

Metastasis-Associated Protein 2 Is a Repressor of Estrogen Receptor α Whose Overexpression Leads to Estrogen-Independent Growth of Human Breast Cancer Cells

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Estrogen receptor (ER) α activity is controlled by the balance of coactivators and corepressors contained within cells that are recruited into transcriptional complexes. The metastasis-associated protein (MTA) family has been demonstrated to be associated with breast tumor cell progression and ER α activity. We demonstrate that MTA2 expression is correlated with ER α protein expression in invasive breast tumors. We show that the MTA2 family member can bind to ER α and repress its activity in human breast cancer cells. Furthermore,

it can inhibit ER α -mediated colony formation and render breast cancer cells resistant to estradiol and the growth-inhibitory effects of the antiestrogen tamoxifen. MTA2 participates in the deacetylation of ER α protein, potentially through its associated histone deacetylase complex 1 activity. We hypothesize that MTA2 is a repressor of ER α activity and that it could represent a new therapeutic target of ER α action in human breast tumors. (*Molecular Endocrinology* 20: 2020–2035, 2006)

NUCLEAR RECEPTORS REPRESENT a superfamily of ligand-dependent transcription factors that provide diverse functions in regulating cell growth, differentiation, development, as well as homeostasis upon ligand binding (1–4). Like other family members, estrogen receptor α (ER α) displays a modular struc-

ture with four well-conserved domains: a DNA-binding domain (DBD), an amino-terminal activation function (AF)-1 domain harboring an autonomous activation function, and a carboxy-terminal AF2 domain, which contains a dimerization interface as well as a ligand-binding structure. ER α also contains a hinge region connecting the DNA and hormone-binding domains (HBDs) that encodes a nuclear localization signal (5), but the overall function of which is less well characterized. In addition to being activated by hormone binding, ER α activity is also regulated by coactivator/corepressor coregulatory protein complexes (reviewed in Ref. 4), diverse growth factor signals (6), and mutation (7).

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Abbreviations: AF, Activation function; AR, androgen receptor; ARE, androgen response element; BAH, bromo-adjacent homology; 8-Br-cAMP, 8-bromo-cAMP; DBD, DNA-binding domain; ER α , estrogen receptor- α ; FBS, fetal bovine serum; GST, glutathione-S-transferase; HAT, histone acetyl transferase; HBD, hormone-binding domain; HDAC, histone deacetylase complex; HEK, human embryonic kidney; IB, immunoblotting; IP, immunoprecipitation; MTA, metastasis-associated protein; NaB, sodium butyrate; N-CoR, nuclear receptor corepressor; NuRD, nucleosome remodeling and histone deacetylation; PID, p53 target protein in the deacetylase complex; PKA, protein kinase A; PR, progesterone receptor; PRE, progesterone response element; qRT-PCR, quantitative RT-PCR; RARE, retinoic acid response element; SDS, sodium dodecyl sulfate; SMRT, silencing mediator of retinoid and thyroid receptor; SRC, steroid receptor coactivator; TIF2, transcriptional intermediary factor 2; WT, wild type.

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In the absence of hormone or in the presence of antiestrogen, it is thought that ER α activity is maintained in an inactive state by its recruitment and binding to constitutively associated corepressor histone deacetylase complexes (HDACs); upon exposure to estrogen, these corepressor complexes are released with subsequent transient association of receptor-associated coactivator proteins (8). Whereas corepressor complexes containing nuclear receptor corepressor (N-CoR) and silencing mediator of retinoid and thyroid receptors (SMRTs) possess histone deacetylase activity, the majority of identified ER α coactivators,

such as p300/CBP, steroid receptor coactivator (SRC)1, and SRC3, encode intrinsic histone acetyltransferase (HAT) activity (9, 10). The steady-state level of acetylation at estrogen-responsive promoters is dictated by the balance of HDACs and HATs recruited to these sites. In addition to acetylating the core histones residing at gene promoters and hence modifying chromatin structure, p300 can also directly acetylate ER α and modulate ER α activity (11). Furthermore, p300-mediated acetylation of the SRC3/activator of thyroid receptor coactivator disrupts promoter-bound ER α , which coincides with the attenuation of hormone-induced transcriptional activity (12). Therefore, it has been hypothesized that the processes of protein acetylation and deacetylation may play important roles in ER α activation and/or repression, and that disruption of ER α protein acetylation could result in altered function (11).

We have previously reported a lysine to arginine somatic mutation in the hinge domain of ER α (called K303R ER α) in human premalignant breast lesions, which exhibited altered growth response to hormone (7). This residue was identified independently as a component of a conserved acetylation motif that serves as a substrate for acetylation by p300 (11). Because we also found that, compared with wild-type (WT) ER α , the K303R ER α mutant showed enhanced association with the SRC2 coactivator in response to hormone (7), we questioned whether the mutation might similarly be differentially regulated by receptor corepressors.

Metastasis-associated protein 2 (MTA2), also known as MTA1-like 1 and PID (p53 target protein in the deacetylase complex) belongs to a highly conserved family of proteins originally identified by differential cDNA screening of rat adenocarcinoma cell lines with low and high metastatic potential (13, 14). MTA2, as well as the prototype family member MTA1 (15), is contained in nucleosome remodeling and histone deacetylation (NuRD) complexes that are involved in both nucleosome remodeling and which possess histone deacetylase activity (16). MTA2 expression enhances p53 deacetylation through targeting p53 to HDACs, thereby strongly repressing p53-dependent transcriptional activation (17). In addition, MTA1 directly interacts with ER α , as well as HDAC 1 and 2, and acts as an ER α corepressor protein, where its overexpression can inhibit ER α transcriptional activity (18). We therefore examined whether MTA2 might also function as an ER α corepressor protein and modulate ER α activity through its associated HDAC activity.

Herein we demonstrate that MTA2 binds to ER α and inhibits the transcriptional activity of WT ER α , functioning as a receptor repressor. However, MTA2 overexpression was unable to similarly inhibit the transcriptional activity of the K303R ER α mutant. We found that MTA2-containing HDAC1 complexes can deacetylate p300-acetylated ER α . Taken together, our results suggest that MTA2 is a component of the

NuRD complex, which serves to modulate the acetylation status and activity of ER α .

RESULTS

MTA2 Inhibits ER α Transcriptional Activity and Colony Formation of ER α -Positive Human Breast Cancer Cells

It has been previously reported that MTA1 expression represses ER α transcriptional activity (18) and that MTA3 transcription is hormonally regulated in breast cancer cells (19, 20). To examine the levels of MTA2 protein, we performed immunoblot analysis for endogenous MTA2 in different human breast cancer cells and quantitated levels relative to actin (Fig. 1A, *upper* and *lower panels*, respectively). The levels of MTA2 were about the same in the ER α -positive MCF-7, MDA-MB-361, and BT474 cells. Higher protein levels were seen in the ER α -positive T47D cell line, but lower levels were expressed in the two ER α -negative cell lines, MDA-MB-231 and MDA-MB-435. We next evaluated the levels of MTA2 in 26 human invasive breast tumors using immunoblot analysis (Fig. 1B, *upper panel*), and expressed the levels of MTA2 in the tumors relative to actin (*lower panel*). MTA2 levels were heterogeneous among tumors and ranged from a relative band intensity of 0.03 to a maximum of 0.98. The mean band intensity of MTA2 in ER α -positive tumors was 0.56 ± 0.07 ; in contrast, the mean MTA2 band intensity in ER α -negative tumors was lower (0.19 ± 0.04). Thus, MTA2 levels were significantly correlated with ER α expression ($P = 0.0002$, *lower panel*), which suggests that higher levels of MTA2 are expressed in ER α -positive breast tumors.

To test for MTA2 effects on ER α transcriptional activity, we transiently transfected a MTA2 expression vector into the MCF-7 and T47D cell lines; transcriptional activity of endogenous ER α was then measured using a cotransfected estrogen-responsive luciferase reporter gene (Fig. 1, C and D, respectively). After exposure to 1 nM 17 β -estradiol (E $_2$), ER α activity was increased in the two cell lines. Cotransfection of MTA2 significantly blocked the ability of estrogen to stimulate ER α transcription. Increasing levels of MTA2 in MCF-7 cells reduced estrogen-induced activity in a dose-dependent manner (Fig. 1C). Levels of ER α activity in T47D cells were also reduced (Fig. 1D). MTA2 overexpression also appeared to affect basal activity when high levels were introduced into these cell lines; however, MTA2 had no effect on transcriptional ER α activity in the presence of the antiestrogen tamoxifen (data not shown). The effects of MTA2 on transcription were also confirmed using small interfering RNA (siRNA) knockdown of MTA2 (Fig. 1E). MCF-7 cells were transfected with either control siRNA or a siRNA specific for MTA2. Fold induction of the estrogen-responsive reporter was significantly enhanced with MTA2 siRNA repression ($P < 0.05$, *upper panel*). Re-

