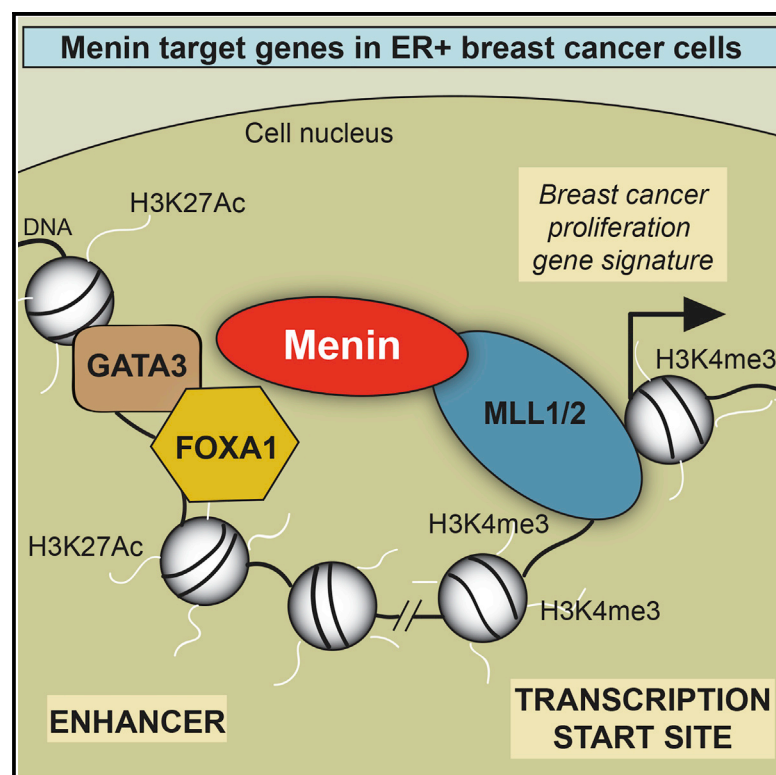# Enhancer-Mediated Oncogenic Function of the Menin Tumor Suppressor in Breast Cancer

Graphical Abstract

Authors

Koen M.A. Dreijerink, Anna C. Groner, Erica S.M. Vos, ..., Prakash K. Rao, Henry W. Long, Myles Brown

Correspondence

myles_brown@dfci.harvard.edu

In Brief

Dreijerink et al. describe the oncogenic actions of the tumor suppressor menin in breast cancer cells. In mammary progenitor cells, menin regulates anti-proliferative genes. Menin is present at FOXA1 and GATA3-bound enhancers that associate with promoters through chromatin looping. Insight into menin's context-dependent function suggests therapeutic strategies.

Highlights

- The *MEN1* gene product, menin, has an oncogenic role in ER$^+$ breast cancer cells

- The breast cancer driver gene *ESR1* is a major menin target gene in ER$^+$ cells

- Menin is present at FOXA1 and GATA3-bound enhancers that are looped to promoters

- Enhancer presence of menin marks target gene H3K4me3 and transcription

Accession Numbers

Enhancer-Mediated Oncogenic Function of the Menin Tumor Suppressor in Breast Cancer

Koen M.A. Dreijerink,[1,2,5] Anna C. Groner,[1,2,6] Erica S.M. Vos,[3] Alba Font-Tello,[1,2] Lei Gu,[4] David Chi,[1,2] Jaime Reyes,[1] Jennifer Cook,[1,2] Elgene Lim,[1,2,7] Charles Y. Lin,[1] Wouter de Laat,[3] Prakash K. Rao,[1,2] Henry W. Long,[1,2] and Myles Brown[1,2,8,*]

[1]Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, 450 Brookline Avenue, Boston 02215, MA, USA
[2]Center for Functional Cancer Epigenetics, Dana-Farber Cancer Institute, Harvard Medical School, 450 Brookline Avenue, Boston 02215, MA, USA
[3]Hubrecht Institute-KNAW and University Medical Center, Uppsalalaan 8, 3584 CT Utrecht, the Netherlands
[4]Division of Newborn Medicine, Children's Hospital Boston and Department of Cell Biology, Harvard Medical School, 300 Longwood Avenue, Boston, MA 02115, USA
[5]Present address: UMC Cancer Center, University Medical Center, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands
[6]Present address: Department of Biosystems Science and Engineering, ETH Zurich, Mattenstrasse 26, Basel 4058, Switzerland
[7]Present address: Garvan Institute of Medical Research and St Vincent's Hospital, 384 Victoria Street, Darlinghurst Sydney, NSW 2010, Australia
[8]Lead Contact
*Correspondence: myles_brown@dfci.harvard.edu
http://dx.doi.org/10.1016/j.celrep.2017.02.025

SUMMARY

While the multiple endocrine neoplasia type 1 (*MEN1*) gene functions as a tumor suppressor in a variety of cancer types, we explored its oncogenic role in breast tumorigenesis. The *MEN1* gene product menin is involved in H3K4 trimethylation and co-activates transcription. We integrated ChIP-seq and RNA-seq data to identify menin target genes. Our analysis revealed that menin-dependent target gene promoters display looping to distal enhancers that are bound by menin, FOXA1 and GATA3. In this fashion, *MEN1* co-regulates a proliferative breast cancer-specific gene expression program in ER+ cells. In primary mammary cells, *MEN1* exerts an anti-proliferative function by regulating a distinct expression signature. Our findings clarify the cell-type-specific functions of *MEN1* and inform the development of menin-directed treatments for breast cancer.

INTRODUCTION

Multiple endocrine neoplasia type 1 (MEN1) is caused by inactivating germline mutations of the *MEN1* gene and is predominantly characterized by parathyroid adenomas, pituitary adenomas, and pancreatic and duodenal neuroendocrine tumors (Chandrasekharappa et al., 1997; Thakker et al., 2012). Previous studies have suggested however that the *MEN1* gene has a dual role in breast tumorigenesis. Female MEN1 patients are at increased risk for developing breast cancer suggesting a tumor suppressive role. Consistent with this, breast tumors in MEN1 patients show complete loss of the *MEN1* gene (Dreijerink et al., 2014). Moreover, genetic loss of function MEN1 mouse models show

increased incidence of both in situ and invasive mammary cancer (Seigne et al., 2013). In contrast, in sporadic breast cancers the *MEN1* gene appears to exert a proliferative function. *MEN1* gene mutations are uncommon and expression of the *MEN1* gene product menin has been reported to be involved in resistance to endocrine therapy (Imachi et al., 2010; TCGA, 2012). Menin is able to interact with and co-activate the estrogen receptor alpha (ERα), a critical driver in approximately 70% of sporadic breast cancer cases (Dreijerink et al., 2006; Imachi et al., 2010). A similar proliferative function of menin has recently been shown in sporadic androgen receptor (AR)-expressing prostate cancer (Malik et al., 2015).

Menin is a ubiquitously expressed nuclear protein that has no intrinsic enzymatic activity. Over the years, many menin-interacting proteins have been reported. Most of the interacting proteins indicate a role for menin in transcriptional regulation, either as a co-activator or a co-repressor (Matkar et al., 2013). Menin was found to be an integral part of mixed-lineage leukemia (MLL)1/MLL2 (lysine methyltransferase [KMT2A/B]) containing protein complexes that have methyltransferase activity directed at trimethylation of lysine 4 of Histone H3 (H3K4me3) (Huang et al., 2012; Hughes et al., 2004; Yokoyama et al., 2004).

Aberrant H3K4me3 is considered to contribute to MEN1 tumorigenesis as simultaneous knockout of the H3K4me3 demethylase Rbp2/Kdm5a resulted in longer survival in a MEN1 mouse model in which mice develop insulinomas (Lin et al., 2011). H3K4me3 is an epigenetic mark of active transcription and is localized primarily to transcription start sites (TSSs) (Santos-Rosa et al., 2002). Menin has also been found to be predominantly present at TSS (Agarwal et al., 2007; Cheng et al., 2014; Scacheri et al., 2006). Reports addressing the genome-wide function of menin have yielded cell-specific results in terms of regulation of H3K4me3 and target gene expression (Agarwal and Jothi, 2012; Li et al., 2013; Lin et al., 2011, 2015).

A similar dual role in oncogenesis has been reported for other epigenetic regulators, such as the enhancer of zeste homolog protein 2 (EZH2 [KMT6]). EZH2 is the catalytic subunit of the polycomb repressive complex 2 that methylates H3K27 (Cao et al., 2002): overexpression of EZH2 has been observed in breast and prostate cancer (Xu et al., 2012). Gain-of-function mutations are present in lymphomas. In contrast, loss-of-function mutations are found in myelodysplastic syndrome and leukemia (reviewed in Lund et al., 2014). These dualities likely reflect differential epigenetic regulation of predefined cell-type-specific transcriptional programs.

