MEN1 Is a Melanoma Tumor Suppressor That Preserves Genomic Integrity by Stimulating Transcription of Genes That Promote Homologous Recombination-Directed DNA Repair

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Multiple endocrine neoplasia type 1 is a familial cancer syndrome resulting from loss-of-function mutations in the *MEN1* gene. We previously identified the tumor suppressor *MEN1* as a gene required for oncogene-induced senescence in melanocytes, raising the possibility that *MEN1* is a melanoma tumor suppressor. Here we show that *MEN1* expression is lost in a high percentage of human melanomas and melanoma cell lines. We find that melanocytes depleted of *MEN1* are deficient in homologous recombination (HR)-directed DNA repair, which is accompanied by increased nonhomologous end-joining activity. Following DNA damage, *MEN1* levels increase as a result of phosphorylation by the DNA damage kinase ATM/ATR. Most importantly, we show that *MEN1* functions by directly stimulating the transcription of several genes, including *BRCA1*, *RAD51*, and *RAD51AP1*, that encode proteins involved in HR. *MEN1* and its coactivator, the mixed-lineage leukemia histone methyltransferase, are recruited to the *BRCA1*, *RAD51*, and *RAD51AP1* promoters by estrogen receptor 1, resulting in increased histone H3-lysine 4 trimethylation and transcription. Collectively, our results indicate that *MEN1* is a melanoma tumor suppressor that functions by stimulating the transcription of genes involved in HR-directed DNA repair.

Multiple endocrine neoplasia type 1 (also called MEN1 syndrome) is an inherited autosomal dominant disease characterized by an increased risk of developing pituitary, parathyroid, and pancreatic islet tumors and, less commonly, thymic carcinoids, lipomas, and benign adrenocortical tumors (reviewed in reference 1). The familial cancer syndrome results from loss-of-function mutations in the *MEN1* gene. *MEN1* (also called menin) is a nucleus-localized protein (2) that has been reported to play a role in a variety of cellular processes, including transcription, cell cycle regulation, and proliferation (1). For example, *MEN1* interacts directly with a number of transcription factors, including JUND (3), NF- κ B (4), and SMAD3 (5). Furthermore, as part of mixed-lineage leukemia (MLL) protein-containing histone methyltransferase complexes, *MEN1* promotes the trimethylation of histone H3 tails at lysine 4 (H3K4me3), thereby activating the transcription of target genes, including homeobox genes *HOXA9*, *HOXC6*, and *HOXC8* and the cyclin-dependent kinase inhibitors *p18*, *p21*, and *p27* (also known as *CDKN2C*, *CDKN1A*, and *CDKN1B*, respectively) (6–9).

Several studies have raised the possibility that *MEN1* is also involved in the maintenance of genome integrity. For instance, lymphocytes isolated from individuals with *MEN1* syndrome exhibit increased chromosomal breakage and pancreatic tumors from *MEN1* syndrome patients fail to maintain DNA integrity and chromosomal stability (10, 11). Furthermore, depletion of *MEN1* from *Drosophila* larvae results in an elevated rate of DNA damage-induced mutation (12). However, the mechanism by which *MEN1* preserves genomic integrity remains largely unknown.

In a previous genome-wide RNA interference screen, we identified *MEN1* as one of 17 genes required for an activated *BRAF* oncogene (BRAFV600E) to induce senescence in melanocytes (13). Oncogene-induced senescence is a cancer prevention mech-

anism, and thus, factors that promote senescence are candidate tumor suppressors (reviewed in reference 14). Here we investigate the possibility that *MEN1* is a melanoma tumor suppressor and go on to study its mechanism of action.

MATERIALS AND METHODS

Cell lines and culture/treatment conditions. Cells and cell lines were obtained as follows: human neonatal primary melanocytes (used in most experiments involving melanocytes) from Invitrogen; human melanocytes (see Fig. 1B) and short-term melanoma cultures YULAC, YUSAC, YUGEN8, YURIF, 501mel, and YULIZ from YSPORE, Yale University; WM3918 cells from the Wistar Institute; and SK-MEL-28, SK-MEL-103, A375, M14, MeWo, primary foreskin fibroblasts (PFFs), and HCT116 cells from the American Type Culture Collection. All cell lines were maintained as recommended. Normal skin, melanocytic nevus, and melanoma samples were provided by the UMass Cancer Center Tissue Bank and the Skin Pathology Laboratory, Boston University School of Medicine. For BRAFV600E/MEL-ST cells, immortalized but nontransformed human MEL-ST cells (provided by Robert Weinberg) were transduced with a BRAFV600E-expressing retrovirus, pBABE-Blast-BRAFV600E (constructed by cloning BRAFV600E from pBABE-Puro-BRAFV600E [Addgene] into pBABE-BLAST [Addgene]), with 10 μ g/ml Polybrene (Sigma) and selected with blasticidin. HCT116/HN5 cells were kindly provided by Mark Meuth.

For gamma irradiation treatment, cells were gamma irradiated with 20 Gy for 6 h unless otherwise noted. For ATM/ATR inhibitor treatment,

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cells were treated with 10 μ M CGK733 (Calbiochem) (15) for 2 to 6 h (see Fig. 5B and F) or 2 h (see Fig. 7B). Ethanol or estradiol (Sigma) was added to the medium at a concentration of 300 nM 45 min before the fixation of cells (see Fig. 8B and C and 9C to E). Ethanol or fulvestrant (also called ICI 182,780; Tocris Bioscience) was added to the medium at a concentration of 1 μ M at 24 h (see Fig. 8D) or 48 h (see Fig. 8E) before the harvesting of cells.

Plasmids. The human MEN1 cDNA clone was provided by S. C. Chandrasekharappa and cloned into pcDNA3.1/hygro (Invitrogen) for transient expression. MEN1 point mutations were generated by the QuikChange site-directed mutagenesis kit (Stratagene) with the following primers (altered nucleotides are underlined): MEN1-H139D (forward, 5'-CTTCAAGGATCGGGCCGACATCCAGTCCCTCTTC-3'; reverse, 5'-GAAGAGGGACTGGATGTCCGGCCGATCCTTGAAG-3'), MEN1-A160P (forward, 5'-CGGTGTGGCCTTCTCTGTGGTTGGGGCCGTG-3'; reverse, 5'-CAGGCCCAACACAGGAAAGGCCACACCG-3'), MEN1-A176P (forward, 5'-GGATGTCCACCTCCCTCTGTCTGAGGATCATGC-3'; reverse, 5'-GCATGATCCTCAGACAGGGGAGGTGGAATCC-3'), and MEN1-S394A/S399A (forward, 5'-GGGGAGCAAGCC CAGGGCACCCAGGCCCAAGGTTCCGC-3'; reverse, 5'-GCGGAACC TTGGGCTTGGGTGCCCTGGGCTTGTCCCC-3'). All mutant forms were confirmed by sequencing. For lentivirus expression, wild-type MEN1 and point mutant forms were cloned into pGIPZ-CMV (Open Biosystems). Plasmids expressing wild-type and kinase-dead FLAG-ATM (16) were provided by M. Kastan.

