LSD1 engages a corepressor complex for the activation of the estrogen receptor α by estrogen and cAMP

Marcela A. Bennesch, Gregory Segala, Diana Wider and Didier Picard[*]

Département de Biologie Cellulaire, Université de Genève, Sciences III, 30 quai Ernest-Ansermet, CH-1211 Genève 4, Switzerland

ABSTRACT

The estrogen receptor α (ERα) is a transcription factor that can be directly activated by estrogen or indirectly by other signaling pathways. We previously reported that activation of the unliganded ERα by cAMP is mediated by phosphorylation of the transcriptional coactivator CARM1 by protein kinase A (PKA), allowing CARM1 to bind ERα directly. This being insufficient by itself to activate ERα, we looked for additional factors and identified the histone H3 demethylase LSD1 as a substrate of PKA and an important mediator of this signaling crosstalk as well as of the response to estrogen. Surprisingly, ERα engages not only LSD1, but its partners of the CoREST corepressor complex and the molecular chaperone Hsp90. The recruitment of Hsp90 to promote ERα transcriptional activity runs against the steroid receptor paradigm and suggests that it might be involved as an assembly factor or scaffold. In a breast cancer cell line, which is resistant to the anti-estrogen tamoxifen because of constitutively activated PKA, some interactions are constitutive and drug combinations partially rescue tamoxifen sensitivity. In ERα-positive breast cancer patients, high expression of the genes encoding some of these factors correlates with poor prognosis. Thus, these mechanisms might contribute to ERα-driven breast cancer.

INTRODUCTION

Estrogen receptors α (ERα) and β are members of the steroid receptor family of nuclear receptors and mediate most estrogen responses in the body (1). They are not only simultaneously hormone receptors and transcription factors, but also integrators of multiple other signals, which has broad physiological and pathological implications (2). The isoform ERα plays a central role in breast carcinogenesis as a driver of proliferation. Hence, adjuvant therapy for ERα-driven breast cancers involves depriving cells of estrogens or blocking ERα activity with anti-estrogens such as tamoxifen (3), replaced in cell culture by its physiologically active form hydroxytamoxifen (OHT). Unfortunately, about one-third of the patients treated with tamoxifen for 5 years develop resistance (4). While there are certainly multiple pathways to OHT resistance (5), signaling pathways leading to the activation of ERα in the absence of cognate ligand can be expected to contribute (2,6–12). Understanding the mechanisms and factors involved in estrogen-independent and possibly OHT-resistant activation of ERα is essential to rescue a treatment that initially works and is tolerated rather well.

ERα activity is influenced by the levels of transcriptional coactivators and corepressors and their post-translational modifications (13–15), and their differential interaction with ERα in the presence of estrogen or anti-estrogens (16,17). Work from our group showed that cAMP-activated protein kinase A (PKA) can phosphorylate the coactivator-associated arginine methyltransferase 1 (CARM1), thereby inducing its direct recruitment to and transcriptional activation of the unliganded ERα irrespective of the presence of OHT (12). We also knew that this cAMP-induced phosphorylation of CARM1 is necessary but not sufficient to activate ERα, indicating that additional factors must mediate the cAMP-induced activation of ERα activity. These could include other coregulators such as the coactivator glucocorticoid receptor interacting protein 1 (Grip1), whose recruitment to ERα had been shown to be stimulated by cAMP, albeit not through direct phosphorylation by PKA (18). We therefore set out to identify additional factors that mediate signaling crosstalk with the ERα and may be relevant to breast cancer progression. Initially, we focused on LSD1 because of its known involvement in the estrogen-dependent activation of ERα (19,20) and its activation of c-Myc-dependent transcription by cAMP-PKA signaling (21).

[*]To whom correspondence should be addressed. Tel: +41 22 379 6813; Email: didier.picard@unige.ch

The lysine-specific histone demethylase 1A (KDM1A; referred to hereafter solely by its alias LSD1) is a flavin adenine dinucleotide (FAD)-dependent amine-oxidase, which specifically demethylates mono- or dimethylated histone H3 at lysine 4 (H3K4) and lysine 9 (H3K9) (21–25). LSD1 is both part of corepressor and coactivator complexes and contributes to regulate the activity of certain transcription factors including nuclear receptors (16,19,26,27). In addition to histone H3, LSD1 has non-histone substrates such as p53, DNA methyltransferase 1 and, in fact, even ERα, regulating their activities and stability (28,29). LSD1 has become a candidate drug target because it is frequently overexpressed and, in most cases, correlated with bad prognosis in a variety of cancers (30–37).

A direct link of LSD1 and LSD1-associated corepressors to the ligand-independent activation of ERα by cAMP and the implications for OHT resistance of breast cancer had not been investigated. In doing so, we discovered several other players whose contributions to the activation of ERα by both estrogen and cAMP could not have been predicted based on their previously established functions.

MATERIALS AND METHODS

Plasmids

Standard expression vectors, reporter plasmids and shRNA constructs were used. Details about them can be found in the Supplementary Material.

Cell culture, transfection, luciferase assays and knockdown experiments

Cells were maintained under standard conditions and cultured in medium with charcoal-stripped serum for induction experiments. cAMP signaling was induced by a combination (indicated as F/I) of 10 μM Forskolin (Cayman) and 100 μM 3-isobutyl-1-methylxanthine (Calbiochem) with dimethyl sulfoxide (DMSO) as the solvent. Geldanamycin (GA), trichostatin A (TSA), GSK-LSD1 and OHT were purchased from Sigma. Firefly luciferase activities were standardized to a Renilla luciferase transfection control. Knockdown in MDA-MB-134 cells were generated by infection with lentiviruses expressing specific shRNA against each target protein. Further details and shRNA sequences are given in Supplementary Material.

Antibodies

The following primary antibodies were used: the mouse monoclonal 1B2E5 (Cell Signaling) and rabbit polyclonal H-220 (Santa Cruz Biotechnologies) against LSD1; the rabbit polyclonal serum A300-421A against CARM1 (Bethyl Laboratories); the mouse monoclonal H90-10 (a gift from David O. Toft) and the rabbit polyclonal PA3-012 (Pierce) against Hsp90β; the rabbit polyclonal antibodies HC-20, H-51, H-300 and H-65 against ERα, HDAC1, HDAC6 and CoREST, respectively (Santa Cruz Biotechnologies); the rabbit polyclonal serum 07–579 (Upstate Biotechnologies) and the mouse monoclonal NRSF (F-3)x (Santa Cruz Biotechnologies) against REST; the rabbit polyclonal antiserum # 9621 against the PKA-phosphorylated motif RRX-phospho-S/T (Cell Signaling); the mouse monoclonals 17–681 against methylated histone H3K9 (Upstate Biotechnologies) and CMA303 against dimethylated H3K4 (Millipore); the mouse monoclonal (6C5) and the rabbit polyclonal antiserum ab1791 against GAPDH and histone H3, respectively (Abcam); the mouse monoclonals FLAG M2, Clone M2 and DM1A against α-tubulin (Sigma); the rabbit polyclonal PA3-013 against Hsp90α (Thermo Scientific).

Immunoprecipitation experiments

The standard protocol for all IP experiments was as follows: cells were lysed by sonication in 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 10% glycerol, 10 mM Na-molybdate and protease inhibitors (Roche). A total of 2 mg of each total protein extract were first incubated with the primary antibody overnight at 4°C, and then 50 μl of protein G-Dynabeads (Life Technologies) were added and incubated for an additional 2 h at 4°C. Beads were washed four times with lysis buffer containing 0.1% Triton X-100 and three more times with lysis buffer without detergent before boiling in reducing sample buffer. A total of 80 μg of corresponding total extracts were loaded as input samples.

Chromatin immunoprecipitation experiments

The recruitment of LSD1, CoREST, HDAC1, REST, HDAC6, Hsp90β and ERα to enhancers/promoters of ERα target genes upon stimulation of MDA-MB-134 cells for 45 min was determined by chromatin immunoprecipitation (ChIP) with specific antibodies. Further details are given in the Supplementary Material.

Quantitative reverse-transcription PCR analysis of ERα target gene expression

MDA-MB-134 cells were induced for 4 to 6 h, and gene expression was analyzed by quantitative reverse-transcription PCR (RT-PCR) with standard procedures described in detail in the Supplementary Material.

