Research Article

# Menin Links Estrogen Receptor Activation to Histone H3K4 Trimethylation

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## Abstract

**The product of the multiple endocrine neoplasia type 1 (*MEN1*) tumor suppressor gene, menin, is an integral component of MLL1/MLL2 histone methyltransferase complexes specific for Lys4 of histone H3 (H3K4). We show that menin is a transcriptional coactivator of the nuclear receptors for estrogen and vitamin D. Activation of the endogenous estrogen-responsive TFF1 (*pS2*) gene results in promoter recruitment of menin and in elevated trimethylation of H3K4. Knockdown of menin reduces both activated TFF1 (pS2) transcription and H3K4 trimethylation. In addition, menin can directly interact with the estrogen receptor-α (ERα) in a hormone-dependent manner. The majority of disease-related MEN1 mutations prevent menin-ERα interaction. Importantly, ERα-interacting mutants are also defective in coactivator function. Our results indicate that menin is a critical link between recruitment of histone methyltransferase complexes and nuclear receptor–mediated transcription.** (Cancer Res 2006; 66(9): 4929-35)

## Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominantly inherited cancer syndrome with high penetrance and variable expression. MEN1 is characterized by the combined occurrence of tumors in the parathyroid glands, the endocrine pancreas/duodenum, and the anterior pituitary gland. Less frequently, adrenal tumors and carcinoid tumors of the thymus, bronchus, or stomach occur (1). The clinical manifestations can result from hormone hypersecretion, mass effect of tumor growth, malignancy, or any combination thereof. The MEN1 syndrome affects about 2 to 3 per 100,000 individuals and is caused by germ line mutations of the *MEN1* tumor suppressor gene (2). Although the majority of these mutations result in truncated MEN1 products, a significant proportion (~20%) are missense mutations, presumably yielding nonfunctional proteins. In MEN1-associated tumors, loss of heterozygosity of the wild-type *MEN1* allele is observed (3), fulfilling the "two-hit" hypothesis of Knudson for a tumor suppressor gene (4).

MEN1 orthologues are found in the genomes of higher eukaryotes, and the gene displays a ubiquitous expression pattern in human tissues (2). The product of the *MEN1* gene, menin, is a nuclear protein (2). Menin interacts with several nuclear proteins [including JunD, nuclear factor-κB (NF-κB), HDAC1/2, and Sin3A], and it can attenuate JunD- and NF-κB-mediated transcription (5–7). Menin was also isolated as a negative regulator of the *hTERT* gene (8). However, the relevance of these interactions for the endocrine phenotype of the MEN1 syndrome is unclear.

Recently, menin was found to be associated in MLL1/MLL2 histone methyltransferase (HMT) complexes, which can direct modification of histone H3 Lys4 (H3K4; refs. 9, 10). Trimethylation of H3K4 (H3K4me3) is linked to gene activity in yeast (11). Consistent with this, H3K4me3 and MLL1 localize at the 5′ ends of actively transcribed genes in human cells (12). Several Hox genes and the genes for the p18 and p27 cyclin-dependent kinase inhibitors are targets for menin-HMT complexes (9, 10, 13), but how these complexes are recruited remains unknown. The activity of the *MEN1* gene is clearly associated with endocrine function. Nuclear receptors play an important role in endocrine processes and in human cancers. The nuclear receptor family represents a class of transcription factors and includes the receptors for steroid and thyroid hormones but also receptors for derivatives of vitamins A and D. Multiple coactivator and corepressor complexes are responsible for regulation of nuclear receptor–mediated transcription (14). In this study, we report that menin can act as a direct coactivator for estrogen receptor α (ERα)–mediated transcription. We provide data indicating that menin serves as a critical link between activated ERα and H3K4 trimethylation and is involved in activation of transcription of the estrogen-regulated *TFF1* gene. These results provide a molecular mechanism for recruitment of H3K4 HMT complexes and could provide an explanation for the clinical manifestations of the MEN1 syndrome.

## Materials and Methods

**Plasmids and mutagenesis.** The baculovirus transfer vector, pFBhMEN-his, was constructed by insertion of a *Bam*HI-*Hin*dIII fragment from pBacM1-H (gift from G. Weber), which carries the human MEN1 cDNA, into pFastBac (Invitrogen, Breda, the Netherlands). pEG202NLS-menin for expression of the LexA-menin fusion protein was constructed by PCR amplification of the MEN1 cDNA from pCDNA3.1M+ (gift of G. Weber) using oligos M2-*Bam*HI-F1, 5′-GATCATCCGGATCCGGGCTGAAGGCCGCC-CAGAA-3′ and M610-B-N-R, 5′-GATCATCCGGATCCGCGGCCGCTCA-GAGGCCTTTGCGCTGCC-3′ and insertion using *Bam*HI and *Not*I of pEG202NLS. pEG202NLS is similar to pEG202 (15) but includes a SV-LT nuclear localization signal introduced in the multiple cloning site. MEN1 missense mutations were introduced by site-directed mutagenesis in the menin expression vector pCDNA3.1M+ and pEG202NLS-menin as described in the QuikChange protocol from Stratagene (La Jolla, CA). Construction of pSG424mERα(AF2) and GST-mERα(AF2), pGEX1λT-hJunD (gift from S. Agarwal), and pGEX-5x/GST-65 (gift from L. Burns) have been described (5, 6, 16). pXJ440hVDR(DE) for expression of the Gal4-fusion with the AF2 domain of human vitamin D receptor (VDR) was provided by G. Mengus (17). B42-mERα(AF2), B42-hERα(AF2), and B42-m ERα(M547A/L548A) (18) were constructed in the pJG4-5 vector.

