THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 38, pp. 26143–26154, September 19, 2014

The Transcription Factor MEF/Elf4 Is Dually Modulated by p53-MDM2 Axis and MEF-MDM2 Autoregulatory Mechanism*

Received for publication, May 9, 2014, and in revised form, July 22, 2014 Published, JBC Papers in Press, July 31, 2014, DOI 10.1074/jbc.M114.580209

Mary Ann Suico[1], **Ryosuke Fukuda, Rui Miyakita, Kosuke Koyama, Manabu Taura, Tsuyoshi Shuto, and Hirofumi Kai**[2]

From the Department of Molecular Medicine, Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan

Background: The ETS transcription factor myeloid elf-1-like factor (MEF) activates some genes including lysozyme, interleukin-8, and MDM2, and also influences the cell cycle.
Results: MEF protein expression and stability is suppressed by MDM2 in p53-dependent and -independent manner.
Conclusion: MEF is targeted by MDM2 for degradation.
Significance: The previously unrecognized MEF-MDM2-p53 axis highlights a regulatory balance of these transcription factors.

Myeloid Elf-1-like factor (MEF) or Elf4 is an ETS transcription factor that activates innate immunity-associated genes such as lysozyme (*LYZ*), human β-defensin 2 (*HβD2*), and interleukin-8 (*IL-8*) in epithelial cells and is also known to influence cell cycle progression. MEF is transcriptionally activated by E2F1, but the E2F1-mediated transcriptional activation is inhibited by p53 through E2F1-p53 protein interaction. Although the transcriptional activation of MEF has been investigated in depth, its post-translational regulation is not well explored. By overexpressing MEF cDNA in human cell lines, here we show that MEF protein expression is suppressed by p53. By screening a number of E3 ligases regulated by p53, we found that MDM2 is involved in the effect of p53 on MEF. MDM2 is transcriptionally activated by p53 and interacts with MEF protein to enhance MEF degradation. MDM2 reduces MEF protein expression, as well as stability and function of MEF as transcriptional activator. Furthermore, MDM2 was able to down-regulate MEF in the absence of p53, indicating a p53-independent effect on MEF. Notably, MEF transcriptionally activates MDM2, which was previously demonstrated to be the mechanism by which MEF suppresses the p53 protein. These results reveal that in addition to the potential of MEF to down-regulate p53 by transcriptionally activating E3 ligase MDM2, MEF participates with MDM2 in a novel autoregulatory feedback loop to regulate itself. Taken together with the findings on the effect of p53 on MEF, these data provide evidence that the p53-MDM2-MEF axis is a feedback mechanism that exquisitely controls the balance of these transcriptional regulators.

The members of the E-twenty-six (ETS)[3] family of transcription factors, which are found exclusively in multicellular organisms, function as transcriptional activators or repressors and play critical roles in development, cellular differentiation, proliferation, and transformation (1). The ETS factors have oncogenic and tumor suppressive activities and their aberrant expressions are associated with many of the processes that lead to cancer progression (2). One member of the ETS family, myeloid elf-1-like factor (MEF) or Elf4, is known for its role in promoting cell cycle progression from G_1 to S phase and driving hematopoietic stem cells from quiescent to G_1 phase (3, 4). MEF may have tumorigenic functions (5), but it has also been shown to possess tumor suppressor activity in lung carcinoma cells (6). As a transcription factor, MEF activates a number of innate immunity-associated genes, notably lysozyme (*LYZ*) (7), human β-defensin 2 (*HβD2*) (8), granulocyte macrophage colony-stimulating factor (9), interleukin (*IL*)-*3* (9), *IL-8* (10), and perforin (11). Besides activating innate immune molecules, it was recently reported that MEF is highly expressed in gliomas and activate *SOX2* expression to promote stemness in glioblastoma (12). MEF also transcriptionally activates *MDM2*, a well known E3 ligase of the tumor suppressor p53. MEF-mediated activation of *MDM2* leads to decreased p53 stability and inhibition of p53-dependent oncogene-induced senescence (13). Given the various target genes of MEF, it is obvious that as a transcription factor, MEF plays diverse roles in the cell.

With regards to the regulation of MEF, our earlier studies have made headway in the understanding of MEF transcription and post-translational modification. MEF transcription is facilitated by the ubiquitous zinc finger transcriptional activator Sp1, which binds to the proximal promoter region of *MEF* (14). Notably, we previously demonstrated that *MEF* transcription is activated by E2F1, but the E2F1-mediated *MEF* activation is suppressed by p53 through E2F1-p53 interaction that sequesters E2F1 away from *MEF* promoter (15). MEF expression and activity are also governed by post-translational modifications.

* This work was supported by Grants-in-Aid for Science Research from the Ministry of Education, Science, Sports, and Culture of Japan (MEXT) 22390015 (to H. K.) and 23590082 (to M. S.) and the Program for Leading Graduate Schools "HIGO" (Health life science: Interdisciplinary and Glocal Oriented), MEXT, Japan.
[1] To whom correspondence may be addressed. E-mail: mann@gpo.kumamoto-u.ac.jp.
[2] To whom correspondence may be addressed: Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. Tel./Fax: 81-96-371-4405; E-mail: hirokai@gpo.kumamoto-u.ac.jp.

[3] The abbreviations used are: ETS, E-twenty-six; MEF, myeloid elf-1-like factor; 5-FU, 5-fluorouracil; DMSO, dimethyl sulfoxide; CHX, cycloheximide; LMB, leptomycin B; P-MT, phosphorylation site mutant; TAD, transactivation domain; ANOVA, one-way analysis of variance.

TABLE 1
siRNA oligonucleotide sequences

Gene	Forward	Reverse
p53 siRNA	5′-gacuccagugguaaucuatt-3′	5′-guagauuaccacuggaguctt-3′
MDM2 siRNA	5′-gccuggcucuguguguaautt-3′	5′-uuacacacagagccaggctt-3′
Siah1 siRNA	5′-gcugauaggaacacgcaagcatt	5′-ugcuugcguguuccuaucagctt-3
GL2 siRNA	5′-cguacgcggaauacuucgatt-3′	5′-ucgaaguauuccgcguacgtt-3′

MEF is SUMOylated, which leads to diminished transactivation functions of MEF (16). MEF activity is enhanced upon interaction with promyelocytic leukemia protein that induces accumulation of MEF in the promyelocytic leukemia nuclear bodies (17, 18). MEF is phosphorylated by the cyclin A-cdk2 complex, ubiquitinated by Skp1/Cul1/F-box (SCF) E3 ubiquitin ligase complex SCF^{Skp2}, and degraded by proteasome at the G_1/S phase transition (19). Skp2 specifically degrades the phosphorylated form of MEF following cyclin A-mediated MEF phosphorylation (19). Aside from Skp2, no other E3 ligase has been reported for MEF.

The *P53* gene is a central integrator of multiple signaling networks that essentially protects the integrity of the genome against DNA damage and oncogenic processes (20). Normally, p53 protein levels are low due to its proteasomal degradation that is mainly directed by MDM2, which is also a transcriptional target of p53 (21). This ensures a tight control of p53 at the basal state. Stabilization of p53 occurs because of post-translational modifications during cellular stress or DNA damage, most notably, phosphorylation of p53 serine residues that preclude p53 interaction with MDM2 (22, 23). After p53 has served its functions as "cellular stress sentinel," it is presumed that p53 reverts to its basal state and kept at low level by MDM2. Aside from p53, MDM2 has many targets and it degrades numerous proteins (24). The seemingly opposite functions of MEF and p53 in cellular proliferation, and the suppression of p53 expression by MEF via MDM2 (13) led us to consider that p53 could reciprocally antagonize MEF protein expression. Here, we show evidence that p53 down-regulates the protein expression of MEF by transcriptionally activating MDM2, which interacts with MEF and leads to MEF protein degradation. Moreover, our results showed that in the absence of p53, MDM2 could still negatively regulate the expression and stability of MEF, revealing MEF as a novel client of MDM2. Because MEF transcriptionally activates *MDM2* (13), these findings also reveal that MEF is linked to MDM2 in an autoregulatory feedback mechanism.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Nutlin-3 (number 430-128-M001) was obtained from Alexis Biochemicals (San Diego, CA). 5-Fluorouracil (5-FU) was purchased from Wako (Osaka, Japan). MG-132 was from Calbiochem (number 474790). Cycloheximide (CHX; number C7698) was obtained from Sigma. Leptomycin B (LMB; sc-358688) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies purchased from Santa Cruz Biotechnology are the following: p53 (DOI; sc-126), Elf-4/MEF (M-20; sc-101947), MDM2 (SMP14; sc-965), Actin (I-19; sc-1616), γ-tubulin (C-20; sc-7396), normal mouse IgG (sc-2025), and normal rabbit IgG (sc-2027). HA tag polyclonal antibody (number 3808-1) was obtained from Clontech (Palo Alto, CA). Anti-Hsc70 antibody (SPA-815) was from Stressgen Bioreagents (Canada). The horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Cell Culture, Transfection, and Treatment—Human colorectal cell lines HCT116 $p53^{+/+}$ (wild-type) and HCT116 $p53^{-/-}$ (knock-out) were kindly provided by Bert Vogelstein (Johns Hopkins University). Cervical carcinoma cells (HeLa), lung adenocarcinoma cells (A549), and human embryonic kidney cells (HEK293) were obtained from the American Type Culture Collection. HCT116 cell lines were cultured in Dulbecco's modified Eagle's medium-Ham's F-12 (DMEM/F-12) medium. HeLa was cultured in minimal essential medium. A549 and HEK293 cell lines were cultured in DMEM. All media were supplemented with 10% fetal bovine serum (FBS) and 2% antibiotics. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Transient transfections of DNAs were performed using TransIT-LT1 reagent (Mirus, Madison, WI) according to the recommended protocol. Briefly, LT1 reagent diluted with reduced serum Opti-MEM (Invitrogen) was incubated with DNA at 1:3 ratio (DNA:LT1) for 20 min at room temperature. The complex was applied to subconfluent cells. Transfection of small interfering RNA (siRNA) was carried out using TransIT-TKO reagent (Mirus). Diluted TKO was mixed with siRNA, and the complex was added to subconfluent cells. GL2-luciferase siRNA (siGL2) was used as control. The siRNA oligonucleotides used in these experiments are listed in Table 1. Forty-eight hours after transfection, samples were collected for analysis. For chase experiments, at 48 h post-transfection cells were treated with 0.5 mM CHX and harvested immediately (0 h), or at 3 or 6 h after CHX treatment. For MG132 treatment, cells were treated with 10 μM MG132 or DMSO (control) and co-treated with 0.2 mM CHX. Cell lysates were collected immediately, at 3 or 6 h after treatment.

