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Menin Associates with FANCD2, a Protein Involved in Repair of DNA Damage[1]

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ABSTRACT

Multiple endocrine neoplasia type I (MEN1) is an inherited tumor syndrome characterized by tumors in multiple endocrine organs including the parathyroids, pancreatic islets, and the pituitary. The gene mutated in MEN1 patients, *Men1*, encodes a protein of 610 amino acid residues, menin, and mutations in the *Men1* gene lead to the MEN1 syndrome. Although the chromosomal instability in the peripheral lymphocytes from the MEN1 patients has been reported previously, it is not clear whether menin is involved in repair of DNA damage. Here we show that menin specifically interacts with FANCD2, a protein encoded by a gene involved in DNA repair and mutated in patients with an inherited cancer-prone syndrome, Fanconi anemia. The interaction between menin and FANCD2 is enhanced by γ-irradiation. Moreover, loss of menin expression in mouse embryonic fibroblasts leads to increased sensitivity to DNA damage. Furthermore, menin is localized to chromatin and nuclear matrix, and the association with nuclear matrix is enhanced by γ-irradiation. Together, these results suggest that menin plays a critical role in repair of DNA damage in concert with FANCD2.

INTRODUCTION

MEN1[4] is an inherited tumor syndrome characterized by tumors in multiple endocrine organs including the parathyroids, pancreatic islets, and the pituitary (1–4). The gene mutated in MEN1 patients, *Men1*, was identified by positional cloning and mapped to chromosome 11 (5). The product of *Men1,* menin, encodes a protein of 610 amino acid residues (5). However, there are no paralogs throughout various genomes. Thus far, >300 individual mutations in *Men1* have been identified (4). The tumors from patients carrying a germ-line mutation in *Men1* often lose the normal allele of *Men1*. Mice heterozygous for *Men1* knockout have been found recently to develop tumors in the parathyroids, β-islets, and the pituitary (6), closely mimicking the human MEN1 syndrome. Ectopic expression of menin inhibits proliferation of Ras-transformed NIH-3T3 cells (7). Menin is a nuclear protein containing two nuclear localization signals in the COOH terminus (8), but its subnuclear distribution is not yet clear.

Menin interacts with a number of transcriptional factors such as JunD and NFκB and, based on luciferase reporter gene assays, inhibits their activities in gene transcription (9, 10). Menin has also been shown to interact with Smad3, a signal transducer downstream of the transforming growth factor β signaling pathway and is involved in transforming growth factor β-induced gene transcription (11). Inhibition of NFκB or activation of Smad3 by menin may lead to inhibition of cell proliferation. Replication protein A2, a subunit of a trimeric protein that binds single strand DNA, has been shown recently to interact with menin (12). Replication protein A is involved in DNA replication, DNA repair, DNA recombination, and potentially gene transcription. However, it is not clear what role these menin-interacting proteins play in the development of MEN1.

Several previous reports show increased chromosome breakage in lymphocytes from MEN1 patients (13, 14). Peripheral blood lymphocytes from MEN1 patients undergo extensive chromosomal breakage, as compared with normal lymphocytes, after treatment with DEB, an agent cross-linking double-strand DNA (15, 16). A genome-wide LOH screening of 13 MEN1 patients indicates that MEN1 pancreatic tumors fail to maintain DNA integrity and chromosomal stability (17). These findings implicate that *Men1* may be involved in the maintenance of genomic stability (15, 18). However, it is not clear whether menin is involved in the maintenance of genomic stability and, if it is, how menin carries out this function. Here we show that menin interacts with FANCD2, a protein involved in a BRCA1-mediated DNA repair pathway (19), and the interaction between menin and FANCD2 is enhanced by γ-irradiation. Moreover, targeted disruption of *Men1* results in increased sensitivity to DNA damage. Furthermore, menin is localized to chromatin and NM, and the association with NM is enhanced by γ-irradiation. Thus, menin may play a critical role in repair of DNA damage in concert with FANCD2.

MATERIALS AND METHODS

Plasmid Construction. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. To construct pcDNA3-F-menin and other constructs expressing various fragments of menin, human cDNA (20) was amplified by PCR and cloned into the *BamH*I/*Not*I site of pcDNA3-Flag with two repeats of a Flag epitope (21). Human menin cDNA was also cloned into the *BamH*I and *Not*I sites of retroviral vector pMX-puro to generate retroviruses expressing menin as described previously (22).

Cell Lines, Tissue Culture, and Transfection. TGP61 cells, a murine insulinoma cell line, HeLa cells, and NIH 3T3 cells were purchased from American Type Culture Collection. HEK 293 and HeLa S3 cells were cultured in DMEM supplemented with 10% (v/v) FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml) as described previously (23). TGP61 cells were cultured in 45% DMEM and 45% Ham's F12 medium supplemented with 10% (v/v) FCS, penicillin (100 units/ml), and streptomycin (100 units/ml). The cells were transfected using the calcium phosphate precipitation method as described previously (21).

