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Cooperative Activation of Gene Expression by Agonists and Antagonists Mediated by Estrogen Receptor Heteroligand Dimer Complexes

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Running title: Activation of gene expression by ER-heteroligand dimers

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Abbreviations: AF-1, activation function-1; AF-2, activation function-2; BAZ, bazedoxifene; CE, conjugated estrogen; DBD, DNA binding domain; DR1, direct repeat spacing with 1bp; ER, estrogen receptor; ERE, estrogen response element; ER-HLD, ERα heteroligand dime; FXR, farnesoid X receptor; GRE, glucocorticoid response element; LBD, ligand binding domain; LXR, liver X receptor, NCoR, nuclear receptor corepressor; NR, nuclear receptor; pC3, promoter region of the human complement C3 gene; PPAR, peroxisome proliferator-activated receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor, SERM, selective estrogen receptor modulator; SERD, selective estrogen receptor degraders; SRC, steroid receptor coactivator; TR, thyroid hormone receptor, TSEC, tissue selective estrogen complexes, VDR, vitamin D receptor.

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Abstract

Estrogen receptor (ER) antagonists are generally thought to inhibit estrogen action through competitive inhibition resulting in receptor binding to antagonist rather than agonist. However, microarray analyses reveal a group of genes for which ER agonist and antagonist cooperatively regulate expression, suggesting additional models of combined agonist/antagonist action must exist. In conjunction with a chimeric reporter gene and two modified ERs, one [ERα(GSCKV)] with a mutation in the DNA binding domain and the other (ERα-G521R) with a ligand binding specificity mutation, we herein demonstrate that ER agonist and antagonist cooperatively active gene expression through an ER heteroligand dimer complex (ER-HLD) consisting of one subunit of the receptor dimer bound to agonist and another occupied by antagonist. Co-immunoprecipitation experiments confirmed interaction between the agonist-bound and antagonist-bound receptors. This cooperative activation of gene expression was enhanced by SRC-3 coactivator and required each ligand-bound subunit of the dimer to bind to DNA, as well as both activation function-1 domains for maximal transcriptional activity. Ligand combinations able to induce ER-HLD transcriptional activity include the agonists 17β-estradiol or conjugated estrogens with the antagonists tamoxifen, raloxifene, bazedoxifene or fulvestrant. Moreover, ER-HLD can activate transcription in the context of a natural promoter. Taken together, this finding broadens our understanding of the complex relationship between ER agonist and antagonist, and suggests a novel model by which cell and tissue selective effects of antiestrogens may be achieved.

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Introduction

Estrogen receptors (ERs) belong to the nuclear receptor (NR) superfamily of transcription factors and are modular proteins consisting of six domains designated A to F (Heldring et al., 2007). The A/B domain contains activation function-1 (AF-1) that influences transcriptional activity in a ligand-independent manner. The DNA binding domain (DBD, domain C) defines the response element specificity; while the ligand binding domain (LBD, domain E), mediates ligand binding, dimerization and contains a ligand-dependent transactivation function referred to as AF-2. The hinge region (domain D) is located between DBD and LBD, while the F domain is located at the extreme C-terminus. There are two ER genes, ERα and ERβ, and the corresponding proteins share approximately 95% and 55% homology in the DBD and LBD, respectively (Thomas and Gustafsson, 2011). In the classical model, both receptors bind to estradiol with high affinity whereupon they undergo changes in conformation, dimerize either as homodimers (ERα/ERα or ERβ/ERβ) or heterodimers (ERα/ERβ), bind to estrogen response elements (ERE) in the regulatory region of estrogen target genes and recruit coactivators to modulate gene expression (Thomas and Gustafsson, 2011;Heldring et al., 2007).

The biological functions of estrogens are important in many tissues including the breast, prostate, bone, brain and reproductive tract and pharmacological regulation of ER function is important in pre- and post-menopausal women. In addition to ER agonists such as 17β-estradiol (E2), there are two classes of ER antagonists. The selective estrogen receptor modulators (SERMs) including tamoxifen, raloxifene and bazedoxifene exert estrogen-like and antiestrogen-like activities in a tissue selective manner, while the selective estrogen

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receptor degraders (SERDs) such as ICI 182,780 downregulate ERα and inhibit ER function in most contexts. Agonists and antagonists for ER bind to the same site within the LBD (Shiau et al., 1998;Brzozowski et al., 1997) and antagonists are therefore able to competitively block estrogens from binding to the receptor and inducing gene expression. Moreover, antagonist-bound ERs adopt a distinct conformation that enables them to preferentially interact with corepressors rather than coactivators (Huang et al., 2010), thereby reinforcing their negative regulatory properties.

In contrast to the model of agonist and antagonist competing for binding to the LBD, ligand binding to heterodimeric NRs such as RXR partnered with either RAR, TR, VDR or PPAR, and regulation of their transcriptional activities is more complex (Pérez et al., 2012;Germain et al., 2006;Germain et al., 2002). Some heterodimers (PPAR/RXR, LXR/RXR, FXR/RXR) are “permissive” whereby a RXR-selective ligand (“rexinoid”) and a NR partner ligand can independently or synergistically activate the transcriptional activity of the heterodimer (Kliewer et al., 1992;Willy et al., 1995;Leblanc and Stunnenberg, 2012). In contrast, “non-permissive” heterodimers (including RAR/RXR, VDR/RXR and TR/RXR) are unresponsive to rexinoids alone and can only be stimulated by ligands that bind to the RXR partner receptor (Forman et al., 1995;Kurokawa et al., 1994;Westin et al., 1998) although rexinoids synergize with partner agonists to activate gene transcription when both ligands are present (Roy et al., 1995;Shulman et al., 2004). In addition, an RXR homodimer antagonist functions as an agonist when RXR is paired to specific partners, including PPAR and RAR (Lala et al., 1996). Thus, the ability of a given receptor ligand to activate or repress gene expression can be influenced by other ligands bound to the dimer partner.

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Unlike RXR-associated heterodimers, ERα is generally thought to form homodimers bound to either agonist or antagonist depending upon the relative ligand concentrations. Thus, antiestrogens (e.g. tamoxifen) block E2 binding to ERα and antagonize estrogen-stimulated gene expression which is highly desirable relative to breast cancer prevention and treatment. However, recent MCF-7 breast cancer cell microarray experiments revealed a group of novel genes cooperatively regulated by ERα agonist and antagonist (Chang et al., 2010;Wardell et al., 2012). This is difficult to reconcile with competitive antagonism, and argues for an additional model for combined agonist/antagonist regulation of ER activity. Based on RXR heterodimer models, it was hypothesized that an antagonist within an ERα heteroligand dimer (ER-HLD) complex, consisting of antagonist-bound and agonist-bound ERα subunits, could stimulate rather than inhibit gene expression. To date, this possibility has not been addressed experimentally, particularly because regulating the binding of agonist and antagonist to homodimers is considerably more difficult than controlling the interaction of two different ligands to RXR-containing heterodimers. Nonetheless, this is an important question that has implications for the pharmacology of SERMs when used to inhibit ERα function in breast tissues where systemic and even locally produced estrogens may be present (Yaghjyan and Colditz, 2011).