Plasmids—The full-length cDNA for human *MEF* (1992 bp) in pCB6 vector and the lysozyme promoter in luciferase reporter pGL2 vector were generated as described previously (7). MEF phosphorylation mutant (P-MT) has mutations at Ser-641, Thr-643, and Ser-648, the potential phosphorylation sites (3), which were all changed to alanine using the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). HA-tagged *MEF* was generated by cloning full-length MEF cDNA into pCMV-HA vector (Clontech). This was done using pCB6-MEF as template, Zero Blunt TOPO PCR cloning kit (Invitrogen), and the following primers: 5′-primer, 5′-gcatggctattaccctacagcccagtg-3′ and 3′-primer, 5′-cacctcgagttatatgtcatggggctc-3′. The *MEF* fragment in TOPO vector was digested with EcoRI and ligated into pCMV-HA vector. The resulting plasmid was sequenced to verify the insert. The *P53* wild-type expression plasmid in pCDM8 vector was gener-

TABLE 2
Primers used for the generation of mutant constructs

Gene	Sequence[a]
p53 TAD1 MT_forward	5′-cattttcagacctatggaaacAaAGtcctgaaaacaac-3′
p53 TAD1 MT_reverse	5′-gttgttttcaggaCTtTgtttccataggtctgaaaatg-3′
p53 TAD2 MT_forward	5′-ggacgatattgaacaaCAgtCcactgaagaccccaggtc-3′
p53 TAD2 MT_reverse	5′-gacctgggtcttcagtgGacTGttgttcaatatcgtcc-3′
MEF P-MT_forward	5′-cttctgacaagagcTcccGccccagccccttt cGccccattcaac-3′
MEF P-MT_reverse	5′-gttgaatggggCgaaaggggctggggCgggAgctcttgtcagaag-3′

[a] Capital letters are mutated bases.

ously provided by Dr. Hideyuki Saya (25). We constructed the *P53* transactivation domain (TAD) mutant, which has mutations in both TADs, using the primers listed in Table 2 and the *P53* wild-type cDNA as template. The point-mutated construct was prepared using the QuikChange II XL site-directed mutagenesis kit (Stratagene). The generated construct was verified by sequencing before DNA was used for experiments. *MDM2* in pcDNA3 (26) was obtained from Addgene (plasmid 16233).

Real-time Quantitative PCR Analysis—Total RNA was isolated from cells using RNAiso Plus (Takara, Japan) according to the recommended protocol. Real-time quantitative RT-PCR analyses were carried out with SYBR Green Master Mix (Applied Biosystems). PCR amplifications were performed as described previously (27). The C_t values for each gene amplification were normalized by subtracting the C_t value calculated for 18 S ribosomal RNA (*18 S* rRNA; internal control). The normalized gene expression values were expressed as the relative quantity of gene-specific mRNA. The oligonucleotide primers used in the real-time quantitative PCR amplifications are listed in Table 3. The human perforin and *HβD2* primer sequences had been previously reported (28, 29).

Luciferase Assay—HCT116 cells were seeded in 12-well plates. At 40–50% confluent cells, DNAs were co-transfected. Specifically, 0.2 μg of lysozyme-luc promoter, 20 ng of phRG-TK *Renilla* reporter (Promega), 0.1 μg of MEF and the indicated amount of p53 were transfected. Empty vector was added when necessary to ensure equal amounts of input DNA. Luciferase activity was determined 48 h after transfection using the dual-luciferase reporter assay system (Promega) and a luminometer (TD-20/20; Promega) according to the manufacturer's instructions. Luciferase activity for each sample was normalized to the *Renilla* reporter as described previously (18).

Western Blotting and Immunoprecipitation Analysis—For analysis of protein expression, cells grown in 6-well plates were lysed on ice for 30 min with lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.5% SDS, 5% deoxycholate, and 5% Nonidet P-40). The lysate was added with 4 volumes of dilution buffer (150 mM NaCl and 50 mM Tris-HCl, pH 8.0) and mildly sonicated. Samples were centrifuged to remove debris, and total protein lysates were collected. For nuclear extraction and isolation of cytoplasmic fraction, HCT116 cells were suspended by gentle pipetting in 100 μl of ice-cold buffer containing 10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA (pH 8.0), and 0.1 mM EGTA (pH 8.0). The cells were allowed to swell on ice for 15 min, then 6.25 μl of 10% Nonidet P-40 solution was added and samples were vortexed vigorously for 10 s. The homogenate was centrifuged at 15,000 × *g* for 1 min at 4 °C. The lysate was collected as the cytoplasmic fraction. The nuclear pellet was resuspended in 25 μl of ice-cold nuclear lysis buffer (20 mM HEPES-KOH (pH 7.9), 400 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM EGTA (pH 8.0)). The samples were vigorously vortexed for 20 min at 4 °C, and centrifuged at 15,000 × *g* for 5 min at 4 °C. The nuclear lysate was collected for analysis. Equal amounts of proteins were subjected to Western blot analysis using the protocol described previously (18, 27). Immunoprecipitation was performed as essentially reported in Ref. 18. Briefly, HCT116 cells were washed with cold PBS(−) twice and cross-linked with 1 mM dithiobis[succinimidylpropionate] for 30 min at room temperature. Tris-HCl (pH 7.5; 20 mM final concentration) was added to stop the cross-linking reaction. Cells were pelleted, and total protein lysates or nuclear extracts were collected using the methods of protein extraction as described above. Lysates were pre-cleaned with Sepharose-G beads (Amersham Biosciences). Pre-cleared lysates were reacted with 2 μg of MDM2, HA, ELF4, normal mouse IgG, or normal rabbit IgG antibodies by gentle rotation at 4 °C for 2 h. Sepharose beads were added and the reaction was continued for 1 h. The beads were washed with wash buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 0.5% Nonidet P-40). Immunoprecipitates were eluted by boiling for 5 min with 2× SDS sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, 12% (v/v) β-mercaptoethanol, and bromphenol blue). Samples were loaded on 7.5% SDS-PAGE gels and blotted onto PVDF membrane (Millipore Corp., Bedford, MA). Blots were probed with appropriate antibodies and visualized using SuperSignal chemiluminescence reagent (Pierce).

Statistical Analysis—Data are presented as mean ± S.E. as indicated in the figure legends. Significance of the difference between 2 groups was assessed using Student's unpaired two-tailed *t* test. For 3 or more group comparisons, we used one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test or with Tukey-Kramer (JMP software; SAS Institute, and Statcel3 3rd edition, OMS Publication, Japan). A p value of <0.05 is considered statistically significant.

RESULTS

p53 Modulates the Level of MEF Protein—To scrutinize the relationship between MEF and p53 at the protein level, we compared the exogenously delivered or endogenous MEF protein expression between HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells. The endogenous MEF could be detected in nuclear extracts although it is mostly undetectable in total cell lysate (Fig. 1, *A* and *B*). Confirming the observation we reported previously (15), endogenous MEF protein was lower in HCT116 $p53^{+/+}$ cells than in HCT116 $p53^{-/-}$ cells (Fig. 1*A*). This difference could be attributed to the inhibitory effect of p53 on E2F1-

TABLE 3
Primers used for real-time quantitative RT-PCR

Gene	Forward	Reverse
LYZ	5′-aaaaccccaggagcagttaat-3′	5′-caaccctctttgcacaagct-3′
Perforin	5′-cgcctacctcaggcttatctc-3′	5′-cctcgacagtcaggcagtc-3′
IL-8	5′-ctggccgtggctctcttg-3′	5′-ccttggcaaaactgcaccctt-3′
Human β-defensin 2	5′-atcagccatgagggtcttgt-3′	5′-gagaccacaggtgccaattt-3′
UBE4B	5′-tcgccctctaatagccttga-3′	5′-tatcactgaggctccgcttt-3′
PIRH2	5′-gacagctggatgatgaagtagcaca-3′	5′-ctcgtcattgctgatccagtgaa-3′
SIAH1	5′-ttgcttaccgacttgagctaaatg-3′	5′-gctgttgcaattccttcatgaa-3′
hMDM2	5′-acctcacagattccagcttcg-3′	5′-tttcatagtataagtgtcttttt-3′
18 S rRNA	5′-cggctaccacatccaaggaa-3′	5′-gctggaattaccgcggct-3′

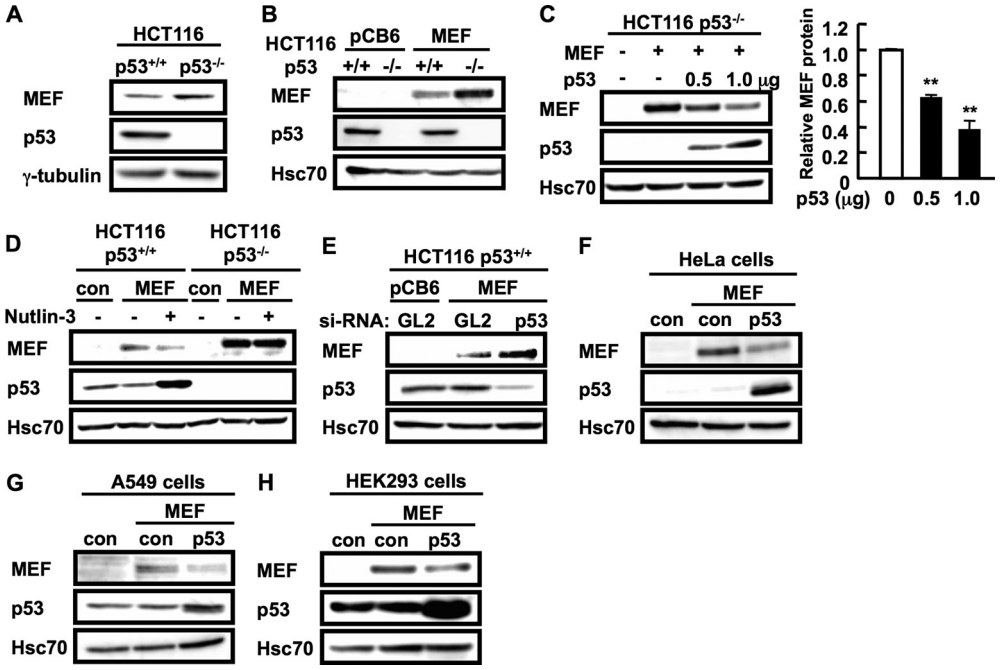

FIGURE 1. **p53 down-regulates MEF protein expression.** *A,* nuclear protein extracts were isolated from HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells, and endogenous MEF expression was assessed by Western blotting. γ-Tubulin was used as loading control. *B*, HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were transfected with MEF DNA or pCB6 vector (control). Forty-eight hours after transfection, total lysates were isolated and analyzed by immunoblotting with the indicated antibodies. *C,* HCT116 $p53^{-/-}$ cells were mock transfected or transfected with MEF and/or p53 as indicated. Protein expression was analyzed by immunoblotting. *Right panel*, decrease in expression of the MEF protein in the presence of p53 was assessed by Image Gauge software (version 4.2; Fujifilm) and plotted as relative expression against MEF-transfected cells. Bar graphs are mean ± S.E. of 3 independent experiments. **, $p < 0.01$ *versus* control, assessed by ANOVA with Dunnett's test. *D,* HCT116 $p53^{+/+}$ or $p53^{-/-}$ cells were transfected with pCB6 or MEF. Twenty-four hours after transfection, cells were treated with DMSO or 10 μM nutlin-3 for 24 h. *E,* HCT116 $p53^{+/+}$ cells were transfected with pCB6 or MEF and co-transfected with si-GL2 (control) or si-p53. *F–H*, the indicated cell lines were transfected with pCB6 or MEF and p53. For *C–H*, total protein lysates were extracted and analyzed by Western blotting using the indicated antibodies. Hsc70 was used as internal control.