Real-time qRT-PCR. Total RNA was isolated, and reverse transcription (RT) was performed as described previously (17), followed by real-time quantitative RT-PCR (qRT-PCR) with Platinum SYBR green qPCR SuperMix-UDG with Rox (Invitrogen). The primers used were human *BRCA1* (forward, 5'-CCACTCAGCAGAGGGATACCA-3'; reverse, 5'-TTATGATGGAAGGGTAGCTGTTAGAAG-3'), mouse *Brca1* (forward, 5'-TGATGTGGGACTGGGTGTTG-3'; reverse, 5'-CTGTACC AGGTAGGCATCCAGAT-3'), human *MEN1* (forward, 5'-CAGGGGCC AGACAGTCAATG-3'; reverse, 5'-GGTGGGCTCCAGCTCCTCTA-3'), mouse *Men1* (forward, 5'-GCCCAAGTCACGCCTGAA-3'; reverse, 5'-T AAGGTCAATCTAGCCCCCAAGT-3'), p21 (forward, 5'-TCCCACCTT GCCAGAGAA-3'; reverse, 5'-GACATCACCAGGATTGGACATG-3'), *RAD1* (forward, 5'-AGTG GACGATGCCCCCTTCT-3'; reverse, 5'-GGC CACAAGGCTGTACTGATC-3'), *RAD9B* (forward, 5'-GCCTTCCGAG CTTGCTTT-3'; reverse, 5'-ACCTGACTGCCGCTCATCA-3'), *RAD17* (forward, 5'-GGTGACTGGAATACAGCTCTTT-3'; reverse, 5'-GGAA TGCATCACACCTCTCGTA-3'), *RAD51* (forward, 5'-TGGATGGAGC AGCGATGTT-3'; reverse, 5'-GCATGGGCGATGATATTTCC-3'), *RAD51API* (forward, 5'-CAGAATCTCAGTCCTTGCCAAA-3'; reverse, 5'-GACTGCGTATTCTAATGGTTTCCTA-3'), *RAD51C* (forward, 5'-ATGCGCGGGAAGACGTT-3'; reverse, 5'-CAGCGGGAAACT CACAAA-3'), *ESR1* (forward, 5'-GTGCCTGGCTAGAGATCCTG-3'; reverse, 5'-GGTTCCTGTCCAAGAGCAAG-3'), *RPL41* (forward, 5'-CA TTAAATAGCCGTAGACGGAACCTT-3'; reverse, 5'-GCGCAGAGGTTT CCAAAAA-3'), and *TUBA* (forward, 5'-GGCCAAGGTACAGAGAGC TG-3'; reverse, 5'-CAGTTTGGCATACATCAGG-3').

Immunohistochemistry. Immunohistochemical analysis was carried out as previously described (13), with a MEN1 antibody (1:100 dilution; Abgent). This study was approved by the Boston University School of Medicine Institutional Review Board (IRB docket H-26837). Archival materials from nevi ($n = 21$) and malignant melanoma ($n = 25$) were retrieved from the pathology files of the Skin Pathology Laboratory, Boston University School of Medicine.

Methylated DNA immunoprecipitation (MeDIP) assay. Genomic DNA was isolated and processed with the MethylMiner methylated DNA enrichment kit (Invitrogen). The DNA eluate was analyzed by qRT-PCR with a primer pair that amplifies the CpG island of the *MEN1* gene (forward, 5'-GCGGTTGACAGCCAGAAAAT-3'; reverse, 5'-CTTGGTGCT GGGCTTCGT-3').

Colony formation assays. SK-MEL-28, SK-MEL-103, or WM3918 cells (5×10^5) stably expressing pGIPZ-MEN1 (described above) or the empty vector (pGIPZ-CMV) were plated in 60-mm dishes and selected with puromycin for 14 days. Colonies were stained with crystal violet and counted.

RNA interference. Cells were stably transduced with the following small hairpin RNA (shRNAs) from Open Biosystems: *ATM* (V2HS-89368 and V2HS-192800), human *BRCA1* (TRCN0000039833), mouse *Brca1* (TRCN0000042558), *ESR1* (TRCN0000003300), human *MEN1* (TRCN0000040138 and TRCN0000040139), mouse *Men1* (TRCN0000034394), *MLL* (TRCN0000005954), and *PSIP1* (TRCN0000074818).

Immunoblot analysis. Protein extracts were prepared by lysis in a buffer containing 50 mM Tris-HCl (pH 7.4), 0.1% Triton X-100, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1 mM Na_3VO_4 , and protease inhibitors (Roche). Blots were probed with the following antibodies: anti-human MEN1 rabbit polyclonal (Santa Cruz), anti-phospho-H2AX (Ser139) mouse monoclonal (Millipore), anti-phospho-Chk1 (Thr317) (Cell Signaling), anti-phospho-p53 (Ser15) (Cell Signaling), anti-p53 (Santa Cruz), antihemagglutinin (anti-HA) mouse monoclonal (Roche), anti-FLAG mouse monoclonal (Sigma), anti- α -tubulin mouse monoclonal (Sigma), anti-green fluorescent protein (anti-GFP) mouse monoclonal (BD Biosciences), and anti-glutathione S-transferase (anti-GST) mouse monoclonal (Santa Cruz), and anti-estrogen receptor 1 (ESR1) rabbit monoclonal (Cell Signaling). For one experiment (see Fig. 5E), 5 mM lactacystin (Calbiochem) was added 8 h before protein extract preparation.

Immunocytochemistry. Immunocytochemical analysis was performed as previously described (18). Briefly, cells were treated in the presence or absence of 0.2 mM hydroxyurea (Sigma) for 24 h. Cells were stained with anti-RAD51 (Santa Cruz) and anti-phospho-H2AX (Ser139) (Millipore) antibodies and 4',6-diamidino-2-phenylindole (DAPI) and then analyzed on a Zeiss AXIO Imager Z2 microscope. Nuclei with >5 foci (for melanocytes) or >20 foci (for melanoma cell lines) were considered positive for hydroxyurea-induced RAD51 foci; nuclei with >10 foci (for melanocytes) and >50 foci (for melanoma cell lines) were considered positive for hydroxyurea-induced γ -H2AX foci. The percentages of cells with positive RAD51 and γ -H2AX foci were determined in at least two separate experiments by counting at least 300 nuclei on each slide.

HR repair assays. A small-plasmid-based homologous recombination (HR) assay was performed as previously described (19). Briefly, plasmids pSV2neoDL, pSV2neoDR, and pRSVed1884 were transfected into melanocytes, isolated at 48 h posttransfection, DpnI digested, and transformed into *Escherichia coli* strain DH10B. The HR frequency was determined by calculating the ratio of kanamycin-resistant colonies to ampicillin-resistant colonies, and the result was normalized to that observed in control melanocytes, which was set to 1. The chromosomal HR assay was performed with HCT116/HN5 cells as previously described (20, 21). The HR frequency was calculated from the ratio of the number of G418-resistant colonies to the total number of cells seeded and normalized for transfection and plating efficiencies.