In this study, by integrating chromatin immunoprecipitation combined with next-generation sequencing (ChIP-seq) and RNA sequencing (RNA-seq), we aimed to investigate the genome-wide function of menin in breast cancer. In addition, we combined our data with publicly available ChIP-seq and chromatin interaction datasets. We show that menin-H3K4me3 target gene preference is associated with the presence of menin at enhancer sites that are found to be involved in looping with their target gene TSS. In this fashion, menin controls a highly luminal breast cancer-specific proliferative gene expression program in breast cancer cells. In contrast, in primary luminal progenitor (LP) cells, menin regulates a different gene signature that is in line with its role as a tumor suppressor.

Our results clarify the proliferative role of the *MEN1* gene in sporadic ER$^+$ breast cancer and provide a potential explanation for the cell-type-specific actions of menin.

RESULTS

Menin Has a Critical Proliferative Role in ERα-Expressing Cell Lines

We chose the MCF-7 breast cancer cell line to study the role of menin in transcriptional regulation. Menin has been shown to co-activate ERα in a ligand-dependent fashion at the canonical ERα target gene *TFF1* in these cells (Dreijerink et al., 2006). MCF-7 cell lines were established stably expressing doxycycline(dox)-inducible small hairpin RNA (shRNA) targeting *MEN1* or a scrambled shRNA construct (Figure 1A). After synchronization in phenol red-free medium containing 10% charcoal dextran-treated fetal bovine serum (charcoal dextran-treated fetal bovine serum [CDT] medium), cells were treated with either estradiol (E2) or epidermal growth factor (EGF) for 96 hr. Both in the E2 and EGF-stimulated cells, growth was severely reduced after *MEN1* gene silencing (Figure 1B). To assess whether this effect could be extrapolated to other cell lines, we studied the ERα positive (ER$^+$) breast cancer cell line MDA-MB-361 and the *ESR1*-expressing αT3 and GH3 pituitary cell lines. In these cell lines, E2-stimulated growth was likewise attenuated after silencing of *MEN1* (Figure 1C).

Fluorescence activated cell sorting (FACS) showed that in the absence of E2, equal numbers of MCF-7 cells are in the G1 phase of the cell cycle both in control cells and *MEN1* silenced cells. After 4 days of treatment with E2, fewer control cells were in G0/G1 compared with shMEN1 cells (Figure 1D). These observations support an essential role for the *MEN1* gene in the ability of E2 to promote cell-cycle progression in ER$^+$ breast cancer cells.

In addition, we assessed the proliferative role of the *MEN1* gene in ER$^+$ breast cancer cells using data from recently performed genome-scale CRISPR knockout (GECKO) screens in MCF-7 cells and also the ER$^+$ T47D breast cancer cell line. In both cell lines, the *MEN1* gene was found to be a high-ranking proliferative gene (M.B., unpublished data).

The Menin Histone Methyltransferase Complex Is Associated with Active Gene Promoters in ER$^+$ Breast Cancer Cells

In order to define the presence of the menin-H3K4me3 methyltransferase complex and its relevance for gene expression in ER$^+$ breast cancer cells, we performed ChIP-seq for menin, MLL1, MLL2, and H3K4me3 and RNA-seq in cells cultured under hormone-free conditions.

Consistent with the published literature, we found menin, MLL1 and MLL2 to be present predominantly at TSS (TSS: defined as the TSS ±5 kb) (menin 83% of peaks; MLL1 82%; MLL2 92%; Figure 2A). The distribution of MLL1, MLL2 and H3K4me3 peaks clustered with the menin-bound TSS (Figure 2B). Transcripts that have menin present at their TSSs were found to be expressed at significantly higher levels compared with transcripts from TSSs that lacked menin binding (Figure 2C). We expanded the menin ChIP-seq analysis to the T47D ER$^+$ and MCF-10A ER$^-$ cell lines. We found that menin-promoter binding was comparable in MCF-7 and T47D cells. However, menin was not found to be present at these ER$^+$-specific TSSs in the MCF-10A cell line (Figure 2D). Thus, in ER$^+$ breast cancer cells, the menin cistrome appears to be localized primarily at a set of transcriptionally active TSSs that are not bound by menin in ER$^-$ breast cancer cells, suggesting distinct roles for menin in ER$^+$ and ER$^-$ breast cancer.

MEN1-Dependent H3K4me3 and Transcription Is Restricted to a Subset of Genes

Global H3K4me3 was assessed by immunoblotting of histone extracts and found not to be affected by *MEN1* silencing, as previously reported (Figure 3A) (Dreijerink et al., 2006). We performed ChIP-seq of H3K4me3 in shCtrl cells versus the shMEN1 cells that showed the most thorough silencing in Figure 1A. There were no major differences in the numbers (20,083 in shCtrl versus 20,066 in shMEN1) or locations of H3K4me3 peaks (Figure 3B). Comparison of peak heights after *MEN1* silencing showed a modest decrease of the average H3K4me3 ChIP-seq signal at menin-bound TSS (Figure 3C). Reduced *MEN1* expression did not affect global mRNA levels (Figure 3D). Assessment of H3K4me3 peaks at individual menin-bound sites identified a limited number of TSSs that showed differential H3K4me3 (Figure 3E). Although global gene expression was not affected after silencing of *MEN1*, a number of genes were expressed at lower levels following *MEN1* silencing (Figures 3F and S1A). Thus, although menin is present at the TSSs of many active genes, it regulates the level of H3K4me3 and subsequent transcription at a limited number of genes.

Menin inhibitors that block the interaction of menin and MLL1 are being developed for the treatment of MLL-driven acute myeloid leukemia (Grembecka et al., 2012). In order to verify the changes in gene expression regulated by *MEN1*, we compared
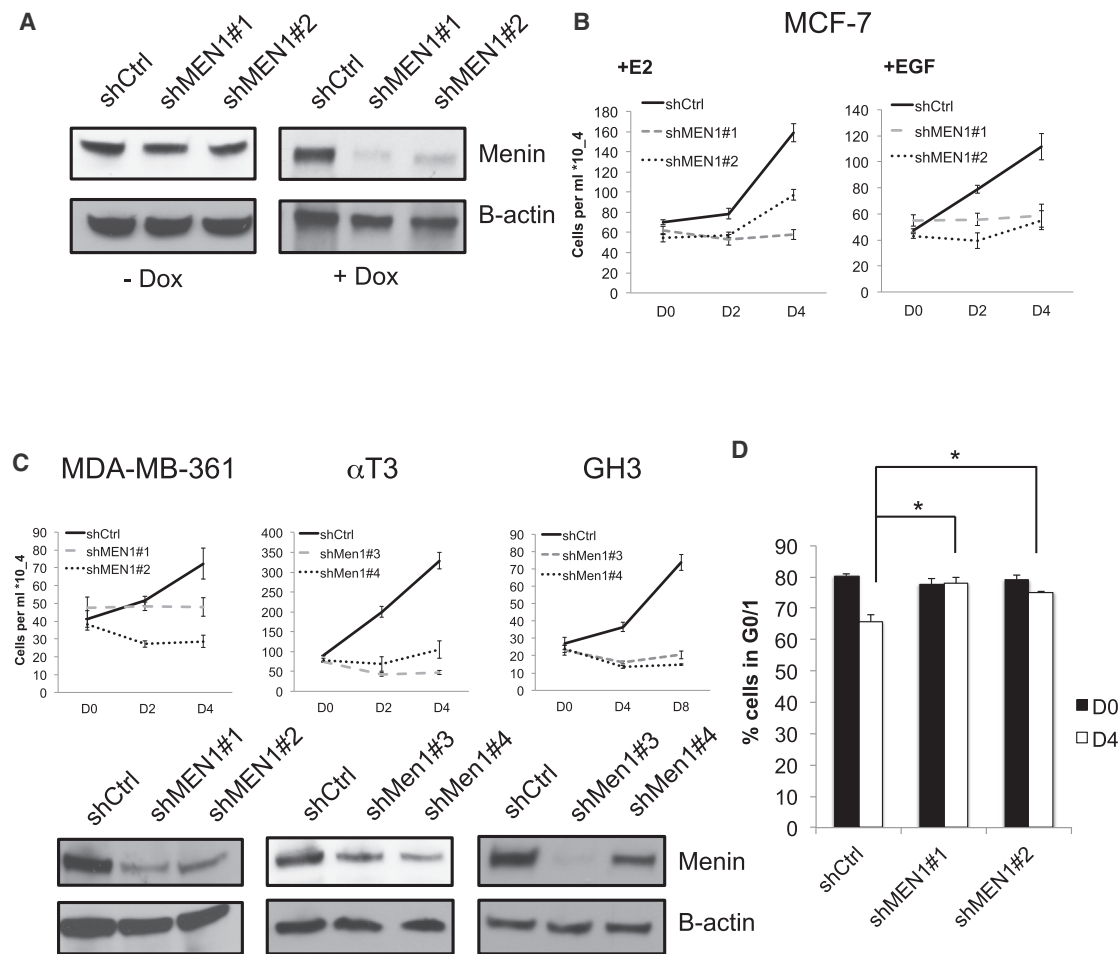
Figure 1. *MEN1* **Silencing Disrupts Proliferation of ERα-Expressing Cell Lines**

(A) Total cell lysate immunoblot of menin after doxycycline-inducible sh-mediated *MEN1* silencing in MCF-7 cells. β-actin was used as a loading control.