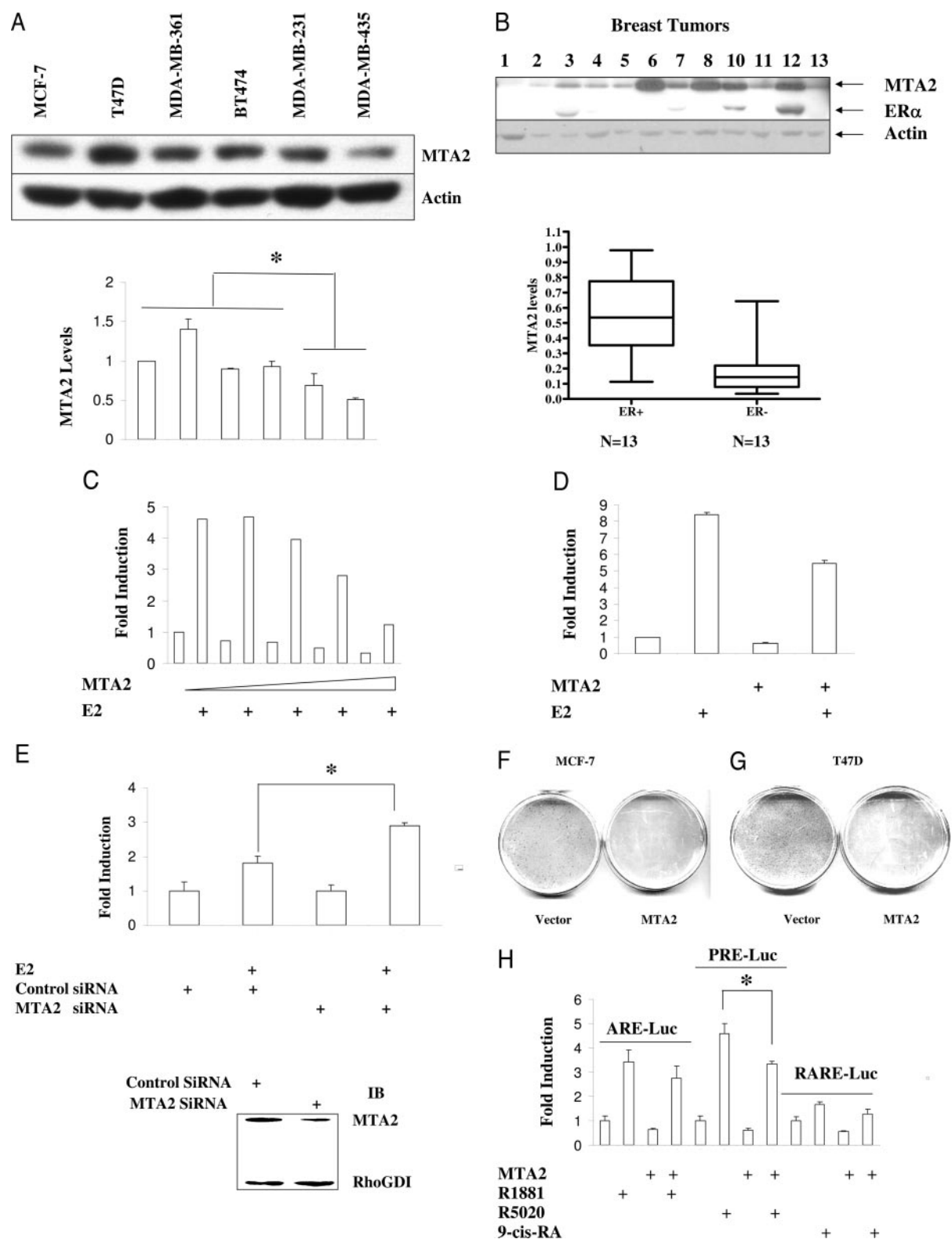


Fig. 1. MTA2 Levels Correlate with ER Expression, and MTA2 Inhibits ER α Transcriptional Activity and Colony Formation in Human Breast Cancer Cells

A, Immunoblot analysis of different breast cancer cell lines with antibodies to MTA2 and β -actin (*upper panel*); the *lower panel* is the relative level of MTA2 normalized to the respective β -actin level and is the average ratio of MTA2/Actin of two independent experiments. Statistical analysis was performed using two-tailed Student's *t* test comparing ER α -positive to negative cell lines ($P = 0.047$). B, Representative immunoblot analysis of 13 invasive breast tumors with antibodies to MTA2, ER α , and β -actin.

duction of MTA2 levels with the use of MTA2 siRNA was confirmed using immunoblot analysis of these cells (*lower panel*). These data demonstrate that MTA2 expression can repress estrogen-induced ER α transcriptional activity in ER α -positive breast cancer cells.

ER α activity is essential for the growth and survival of normal breast epithelium and of hormone-dependent human breast tumor cells. Because MTA2 expression inhibits endogenous ER α transcriptional activity in the hormone-dependent MCF-7 and T47D cells, we next examined the effects of MTA2 on colony formation (Fig. 1, F and G). This assay has previously been used to test the effect of another ER α repressor, the BRCA1 tumor suppressor gene product (21). Either a MTA2 expression vector or a control vector was transfected into MCF-7 and T47D cells, and its ability to form colonies in full serum-containing media was assessed after 14 d of selection with G418 antibiotic. Overexpression of MTA2 inhibited colony formation by 96% and 94%, compared with the vector control groups in MCF-7 and T47D cells, respectively.

To test whether the effects of MTA were specific for ER α , we performed transient transactivation assays using a number of different nuclear receptor luciferase reporters in MCF-7 cells (Fig. 1H), as well as transient transactivation and colony formation assays in the ER α -negative MDA-MB-231 breast cancer cell line (Fig. 1, I–L). Although MTA2 exhibits strong transcriptional repression of promoters containing Gal4-binding sites when it is fused to a Gal4 DNA binding domain (22), two other nuclear receptors, androgen receptor (AR) activity measured with an androgen response element (ARE)-Luc and retinoic acid receptor activity with a retinoic response element (RARE)-Luc reporter, were not modulated after MTA2 overexpression in MCF-7 cells, although basal activity was affected (Fig. 1H). However, progesterone-induced and basal transactivation, as assayed with a progesterone response element (PRE)-Luc reporter, was reduced with MTA2 overexpression (Fig. 1H).

In contrast, there was no estrogen-induced ER α transcriptional activity, or MTA2-induced repression in ER α -negative MDA-MB-231 cells (Fig. 1I), whereas there was a slight decrease (13–15%) in colony-forming ability when MTA2 was expressed in these cells

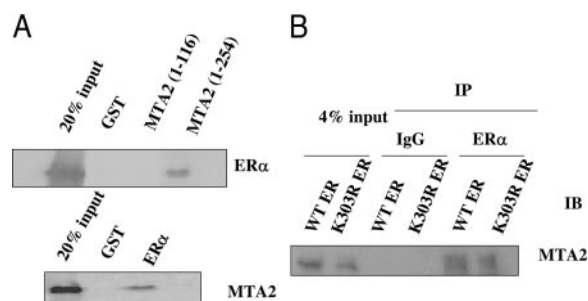


Fig. 2. MTA2 Is an ER α -Binding Protein

A, *In vitro* interaction: ER α and MTA2 were labeled with [³⁵S]methionine by *in vitro* translation and tested for interaction with GST-MTA2 and GST-ER α , respectively, in the absence of ligand. B, *In vivo* interactions: HeLa cells were transiently transfected with Flag-MTA2 and expression vectors for either HA-tagged WT or K303R ER α . The cell lysates were immunoprecipitated (IP) with either anti-HA monoclonal antibody-conjugated Sepharose (ER α) or protein G Sepharose (IgG), negative control. The immunoprecipitates were then subjected to immunoblot analysis (IB) with an anti-M2 antibody.

(Fig. 1, J and L). When ER α was coexpressed with MTA2 into these cells, estrogen-induced activity was reduced (Fig. 1K). Furthermore, ER α expression greatly enhanced the effect of MTA2 on colony formation in MDA-MB-231 cells; colony formation was reduced by 95% when ER α was coexpressed with MTA2 (Fig. 1L), similar to the reduction in colonies observed in MCF-7 and T47D cells when MTA2 was overexpressed (Fig. 1, F and G). Therefore, the major effects of MTA2 on estrogen-induced activity and growth were related to coexpression of ER α .

MTA2 Binds to ER α and Mapping of Their Interaction Sites

The SMRT and N-CoR ER α transcriptional repressors bind directly to nuclear receptors (23, 24), as do several of the components of the NuRD complex, e.g. HDAC1 (25, 26). To determine whether MTA2 can bind ER α , we performed *in vitro* glutathione S-transferase (GST)-pull-down binding experiments, which give a

Levels of MTA2 are normalized for the levels of β -actin in each tumor, with the entire cohort of ER α -positive ($n = 13$) and ER α -negative ($n = 13$) tumors displayed in a Whisker-box plot in the *lower panel* ($P = 0.0002$). Boxes and whiskers indicate minima, maximum, and means of expression. C, Transfection of 1 μ g ERE₂-tk-Luc with increasing amounts of expression vector plasmids of either MTA2 (50, 100, 200, 800 ng) or empty vector into MCF-7. D, Transfection of 1 μ g ERE₂-tk-Luc with expression vector plasmids of either MTA2 (800 ng) or empty vector into T47D, or MDA-MB-231 cells (panels I and K), treated with 10^{-9} M E₂ for 18–24 h; in panel K, increasing amounts of an expression plasmid for ER α (10, 50, 200 ng) was co-expressed. E, Transfection of MCF-7 cells with 1 μ g ERE₂-tk-Luc plus 200 ng control siRNA or a siRNA specific for MTA2 (*, $P < 0.05$). An immunoblot of transfected cells with MTA2 and RhoGDI as a loading control is shown in the *lower panel*. Panels F, G, J, and L represent the colony formation assays of either MTA2 expression plasmid or control vectors transfected into MCF-7 (panel F), T47D (panel G), or MDA-MB-231 (panels I and K) cells; in panel L, either vector alone, MTA2 (1 μ g), and MTA2 (1 μ g) plus ER α expression plasmid (4 μ g) were transfected. H, Transfection of 1 μ g ARE-Luc, PRE-Luc, or RARE-Luc with 0.8 μ g of expression vector plasmids of either MTA2 or empty vector into MCF-7 cells, treated with 10^{-9} M R1881, R5020, or 9-*cis*-RA, respectively, for 18–24 h. Luciferase activity was normalized to the cotransfected pCMV- β -gal vector and represented as fold induction. The data shown represent the mean \pm SEM for three independent experiments (*, $P < 0.05$). 9-*cis*-RA, 9-*cis*-Retinoic acid.

qualitative assessment of direct interaction (Fig. 2). Incubation of *in vitro* translated ER α with two GST-MTA2 fragments bound to glutathione-Sepharose beads (Fig. 2A, *top panel*), or *in vitro* translated full-length MTA2 with GST-ER α (Fig. 2A, *lower panel*) demonstrated an interaction between the two proteins. All incubations were performed in the absence of estrogen. There was no binding when ER α or MTA2 was bound to GST only, and an ER α interaction domain exists in MTA2 between residues 116–254.

