Molecular Biology of the Cell
Vol. 18, 1701–1709, May 2007

Dynamic Regulation of p53 Subnuclear Localization and Senescence by MORC3[D]

Keiko Takahashi,*† Naofumi Yoshida,* Naoko Murakami,*‡ Kiyo Kawata,* Hiroyuki Ishizaki,§‖ Miki Tanaka-Okamoto,§ Jun Miyoshi,§ Andrew R. Zinn,¶ Hiroaki Shime,* and Norimitsu Inoue*

Departments of *Molecular Genetics and §Molecular Biology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Osaka 537-8511, Japan; †Division of Molecular Biology, Nara Institute of Science and Technology, Ikoma, Nara 630-0101, Japan; ‖Kan Research Institute, Kyoto, Kyoto 600-8815, Japan; and ¶Department of Internal Medicine and McDermott Center for Human Growth and Development, The University of Texas Southwestern Medical School, Dallas, TX 75390

Submitted August 25, 2006; Revised February 9, 2007; Accepted February 16, 2007
Monitoring Editor: A. Gregory Matera

The tumor suppressor p53 is a key transcriptional factor regulating the induction of cellular senescence by oncogenic signals. The activity of p53 is regulated by recruitment into promyelocytic leukemia (PML)-nuclear bodies (NBs) as well as by stabilization through posttranslational modifications such as phosphorylation and acetylation. Here we found that MORC3 (microrchidia3)-ATPase activated p53 and induced cellular senescence in normal human and mouse fibroblasts but not *p53*−/− fibroblasts. Conversely, genotoxic stress–induced phosphorylation and stabilization of p53 but barely increased its transcriptional activity in *Morc3*−/− fibroblasts. MORC3 localized on PML-NBs in presence of PML and mediated recruitment of p53 and CREB-binding protein (CBP) into PML-NBs. In contrast, expression of ATPase activity-deficient mutant MORC3-E35A or siRNA repression of MORC3 impaired the localization of p53 and Sp100 but not CBP on PML-NBs. These results suggest that MORC3 regulates p53 activity and localization into PML-NBs. We identified a new molecular mechanism that regulates the activity of nuclear proteins by localization to a nuclear subdomain.

INTRODUCTION

Cellular senescence functions as an initial barrier to tumorigenesis in response to the activation of oncogenes (Braig *et al.*, 2005; Chen *et al.*, 2005). Tumor suppressor proteins p53 and pRB both act in pathways that regulate the cellular senescence induced by oncogenic signals (Serrano *et al.*, 1997; Campisi, 2005). The activity of p53 is modulated mostly by stabilization through posttranslational modifications, including phosphorylation and acetylation (Bode and Dong, 2004). In addition to these posttranslational modifications, recruitment of p53 into promyelocytic leukemia (PML)-nuclear bodies (NBs) also plays an important role in its activation in response to genotoxic stress (Fogal *et al.*, 2000; Pearson *et al.*, 2000). PML-NBs are highly dynamic nuclear structures containing an essential component, PML, to which many proteins are recruited to regulate transcription or DNA repair (Borden, 2002). Expression of PML IV, one of the PML isoforms encoded by several alternative spliced transcripts, recruits p53 and CBP to PML-NBs, activates them and induces cellular senescence (Pearson *et al.*, 2000). However, little is known about how p53 or other proteins are recruited into PML-NBs or other nuclear subdomains.

MORC3 (microrchidia3), also called KIAA0136, ZCWCC3, and NXP-2 (Kimura *et al.*, 2002), is a member of the MORC protein family characterized by conserved structures consisting of a GHL (Gyrase B, Hsp90 and MutL)-ATPase domain (Dutta and Inouye, 2000) at the amino-terminus, a zinc finger type CW domain containing conserved four cysteines and two tryptophans (Perry and Zhao, 2003), a nuclear localization signal (NLS) and coiled-coil domains at the carboxy-terminus (Supplementary Figure 1). We previously identified one of MORC family proteins, mouse Morc1 (Inoue *et al.*, 1999), whose deficiency causes arrest of spermatogenesis before the pachytene stage of meiosis prophase I (Watson *et al.*, 1998). There are four predicted MORC family proteins (MORC1, MORC2 [KIAA0852, ZCWCC1], MORC3, and MORC4 [ZCWCC2]) in human and five (Morc1, Morc2a, Morc2b, Morc3, and Morc4) in mice (Supplementary Figure 1 and Supplementary Table 1). The MORC family proteins belong to CW-domain–containing subfamilies I (MORC1 and MORC2) or IX (MORC3 and MORC4) (Perry and Zhao, 2003). MORC1 is expressed specifically in male germ cells (Inoue *et al.*, 1999), whereas MORC2 and MORC3 in human

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06–08–0747) on March 1, 2007.

[D] The online version of this article contains supplemental material at *MBC Online* (http://www.molbiolcell.org).

‡ Present address: Department of Pediatrics, University of Michigan Health System, Ann Arbor, MI 48109-0656.

Address correspondence to: Norimitsu Inoue (inoue-no@mc.pref.osaka.jp).

Abbreviations used: MORC, microrchidia; PML, promyelocytic leukemia; NBs, nuclear bodies; CBP, CRE-binding protein; MEFs, mouse embryonic fibroblasts; SA-β-Gal, senescence–associated β-galactosidase; bp, base pairs; I-P, internal ribosomal entry site-puromycin resistance gene; dpc, days postcoitum; ADR, adriamycin; ATRA, all-*trans*-retinoic acid.

are ubiquitously expressed (The HUGE database, http://www.kazusa.or.jp/huge/index.html). *Caenorhabditis elegans* has only one MORC family member, ZC155.3, whose knockdown by siRNA is embryonic lethal (Wormbase, http://www.wormbase.org/).

Although MORC family proteins are conserved in higher eukaryotes, no molecular function of any MORC family protein has yet been clarified. Here we show that MORC3 is involved in recruiting p53 and Sp100 but not PML or CBP from the nucleoplasm to PML-NBs. In addition, MORC3 regulates p53 activation in a manner dependent on MORC3-ATPase activity.

MATERIALS AND METHODS

Plasmids

All plasmids used in this study were constructed by standard procedures. pMEPy-FLAG vector and pMEPy-hemagglutinin (HA) vector were previously constructed for the expression of proteins tagged with FLAG and HA at the amino-terminus (Ohishi *et al.*, 2000). pMEPy-enhanced green fluorescent protein (EGFP)-tag vector was generated by introduction of an EGFP fragment in which the stop codon was replaced with SalI site. pME-I-P vector was constructed by subcloning the internal ribosomal entry site-puromycin resistance gene (I-P) fragment derived from pIRESpuro2 (Clontech, Mountain View, CA) into the pME vector. We replaced the initiation codon of human MORC3 with a SalI site and inserted the resulting construct into the expression vectors. MORC3 mutants were generated by the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). p53, PML IV, and Maltose-binding protein (MBP) cDNA carrying a SalI site instead of the initiation codon were subcloned into the FLAG- or HA-tag vectors. A knockdown vector, pH1′-DsRed2-I-P and pH1′-SV40-puro was constructed by the introduction of HindIII-KpnI fragment of pME-DsRed2-I-P and a fragment of *puromycin resistance* gene transcribed from SV40 promoter, respectively, into pH1′ vector (Hasuwa *et al.*, 2002). MORC3 knockdown vectors, pH1′-shRNA732-DsRed2-I-P (shRNA732 [short hairpin RNA]) were designed to contain the following inserts: 5′-CCCGGGGTACAAGAAGCAGGAAAGTTCAAGAGACTTTCCTGCTTCTTGTACCCCTTTTTGGAAA-3′ (the target sense and antisense sequences are underlined). As a negative control, we replaced shRNA732 with the following shRNA695 sequence to target the same region of mouse *Morc3*: 5′-CCCaGGGTACAAGAAaCAaGAAAGTTCAAGAGACTTTCtTGtTTCTTGTACCCtTTTTTGGAAA-3′ (the mismatch nucleotides are lowercased). PML-knockdown vector pH1′-shRNA-PML1555 (shRNA-PML1555) was designed to contain the following inserts: 5′-CCCGGGGTACAAGAAGCAGGAAAGTTCAAGAGACTTTCCTGCTTCTTGTACCCCTTTTTGGAAA-3′. Proper construction of all plasmids was confirmed by restriction digestions and sequencing. These plasmids and their sequences are available upon request.

