

# A Distinct Mechanism for Coactivator *versus* Corepressor Function by Histone Methyltransferase G9a in Transcriptional Regulation<sup>\*[5]</sup>

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**Background:** The transcriptional corepressor G9a also activates genes by an unknown mechanism.

**Results:** The N-terminal region of G9a binds estrogen receptor  $\alpha$  and is necessary and sufficient for enhancing estrogen-induced gene activation and for occupancy of G9a on target genes.

**Conclusion:** The domains of G9a responsible for activation and inhibition of transcription are different.

**Significance:** G9a has inherent coactivator as well as corepressor activity.

Histone methyltransferase G9a has been understood primarily as a corepressor of gene expression, but we showed previously that G9a positively regulates nuclear receptor-mediated transcription in reporter gene assays. Here, we show that endogenous G9a contributes to the estradiol ( $E_2$ )-dependent induction of some endogenous target genes of estrogen receptor (ER) $\alpha$  in MCF-7 breast cancer cells while simultaneously limiting the  $E_2$ -induced expression of other ER $\alpha$  target genes. Thus, G9a has a dual and selective role as a coregulator for ER $\alpha$  target genes. The ER $\alpha$  binding regions associated with the pS2 gene, which requires G9a for  $E_2$ -induced expression, are transiently occupied by G9a at 15 min after beginning  $E_2$  treatment, suggesting that G9a coactivator function is by direct interaction with ER $\alpha$  target genes. Transient reporter gene assays with deletion mutants of G9a demonstrated that domains previously associated with the corepressor functions of G9a (C-terminal methyltransferase domain, ankyrin repeat domain, and cysteine-rich domain) were unnecessary for G9a coactivator function in ER $\alpha$ -mediated transcription. In contrast, the N-terminal domain of G9a was necessary and sufficient for enhancement of ER $\alpha$ -mediated transcription and for  $E_2$ -induced occupancy of G9a on ER $\alpha$  binding sites associated with endogenous target genes of ER $\alpha$ . In addition to a previously identified activation domain, this region contains a previously uncharacterized ligand-dependent ER $\alpha$  binding function, indicating how G9a is recruited to the target genes. Therefore, the coactivator and corepressor functions of G9a involve different G9a domains and different molecular mechanisms.

Transcription regulation is directed by thousands of transcriptional activator and repressor proteins, which bind to a

wide variety of specific regulatory DNA sequences that control transcription of associated genes. Once bound to DNA, these activator and repressor proteins recruit a large number of coregulator proteins, which carry out the processes of chromatin remodeling and transcription complex assembly/disassembly and regulation, resulting in increased or decreased transcription from the associated transcription start site (1–3). As suggested by the amazing number of different posttranslational modifications of histones and other proteins involved in transcriptional regulation, many coregulators are enzymes that add or remove these modifications (4). One of the most heavily studied histone-modifying enzymes is G9a, which methylates histone H3 at Lys-9 (H3K9),<sup>2</sup> a modification which is generally associated with repressed genes (5–7). G9a plays critical roles in regulation of genes required for normal embryonic development (8). In addition, G9a is one of the major H3K9 methyltransferases and is localized in euchromatin, where it is recruited to specific genes by DNA-binding transcriptional repressor proteins and non-coding RNAs; it is associated with corepressor complexes and plays a major role in repression of genes that are potentially active (8–13). G9a exists in cells primarily in a complex with G9a-like protein, a highly homologous H3K9 methyltransferase (6). The corepressor function of G9a has been well characterized. Repression of some genes by G9a involves methylation of H3K9 by G9a, leading to recruitment of proteins such as HP1, which bind preferentially to histone H3 methylated at Lys-9 and establish a repressive chromatin conformation (8). However, more recently, it has been shown that G9a contributes to repression of other genes by a mechanism that does not require G9a methyltransferase activity but instead involves the ankyrin repeat domain and its ability to bind DNA methyltransferases (14).

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table 1, Figs. 1–5, and additional references.

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<sup>2</sup> The abbreviations used are: H3K9, histone H3 Lys-9; ER $\alpha$ , estrogen receptor  $\alpha$ ; mG9a, mouse G9a; MMTV, mouse mammary tumor virus; ERE, estrogen responsive enhancer element; GRIP1, glucocorticoid receptor interacting protein 1; CARM1, coactivator-associated arginine methyltransferase 1; ANK, ankyrin repeats; hG9a, human G9a; qRT-PCR, quantitative reverse transcription-PCR; PgR, progesterone receptor; LBD, ligand binding domain.

## Mechanism of G9a Coactivator Function for Estrogen Receptor

Although the corepressor functions of G9a have been thoroughly documented, there have also been several reports indicating that G9a contributes to gene activation. We previously demonstrated that transiently overexpressed G9a cooperates synergistically with two other coactivators, GRIP1 and CARM1, as a coactivator for steroid hormone receptors in transient reporter gene assays (15). Subsequently, several other groups reported that reduction or loss of endogenous G9a expression resulted in increased expression of some endogenous genes (suggesting that G9a functions as a repressor of these genes) but reduced expression of other genes (suggesting a positive contribution by G9a to the expression of these genes) (16–18). It is possible some of the positive effects of G9a on expression of endogenous genes may be due to an indirect effect where G9a acts as a corepressor of a second gene which produces a protein that represses expression of the gene of interest. However, one group has shown that G9a associates with a broad region containing an active gene that requires G9a for maximum expression, indicating that G9a may act directly on this target gene. G9a also binds to RNA polymerase II, suggesting that it may play a role in establishing a preinitiation complex at the promoter of the gene (16).

To investigate further the mechanism by which G9a enhances gene expression, we have employed the MCF-7 breast cancer cell culture model, a very well characterized experimental system for studying the mechanism of estrogen-regulated gene expression mediated by estrogen receptor (ER) $\alpha$ . ER $\alpha$  is a steroid hormone receptor and a member of the nuclear receptor family of proteins, many of which function as ligand-activated transcription factors which bind specific regulatory DNA sequences associated with target genes and recruit coregulators to those genes to activate or repress transcription (1–3, 19). The natural estrogen estradiol (E<sub>2</sub>) binds to ER $\alpha$  and causes an altered conformation of ER $\alpha$ , which allows it to bind DNA and recruit coregulators, resulting in activation or repression of transcription of well characterized sets of target genes (20). In the current study, we identified specific genes that are regulated positively by E<sub>2</sub> and that also require G9a for their E<sub>2</sub>-induced expression. To explore the mechanism of G9a action on these genes, we tested whether G9a is recruited to the regulatory sites of these genes; this addresses the question of whether G9a action on these genes is direct or indirect. We also defined the specific domains of G9a, which are required for its ability to enhance E<sub>2</sub>-induced gene expression. Our results indicate G9a acts directly on genes that it regulates positively, and the mechanism of positive gene regulation by G9a on E<sub>2</sub>-induced genes is completely different from the previously defined mechanisms of corepressor function by G9a.

### EXPERIMENTAL PROCEDURES

**Plasmids**—Protein expression vectors for mouse G9a (mG9a) and G9a fragments, ER $\alpha$ , MMTV(ERE)-luciferase, GRIP1, and CARM1 were described previously (15) except as indicated below. Briefly, proteins expressed with HA or FLAG epitope tags in human cells or *in vitro* were encoded by pSG5.HA or pSG5.FLAG vectors containing SV40 and T7 promoters. cDNAs encoding the mG9a  $\Delta$ ANK ( $\Delta$ 734–934),  $\Delta$ Cys ( $\Delta$ 518–591), and  $\Delta$ 252 ( $\Delta$ 1–252) deletion mutants were cloned

into the EcoRI and XhoI sites of pSG5.HA. cDNA encoding mG9a fragments 1–730 and 1–600 were cloned in between the EcoRI and XhoI sites of pSG5.HA. cDNA encoding human G9a (hG9a) 93–211 with an N-terminal His-tag was cloned into pMCSG7 between the SspI and BamHI restriction sites. The cDNA encoding full length hG9a was obtained from Invitrogen and cloned between the EcoRI and XhoI sites of pSG5.HA. cDNA encoding FLAG-hG9a and FLAG-hG9a 1–280 was PCR amplified and cloned between the EcoRI and XhoI sites of pSG5.FLAG to result in a 2 $\times$ FLAG epitope.

