse immunoprecipitation using anti-p53 antibodies also showed, although in a lesser amount, the coimmunoprecipitation of HERC2 in U2OS cells but not in H1299 human cells, which do not express p53 (Fig. 2*B*). To

discard the theory that anti-HERC2 antibodies cross-react with p53, immunoprecipitations were performed in U2OS cells transfected with HERC2 siRNA. NT siRNA was used as negative control. As shown in Fig. 2*C*, the amount of p53 in the immunoprecipitates decreased when HERC2 was knocked down, indicating that there is no cross-reactivity. In addition, the HERC2–p53 interaction was also confirmed with pull-down experiments using glutathione beads in lysates from H1299 cells transfected with plasmids expressing GST or GST–p53. As shown in Fig. 2*D*, GST–p53 specifically interacts with HERC2. This interaction was also observed in other human cell lines expressing wild-type p53, such as HEK-293 and HeLa (Fig. 2*E*). As expected, this interaction was not observed in H1299 cells (Fig. 2*E*). In mouse tissues such as brain or kidney we also observed the coimmunoprecipitation of p53 with HERC2 (Fig. 2*F* and results not shown). Anti-HERC1 antibodies could not

# HERC2 Regulates p53 Oligomerization



coimmunoprecipitate p53, indicating the specificity of the HERC2-p53 interaction (Fig. 2F).

CUL7, PARC, and HERC2 share a CPH domain (6, 7). Because the CPH domain of CUL7 and PARC is sufficient for p53 binding (6–8), we tested whether the CPH domain of HERC2 was also sufficient to mediate the interaction with p53. We performed pulldown assays with GST or GST fusion constructs of HERC2 purified from bacteria (GST-CPH (residues 2547–2640) and GST-HERC2 (carboxyl-terminal residues 4785–4834)) and lysates from HEK-293 cells. Fig. 2G shows that the CPH domain of HERC2 was sufficient for this interaction. No interaction was observed with GST-HERC2 (residues 4785–4834) nor with the negative control GST.

To map the HERC2-binding domain of p53, we expressed a series of GST-p53 deletion mutants in H1299 cells (6). Anti-HERC2 antibodies coimmunoprecipitated all the GST-p53 fusion proteins expressed except the GST-p53<sup>ΔN300ΔC43</sup> fusion protein (Fig. 2H), indicating that the last 43 amino acid residues of p53 are essential for the interaction with HERC2. Similar results were obtained using HEK-293 cells.

To analyze whether HERC2 can directly interact with p53, we purified His-p53 (wild-type, residues 1–393), deleted His-p53 (residues 1–320), and the GST-CPH fusion protein from bacteria. With these purified protein, we observed a direct and specific interaction between His-p53 (wild-type) and GST-CPH (Fig. 2I).

*HERC2 Regulates the Transcriptional Activity of p53*—HERC2 belongs to the E3 ubiquitin ligase family (12). To determine whether HERC2 regulates p53 levels in a similar manner to other E3 ubiquitin ligases such as MDM2, we depleted U2OS cells of HERC2 using interference RNA experiments. Cells were transfected independently with two different specific siRNAs of HERC2 (H2.2 or H2.4) or with NT siRNA as a negative control. HERC2 knockdown did not significantly modify p53 protein levels (Fig. 3A). The content of other proteins, such as Ras-related nuclear protein (Ran), was not altered either.

p53 functions as a transcriptional factor regulating gene expression. One of the genes most studied is the cell cycle inhibitor *p21* (3). In this context, we analyzed p21 levels by immunoblotting, observing a great decrease after HERC2 depletion (Fig. 3B). To rule out the possibility that a posttranslational mechanism could be involved in the regulation of p21 levels by HERC2, experiments were performed using the translational inhibitor cycloheximide. Time course experiments with cycloheximide after siRNA transfection showed that the half-life of p21 was not modified significantly by HERC2 depletion (Fig. 3C), suggesting that HERC2 could be involved in the transcrip-

tional regulation of p21 by p53. To show that a transcriptional mechanism could be involved in the regulation of p21 by HERC2, luciferase reporter assays were performed in U2OS cells transfected with the *p21* promoter. We observed a great decrease in *p21* promoter activity after HERC2 depletion in U2OS cells (Fig. 3D). Endogenous p21 mRNA levels were also decreased by HERC2 knockdown (Fig. 3E). Similar decreases were observed using p53 siRNA in U2OS cells (Fig. 3, D and E). No variations were observed in p53-null H1299 cells (Fig. 3, D and E). These results were also confirmed with other genes regulated by p53, such as *p53R2* or *p53AIP1*. Thus, the promoter activity of *p53R2* or *p53AIP1* was reduced significantly after HERC2 depletion (Fig. 3F).