NHEJ assays. The small-plasmid-based nonhomologous end-joining (NHEJ) assay was performed as previously described (22). Briefly, melanocytes were cotransfected with a linearized small ampicillin plasmid (pGL-4.14) and a circularized kanamycin plasmid (pDSRed-N1). At 48 h later, plasmids were isolated and transformed into *E. coli* strain DH10B. The NHEJ frequency was determined by calculating the ratio of ampicillin-resistant colonies to kanamycin-resistant colonies, and the result was normalized to that observed in control melanocytes, which was set to 1. The chromosomal NHEJ assay was performed with HEK293/pPHW1 cells as previously described (23). The NHEJ frequency was calculated from the ratio of the number of XHATM-resistant colonies to the total number of cells seeded and normalized for transfection and plating efficiencies.

Spontaneous mutation frequency. Cells expressing a nonsilencing (NS) or MEN1 shRNA were seeded at 5,000/well into 48-well plates and selected with 0.25 μ g/ml (for melanocytes) or 0.75 μ g/ml (for HCT116 cells) 6-thioguanine (Sigma) for 4 weeks. For each line, 500 cells were seeded into a well of a six-well plate for plating efficiency determination. The mutation rate was calculated from the ratio of the number of 6-thioguanine-resistant colonies to the total number of cells seeded and normalized for plating efficiency.

Identification of potential ATM/ATR phosphorylation sites in MEN1. The sequences with the following accession numbers were aligned with ClustalW: NP_570711 (*Homo sapiens*), XP_001166985 (*Pan troglodytes*), ACA64878 (*Callicebus moloch*), AAI20006 (*Bos taurus*) ACF98284 (*Mustela putorius furo*), and NP_001074977 (*Canis lupus familiaris*).

ATM kinase assays. A peptide of 126 amino acids (from N331 to V455) was amplified from a plasmid expressing wild-type or mutant MEN1 and cloned into pGEX-3T (GE Healthcare). The GST-MEN1 proteins were purified from *E. coli*. *In vitro* ATM kinase assays were performed as described previously (16). Briefly, 293T cells were transfected with plasmids expressing FLAG-tagged ATM or the kinase-dead mutant form (ATM-KD). ATM was immunoprecipitated with anti-FLAG M2 beads (Sigma), incubated with 10 μ Ci [γ -³²P]ATP and 1 μ g of GST fusion substrates for 20 min at 30°C, and the reaction was stopped by the addition of SDS-PAGE protein sample buffer. Proteins were separated by SDS-PAGE and detected by autoradiography.

ChIP assay. Chromatin immunoprecipitation (ChIP) assays were performed as previously described (24), with a MEN1 (Bethyl Laboratories), ESR1 (Cell Signaling Technology), MLL (Bethyl Laboratories), or H3K4me3 (Abcam) antibody and the following quantitative PCR primer pairs: *BRCA1* promoter (forward, 5'-GAGGGAAGCGCTGAGGATCA-3'; reverse, 5'-CTAGCGATTCTCCTGCCTCA-3'), *BRCA1* last exon (forward, 5'-CATTGGGGAGGAAATTCTGA-3'; reverse, 5'-CTCCCCAAGTGCTGGGATTA-3'), *RAD51* promoter (forward, 5'-ATGGCGTGA TCTCGGCTCA-3'; reverse, 5'-AGAGTGAGAGACGGTCTCAA-3'), *RAD51* last exon (forward, 5'-AAGGAAGAGGGGAAACCAGA-3'; reverse, 5'-ATCTCCCACTCCATCTGCAT-3'), *RAD51API* promoter (forward, 5'-AAGGTCTGGACCGAACACAC-3'; reverse, 5'-CGGCTGA AGTTTCTCAGCTC-3'), and *RAD51API* last exon (forward, 5'-GCCCA CTGGTGGTAGTGTCT-3'; reverse, 5'-GGTGCTAGTGGCATTGGA T-3').

ELISA. Unirradiated and gamma-irradiated melanocytes were harvested and analyzed for estradiol levels with a 17 β -estradiol enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences) according to the manufacturer's instructions.

Statistical analysis. Statistical analysis, carried out in R (25), was performed only for the experiments in Fig. 4. HR frequency and mutation rate assay results were arcsine transformed, and NHEJ assay results were log transformed prior to the application of analysis of variance for a randomized block design ($n = 3$).

RESULTS

MEN1 expression is frequently lost in human melanomas and melanoma cell lines. To evaluate the possibility that MEN1 is a melanoma tumor suppressor, we first analyzed *MEN1* expression in human melanoma samples. The real-time qRT-PCR results in Fig. 1A show that the levels of *MEN1* mRNA in cultured human melanocytes, normal skin, and melanocytic nevi were roughly equivalent. However, the *MEN1* mRNA levels were substantially reduced in 9/11 human melanoma samples analyzed. The *MEN1* mRNA levels were also reduced in 6/7 short-term melanoma cultures and 6/6 established melanoma cell lines (Fig. 1B).

To verify the qRT-PCR results, we also analyzed MEN1 protein levels in human melanoma samples by immunohistochemistry analysis. Figure 1C shows that MEN1 staining was strong in nevi, consistent with the qRT-PCR results. In contrast, MEN1 staining

was not detectable in 88% of the human melanoma samples analyzed (Fig. 1C and D). In the human melanoma samples, the frequencies of wild-type BRAF and BRAFV600E were roughly comparable and the loss of MEN1 expression was independent of BRAF status (Fig. 1D). Immunoblot analysis of several established melanoma cell lines also confirmed the absence of MEN1 at the protein level (Fig. 1E).

In many instances, tumor suppressor genes are inactivated by epigenetic silencing, which involves repressive histone modifications and DNA hypermethylation (26). We found that *MEN1* contains an atypical CpG island located in the intron 1-exon 2 region and analyzed methylation in this region with a MeDIP assay. The results in Fig. 1F show that methylation was greater in human melanoma samples and the human melanoma cell line SK-MEL-28 than in melanocytes and normal skin.

Restoration of a tumor suppressor gene in a cancer cell typically results in growth inhibition or the induction of apoptosis (27). Figure 1G shows that ectopic expression of MEN1 inhibited the growth of the SK-MEL-28 (harboring an activated BRAFV600E mutation), SK-MEL-103 (harboring an activated NRASQ61R mutation), and WM3918 (harboring wild-type BRAF and RAS) cell lines.

Loss of MEN1 results in decreased efficiency of DNA double-strand break repair. As described above, several previous studies have suggested that MEN1 is involved in the maintenance of genome integrity (10–12). To further investigate this possibility, we used an shRNA to stably knock down *MEN1* in primary human melanocytes. As a control, we used an NS shRNA. Immunoblot and qRT-PCR analyses confirmed loss of MEN1 protein (Fig. 2A) and mRNA (Fig. 2B), respectively, in MEN1 knockdown melanocytes.