Proliferation assays

A total of 2000 cells per well were seeded into 96-well plates. The day after, cells were treated as indicated for 72 h. After 96 h, MTT assays were performed using a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (BioChemica) in phosphate buffered saline (PBS) to a final concentration of 0.5 mg/ml per well and incubation of the cells for 2 h. After removal of the medium, DMSO was added to each well to dissolve the formazan crystals. The absorbance at 600 nm was determined using a Sunrise plate reader (Tecan). Triplicate wells were assayed for each condition in three independent experiments.

Kaplan–Meier analyses

Kaplan–Meier curves (38) were generated using the GOBO tool (http://co.bmc.lu.se/gobo) (39) and the following gene set: *KDM1A* (LSD1) (Entrez Gene ID: 23028), *HDAC1* (Entrez Gene ID: 3065), *HDAC6* (Entrez Gene ID: 10013), *HSP90AB1* (Hsp90β) (Entrez Gene ID: 3326) and *CARM1* (Entrez Gene ID: 10498).

Statistical analyses

Unless indicated otherwise, the data shown are averages of three independent experiments made in triplicates, with error bars indicating the standard error of the mean. A two-sided non-paired Student's *t*-test was used to determine differences between two groups with $P < 0.05$ considered statistically significant.

RESULTS

CARM1 and LSD1 are major substrates of PKA phosphorylation for the activation of ERα by cAMP

We knew from our previous work that CARM1 is a necessary but insufficient PKA target for the activation of ERα (12). We decided to study the importance of LSD1, since it was already known to act as a coactivator for the estrogen-dependent activation of ERα (19,23,29). In addition, other studies had suggested that LSD1 is recruited to chromatin upon activation of PKA by waves of cAMP generated in response to rapid non-genomic signaling by membrane-associated ERα activated with estrogen (20). We first sought to identify potential PKA phosphorylation sites in LSD1 using the pkaPS algorithm (http://mendel.imp.ac.at/pkaPS). We found one high score site at S111 within the sequence RRTSRRK of LSD1, immediately preceded by a threonine. To determine whether LSD1 can be phosphorylated by PKA and whether these predicted sites are relevant for phosphorylation *in vivo*, we transfected the ERα-positive breast cancer cell line MDA-MB-134 (40) with expression vectors for wild-type LSD1 or for the phosphothreonine/serine LSD1 double mutant T110/S111A. Following immunoprecipitation of the exogenous LSD1 proteins using an anti-Flag antibody, we revealed phosphorylation with an antibody recognizing the PKA-phosphorylated motif RRX-phospho-S/T (Figure 1A). Only wild-type LSD1 but not the double mutant T110/S111A showed increased phosphorylation upon activation of PKA by a cocktail of the adenylate cyclase activator forskolin and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (F/I), supporting the prediction of PKA-mediated phosphorylation of LSD1 at T110 and/or S111.

Their functional importance was tested by coactivation assays in HeLa cells with exogenously expressed ERα. In response to cAMP signaling, only wild-type LSD1 and the phosphoserine mimic mutant showed coactivator effects while the double mutant T110/S111A failed to do so and even reduced ERα activity (Figure 1B). We determined which transcriptional activation domain of ERα mediates the activation by LSD1 by testing the activation functions (AF) 1 and 2 separately as fusion proteins with the Gal4
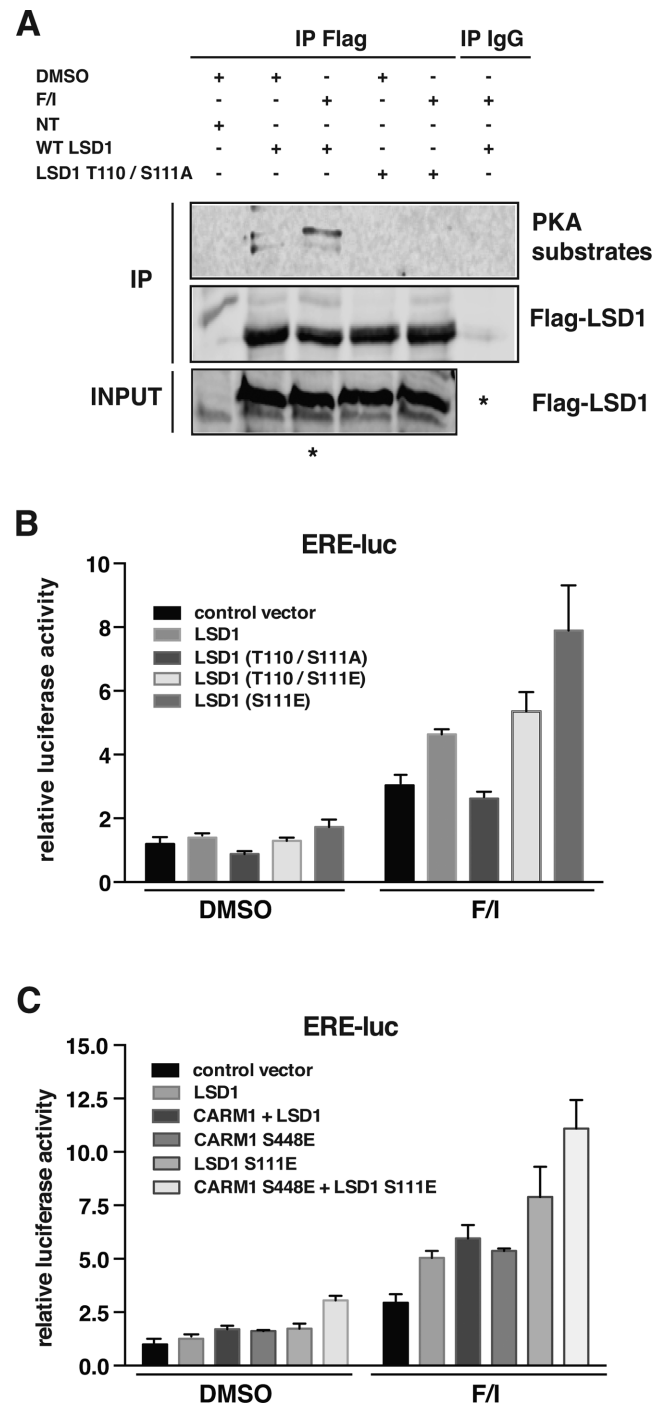

Figure 1. The phosphorylation of LSD1 by PKA promotes its activation of ERα. (**A**) LSD1 is a substrate of PKA *in vivo*. MDA-MB-134 cells transfected with vectors for wild-type LSD1 or phosphothreonine/serine mutant T110A/S111A were stimulated with F/I for 2 h before immunoprecipitation (IP) and immunoblotting with indicated antibodies. NT, not transfected; F/I, two-compound mixture increasing cAMP levels. The asterisk points out which input extract was used in parallel for the control IP. (**B** and **C**) Overexpression of wild-type and phosphoserine mimics of LSD1 and CARM1 increases ERα activation by cAMP. Luciferase reporter assays in Hela cells with exogenously expressed ERα. The bar graphs show averages of three replicate experiments with triplicates, standardized to the values obtained with the control transfections with vector and treated with the solvent (DMSO), and the error bars represent the standard error of the mean.

DNA binding domain by transfection into HeLa cells. In response to cAMP signaling and the physiological estrogen 17β-estradiol (E2), LSD1 further stimulates ERα AF2 activity; in contrast, constitutive AF1 activity is barely affected by LSD1, but blunted by cAMP signaling. The latter is most likely due to the interference of cAMP signaling with MAPK signaling, which by itself is known to stimulate AF1 activity (7,41) (Supplementary Figure S1A and B). Additional support for a role of phosphorylation of more than one coregulator by PKA comes from genetic experiments in which we explored the coactivator activity of combinations of CARM1 and LSD1 and phosphoserine mimic mutants thereof (Figure 1C). The strongest effect could be seen with the combination of the mutants CARM1 S448E and LSD1 S111E. This combination even induced a substantial activity of ERα in the absence of any treatment. These results support the idea that both CARM1 (12) and LSD1, as direct substrates of PKA, are essential and partially sufficient mediators of the activation of ERα by cAMP signaling.