Western Blotting and Immunoprecipitation. Proteins in cell lysates or in various cellular fractions were separated on SDS-PAGE gels, transferred to polyvinylidene difluoride membranes (Millipore), and blotted with various primary antibodies. The blotted proteins were visualized using the enhanced chemiluminescence detection system (Amersham-Pharmacia Biotech). To immunoprecipitate menin, cells were lysed in lysis buffer [50 mM HEPES (pH 7.5), 0.15 M NaCl, 0.5% NP40, 1 mM EDTA, 1 mM EGTA, 1 mM glycerophosphate, 0.5 mM vanadate, and 10% glycerol] supplemented with 1 mM phenylmethylsulfonyl fluoride and 4 μg/ml protease inhibitor mixture (Calbiochem). The lysates were incubated with antimenin antibody (#80) or with agarose beads coupled with α-Flag M_2 (Sigma) at 4°C for 2 h. For immunoprecipitation with the antimenin antibody, the lysates were additionally incubated with the antirabbit IgG secondary antibody, followed with agarose beads coupled with protein G (Amersham-Pharmacia Biotech) at 4°C for 3 h.

Isolation of Menin-interacting Proteins. HEK 293 cells transfected with vector or pcDNA3-F-menin were lysed in the high salt lysis buffer [50 mM

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[4] The abbreviations used are: MEN1, multiple endocrine neoplasia type I; NFκB, nuclear factor κB; DEB, diepoxybutane; LOH, loss of heterozygosity; NM, nuclear matrix; HEK, human embryonic kidney; MEF, mouse embryonic fibroblast; MMC, mitomycin C; NHS, *N*-hydroxysuccinimide; FA, Fanconi anemia.

HEPES (pH 7.5), 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% NP40, 0.3% Triton X-100, 1 mM β-glycerophosphate, 0.5 mM vanadate, 0.5 mM phenylmethylsulfonyl fluoride, 4 μg/ml of aprotinin, pepstatin A, and leupeptin] by Dounce homogenization. The samples were diluted with the above lysis buffer without NaCl to reach a final concentration of 150 mM NaCl. The diluted samples were centrifuged at $10^5 \times g$, and the supernatant was incubated with 100 μl of the equilibrated agarose beads coupled with the anti-Flag antibody (M_2; Sigma) at 4°C for 16 h. The beads were thoroughly washed and then incubated with 150 μl of the elution buffer (the lysis buffer with 150 mM NaCl and 0.5 mg/ml Flag peptide from Sigma) at 4°C for 20 min. The purified proteins from the control cells and the cells expressing F-menin were separated by 6% SDS-PAGE, stained with a Novex Colloid Coomassie Staining kit (Invitrogen), and excised under a sterile hood. The peptide sequences from these proteins were identified using mass spectrometry at the Harvard Microchemistry Facility.

Isolation of *Men1*$^{-/-}$ MEFs and Complementation of the Cells with Menin. *Men1* $\Delta^{N3-8/+}$ mice heterozygous for the *Men1* locus (*Men1*$^{+/-}$) were a gift from Dr. Francis Collins at NHGRI and were maintained on a 129S6/SvEvTac background (Taconic, Germantown, NY; Ref. 6). *Men1*$^{+/-}$ male and female mice were mated, and 9.5 days after plugging the females were euthanized. The embryos were placed in gelatin-coated 12-well plates with trypsin buffer and dissociated into single cells by repeated pipetting. LXSN16E6E7 retroviruses that express the human papillomaviral E6 and E7 open reading frame (24) were used to infect the primary MEFs to immortalize the cells. The infected cells were subjected to selection with 500 μg/ml G418. Immortalized *Men1*$^{-/-}$ cells (< passage 12) were seeded in six-well plates on day 0 and were infected with vector retroviruses (+ vector) or menin-expressing retroviruses (+ menin). Cells were subjected to selection with 2 μg/ml puromycin 72 h after changing medium. Loss of menin expression and complementation by menin were confirmed by immunoblotting.

Clonogenicity and Chromosome Breakage Assays. *Men1*$^{-/-}$ MEFs and *menin*-complemented cells at the exponential growth phase were seeded in 100-mm dishes. After 24 h the desired amounts of DEB were added directly to the medium in the appropriate dishes. Nine days after the addition of the DEB the medium was removed from the dishes, and the cells were fixed with ethanol and then stained with Crystal Violet staining solution. Cell colonies with ~25 or more cells were counted to determine the percentage of surviving cells. To examine the chromosomal aberrations in response to DNA damage, *Men1*$^{-/-}$ MEFs and menin-complemented MEFs were cultured for 24 h in normal medium with phytohemagglutinin and then exposed to MMC for an additional 48 h for chromosome breakage assay as described previously (25).

Antibody Production and Immunofluorescent Staining. Two peptides (#77, EAREGRRRGPRRESKPEE, and #80, STPSDYTLSFLKRQRKGL, corresponding to amino acid residues 472–491 and 593–610 of human menin, respectively) were synthesized (Boston Biomolecules, Inc.) and conjugated to KLH (26). The KLH-conjugated peptides were injected into rabbits for antibody production (Covance). Peptide #80 was cross-linked to NHS-activated Sepharose 4 Fast Flow beads (Amersham-Pharmacia Biotech) as instructed by the manufacturer, and the beads were used to affinity purify the antimenin serum. TGP61 cells were set up on coverslips, and processed for incubation with the primary antibodies and the FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) as described previously (26) and the stained cells were photographed under a fluorescence microscope (Nikon). NM was prepared as described previously (26) and stained with primary antimenin antibody (#80), followed by the FITC-conjugated antirabbit IgG secondary antibody.