(B) Growth curves of synchronized MCF-7 cells induced with E2 or EGF-expressing shCtrl or shMEN1 shRNAs.

(C) Growth curves and immunoblots of ERα-expressing cell lines that were stably infected with inducible shRNAs and treated with E2.

(D) Propidium iodine staining of sh cells before and after 4 days of treatment with E2, after the induction of shRNAs. Percentages of MCF-7 cells in G0/1 phase are shown. Data are represented as mean ± SEM (*p < 0.05 in an unpaired two-tailed Student's t test).

gene expression by RNA-seq following *MEN1* silencing with gene expression in cells treated with the menin inhibitor MI-2. We found a set of 57 genes downregulated under both conditions (Figures 3G and 3H). Gene ontology (GO) analysis revealed that this gene list was most significantly enriched for oncogenic genes involved in estrogen and steroid hormone response such as the ERα gene (*ESR1*), carbonic anhydrase (*CA2*), neuropeptide y receptor Y1 (*NPY1R*), and the sonic hedgehog homolog (*SHH*) genes (Figure S1B).

Integrative Genomics in ER[+] Breast Cancer Cells Identifies *ESR1* as a Major Menin-H3K4me3 Target Gene

To identify the direct menin-H3K4me3 target genes responsible for the *MEN1*-dependent growth differences observed in breast cancer cells, the results of RNA-seq following *MEN1* silencing were integrated with the menin and H3K4me3 ChIP-seq data (Figure 4A). The 6,403 TSSs with menin ChIP-seq peaks were

filtered for those showing a significant reduction in H3K4me3 (>0.5 log2 fold down in Figure 3E) following *MEN1* silencing resulting in 104 potential target genes. When these genes were further refined for those also showing a significant decrease in mRNA levels (>0.5 log2 fold down, p < 0.05) four differentially expressed menin-H3K4me3 target genes were identified: *ESR1*, *AGR3*, *AGR2*, and *VAV3* (Figure 4A). These genes have been implicated in breast tumorigenesis and are typically expressed in ER[+] breast cancer (Figure 4B) (Curtis et al., 2012).

A detailed examination of the *ESR1* gene locus revealed that menin is present at multiple sites at the *ESR1* gene locus, along with MLL1 and MLL2 (Figure 4C). Following *MEN1* silencing, H3K4me3 is reduced, especially at an upstream site (labeled 1, Figures 4C and 4D) and at the TSS of the transcription variant 4 (labeled 2, Figures 4C and 4D). By immunoblotting, we could demonstrate that at the protein level total ERα was reduced after *MEN1* silencing (Figure 4E). ERα was also reduced by *MEN1*
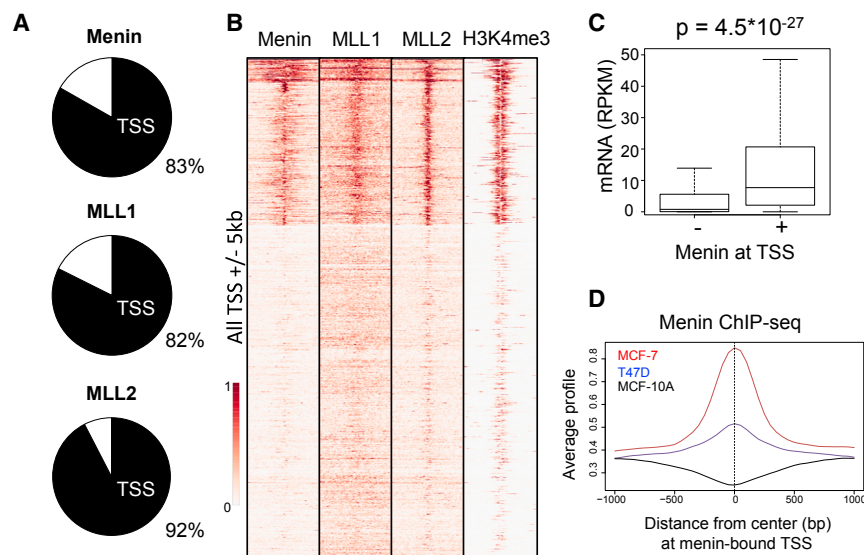
A Menin

B Menin | MLL1 | MLL2 | H3K4me3

83%

MLL1

82%

MLL2

92%

All TSS +/- 5kb

1

0

C

p = 4.5*10^{-27}

mRNA (RPKM)

50
40
30
20
10
0

Menin at TSS
−   +

D

Menin ChIP-seq

Average profile

0.8
0.7
0.6
0.5
0.4
0.3

MCF-7
T47D
MCF-10A

−1000   −500   0   500   1000

Distance from center (bp)
at menin-bound TSS

Figure 2. Menin Is Predominantly Present at Actively Transcribed TSS

(A) Distribution of menin, MLL1 and MLL2 peaks at transcription start sites (TSS; black; percentages at TSS indicated) and outside TSS (white).

(B) K-means clustering, based on menin binding intensity, of menin, MLL1, MLL2, and H3K4me3 signals.

(C) Boxplot of average expression of transcripts at TSS that do not have menin bound versus menin-bound sites, measured by RNA-seq. The plot represents averages of RNA-seq duplicates in quartiles analyzed using a paired two-tailed Student's t test.

(D) Average menin ChIP-seq signals centered around the menin-bound TSS in MCF-7 cells, in MCF-7 and T47D ER$^+$ cells and the MCF-10A ER$^−$ cell line.

silencing in the other cell lines that exhibited similar E2-dependent growth differences (Figure S2). Inducible re-expression of an shMEN1#1-resistant *MEN1* cDNA interfered with downregulation of ERα and could also rescue the attenuated growth of the shMEN1#1 cells, supporting the specificity of the observed effects (Figure 4E). Several *ESR1* mRNA transcripts were also expressed at reduced levels after MI-2 treatment (Figure 3H). MI-2 treatment caused downregulation of ERα protein levels (Figure 4F). The results substantiate the finding that menin directly regulates *ESR1* gene expression.
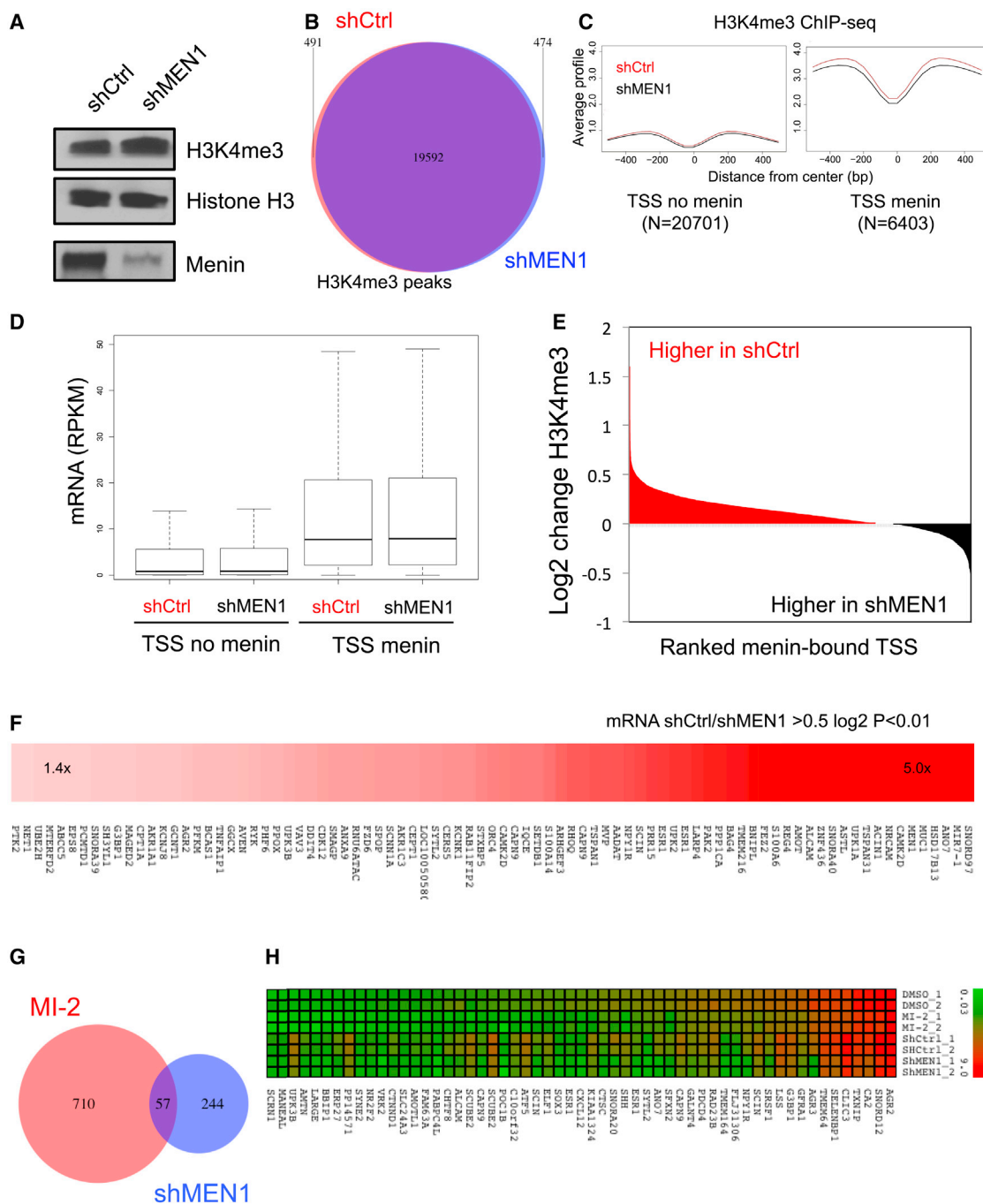
Mammary cells from MEN1 patients lack one functional *MEN1* allele from the initiation of cell differentiation. Therefore, in order to mimic the loss of *MEN1* in MEN1 patients, we studied the effect of *MEN1* silencing in primary LP cells derived from healthy individuals undergoing reduction mammoplasty. LP cells represent a differentiation stage between the mammary stem cell and differentiated mature luminal cells and express ERα only at very low levels. Differential gene expression was studied in FACS-sorted CD49f$^+$, EpCAM$^+$ mammary LP cells (Lim et al., 2009). After harvesting and cell sorting, cells were treated with lentiviral particles containing sh constructs directed against *MEN1* or a control sequence and subjected to puromycin selection. Treatment of the cells with shMEN1 viruses resulted in lower menin protein levels (Figure 4G). Total RNA from these cells was subjected to gene expression analysis and clusters of up and down-regulated genes were identified. GO and gene set enrichment analysis were performed on the differentially expressed genes. The most significantly enriched expression profiles included genes that were upregulated after *MEN1* silencing, in particular, genes that are involved in extracellular matrix formation such as the matrix metalloproteinase genes 9 (*MMP9*) and also *MMP3* (Figure S1C). Accordingly, in addition to the extracellular matrix GO term, gene set enrichment analysis also gave a significant match with an invasive breast cancer signature (Figure 4H) (Schuetz et al., 2006). There was no significant overlap of the differentially regulated genes after *MEN1* silencing in the