Convergence of the LSD1 and ERα complexes

Having found that both CARM1 and LSD1 may be required for full activation of ERα by cAMP, we investigated the interaction of LSD1 with the core ERα-CARM1 transcriptional complex (12) in more detail. We began with immunoprecipitation experiments to examine the interaction between endogenous ERα, CARM1 and Hsp90β (one of the two cytosolic Hsp90 isoforms) and exogenously expressed Flag-tagged LSD1 in MDA-MB-134 cells (Figure 2A). We observed an increased recruitment of LSD1 in cells treated with F/I, paralleling that of CARM1, and more surprisingly, that of Hsp90, which was not known to be associated with LSD1 complexes. Some co-immunoprecipitation (coIP) of LSD1, CARM1 and Hsp90 with ERα could be seen even in untreated cells, most likely because of overexpression. The association of LSD1 with ERα could be confirmed for the endogenous proteins both in response to cAMP and E2 (Figure 2B). Unexpectedly, under these same conditions, we discovered that both ERα-activating signals increase the association of several other components of a corepressor complex with LSD1 (42), which are well known for their role in silencing neuronal genes (43,44). These notably include the RE1-silencing transcription factor (REST; also known as neuron-restrictive silencer factor), the REST corepressor 1 (CoREST) and histone deacetylase 1 (HDAC1). Considering the link between OHT resistance and cAMP signaling (12), we performed the same type of immunoprecipitation of ERα with extracts from LCC2 cells, an OHT-resistant variant of MCF7 cells (45) (Figure 2C). Similarly to what we had found for CARM1 (12), the interactions of ERα, especially with HDAC1, but also with LSD1 and Hsp90 are already present in the absence of any treatment. By and large, we made the same observations when we interrogated the complexes by immunoprecipitation of CARM1 in both MDA-MB-134 and LCC2 cells (Supplementary Figure S2A and B).

In addition, we found that another histone deacetylase, HDAC6, associates with ERα and CARM1 complexes (Figure 2C, Supplementary Figure S2B). It is noteworthy that HDAC1 and HDAC6 are both known to have non-

histone substrates as well; these include Hsp90, which is dependent on deacetylation for normal chaperone function (46,47). We speculate that Hsp90 may act as a scaffold protein for the assembly of the ERα transcriptional complex, which may in part explain why HDACs are recruited to this complex. Therefore, ERα appears to be at the center of a novel transcriptional activation complex that involves the association of several unexpected partners (Figure 2D).

We dissected LSD1 to determine the minimal functional domain of LSD1 required for the interaction with ERα and for coactivation (Figure 2E and Supplementary Figure S2C). LSD1 has three main domains: the longest is the C-terminal catalytic center, which is a FAD-dependent amine-oxidase-like domain (AOL); the N-terminal part contains a flexible region (NFR) and the SWIRM domain, which mediates the interactions with chromatin and histones; the large insertion in the catalytic domain, the Tower domain, is indispensable for the interaction with CoREST and to increase LSD1 enzymatic activity (48,49). As can be seen in Figure 2E, the immunoprecipitation of ERα from HEK 293T cells transfected with the series of LSD1 deletion mutants showed that the AOL domain with the inserted Tower domain is sufficient to bind ERα, even though additional contacts in the N-terminal domain may stabilize the interaction. In coactivation assays, the AOL domain also proved to be sufficient to account for all of the activity of full-length LSD1 (Supplementary Figure S2C).

The LSD1-CoREST-HDAC-Hsp90 connection is necessary for ERα transcriptional activity

To explore the functional importance of the CoREST complex proteins, HDAC6 and Hsp90 for ERα transcriptional activity, we stably knocked down proteins of interest in MDA-MB-134 cells (Supplementary Figure S3A) and transfected these cells with the ERE-luciferase reporter. Note that cAMP signaling suppressed the inhibitory effects of OHT in this experimental system, as expected from previous work of ours and others (12). The knockdowns of LSD1, CoREST, HDAC1, HDAC6 and either of the two Hsp90 isoforms Hsp90α and Hsp90β, and thereby a reduction of the overall amount of Hsp90, significantly reduced ERα activity independently of how it was activated (Figure 3A–F, Supplementary Figure S3B and C). Unexpectedly, the REST knockdown also reduced ERα activity, which suggests that REST could be a functional component of this ERα transcriptional complex. Conversely, the overexpression of LSD1, HDAC1 and HDAC6 in MDA-MB-134 cells resulted in increased ERα activity (Figure 3G). ERα protein levels were at most slightly reduced by some of the knockdowns whereas ERα mRNA levels were not coordinately affected (Supplementary Figure S3D and E). The overexpression of LSD1, HDAC1 or HDAC6 did not affect ERα protein levels (Supplementary Figure S3D).

Recruitment and functional requirement of the LSD1 corepressor complex for activation of endogenous ERα target genes

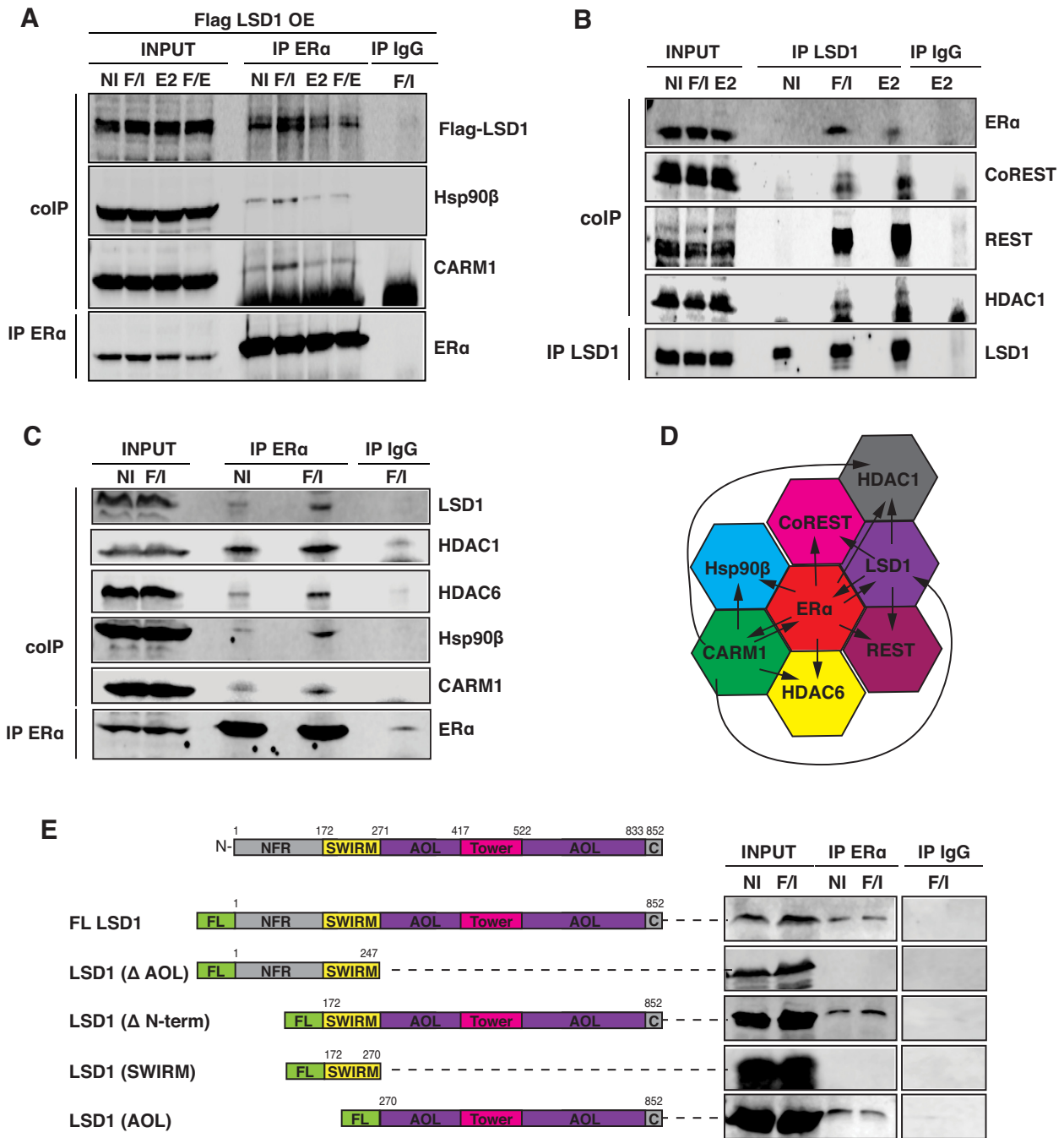Having confirmed that LSD1, REST, CoREST, HDAC1, HDAC6 and Hsp90 are important for the activation of