mediated transcriptional activation of *MEF* as demonstrated before (15). To exclude the effect of p53 on *MEF* transcription, we overexpressed *MEF* DNA in HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells, and in the succeeding experiments *MEF* was exogenously introduced. Here, we observed that the MEF protein level is clearly down-regulated in HCT116 $p53^{+/+}$ cells compared with that in $p53^{-/-}$ cells (Fig. 1*B*). This result was verified by the quantified dose-dependent effect of p53 on MEF protein expression (Fig. 1*C*). Activating the endogenous p53 by nutlin-3 treatment substantially reduced the level of MEF protein in HCT116 $p53^{+/+}$ cells but nutlin-3 treatment had no effect on the level of MEF in HCT116 $p53^{-/-}$ cells (Fig. 1*D*). Conversely, the knockdown of *P53* by siRNA in HCT116 $p53^{+/+}$ cells increased the expression of MEF protein (Fig. 1*E*). We examined several cell lines to verify our observations. In HeLa, A549, and HEK293 cells, exogenous addition of p53 reduced the protein level of MEF (Fig. 1, *F–H*), confirming the above observations.

p53 Negatively Influences MEF Activation Function—We next asked whether the decrease in MEF protein expression induced by p53 correlates with a decrease in MEF transcriptional activating function. HCT116 $p53^{-/-}$ cells were transiently transfected with empty vectors, p53 and/or MEF. Total RNA was isolated for quantitative PCR analysis. By itself, p53 did not affect the transcription of MEF target genes *LYZ*, *IL-8*, perforin, and *HβD2* (Fig. 2, *A–D*). However, the MEF-induced activation of these genes was dose dependently suppressed by p53 (Fig. 2, *A–D*). Consistently, we found that MEF-induced lysozyme promoter activation was titrated away by the increasing amount of p53 (Fig. 2*E*). Taken together with the results in Fig. 1, these data demonstrate that p53 reduces MEF protein expression and transactivating function.

p53 Enhances the Degradation of MEF Protein—To probe into the effect of p53 on the stability of MEF protein, we monitored the degradation of MEF in HCT116 $p53^{+/+}$ and $p53^{-/-}$

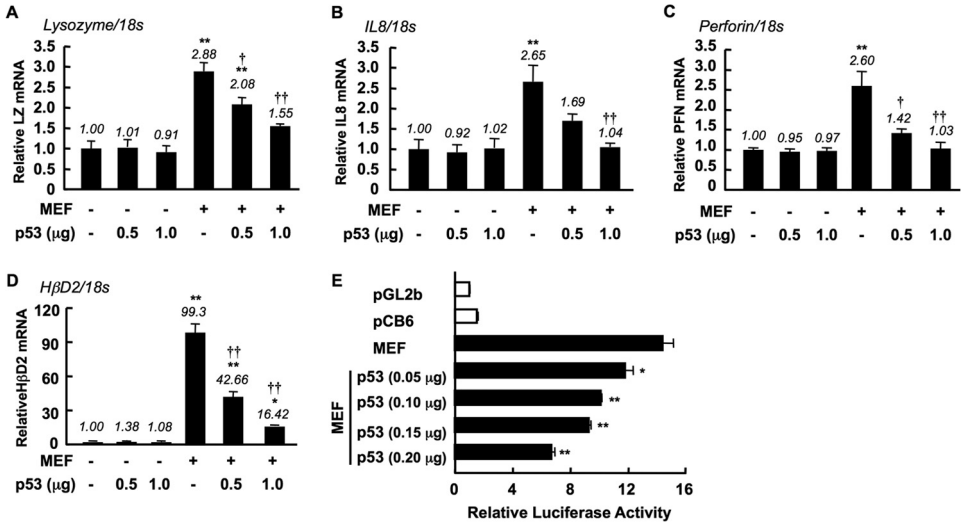

FIGURE 2. **p53 down-regulates MEF activation function.** *A–D*, HCT116 $p53^{-/-}$ cells were transfected with MEF and/or the indicated amount of p53. Forty-eight hours post-transfection, total RNA was isolated. Lysozyme, *IL-8*, perforin, and *HβD2* mRNA expressions were analyzed by quantitative RT-PCR. mRNA levels were normalized to *18 S* rRNA. Values are mean ± S.E. ($n = 3$). *, $p < 0.05$ and **, $p < 0.01$ *versus* control. †, $p < 0.05$ and ††, $p < 0.01$ *versus* MEF-transfected cells, assessed by ANOVA with Dunnett's test. *E*, HCT116 $p53^{-/-}$ cells were co-transfected with lysozyme promoter construct, MEF, or pCB6 and the indicated amount of p53. Luciferase assay was performed 48 h after transfection to assess the lysozyme promoter activity. Luciferase activity normalized to *Renilla* (phRG-tk; Promega) is expressed as fold-activation over the pGL2 basic vector. Values are mean ± S.E. ($n = 3$). *, $p < 0.05$, **, $p < 0.01$ *versus* MEF. *p* values were assessed by ANOVA with Dunnett's multiple comparison test.

cells that were treated with the protein synthesis inhibitor CHX. The MEF protein was degraded to half of its initial level at 6 h of CHX treatment in HCT116 $p53^{+/+}$ cells, but was relatively unchanged in HCT116 $p53^{-/-}$ cells (Fig. 3, *A* and *B*). We then tested the effect of proteasome inhibitor MG132 on MEF degradation in HCT116 $p53^{+/+}$ cells. The MEF protein level was down-regulated in control cells during CHX chase but co-treatment with MG132 suppressed this reduction (Fig. 3, *C* and *D*). These observations indicated that MEF is subject to enhanced proteasomal degradation in the presence of p53. When we checked for protein-protein interaction between p53 and MEF by immunoprecipitation, we could not detect their association (data not shown). Thus, another factor might be involved in the p53-mediated suppression of MEF.

p53-regulated E3 Ligase MDM2 Influences MEF Protein Expression—Identifying the p53-regulated E3 ligase that modulates the MEF protein level is important in understanding the molecular underpinning of the effect of p53 on MEF. The E3 ligase Skp2 was previously identified by Nimer's group (19) as the ubiquitin ligase that degrades MEF, and that Skp2-mediated MEF proteolysis is dependent on MEF phosphorylation status. Interestingly, however, we found that the P-MT MEF could still be subjected to proteasomal degradation because treatment with MG132 significantly increased the expression of MEF P-MT (Fig. 4*A*). In addition, *SKP2* transcription is suppressed by p53 as shown in Fig. 4*B* and as previously reported by Barré *et al.* (30), suggesting that Skp2 is not involved in the p53-mediated down-regulation of MEF. Among the E3 ligases previously indicated to be activated by p53 such as *UBE4B* (31), *PIRH2* (32), *SIAH1* (33), and *MDM2* (34–36), only MDM2 transcription was significantly diminished in HCT116 $p53^{-/-}$ cells compared with $p53^{+/+}$ cells (Fig. 4, *C–F*). The MDM2 protein level is also lower in $p53^{-/-}$ cells (Fig. 4*G*). Because the TAD of p53 is important for the induction of *MDM2* by p53 (37, 38), we checked the mRNA level of *MDM2* in the presence of

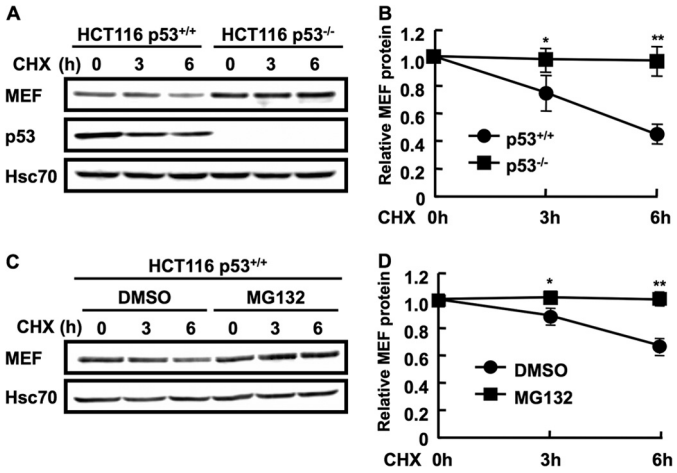

FIGURE 3. **MEF protein stability is decreased in the presence of p53.** *A*, HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were transfected with MEF cDNA. Forty-eight hours after transfection, cells were treated with 0.5 mM CHX for the indicated time, and protein lysates were collected for analysis. *B*, MEF blots in *A* were subjected to densitometric analysis using Image Gauge software and normalized to Hsc70 (internal control). Values are expressed relative to protein expression at 0 h. *C*, HCT116 $p53^{+/+}$ cells were transfected with MEF. After 48 h, cells were treated with DMSO (control) or 10 μM MG132 and co-treated with CHX for the indicated times. Lysates were extracted and analyzed by immunoblotting. *D*, relative expression of MEF was assessed using Image Gauge software and normalized to Hsc70. For *B* and *D*, values are mean ± S.E. ($n = 3$). *, $p < 0.05$, **, $p < 0.01$ *versus* HCT116 $p53^{+/+}$ cells (*B*) or DMSO-treated cells (*D*) at each indicated time. *p* values were assessed using *t* test.