To examine whether the interaction between MTA2 and ER α occurs in cells, we transiently transfected HeLa cells with Flag-tagged MTA2, and either WT or the K303R mutant ER α (Fig. 2B). Immunoprecipitation (IP) of ER α followed by immunoblotting (IB) for MTA2 with an antibody to the Flag peptide tag revealed a band with the molecular mass of MTA2; MTA2 bound to both WT and the K303R ER α mutant. MTA2 was not present in IgG control-precipitated complexes. Thus, MTA2 is an ER α binding protein.

ER α contains at least four functional domains, and the ability of MTA2 to interact with these different domains was next examined using GST-pull-down assays. The different GST-ER α fusion proteins were separated by SDS-PAGE and stained with Coomassie blue, to ensure that the input of immobilized GST fragments was equal (data not shown). We examined MTA2 interactions with GST alone, ER α AF1, DBD, Hinge, and AF2 domains (Fig. 3A). All incubations were done in the absence of hormone. We found that MTA2 interacted with all of the ER α domains except the amino-terminal AF1 region. Thus, as described for a number of repressors including MTA1, MTA2 interacts with multiple regions of ER α . Figure 3B (*upper panel*), examines the binding of MTA2 to the isolated hinge domain of ER α and the K303R mutant (7); the input is shown in the *lower panel*. Binding of WT and the K303R ER α mutant to MTA2 were equivalent. In addition, using IP we examined the binding of MTA2 to an ER α AF2 domain mutation (ER α -3X), which abolishes ER α transcriptional activity (Fig. 3C). We observed that MTA2 bound similarly to this mutant as well. These data suggest that the acetylation status of the ER α hinge domain and the transactivation potential of the AF2 domain are not necessary for MTA2 interactions with ER α (shown schematically in Fig. 3D).

MTA2 also encodes several functional domains (27). We used a GST-ER α hinge/AF2 fusion fragment (residues 251–595) immobilized on glutathione beads, and incubated this with a number of deletion fragments of MTA2, and we also analyzed several MTA2 mutants for their effects on ER α transactivation (Fig. 3, E–G, and summarized in Fig. 3H). Only the MTA2 1–116 fragment failed to interact with ER α (Fig. 3E). A MTA2 construct containing an internal deletion between residues 115–254 was capable of repressing ER α activity comparable to full-length MTA2, and a MTA2 deletion construct containing residues 1–116 blocked its ability to act as an ER α repressor (Fig. 3F, *upper panel*). These deletion constructs were expressed equivalent

to full-length MTA2 (*lower panel*). Thus, the MTA2 leucine zipper motif is not essential for ER α interaction because its removal in the internal deletion construct did not affect binding or activity. Because the MTA2 1–116 region containing only the bromo-adjacent homology (BAH) domain failed to bind or repress ER α activity, binding may be necessary for MTA2's ER repressor function. Also, it appears that binding probably occurs across multiple regions of MTA2.

The ER α AF2 domain contains a conserved amphipathic α -helix structure in helix 12, which is essential for hormone-inducible function. Upon hormone binding, this helix folds back and generates a transcriptionally active conformation (28). We next investigated whether hormone would affect ER α -MTA2 interactions using GST-pulldown of the ER α hinge and AF2 residues 251–595, and by co-IP of ER α and MTA2 in cells. Figure 4A demonstrates that binding was ligand independent and that the K303R ER α mutation within the hinge domain does not alter this binding. In Fig. 4B, estrogen also had no effect on *in vivo* binding as assessed by IP; the coactivator transcriptional intermediary factor 2 (TIF2) was used as a positive control of estrogen-induced ER α binding in this assay. These results suggest that MTA2 binding is independent of estrogen.

MTA2 Is an Inadequate Repressor of the K303R ER α Acetylation Mutant

MTA2 is a component of the core NuRD complex (29) and inhibits the transcriptional activity of WT, but not a p53 mutant harboring mutations in potential acetylation sites (17). This raises the possibility that MTA2 may only function on acetylated or acetylation-competent p53 protein in addition to its effects on chromatin. We have previously reported that the K303R hinge mutation disrupts an acetylation site of ER α , generating a hypoacetylated form of the receptor (11). To address whether this mutation could compromise the effects of MTA2 on ER α function, we performed transient transactivation assays (Fig. 5). MTA2 expression vectors were cotransfected with either WT or the K303R ER α mutant DNA into both MCF-7 and T47D breast cancer cells. In the conditions of this assay, using coexpression of exogenous ER and MTA2, the mutant exhibits a lower fold of induction than wild type. As was observed with endogenous ER α in Fig. 1, overexpression of MTA2 repressed exogenous WT ER α activity by 50% and 62% in MCF-7 and T47D cells (Fig. 5, panels A and B, respectively). However, MTA2 expression had no effect on the K303R ER α mutant's transcriptional activity, showing that the mutation impaired MTA2-mediated repression. A similar lack of repression of this mutant was seen with two other ER α corepressors, BRCA-1 and N-CoR (results not shown). Similar levels of MTA2 and ER α protein were expressed in transient transfections as demonstrated by immunoblot analysis (Fig. 5C). These results suggest that the acetylation potential of the receptor is