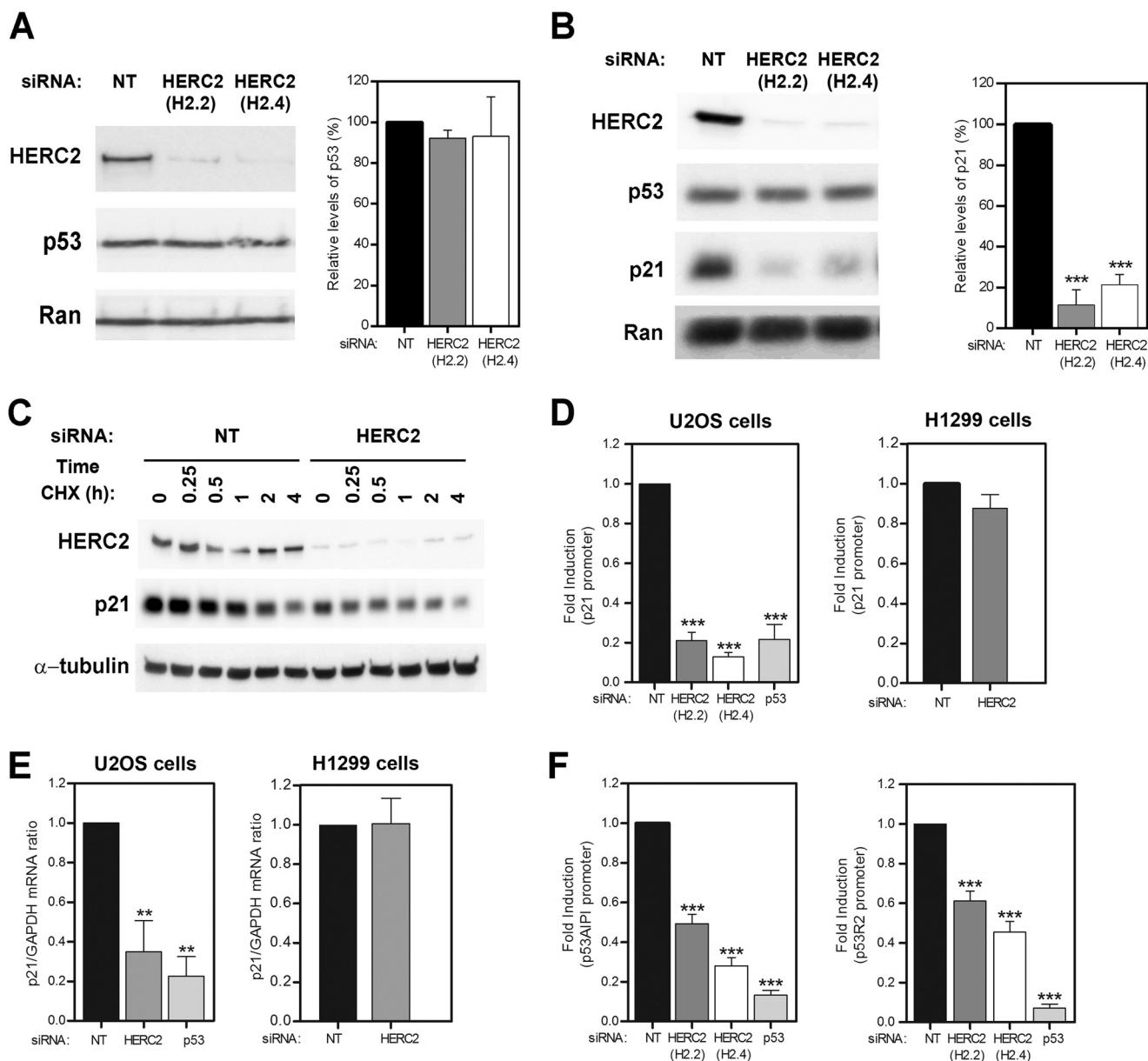
Cellular processes such as growth arrest, apoptosis, and DNA damage responses are regulated by p53. The above results demonstrate that HERC2 regulates the transcriptional activity of p53. We wondered whether HERC2 could also regulate cellular processes. To address this question, we analyzed the growth of U2OS cells. We observed a significant increase in growth after HERC2 depletion (Fig. 4A). These results were confirmed via a clonogenicity assay. Thus, after 15 days, the HERC2 knockdown tripled the colony number (Fig. 4B). Similar increases were observed using p53 siRNA in U2OS cells (Fig. 4, A and B). No variations were observed in H1299 cells (Fig. 4, A and B).

*HERC2 Regulates p53 Activity in a Proteasome- and MDM2-independent Manner*—p53 levels are controlled by E3 ubiquitin ligases that target p53 for proteasomal degradation. It is well known that the inhibition of proteasome activity increases p53 levels. We wondered whether the regulation of p53 activity by HERC2 was dependent on the proteasome. To this end, we first confirmed the increase in p53 levels in the presence of the proteasome inhibitor MG132 in U2OS cells transfected with control siRNA (Fig. 5A, *first* and *fourth* lanes). This increase correlated with the increase in p21. Knockdown of HERC2 did not affect p53 levels in the presence of MG132 (Fig. 5A). However, p21 levels were reduced to a similar extent in the absence of MG132, suggesting that the inhibition of p53 activity by HERC2 was independent of proteasome activity.

It has been reported that inhibition of MDM2 activity with inhibitors such as nutlin increases p53 signaling (45). We wondered whether MDM2 activity could be involved in the regulation of p53 activity by HERC2. Immunoblotting experiments in U2OS cells showed that the inhibition of MDM2 activity in the presence of nutlin increased MDM2 levels (Fig. 5B, *first* and *fourth* lanes). Under these conditions, the levels of p53 were barely affected. In contrast, p21 levels were increased greatly in the presence of nutlin, suggesting an increase in the transcrip-

FIGURE 2. **HERC2 binds to p53.** Lysates from U2OS (A–C and E), H1299 (B and H), HEK-293 (E and G), and HeLa (E) cells or from mouse brain (F) were subjected to IP with preimmune serum (PI) or with the indicated antibodies and analyzed by immunoblotting with antibodies against the indicated proteins. C, lysates from transfected U2OS cells with the indicated siRNAs were subjected to immunoprecipitation and analyzed by immunoblotting as above. D, pulldown experiments with H1299 cells transfected with GST or GST-p53 constructs. 48h post-transfection, lysates from these cells were incubated with glutathione-Sepharose, and proteins retained on Sepharose were analyzed by immunoblotting. F, lysates from mouse brain were immunoprecipitated with antibodies against HERC2 (bvg3) or HERC1 (410) and analyzed by immunoblotting with antibodies against the indicated proteins. G, pulldown experiments with purified GST and GST fusion proteins coupled to glutathione-Sepharose in lysates from HEK-293 cells. Proteins retained on Sepharose were analyzed by immunoblotting with antibodies against the indicated proteins. H, H1299 cells transfected with the indicated constructs were immunoprecipitated with anti-HERC2 polyclonal antibody and analyzed by immunoblotting with antibodies against the indicated proteins. CHC, clathrin heavy chain. I, direct interaction. Beads with purified proteins (His-p53 (wild-type, residues 1–393) or deleted His-p53 (residues 1–320)) were incubated with purified GST-CPH (residues 2547–2640). Interacting proteins were pulled down and analyzed by immunoblotting or staining with Amido Black. In all experiments, input represents 5% of the extract used.