**Cell Culture and Reporter Gene Assay**—CV-1, Cos-7, and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 37 °C and 5% CO<sub>2</sub>. For reporter gene assays, CV-1 cells were plated in hormone-free medium (phenol red-free Dulbecco's modified Eagle's medium with 5% charcoal-stripped serum) at 1  $\times$  10<sup>5</sup> cells/well in 12-well plates the day before transfection. Transfection of expression plasmids was performed using BioT transfection agent (Bioland) according to the manufacturer's protocol. Molar amounts of total transfected promoters were balanced with empty pSG5 vector, and total DNA mass was balanced with the promoterless pCAT basic plasmid. After transfection, the cells were grown in hormone-free medium for 48 h in the presence or absence of 100 nM E<sub>2</sub>. Cell lysis and luciferase assays on cell extracts were performed with Promega luciferase assay kit. Internal controls were not used to normalize results because expression of so-called constitutive reporter genes is enhanced by overexpression of many coactivators, including GRIP1 and CARM1, which were used in these studies. To control for variations in transfection efficiency, multiple independent transfection experiments were performed, and multiple plasmid preps for key plasmids were tested in these assays. The results are presented as the mean  $\pm$  S.D. of three transfected wells and are representative of at least three independent experiments. An aliquot of the cell lysate was reserved for immunoblot analysis.

**Coimmunoprecipitation and Immunoblot**—For coimmunoprecipitation, Cos-7 cells were seeded 1  $\times$  10<sup>6</sup> cells/10-cm dish the day before transfection. Transfection of 3  $\mu$ g of expression vectors was performed with Bio-T (Bioland) according to the manufacturer's protocol. Cells were incubated for 48 h in the presence of the indicated ligand (100 nM 17 $\beta$ -estradiol (E<sub>2</sub>), 4-hydroxytamoxifen, or ICI164,384, or the comparable volume of ethanol as vehicle) before whole cell extracts were made in 1 ml of radioimmune precipitation assay buffer (50 mM Tris·HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). Coimmunoprecipitation and immunoblotting were performed as described previously (21). The lysate was precleared by incubation with protein A/G beads for 1 h and then immunoprecipitated overnight with 1  $\mu$ g of the indicated antibodies and A/G beads. Immunoblotting was conducted with primary antibodies against HA epitope (3F10, Roche Applied Science), G9a (Sigma), ER $\alpha$  (HC20, Santa Cruz Biotechnology), RNA polymerase II (CTD4H8, Millipore), actin (Santa Cruz Biotechnology), and FLAG epitope (M2, Sigma). Secondary antibodies from Santa Cruz Biotechnology (anti-rat) and Promega (anti-rabbit and anti-mouse) were used for chemiluminescence detection by film, or antibodies were

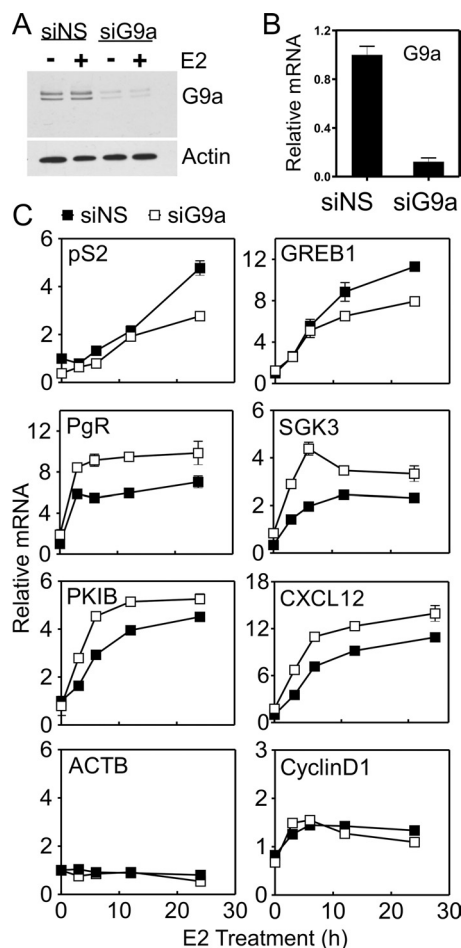
purchased from LI-COR Biosciences for quantitative infrared imaging.

**RNA Isolation and Quantitative Reverse Transcriptase PCR (qRT-PCR)**—For RNA extraction, cells were lysed in TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription was accomplished using iScript (Bio-Rad) according to specifications with 0.8  $\mu\text{g}$  of total RNA for cDNA template. Real-time PCR amplification of this cDNA was performed on a Roche Lightcycler 480 using SYBR Green I mastermix (Roche Applied Science) and primers specific for the indicated mRNAs (see supplemental Table 1). All mRNA levels were normalized to the level of GAPDH mRNA.

**G9a Depletion**—For depletion of G9a with siRNA, SMART-pool siRNA targeting G9a (Dharmacon) was transfected into MCF-7 cells using Oligofectamine (Invitrogen) according to the manufacturer's protocol. Transfected MCF-7 cells were cultured for 72 h in hormone-free medium prior to  $\text{E}_2$  treatment. Cells were harvested either in TRIzol (Invitrogen) for qRT-PCR or radioimmune precipitation assay buffer for immunoblot analysis.

**Chromatin Immunoprecipitation (ChIP)**—ChIP was performed as described previously (15). Briefly, MCF-7 cells were cultured in hormone-free medium for 3 days before addition of  $\text{E}_2$ . After exposure to 100 nM  $\text{E}_2$  for the indicated time, cells were fixed in formaldehyde for 15 min before addition of glycine solution to stop cross-linking. After washing with PBS, cells were pelleted by centrifugation and resuspended in hypotonic buffer before being passed through a 25-gauge needle three times. Chromatin was pelleted and resuspended for sonication in the Bioruptor apparatus (Diagenode) for 15 min (30 s on, then 30 s off, repeated) until chromatin fragment sizes were  $<0.5$  kb as determined by agarose gel electrophoresis. Sonicated chromatin was diluted 1:10 for immunoprecipitation and precleared by incubating with AG-agarose beads. Immunoprecipitation was performed with the indicated antibodies by incubation overnight with AG-agarose beads. Washing and elution of the beads occurred the next day followed by overnight incubation at 65  $^\circ\text{C}$  to reverse cross-linking. DNA purification was performed by extraction with phenol and chloroform followed by ethanol precipitation, and DNA was resuspended for quantitative PCR (qPCR) using primers specific for the indicated DNA sites (see supplemental Table 1). G9a antibodies used were as follows: 1  $\mu\text{g}$  (Sigma); 0.5  $\mu\text{g}$  (R&D Systems); and Abcam whole antiserum, 10  $\mu\text{l}$ . The signal from the immunoprecipitated DNA was normalized to the signal from DNA prepared from the same amount of chromatin before immunoprecipitation (input).