Cell Culture and Transfection

Cells were cultured in DMEM (Sigma, St. Louis, MO) for HeLa and mouse embryonic fibroblasts (MEFs), McCoy's 5A (Sigma) for U-2 OS (ATCC, Manassas, VA) and Saos-2, RPMI 1640 (Sigma) for H1299 (ATCC) and NB4, and Minimum essential medium (Invitrogen, Carlsbad, CA) for WI38 (RIKEN cell bank) at 37°C under 5% CO_2 atmosphere. All media were supplemented with 10% fetal calf serum (FCS; 15% FCS for Saos-2), 100U/ml penicillin, and 100 μg/ml streptomycin. Plasmids were transfected into cells with PolyFect (Qiagen, Chatsworth, CA) or FuGENE6 (Roche, Indianapolis, IN) for HeLa, FuGENE6 (Roche) for U-2 OS and Saos-2, and Lipofectamine 2000 (Invitrogen) for H1299, according to the manufacturers' instructions.

Retroviral Infection and Senescence Analysis

The retroviral vectors, pLNCX2-EGFP-MORC3-I-P and pLNCX2-FLAG-MORC3-I-P were constructed by inserting EGFP-MORC3-I-P and FLAG-MORC3-I-P fragments, respectively, between the EcoRI and NotI sites of pLNCX2 vector (Clontech). pLNCX2-EGFP-PML IV-I-P and pLNCX2-FLAG-PML IV-I-P were generated from pLNCX2-EGFP-MORC3-I-P and pLNCX2-FLAG-MORC3-I-P, respectively, by replacing their MORC3 fragments with PML IV fragment. pLNCX2-EGFP-I-P was generated from pLNCX2-EGFP-MORC3-I-P by replacing its EGFP-MORC3 fragment with EGFP fragment derived from pEGFP vector. Plat-E or Plat-A packaging cells were used for production of the retrovirus, as described previously (Morita *et al.*, 2000). Infected cells were selected with puromycin (2 μg/ml for WI38 and 3 μg/ml for MEFs) for 4 d. Day 0 is the first day after the selection. At day −1, cells were plated in 15 wells of 12-well plates at 2×10^4 cells/well. At the indicated time points, the numbers of cells in three wells were counted using a Z1 particle counter (Beckman Coulter, Fullerton, CA). The population doublings were calculated as described previously (Bischof *et al.*, 2002). When cells became semiconfluent, 25% of the cells were further subcultured. Senescence-associated β-galactosidase (SA-β-Gal) activity was detected as previously described (Dimri *et al.*, 1995).

Luciferase Assay

The pGL3-PG12S reporter vector derived from pGL3-Promoter (Promega, Madison, WI) contains 12 repeats of a p53-binding site (5′-CCTGCCTGGACTTGCCTGG-3′; el-Deiry *et al.*, 1993) upstream of an SV40 promoter. As a negative control, the pGL3-MG14S vector was generated by replacing the p53-binding sites with 14 repeats of mutated p53-binding site (5′-CCTTAATGGACTTTAATGG-3′). Transfection efficiency was normalized using Renilla luciferase activity expressed from pGL3-Renilla, containing the *Renilla luciferase* gene derived from phRL-TK vector (Promega).

pCBG99-p21 and pCBG99-Bax reporter vectors were constructed by inserting the 2.4-kbp p21 promoter region (el-Deiry *et al.*, 1993) and the Bax promoter (−690 base pairs to −317 base pairs; Miyashita and Reed, 1995), respectively, upstream of the *green-emitting luciferase* gene of pCBG99-Basic (Promega). An internal control vector, pCBR-SV40pro, was generated using an SV40 promoter derived from pGL3-Control (Promega). The total amount of transfected DNA was kept constant by adjusting with empty vectors or pMEPy-GST. One or two days after transfection, luciferase activities were measured using a Dual-Glo Luciferase Assay System (Promega) or a Chroma-Glo Luciferase Assay System (Promega). Means were calculated from the results of three independent experiments.

Generation of Morc3-Knockout Mice

We obtained *Morc3* genomic BAC clones by screening the RPCI-22 129s6/SvEvTAC mouse BAC library (BACPAC Resources Center, Oakland, CA). A 3-kbp fragment containing *Morc3* exon 3 and exon 4 (103 base pairs), *hrGFP* gene (Stratagene) fused in frame to *Morc3*, and a rabbit β-globin polyadenylation signal was cloned into the SmaI site of the pBluescript-neo/Diphtheria toxin fragment A (DT-A)/loxP vector (Ikeda *et al.*, 1999). A 4-kbp fragment containing *Morc3* exon 6 was cloned into the EcoRV site of the same vector. Part of *Morc3* exon 4 (112 base pairs) and exon 5 encoding GHL-ATPase motifs III and IV were replaced by the *hrGFP* gene and *neomycin resistance* gene flanked by loxP sites. RW4 Embryonic stem (ES) cells transfected with the NotI-linearized vector were selected with G418 and screened with a 1-kbp probe (see Figure 3A) using the ALKphos direct labeling and detection system with CDP-*Star* (GE Healthcare, Waukesha, WI). Generation of *Morc3*-knockout mice from two independent targeted ES clones was carried out as described previously (Ikeda *et al.*, 1999). MEFs were generated by intercrossing *Morc3*+/− mice backcrossed to C57/BL6J one to three times. Animal experiments were conducted in accordance with our institutional guidelines.

Antibodies

The following antibodies and antisera were used: mouse anti-FLAG M2 (Sigma), rabbit polyclonal anti-FLAG (Sigma), rabbit polyclonal anti-HA (Sigma), mouse monoclonal anti-PML (PG-M3; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-PML (H-238; Santa Cruz), goat polyclonal anti-mouse Pml (E-15; Santa Cruz), rabbit polyclonal anti-Sp100 (Chemicon, Temecula, CA), mouse monoclonal anti-human p53 (DO-1; Santa Cruz), rabbit polyclonal anti-mouse p53 (CM5; Novocastra, Newcastle, United Kingdom), rabbit polyclonal anti-p21 (C-19; Santa Cruz), mouse monoclonal anti-p16 (DCS-50; Sigma), rabbit anti-CBP (A-22; Santa Cruz), and mouse monoclonal anti-β-tubulin (TUB 2.1; Sigma) antibodies. The following rabbit polyclonal antibodies were used to assay posttranslational modifications of p53: anti-phospho-p53 (Ser15) (9284; Cell Signaling, Beverly, MA), anti-phospho-p53 (Ser20) (9287; Cell Signaling), and anti-acetyl-p53 (Lys373, Lys382) (06–758; Upstate Biotechnology, Lake Placid, NY) antibodies. Anti-MORC3 rabbit polyclonal antiserum was raised against recombinant Histidine-tagged-MORC3 (His-MORC3) purified from Rosetta DE3 (Novagen, Madison, WI) carrying pET16b-His-MORC3. We established a hybridoma clone (17A9) from mice immunized with His-MORC3 and purified monoclonal anti-MORC3 antibody from it by standard methods.

Western Blotting

Proteins were transferred from SDS-PAGE gels to Hybond-P (GE Healthcare) or Immobilon-P (Millipore, Bedford, MA) membranes and visualized by incubation with various primary antibodies and peroxidase-conjugated secondary antibodies (Sigma), using ECL or ECL-Plus detection reagents (GE Healthcare).

Immunofluorescence

Immunofluorescence studies were performed using the standard procedure previously described (Watanabe *et al.*, 1996). Briefly, cells grown on Lab-Tek II Chamber Slides (Nalge Nunc, Naperville, IL) were fixed and permeabilized. Cells were then incubated with the indicated antibodies and further incubated with appropriate Alexa Fluor-488– or Alexa Fluor-594–conjugated, cross-adsorbed secondary antibodies (Invitrogen). Confocal imaging was performed using a Radiance 2000 laser-scanning system (Bio-Rad, Richmond, CA) connected to a BX51 microscope (Olympus, Melville, NY). When cells were permeabilized before fixation, they were treated with CSK buffer (10

mM PIPES, pH 6.8, 100 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose, and complete protease inhibitor cocktail [Complete Mini, EDTA free; Roche]) with 0.5% Triton X-100 for 5 min and fixed with 4% paraformaldehyde at 4°C overnight. The fixed cells were stained and observed as described above. NB4 cells were treated with 1 μM of all-*trans*-retinoic acid (ATRA, Sigma) or dimethyl sulfoxide (DMSO) as vehicle for 3 d, attached to slide glasses with Shandon Cytospin 4 (Thermo Electron, Pittsburgh, PA), and stained as described above.