Figure 2. LSD1 interacts with both ERα and components of the CoREST transactivation complex. (**A**) Endogenous ERα co-immunoprecipitates with overexpressed LSD1 and endogenous Hsp90β and CARM1. MDA-MB134 cells transfected to express Flag-tagged LSD1 were stimulated with F/I or E2 or a combination thereof for 2 h before cell lysis and immunoprecipitation with an antibody against ERα or a control antibody (IgG). coIP, co-immunoprecipitation; NI, non-induced. (**B**) LSD1 co-immunoprecipitates with both ERα and CoREST complex proteins. Co-immunoprecipitation experiment with an antibody against endogenous LSD1 or control IgG with extracts from MDA-MB-134 cells treated as indicated. (**C**) Co-immunoprecipitation experiment indicating constitutive interactions of ERα with LSD1 and other factors in OHT-resistant LCC2 cells. (**D**) Schematic representation of interactions. The arrows indicate both previously published and new interactions. Note that interactions assessed by co-immunoprecipitation may not be direct and that the exact arrangement of the hexagons is mostly arbitrary. (**E**) Analysis of interaction of LSD1 truncations with ERα. Indicated proteins were overexpressed together with ERα in 293T cells. FL, Flag tag; NFR, N-terminal flexible region; SWIRM, SWIRM domain; AOL, Amine-oxidase-like domain; Tower, Tower domain; C, C-terminal region. Co-immunoprecipitated proteins were displayed with an anti-Flag antibody.
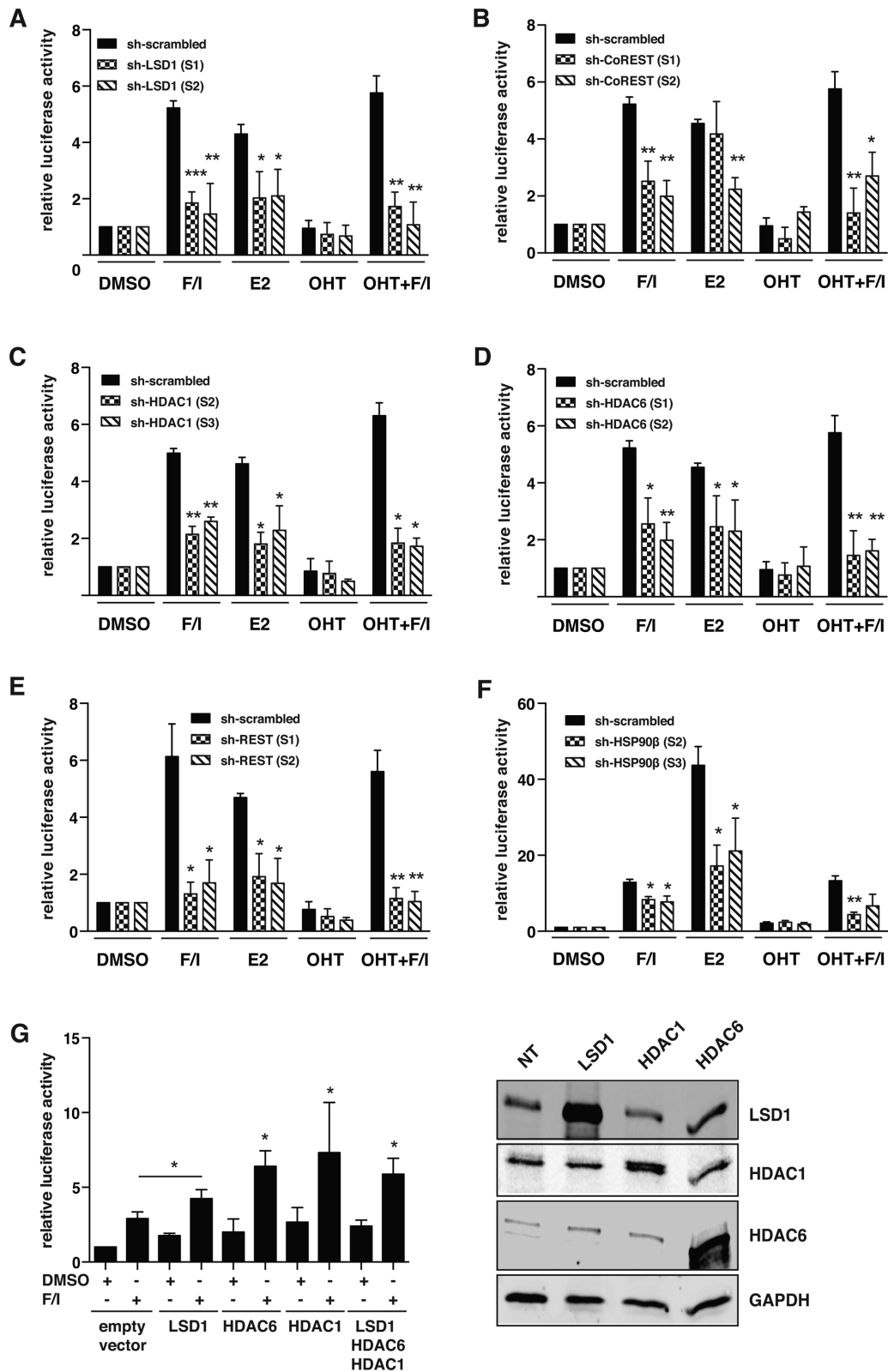
Figure 3. The Hsp90-HDAC6-CoREST complexes are required for ERα transcriptional activity. (**A-F**) Luciferase reporter gene assays for ERα activity in transfected MDA-MB 134 cells with stable shRNA-mediated knockdowns of LSD1, CoREST, HDAC1, HDAC6, REST and Hsp90β, respectively. Two different shRNA sequences were used for each target. (**G**) Overexpression of LSD1 and HDACs increases cAMP-induced ERα transcriptional activity. MDA-MB-134 cells were transiently transfected and the activity of ERα was assessed with a luciferase reporter (left panel). The immunoblot on the right confirms the overexpression of the indicated proteins relative to the loading control GAPDH. These data points are averages of three experiments with triplicates and the error bars represent the standard error of the mean. Relevant statistically significant values are highlighted by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

ERα, we evaluated whether these proteins are recruited to chromatin at endogenous ERα target genes. ChIP experiments were performed with MDA-MB-134 cells induced for 45 min with F/I or E2 or treated with vehicle alone (Figure 4A–G). We indeed observed increased recruitment of these factors after stimulation with either cAMP or E2. This correlated with a functional requirement for these factors for the transcriptional activation of the ERα target genes *TFF1*, *GREB1* and *BCL2*, since they were all reduced by knocking down the factors of interest (Supplementary Figure S4).

LSD1-HDAC-Hsp90 inhibition blocks ERα-mediated transcription and proliferation of breast cancer cells

We used pharmacological inhibitors to further validate the knockdown experiments. To inhibit HDACs we used the well-characterized pan-HDAC inhibitor trichostatin A (TSA), which has an IC50 in the nM range and established antitumor activity (50). For LSD1, we used GSK-LSD1, which is an irreversible inhibitor of LSD1 with an IC50 of 16 nM and a more than 1000-fold selectivity over other closely related FAD-dependent enzymes such as LSD2, MAO-A and MAO-B; it has been found to inhibit the *in vitro* proliferation of certain cancer cell types (51) and is currently in clinical trails for the treatment of refractory small cell lung carcinoma and acute myeloid leukemia (ClinicalTrials.gov identifiers NCT02034123 and NCT02177812, respectively). Since HDAC inhibitors can affect ERα protein levels (52–56), we first established a treatment regimen that would not affect ERα levels. The dose- and time-response experiment of Supplementary Figure S5A indicates that ERα protein levels are preserved when cells are treated with only 250 nM TSA for 24 h. Under these conditions, while the levels of other proteins of interest were not affected either, the activation of the ERα reporter gene transfected into MDA-MB-134 cells was dramatically impaired (Figure 5A). In the same line of experiments, we then tested the effects of the inhibitor GSK-LSD1. The results showed a dose-dependent increase in LSD1 inhibition as judged by the global, albeit modest, increase in dimethylation of H3K9 (H3K9me2) (Supplementary Figure S5B) compared with untreated cells or with MDA-MB-134 cells overexpressing LSD1. In accordance with increased levels of H3K9me2 with low concentrations of LSD1 inhibitor, activation of the ERα reporter gene was greatly reduced (Figure 5B). The inhibitory chromatin mark H3K9me2 also failed to be decreased to the same extent by activation of ERα at both enhancer and promoter sequences of the genes *TFF1 and GREB1* when LSD1 was knocked down (Supplementary Figure S5C).