MCF-7 breast cancer cells compared with the LP cells (Figure 4I). The expression of the *ESR1* gene, which is transcribed at low levels in LP cells, was not affected by *MEN1* silencing. However, in the ZR-75-1 ER$^+$ cell line that lacks menin protein expression, reintroduction of a *MEN1* cDNA construct resulted in increased *ESR1* mRNA levels, further supporting the relevance of this mechanism in ER$^+$ cells (Figure 4J) (Barretina et al., 2012). These results indicate that *MEN1* suppresses the expression of genes associated with invasion in normal LP cells consistent with its tumor suppressive properties while it stimulates the expression of growth promoting genes in ER$^+$ breast cancer cells consistent with an oncogenic activity.

Estradiol Treatment of ER$^+$ Breast Cancer Cells Leads to Recruitment of Menin to ERα-Bound Enhancer Sites

Using candidate gene approaches, we have previously shown that menin can be recruited to nuclear receptor binding sites after ligand treatment (Dreijerink et al., 2006, 2009). We used E2 stimulation of MCF-7 cells to study genome-wide menin dynamics. Incubation of shCtrl cells with 10 nM E2 for 45 min resulted in a shift of the menin cistrome (Figure 5A). Motif analysis at 1,464 menin ChIP peaks gained after E2 treatment showed enrichment for the ERα binding motif (Figure 5B). ERα typically binds to enhancer regions and although the most common sites of menin binding after E2 stimulation were at TSS, overlapping the gained menin peaks with known ERα binding sites indeed showed recruitment to non-TSS sites (only 14% of menin and ERα−bound sites after E2 treatment are at TSS) (Figures 5C and 5D) (Carroll et al., 2006). Recruitment of menin to the regulatory sites of the canonical ERα target gene *TFF1* has been shown using ChIP-PCR previously (Dreijerink et al., 2006). In the ChIP-seq experiment shown, stimulation of the cells with E2 demonstrated recruitment of menin to the *TFF1* TSS and a *TFF1* upstream enhancer site (Figure 5E) (Kong et al., 2011).

These changes in the menin cistrome following E2 treatment suggest that recruitment to enhancer sites plays a role in menin's co-activator function.
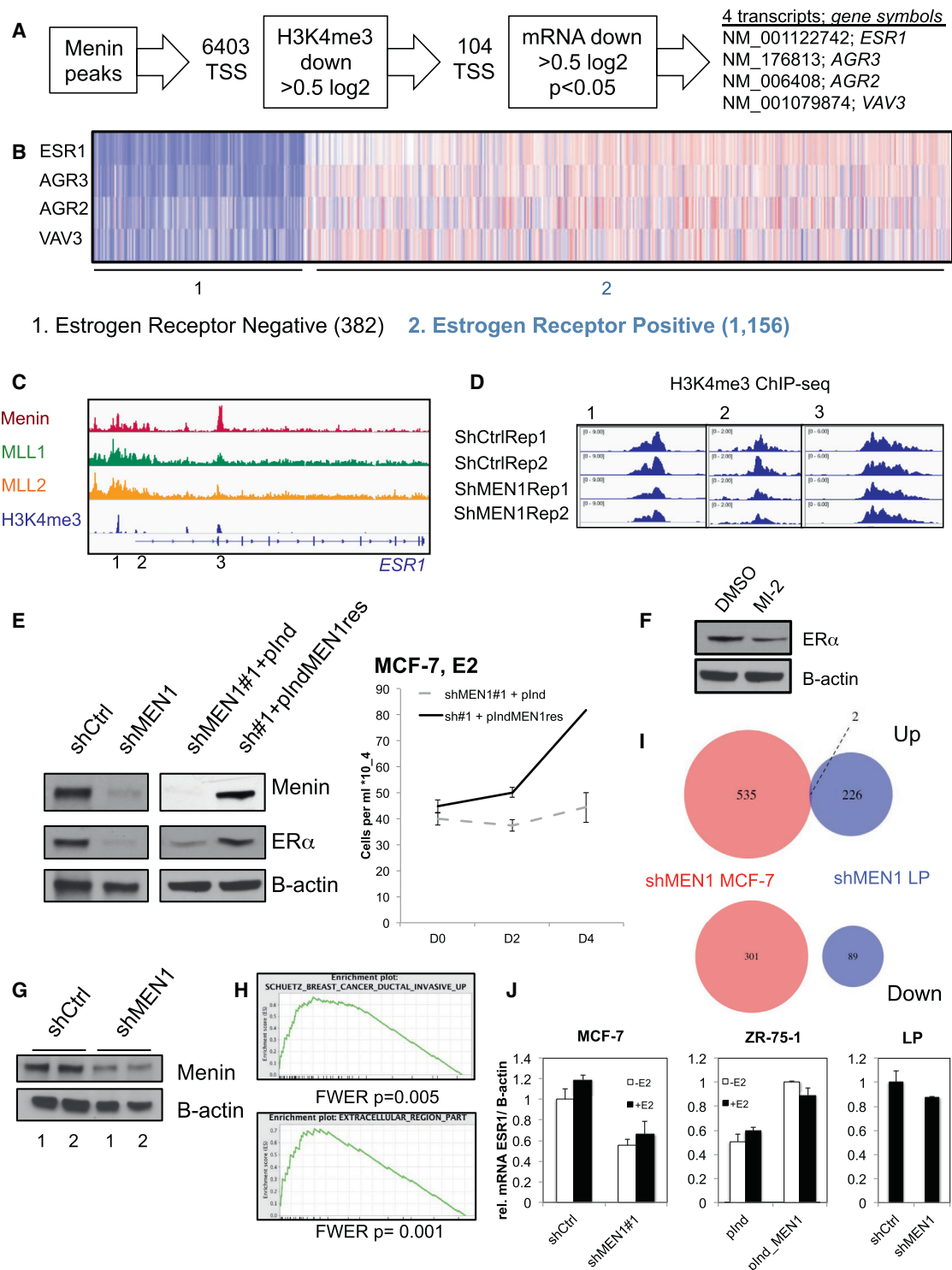
Figure 3. *MEN1*-Dependent H3K4me3 and Differential Expression at Selected Target Genes

(A) Immunoblot of H3K4me3 in histone extracts from shCtrl and shMEN1#1 cells. Histone H3 protein levels were used as an internal control. Silencing of *MEN1* in the corresponding total cell lysate is also shown.

(B) Venn diagram of H3K4me3 ChIP-seq peaks after shCtrl and shMEN1 in vehicle-treated cells.