The immunoblot assay in Fig. 2A shows that depletion of MEN1 increased the level of γ -H2AX, a marker of DNA double-strand breaks (28). Notably, in MEN1 knockdown melanocytes, the levels of phospho-CHK1 and TP53 (both phosphorylated and total proteins) were also increased, consistent with the elevated level of DNA double-strand breaks and indicative of an active DNA damage response. Knockdown of MEN1 in another cell type, human PFFs, with a second, unrelated MEN1 shRNA also resulted in increased levels of γ -H2AX, phospho-CHK1, and TP53 (Fig. 2C).

The increased level of DNA double-strand breaks in MEN1 knockdown cells suggested that DNA repair was inefficient. To further investigate this possibility, we analyzed RAD51 focus formation, a characteristic marker of HR-directed DNA repair (29). Melanocytes were subjected to DNA replication stress by the addition of hydroxyurea, a DNA replication inhibitor, and 24 h later, γ -H2AX and RAD51 foci were detected by immunofluorescence assay. Figure 2D shows that following MEN1 knockdown, there was a modest increase in the percentage of γ -H2AX focus-positive cells accompanied by a substantial decrease in the percentage of RAD51 focus-positive cells. Similar results were obtained with a second, unrelated MEN1 shRNA in PFFs and HCT116 cells (Fig. 2E and F).

As described above, human melanoma cell lines express very low levels of *MEN1*, in contrast to melanocytes (Fig. 1B). On the basis of the results described above, we predicted that human melanoma cell lines would be relatively inefficient at RAD51 focus formation. To test this prediction, WM3918, SK-MEL-103, and SK-MEL-28 cells and, as a control, melanocytes were treated with

FIG 1 *MEN1* expression is frequently lost in human melanomas and melanoma cell lines. (A and B) qRT-PCR analysis monitoring the expression of *MEN1* in human melanocytes, normal skin, melanocytic nevi, and melanoma samples (A) and in human short-term melanoma cultures and established melanoma cell lines (B). Expression was normalized to that observed in melanocytes, which was set to 1. (C) Immunohistochemical analysis showing *MEN1* staining in a human nevus, BRAFV600E melanoma, and wild-type BRAF (BRAF-WT) melanoma. (D) Summary of the *MEN1* immunohistochemistry analysis and BRAF status of 25 human melanoma samples. (E) Immunoblot analysis monitoring *MEN1* levels in melanocytes and human melanoma cell lines WM3918, SK-MEL103, and SK-MEL28. α -Tubulin (TUBA) was monitored as a loading control. (F) (Top) Schematic of the CpG island located in the intron 1-exon 2 region of the *MEN1* gene. TSS, transcription start site; 1 and 2, exons 1 and 2, respectively. (Bottom) MeDIP analysis of the *MEN1* promoter in human melanocytes, normal skin, melanoma samples, and SK-MEL-28, a melanoma cell line. Methylation was normalized to that observed in melanocytes, which was set to 1. (G) Colony formation assay. SK-MEL-28, SK-MEL-103, and WM3918 cells stably expressing *MEN1* or, as a control, the empty vector were analyzed for colony formation. Colony formation was normalized to that observed in cells expressing the empty vector, which was set to 1. Error bars show standard deviations.

hydroxyurea and RAD51 focus formation was quantified. **Figure 3A** shows that in the absence of hydroxyurea, the percentage of γ -H2AX focus-positive cells was much higher in the three human melanoma cell lines than in melanocytes, suggesting that human melanoma cell lines were relatively inefficient at repairing basal levels of DNA double-strand breaks. Moreover, following hydroxyurea treatment, the percentage of RAD51 focus-positive cells was much lower in the three melanoma cell lines than in melanocytes, indicative of inefficient DNA double-strand break repair.

To confirm that the reduced capacity to carry out DNA dou-

ble-strand break repair was due to the absence of *MEN1*, we performed a “rescue” experiment. The three human melanoma cell lines were stably transduced with a plasmid expressing *MEN1* and treated in the presence or absence of hydroxyurea, and 24 h later, γ -H2AX and RAD51 foci were measured. The results in **Fig. 3B** show that ectopic expression of *MEN1* substantially decreased the percentage of cells containing γ -H2AX foci in the absence of hydroxyurea treatment and increased the percentage of cells containing RAD51 foci following hydroxyurea treatment.

Loss of *MEN1* results in decreased HR-directed DNA repair and increased NHEJ. To verify that loss of *MEN1* results in inef-

FIG 2 Loss of *MEN1* results in decreased efficiency of DNA double-strand break repair. (A) Immunoblot analysis monitoring levels of MEN1, γ -H2AX, phospho-CHK1(S317), phospho-TP53(S15), and total TP53 in primary human melanocytes expressing a MEN1 or NS shRNA. α -Tubulin (TUBA) was monitored as a loading control. (B) qRT-PCR analysis monitoring *MEN1* expression in primary human melanocytes stably expressing a MEN1 or NS shRNA. Expression was normalized to that obtained with the NS shRNA, which was set to 1. (C) Immunoblot analysis monitoring levels of MEN1, γ -H2AX, phospho-CHK1(S317), phospho-TP53(S15), and total TP53 in PFFs expressing a second, unrelated MEN1 shRNA or NS shRNA. (D) Quantification of γ -H2AX (left) and RAD51 (right) focus formation in primary human melanocytes expressing a MEN1 or NS shRNA and treated in the absence or presence of hydroxyurea (HU). (E and F) PFFs (E) or HCT116 cells (F) expressing a second, unrelated MEN1 shRNA or NS shRNA were treated in the absence or presence of HU and monitored for γ -H2AX (left) and RAD51 (right) focus formation. Error bars show standard deviations.

efficient HR-directed DNA repair, we specifically measured HR activity in MEN1 knockdown and NS melanocytes with a well-established assay based on HR-directed repair of a plasmid-borne kanamycin resistance reporter gene (19). Briefly, two plasmids carrying nonoverlapping deletions within the coding region of the

kanamycin resistance gene were cotransfected with a plasmid encoding an ampicillin resistance gene into MEN1 knockdown and NS melanocytes. Generation of a functional kanamycin gene is dependent on interplasmid HR. Following plasmid isolation and transformation into bacteria, the HR frequency was calculated as

FIG 3 Human melanoma cell lines are inefficient at DNA double-strand break repair because of *MEN1* deficiency. (A) Quantification of γ -H2AX (left) and RAD51 (right) focus formation in WM3918, SK-MEL-103, and SK-MEL-28 cells treated in the absence or presence of hydroxyurea (HU). (B) Quantification of γ -H2AX (left) and RAD51 (right) focus formation in WM3918, SK-MEL-103, and SK-MEL-28 cells expressing MEN1 or, as a control, the empty vector and treated in the absence or presence of HU. Error bars show standard deviations.