These drugs could affect ERα signaling at a variety of levels. We therefore tested whether ERα recruitment to endogenous target genes is affected when HDAC1 and HDAC6 are knocked down or inhibited with TSA in MDA-MB-134 cells. Both the genetic and the pharmacological inhibition of HDACs severely reduced the binding of ERα to its target genes (Figure 5C and Supplementary Figure S5D). Pharmacological inhibition of Hsp90 with geldanamycin (GA) or of HDACs with TSA impairs the assembly of the complex of ERα with LSD1 and CARM1 (Figure 5D) indicating that ERα signaling is indeed impaired at multiple different levels.

Having discovered new coregulators, we determined their impact on the ERα-dependent and OHT-resistant proliferation of LCC2 cells (Figure 5E). When assayed over a period of 4 days, inhibitors tested at relatively low concentrations had no or at most a relatively modest inhibitory effect. These inhibitors were then combined with OHT at concentrations (1–10 μM) to which LCC2 cells are resistant or partially resistant. Significant combinatorial toxicities could be observed for 1 μM OHT with GA and for 10 μM OHT with GSK-LSD1 and TSA. For TSA, our results confirm previous results (57), and mirror additional results with the HDAC inhibitors valproic acid (58) and varinostat in the treatment of advanced breast cancer patients (59). No significant drug–drug interactions could be seen with ERα-negative SK-BR-3 cells (Supplementary Figure S5E) supporting the idea that the OHT-sensitizing effects of these inhibitors in LCC2 cells are specific and mediated by an impact on ERα signaling.

High expression of the gene set LSD1-HDAC-CARM1-Hsp90 correlates with bad prognosis in breast cancer

To provide further evidence for a role of LSD1, HDAC, CARM1 and Hsp90 in ERα-modulated breast carcinogenesis, we searched public databases for a correlation between clinical outcome and gene expression. This was done with the 'Gene expression-based Outcome for Breast cancer Online tool' (GOBO) (39). Initially, we chose a gene set with *KDM1A* (LSD1) *HDAC1*, *HDAC6* and *HSP90AB1* (Hsp90β) (Figure 6A). Comparing all breast tumors, ERα-positive (ER+) breast cancer patients who expressed higher levels of this gene set had lower relapse-free survival (RFS) (Figure 6B). In contrast, there was no correlation in RFS for patients with ERα-negative (ER−) breast tumors. Even more striking differences in RFS could be seen for patients with metastases in lymph nodes (LN+), patients with aggressive grade 3 ERα-positive tumors and patients who had received tamoxifen treatment (TAM) (Figure 6C). This correlation could not be found with grade 1 and 2 tumors (data not shown). Moreover, if *CARM1* is included in the gene set, expression also correlates with tumor grade (Supplementary Figure S6A). Patients with ER+ tumors, classified as grade 3 and presenting higher expression of the gene set had less distant metastasis and lower relapse free survival (DMSF_mixed) (Supplementary Figure S6B). Interestingly, focusing on TAM-treated tumors as an example, expression of the selected gene set correlates with the expression of genes related to cell proliferation, notably checkpoint control, M-phase and steroid response (Supplementary Figure S6C). Finally, reduced overall survival and not just reduced RFS correlates significantly with high expression of the gene set in patients with ERα-positive tumors (Figure 6D).