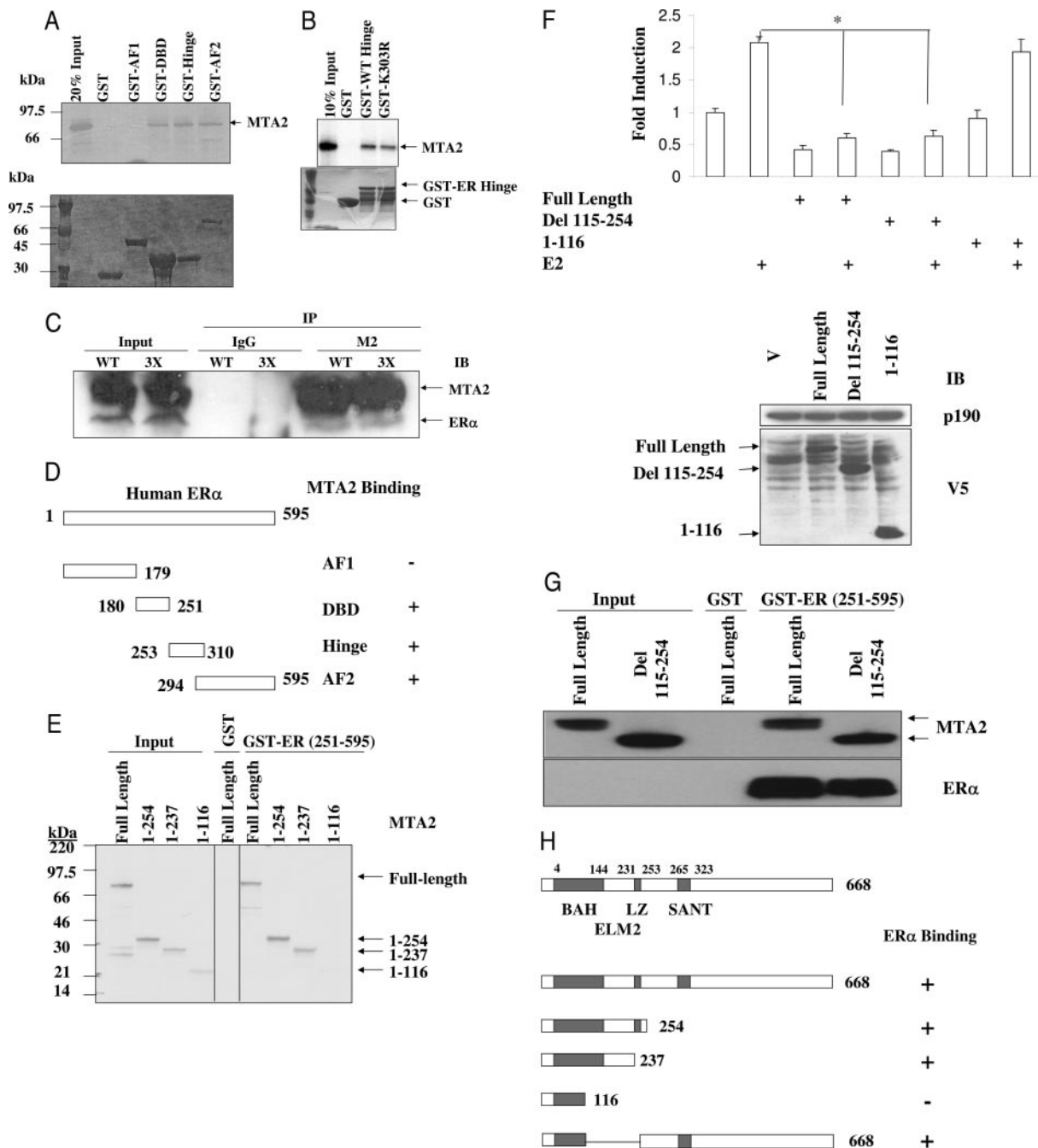


Fig. 3. Mapping of the Interaction Sites between ER α and MTA2

A, To map ER α binding sites in MTA2, different GST-fusion fragments of ER α were incubated with *in vitro* translated full-length MTA2 in the absence of ligand and analyzed by both autoradiography and Coomassie blue staining. B, GST-WT and K303R mutant ER α hinge fragments were incubated with full-length MTA2 and analyzed by both autoradiography and Coomassie blue staining. C, HEK293 cells were cotransfected with MTA2 and either WT ER α or ER α -3X, followed by IP with an anti-M2 antibody to recognize the Flag tag, and immunoblot analysis for ER α . D, Schemata of MTA2 binding to ER α regions. E, To map MTA2 binding sites in ER α , full-length and different fragments of MTA2 were labeled with [³⁵S]methionine using *in vitro* translation and tested for interaction with equal amounts of a GST-ER α hinge-AF2 domain region fragment in the absence of ligand, and analyzed by autoradiography. F, MTA2 full-length (FL) or a mutant deleting residues 115–254 or a mutant containing only residues 1–116 was used in a transactivation assay in MCF-7 cells (*upper panel*) (*, *P* = 0.001); levels of introduced MTA2 protein are shown in the immunoblot analysis (V5) in the *lower panel*; p190 was used a loading control. G, GST pull-down assay with ER fragment 251–595 and MTA2 full length or deleted for 115–254. H, Schemata showing MTA2 structural domains and summarization of MTA2 binding to ER α . Del, Deletion of; LZ, leucine zipper; ELM2, EGL-27 and MTA1 homology.

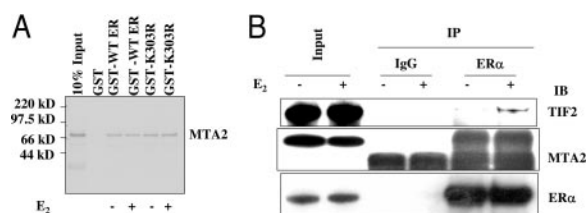


Fig. 4. Hormone Binding Does Not Alter the Interaction between ER α and MTA2

A, Full-length MTA2 was labeled with [35 S]methionine using *in vitro* translation and interacted with equal amounts of either GST-WT ER α or GST-K303R ER α (residues 251–595) in the absence or presence of 1 nM E $_2$. B, MCF-7 cells were treated with 10^{-7} M E $_2$ for 30 min, and cell lysates were prepared, after which they were immunoprecipitated with anti-ER α polyclonal antibody H184 or rabbit IgG as a negative control. The immunoprecipitates were then subjected to electrophoresis and staining with an anti-M2 antibody to detect MTA2, anti-TIF2 antibody, and anti-ER α 6F11. TIF2-ER α co-IP was used as a positive control.

important for MTA2's repressor effect on ER α transcriptional activity in breast cancer cells.

MTA2-Associated HDAC1 Deacetylase Activity Mediates ER α Deacetylation

Experiments were next performed to investigate whether MTA2 participates in ER α deacetylation. We first established a T47D cell line overexpressing Flag-MTA2 (Fig. 6A) to obtain purified MTA2-associated deacetylase complexes for functional analysis on ER α protein. Although stable transfection of MTA2 into these cells resulted in a low yield of G418-resistant colonies (data not shown), we were successful in obtaining a number of T47D subclones that stably overexpressed MTA2. High-salt cell extracts from these cells were then subjected to affinity chromatography on M2-sepharose and the Flag-MTA2 deacetylase complexes were eluted with Flag peptide. This technique has been previously used to demonstrate the effect of MTA2 on p53 acetylation (17). Western blot analysis indicated that both HDAC1 and ER α were contained in the MTA2-purified complex (Fig. 6B, IP M2), but not in the control (Protein G IP complex). This demonstrates that both HDAC1 and ER α are specifically associated with overexpressed MTA2 protein in these cells.

These complexes were then tested for deacetylation activity on ER α previously acetylated *in vitro* by p300 HAT (Fig. 6C). As expected, WT ER α was efficiently acetylated by GST-p300, but the K303R ER α mutant was deficient in acetylation (acetylation control). Incubation of WT or the K303R ER α with the control eluate had no effect on the acetylation status of the receptors. However, WT ER α was deacetylated by the purified MTA2-associated complex (MTA2 eluate), suggesting that the MTA2 complex can indeed deacetylate ER α , probably through its associated HDAC1 deacetylase activity.

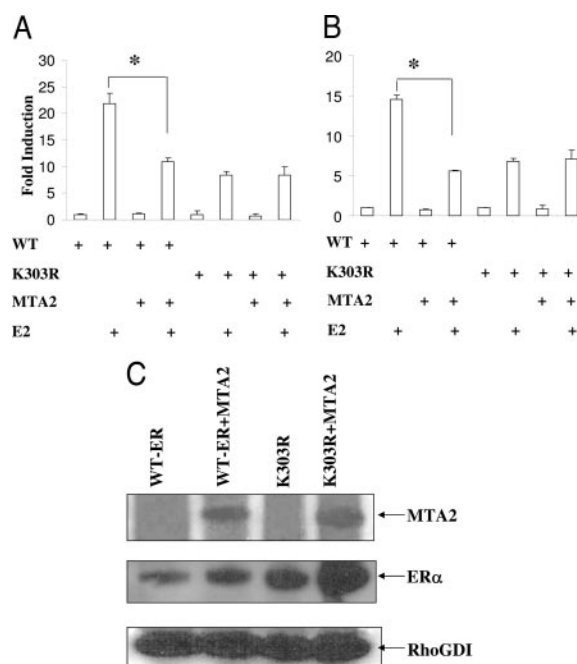


Fig. 5. MTA2 Is an Insufficient Repressor of the K303R ER α Mutant

Cotransfection of ERE-tk-Luc (1 μ g), and either WT or K303R ER α vectors (25 ng) with expression vectors MTA2 or empty vector into MCF-7 (panel A) or T47D cells (panel B) treated with 1 nM E $_2$ for 18–24 h (*, $P < 0.0001$). C, An immunoblot analysis of MCF-7 cells transfected with the ER α and MTA2 vectors is shown to demonstrate that the exogenous proteins were similarly expressed. Luciferase values were normalized to a cotransfected pCMV- β -gal control vector and expressed as fold induction relative to control. Transactivation data shown represent the mean \pm SEM for three separate transfections for each experiment.

We next coexpressed ER α and MTA2 siRNA into C4–12–5 cells, a subline of MCF-7 cells that are devoid of ER α expression (30), and used IP with an antiacetylated-lysine antibody, followed by IB analysis with an antibody to ER α (Fig. 6D). When MTA2 siRNA was coexpressed with ER α , we observed an increase in ER α acetylation (Ac-K siMTA2 lane), compared with the siRNA control (siCtrl) without affecting the total levels of ER α (Input lanes). Confirmation of MTA2 knockdown is shown in the IB in the *lower panel*. To block MTA2-associated HDAC1 activity we next used the HDAC inhibitor sodium butyrate (NaB) in transactivation assays in HeLa cells (Fig. 6E). As expected NaB enhanced estrogen-induced activity of WT ER α (11), enhanced activity of the K303R mutant receptor, and abrogated MTA2's repressor effects. In these cells, WT and mutant activity in the presence of estrogen was about the same, highlighting the differential results that can be obtained in transactivation experiments depending on the cellular background. The relationship between ER α activity and the selective knockdown of MTA2 by siRNA, or inhibition of HDAC activity, further supports our conclusion that MTA2 is

