Mutant p53-induced Up-regulation of Mitogen-activated Protein Kinase Kinase 3 Contributes to Gain of Function*§

Received for publication, December 14, 2009, and in revised form, March 9, 2010. Published, JBC Papers in Press, March 11, 2010, DOI 10.1074/jbc.M109.094813

Aymone Gurtner§, Giuseppe Starace§, Giuseppe Norelli§, Giulia Piaggio§, Ada Sacchi‡, and Gianluca Bossi††

From the §Molecular Oncogenesis Laboratory, Department of Experimental Oncology, Regina Elena Cancer Institute, Via delle Messi D’Oro 156, 00158 Rome, Italy and the †National Council of Research, Istituto di Neurobiologia e Medicina Molecolare, 00133 Rome, Italy

Mitogen-activated protein kinase kinase 3 (MAP2K3) is a member of the dual specificity kinase group. Growing evidence links MAP2K3 to invasion and tumor progression. Here, we identify MAP2K3 as a transcriptional target of endogenous gain-of-function p53 mutants R273H, R175H, and R280K. We show that MAP2K3 modulation occurred at the mRNA and protein levels and that endogenous mutant p53 proteins are capable of binding to and activate the MAP2K3 promoter. In addition, we found that the studied p53 mutants regulate MAP2K3 gene expression through the involvement of the transcriptional cofactors NF-Y and NF-κB. Finally, functional studies showed that endogenous MAP2K3 knockdown inhibits proliferation and survival of human tumor cells, whereas the ectopic expression of MAP2K3 can rescue the proliferative defect induced by mutant p53 knockdown. Taken together, our findings define a novel player through which mutant p53 exerts its gain-of-function activity in cancer cells.

TP53 gene mutations are the most frequent genetic alterations in human cancers; >50% of all human cancer cases carry mutations within the TP53 locus (1). Most of these are missense point mutations and are localized in the core DNA-binding domain (2). These alterations disrupt the normal transcriptional capacity of p53 and compromise its tumor suppressor properties by abrogating its transcriptional activity on genes connected with cell cycle arrest, apoptosis, or DNA repair, in response to a variety of stress signals (3, 4). Recent studies have shown that mutations of the TP53 gene can confer additional functions (gain of function, GOF) that are exerted in a variety of ways, ranging from enhanced proliferation in culture, increased tumorigenicity in vivo, and enhanced resistance to a variety of commonly used anti-cancer drugs (5, 6). The GOF hypothesis has recently been reinforced by studies employing mutant p53 (mutp53) “knock-in” mice, which show a higher frequency of tumor development and increased metastatic potential, compared with p53-deficient mice (7, 8). Furthermore, RNA interference (RNAi) studies demonstrated that depletion of mutp53 renders cancer cells more sensitive to DNA-damaging chemotherapeutic agents in vitro (9, 10) and reduces tumor malignancy both in vitro and in vivo (10). In agreement with these results, tumor growth delay studies, performed in the HT29 xenograft model, showed that conditional silencing of mutp53 does not only impact on tumor growth but leads to tumor architecture modifications, with consistent reduction in stromal invasion and tumor angiogenesis (11). At the molecular level, these GOF effects were shown to be linked to the ability of mutp53 to modulate the expression of several genes, such as MDRI (12), c-MYC (13), CD95 (Fas/APO-J) (14), EGR1 (9), MSP/MST-1 (15), GEF-H1 (16), ID2 (17), GRO1 (18), PPARGC1A, FRMD5 (19), and ID4 (20), supporting the hypothesis that mutp53-specific transcriptional activity is required for at least some of the mutp53 GOF effects. However, the molecular mechanisms underlying the GOF of mutp53 proteins are still far from being understood. Two different and not mutually exclusive possibilities are currently considered: (i) mutp53 retains residual transcriptional activity and acts as regulator of transcription (19, 21–24); and (ii) mutp53 can no longer bind DNA but interacts with other transcription factors and modulates their activities (25–27).

The mitogen-activated protein kinase-kinase 3 (MAP2K3) belongs to a dual specificity kinase group (MKK7) and is activated by MKKK proteins (MEKK1–4) through Ser-189 and Thr-193 phosphorylation (28). MAP2K3 is a specific upstream activator of the p38 MAPK protein (28). Recent studies found that MAP2K3 up-regulation was involved in invasion and progression of gliomas and breast tumors (29). Our recent quantitative PCR validation of microarray data, from cells expressing endogenous mutp53 (HT29 and SKBR3) and xenograft tumors, indicated that MAP2K3 (also known as MK3) is a mutp53 target gene (11). To unveil a possible role of MAP2K3 up-regulation in mutp53 GOF activity, we studied the molecular mechanisms through which different mutp53 proteins up-regulate MAP2K3 expression and the biologic effects of its up-regulation in different cell lines.

