Identification of an Aberrantly Spliced Form of HDMX in Human Tumors: A New Mechanism for HDM2 Stabilization

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Abstract

The HDMX protein is closely related to HDM2 with which it shares different structural domains, particularly the p53 binding domain and the ring finger domain, where the two HDM proteins interact. Several oncogenic forms derived from splicing of HDM2 have been described in cancer. This work aimed at investigating whether analogous forms of HDMX exist in human tumors. Here, we report the characterization of an aberrantly spliced form of HDMX, HDMX211, isolated from the thyroid tumor cell line, ARO. HDMX211 binds and stabilizes the HDM2 protein. Although it lacks the p53 binding domain, HDMX211 also stabilizes p53 by counteracting its degradation by HDM2. However, the resulting p53 is transcriptionally inactive and increasingly associated to its inhibitor HDM2. Expression of HDMX211 strongly enhances the colony-forming ability of human cells in the presence or absence of wild-type p53. Conversely, depletion of HDMX211 by small interfering RNA significantly reduces the growth of ARO cells and increases their sensitivity to chemotherapy. Screening of lung cancer biopsies shows the presence of HDMX211 in samples that overexpress HDM2 protein, supporting a pathologic role for this new protein. This is the first evidence of a variant form of HDMX that has oncogenic potential independently of p53. HDMX211 reveals a new mechanism for overexpression of the oncoprotein HDM2. Most interestingly, it outlines a possible molecular explanation for a yet unclarified tumor phenotype, characterized by simultaneous overexpression of HDM2 and wild-type p53. (Cancer Res 2005; 65(21): 9687-94)

Introduction

The MDM2 (and its human analogue HDM2) protein has been mainly characterized as a p53-binding protein able to bring this oncosuppressor to degradation and to inhibit its transcriptional function masking the p53 activation domain (1–3). In agreement with this function, *HDM2* gene is amplified in a large cohort of human tumors (about 7% in a survey of 28 tumor types) and specifically in certain histotypes (osteosarcomas and soft tissue tumors, 20% and 16%, respectively; ref. 4). In addition, HDM2 levels can be up-regulated independently of gene amplification (5–10). Particularly, enhanced HDM2 protein levels have been reported in thyroid (43%) and lung tumors (45%; ref. 11). However, the molecular mechanisms underlying such alterations are not completely characterized. Besides full-length mRNA, more than 40 different splice variants of *HDM2* transcripts have been detected in tumors and normal tissues, and tumorigenicity of some of these variants has been assessed (12). Interestingly, many variants show a p53-independent function as they have lost, at least in part, the p53-binding domain. Accordingly, the oncogenic potential of full-length MDM2 has been assessed in the absence of p53: Different mouse models overexpressing a transgenic MDM2 develop tumors in a $p53^{+/+}$ as well as in a $p53^{-/-}$ background (13, 14).

MDMX (also named MDM4) was discovered in 1996 and its similitude with MDM2 evidenced (15–17). The proteins share an NH_2-terminal p53 binding domain and a COOH-terminal ring finger domain. The latter mediates the interaction of the two MDM proteins. In analogy to HDM2, human MDMX (HDMX) is overexpressed in some human tumors (18, 19). Ramos et al. (20) have described HDMX forms with higher mobility in SDS-PAGE in different tumor cell lines. Additionally, an *HDMX* alternatively spliced form was isolated from normal and tumor cell lines (21). These data suggest that, in analogy to HDM2, HDMX may undergo differential splicing.

To assess whether *HDMX* spliced variants exist in human tumors, we analyzed a panel of cell lines derived from thyroid tumors. From one of these (ARO cell line), we isolated an mRNA derived from an aberrant splicing event that generates a protein, named HDMX211, which contains the first 26 amino acids and the entire COOH terminus of the full-length HDMX. As a consequence, this form lost the ability to bind p53 and, thus, does not directly affect such protein. However, HDMX211 binds HDM2 and inhibits its degradative activity, stabilizing the HDM2 protein itself and its target p53. The resulting p53 is transcriptionally inactive due, at least in part, to its increased association to HDM2. Biologically, HDMX211 promotes colony formation independently of p53 whereas HDMX211 depletion by siRNA strongly suppresses survival and proliferation of the cells from which it was isolated. Noteworthy, HDMX211 is present in a group of lung carcinoma overexpressing HDM2 protein, supporting the hypothesis of a role of this protein in human tumorigenesis.

Our data show that in analogy to HDM2, HDMX undergoes aberrant splicing that can generate proteins with oncogenic potential independently of p53. Moreover, HDMX211 provides the first evidence of a mechanism able to enhance HDM2 at the protein

Note: S. Giglio, F. Mancini, and F. Gentiletti contributed equally to this work.

Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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levels. Interestingly, the existence of this protein may explain the overexpression of HDM2 and wild-type (wt) p53 observed in different tumor types, which is still unexplainable with our current understanding of MDM2-p53 feedback loop.