## HERC2 Regulates p53 Oligomerization

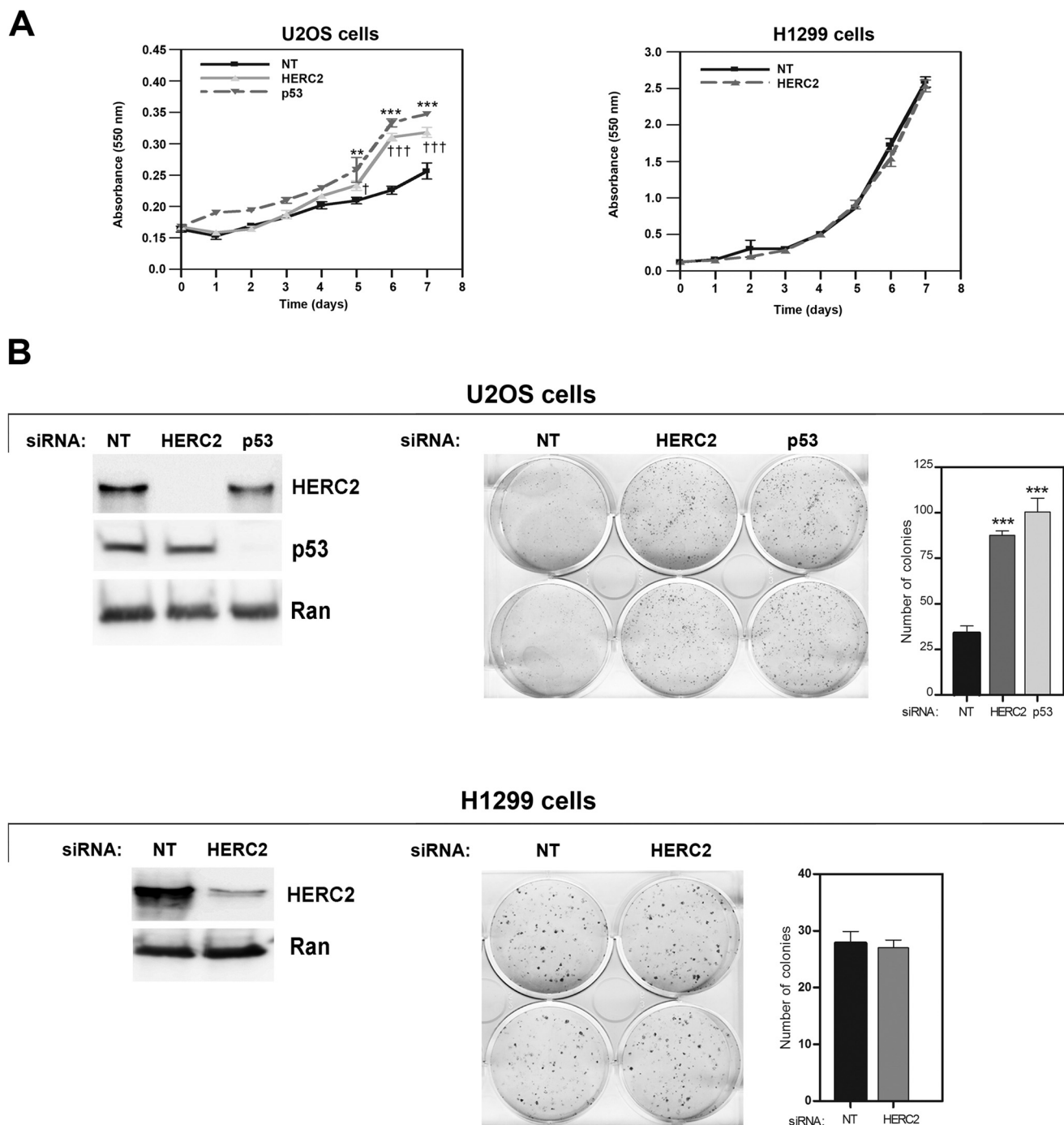


**FIGURE 3. HERC2 is required for transcriptional activity of p53.** *A* and *B*, lysates from transfected U2OS cells with NT or HERC2 siRNAs were analyzed by immunoblotting with antibodies against the indicated proteins. Levels of p53 or p21 were quantified and normalized with respect to Ran levels. *C*, similar to the above, but cells were treated with cycloheximide (CHX) for the indicated times. *D*, U2OS or H1299 cells were transfected with the p21 promoter (p21WAF1) and the indicated siRNAs. The luciferase activity was quantified as indicated under "Experimental Procedures." (E) RT quantitative PCR analysis was performed in U2OS or H1299 cells to quantify gene expression of p21. The levels of expression were normalized with respect to GAPDH gene expression. (F) U2OS cells were transfected with the promoter of p53R2 or p53AIP1 and the indicated siRNAs. The luciferase activity was quantified as indicated under "Experimental Procedures." Data are expressed as mean  $\pm$  S.E. Statistical analysis was carried out as described under "Experimental Procedures." The differences are shown with respect to NT siRNA. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

tional activity of p53. In these conditions, knockdown of HERC2 decreased p21 levels to a similar extent as in the absence of nutlin (Fig. 5B), suggesting that the inhibition of p53 activity by HERC2 was independent of MDM2 activity. This suggestion was confirmed by knockdown experiments of MDM2. Depletion of MDM2 slightly increased p53 and p21 levels (Fig. 5C, first and third lanes). Under these conditions, HERC2 depletion decreased p21 levels independently of MDM2 (Fig. 5C, second and fourth lanes). Because endogenous levels of MDM2 were very low in U2OS cells, we repeated all of these experiments in the presence of the proteasome inhibitor MG132. Under these conditions, we obtained similar results for

p53 signaling, and higher levels of MDM2 were detected (Fig. 5C, fifth through eighth lanes).