Here, we find that different mutp53 proteins bind the MAP2K3 promoter, enhancing its transcriptional activity. Moreover, by dissecting the promoter region, we identify co-activators...
required by mutp53 to up-regulate MAP2K3 expression. Finally, biological studies indicate that MAP2K3 up-regulation plays an important role in cell proliferation and survival. Overall, our study defines MAP2K3 as a novel target of mutp53 and provides new insights for the understanding of mutp53 GOF activity.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Non-small cell lung carcinoma H1299 (p53-null), colon adenocarcinoma HT29 (mutp53R273H), breast carcinoma SKBR3 (mutp53R175H) (10), breast adenocarcinoma MDA-MB468 (mutp53R273H) (providing by Dr. G. Blan- dino), and colorectal carcinoma HCT116 (wt p53) (provided by Dr. M. Fanciulli) human cell lines were maintained in Dulbecco’s modified Eagle’s medium (Eurobio, Les Ulis, France). Human breast carcinoma cell line MDA-MB231 (mutp53R280K) (provided by Prof. S. Ando) was maintained in Dulbecco’s modified Eagle’s medium-F12 1:1. All cell cultures were supplemented with 10% fetal bovine serum (GIBCO/Invitrogen, Grand Island, NY), 2 mM l-glutamine (Invitrogen), 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). HEK293T packaging cell lines (11) were maintained in Dulbecco’s modified Eagle’s medium high glucose (GIBCO/Invitrogen) 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 1× nonessential amino acids (GIBCO/Invitrogen). All cell lines were maintained at 37 °C in a humidified environment of 5% CO2.

**Western Blotting**—Cells were washed twice in ice-cold phosphate-buffered saline, harvested by scraping into 1× radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 0.1% SDS, 50 mM Tris/HCl, pH 8.0, and 20 mM EDTA) supplemented with 1× protease and phosphatase inhibitor mixture (Sigma-Aldrich), 1× phenylmethylsulfonyl fluoride (Sigma-Aldrich), 50 µM sodium fluoride (Sigma), and 50 µM dithiothreitol (Bio-Rad). Lysates were incubated with the following antibodies: anti-p53 (Cell Signaling), phospho-ATF2 rabbit polyclonal antibody (DOI) (6), MAP2K3 rabbit polyclonal antibody (Cell Signaling), phospho-PAN-H4ac mouse monoclonal antibody (Ab-1, Calbiochem, San Diego, CA), and anti-NF-YB (3) antibody (Cell Signaling, C-19 SC-1190), anti-PAN-H4ac mouse monoclonal antibody (Ab-1, Calbiochem, San Diego, CA), Secondary horseradish peroxidase-conjugated antibodies (anti-mouse [Santa Cruz Biotechnology] or anti-rabbit [Calbiochem, respectively) were used. Detection of immuno-reactions was performed by ECL kit (Amersham Biosciences). Densitometric analyses were performed by Scion Image software.

**MAP2K3 Promoter Constructs**—A fragment of 1.0 kb of the MAP2K3 gene 5′-regulatory region (−989 to −2 with respect to the translational start site) was PCR-amplified from HT29 genomic DNA by using information obtained from the NCBI database. Primers were designed as follows. The forward primer includes a Sac1 restriction enzyme sequence plus a short tail (MAP2K3-For, 5′-tataagatct-TGC-AAGTTGGTCTCCTGGAC-3′). The reverse primer includes a BglII restriction enzyme sequence plus a short tail (MAP2K3-Rev, 5′-tataagatct-TGC-AAGTTGGTCTCCTGGAC-3′). PCR reaction was performed with HOT-MASTER Taq (Eppendorf). Amplified fragment was digested with SacI/BglII restriction enzymes (Invitrogen) and cloned into digested pGL3-Luc promoter-less vector generating pMAP2K3-Luc. The new vector was then sequence analyzed. Deletion derivatives of the MAP2K3 cloned promoter were generated as follows. pdel1-Luc (deletion −989 to −716) was generated by cutting cloned promoter (pMAP2K3-Luc) with SacI restriction enzyme, T4 DNA polymerase blunt-ended (Biolabs), PvuII-digested (Invitrogen), and self-ligated (Takara DNA ligation kit). pdel2-Luc (deletion −782 to −499) was generated by cutting with SmaI (Invitrogen) dual restriction enzyme sites and self-ligated. pdel3-Luc (deletion −711 to −167) was generated by cutting with PstI restriction enzyme (Roche Applied Science) and self-ligated.

**Transactivation Assay**—Cells were plated in 6-well plates (5×10⁴ cells/well), and on the following day, cells were transiently transfected with vector reporter (0.9 µg/well) and pCMVβ-gal (0.1 µg/well) (transfection efficiency) vector by following Lipofectamine Plus guidelines (Invitrogen). Luciferase and β-galactosidase assays were performed 24 h later on whole-cell extract, as described (30). Luciferase values were normalized to β-galactosidase activity and protein contents. H1299 cells were plated in 6-well plates (5×10⁴ cells/well), and 24 h later, cells were transiently co-transfected with either the pMAP2K3-Luc or the partial deleted promoters (0.8 µg/well) along with the pCMVβ-gal (0.1 µg/well) and p53-expressing (0.1 µg/well) vectors. Luciferase and β-galactosidase assays were performed 24 h later as reported.