Materials and Methods

Cell culture, plasmids, and transfections. Mouse NIH 3T3 fibroblasts were cultured at 37°C in DMEM high glucose with 10% donor calf serum (FBS; Invitrogen, Paisley, United Kingdom). MEF *p53*$^{-/-}$ *MDM2*$^{-/-}$ (kindly provided by Dr. G. Lozano, Department of Molecular Genetics, University of Texas M.D. Anderson Cancer Center, Houston, TX) and *p53*$^{-/-}$ *MDM4*$^{-/-}$ (kindly provided by Dr. C. Marine, Laboratory of Molecular Cancer Biology, University of Ghent, Ghent, Belgium), derived from the knockout mice, were cultured in DMEM high glucose plus 10% fetal serum (FBS; Invitrogen). All experiments were done between passage 4 and 8. MCF7 and H1299 cells were cultured in DMEM, and ARO, NPA, WRO, and FRO cells were cultured in RPMI with 10% FBS (Invitrogen). The *Apa*I/*Hin*dIII fragment of cDNA encoding HDMX211 was obtained by PCR using the following primers: HMXstart2, 5′-AAAAAAGGGCCCCTCATG-GAGCTGCCGTAAGTTT-3′; HMX211HindIII, 5′-AAAAAAAAGCTTGCTGC-TATAAAAACCTTAATAACCAGCTGAA-3′. The fragment was cloned into pcDNA3.1/*Myc*-His(−)B expression vector (Invitrogen) in frame with the myc and hystidine tags and checked by sequence. Alternatively, the *Apa*I/*Eco*RI fragment of HDMX211 cDNA, obtained by PCR using the following primers: HMXstart2 and 211RVStop, 5′-AAAAGGATCCTTATGCTA-TAAAAACCTTAATAAC-3′, was cloned into pcDNA3.1/*Myc*-His(−)B expression vector (Invitrogen), not in frame with tags. The tagged HDMX211 was used in all experiments unless differently indicated. Transient transfections were with Lipofectamine Plus (Invitrogen). In 60-mm plates, a fixed number of cells were transfected with 2 μg of pcDNA3.1HDMX211 and CMVHDM2, 0.5 μg of PCAGp53 plus 0.3 μg of pEGFP plasmid (Invitrogen), as internal control of transfection efficiency. For transcriptional assays, NIH3T3 cells were transfected with 2 μg of HDMX211, 2 μg of HDM2, and 1 μg of PG13Luc plasmid plus 0.25 μg of CMV β-gal plasmid, as internal control of transfection efficiency. In MEF *p53*$^{-/-}$ *MDM2*$^{-/-}$, 0.15 μg of p53 was added. Cells were harvested at 48 hours and Luc activity was assayed on whole-cell extracts (Promega, Madison, WI). HDMX211 small interfering RNA (siRNA; UCAAUCAGGAAAAUGAAGGtt) and control antisense siRNA (GGAAGUAAAAGGACUAACUtt) were generated by MWG, Inc. (High Point, NC). Cells were transfected using Oligofectamine (Invitrogen).

Clonogenicity assay. MCF7 (1×10^3, 5×10^3, 10×10^4 cells) or H1299 (1×10^3, 2×10^3, 5×10^3 cells) was plated in triplicate in 6 mm dishes and grown in the presence or absence of G418. Fourteen days after plating, dishes were stained by crystal violet and colonies (≥ 1 mm diameter) were counted.

Proliferation assay and cell cycle analyses. Cell proliferation for ARO cells was determined by Cell Titer Blue colorimetric assay (Promega). Briefly, 2 hours after transfection, cells were trypsinized and plated in octuplicate in 96 multiwell plates. At different time points, Cell Titer Blue was added to the cells for 3 hours at 37°C. Cell viability was then measured by spectrophotometric determination at 570 and 620 nm. Cell cycle profiles were evaluated by fixing 5×10^5 cells in 4% paraformaldehyde and 0.1% Triton X-100 for 10 minutes on ice and staining DNA for 30 minutes at room temperature with 50 μg/mL propidium iodide in PBS containing 1 mg/mL RNase A. Cell percentages in the different phases of the cycle were measured by flow cytometric analysis of propidium iodide–stained nuclei using Multicycle Software (Phoenix Flow System) Epics XL (Coulter, Fullerton, CA).

Immunoprecipitation and Western blot analysis. Immunoprecipitation and Western blot were done according to Gentiletti et al. (22). The following primary antibodies were used: rat anti-HDMX polyclonal antibody C-2 (against full-length HDM4), mouse anti-Myc monoclonal antibody 9E10, rabbit anti-p53 polyclonal antibody FL393 (Santa Cruz Biotechnology, Santa Cruz, CA), sheep anti-p53 polyclonal antibody Ab-7 (Oncogene, San Diego, CA), mouse anti-MDM2 monoclonal antibody 2A10, mouse anti-MDM2 monoclonal antibody Ab-1 (Oncogene), mouse anti–α-tubulin monoclonal antibody (Sigma, St. Louis, MO), rabbit anti–green fluorescent protein (GFP) polyclonal antibody (Invitrogen), and mouse anti–β-galactosidase monoclonal antibody (Roche Applied Science, Indianapolis, IN).

Reverse transcription-PCR analysis. Reverse transcription-PCR (RT-PCR) was done according to Moretti et al. (23). Analysis of *HDMX* transcripts was carried out by PCR amplification using the following primers: *HDMXfor*, 5′-CTCATGGAGCTGCCGTAAGTTT-3′; *HDMXrev2*, 5′-GAACATTCTCTGACAGGTTGGA-3′. HDMX mRNA analysis in the other cases was carried out accordingly to Rallapalli et al. (21). HDMX211 mRNA analysis was carried out by PCR amplification of a fragment of 170 bp using the following primers: *Hdmx211f*, 5′-GGACAAATCAATCAGAGGAAAAT-GAAGGAA-3′; *HDMXrev2*, 5′-GAACATTCTCTGACAGGTTGGA-3′. The housekeeping aldolase mRNA was used as external standard.

Real-time reverse transcription-PCR analysis. The expression of *HDM2, HDMX*, and *HDMX211* in tumors and matching normal lung samples was measured by real-time quantitative RT-PCR, based on TaqMan methodology, using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) according to Marchetti et al. (24). To normalize the amount of total RNA present in each reaction, we amplified the housekeeping gene *β-actin*. The primer/probe set for *HDM2* was as follows: forward, 5′-ATATACCATGATCTACAGGAACTTGGTAGT-3′; reverse, 5′-GGTGACACCTGTTCTCACTCACA-3′; TaqMan probe: Hdm2, ATCAGCAG-GAATCATCGGACTCAGGTACAT. The primer/probe set for *HDMX* was as follows: forward, 5′-TCGCACAGGATCACAGTATGG-3′; reverse, 5′-TTCTTTTTCTGGAAGTGGAACTTTC-3′; TaqMan probe: Hdmx, ATTC-CAAGTCAAGACCAACTGAAGCAAAGTGC. The primer/probe set for *HDMX211* (GenBank accession number AY923176) was as follows: forward, 5′-ACATCATTTTCCACCTCTGCTCA-3′; reverse, 5′-CATCATTTCCTT-CATTTTCCTGATT-3′; TaqMan probe, (FAM)-ACATCTGACAGTGCTTG-CAGGATCTCTCC-(TAMRA). Primers and probe for *β-actin* mRNA (GenBank accession number X00351), chosen using the computer program Primer Express (Applied Biosystems), were as follows: forward, 5′-TCCTT-CCTGGGCATGGAG-3′; reverse, 5′-AGGAGGAGCAATGATCTTGATCTT-3′; TaqMan probe, 5′(FAM)-CCTGTGGCATCCACGAAACTACCTTC-(TAMRA) 3′. All probes and reagents used for RT-PCR were purchased from Applied Biosystems. β-Actin and each target amplification were done in duplicate. Each sample was previously treated with DNase I, amplification grade (Invitrogen). Real-time PCR was done on each sample before and after reverse transcription.