**HERC2 Interacts with p53 in Cytoplasmic and Nuclear Fractions**—In stressed cells, p53 activation generally consists of three sequential activating steps: stress-induced stabilization mediated by phosphorylation, DNA binding, and recruitment of the general transcriptional machinery (2–4). We wondered whether HERC2 could be involved in the regulation of these steps. To this end, we investigated whether the phosphorylation of p53 could be affected by HERC2 depletion. To activate p53 phosphorylation, we used bleomycin, a radiomimetic chemical used as a chemotherapeutic agent in cancer treat-



**FIGURE 4. HERC2 regulates cellular growth.** *A*, U2OS or H1299 cells were transfected with the indicated siRNAs, and cell proliferation was analyzed as indicated under "Experimental Procedures." *B*, colony formation assays were performed in U2OS or H1299 cells as indicated under "Experimental Procedures." The protein levels at 72 h post-transfection are shown in the *left panel*, and the quantification of the number of colonies is shown in the *right panel*. Data are expressed as mean  $\pm$  S.E. Statistical analysis was carried out as described under "Experimental Procedures." The differences are shown with respect to NT siRNA. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; T,  $p < 0.05$ ; TTT,  $p < 0.001$ .

ment. Bleomycin interacts with DNA to directly produce double strand breaks (46, 47). As expected, bleomycin activated p53 phosphorylation on serine 15 (*P-S15-p53*) in U2OS cells transfected with non-targeting siRNA (Fig. 6A). This phosphorylation was clearly detected after 1 h of treatment and increased for at least the next 6 h (Fig. 6A). During this time, the p53 level also increased (stabilization), but not that of other proteins tested, such as Ran (negative control). Under these conditions, down-regulation of HERC2 did not prevent the phosphoryla-

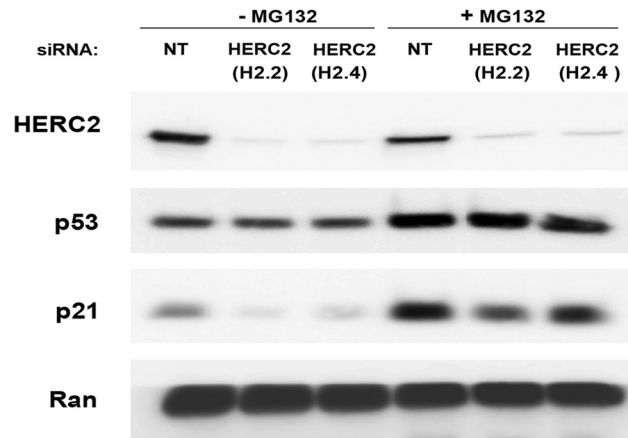
tion and stabilization of p53 (Fig. 6A). Thus, the phospho-Ser-15-p53/p53 ratio was similar in cells transfected with NT or HERC2 siRNAs, suggesting that HERC2 does not regulate this step.

To bind DNA and activate the transcriptional machinery, p53 is translocated from the cytoplasm to the nucleus. Because proteins such as PARC and CUL7 have been reported to be involved in this process (6–8), we tested whether HERC2 also regulates the subcellular localization of p53. Cytoplasmic and