In order to evaluate whether antagonists could positively regulate the transcriptional activity of an ER-HLD complex, a chimeric luciferase reporter system was developed in conjunction with receptor mutations that regulate the specificity of ligand binding as well as DNA interaction. This model system demonstrates that ERα agonist and antagonist can cooperatively activate gene expression through an ER-HLD complex and has implications for understanding the molecular pharmacology of clinically important estrogen receptor

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antagonists.

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Materials and Methods

Cell Culture and Reagents

The HeLa human cervical carcinoma and HepG2 human hepatoma cell lines were obtained from American Type Culture Collection. HeLa cells were cultured in Dulbecco's Modified Eagle (DME) media containing 10% fetal bovine serum (FBS). HepG2 cells were maintained in minimum essential medium (MEM) supplemented with 10% FBS. 17β-Estradiol, 4-hydroxytamoxifen and raloxifene were purchased from Sigma Chemical Company (St. Louis, MO). Bazedoxifene was provided by Pfizer Inc. (New York, NY). The pure antiestrogen ICI 182,780 was obtained from Tocris (Ellisville, MO). The mixture of the unconjugated forms of the 10 most abundant conjugated estrogen (CE) components of Premarin was prepared in the same relative proportions as is present in the Premarin formulation (Chang et al., 2010). Thus, a 1 mM CE stock solution was prepared from 1 mM solutions of each of the 10 components mixed proportionally according to their percentage ratios.

Plasmids

The expression plasmid pCR3.1-ERα and corresponding reporter gene ERE-e1b-Luc have been described previously (Nawaz et al., 1999a). The pCR3.1-ERα(GSCKV) plasmid was generated by PCR mutagenesis in which primers containing the DNA binding domain mutation region (5'-GGAAGCTGTAAAGTT-3') were used in two PCR reactions, generating ER cDNA fragments between the SmaI site and the mutation and between the mutation and the BglII site. The two PCR products were then used as a template in a third PCR reaction to

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create a fragment that was then digested with SmaI and BglII and subcloned back into the pCR3.1-ERα vector. The pCR3.1-ERα-G521R-Flag was generated by PCR with primers (5'-GGGGTACCCGGTCTGCACCCTGC-3' and 5'-CCGCTCGAGCGGACCGTGGCAGGGAA-3') using $pCMV_5$-ERα-G521R as a template (kindly provided by Dr. Benita Katzenellenbogen (Schodin et al., 1995)) and subcloned into pCR3.1 vector upstream of the sequence for a Flag epitope. The pcDNA3-ERα(GSCKV)-HA plasmid was generated by PCR with primers (5'-CGGAATTCCGGTCTGCACCCTGC-3' and 5'-CCGCTCGAGGACCGTGGCAGGGAA-3') using pCR3.1-ERα(GSCKV) as a template and subcloned into pcDNA3-HA vector (kindly provided by Dr. Hank H. Qi from Harvard Medical School, Boston, MA (Qi et al., 2010)). GRE-Luc and pC3-Luc have been described previously (Tzukerman et al., 1994;Nawaz et al., 1999b). The GGGTCAcagTGACCT estrogen response element of ERE-e1b-Luc was mutated to GGGTCAcagTG**TT**CT or **TGTA**CAcagTGACCT for the EGRE-e1b-Luc and the GERE-e1b-Luc reporter genes, respectively, by site directed mutagenesis. The $^{1}/_{2}$ERE-Luc reporter was generated by mutating the GRE half site in EGRE-Luc construct from TGTTCT to TTTTTT by using mutagenesis primers (sense 5'-CTGCGATCTAAGTAAGCTTGGGTCACAGTTTTTTGATCAAAGTTAATGT-3' and antisense 5'-ACATTAACTTTGATCAAAAAACTGTGACCCAAGCTTACTTAGATCGCAG-3') following the instruction of Stratagene QuikChange® Site-Directed Mutagenesis Kit. The AF-1 mutant constructs 179C- ERα-G521R and 179C- ERα-GSCKV were generated by PCR amplifying the fragments of 179-595AA of the pCR3.1-ERα-G521R and

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pCR3.1-ERα(GSCKV) vectors, respectively; and subcloning them into pCR3.1 vectors, respectively. All constructs were verified by DNA sequencing.

Luciferase Assay

For the HeLa cell line, 200,000 cells/well were seeded in 6-well plates in phenol red-free DME media containing 5% charcoal-stripped FBS (sFBS) one day before transfection. Cells were transfected with 1 µg reporter gene and indicated amounts of expression vectors using Lipofectamine reagent (Invitrogen) in Opti-MEM (Invitrogen) following the manufacturer's instructions. Four to six hours later, Opti-MEM was replaced with phenol red-free DME media containing 5% sFBS. For the HepG2 cell line, 750,000 cells/well were seeded in 6-well plates in phenol red-free MEM supplemented with 10% sFBS. The next day media was removed. Cells were transfected with 1 µg reporter gene and indicated amounts of expression vectors using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen) following the manufacturer's instructions. Four to six hours later, Opti-MEM was replaced with phenol red-free MEM supplemented with 10% sFBS. The following day, cells were treated with the indicated ligands at the specified concentrations, and 24 h thereafter were harvested using TEN buffer (40 mM Tris, pH 7.5, 150 mMNaCl and 1 mM EDTA). Cell pellets were resuspended in Reporter Lysis Buffer (Promega) and lysed according to the manufacturer's instructions. Luciferase activity was determined using a Luminoskan Ascent Microplate Luminometer (Thermo Labsystems) with Luciferase Assay Reagent (Promega) and normalized to protein concentration determined by Bio-Rad Protein Assay reagent (Bio-Rad Laboratories). All experiments were done in duplicate and repeated for three times.

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Western blot

Cells were collected using TEN buffer and resuspended in ER extraction buffer (50 mM Tris, pH 8.0, 400 mM NaCl, 5 mM EDTA, 1% NP40, 0.2% Sarkosyl) supplemented with an EDTA-free Complete Mini protease inhibitor (Roche Applied Science, Indianapolis, IN). Cells were lysed on ice for 30 min after which protein concentrations were determined as above. Twenty micrograms of protein was mixed with LDS Sample Buffer containing Sample Reducing Agent (both Invitrogen), then heated to 75°C for 10 min. Proteins were separated using a 10% Bis-Tris gel and transferred in NuPAGE Transfer Buffer (Invitrogen) to a nitrocellulose membrane (GE Water & Process Technologies). The membrane was blocked in PBST (1×PBS plus 0.1% Tween-20) containing 5% dry milk powder (w/v). Primary antibodies were diluted in PBST with 1% milk powder and detected using HRP-conjugated secondary antibodies and the ECL Plus Western Blotting Detection System (both from GE Healthcare). Primary antibodies include ERα (HC-20, Santa Cruz Biotechnology), Actin (Clone C4, Millipore), anti-FLAG (F7425, Sigma) and HA (Y-11, Santa Cruz Biotechnology).

Co-immunoprecipitation

Following transfection, HeLa cells were cultured in phenol red-free DME media containing 5% sFBS for 48 h before treated with the indicated ligands for 1 h. Cells were harvested on ice in 1×PBS containing Complete Mini protease inhibitor (Roche). Cell pellets were lysed at 4 °C for 30 min in IP lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and Complete Mini protease inhibitor), and lysate protein concentrations were determined as described above. One mg of lysate was pre-cleared for 2 h at 4 °C with 4 μg

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mouse IgG and pre-washed protein G PLUS agarose beads (both from Santa Cruz Biotechnology) under constant rotation. Thereafter, lysates were incubated with 40 µL anti-Flag M2 affinity gel suspension overnight at 4 °C. Immune complexes were washed four times with IP lysis buffer, then eluted using LDS sample buffer, and analyzed by Western blot analysis as described above.