**Chromatin Immunoprecipitations**—Chromatin immunoprecipitation assays were performed as described previously (31). In brief, cells were incubated in 1% of formaldehyde for 10 min at 22 °C. The reaction was stopped by addition of glycine to 125 mM final concentration. Sonicated chromatin were incubated with the following antibodies: anti-p53 (6 µg/reaction) (ab-7, Oncogene PC35); anti-NF-YB (3 µg/reaction) (generous gift from Dr. R. Mantovani); anti-NF-kB (10 µg/reaction) (p50) (Santa Cruz Biotechnology, C-19 SC-1190), anti-PAN-H4ac (10 µg/reaction) (Upstate, 06-598). For PCR analyses, 2 µl of template in 20–30 µl of total reaction were used. PCR was performed with HOT-MASTER Taq (Eppendorf) by using the following primers: hMAP2K3-For (5′-CCCTTAGGGATCTCTGTTTTT-3′), hMAP2K3-Rev (5′-TCCCGCTCTCTGAACTCAA-3′), hCycB2-For (5′-AGAGGCGTCCTACGTCTGC-3′), and hCycB2-Rev (5′-TGCCACAGGGTCCGTCTTCTT-3′). Primer sequences of the adjacent region to hMAP2K3 promoter as negative control (−3200 bp) were: −3200-For 5′-TCTTACGTGGCAGTCACACAGTAA-3′ and −3200-Rev 5′-GGCAGCTTCCTTACATGAC-3′.

**Viral Vectors**—Lentiviral vectors were produced in HEK293T cells by transient transfection as described previously (11). Lentiviruses were harvested 48 h later, centrifuged for 5 min at 3,000 rpm, aliquoted, and stored at −80 °C. Lentiviral stocks were titered following standard protocols (32). Routinely, a viral titer of 106 transducing units per ml was achieved.

**Design and Cloning of shRNA**—The short hairpin RNA (shRNA) sequence specific to hMAP2K3 (lab_hairpin_id 1:v2HS_170539) was identified by the RNAi codex portal/data base (33). The selected sequence was then adapted for cloning.
MAP2K3 Up-regulation in Mutant p53 Gain of Function

A

|          | cDNA3 | wt p53 | R175H | R273H |
|----------|-------|--------|-------|-------|
| p33null  | -     | -      | -     | -     |
| H1299    | -     | -      | -     | +     |
| p33null  | -     | +      | -     | +     |
| H1299    | -     | +      | -     | -     |

- p53
- MAP2K3
- actin

H1299 (p53-null)

B

|          | 1.0  | 0.47 |
|----------|------|------|
| sh/scr   | -    | +    |
| sh/p53   | +    | -    |

- p53
- MAP2K3
- actin

HT29
SKBR3
MDA-MB231
MDA-MB468
HCT116

HT29 (R273H)
SKBR3 (R175H)
MDA-MB231 (R208K)
MDA-MB468 (R273H)
HCT116 (wt/p53)

FIGURE 1. Mutant proteins but not wild-type p53 contribute in MAP2K3 protein up-regulation. A, p33-null H1299 cells were transiently transduced with empty (pcDNA3), wild-type p53-, mutp53R175H-, or mutp53R273H-expressing vectors. Then, 24 h later, cells were processed, and MAP2K3 protein level was established by Western blot analysis. Protein lysate (30 μg/lane) were resolved and probed with specific antibodies: anti-p53 (DO1), anti-MAP2K3, and anti-actin (loading control). B, human cancer cell lines harboring p53 mutations or wild-type p53 proteins were infected with either the sh/p53 or sh/scr lentivirus, and then 96 h later, cells were processed, and Western blot analysis was performed as reported. Intensities of MAP2K3 bands were quantified by Scion Image software and normalized to actin protein bands.

For stable ectopic expression of MAP2K3, cells were transfected with either eukaryotic pcDNA3 (empty) or pRc/RSV-FLAG-MAP2K3 (Addgene) following Lipofectamine Plus guidelines (Invitrogen). Cells were selected by Geneticin (G418 sulfate, Invitrogen), and stable transfected cells were analyzed by Western blot to monitor ectopic expression of MAP2K3 protein.

Flow Cytometry Analysis—Infected HT29 cells were maintained in either ±Dox conditions (1.0 μg/ml). At different time points, cells were harvested, washed once in phosphate-buffered saline/NaN3 1×, and fixed in MetOH/acetic acid solution (4:1) for 60 min at 4°C. Then, cells were centrifuged, resuspended in phosphate-buffered saline/NaN3 1× supplemented with 2 μg/ml RNase (150 units/ml), and incubated, during the last 30 min, in the dark with 0.1 μg/ml of propidium iodide. Subsequently, cells were analyzed by flow cytometry.

Quantitative PCR—Quantitative PCR was performed using SYBR Green (Applied Biosystems) as a marker for DNA amplification on an ABI Prism 7500 apparatus (Applied Biosystems), with 50 cycles of two-step amplification. Samples were quantified in triplicate from two independent immunoprecipitations. The relative proportions of immunoprecipitated promoter fragments were determined based on the comparative threshold (Ct) method (31). Primers sequences of the hMAP2K3 promoter used in the quantitative PCR reactions were: forward, 5′-TGAACCGGGCCCGACCTTC-3′ and reverse, 5′-TACAAGCGGTTGGGGAAC-3′.

Statistical Analysis—All experiments were performed in triplicate. Numerical data were reported as means ± S.D. Significance was assessed by Student’s t test analysis.