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**Antibodies and immunoblotting.** Polyclonal menin antibodies (20.145-436 for chromatin immunoprecipitation experiments) were generated by immunization of a rabbit with purified recombinant menin. After three immunizations, plasma was obtained by plasmapheresis. Antibodies were purified by binding to an affinity-matrix consisting of recombinant menin (4 mg/mL) attached to Affi-Gel 10 beads (Bio-Rad, Veenendaal, the Netherlands) and eluted with 100 mmol/L glycine (pH 2.5).

Antibodies against menin were purchased from Bethyl Laboratories (Montgomery, TX; A300-105A), against ERα from Santa Cruz Biotechnology (Santa Cruz, CA), and against the histone H3 COOH terminus, H3K4me2, and H3K4me3 from Abcam (Cambridge, United Kingdom). For TATA-binding protein (TBP) detection, 1F8 monoclonal antibodies were used, and for the chromatin immunoprecipitation of RNA polymerase II, 8WG16 antibodies were used. Control rabbit immunoglobulins were purchased from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands).

For immunoblotting, proteins were separated by SDS-PAGE and blotted to nitrocellulose membrane (Protran BA83, Schleicher and Schuell, Dassel, Germany). Blots were blocked for >1 hour in TBST containing 5% dried milk (ELK, Campina, Woerden, the Netherlands) and subsequently incubated with appropriate primary and secondary antibodies. Bands were visualized by enhanced chemiluminescence (Western lightning, Perkin-Elmer, Wellesley, MA).

**Cell lines and transient transfections.** Cos7 (African green monkey kidney), 293T (human embryonic kidney), and MCF-7 (human breast cancer, provided by R. Bernards, NKI) cells were routinely cultured in DMEM (Cambrex, East Rutherford, NJ) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. For reporter experiments, Cos7 cells were seeded at 8 to 10 $\times$ $10^4$ cells per well in DMEM and 293T cells at 1.5 $\times$ $10^5$ cells per well in DMEM without phenol red (Life Technologies, Breda, the Netherlands) containing 5% dextran-coated, charcoal-treated serum, L-glutamine, penicillin, and streptomycin, in 12-well plates. All manipulations were done at 24-hour intervals. In Gal4-ERα reporter experiments, Cos7 cells were washed with PBS, and medium was changed to DMEM without phenol red on day 2. DNA (750 ng per well) was transfected using FuGene 6 reagent (Roche Diagnostics, Almere, the Netherlands). Transfection mixtures consisted of 200 ng luciferase reporter, 25 ng pCMV-*Renilla* and 5 ng pSG424mERα, pXJ440hVDR(DE), or pGal4-VP16 expression vector and supplemented with 520 ng pCDNA3.1M+ and/or empty pCDNA3 plasmid. Twenty-four hours after transfection, the medium was changed to medium containing the appropriate ligand [10 nmol/L 17β-estradiol ($E_2$; Sigma), 100 nmol/L 1α,25-dihydroxyvitamin $D_3$ (Sigma), or ethanol vehicle]. Luciferase and *Renilla* activities were measured on a Lumat LB9507 luminometer (EG&G Berthold, Bad Wildbad, Germany) using the Dual Luciferase kit (Promega, Leiden, the Netherlands). Luciferase activities were corrected for CMV-*Renilla* values and are expressed as relative to transfections without menin. Each transfection was done at least thrice to ensure reproducibility of the observations.

**Protein expression and purification.** Recombinant baculoviruses expressing 6xHis-tagged menin protein were obtained using pFBhMENhis in the Bac-to-Bac baculovirus system (Invitrogen). Sf9 cells were grown in a 5-liter bioreactor (Applikon, Schiedam, the Netherlands). At a density of 5 $\times$ $10^5$ cells per mL, recombinant virus was added. Cells were harvested 5 days after infection and resuspended in lysis buffer [50 mmol/L Tris-HCl (pH 8), 300 mmol/L KCl, 5 mmol/L β-mercaptoethanol, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.1% NP40, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A].

The lysate was loaded onto a 40-mL Ni-NTA agarose (Qiagen, Venlo, the Netherlands) column, which was washed with lysis buffer containing 1 mmol/L imidazole and subsequently with wash buffer [20 mmol/L Tris-HCl (pH 8), 20% glycerol, 100 mmol/L KCl, 2 mmol/L β-mercaptoethanol, and 16 mmol/L imidazole]. Bound proteins were eluted with three column volumes of wash buffer containing 300 mmol/L imidazole. Menin-containing fractions were pooled and dialyzed against buffer T50 [20 mmol/L Tris-HCl (pH 8), 50 mmol/L KCl, 20% glycerol, 0.5 mmol/L EDTA, 0.5 mmol/L PMSF, and 1 mmol/L DTT]. The protein was subsequently applied to a MonoQ HR10/10 anion exchange column (Amersham Biosciences, Roosendaal, the Netherlands), which was developed by a 50 to 1,000 mmol/L KCl linear gradient in buffer T over 15 column volumes. The menin protein elutes at a conductivity of 100 to 200 mmol/L KCl.