**GST Pulldown Experiments**—Protein fragments labeled with  $^{35}\text{S}$  were synthesized by transcription and translation *in vitro* using TNT T7 Quick kit (Promega) and the appropriate plasmid as template. mG9a protein fragments 72–200, 150–265, and 200–333 were synthesized using PCR amplification products as the DNA templates. The GST fusion protein attached to beads was incubated with the  $^{35}\text{S}$ -labeled proteins overnight at 4  $^\circ\text{C}$ . After washing with NETN buffer (22) containing 0.5% and 0.01% Nonidet P40, the beads were boiled in SDS lysis buffer for analysis by SDS-PAGE and autoradiography. For pull-down of a His-tagged protein fragment, hG9a 93–211 was bacterially



**FIGURE 1. Selective positive and negative effects of G9a depletion on  $\text{E}_2$ -regulated gene expression.** MCF-7 cells were transfected with SMART-pool siRNA targeting G9a (siG9a) or siRNA against a nonspecific sequence (siNS). Cells transfected with siRNA were cultured for 3 days in hormone-free medium prior to addition of 100 nM  $\text{E}_2$  for the indicated time. *A*, whole cell extracts from cells at the zero time point of  $\text{E}_2$  treatment were analyzed by immunoblot with antibodies for G9a and actin. *B*, total cellular RNA was extracted from cells at the zero time point of  $\text{E}_2$  treatment and used to measure G9a mRNA level by qRT-PCR. *C*, total cellular RNA was extracted from cells at the indicated time point of  $\text{E}_2$  treatment and analyzed by qRT-PCR to determine levels of the indicated mRNAs, which are normalized to the level of GAPDH mRNA. Results shown are the mean and S.D. for three PCR reactions performed with cDNA samples from a single experiment, and are representative of at least three independent biological experiments performed on different days.

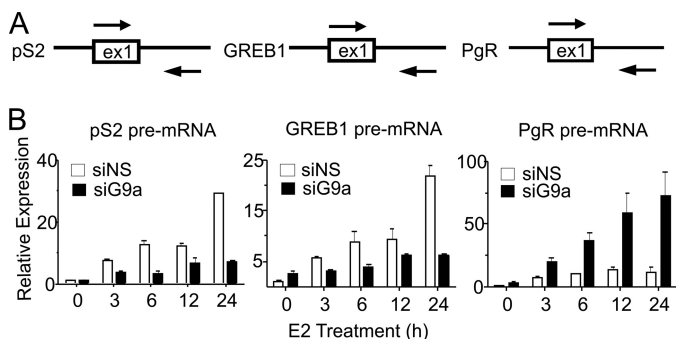
expressed and purified using a His Bind purification kit (Novagen). The His-tagged G9a protein was incubated with the GST fusion protein in the same manner as above.

## RESULTS

**G9a Depletion Differentially Affects Expression of ER $\alpha$  Target Genes**—To determine the importance of G9a for ER $\alpha$ -mediated gene expression, we depleted G9a in MCF-7 human breast cancer cells using a pool of four siRNAs (Fig. 1, *A* and *B*) and measured mRNA levels of several well known ER $\alpha$  target genes at various time points after beginning  $\text{E}_2$  treatment (Fig. 1*C*).  $\text{E}_2$ -induced levels of pS2 and GREB1 mRNA were reduced by G9a deficiency, indicating a positive regulatory effect of G9a. In contrast, induction of Protein Kinase Inhibitor  $\beta$  (PKIB), SGK3, PgR, and CXCL12 mRNAs by  $\text{E}_2$  in the absence of G9a was



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**FIGURE 2. G9a affects ER $\alpha$  target gene expression at the level of transcription.** *A*, to detect pre-mRNA for the indicated gene, primers were designed to amplify a DNA sequence that spans the boundary of exon 1 (*ex1*) and intron 1 for each gene. *B*, total RNA samples prepared as described in the legend to Fig. 1C were analyzed by qRT-PCR, using primers designed as illustrated in *A*. Results are presented relative to GAPDH mRNA levels as the mean and S.D. for three PCR reactions performed with the cDNA samples from a single experiment and are representative of at least three independent biological experiments performed on different days. *siG9a*, siRNA targeting G9a; *siNS*, siRNA against a nonspecific sequence.

greater than in non-depleted cells. However, induction of cyclin D1 mRNA by E<sub>2</sub> was not altered by G9a depletion. These results indicate that endogenous G9a is required for full induction of some endogenous genes by E<sub>2</sub>, limits the extent of induction of other ER $\alpha$  target genes, and has no effect on E<sub>2</sub>-induced expression of a third subset of ER $\alpha$  target genes.

**Measurement of Pre-mRNA Reveals Effect of G9a Depletion on Transcription**—The mRNA level for a particular gene depends on the rate of synthesis and the rate of degradation. In contrast, the level of primary transcript provides a more accurate measure of the instantaneous transcriptional activity or rate for a given gene. Therefore, we measured pre-mRNA levels of pS2, GREB1, and PgR at different time points after E<sub>2</sub> addition. Pre-mRNA levels for pS2 and GREB1 (which require G9a for full E<sub>2</sub> induction) increased substantially after E<sub>2</sub> treatment and were substantially inhibited by G9a depletion (Fig. 2). In contrast, in the same cells, G9a depletion increased the E<sub>2</sub>-induced levels of pre-mRNA for PgR (Fig. 2), matching the effect of G9a depletion on PgR mRNA (Fig. 1C). Similar results were obtained when the pool of four siRNAs was split into two pairs of siRNAs (supplemental Fig. 1). Thus, endogenous G9a contributes to the transcriptional activation of some ER $\alpha$  target genes while simultaneously limiting the E<sub>2</sub>-induced transcriptional activation of other ER $\alpha$  target genes.

**ER $\alpha$  Binding Regions of pS2 Gene Are Occupied by G9a in Hormone-dependent Manner**—The actions of G9a as a corepressor involve direct G9a occupancy of regulatory sites on the regulated gene, resulting in methylation of Lys-9 of histone H3 by G9a or recruitment of other corepressor proteins (8, 14). In contrast, the mechanism by which G9a contributes to gene activation is unknown. G9a could occupy regulatory regions of the target gene and thereby contribute directly to enhanced transcription. Alternatively, positive effects on transcription of a specific gene by G9a could be indirect: G9a could occupy and repress a second gene that encodes a repressor protein for the first gene. Direct action of G9a as a coactivator would require that G9a occupy regulatory sites of the gene. We therefore tested for E<sub>2</sub>-regulated occupancy of G9a on the ER $\alpha$  binding

sites of the pS2 gene in MCF-7 cells. ER $\alpha$  binding sites are located 9.9, 9.1, and 0.3 kb upstream of the transcription start site of the pS2 gene (Fig. 3A) (23). Using ChIP assays, we observed E<sub>2</sub>-induced binding of ER $\alpha$  to these three sites, but not to a control site at -3.1 kb, beginning at 5 min and continuing through 30 min of E<sub>2</sub> treatment (Fig. 3B). At ~15 min after beginning E<sub>2</sub> exposure, G9a occupancy of the pS2 gene also increased sharply and transiently at the ER $\alpha$  binding regions but not at the -3.1 kb control region (Fig. 3C). By 25 min of E<sub>2</sub> exposure, G9a occupancy returned to pre-E<sub>2</sub> treatment levels. These results were obtained with G9a antibodies from R&D Systems, and similar results were obtained with G9a antibodies from Sigma-Aldrich and Abcam (data not shown). The specificity of the G9a antibodies was further confirmed by repeating the ChIP analysis after depletion of G9a. Upon G9a depletion, the E<sub>2</sub>-induced occupancy of G9a at the ER $\alpha$  binding sites of the pS2 gene after 15 min of E<sub>2</sub> exposure was abolished (Fig. 4A). G9a depletion also caused diminished E<sub>2</sub>-induced occupancy of RNA polymerase II at the pS2 gene measured after 45 min of E<sub>2</sub> exposure (Fig. 4B), which is near the peak time for RNA polymerase II occupancy. This result is consistent with the reduced expression of pS2 mRNA and pre-mRNA after depletion of G9a. The E<sub>2</sub>-induced occupancy of G9a at ER $\alpha$  binding sites indicates that G9a acts directly on the target gene in a hormone-dependent manner.