RESULTS

Mutf53 Up-regulates MAP2K3 Protein Expression—We previously showed (11) that exogenous expression of mutp53R175H, in p53-null H1299 cells, significantly increases MAP2K3 mRNA. Conversely, depletion of endogenous mutp53R175H or mutp53R273H proteins by RNAi induced a sizable reduction of MAP2K3 mRNA levels in human cancer cell lines (11). We wished to verify whether the observed up-regulation of MAP2K3 gene expression by mutp53 translates into increased MAP2K3 protein levels. To this aim, we first explored its modulation in p53-null H1299 cells upon ectopic expression of the wild-type (wt) p53, mutp53R175H, or
mutp53R273H proteins. As shown in Fig. 1A, ectopic expression of the p53 mutants induced a significant increase in MAP2K3 proteins levels, compared with empty-vector (pcDNA3) transfection. On the contrary, no significant effects were observed upon ectopic expression of wt p53 (Fig. 1A). To confirm these results in a more relevant cellular context, similar experiments were performed with a panel of human cancer cell lines naturally harboring mutations in the TP53 locus. MAP2K3 modulation was assessed upon RNAi depletion of endogenous mutp53 in SKBR3 (mutp53R175H), MDA-MB468 (mutp53R273H), MDA-MB231 (mutp53R280K), and HT29 (mutp53R273H) cell lines. The cells were transduced with either lentiviral vectors carrying shRNAs specific to p53 (sh/p53) or control scrambled shRNA (sh/scr) (11). mutp53 depletion induced a significant reduction in MAP2K3 protein levels in all tested cell lines (Fig. 1B, lanes 2, 4, 6, and 8). In contrast, similar experiments performed with wt p53-expressing cells (HCT116) showed no modulation of MAP2K3 protein levels (Fig. 1B, lane 10). Overall, these data indicate that, in our cancer cell line panel, MAP2K3 is likely to be a common target of mutp53R273H, mutp53R175H, and mutp53R280K proteins.

p53 Mutants Transactivate the MAP2K3 Promoter—To determine whether mutp53 up-regulates MAP2K3 expression through transactivation of its promoter, we cloned the MAP2K3 gene 5'-regulatory region (−989 to −2) upstream of a luciferase gene, generating the pMAP2K3-Luc vector (see “Experimental Procedures”). The effect of mutp53 on MAP2K3 promoter activity was evaluated by transient transfection assays in our cohort of human cancer cells. Cells infected with either the sh/p53- or the sh/scr-bearing lentiviral vector were transiently transfected 96 h later with either the pBasic-Luc (promoter-less) or the pMAP2K3-Luc vector. Promoter activity was evaluated after 24 h by luciferase assay. Fig. 2A shows that mutp53 actively contributes to MAP2K3 gene expression, though to different extents, in all tested cell lines. Consistently, depletion of endogenous mutp53 induces a significant reduction in luciferase activity. Similar experiments were then performed with p53-null H1299 cells, in which MAP2K3 promoter activity was assessed upon transient expression of either wt or mutp53 proteins (R175H, R273H). The results showed that the baseline MAP2K3 promoter activity, present in control cells, was not affected by wt p53 protein, whereas the ectopic expression of the R175H or R273H mutant induced significant increases in MAP2K3 promoter activity (Fig. 2B). These data indicate that the analyzed cancer-associated p53 mutants can up-regulate MAP2K3 expression via transactivation of the MAP2K3 promoter.

Mutp53 Proteins Are Recruited onto the MAP2K3 Promoter and Modulate Its Activity through the −499 to −167 Regulatory Region—To identify the promoter region required for mutp53 to up-regulate MAP2K3 gene expression, we generated three
MAP2K3 Up-regulation in Mutant p53 Gain of Function

Next, we asked whether mutp53 proteins contribute directly to MAP2K3 up-regulation via physical recruitment onto the MAP2K3 promoter. To this end, chromatin immunoprecipitation assays were performed on chromatin isolated from our human cancer cell lines infected with either the sh/p53 or the sh/scr lentivirus. Cyclin B2 was included in our experiments as a positive control, because it has been reported that the p53R175H mutant protein is recruited onto cell cycle-related gene promoters (25). All endogenous mutants investigated (R273H, R175H, and R280K) were found to be recruited onto the MAP2K3 promoter. To this end, chromatin immunoprecipitation assays were performed on chromatin isolated from our panel of cancer cell lines either transduced with the mutp53R175H- or the mutp53R273H-expressing vector, and transcriptional activity was evaluated 24 h later. Full-length MAP2K3 promoter activity increased significantly upon expression of either p53 mutant protein (Fig. 3C). These two mutants showed similar activities on the pdel1-Luc and pdel2-Luc reporter constructs, whereas the pdel3-Luc construct showed transcriptional activity independent of the presence of p53 mutant proteins (Fig. 3C). In conclusion, our results show that the response to mutp53 proteins maps to the −499 to −167 promoter region.