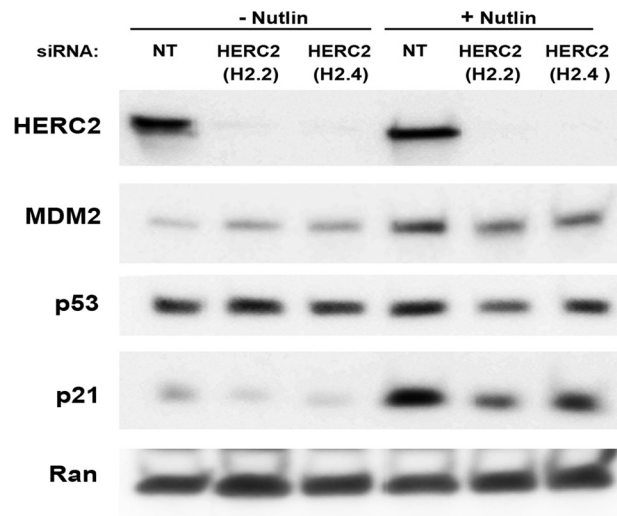


## HERC2 Regulates p53 Oligomerization

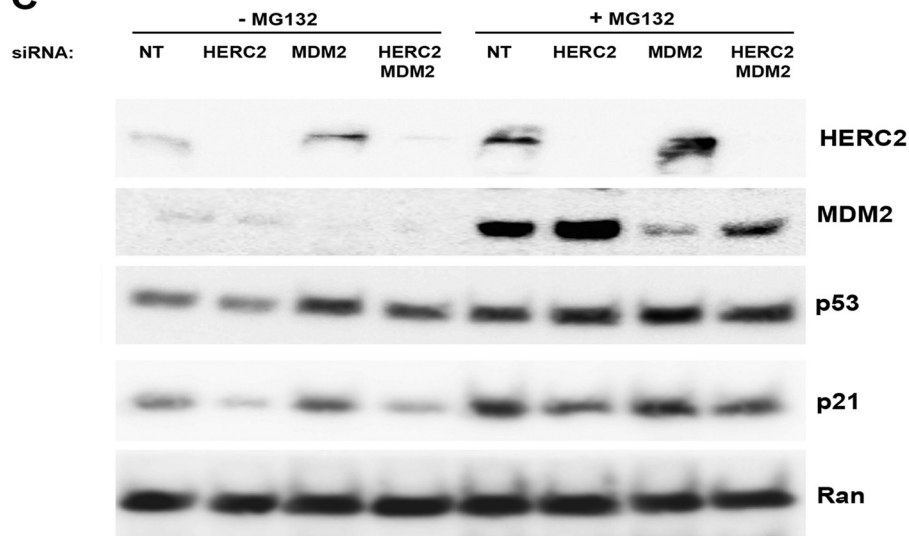
**A**



**B**



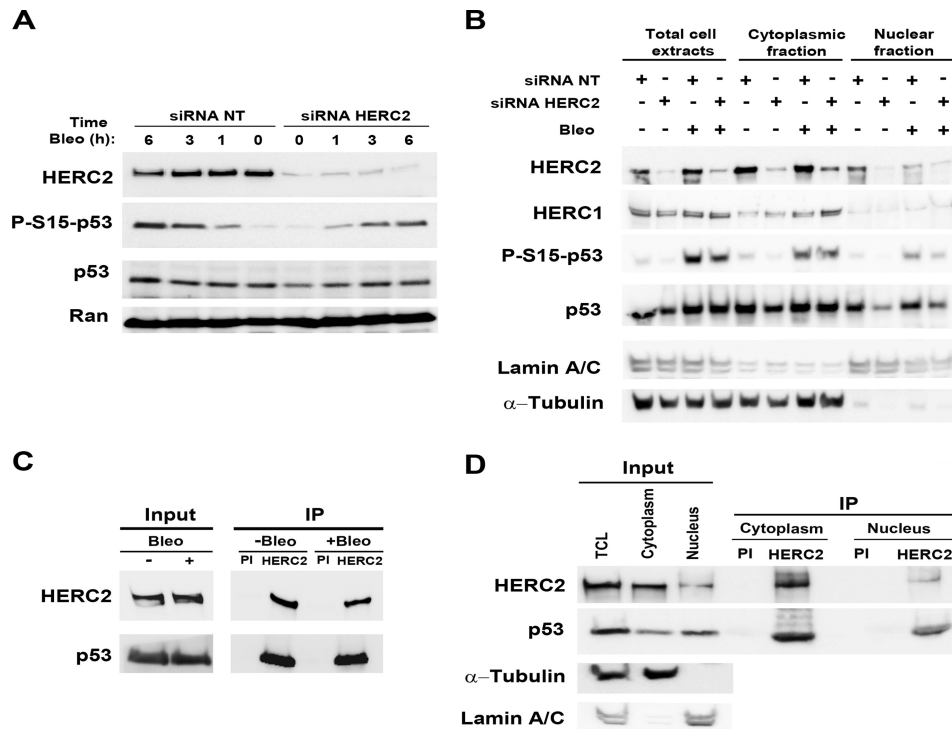
**C**



**FIGURE 5. HERC2 regulates p53 activity in a proteasome- and MDM2-independent manner.** Lysates from transfected U2OS cells with NT or HERC2 siRNAs were analyzed by immunoblotting with antibodies against the indicated proteins. Before lysis, cells were treated with the proteasome inhibitor MG132 (A and C) or with the MDM2 inhibitor Nutlin 3a (B) for 6 and 16 h, respectively. Data are representative of at least three independent experiments.

nuclear fractions were separated and analyzed in the presence or absence of bleomycin. Interestingly, HERC2 was present in both fractions, whereas the homolog HERC1 was restricted to

the cytoplasmic fraction (Fig. 6B). Phosphorylation and stabilization of p53 and its nuclear translocation were observed after treatment with bleomycin (Fig. 6B). p53 translocation was not



**FIGURE 6. HERC2 interacts with p53 in cytoplasmic and nuclear fractions.** *A* and *B*, U2OS cells transfected for 72 h with NT or HERC2 siRNA were treated with bleomycin (*Bleo*) for the indicated times. Lysates were analyzed by immunoblotting with antibodies against the indicated proteins. *B*, transfected U2OS cells were treated with bleomycin for 3 h. Lysates were subjected to subcellular fractionation as indicated under “Experimental Procedures” and analyzed by immunoblotting with antibodies against the indicated proteins. *C*, U2OS cells were treated with bleomycin for 3 h, and lysates were immunoprecipitated (*IP*) with preimmune serum (*PI*) or with anti-HERC2 polyclonal antibodies and analyzed by immunoblotting with antibodies against the indicated proteins. Lysates from non-treated cells were used as a control. *D*, lysates from U2OS cells were fractionated as indicated under “Experimental Procedures.” The fractions corresponding to the cytoplasm and nucleus were subjected to immunoprecipitation as in *C*.  $\alpha$ -Tubulin and Lamin A/C were used as controls of subcellular fractionation. Input represents 5% of the extract used. *TCL*, total cell lysates.

impaired by HERC2 depletion. Lamin A/C and  $\alpha$ -tubulin are shown as controls of the nuclear and cytoplasmic fractions, respectively (Fig. 6*B*). Furthermore, HERC2-p53 interaction was maintained after treatment with bleomycin (Fig. 6*C*).