**N-terminal Region of G9a Is Necessary and Sufficient for G9a Coactivator Function in Transient Reporter Gene Assays**—To explore further the mechanism of G9a recruitment and action as a coactivator, we tested which domains of G9a are required for its coactivator function with ER $\alpha$ . We previously observed enhancement of steroid hormone-induced reporter gene expression by G9a and found that the SET (Su(var), enhancer of Zeste, trithorax) domain of G9a, which contains the methyltransferase activity, was not required (15). Robust G9a coactivator function required the co-expression of p160 coactivator GRIP1/SRC-2, and G9a also exhibited dramatic three-coactivator synergy in cooperation with GRIP1 and protein arginine methyltransferase CARM1. GRIP1, a member of the p160 or steroid receptor coactivator family, binds ER $\alpha$  in a hormone-dependent manner and serves as a scaffold protein for recruitment of other coactivators, including CARM1 (24). Because GRIP1 can bind to the ankyrin repeat domain of G9a (25), we tested whether the ankyrin repeat domain is required for G9a coactivator function with ER $\alpha$ . When ER $\alpha$  and GRIP1 were overexpressed by transient transfection of CV-1 cells, E<sub>2</sub>-induced expression of a transiently transfected ER $\alpha$  reporter gene was enhanced greatly by coexpression of full-length mG9a (Fig. 5A, bars 1–6). The enhancement by G9a was only observed in the presence of E<sub>2</sub> (supplemental Fig. 2D), indicating that G9a coactivator activity depends upon the presence of hormone-activated ER $\alpha$ . A mG9a mutant lacking the ankyrin repeat domain (G9a $\Delta$ ANK, lacking amino acids 730–965) and expressed at levels similar to full-length mG9a, exhibited coactivator activity similar to that of full-length mG9a (Fig. 5A, bars 10–12). Thus, neither the C-terminal SET domain nor the ankyrin repeat domain is required for the coactivator function of G9a in this assay, suggesting that neither the methyltransferase activity nor the ability to bind GRIP1 is required for G9a

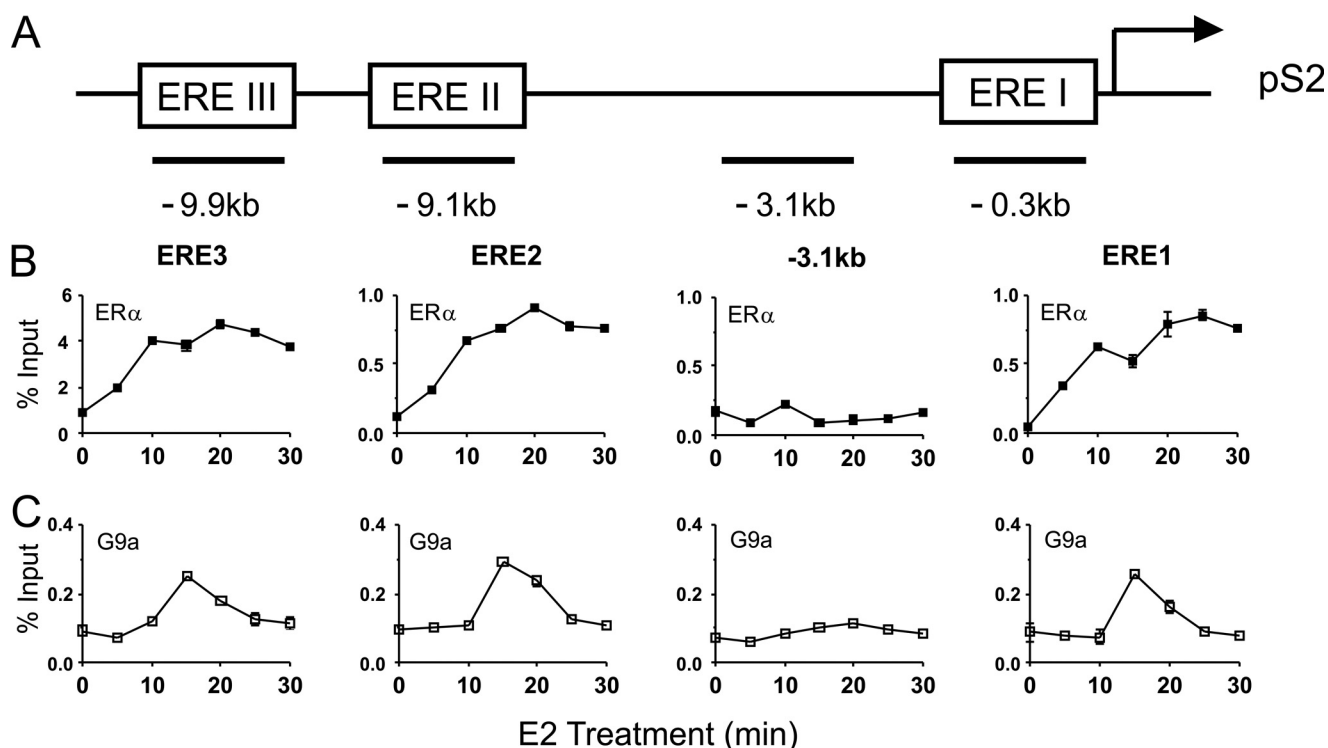


FIGURE 3. **E<sub>2</sub>-induced recruitment of G9a to ER $\alpha$  binding sites associated with the pS2 gene.** *A*, the upstream regulatory region of the pS2 gene, showing three ER $\alpha$  binding sites (ERE), the transcription start site (arrow), and the locations of amplicons used for qPCR analysis of DNA from ChIP (horizontal bars with distance from the transcription start site indicated in kb). *B* and *C*, MCF-7 cells were treated with E<sub>2</sub> for the indicated time before cross-linking of chromatin with formaldehyde. ChIP was performed with antibodies against ER $\alpha$  (*B*) and G9a (*C*), and the indicated amplicons were quantified by qPCR in the immunoprecipitated DNA and the input DNA before immunoprecipitation. Results shown for G9a were performed with antibodies from R&D Systems; similar results were obtained with anti-G9a antibodies from Abcam and Sigma. Results are shown as percentage of input, are the mean and S.D. of three PCR reactions performed with the DNA samples from a single experiment, and are representative of at least three independent biological experiments performed on different days.

coactivator function. We also tested a G9a mutant lacking the cysteine-rich region (amino acids 518–591), which is homologous to cysteine ring domains known to bind other transcription factors. Again, the coactivator function of this deletion mutant, in cooperation with GRIP1, was similar to that of full-length G9a (Fig. 5B).

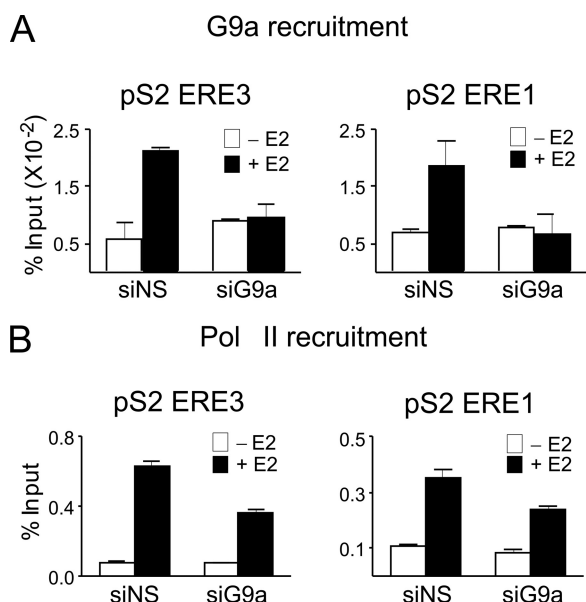
We then focused on the only remaining untested region, the N terminus, which contains a previously characterized activation domain (15). A mG9a mutant lacking the N-terminal 252 amino acids, which was also expressed at levels similar to full-length mG9a, exhibited little or no activity as a coactivator in cooperation with GRIP1 (Fig. 5A, bars 7–9), indicating that the N-terminal region is required for coactivator function. Because the N-terminal region of G9a, but none of the other regions tested, was required for coactivator function, we tested whether the N-terminal region alone is sufficient for coactivator function. Indeed, full-length mG9a and an N-terminal mG9a fragment consisting of amino acids 1–333, expressed at similar levels, enhanced E<sub>2</sub>-induced reporter gene activity to a similar extent in cooperation with GRIP1 (Fig. 5C). Note that approximately half as much of the mutant mG9a plasmid, compared with the wild type plasmid, was required to achieve equivalent expression levels. For all of the above mG9a mutants, similar results were obtained when both GRIP1 and CARM1 were coexpressed with mG9a or the mG9a mutants (supplemental Fig. 2). However, under these conditions where GRIP1, CARM1, and G9a cooperated synergistically as coactivators, the mG9a 1–333 fragment was quite active but exhibited some-

what less activity than full-length mG9a at the higher levels of expression.