B. HT29 cells were transiently transfected with promoter-less (pBasic-Luc), full-length (pMAP2K3-Luc), or partially deleted mutant constructs along with pCMVβ-gal vector (internal control). C. p53-null H1299 cells were transiently co-transfected with full-length (pMAP2K3-Luc) or partial deleted mutant and pCMVβ-gal (internal control) constructs along with either the mutp53R175H or mutp53R273H expressing vectors. All luciferase and β-galactosidase assays were performed 24 h later, and transcriptional activities were measured 24 h later. pMAP2K3-Luc vector showed an activity ~10-fold greater than the promoterless construct. Similar activities were displayed by the pdel1-Luc and pdel2-Luc constructs. However, the −711 to −167 deletion completely abrogated promoter activity (pdel3-Luc, Fig. 3B). For confirmation, experiments were then performed with p53-null H1299 cells where the transcriptional activity of mutp53 proteins on the MAP2K3 promoter was explored by exogenously expressing different p53 mutants. The Luc reporter vectors already described were co-transfected along with either the mutp53R175H- or the mutp53R273H-expressing vector, and transcriptional activity was evaluated 24 h later. Full-length MAP2K3 promoter activity increased significantly upon expression of either p53 mutant protein (Fig. 3C). These two mutants showed similar activities on the pdel1-Luc and pdel2-Luc reporter constructs, whereas the pdel3-Luc construct showed transcriptional activity independent of the presence of p53 mutant proteins (Fig. 3C). In conclusion, our results show that the response to mutp53 proteins maps to the −499 to −167 promoter region.

FIGURE 3. mutp53 proteins are physically recruited on MAP2K3 regulatory region (−499 to −167). A, partially deleted constructs were produced by cutting restriction enzymes (see under “Experimental Procedures”). Maps of partially deleted promoter with restriction enzymes sites are reported.

C. H1299 (p53-null) cells were transiently transfected with pCMVβ-gal (internal control), p53-null H1299 cells were transiently co-transfected with full-length (pMAP2K3-Luc) or partial deleted mutants and pCMVβ-gal (internal control) constructs along with either the mutp53R175H or mutp53R273H expressing vectors. All luciferase and β-galactosidase assays were performed 24 h later, and transcriptional activities (Luc) were normalized as reported. White bars, vector reporter alone; black bars, vector reporter and mutp53R175H-expressing vector; gray bars, vector reporter and mutp53R273H-expressing vector. Means and S.D. of three independent experiments are reported. *, p < 0.05 and **, p < 0.1. D, multiple mutp53 proteins are physically recruited on the MAP2K3 regulatory region. Chromatin derived from our panel of cancer cell lines either transduced with the sh/p53 or sh/scr lentivirus were immunoprecipitated with anti-p53 (p53, Ab7) or no antibody (No-ab) as negative control. PCR analyses were performed on immunoprecipitated DNA samples, by using a specific set of primers for MAP2K3 and cyclin B2 promoters (see under “Experimental Procedures”). Similar PCR analyses were performed in an adjacent region (−3200 bp, negative control probe) to MAP2K3 regulatory region chosen to verify the specificity of achieved results. A representative panel of several analyses is reported.
MAP2K3 Up-regulation in Mutant p53 Gain of Function

Mutp53 Regulates MAP2K3 Gene Expression through the NF-Y and NF-κB Transcriptional Cofactors—Studies published in the past few years indicate that mutp53 may exert its GOF activity via transcriptional regulation of target genes through the formation of large transcriptional complexes (reviewed in Ref. 34). To identify transcriptional cofactors through which mutp53 might regulate MAP2K3 expression, we searched for consensus binding sites using the MatInspector software tool. An analysis of the mutp53-responsive promoter region (−499 to −167) showed that it includes consensus sequences for several transcription factors, but no TATA box. Therefore, among the transcription factors potentially binding this region, we focused on those known to functionally interact or form complexes with mutp53, such as NF-Y (25) and NF-κB (20, 26, 27) (Fig. 4A). To ascertain their involvement in mutp53-mediated MAP2K3 up-regulation, we first analyzed the in vivo recruitment of NF-Y and NF-κB on MAP2K3 regulatory regions. Chromatin immunoprecipitation analyses were performed on chromatin from HT29 cells transduced with either the sh/p53 or sh/scr lentivirus. Chromatin was immunoprecipitated with antibodies to NF-YB, NF-κB (p50), or acetylated histone H4 (H4ac). Acetylated histone H4 was chosen to assess the accessibility of the chromatin regions analyzed. The results showed that both transcriptional cofactors are recruited to the MAP2K3 regulatory region. In particular, NF-Y occupancy did not vary between the analyzed conditions (sh/p53 and sh/scr cells) (Fig. 4B, left panel, lane 1), in agreement with published data (24). In contrast, a reduced recruitment of NF-κB (p50) was observed in sh/p53, compared with sh/scr cells (Fig. 4B, left panel, lane 3). Acetylated histone 4 behaved similarly to NF-κB (Fig. 4B, left panel, lane 2), further confirming that the MAP2K3 promoter activity was significantly reduced by mutp53 depletion. Quantitative PCR measurements confirmed quantitatively the observed effect (Fig. 4B, right panel). The absence of signal in an adjacent

Furthermore, no amplification of an adjacent region (−3200 bp) on the MAP2K3 promoter was detected. These findings indicate that up-regulation of MAP2K3 occurs through in vivo recruitment of mutp53 proteins onto specific MAP2K3 regulatory regions.