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Results

To test whether ER dimers in which each subunit binds to a different ligand can regulate gene expression in response to combined agonist/antagonist treatments, a chimeric receptor and reporter system was employed. First, a mutant form of ERα, ERα-G521R (Schodin et al., 1995) that possesses a ligand binding domain mutation permitting high affinity antagonist but not agonist binding was generated (**Fig. 1A**). As shown in **Fig. 1B**, estradiol (E2) and conjugated estrogens (CE) but not bazedoxifene (BAZ) activated wild-type ERα transcriptional activity measured by pC3-Luc reporter in HepG2 cells. In contrast, the ERα-G521R mutant was strongly activated by BAZ but not E2 or CE, demonstrating a complete change in the ligand specificity for inducing gene expression. Bazedoxifene was unable to increase pC3-Luc activity in cells co-transfected with pCR3.1 empty vector, indicating that the BAZ-induced luciferase expression was ERα-G521R–dependent (data not shown). The relative ability of CE and BAZ to regulate the transcriptional activity of ERα-G521R was further evaluated in dose response studies. Increasing concentrations of CE from 1 nM to 100 nM were unable to stimulate ERα-G521R activity, while BAZ stimulated the activity of ERα-G521R in a dose-responsive manner (**Fig. 1C**).

The second objective was to establish conditions where wild-type ERα would be activated by CE in the presence of a concentration of BAZ sufficient to activate ERα-G521R. A dose response study in cells transfected with wild-type ERα shows comparable activation of the pC3-Luc reporter gene by 1 to 100 nM CE, while BAZ failed to activate luciferase activity (**Fig. 1D**). As expected, BAZ activation of ERα-G521R was not influenced by co-treatment with CE, consistent with the inability of this receptor to be activated by estrogens (**Fig. 1E**). In contrast, antagonism studies demonstrated that BAZ at a concentration that is equal to or greater than the concentration of CE inhibited wild type ERα transcriptional activity (**Fig. 1F**). Thus, BAZ at a concentration of 1 nM or 10 nM does not

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antagonize 10 nM CE or 100 nM CE induced wild-type ERα activity, respectively. Since 10 nM but not 1 nM BAZ was sufficient to activate ERα-G521R activity alone (Fig. 1C) or in combination with 1 to 100 nM CE (Fig. 1E), a combined treatment dosage of 100 nM CE and 10 nM BAZ was selected based on their abilities to effectively activate wild-type ERα and ERα-G521R, respectively.

Third, a DNA binding specificity mutation was introduced into wild-type ERα and a modified ER responsive reporter was generated. These chimeric reporter genes, GERE-Luc or EGRE-Luc consist of a luciferase cDNA downstream of a composite response elements consisting of a GRE half-site fused 5’ to an ERE half-site and *vice versa* (**Fig. 2A**). To generate a receptor mutant able to bind to the GRE half-site, three amino acid residues located at the P-box, implicated in specific DNA interaction, in the first zinc finger module of the wild-type ERα DNA binding domain (DBD) were mutated to the corresponding amino acids in the glucocorticoid receptor (GR) DBD (**Fig. 2B**) thereby generating ERα(GSCKV) which had been shown previously to enable binding to GREs (Mader et al., 1989). Luciferase assays were employed to evaluate the function of ERα(GSCKV). As shown in **Fig. 2C**, ERα(GSCKV) was unable to activate ERE-Luc reporter activity in response to E2 or CE treatment while expression of the GRE-Luc reporter was strongly stimulated. This was consistent with prior testing reports (Tremblay et al., 1999). In contrast, wild-type ERα strongly stimulated expression of luciferase from the ERE-Luc but not the GRE-Luc reporter. To test whether wild type ERα and ERα(GSCKV) can work together to regulate the activity of the chimeric EGRE-Luc reporter, HepG2 cells were transfected with EGRE-Luc as well as wild type ERα and ERα(GSCKV), alone or in combination. As shown in **Fig. 2D**, 10 nM CE induced EGRE-Luc activity in the presence of either wild-type ERα or ERα(GSCKV) single receptor to a variable extent. However, greater activity was observed when both receptors

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were co-expressed indicating that they work together to activate expression of the EGRE-Luc reporter.

With the successful generation of the chimeric reporter genes and receptor mutations, the ability of ERα dimers bound to agonist and antagonist to activate gene expression was tested. First, HepG2 cells were co-transfected with EGRE-Luc as well as ERα-G521R and ERα(GSCKV), alone or in combination (**Fig. 3A**). In cells expressing ERα-G521R, luciferase activity was stimulated by BAZ but not CE treatment, and CE+BAZ did not further increase luciferase. In cells expressing ERα(GSCKV), CE but not BAZ induced reporter activity, while the combination of CE and BAZ showed no further induction. When both forms of ERα were expressed in the cells, the combined CE+BAZ treatment induced maximal reporter gene expression while exposure to only agonist or antagonist yielded partial luciferase activity. The cooperative effect of CE+BAZ was also observed on the GERE-Luc reporter (**Fig. 3B**). In order to test whether this phenomenon is cell-type specific, similar luciferase assays were conducted in HeLa cells which demonstrated that combined CE and BAZ treatment induced greater activity of the chimeric reporters than either agent alone (**Fig. 3C**).

The above results indicate that ERα agonist and antagonist can work cooperatively to induce the maximal transcriptional activity. To test whether coactivators such as SRC-3, which are known to enhance ERα transcriptional activity (Suen et al., 1998), could enhance CE+BAZ stimulated expression of the chimeric reporter, HeLa cells were transfected with GERE-Luc, ERα-G521R and ERα(GSCKV) in the absence or presence of SRC-3 expression vector. Exogenous SRC-3 significantly enhanced CE+BAZ-induced luciferase activity, indicating that this coactivator can functionally interact with these receptors following combined agonist/antagonist treatment and suggesting a role for SRC-3 in mediating the gene

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expression induced by this combined ligand treatment (**Fig. 3D**). Additionally, transfection of SRC-1or SRC-2 can also boost the cooperative activation of CE+BAZ (data not shown). Lastly, to exclude the effect of differential receptor expression levels on reporter activity, Western blots were performed. HepG2 or HeLa cells transfected with single receptors [ERα-G521R or ERα(GSCKV)] expressed similar levels of receptor in comparison to ERα-G521R and ERα(GSCKV) co-transfected cells, indicating that the greater activity observed in the latter condition is not due to increased receptor expression (**Fig. 3E & F**). Taken together, the above data demonstrate the ability of an antagonist treatment to work together with agonist to activate gene expression in the chimeric model system. This cooperative effect requires the presence of both forms of receptor and can be co-activated by SRC-3.