Results

Identification of an aberrantly spliced form of HDMX. To assess the presence of HDMX spliced forms in human tumors, we did RT-PCR of *HDM4* mRNAs in four thyroid tumor cell lines. To identify all splicing events, we used a set of oligonucleotides pairing with the 5′ and 3′ untranslated regions of full-length *HDM4* mRNA. In ARO cells, derived from an anaplastic thyroid tumor, RT-PCR showed shorter bands besides the expected 1,500 bp fragment corresponding to the full-length mRNA (Fig. 1*A*). Sequence analysis revealed that the 740 bp fragment derives from aberrant splicing between the canonical donor site of exon 2 and a cryptic acceptor site into exon 11 (Fig. 1*B*). The resulting mRNA maintains the reading frame of full-length mRNA and codes for a protein containing the first 26 amino acids and the last 138 amino acids of the HDM4 protein. We named this protein HDMX211 (Fig. 1*C*). RT-PCR of *HDMX211* in thyroid tumor cell lines confirmed its presence in ARO cells and revealed it in FRO, but not in NPA and WRO cells. HDMX211 was also not detected in human fibroblasts and human embryonic kidney cells, suggesting its specific presence in tumor cells (Fig. 1*D*).

The cDNA of *HDMX211*, cloned in frame with the myc and hystidine tags and expressed in NIH 3T3 cells, yielded a protein of ~30 kDa (data not shown). The predicted molecular weight by ExPASy is 18,194 kDa, corresponding to the 164-amino-acid sequence. Despite the tag contribution, the discrepancy between predicted and observed molecular weights was not surprising. It is

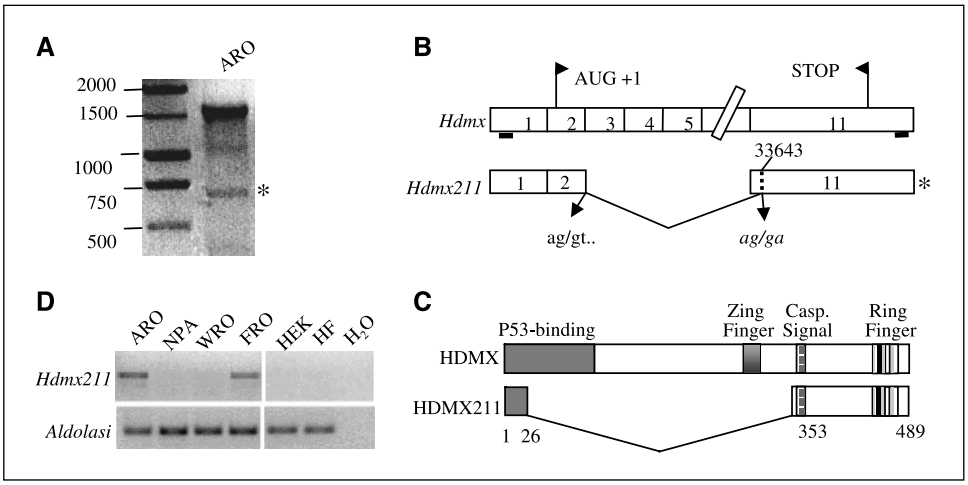

Figure 1. Characterization of the aberrantly spliced form *HDMX211*. *A,* RT-PCR for *HDMX* transcripts in total RNA extracted from ARO cell line. *, the mRNA of which the scheme is reported in *B*. *B,* schematic representation of *HDMX* and *HDMX211* mRNAs showing the sequences of the aberrant splicing. *Black dashes,* relative position of primers used for RT-PCR reported in *A*. *1* to *11,* exons; *33643,* base number corresponding to sequence AY207458, where the aberrant splicing takes place. *C,* protein structure and domains of HDMX and HDMX211. *D,* RT-PCR for HDMX211 in thyroid tumor cell lines ARO, NPA, WRO, and FRO, and in human embryonic kidney cells (*HEK*) and human fibroblasts (*HF*). Aldolase was used as loading control.

analogous to what was observed with the full-length HDMX (17) and is consistent with the COOH terminus of HDMX being covalently modified in the cell (25).

HDMX211 binds and stabilizes HDM2. HDMX211 almost completely lost the NH_2 terminus containing the p53 binding domain, but maintained the ring finger domain where HDM2 binding was mapped. Based on these observations, we tested whether HDMX211 maintained the ability to bind HDM2. We coexpressed HDMX211 and HDM2 in MEF *p53*$^{-/-}$ *MDM2*$^{-/-}$ and immunoprecipitated HDM2. HDMX211 was coimmunoprecipitated only in the presence of HDM2 (Fig. 2*A*). Similarly, in MCF7 cells that express high levels of HDM2 (26), immunoprecipitation of HDM2, either endogenously or exogenously expressed, coimmunoprecipitated HDMX211 in quantities proportional to the amount of HDM2 (Fig. 3*D*).