DISCUSSION

Our work demonstrates the importance of an ERα activation complex that includes corepressor proteins of the CoREST complex. This counterintuitive finding further underlines the fact that a vast variety of factors, including seemingly dedicated corepressors, collaborate in ERα signaling;
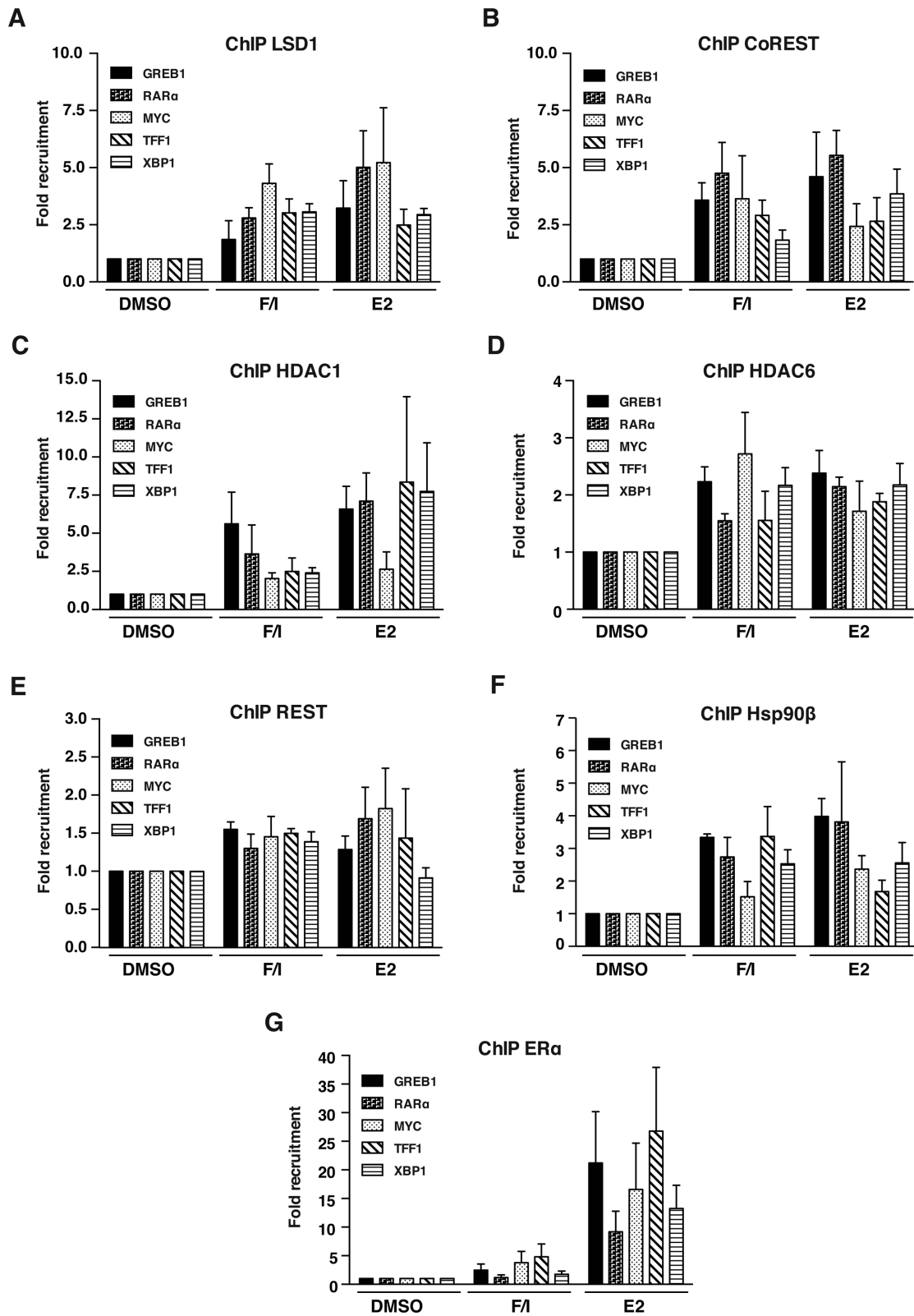
Figure 4. The core components of the ERα-Hsp90-HDAC6-CoREST complex are recruited to endogenous ERα target genes. (**A-G**) ChIP experiments with MDA-MB-134 cells stimulated with F/I or E2 for 45 min. The data points are averages of three independent experiments and the error bars represent the standard error of the mean.
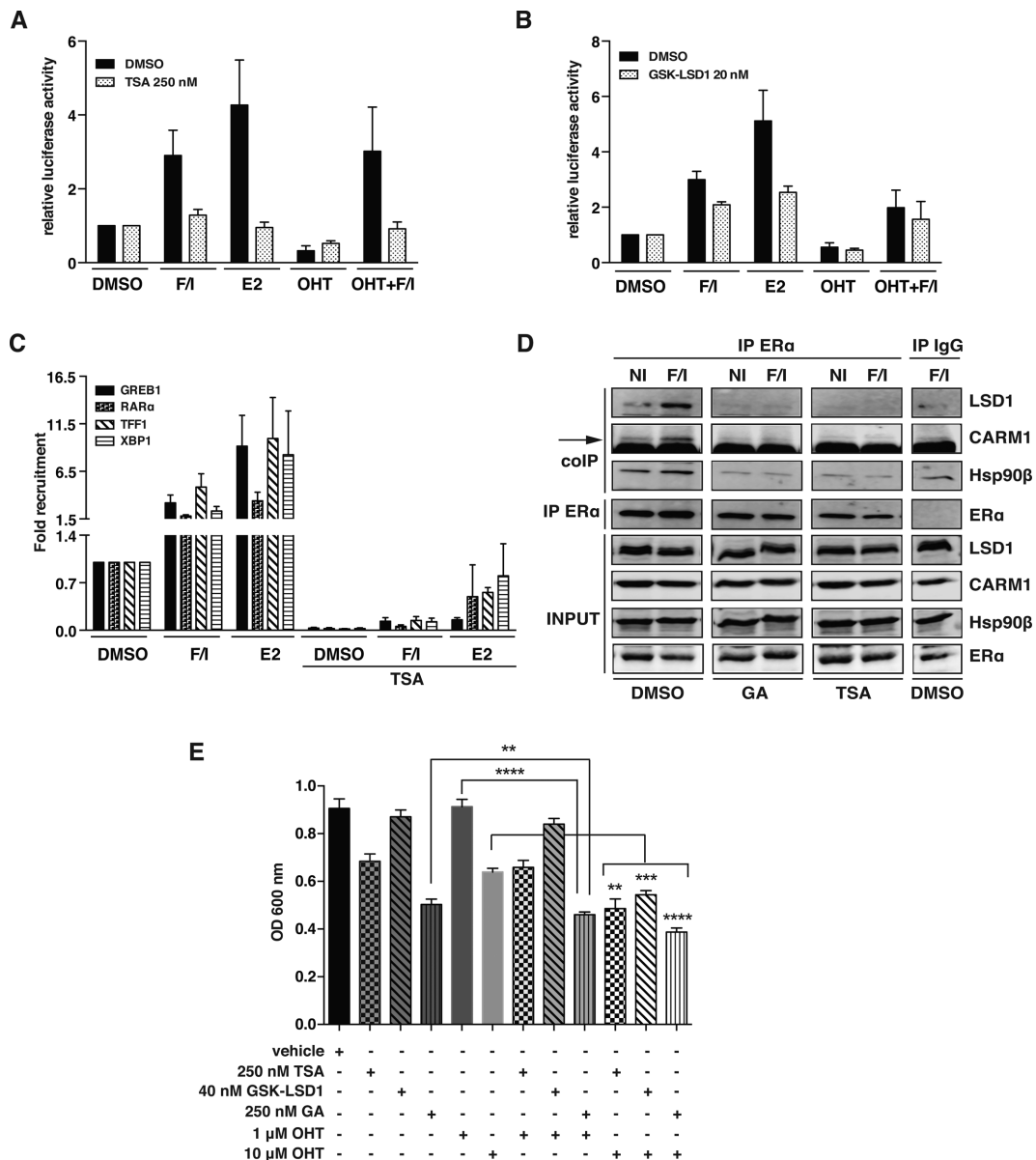
Figure 5. The LSD1 demethylase and HDAC deacetylase activities are required to stimulate ERα-dependent transcription and breast cancer cell proliferation. (**A** and **B**) The inhibition of HDAC and LSD1 decreases ERα activation. Reporter gene assays with MDA-MB-134 cells treated with the pan-HDAC inhibitor trichostatin A (TSA) or the LSD1 inhibitor GSK-LSD1, respectively, were stimulated with F/I, E2 or OHT for 24 h. (**C**) HDAC inhibition decreases ERα binding to endogenous target genes. MDA-MB 134 cells were treated overnight with TSA before stimulation for 45 min and processing for a ChIP assay with an antibody against ERα. (**D**) Drug inhibition of Hsp90 and HDACs abolishes the assembly of the ERα transactivation complex. Co-immunoprecipation experiment with MDA-MB-134 cells were treated for 2 h with vehicle (DMSO) or the indicated inhibitors. GA, geldanamycin. (**E**) Drug inhibition of LSD1, HDAC and Hsp90 increases inhibitory effects of OHT in ER-positive OHT-resistant LCC2 breast cancer cells. The proliferation of LCC2 cells was measured by MTT assay after 96 h of culture. The data points are averages of three independent experiments made in triplicates and the error bars represent the standard error of the mean. Relevant statistically significant values are highlighted by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

they do both in response to cognate ligand and to ligand-independent activation pathways such as cAMP signaling. Despite the substantial differences between these pathways, they end up inducing the formation of rather similar transcriptional complexes; with slightly different assembly rules, most of the components are the same and include chromatin remodelers, scaffold proteins such as Grip1 and Hsp90, and enzymes, which post-translationally modify the chromatin and other factors (Figure 7). From a therapeutic perspec-

tive, considering the pathological role of ERα in breast cancer, it is interesting to note that the involvement of the CoREST complex brings in several druggable factors.

CARM1 and LSD1 are necessary coactivators of the cAMP- and ligand-dependent ERα activation pathways

Earlier studies of our laboratory identified CARM1 as an essential link between PKA and ERα, but revealed a differ-
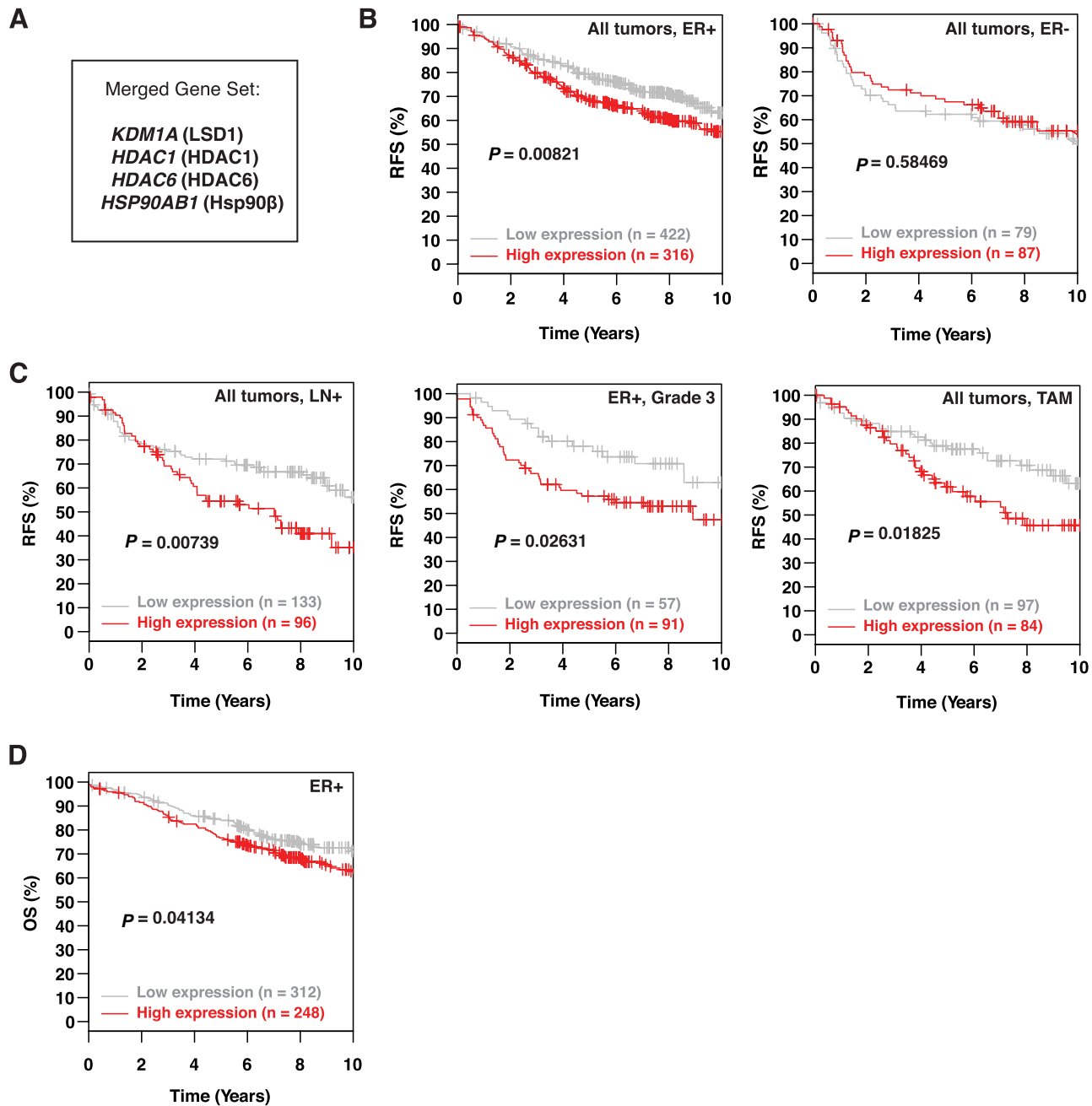
Figure 6. High expression levels of the gene set encoding LSD1-HDAC-Hsp90β correlate with poor breast cancer outcome. (**A**) Merged gene set used for the Kaplan–Meier survival analyses of different breast cancer types. The prognosis-linked gene expression data were obtained using the tool GOBO. (**B**) Relapse-free survival (RFS) analysis of patients with ERα-positive (ER+) versus ERα-negative (ER−) breast tumors. (**C**) RFS analysis of patients with positive lymph nodes (LN+), ERα-positive grade 3 tumors and tamoxifen-treated (TAM) tumors. (**D**) Analysis of overall survival (OS) of patients with ERα-positive tumors.