FIG 4 Loss of *MEN1* results in decreased HR-directed DNA repair and increased NHEJ. (A) Plasmid-based (left) and chromosome-based (right) HR assays with melanocytes and HCT116/HN5 cells, respectively, expressing a *MEN1* or NS shRNA. (B) Plasmid-based (left) and chromosome-based (right) NHEJ assays with melanocytes and HEK293/pPHW1 cells, respectively, expressing a *MEN1* or NS shRNA. (C) Spontaneous mutation frequencies in melanocytes expressing a *MEN1* or NS shRNA. (D) *HPRT* gene mutation assay of HCT116 cells expressing a *MEN1* or NS shRNA. Error bars show standard deviations (*, $P < 0.01$; **, $P < 0.001$).

the ratio of kanamycin-resistant (indicative of recombination) to ampicillin-resistant (indicative of total plasmid recovery) colonies. **Figure 4A** (left) shows that the HR frequency was 5.0-fold lower in *MEN1* knockdown melanocytes than in control NS melanocytes. To confirm this result, we also carried out a chromosome-based HR assay. We knocked down *MEN1* in HCT116/HN5 cells, a reporter cell line that contains two chromosomally integrated nonfunctional portions of a neomycin resistance gene (20, 21). HR can generate an intact neomycin resistance gene, enabling colony formation in the presence of neomycin. **Figure 4A** (right) shows that *MEN1* knockdown resulted in a 4.0-fold decrease in HR efficiency.

DNA double-strand breaks can also be repaired by NHEJ, and a number of studies have shown that HR and NHEJ are competing processes (30, 31). Therefore, we predicted that loss of *MEN1* would result in increased NHEJ and tested this idea with several NHEJ assays. First we used a plasmid-based assay similar to that described above (22). **Figure 4B** (left) shows that NHEJ activity in *MEN1* knockdown melanocytes was 2.8-fold greater than that in control NS melanocytes. We also performed a chromosome-

based NHEJ assay in which *MEN1* was knocked down in HEK293/pPHW1 cells, which contain a nonfunctional integrated *GPT* reporter gene that can be repaired by NHEJ (23). **Figure 4B** (right) shows that *MEN1* knockdown resulted in a 1.8-fold increase in NHEJ activity. Thus, loss of *MEN1* results in a decrease in HR and a corresponding increase in NHEJ.

Compared to HR-directed DNA repair, NHEJ is error prone and can lead to mutations (32). To test whether loss of *MEN1* results in an increased mutation rate, we measured the spontaneous mutation rate of the hypoxanthine guanine phosphoribosyl-transferase gene (*HPRT*) in *MEN1* knockdown and NS melanocytes. Equal numbers of *MEN1* knockdown and NS melanocytes were plated in the presence of 6-thioguanine, and 4 weeks later, the *HPRT* mutation rate was determined from the ratio of the number of 6-thioguanine-resistant colonies to the total number of cells seeded. **Figure 4C** shows that the mutation rate in *MEN1* knockdown melanocytes was 2.4-fold higher than that in control NS melanocytes. Similarly, the mutation rate in *MEN1* knockdown HCT116 cells was 2.2-fold higher than that in control NS HCT116 cells (**Fig. 4D**).

MEN1 is phosphorylated by ATM/ATR and stabilized following DNA damage. Following DNA damage, many proteins involved in the DNA damage response are phosphorylated by the DNA damage kinases ATM and ATR, which results in their protection from proteolytic degradation (reviewed in reference 33). Previous studies, including a large-scale proteomic analysis, identified *MEN1* as a substrate of ATM/ATR (34, 35). We therefore investigated the role of *MEN1* in the DNA damage response. To confirm the phosphorylation of *MEN1* by ATM/ATR, we performed an *in vitro* kinase assay. Briefly, FLAG-tagged ATM was immunoprecipitated from 293T cells and incubated in the presence of purified, GST-tagged *MEN1* and [γ -³²P]ATP. The results in **Fig. 5A** show that wild-type ATM, but not a catalytically inactive mutant form of ATM, phosphorylated GST-*MEN1*. In contrast, a mutant form of *MEN1* in which two putative ATM phosphorylation sites (SQ motifs) at serines 394 and 399 were both mutated to alanine (*MEN1*-S394A/S399A) failed to be phosphorylated.

Phosphorylation by ATM/ATR protects proteins from ubiquitin-mediated degradation (36), and we therefore performed a series of experiments to analyze the consequence of ATM phosphorylation for *MEN1* levels. Wild-type *MEN1* was ectopically expressed in SK-MEL-28 cells, which contain very low levels of endogenous *MEN1* (**Fig. 1B**). **Figure 5B** shows that gamma irradiation of these cells resulted in increased levels of wild-type *MEN1* protein (compare lanes 1 and 2 to lanes 5 and 6). Notably, gamma irradiation did not result in an increase in *MEN1* mRNA levels (**Fig. 5C**), consistent with the notion that increased levels of *MEN1* following irradiation occur by a posttranscriptional mechanism. **Figure 5B** shows that knockdown of ATM in unirradiated cells resulted in reduced *MEN1* levels (compare lanes 1 and 2 to lanes 3 and 4), indicating that ATM stabilizes *MEN1* under basal conditions. In addition, a pharmacological inhibitor of ATM/ATR resulted in reduced *MEN1* levels following irradiation (compare lanes 5 and 6 to lanes 7 and 8), indicating that ATM/ATR stabilizes *MEN1* following DNA damage. **Figure 5D** shows that the level of ectopically expressed wild-type *MEN1* was substantially increased by gamma irradiation, whereas the *MEN1*-S394A/S399A mutant form, which cannot be phosphorylated by ATM/ATR, was relatively unaffected. Like wild-type *MEN1*, the *MEN1*-

FIG 5 MEN1 is phosphorylated by ATM/ATR and stabilized following DNA damage. (A) (Left) Schematic of the MEN1 protein and sequence alignment of putative ATM/ATR phosphorylation sites (SQ motifs) in different species. The wild-type (WT) and mutant MEN1 protein sequences at the SQ motifs are shown. (Right) *In vitro* kinase assay monitoring phosphorylation of GST-tagged wild-type MEN1 (WT) or mutant MEN1-S394A/S399A in the presence of FLAG-tagged wild-type ATM (ATM-WT) or a kinase-dead (ATM-KD) mutant form. (B) Immunoblot analysis monitoring levels of ectopically expressed MEN1, phospho-TP53(S15), and GFP in SK-MEL-28 cells expressing an NS or ATM shRNA either unirradiated (–) or 2 or 6 h following gamma irradiation (IR) and treated in the presence or absence of the ATM/ATR inhibitor CGK733. (C) qRT-PCR analysis of *MEN1* expression in SK-MEL-28 cells and melanocytes. Expression was normalized to that observed in unirradiated cells, which was set to 1. Error bars show standard deviations. TUBA, α -tubulin. (D) Immunoblot analysis monitoring levels of MEN1, phospho-TP53(S15), and GFP in SK-MEL-28 cells ectopically expressing either wild-type or mutant MEN1 and either unirradiated or 2 or 6 h following gamma irradiation. (E) Ubiquitination assay. Extracts from unirradiated or gamma-irradiated SK-MEL-28 cells coexpressing MEN1 and HA-tagged ubiquitin were immunoprecipitated with an HA antibody, and the immunoprecipitate (IP) was analyzed by immunoblotting for MEN1. WB, Western blotting. (F) Immunoblot analysis monitoring levels of endogenous MEN1 and phospho-TP53(S15) in melanocytes either unirradiated or 2 or 6 h following irradiation and treated in the presence or absence of CGK733.