p53 TAD mutant. Although wild-type p53 induced *MDM2* transcription, p53 TAD mutant (MT) did not increase the *MDM2* mRNA level (Fig. 4*H*), congruent with previous observations (37, 38). In contrast, *SIAH1*, another p53-regulated E3 ligase, was not activated by p53 or affected significantly by the p53 TAD mutant (Fig. 4*I*). Introduction of wild-type p53, but not p53 TAD mutant, in HCT116 $p53^{-/-}$ cells suppressed MEF protein expression in a statistically significant manner com-

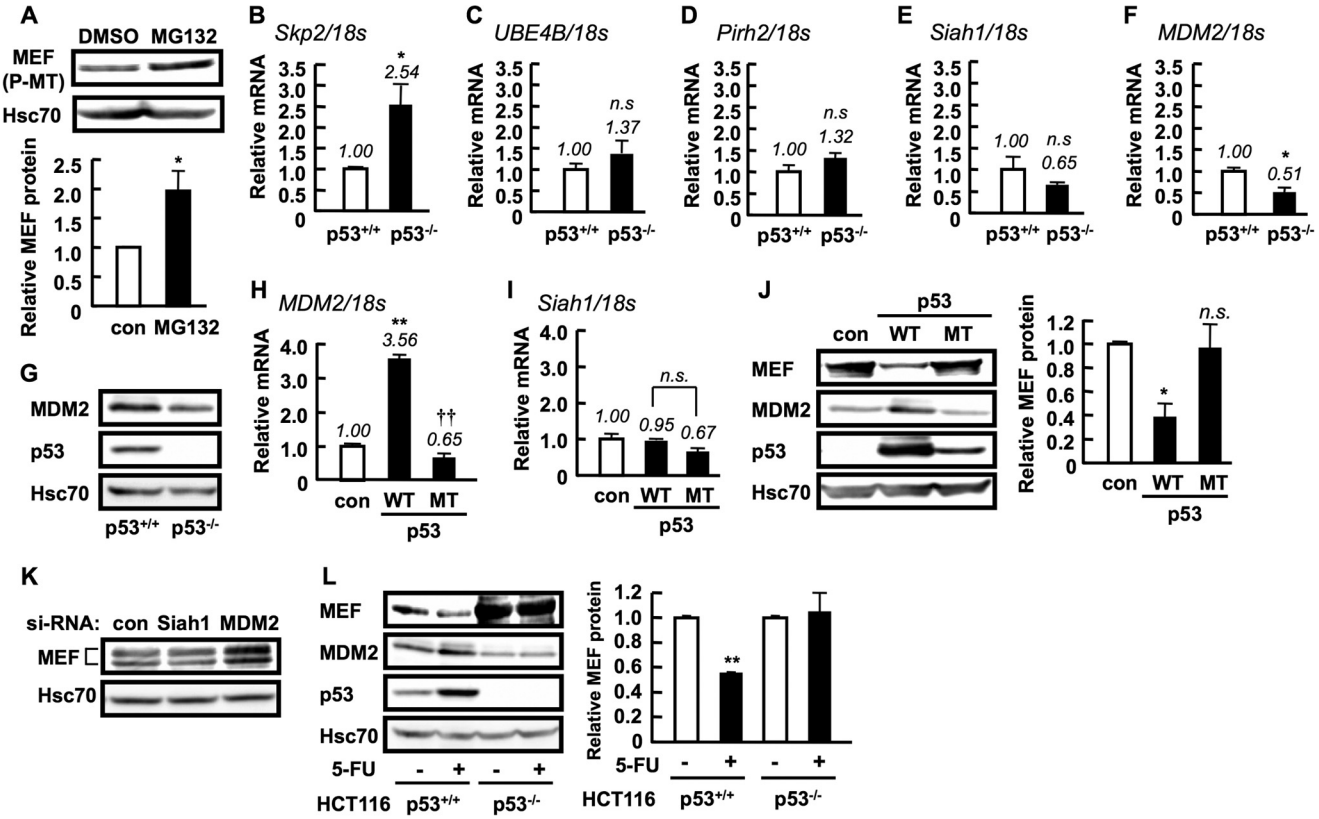

FIGURE 4. **The p53-regulated E3 ligase MDM2 affects MEF expression.** *A*, HCT116 $p53^{+/+}$ cells were transfected with MEF phosphorylation site mutant (*P-MT*). Forty-eight hours post-transfection, cells were treated with DMSO or 2.5 μM MG132 for 24 h. Total protein lysates were extracted and analyzed by immunoblotting using MEF and Hsc70 antibodies. *Lower panel*, MEF blot intensity was assessed by Image Gauge and normalized to Hsc70. The level of MEF protein in MG132-treated cells was compared with that in DMSO-treated cells. Values are mean ± S.E. of 3 independent experiments. *, $p < 0.05$ *versus* control, determined by Student's *t* test. *B–F*, total RNA was isolated from HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells, and the mRNA level of the indicated p53-regulated E3 ligases was assessed by quantitative RT-PCR. mRNA expression was normalized to *18 S* rRNA, and values are presented as mean ± S.E. ($n = 3$). *, $p < 0.05$ *versus* $p53^{+/+}$ cells, determined by Student's *t* test. *n.s.*, not significant. *G*, total protein lysates from HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were isolated for immunoblotting analysis of MDM2 and p53. *H* and *I*, HCT116 $p53^{-/-}$ cells were transfected with p53 wild-type (*WT*) or transactivation domain-mutated (*MT*) p53 constructs. Total RNA was extracted, and mRNA levels of *MDM2* (*H*) and *SIAH1* (*I*) were analyzed by quantitative RT-PCR. mRNA was normalized to *18 S* rRNA. Bar graphs are mean ± S.E. ($n = 3$). **, $p < 0.01$ *versus* control. ††, $p < 0.001$ *versus* p53 WT, assessed by ANOVA with Tukey-Kramer comparison test. *J*, HCT116 $p53^{-/-}$ cells were co-transfected with MEF and pcDNA3.1 (*con*), p53 wild-type, or p53 mutant DNAs. Total lysates were extracted 48 h post-transfection and analyzed by Western blotting. *Right panel*, immunoblots were assessed using Image Gauge software and normalized to Hsc70. Values are mean ± S.E. of 3 independent experiments. *, $p < 0.05$ *versus* con, determined by ANOVA with Tukey-Kramer test. *K*, HCT116 $p53^{+/+}$ cells were co-transfected with *MEF* cDNA and si-Siah1, si-MDM2, or si-control nucleotides. Total protein lysates were extracted for immunoblotting analysis 48 h post-transfection. Hsc70 served as internal control. *L*, HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were transfected with MEF. Forty-eight hours after transfection, cells were untreated or treated with 10 μM 5-FU for 24 h. Total protein lysates were extracted for immunoblotting analysis with the indicated antibodies. Data shown are representative of 2 independent experiments. *Right panel*, immunoblots were analyzed by Image Gauge software and normalized to Hsc70. Values are mean ± S.E. of 3 independent experiments. **, $p < 0.01$ *versus* non-treated HCT116 $p53^{+/+}$ cells, determined by Student's *t* test.

pared with control (Fig. 4*J*). These data point out the importance of the p53 transactivation domain on the inhibitory effect of p53 on MEF probably via MDM2. Admittedly, protein expression of the p53 TAD mutant is noticeably lower than wild-type p53 (Fig. 4*J*, *p53*). This is due to the mutation of amino acid residues 25 and 26 at the p53 TAD site, which decreased the efficiency of antibody recognition of epitopes between 11 and 25 amino acid residues of p53. Consistent with the involvement of MDM2 on MEF protein down-regulation, knocking down *MDM2*, but not *SIAH1* by siRNA resulted in the increase of MEF protein level (Fig. 4*K*). During siRNA transfection, we invariably detected 2 bands of MEF, which could be due to protein modification. The precise identity and the reason for their appearance are unknown, but we considered both bands to be that of MEF, indicated by a bracket. In addition, we found that activating endogenous p53 by treatment with 5-FU increased MDM2 expression and decreased the MEF protein level in HCT116 $p53^{+/+}$ cells. But the effect of 5-FU on MDM2 and MEF was not apparent in HCT116 $p53^{-/-}$ cells (Fig. 4*L*). This result indicated that p53 induces MDM2, which in turn suppresses MEF protein.

MDM2 Interacts with and Down-regulates MEF Expression and Stability—To clarify the effect of MDM2 on MEF protein expression, we overexpressed MDM2, MEF, and ubiquitin in HCT116 $p53^{+/+}$ cells. MDM2 dose-dependently reduced the steady-state expression of MEF (Fig. 5, *A* and *B*). Conversely, siMDM2 up-regulated the level of MEF protein (Fig. 5, *C* and *D*). Knockdown of MDM2 in HEK293 cells also increased the MEF protein level (Fig. 5, *E* and *F*). We next examined the possibility of interaction between MEF and MDM2. HCT116 $p53^{+/+}$ cells were transfected with MEF and MDM2. Total lysates were isolated from the transfected cells after cross-linking. Immunoprecipitation was performed using MDM2 antibody or control IgG (mock immunoprecipitation) and immu-