ATP Depletion Analysis

ATP depletion analysis was performed as previously described (Platani *et al.*, 2002). HeLa cells transiently transfected with pME-FLAG-MORC3-I-P were incubated in glucose-free medium containing 10 mM sodium azide (Wako, Richmond, VA) and 50 mM 2-deoxyglucose (Sigma) for 30 min and then fixed and stained with anti-FLAG antibodies and anti-PML antibodies as described above. To determine the time course of MORC3 localization, HeLa cells were transfected with pME-EGFP-MORC3-I-P and pME-FLAG-MORC3-I-P (1:8) to prevent EGFP aggregation. Live cells were imaged using an IX70 microscope (Olympus) and CoolSnap cf CCD camera (Roper Scientific, Tucson, AZ).

RESULTS

MORC3 Induces p53-dependent Premature Senescence

To analyze the function of MORC3, we first expressed EGFP-MORC3 in WI38 human normal fibroblasts using the pLNCX2 retrovirus vector (Supplementary Figure 2A). Expression of MORC3 severely inhibited proliferation of infected cells (Figure 1A). Most cells expressing MORC3 exhibited a large flat morphology, suggesting premature senescence, like PML IV-expressing cells (Bischof *et al.*, 2002). To clarify whether MORC3 induces premature senescence, infected cells were stained for SA-β-Gal activity at day 6 (Figure 1, B and C). The percentage of SA-β-Gal–positive cells infected with the MORC3-retrovirus vector (51.0 ± 5.1%) was significantly greater than that of controls infected with vector alone (2.3 ± 0.8%). MORC3 expression also strongly induced p16, another senescence marker (Figure 1D).

Because p53 plays important roles in preventing cell proliferation by inducing premature senescence (Campisi, 2005), we next examined the effect of MORC3 expression in wild-type and p53-deficient MEFs (Gondo *et al.*, 1994) (Supplementary Figure 2B). Wild-type MEFs (*p53*+/+) infected with the MORC3-retrovirus vector ceased proliferating (Figure 1E) and exhibited the same large flat morphology as MORC3-infected WI38 cells (Supplementary Figure 2C). However, MORC3 did not impair proliferation or induce the flat morphology of *p53*−/− MEFs derived from littermates of *p53*+/+ mice (Figure 1E and Supplementary Figure 2C).

Expression of MORC3 Activates p53

To examine whether MORC3 regulates p53 activity, we measured the effect of MORC3 expression on p53-mediated trans-

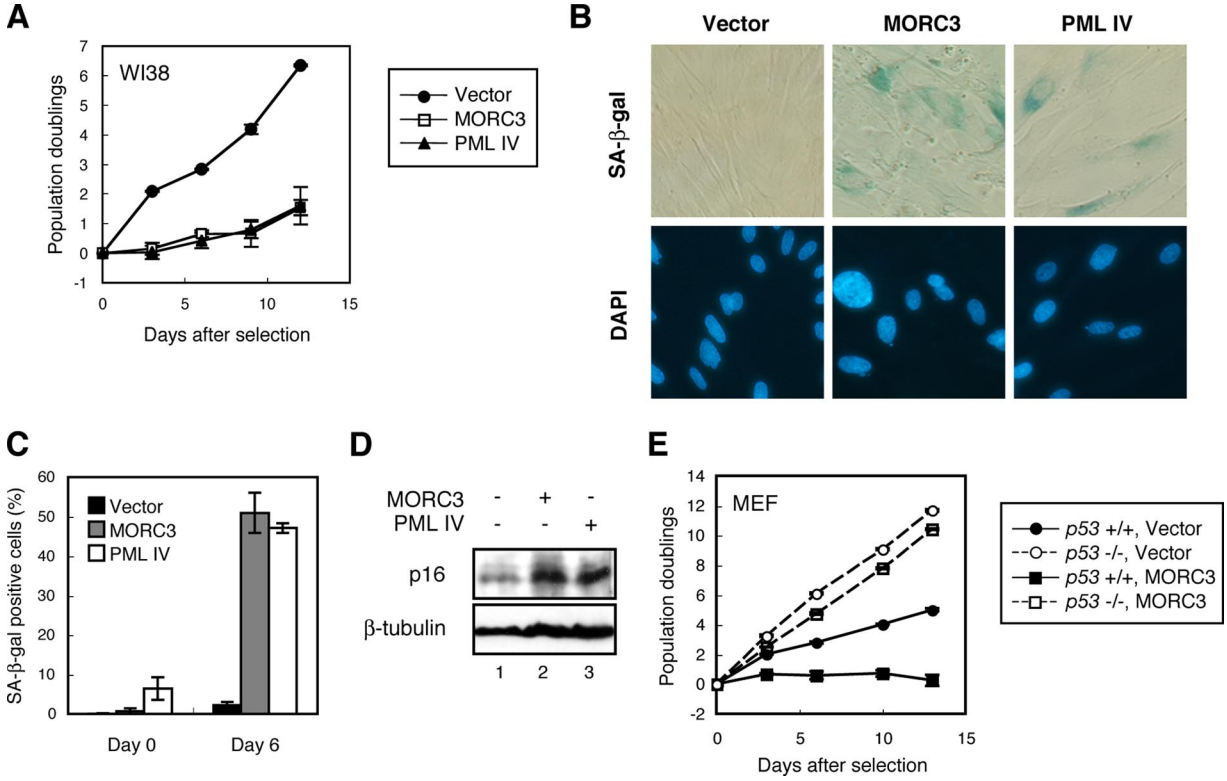

Figure 1. MORC3 induces p53-dependent premature senescence. Data in A, C, and E represent mean ± SD of triplicate experiments. (A) Human normal fibroblasts WI38 infected with a retroviral vector carrying EGFP (Vector, ●), EGFP-MORC3 (MORC3, □) or EGFP-PML IV (PML IV, ▲) were selected with puromycin for 4 d, and the number of the cells was counted at days 3, 6, 9, and 12. Day 0 indicates the first day after the selection. (B) Representative senescence-associated β-galactosidase (SA-β-Gal) staining in WI38 cells infected with a vector carrying EGFP (Vector, left), EGFP-MORC3 (MORC3, middle) or EGFP-PML IV (PML IV, right). SA-β-Gal–positive cells were blue (top images). Nuclei were stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI, bottom images). (C) Quantitation of SA-β-Gal–positive cells as shown in B. More than 249 cells were counted in each experiment. (D) Induction of p16 in WI38 cells infected with vector (lane 1), vector carrying MORC3 (lane 2), or PML IV (lane 3). Lysates were collected at day 6 and immunoblotted with anti-p16 or anti-β-tubulin antibody. (E) The retrovirus control vector (circles) or the vector carrying MORC3 (squares) was infected into *p53*+/+ (filled) or *p53*−/− (open) mouse embryonic fibroblasts (MEFs), and the population doublings of these cells were measured. *p53*+/+ and *p53*−/− MEFs were generated from littermate embryos.