**Yeast two-hybrid analysis.** EGY48 cells were transformed with the B42-ERα(AF2) constructs and the indicated LexA-menin constructs. Cells were grown overnight at 30°C in 2% galactose/1% sucrose containing SC medium lacking the appropriate amino acids and in the presence of vehicle, 1 μmol/L $E_2$, or 1 μmol/L 4OH-tamoxifen. Lysates were prepared, and the LacZ activity was determined by a liquid β-galactosidase assay, as described previously (19). Values were corrected for the total amount of protein present in the extract.

**Protein-binding experiments.** GST-NF-κB (p65-RelA) and GST-JunD proteins were expressed in *Escherichia coli* strain BL21(DE3); glutathione *S*-transferase (GST) and GST-mERα(AF2) were expressed in strain DH5α. Expression and lysis procedures have been described previously (19). Purified GST-mERα(AF2) or GST and soluble lysates containing equivalent amounts of GST-p65 or GST-JunD were bound to glutathione agarose beads (Sigma) for 4 hours at 4°C in binding buffer [50 mmol/L Tris-HCl (pH 8), 10% glycerol, 100 mmol/L NaCl, 10 mmol/L $MgCl_2$, 0.5 mmol/L PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 1 mmol/L NaF, 1 mmol/L $Na_3VO_4$, and 0.5 mmol/L DTT]. After washing with binding buffer, purified menin protein (8 μg/mL) was added. Binding experiments with GST and GST-mERα(AF2) were carried out in the presence of 1 μmol/L $E_2$ or ethanol for 4 hours at 4°C. Glutathione beads were washed thrice with excess binding buffer, and eluted proteins were analyzed by immunoblotting.

**Small interfering RNA knockdown and analysis of *TFF1/pS2* gene expression.** MCF-7 cells were trypsinized and resuspended in electroporation buffer [2 mmol/L HEPES (pH 7.2), 15 mmol/L phosphate buffer (pH 7.2), 250 mmol/L mannitol, and 1 mmol/L $MgCl_2$]. Resuspended cells ($\sim 10^6$) were mixed with 2 μg of duplex small interfering RNAs (siRNA; Dharmacon, Chicago, IL) against Lamin (Lamin-SiGLO), or menin [Menin#1 (10) and Menin#2, which has been published as MEN1-10 (20)] and electroporated in 1-mm cuvettes at 140 V, 16 pulses of 1.5 milliseconds (Gene Pulser X-cell, Bio-Rad). Cells were grown in DMEM without phenol red. After 72 hours, medium containing 10 nmol/L $E_2$ or ethanol vehicle was added for 3 hours before cell harvesting. Total RNA was extracted using the RNeasy kit (Qiagen) and subsequently treated with DNase (DNA-free, Ambion, Huntingdon, United Kingdom); 120 ng of total RNA was used for cDNA synthesis (Superscript II, Invitrogen). Expression of TFF1/pS2 and β-actin mRNA expression were analyzed by quantitative PCR on a Chromo4-equipped PCR cycler (MJ Research, Bio-Rad) using primers RT-PS2-FOR, 5′-ATACCATCGACGTCCCTCCA-3′; RT-PS2-REV, 5′-AAGCG-TGTCTGAGGTGTCCG-3′; RT-B-ACTIN-F, 5′-AGAAAATCTGGCACCACACC-3′; and RT-B-ACTIN-R, 5′-AGAGGCGTACAGGGATAGCA-3′ and normalized against a standard reference cDNA from untreated MCF-7 cells. The experiment shown is a representative experiment done in duplicate and analyzed by reverse transcription-PCR in duplicate.

**Chromatin immunoprecipitation experiments.** Subconfluent cultures of MCF-7 cells ($\sim 5 \times 10^6$ cells) were cross-linked by addition of 1% formaldehyde in PBS for 10 minutes at 37°C. Cells were lysed in buffer [50 mmol/L Tris-HCl (pH 7.9), 1% SDS, 10 mmol/L EDTA, 1 mmol/L DTT, and protease inhibitors]. The lysate was sonicated thrice for 15 seconds in a Bioruptor (Diagenode, Liege, Belgium). These mild sonication conditions result in DNA fragments of 2 to 10 kb. Soluble material was diluted 10 times in dilution buffer [20 mmol/L Tris-HCl (pH 7.9), 2 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.5% Triton X-100, and protease inhibitors] and incubated with appropriate antibody or control rabbit IgG overnight at 4°C. Protein A and protein G beads (1:1) were blocked in buffer TE [10 mmol/L Tris-HCl (pH 8), 1 mmol/L EDTA] containing 1 mg/mL herring sperm and 1 mg/mL bovine serum albumin overnight at 4°C. Beads were washed four times in buffer TE and incubated with the chromatin for 3 hours at 4°C. Subsequently, the beads were washed four times with wash buffer [20 mmol/L Tris-HCl (pH 8), 250 mmol/L NaCl, 2 mmol/L EDTA, 0.05% SDS, 0.25% NP40, and protease inhibitors] and once in buffer TE. The beads were resuspended in elution buffer (100 mmol/L $NaHCO_3$, 1% SDS) and placed at 65°C overnight to reverse DNA/protein cross-links.