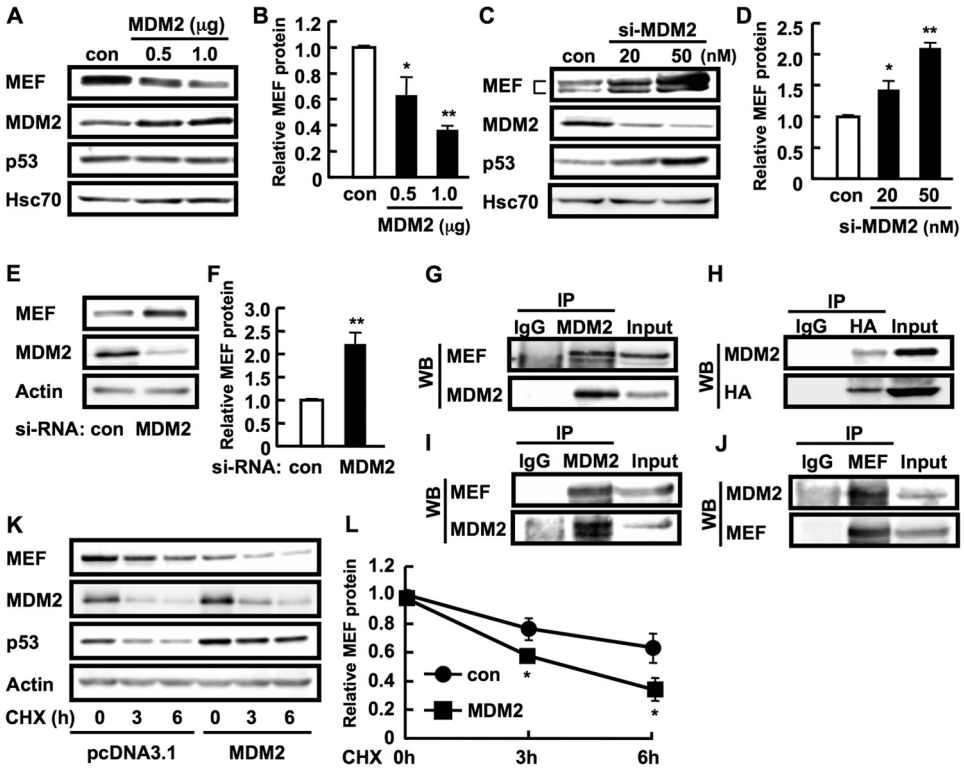

FIGURE 5. **MDM2 interacts with and down-regulates MEF expression and stability.** *A* and *C*, HCT116 p53$^{+/+}$ cells were co-transfected with MEF, ubiquitin, and the indicated amount of MDM2 or pcDNA3.1 (*con*) (*A*), or with MEF and the indicated amount of si-MDM2 or si-control (*C*). After 48 h, total protein lysates were extracted for immunoblotting analysis. *B* and *D*, immunoblots were assessed using Image Gauge software and normalized to Hsc70. Values are mean ± S.E. of 3 independent experiments. *, $p < 0.05$, **, $p < 0.01$ *versus* con, determined by ANOVA with Dunnett's comparison test. *E*, HEK293 cells were co-transfected with MEF and si-MDM2 or si-control. Total protein lysates were extracted 48 h after transfection. *F*, immunoblots were quantified using Image Gauge software and normalized to actin (internal control). Values are mean ± S.E. of 3 independent experiments. **, $p < 0.01$ *versus* control, determined by Student's *t* test. *G-J*, HCT116 p53$^{+/+}$ cells were transfected with MEF and MDM2 DNAs (*G*), with HA-tagged MEF and MDM2 (*H*), or nontransfected (*I* and *J*). Cross-linking was performed prior to protein recovery. Total lysates (*G* and *H*) or nuclear extracts (*I* and *J*) were isolated for analysis of the MEF-MDM2 interaction by immunoprecipitation using the indicated antibody or the appropriate control IgG. Immunoprecipitates were loaded onto SDS-PAGE gel, blotted, and probed with the indicated antibody, and immunoblots were visualized using chemiluminescence. Data shown are representative of 2 independent experiments. *K*, MEF and MDM2 or pcDNA3.1 were co-transfected in HCT116 p53$^{+/+}$ cells. Forty-eight hours post-transfection, cells were treated with 0.5 mM cycloheximide and total protein lysates were extracted immediately (0 h), 3 or 6 h after treatment. *L*, blots were quantified using Image Gauge and normalized to internal control. Values are mean ± S.E. ($n = 3$). *, $p < 0.05$, *versus* control at each indicated time. *p* values were assessed using *t* test. *WB*, Western blot.

noprecipitates were probed with MEF antibody. We detected exogenous interaction between MEF and MDM2 (Fig. 5*G*). We performed reverse immunoprecipitation by transfecting HA-tagged MEF and MDM2 in HCT116 cells. MEF was immunoprecipitated using HA antibody, and the immunoprecipitants were blotted with MDM2 antibody. Interaction between MEF and MDM2 was also detected in this condition (Fig. 5*H*). To determine whether these proteins associate endogenously, we isolated nuclear extracts from cross-linked, non-transfected HCT116 p53$^{+/+}$ cells. The nuclear extracts were subjected to immunoprecipitation analysis using MDM2 antibody, and immunoprecipitants were probed with MEF antibody. The MDM2-immunoprecipated sample but not the IgG-precipitated sample was positive for MEF immunoblotting (Fig. 5*I*). Consistent with our observations above, endogenous MDM2 was detectable in the MEF-immunoprecipitated fraction (Fig. 5*J*). Importantly, MDM2 clearly decreased the stability of the MEF protein as determined by CHX chase analysis (Fig. 5, *K* and *L*). These results showed that MDM2 interacts with the MEF protein and decreases its stability.

MDM2 Degrades MEF in the Nucleus—Given that MDM2 shuttles between the nucleus and cytoplasm (39), whereas MEF is mainly a nuclear protein (40), we wanted to determine whether MEF is mainly degraded in the nucleus or cytoplasm. Interestingly, the expression of MEF in the nuclear fraction was substantially decreased in the presence of MDM2 (Fig. 6, *A* and *B*). Consistent with what we previously showed (40), MEF was strongly expressed in the nucleus, whereas it was barely detected in the cytoplasmic fraction despite that the protein amount loaded for the latter was 10 times more than the nuclear extract. At longer exposures, we observed that MEF expression in the cytoplasm was also down-regulated by MDM2 (Fig. 6*A*, *MEF*$^{\#}$, *Cyto*). As expected, in total lysate, the MEF protein level was reduced in the presence of MDM2 (Fig. 6, *A* and *B*). When MDM2-transfected cells were treated with MG132, the MEF protein level was increased in nuclear and cytoplasmic fractions as well as in the total lysate compared with their respective controls (Fig. 6, *C* and *D*). From these data, we surmised that MDM2 degrades MEF both in the cytoplasm and nucleus. But being a mainly nuclear protein, the degradation of MEF by MDM2 in the nucleus is likely predominant. Last, to determine the effect of MDM2 on MEF stability in the nucleus, we pre-treated HCT116 p53$^{+/+}$ cells for 2 h with 20 nM LMB, an inhibitor of nuclear export. The cells were then subjected to CHX chase with or without LMB, and the nuclear and cytoplasmic extracts were isolated. The degradation rate of

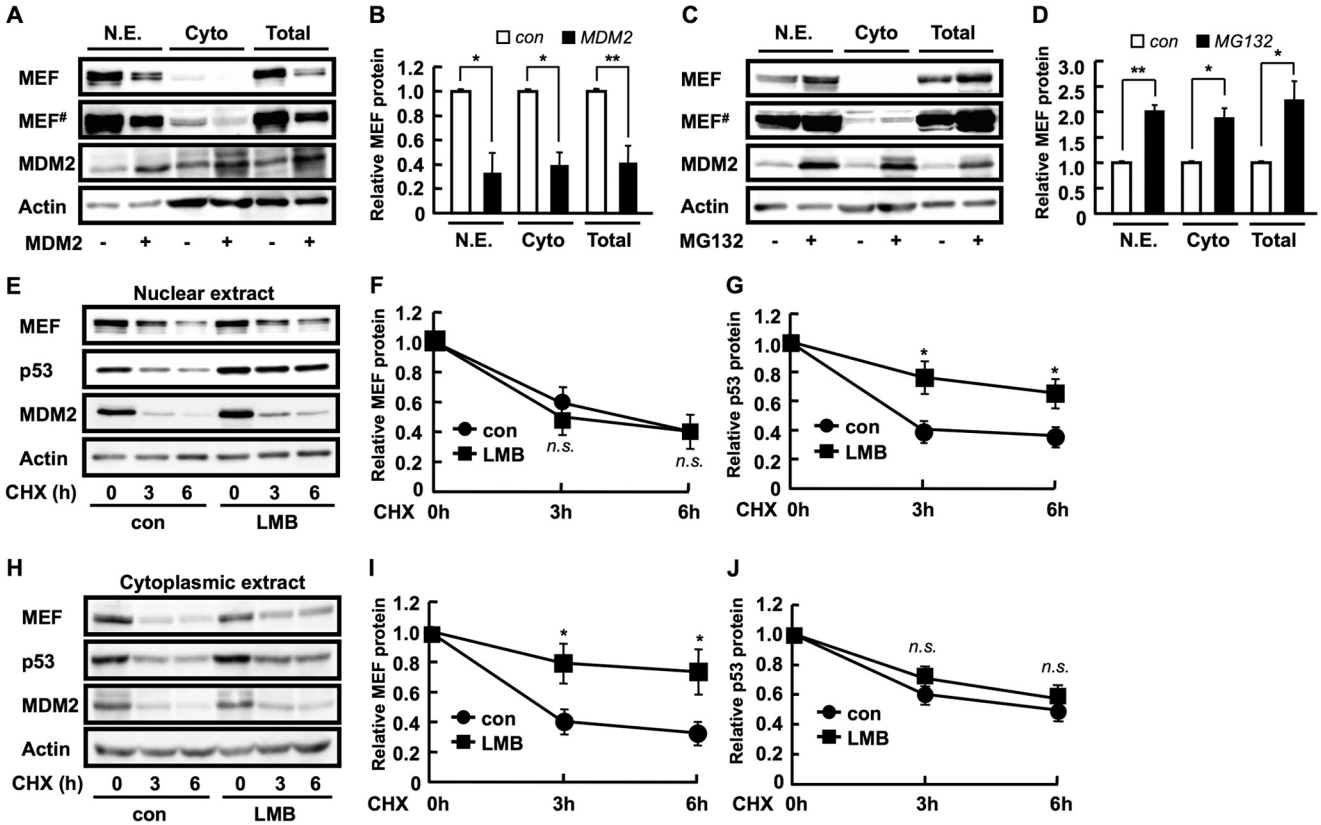

FIGURE 6. **MDM2 leads to nuclear degradation of MEF.** *A*, MEF and MDM2 or pcDNA3.1 (*con*) were co-transfected in HCT116 $p53^{+/+}$ cells. Forty-eight hours after transfection, nuclear extracts, cytoplasmic fraction, and total lysates were obtained for immunoblotting analysis of MEF expression. *B*, immunoblot of MEF was assessed using Image Gauge and normalized to internal control. Values are mean ± S.E. ($n = 3$). *, $p < 0.05$, **, $p < 0.01$ *versus* pcDNA3.1-transfected cells in each fraction. *p* values were assessed using *t* test. *C*, MEF and MDM2-transfected cells were treated with DMSO (con) or 2.5 μM MG132 for 20 h. Nuclear extracts, cytoplasmic fraction, and total lysates were obtained for immunoblotting analysis of MEF. *D,* immunoblot of MEF was assessed using Image Gauge and normalized to internal control. Values are mean ± S.E. ($n = 3$). *, $p < 0.05$, **, $p < 0.01$ *versus* DMSO-treated cells in each fraction. *p* values were assessed using *t* test. *A* and *C,* for the amount of protein, 2.5, 25, and 5 μg of lysates were loaded for nuclear extract, cytoplasmic fraction, and total lysate, respectively. # indicates longer time and high exposure during chemiluminescence detection. *E* and *H,* MEF and MDM2-transfected cells were pre-treated with ethanol (control) or 20 nM LMB for 2 h. Cells were then co-treated with CHX (0.5 mM) and LMB (20 nM). Nuclear extracts (*E*) and cytoplasmic lysates (*H*) were obtained at the indicated time for analysis of MEF expression. p53 was used as control for LMB treatment. Actin was used as internal control. The loading amounts of nuclear extract and cytoplasmic fraction for MEF were 2.5 and 25 μg, respectively. Blots in *E* and *H* were quantified and normalized to the internal control using Image Gauge software. Quantification of MEF (*F* and *I*) and p53 (*G* and *J*) immunoblots in the nuclear extract (*F* and *G*) and cytoplasmic fraction (*I* and *J*) are shown. Values are mean ± S.E. ($n = 3$). *, $p < 0.05$ *versus* control at each indicated time, assessed using Student's *t* test. *n.s.*, not significant.