Because HERC2 and p53 are present in the cytoplasmic and nuclear fractions, we investigated whether their interaction was maintained in both fractions. Coimmunoprecipitation studies with HERC antibodies showed that the HERC2-p53 interaction occurs in both fractions (Fig. 6*D*).

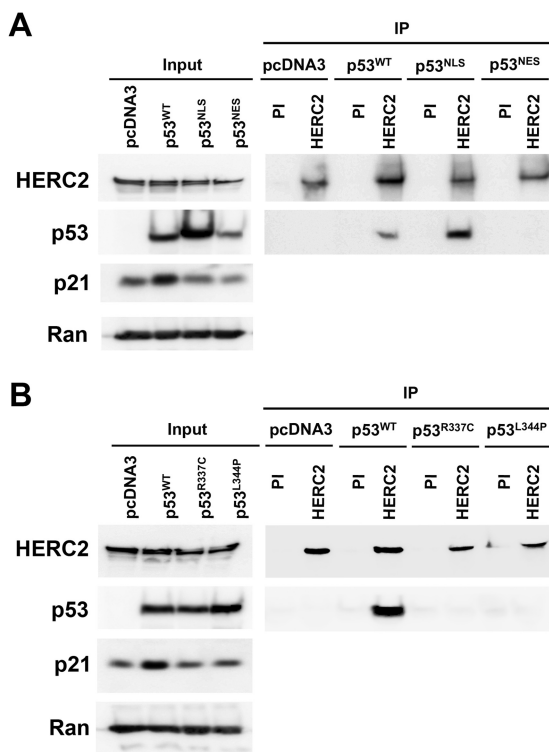
*Mutations That Affect the Tetramerization Domain of p53 Disrupt the Interaction with HERC2*—We observed that the deletion of the last 43 amino acid residues of p53 (residues 350–393) impaired its interaction with HERC2 (Fig. 2*H*). Because this domain contains part of the tetramerization domain of p53 (3), we wondered whether this p53 domain was required for the HERC2-p53 interaction. To this end, we performed coimmunoprecipitation experiments with anti-HERC2 antibodies in p53-null human H1299 lung cancer cells. H1299 cells were transfected with different mutants of p53. Cells transfected with wild-type p53 were used as controls. First, we expressed the p53<sup>NLS</sup> and p53<sup>NES</sup> mutants, which fail in nuclear import or nuclear export activities, respectively (38). The p53<sup>NLS</sup> mutant confined to the cytoplasm is comparable with the wild-type p53 because it maintains its capacity for oligomerization and can be acetylated. In contrast, the p53<sup>NES</sup> mutant confined to the nucleus fails to oligomerize because the C-terminal NES overlaps with the tetramerization domain (38).

As shown in Fig. 7*A*, we observed that the HERC2-p53 interaction is disrupted when the mutant p53<sup>NES</sup> is expressed, suggesting that an intact tetramerization domain of p53 is required for its interaction with HERC2.

To confirm this observation, we used two p53 mutants in which the tetramerization domain was affected. These mutants were p53<sup>R337C</sup> and p53<sup>L344P</sup>. Individuals with these mutations suffer from Li-Fraumeni syndrome (38). Plasmids containing these mutations were transfected into H1299 cells, and the p53 mutants that were expressed were analyzed. We found that neither mutant was able to associate with HERC2 (Fig. 7*B*), confirming the previous observation that an intact oligomerization domain of p53 is required for its interaction with HERC2. As expected, p21 levels were induced by wild-type p53 expression but not by the expression of p53 mutants (Fig. 7).

*HERC2 Regulates p53 Oligomerization*—Our data show that HERC2 interacts with p53 and regulates its transcriptional activity. Because this interaction is mediated by an intact oligomerization domain in p53 and because p53 oligomerization has been reported as an essential step for its transcriptional activity (48), we wondered whether HERC2 could regulate p53 oligomerization. To answer this question, we studied p53 oligomerization using a protein cross-linking assay (38). H1299 cells were transfected with wild-type p53 together with siRNAs and analyzed 72 h later. Cell lysates were isolated, treated with increasing amounts of glutaraldehyde, and analyzed by SDS-PAGE and immunoblotting. As shown in Fig. 8*A* (left panel),

## HERC2 Regulates p53 Oligomerization



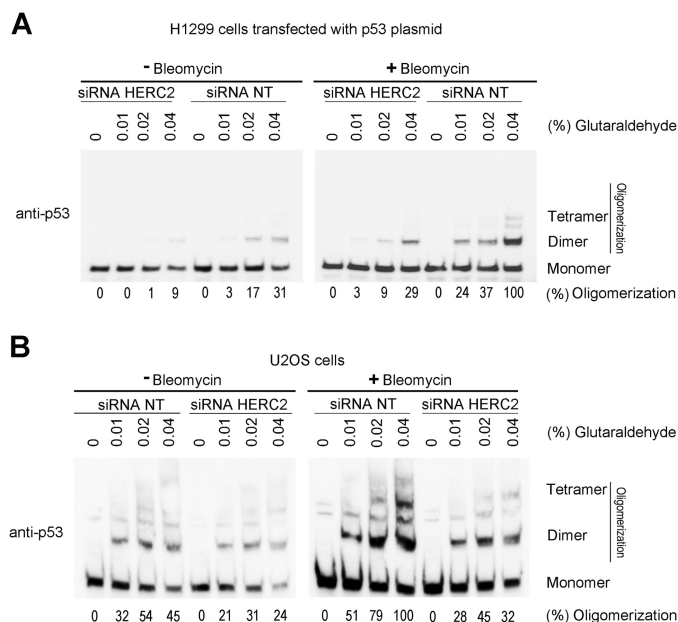
**FIGURE 7. The tetramerization domain of p53 is required for the HERC2-p53 interaction.** H1299 cells were transfected with the indicated plasmids for 48 h. Lysates were then subjected to immunoprecipitation with anti-HERC2 polyclonal antibody and analyzed by immunoblotting with antibodies against the indicated proteins. *A*, p53 mutants that fail in nuclear import ( $p53^{NLS}$ ) or nuclear export ( $p53^{NE5}$ ). *PI*, preimmune serum. *B*, p53 mutants ( $p53^{R337C}$  and  $p53^{L344P}$ ) associated with Li-Fraumeni syndrome. *pcDNA3*, negative control of transfection;  $p53^{WT}$ , p53 wild-type. Data are representative of at least three independent experiments. Input represents 5% of the extract used.