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Proteinase K was added for 30 minutes at 37°C. Eluted DNA was purified by Qiaquick PCR purification columns (Qiagen). Binding of TFF1/pS2 promoter DNA was assessed by quantitative PCR using primers PS2-FOR, 5′-CCTGGATTAAGGTCAGGTTGGA-3′ and PS2-REV, 5′-TCTTGGCTGAGG-GATCTGGAGA-3′ and normalized against input samples from the same experiment. Analysis of exon 2 DNA of *myoglobin*, a non–$E_2$-responsive gene, was used as an internal control. Primers used were MYO EX2_FW, 5′-AAGTTTGACAAGTTCAAGCACCTG-3′ and MYO EX2_RV, 5′-TGGCAC-CATGCTTCTTTAAGTC-3′ (sequences from S. Denissov).

## Results

**Menin is a coactivator of nuclear receptor mediated transcription.** Association of menin activity with endocrine functions and the presence of sequence motifs involved in recruitment of cofactors (see below) suggested that menin can act as a cofactor for nuclear receptors. We tested this hypothesis in a luciferase reporter assay measuring activation by the ERα. A reporter construct bearing multiple binding sites for the yeast Gal4 activator and an expression vector for a Gal4 DNA-binding domain fusion with the ligand binding (AF2) domain of ERα was used. We found that menin overexpression in Cos7 cells augmented ERα-mediated transcription in an $E_2$-dependent manner (Fig. 1*A*). Menin also increased VDR-activated transcription to about 2-fold (Fig. 1*B*). Transient transfection assays using 293T human embryonic kidney cells showed a similar stimulation of ERα-activated transcription (Fig. 1*C*). Whereas coactivation of ERα by menin in Cos7 cells showed a clear optimum, 293T cells displayed a linear dose-response curve. Possibly, the optimum observed in Cos7 cells resulted from titration of limiting factors by excessive overexpression of menin. The nonrelated activator Gal4VP16 was included in these assays as a negative control for coactivation by menin. As reported previously (6), menin did not affect activation by VP16 (Fig. 1*D*), indicating that in our transfections, menin displayed transcription activator specificity. Thus, the transient reporter assays indicate that menin can act as a coactivator for the nuclear receptors for estrogen and vitamin D.

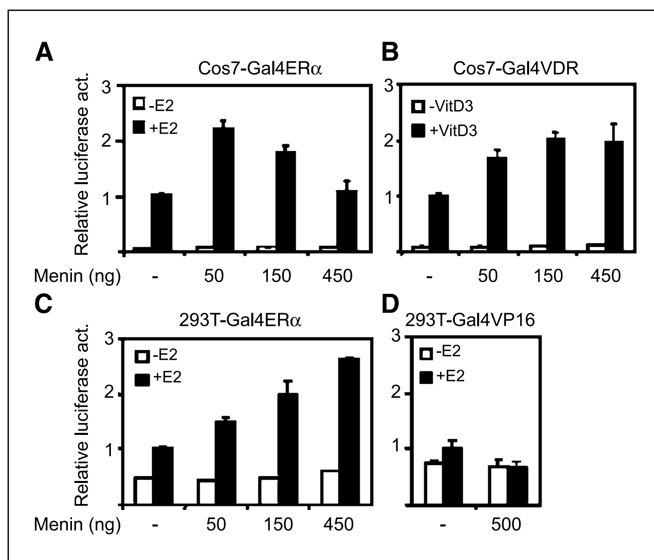


**Figure 1.** Menin is a coactivator of ERα-mediated transcription. *A,* Cos7 cells were transiently transfected with 5xGalTKLuc and Gal4-mERα plasmids and subsequently treated with 10 nmol/L $E_2$ or ethanol. Increasing amounts of menin expression vector were cotransfected, and a CMV promoter–driven *Renilla* luciferase plasmid was included for normalization purposes. *B,* Cos7 cells were cotransfected with a Gal4-hVDR plasmid and increasing amounts of menin expression vector; 100 nmol/L vitamin $D_3$ was added as indicated. *C,* 293T cells were cotransfected with a Gal4-mERα plasmid and treated with 10 nmol/L $E_2$ or ethanol. *D,* Gal4-VP16 plasmid was cotransfected into 293T cells and treated with 10 nmol/L $E_2$ or ethanol. Luciferase activities (*A-D*) relative to transfections without menin. *Columns,* mean; *bars,* SE.