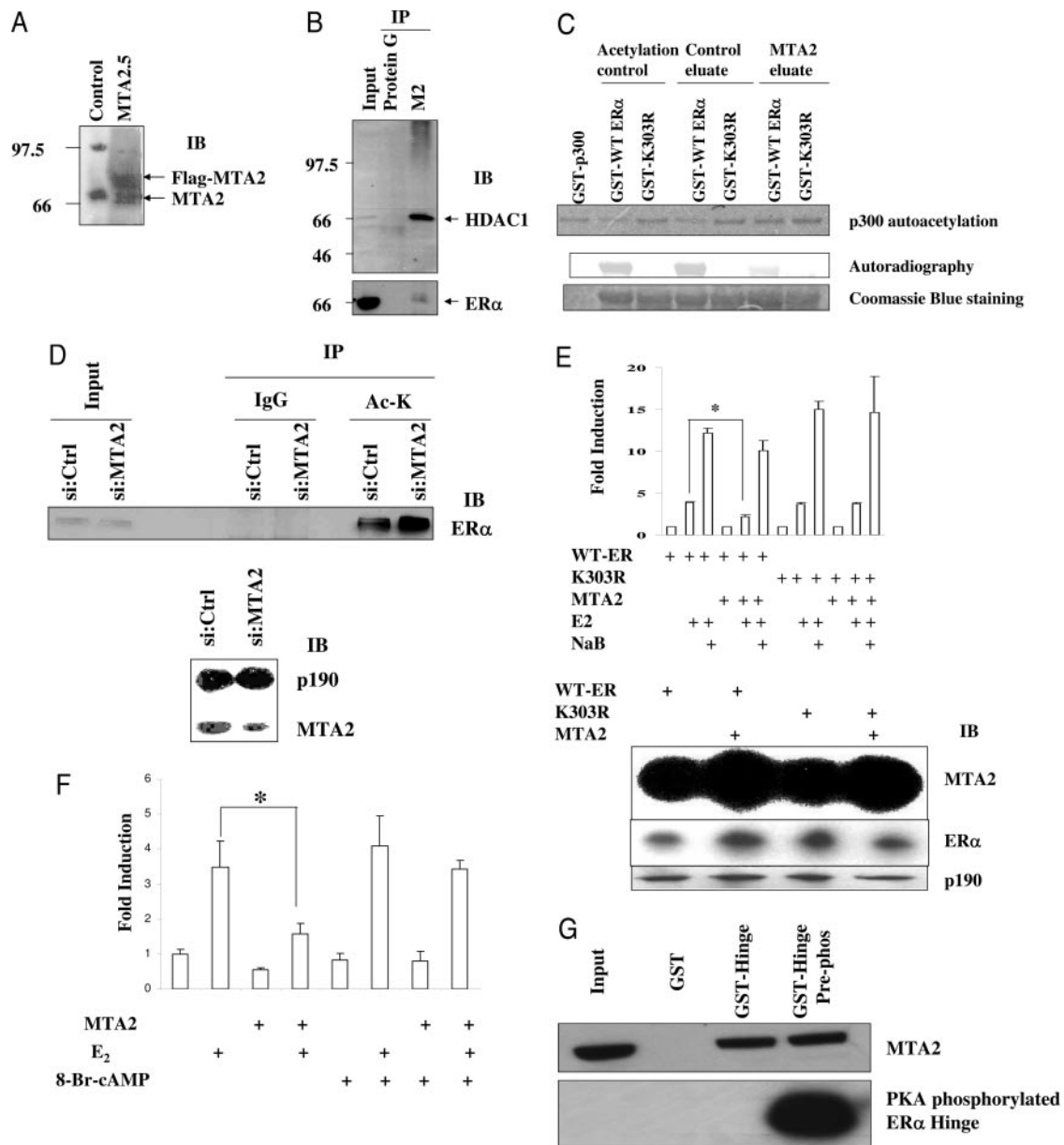


Fig. 6. MTA2-Associated Complexes Participate in the Deacetylation of ER α

A, T47D cells were engineered to overexpress either Flag-tagged MTA2 or a control vector. Cell lysates from one control and a MTA2-overexpressing clone were resolved by electrophoresis, and an anti-PID polyclonal antibody was used to detect the expression of Flag-MTA2. Endogenous MTA2 is shown *below* the exogenous Flag-MTA2, as denoted with *arrows*. B, Cell lysates from T47D cells overexpressing MTA2 were subjected to IP with either protein G Sepharose as a negative control, or anti-M2 Sepharose. The immunoprecipitates were then eluted with Flag peptide and resolved by electrophoresis. Anti-HDAC1 antibody was used to detect the presence of HDAC1. The same immunoblot membrane was then stripped, and the presence of ER α was detected with a specific anti-ER α antibody. C, Purified GST-WT or K303R ER α hinge fragments were acetylated by purified p300 HAT protein and then deacetylated using the control and MTA2 eluates. The reactions were resolved by electrophoresis and analyzed by both Coomassie blue staining and autoradiography. D, The MCF-7 subline, C4-12-5, which is devoid of endogenous ER α , was cotransfected with 1 μ g ER α , and either a control siRNA or an siRNA to MTA2 (4 μ g), immunoprecipitated (IP) with an antibody to acetylated lysine (Ac-K), and immunoprecipitates were immunoblotted with an antibody specific for ER α (*upper panel*). An immunoblot of MTA2 is shown in the *lower panel*; p190 levels were used as a loading control. E, HeLa cells were transfected with 1 μ g of an ERE₂-tk-Luc reporter, 100 ng WT or K303R ER α , and 200 ng MTA2 in the presence or absence of NaB and 10⁻⁹ M E₂ (*, $P < 0.001$). An immunoblot demonstrating the levels of exogenous MTA2 and ER α is shown in the *lower panel*; p190 levels were used as a loading control. F, Transfection of 1 μ g of an ERE₂-tk-Luc reporter and 0.8 μ g of a MTA2 expression vector or control empty vector into MCF-7 cells. The cells were treated 24 h later with 10⁻⁹ M E₂ and/or 100 nM 8-Br-cAMP for 18–24 h. Luciferase activities were normalized to the cotransfected β -galactosidase activity and are expressed as fold induction compared with control. The data shown represent the mean \pm SEM for three independent experiments (*, $P < 0.001$). G, GST-hinge fragments were prephosphorylated with PKA, used for GST pull-down with *in vitro*-translated MTA2, and analyzed by autoradiography and IB with antibody to MTA2. si:Ctrl, control siRNA; Pre-phos, prephosphorylated.

involved in ER α transcriptional activity in breast cancer cells.

We have recently demonstrated that activation of protein kinase A (PKA) signaling by the cell-permeable cAMP analog 8-bromo-cAMP leads to phosphorylation of ER α at serine 305, which blocks protein acetylation at lysine 303 (30). We therefore used 8-bromo-cAMP (8-Br-cAMP) treatment and examined the effect of MTA2 expression on ER α transactivation in MCF-7 cells (Fig. 6F). As expected, MTA2 overexpression inhibited estrogen-induced ER α transcriptional activity in these cells. Blocking ER α acetylation using 8-Br-cAMP treatment blocked MTA2 repression of ER α activity.

We also used a GST-ER α hinge construct containing the S305 phosphorylation site and tested the ability of MTA2 to bind this fragment after phosphorylation by PKA (Fig. 6G). MTA2 can still bind to the hinge region when S305 is phosphorylated (compare GST hinge *vs.* GST-hinge Prephos). Therefore, with this cumulative data we hypothesize that MTA2's repressor effect on WT ER α transcriptional activity may be due to its ability to modulate ER α deacetylation, and that the K303R ER α mutant was refractory to this inhibition because of its hypoacetylated status. Phosphorylation and acetylation of the receptor do not appear to modulate MTA2 binding; however, these results suggest that MTA2 may function as an ER α transcriptional repressor via its effects on acetylation.

MTA2 Overexpression Renders Breast Cancer Cells Unresponsive to Hormone

Expression of both MTA1 and MTA3 correlates with the invasive growth potential of breast cancer cells (18, 31). Unfortunately, there are no knockout models of MTA2 available to examine for effects without background levels of endogenous WT MTA2 protein. Therefore, to delineate the potential effects of MTA2 on growth, we had to examine the anchorage-independent growth of T47D (endogenous ER $^+$ /MTA2 $^+$) cells stably overexpressing exogenous MTA2 (Fig. 7A). E $_2$ enhanced the ability of T47D vector control-transfected cells to form colonies in soft agar (labeled V1 and V2, E $_2$), and the antiestrogen tamoxifen inhibited this increase in colony formation (V1 and V2, E $_2$ +Tam). In contrast, overexpression of MTA2 markedly enhanced anchorage-independent growth in the absence of estrogen (clones MTA2-2.5 and 2.8). Interestingly, the two MTA2-overexpressing clones were also resistant to the growth-inhibitory effects of tamoxifen (Tam and E $_2$ +Tam). To explore the role of ER α in the tamoxifen-resistant growth of MTA2-overexpressing cells, we examined the effect of the pure steroidal antiestrogen, ICI 182,780, on colony formation and found that ICI inhibited the growth of the MTA2 transfectants in the presence of tamoxifen (Fig. 7B). The ICI compound is known to induce ER α degradation (32), and, indeed, ER α levels were reduced in both vector and MTA2.5 cells when they were treated

with ICI (Fig. 7C). We conclude that ER α is involved in MTA2-induced hormone-independent growth.

An important question is whether the hormone independence observed with MTA2 overexpression was associated with a concomitant loss in ER α expression and/or gene regulation. To examine this, we treated vector-control, and T47D-MTA2 stable transfectants with estrogen or tamoxifen and performed immunoblot analysis for MTA2, ER α , and the endogenous estrogen-induced proteins, progesterone receptor (PR) A and B isoforms and the sodium/hydrogen exchanger, solute carrier family 9, isoform 3 regulator factor 1 (SLC9A3R1; also called EBP50 or NHERF) (Fig. 7D). Both of these proteins contain estrogen-responsive elements in their promoters that confer estrogen inducibility (33, 34). The levels of endogenous MTA2 and exogenously expressed Flag-tagged MTA2 are shown in the first and second panels, respectively. As previously demonstrated by others (32, 35), the levels of endogenous ER α protein were down-regulated with estrogen, and tamoxifen treatment increased ER α levels in vector-control cells (V1 and V2, E or T, respectively). In contrast, estrogen-induced degradation was not observed in the MTA2 transfectants (MTA2.5 and MTA2.8). Thus, although ER α expression is maintained in these cells, its regulation appears to be altered. In addition, the MTA2 transfectants have dramatically reduced induction of the PR-A and B isoforms, as well as SLC9A3R1. Of particular note is that the levels of the PR-A and B isoforms are altered, resulting in a relative excess of PR-A in the transfectants compared with the vector-control cells. Actin was used as the loading control in this immunoblot analysis.

The pS2 gene contains a relatively simple promoter with classical estrogen response elements, compared with the PR promoter, which contains heterogeneous elements (36). Thus, we also examined the effect of MTA2 on the levels of pS2 mRNA using quantitative RT-PCR (qRT-PCR) (Fig. 7E). pS2 mRNA levels were up-regulated 12-fold with estrogen treatment in vector control cells (V2), but only 3-fold in MTA2-overexpressing cells (MTA2.5 and 2.8). Basal pS2 mRNA levels were reduced to approximately 20% of control untreated levels, and estrogen-induced levels were repressed to about 7% of control-treated levels in the two MTA2 transfectants; thus, the effect of MTA2 was more pronounced on the estrogen-induced activity. These results suggest that the estrogen- and tamoxifen-unresponsive phenotype associated with MTA2 overexpression could be mediated through a repression of endogenous ER α -induced gene expression.