(C) Average H3K4me3 ChIP-seq signals at TSS without menin and with menin peaks after shCtrl or shMEN1 induction.

(D) Gene expression at TSS without menin and bound by menin after *MEN1* silencing (no significant differences between shCtrl and shMEN1).

(E) Waterfall plot of H3K4me3 ChIP-seq fold changes after *MEN1* silencing at menin-bound TSS. TSSs that have higher H3K4me3 levels in shCtrl cells are potential menin-H3K4me3-dependent genes.

(F) Heatmap of average fold change of significantly downregulated transcripts after *MEN1* silencing (Limma, < 0.5 log2 fold down, p < 0.01, RPKM > 1).

(G) Overlap of downregulated transcripts (Limma, < −0.5 log2, p < 0.05) after treatment with the menin-MLL1 inhibitor MI-2 or shMEN1#1.

(H) Heatmap showing transcripts of 57 genes that are significantly downregulated by both MI-2 and shMEN1#1 (experiments in duplicate).

Figure 4. Integrative Strategy to Identify Menin-H3K4me3 Target Genes

(A) By focusing on TSS that have menin peaks and both downregulated H3K4me3 and gene expression after *MEN1* silencing four target genes were identified.

(B) The top menin-H3K4me3 target genes are highly expressed in ER positive versus ER negative breast cancer. Colors indicate log2 median-centered intensity: blue, least expressed; red, most expressed (https://www.oncomine.org/resource/login.html).

(C) Menin, MLL1, MLL2, and H3K4me3 ChIP-seq tracks at the *ESR1* locus: (1) upstream enhancer, (2) transcript variant four TSSs, (3) transcript variant one to three TSSs, first ATG.
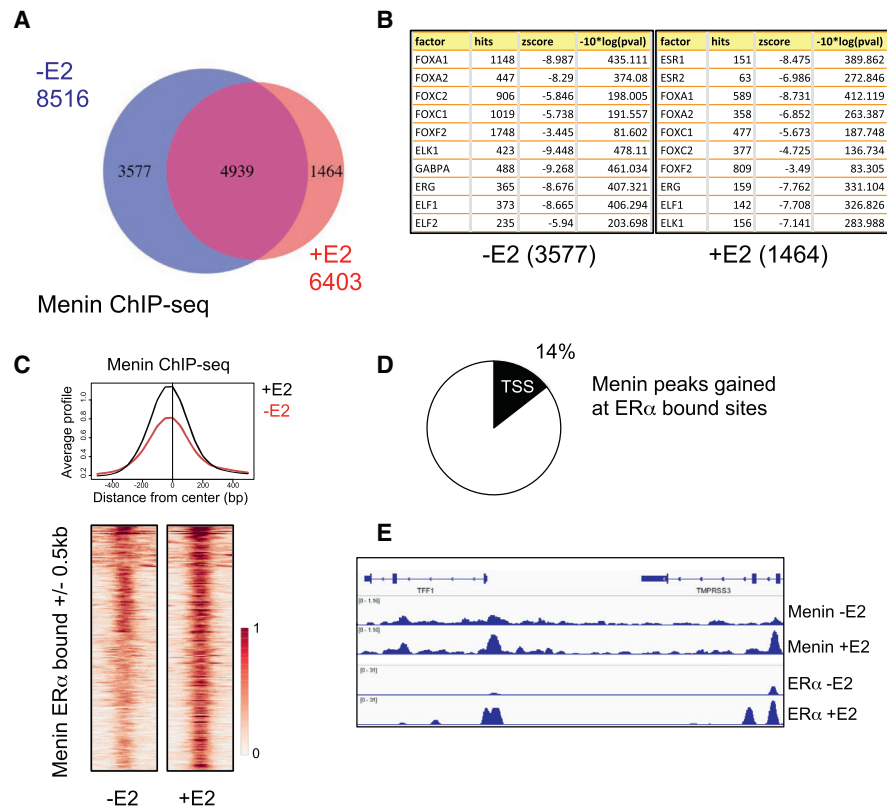
(legend continued on next page)

A

-E2
8516

3577 4939 1464

+E2
6403

Menin ChIP-seq

B

factor	hits	zscore	-10*log(pval)	factor	hits	zscore	-10*log(pval)
FOXA1	1148	-8.987	435.111	ESR1	151	-8.475	389.862
FOXA2	447	-8.29	374.08	ESR2	63	-6.986	272.846
FOXC2	906	-5.846	198.005	FOXA1	589	-8.731	412.119
FOXC1	1019	-5.738	191.557	FOXA2	358	-6.852	263.387
FOXF2	1748	-3.445	81.602	FOXC1	477	-5.673	187.748
ELK1	423	-9.448	478.11	FOXC2	377	-4.725	136.734
GABPA	488	-9.268	461.034	FOXF2	809	-3.49	83.305
ERG	365	-8.676	407.321	ERG	159	-7.762	331.104
ELF1	373	-8.665	406.294	ELF1	142	-7.708	326.826
ELF2	235	-5.94	203.698	ELK1	156	-7.141	283.988

-E2 (3577)   +E2 (1464)

C

Menin ChIP-seq

+E2
-E2

Average profile

Distance from center (bp)

Menin ERα bound +/- 0.5kb

-E2   +E2

D

14%

TSS

Menin peaks gained
at ERα bound sites

E

TFF1   TMPRSS3

Menin -E2
Menin +E2
ERα -E2
ERα +E2

Figure 5. E2 Treatment Leads to Recruitment of Menin to ERα-Bound Enhancer Sites
(A) Venn diagram showing change of the menin cistrome after E2 stimulation versus vehicle.
(B) Ranked list of clustered motifs at the menin sites lost and gained after treatment with E2.
(C) Menin ChIP-seq peaks gained after E2 treatment at known ER binding sites, shown as average signals versus peaks lost or as locus-specific signals in a heatmap.
(D) Menin peaks that are gained during treatment that overlap with known ERα binding sites are mostly located at enhancer sites (white) or TSS (black; percentage indicated).
(E) Menin and ERα tracks at the *TFF1* locus in MCF-7 cells treated with vehicle or E2.

MEN1-Dependent Target Genes Are Characterized by the Presence of Menin at Enhancers that Are Bound by FOXA1 and GATA3 and Display Looping

We revisited the menin ChIP-seq results at the *MEN1*-regulated genes *ESR1*, *AGR3*, *AGR2*, and *VAV3* in order to further identify determinants of differentially expressed menin-H3K4me3 target genes. At these loci, we found that in addition to the TSS, menin was also present at non-TSS sites, as it was upstream of the *ESR1* TSS (Figure 4C). In the menin ChIP-seq experiment, 1,428 peaks were called outside TSS ±5-kb regions. The average ampli-

tude of these non-TSS or enhancer menin peaks was similar to menin peak heights at TSS (Figure 6A). Clustered motif analysis of the 1,428 menin enhancer peaks showed enrichment of forkhead and GATA transcription factor binding sites (Figure 6B). In order to assess true enhancer TSS interactions, we intersected the menin ChIP-seq data with available looping data from ChIA-PET experiments at CTCF and RNA polII-bound loci in MCF-7 cells (ENCODE Project Consortium, 2012; Li et al., 2012).

Three of the top candidate menin-H3K4me3 target genes showed presence of menin at sites that could also be bound by