Preparation of Chromatin and NM. The methods used to isolate the soluble fraction, the chromatin fraction, and the NM were described by Qiao *et al.* (27). Briefly, 293 cells (5×10^7) treated with or without irradiation were permeabilized in 0.5 ml of the low salt buffer [10 mM HEPES, (pH 7.4), 10 mM KCl, and 50 μg/ml digitonin] containing the protease and phosphatase inhibitors as described above for 15 min at 4°C. The permeabilized nuclei were collected by brief centrifugation to obtain the soluble fraction (supernatant). The nuclei were washed for two additional times with the same buffer, and then resuspended in 100 μl of the permeabilization buffer containing 300 units of DNase I/ml (RNase free; Roche Molecular Biochemicals) at room temperature for 15 min. The nuclei were incubated for 20 min at 37°C to extract the chromatin fraction (supernatant fraction). The pellet was additionally extracted in urea buffer [8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris (pH 8.0)] to obtain the NM fraction.

RESULTS

Menin Interacts with FANCD2. To identify menin-interacting proteins that might shed light on the biochemical function of menin, we first stably transfected 293 cells with either vector DNA or a construct expressing a Flag-tagged menin. Expression of the tagged menin in the cells was confirmed by Western blotting analysis using an anti-Flag antibody (Fig. 1*A*). Cell lysates from the control cells and the cells expressing the epitope-tagged menin were prepared and then incubated with agarose beads covalently conjugated to the anti-Flag antibody. The tagged menin and its associating proteins were separated by SDS-PAGE and stained with a sensitive silver staining method (Fig. 1*B*). A large preparation of the menin-interacting proteins from 100 × 150-mm dishes of 293 cells were separated by SDS-PAGE and stained with a Colloid Coomassie Blue staining kit. The stained protein band was excised from the gel and digested with trypsin for sequence analysis using mass spectrometry. A single peptide sequence, SEDKESLTEDASK, was identified and found to be identical to an internal peptide in a recently cloned gene, *FANCD2* (28).

FANCD2 is one of seven genes mutated in FA, a human cancer-prone genetic disease (29). This is a recessive genetic disease, char-

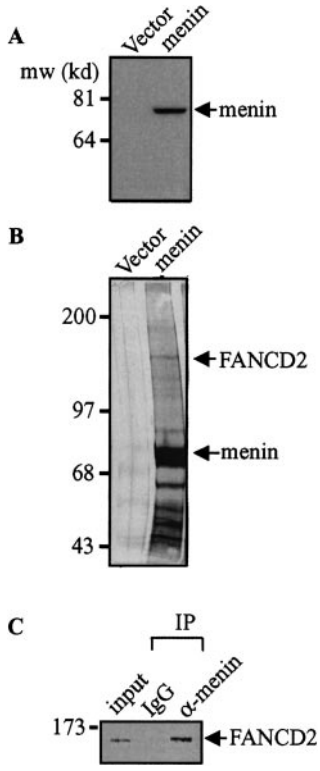

Fig. 1. Menin interacts with FANCD2. *A,* expression of Flag-tagged menin in 293 cells. 293 cells were stably transfected with either vector DNA or pcDNA3-F-menin. Expression of Flag-tagged menin was determined by blotting with the anti-Flag antibody (M_2, Sigma). *B,* detection of association between menin and FANCD2. The whole cell lysates were prepared from 293 cells stably transfected with vector (*Lane 1*) or pcDNA3-F-menin (*Lane 2*). The cell lysates were incubated with agarose beads coupled with anti-Flag antibody (M_2), and the menin-containing complex was isolated and visualized by silver staining as described in "Materials and Methods." *C,* detection of the association between endogenous menin and FANCD2. The 293 whole cell lysates were subjected to immunoprecipitation with control rabbit IgG or the affinity-purified antimenin antibody (#80). The immunoprecipitated samples were separated and immunoblotted with a monoclonal anti-FANCD2 antibody. The input represents 0.5% of the lysates used for immunoprecipitation.

acterized by congenital abnormalities, bone marrow failure, and predisposition to acute myeloid leukemia (29). Peripheral lymphocytes from FA patients are hypersensitive to DNA damage induced by DNA cross-linking agents, such as DEB and MMC (29). To additionally confirm the interaction between menin and FANCD2, we isolated cell lysates from 293 cells and immunoprecipitated the endogenous menin, using a combination of the antimenin antibodies (#77 and #80) or a control IgG. The immunoprecipitated menin and its associating proteins were separated by SDS-PAGE and blotted with a monoclonal anti-FANCD2 antibody (F17). Fig. 1*C* shows that FANCD2 was specifically immunoprecipitated by the antimenin antibody but not by the control IgG.

To determine which region is responsible for mediating interaction with FANCD2, the full-length menin and its various fragments were transiently expressed in 293 cells (Fig. 2*A*). The expressed proteins were immunoprecipitated, and the associated FANCD2 was detected with an anti-FANCD2 antibody (Fig. 2*B*). The results show that a protein fragment, F2, which includes amino acid residues 219–395 of menin, is capable of interacting with FANCD2 (Fig. 2, *Lane 4*). However, the amount of FANCD2 associating with F2 is much less as compared with that associated with the full-length menin (Fig. 2, *Lanes 4* and *6*). Thus, although the middle region of menin is capable of interacting with FANCD2, additional sequence in other parts of menin also contributes to the interaction between menin and FANCD2.