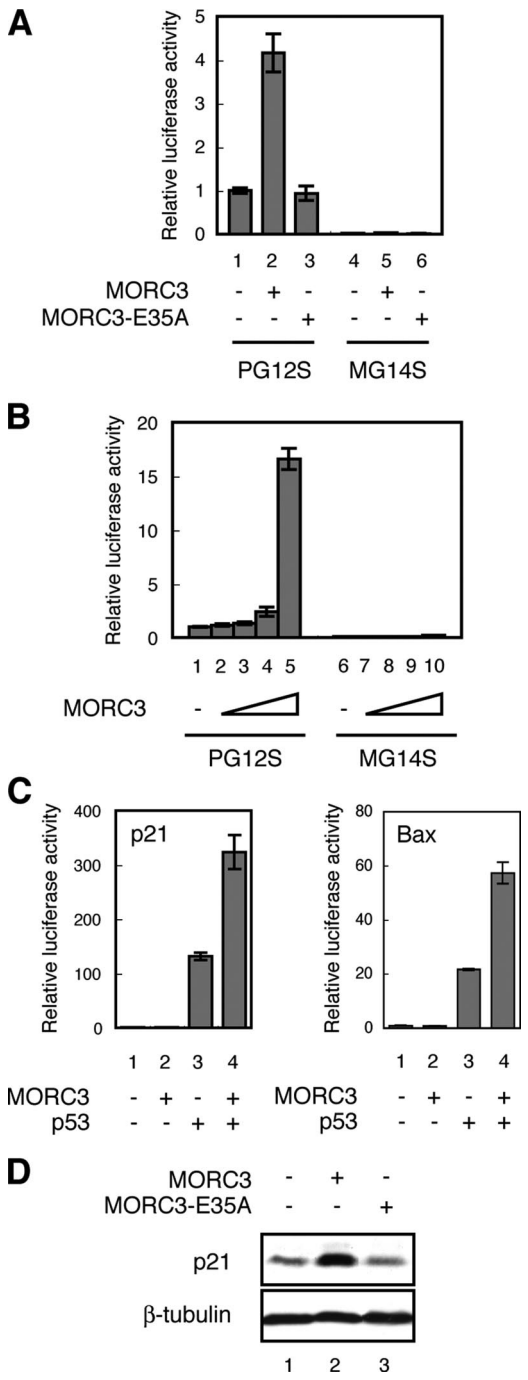

Figure 2. MORC3 transactivates p53. (A) U-2 OS cells were cotransfected with the control vector, pMEPy-FLAG-MORC3, or pMEPy-FLAG-MORC3-E35A, and firefly luciferase reporter constructs (PG12S-Luc containing 12 p53-binding elements or MG14S-Luc containing 14 mutated p53-binding elements). Relative activities were calculated from arbitrary light units of firefly luciferase activity normalized for transfection efficiency (see *Materials and Methods*). Data represent mean ± SD of triplicate transfections. (B) U-2 OS cells were cotransfected with firefly luciferase constructs (PG12S-Luc or MG14S-Luc, 35 ng) and increasing amounts of the MORC3 expression vector (0, 8.75, 17.5, 35, and 70 ng). Total amount of plasmid DNA was kept constant by the addition of empty vector. Luciferase activities were measured as described in A. (C) MORC3 induces p53-transactivation of the p21 (left) and Bax (right) promoters. H1299 cells were transfected with p21-CBG99luc containing the p21 promoter or Bax-CBG99luc containing the Bax promoter together with pMEPy-FLAG-MORC3 and pMEPy-HA-p53. The CBG99 luciferase reporter construct, pMEPy-FLAG-MORC3, pMEPy-HA-p53, and CBR luciferase control vector were transfected at amount ratios of 40:80:1:320 in the p21 promoter assay and 20:12.5:1:10 in the Bax promoter assay, respectively. (D) U-2 OS cells were transfected with the pMEPy vector (lane 1), pMEPy-FLAG-MORC3 (lane 2), or its E35A mutant (lane 3). Three days after transfection, total lysates of transfectants were immunoblotted for p21 and β-tubulin.

activation by luciferase reporter analyses. FLAG-MORC3 expression enhanced transcriptional activation by endogenous p53 of a reporter containing 12 p53-responsive elements (PG12S) in a dose-dependent manner, but not activation of a reporter containing 14 mutated responsive elements (MG14S) (Figure 2, A and B). In contrast, FLAG-MORC3-E35A, a mutant of MORC3 lacking ATPase activity but retaining ATP-binding activity generated by mutation of the conserved catalytic glutamic acid 35 in N-box of the GHL-ATPase domain to alanine (Supplementary Figure 1; Panaretou *et al.*, 1998; Ban *et al.*, 1999) showed no transcriptional enhancement (Figure 2A). To examine whether MORC3 also affects transactivation of the promoters of p53-inducible genes, MORC3 was expressed together with p53, and *luciferase* was transcribed from p21 and Bax promoters in p53-deficient H1299 cells. MORC3 enhanced the p53-dependent activation of p21 (2.5-fold, Figure 2C, left) and Bax (2.6-fold, Figure 2C, right) promoters. MORC3 but not MORC3-E35A also induced the expression of endogenous p21 in U-2 OS cells expressing wild-type p53 (Figure 2D).

Transcriptional activity of p53 is regulated mainly by protein stability and DNA-binding activity through post-translational modifications including phosphorylation and acetylation (Bode and Dong, 2004). Therefore, we examined whether overexpression of MORC3 induces p53 stabilization by the phosphorylation and acetylation of p53. In MORC3-overexpressed WI38 cells, p53 was stabilized and phosphorylated at serine 15 but barely acetylated at lysine 373 and 382 (Supplementary Figure 3).

Deficiency of Morc3 Reduces p21 Induction by Adriamycin in MEFs

To investigate whether p53 is activated by genotoxic signals in MORC3 deficient cells, we produced *Morc3*-knockout mice by homologous recombination in murine ES cells (Figure 3, A–C). We generated two lines of *Morc3*-targeted mice from independently targeted ES cell clones as described previously (Ikeda *et al.*, 1999). All mice homozygous for the *Morc3*-disrupted allele (*Morc3*−/−) died at birth or within a day thereafter for unknown reasons (Yoshida, Takahashi, Mimura, Ishizaki, Tanaka-Okamoto, Miyoshi, Shime, and Inoue, unpublished results). Wild-type, heterozygous and homozygous embryos at 18.5 d postcoitum (dpc) were present at almost the expected Mendelian ratio. MEFs of *Morc3*+/+ and *Morc3*−/− established from littermate embryos at 14.5 dpc were morphologically indistinguishable.

Treatment of both *Morc3*+/+ and *Morc3*−/− MEFs with the genotoxic drug adriamycin (ADR) increased the expression of p53 protein (Figure 3, D and E). In both MEFs, p53 was efficiently phosphorylated at residues homologous to human serine 15 and 20 (mouse S18 and S23; Figure 3E) and stabilized by ADR treatment. However, in the absence of Morc3, p21 was barely induced (Figure 3, D and E). Acetylation of p53 at residues homologous to human lysine 373 and lysine 382 (mouse K370 and K379) was slightly reduced in *Morc3*−/− MEFs compared with *Morc3*+/+ MEFs (Figure 3, D and E).