(D) Differential H3K4me3 at the *ESR1* locus after *MEN1* silencing. Tracks as indicated in (C) (duplicate ChIP-seq experiment).
(E) Immunoblot analysis of ERα after shMEN1 induction and after rescue of *MEN1* expression. β-actin was used a loading control. Growth analysis after rescue of *MEN1* expression in shMEN1#1 cells. Data are represented as mean ± SEM.
(F) Immunoblot of total MCF-7 cell lysates with an ERα antibody after treatment with vehicle or 1 mM MI-2.
(G) Menin immunoblot after induction of shMEN1 versus shCtrl-treated LP cells (duplicate experiment).
(H) Gene set enrichment analysis of upregulated genes in LP cells after *MEN1* depletion. FWER, family wise error rate.
(I) Overlap of significantly up- or downregulated genes after shMEN1 treatment in MCF-7 cells versus LP cells.
(J) RT-PCR analysis of *ESR1/ACTB* gene expression in MCF-7 cells after induction of shCtrl or shMEN1#1 in the presence of vehicle or 10 nM estradiol, in ZR-75-1 menin-negative cells either expressing a control construct or a wild-type (WT) *MEN1* cDNA and in LP cells after treatment with shCtrl or shMEN1 viral particles (experiments in triplicate, represented as mean ± SEM).
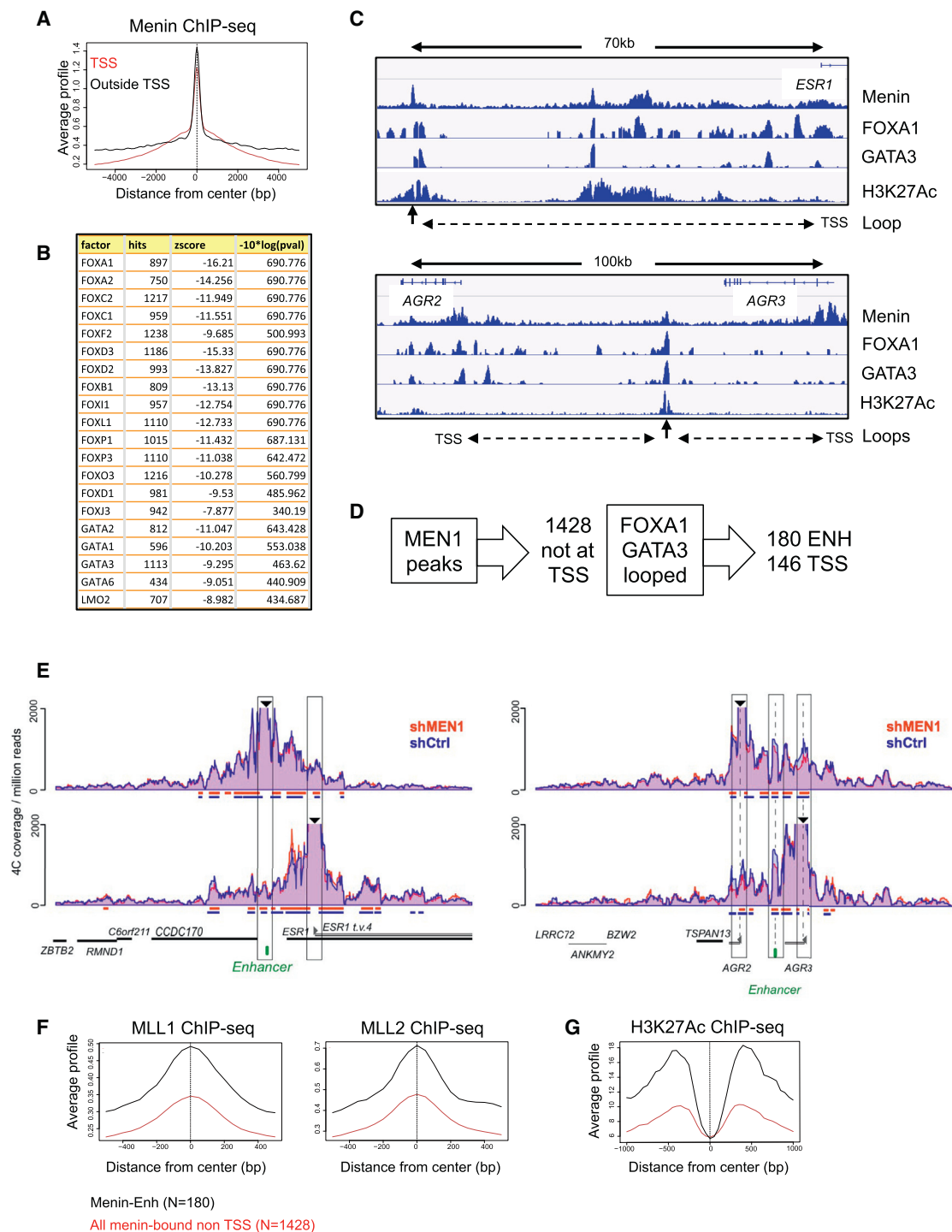
A Menin ChIP-seq

B

factor	hits	zscore	-10*log(pval)
FOXA1	897	-16.21	690.776
FOXA2	750	-14.256	690.776
FOXC2	1217	-11.949	690.776
FOXC1	959	-11.551	690.776
FOXF2	1238	-9.685	500.993
FOXD3	1186	-15.33	690.776
FOXD2	993	-13.827	690.776
FOXB1	809	-13.13	690.776
FOXI1	957	-12.754	690.776
FOXL1	1110	-12.733	690.776
FOXP1	1015	-11.432	687.131
FOXP3	1110	-11.038	642.472
FOXO3	1216	-10.278	560.799
FOXD1	981	-9.53	485.962
FOXJ3	942	-7.877	340.19
GATA2	812	-11.047	643.428
GATA1	596	-10.203	553.038
GATA3	1113	-9.295	463.62
GATA6	434	-9.051	440.909
LMO2	707	-8.982	434.687

C

D

MEN1 peaks → 1428 not at TSS → FOXA1 GATA3 looped → 180 ENH 146 TSS

E

F MLL1 ChIP-seq   MLL2 ChIP-seq   **G** H3K27Ac ChIP-seq

Menin-Enh (N=180)
All menin-bound non TSS (N=1428)

Figure 6. Looped Enhancers Containing Menin, FOXA1, and GATA3 Mark Menin-H3K4me3 Target Genes
(A) Menin ChIP-seq peaks outside TSS are of equal height compared with menin peaks at TSS.
(B) Ranked list of clustered motifs at 1,428 non-TSS menin peaks.
(C) The *ESR1* and *AGR* gene loci that are regulated by menin have menin, FOXA1, and GATA3 at distal sites (indicated by arrows) that carry the H3K27Ac mark and can be looped to their TSS (indicated by dotted lines).
(D) Overlapping of menin peaks outside TSS with FOXA1 and GATA3 peaks and looping data resulted in the identification of 180 potential enhancer loci linked to 146 TSS.

(legend continued on next page)

FOXA1 and GATA3 and displayed looping by ChIA-PET (Figure 6C) (Hua et al., 2009; Schmidt et al., 2010). By overlapping menin peaks outside TSS regions with publicly available FOXA1 and GATA3 ChIP-seq data and ChIA-PET looping data, 180 potential enhancers could be identified that are linked to 146 unique TSSs (Figure 6D). GO analysis revealed that among the 146 transcripts from these TSSs there is a significant enrichment for genes encoding transcriptional regulatory proteins (Figure S1D).

The loops at the *ESR1* and *AGR* gene loci identified by ChIA-PET could be confirmed by 4C-seq chromatin conformation capture (Figures 6E and S3). The looped enhancers that are bound by menin, FOXA1, and GATA3 are also bound by MLL1 and MLL2 as indicated by an enrichment of MLL1 and MLL2 ChIP-seq signals at these sites compared with all menin peaks outside TSS (Figure 6F). The 180 menin/FOXA1/GATA3 sites that are associated with gene looping show clear enrichment of the canonical enhancer histone mark H3K27Ac, supporting that these are bona fide enhancer sites (Theodorou et al., 2013) (Figure 6G).

MEN1-Dependent H3K4me3 and Expression of Regulated Genes Is Associated with FOXA1 Enhancer Binding

We assessed the effect of *MEN1* silencing on H3K4me3 and gene expression of the 146 enhancer-linked TSSs. Average H3K4me3 at the 146 TSSs was clearly reduced in the shMEN1-expressing cells (Figure 7A). By integrating the 146 menin/FOXA1/GATA3 looping TSSs with the *MEN1*-dependent H3K4me3 ChIP-seq data, we could identify six transcripts from this group that showed >0.50 log2 fold *MEN1*-dependent H3K4me3: in addition to the *ESR1*, *AGR2*, and *AGR3* transcripts, we also identified the *GREB1* and *NR2F2* TSSs as direct menin-H3K4me3 targets. Out of the nine TSSs showing the largest overall H3K4me3 decrease (>0.75 log2 fold) after *MEN1* silencing four TSSs displayed looping to menin/FOXA1/GATA3 sites (Figure 7B). This represents a significant enrichment of menin/FOXA1/GATA3 enhancer-linked TSSs versus all other menin-bound TSSs (p < 0.0001 using a Fisher's exact test). To verify whether looping at the *ESR1* and *AGR* loci was affected by *MEN1* silencing, we compared the 4C-seq signals after induction of shCtrl or shMEN1 at the *ESR1* and *AGR* loci and found that looping at these sites is not *MEN1*-dependent (Figure 6E).

Although *MEN1* silencing did lead to lower average H3K4me3 at the 146 TSSs, average mRNA levels were not affected (Figure 7C, left panel). However, the six TSSs that had the most substantial *MEN1*-dependent H3K4me3 did show significantly reduced mRNA levels after *MEN1* depletion (Figure 7C, right panel).

To further investigate the mechanism of menin-H3K4me3 target gene regulation and the role of FOXA1, we interrogated gene expression data after siRNA-mediated knockdown of *FOXA1* in MCF-7 cells and compared the results with our RNA-seq data (Hurtado et al., 2011). We found a common subset of genes showing reduced expression both after *MEN1* and *FOXA1* silencing, which includes the *ESR1*, *AGR2*, and *AGR3* genes (Figure S4). In addition, we performed FOXA1 ChIP-seq in shCtrl and shMEN1-expressing MCF-7 cells. *FOXA1* mRNA levels were not affected by *MEN1* silencing. We found that FOXA1 presence at the menin-bound enhancer sites was severely impaired after menin depletion, suggesting that FOXA1 DNA-binding is connected to menin's function in MCF-7 cells (Figure 7D).