Interaction of Menin with FANCD2 Is Enhanced by γ-Irradiation. Because *FANCD2* is involved in repair of DNA damage induced by γ-irradiation and DNA cross-linking agents (29), we tested whether γ-irradiation modulates the interaction between menin and FANCD2. To this end, cells expressing the Flag-tagged menin were exposed to γ-irradiation. After various periods of time postirradiation, cell lysates were isolated from treated cells and were incubated with the agarose beads conjugated with the anti-Flag antibody. Finally, menin and its associating proteins were eluted with a buffer containing the Flag peptide, and the amount of FANCD2 associated with menin was determined by Western blotting using an anti-FANCD2 antibody (Fig. 3*A, top*).

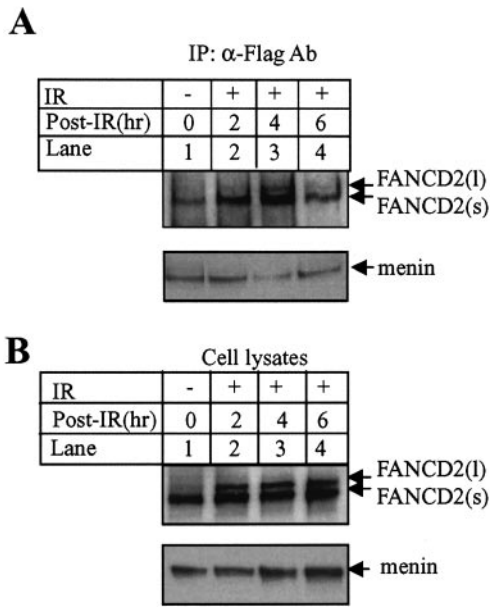

Fig. 3. Induction of association between FANCD2 and menin by γ-irradiation (*IR*). *A,* HeLa cells expressing Flag-menin were exposed to 2500 rads of irradiation and harvested at the indicated time points. The whole cell lysates were prepared and then subjected to immunoprecipitation with the anti-Flag agarose beads. The proteins bound to the beads were separated and immunoblotted with anti-FANCD2 antibody (*Lanes 1–4*, *top*). The bottom panel in *Lanes 1–4* shows the amount of the menin equivalent to 0.1% of the beads used in the *top*. *B,* the level of the FANCD2 and menin in the cell lysates. Cell lysates from each sample (2.5 μg) were separated by SDS-PAGE and detected using the anti-FANCD2 antibody (F17; *top*) and the anti-Flag antibody (*bottom*).

As a control, a fraction of the agarose beads from each sample was also analyzed by Western blotting analysis to ensure that the amounts of immunoprecipitated menin were equal (Fig. 3*A, bottom*). Notably, after exposure to γ-irradiation, the amount of FANCD2 associating with menin increased, reaching a peak after 4 h and almost returning to the normal level after 6 h. At the peak level (Fig. 3*A, bottom, Lane 3*), FANCD2 (l), a monoubiquinated form of FANCD2 (29), also associated with menin. As a control to make sure that the amounts of FANCD2 and menin used for the immunoprecipitation in each sample were equal, a fraction of cell lysates used for immunoprecipitation was analyzed by Western blotting analysis using anti-FANCD2 and anti-Flag antibody, respectively (Fig. 3*B*). Monoubiquitination of FANCD2 was induced by γ-irradiation (Fig. 3*B, top*), as reported previously (19). However, monoubiquitination of FANCD2 did not enhance the menin/FANCD2 interaction, because the amount of the monoubiquitinated FANCD2 (l) bound to the menin beads was not proportionally higher than that of the unmodified menin FANCD2 (s; Fig. 3*A, top*). Together, these results suggest that menin may be involved in DNA damage repair in cooperation with FANCD2.

Loss of Menin Expression Increases Cell Sensitivity to DNA Damage. Fig. 4*A* shows that menin-complemented cells, but not the vector-infected *Men1*$^{-/-}$ cells, express menin. These cells were cultured in increasing concentrations of DEB, an intrastrand DNA cross-linker (15, 16). The number of colonies from these cells was counted

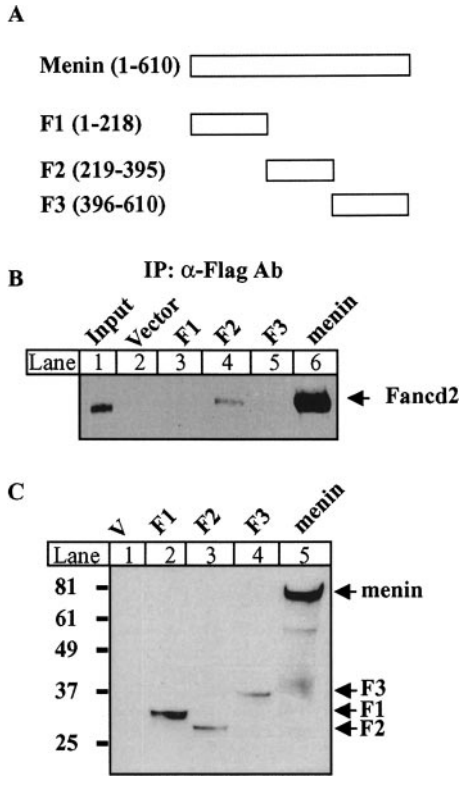

Fig. 2. The middle fragment of menin is capable of interaction with FANCD2. *A,* a diagram for full-length menin and its various fragments expressed in 293 cells. *B,* these various menin constructs were transfected into 293 cells, and the resulting whole cell lysates were incubated with anti-Flag agarose beads. After extensive washing the protein complexes were eluted by the Flag peptide and subjected to SDS-PAGE followed by immunoblotting with anti-FANCD2 (F1–17 antibody). *C,* the expression level of menin or its various fragments was detected by Western blot analysis with 0.5% cell lysate used for immunoprecipitation.