Amino acids 72–333 of mG9a are highly conserved with amino acids 15–281 of hG9a (supplemental Fig. 3). Therefore, we tested the coactivator function of the hG9a N-terminal fragment (amino acids 1–280), which is analogous to mG9a 1–333. As with mG9a, comparable levels of full-length hG9a and hG9a 1–280 exhibited similar levels of coactivator activity in cooperation with GRIP1 (supplemental Fig. 4). Thus, the N-terminal region of G9a (mouse or human) was both necessary and sufficient for G9a coactivator function in this transient reporter gene assay.

**G9a Exhibits Ligand-dependent ER $\alpha$  Binding Activity**—To understand further the mechanism of coactivator function by G9a and its N-terminal region in ER $\alpha$ -mediated transcription, we investigated how G9a is recruited to the target genes of ER $\alpha$ . Because our results suggested that G9a association with GRIP1 is not required for coactivator function (Fig. 5A), we considered the possibility of G9a recruitment directly by interaction with ER $\alpha$ . When transiently expressed ER $\alpha$  was immunoprecipitated from Cos-7 cells, transiently expressed HA-tagged mG9a was co-precipitated in the absence of E<sub>2</sub>, but the co-precipitation was further enhanced when cells were treated with E<sub>2</sub> prior to harvest (Fig. 6A). ER $\alpha$  antagonists 4-hydroxytamoxifen and ICI164,384 failed to enhance the ER $\alpha$ -G9a interaction. To test whether this apparent hormone-dependent interaction of mG9a with ER $\alpha$  was direct, <sup>35</sup>S-labeled mG9a was translated *in vitro* and incubated with GST-tagged ER $\alpha$  LBD bound to aga-

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**FIGURE 4. G9a depletion reduces occupancy of G9a and RNA polymerase II at ER $\alpha$  binding sites associated with the pS2 gene.** MCF-7 cells were transfected with siNS or siG9a, grown for 3 days in hormone-free medium, and treated with 100 nM E<sub>2</sub> for 0, 15, or 45 min as described in the legend to Fig. 1. *A*, ChIP was performed on chromatin from the 0- and 15-min E<sub>2</sub>-treated cells using antibodies against G9a. *B*, ChIP was performed on chromatin from the 0- and 45-min E<sub>2</sub>-treated cells using antibodies against RNA polymerase II (Pol II). DNA regions including the indicated pS2 EREs were amplified by qPCR from the DNA isolated by ChIP. Results are shown as percentage of input before ChIP, are the mean and S.D. for three PCR reactions from the DNA samples from a single experiment, and are representative of at least three independent biological experiments performed on different days. *siG9a*, siRNA targeting G9a; *siNS*, siRNA against a nonspecific sequence.

rose beads. Binding of mG9a to ER $\alpha$  LBD in this assay was highly dependent on E<sub>2</sub> (Fig. 6B), suggesting that the interaction was probably direct. The small amount of mG9a binding to GST alone may be due to the overloading of GST on the beads compared with GST-ER $\alpha$  LBD. In similar GST pulldown assays, mG9a 1–600 also bound to the ER $\alpha$  N-terminal AF-1 fragment and to the ER $\alpha$  DNA binding domain fragment (supplemental Fig. 5). However, in our experiment, the ER $\alpha$  DNA binding domain binds to many proteins, casting doubt on whether the interactions observed are specific or are instead *in vitro* artifacts of this fragment.

Although we cannot rule out contributions from the G9a interactions with the ER $\alpha$  DNA binding domain and AF-1 regions, we focused on the hormone-dependent interaction of G9a with the ER $\alpha$  LBD, as the coactivator function of G9a also depends upon hormonal activation of ER $\alpha$ . To determine the sequences of G9a necessary for ligand-dependent binding to ER $\alpha$  LBD, multiple deletion mutants of G9a were translated *in vitro* and tested for their ability to bind GST-ER $\alpha$  LBD in the presence and absence of E<sub>2</sub>. C-terminal truncations of various sizes or deletion of the ankyrin repeat domain failed to reduce the E<sub>2</sub>-dependent binding of mG9a to ER $\alpha$  LBD (Fig. 6C). In contrast, deletion of the N-terminal 252 amino acids eliminated E<sub>2</sub>-dependent binding to the ER $\alpha$  LBD, although some fragments lacking the N-terminal end exhibited weak E<sub>2</sub>-independent binding not observed with full-length mG9a. Finally, the N-terminal mG9a fragment consisting of amino acids 1–333, which was active as a coactivator (Fig. 5), retained the ability to

bind ER $\alpha$  LBD in an E<sub>2</sub>-dependent manner (Fig. 6C). Similar E<sub>2</sub>-dependent binding to ER $\alpha$  LBD was obtained with hG9a 1–280, which is the homologous fragment for mG9a 1–333 (data not shown).

Because the N-terminal sequence of G9a contained no known ER $\alpha$  binding motif (e.g. LXXLL where L is leucine and X is any amino acid), we sought to further define the region necessary for this novel ER $\alpha$  LBD binding activity. Three overlapping fragments of the N terminus were translated *in vitro* and incubated with GST ER $\alpha$  LBD in the presence and absence of E<sub>2</sub>. Of the three fragments, only the central region, consisting of mG9a amino acids 150–265, bound ER $\alpha$  LBD and bound in a ligand-dependent manner (Fig. 6D). As a stringent test for direct G9a binding to ER $\alpha$  LBD, hG9a 93–211 (which corresponds to mG9a 150–265) containing a His tag was bacterially expressed and incubated with GST-ER $\alpha$  LBD. The hG9a (93–211) fragment produced in bacteria was bound to ER $\alpha$  LBD in an E<sub>2</sub>-dependent manner (Fig. 6E). Thus, the N-terminal region of G9a, which is sufficient for coactivator function, can bind directly to ER $\alpha$  LBD in response to hormone, providing a potential mechanism for recruitment of G9a to the target genes of ER $\alpha$ .