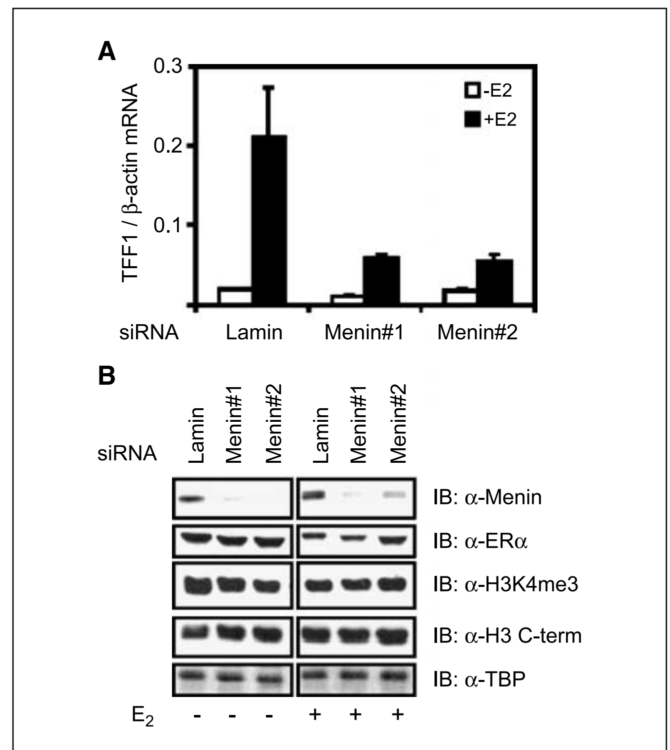


**Figure 2.** Menin is critically involved in activation of the ERα target gene *TFF1* (formerly pS2). *A,* MCF-7 cells were electroporated with siRNAs against menin, which were previously shown to be effective (10, 20), or nuclear lamin (as a control). After 72 hours, cells were treated with 10 nmol/L $E_2$ or ethanol for 3 hours, and RNA was extracted. mRNA levels of TFF1 relative to β-actin were determined by quantitative RT-PCR. *Columns,* mean; *bars,* SE. *B,* Efficiency of menin knockdown was determined by immunoblotting of total cell lysates from siRNA-treated MCF-7 cells (as in *A*). Additionally, protein levels of ERα, H3K4me3, and total histone H3, both from nonstimulated and stimulated cells, were determined. TBP was used as loading control.

**Menin is an activator of ligand-induced TFF1 gene expression.** To investigate whether menin is involved in regulation of endogenous nuclear receptor–responsive genes, we analyzed transcriptional activation of the *TFF1/pS2* gene after siRNA-mediated knockdown of menin. The *TFF1* gene (*pS2*) has served as a model to study effects of coactivators of ERα (21, 22). Two different effective siRNAs against menin or nuclear lamin (as a control) were transfected into breast carcinoma–derived MCF-7 cells. Quantitative mRNA analysis of the transfected cells indicated that menin knockdown reduces $E_2$-dependent transcription of TFF1 about 4-fold (Fig. 2*A*). Immunoblot analysis of transfected cell lysates indicated a specific decrease of menin levels (~75%; Fig. 2*B*). Importantly, siRNA treatment did not affect expression of ERα or TBP, which was included as a loading control (Fig. 2*B*). This analysis indicates that menin is involved in hormone-dependent transcription of the ERα-responsive *TFF1* gene.

**Ligand-induced recruitment of menin is required for H3K4me3 of the TFF1 promoter.** The above experiments indicated that menin is important for regulation of $E_2$-dependent TFF1 promoter activity. We investigated direct involvement of

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menin by chromatin immunoprecipitation experiments. Using menin-specific antibodies, we found that the menin protein was recruited to the activated TFF1 promoter along with ERα and RNA polymerase II (Fig. 3*A*). Because menin is associated with HMT-complexes (9, 10), we examined the methylation status of H3K4 in chromatin immunoprecipitation assays. Interestingly, H3K4 trimethylation (H3K4me3) but not dimethylation (H3K4me2) was elevated after $E_2$ treatment (Fig. 3*B*). It is important to note that upon $E_2$ stimulation, histone H3 levels on the *TFF1* gene are lowered. This suggests that removal of nucleosomes is involved in TFF1 activation as was also observed for activated yeast promoters (23).

We used our siRNA knockdown approach to investigate whether menin is also critical for elevated H3K4me3 levels at the TFF1 promoter. MCF-7 cells were treated with menin siRNAs before $E_2$ stimulation and formaldehyde cross-linking. In these cells, but not in control siRNA-treated cells, induced H3K4me3 levels were severely diminished (Fig. 3*D*). In this setup, the reduction is not due to a global effect on H3K4me3 levels (Fig. 2*B*). Interestingly, upon $E_2$ stimulation, we observed a reduction in total histone H3 associated with the TFF1 promoter in lamin knockdown cells but not in menin knockdown cells (Fig. 3*C*). Together, our experiments indicate that the menin tumor suppressor protein links activating histone H3K4 methylation to transcription stimulation of the endogenous *TFF1* gene.