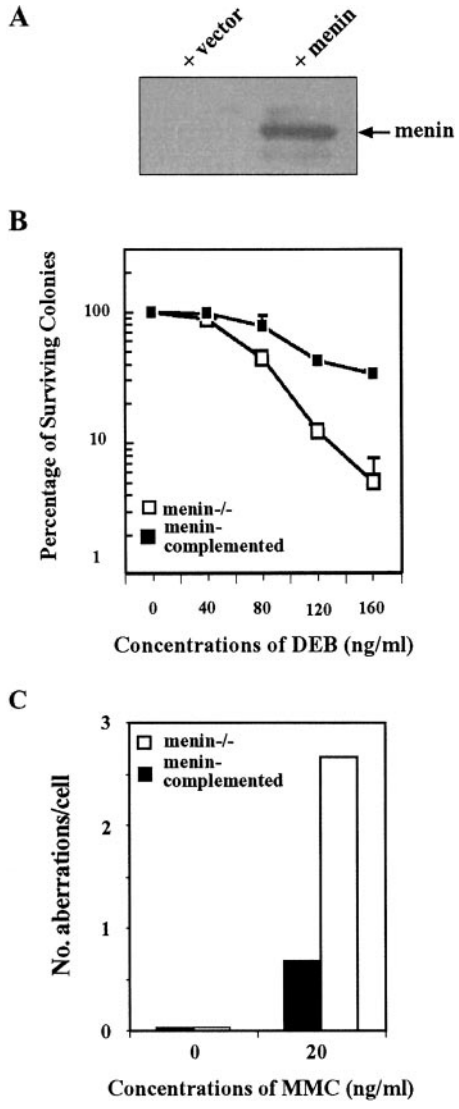

Fig. 4. Loss of menin increases cell sensitivity to DNA damage. *A,* detection of menin expression in *menin*$^{-/-}$ MEFs infected with either vector viruses or viruses expressing menin by Western blot analysis using antimenin antibody (#80). *B,* increased sensitivity of *menin*$^{-/-}$ MEFs to DEB. The cells were seeded on 100-mm dishes, and treated with increasing concentrations of DEB as indicated. On day 9 of culture, the cells were fixed with ethanol and stained with crystal violet as described previously (22), and surviving colonies were counted. This is a representative of six independent experiments. *C,* increased chromosomal aberrations in *menin*$^{-/-}$ cells after treatment with MMC. Chromosome aberrations included breaks and quadriradial forms. This is a representative of five experiments.

after 9 days of culture. Fig. 4*B* shows that at concentrations of 120 and 160 ng/ml of DEB, loss of menin expression decreases the survival of *Men1*$^{-/-}$ cells modestly yet significantly ($P = 0.04$ and 0.005, respectively). Moreover, cytogenetic analysis demonstrates that the *Men1*$^{-/-}$ cells had more chromosomal aberrations in the presence of MMC, a DNA cross-linking agent, as compared with the menin-complemented cells (Fig. 4*C*). The chromosomal aberrations were similar to those observed in FA cells, although FA cells are generally more sensitive to DEB than *Men1*$^{-/-}$ cells. These results suggest that cells with defects in *Men1* are sensitive to DNA damage, and menin and FANCD2 may interact in a common DNA repair pathway.

Menin Is Localized to Nuclear Body-like Structures. To examine whether the subcellular distribution of menin is altered by DNA damage, we generated specific antibodies against human menin. Affinity-purified antibody #80 was able to specifically detect human menin transiently expressed in NIH 3T3 cells (Fig. 5*A*) as well as the endogenous menin from the nuclear extract of a murine insulinoma cell line, TGP61 (Fig. 5*B*). To test whether the purified menin antibody could specifically detect endogenous menin by immunofluorescent staining, TGP61 cells were stained with the purified antibody, followed by staining with the FITC-conjugated secondary antibody. The antibody stained dot-like nuclear structures in the cells (Fig. 5*C, top*), and the staining was competed away by a specific peptide (5*C, middle*) but not by a nonspecific peptide (5*C, bottom*). These results show that the menin antibody (#80) specifically recognizes endogenous menin in the nuclear body-like structures. Similarly, antibody #77 also specifically stained menin in a similar pattern (data not shown). Fig. 5*D* is a higher magnification image of the nuclear body-like structures (5*C, top*), and these structures are within the area stained by 4′,6-diamidino-2-phenylindole, which stains nuclear DNA (5*C, middle* and *bottom*). Together, these results suggest that menin is localized to nuclear body-like structures in the nucleus.