Furthermore, HDMX211 expression enhanced the levels of both exogenous and endogenous HDM2 proteins (Figs. 2*A* and 3*D*). Full-length HDMX binds and stabilizes HDM2 by counteracting its autodegradation (27, 28). To evaluate whether HDMX211 works in an analogous manner and to exclude any interference from p53-dependent activation of *HDM2* transcripts, we coexpressed HDMX211 and HDM2 in MEF *p53*$^{-/-}$ *MDM2*$^{-/-}$. Western blot showed a strong increase of HDM2 levels in the presence of HDMX211 compared with cotransfected GFP protein (Fig. 2*B*). Addition of p53 did not alter this effect, indicating that HDMX211 enhances HDM2 protein levels independently of p53. To ascertain whether this effect is due to HDM2 increased stabilization, we analyzed HDM2 levels following cycloheximide treatment (Fig. 2*C*). According to previous reports, the half-life of exogenous HDM2 protein was estimated to ~20 minutes in the control cells (Fig. 2*C*,

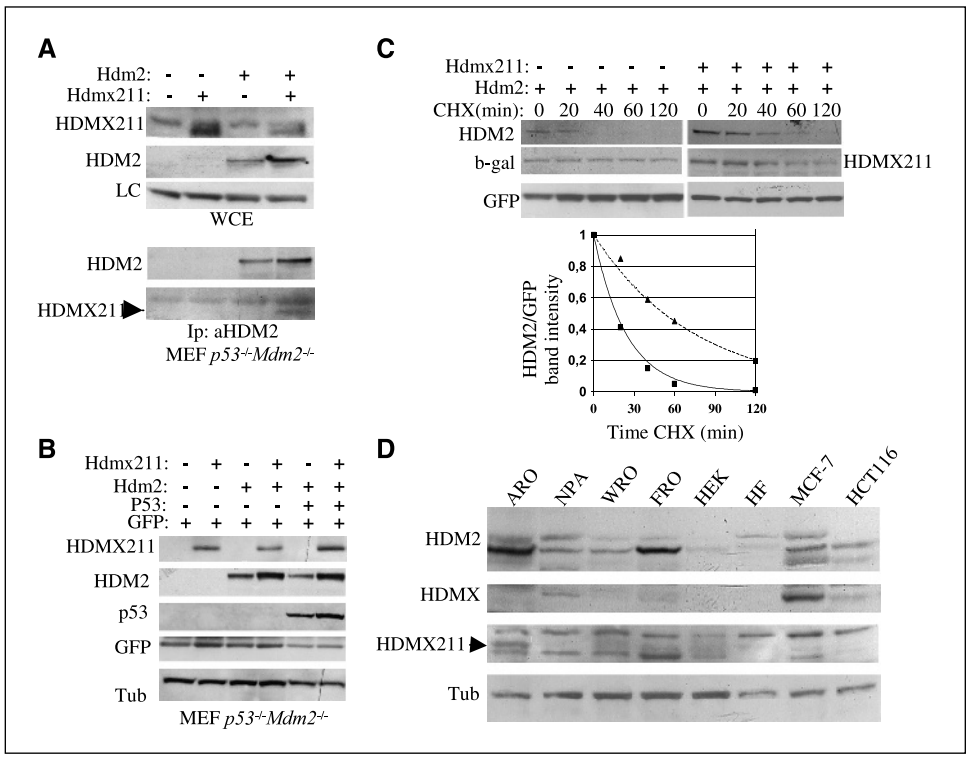

Figure 2. HDMX211 increases HDM2 stability. *A,* MEF *p53*$^{-/-}$ *MDM2*$^{-/-}$ were transfected with the indicated genes and analyzed by Western blot of the whole-cell extract (*top*) or of immunocomplexes obtained by anti-HDM2 antibodies 2A10/Ab1 (*bottom*). *B,* Western blot of the indicated proteins expressed in MEF *p53*$^{-/-}$ *MDM2*$^{-/-}$ transfected with the expression vectors shown on top. Tubulin (*Tub*) immunostaining was used as a loading control. GFP was used as marker of transfection efficiency. *C,* half-life of exogenous HDM2 protein. *Left,* Western blot of the indicated proteins in MEF *p53*$^{-/-}$ *MDM2*$^{-/-}$ transfected with HDM2 and HDMX211 or β-gal expression vectors and then treated with cycloheximide (*CHX*; 50 μg/mL) for the indicated times. *Right,* quantitation of HDM2 destabilization shown on the left. Each HDM2 band intensity was normalized to GFP, then normalized to the *t* = 0 value, set to 1. *Filled* and *dotted lines,* interpolation of HDM2/GFP band intensity in the presence of β-gal and HDMX211, respectively. *D,* Western blot of the endogenous proteins HDM2, HDMX, HDMX211, and tubulin in different cell lines as indicated. *Arrow,* HDMX211 band.

filled line; ref. 29). In the presence of HDMX211, the steady-state levels of HDM2 were increased and the half-life of HDM2 was about 50 minutes (Fig. 2*C, dotted line*), confirming that HDMX211 increases HDM2 protein levels by enhancing its stability.

To confirm the positive correlation between HDMX211 and HDM2 increase, levels of endogenous HDM2 protein in ARO cells—from which we isolated HDMX211—and of other thyroid tumor cell lines were compared with those of other cells. Indeed, ARO and FRO cells, positive for HDMX211 by RT-PCR, expressed HDM2 levels comparable to those of HCT116 and MCF7, two cell lines reported to highly express HDM2 (26). In comparison, human embryonic kidney cells and human fibroblasts expressed lower levels of HDM2 (Fig. 2*D*). Anti-HDMX polyclonal antibody C2, which recognizes an epitope in the COOH terminus of full-length HDMX, detected a band of the size of untagged HDMX211 in ARO cells, in agreement with RT-PCR data. In these cells, HDMX was barely detectable, indicating no correlation between HDMX211 presence and HDMX levels.

HDMX211 stabilizes inactive p53 in a HDM2-dependent manner. In the previous experiments, despite increased levels of HDM2, p53 protein levels were enhanced by HDMX211 expression (Fig. 2*B*). This was unexpected given that HDM2 mediates degradation of p53. To evaluate whether HDMX211 has a direct effect on p53, we coexpressed HDMX211 and p53 in MEF *p53*$^{-/-}$ *MDM2*$^{-/-}$. Under these conditions, HDMX211 did not significantly alter p53 levels (Fig. 3*A, first two lanes*) whereas, on HDM2 reconstitution, it enhanced them (Fig. 3*A, last two lanes*). Thus, HDMX211 affects p53 indirectly only in the presence of HDM2.