nuclear MEF protein was similar between LMB-treated and control cells (Fig. 6, *E* and *F*), indicating that MDM2 could degrade MEF in the nucleus. On the other hand, in the nuclear fraction, the stability of the p53 protein, which we used here as control for LMB treatment, was lower in the non-treated cells (*con*) than in LMB-treated cells (Fig. 6, *E* and *G*). This result suggested the importance of cytoplasmic degradation for p53 (41). Notably, cytoplasmic MEF had lower stability in control cells than in LMB-treated cells, whereas cytoplasmic p53 was decreased at a similar rate in control and LMB-treated cells (Fig. 6, *H–J*). These observations indicated that MDM2 facilitates MEF degradation mostly in the nucleus.

MDM2 Down-regulates MEF Expression and Stability in the Absence of p53—The question that we next addressed is whether MDM2 can decrease the MEF protein level independently of p53. In HCT116 $p53^{-/-}$ cells, overexpression of MDM2 dose-dependently suppressed MEF protein expression (Fig. 7, *A* and *B*). Conversely, knockdown of MDM2 by siRNA increased the MEF expression (Fig. 7, *C* and *D*). These results were confirmed in HeLa cells (Fig. 7, *E–H*), which do not have functional p53 (Fig. 1*F*) due to inactivation by the HPV E6 protein (42). MDM2 interacted with the MEF protein in HCT116 $p53^{-/-}$ cells as determined by immunoprecipitation analysis (Fig. 7, *I* and *J*). CHX chase analysis revealed that MDM2 overexpression decreased stability of the MEF protein compared with control (Fig. 7, *K* and *L*). Together, these results point out that MDM2 suppresses MEF protein expression and stability independently of p53.

MDM2 Decreases the Induction of MEF Target Genes—To determine the effect of MDM2 on the function of MEF, we examined the mRNA expression levels of MEF target genes in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells. MDM2 alone did not significantly affect the induction of *LYZ*, *IL-8*, and *HβD2* mRNA in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells (Fig. 8, *A–F*). As expected, MEF increased the expression level of its target genes, but the exogenous addition of MDM2 inhibited the MEF-induced activation of *LYZ*, *IL-8*, and *HβD2* genes in both cell lines (Fig. 8, *A–F*). These results suggest that MDM2 suppresses MEF function. Importantly, congruent with a previous report (13), we found that MEF increased the mRNA and protein expression

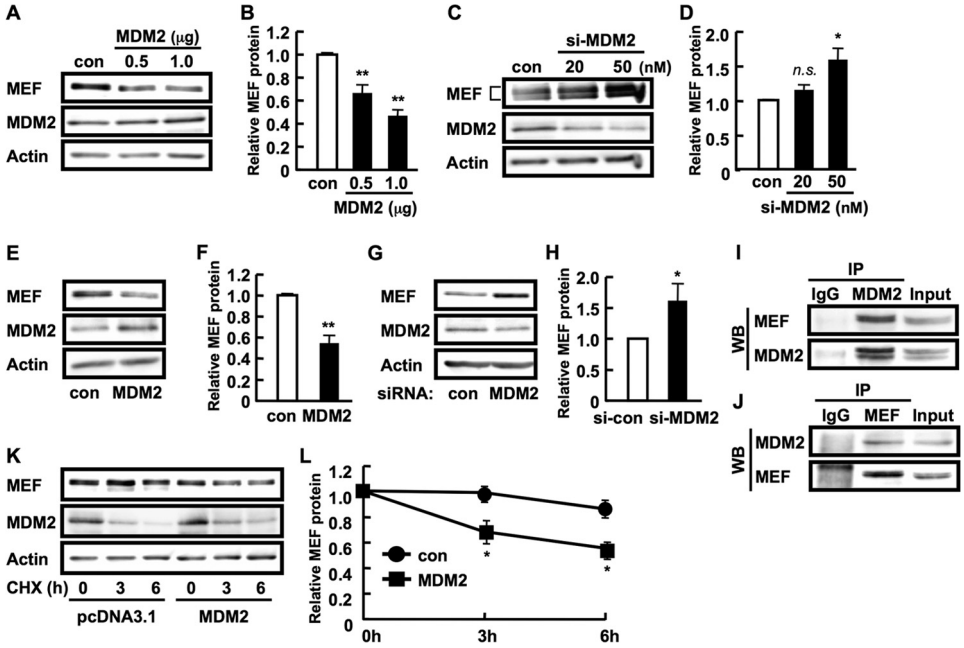

FIGURE 7. **MDM2 down-regulates MEF expression and stability independent of p53.** *A* and *C*, HCT116 p53$^{-/-}$ cells were co-transfected with MEF, ubiquitin, and the indicated amount of MDM2 or pcDNA3.1 (con) (*A*), or with MEF and the indicated amount of si-MDM2 or si-control (*C*). After 48 h, total protein lysates were extracted for immunoblotting analysis. *B* and *D*, immunoblots were assessed using Image Gauge software and normalized to internal control. Values are mean ± S.E. of 3 independent experiments. *, $p < 0.05$; **, $p < 0.01$ *versus* con, determined by ANOVA with Dunnett's comparison test. *n.s.*, not significant. *E* and *G*, HeLa cells were co-transfected with MEF and MDM2 or pcDNA3.1 (*E*) or with MEF and si-MDM2 or si-control (*G*). Total protein lysates were extracted 48 h after transfection. *F* and *H*, immunoblots were quantified using Image Gauge software and normalized to actin. Values are mean ± S.E. of 3 independent experiments. *, $p < 0.05$, **, $p < 0.01$ *versus* con, determined by Student's *t* test. *I* and *J*, HCT116 p53$^{-/-}$ cells were transfected with MEF and MDM2. Cross-linking was performed prior to protein recovery. Total protein lysates were isolated for analysis of the MEF-MDM2 interaction by immunoprecipitation using the indicated antibody or the appropriate control IgG. Immunoprecipitates were loaded onto SDS-PAGE gel, blotted, and probed with the indicated antibody, and immunoblots were visualized using chemiluminescence. Data shown are representative of 2 independent experiments. *K*, MEF and MDM2 or pcDNA3.1 were co-transfected in HCT116 p53$^{-/-}$ cells. Forty-eight hours post-transfection, cells were treated with 0.5 mM cycloheximide and total protein lysates were extracted at the indicated time. *L*, blots were quantified using Image Gauge and normalized to internal control. Values are mean ± S.E. ($n = 3$). *, $p < 0.05$, *versus* control at each indicated time. *p* values were assessed using Student's *t* test. *WB*, Western blot.

levels of MDM2 (Fig. 8, *G* and *H*). Taken together with the above observations, this result indicates that a negative feedback signaling exists for MEF via MDM2.

DISCUSSION

The regulation of MEF, especially at the protein level, is important for its function as a transcription factor. Our previous work had implicated an indirect negative effect of p53 on the transcription of MEF via E2F1 (15). However, it was unclear whether p53 could affect MEF directly at the protein level, and if so, through which mechanism. Here, we clarified that p53 down-regulates MEF protein expression. Furthermore, our results revealed that MDM2 is the E3 ligase that mediates the effect of p53. MEF is also a client of SCF^{Skp2}, but the Skp2-mediated degradation of MEF is dependent on the phosphorylation status of MEF (19). However, the MEF construct whose phosphorylation sites have been mutated (P-MT) could still be subjected to proteasomal degradation (Fig. 4*A*). Proteins could be degraded by more than one E3 ligase, therefore it is not surprising that MEF is targeted by an E3 ligase other than Skp2. It is most likely that Skp2 is the specific E3 for phosphorylated MEF, which is accumulated and degraded at the G_1/S boundary (3), whereas MDM2 may target non-phosphorylated MEF. Because *MDM2* is a gene target of p53, whereas *SKP2* is not, MDM2 might be well placed to influence MEF degradation. This process may be important especially when cells are exposed to DNA damage, and proliferative factors such as MEF need to be degraded regardless of its phosphorylation status. Because MEF has been shown to facilitate cell cycle progression from the G_1 to S phase (3), the ability of MDM2 to suppress the MEF protein might also be a cellular mechanism that is part of the repertoire of p53 of cell cycle modulation in response to cellular stress and damage. As shown in Fig. 4*L*, treatment with the DNA-damaging reagent 5-FU activated p53 and increased MDM2 expression leading to the decrease of MEF protein level in HCT116 p53$^{+/+}$ cells. Admittedly, however, we were not able to detect an increase in cell fraction at the S phase in MEF-transfected cells compared with control when the cells were treated with 5-FU (data not shown). It is probable that the effect of MEF on proliferation is more detectable in hematopoietic stem cells, in which MEF is highly endogenously expressed (4), rather than in epithelial cells. The functional impact of the MEF-p53 relationship especially in the context of cellular proliferative control might also be more obvious in cells with "stemness" characteristics, such as the glioblastoma stem cells (12), than in epithelial cells wherein MEF is mostly known for its transactivation function on innate immunity-associated genes (7, 8, 10).