Menin Associates with Chromatin and NM. TGP61 cells were treated first with a detergent to remove soluble proteins, and then with DNase I to collect chromatin DNA and associating proteins using an established procedure (30). The remaining part is NM fraction. Fig. 6*A* shows that menin is localized to nuclear body-like structures in the nucleus (Fig. 6*A, top*). After digestion with DNase I, the small foci, which may associate with chromatin, disappear, but the relatively larger foci (Fig. 6*A, bottom*) associating with NM still remain. To additionally confirm the association of menin with chromatin and NM, 293 cells expressing Flag-tagged menin or control cells were permeabilized with a detergent. The soluble fraction, the chromatin fraction, and the NM were isolated. The level of menin in each of these fractions was determined by Western blotting using the anti-Flag antibody (Fig. 6*B*). Similarly, the distribution of the endogenous menin in TGP61 cells was determined by Western blot analysis using the antimenin antibody (Fig. 6*C*). The level of signal in each band was quantified, and the results show that ~5% of menin is in NM, 80% is in the chromatin fraction, and 15% is in the soluble fraction. These results indicate that endogenous menin is associated with both chromatin and NM.

Association of Menin with NM Is Induced by γ-Irradiation. We next tested whether the DNA damage induced by γ-irradiation changes the distribution of menin in chromatin and NM. HEK 293 cells expressing Flag-tagged menin were treated with γ-irradiation, and the soluble, chromatin, and NM fractions were isolated. Fig. 7*A* shows that after γ-irradiation, association of menin with NM is enhanced ~6-fold (Fig. 7*A, Lane 2*, *bottom*), whereas the amount of the NM protein Lamin B does not change (Fig. 7*A, middle*). The distribution of menin in the chromatin fraction does not change dramatically (data not shown). To additionally confirm these results, 293 cells were treated with various doses of γ-irradiation; at different time points after irradiation, the cells were harvested, and the distribution of the endogenous menin was determined by Western blotting analysis. Fig. 7*B* shows that the distribution of menin in NM increases 3-fold 2 h after the irradiation, whereas the menin in the soluble fraction decreases (Fig. 7*B, top*). The level of menin in chromatin does not change (data not shown). FANCD2 is also associated with chromatin.[5] Collectively, these results indicate that part of menin redistributes to the NM in response to DNA damage, suggesting a potential function of menin in DNA repair in concert with FANCD2.

DISCUSSION

Through affinity chromatography coupled to protein sequencing using mass spectrometry, we show that menin specifically interacts

[5] A. D'Andrea, unpublished observations.

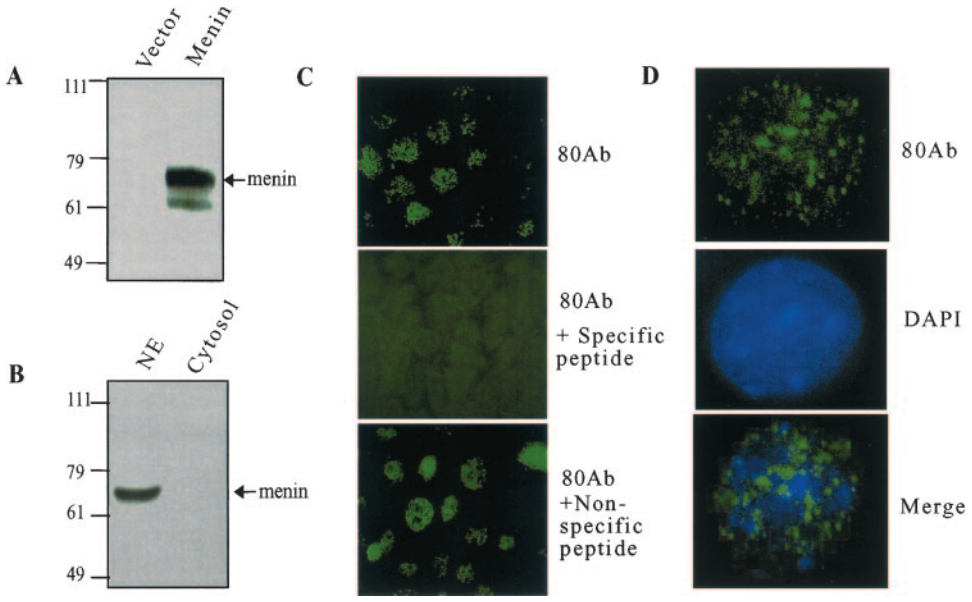

Fig. 5. Menin is localized to nuclear body-like structures. *A,* detection of ectopically expressed menin using an antimenin antibody (#80). NIH 3T3 cells were transiently transfected with either vector DNA or pcDNA3-F-menin and the resulting cell lysates were separated and blotted with an antimenin antibody (#80). *B,* detection of endogenous menin in the nuclear extracts. Nuclear extracts (30 μg) and cytosol (30 μg) from TGP61 cells were separated and blotted with the antimenin antibody. *C,* the antimenin antibody specifically recognizes menin. *Top,* TGP61 cells were stained with the antimenin antibody (#80), followed by staining with the FITC-conjugated secondary antibody. *Middle,* the cells were stained with the antimenin antibody in the presence of the specific peptide (#80,10 μg/ml) that was used to produce the #80 antibody. *Bottom,* the cells were stained with the antimenin antibody (#80) in the presence of the nonspecific peptide (#77, 10 μg/ml). *D,* menin is located in nuclear body-like structures. *Top,* the cells were first stained with the antimenin antibody, followed by the FITC-conjugated secondary antibody, and then additionally stained with 4′,6-diamidino-2-phenylindole for the nuclear DNA (*middle*). *Bottom* shows the merged pictures from the *top* and *middle*.

with endogenous FANCD2 (Fig. 1), a protein encoded by a gene mutated in patients with FA and involved in DNA repair (28, 29). Moreover, γ-irradiation increases the interaction between menin and FANCD2 (Fig. 3). Furthermore, loss of menin expression rendered the cells hypersensitive to DNA damage mediated by DEB, to which FA cells are also characteristically sensitive (Fig. 4). A role for menin has been implicated in maintaining genome stability (13–16, 18). For example, extensive chromosomal breakage has been observed in lymphocytes from MEN1 patients but not in lymphocytes from normal individuals, after treatment with an agent cross-linking double-strand DNA (15, 16). A genome-wide LOH screening of 13 MEN1 patients indicates that MEN1 pancreatic tumors fail to maintain DNA integrity and chromosomal stability (17). Hence, the current results are consistent with the notion that DNA repair might be one of the functions for menin.