We then asked whether the increased p53 is transcriptionally functional. We transfected p53 along with the p53-responsive PG13 promoter driving luciferase transcription in MEF *p53*$^{-/-}$ *MDM2*$^{-/-}$ and tested its activity in the presence or absence of HDMX211 and HDM2. Promoter activity was not altered by the presence of HDMX211 (Fig. 3*B*) both in the presence and absence of HDM2 despite different levels of p53 (see Fig. 3*A*), indicating that HDMX211, although enhancing p53 levels, does not activate its transcriptional function. Similar results were obtained in NIH3T3 with endogenous HDM2 and p53 proteins (Fig. 3*C*) despite enhanced levels of p53 and MDM2 by HDMX211 in this cell context, too (data not shown). In the same cells, p53 function is altered by HDM2 overexpression or Adriamycin treatment (known to affect p53 levels), confirming the specificity of the effect by HDMX211 (Fig. 3*C*). Because p53 transcriptional function is inhibited by binding of HDM2 to the NH_2-terminal transactivation domain, we analyzed levels of p53 coimmunoprecipitated with HDM2 in MCF7. Indeed, in the presence of HDMX211, increased levels of p53 corresponded to an increase of HDM2-conjugated p53 (Fig. 3*D*), indicating that p53 enhancement correlates with its association with HDM2. Similar results were obtained in NIH 3T3 cells (data not shown). These data support a model by which HDMX211 activity results in stabilization of HDM2 and p53 levels in association with increased binding between p53 and HDM2.

HDMX211 counteracts HDM2-mediated p53 degradation. A possible explanation for HDM2 and p53 enhancement by HDMX211 is that HDMX211 inhibits HDM2 degradative activity on itself and on p53.

HDM2-mediated p53 degradation is dependent on p53-HDM2 binding (2). Accordingly, p53$^{22/23}$, a p53 mutant unable to bind HDM2, is not degraded by HDM2 (30, 31). p53$^{22/23}$ was not stabilized by HDMX211 in comparison with wt p53, confirming that p53-HDM2 binding is required for HDMX211-mediated p53 stabilization (Fig. 4*A*). Noteworthy, HDMX211 expression resulted in p53 levels higher than those observed in the absence of HDM2 (see Figs. 3*A* and 4*A*). This suggests that increased binding to HDM2 may protect p53 from degradation by the other p53-ubiquitinating enzymes, COP1 and Pirh2 (32, 33).

To further test whether HDMX211 inhibits HDM2-mediated degradation of p53, we used a system where p53 degradation is impaired. Leptomycin B is reported to protect p53 from HDM2-mediated degradation leading to stabilization of the nuclear protein (34). We expressed HDMX211 in NIH3T3 cells and treated them with leptomycin B. As previously reported, endogenous p53 was strongly stabilized by leptomycin B. However, under these conditions, HDMX211 did not further increase p53 levels (Fig. 4*B*), supporting the hypothesis that HDMX211 counteracts HDM2-mediated p53 degradation. In the same conditions, endogenous MDM2 was still stabilized, although to a lesser extent than in the absence of leptomycin B (Fig. 4*B, left two lanes*). Indeed, leptomycin B only partially blocks MDM2 shuttling between nucleus and cytoplasm and its consequent degradation (35).

p53 degradation is cooperatively regulated by heterodimerization of HDM2 to HDMX (36). We asked whether the increase

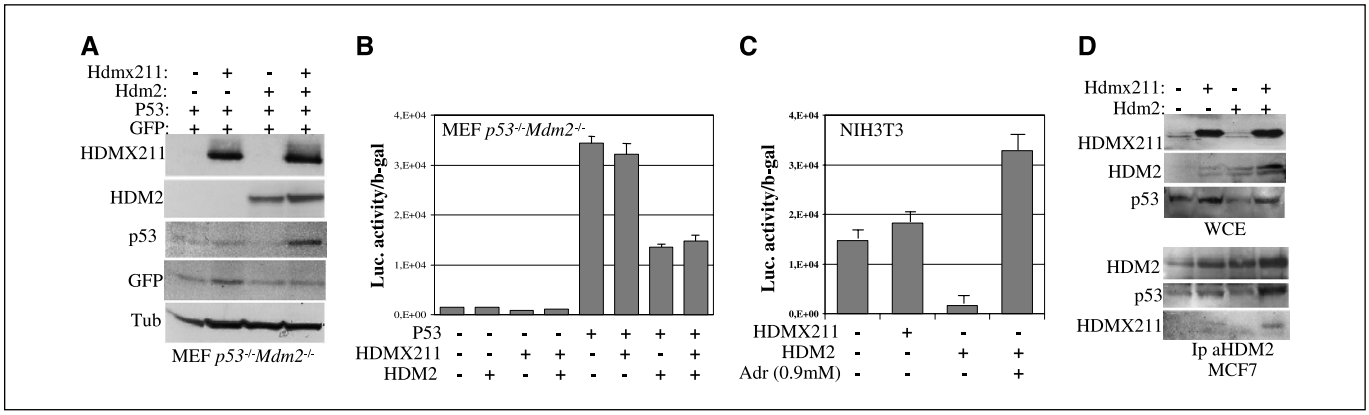

Figure 3. HDMX211 stabilizes inactive p53 in a HDM2-dependent manner. *A,* Western blot of the indicated proteins in MEF *p53*$^{-/-}$ *MDM2*$^{-/-}$ transfected with the expression vectors shown on top. *B* and *C,* transcriptional assay for p53 activity in MEF *p53*$^{-/-}$ *MDM2*$^{-/-}$ (*B*) and NIH 3T3 (*C*) transfected with the indicated genes and the p53-responsive PG13Luc reporter plasmids (1 μg/dish) plus β-gal (0.25 μg/dish). Cells were assayed 48 hours after transfection. *Columns,* mean of two independent experiments done in triplicate; *bars,* SD. *D,* analysis of p53, HDMX211, and HDM2 immunocomplexes in MCF7 cells following HDMX211 expression. Western blot analysis of the indicated proteins in whole-cell extract (*top*) and in immunocomplexes obtained by anti-HDM2 antibodies 2A10/Ab1 (*bottom*).