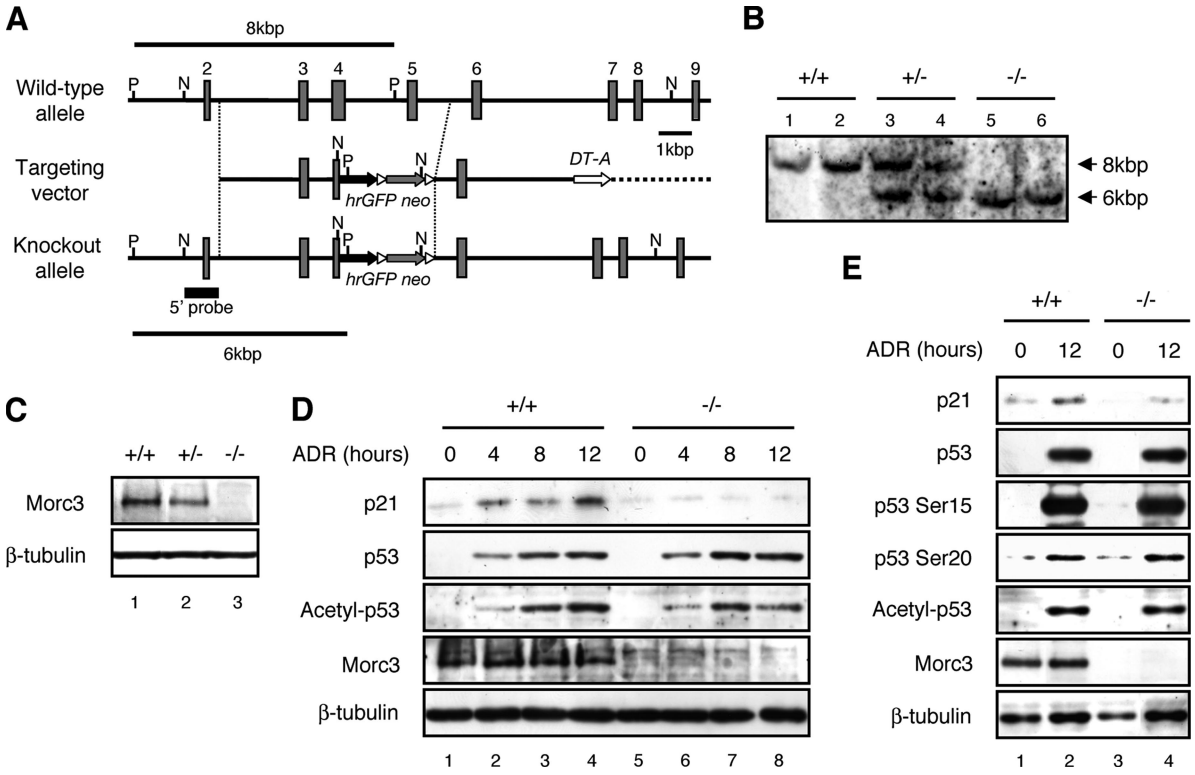

Figure 3. Impairment of p53 activation by adriamycin (ADR) treatment in MEFs derived from *Morc3*−/− mice. (A) Organization of mouse *Morc3* gene, targeting vector, and knockout allele. Gray boxes denote exons (2–9). Black, gray, and white arrows indicate *hrGFP* (Stratagene), *neomycin resistance* gene (*neo*) and *Diphtheria toxin fragment A* (*DT-A*) expression cassette, respectively. White arrowheads indicate loxP sites. P, PvuII; N, NcoI. (B) Southern blot analysis of genomic tail DNA digested with PvuII using the 5′ probe shown in A. An 8-kbp band in wild-type mice (lanes 1 and 2), a 6-kbp band in homozygous mice (lanes 5 and 6), and both bands in heterozygous mice (lanes 3 and 4) were detected. (C) Western blot analysis of lysates of MEFs from wild-type (lane 1), heterozygous (lane 2), and homozygous (lane 3) mice. Morc3 protein was detected with a rabbit polyclonal antibody. (D) *Morc3*+/+ and *Morc3*−/− MEFs were treated with 1 μg/ml adriamycin (ADR) for 0–12 h. Total cell lysates at 0, 4, 8, and 12 h were immunoblotted for p21, p53, p53 with acetylated lysine 373 and lysine 382 (Acetyl-p53; mouse K370 and K379), Morc3, and β-tubulin. This experiment was performed at least three times, with similar results. (E) *Morc3*+/+ and *Morc3*−/− MEFs were treated with ADR for 12 h. Total cell lysates from treated or untreated MEFs were separated by SDS-PAGE and immunoblotted with antibodies for p21, p53, p53 with phosphorylated serine 15 (p53 Ser15; mouse S18) and serine 20 (p53 Ser20; mouse S23), Acetyl-p53, Morc3, and β-tubulin.

MORC3 Localizes on PML-NBs

In addition to posttranslational modification, recruitment of p53 into PML-NBs is important for its activation (Fogal *et al.*, 2000; Pearson *et al.*, 2000). The mechanism of this recruitment is poorly understood. Because p53 was stabilized but not activated in ADR-treated *Morc*3−/− cells, we hypothesized that MORC3 could be involved in p53 localization into PML-NBs.

To test this hypothesis, we first determined the subcellular localization of MORC3. An anti-MORC3 mAb specifically stained entire nuclei and a few small nuclear foci together with Pml, except for the nucleolus, in *Morc*3+/+ but not *Morc*3−/− MEFs (Figure 4A and Supplementary Figure 4, A and B). In human cell lines, Saos-2 (Figure 4B) and HeLa cells (Supplementary Figure 4C), endogenous MORC3 was detected primarily on nuclear foci, together with PML (Figure 4B, top images) and Sp100, which is constitutively localized on PML-NBs (Figure 4B, bottom images) and also in entire nuclei. These results indicate that MORC3 localizes to both PML-NBs and the nucleoplasm. Although we detected MORC3 nuclear foci in almost all PML-NBs of most human and mouse cells, the size and the number of these nuclear foci were different in cell lines. When FLAG-MORC3 was expressed in HeLa cells, endogenous PML was localized within almost all of the nuclear foci formed by overexpressed FLAG-MORC3 in all cells (Figure 4C, top images), and endogenous Sp100 was completely colocalized with FLAG-MORC3 in 44% of the FLAG-MORC3–expressing cells (Figure 4C, bottom images).

PML-mediated Localization of MORC3 on PML-NBs

To investigate whether PML is required for the localization of MORC3 on PML-NBs, we examined the localization of MORC3 in NB4 cells, an acute promyelocytic leukemia (APL) cell line expressing PML-retinoic acid receptor α (RARα) (Dyck *et al.*, 1994; Weis *et al.*, 1994). In these cells, PML-NB structure is disrupted but is restored by treatment with all-*trans*-retinoic acid (ATRA; Figure 4D). MORC3 was diffusely distributed in the entire nucleus of untreated NB4 cells, and nuclear foci were detected in only 2.86% of the cells (Figure 4D, top images). ATRA treatment induced PML-NB formation and recruitment of MORC3 (Figure 4D, bottom images) as well as Sp100 and CBP (Supplementary Figure 5, A and B) to the reorganized PML-NBs in 15.4% of the cells. The localization of MORC3 to nuclear foci was also suppressed in PML-knockdown cells (Figure 4E). Furthermore, in WI38 cells, overexpression of PML IV increased the localization of endogenous MORC3 to PML-NBs (Figure 4F).