To further characterize the ability of these receptors to mediate the cooperative effect of combined agonist/antagonist treatment, the ability of epitope tagged ERα-G521R-Flag and ERα(GSCKV)-HA receptors to interact with one another was tested in a co-immunoprecipitation assay. In cells treated with CE+BAZ, Flag antibody is able to immunoprecipitate ERα-G521R-Flag and ERα(GSCKV)-HA indicating an interaction between the receptors (**Fig. 4A**). A low level of interaction between the two forms of receptor is detected in the absence of any ligand, and is enhanced upon treatment with CE+BAZ (**Fig. 4B, compare lanes 5 to 6**), indicating that a complex can be formed between CE-bound ERα(GSCKV)-HA and BAZ-bound ERα-G521R-Flag *in vivo*. Receptor-receptor interaction was also promoted by BAZ alone (compare lanes 5 to 8), consistent with the ability of this ligand to bind to both receptors. In contrast, the interaction between ERα-G521R-Flag and ERα(GSCKV)-HA was inhibited by CE (compare lanes 5 to 7) presumably due to the ability of CE to promote formation of ERα(GSCKV)-HA homodimers and consequently reducing

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the amount of this receptor available for heterodimer formation with ERα-G521R-Flag. To test whether the epitope-tagged receptors are functional, HeLa cells transfected with GERE-Luc, ERα-G521R-Flag and ERα(GSCKV)-HA were treated with CE and BAZ, alone or in combination. The epitope tagged vectors were similar to ERα-G521R and ERα(GSCKV) in their ability to cooperatively activate luciferase activity in response to CE+BAZ treatment (data not shown). These data demonstrate the possibility that agonist-bound ERα(GSCKV)-HA and antagonist-bound ERα-G521R can form a ERα heteroligand dimer (ERα-HLD) complex in cells that can cooperatively stimulate gene expression.

In order to test whether the CE+BAZ cooperative activation of chimeric reporter activity requires each monomer within the ERα-HLD to bind to DNA, a ½ERE-Luc reporter was generated in which the GRE half-site of the chimeric reporter was mutated. Activities induced by CE+BAZ treatment in cells transfected with ERα-G521R and ERα(GSCKV) were compared for the ½ERE-Luc *versus* the EGRE-Luc reporter (**Fig. 5A & B**). No cooperative effect of CE+BAZ treatment was observed for the ½ERE-Luc reporter in either HepG2 or HeLa cells. This indicates that CE-bound ERα(GSCKV) and BAZ-bound ERα-G521R must both bind to DNA to cooperatively activate gene expression, rather than a single receptor binding to the ERE half site and interacting with a non-DNA binding receptor partner solely via protein-protein interactions.

As the agonist activity of SERMs is dependent on AF-1 activity (Berry et al., 1990), the role of each receptor's AF-1 domain on the activity achieved by the ERα-HLD complex was evaluated on the EGRE-Luc and GERE-Luc reporters. Deletion of the AF-1 domain of either of the ERα receptors attenuated the cooperative effect of CE+BAZ, regardless of the order in which the receptors bound to the target gene (**Fig. 6A & B**). Moreover, removal of both AF-1 domains from the heteroligand dimer complex completely blocked the ability of

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CE+BAZ to stimulate luciferase gene expression, indicating that the AF-1 domains from each ERα subunit make important contributions to the transcriptional activity of the ERα heteroligand dimer.

To extend these observations beyond the chimeric reporter model system, the ability of CE+BAZ to cooperatively activate a reporter gene regulated by a natural ERα target promoter was investigated. HepG2 cells were co-transfected with the pC3-Luc reporter, consisting of the complement 3 promoter linked to luciferase (Tzukerman et al., 1994) along with wild-type ERα and ERα-G521R, alone or in combination. For cells expressing only wild-type ERα or ERα-G521R, the activity from CE or BAZ single treatment was equivalent to the CE+BAZ combination treatment (**Fig. 7**). However, when both receptors are co-expressed, luciferase activity is greater in cells treated with CE+BAZ than either of the single ligands alone. These data clearly indicate that CE-bound wild-type ERα and BAZ-bound ERα-G521R can cooperatively work to promote a transcriptional response.

In addition to the combination of CE and BAZ, the ability of different combinations of estrogens and SERMs to cooperatively activate expression of the chimeric reporter was tested. As shown in **Fig. 8A,** E2 cooperated with BAZ to stimulate the activity of the GERE-Luc reporters in HeLa cells at a level comparable to CE+BAZ combined treatment indicating that estradiol, the most potent naturally occurring estrogen, can work in combination with antagonists to induce gene expression. This effect is not limited to BAZ as CE could cooperate with a variety of SERMs including 4-hydroxytamoxifen, raloxifene and ICI 182,780 to activate gene expression (**Fig. 8B**). Similar amounts of receptor expression were detected for all treatment groups, indicating that the greater activities in the combination conditions are not due to a corresponding change in receptor levels (**Fig. 8B, inset**). Thus, these data indicate that the agonist/antagonist cooperative transcriptional effects mediated by

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an ERα heteroligand dimer complex can be achieved by a variety of estrogen and anti-estrogen combinations.

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Discussion

ER antagonists are generally thought to inhibit estrogen action through a competitive mechanism. However, microarray analyses reveal a group of genes for which ER agonist and antagonist cooperatively regulate expression, suggesting additional models of combined agonist/antagonist action must exist (Chang et al., 2010;Wardell et al., 2012). We herein demonstrate that ER agonist/antagonist combined treatment can cooperatively activate gene expression through an ER-HLD complex consisting of one receptor monomer bound to agonist and another occupied by antagonist. This cooperative activation of gene expression can be enhanced by SRC-3 coactivator, requires both ligand-bound subunits to bind to DNA and both AF-1 domains within the ER-HLD for maximal transcriptional activity. Moreover, ER-HLD complexes can activate transcription in the context of a natural promoter, and taken together demonstrates that ERs bound to different classes of ligands can form dimers that promote gene expression.

The ability of the CE-bound ERα(GSCKV)-HA and BAZ-bound ERα-G521R-Flag to form a dimer complex in cells, as demonstrated by co-immunoprecipitation experiments, revealed a weak level of basal interaction that was significantly enhanced upon treatment with CE+BAZ. This is analogous to the ability of wild-type ERα to exist as a dimer in the absence of ligand and for E2 to promote dimer interaction (Tamrazi et al., 2002). Individual ER subunits come together to form dimers through two domains, one in the DBD and a second in the LBD (Bai and Gust, 2009). The weak dimerization interface located in the DBD is ligand-independent and responsible for the selection of the spacing distance between the two ERE half-sites (Bai and Gust, 2009;Kuntz and Shapiro, 1997). The latter constitutes

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the principal ERα dimerization interface with head-to-head contacts between the two subunits mediated primarily through helix 11 (H11) to H11 interactions (Shiau et al., 1998;Brzozowski et al., 1997). Crystal structures of the ERα LBDs bound to either E2 or raloxifene reveal that both ligands bind to the same pocket, and that the overall homodimeric arrangement is the same regardless of whether the LBD is agonist or antagonist bound (Shiau et al., 1998;Brzozowski et al., 1997). Indeed, with the possible exception of ICI-bound ERα, the relative monomer orientation at the H11 dimer interface does not vary substantially for receptors bound to agonist or antagonist (Pike et al., 2001), and it is therefore reasonable that these interaction surfaces can mediate dimer formation even when each subunit is occupied by a different ligand.