Thus, the combination of the presence of menin, FOXA1, and GATA3 at non-TSS sites that display looping marks genes that are specifically co-activated by menin as part of a H3K4 methyltransferase complex (Figure 7E).

DISCUSSION

The *MEN1* gene has a dual role in breast cancer. In the normal mammary epithelium it exerts an anti-proliferative and tumor suppressive role consistent with the increased risk of breast cancer in women with the MEN1 syndrome. In contrast, in sporadic ER[+] breast cancer *MEN1* is oncogenic and promotes proliferation. This study provides important insights into this duality. The proliferative role of *MEN1* in sporadic breast cancer is consistent with the function of menin in ER[+] breast cancer cells both as a regulator of *ESR1* gene expression and as a co-activator of ERα. *MEN1* silencing also inhibited EGF-dependent growth, which has previously been demonstrated to rely on the presence of ERα (Lupien et al., 2010). In addition, the *AGR2*, *AGR3*, and *VAV3* genes that were identified as menin-H3K4me3 targets are also involved in breast tumorigenesis. The anterior grade homology 2 protein (*AGR2*) gene was reported in a regulatory pathway with ERα and FOXA1 and has been associated with metastatic breast cancer (Liu et al., 2005; Wright et al., 2014). *AGR3* may serve as a biomarker in luminal breast cancer (Garczyk et al., 2015). The guanidine nucleotide exchange factor gene *VAV3* was recently found to be involved in resistance to endocrine therapy in breast cancer (Aguilar et al., 2014). In addition to *ESR1*, *AGR2*, and *AGR3*, the *GREB1* and *NR2F2* genes were identified as menin/FOXA1/GATA3 enhancer linked target genes. Interestingly, the *GREB1* gene has recently been reported as an ERα interacting transcriptional co-activator (Mohammed et al., 2013). The *NR2F2* gene encodes the COUP-TFII nuclear receptor that has been shown to have a role in breast cancer progression as well (Zhang et al., 2014).

In contrast to the findings in sporadic breast cancer, female MEN1 patients have an increased risk to develop mostly luminal type breast cancer. Most breast tumors in MEN1 patients express ERα or the progesterone receptor (Dreijerink et al., 2014). We analyzed gene expression after *MEN1* silencing in

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(E) Chromatin conformation capture analysis using 4C-seq at the *ESR1* (left) and *AGR* (right) gene loci, in MCF-7 cells after induction of shCtrl (blue lining) or shMEN1#1 (red lining). Top left: the viewpoint from the *ESR1* enhancer sequence identifies a peak at the *ESR1* TV4 TSS. Bottom left: the reverse experiment using the TSS as the viewpoint. Top right: using the *AGR2* TSS as the viewpoint, peaks can be identified both at the enhancer and *AGR3* TSS. Conversely, the *AGR2* TSS and the enhancer are found using the *AGR3* TSS as the starting point (bottom right) (see also Figure S3).
(F) Average MLL1 and MLL2 ChIP-seq presence at menin/FOXA1/GATA3 enhancers (menin-Enh) versus all menin-bound non-TSS sites.
(G) Average H3K27Ac ChIP-seq signals at menin/FOXA1/GATA3 enhancers (menin-Enh) versus all menin-bound non-TSS sites.
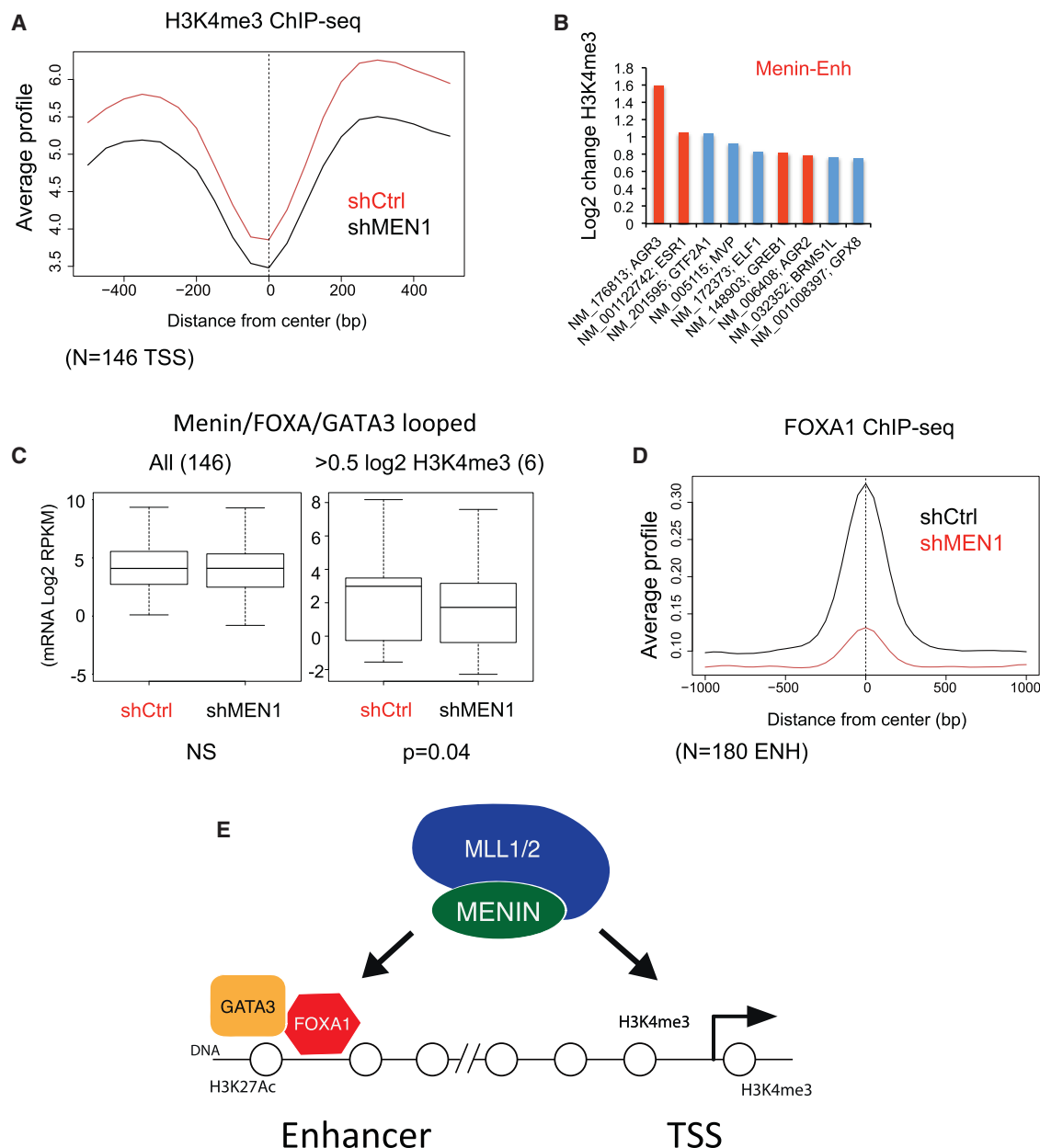
Figure 7. *MEN1*-Dependent H3K4me3 and Expression of Regulated Genes Is Associated with FOXA1 Enhancer Binding

(A) Average H3K4me3 ChIP-seq signals at menin/FOXA1/GATA3 enhancer looped TSS after induction of shCtrl or shMEN1.

(B) Top differentially *MEN1*-regulated H3K4me3 at TSS. Red bars indicate TSS looped to menin/FOXA1/GATA3-bound sites (menin-Enh).

(C) *MEN1*-dependent log2 mRNA expression of all menin/FOXA1/GATA3 looped TSS (n = 146; p = 0.83) and menin/FOXA1/GATA3 looped TSS that show > 0.5 log2 fold *MEN1*-dependent H3K4me3 (n = 6; p = 0.04 in a paired two-tailed Student's t test).

(D) Average FOXA1 ChIP-seq peak height at menin/FOXA1/GATA3 looped sites (n = 180) after induction of shCtrl or shMEN1.

(E) Model of menin-H3K4me3-dependent gene regulation marked by menin-bound TSS connected to FOXA1 and GATA3-bound enhancers.

primary mammary LP cells from non-MEN1 patients. In the LP cells, a distinct gene expression program was regulated by *MEN1*. The differentially expressed genes were mostly upregulated and mainly included genes involved in maintenance of the extracellular matrix. Both *MMP9* and *MMP3* have been reported to be important for breast tumorigenesis (Kessenbrock et al., 2013; Qin et al., 2008). In addition, the metastasis promoting periostin (*POSTN*) and the platelet-derived growth factor receptor (*PDGFRA*) genes that have been implicated in endocrine resistance were among the upregulated genes (Malanchi et al., 2011; van Agthoven et al., 2010). Both the *MMP3* and *POSTN* genes are involved in Wnt/β-catenin signaling (Kessenbrock

et al., 2013; Malanchi et al., 2011). Interestingly, menin has been reported to be able to interact with β-catenin and suppress Wnt signaling (Cao et al., 2009). The upregulated genes identified could be direct menin target genes since menin, besides its H3K4me3 function, is also known to play a role in transcriptional repression (Kim et al., 2003). In LP cells, *ESR1* is expressed at very low levels, suggesting that different enhancer signatures and transcriptional programs are important in these cells (Lim et al., 2009). *MEN1* may thus exert its breast tumor suppressive function in LP cells independent of ERα.