FIGURE 4. mutp53R273H and mutp53R175H proteins regulate MAP2K3 gene expression through NF-Y and NF-κB transcriptional cofactors. A, a map of identified MAP2K3 regulatory region responsive to mutp53 with consensus sequences for transcription cofactors NF-Y (−281 and −267 with respect to the translational start site) and NF-κB (−231 and −219 with respect to the translational start site). Restriction enzymes (SmaI and PstI) refer to the isolated regulatory region. B, left panel, NF-Y and NF-κB transcription cofactors are physically recruited on the MAP2K3 promoter. Chromatins from HT29 cells transduced with either the sh/p53 or sh/scr lentivirus were immunoprecipitated with the following antibody: anti-NF-YB, or anti-NF-κB super-repressor, or anti-H4ac or no antibody (No-Ab) as a negative control. PCR analyses were performed on immunoprecipitated DNA samples by using specific set of primers for MAP2K3 promoter. An adjacent region (−3200 bp, negative control probe) was included to demonstrated the specificity of the achieved results. B, right panel, quantitative PCR analyses (see under “Experimental Procedures”). C, NF-Y (YA13m29) and NF-κB (IκBα super-repressor, IκB-SR) dominant-negative mutants abrogate mutp53-mediated MAP2K3 promoter activity. p53-null H1299 cells were transiently co-transfected with the full-length promoter construct (pMAP2K3-Luc) along with empty (pcDNA3, black bar), or mutp53R75H (gray bars) or mutp53R273H (white bars) expressing vectors and increasing amount (µg) of either YA13m29 (upper panel) or IκBα super-repressor (IκBα SR, lower panel) dominant-negative-expressing vectors. Either the pCMVβ-gal or pRSVβ-gal vectors (internal control) were included in described co-transfection experiments. Transcriptional activity was then monitored 24 h later by luciferase and β-galactosidase assay as reported. Means and S.D. of three independent experiments are reported.
region (−3200 bp) demonstrated the specificity of the results. To rule out the possibility that mutp53 affects the steady state levels of NK-κB, thereby altering MAP2K3 expression, we monitored NF-κB protein in H1299 cells upon exogenous expression of either the mutp53R175H or mutp53R273H protein. Supplemental Fig. 1 shows that ectopic expression of the mutants does not modify NF-κB protein levels with respect to control cells.

To evaluate the functional involvement of NF-Y and NF-κB in mutp53-mediated MAP2K3 up-regulation, we exploited dominant-negative mutants of NF-Y (YA13m29) (35) or NF-κB (IκBα super-repressor) (36). p53-null H1299 cells were transiently co-transfected with the full-length promoter (pMAP2K3-Luc) along with the mutp53R175H or mutp53R273H or empty (pcDNA3) vector and increasing amounts of either YA13m29- or IκBα super-repressor-expressing vector. Transcriptional activity was measured 24 h later. These experiments showed that the increased MAP2K3 promoter activity induced by p53 mutants was significantly repressed in the presence of either dominant-negative mutant (Fig. 4C). Taken together, our findings indicate that mutp53 up-regulates MAP2K3 expression through the involvement of NF-Y and NF-κB transcriptional cofactors.

Knockdown of Endogenous MAP2K3 Protein Interferes with mutp53 GOF Effects—To evaluate whether MAP2K3 contributes to mutp53 GOF activity, we explored the biologic effects of depleting the endogenous MAP2K3 protein by RNAi. To this end, we constructed a lentiviral vector bearing a MAP2K3 shRNA (see “Experimental Procedures”). HT29 cells conditionally expressing the MAP2K3 or the scr shRNA were generated as described previously (11). The engineered cell lines were then challenged with doxycycline (+Dox), and MAP2K3 depletion efficiency was monitored. Maximal depletion of endogenous MAP2K3 was achieved after 144 h of +Dox treatment (Fig. 5A, left panel). In addition, because MAP2K3 is a specific upstream activator of p38 MAPK (28), we evaluated whether silencing MAP2K3 compromises its signaling cascade by monitoring the phosphorylation status of ATF2, a downstream factor of p38 MAPK. Indeed, depletion of MAP2K3 causes a significant reduction in phospho-ATF2 at 144 h of +Dox treatment (Fig. 5A, left panel). Accordingly, depletion of the endogenous mutp53R273H protein sharply reduced ATF2 phosphorylation (Fig. 5A, right panel). Together, these results suggest that the knockdown of mutp53 and the ensuing reduc-
MAP2K3 Up-regulation in Mutant p53 Gain of Function

Depletion of MAP2K3 impairs cell proliferation and survival of other human cancer cell lines. MDA-MB468 (A), MDA-MB231 (B), and SKBR3 (C) cells were transduced with either the sh/MAP2K3 or sh/scr lentivirus. Then, 96 h later, cells were plated in 6-well plates (2 × 10^4 cells/well), and proliferation (left panels) and survival (right panel) assays were performed at different time points as reported. Means and S.D. of three independent experiments are reported.

FIGURE 6. Depletion of MAP2K3 impairs cell proliferation and survival of other human cancer cell lines. MDA-MB468 (A), MDA-MB231 (B), and SKBR3 (C) cells were transduced with either the sh/MAP2K3 or sh/scr lentivirus. Then, 96 h later, cells were plated in 6-well plates (2 × 10^4 cells/well), and proliferation (left panels) and survival (right panel) assays were performed at different time points as reported. Means and S.D. of three independent experiments are reported.

Depletion of MAP2K3 levels impair p38 MAPK signaling. We previously reported that the knockdown of endogenous mutp53 impacts on the proliferation and survival of HT29 and SKBR3 cells (10). To explore whether MAP2K3 depletion affects mutp53 GOF activities on cell proliferation and cell survival, engineered HT29 cells were plated in the presence (+Dox) or absence (−Dox) of doxycycline and total cell numbers, cell viability, and cell cycle profiles were analyzed in time. MAP2K3 depletion strongly impaired cell proliferation (Fig. 5B, left panel) and cell survival (Fig. 5B, right panel) and induced significant cell accumulation in G2/M phase (Fig. 5C).