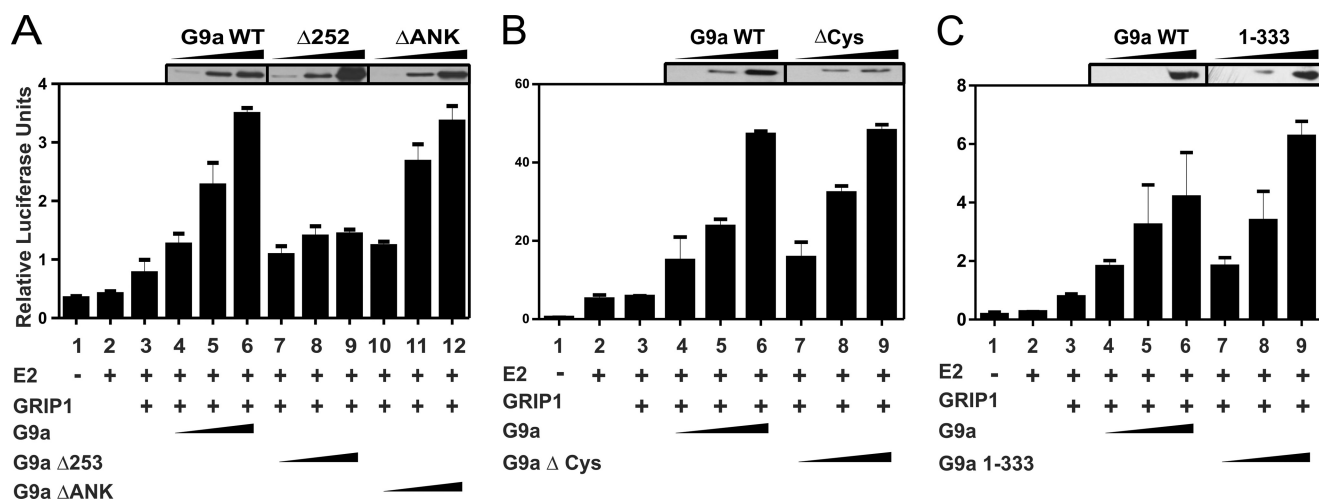
*G9a N Terminus Is Sufficient for Recruitment to ER $\alpha$  Target Genes*—The presence of a ligand-dependent ER $\alpha$  binding domain and coactivator function within the N-terminal region of G9a led us to investigate whether the same N-terminal region of G9a is responsible for the E<sub>2</sub>-induced occupancy of G9a on ER $\alpha$  binding sites associated with ER $\alpha$  target genes. MCF-7 cells were transfected with a plasmid encoding hG9a full-length or hG9a 1–280 containing two N-terminal FLAG tags. We observed E<sub>2</sub>-induced association of full length G9a and G9a 1–280 with the ER $\alpha$  binding sites associated with the pS2 and GREB1 genes (Fig. 7). Thus, the N-terminal region of G9a is sufficient for coactivator function, ligand-dependent binding to ER $\alpha$ , and recruitment of G9a to ER $\alpha$  binding sites associated with E<sub>2</sub>-regulated genes.

## DISCUSSION

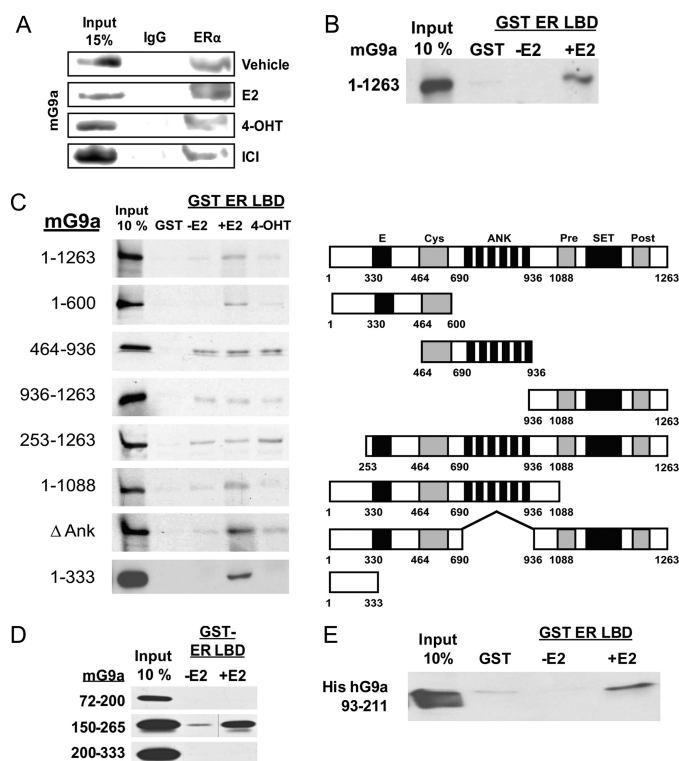
*Selective Positive and Negative Regulation of Target Genes of ER $\alpha$  by Coregulator G9a*—By examining mRNA levels of multiple genes, which are up-regulated in response to E<sub>2</sub> in MCF-7 cells, we found that the effects of G9a depletion fell into three different categories (Fig. 1C). 1) E<sub>2</sub> caused a stronger induction of some genes after G9a depletion, indicating that G9a acts negatively to limit the extent of E<sub>2</sub>-induced expression for these genes. 2) G9a depletion restricted the extent of induction of a second set of ER $\alpha$  target genes, indicating a positive role for G9a in E<sub>2</sub>-induced expression of these genes. 3) Depletion of G9a had no effect on the E<sub>2</sub>-induced expression of a third subset of genes. For both subsets of ER $\alpha$  target genes regulated by G9a, the effect of G9a on the mRNA level was transcriptional, as depletion of G9a caused similar effects on the levels of E<sub>2</sub>-induced pre-mRNA and mature mRNA for the target genes (Fig. 2).

What are the mechanisms that determine whether G9a acts to enhance or inhibit gene expression, after it is recruited to the gene in response to E<sub>2</sub>? Promoter-specific effects on gene regulation by various transcription factors and coregulators is a





**FIGURE 5. Enhancement of E<sub>2</sub>-dependent reporter gene expression by G9a and G9a protein fragments in cooperation with GRIP1.** CV-1 cells in 12-well dishes were transfected with MMTV(ERE)-luciferase reporter plasmid (200 ng) and expression vectors encoding ER $\alpha$  (0.2 ng); GRIP1 (100 ng); HA-tagged mG9a full-length (FL), mG9a $\Delta$ 252, mG9a $\Delta$ ANK, or mG9a $\Delta$ Cys (100, 200 or 400 ng); and mG9a 1–333 (50, 100, or 200 ng). Cells were grown in the presence or absence of E<sub>2</sub> for 48 h before measurement of luciferase activity and immunoblot analysis with antibodies against HA. Results shown are mean and S.D. from triplicate transfected cultures for each condition, and each experiment shown is representative of three or more independent experiments performed on different days.



**FIGURE 6. E<sub>2</sub>-dependent binding of ER $\alpha$  to the N-terminal region of G9a.**

**A.** Cos-7 cells were transfected with 3  $\mu$ g each of expression vectors encoding ER $\alpha$  and HA-tagged full-length mG9a. Cells were treated with the indicated ER $\alpha$  ligand (4-OHT, 4-hydroxytamoxifen; ICI, ICI164,384) for 48 h, cell extracts were immunoprecipitated with anti-ER $\alpha$  antibody, and immunoblot was performed with anti-HA antibody. **B.** <sup>35</sup>S-labeled full-length mG9a was synthesized *in vitro* and incubated in the presence and absence of E<sub>2</sub> with GST or GST-ER $\alpha$  LBD bound to glutathione-agarose beads. The bound protein fraction was eluted and analyzed by SDS-PAGE and autoradiography. A 10% input sample was loaded for comparison. **C and D.** <sup>35</sup>S-labeled mG9a fragments were synthesized *in vitro* and incubated with GST or GST-ER $\alpha$  LBD bound to glutathione-agarose beads in the presence and absence of E<sub>2</sub> or 4-hydroxytamoxifen. Bound proteins and 10% input samples were analyzed as described in **B**. The vertical line in the G9a 150–265 gel image is a scratch on the film. **E.** Bacterially expressed His-tagged hG9a 93–211 was incubated in the presence and absence of E<sub>2</sub> with GST or GST-ER $\alpha$  LBD bound to glutathione-agarose beads. Input and bound samples were analyzed by immunoblot with an anti-His tag antibody.

well known but poorly understood phenomenon. We speculate that the specific protein-protein interactions or post-translational modification that G9a experiences on a particular target gene of ER $\alpha$  determine whether G9a acts as a coactivator or corepressor.