DISCUSSION

Breast cancer is one of the most common and lethal female cancers in the United States. It is known that ER α plays a central role in breast carcinogenesis (37),

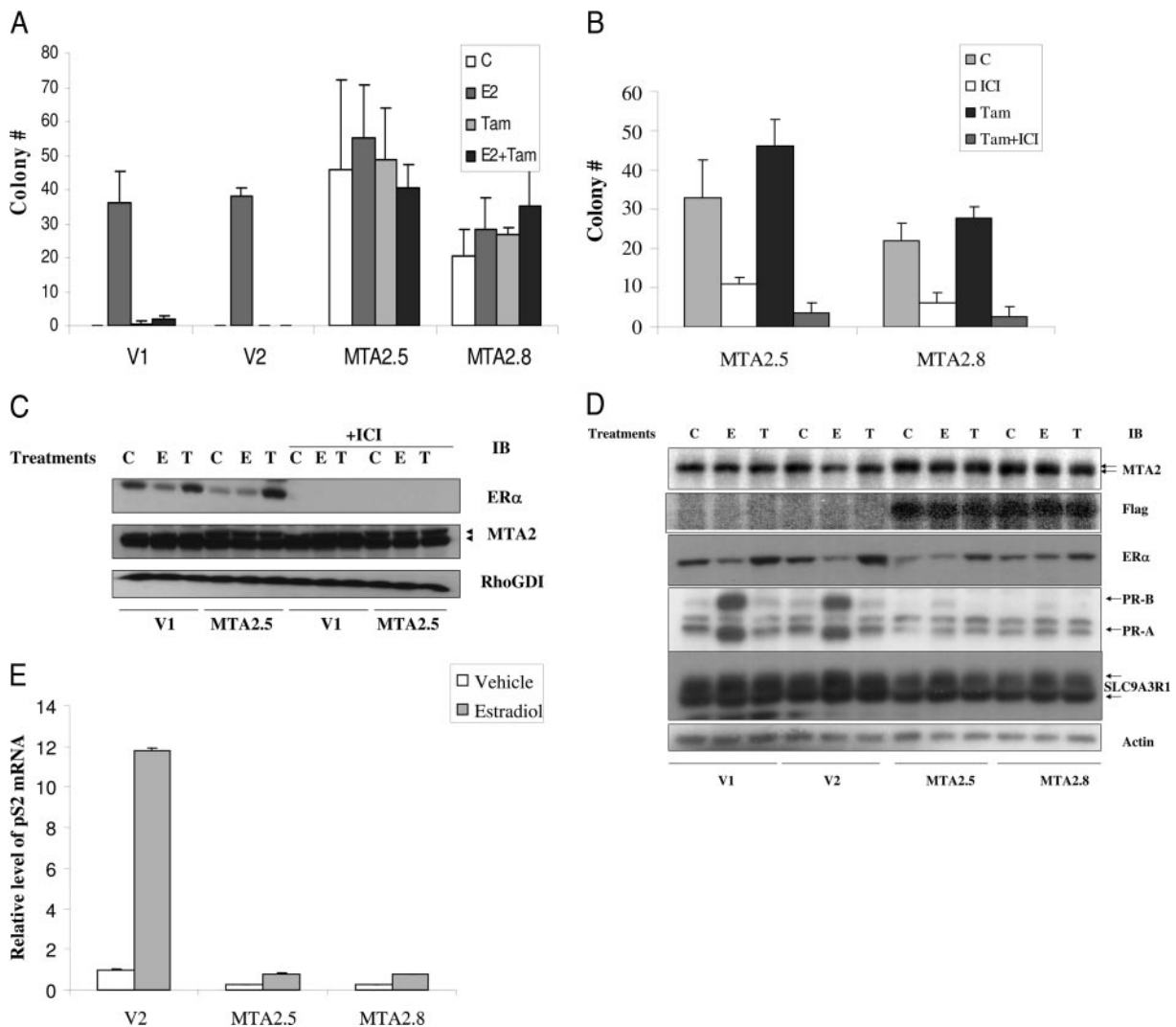


Fig. 7. MTA2 Overexpression Facilitates Hormone-Independent Growth

A, Soft agar cloning assay of two vector control-transfected (V1 and V2), and two MTA2-transfected T47D clones (MTA2.5 and MTA2.8) in the presence of vehicle (C), 1 nM E₂, 100 nM Tam, or E₂+ Tam. B, Soft agar cloning assay of MTA2.5 and 2.8 treated with vehicle, 10 nM ICI 182,780 (ICI), Tam, or Tam+ICI. C, Immunoblot analysis of vector control (V1) or MTA2.5 after treatment with E₂ or Tam +/- ICI. Immunoblots were performed using antibodies to ER α , MTA2, or RhoGDI as a loading control. D, Immunoblot analysis of the T47D MTA2.5 and 2.8 transfectants with antibodies to MTA2, Flag, and ER α and the estrogen-inducible proteins PR-A, PR-B, and SLC9A3R1, with β -actin as a loading control. E, qRT-PCR of pS2 RNA in control or E₂-treated vector (V2), MTA2.5, and MTA2.8 transfectants. The vehicle-treated and the E₂-treated groups of four replicates were compared by Student's *t* test, *P* < 0.0001. Tam, Tamoxifen.

and this forms the foundation for the use of various hormonal treatment strategies directed toward estrogen depletion by the use of aromatase inhibitors, or estrogen antagonism via selective ER modulators, such as tamoxifen. However the successful use of these strategies is often limited by the development of resistance through the occasional loss of ER α expression (38), or altered growth factor cross-talk and cellular signal transduction pathways (39, 40). It is hoped that a thorough understanding of the molecular basis for estrogen signaling will help us to identify new strategies to thwart the development of resistance in breast cancer patients.

Generally, when ER α is bound to tamoxifen, it recruits corepressors and different HDAC complexes to the promoter of estrogen-responsive genes, and thereby attenuates promoter activity (26). It is thought that the steady-state level of acetylation, either at the promoter or of the transcription factor itself, is dictated by the balance of repressors and activator proteins present. The pivotal discoveries that members of the MTA family of proteins, which are present in distinct NURD complexes (16, 29), act as direct modulators of estrogen action and breast cancer growth pathways (18, 31), provides another level of complexity to the negative control of estrogen action.

In this report, we demonstrate that MTA2 is an ER α -binding protein and repressor of estrogen action. MTA2 protein levels are significantly higher in ER α -positive invasive breast tumors and cell lines. In addition, overexpression of exogenous MTA2 rendered ER α -positive breast cancer cells unresponsive to hormonal stimulation, the molecular basis of which we do not yet understand, but which is associated with a loss in estrogen-inducibility of estrogen-regulated genes. Furthermore, we provide evidence that MTA2 overexpression can enhance ER α deacetylation. ER α corepressors, such as N-CoR, are recruited to HDAC complexes (41), probably through their conserved SW13, ADA2, NcoR, and TFIIIB domains (42), suggesting that their repressor functions are related to their associated HDAC activity. Because we are unable to differentiate the effects of MTA2 on ER α activity from its known effects on chromatin in these experiments, it is probable that some of the estrogen-independent effects we observed could be indirect, and occur through its associated HDAC activity. It is also possible that some of the observed biological effects, such as reduced colony formation and hormone-independent growth, could be attributed to other nonspecific transcriptional repressor effects of MTA2. We hypothesize that MTA2 represses ER α activity, at least in part, through its enhancement of ER α deacetylation. It must be stressed, however, that until a knockout model of MTA2 becomes available, it is difficult to assess the complete functional effects of MTA2 within the confounding background of endogenous protein. However, even with this potential limitation, our results suggest that altered MTA2 levels, as well as ER α deacetylation, could prove to play important roles in breast tumor progression.

The HBD of ER α contains a large hydrophobic cleft composed of helices H3, H5, and H12 that binds coregulatory proteins (43). It has been proposed that corepressors such as N-CoR are also capable of binding HBD residues outside of the AF2 core region contained within helix 12 (44, 45). It is well known that receptor coactivators and corepressors compete for binding to the AF2 surface, and hormone binding influences the exchange of these coregulators (reviewed in Ref. 46). We show that MTA2 interacts with several discrete regions of ER α , including the receptor hinge domain, in a constitutive manner. Although evidence implicates N-CoR and SMRT in ER α antagonist activity, it has been difficult to demonstrate a direct interaction between these two repressors and ER α in the presence of tamoxifen, leading to one hypothesis that other corepressors might be more central for ER α signaling (45). Thus, the MTA family of proteins may prove to serve a fundamental role in the process of breast tumor progression.

There are limited examples in which coregulatory proteins bind to separate ER α domains, as we demonstrate here for the MTA2 repressor. Two recently identified coregulators, the coactivator ER-binding protein (47) and the corepressor Hsp27-ERE-TATA-

binding protein/scaffold attachment factor B (48), bind to residues along the combined DNA-binding and hinge domains. The L7/SPA coactivator interaction domain maps to the ER α hinge domain, and binding of corepressors can interfere with its ability to increase the transcription of tamoxifen-occupied receptors (49). Similarly, the peroxisome proliferator-activated receptor- γ coactivator interacts with the hinge domain (50). Interestingly, a MTA1-interacting protein named MICOA, which can enhance ER α transcriptional activity, has also been shown to bind to the ER α hinge region, implying a competition mechanism of action with MTA1 (51).

The exact mechanism of MTA2 binding to ER α is currently not known; MTA2 does not appear to contain a typical corepressor nuclear receptor motif (52). It has been shown that MTA1 binds to ER α through a corepressor nuclear receptor motif within MTA1 (18, 53) in a region different from that found for MTA2, which further highlights the differences between these two MTA family members (22, 54). It has not been shown that MTA3 binds directly to ER α ; however, its expression is related to ER α expression in breast cancer cells (19, 31). The BAH domain (55) of MTA2 interacts with ER α , and this domain is present in a number of proteins involved in protein-protein interactions within protein complexes functioning in chromatin replication and transcriptional repression, possibly through its role in DNA methylation (56, 57). Our demonstrated ER α -MTA2 binding is the first eukaryotic example of a protein-protein interaction that involves the BAH domain. However, our study suggests interactions between ER α and other MTA2 domains.