S394A/S399A mutant form was nucleus localized (data not shown).

It seemed likely that phosphorylation by ATM/ATR would protect MEN1 from ubiquitin-mediated degradation. To confirm this possibility, SK-MEL-28 cells were cotransfected with plasmids expressing MEN1 and HA-tagged ubiquitin, and the anti-HA immunoprecipitate was analyzed by immunoblotting for MEN1. Figure 5E shows that following gamma irradiation, the levels of full-length MEN1 increased whereas the amount of MEN1 ubiquitinated degradation products decreased. In contrast, full-length MEN1-S394A/S399A levels were relatively constant and the amount of MEN1-S394A/S399A ubiquitinated degradation products was unaffected by irradiation.

The experiments described above were performed with ectopically expressed MEN1. We therefore also tested whether DNA damage resulted in the stabilization of endogenous MEN1. Figure 5F shows that gamma irradiation of melanocytes also resulted in an ATM/ATR-dependent increase in endogenous MEN1 levels.

MEN1 stimulates the transcription of *BRCA1* and other genes involved in HR. We next sought to investigate the mechanism by which MEN1 promotes HR-directed DNA repair. In a previous genome-wide occupancy study, MEN1 was found to be associated with ~2,000 human promoters (37). We mined this data set, found seven genes with well-established roles in HR-directed DNA repair, and investigated whether MEN1 is a tran-

scriptional activator of these genes. Figure 6A shows that knockdown of MEN1 in melanocytes substantially decreased the expression of three genes, *BRCA1*, *RAD51*, and *RAD51AP1*. Similar results were obtained with PFFs (Fig. 6B). To confirm that these genes are direct targets of MEN1, we performed ChIP experiments. The ChIP results in Fig. 6C show that in melanocytes, MEN1 was bound to the promoters of *BRCA1*, *RAD51*, and *RAD51AP1* but not to a downstream region, indicating that these genes are direct MEN1 targets.

To further establish the functional relationship between MEN1 and the expression of HR target genes, we analyzed the ability of MEN1 loss-of-function mutant forms found in individuals with multiple endocrine neoplasia type 1 to stimulate *BRCA1* expression. SK-MEL-28 cells were transfected with a plasmid expressing wild-type MEN1 or one of three common mutant forms of MEN1, i.e., MEN1-H139D, -A160P, or -A176P. All three mutant forms of MEN1 were expressed at levels roughly comparable to that of wild-type MEN1 (Fig. 7A) and were also, like wild-type MEN1, nucleus localized (data not shown). Figure 7B shows that following gamma irradiation, the levels of these ectopically expressed mutant forms, like that of wild-type MEN1, markedly increased. Moreover, this increase was substantially smaller upon treatment with an ATM/ATR inhibitor.

The qRT-PCR results in Fig. 7C show that ectopic expression of wild-type MEN1 resulted in increased *BRCA1* mRNA levels,

FIG 6 MEN1 stimulates the transcription of *BRCA1* and other genes involved in HR. (A and B) qRT-PCR analysis monitoring the expression of genes involved in HR-directed DNA repair in melanocytes (A) or PFFs (B) expressing a MEN1 or NS shRNA. *RPL41* (a ribosomal gene) and *TUBA* were monitored as negative controls. Expression was normalized to that obtained with the NS control shRNA, which was set to 1. (C) ChIP assay monitoring binding of MEN1 to the *BRCA1*, *RAD51*, and *RAD51AP1* promoters (P) or last exons (E) in melanocytes. The result was normalized to that observed in the no-antibody (No ab) control, which was set to 1.

whereas MEN1-H139D, -A160P, and -A176P had no effect on *BRCA1* expression. Likewise, the ChIP results in Fig. 7D show that wild-type MEN1 was bound to the *BRCA1* promoter, whereas MEN1-H139D, -A160P, and -A176P were not. We also analyzed the abilities of the mutant forms of MEN1 to promote HR-directed DNA repair. Figure 7E shows that in a plasmid-based HR assay, ectopic expression of wild-type MEN1 in SK-MEL-28 cells increased HR activity, consistent with the results described above (Fig. 4A). In contrast, the ectopic expression of MEN1-H139D, -A160P, or -A176P had no significant effect on HR activity. Likewise, unlike the ectopic expression of wild-type MEN1, that of the mutant forms of MEN1 was unable to decrease the percentage of cells containing γ -H2AX foci in the absence of hydroxyurea treatment or increase the percentage of cells containing RAD51 foci following hydroxyurea treatment (Fig. 7F). Collectively, the results in Fig. 7C to F demonstrate that, in contrast to wild-type MEN1, MEN1-H139D, -A160P, and -A176P are unable to stimulate the transcription of HR target genes and promote HR-directed DNA repair.

MEN1 and MLL are recruited to the *BRCA1*, *RAD51*, and *RAD51AP1* promoters by ESR1. We next sought to determine the mechanism by which MEN1 is recruited to the promoters of HR genes. Previous studies have shown that MEN1 is recruited to target promoters through interactions with a variety of proteins, including nuclear receptors such as ESR1 (also called estrogen receptor α), the chromatin-associated protein PSIP1 (also called LEDGF), and β -catenin (reviewed in reference 38). In a previous genome-wide expression profiling study, *BRCA1*, *RAD51*, and *RAD51AP1* were found to be estrogen-responsive genes (39), and we therefore investigated the possible role of ESR1, which has been shown to interact directly with MEN1 (40), in MEN1 recruitment. The qRT-PCR results in Fig. 8A show that knockdown of ESR1 in melanocytes decreased the expression of *BRCA1*, *RAD51*, and *RAD51AP1* to a level comparable to that observed following the knockdown of MEN1. As expected, knockdown of the MEN1-associated H3K4 histone methyltransferase MLL led to a comparable reduction of *BRCA1*, *RAD51*, and *RAD51AP1* expression. In contrast, knockdown of another MEN1-interacting protein, PSIP1, had no effect on *BRCA1*, *RAD51*, and *RAD51AP1* expression levels.