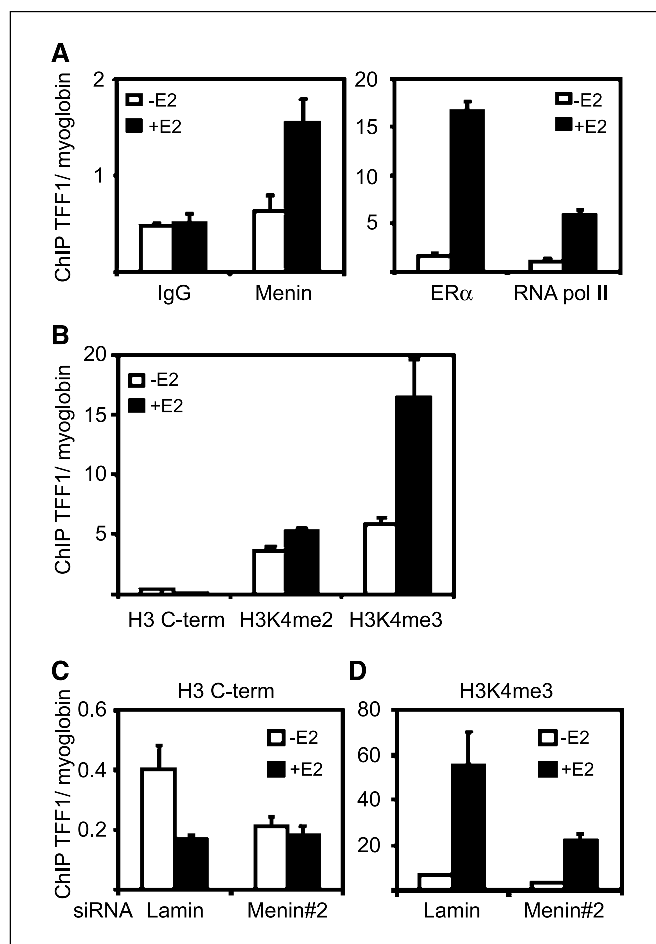


**Figure 3.** H3K4 trimethylation of TFF1 is increased upon $E_2$ activation and requires menin protein. Transcription factor association (*A*) and histone modification (*B*) were determined by chromatin immunoprecipitation analysis. MCF-7 cells were stimulated for 3 hours with 10 nmol/L $E_2$ or ethanol before cross-linking. Ratios of immunoprecipitated TFF1 promoter DNA and heterochromatic myoglobin exon 2 DNA. The values were determined by quantitative PCR relative to input reference curves. Representative experiment analyzed in triplicate. *Columns,* mean; *bars,* SE. *C* and *D,* chromatin immunoprecipitation analysis of total histone H3 and H3K4me3 bound to the TFF1 promoter upon menin knockdown. Cross-linked chromatin was prepared from cells treated with the indicated siRNAs and subsequent hormone stimulation for 3 hours.

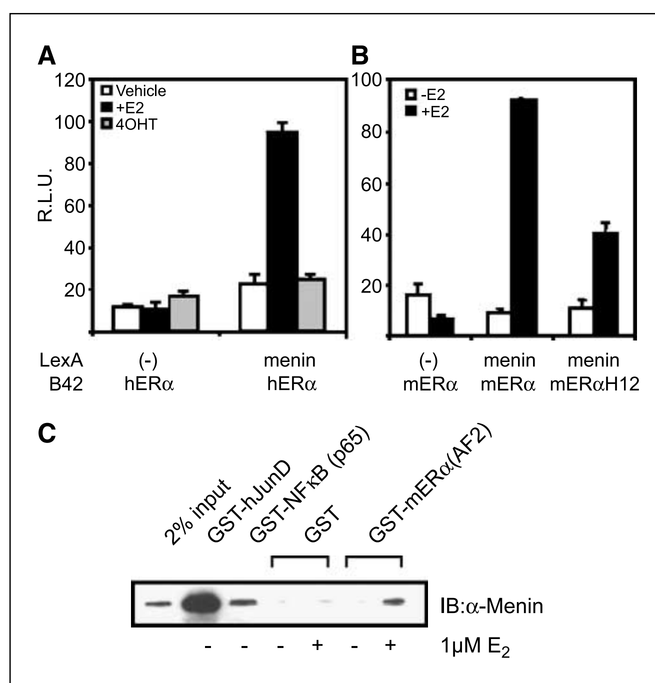


**Figure 4.** Menin interacts directly with activated ERα. *A* and *B,* binding of menin to hERα-AF2 or the M547A/L548A helix 12 mutant was investigated in a yeast two-hybrid experiment in the presence of ethanol, 1 μmol/L $E_2$, or 4OH-Tamoxifen. Relative β-galactosidase activities. *Columns,* mean; *bars,* SE. Testing LexA-menin in combination with B42 alone resulted in 8-fold lower levels than LexA-menin and B42-hERa in the presence of $E_2$ (data not shown). *C,* menin interacts directly with activated ERα. Glutathione beads were coated with purified GST, GST-mERα-AF2, GST-hJunD, and GST-NF-κB proteins and incubated with recombinant menin in the absence or presence of $E_2$. Retention of menin protein was determined by immunoblotting.