monomers, dimers, and tetramers of p53 were detected with anti-p53 antibody. Under these conditions, HERC2 depletion inhibited p53 oligomerization.

Next, we hypothesized that this inhibition would be more obvious in cells with activated p53. Given that in the previous experiments we showed that bleomycin stimulated p53 activity, we used the same drug. Thus, in H1299 cells transfected with wild-type p53, we observed the stimulation of p53 oligomerization by bleomycin and its inhibition by the knockdown of HERC2 (Fig. 8*A*, right panel). All of these results were confirmed in another human cell line with endogenous p53. In U2OS cells, HERC2 depletion inhibited p53 oligomerization (Fig. 8*B*). These results could indicate that HERC2 is necessary to maintain p53 oligomerization. We wondered whether HERC2 could also promote p53 oligomerization. To this end, H1299 cells were transfected with wild-type p53 and with a HERC2 construct (Myc-HERC2 F3) expressing residues 2292–2923, which include the CPH domain (39). We observed a great stimulation of p53 oligomerization by the expression of Myc-HERC2 F3 (Fig. 9, *A* and *B*). Luciferase reporter assays showed that this stimulation correlated with a significant increase of its transcriptional activity (Fig. 9*C*).

## DISCUSSION

This study identified HERC2 as a protein that binds p53, thus regulating cellular events mediated by p53. HERC2 interacts



**FIGURE 8. HERC2 regulates p53 oligomerization.** *A*, H1299 cells transfected with the wild-type p53 plasmid and the indicated siRNAs for 72 h were treated with bleomycin for 3 h. Lysates were incubated on ice with glutaraldehyde at the indicated concentrations (percent) for 30 min. Samples were analyzed by immunoblotting with anti-p53 antibody. Monomers, dimers, and tetramers of p53 are indicated. The percentage of oligomerization was calculated with dimers and tetramers. The condition with the higher levels of oligomers was considered as 100%. *B*, oligomerization of endogenous p53. U2OS cells were analyzed as in *A*. Data are representative of at least three independent experiments.

with p53 through its CPH domain. Consistent with this, HERC1, a structural homolog of HERC2 that does not contain a CPH domain, did not interact with p53. Moreover, and in contrast to what has been described for smaller members of the HERC family (17), the largest members, HERC1 and HERC2, did not form heteromers. The HERC2-binding domain of p53 was located in its carboxyl terminus, similar to other p53-binding proteins with CPH domains, such as PARC and CUL7 (6–8). Structural studies of the CPH domain of PARC and CUL7 indicated that this domain interacts with the tetramerization domain of p53 (residues 310–360) (49). In agreement with these data, deletion of the last 43 amino acid residues of p53 (residues 350–393) impaired the interaction with HERC2 (Fig. 2*H*), suggesting that an intact tetramerization domain in p53 is required for this interaction. These results led us to check whether HERC2 could interact with oligomerized p53. Using p53 mutants, we found that HERC2 does not interact with p53 mutants defective in oligomerization (Fig. 7). These observations could explain the large amount of p53 in HERC2 immunoprecipitates (Fig. 2). Interestingly, we demonstrated that HERC2 regulates the transcriptional activity of p53 (Figs. 3 and 9). Thus, we show that, in the absence of HERC2, the transcriptional activity of p53 decreases in unstressed cells. These observations were also confirmed in stressed cells. Interestingly, after genotoxic stress induced with bleomycin, the interaction between HERC2 and p53 was maintained.

To shed light on the molecular mechanism involved in the regulation of transcriptional activity of p53 by HERC2, we showed that, in the absence of HERC2, phosphorylation and