In further support of the role of ESR1, addition of estradiol, an ESR1 ligand, resulted in an increase in *BRCA1*, *RAD51*, and *RAD51AP1* expression levels (Fig. 8B), consistent with the previous genome-wide expression profiling study (39). Significantly, addition of estradiol also resulted in increased recruitment of MEN1, ESR1, and MLL to all three promoters (Fig. 8C). We also performed reciprocal experiments with the ESR antagonist fulvestrant (41). Figure 8D shows that addition of fulvestrant resulted in decreased *BRCA1*, *RAD51*, and *RAD51AP1* expression levels. Furthermore, fulvestrant treatment also resulted in decreased HR activity and increased NHEJ activity (Fig. 8E), consistent with our findings on MEN1 knockdown cells (Fig. 4A and B).

Finally, we analyzed the effect of ESR1 depletion on the recruitment of MEN1 and MLL to the promoters of HR genes. The ChIP experiments in Fig. 8F show that knockdown of ESR1 resulted in the decreased association of MEN1, as well as MLL, with the *BRCA1*, *RAD51*, and *RAD51AP1* promoters. In contrast, knockdown of MEN1 did not affect the association of ESR1 with the three promoters. As expected, the binding of both MEN1 and ESR1 was unaffected by the knockdown of MLL. Also, as expected, the knockdown of MEN1, ESR1, or MLL substantially reduced H3K4me3 at the *BRCA1*, *RAD51*, and *RAD51AP1* promoters.

DNA damage and estradiol promote the MEN1-dependent expression of *BRCA1*, *RAD51*, and *RAD51AP1*. To assess the role of DNA damage in the MEN1-mediated transcription pathway, we performed a series of experiments with melanocytes treated in the presence or absence of gamma irradiation. The qRT-PCR analysis in Fig. 9A shows that following gamma irradiation, the expression levels of *BRCA1*, *RAD51*, and *RAD51AP1* increased, consistent with the finding that gamma irradiation increases MEN1 levels (Fig. 5F). As expected, the increase in MEN1 target gene expression following gamma irradiation was dependent on MEN1 and ATM. Consistent with these results, binding of MEN1 and MLL to the promoters of *BRCA1*, *RAD51*, and *RAD51AP1* also increased following gamma irradiation, which was dependent on ATM (Fig. 9B). In contrast, the recruitment of ESR1 was unaffected by gamma

FIG 7 Mutant forms of MEN1 are unable to stimulate the transcription of HR target genes and promote HR-directed DNA repair. Immunoblot analysis monitoring levels of wild-type (WT) and mutant MEN1 proteins following ectopic expression in SK-MEL-28 cells. (B) Immunoblot analysis monitoring levels of wild-type and mutant MEN1 proteins following ectopic expression in SK-MEL-28 cells left unirradiated, gamma irradiated (IR), or treated with gamma irradiation and CGK733. (C) qRT-PCR analysis monitoring *BRCA1* expression in SK-MEL-28 cells ectopically expressing wild-type or mutant MEN1 protein. Expression was normalized to that observed in SK-MEL-28 cells expressing the empty vector, which was set to 1. (D) ChIP assay monitoring the binding of wild-type or mutant MEN1 protein to the *BRCA1* promoter in SK-MEL-28 cells. The result was normalized to that observed in SK-MEL-28 cells expressing the empty vector, which was set to 1. (E) Plasmid-based HR assay of SK-MEL-28 cells expressing wild-type or mutant MEN1 protein. The result was normalized to that observed in SK-MEL-28 cells expressing the empty vector, which was set to 1. (F) Quantification of γ -H2AX (left) and RAD51 (right) foci formation in SK-MEL-28 cells expressing wild-type or mutant MEN1 and treated in the absence or presence of hydroxyurea (HU). Error bars show standard deviations.

irradiation or ATM knockdown, consistent with the finding that ESR1 binding is MEN1 independent (Fig. 8F).

We also analyzed the relationship between gamma irradiation-mediated and estradiol-mediated increases in MEN1 target gene expression. Figure 9C shows, consistent with the results in Fig. 8B, that estradiol treatment resulted in the increased expression of MEN1 target genes. Notably, the expression of MEN1 target genes increased even further when estradiol treatment was combined with gamma irradiation. ESR1 mRNA and protein levels, as well as estradiol levels, were unaffected by gamma irradiation (Fig. 9D). Thus, the increase in MEN1 target gene expression following gamma irradiation was not due to an increase in the level of ESR1 or its ligand. To rule out the possibility of an ESR1-dependent, MEN1-independent mechanism of gene regulation, we asked whether estradiol-induced gene expression in melanocytes is affected by MEN1 knockdown. The results in Fig. 9E show that estradiol-induced expression of the MEN1 target genes was decreased following MEN1 knockdown, indicating that MEN1 is required for ESR1-mediated transcription activation.

Collectively, our results reveal a model, shown in Fig. 9F, in

which MEN1 is phosphorylated by ATM/ATR and stabilized following DNA damage. MEN1 and its coactivator MLL are then recruited to the *BRCA1*, *RAD51*, and *RAD51AP1* promoters by ESR1, resulting in increased H3K4me3 and transcription.

Correlation between MEN1 and *BRCA1* expression in human melanoma samples and cell lines. As shown in Fig. 1, many human melanomas and melanoma cell lines have decreased levels of MEN1. On the basis of the results described above, we predicted that in these cells there would be a corresponding decrease in the expression of MEN1 target genes involved in HR. To test this prediction, we measured the expression of *BRCA1* and, as a control, *p21*, a previously established MEN1 target gene (8), in a subset of the human melanoma samples and melanoma cell lines analyzed in Fig. 1. The qRT-PCR results in Fig. 10A show that in melanocytic nevi, *MEN1*, *BRCA1*, and *p21* were expressed at levels comparable to those found in cultured melanocytes. In contrast, in the majority of human melanoma samples and melanoma cell lines, *BRCA1* and *p21* levels were, like *MEN1* levels, substantially decreased (Fig. 10A and B). Notably, in atypical human melanomas, such as sample 4, and short-term melanoma cultures, such as YURIF, in which

FIG 8 MEN1 and MLL are recruited to the *BRCA1*, *RAD51*, and *RAD51AP1* promoters by ESR1. (A) qRT-PCR analysis monitoring the expression of *BRCA1*, *RAD51*, and *RAD51AP1* in melanocytes expressing an NS, MEN1, ESR1, MLL, or PSIP1 shRNA. Expression was normalized to that observed in NS control melanocytes, which was set to 1. (B) qRT-PCR analysis monitoring the expression of *BRCA1*, *RAD51*, and *RAD51AP1* in melanocytes treated with estradiol or, as a control, ethanol. (C) ChIP assay monitoring the binding of MEN1, ESR1, and MLL to the *BRCA1*, *RAD51*, and *RAD51AP1* promoters in melanocytes treated in the presence or absence of estradiol. The result was normalized to that observed in a no-antibody control, which was set to 1. (D) qRT-PCR analysis monitoring the expression of *BRCA1*, *RAD51*, and *RAD51AP1* in melanocytes treated with fulvestrant or, as a control, ethanol. (E) Plasmid-based HR (left) and NHEJ (right) assays with melanocytes treated in the presence or absence of fulvestrant. (F) ChIP assays monitoring the binding of MEN1, ESR1, MLL, and H3K4me3 to the *BRCA1*, *RAD51*, and *RAD51AP1* promoters in melanocytes expressing an NS, MEN1, ESR1, or MLL shRNA. The results were normalized as described in panel C. Error bars show standard deviations.