We show that MTA2 overexpression results in a reduction in the levels of ER α -responsive genes such as pS2, similar to that which has been demonstrated for MTA1 overexpression in MCF-7 cells (18). We also observed a relative increase in the PR-A to PR-B isoform ratio in the MTA2 transfectants. We recently reported that an excess of the PR-A isoform in invasive breast cancers was significantly associated with a failure to respond to tamoxifen (58); thus, future experiments will be focused on exploring the potential association between PR and MTA2, and response to antiestrogens in these cells. Furthermore it has been shown that MTA1 and MTA3 overexpression results in an increase in invasive properties (18, 31). We have not yet explored invasive pathways in the MTA2 transfectants; however, we did observe that these cells were rendered hormone independent, and exhibited enhanced anchorage-independent growth *in vitro*. MTA2 overexpression thus could drive cells to the first steps in the progression of estrogen independence, via a loss in the ability to regulate critical estrogen-induced gene expression. It has been observed that in the progression to hormone independence, different growth factor receptors and possible ligands can be up-regulated, leading to autocrine growth-regulatory mechanisms. We did not observe increased expression of either the c-Erb-B2 or the epidermal growth

factor receptors in the MTA2 transfectants (data not shown). Our results suggest that similar to MTA1 and the MTA1 short form (53), MTA2 may be a potent regulator of nuclear ER α function. As suggested (59), down-regulation of estrogen receptor signaling by these family members could then be implicated in the loss of MTA3 expression, and subsequent enhanced invasive properties of breast cancer cells. Because the MTA1 and 2 proteins appear to form complexes with a different set of transcription factors (22, 54), and because MTA3 has been associated with the Mi2/NuRD complex (31), the functions of this family of proteins may be distinct. Our observed difference in the region of MTA2 interacting with ER α also supports this supposition. Future studies are required to fully understand the discrete functions and regulatory effects of the MTA family members on the ER-signaling pathway.

We also examined the effects of MTA2 expression on the K303R ER α somatic mutation that we identified in premalignant breast lesions (7). This specific alteration appears to be a gain of function mutation, with activation at low concentrations of hormone and altered binding to ER α HAT coactivators. Our previous finding, that this ER α lysine 303 residue is acetylated (11), suggests that the K303R somatic mutation might be a useful tool to dissect the role of MTA2 and HDAC in the regulation of ER α acetylation. Our data herein, showing that the K303R ER α mutant was refractory to MTA2 inhibition, suggests that acetylation may be one component of the altered phenotype associated with this mutation. It has been reported that the K303R ER α mutation responds to MTA1-mediated transcriptional repression (51), a difference in function compared with that reported here for the MTA2 family member. This reinforces the concept that the different MTA family members may have distinct effects on ER α signaling.

It is possible that the simply named receptor hinge domain may perform important roles in ER α signaling. Mutations in the hinge domain of the androgen receptor (AR) are frequently present in prostate cancer (60, 61), and this region binds corepressors that govern hormone-dependent AR activity (62). Studies are currently underway in our laboratory to determine the frequency of the K303R ER α mutation in invasive breast cancers arising in the United States. Although the presence of the mutation is controversial because a number of investigators have failed to detect the mutation using fluorescent dideoxysequencing (63, 64), we and another group have reported its presence in invasive tumors using more sensitive microsequencing techniques (65, 66). Our data suggest that the ER α hinge domain appears to be more important than once appreciated, and we suggest that this region denotes a pivotal role between other functional domains. The K303R ER α mutation demonstrates this bifunctional activity with a gain in sensitivity to coactivators (7), but a loss in sensitivity to repressors, such as MTA2. The ER α hinge domain also appears to be a site of phosphorylation by signaling molecules p21-

activated kinase 1 (67) or PKA (68), and a binding site for signal transducer and activator of transcription 5b (69) and JUN transcription factor proteins (70). Thus, the ER α hinge region may also play a key role in regulation of receptor-mediated events. Because several ER α corepressors are also targets of cellular signaling molecules, the process of receptor acetylation, along with coordinate signaling to ER α coregulatory proteins, may be key targets for future therapeutic intervention.

MATERIALS AND METHODS

Plasmids and Chemicals

The *Xenopus* vitellogenin ERE₂-tk-luciferase (Luc) reporter, pCMV- β -galactosidase (β -gal) (48), the hemagglutinin (HA)-tagged WT and K303R ER α vectors (48), the Flag sequence (DYKDDDDK)-tagged MTA2 plasmid (a kind gift from Wei Gu, Columbia University, New York, NY) (17), glutathione-S-transferase (GST)-fused ER α AF2, DBD, and AF2/hinge domain vectors (48), K302R/K303R ER α (11), ER α -3X mutation [residues 538, 542, 545 (a gift from Donald McDonnell at Duke University, Durham, NC) (71)], and the GST-p300 HAT vector (72) have been previously described. The amino-terminal truncated MTA2 expression vector (residues 116–668) was generated by *EcoRV* and *BamHI* digestion of the Flag-MTA2 vector, and insertion into the pCin4 vector. GST-ER α hinge domain constructs (residues 253–310) with or without the 908 A to G ER α mutation (K303R) were generated through PCR and inserted into the pGEX 4T-1 vector (Pharmacia Biotech, Piscataway, NJ) at the *BamHI* and *EcoRI* cloning sites. To generate the GST fusion vectors of MTA2, full-length MTA2 cDNA was prepared by performing RT-PCR with RNA from MB-MDA-231 breast cancer cells as template. The cDNA was then cut by *XhoI* combined with *Asp718* or *EcoRV* and inserted into pGEX 4T-3 vector through the *XhoI* and *NotI* sites. The cDNA was also cut with *SpeI* and *NotI* (these restriction sites were designed in the PCR primers) or with *SpeI* and *EcoRV*, and inserted into pEF-Tracer-V5-B (Invitrogen, Carlsbad, CA) via the same restriction sites to generate the mammalian expression vector, pEF-MTA2, and the amino-terminal MTA2 expression vector, pEF-MTA2 1–116. pEF-MTA2 was partially digested with *Asp718* and then blunted with Klenow polymerase followed by digestion with *NotI*, the resultant 1.3-kb fragment was then inserted into pEF-MTA2, which had been digested with *EcoRV* and *NotI*, and the internal deleted MTA2 expression vector (Del 115–253) was generated. These two vectors were also used for *in vitro* translation. The other MTA2 constructs used for *in vitro* translation were cut from Flag-MTA2 by *NdeI* and *BamHI* (full-length MTA2), *Asp718* (residues 1–254 MTA2), *HindIII* (residues 1–237 MTA), or *EcoRV* (residues 1–115 MTA), and inserted into the pET-15b vector (Novagen, Madison, WI) through the *NdeI* and *BamHI* sites. The pG5-Luc reporter and pBinder vectors were purchased from Promega Corp. (Madison, WI). The Gal-4 ER α (residues 251–595) with or without the K303R mutation were generated through PCR and inserted into pBinder through the *BamHI* and *ASP718* sites. The MTA2 siRNA oligonucleotides containing the sequence GTCACCTTGCCAGCATAGTC, corresponding to the MTA2 coding region from nos. 1102–1120, was inserted into RNAi-Ready pSiren-Retro-Q vector (CLONTECH Laboratories, Inc., Palo Alto, CA). The control RNA interference oligonucleotide was purchased from CLONTECH. The progesterone response element (PRE)-Luc and androgen response element (ARE)-Luc reporters were kindly provided by Dr. Nancy Wiegel (Baylor College of Medicine, Houston, TX), and the

RARE-Luc was provided by Dr. Powel Brown (also at Baylor College of Medicine). NaB and 8-Br-cAMP were purchased from Calbiochem (La Jolla, CA).

Tumor Specimens

A cohort of frozen invasive breast tumor specimens from 26 patients was selected from the tumor bank of The Breast Center, Baylor College of Medicine, for use in the immunoblot study. These specimens did not have long-term clinical follow-up. This study was approved by the Baylor College of Medicine Institutional Review Board. Proteins were extracted from the tumors as previously described (73). Briefly, 30 mg of pulverized tumor powder was solubilized in 300 μ l of 5% sodium dodecyl sulfate (SDS) at 90 C for 5 min and centrifuged at 13,000 \times g for 5 min. Protein concentrations of the supernatants were determined using the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL). Typical protein yields were 2–5 μ g/ μ l. Supernatants were then stored at –70 C until use in the immunoblot analysis. Total ER levels were previously measured by ligand-binding assay. Briefly, cytosolic proteins were extracted from tumor tissues that had been pulverized in liquid nitrogen. ¹²⁵I-labeled E₂ (Amersham Pharmacia Biotech, Arlington Heights, IL) addition allowed for the determination of ER levels in a standard multipoint dextran-coated charcoal assay. Tumors with an ER content of at least 3 fmol/mg protein were considered positive for ER and PR, respectively.

Cell Culture and Transfection

The ER α -positive T47D, MDA-MB-361, BT474, and ER α -negative MDA-MB-231 MDA-MD-435 human breast cancer cells, HeLa cervical cancer cells, and human embryonic kidney (HEK)293 cells were obtained from American Type Tissue Culture Collection (Manassas, VA). ER α -positive MCF-7 human breast cancer cells were previously described (7). MCF-7 cells lacking ER expression (C4-12-5) have been previously described (30). All the cell lines were maintained in MEM (Invitrogen) supplemented with 5% fetal bovine serum (FBS, Summit Biotechnology, Fort Collins, CO), 6 ng/ml insulin, 200 units/ml penicillin, and 200 μ g/ml streptomycin. Cells were incubated at 37 C in 5% CO₂.