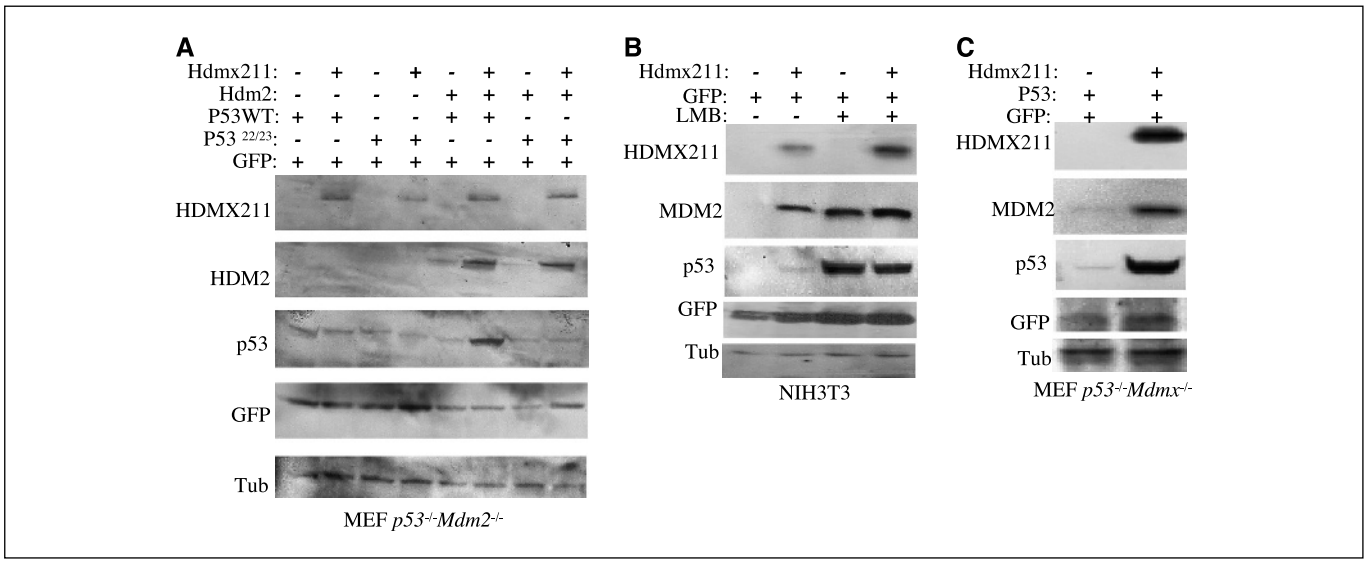

Figure 4. HDMX211 counteracts HDM2-mediated degradation of p53. *A,* Western blot of the indicated proteins in MEF $p53^{-/-}$ $MDM2^{-/-}$ transfected with the expression vectors shown on top. *B,* Western blot of endogenous MDM2 and p53 levels in NIH 3T3 cells following expression of HDMX211 and treatment with leptomycin B (10 nmol/L) for 24 hours. *C,* Western blot of the indicated proteins in MEF $p53^{-/-}$ $MDMX^{-/-}$ transfected as in *B* with the exception of HDM2, which represents the endogenous protein.

of p53 levels could be related to HDMX211 interference on this cooperation. In MEF $p53^{-/-}$ $MDMX^{-/-}$, HDMX211 stabilized endogenous MDM2 and exogenous p53 (Fig. 4*C*), demonstrating that the HDMX211 effects are not dependent on squelching of HDMX from the heterodimer HDMX/HDM2.

All together, these data support the hypothesis that HDMX211 stabilizes p53 by inhibiting HDM2 degradative activity without interfering with the association between p53 and HDM2.

HDMX211 enhances cell clonogenicity. Based on the cell context, HDM2 possesses oncogenic activities dependently on and independently of p53 (37, 38). Because HDMX211 stabilizes HDM2, we asked whether HDM2, in the presence of HDMX211, enhances cell clonogenicity, as a measure of its transforming potential. We expressed HDMX211 in H1299 cells that are p53 negative and responsive to the oncogenic function of HDM2 (39). A significant increase in colony-forming ability was observed in HDMX211-expressing cells compared with controls (Fig. 5*A*). Consistent with these results, whole-cell extract from the H1299 transfected cells showed expression of HDMX211 and, correspondingly, increased levels of HDM2 (Fig. 5*B*). Similar results were obtained in a wt p53 context, too. As shown in Fig. 5*C*, when expressed in MCF7 cells containing wt p53, HDMX211 significantly increased colony number, particularly at the lowest cell density. Enforced expression of HDM2 also sustained colony growth, indicating that HDM2 is oncogenic in this cell context, too (data not shown). Again, the analysis of whole-cell extract showed that expression of HDMX211 correlated to increased levels of HDM2 and p53 (Fig. 5*D*).

These data provide evidence that HDMX211 possesses growth-altering properties *in vitro*, tightly connected with enhancement of HDM2 levels. These properties are independent of and not suppressed by p53, further supporting the notion that HDMX211, although stabilizing p53 levels, does not activate its growth inhibitory function.

HDMX211 controls ARO cell growth properties. To further test whether HDMX211 regulates cell growth, protein expression was inhibited by siRNA in ARO cells. We used an siRNA oligo matching the aberrant spliced junction (siHDMX211) and analyzed by RT-PCR the mRNA levels of *HDMX211* and *HDM4* as internal control. As a further control, the reverse oligo was used (siCTRL). The siHDMX211 oligo decreased *HDMX211* mRNA but not *HDM4* mRNA (Fig. 6*A*). Real-time RT-PCR further confirmed this reduction: *HDMX211/b-actin* ratio was reduced in siHDMX211