Through the use of chimeric reporters which directed CE-bound ERα(GSCKV) to bind to DNA either upstream or downstream of the BAZ-bound ERα-G521R, we were able to demonstrate that the relative position of the receptors does not impact cooperative activation by combined agonist/antagonist treatment. This is different from RXR-associated heterodimers in which the polarity of receptor binding to DNA impacts transcriptional activity (Orlov et al., 2012;Jimenez-Lara and Aranda, 1999;Chandra et al., 2008). For direct repeat (DR) response elements, RXR is generally located on the upstream half-site and this arrangement permits the ligand-bound NR partner to attain an active conformation that facilitates coactivator recruitment (Mangelsdorf and Evans, 1995;Orlov et al., 2012). However, the RAR/RXR heterodimer binds to DNA with a reversed polarity on DR1 (direct repeat spacing with 1bp) response elements, and in most contexts this heterodimer constitutively represses transcription because the NCoR corepressor remains associated with

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the RAR/RXR heterodimer even in the presence of RAR or RXR ligands (Kurokawa et al., 1994;Kurokawa et al., 1995). Thus, RAR/RXR experiments suggest that the relative orientation of half-sites within the response element can allosterically regulate receptor interaction with coregulators leading to either activation or repression of gene expression, but this is not the case for ER-HLDs since they interacted with SRC-3 coactivator and induced gene expression in both DNA-binding orientations.

In the classical model for ER dimers binding to EREs, each receptor partner binds to one of the ERE half-sites with the 3-bp spacer influencing stability of the dimer interface and DNA binding specificity (Schwabe et al., 1993b;Schwabe et al., 1993a). In contrast, evidence from multiple whole genome studies indicates that a majority of ERα binding sites do not correspond to consensus EREs but rather encompass one or more half-site EREs (Carroll et al., 2005;Lin et al., 2007). This was somewhat surprising as individual half EREs were considered to be non-functional because early *in vitro* binding experiments including EMSA assays did not detect binding to these half-sites (Klinge, 2001). However, a recent report demonstrates ERα binding to half-site EREs, particularly in conjunction with elevated high-mobility group protein B1 expression, and stimulation of luciferase reporter activity (Joshi et al., 2011). Our data also indicate that ERα can weakly activate gene transcription through a half-site ERE site in response to ligand treatment, but without a cooperative effect of combined agonist/antagonist treatment indicating that effective ER-HLD promotion of gene expression requires both ER subunits to bind to DNA rather than one receptor tethering a second ER molecule to the gene. Whether ER-HLD can mediate transcriptional effects via multiple half-site EREs, as have been found in numerous genes,

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remains to be determined.

Activation of gene expression by ERs is dependent upon coactivators, such as SRC family coactivators, which make their primary contact through interaction of one or more LXXLL motifs with the AF-2 domain of agonist-bound receptors (Heery et al., 1997). Conversely, antagonist-bound ERα adapts a conformation in which the LXXLL-like sequence within helix H12 binds against the coactivator docking surface and thereby prevents coactivators from effectively binding to receptor (Shiau et al., 1998). It has been reported that SRC-1 and SRC-2 bind to the liganded ERα LBD with a stoichiometry of one coactivator per homodimer (Margeat et al., 2001;Osz et al., 2012), and a similar mode of binding was observed for SRC-1 and SRC-2 binding to RAR-RXR heterodimers (Osz et al., 2012). Moreover, 1,25-dihydroxyitamin D3 and 9-cis-retinoic acid synergistically activate VDR/RXR heterodimers by facilitating a concerted interaction between both receptors with distinct NR boxes of one molecule of SRC-1 (Zhang et al., 2011). Enhancement of agonist/antagonist-stimulated ER-HLD activity by SRC-3 indicates that this coactivator can interact with the heteroliganded dimer to active gene expression. While it is highly likely that SRC-3 binds to the agonist-bound LBD, it is less certain that this coactivator can bind to antagonist-bound ER-G521R and whether one molecule of SRC-3 can bind to the LBD of each member of the ER-HLD is therefore unclear.

Relative to co-stimulation by agonist/antagonist, the AF-1 domain is important for the partial agonist activity of SERMs such as tamoxifen (McInerney et al., 1996), and SRC-1 can bind to both the AF-1 and AF-2 domains of ERα through the coactivator's Q-rich region and LXXLL motifs, respectively (Mérot et al., 2004). This raises the possibility that SRC-3

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interacts with ER-HLDs through different AF domains on distinct subunits (*e.g.* AF-2 on agonist-bound ER(GSCKV) and AF-1 on antagonist-bound ER-G521R) to induce maximal transcriptional activity. Indeed, while our data revealed that AF-1 deletion for either ERα-G521R or ERα(GSCKV) attenuated transcriptional activity, loss of a single AF-1 domain had a greater impact on the ERα-G521R than the ERα(GSCKV) receptor, suggesting a greater dependence on AF-1 activity by the former component of the ER-HLD. The near complete loss of transcriptional activity when both AF-1 domains were deleted further demonstrates the importance of the AF-1 region to ER-HLD transcriptional activity, and suggests that ER-HLD activity may be more prominent in cell and tissue environments favorable to AF-1 activity (*e.g.* during elevated growth factor signaling).

Agonist and antagonist combinations can cooperatively activate gene expression mediated through ER-HLDs on chimeric reporter genes as well as on a target gene regulated by a natural promoter, indicating the potential of this novel mode of ERα function to regulate endogenous gene expression. Two recent microarray experiments demonstrate that in MCF-7 cells treated with estrogen and SERMs, alone or in combination, there is a subset of genes induced to a greater extent by combined estrogen and SERM treatment than by either single agent (Chang et al., 2010;Wardell et al., 2012). The ability of ER-HLD to cooperatively regulate gene expression induced by combined agonist/antagonist treatments provides a possible mechanistic explanation for the induction rather than the inhibition of the expression of these genes. On a global level, the features of these potential ER-HLD regulated genes that enables antagonists to cooperate with, rather than antagonize agonists in the stimulation of their expression are unknown. Of potential significance, the existing gene microarray data

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also reveal unique gene sets induced by different antagonist/estrogen combinations, suggesting that the regulatory (*e.g.* promoter) regions of endogenous genes encode information that enables differential responses to distinct ER-HLD complexes (*e.g.* following bazedoxifene/estrogen *versus* raloxifene/estrogen exposure) perhaps through alterations in ER-HLD conformation and resultant recruitment of distinct coactivator complexes.

Our novel finding of ER-HLD mediating the cooperative effect of ER agonist and antagonist within a dimer context not only broadens our understanding of the complex mechanisms of action of ER agonists and antagonists, but also is of significant clinical interest. Combined administration of a SERM and an estrogen has been evaluated clinically, and these tissue selective estrogen complexes (TSECs) alleviate post-menopausal symptoms without the increased risks associated with estrogen monotherapy (Archer, 2010;Lobo et al., 2009;Lindsay, 2011;Pinkerton et al., 2009). In addition, the well-established use of ER antagonists such as tamoxifen for breast cancer prevention and treatment is based on their demonstrated ability to inhibit E2-induced effects as well as their clinical efficacy (Osborne, 2012). Thus, antagonists are routinely employed in settings where endogenous estrogens, be they systemic or locally produced by breast and/or tumor cells (Yaghjyan and Colditz, 2011), are available to generate ER-HLD complexes. The contribution of these types of complexes to the *in vivo* biological activities of ER antagonists remains to be fully elucidated, but these data suggest a potential role for ER-HLDs in the gene-, cell- and tissue-specific of SERMs and TSECs, as well as the ability of breast tumors to become resistant to SERM therapies.