For transient transactivation assays, cells were maintained in phenol red-free MEM supplemented with 5% charcoal-stripped FBS (HyClone Laboratories, Inc., Logan, UT) for 5–7 d. One day before transfection, cells were plated in 2 ml media at 2.5 \times 10⁵ (MCF-7 and T47D cells) or 0.5 \times 10⁵ (HeLa, MDA-MB-231, and HEK293 cells) per well in six-well plates, and then transfected using Fugene 6 reagent (Roche, Indianapolis, IN) following the manufacturer's protocol. Each well was transfected with 1 μ g different luciferase reporters, 100 ng pCMV- β -gal vector, and MTA2 and/or ER α expression vectors as indicated. Cells were treated with E₂ (10^{–9} M), or 1 nM nuclear receptor ligand as indicated in the figure legend, or vehicle for an additional 18–24 h, after which the cells were washed twice with PBS, harvested into 1 \times reporter lysis buffer (Promega), and luciferase values as well as β -gal activities were assayed as described elsewhere (48). ER luciferase activity was normalized by dividing by the β -gal activity to give relative luciferase units. Experiments were performed in triplicate; the data are presented as the average \pm SEM and are representative of three independent experiments. Data are presented as fold induction.

To generate T47D cells stably overexpressing MTA2 or empty vector, 5 μ g Flag-MTA2 plasmid or empty vector was transfected with Fugene 6 reagent into 100-mm tissue culture dishes following the manufacturer's protocol. Stable clones overexpressing MTA2 were selected as described (7), and positive clones were identified using immunoblot analysis with an anti-PID antibody (17).

Colony Formation and Soft Agar Assay

For colony formation assays, 2 \times 10⁶ cells (MCF-7 or T47D) and 0.5 \times 10⁶ cells (MDA-MB-231) were plated in 100-mm tissue culture plates 24 h before transfection to allow for attachment. DNA (5 μ g), as indicated in the individual figures legends, was transfected into each plate with Fugene6 reagent following the manufacturer's protocol. The cells were incubated, 24 h after transfection, with 800 μ g/ml G418 antibiotic (Invitrogen) in 5% FBS for 14 d, the colonies were stained with 1% crystal violet, and the colonies were counted under a microscope and photographed. The data are representative of two independent experiments done in triplicate.

Soft agar assays were performed in six-well plates. Into each well, 1.5 ml of prewarmed (50 C) 0.7% SeaPlaque agarose (FMC, Rockland, ME) was added and dissolved in phenol red-free MEM (Life Technologies, Gaithersburg, MD) supplemented with 5% charcoal-stripped FBS (Summit Biotechnology) to serve as the bottom layer, and allowed to solidify at 4 C until to use. Cells (0.5 \times 10⁴) as a single cell suspension were suspended in prewarmed (37 C) same media, 4 ml of 0.5% SeaPlaque agarose was then added, and this suspension was plated as the top layer by adding it dropwise to the solidified bottom layer plates. Plates were cooled for 2 h and then transferred to a 37 C humidified incubator. Two days after plating, media containing control vehicle, 1 nM E₂, 100 nM 4-hydroxy tamoxifen, 10 nM ICI 182,780 (ICI), or 1 nM E₂ plus 100 nM 4-hydroxy tamoxifen or 10 nM ICI was added to the top cell layer, and the appropriate media was replaced every 2 d. After 14 d, the colonies were fixed, and the colony number from quadruplicate assays was then counted. The data shown are representative of two independent experiments.

GST Pulldown

GST protein expression and purification procedures on glutathione-Sepharose (Pharmacia Biotech) have been described previously (48). *In vitro* translation using the TNT transcription-coupled translation system from Promega, as well as GST pull-down procedures, were performed as described elsewhere (7).

Phosphorylation-Coupled GST-Pulldown

Purified GST-ER α Hinge fragment was phosphorylated by PKA as previously described (30). The reaction was stopped by the addition of 10 mM EDTA and used for GST pulldown as described (7).

IP

Co-IP assays were performed as described (74) with some modifications. Briefly, indicated cells were transfected with expression vectors and incubated in the absence or presence of 1 nM E₂ for 24 h as indicated in the figure legends. High-salt cell lysis buffer (7) with 1:100 diluted complete proteinase inhibitor III solution (Roche) was used to prepare cellular extracts, after which these extracts were adjusted to 150 mM NaCl plus 0.2% Nonidet P-40 binding buffer. The extracts were then incubated with the appropriate antibody as indicated. The precipitated components were eluted by boiling in SDS loading buffer [125 mM Tris (pH 6.8), 20% glycerol (vol/vol), 4% SDS, 0.001% Bromophenol blue, 2% 2-mercapto ethanol (vol/vol)] resolved by SDS-PAGE, and analyzed by immunoblot analysis.

IB

Cell extracts or immunoprecipitated proteins were resolved by SDS-PAGE and electrophoretically transferred to nitrocel-

lulose membranes. The blots were blocked in TBST [20 mM Tris (pH 7.6), 150 mM NaCl, and 0.1% Tween 20] supplemented with 5% nonfat milk (wt/vol) for 1 h at room temperature. Anti-PID (MTA2) was a gift from Wei Gu (1:400) (17); anti-ER α clone 6F11 (1:200) was obtained from Vector Laboratories (Newcastle, UK); anti-ER α hinge domain-specific clone SPA-1000 (1:1000) was purchased from Stressgen (Victoria, British Columbia, Canada); anti-EBP50 (1:250) and anti-TIF2 (1:200) were obtained from BD Transduction Laboratories, San Diego, CA; anti-HDAC1 clone A19 (1:500) and anti-PR clone C19 (1:200) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti- β -Actin AC-15(1:3000) was from Sigma Chemical Co. (St. Louis, MO); anti-V5 (1:5000; Invitrogen) and anti-Flag M2 (1:2000; Sigma) antibodies were diluted in blocking buffer and incubated with the membranes for 1 h, after which the membranes were then washed extensively with TBST buffer and incubated with horseradish peroxidase-linked antimouse IgG (1:4000); antirabbit IgG (1:4000), or antigoat IgG (1:7500) (Santa Cruz Biotechnology), respectively, diluted in TBST. After washing five times with TBST, the immunoblot signals were visualized by enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) following the manufacturer's protocol. A MCF-7 cell line extract was used for normalization of MTA2 levels in tumors and was included on all gels for standardization across gels. Antibodies to p190 (1:1000; BD Transduction Laboratory) and RhoGDI (1:500; Santa Cruz Biotechnology) were used as loading controls.

In Vitro Acetylation and Deacetylation

GST-p300 HAT and GST-ER α hinge domain proteins were purified as described elsewhere (75). Briefly, 25 μ g GST-ER α hinge protein was acetylated using 400 ng p300 HAT in the presence of 50 nCi [14 C]acetyl-coenzyme A (ICN Pharmaceuticals, Inc., Costa Mesa, CA) in 30 μ l acetylation buffer (72) at 30 C for 30 min (76). The acetylated proteins were immobilized on glutathione-Sepharose (Amersham) and washed twice with deacetylation buffer (17). HDAC activity was obtained by eluting with Flag peptide (Sigma). 14 C-acetylated protein was incubated with purified Flag-MTA2-associated complexes or control eluate (cell lysates precipitated with only Protein G beads) at 30 C for 60 min in 1 \times deacetylation buffer as described by (17). The reactions were resolved by SDS-PAGE, after which the gels were stained with Coomassie blue dye to evaluate input and developed using autoradiography.

qRT-PCR

We performed qRT-PCR for pS2 following the protocol previously described (36) with minimal modifications. Briefly, T47D vector control (V2) and MTA2 overexpressing (MTA2.5 and 2.8) cells were grown in serum-free medium for 3 d. The cells were then treated with E $_2$ (1 nM) for 24 h. Total RNA was prepared with a RNeasy Mini Kit (QIAGEN, Valencia, CA), according to the manufacturer's protocol. RNA was reverse transcribed, and the resulting cDNA was used in subsequent qRT-PCR reactions, performed in 1 \times iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA) with 5 pmol forward and reverse primers. The primers used were pS2: forward primer, 5'-ATACCATCGACGTCCCTCCA-3'; and reverse primer, 5'-AAGCGTGTCTGAGGTGTCCG-3'; cyclophilin A: forward primer, 5'-TGCGTGACATTAAGGAGAAG-3'; and reverse primer, 5'-GCTCGTAGCTCTTCTCCA-3'. qRT-PCR was performed in 96-well optical plates (Bio-Rad) using an iCycler system (Bio-Rad) for 40 cycles (94 C for 12 sec, 60 C for 40 sec), after an initial 3-min denaturation at 94 C. The relative concentration of RNA was calculated using the $\Delta\Delta$ Ct method according to Relative Quantitation of Gene Expression (Applied Biosystems User Bulletin) with cyclophilin A mRNA as an internal control. Results were expressed as relative RNA levels standardized such that values obtained in

vector control cells treated with vehicle (ethanol) only were set to 1.

Statistical Analyses

Continuous levels of MTA2 were calculated as the ratio of band intensities measured in densitometry units from Western blots of individual samples normalized to the MCF-7 reference standards and actin. Correlations between continuous levels of MTA2 and ER α status are displayed using a box-and-whisker plot and compared using a Wilcoxon rank sum test. All other statistical analyses were performed using Student's *t* test, comparing the difference between control and experimental values.

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