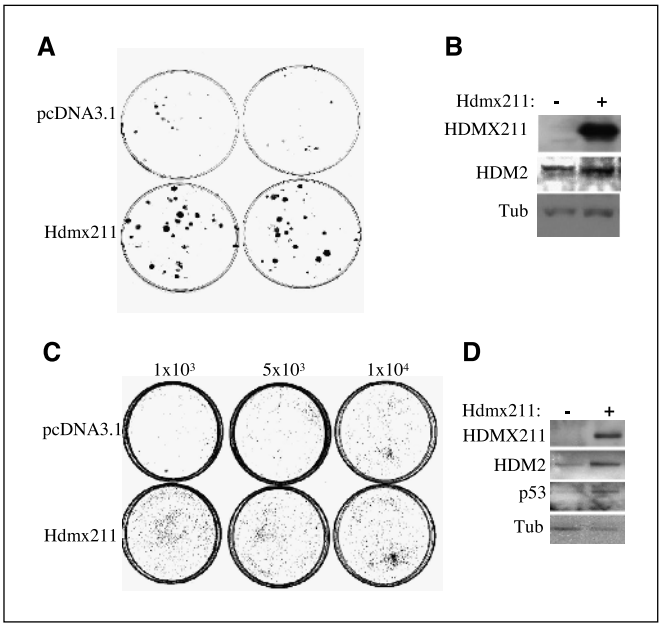

Figure 5. HDMX211 increases the colony-forming ability of tumor cells. *A,* p53-null H1299 cells were transfected with HDMX211 or control (pcDNA3.1) vectors. Eight hours after transfection, cells were plated at different cell densities. Colonies formed at the lowest density (1×10^3) in two independent plates. Representative of two different experiments done in triplicate. *B,* Western blot of whole-cell extract from a portion of the cells used in *A* and collected 48 hours after transfection. *C,* wt p53 carrier MCF7 cells were transfected with HDMX211 or control (pcDNA3.1) vectors. Eight hours after transfection, cells were plated by serial dilutions at different cell densities as indicated. Representative of three different experiments done in triplicate. *D,* Western blot of whole-cell extract from a portion of the cells used in *C* and collected 48 hours after transfection.

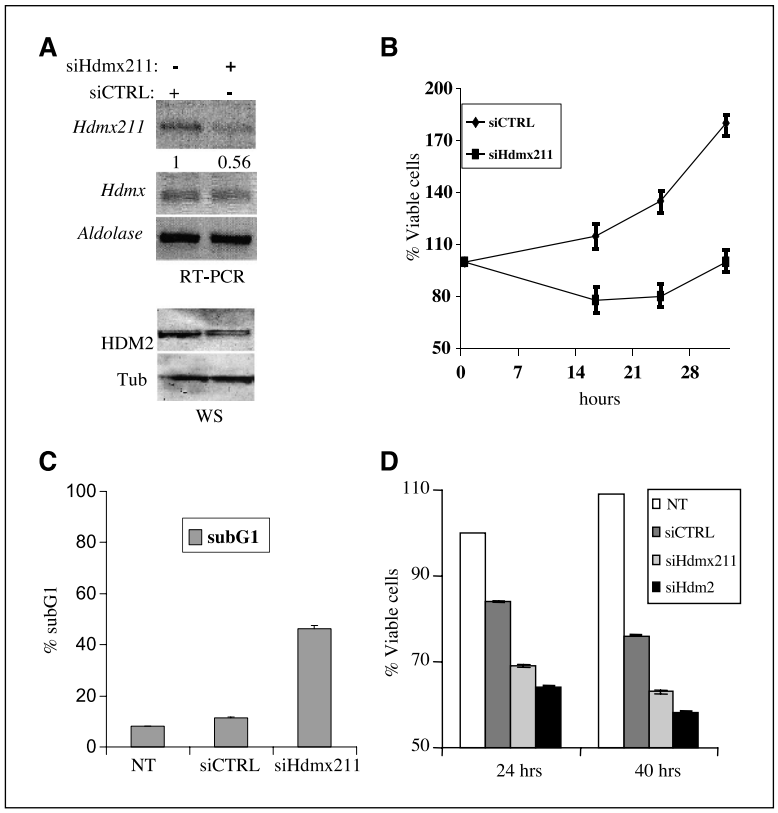

Figure 6. HDMX211 depletion in ARO cells affects their growth properties. *A,top,* RT-PCR analysis of *Hdmx211* and full-length *Hdmx* mRNAs following small interference of *HDMX211* mRNA (siHDMX211) or small interference with the inverted oligo (siCTRL). Aldolase was used as a loading control. The numbers indicate percentage densitometric values. *Bottom,* Western blot of endogenous HDM2 levels following siHDMX211 or siCTRL. *B,* cell viability of siHDMX211 or siCTRL plus 1 μg of GFP transfected cells. Mean of two independent experiments done in octuplicate. Cell viability was evaluated by spectrophotometric quantitation of Cell Titer Blue incorporated into viable cells. All values were normalized to the starting number of cells, set to 100. *C,* percentage of sub-G_1 area in fluorescence-activated cell sorting analysis of cells transfected as in *B*, and compared with nontransfected cells (*NT*) or cells transfected with siCTRL. *D,* percentage of cell viability of ARO cells interfered with siCTRL, siHDMX211, or siHDM2, and treated with Adriamycin (4 μg/mL) for the indicated times. Cell viability was evaluated by Cell Titer Blue assay as in *B*, at two different times points (24 and 40 hours). *Columns,* mean of two experiments done in octuplicate; *bars,* SD. The value for each group was normalized to that of nontreated cells (*NT*) at 24 hours, set to 100.

versus siCTRL cells (0.3 versus 1) but not the *HDM4/b-actin* ratio (1.1 versus 1). A clear decrease in HDM2 levels in whole-cell extract from siHDMX211-expressing cells in comparison with the siCTRL was observed by Western blot (Fig. 6*A, bottom*), confirming that expression of HDMX211 correlates with HDM2 levels. To test whether this effect was accompanied by alterations of growth, cell viability of siHDMX211 transfected cells was measured. ARO cells depleted of HDMX211 showed a significant decrease of cell viability compared with siCTRL control cells (Fig. 6*B*). Analysis of cell death done 24 hours after transfection showed a strong increase of the sub-G_1 fraction in the population transfected with siHDMX211 (Fig. 6*C*). Moreover, cell cycle of GFP-positive transfected cells showed a significant increase of G_1 phase in siHDMX211 cells versus siCTRL or nontransfected cells (52 ± 4% versus 38 ± 3% or 40 ± 3%, respectively). In agreement with these data, the sensitivity to adriamycin (Fig. 6*D*) or cisplatin (data not shown) of HDMX211-depleted cells was significantly higher than the control. Interference of endogenous HDM2 by siRNA gave similar results to those obtained with siHDMX211, supporting the tight correlation between HDMX211 and HDM2 activities (Fig. 6*D, siHDM2*). These data are in accord with previous results from clonogenicity experiments. They point to a possible role of HDMX211 in the control of cell growth properties, mainly through the control of HDM2 protein levels.

HDMX211 is present in human tumors. High levels of HDM2 protein independently of gene amplification have been reported in human tumors (6–8, 11). A stabilizing factor has been invoked but not yet identified. To ascertain whether HDMX211 could be such factor, its presence in human tumors with high levels of HDM2 protein was investigated.