ent mode of recruitment and a differential requirement for CARM1 for E2- versus cAMP-induced ERα signaling (12). Moreover, whereas the enzymatic activity of CARM1 is important for the transcriptional activation of ERα by E2 (60), it is dispensable for the activation by cAMP (12). As we have demonstrated here, LSD1 is similar to CARM1 in some aspects while being clearly distinct in others. The histone lysine demethylase LSD1 had already been linked to ligand-induced activation of androgen receptor (AR) and ERα

(19,23,27). In view of the established role of FoxA1 as a pioneer factor for ERα (61–63), it is difficult to reconcile these published findings and ours with the claim that LSD1 over-expression compromises FoxA1 recruitment (64). However, in a subsequent publication including some of the same authors, the order of events was proposed to be the other way around (65). Although the exact order and dynamics of pioneer factor recruitment, demethylation of specific histone marks by LSD1 and ERα binding remain to be sorted out,
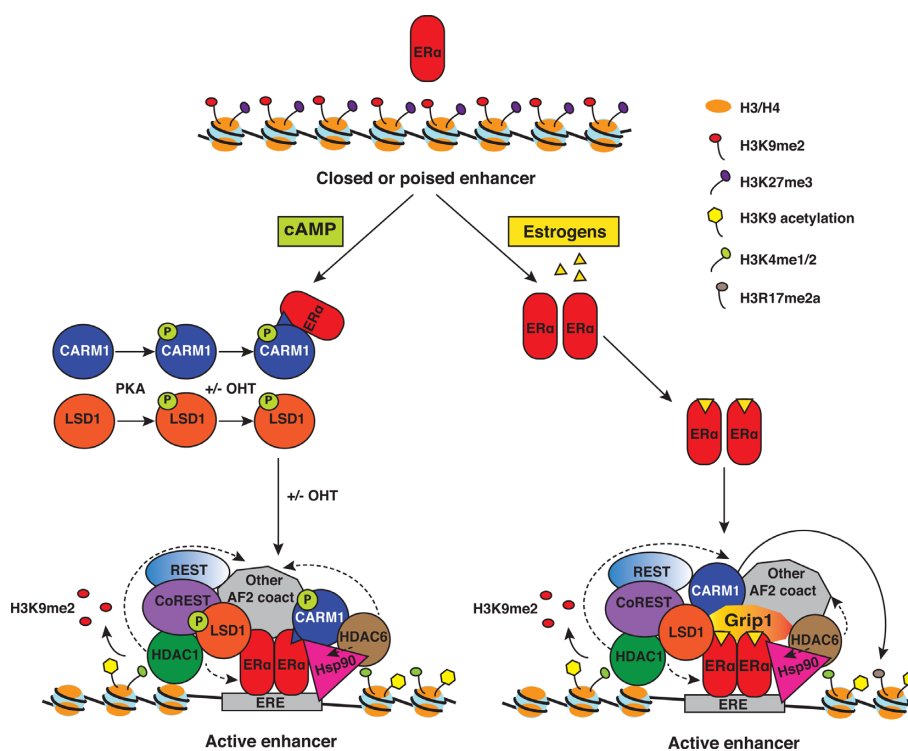
Figure 7. Comparative model of transcriptional activation of ERα by cognate ligand versus cAMP signaling in the absence of estrogen. Note that in the estrogen-dependent pathway the recruitment of CARM1 to ERα is indirect and mediated by Grip1 whereas upon cAMP signaling and phosphorylation of CARM1 and LSD1 by PKA, at least CARM1 is directly recruited to the unliganded ERα. This signaling crosstalk may favor OHT resistance by rendering some steps OHT-insensitive. Additional AF2-directed coactivators are likely to be involved.

it is very clear that LSD1 can promote the activation of ERα. The specifics of the LSD1-targeted histone mark is important as well: H3K9 demethylation is required for activation of hormone-responsive genes by AR and ERα; this is in contrast to the repressive effect of H3K4 demethylation by LSD1 as a member of the CoREST complex and the resulting silencing of a specific set of genes (22,26). Note that we see this ying-yang relationship between H3K9me2 and H3K4me2 upon activation of ERα by E2 or cAMP as well (Supplementary Figures S5B, C and S7). Intriguingly, the increase in the activation mark H3K4me2 at specific ERα targets is itself dependent on LSD1, despite the fact that a modest global increase in H3K4me2 can indeed be observed upon inhibition of LSD1 (Supplementary Figure S7). These results and the previous demonstration that the histone methyltransferase SMYD3 is required for ERα-mediated gene activation (66) lead us to speculate that LSD1 could contribute to the recruitment of specific H3K4 methyltransferases. It should also be mentioned that LSD1 is not the only demethylase that is required for E2-induced ERα activity, albeit not as part of the CoREST complex. KDM4B controls the expression of ERα and FoxA1, interacts with ERα (67–69) and KDM3A is required for the recruitment of ERα to target sites (70). A full understanding of the complex interplay between demethylases and methyltransferases will require further studies.

Mimicking the phosphorylation of both CARM1 and LSD1 with phosphoserine mutants proved to be sufficient to activate ERα in the absence of any stimulus, at least to some extent. This argues that the activation of unliganded ERα by cAMP may be mediated in part by the phosphorylation of both CARM1 and LSD1. This is not to say that cAMP signaling does not synergize with E2. For example, E2-induced non-genomic signaling by membrane-associated ERα triggers the production of cAMP (20,71), and the inhibition of ligand-bound ERα by retinoic acid involves the reversal of LSD1-mediated transcription of ERα target genes by sequestration of PKA to RAR/RXR target sites (20). Further evidence for the role of LSD1 phosphorylation for its coactivator activity comes from a recent study that identified the same phosphorylation site, which we have found for PKA, as a target of PKCα. It was demonstrated that the phosphorylation of this site does not affect the demethylase activity, but is essential for circadian clock-mediated transcriptional activation (72). In this case, the phosphorylation of LSD1 promotes its interaction with the CLOCK:BMAL1 transcription factor complex. Similarly, we had found that the phosphorylation of CARM1 allows its direct interaction with ERα (12). Whether the phosphorylation of LSD1 has a similar effect in the context of the activation of ERα or whether it affects ERα activity indirectly by impacting the recruitment of other factors remains to be elucidated.