The physical interaction between menin and FANCD2 could link menin to a FANCD2-mediated DNA repair pathway. FANCD2 is one of the critical genes of which the mutations lead to FA, a recessive genetic disease characterized by congenital defects, progressive bone marrow failure, and predisposition to myeloid leukemia (29, 31–33). Most of the other FA genes function upstream of *FANCD2*, leading to monoubiquitination of FANCD2 in response to DNA damage (29, 34, 35). Mutations in *FANCD2* and several other FA genes render the cells hypersensitive to DNA damage induced by DEB and MMC (36–39). FANCD2 is also phosphorylated at serines 222 and 1404 by ATM in response to γ-irradiation (39). Moreover, FANCD2 has been shown recently to associate with BRCA1 in response to γ-irradiation (19), and biallelic inactivation of BRCA2 is responsible for the phenotypes in FANCB and FANCD1 cells (40). Because monoubiquitination does not seem to enhance the interaction between menin and FANCD2, it is possible that phosphorylation of FANCD2 regulates the interaction between menin and FANCD2, and additionally enhances their function in DNA repair.

Demonstration of an interaction between menin and FANCD2 provides new insights into the molecular pathogenesis of MEN1. This is consistent with reports that peripheral blood lymphocytes from MEN1 patients are hypersensitive to DEB (14–16, 18). It is possible that menin and FANCD2 cooperate in repairing DNA damage and maintaining genome stability. Perhaps a mutation in *Men1* may compromise its role in DNA repair and lead to either activation of an oncogene or suppression of a tumor suppressor gene in endocrine cells. In this regard, loss-of-function mutations in both alleles of *Men1* may be essential for tumorigenesis in MEN1, because LOH in the menin loci is observed frequently in tumors from MEN1 patients as well as from *Men1*$^{+/-}$ knockout mice. However, it is not clear how mutations in *Men1* preferentially lead to development of tumors in endocrine organs; these tumors are uncommon in FA patients. One possibility could be that chromosomal abnormalities in *Men1*$^{-/-}$ cells result in a change of an endocrine organ-specific protein that either promotes or suppresses tumorigenesis, leading to MEN1. Alternatively, the functional interaction between menin and FANCD2 may rely on endocrine organ-specific proteins. Furthermore, other functions, for example, regulation of transcription factors such as Jun D and NFκB (9, 10) may also be critical for tumorigenesis in endocrine organs. However, lack of sustainable cell lines that naturally lose menin hinders efforts to understand the tissue-specific role of menin in endocrine cells.

That menin associates with chromatin and NM provides insights into the function of menin in the nucleus. Tumor suppressor genes such as *p53* and *PML* associate with NM (41–45), a scaffolding structure anchoring the cellular DNA that is involved in regulation of transcription and DNA repair, among other functions (43, 46–49). For example, Cockayne syndrome group A protein, a protein involved in transcription-coupled repair, translocates to the NM after DNA damage (50). Proteins associating with chromatin, a structure consisting of cellular DNA, histones, and other nonhistone proteins such as transcription factors (51–53), are usually involved in gene transcription, DNA replication, and DNA repair (46, 53). Although it has been reported that menin is primarily a nuclear protein (8, 11), its subcellular localization in the nucleus is not clear. The specific antibodies we generated reveal that menin is associated with nuclear body-like structures (Fig. 5). These structures are similar to the PML nuclear body structures (54). Additional fluorescent staining and biochemical fractionation have shown that menin associates with chromatin and NM (Fig. 6). Interestingly, γ-irradiation of the cells enhances accumulation of menin in the NM (Fig. 7) and decreases the amount of menin in the soluble fraction, whereas the amount of lamin B, a protein in NM, remains constant (Fig. 7). This suggests that DNA damage induced by γ-irradiation leads to translocation of menin to NM. Collectively, the current data suggest that menin plays a role in repairing DNA damage. It is likely that by associating with chromatin and NM, where cellular DNA resides, menin may aid in sensing DNA

damage and/or repairing DNA damage. It is also likely that association of menin with chromatin and NM is also critical for other function of menin, such as regulation of gene transcription. However, it is not clear how the association of menin with chromatin and NM affects its role in DNA repair.

The current studies demonstrate that menin interacts with FANCD2, a recently identified protein that is involved in repairing DNA damage induced by interstrand DNA cross-linking and γ-irradiation, and loss of menin expression leads to increased sensitivity to DNA damage. These results suggest a role for menin in DNA repair in concert with FANCD2. It is possible that menin modulates the function of FANCD2 in association with chromatin and NM. However, additional investigation is required to understand the precise mechanism for the potential role of menin in repair of DNA damage.