Overexpression of HDM2 not sustained by gene amplification has been observed in lung cancer and particularly in non–small-cell lung cancer (11, 40) where overexpressed wt p53 has also been reported (40). A panel of 16 non–small-cell lung cancer samples and of matching normal lung samples were analyzed at RNA and protein levels. The presence of *HDMX211* and the levels of *HDM2* and *HDMX* mRNA were analyzed by real-time RT-PCR. Protein levels of HDM2 and p53 were determined by densitometry of Western blot of each tumor sample compared with the matching normal lung samples (Supplementary Fig. 2). Lastly, p53 status was ascertained by sequencing of the entire coding sequence. The results (Table 1) show that, compared with control samples, *HDM2* mRNA was overexpressed in 8 of 16 cases, whereas HDM2 protein was overexpressed in 13 of 16 samples. All samples overexpressing the RNA overexpressed the protein. Quantitative RT-PCR revealed that two of the five cases overexpressing HDM2 protein but not mRNA possess *HDMX211* RNA. Interestingly, one of them showed enhanced levels of wt p53. *HDMX* mRNA and protein levels were not concomitantly increased in the same samples (data no shown). None of the normal lung samples showed the presence of HDMX211.

These data show that HDMX211 is present in human tumors. The tight association of its presence with enhanced levels of HDM2 protein strongly supports its role in HDM2 stabilization. Lastly, overexpression of wt p53 in one case expressing HDMX211 supports a model of inhibition of p53 oncosuppressive function by HDMX211.

Discussion

We have investigated the presence of spliced forms of HDMX that may possess oncogenic potential in tumor cell lines. We have isolated an aberrantly spliced mRNA that generates a shorter HDMX protein, named HDMX211. This protein retains the ring finger domain of the wt HDMX but lacks the NH_2 terminus and part of the central region, a deletion resulting in loss of the p53-binding domain.

Table 1. Frequency of HDMX211 in 16 non–small-cell lung tumors with different HDM2 status

	Enhanced levels of HDM2 protein*		HDM2 normal levels†
	Enhanced levels of HDM2 mRNA‡	Normal levels of HDM2 mRNA§	
Presence of HDMX211	0	2	0
Absence of HDMX211	8	3	3

*Densitometric value of HDM2 protein in tumor sample/matching normal sample > 3.
†Densitometric value of HDM2 protein in tumor sample/matching normal sample < 2.
‡Quantitative RT-PCR value in tumor sample/matching normal sample > 2.
§Quantitative RT-PCR value in tumor sample/matching normal sample < 2.

Accordingly, direct activity of HDMX211 on the p53 oncosuppressor was not observed. However, HDMX211 exerts its activity on HDM2, enhancing its protein levels and counteracting its degradative function. Indeed, the presence of HDMX211 in ARO cells coincides with high levels of HDM2. Inhibition of HDMX211 expression in these cells decreases HDM2 protein levels and concomitantly strongly reduces their growth properties and increases their sensitivity to chemotherapeutic drugs. Conversely, expression of HDMX211 in H1299 cells increases HDM2 levels and enhances their colony-forming ability. All these data are compatible with the transforming potential of HDMX211 exerted through enhancement of HDM2 independently of wt p53. Indeed, both ARO and H1299 cell lines are devoid of wt p53 (23, 39).

Overexpression of HDM2 in human tumors has been linked primarily to gene amplification, although high protein levels in the absence of gene amplification have been detected in different tumors and are considered almost as relevant as gene amplification (6–8, 40). Notably, thyroid tumors, from one of which ARO cells derived, are a histotype with high levels of HDM2 protein (11). Different reports have indicated that overproduction of HDM2 may also result from chromosomal translocations, increased transcription, or enhanced translation, and some of these mechanisms have been evidenced in human tumors (5–10). No data have been reported on mechanisms acting at the HDM2 protein levels in cancer. HDMX211 may represent a new molecule capable of inducing overproduction of HDM2 in human tumors. Its presence in two lung tumors overexpressing only HDM2 protein strongly supports this hypothesis.

In addition, our data showed that HDMX211 acts also on wt p53, albeit indirectly, by counteracting its HDM2-mediated degradation and thereby increasing its levels. Interestingly, HDMX211 does not activate p53 transcriptional function nor enhance its growth inhibitory properties, as indicated by our results with MCF7 cells. Increased association of p53 to its inhibitor HDM2 may explain the lack of increased p53 activity in these cells. The increased levels of wt p53 in the non–small-cell lung tumors expressing HDMX211 and high levels of HDM2 support the notion of HDMX211 activity toward the oncosuppressor p53. As reported by Keleti et al. (41), simultaneous overexpression of HDM2 and wt p53 has been detected in some rhabdomyosarcoma-derived cell lines. Interestingly, the authors showed that overexpression of wt p53 was mediated by overexpressed HDM2 and coincides with the association of the two proteins, a mechanism closely resembling what we observed on expression of HDMX211. Overexpression of both HDM2 and wt p53 was observed also in a fraction of human tumor cells (5) and in non–small-cell lung cancer (40), the same tumor histotype where we detected HDMX211.

HDMX211 activity toward HDM2 and p53 is independent of HDMX as shown by the results obtained in MEF $p53^{-/-}$ $MDM4^{-/-}$. In agreement with these data, overexpression of HDMX in tumor samples expressing HDMX211 was not observed. Further, HDMX211 activity differs from that of full-length HDMX. HDMX cooperates with HDM2 in the degradation of p53, an effect not observed with HDMX211 (Supplementary Fig. 1). Moreover, HDMX activates p53 functions under stress conditions (42), as well as its antiproliferative effects (43). Thus, expression of HDMX211 in a transformed cell context could amount to a gain of function relative to HDMX due to loss of p53-activating capability.

In summary, our study describes a new spliced variant of *HDMX* that functions independently of p53. Interestingly, its direct effect on the HDM2 protein prefigures a new mechanism for overproduction of the HDM2 protein. Moreover, the existence of this variant may provide the molecular explanation for the phenotype of overexpressed HDM2 and wt p53 in various tumor samples and cell lines, the causes of which were hitherto unclear.

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Identification of an Aberrantly Spliced Form of HDMX in Human Tumors: A New Mechanism for HDM2 Stabilization

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