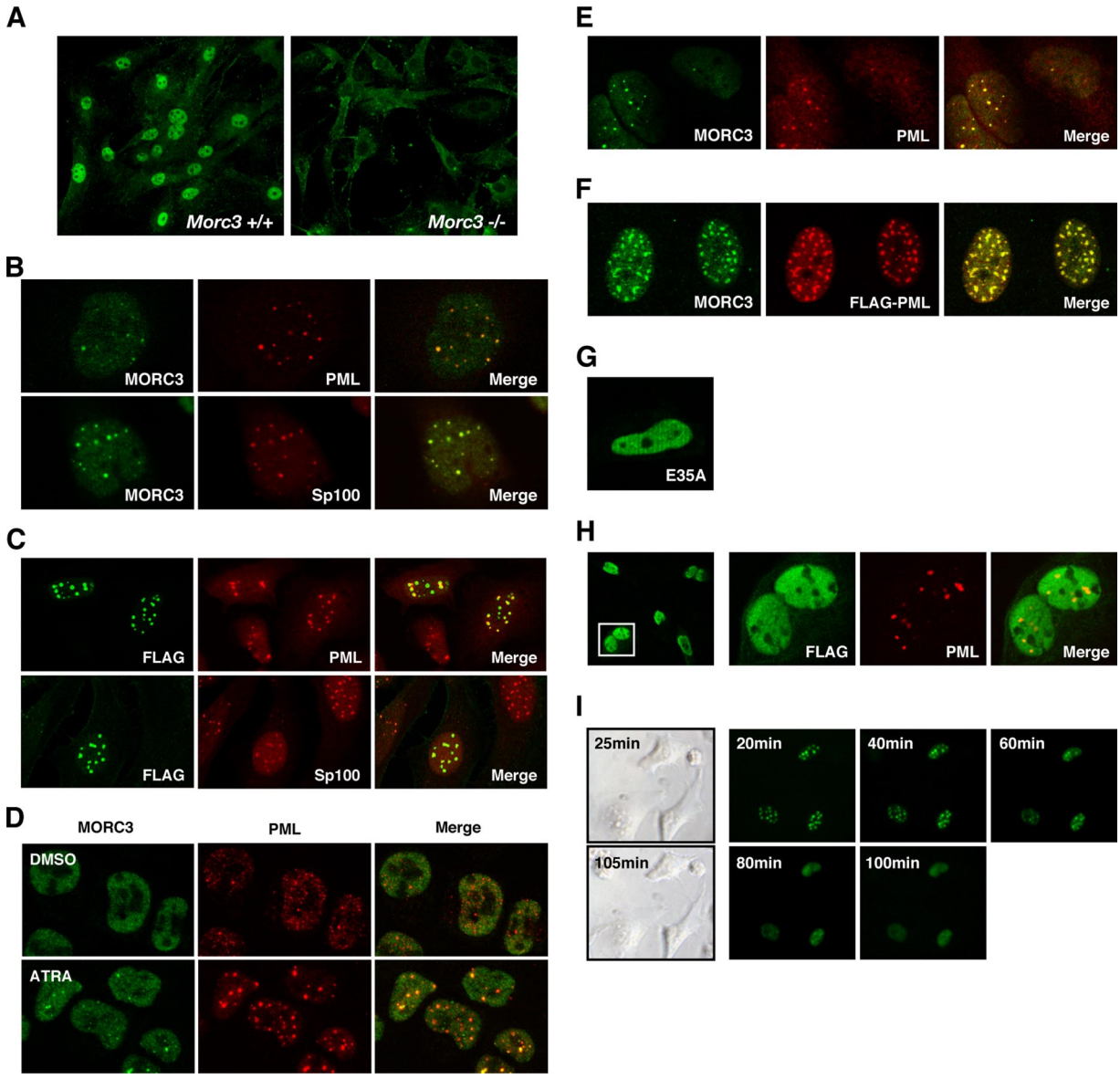

Figure 4. MORC3 localization to PML-NBs depends on its GHL-ATPase activity. Overlapping images are shown in the right side (Merge) of B–F and H. (A) *Morc3*+/+ and −/− MEFs were stained by indirect immunofluorescence with anti-MORC3 monoclonal antibody (17A9; green). This antibody specifically detected nuclei of *Morc3*+/+ (right image) but not *Morc3*−/− (left image) MEFs. (B) Representative images of Saos-2 cells stained with anti-MORC3 antibody (17A9; green), anti-PML antibody (red, top images) and anti-Sp100 antibody (red, bottom images). MORC3 colocalized with PML and Sp100 on PML-NBs. (C) HeLa cells were transiently transfected with the FLAG-MORC3 expression vector and stained for FLAG (green), and PML (red, top image), or Sp100 (red, bottom image). Overexpressed MORC3 colocalized with PML and with Sp100. (D) Acute promyelocytic leukemia NB4 cells were incubated in the presence of 1 μM of all-*trans*-retinoic acid (ATRA, bottom images) or vehicle (DMSO, top images) and stained for MORC3 (green) and PML (red). In the absence of ATRA, MORC3 was diffusely localized to entire nuclei except for nucleoli. Treatment with ATRA-mediated localization of MORC3 into PML-NBs in cells with reorganized PML-NBs. (E) PML-knockdown vector-transfected Saos-2 cells were stained for MORC3 (green) and PML (red). MORC3 was completely dispersed or localized to two or fewer obvious nuclear foci in 73.3% of cells in which PML was repressed, as indicated by two or fewer obvious PML-nuclear foci. In contrast, we detected cells containing two or fewer MORC3 nuclear foci in 38.5% of cells transfected with a control vector (data not shown). (F) WI38 cells were infected with a retrovirus vector carrying FLAG-PML IV, selected with puromycin, and stained for MORC3 (green) and for FLAG (red) at day 1. PML-NBs localization of endogenous MORC3 was enhanced in FLAG-PML IV expressing cells. (G) ATPase-activity–deficient mutant (FLAG-MORC3-E35A)-expressing HeLa cells were stained for FLAG (E35A). MORC3-E35A did not accumulate in PML-NBs and diffusely localized to entire nuclei except for nucleoli. (H) FLAG-MORC3-expressing HeLa cells were fixed after ATP depletion for 30 min and stained for FLAG (green) and PML (red). Left image shows low-power view; other images show magnified view of boxed region. ATP depletion caused nuclear dispersion of MORC3 but not PML from PML-NBs. (I) ATP in HeLa cells transfected with pME-EGFP-MORC3-I-P and pME-FLAG-MORC3-I-P at a 1:8 ratio was depleted by the addition of sodium azide and 2-deoxyglucose to the culture medium. Cells in the same field were observed by phase contrast (left) or epifluorescence microscopy after the indicated times. EGFP-MORC3 gradually dispersed from nuclear foci with ATP depletion.