Other E3 ligases that we looked into, such as *UBE4B*, *PIRH2*, and *SIAH1* are also transcriptional targets of p53 and are E3 ligases of p53. Ube4b is overexpressed in brain tumors, leading

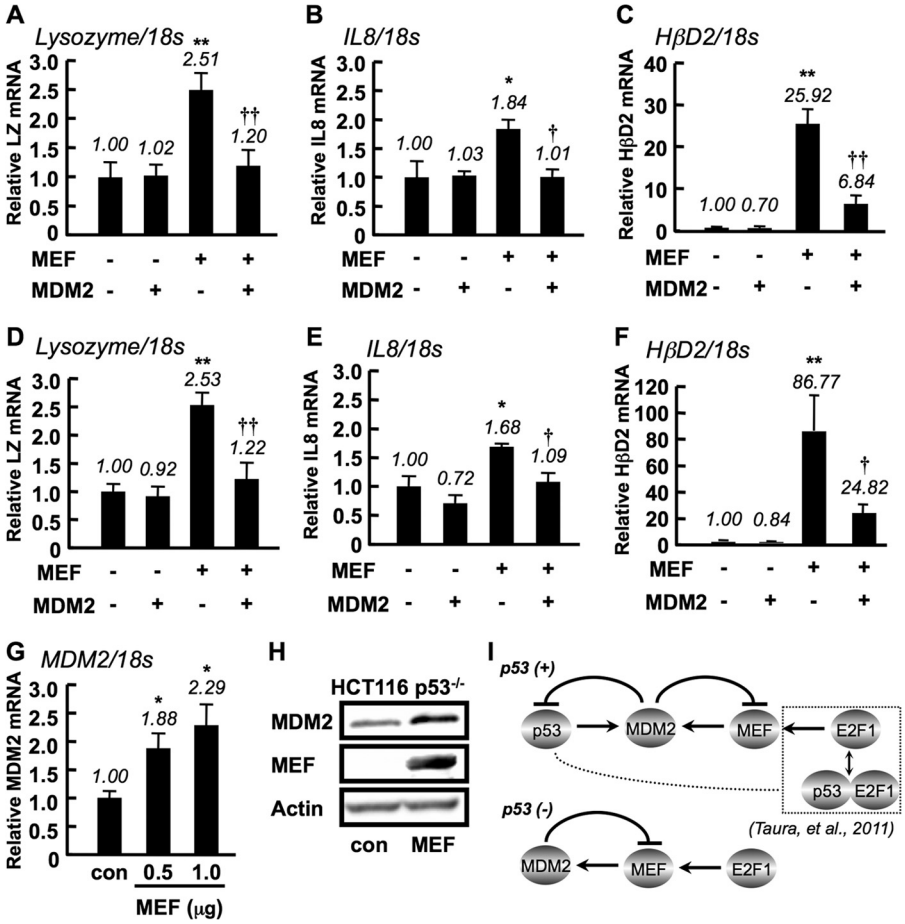

FIGURE 8. **MEF-induced activation of target genes is suppressed by MDM2.** HCT116 $p53^{+/+}$ (*A–C*) and HCT116 $p53^{-/-}$ (*D–F*) cells were mock-transfected (*con*) or transfected with MEF and/or MDM2 as indicated. Total RNA was isolated 48 h post-transfection, and analyzed for the expression of the indicated genes by quantitative RT-PCR. mRNA levels were normalized to *18 S* rRNA. Values are mean ± S.E. ($n = 3$). *, $p < 0.05$; **$p < 0.01$ *versus* non-transfected control. †, $p < 0.05$; ††, $p < 0.01$ *versus* MEF-transfected cells, assessed by ANOVA with Tukey-Kramer test. *G*, HCT116 $p53^{-/-}$ cells were transfected with pCB6 (con) or the indicated amount of MEF. Total RNA was isolated and analyzed for the expression of *MDM2*. mRNA level was normalized to *18 S* rRNA. Values are mean ± S.E. ($n = 3$). *, $p < 0.05$ *versus* control, assessed by ANOVA with Dunnett's test. *H*, HCT116 $p53^{-/-}$ cells were transfected with MEF or pCB6. Forty-eight hours after transfection, total protein lysates were isolated and analyzed by Western blotting. *I*, schematic representation of MEF and p53 regulation via MDM2. *MDM2* is transcriptionally activated by MEF or p53, and targets MEF and p53 for degradation. Included in the scheme is the previously reported transcriptional activation of *MEF* exerted by E2F1, which could be inhibited by p53 through E2F1-p53 interaction (15). *Arrows* represent transcriptional activation. *Lines* represent regulation at the protein level.

to the inactivation of p53. Ube4b contains functional p53 DNA binding sites and are efficiently transactivated by p53 (31). *PIRH2* is induced during UV irradiation in a p53-dependent manner (43). *SIAH1* is activated by p53 during telomere damage and cellular senescence (33). The mRNA level of these genes in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells, however, had no significant difference between these cell lines (Fig. 4, *C–E*). It could be that although p53 is important for their activation during certain cellular stress conditions, such as UV-induced damage and telomere attrition, p53 is not critical for the basal regulation of these genes. With regards to the activation of *MDM2* by p53, previous reports have shown that the p53 transactivation domain is important (37, 38). In line with these studies, the wild-type p53, but not the TAD mutant, was able to transactivate the *MDM2* gene (Fig. 4*H*). Consequently, the p53 TAD mutant was unable to down-regulate the MEF protein (Fig. 4*J*), highlighting the role of p53 TAD in the effect of p53 on MEF via MDM2.

Most nuclear proteins are exported to the cytoplasm for efficient degradation, however, recent studies have shown that nuclear proteins are also substrates for proteasomal degradation in the nucleus (reviewed in Ref. 44). Because MDM2 is known to shuttle its substrate such as p53 to the cytoplasm for degradation, we investigated whether MDM2 would facilitate the export of MEF from the nucleus to be degraded mainly in the cytoplasm. Although MEF was detected in the cytoplasmic fraction and the addition of MDM2 decreased MEF expression (Fig. 6, *A–D*), MEF was mainly expressed and down-regulated by MDM2 in the nuclear extracts. Furthermore, the stability of nuclear MEF was decreased by MDM2 in cells treated with LMB, an inhibitor of nuclear export. In addition, cytoplasmic MEF was more stable in LMB-treated cells than in control cells. Given that LMB inhibited the export of nuclear MEF, which was degraded, whereas cytoplasmic MEF had relatively high stability in the presence of LMB, it can be deduced that MEF was degraded by MDM2 mainly in the nucleus. In contrast, we detected that p53 is preferentially degraded in the cytoplasm, which is consistent with the report demonstrating that shuttling of p53 from the nucleus to the cytoplasm is essential for its degradation (45). MEF being degraded in the nucleus is a logical

observation considering that MEF is known to be constitutively nuclear localizing and is barely detected in the cytoplasm (18, 40) (Fig. 6, *A* and *C*). This adds MEF to a relatively short but growing list of transcription factors that are degraded in the nucleus (44). The mammalian nucleus harbors numerous genes but only a handful are expressed at a given time point to allow cells to respond to environmental stimuli. It might then be imperative that a system for tight regulation of nuclear function exists within the nucleus itself for rapid response to cellular demands.

We noted that MDM2 could down-regulate MEF expression and stability independently of p53. Taken together with the finding that MEF is a transcriptional activator of *MDM2* (Fig. 8, *G* and *H*, and Ref. 13), our results also revealed a p53-independent network in which MEF regulates its own fate by activating its own E3 ligase akin to the way p53 regulates itself through MDM2. This intriguing observation adds a layer of complexity to the mutual and seemingly symmetrical regulation of these proteins. MDM2 is an E3 ligase of p53 and MEF, capable of degrading p53 and MEF proteins. As transcription factors, p53 and MEF activate *MDM2* transcription, providing an autoregulatory negative feedback mechanism (Fig. 8*I*). It was previously shown that transcriptional activation of *MDM2* by MEF leads to the degradation of p53 (13) and conversely, here we have shown that transcriptional activation of *MDM2* by p53 leads to the degradation of MEF. Based on previous and current results, a picture emerges in which the known roles of MEF and p53 as positive and negative cell cycle regulators, respectively, are reflected on their inhibitory effects on each other via MDM2. MEF may facilitate cell cycle progression, in part, by activating MDM2, which interacts with and suppresses p53. During DNA damage, however, when p53 is activated, *MDM2* transcription could be abetted by p53. Because activated p53 has minimal interaction with MDM2 due to the p53 phosphorylated status (46), MDM2 might interact mostly with MEF to promote MEF degradation. Affinity of E3 ligases to its client proteins changes according to the post-translational modification status of the client protein (47), which is influenced by cellular conditions. Thus, MDM2 interaction with p53 or MEF could be affected by the state of the cellular environment. Beyond the effect that MEF and p53 have on each other by activating MDM2, these proteins could turn that same mechanism on themselves (*i.e.* activating MDM2) for negative feedback autoregulation. These findings give evidence that the p53-MDM2-MEF axis is a feedback mechanism that provides exquisite control on the maintenance and balance of these transcriptional regulators (Fig. 8*I*). It is likely that dysfunction of one of these factors could affect a fragile balance that could lead to the dysregulation of some cellular processes.

Acknowledgments—HCT116 $p53^{+/+}$ and $p53^{-/-}$ cell lines were provided by Dr. Bert Vogelstein. The p53 wild-type DNA plasmid was a kind gift from Dr. Hideyuki Saya (Keio University, Japan).