The selective actions of G9a on different subsets of ER $\alpha$  target genes suggest the interesting possibility that regulation of G9a levels, localization, or activity could alter the overall response to E<sub>2</sub> and thus alter the physiological response elicited by E<sub>2</sub>. This may be a mechanism for fine tuning of the response to E<sub>2</sub>. Future work to determine the effects of G9a depletion or inhibition on global gene regulation in response to E<sub>2</sub> will examine this hypothesis.

**Mechanism by Which G9a Is Recruited to ER $\alpha$  Target Genes and Enhances E<sub>2</sub>-induced Transcription**—We found that the specific domains of G9a required for the ability of G9a to enhance E<sub>2</sub>-induced transcription are completely different from the domains implicated previously in G9a-mediated repression. Repression can be achieved through methylation of H3K9 by the C-terminal SET domain of G9a, leading to recruitment of HP1 and establishment of a repressive chromatin environment (8). Methyltransferase-independent repression has been assigned to the ankyrin repeat domain and correlated with the ability of that domain to interact with DNA methyltransferases (6, 14). The cysteine-rich domain is also apparently required for repression in some cases because it is the site of interaction for some DNA-binding repressor proteins that recruit G9a to specific genes (11). We found that none of these three domains is required for the coactivator function of G9a in transient transfection assays (Fig. 5). Instead, only the N-terminal region of G9a, which contains no recognizable amino acid motifs, was necessary and sufficient for enhancement of transcription of a transient reporter gene by E<sub>2</sub>-activated ER $\alpha$ .

Because G9a acted synergistically with GRIP1 as a coactivator for steroid receptors (Fig. 5) (15), we proposed that G9a is recruited to the target gene by interaction with GRIP1. However, GRIP1 interacts with G9a through the ankyrin repeat

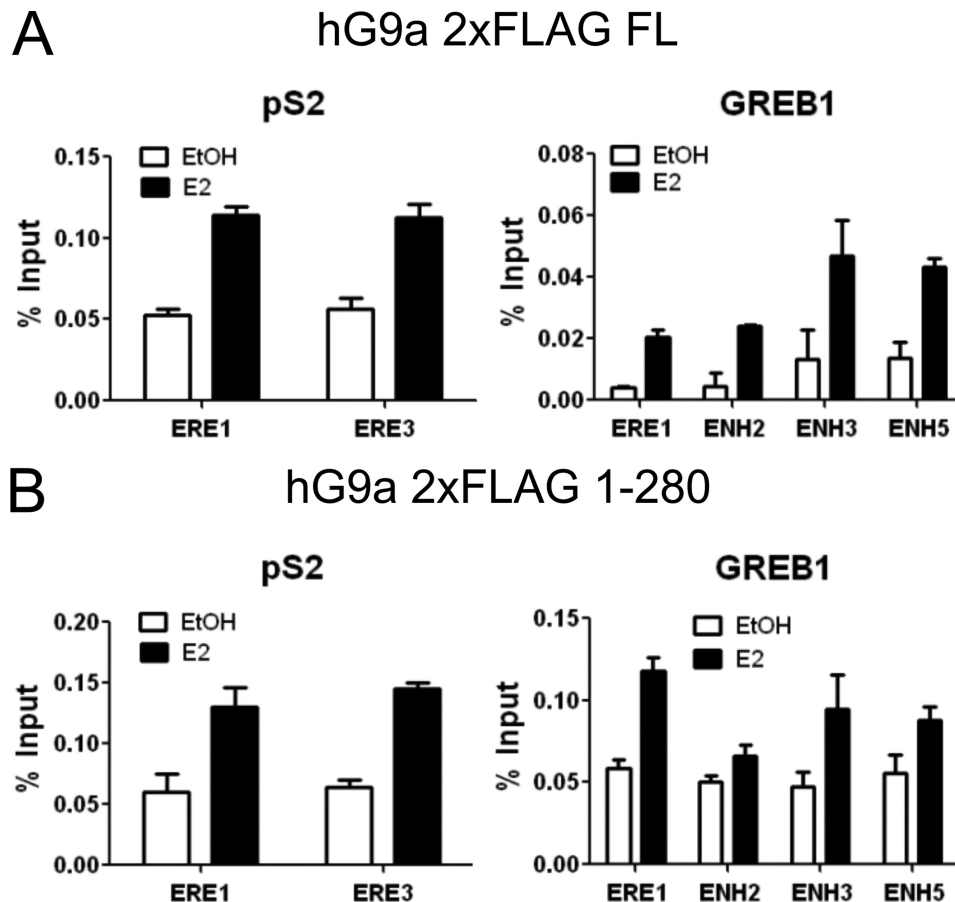


FIGURE 7. **The N-terminal region of G9a is sufficient for E<sub>2</sub>-induced recruitment to endogenous ER $\alpha$  target genes.** MCF-7 cells were transfected with pSG5.2 $\times$ FLAG-hG9a (A) or pSG5.2 $\times$ FLAG-hG9a 1–280 (B), grown for 3 days in hormone-free medium, and treated with 100 nM E<sub>2</sub> for 0 or 15 min. ChIP was performed using antibodies against FLAG and qPCR primers for the ER $\alpha$  binding sites associated with the pS2 and GREB1 genes. Results are shown as percentage of input, are the mean and S.D. for three PCR reactions from the DNA samples from a single experiment, and are representative of two independent biological experiments performed on different days.

domain (25), which is not required for the coactivator function of G9a (Fig. 5). What then is the mechanism by which the N-terminal region of G9a enhances transcription by E<sub>2</sub>-activated ER $\alpha$ ? The N-terminal region of G9a binds to ER $\alpha$  LBD in a hormone-dependent manner (Fig. 6), and it contains a transcriptional activation domain (15). These two activities satisfy the minimum requirements for a transcriptional coactivator: a mechanism for recruitment to regulatory sequences of target genes (in this case through interaction with ER $\alpha$ ) and a mechanism for contributing to transcriptional activation after recruitment to the gene (through the action of an activation domain, which can recruit other coactivators, make contact with other components of the chromatin or transcription machinery, or carry out some enzyme reaction, if the activation domain contains such an activity). Furthermore, we confirmed that the N-terminal fragment of hG9a, which binds ER $\alpha$  and is sufficient for coactivator function in transient reporter gene assays, is also sufficient for E<sub>2</sub>-induced recruitment of G9a to ER $\alpha$  binding sites associated with E<sub>2</sub>-regulated genes (Fig. 7). Thus, we have identified a new set of functions for G9a that allow it to serve as a coactivator. The domains required for positive regulation of transcription are located in a small region at the extreme N terminus of G9a, whereas the domains

required for corepressor function occupy the C-terminal three-quarters of the G9a polypeptide.

*Does G9a Enhance E<sub>2</sub>-induced Transcription by Direct or Indirect Action on Target Genes of ER $\alpha$ ?*—Enhancement of E<sub>2</sub>-induced transcription by G9a could be accomplished by direct action of G9a on the target gene or by indirect actions of G9a. The indirect mechanism could involve repressive action of G9a on another gene encoding a protein that represses expression of the ER $\alpha$  target gene. If the indirect mechanism is correct, we would expect it to involve the previously defined repression mechanisms of G9a carried out by some combination of the SET, ankyrin repeat, and cysteine-rich domains. Because none of these domains was required for G9a coactivator function with ER $\alpha$  on a transient reporter gene (Fig. 5), our results do not support the indirect repressive mode of action by G9a to enhance E<sub>2</sub>-induced expression of some ER $\alpha$  target genes. Furthermore, the N-terminal domain of G9a, which is necessary and sufficient for the coactivator function of G9a, has never been implicated in repression by G9a.