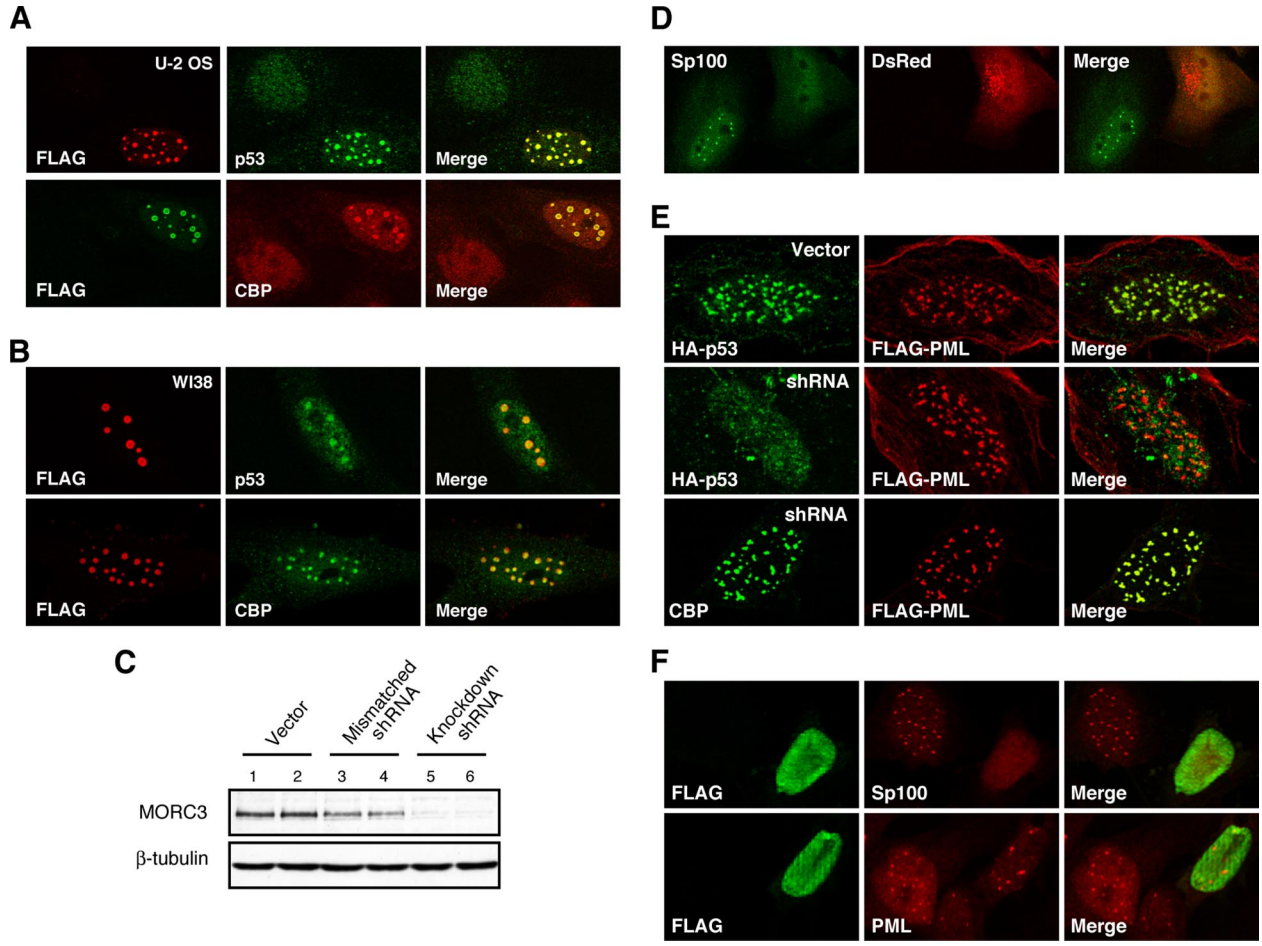

Figure 5. ATPase-dependent recruitment of Sp100, CBP, and p53 into PML-NBs by MORC3. (A) and (B) FLAG-MORC3–expressing U-2 OS cells (A) and WI38 cells (B) were stained for FLAG, and endogenous p53 (top images) or CBP (bottom images). WI38 cells infected with pLNCX2-FLAG-MORC3 retrovirus vector were selected with puromycin for 4 d and stained at day 1. Both p53 and CBP were completely colocalized with FLAG-MORC3 in all FLAG-MORC3–expressing U-2 OS cells. In WI38 cells, CBP accumulated in PML-NBs of all FLAG-MORC3–expressing cells, but p53 accumulated in only a portion of FLAG-MORC3-expressing cells (14.3% of cells at day 1 and 47.4% of cells at day 3). (C) HeLa cells transfected with control vector pH1′-DsRed2-I-P (Vector, lanes 1 and 2), mismatched shRNA vector pH1′-shRNA695-DsRed2-I-P containing three mismatched nucleotides (Mismatched shRNA, lanes 3 and 4) or knockdown shRNA vector pH1′-shRNA732-DsRed2-I-P (Knockdown shRNA, lane 5 and 6) were selected with 1 μg/ml puromycin for 2 d. Lysates of the transfected cells were immunoblotted with anti-MORC3 rabbit polyclonal antibody or anti-β-tubulin antibody. MORC3 expression was assayed in two independent shRNA transfections. (D) HeLa cells were transfected with knockdown shRNA vector shRNA732. After selection with puromycin, the transfected cells were stained for Sp100 (green). Sp100 was dispersed in 41.3% of DsRed2-positive cells (DsRed) in which expression of MORC3 should be suppressed by shRNA. (E) Saos-2 cells were transfected with the MORC3-knockdown shRNA vector shRNA732 (shRNA, middle and bottom images) or the control vector pH1′-DsRed2-I-P (Vector, top images) (1 μg), and the HA-p53 (1 μg) and FLAG-PML IV (3 μg) expression vectors, selected with puromycin for 2 d and treated with 1 μg/ml ADR for 6 h. These cells were permeabilized before fixation and stained for HA (green, top and middle images) or CBP (green, bottom images), and FLAG (red). After ADR treatment, HA-p53 accumulated in nuclear bodies containing FLAG-PML IV in vector-only–transfected cells (Vector, top images) but not in knockdown shRNA vector-transfected cells (middle images). Endogenous CBP colocalized with FLAG-PML IV in both vector-only and MORC3-knockdown vector-transfected cells (bottom images). (F) Localization of endogenous Sp100 (red, top image) and endogenous PML (red, bottom image) in HeLa cells expressing FLAG-MORC3-E35A (green). Expression of MORC3-E35A caused complete dispersion of Sp100 in 91% of cells, whereas PML was still localized in NBs.

Role of GHL-ATPase Domain in Localization of MORC3 into PML-NBs

Because expression of the ATPase-deficient MORC3-E35A mutant protein did not activate p53, we examined the effect of this mutation on MORC3 localization. FLAG-MORC3-E35A showed diffuse nuclear localization in HeLa cells (Figure 4G). Furthermore, depleting HeLa cells expressing wild-type FLAG-MORC3 of ATP by adding sodium azide and 2-deoxyglucose to the medium caused MORC3 to disperse from PML-NBs within 30 min in most cells, whereas endogenous PML remained localized to PML-NBs (Figure 4H). To study the time course of this dispersion in living cells, we expressed EGFP-MORC3 in HeLa cells. EGFP-MORC3 gradually dispersed after ATP depletion (Figure 4I).

MORC3 Induces Localization of p53 and Sp100 into PML-NBs

In premature senescence induced by expression of PML IV or oncogenic Ras, p53 and CBP are recruited into PML-NBs to form a ternary complex with PML (Pearson *et al.*, 2000; Bischof *et al.*, 2002). To test whether MORC3 mediates the recruitment of p53 and CBP into PML-NBs, we analyzed the

effect of MORC3 overexpression on p53 and CBP localization. Overexpressed FLAG-MORC3 colocalized with endogenous p53 (Figure 5, A and B, top images) and CBP (Figure 5, A and B, bottom images) in U-2 OS cells (Figure 5A) and WI38 cells (Figure 5B), but p53 showed diffuse nuclear localization in nontransfected and FLAG-MORC3-E35A–transfected cells (Supplementary Figure 6). Furthermore, p53 and PML IV specifically formed a complex with MORC3 (Supplementary Figure 7). We then examined the effect of MORC3 repression on Sp100, p53, and CBP subnuclear localization. MORC3 was efficiently repressed by shRNA732 in HeLa cells (Figure 5C). Endogenous Sp100 was completely dispersed or localized to two or fewer nuclear foci in DsRed2-positive cells (41.3%) in which MORC3 was simultaneously repressed (Figure 5D). In contrast, fewer than 10% of cells transfected with a mutant shRNA695 (8.7%) or a control vector (5.3%) showed diffuse localization of Sp100 (Supplementary Figure 8A). Notably, subcellular localization of PML was not altered by shRNA knockdown of MORC3 (supplementary Figure 8B). We also examined the effect of MORC3 repression in p53-expressing Saos-2 cells treated with ADR. In cells transfected with the shRNA control vector, p53 was recruited into PML-NBs 6 h after ADR treatment (14% of PML IV–expressing cells; Figure 5E, top images). However, we could not detect ADR-induced recruitment of p53 into PML-NBs in MORC3-repressed cells (Figure 5E, middle images). In contrast, endogenous CBP localized on PML-NBs in both MORC3-knockdown cells (Figure 5E, bottom images) and cells transfected with control vector (data not shown). To clarify the role of ATPase domain of MORC3 in the localization of Sp100 and p53 to PML-NBs, we transfected HeLa cells with the vector carrying FLAG-MORC3-E35A. Overexpression of the mutant MORC3 protein caused dispersion of Sp100 in 91% of cells (Figure 5F, top images), whereas PML was still localized in NBs (Figure 5F, bottom images).

DISCUSSION

Novel Mechanism of Subnuclear Localization

In this study, we show that MORC3 is involved in p53 activation and localization of conditionally localized p53 and constitutively localized Sp100 to PML-NBs through its GHL-ATPase activity. Although a GTP-driven mechanism regulates dynamic shuttling of Nucleostemin between nucleolus and the nucleoplasm (Tsai and McKay, 2005), little is known about other machinery involved in localizing proteins from the nucleoplasm to subnuclear domains. This is the first reported ATP-dependent molecular mechanism of recruitment of proteins to a subnuclear domain essential for an important biological response.

Induction of Premature Senescence and Recruitment of Nuclear Proteins into PML-NBs by MORC3

Both the p53 and pRB pathways are essential for cells to establish and maintain the senescent state (Serrano *et al.*, 1997; Campisi, 2005). We show that expression of MORC3 induced premature senescence (Figure 1) through activation of p53 (Figure 2). Formation of a ternary complex of p53, CBP, and PML in PML-NBs is essential for this process (Pearson *et al.*, 2000; Bischof *et al.*, 2002). MORC3 mediated the localization of both p53 and CBP into PML-NBs in U-2 OS cells and WI38 cells (Figure 5, A and B) and formed a complex with p53 and PML IV (Supplementary Figure 7). In MORC3-repressed cells treated with ADR, CBP but not p53 localized into PML-NBs (Figure 5E). These results explain why p53 was not efficiently activated in ADR-treated *Morc3*−/− cells despite its stabilization and suggest that MORC3 mediates p53 recruitment to a CBP and PML complex in the process of premature senescence and p53 activation induced by genotoxic stress. MORC3 was partially localized in PML-NBs by ATRA treatment of NB4 cells (Figure 4D). The partial rather than complete localization of MORC3 could be due to incomplete reorganization of PML-NBs or to the expression of the fusion protein, PML-RARα. Furthermore, overexpression of PML IV also recruited MORC3 (Figure 4F) as well as CBP and p53 into PML-NBs (Pearson *et al.*, 2000). These results suggest that MORC3 recognizes PML to recruit p53 into PML-NBs.

Effect of MORC3 on Posttranslational Modification of p53

Localization of p53 on PML-NBs is thought to mediate its posttranslational modifications, including acetylation of lysine residue 382 (mouse K379) by p300/CBP (Pearson *et al.*, 2000) and phosphorylation of human p53 serine residue 20 (mouse S23) by CHK2 (Louria-Hayon *et al.*, 2003) and serine residue 46 (no equivalent residue in mice) by HIPK2 (D'Orazi *et al.*, 2002; Hofmann *et al.*, 2002). In *Morc3*−/− cells treated with ADR, p53 acetylation on K373 (mouse K370) and/or K382 but not phosphorylation of S20 was slightly but reproducibly reduced compared with wild type (Figure 3E). However, the magnitude of this reduction does not appear sufficient to explain the lack of p53 activation. Although PML is essential for acetylation of p53 (Fogal *et al.*, 2000; Pearson *et al.*, 2000), MORC3-dependent localization of p53 into PML-NBs is not necessary for its stabilization through phosphorylation and acetylation but is required for its activation in response to genotoxic stress. The relationship between acetylation, phosphorylation, and subnuclear localization of p53 requires further study.