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Participated in research design: Shuang Liu, Carolyn L Smith

Conducted experiments: Shuang Liu

Contributed new agents or analytic tools: Sang Jun Han

Performed data analysis: Shuang Liu, Carolyn L Smith

Wrote and contributed to the writing of the manuscript: Shuang Liu, Carolyn L Smith

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Footnotes

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Figure Legends

Figure 1. Activation of ERα-G521R with bazedoxifene. (A) Schematic diagram showing wild type ERα (ERα-wt) and mutant ERα (ERα-G521R) used in these experiments. ERα consists of six domains (A-F). The A/B domains contain the ligand-independent AF-1 domain, the C domain is the DNA-binding domain (DBD), D is the hinge and the EF domain encompasses the ligand binding domain (LBD). The arrow indicates the position of the G521R point mutation. (B) HepG2 cells were co-transfected with 1μg pC3-Luc reporter and 50 ng expression vectors for either ERα-G521R or ERα-wt. Cells were treated with the indicated concentrations of estradiol (E2), bazedoxifene (BAZ) or conjugated estrogen (CE) for 24 hr, and then harvested for luciferase assay. The activity of 10 nM E2 treated ERα-wt transfected cells was set to '100'. (C, E) HepG2 cells co-transfected with 1μg pC3-Luc reporter and 50 ng ERα-G521R were treated with the indicated concentrations of ligands for 24 hr. The luciferase activity of cells treated with 1000 nM BAZ (panel C) or 100 nM BAZ + 1 nM CE (panel E) was set to '100'. (D, F) HepG2 cells co-transfected with 1μg pC3-Luc reporter and 50 ng ERα-wt were treated with the indicated concentrations of CE or BAZ alone or in combination for 24 hr. The luciferase activity of 1 nM CE treated cells was set to a value of '100' (panels D & F). Values represent the average ± SEM of 3 independent experiments.

Figure 2. The DNA-binding mutant ERα(GSCKV) activates expression of a GRE-containing reporter gene. (A) Schematic diagram showing the ERE-Luc, GRE-Luc and two chimeric reporter genes. (B) The amino acid sequence of the DNA-binding domain

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(DBD) of wild type ERα and DNA-binding mutant ERα(GSCKV). The grey circles indicate the three amino acids that were mutated to enable binding to a glucocorticoid responsive element (GRE). (C) HepG2 cells co-transfected with 1 µg of the indicated reporter genes and 50 ng expression vectors as indicated were treated with vehicle (0.1% ethanol, Veh), 10 nM E2 or 10 nM CE for 24 hr. Data are presented as relative luciferase activity to their respective vehicle-treated control. (D) HepG2 cells were co-transfected with 1 µg EGRE-Luc and ERα-wt (25 ng), ERα(GSCKV) (25 ng) or both [25 ng ERα-wt + 25 ng ERα(GSCKV)] plasmids, and treated with vehicle or 10 nM CE for 24 hr. The luciferase activity of 10 nM CE treated ERα-wt + ERα(GSCKV) co-transfected cells was set to '100'. Values represent the average ± SEM (n=3) for all experiments.

Figure 3. Cooperative activation of chimeric reporter gene expression by combined ERα agonist and antagonist treatment. (A & B) HepG2 cells co-transfected with 1 µg EGRE-Luc or GERE-Luc reporter genes with 50 ng ERα-G521R, 50 ng ERα(GSCKV), or combination [25 ng ERα- G521R + 25 ng ERα(GSCKV)] of the two were treated with vehicle (0.1% ethanol), 100 nM CE, 10 nM BAZ or 100 nM CE + 10 nM BAZ for 24 hr. The luciferase activity of 100 nM CE + 10 nM BAZ treated ERα-wt + ERα(GSCKV) co-transfected cells was set to '100'. The cartoon above each graph is a schematic representation of the respective chimeric reporter gene (EGRE-Luc or GERE-Luc) and transfected forms of ERα. (C) HeLa cells were co-transfected with 1 µg EGRE-Luc or GERE-Luc along with 5 ng ERα-G521R + 5 ng ERα(GSCKV) and treated as indicated for 24 hr. The luciferase activity of 100 nM CE+ 10 nM BAZ treated cells was set as value of

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'100'. (D) HeLa cells were co-transfected with 1 μg GERE-Luc, 5 ng ERα-G521R and 5 ng ERα(GSCKV) along with either 100 ng pCR3.1 or SRC-3 expression vector, and treated with agonist and antagonist as described above. The luciferase activity of 100 nM CE+10 nM BAZ treated pCR3.1 + ERα-wt + ERα(GSCKV) transfected cells was set to '100'. Values represent the average ± SEM of 3 independent experiments for all luciferase assays. (E & F) Representative Western blots show the corresponding expression level of transiently transfected ERα-G521R and ERα(GSCKV), alone or in combination in HepG2 and HeLa cells. HepG2 cells were transfected with 50 ng ERα-G521R, 50 ng ERα (GSCKV), or 25 ng ERα-G521R + 25 ng ERα(GSCKV). HeLa cells were transfected with 10 ng ERα-G521R, 10 ng ERα(GSCKV), or 5 ng ERα-G521R + 5 ng ERα(GSCKV). Actin was used as loading control.

Figure 4. Ligand-regulated interaction of ERα-G521R and ERα(GSCKV). HeLa cells were transfected with 5 ng ERα-G521R-Flag and 5 ng ERα(GSCKV)-HA, either alone or in combination; pCR3.1 empty vector was added to make equal amounts of DNA for each transfection. Forty-eight hour thereafter, cells were treated with 100 nM CE + 10 nM BAZ (panel A), or vehicle, 100 nM CE, 10 nM BAZ, or 100 nM CE+ 10 nM BAZ (panel B) for 1 hr. Immunocomplexes were precipitated with anti-Flag antibody, and Western blots were performed with either Flag or HA antibody, as indicated.

Figure 5. Cooperative activation by agonist and antagonist requires receptor dimer to bind to DNA**.** HepG2 (panel A) and HeLa (panel B) cells co-transfected with ½ERE-Luc or

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EGRE-Luc reporter genes as well as the indicated ER expression vectors were treated with vehicle (0.1% ethanol), 100 nM CE, 10 nM BAZ, or 100 nM CE + 10 nM BAZ for 24 hr. Data are presented as relative luciferase activity comparing to EGRE-Luc plus ERα-G521R + ERα(GSCKV) co-transfected samples treated with 100 nM CE + 10 nM BAZ. Values represent the average ± SEM of 3 independent experiments.

Figure 6. AF1 domain is required for maximal activation by combined ERα agonist and antagonist treatment. HeLa cells were co-transfected with EGRE-Luc (panel A) or GERE-Luc (panel B) and 5 ng each of the indicated plasmids. One day after transfection, cells were treated with vehicle, 100 nM CE, 10 nM BAZ, or 100 nM CE+ 10 nM BAZ for 24 hr and harvested for luciferase assay. Data are presented as relative luciferase activity comparing to EGRE-Luc plus ERα-G521R + ERα(GSCKV) co-transfected samples treated with 100 nM CE + 10 nM BAZ. Values represent the average ± SEM of 3 independent experiments.