We found that menin is present at TSS of a large proportion of active genes in MCF-7 cells and T47D cells. However, the functional relevance of its presence appears to be restricted to a small number of target genes that are linked to distant regulatory enhancer sites. These results reconcile the findings in published reports that have addressed the function of menin. In the ER-expressing cell lines MCF-7 and T47D, we found that menin is present mostly at TSS, as has been reported in other cell types (Agarwal et al., 2007; Cheng et al., 2014; Scacheri et al., 2006). In accordance with previous findings, *MEN1* silencing leads to downregulation of H3K4me3 and mRNA of a selected number of genes in a tissue-specific manner (Agarwal and Jothi, 2012; Li et al., 2013; Lin et al., 2011, 2015; Scacheri et al., 2006). Using the well-established MCF-7 breast cancer cell line enabled us to combine our results with published datasets and to take a comprehensive look at the genes regulated by *MEN1*. This analysis shows that a small number of menin-bound sites at specific enhancer regions mark *MEN1*-dependent target gene H3K4me3 and expression.

A group of regulatory DNA regions with high enhancer density termed super enhancers appear to include especially critical regulatory hotspots (Loven et al., 2013). On average, cells harbor 300–500 of such super enhancer regions.

We used the combination of binding of menin, FOXA1, and GATA3 and looping to identify 180 enhancer sites. This number indicates that the use of these criteria is a valid approach to select active enhancers. The menin/FOXA1/GATA3 enhancers carry the H3K27Ac mark. Indeed, about 50% of the menin/FOXA1/GATA3-bound enhancers that displayed looping, including the sites near the *ESR1*, *AGR2*, and *AGR3* genes, are within predicted super enhancer regions, indicating functional overlap of these menin-bound sites with super enhancers (data not shown) (Hnisz et al., 2013). Recently, a core transcriptional regulatory circuitry has been proposed in MCF-7 cells, consisting of eight transcriptional activating genes (Saint-André et al., 2016). Five of the eight reported transcriptional regulators: *ESR1*, *GATA3*, *NR2F2*, *TBX2*, and *ZNF217*, are among the 146 genes potentially connected to menin/FOXA1/GATA3 enhancers. Although we found the highest correlation of menin-dependent H3K4me3 and expression at genes looped to enhancers bound by both the FOXA1 and GATA3 DNA binding transcription factors, it is very likely that there are additional combinations of factors that could confer a comparable level of regulation. For example, the menin-bound putative enhancer looped to the *VAV3* gene is bound by FOXA1 but not GATA3. Conversely, the *ELF1* locus that showed menin-dependent H3K4me3 is connected to a looped menin-bound enhancer that is bound by GATA3 but not FOXA1.

Menin does not bind to DNA directly and is recruited to the DNA through interactions with numerous DNA-binding transcription factors (Matkar et al., 2013). For technical reasons, we used relatively mild lysis conditions in the menin, MLL1 and MLL2 ChIP-seq experiments. Therefore, we cannot exclude the possibility that the menin signals detected at enhancers are in fact derived in *trans* from TSS-bound menin that is looped to distal sites. However, a model in which menin plays an important role at enhancer-looped TSS fits with previous reports that menin is able to interact with DNA binding transcription factors that are mostly present at enhancers but also the MLL1/MLL2 histone methyltransferase complexes that are predominantly located at TSS.

We demonstrate that menin has an important role at FOXA1 and GATA3-bound enhancers, as supported by the observation that *MEN1* silencing leads to reduced FOXA1 binding at menin-bound enhancers. Re-evaluation of FOXA1-dependent gene expression identified *ESR1* as a FOXA1-dependent gene. *ESR1* gene expression has previously been reported to be downregulated after GATA3 silencing (Eeckhoute et al., 2007). In immunoprecipitation studies in Th2 cells, menin has been shown to co-precipitate with GATA3, suggesting that the presence of menin on GATA3 binding sites is mediated through a direct protein interaction (Nakata et al., 2010). Menin is also able to bind to the forkhead transcription factors CHES1/FOXN3 and FOXO1 (Busygina and Bale, 2006; Wuescher et al., 2011).

The presence of menin at FOXA1/GATA3 bound enhancers that by analysis of ChIA-PET data are looped to nearby promoters is not E2-dependent (data not shown). Overlap of these menin/FOXA1/GATA3-bound enhancer sites with ERα ChIP-seq data did, however, indicate the presence of ERα at 89% of these sites (data not shown) (Kong et al., 2011). Menin-bound enhancer sites containing FOXA1 and GATA3 were also enriched for MLL1 and MLL2. Regulation of enhancer-mediated transcription by MLL1 or MLL2 has been reported (Kaikkonen et al., 2013; Won Jeong et al., 2011). Since MLL1 has been shown to be a pioneer factor for FOXA1 and ERα DNA binding in MCF-7 cells, the ERα binding may occur following recruitment of the menin methyltransferase complexes (Won Jeong et al., 2012).

The insight gained from these experiments into the proliferative role of *MEN1* may be useful for sporadic breast cancer from a therapeutic point of view. Activating *ESR1* gene mutations have regained attention as these were found to be present in a significant proportion of ERα-expressing metastatic breast tumors (Jeselsohn et al., 2014; Robinson et al., 2013; Toy et al., 2013). The MI-2 menin inhibitor tested is an early generation compound designed to disrupt the interaction between menin and MLL1 (Grembecka et al., 2012). Toxicity of this compound did not allow its use in cell growth assays. However, more specific and potent menin-MLL inhibitors have been developed (Cierpicki and Grembecka, 2014). Such compounds have been shown to suppress growth in AR-expressing prostate cancer models (Malik et al., 2015). Next-generation menin inhibitors or related compounds could be used to downregulate the expression of *ESR1* and may hold promise for the treatment of advanced ER+ breast cancer.

EXPERIMENTAL PROCEDURES

Detailed experimental procedures can be found in the Supplemental Information.

Cell Growth Assays

MCF-7, MDA-MB-361, T47D, ZR-75-1 (human *ESR1* gene-expressing breast cancer cell lines), αT3 (mouse *Esr1*-expressing pituitary), and GH3 (rat *Esr1*-expressing pituitary) were grown in phenol red-free medium containing 10% charcoal dextran-treated fetal bovine serum (CDT medium) containing 10 nM E2 or 100 ng/mL EGF. Cells were counted at the indicated time points. Experiments were repeated multiple times in triplicate.

Propidium Iodine Cell-Cycle Analysis

For cell-cycle analysis cells were resuspended in PBS containing 100 μg/mL RNase, 40 μg/mL propidium iodide (Sigma). The PI profile was measured using an LSR Fortessa machine (BD Biosciences) at the DFCI Flow Cytometry Facility. For data analysis, FlowJo version 7.6.5 was used.

ChIP-Seq

For ChIP with H3K4me3 and FOXA1 antibodies a standard SDS-based protocol was used, as has been described (Carroll et al., 2005).

For menin, MLL1 and MLL2 ChIP experiments a sarkosyl-based protocol was used, essentially as described (Lee et al., 2006). For sequencing, we used the NextSeq 500 sequencing platform (Illumina).

RNA Microarrays and RNA-Seq

Starting with total RNA, poly-A selected, non-stranded RNA-seq libraries were constructed on the Sciclone liquid handler (Perkin Elmer). 40-bp single-end reads were obtained using Illumina HiSeq machine (Illumina). For microarray analysis, total LP RNA was applied to Human Gene 1.0 ST Arrays (Affymetrix).

4C-Seq

Two independent 4C experiments were performed in both shMEN1 and shCtrl-expressing MCF-7 cells, as previously described (Splinter et al., 2012).

ACCESSION NUMBERS

The accession numbers for the microarray, RNA-seq, and ChIP-seq data reported in this paper are GEO: GSE85099, GSE85315, GSE86316, GSE85317, GSE94001, and GSE94009.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.02.025.

AUTHOR CONTRIBUTIONS

K.M.A.D., A.C.G., E.S.M.V., E.L., W.d.L., P.K.R., and M.B. conceived and designed the experiments. K.M.A.D., A.C.G., E.S.M.V., A.F.-T., D.C., and P.K.R. performed the experiments. K.M.A.D., A.C.G., E.S.M.V., L.G., J.R., C.Y.L., and W.d.L. analyzed the data. L.G., E.L., C.Y.L., P.K.R., and H.W.L. contributed reagents, materials, and analysis tools. K.M.A.D. and M.B. wrote the paper.

ACKNOWLEDGMENTS

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