**Ligand-dependent interaction of menin and ERα.** We noticed that menin contains an evolutionarily conserved LXXLL (amino acids 263-267) and several LXXLL-like motifs, which are involved in binding of transcriptional coactivators to liganded nuclear receptors (16). As our experiments indicated that menin can provide a critical link in the activation of nuclear receptors, we did two different assays to determine whether menin can interact directly with ERα. First, we found that menin interacted with the AF2 domain of ERα in a yeast two-hybrid assay in an agonist-dependent manner (Fig. 4*A*). Moreover, this menin-ERα interaction was severely compromised by the transcription-defective ERα mutant M547A/L548A (Fig. 4*B*) in activation helix 12 (24). Using the yeast two-hybrid system, we also found a ligand-dependent interaction with the retinoid X receptor (RXR; data not shown). Second, recombinant menin was retained by a GST-ERα AF2 fusion protein in the presence of $E_2$, showing a direct interaction (Fig. 4*C*). Fusions of the NF-κB family member p65/RelA and of JunD were included as positive controls (5, 6). Menin-ERα binding was comparable with the menin-NF-κB interaction but not as strong as JunD binding. We failed to observe coimmunoprecipitation of

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the transfected proteins in $E_2$-treated 293T cells, which would be in agreement with a low-affinity interaction. Nevertheless, the experiments of Fig. 4 show that menin can act interact directly with ERα in a hormone-dependent manner.

**MEN1 mutations can disrupt ERα association and activation.** To determine the clinical relevance of the menin-ERα interaction, 11 disease-related MEN1 mutants were tested in the yeast two-hybrid assay. These mutations have all been reported in the literature (25–30). Most mutations disrupted the ligand-dependent interaction between menin and ERα (Fig. 5*A*). For example, the L264P and L267P mutations in the putative LXXLL-motif were completely defective for ERα interaction as expected. However, other disease-related mutants like G305D and H317R displayed no defect.

We decided to analyze the coactivator properties of four MEN1 mutants. Interestingly, none of these selected mutants increased $E_2$-dependent ERα activity in the luciferase reporter assay (Fig. 5*B*). Previously, it was shown that MEN1 mutations can result in reduced steady-state levels of overexpressed menin (31). However, analysis of transfected cell lysates indicated the menin proteins were expressed to similar levels in our experiment (Fig. 5*B*).

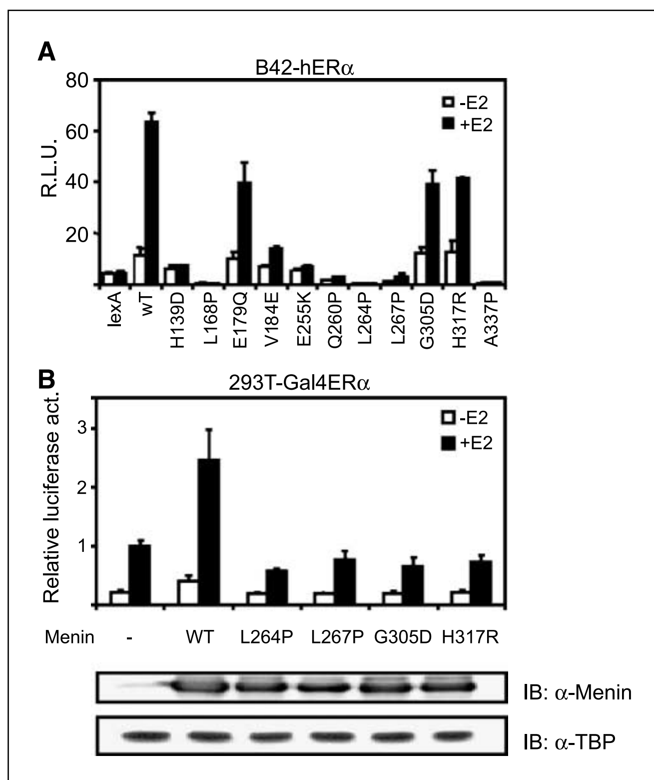


**Figure 5.** Several disease-related *MEN1* gene mutations disrupt the two-hybrid interaction of menin and ERα. *A,* eleven different MEN1 gene mutations were introduced into the LexA-menin yeast expression vector. The interaction of menin mutants with activated ERα was tested in the yeast two-hybrid setup and compared with the interaction of wild-type menin. Relative β-galactosidase activities. *Columns,* mean; *bars,* SE. *B,* four selected menin mutations were introduced into the pCDNA3.1M+ expression vector for human menin; 500 ng of the expression vectors were cotransfected into 293T cells together with an expression plasmid for Gal4-mERα and luciferase reporter constructs. *Top*, luciferase values are expressed relative to the internal *Renilla* control as described for Fig. 1. *Bottom*, total cell lysates from transfected cells were subjected to immunoblotting to determine expression levels of mutant menin proteins. Endogenous TBP was used as loading control.

Taken together, the MEN1 mutant analysis indicates that besides disruption of the ERα interaction, *MEN1* gene mutations may also impair ERα-mediated transcription via alternative mechanisms. Possibly, these mutants are defective in interaction with other components of the histone methyltransferase complexes (9).