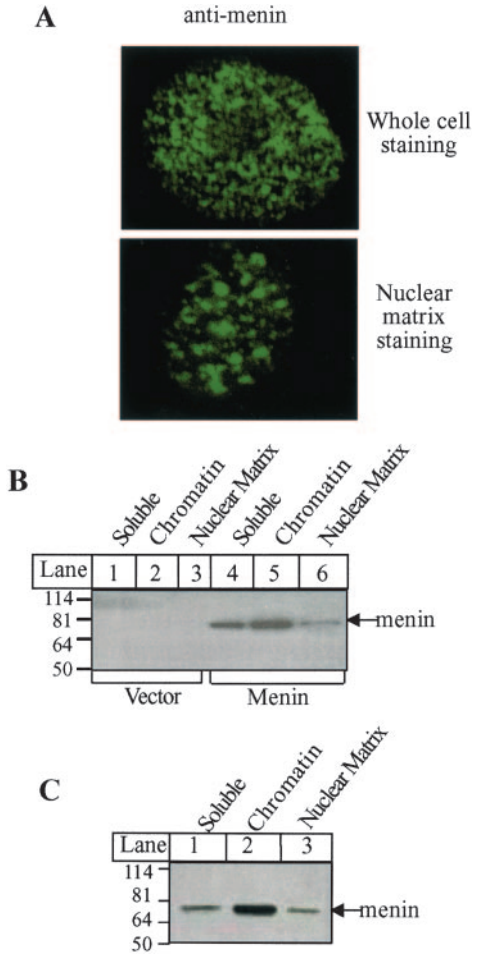

Fig. 6. Menin associates with chromatin and NM. *A,* TGP61 cells were stained with an antimenin antibody (#80; *top*) as described in Fig. 5*C*. *Bottom,* the NM was stained with the affinity-purified antimenin antibody (#80), followed by the FITC-conjugated secondary antibody as described in "Materials and Methods." *B,* detection of epitope-tagged menin in the soluble fraction, the chromatin, and the NM. 293 cells stably transfected with vector (*Lanes 1–3*) or pcDNA3-F-menin (*Lanes 4–6*) were harvested and processed to prepare the soluble fraction, the chromatin fraction, and the NM as described in "Materials and Methods." Total amounts of proteins from the soluble fraction, the chromatin fraction, and the NM were 4.0 mg, 3.4 mg, and 1.2 mg, respectively. Equal amounts of proteins (5 μg) from each fraction were separated by SDS-PAGE, and blotted with the anti-Flag antibody (M_2; Sigma; 5 μg/ml). *C,* detection of endogenous menin in the soluble fraction, the chromatin fraction, and the NM. Equal amounts of proteins (140 μg) from each fraction of TGP61 cells were separated and blotted with the antimenin antibody (#80). The total amounts of proteins isolated from each of the fractions were 3.5 mg for the soluble fraction, 2.25 mg for the chromatin fraction, and 1.1 mg for the NM.

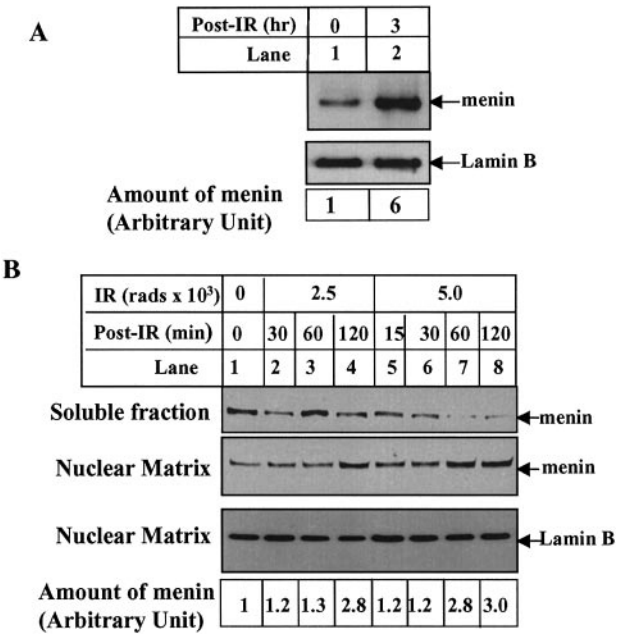

Fig. 7. γ-Irradiation induces the association of menin with the NM. *A,* 293 cells stably expressing Flag-tagged menin were treated with or without ionizing radiation (2500 rads) and were then harvested for isolating the NM. The NM (10 μg/lane) was separated and blotted with either the anti-Flag antibody (M_2; *top*) or antilamin B antibody (*bottom*). The density of the signal in each *lane* was scanned on a Microtek scanner and quantified using NIH Image 1.62 software as arbitrary units. The quantity of menin is normalized by staining for lamin B in the NM. *B, top,* 293 cells (10^7) were treated with the indicated doses of irradiation and harvested at the indicated time points. The soluble fraction and the NM (140 μg/lane) were separated and blotted with the antimenin antibody (#80; *top* and *middle*). *Bottom,* the NM protein lamin B was detected in the NM as a control. Equal amounts of proteins (140 μg/lane) from the NM were separated and blotted with antilamin B antibody (Oncogene Research Products). The amount of menin in NM is normalized by the lamin B signal as arbitrary units as described in Fig. 6.

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