REFERENCES

1. Sharrocks, A. D. (2001) The ETS-domain transcription factor family. *Nat. Rev. Mol. Cell Biol.* **2,** 827–837
2. Turner, D. P., and Watson, D. K. (2008) ETS transcription factors: oncogenes and tumor suppressor genes as therapeutic targets for prostate cancer. *Expert Rev. Anticancer Ther.* **8,** 33–42
3. Miyazaki, Y., Boccuni, P., Mao, S., Zhang, J., Erdjument-Bromage, H., Tempst, P., Kiyokawa, H., and Nimer, S. D. (2001) Cyclin A-dependent phosphorylation of the ETS-related protein, MEF, restricts its activity to the G_1 phase of the cell cycle. *J. Biol. Chem.* **276,** 40528–40536
4. Lacorazza, H. D., Yamada, T., Liu, Y., Miyata, Y., Sivina, M., Nunes, J., and Nimer, S. D. (2006) The transcription factor MEF/ELF4 regulates the quiescence of primitive hematopoietic cells. *Cancer Cell* **9,** 175–187
5. Sashida, G., Bazzoli, E., Menendez, S., Liu, Y., and Nimer, S. D. (2010) The oncogenic role of the ETS transcription factors MEF and ERG. *Cell Cycle* **9,** 3457–3459
6. Seki, Y., Suico, M. A., Uto, A., Hisatsune, A., Shuto, T., Isohama, Y., and Kai, H. (2002) The ETS transcription factor MEF is a candidate tumor suppressor gene on the X chromosome. *Cancer Res.* **62,** 6579–6586
7. Kai, H., Hisatsune, A., Chihara, T., Uto, A., Kokusho, A., Miyata, T., and Basbaum, C. (1999) Myeloid ELF-1-like factor up-regulates lysozyme transcription in epithelial cells. *J. Biol. Chem.* **274,** 20098–20102
8. Lu, Z., Kim, K. A., Suico, M. A., Shuto, T., Li, J. D., and Kai, H. (2004) MEF up-regulates human β-defensin 2 expression in epithelial cells. *FEBS Lett.* **561,** 117–121
9. Miyazaki, Y., Sun, X., Uchida, H., Zhang, J., and Nimer, S. (1996) MEF, a novel transcription factor with an Elf-1 like DNA binding domain but distinct transcriptional activating properties. *Oncogene* **13,** 1721–1729
10. Hedvat, C. V., Yao, J., Sokolic, R. A., and Nimer, S. D. (2004) Myeloid ELF1-like factor is a potent activator of interleukin-8 expression in hematopoietic cells. Erratum in *J. Biol. Chem.* (2006) Mar. 31, 281, 8996. *J. Biol. Chem.* **279,** 6395–6400
11. Lacorazza, H. D., Miyazaki, Y., Di Cristofano, A., Deblasio, A., Hedvat, C., Zhang, J., Cordon-Cardo, C., Mao, S., Pandolfi, P. P., and Nimer, S. D. (2002) The ETS protein MEF plays a critical role in perforin gene expression and the development of natural killer and NK-T cells. *Immunity* **17,** 437–449
12. Bazzoli, E., Pulvirenti, T., Oberstadt, M. C., Perna, F., Wee, B., Schultz, N., Huse, J. T., Fomchenko, E. I., Voza, F., Tabar, V., Brennan, C. W., DeAngelis, L. M., Nimer, S. D., Holland, E. C., and Squatrito, M. (2012) MEF promotes stemness in the pathogenesis of gliomas. *Cell Stem Cell* **11,** 836–844
13. Sashida, G., Liu, Y., Elf, S., Miyata, Y., Ohyashiki, K., Izumi, M., Menendez, S., and Nimer, S. D. (2009) ELF4/MEF activates MDM2 expression and blocks oncogene-induced p16 activation to promote transformation. *Mol. Cell. Biol.* **29,** 3687–3699
14. Koga, T., Suico, M. A., Nakamura, H., Taura, M., Lu, Z., Shuto, T., Okiyoneda, T., and Kai, H. (2005) Sp1-dependent regulation of Myeloid Elf-1 like factor in human epithelial cells. *FEBS Lett.* **579,** 2811–2816
15. Taura, M., Suico, M. A., Fukuda, R., Koga, T., Shuto, T., Sato, T., Morino-Koga, S., Okada, S., and Kai, H. (2011) MEF/ELF4 transactivation by E2F1 is inhibited by p53. *Nucleic Acids Res.* **39,** 76–88
16. Suico, M. A., Nakamura, H., Lu, Z., Saitoh, H., Shuto, T., Nakao, M., and Kai, H. (2006) SUMO down-regulates the activity of Elf4/myeloid Elf-1-like factor. *Biochem. Biophys. Res. Commun.* **348,** 880–888
17. Suico, M. A., Lu, Z., Shuto, T., Koga, T., Uchikawa, T., Yoshida, H., Matsuzaki, K., Nakao, M., Li, J. D., and Kai, H. (2004) The regulation of human β-defensin 2 by the ETS transcription factor MEF (myeloid Elf-1-like factor) is enhanced by promyelocytic leukemia protein. *J. Pharmacol. Sci.* **95,** 466–470
18. Suico, M. A., Yoshida, H., Seki, Y., Uchikawa, T., Lu, Z., Shuto, T., Matsuzaki, K., Nakao, M., Li, J. D., and Kai, H. (2004) Myeloid Elf-1-like factor, an ETS transcription factor, up-regulates lysozyme transcription in epithelial cells through interaction with promyelocytic leukemia protein. *J. Biol. Chem.* **279,** 19091–19098
19. Liu, Y., Hedvat, C. V., Mao, S., Zhu, X. H., Yao, J., Nguyen, H., Koff, A., and Nimer, S. D. (2006) The ETS protein MEF is regulated by phosphorylation-dependent proteolysis via the protein-ubiquitin ligase SCF^{Skp2}. *Mol. Cell. Biol.* **26,** 3114–3123
20. Vousden, K. H., and Lane, D. P. (2007) p53 in health and disease. *Nat. Rev. Mol. Cell Biol.* **8,** 275–283
21. Carter, S., and Vousden, K. H. (2009) Modifications of p53: competing for

the lysines. *Curr. Opin. Genet. Dev.* **19,** 18–24
22. Kruse, J. P., and Gu, W. (2008) SnapShot: p53 posttranslational modifications. *Cell* **133,** 930–30.e1
23. Vousden, K. H., and Lu, X. (2002) Live or let die: the cell's response to p53. *Nat. Rev. Cancer* **2,** 594–604
24. Riley, M. F., and Lozano, G. (2012) The many faces of MDM2 binding partners. *Genes Cancer* **3,** 226–239
25. Tamura, T., Aoyama, N., Saya, H., Haga, H., Futami, S., Miyamoto, M., Koh, T., Ariyasu, T., Tachi, M., and Kasuga, M. (1995) Induction of Fas-mediated apoptosis in p53-transfected human colon carcinoma cells. *Oncogene* **11,** 1939–1946
26. Zhou, B. P., Liao, Y., Xia, W., Zou, Y., Spohn, B., and Hung, M. C. (2001) HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. *Nat. Cell Biol.* **3,** 973–982
27. Taura, M., Eguma, A., Suico, M. A., Shuto, T., Koga, T., Komatsu, K., Komune, T., Sato, T., Saya, H., Li, J. D., and Kai, H. (2008) p53 regulates Toll-like receptor 3 expression and function in human epithelial cell lines. *Mol. Cell. Biol.* **28,** 6557–6567
28. Morissette, M. C., Parent, J., and Milot, J. (2007) Perforin, granzyme B, and FasL expression by peripheral blood T lymphocytes in emphysema. *Respir. Res.* **8,** 62
29. Lehmann, J., Retz, M., Harder, J., Krams, M., Kellner, U., Hartmann, J., Hohgräwe, K., Raffenberg, U., Gerber, M., Loch, T., Weichert-Jacobsen, K., and Stöckle, M. (2002) Expression of human β-defensins 1 and 2 in kidneys with chronic bacterial infection. *BMC Infect. Dis.* **2,** 20
30. Barré, B., and Perkins, N. D. (2010) The Skp2 promoter integrates signaling through the NF-κB, p53, and Akt/GSK3β pathways to regulate autophagy and apoptosis. *Mol. Cell* **38,** 524–538
31. Wu, H., Pomeroy, S. L., Ferreira, M., Teider, N., Mariani, J., Nakayama, K. I., Hatakeyama, S., Tron, V. A., Saltibus, L. F., Spyracopoulos, L., and Leng, R. P. (2011) UBE4B promotes Hdm2-mediated degradation of the tumor suppressor p53. *Nat. Med.* **17,** 347–355
32. Leng, R. P., Lin, Y., Ma, W., Wu, H., Lemmers, B., Chung, S., Parant, J. M., Lozano, G., Hakem, R., and Benchimol, S. (2003) Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. *Cell* **112,** 779–791
33. Fujita, K., Horikawa, I., Mondal, A. M., Jenkins, L. M., Appella, E., Vojtesek, B., Bourdon, J. C., Lane, D. P., and Harris, C. C. (2010) Positive feedback between p53 and TRF2 during telomere-damage signalling and cellular senescence. *Nat. Cell Biol.* **12,** 1205–1212
34. Kubbutat, M. H., Jones, S. N., and Vousden, K. H. (1997) Regulation of p53 stability by Mdm2. *Nature* **387,** 299–303
35. Honda, R., Tanaka, H., and Yasuda, H. (1997) Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett.* **420,** 25–27
36. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) Mdm2 promotes the rapid degradation of p53. *Nature* **387,** 296–299
37. Brady, C. A., Jiang, D., Mello, S. S., Johnson, T. M., Jarvis, L. A., Kozak, M. M., Kenzelmann Broz, D., Basak, S., Park, E. J., McLaughlin, M. E., Karnezis, A. N., and Attardi, L. D. (2011) Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. *Cell* **145,** 571–583
38. Ohki, R., Kawase, T., Ohta, T., Ichikawa, H., and Taya, Y. (2007) Dissecting functional roles of p53 N-terminal transactivation domains by microarray expression analysis. *Cancer Sci.* **98,** 189–200
39. Roth, J., Dobbelstein, M., Freedman, D. A., Shenk, T., and Levine, A. J. (1998) Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein. *EMBO J.* **17,** 554–564
40. Suico, M. A., Koyanagi, T., Ise, S., Lu, Z., Hisatsune, A., Seki, Y., Shuto, T., Isohama, Y., Miyata, T., and Kai, H. (2002) Functional dissection of the ETS transcription factor MEF. *Biochim. Biophys. Acta* **1577,** 113–120
41. Freedman, D. A., and Levine, A. J. (1998) Nuclear export is required for degradation of endogenous p53 by MDM2 and human papillomavirus E6. *Mol. Cell. Biol.* **18,** 7288–7293
42. Hoppe-Seyler, F., and Butz, K. (1993) Repression of endogenous p53 transactivation function in HeLa cervical carcinoma cells by human papillomavirus type 16 E6, human mdm-2, and mutant p53. *J. Virol.* **67,** 3111–3117
43. Jung, Y. S., Liu, G., and Chen, X. (2010) Pirh2 E3 ubiquitin ligase targets DNA polymerase η for 20 S proteasomal degradation. *Mol. Cell. Biol.* **30,** 1041–1048
44. von Mikecz, A. (2006) The nuclear ubiquitin-proteasome system. *J. Cell Sci.* **119,** 1977–1984
45. O'Keefe, K., Li, H., and Zhang, Y. (2003) Nucleocytoplasmic shuttling of p53 is essential for MDM2-mediated cytoplasmic degradation but not ubiquitination. *Mol. Cell Biol.* **23,** 6396–6405
46. Kruse, J. P., and Gu, W. (2009) Modes of p53 regulation. *Cell* **137,** 609–622
47. Deshaies, R. J., and Joazeiro, C. A. (2009) RING domain E3 ubiquitin ligases. *Annu. Rev. Biochem.* **78,** 399–434