Similar experiments were performed with our other cancer cell lines. We first established that mutp53 enhances proliferation and survival of MDA-MB231 (R280K) and MDA-MB468 (R273H) cell lines. Similarly to HT29 and SKBR3 cells (10), depletion of mutp53R280K and mutp53R273H, respectively, in MDA-MB231 and MDA-MB468 cells compromises cell proliferation and survival (supplemental Fig. 2). Afterward, we explored whether depletion of MAP2K3 compromises proliferation and survival of our other cancer cell lines. Efficient knockdown of MAP2K3 (supplemental Fig. 3, A–C) affects proliferation of all studied cell lines (Fig. 6, A–C, left) and survival of MDA-MB468 and SKBR3 cells (Fig. 6, A–C, right). One possible reason of the different sensitivity to survival of MDA-MB231 may be due to additional survival signals provided by expression of activated mutant Ras protein in these cells (37). Overall, our findings suggest that, in our panel of cancer cell lines, MAP2K3 depletion strongly impairs cell proliferation and cell survival (Fig. 5B, left panel) and cell survival (Fig. 5B, right panel), and induced significant cell accumulation in G2/M phase (Fig. 5C).

One possible reason of the different sensitivity to survival of MDA-MB231 may be due to additional survival signals provided by expression of activated mutant Ras protein in these cells (37). Overall, our findings suggest that, in our panel of cancer cell lines, MAP2K3 depletion strongly impairs cell proliferation and cell survival (Fig. 5B, left panel) and cell survival (Fig. 5B, right panel), and induced significant cell accumulation in G2/M phase (Fig. 5C).

Similar experiments were performed with our other cancer cell lines. We first established that mutp53 enhances proliferation and survival of MDA-MB231 (R280K) and MDA-MB468 (R273H) cell lines. Similarly to HT29 and SKBR3 cells (10), depletion of mutp53R280K and mutp53R273H, respectively, in MDA-MB231 and MDA-MB468 cells compromises cell proliferation and survival (supplemental Fig. 2). Afterward, we explored whether depletion of MAP2K3 compromises proliferation and survival of our other cancer cell lines. Efficient knockdown of MAP2K3 (supplemental Fig. 3, A–C) affects proliferation of all studied cell lines (Fig. 6, A–C, left) and survival of MDA-MB468 and SKBR3 cells (Fig. 6, A–C, right). One possible reason of the different sensitivity to survival of MDA-MB231 may be due to additional survival signals provided by expression of activated mutant Ras protein in these cells (37). Overall, our findings suggest that, in our panel of cancer cell lines, MAP2K3 depletion strongly impairs cell proliferation and cell survival (Fig. 5B, left panel) and cell survival (Fig. 5B, right panel), and induced significant cell accumulation in G2/M phase (Fig. 5C).

Similar experiments were performed with our other cancer cell lines. We first established that mutp53 enhances proliferation and survival of MDA-MB231 (R280K) and MDA-MB468 (R273H) cell lines. Similarly to HT29 and SKBR3 cells (10), depletion of mutp53R280K and mutp53R273H, respectively, in MDA-MB231 and MDA-MB468 cells compromises cell proliferation and survival (supplemental Fig. 2). Afterward, we explored whether depletion of MAP2K3 compromises proliferation and survival of our other cancer cell lines. Efficient knockdown of MAP2K3 (supplemental Fig. 3, A–C) affects proliferation of all studied cell lines (Fig. 6, A–C, left) and survival of MDA-MB468 and SKBR3 cells (Fig. 6, A–C, right). One possible reason of the different sensitivity to survival of MDA-MB231 may be due to additional survival signals provided by expression of activated mutant Ras protein in these cells (37). Overall, our findings suggest that, in our panel of cancer cell lines, MAP2K3 depletion strongly impairs cell proliferation and cell survival (Fig. 5B, left panel) and cell survival (Fig. 5B, right panel), and induced significant cell accumulation in G2/M phase (Fig. 5C).

To assess whether MAP2K3 is capable to mediate mutp53 GOF activities, we stably expressed exogenous MAP2K3 in our cohort of human cancer cell lines. Results show MDA-MB468 cells as more suitable hosts for stable and sustained expression of ectopic MAP2K3 (Fig. 7A, left panel). To measure the effect of MAP2K3 on mutp53 GOF activities, stable transfected cells were infected with either the sh/p53 or sh/scr lentivirus, and cell proliferation was assessed. Depletion of mutp53R273H markedly reduces proliferation of empty vector-transfected cells (pcDNA3) (Fig. 7A, right panel). In contrast, upon stable ectopic expression of MAP2K3, depletion of mutp53 had little or any effect on cell proliferation of MDA-MB468 cells (Fig. 7A, right panel). These findings suggest that mutp53 is required for cell proliferation of MDA-MB468 cells and ectopic expression of MAP2K3, as a target of mutp53, is sufficient to compensate for mutp53 knockdown.