Roles of MORC3 GHL-ATPase Domain

We showed that the ATPase activity-deficient MORC3 mutation, glutamic acid 35 to alanine, caused diffuse nuclear localization of MORC3 and could not increase the activation of p53. We also showed by an ATP-depletion experiment that ATP is necessary for the PML-NBs localization of MORC3. The glutamic acid is conserved in all GHL-ATPase family proteins but not in Histidine kinases of the GHKL-ATPase/kinase superfamily and functions as a base for water activation in ATP hydrolysis (Dutta and Inouye, 2000). GHL-ATPase family proteins exist as homodimers, and hydrolysis of ATP causes a conformational change that regulates intermolecular interaction and association with other molecules. By immunoprecipitation analysis, MORC3 also forms at least a homodimer (Supplementary Figure 7). MORC3 may associate with other molecules in the nucleoplasm or PML-NBs in an ATP-dependent conformation.

Other MORC Family Proteins

It is likely that other MORC family proteins play important roles in shuttling various proteins between nuclear subdomains and the nucleoplasm. In fact, Spo11, which mediates double-strand chromosomes breaks during the first meiotic stage, cannot form foci on chromosomes in *Morc1*-deficient mice (Romanienko and Camerini-Otero, 2000). Morc1 may thus serve a function analogous to MORC3 in recruiting proteins such as Spo11 onto foci of meiotic chromosomes. The ancient phylogeny of MORC proteins (Inoue *et al.*, 1999) suggests that other members are also involved in intranuclear shuttling mechanisms that remains to be elucidated.

ACKNOWLEDGMENTS

We thank T. Yasuda and C. Kozai for technical assistance, Dr. T. Nakano for critical suggestions and Drs. M. Okabe and H. Hasuwa (Osaka University) for pH1′ vector, M. Katsuki (National Institute for Basic Biology) for *p53* knockout mice, T. Nagase (Kazusa DNA Research Institute) for MORC3 (KIAA0136) cDNA, and T. Kitamura (The University of Tokyo) for Plat-A and Plat-E. This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and the Uehara Memorial Foundation.

REFERENCES

Ban, C., Junop, M., and Yang, W. (1999). Transformation of MutL by ATP binding and hydrolysis: a switch in DNA mismatch repair. Cell *97*, 85–97.

Bischof, O., Kirsh, O., Pearson, M., Itahana, K., Pelicci, P. G., and Dejean, A. (2002). Deconstructing PML-induced premature senescence. EMBO J. *21*, 3358–3369.

Bode, A. M., and Dong, Z. (2004). Post-translational modification of p53 in tumorigenesis. Nat. Rev. Cancer *4*, 793–805.

Borden, K. L. (2002). Pondering the promyelocytic leukemia protein (PML) puzzle: possible functions for PML nuclear bodies. Mol. Cell Biol. *22*, 5259–5269.

Braig, M., Lee, S., Loddenkemper, C., Rudolph, C., Peters, A. H., Schlegelberger, B., Stein, H., Dorken, B., Jenuwein, T., and Schmitt, C. A. (2005). Oncogene-induced senescence as an initial barrier in lymphoma development. Nature *436*, 660–665.

Campisi, J. (2005). Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell *120*, 513–522.

Chen, Z. *et al.* (2005). Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature *436*, 725–730.

D'Orazi, G. *et al.* (2002). Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. Nat. Cell Biol. *4*, 11–19.

Dimri, G. P. *et al.* (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl. Acad. Sci. USA *92*, 9363–9367.

Dutta, R., and Inouye, M. (2000). GHKL, an emergent ATPase/kinase superfamily. Trends Biochem. Sci. *25*, 24–28.

Dyck, J. A., Maul, G. G., Miller, W. H., Jr., Chen, J. D., Kakizuka, A., and Evans, R. M. (1994). A novel macromolecular structure is a target of the promyelocyte-retinoic acid receptor oncoprotein. Cell *76*, 333–343.

el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817–825.

Fogal, V., Gostissa, M., Sandy, P., Zacchi, P., Sternsdorf, T., Jensen, K., Pandolfi, P. P., Will, H., Schneider, C., and Del Sal, G. (2000). Regulation of p53 activity in nuclear bodies by a specific PML isoform. EMBO J. *19*, 6185–6195.

Gondo, Y., Nakamura, K., Nakao, K., Sasaoka, T., Ito, K., Kimura, M., and Katsuki, M. (1994). Gene replacement of the p53 gene with the lacZ gene in mouse embryonic stem cells and mice by using two steps of homologous recombination. Biochem. Biophys. Res. Commun. *202*, 830–837.

Hasuwa, H., Kaseda, K., Einarsdottir, T., and Okabe, M. (2002). Small interfering RNA and gene silencing in transgenic mice and rats. FEBS Lett. *532*, 227–230.

Hofmann, T. G., Moller, A., Sirma, H., Zentgraf, H., Taya, Y., Droge, W., Will, H., and Schmitz, M. L. (2002). Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2. Nat. Cell Biol. *4*, 1–10.

Ikeda, W. *et al.* (1999). Afadin: a key molecule essential for structural organization of cell-cell junctions of polarized epithelia during embryogenesis. J. Cell Biol. *146*, 1117–1132.

Inoue, N., Hess, K. D., Moreadith, R. W., Richardson, L. L., Handel, M. A., Watson, M. L., and Zinn, A. R. (1999). New gene family defined by MORC, a nuclear protein required for mouse spermatogenesis. Hum. Mol. Genet. *8*, 1201–1207.

Kimura, Y., Sakai, F., Nakano, O., Kisaki, O., Sugimoto, H., Sawamura, T., Sadano, H., and Osumi, T. (2002). The newly identified human nuclear protein NXP-2 possesses three distinct domains, the nuclear matrix-binding, RNA-binding, and coiled-coil domains. J. Biol. Chem. *277*, 20611–20617.

Louria-Hayon, I., Grossman, T., Sionov, R. V., Alsheich, O., Pandolfi, P. P., and Haupt, Y. (2003). The promyelocytic leukemia protein protects p53 from Mdm2-mediated inhibition and degradation. J. Biol. Chem. *278*, 33134–33141.

Miyashita, T., and Reed, J. C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell *80*, 293–299.

Morita, S., Kojima, T., and Kitamura, T. (2000). Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther. *7*, 1063–1066.

Ohishi, K., Inoue, N., Maeda, Y., Takeda, J., Riezman, H., and Kinoshita, T. (2000). Gaa1p and gpi8p are components of a glycosylphosphatidylinositol (GPI) transamidase that mediates attachment of GPI to proteins. Mol. Biol. Cell *11*, 1523–1533.

Panaretou, B., Prodromou, C., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1998). ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. EMBO J. *17*, 4829–4836.

Pearson, M. *et al.* (2000). PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. Nature *406*, 207–210.

Perry, J., and Zhao, Y. (2003). The CW domain, a structural module shared amongst vertebrates, vertebrate-infecting parasites and higher plants. Trends Biochem. Sci. *28*, 576–580.

Platani, M., Goldberg, I., Lamond, A. I., and Swedlow, J. R. (2002). Cajal body dynamics and association with chromatin are ATP-dependent. Nat. Cell Biol. *4*, 502–508.

Romanienko, P. J., and Camerini-Otero, R. D. (2000). The mouse Spo11 gene is required for meiotic chromosome synapsis. Mol. Cell *6*, 975–987.

Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593–602.

Tsai, R. Y., and McKay, R. D. (2005). A multistep, GTP-driven mechanism controlling the dynamic cycling of nucleostemin. J. Cell Biol. *168*, 179–184.

Watanabe, R., Kinoshita, T., Masaki, R., Yamamoto, A., Takeda, J., and Inoue, N. (1996). PIG-A and PIG-H, which participate in glycosylphosphatidylinositol anchor biosynthesis, form a protein complex in the endoplasmic reticulum. J. Biol. Chem. *271*, 26868–26875.

Watson, M. L., Zinn, A. R., Inoue, N., Hess, K. D., Cobb, J., Handel, M. A., Halaban, R., Duchene, C. C., Albright, G. M., and Moreadith, R. W. (1998). Identification of morc (microrchidia), a mutation that results in arrest of spermatogenesis at an early meiotic stage in the mouse. Proc. Natl. Acad. Sci. USA *95*, 14361–14366.

Weis, K., Rambaud, S., Lavau, C., Jansen, J., Carvalho, T., Carmo-Fonseca, M., Lamond, A., and Dejean, A. (1994). Retinoic acid regulates aberrant nuclear localization of PML-RAR alpha in acute promyelocytic leukemia cells. Cell *76*, 345–356.