Instead, our results support a direct mechanism of action. The N-terminal domain is capable of binding directly to ER $\alpha$  LBD in a hormone-dependent manner (Fig. 6), occupying genes that are induced by E<sub>2</sub> at the same sites occupied by ER $\alpha$  in



response to E<sub>2</sub> treatment (Fig. 7) and enhancing E<sub>2</sub>-induced expression of a transient reporter gene (Fig. 5). The ER $\alpha$  binding activity (Fig. 6) and transcriptional activation function (15) contained in the N-terminal region provide a plausible mechanism for the coactivator function of G9a. E<sub>2</sub> causes increased occupancy by G9a on the regulatory sites of endogenous target genes of ER $\alpha$ , which require G9a for full E<sub>2</sub>-induced expression. The occupancy of G9a on the target gene thus satisfies the minimum condition for a direct mechanism of positive regulation by G9a. In addition, the sites occupied by G9a are the exact sites occupied by ER $\alpha$  in an E<sub>2</sub>-dependent manner. The E<sub>2</sub>-dependent occupancy of G9a on DNA sites occupied by ER $\alpha$  (Fig. 3) strongly suggests that G9a is recruited by ER $\alpha$ . Although we have not tested whether G9a also interacts with ER $\beta$ , ER $\beta$  is not expressed in MCF-7 cells (26). Our demonstration that G9a directly binds to ER $\alpha$  LBD in a hormone-dependent manner (Fig. 6) provides an explanation of why G9a occupies the same genomic sites occupied by ER $\alpha$  (Figs. 3 and 7). We cannot rule out some additional indirect contribution by G9a in the E<sub>2</sub>-induced expression of ER $\alpha$  target genes, but our results clearly show that G9a is recruited to the endogenous genes in an E<sub>2</sub>-dependent manner and thus can act directly on the target genes.

Because G9a binds *in vitro* to the ER $\alpha$  DNA binding domain and AF-1 regions as well as the LBD, further work will be required to determine whether these additional *in vitro* interactions are functionally relevant *in vivo*. However, the relatively strong hormone-dependent interaction with the ER $\alpha$  LBD would appear to be most relevant and sufficient for recruitment of G9a to target genes by ER $\alpha$ . The most commonly documented mechanism for binding of coactivators to the LBD of a nuclear receptor, including ER $\alpha$ , is through an LXXLL motif in the coactivator, which forms an amphipathic  $\alpha$  helix that binds to a hydrophobic groove in the surface of the ligand-activated receptor (27, 28). However, the region of G9a responsible for binding to ER $\alpha$  LBD does not contain a motif resembling the LXXLL motif, suggesting that G9a binds to the ER $\alpha$  LBD via a novel mechanism. The small ER $\alpha$ -binding fragment of G9a (hG9a amino acids 93–211) defined here may be amenable to structural studies that can define the structural basis of this novel type of coactivator interaction with the ER $\alpha$  LBD.

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## REFERENCES

- Glass, C. K., and Rosenfeld, M. G. (2000) *Genes Dev.* **14**, 121–141
- Métivier, R., Penot, G., Hübner, M. R., Reid, G., Brand, H., Kos, M., and Gannon, F. (2003) *Cell* **115**, 751–763
- Métivier, R., Gallais, R., Tiffocche, C., Le Péron, C., Jurkowska, R. Z., Carmouche, R. P., Ibberson, D., Barath, P., Demay, F., Reid, G., Benes, V., Jeltsch, A., Gannon, F., and Salbert, G. (2008) *Nature* **452**, 45–50
- Lee, D. Y., Teysier, C., Strahl, B. D., and Stallcup, M. R. (2005) *Endocr. Rev.* **26**, 147–170
- Tachibana, M., Sugimoto, K., Fukushima, T., and Shinkai, Y. (2001) *J. Biol. Chem.* **276**, 25309–25317
- Shinkai, Y., and Tachibana, M. (2011) *Genes Dev.* **25**, 781–788
- Milner, C. M., and Campbell, R. D. (1993) *Biochem. J.* **290**, 811–818
- Tachibana, M., Sugimoto, K., Nozaki, M., Ueda, J., Ohta, T., Ohki, M., Fukuda, M., Takeda, N., Niida, H., Kato, H., and Shinkai, Y. (2002) *Genes Dev.* **16**, 1779–1791
- Nishio, H., and Walsh, M. J. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101**, 11257–11262
- Gyory, I., Wu, J., Fejér, G., Seto, E., and Wright, K. L. (2004) *Nat. Immunol.* **5**, 299–308
- Roopra, A., Qazi, R., Schoenike, B., Daley, T. J., and Morrison, J. F. (2004) *Mol. Cell* **14**, 727–738
- Duan, Z., Zarebski, A., Montoya-Durango, D., Grimes, H. L., and Horwitz, M. (2005) *Mol. Cell Biol.* **25**, 10338–10351
- Nagano, T., Mitchell, J. A., Sanz, L. A., Pauler, F. M., Ferguson-Smith, A. C., Feil, R., and Fraser, P. (2008) *Science* **322**, 1717–1720
- Epsztejn-Litman, S., Feldman, N., Abu-Remaileh, M., Shufaro, Y., Gerson, A., Ueda, J., Deplus, R., Fuks, F., Shinkai, Y., Cedar, H., and Bergman, Y. (2008) *Nat. Struct. Mol. Biol.* **15**, 1176–1183
- Lee, D. Y., Northrop, J. P., Kuo, M. H., and Stallcup, M. R. (2006) *J. Biol. Chem.* **281**, 8476–8485
- Chaturvedi, C. P., Hosey, A. M., Palii, C., Perez-Iratxeta, C., Nakatani, Y., Ranish, J. A., Dilworth, F. J., and Brand, M. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106**, 18303–18308
- Kondo, Y., Shen, L., Ahmed, S., Boumber, Y., Sekido, Y., Haddad, B. R., and Issa, J. P. (2008) *PLoS One* **3**, e2037
- Lehnertz, B., Northrop, J. P., Antignano, F., Burrows, K., Hadidi, S., Mul-laly, S. C., Rossi, F. M., and Zaph, C. (2010) *J. Exp. Med.* **207**, 915–922
- Tsai, M. J., and O'Malley, B. W. (1994) *Annu. Rev. Biochem.* **63**, 451–486
- Tee, M. K., Rogatsky, I., Tzagarakis-Foster, C., Cvorro, A., An, J., Christy, R. J., Yamamoto, K. R., and Leitman, D. C. (2004) *Mol. Biol. Cell* **15**, 1262–1272
- Lee, Y. H., Koh, S. S., Zhang, X., Cheng, X., and Stallcup, M. R. (2002) *Mol. Cell Biol.* **22**, 3621–3632
- Koh, S. S., Chen, D., Lee, Y. H., and Stallcup, M. R. (2001) *J. Biol. Chem.* **276**, 1089–1098
- Pan, Y. F., Wansa, K. D., Liu, M. H., Zhao, B., Hong, S. Z., Tan, P. Y., Lim, K. S., Bourque, G., Liu, E. T., and Cheung, E. (2008) *J. Biol. Chem.* **283**, 32977–32988
- Ma, H., Hong, H., Huang, S. M., Irvine, R. A., Webb, P., Kushner, P. J., Coetzee, G. A., and Stallcup, M. R. (1999) *Mol. Cell Biol.* **19**, 6164–6173
- Collins, R. E., Northrop, J. P., Horton, J. R., Lee, D. Y., Zhang, X., Stallcup, M. R., and Cheng, X. (2008) *Nat. Struct. Mol. Biol.* **15**, 245–250
- Paruthiyil, S., Parmar, H., Kerekatte, V., Cunha, G. R., Firestone, G. L., and Leitman, D. C. (2004) *Cancer Res.* **64**, 423–428
- Darimont, B. D., Wagner, R. L., Apriletti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998) *Genes Dev.* **12**, 3343–3356
- Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) *Cell* **95**, 927–937