Figure 7. Cooperative activation of pC3-Luc by combined ERα agonist and antagonist treatment. HepG2 cells were co-transfected with the pC3-Luc reporter gene and the indicated amounts of expression vectors for ERα-wt and ERα-G521R alone or in combination. Treatments were with vehicle, 100 nM CE, 10 nM BAZ, or 100 nM CE + 10 nM BAZ for 24 hr. The level of activity achieved with 100 nM CE alone in each transfection was set to ‘100’. Values represent the average ± SEM of 3 independent experiments.

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Figure 8. Different estrogens and SERMs synergistically activate chimeric reporter genes. HeLa cells were co-transfected with 1 μg GERE-Luc reporter gene and 5 ng each of the expression vectors for ERα-G521R and ERα(GSCKV). One day after transfection, cells were treated with ERα agonist (either 100 nM CE or 100 nM E2) alone or in combination with 10 nM BAZ (panel A) or 100 nM CE alone or combined with 10 nM of the indicated SERMs (bazedoxifene, B; 4-hydroxytamoxifen, T; raloxifene, R; and ICI 182,780, I) (panel B) for 24 hr. Data are presented as relative luciferase activity comparing to 100 nM E2 + 10 nM BAZ treated samples. Values represent the average ± SEM of 3 independent experiments. Inset in panel B, Western blot showing ER expression levels after 24 hr treatment with the indicated ligands.

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Figure 1

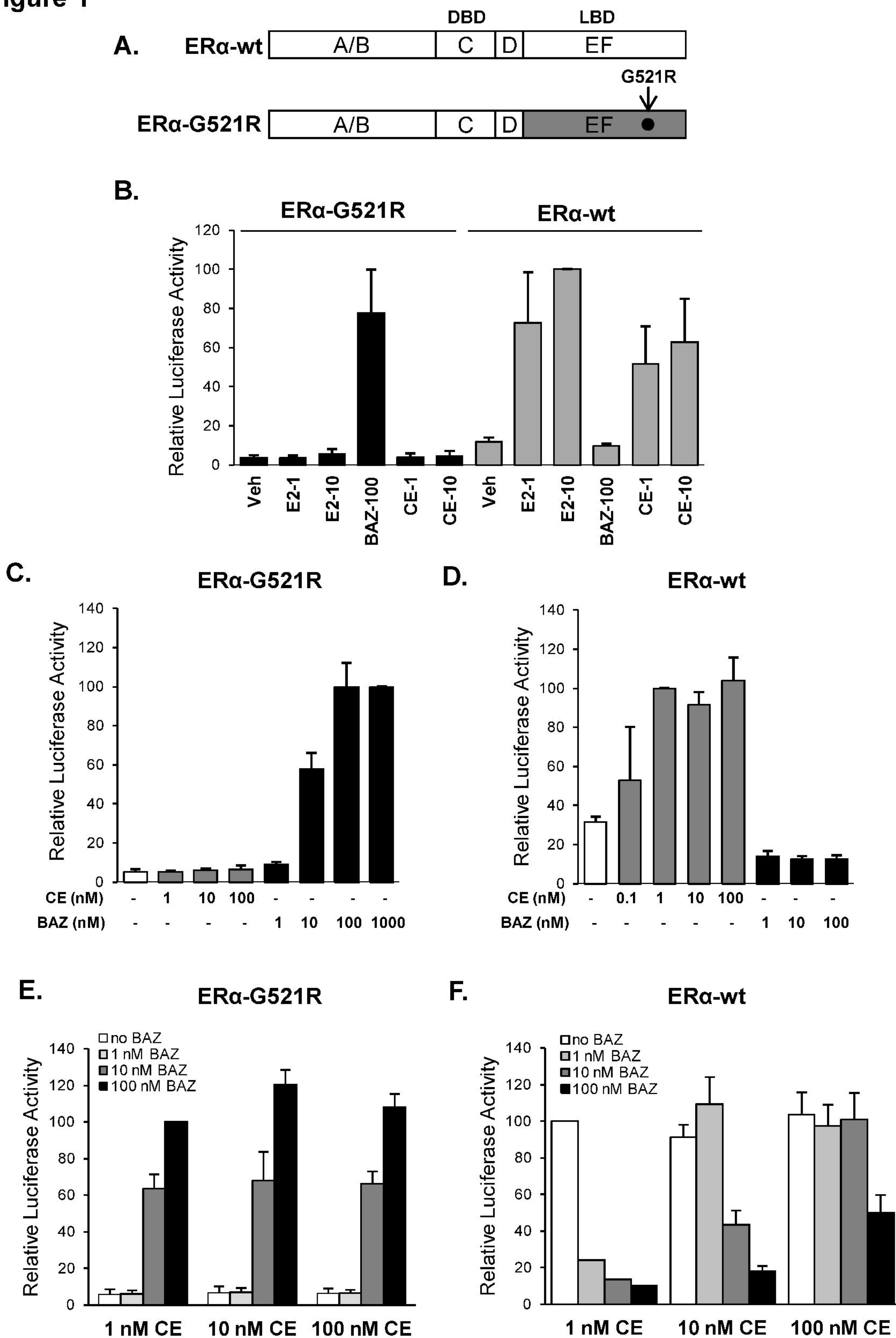


Figure 2

A.

½ ERE | ½ERE | TATA | Luciferase — ERE-Luc
½ GRE | ½GRE | TATA | Luciferase — GRE-Luc
½ ERE | ½ GRE | TATA | Luciferase — EGRE-Luc
½ GRE | ½ ERE | TATA | Luciferase — GERE-Luc

B.

ERα-wt
MKETRY A V C N D Y A S G Y H Y G V W S C E G C K A FF
Zn

ERα(GSCKV)
MKETRY A V C N D Y A S G Y H Y G V W S C G S C K V FF
Zn

C.

ERE-Luc
Relative Luciferase Activity
0 200 400 600 800 1000 1200
Veh
10 nM CE
10 nM E2
ERα-wt ERα(GSCKV)

GRE-Luc
0 50 100 150 200 250 300
Veh
10 nM CE
10 nM E2
ERα-wt ERα(GSCKV)

D.

EGRE-Luc
Relative Luciferase Activity
0 20 40 60 80 100
Veh
10 nM CE
ERα-wt ERα(GSCKV) ERα-wt + ERα(GSCKV)