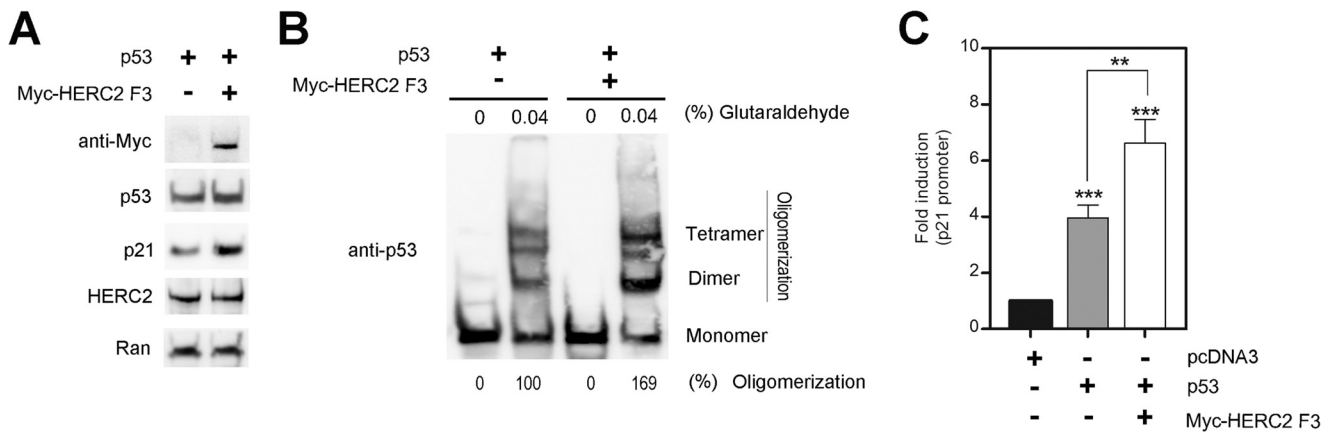


FIGURE 9. **HERC2 promotes p53 oligomerization.** H1299 cells were transfected with wild-type p53 and Myc-HERC2 F3 (residues 2292–2923) constructs and analyzed by immunoblotting with antibodies against the indicated proteins (A) or treated with glutaraldehyde at the indicated concentrations (percent) for 30 min to analyze p53 oligomerization as in Fig. 8 (B). C, H1299 cells were transfected with the *p21* promoter (*p21WAF1*) and the indicated plasmids. The luciferase activity was quantified as indicated under "Experimental Procedures." Data are expressed as mean  $\pm$  S.E. Statistical analysis was carried out as described under "Experimental Procedures." \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

nuclear translocation of p53 were not impaired. Bearing the above data in mind, and also bearing in mind that p53 acetylation is essential for p53 activation (50) and that p53 oligomerization is essential for the carboxyl terminal lysine acetylation of p53 (38), we were finally able to show that HERC2 regulates p53 oligomerization. All these results led us to propose the next working model. HERC2 interacts with oligomerized p53 and regulates its oligomerization. In response to cellular stress, such as DNA damage produced by bleomycin, p53 oligomerization is stimulated, and p53 is phosphorylated and translocated to the nucleus to activate transcription of its specific gene targets, such as *p21*. Under these conditions, p53 oligomerization is regulated by HERC2. This model is consistent with published data showing that p53 oligomerization is sufficient to activate p53 transcriptional targets (51), but it does not explain why bleomycin does not increase HERC2-p53 interaction (Fig. 6C). Although a possible explanation could be that initially all HERC2 is bound to oligomerized p53, more studies will be necessary to shed light on these steps.

HERC2 belongs to the E3 ubiquitin ligase family. E3 ubiquitin ligases have been classified into two main types: HECT and RING (12, 13, 52). HERC2 belongs to the HECT family and PARC and CUL7 to the RING family. p53 levels are regulated by ubiquitin-dependent proteasomal degradation. E6AP and MDM2 are E3 ubiquitin ligases of the HECT and RING families, respectively, which bind and ubiquitinate p53, thus regulating its levels (12, 53). However, this does not seem to be the case for CPH domain-containing E3 ubiquitin ligases. Knock-down of PARC, CUL7, or HERC2 proteins did not increase p53 levels (Refs. 7, 8) and our data). In fact, substrates other than p53 have been reported for these proteins. For example, Cyclin D1 and insulin receptor substrate 1 (IRS-1) are targeted by CUL7 E3 ubiquitin ligase complex for ubiquitin-dependent degradation (9, 54). For HERC2, several substrates have been identified in recent years. Thus, it has been reported that HERC2 ubiquitinates xeroderma pigmentosum A protein, thus promoting its proteasomal degradation (34). Xeroderma pigmentosum A is a limiting factor in the mechanism of DNA repair known as nucleotide excision repair. HERC2 had been

implicated previously in the machinery of DNA repair in response to ionizing radiation (32). Interestingly, BRCA1, a protein that also participates in this repair mechanism, has also been reported to be a substrate of HERC2 (35). In this context, down-regulation of HERC2 improved the activation of the nucleotide excision repair mechanism by the chemotherapeutic drug cisplatin (34). Cisplatin produces intra- and interstrand DNA diadducts that are repaired by nucleotide excision repair (55). Bleomycin and ionizing radiation induce double strand DNA breaks that are mainly repaired by homologous recombination and non-homologous end joining machineries (47). Cisplatin and bleomycin are potent anticancer agents used in the chemotherapy of various types of cancer. For example, cisplatin is the drug of choice in testicular and ovarian cancers and a main component of combination therapy regimens for many other cancers, including head and neck, lung, gastric, and colorectal cancers (55). Bleomycin is given in testicular cancer, lymphoma, and cancers of the head and neck (56–58). Knowing the molecular mechanism involved in the action of these drugs will lead to a better understanding of their effects on tumor cells, which will, in turn, allow the design of more efficient trials (combination therapy) as well as the development of specific inhibitors against regulatory proteins that increase the efficiency of these drugs. Because HERC2 regulates both DNA repair mechanisms (32, 34), it seems plausible that specific inhibitors of this protein might increase the cytotoxic action of cisplatin or bleomycin. However, one must bear in mind that HERC2 could also function as a tumor suppressor protein and that inhibition of its activity would cause a decrease in p53 activity.

p53 mutations that affect its oligomerization have been associated with Li-Fraumeni syndrome and Li-Fraumeni-like syndromes (38, 59, 60). In these cases, a decrease in p53 activity has been observed. This study shows the regulation of p53 oligomerization by HERC2 and suggests that HERC2 mutations that affect p53 oligomerization could also be associated with these syndromes. In addition to this putative role in cancer, HERC2 has also been associated with neurological disorders. Thus, a HERC2 mutation associated with a neurodevelopmental delay

## HERC2 Regulates p53 Oligomerization

similar to Angelman syndrome has been reported (28, 29). In this case, the HERC2 mutation was associated with a decrease in E6AP (UBE3A) activity (28, 37). In summary, although more remains to be learned about HERC2 biology and its role in human diseases, our results establish HERC2 protein as an important regulator of p53 signaling.

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