In addition, LSD1 may demethylate other substrates, including ERα. Indeed, it was found that LSD1 reverses an inhibitory methylation by SMYD2 of a residue in the DNA binding domain of ERα (29). Whether this is also at play

for the activation of ERα by cAMP and whether other key factors are targeted remain to be investigated.

CoREST complex is co-opted for the regulation of ERα target genes

We have found that proteins of the CoREST complex are recruited to ERα target genes and that their knockdown reduces ERα transactivation and the expression of ERα target genes. These findings strongly emphasize that the function of the CoREST complex is not limited to repressing neuronal genes (43,73,74), regulating developmental genes of early embryogenesis, such as brachyury and homeodomain genes (75–77), the regulation of pluripotency of embryonic stem cells (78) and hematopoietic differentiation (79). When this study was initiated, it was already known that LSD1 could switch from the substrate H3K4 in gene repression to H3K9 in the activation of target genes of steroid-activated AR and ERα (19,23,27). However, it was unknown whether LSD1 plays any role in the ligand-independent activation of ERα by cAMP and whether other components of the CoREST complex are required for activation of ERα. We discovered not only that LSD1 is required for the activation of ERα by cAMP, but that it is accompanied by other components of the corepressor CoREST complex, and even REST, for the activation of ERα by either E2 or cAMP. Even though CoREST was known to be essential for LSD1 demethylase activity *in vivo* and to protect LSD1 from proteasomal degradation (26), this is the first time that CoREST components and REST are shown to contribute to LSD1-mediated ERα activation (Figure 7). It is interesting to speculate that they could assist in promoting or maintaining a certain differentiation state of breast cancer cells. Future work will address how the genetic programs of ERα and CoREST/REST intersect and influence each other.

Requirement for Hsp90 and HDACs - beyond cytoplasmic functions

Our findings that Hsp90 is recruited to the ERα transcription complex on chromatin and that the Hsp90 inhibitor GA disrupts the interaction of ERα with CARM1 and LSD1 are unexpected and run against the standard paradigm for steroid receptors. Work primarily with other steroid receptors has led to the view that the Hsp90 complex is required before activation for the correct folding and maturation, for maintaining the inactive state in the absence of ligand and for efficient response to ligand binding (80,81). Following the ligand-induced or -stabilized release of Hsp90, the active state is thought to be independent of Hsp90. After activation, distinct molecular chaperone functions of Hsp90 and the co-chaperone p23 become relevant to facilitate the disassembly of transcription complexes of hormone-bound steroid receptors and to allow cells to respond dynamically to changes in hormone levels (82,83). A number of other functions have emerged for Hsp90 in the nucleus and even on chromatin in the last few years (84–86). Intriguingly, some 10 years ago it was found that anti-inflammatory effects of estrogens may involve the disruption of a transactivation complex containing Hsp90, the unliganded ERα and several other transcription factors (87).

Based on our results, it now appears that Hsp90 may also provide assistance in assembling or stabilizing certain transcription complexes of signal-activated ERα. The fact that Hsp90 can interact with CARM1 independently of ERα (88) could allow it to serve as a scaffold. Although we could detect the recruitment of Hsp90 to ERα target sites in response to both E2 and cAMP, it is conceivable that the signal-specific differences in the architecture of these complexes may impose subtly distinct requirements for chaperone assistance.

Our discovery that at least two HDACs are involved with active ERα at chromatin is similarly unexpected. HDACs are more typically associated with repression and HDAC6 is better known for its cytoplasmic functions, including as an Hsp90 deacetylase. We find that both HDAC1 and HDAC6 are recruited to the ERα transcription complex and bind to ERα target genes; their knockdown or pharmacological inhibition with TSA correlates with reduced ERα binding to chromatin, a reduction of transactivation in reporter gene assays, and transcription of endogenous target genes. The inhibition of HDACs also disrupts the recruitment of CARM1, LSD1 and Hsp90 to ERα. Consistent with the idea that Hsp90 could act as a scaffold, we can speculate that HDAC inhibitors in this context impair Hsp90 function since this is dependent on deacetylation (46,47). However, we cannot exclude that the inhibition is due to hyperacetylation of ERα itself or of some of its coactivators, as seen for other steroid receptors (89). It is well known that the direct acetylation of ERα affects its activity and that deacetylases reverse the effects: the acetylation of the DNA binding domain at K266 and K268 by p300 and acetylation-mimicking mutants increase ERα DNA binding and activity *in vitro* (90); in contrast, the acetylation of the hinge region at K302 and K303 decreases ERα activity, most probably because these are also sites of regulation by sumoylation, methylation and ubiquitination (89,91,92); the p300/CBP-mediated hyperacetylation of the coactivator steroid receptor coactivator-3 (SRC-3; also known as AIB1) decreases its recruitment to ERα and attenuates hormone-induced gene activation (93). Interestingly, a recent study argues that HDACs promote transcription by stimulating the production of enhancer RNAs that in turn facilitate the release of RNA polymerase II from NELF (94). Recognizing that HDACs affect ERα signaling at multiple different levels, from influencing ERα levels to participating as components of ERα transcription complexes at target sites, we propose that they might also function as co-activators.

Pathological significance

ERα-dependent breast tumors are amenable to endocrine therapy, but many eventually become resistant to it. There are clearly multiple pathways that can lead to endocrine therapy resistance (5), and gaining a better understanding of them might open up new therapeutic avenues. Pathways that can activate ERα in the absence of estrogens can contribute to OHT-resistance (2). Indeed, upon activation of PKA, ERα activity becomes resistant to the inhibitory effects of OHT (9,12,17). Our results obtained with the OHT-resistant MCF7 variant LCC2 indicate that the convergence of the ERα-CARM1-Hsp90 and LSD1-CoREST

complexes could result in the formation of a constitutively active and OHT-resistant ERα transcription complex (Figure 7). The combination of ERα antagonists with inhibitors targeting the main catalytic activities of this complex(es), notably LSD1, the HDACs and Hsp90, may considerably increase their effectiveness.

For HDACs, we confirmed published results with cell lines showing that HDAC inhibitors are able to reestablish OHT-sensitivity (57,95). This is mirrored by promising clinical results obtained with the combinatorial treatment of advanced breast cancer patients (59,96). Although HDACs undoubtedly act at many different levels, we believe their function in transcriptional coactivation by ERα contributes and may ultimately be targeted separately from their other functions, most notably in ERα-unrelated cellular processes.

It is noteworthy that the LSD1 inhibitor GSK-LSD1 is currently in clinical trials for the treatment of refractory small cell lung carcinoma and acute myeloid leukemia. Our results suggest it might be worth testing it against ERα-dependent and OHT-resistant breast cancer. It has been reported that the LSD1 inhibitor pargyline in combination with OHT could reduce the proliferation of OHT-resistant breast cancer cells (97). However, it must be pointed out that the antidepressant drug pargyline is a non-specific inhibitor of monoamine oxidases and that the very high concentrations used to inhibit the demethylation of histone substrates *in vitro* are not likely attainable *in vivo* without considerable off-target effects (98,99). In contrast, GSK-LSD1 is far more potent and specific, despite the fact that LSD1 inhibition has known cytostatic effects (99–101); these may be related to the fact that LSD1 is also highly expressed and a marker of an aggressive phenotype in ERα-negative breast cancer (102).

Hsp90 inhibitors have already been extensively tested in clinical trials for the therapy of a variety of cancers. Whereas single agent treatments have not yet been successful, there is a growing sense that these inhibitors may have potential in combination with drugs against other important targets (103). For OHT-resistant breast cancers, our new mechanistic insights suggest that a renewed and more directed effort might be worthwhile to move beyond earlier reports on the combination of OHT with Hsp90 inhibitors; these had notably suggested that the ERα-stabilizing effects of OHT compared to those of other anti-estrogens might compromise the impact of Hsp90 inhibitors (104,105).

Finally, even if the new players for ERα activity may not all turn out to be good drug targets, they may have value as markers. In this regard, our very limited gene set consisting of only the six genes *KDM1A (LSD1), HDAC1, HDAC6, Hsp90AB, CARM1* revealed potentially useful ERα-related correlations with relapse-free and overall survival, extending previous studies with some of the same markers (32,36,106). Such a gene signature could eventually be refined, possibly combined with others, and applied for a better stratification of breast cancer patients.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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