Finally, to highlight the relevance of our studies, we evaluated the MAP2K3 expression of primary tumors by querying public gene expression data repositories (Oncomine 4 research edition) (38). Analysis of data sets obtained from specimens of primary tumors revealed that the MAP2K3 transcription is significantly higher in breast (39) and colon (40) cancers carrying p53 mutations in comparison with those carrying wt p53 protein (Fig. 7B). In conclusion, our
results confirmed MAP2K3 as a mutp53 target gene and provided evidence that one of the mechanisms by which mutp53 exerts its GOF activity is through the up-regulation of MAP2K3 expression.

**DISCUSSION**

Recently reported data indicate that the MAP2K3 up-regulation is involved in invasion and progression of glioma and breast tumors. In this study, we show the first evidence that different endogenous mutp53 proteins transcriptionally up-regulate MAP2K3 expression in diverse human tumor cell lines. Ample data indicate that mutp53 proteins do not lose only their tumor suppressive functions, but they do gain new abilities that promote tumorigenesis by influencing cancer cell transcriptome and phenotype. In particular, we found that knockdown of mutp53 inhibits, whereas ectopic expression of mutp53 increases MAP2K3 transcription. Contrarily, no significant effects were observed upon ectopic expression of wt p53 in p53-null H1299 cells or upon depletion of endogenous wt p53 in the HCT116 cell line. These data showed that, in our panel of cancer cell lines, MAP2K3 is likely to be a common target of mutp53 proteins.

By analysis for deletions, we identified a MAP2K3 regulatory region (−499 to −167) required to mutp53 proteins to modulate MAP2K3 expression. We found that MAP2K3 up-regulation occurs by physical recruitment of p53 mutant proteins...
onto the MAP2K3 regulatory region. Moreover, we found that NF-Y and NF-κB transcriptional cofactors are recruited on MAP2K3 promoter and thus required for MAP2K3 up-regulation. Consistently with data found in the literature, our findings are indicative that both transcriptional cofactors are relevant players in the mutp53 transcriptome. Their involvement in biologic processes such as cell proliferation (NF-Y) and inflammation (NF-κB) highlight the relevance of their contribution in mutp53 GOF activity.

To better understand the involvement of MAP2K3 up-regulation in the mutp53 GOF activity, we explored biologic effects linked to modulation of the MAP2K3 expression in our panel of cancer cell lines. We found that knockdown of endogenous MAP2K3 inhibits cell proliferation and survival. In contrast, the exogenous expression of MAP2K3 is sufficient to compensate alterations of cell proliferation upon silencing of mutp53.

Moreover, by querying public gene expression data repositories, we showed a higher expression of MAP2K3 mRNA in specimens of human primary tumors harboring p53 mutations in respect to those expressing wt p53 protein. In conclusion, our present study suggests that one possible mechanism through which mutants of p53 may acquire their GOF activities is via up-regulation of MAP2K3.

Acknowledgments—We thank Dr. Marco Cippitelli for providing reagents and valuable suggestions and Dr. Silvia Soddu and Dr. Marco Crescenzi for helpful discussions and critical reading of the manuscript.

REFERENCES

1. Hainaut, P., and Hollstein, M. (2000) Adv. Cancer Res. 77, 81–137
2. Bullock, A. N., and Fersht, A. R. (2001) Nat. Rev. Cancer 1, 68–76
3. Harris, S. L., and Levine, A. J. (2005) Oncogene 24, 2899–2908
4. Oren, M. (2003) Cell Death Diff. 10, 413–442
5. Aas, T., Borresen, A. L., Geisler, S., Smith-Sørensen, B., Johnsen, H., Varhaug, J. E., Akslen, L. A., and Lønning, P. E. (1996) Nature Med. 2, 811–814
6. Blandino, G., Levine, A. J., and Oren, M. (1999) Oncogene 18, 477–485
7. Lang, G. A., Iwakuma, T., Suh, Y. A., Liu, G., Rao, V. A., Parant, J. M., Varhaug, J. E., Akslen, L. A., and Lønning, P. E. (1996) Cancer Res. 56, 13550–13555
8. Kozma, S. C., Bogaard, M. E., Buser, K., Saurer, S. M., Bos, J. L., Groner, B., Charro, T. M., Krammer, P. H., Rotter, V., and Oren, M. (2003) Oncogene 22, 5667–5676
9. Weisz, L., Zalcenstein, A., Stambolsky, P., Weisz, L., Müller, M., Wallach, D., Goncharov, T. M., Krammer, P. H., Rotter, V., and Oren, M. (2003) Oncogene 22, 5667–5676
10. Bossi, G., Lapi, E., Strano, S., Rinaldo, C., Blandino, G., and Sacchi, A. (2006) Oncogene 25, 359–369
11. Mizuari, S., Yamanaka, K., and Kotani, H. (2006) Cancer Res. 66, 6319–6326
12. Frazier, M. W., He, X., Wang, J., Gu, Z., Cleveland, J. L., and Zambetti, G. P. (1998) Mol. Cell Biol. 18, 3735–3743
13. Zalcenstein, A., Stambolsky, P., Weisz, L., Müller, M., Wallach, D., Goncharov, T. M., Krammer, P. H., Rotter, V., and Oren, M. (2003) Oncogene 22, 5667–5676
14. Frazier, M. W., He, X., Wang, J., Gu, Z., Cleveland, J. L., and Zambetti, G. P.