## Discussion

In this study, we show that menin, the product of the *MEN1* tumor suppressor gene, is a coactivator for ERα-mediated transcription by increasing H3K4 methylation of the $E_2$-responsive TFF1 promoter. ERα and VDR were chosen as model nuclear receptors for our interaction studies. We propose that a direct interaction between menin and ligand-activated nuclear receptors is responsible for enhanced H3K4 trimethylation. This is most clearly shown by the siRNA knockdown experiment of Fig. 3. Taken together, our results reveal a specific pathway for alterations in histone methylation, and they bear important implications for the etiology of the MEN1 syndrome.

We show for the first time that $E_2$-dependent activation of the TFF1 promoter is accompanied by increased levels of H3K4me3 and a decrease in histone H3 association. Previous analyses indicated that H3K4me3 correlates with the transcriptional activity of genes and occurs at their 5′ end (11, 12). The direct functional consequences of increased H3K4Me3 levels are not known yet. Our observations that menin knockdown prevents both H3K4me3, and a reduction in total H3 (Fig. 3) suggests a functional link between these events. Possibly, chromatin remodelers analogous to the yeast Chd1 or Isw1 proteins (32, 33) recognize the H3K4me3 mark and are involved in nucleosome removal from the activated promoter. Alternatively, H3K4 methylation may be linked to H4K16 modification by the acetyltransferase MOF (34). Recently, H4-K16 acetylation has been shown to inhibit chromatin compaction (35).

Recruitment of transcription cofactors and basal transcription factors was shown to occur in a cyclical pattern during ligand-dependent transcription of the TFF1 gene (14, 21, 22). This also involved dimethylation of H3R17 and H4R3 and acetylation of H3K14 and H4K16 (22). Menin association and H3K4me3 could also be cyclical. However, H3K4me3 marks are believed to be relatively persistent (36). Consistent with this, for androgen receptor (AR) target promoters, ligand-dependent increases in H3K4me3 do not display a cyclical pattern (37). In addition to this, it would be interesting to test menin involvement in AR function, as a reduced level of H3K4me2 (H3K4me3 was not tested) is one of the markers for an increased risk of prostate tumor recurrence (38).

Several mammalian SET-domain containing proteins, including MLL1, MLL2, SET7/9, and SMYD3, can methylate H3K4 (reviewed in ref. 39). We propose that the SET-domain containing MLL1 (mixed lineage leukemia) and/or MLL2/TRX2 (trithorax homologue 2) proteins are responsible for the elevated H3K4me3 levels at the *TFF1* locus. As an integral component (9, 10), menin may act to link MLL1/MLL2 complexes to activated nuclear receptors in general. In accordance with this proposal, experiments in *Drosophila* showed that the activated ecdysone receptor (EcR) recruits the HMT activity of the trithorax-related gene product TRR (40). Interestingly, the RXR orthologue Ultraspiracle is the EcR heterodimerization partner, and we observed that menin can interact with the murine RXR in a ligand-dependent manner (data not

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shown). The model of menin connecting an activated nuclear receptor with MLL1/MLL2 complexes predicts that the G305D and H317R mutations, which are not defective for nuclear receptor interaction (Fig. 5*A*), will be disturbed in MLL1/MLL2-menin complex formation as was suggested by the analysis of other menin mutants (9).

A consistent reduction of global H3K4me3 levels was observed in pancreatic tumors from MEN1$^{+/-}$ transgenic mice (41). This suggests that other transcriptional activators besides activated nuclear receptors may also require menin for efficient H3K4 trimethylation. Most likely, this includes previously identified menin interactors. The fact that a reduction in H3K4me3 was not apparent in menin knockdown cells (Fig. 2*B*) indicates either persistence of the H3K4me3 mark during the time course of knockdown experiments, or that menin/MLL complexes do not represent the predominant H3K4me3 activity in MCF-7 cells. It should be noted that a reduction of global MLL levels as described by Milne et al. (13) in MEN1-associated pancreatic adenomas may contribute to the reduction of global H3K4Me3.

The MEN1 syndrome is rather diverse in its clinical manifestations, and nuclear receptors have many functions in organs affected in MEN1 patients. For example, elevated levels of parathyroid hormone are very common in these patients. In the normal situation, activation of VDR will inhibit production and release of PTH. As reported here, menin is a regulator of ERα and VDR function, and inactivation of menin leads to disruption of ERα-mediated transcription. In the case of VDR, our findings could help explain elevated PTH production in MEN1 patients. Interestingly, polymorphisms of the *VDR* gene have been associated with hyperparathyroidism (reviewed in ref. 42). Prolactinomas are also a common manifestation of MEN1. ERα has a direct effect on prolactin production in the pituitary gland and is expressed in pituitary adenomas (43).

At least two mechanisms have been proposed for MEN1-associated tumorigenesis. First, inactivation of menin may allow JunD to promote tumor growth (44). Second, loss of regulation by menin of the *p18* and *p27* tumor suppressor genes may lead to the development of MEN1 tumors (13). Our finding that menin is critical for linking hormone action to H3K4 trimethylation and gene activation provides additional clues for the etiology and tissue specificity of the manifestations of the MEN1 syndrome. Further studies aimed at restoring nuclear receptor function in MEN1 tumors may eventually yield novel therapeutic options.

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