MEN1 is expressed normally (Fig. 1A and B), *BRCA1* levels were not decreased. Collectively, these results reveal a strong correlation between *MEN1* levels and *BRCA1* expression, consistent with our finding that *BRCA1* is a *MEN1* target gene.

DISCUSSION

MEN1 is a known tumor suppressor in several cancer types (1), but a role for *MEN1* in melanoma has not been well established. Here we provide multiple lines of evidence that *MEN1* is a melanoma tumor suppressor. First, *MEN1* is expressed in melanocytes

and melanocytic nevi but is lost or substantially decreased in a high percentage of human melanoma samples and melanoma cell lines (Fig. 1A to D). Second, ectopic expression of *MEN1* inhibits the proliferation of human melanoma cell lines, confirming the growth-suppressive activity of *MEN1* in this cancer type (Fig. 1G). The finding that *MEN1* is a melanoma tumor suppressor is consistent with our previous demonstration that *MEN1* is involved in BRAFV600E-induced senescence in melanocytes (13). In support of our results, previous studies have identified *MEN1* (42) and *BRCA1* (43) mutations in a subset of melanomas. In addition,

FIG 9 DNA damage and estradiol promote MEN1-dependent expression of *BRCA1*, *RAD51*, and *RAD51AP1*. (A) qRT-PCR analysis monitoring the expression of *BRCA1*, *RAD51*, and *RAD51AP1* in melanocytes expressing an NS, MEN1, or ATM shRNA and treated in the presence or absence of gamma irradiation (IR). (B) ChIP analyses monitoring the binding of MEN1, MLL, and ESR1 to the *BRCA1*, *RAD51*, and *RAD51AP1* promoters in melanocytes expressing an NS or ATM shRNA and treated in the presence or absence of gamma irradiation. The result was normalized to that observed in a no-antibody control, which was set to 1. (C) qRT-PCR analysis monitoring the expression of *BRCA1*, *RAD51*, and *RAD51AP1* in melanocytes treated in the presence or absence of estradiol and gamma irradiation. (D) Melanocytes were treated in the presence or absence of gamma irradiation and monitored for ESR1 protein levels by immunoblotting (left), *ESR1* mRNA levels by qRT-PCR (middle), or estradiol levels by ELISA (right). (E) qRT-PCR analysis monitoring the expression of *BRCA1*, *RAD51*, and *RAD51AP1* in melanocytes expressing an NS or MEN1 shRNA and treated in the presence or absence of estradiol. Expression was normalized to that observed in ethanol-treated NS control melanocytes, which was set to 1. (F) Model showing the phosphorylation and stabilization of MEN1 by ATM/ATR and ESR1-mediated recruitment of MEN1-MLL to the promoter of an HR gene. Recruitment of MEN1-MLL results in increased H3K4me3 and transcription. Error bars show standard deviations. ERE, estrogen response element.

carboxyl-terminal binding protein 1, a transcriptional repressor of *BRCA1*, is overexpressed in many human melanomas (44).

MEN1 is a complex protein with reported roles in diverse cellular processes. A role for MEN1 in genome integrity has been suggested by several previous findings, including the observations that *Men1*-deficient *Drosophila* larvae (12) and mouse embryonic fibroblasts (45) are hypersensitive to certain types of DNA damage and the increased chromosome breakage in lymphocytes from individuals with MEN1 syndrome (11). Several mechanisms for how MEN1 preserves genome integrity have been proposed, in-

cluding promotion of DNA damage checkpoints (8) and interaction with proteins involved in DNA repair, such as FANCD2 (45) and CHK1 (46).

Here we describe a new mechanism by which MEN1 promotes genome integrity. We show that depletion of MEN1 from melanocytes results in decreased efficiency of DNA double-strand break repair. Consistent with our results, a previous genome-wide small interfering RNA screen identified MEN1 as a protein whose knockdown resulted in increased levels of γ -H2AX (47). In addition, we find that depletion of MEN1 from melanocytes results in

FIG 10 Correlation between *MEN1* and *BRCA1* expression in human melanoma samples and cell lines. (A and B) qRT-PCR analyses monitoring the expression of *MEN1*, *BRCA1*, and *p21* in human melanocytes, nevi, and melanoma samples (A) and in human short-term melanoma cultures and melanoma cell lines (B). The expression of each gene was normalized to that observed in melanocytes, which was set to 1. Error bars show standard deviations.

decreased HR-directed DNA repair and a corresponding increase in NHEJ. Conversely, ectopic *MEN1* expression increases HR-directed DNA repair in melanoma cells lacking *MEN1*. Also consistent with our results, HR-directed DNA repair was reported to increase following the overexpression of *MEN1* (46).

Most importantly, we show that *MEN1* functions by directly stimulating the transcription of genes involved in HR. Specifically, *MEN1* and *MLL* are recruited to the *BRCA1*, *RAD51*, and *RAD51AP1* promoters by *ESR1*, resulting in increased H3K4me3 and transcription. The functional relevance of our findings is supported by several additional results. First, we find that patient-derived mutant forms of *MEN1* fail to bind to the promoter and stimulate the transcription of the HR gene *BRCA1* (Fig. 7C and D). Interestingly, one of these mutant forms, *MEN1*-H139D, has been previously shown to be defective for interaction with *ESR1* (40). Second we find that patient-derived mutant forms of *MEN1* are unable to promote HR-directed DNA repair (Fig. 7E and F). Third, we show that there is a correlation between *MEN1* and *BRCA1* expression levels in human melanoma samples (Fig. 10A and B).

We have also found that phosphorylation by ATM/ATR stabilizes *MEN1* following DNA damage. Collectively, our results suggest that DNA damage activates ATM/ATR, which phosphorylates *MEN1*, resulting in stabilization and increased *MEN1* levels. *MEN1* then stimulates the transcription of genes such as *BRCA1*, *RAD51*, and *RAD51AP1*, which facilitates HR-directed DNA repair (Fig. 9F). Although our study focused on melanoma, it is likely that the mechanism we have described is relevant to other cancers in which *MEN1* is also a tumor suppressor.

Previous studies have shown that DNA repair proteins, including TP53 and *BRCA1*, which is encoded by a *MEN1* target gene, can affect the responsiveness of tumors to chemotherapeutic agents (48–50). Notably, depending upon the specific

agent, the loss of a DNA repair protein can be associated with either sensitivity or resistance to chemotherapy. Therefore, it will be interesting to determine whether the presence or absence of *MEN1* influences the responsiveness of melanomas to various chemotherapeutic agents, which could have prognostic value and help guide melanoma treatment.

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