Figure 3

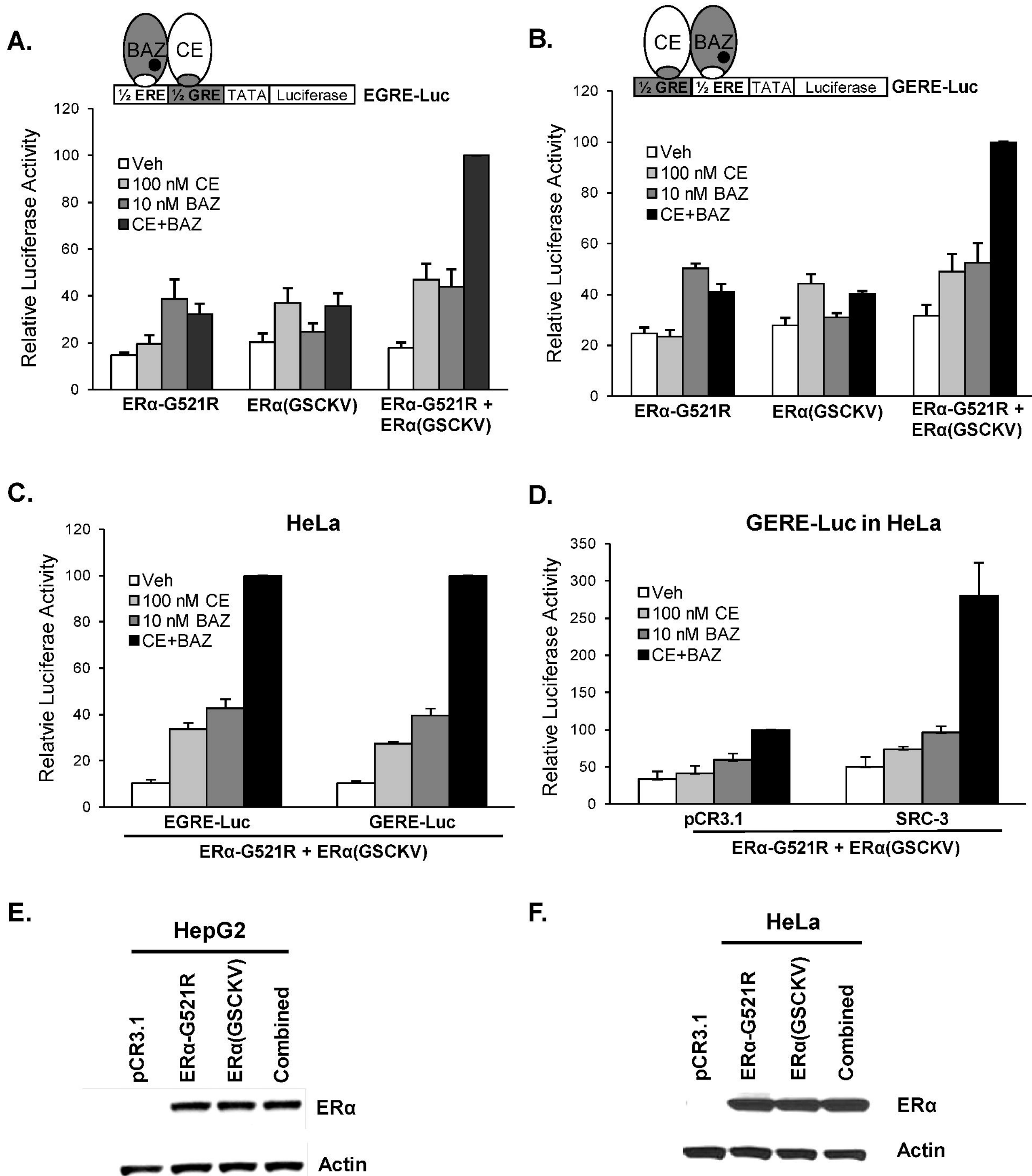
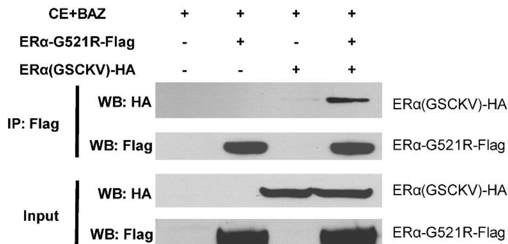


Figure 4

A.



B.

Figure 5

A.

CE
BAZ
½ ERE ½ GRE TATA Luciferase ½ ERE-Luc

Relative Luciferase Activity
100
80
60
40
20
10
8
6
4
2
0

Veh
100 nM CE
10 nM BAZ
CE + BAZ

ERα-G521R
ERα(GSCKV)
ERα-G521R + ERα(GSCKV)
ERα-G521R + ERα(GSCKV)
½ ERE-Luc
EGRE-Luc

B.

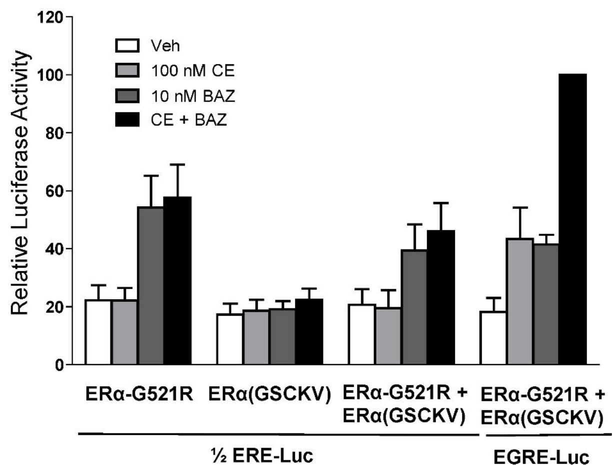
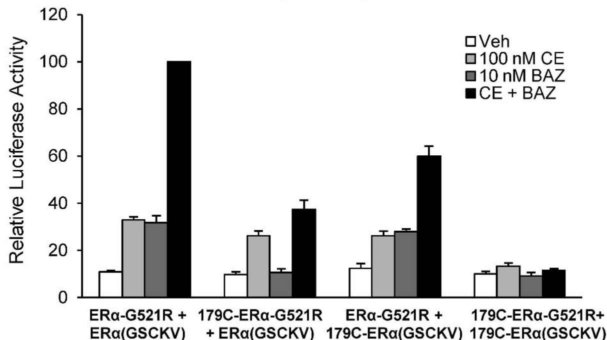


Figure 6

A.



B.

GERE-Luc

120
100
80
60
40
20
0

Relative Luciferase Activity

Veh
100 nM CE
10 nM BAZ
CE + BAZ

ERα(GSCKV) + ERα-G521R
ERα(GSCKV)+ 179C-ERα-G521R
179C-ERα(GSCKV) +ERα-G521R
179C-ERα(GSCKV) +179C-ERα-G521R

Figure 7

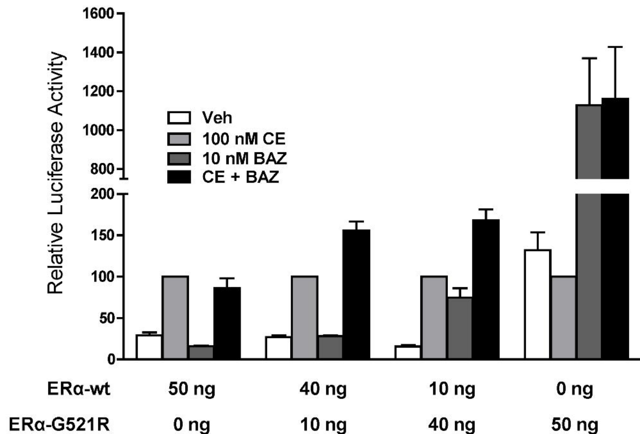


Figure 8

A.

Relative Luciferase Activity

0 20 40 60 80 100 120

Estrogen - CE - CE - E2 - E2

BAZ - - + + - - + +

B.

CE - + + + + +

SERM - - B T R I

ER

Actin

Relatvie Luciferase Activity

0 20 40 60 80 100 120

CE - + - + - + - + - + - + - + - +

SERM - - B B - - T T - - R R - - I I